PHYCOREMEDIATION OF HERBAL PHARMACEUTICAL EFFLUENT USING *Chlorella vulgaris* AND DETERMINATION OF BIOCHEMICAL COMPOSITION OF ITS BIOMASS

BY

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ABSTRACT

Chlorella vulgaris was used to remediate herbal pharmaceutical effluent and the proximate composition of the extracted biomass was analyzed. *Chlorella vulgaris* did not survive high concentration of the effluent thus, dilution was made and remediation carried out at 20% of effluent concentration. The control was carried out using 20% of bold basal media. Algae growth was monitored by dry weight determination and peak growth was recorded on 15^{th} day for algae cultured in effluent with a biomass concentration of 22.211mg/L while the control (20% BBM) recorded peak growth at day 21 with biomass concentration of 26.371mg/L. Physicochemical analysis of the effluent was carried out every 5 days for 30 days and treatment quality was satisfactory when compared with WHO standard for drinking water quality and USEPA standard for effluent discharge. High removal efficiency was also obtained as treatment days progresses. The proximate composition of extracted biomass gave 11.67% protein, 24.36% carbonhydrate, and 8.28% lipids for algae cultured in effluent while the control sample had 9.68% protein, 23.14% carbohydrate and 10.36% lipid. The result of the GC-MS showed the following fatty acids Palmitic acid, Stearic acid, Linoleic acid and Oleic acid (C16:0, C18:0, C18:2, and C18:1) with 22.95%, 7.69%, 21.98%, 27.35% for effluent and 15.63%, 1.87%, 20.51% and 29.58 for control sample. FT-IR analysis revealed O-H stretch of carboxylic acids at 3450.77cm^{-1} and C=O esters at 1734.06cm^{-1} common in triglyceride. This shows that *Chlorella vulgaris* UTEX 259 remediated herbal effluent with satisfactory quality and produced biomass that can be used in agriculture as fertilizers and as feed for biofuel production.

CHAPTER ONE

INTRODUCTION

1.1 Background of Study

Herbal pharmaceutical industries have grown tremendously in the last few decades. In Nigeria, the increase in demand of herbal and bulk drug medicines have resulted in a consequent increase in pharmaceutical manufacturing companies and hence, increased pharmaceutical waste which contains both organic and inorganic pollutants. Drugs of herbal origin have become popular due to its safety, efficacy, low side effects, low cost and cultural acceptabilityas compared to orthodox drugs (Sitre*et al.,* 2009).

Herbal medicines include herbs, herbal preparation and finished herbal products that contains active ingredient from plants. Some of the active plant chemicals can be classified into major groups such as acid, steroids, tannins, saponins, alkaloids and essential oil (WHO, 2006). The production of herbal medicines involves several processes such as drying, passage through cutters or ball mills as required. Further processes such as extraction, fermentation, distillation, decoction preparationand percolation may be used.Though the product is herbal,synthetic chemicals are also used during the manufacturing process.Chemicals utilizedinclude sugar,alcohol,lactose,organic solvent,gelatin,mineral salt,trace metals and edible oil (Vanerkar*et al*., 2015).

Herbal pharmaceuticals generate large volumes of wastewater during the manufacturing processes ranging from crushing, mixing, extraction, fermentation, distillation and other production processes along with physical washing. The quality of the wastewater depends on the type of medicine produced, raw materials utilized and also on the market

demands (Garcia *et al.*, 1995).Herbal pharmaceutical wastewater poses pollution problem due to its high chemical oxygen demand and biological oxygen demand.The nitrogen and phosphorus content of this wastewater are high, leading to eutrophication problems when discharged into the environment.Water quality legislation has increased the standards regarding nutrient removal in order to overcome eutrophication. However, nutrient removal from wastewaters is still a significant concern in many countries due to its high cost. Effective and low cost technologies for nutrient removal from wastewaters are therefore in great demand (Boonchai*et al*.,2012).Untreated pharmaceutical wastewater discharged into the natural environment contributes to environmental pollution and the addition of solvent and oils as per unit requirement also add to pollution load.

Herbal pharmaceutical waste is acomplex constituent of alkaloids,plant extracts,toxic solutes and heavy metal ions (Vanerkar*et al*.,2015).Treatment of the effluent is necessary to bring down the concentration of the pollutant to desired limits before they are finally discharged into the natural systems(UNESCO,2005).The ancient methods employed for the treatment of waste include physical,chemical and thermal method. These treatment methods have drawbacks which includes huge labour requirement, high maintenance cost, low efficiency and huge equipment.The conventional chemical method of pharmaceutical effluent treatment has not been found successful in overcoming the complex pollution load of industrial effluent (Rana*et al.*,2014).Chemical or physicochemical purification methods utilize costly chemicals and treatment units which are difficult to manage at the industrial unit level (Amin *et al*., 2013).One major advantage of algal processes over conventional treatment is the ability to recycle the nutrients forming high value products,such as

fertilizers,pharmaceutical product,food additives and biofuel (Mata *et al*.,2010; Rawat*et al*.,2011).

1.2Microalgae *Chlorella vulgaris*

Chlorella vulgaris is a spherical, eukaryotic, unicellular alga containing chlorophyll, with a cell diameter of approximately 5-10µm(Myers and Cramer, 1947; Miranda *et al*., 2012). The cell wall contains hemicelluloses, which accounts for the stability and rigidity of the cells. It has an asexual reproductive cycle, with the production of autosphores from the mature large cell by dividing the cell into smaller units. One mature cell divides into four new ones every 16-20hours. The algal cells utilize sunlight for photosynthesis. The photosynthetic rate exceeds the respiration rate in *Chlorella* cells by 10-100times. The final harvested algal biomass depends on the cultivation procedure, the growth medium composition, lighting regime, temperature, supply of gases and harvesting time. Maximum annual yields can be in the region of twenty five tonnes or hectares (Myers and Cramer, 1947; Oh-Hama and Miyachi, 1993). The lipid content of *Chlorella vulgaris* is between 6.5-12.5% with very good fatty acid ratio, containing high amount of balanced unsaturated fatty acid (n=6 and n=3) (Yukino *et al*., 2005; Miranda *et al*., 2012).

1.3 Algae for Wastewater Treatment

Treatment of wastewater using different species of algae has been reported in literature (Barlett and Rabe, 1974, Aksu, 2002., Chojnacknaand Gorecka, 2004).The choice of microalgae to be used in wastewater treatment is determined by their robustness against wastewater and by their efficiency to grow and take up nutrients from wastewater (Olguin,2003).*Chlorella vulgaris* is robust and fast growing microalga specie commonly cultivated and interesting regarding the production of secondary metabolite

such as protein, carbonhydrate and lipids (Masson,2012). Factors like CO_2 and intensity of light wavelength affects thegrowth rate of *Chlorella vulgaris* (Sankar and Ranasubramanian,2012)often carbon and nitrogen are the most important nutrients contributing to the biomass produced(Prabakaran and David, 2012).*Chlorella* was used widely to treat wastewater as it removes nitrogen, phosphorus, BOD and COD very efficiently with different retention time ranging from 10hr to 42 days (Wang *et al*., 2010).

Phycoremediation, a type of bioremediation is the use of microalgae for the removal or biotransformation of pollutants including nutrients and xenobiotic from wastewater and CO_2 from waste air (Olguin, 2003).This novel technique utilizes algae to cleanup polluted water and soil thereby improving its quality. Algae growth can improve water quality and make natural waters more suitable for human consumption. This technique makes use of the ability of these microalgae to utilize, accumulate and breakdown the constituent that are present in their growth environment. Algae can utilize CO_2 by photosynthesis and remove excess nutrient effectively at minimal cost. The oxygen produced during this process relieves biological oxygen demand (BOD) in the wastewater. Microalgae are superior in remediation processes as a wide range of toxic and other waste can be treated with algae and they are non-pathogenic (Dominic *et al*., 2009). Algae utilize the wastes as nutritional sources and enzymatically degrade the pollutants. The xenobiotic and heavy metals are detoxified, transformed or volatilized by algal metabolism.Microalgae have a potential to reduce the metal contamination in aquatic system. Initially, the metal ions are physically adsorbed over the algal cell surfaces rapidly. Then, these metals ions move slowly into the cytoplasm of the algae (Omar, 2002). The biomass of microalgae rises during wastewater treatment, and has the potential to remove nitrogen and phosphorus from different sources of wastewater.

However, nutrients are removed from wastewater through a direct uptake by the algal cells (Hoffman, 1998).Studies have shown that algae can remove toxic substances by absorption, accumulation, extracellular secretion and enzymatic degradation and can slowly utilize the herbal pharmaceutical wastewater as nutrient source and make it available for reuse. Several researchers have established that metals such as Ti, Pb, Mg, Zn, Cd, Sr, Co, Hg, Ni, and Cu are sequestrated in polyphosphate bodies in green algae. The polyphase bodies serve as a storage pool for metals and also act as detoxification agents (Yu and Wang, 2004). The algal process is an effective and low technology process which offers inherent cost savings and provides a more appropriate method of water treatment for developing countries (Pittman *et al*, 2011). Algal bioassay is an important tool in water pollution monitoring since algae are the primary producers of the food chain and they are more sensitive to contaminant (Kshirsagar, 2013).

1.4Justification for the Study

Environmental pollution is one major challenge of today's civilization (Kaushik*et al*., 2012; Spina*et al*.,2012).The complex constituent of alkaloids, plant extracts, heavy metal ions and toxic solutes from herbal pharmaceutical wastes contributes to environmental pollution. Industrial wastewater presents a potential hazard to natural water system (Modak*et al*., 1990; Kansal*et al.,* 2011; Lokhande*et al*., 2011; Deepali, 2012). This wastewater contains many inorganic and organic matters which are toxic to the various life forms of the ecosystem (Spina*et al*.,2012). About, 3.4 million people die each year in the world from water borne diseases owing to rapid industrialization (Khan and Noor, 2002; Rajaram and Das, 2008). The reduction of pollution loads from effluent is a serious concern for wastewater treatment plants. Aside the fact that conventional treatment systems are expensive, enormous sludge is being generated which creates disposal problems.Chemical methods utilize costly chemicals and

treatment units,which are difficult to manage at industrial unit or level(Amin *et al*.,2013).Biological treatment processes using algae have shown interesting advantages such as very little sludge,low energy requirement,high organic loading rate,production of biogas which can be used for energy production in the treatment process(Nandy*et al*., 1998).

1.5Aim and Objectives of the Study

This study is aimed at remediating herbal pharmaceutical effluent using *Chlorella vulgaris* and to determine the biochemical composition of its biomass.

The above aim will be achieved through the following objectives;

(i) Culture *Chlorella vulgaris* and harvest the algaefor use in herbal effluent treatment.

(ii) Determine some physicochemical parameters of the pharmaceutical effluent sample; pH,Dissolved Oxygen,COD,BOD,Sulphate,Phosphate,Nitrate and heavy metals (Cu,Ni,Pb,Zn,Fe, and Cd).

(iii) Monitor the physicochemical parameters of the effluent sample at an interval of 5days for 30days after inoculating with *Chlorella vulgaris*.

(iv) Conduct proximate analysis of harvested biomass and fatty acid composition of lipid.

CHAPTER TWO

LITERATURE REVIEW

2.1 Herbal Pharmaceutical Effluent

According to WHO, (2006) up to 80% of the population in Africa depends on alternative medicine for primary healthcare and in china,herbal medicine account for 30-50% of total medicinal consumption.In Europe,North America and other industrialized regions over 50% of the population have used complementary or alternative medicine at least once (Jaggi,1984).

Many materials used in pharmaceuticals are derived from natural sources such as the roots and leaves of plants,animal glands, and parasitic fungi.These pharmaceuticals typically exhibit unique pharmacological properties including allergy relief medicines,insulin,morphine,alkaloids and papaverine.Despite their diversity, all extractive pharmaceuticals are too complex to synthesize commercially.Extraction is an expensive manufacturing process which entails collecting and processing large volumes of specialized plant or animal matter to produce small quantities of products.Sections in pharmaceutical industries include research and development,chemical synthesis,natural product extraction and formulation (Shalini*et al*., 2010).Residual wastes from an extraction plant essentially will be equal to the weight of raw material,since the active ingredients extracted are generally present in the raw materials at very low levels.Solid wastes are the greatest source of the pollution load.However, solvent used in the processing steps can cause both air and water pollution.Detergents and disinfectants

used in equipment cleaning operations are normally found in the wastewater (USEPA, 1983).

Pharmaceutical effluents are waste generated by pharmaceutical industryduring the process of drugs manufacturing.Generally,pharmaceutical industries do not generate uniform waste streams,due to the variety of medicines produced during any given processing period (Frick,2001).Typical waste streams include spent fermentation broths,process liquors,solvents,equipment wash waters,spilled materials and used processing aids(USEPA, 1983).An important pollution index of industrial wastewaters is the oxygen function measured in terms of chemical oxygen demand(COD) and biological oxygen demand (BOD).The chemical oxygen demand is a measure of oxygen equivalent to the requirement of oxidizing organic matter contents by a strong chemical agent (Yadav and Kumar, 2011) while biological oxygen demand is usually defined as the amount of oxygen required by bacteria in stabilizing the decomposable organic or biologically oxidizable matter (Yadav and Kumar, 2011). The nutrient status of the wastewater ismeasured in terms of nitrogen and phosphorus. In addition, other important quality parameters include pH, temperature and total suspended solids (Ezenobiand Okpokweshi,2004).The wastewater produced by the pharmaceutical industry has a very bad quality. Usually the concentration of COD is around 5,000-15,000mg/L,but that of BOD is relatively low,and the ratio B0D/COD is lower than 30% which means the wastewater has a poor biodegradability.

Adebayo*et al*., (2010) studied the effluent discharge from a pharmaceutical company in illorin by analyzing for the BOD,COD,TS,SS,colour intensity and heavy metals using biological treatment. The result of the treatment revealed significant reduction in COD, BOD and TS.

Herbal pharmaceutical wastewater is moderately strong with COD and BOD concentration in the range of 21960-15660mg/L respectively and equally high concentration of suspended solids of 5460-7370mg/L (Vanerkar*et al*., 2013).

2.2 Treatment Method Used for Pharmaceutical Effluent

Wastewater characteristics play an important role in the selection of treatment process of wastewater (Deegan*et al*., 2011).Biological treatment methods have traditionally been used for the management of pharmaceutical wastewater (SumanandAnjaneyulu, 2005).They may be subdivided into aerobic and anaerobic processes.Aerobic applications include activated sludge,membrane batch reactors and sequence batch reactors (Laparа*et al*., 2002; Sumanand Anjaneyulu2005;Noble 2006,Chang *et al*., 2008 and Chen *et al*., 2008).Anaerobic methods include anaerobic sludge reactors,anaerobic film reactors and anaerobic filters(Enright *et al*., 2005,Oktem*et al*., 2007Chelliapan*et al*.,2011;Sreekanth*et al*.,2014).Activated sludge treatment is unsuitable for the treatment of wastewater where the COD levels are greater than 4000mg/L (Suman and Anjaneyulu,2005).Conventional activated sludge with a long hydraulic retention time has historically been the method of choice for the treatment of pharmaceutical industry wastewater (El Gohary*et al*,1995;Oz *et al*.,2004). It has a lower capital cost than more advanced treatment methods and alimited operational requirement; it is generally more environmentally friendly than chlorination.However,high energy consumption,the production of large amounts of sludge (Sreekanth*et al*.,2014) and operational problems including colour,foaming and bulking in secondary clarifiers are associated with activated sludge plant(Oz *et al*.,2004).

Physicochemical treatment technique was carried out on herbal pharmaceutical wastewater using conventional coagulants individually and in combination with different polyelectrolyte of three different charges (Vanerkar*et al*., 2013).Among ten combinations used in the study, 300mg/L Alum + Oxyfloc-Fl-11 was found to be the best combination with respect toCOD,BOD reductionand SS removalof 62.66mg/l (64.00%);2867mg/l (69.40%) and 637mg/l (80.82%) respectively.The treated effluent was selected for secondary biological activated sludge process (ASP) using optimal parameters like organic/ hydraulic loadings.Removals of organics in terms of absolute value of COD,BOD and SS were found in therange with an absolute value of 896-944mg/L, 156-174mg/L and 66-74mg/L respectively.Finally activated sludge process treated effluent was subjected to tertiary fenton's oxidation process where the removal's of COD, BOD,SS and TOC were found to be138mg/L (85.19%),20mg/L (88.10%), 21mg/L (70.00%) and 98mg/L (78.22%) respectively (Vanerkar*et al*., 2013).

2.3Microalgae for Bioremediation

Cultivation of algae started early in the 1970's. Algae were grown in wastepond to treat secondary effluent and to prevent eutrophication. It was then employed as secondary rather than tertiary treatment option (Tam and Wong,1989).*Chlorella vulgaris* was used to bioremediate textile wastewater using four batches of culture in high rate algae ponds containing textiledye (Lim *et al*., 2010).Colour removal ranged from 41.8%-50.0%,NH_4-N reduction (44.4-45.1%), PO_4-P (33.1-33.3%) and COD (38.3-62.3%) in the textile wastewater. *Chlorella vulgaris*treatment offers a good system for treating textile wastewater before final discharge.

Saline water algae *Spirulinaplatensis* was used to treat both herbal and bulk drug wastewater(Kshirsagar, 2010). Herbal pharmaceutical wastewater with high concentration of 70% effluent dilution was efficiently treated and a COD reduction of

70% and BOD reduction of 73% were achieved while bulk drug wastewater showed high toxicity and noalgal growth was noted at high concentration of 5% and 10% effluent dilutions respectively.Only 1% concentrations of bulk drug wastewater promoted algal growth and resulted in COD/BOD reduction of 61.3% and 64.6% respectively.

*Chlorella*is widely used to treat wastewater as it removes nitrogen, phosphorus, BOD and COD very efficiently with different retention time ranging from 10h to 42days (Wang *et al*., 2010).Microalgae can be used for remediation to reduce the nutrient content in the wastewater due to the algae's ability to assimilate nutrients into the cells. *Chlorella vulgaris* was used to reducetotal nitrogen (TN) and total phosphorus (TP).The algae were cultured in shake flasks containing the wastewater in the presence of artificial light under laboratory condition and it reduced TN and TP with 82.1% and 90.9% respectively.*Chlorella vulgaris* did not only bioremediate the wastewater,but also produced biomass that could be exploited for fertilizers,feed additives and biofuel.The optimum detention period for the phytoremediation varied within 10 and 14 days (Gopinath*et al*., 2014).

The capability of *Chlorella vulgaris* to remove nitrogen in the form of ammonia or ammonium ion from wastewater effluent was investigated by Kim *et al*., (2010). The wastewater was found to contain high concentration of nitrogen (7.7±0.19mg/L) and total inorganic carbon (58.6±0.28mg/L) at pH 7 and was suitable for growing *Chlorella vulgaris*.When *Chlorella vulgaris* was cultured in a batch mode under a closed system,half of the nitrogen concentration was removed in 48h after a 24h lag-phase period.Total inorganic carbon concentration also concomitantly decreased during the rapid growth-phase.The total biomass weight gained during the entire cultivation period balanced well with the amount of inorganic carbon and nitrogen removed from the

culture medium whichsuggests that *Chlorella vulgaris* can be synergistically used to remediate residual nutrient in wastewater as well as to cultivate microalgae for biofuel production.

Phycoremediation of partially treated wastewaterby *Chlorella vulgaris*was carried out by Mamun*et al.*, (2012). Thetotal nitrogen and total phosphorus werereduced by 83.1% and 91.2% respectively.The optimum detention period was found tovary from 10 to 14 days.In another study by Ahmad *et al*., (2013),*Chlorella vulgaris* was used to treat municipal wastewater and harvested biomass was converted to biodiesel. After treatment with *Chlorella vulgaris* there was 99.9% reduction in COD, BOD (100%), Nitrate (99.98%),Phosphate (99.96%) and Total Carbon 100%. However, the biochemical composition of the biomass was not analyzed for other applications.

Chlorella vulgaris was used for bio sorption of lead (Aung*et al.*, 2013).The biosorbent was prepared using the harvested biomass of the algae initially cultured in cornmay medium.The biosorption isotherm of lead on *Chlorella vulgaris* follows Langmuir isotherm model. The unicellular microalgae,*Chlorella vulgaris* and *Scenedesmusdimorphus* are capable of removing half of the phosphate and most of the ammonium from dairy and pig farm wastewaterin India (Nouede la and De pauw, 1988, Gonzalez *et al*., 1997; Bashan *et al*.,2004,) in these studies, microalgae were in suspension.

Bioremediation of wastewater usually requires large population of microalgae and application of this method is servely limited by the difficulties of harvesting enormous microalgae populations developed in wastewater after treatment (Basha*et al*.,2008).The phycoremediation efficiency of three microalgae *Chlorella vulgaris,Synechocystissalina* and *Gloeocapsagelatinosa* was studied on industrially polluted wastewater by Dominic *et al.*,(2009).The wastewater was sterilized and treated with these algae and the water

quality was assessed at regular interval of 5days for a period of 25days.Result showed high removal efficiency of pollutantsalthough;*Gloeocapsagelatinosa* showed more efficiency than *Chlorella vulgaris* and *Synechocystissalina* in the bioremediation.*Chlorella vulgaris* were cultured in walne's medium while *Synechocystissalina* and *Gloecapsagelatinosa* were cultured inllen and nelson medium at a temperature of 23±1˚C and 2,000 lux light with a 16:8hour light/dark cycle to obtain synchronous culture.*Chlorella vulgaris* showed a drift toward alkalinity as pH of the medium changed from 6.0 to 8.1 while *Synechocystissalina* changed the pH of the medium from 6.0 to 8.0 and *Gloeocapsagelatinosa* from6.0 to 7.9 after treatment.Phosphate uptake of *Chlorella* was 69.23%,*Synechocystissalina*64.52% and *Gloecapsagelatinosa*74.19%.*Chlorella* showed 84% reduction in nitrate content,*Syneochocystissalina* 82.5% and *Gloecapsagelatinosa* had 80.9%.Dissolved oxygen of the water sample treated with *Chlorella vulgaris* increased from 2.3mg/L to 8mg/L,*Synechocystissalina*showed an increase from 2.3mg/L to 6.4mg/L while *Gloecapsa* gelatinous had an increasefrom 2.3mg/L to 5.9mg/L.

The evaluation of the toxicity of herbal pharmaceutical wastewater to a freshwater inDubai was investigated by Sitre*et al.,* (2009). This investigation was carried out on raw and treated effluent under laboratory conditions.Physicochemically treated effluent reduced the toxicity by 25% while biological treatment with algae reduced the toxicity completely.Statistical analysis showed regression coefficient of more than 0.9 indicating good correlation between the mortality rate of algae and effluent concentration.

The toxicity of effluent was studied using *Scenedesmusquadricauda*with physicochemically and biologically treated effluent respectively (Vanerkar*et al.,* 2015).Although growth rate increased in both medium, highest yield of algae was observed in the biologically treated effluent for up to 15days of incubation by synthesis

of chlorophyll and cell metabolites with 10-100% dilution of effluent.Cultures were maintained for 21 days,biomass concentration was determined by measuring optical density at 660nm using a spectrophotometer.The growth rate of green algae increased throughout the 21days in 10%,20% and 30% dilution for physicochemically treated effluent.No growth was observed in 40%-100% dilution for the physicochemially treated effluent.The maximum growth rate of these green algae in biologically treated effluent increased up to 15^{th} day of incubation,on 18^{th} and 21^{st} day of incubation,the growth rate slowly decreased. Differential tolerance of these algae to the effluents shows that there is great scope for algal treatment in industrial wastewater.

The growth of green algae *Chlorella* specie on wastewater sampled from four different stages of a treatment process flow of a local municipal wastewater treatment plant and the efficiency of these algae to remove nitrogen,phosphorus,COD and metal ions from these sources were investigated by Wang *et al.,* (2010).Result showed a significant nutrient uptake by these green algae and a new option of applying algal process in wastewatertreatment.

Chlorella vulgaris was characterized using scanning electron microscope and FT-IR. Result of the FT-IR showed high amount of protein, carbohydrate and nuclei acid assigned from the various peaks $1656cm^{-1}$,1079 and $1047cm^{-1}$and $1243cm^{-1}$.The green algae also showed high efficiency when applied for hostel wastewater treatment (Shabudeen*et al*., 2013).The potential of the microalgae for pollutant removal from wastewater also showed high efficiency compared to *Chlamydomonas*(Nandini*et al*., 2013).Microalgae ability to remove nitrogen and phosphorus during its cultivation in aquaculture wastewater was investigated by Velichkova*et al*., (2014). The algae were cultured at room temperature (25-27˚C) using a fluorescent tube to supply light with a photoperiod of 12:12hours light/dark cycle, using two different carbon sources,2%

carbon(iv)oxide and 1.125g/L sodium carbonate.*Chlorella vulgaris* showed better growth in wastewater supplemented with bicarbonate as carbon source.

CHAPTER THREE

MATERIALS AND METHOD

3.1Materials

The materials used in this work include; Beakers, conical flasks, volumetric flasks, kjeldahl flask, foil paper, crucible, filter paper, reflux condenser, soxhlet apparatus, heating mantle, separating funnel and watch glass.

All used reagents were of analytical grade and purchased from Sigma-Aldrich Company.

Table 3.1:Equipment

S/No	Name	Model	Manufacturer/Address
1	Atomic absorption spectrophotometer	Varian AA240FS	Agilent Technology/US
2	GC-MS	QP2010	Shimadzu/Japan
3	FT-IR	8400F	Shimadzu/Japan
4	Spectrophotometer	DR 5000	Hach/US
5	pH meter	Eco testr pH1	Oaklon/US
6	DO meter	Jenway 9150	Keison ltd/US
7	Illuminated cooled incubator	Gallenkamp	UK
8	Stainless steel sterilizer	YX-280A	Bluestone ltd/China

3.1.1 Chemicals

S/No	Name	Formula	% Purity	Specific gravity
1	Hydrochloric acid	HCl	37%	1.19
2	Nitric acid	HNO_3	65%	1.41
3	Sulphuric acid	H_2SO_4	98%	1.85
4	Chloroform	$CHCl_3$	99.9%	1.48
5	Methanol	CH_3OH	99%	0.79
6	Distilled water	H_2O	100%	1
7	Sodium hydroxide	NaOH	98.5%	2.13
8	Potassium hydroxide	KOH	99.99%	2.04
9	Cobalt(II)nitrate hexahydrate	$Co(NO_3)_2.6H_20$	99.99%	1.88
10	Boric acid	H_3BO_3	99%	1.44
11	EDTA disodium salt	$C_{10}H_{14}N_2Na_2O_8$	99.4%	0.77
12	Calcuim chloride	$CaCl_2$	99.99%	2.15
13	Sodium nitrate	$NaNO_3$	99.99%	2.26
14	Potassium dichromate	$K_2Cr_2O_7$	99.7%	2.68
15	Ferrous ammonium sulphate	$Fe(NH_4)_2(SO_4)_2.6H_2O$	99%	1.86

16	Ferroin indicator	$C_{36}H_{24}FeN_6O_4S$	99%	0.99
17	Silver sulphate	Ag_2SO_4	99.99%	5.45
18	Sodium chloride	NaCl	99.5%	2.17

3.2Sample Collection

The microalgae strain *Chlorella vulgaris* UTEX 259used in these studies was obtained from National Research Institute for Chemical Technology(NARICT)Bassawa, Zaria.The culture was collected as inoculum in sterilized BOD bottles. Grab pharmaceutical effluent used for this study was collected fromthe drain at National Institute for Pharmaceutical Research and Development,Idu(NIPRD) industrial Area, Abuja using a four litre plastic container that was previously washed with hot water and detergent, followed by 2%v/v nitric acid and finally rinsed with distilled water.

3.3Determination of Physicochemical Parameters.

3.3.1pH

The pH of the sample was determined using a portable pH meter after being standardized with buffers pH 4.0 and pH 10.0 (Ademoroti, 1996).

3.3.2 Dissolvedoxygen (DO)

The dissolved oxygen was taken using a Dissolved Oxygen meter.The meter was calibrated according to the manual and the probe filled with the polarographic dissolved oxygen filling solution. The DO probe was inserted mid-way intothe sample and then stirred until a stabilization graph appeared and the DO value recorded.

3.3.3 Biological oxygen demand (BOD)(Ademoroti,1996)

A fresh sample was incubated at 20^{o}C for five days and the above procedure for the determination of dissolved oxygen was then repeated. The difference between DO for incubated sample and DO not incubated was determined.

Calculation

$$BOD(mg/L) = \frac{D_1 - D_5}{P} \quad (3.1)$$

D_1 = Dissolved oxygen before incubation of sample (mg/L)

D_5= Dissolved oxygen after 5days incubation (mg/L)

P = Decimal volumetric fraction of sample used; 1/P = dilution factor.

Dilution factor is given by the volume of bottle divided by the volume of sample.

3.3.4 Chemical oxygen demand (COD)(Ademoroti, 1996)

0.4 ml of H_2SO_4 was placed in a refluxing flask. 20ml of the sample was diluted with 20 ml of distilled water. 10 ml standard solution of $K_2Cr_2O_7$ was then added to glass beads already heated to 600^{o}C for 1 hour. The flask was then attached to the reflux condenser and about 30ml of concentrated H_2SO_4 containing Ag_2SO_4 was added through the open end of the condenser. The resulting solution was thoroughly mixed by switching. The mixture was refluxed for 1hour and the condenser washed with 25ml of distilled water. The mixture was diluted with 150ml of distilled water and cooled to room temperature. About 3 drops of ferroin indicator was added. The mixture was then titrated with $Fe(NH_4)_2(SO_4)_2$ taking as the end point the sharp colour change from blue-green to reddish brown. In the same manner blank containing 20 ml distilled water was refluxed with the reagent.

Calculation

$$COD(mg/L) = \frac{(a-b)\times M \times 8000}{vol\,of\ sample} \quad (3.2)$$

Where: COD=COD from potassium dichromate

a = vol. in mlFe $(NH_4)_2(SO_4)_2$ used as blank

b = vol. inml Fe $(NH_4)_2(SO_4)_2$ used for sample.
M= morality of Fe $(NH_4)_2$ $(SO_4)_2$.

3.3.5 Nitrate determination

Nitrate concentration was determined using NitraVer reagent powder pillow. One reagent pillow was added to 10 ml of the sample and the concentration of nitrate was read at 400nm wavelength using a spectrophotometer.The sample was used as blank.

3.3.6 Sulphate determination

The concentration of sulphate was determined using SulfaVer reagent powder pillow.One reagent pillow was added to 10 ml of the sample and the concentration read at 450nm using a spectrophotometer.

3.3.7Phosphate determination

The concentration of phosphate was determined using PhosVer reagent powder pillow. One reagent pillow was added to 10 ml of the sample and then the concentration was read at 880nm with a spectrophotometer.

3.3.8 Iron determination

Iron was determined using FerroVer Iron reagent pillow. One reagent pillow was added to 10 ml of the sample and the concentration read at 510nm with a spectrophotometer.

3.3.9 Heavy metal determination

The concentration of heavy metals was determined using AAS. The sample was digested by adding 10 ml of concentrated HNO_3 to 50 mlof the sample in a 100 ml beaker. The sample was then covered with a watch glass and heated on a hot plate until the solution reduced to about 10 ml.The solution was allowed to cool, filtered and quantitatively transferred into a 50 ml standard volumetric flask and made up to mark with distilled water and further analysed using AAS(Anyakora*et al.*, 2011).

3.4Algal Culture

The medium for the algal culture was prepared from reagent given in Table 3.2

Table 3.2: COMPOSITION OF BOLD BASAL MEDIUM(Mamun*et al.*, 2012)

Reagent	A	Per 400 ml of deionized water for each salt
	$NaNO_3$	10.0g
	$MgSO_4.7H_2O$	3.0g
	K_2HPO_4	4.0g
	KH_2PO_4	6.0g
	$CaCl_2$	1.0g
	NaCl	1.0g
Reagent	B	All in a liter of deionized water
	$ZnSO_4$	8.82g
	MoO_4	0.71g
	$Co(NO_3).6H_2O$	0.49g
	$CuSO_4.5H_2O$	1.57g
		Autoclave to dissolve
Reagent	C	Per 100 ml of deionized water

	H_3BO_3	1.14g
Reagent	D	100ml of deionized water
	$EDTA.Na_2$	5.0g
		+
	KOH	3.1g
		Per 100ml of deionized water
E	$FeSO_4.7H_2O$	4.98g + 1.0ml concHCl

1 liter bold basal medium(BBM) was prepared by measuring 10 mleach of reagent A, 1.0 cm^3each of reagent B, C, D and E,then made up to mark in a liter volumetric flask with deionized water. The pH was adjusted to 6.8-7.0 using 1M KOH. The media was autoclaved at 121˚C for 15minutes after which it was allowed to cool to room temperature. 10% of the algal culture was prepared using the BBM.This was done by measuring 100 ml of algae inoculaand 900ml of BBM in a litre conical flask which was covered with aluminium foil. The inoculation was done aseptically and cultures were maintained at room temperature in 12:12hours light/dark cycle.Samples were shaken 2-3 times a day to prevent algal cells from sedimenting to the bottom of the flask. The growth rate of the algae was monitored by taking absorbance at 686nmevery 48 hours and algae inoculum was ready for transfer at exponential phase.

3.4.1 Dry weight of algae (Harvard *et al.*, 2003)

The absorbance of the algae at 750nm was taken using a spectrophotometer and the dry weight of the algae was calculated as

$$Dry\ weight = 3.31 \times A_{750} + 617.45\,(A_{750})^2 \quad (3.3)$$

Where $A_{750}$is absorbance at 750nm

3.5Inoculation of Algae Culture to Diluted Effluent Samples and Control Experiment.

A set of six dilutions, 10%, 20%, 40%, 60%, 80%, and 100% of the effluent was prepared using deionized water in 1000 ml conical flasks. 10% algae culture was inoculated(Vanerkar*et al.,* 2015).Growth of the algae in the effluent was monitored by taking absorbance at 686nm every 2days.The physicochemical parameters of the diluted effluent sample were determined every 5days interval for 30days.This was done by allowing the algae to sediment and aliquot of the effluent was obtained for analysis. The effluent was filtered using whatmann filter No 42 before analysis to remove residual algal cell. Each experiment was performed in triplicate and Scale up cultures was also prepared.The Control for this experiment was prepared usingthe standard nutrient medium (BBM) with dilution. Growth monitoring of the algae and physicochemical analysis of the medium was carried out.

3.6Algae Harvesting and ProximateComposition of Biomass

The algae biomass was harvested using gravity sedimentation technique and then dried at 40˚C in an oven.

3.6.1Determination of moisture content of biomass(AOAC, 1984)

A clean crucible was dried to constant weight in an oven at 105˚C, cooled in a desiccator and weighed (W_1).2g of sample was accurately weighed into the crucible and then weighed (W_2).The crucible was dried in an oven to a constant weight (W_3) at 105˚C.The percentage moisture content was calculated as

$$\%\,Moisture\ content = \frac{W_3 - W_2}{W_2 - W_1} \qquad (3.4)$$

3.6.2 Determination of ash content of biomass(AOAC, 1984)

A porcelain crucible was dried in an oven at 100˚C for 10minutes, cooled in a desiccator and weighed (W_1).2g of the sample was placed into the previously weighed porclain crucible and weighed (W_2).The sample was first ignited and later transferred into

furnace which was then set at 550˚C.The sample was left in the furnace for eight hours to ensure proper ashing. The crucible containing the ash was then removed cooled in the desiccator and weighed (W_3).The percentage ash content was calculated as

$$\%\, Ash\, content = \frac{W_3 - W_1}{W_2 - W_1} \quad (3.5)$$

3.6.3 Crude lipid content determination(AOAC, 1984)

A clean, dry 500 ml round bottom flask containing few anti-bumping granules was weighed (W_1) and 300 ml of petroleum ether (40˚C-60˚C) was poured into the flask fitted with soxhlet extraction unit. The extractor thimble containing 2g of the sample was fixed into the soxhlet extraction unit. The round bottom flask and a condenser were connected to the soxhlet extractor and cold water circulation was turned on. The mantle was switched on and the heating rate adjusted until the solvent was refluxing at a steady rate. Extraction was carried out for six hours. The solvent was recovered and the oil was dried in the oven at 70˚C for one hour. The round bottom containing the oil was cooled in the desiccator and weighed W_2.

$$\%\, Crude\ lipid\ content = \frac{W_2 - W_1}{Weight\ of\ sample} \times 100 \quad (3.6)$$

3.6.4 Crude fibrecontent determination (AOAC, 1980)

2g of sample was weighed out into a round bottom flask.100 ml of 0.25M H_2SO_4 solution was added and the mixture boiled under reflux for 30minutes.The hot solution was quickly filtered under suction. The insoluble matter was washed several times with hot water until it was acid free. It was quantitatively transferred into the flask and 100 ml of hot 0.31M NaOH solution was added and the mixture boiled again under reflux for 30minutes and quickly filtered under suction.The insoluble residue was washed with

boiling water until it was base free.It was dried to constant weight in the oven at 100˚C, cooled in a desiccator and weighed (C_1). It was then incinerated in a muffle furnace at 550˚C for 2hours, cooled in the desiccator and weighed (C_2)

Calculation

$$The\ loss\ of\ weight\ on\ incineration = \frac{C_1 - C_2}{Weight\ of\ sample} \times 100 \quad (3.7)$$

3.6.5 Nitrogen and crude protein determination (Onyeike and Osuji, 2003)

Preparation of reagents

(i) Preparation of mixed catalyst

The mixed catalyst comprises of 1g of $CuSO_4$ and 10g of Na_2SO_4

(ii) Preparation of Sodium hydroxide NaOH (40%) solution

Sodium hydroxide (40g) was dissolved with 20 ml distilled water in a beaker and was transferred to a volumetric flask and made up to 100 mlmark with distilled water to give 40 % solution.

(iii) Preparation of Boric acid (2%) solution

Boric acid (2g) was dissolved with 20 ml distilled water in a beaker and was transferred to a volumetric flask and made up to 100 mlmark with distilled water to give 2 % solution.

(iv) Preparation of Mixed indicator

0.2g of methyl red was diluted with 100 ml of 95% ethanol. 1.0g bromocresol green was diluted with 500 ml of 95% ethanol. 1 part methyl red solution was mixed with 5 parts bromocresol green solution.

(v) Preparation of 0.1M HCl

Hydrochloric acid (83.3 ml) was diluted with 100 ml of distilled water in a beaker and made up to 1000 ml mark with distilled water in a volumetric flask. Working

solution of 0.1M HCl was prepared by diluting 8.33 ml of the stock solution in 40ml of distilled water and made up to 1000 ml mark with distilled water in a volumetric flask.

Procedure

1.5g of the defatted sample was weighed on an ashless filter paper and dropped into 300 ml Kjeldahl flask. 25 ml of H_2SO_4 and 3g of digesting mixed catalystwere dropped into the Kjeldahl flask and the contents were then transferred to the Kjeldahl digestion apparatus. The sample was digested until a clear greenish colour was obtained.The digest was cooled to room temperature and diluted to 100 ml with distilled water. 20 ml of the diluted digest was measured into 500 ml kjeldahl flask containing antibumping chips and 40 ml of 40% NaOH was slowly added by the side of the flask. 250ml conical flask containing a mixture of 50 ml of 2% boric acid and 4drops of mixed indicator (1:5 of methyl red and bromocresol green) was used to trap the ammonia liberated.The conical flask content were heated and the distillate from the Kjeldahl distillation apparatus was collected into the boric acid solution from the point when the boric acid solution turned green, 10minute was allowed for complete distillation of the ammonia present in the digest. The distillate was titrated with 0.1MHCl.

$$\%\,Nitrogen = \frac{14 \times M \times V_t \times T_v}{Weight\ of\ sample\,(mg) \times V_a} \times 100 \qquad (3.8)$$

$$\%\,Crude\ protein = \%\,Nitrogen\ (N_2) \times 6.25 \qquad (3.9)$$

Where M = Molarity of HCl

Tv = Titre volume of HCl used

Vt = Total volume of diluted digest

Va = Aliquot volume distilled

3.6.6 Carbonhydrate(Muller and Tobin, 1980)

The total % carbonhydrate content was determined by the difference between the sum of the% moisture, % ash, % crude lipid, % crude protein and % crude fibre content and 100.

Calculation

$$\%Carbonhydrate = 100 - (\%\, moisture + \%\, ash + \%\ fat + \%\ protein + \%\, fibre) \quad (3.10)$$

3.7Extractionof oil from*Chlorella vulgaris*

Lipids were extracted from 500mg of biomass with a solvent mixture of chloroform, methanol and water (2:1:0.75, by volume) (Christie, 2003). The solvent mixture containing the algae was homogenized with a Teflon rod. The mixture was poured into a separating funnel and agitated for 20-30minutes and was allowed to stand for phase separation. The lower chloroform layer containing the lipid fraction was collected on a preweighed glass vial and then concentrated by drying the sample in an oven at 50˚C-60˚C before being reweighed. The difference between the preweighed glass vial (empty) without the lipid extract and that with lipid after drying at 50˚C-60˚C gives the total lipid concentration

$$\%Lipid\ content = \frac{weight\ of\ oil}{weight\ of\ sample} \times 100 \quad (3.11)$$

3.8Fatty Acid

Profile

The profile of fatty acids present in the sample was analyzed using GC-MS. This technique comprise of gas chromatography which separates the components in the mixture while the mass spectroscopy characterized each of the components individually based on the mass-to-charge ratio.

3.9 Determination of Functional Groups

The position and presence of functional groups in the oil was carried out using FTIR.The sample was mixed with Potassium bromide (KBr) and then smeared on a small transparent disc of 13 mm diameter. A background spectrum of pure KBr was run

before the spectrum of any sample to account for any absorption due to impurities in the KBr. The disc containing the sample was then placed in the infrared spectrometer and the spectrum of the samples were collected between the spectral range of 4000-400 cm^{-1} with a resolution of 4 cm^{-1}.

3.10 Statistical Analysis

The values of the physicochemical analysis are expressed as the arithmetic mean with standard deviation of the triplicate analysis and tabulated. Some of the results are presented as line graphs to give a clearer and simpler picture of the relationship between each parameter and treatment days in both the effluent and control samples. The statistical significance of the mean value between each treatment stage was determined by applying the one way Anova at $p<0.05$.

All statistical analyses were performed using Microsoft Excel 2010 and SPSS 20.0 version.

CHAPTER FOUR

RESULTS

4.1 Algal Growth in Effluent and Control Sample

Higher dilution of the effluent sample (40%-100%) did not favour algae growth with time as no chlorophyll colouration was observed at this dilution rate as time progresses. Therefore, remediation studies were carried out at 20% dilution of the effluent sample and same dilution rate was used for the control. Figure 4.1 and Figure 4.2 shows the optical densities and dry weights of the algae in the effluent and control samples respectively. The algae recorded peak growth in the effluent sample on day 15 with a biomass concentration of 22.21mg/L and on day 21 for the control sample with a biomass concentration of 26.371mg/L. This biomass concentration is recorded for the 20% dilution used in the remediation

4.2 Effect of Phycoremediation on the Physicochemical Properties of Effluent and Control samples

Figure 4.3 shows the variation of pHin the effluent and control samples. The pH of the effluent sample increases from 6.6 on day 1 to 8.4 on day 15 and then dropped from 8.4 to 7.1 on day 30 and similar effect was observed in the control sample. The mean and standard deviation of the pHwith treatment days is shown on appendix 1 and 2 for the effluent and control sample respectively.

Figure 4.3 shows the variation in dissolved oxygen content of the effluent and control sample. The dissolved oxygen increased from 0.92mg/L on day 1 to 10.38mg/L on day 15 and the dropped to 9.24mg/L on day 30 and similar trend was observed in the control sample. The mean and standard deviation of the dissolved oxygen with treatment days is shown on appendix 1 and 2 for the effluent and control sample respectively.

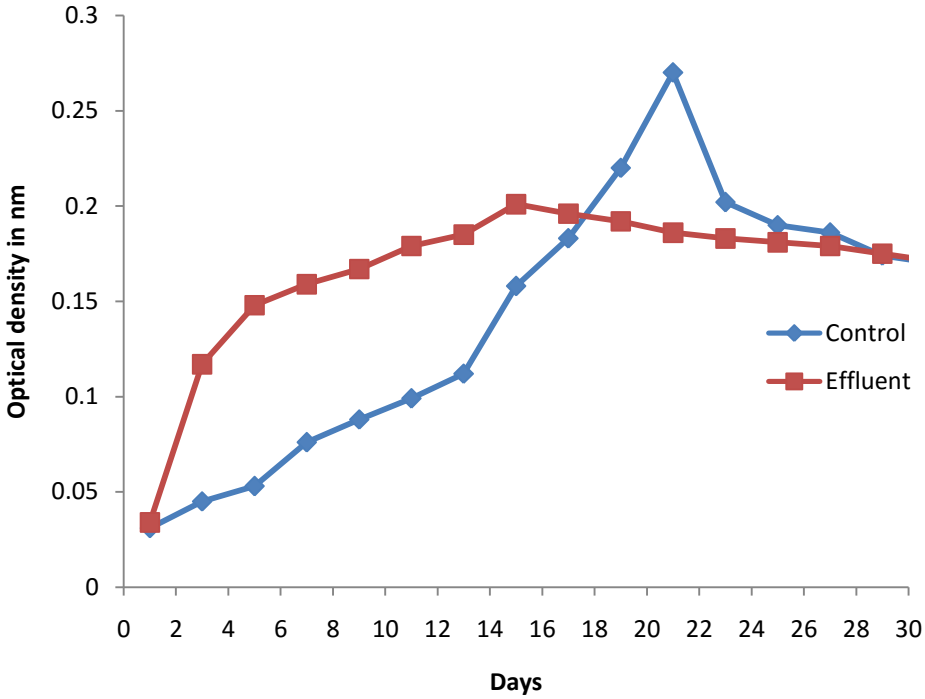

Figure 4.1: Variation in the optical densities of algae in control and effluent samples with days.

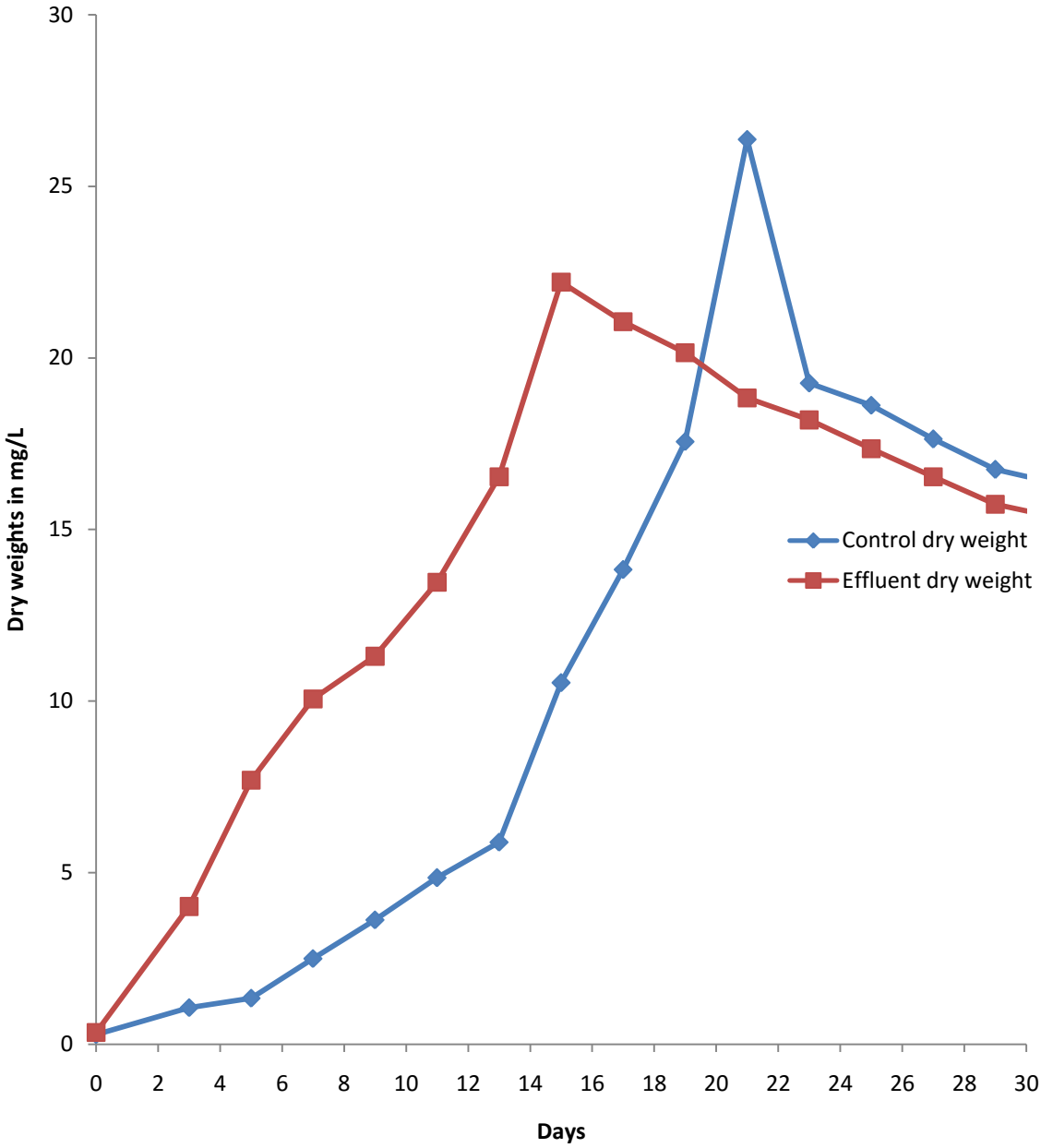

Figure 4.2: Variation in the dry weights of algae with days for control and effluent sample

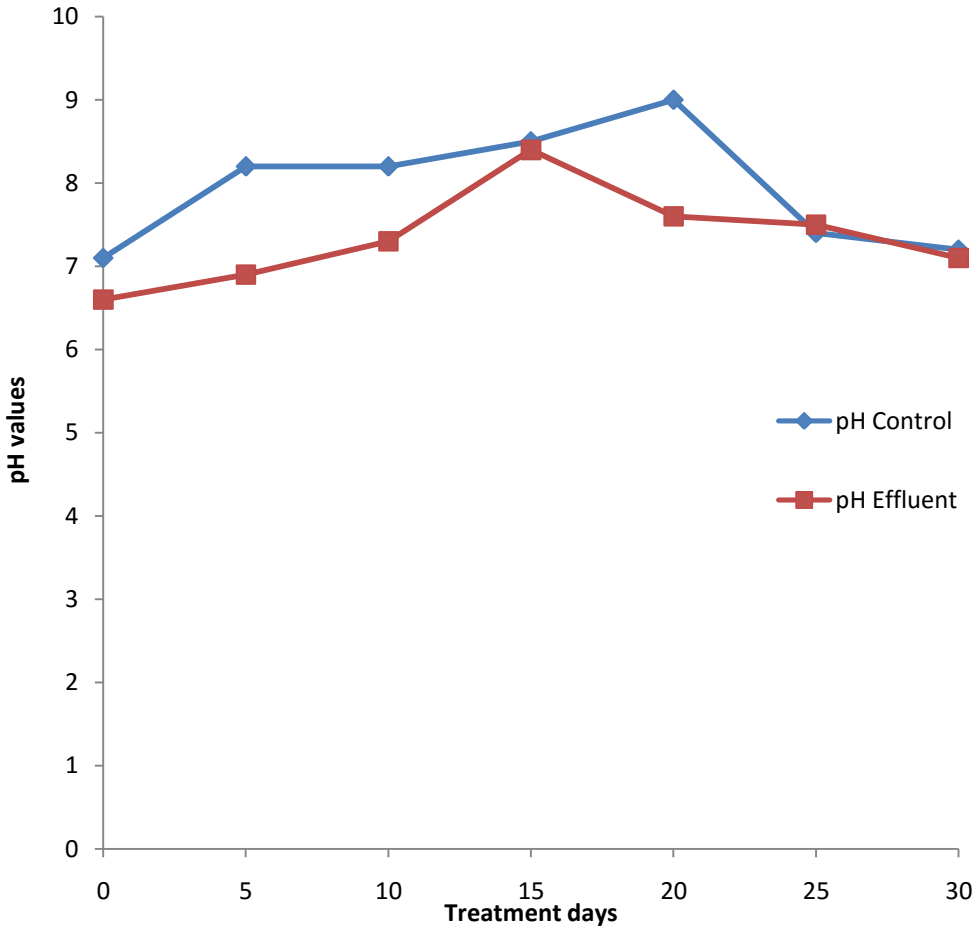

Figure 4.3: Variation of the pH of the control and effluent samples with treatment time (days).

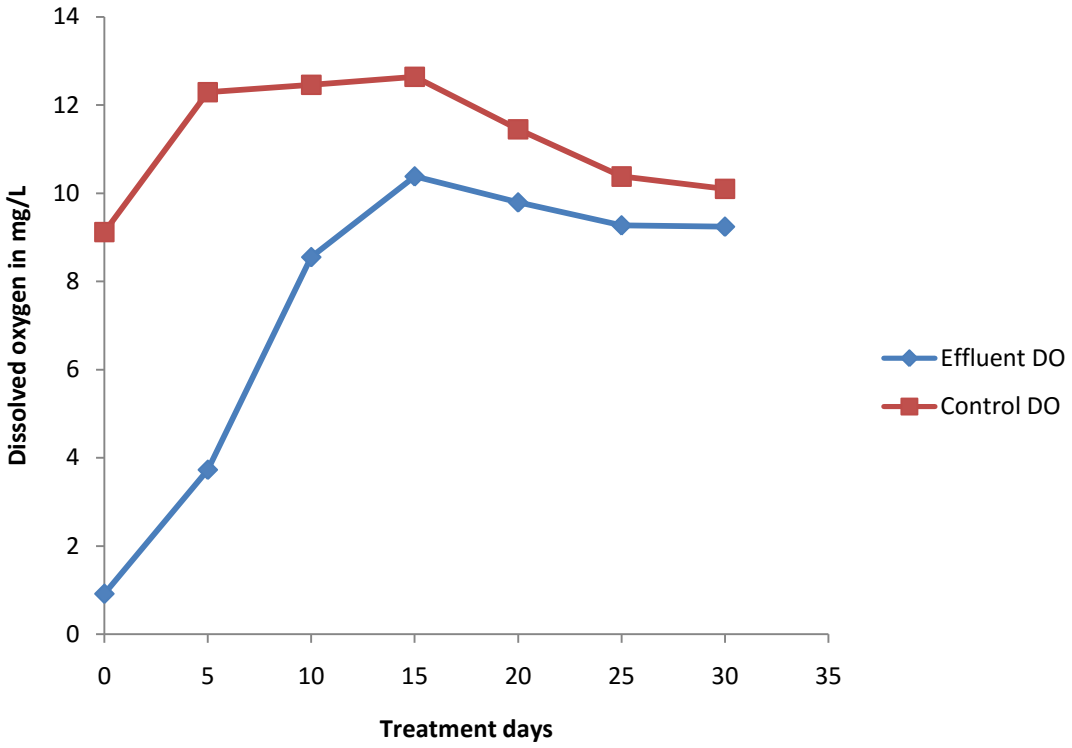

Figure 4.4: Variation of the dissolved oxygen content of control and effluent samples with treatment time (days).

Figure 4.5 shows the variation of BOD in control and effluent sample with treatment days. There was high percentage removal efficiency in BOD as shown in appendix 8 and 9 for control and effluent samples respectively.

Figure 4.6 shows the variation of COD in control and effluent sample with treatment days. The percentage removal efficiency in the COD content is shown in appendix 8 and 9 for control and effluent samples respectively.

Figure 4.7, 4.8, and 4.9 shows the variation of phosphate, nitrate, and sulphate with treatment days in control and effluent samples respectively. High percentage removal efficiency was attained at the end of the remediation period as shown in appendix 8 and 9 for control and effluent samples respectively.

Figure 4.10 shows the variation of iron content with treatment days in control and effluent samples respectively. The metal concentration decreases gradually throughout the treatment period with high removal efficiency attained at the end of the remediation period as shown in appendix 8 and 9 for both samples. Similar effect was observed in the metal concentration of copper, zinc, cadmium and lead as shown in figure 4.11, 4.12, 4.13 and 4.14 respectively except for zinc concentration that increased in the control sample on day 20 when the pH of the sample was 9.0.Lead and cadmium were not detected in the control. The dilution factor was included in all the parameters analysed aside the pH and dissolved oxygen.

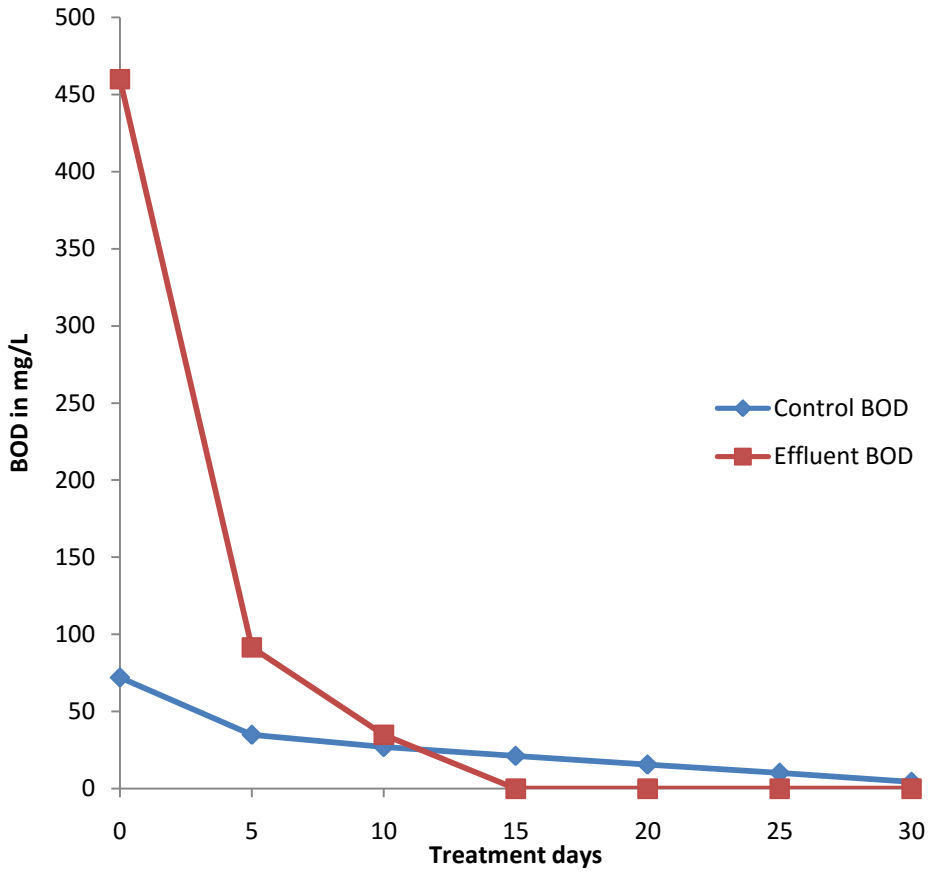

Figure 4.5: Variation of the BOD content of control and effluent sample with treatment time (days).

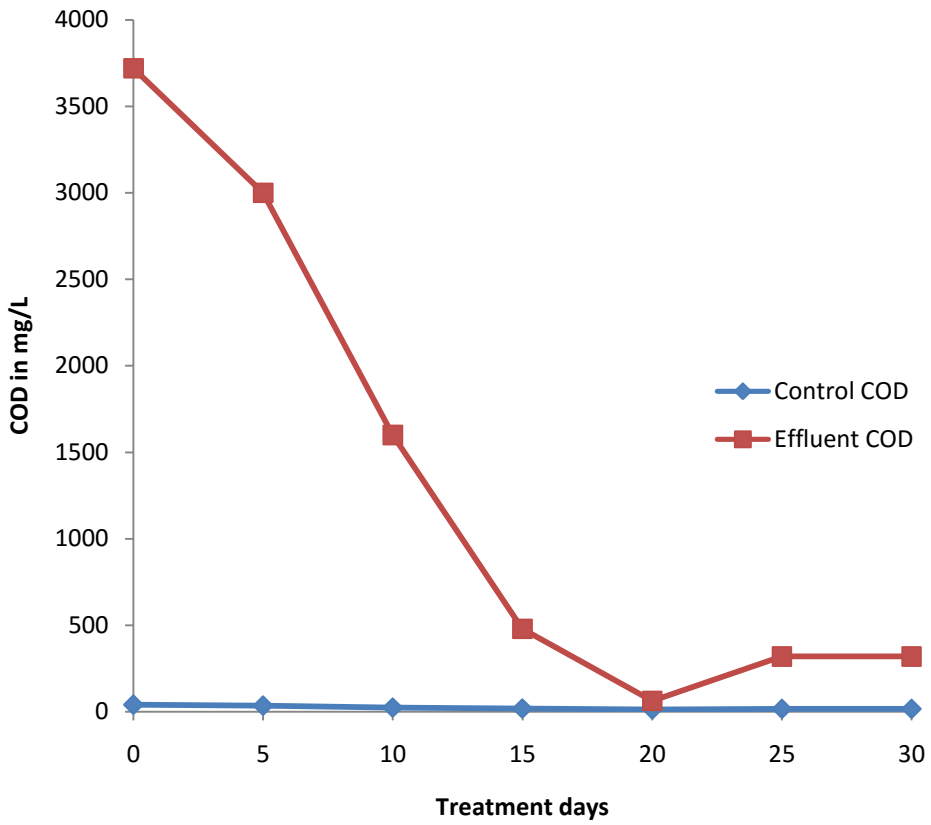

Figure 4.6: Variation of the COD content of control and effluent sample with treatment time (days).

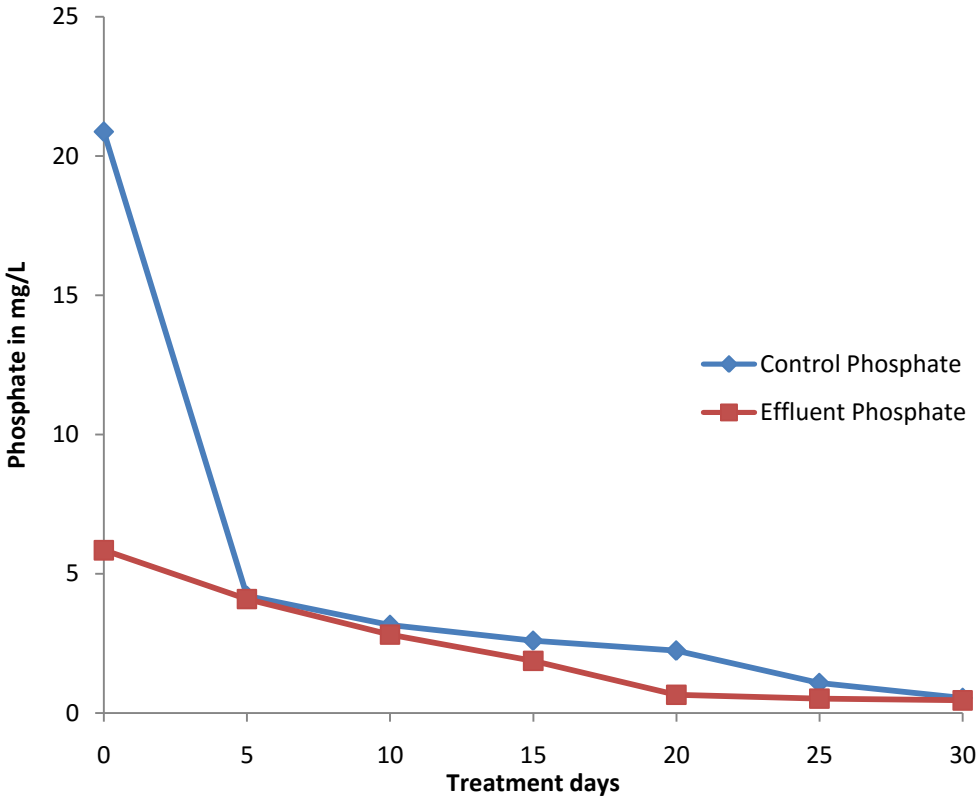

Figure 4.7: Variation of the phosphate content of control and effluent sample with treatment time (days).

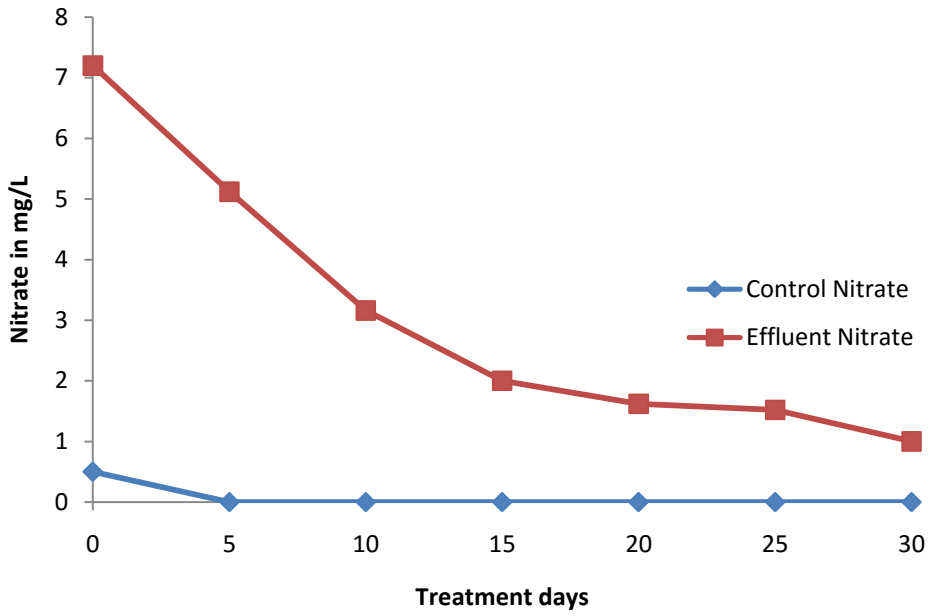

Figure 4.8: Variation of the nitrate content of control and effluent sample with treatment time (days).

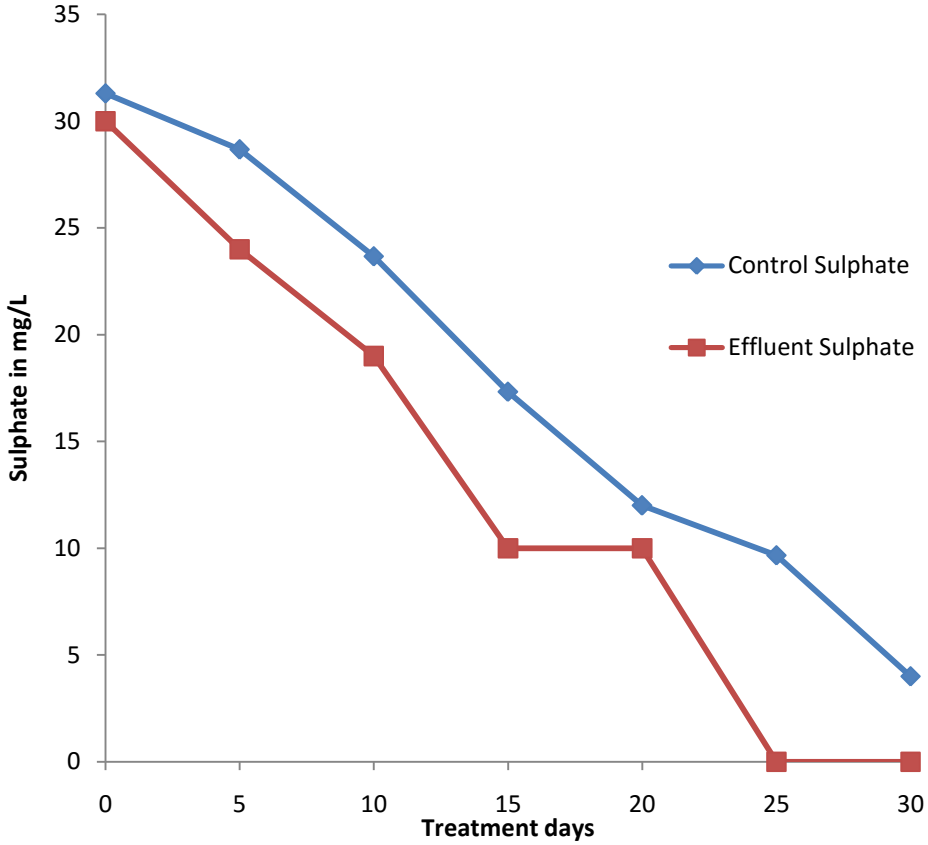

Figure 4.9: Variation of the sulphate content of control and effluent samplewith treatment time (days).

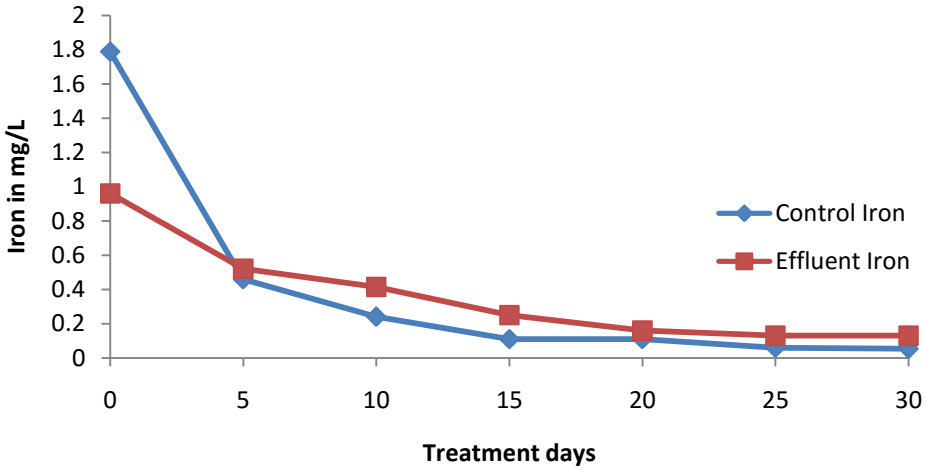

Figure 4.10: Variation of the Iron content of control and effluent sample with treatment time (days).

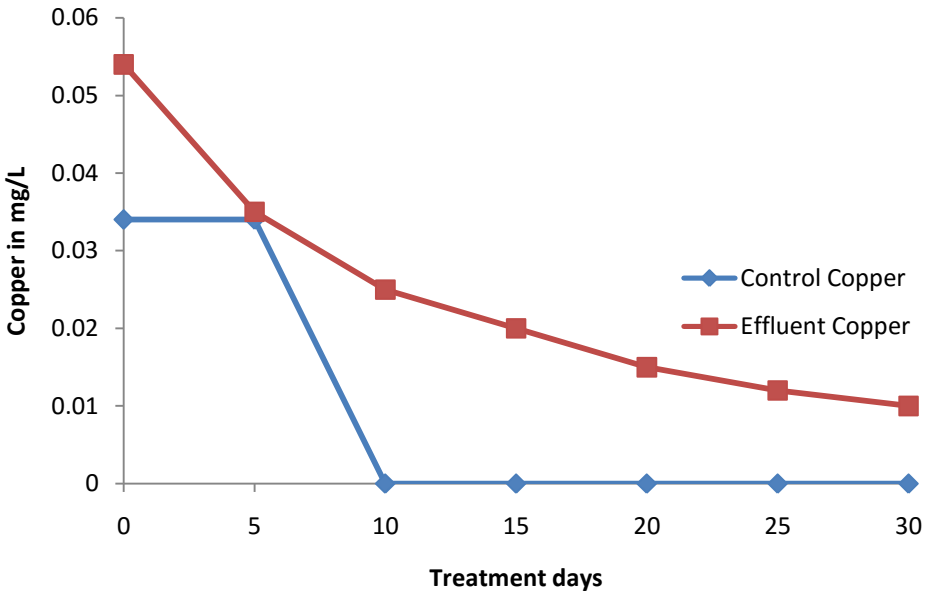

Figure 4.11: Variation of the Copper content of control and effluent sample with treatment time (days).

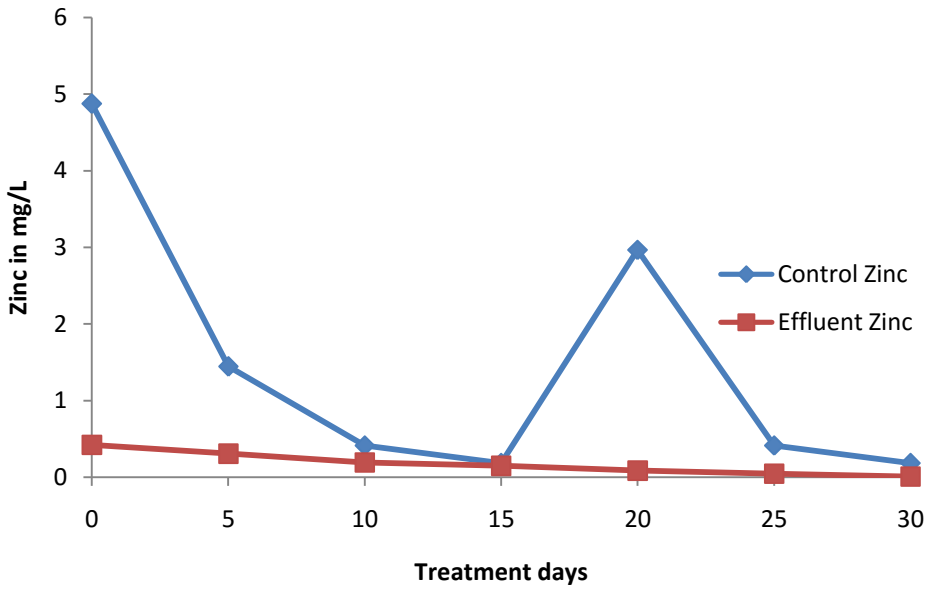

Figure 4.12: Variation of the Zinc content of control and effluent sample with treatment time (days).

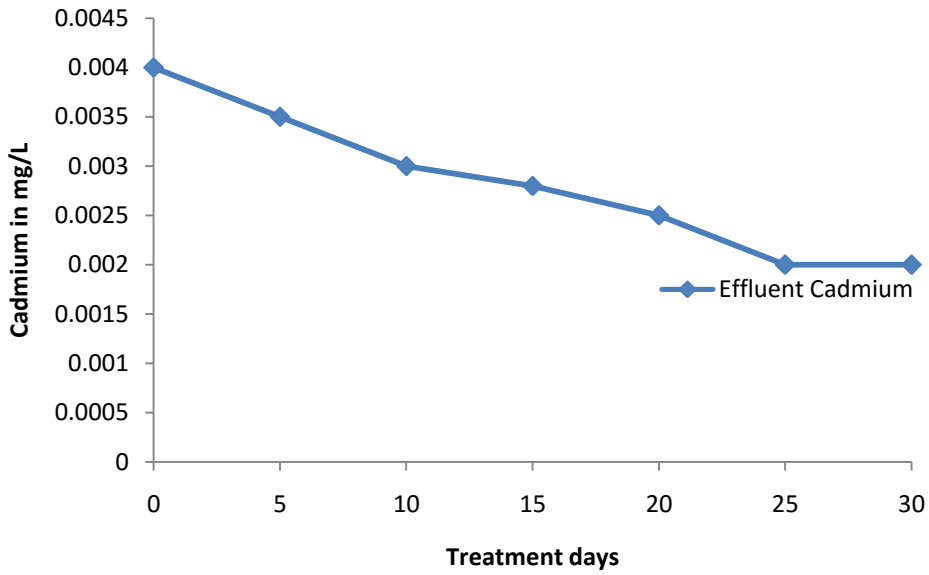

Figure 4.13: Variation of the Cadmium content of effluent sample with treatment time (days).

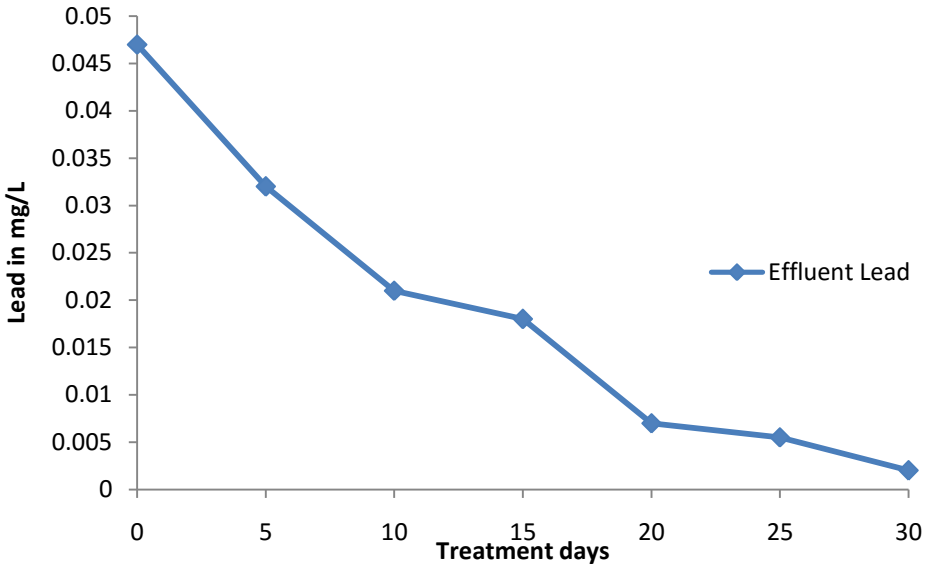

Figure 4.14: Variation of theLead content of effluent sample with treatment time (days).

4.3Fourier Transformed Infrared Spectroscopy

Appendix 7 shows the fuctional groups present in the extracted oil. The major bands are the C-H absorption band at 2868cm^{-1}, C=O stretch of carboxylic acids at 2936cm^{-1} and OH stretch in the region of 3450cm^{-1}.

4.4 GC-MS of the Extracted oil

Table 4.1 shows the fatty acid profile of the extracted oil whilethe GC-MS spectra for effluent and control samples are shown in appendix 5 and 6 respectively. The result suggest that microalgae lipids contain both saturated and unsaturated fatty acid. The major fatty acids were Palmitic and Stearic for saturated and Linoleic and Oleic for unsaturated fatty acids. Oil extracted from effluent sample had 30.64% saturated and 49.33% unsaturated fatty acids while the control sample recorded 17.5% saturated and 50.9% unsaturated fatty acid respectively revealing a higher level of unsaturated to saturated fatty acid.

4.5 Proximate Composition of Biomass

Table 4.2 shows the biochemical compostion of the extracted biomass in the control and effluent samples. Result showed the followingcontent ofprotein 11.67%, carbonhydrate 24.36% and lipid 8.28% for the algae cultured in the effluent while the control sample recorded protein 9.68%, carbonhydrate 23.14% and lipid 10.36%.

4.6Percentage Removal efficiency in Control and Effluent samples

Appendix 8 and 9shows the percentage removal efficiency of the phycoremediation process. There was gradual increase in the removal efficiency of the physicochemical properties analyzed except pH and dissolved oxygen where increment was observed.

Table 4.1Fatty Acid Profile of the Extracted Oil

Fatty Acids	Percentage composition effluent	Percentage composition control
Palmitic acid	22.95	15.63
Linoleic acid	21.98	20.51
Oleic acid	27.35	29.58
Stearic acid	7.69	1.87

Table 4.2Proximate compositionof harvested algal biomass

Parameters	Effluent	Control
Moisture (%)	8.55±0.05	6.45±1.70
Ash (%)	45.65±0.15	47.85±0.25
Fibre (%)	2.60±0.02	2.52±0.30
Lipid (%)	8.28±0.05	10.36±1.90
Protein (%)	11.67±0.14	9.68±2.50
Carbonhydrate (%)	24.36±1.50	23.14±2.00

4.7 Treatment Quality

Table 4.5 shows comparision between remediated sample and regulatory standards WHO standard for drinking water quality and USEPA standard for effluent discharge. Results indicated satisfactory treatment quality after remediation.

Table 4.3: Treatment Quality

Parameters	Effluent Before	Effluent After	Control Before	Control After	WHO	USEPA
pH	6.6	7.1	7.1	7.2	6.5-8.5	6.0-9.0
DO_2	0.92	9.24	9.12	10.10	>5.0	-
BOD	460	0	72.15	4.53	-	40
COD	3720	64	40	16.63	-	120
Nitrate	7.20	1.00	0.5	0	50	10
Sulphate	30	0	31.3	4	400	1500
Phosphate	5.85	0.46	20.87	0.54	6.5	-
Iron	0.961	0.13	1.79	0.053	0.3	2.0
Copper	0.054	0.01	0.034	0	2	0.5
Cadmium	0.004	0.002	ND	ND	0.003	0.01
Lead	0.047	0.002	ND	ND	0.01	0.05
Zinc	0.424	0.009	4.873	0.184	3	2

ND means not detected. All units are in mg/L except pH

CHAPTER FIVE

DISCUSSION

5.1 Physicochemical properties

5.1.1 pH

Figure 4.3 shows the trend in the pH of the effluent and control samples. The result depicted in this figure agrees with the work of Dominic *et al.*, (2009);Khemkaand Meenu, (2015); Kotteswari*et al*, (2007); Manoharanand Subrahmanian, (1992); Nandini*et al*,(2013)and Gopinath*et al*, (2014). Both the effluent and control samples increased in pH with algal growth, attaining their peak on the day optimal growth was recorded for the algae and then decreased as algal growth decreased. The increase in pH may be attributed to increase in photosynthesis, enhanced algal growth and decrease in carbonates and bicarbonate level of effluent medium by Khemkaand Meenu, (2015) while the decrease in pH indicates that organics were excreted out instead of being taken up (Eny 1951; Wang *et al*., 2010). The pH was within the permissible limit set by WHO for drinking water and USEPA standard for effluent discharge as shown in Table 4.3. Analysis of variance showed that there was a significant difference in pH after remediation with $p<0.05$ meaning, the difference in the mean score did not occur by chance but could be attributed to a process. Appendix 3 and 4 shows the anova table for the effluent and control samples respectively.

5.1.2 Dissolved Oxygen

Figure 4.4 shows how dissolved oxygen is affected by the phycoremediation process. Oxygen drops during respiration and decomposition and rises with photosynthetic activity (Aarti*et al*., 2008). This explains the increase in dissolved oxygen as growth increases and decrease in the dissolved oxygen content as the growth drops. These results agree with Dominic *et al*., (2009) who reported an increase in dissolved oxygen

from 2.3mg/L to 8mg/L using *Chlorella vulgaris* after 25 days of treatment. In a similar study,Sengar(2011) reported an increase in dissolved oxygen from 0.7mg/L to 7.8mg/L when mixed algal culture was used to treat sewage water. Algae releases oxygen during their exponential phase of growth and this high level of dissolved oxygen helps in self-purification of water. Kshirsaga, (2010) and Nandini*et al*., (2013) also reported similar results in dissolved oxygen pattern with algal growth. Dissolved oxygen was within the recommended limit of WHO and USEPA standards. There was significant difference between each stage of treatment at $p<0.05$.

5.1.3 Biological oxygen demand (BOD)

Figure 4.5 shows the effect of phycoremediation on the BOD content of the effluent and control samples. 100% removal was achieved form the effluent and 93.72% reduction in the control as shown in appendix 8 and 9 respectively. This result agreed with Kshirsaga, (2010) who reported 73% reduction in BOD from herbal pharmaceutical wastewater using *Spirulinaplatensis.* In another study Ahmad *et al*., (2013) reported 100% BOD removal from his biological treatment plant design using *Chlorella vulgaris* and Murugesan*et al*., (2010) reported 77.89% BOD removal from poultry wastewater on day 15 using *Chlorella vulgaris*as treatment agent. Microalgae utilize the organic materials as nutrients for their growth.The BOD was within the recommended limit of WHO and USEPA standards. There was significant difference in the BOD values between each treatment stage at $p<0.05$.

5.1.4 Chemical oxygen demand (COD)

Figure 4.6 shows the effect of phycoremediation on the COD content of the effluent and control samples respectively. 98.28% reduction in COD was achieved in the effluent while 58.43% was achieved in the control sample as shown in appendix 8 and 9 respectively.An increase in COD indicates that organics were excreted out instead of

being taken up by algae grown in effluent. Wang *et al.,* (2010) found that the metabolic pathway of *Chlorella* can alter with the supply of organic substrate such as organic acid or glucose, which means they perform heterotrophic growth besides the common autotrophic mode of using carbondioxide as the sole carbon source. The organic substances may function directly as essential nutrient (Sachdev and Clesceri, 1978) or act as accessory growth factor (Saunders, 1957; Wang *et al.,* 2010). When organic substrate is not available autotrophic growth uses carbon (iv) oxide as the carbon source, excreting small molecular organic substances such as glycolic acid to the environment as a product of photosynthetic carbon cycle (Merettand Lord, 1975). This explains why COD in the effluent increased during remediation at the retarded growth phase. This result agreed with Mamun*et al*., (2012) who reported 77.8% COD reduction on 14th day of algal growth. Ahmad *et al*., (2013) also reported 99.9% COD reduction whileWang *et al*., (2010) reported 50.9-83.0% reduction from different wastewater. Kshirsagar, (2010) reported 80.04% reduction on 15th day and Sreekanth*et al*., (2014) reported 90% reduction of COD using *Chlorella vulgaris* from indoor cultures of diary wastewater. Treatment quality was found to be satisfactory as seen in Table 4.3. Analysis of variance also showed a p value of 0.00 revealing that a significant difference existed between each stage of treatment.

5.1.5 Phosphate

There was gradual reduction in phosphate concentration throughout the remediation period with high removal efficiencies as shown in appendix 8 and 9 for effluent and control samples respectively. Algae utilize phosphate for their growth and forms part of its essential nutrient. This result agrees with (Shabudeen*et al*., 2013) who reported 100% removal of phosphate. Dominic *et al*., (2009) 69.25% on the 25th day; Kotteswari*et al*., (2007) 88.82% on the 15th day and Sreekaiand Pakpain,(2007) 68%

under natural light and his result showed that removal rate continued even after the peak of *Chlorella vulgaris* growth on day 5 of treatment since phosphate is essential for algal metabolism. Treatment quality was satisfactory as seen in table 4.3 and there was significant difference at $p<0.05$ between each stage of treatment as shown in appendix 3 and 4 of the anova table for the effluent and control samples respectively.

5.1.6 Nitrate

There was reduction in the nitrate content which is an essential algal growth nutrient in the effluent and control samples as shown in Figure 4.8. High removal efficiencies were achieved as shown in appendix 8 and 9. This result agrees with Shabudeen*et al*., (2013) who reported 100% nitrate removal from hostel wastewater after 7 days culture period. Dominic et al., (2009) reported 84% nitrate reduction on the 25th day from industrial wastewater, Ahmad *et al*., (2013) also reported 99.98% nitrate reduction and Jalal *et al*., (2011) reported 78.43% nitrate reduction after 7days using *Chlorella*to treat municipal sludge. Treatment quality was satisfactory as shown in Table 4.3 and there was significant difference at $p<0.05$.

5.1.7 Sulphate

There was high removal efficiency of sulphate in both the effluent and control samples respectively as shown in appendix 8 and 9.Algae utilize sulphate by reduction (Robert and Jerome, 1971). This result agrees with Sreekanth*et al*., (2014) that reported 93.13% reductionin sulphate content from diary wastewater using *Chlorella* after 13thday culture period. Treatment quality was satisfactory as shown in Table 4.3 and there was significant difference at $p<0.05$.

5.1.8 Heavy metals (Zn, Cu, Fe, Ni, Cd, and Ni)

The concentration of these metals reduced gradually throughout the remediation period in both the effluent and control samples as shown in Figure 4.10-4.14. The absorption of heavy metal depend on parameters like pH (Dwivedi, 2012), Size, Shape and Cell composition (Tam *et al.*, 1997), Surface area and binding affinity (Roy *et al*, 1993) of the microalgae. Among several factors pH plays a major role as values above 9.0 can lead to precipitation of various salts which can lead to nutrient deficiency for the microalgae and also increase the metal concentration in the solution (Beckar, 1994). The high removal efficiencies of the metal concentration shown on Table 4.3 and 4.4 for control and effluent sample agree with several works in heavy metals bioremediation by microalgae species. Sengar, (2011) reported reduction in heavy metals (Fe, Zn and Cu) from sewage wastewater using mixed algal cultures with 100% removal efficiency from day 20^{th} -$25^{th}$of culture within a pH range of 7.1-8.1 whileShabudeen*et al*(2013) reported 93.57% removal efficiency of Iron after 10day culture period. *Chlorella pyrenoidosa* reduced copper in plastic wastewater to about 44.83% within 8 day incubation period (Kenanga*et al*., 2014). It reduced Cu, Pb and Zn from wastewater with removal efficiencies of 77.1%, 43.8%, and 68.9% respectively as quoted by Ajayan*et al*,(2011). Treatment quality was satisfactory as shown on Table 4.3 and there was significant difference at $p<0.05$.

5.2 Biomass production

The pattern of dry weight (biomass) of the algae is shown in Figure 4.2 for the control and effluent samples respectively. The reason for decrease in growth could be due to decreased nutrient and other growth limiting condition. The other limiting factor that might have affected growth is the availability of a carbon source (CO_2). In this study, carbon source was not supplemented in the medium which could help enhance or

optimize *Chlorella vulgaris* growth. This result is similar to previous study by Sengar, (2011) who observed that algal biomass was less at the initial phase but increased with time due to multiplication and uptake of necessary nutrient from wastewater. The maximum biomass in his study was achieved on 25^{th} day of culture after which there was decline in growth. This growth pattern is also similar to the findings of Mamun*et al*., (2012) who observed peak growth on day 5 for sample and day 4 for control after which there was growth declination. Biomass was produced from *Chlorella vulgaris* as shown in this study and similar studies reported by Velichkova*et al.,* (2014), and Shabudeen*et al.,* (2013). Yield can be optimized for better productivity by improving culture conditions i.e aeration and lighting regime Sharma and Khan, (2013);Agwa*et al.,* (2014). Biomass can be harvested after the day of optimum growth and can be used in agriculture as animal feed, soil conditioner and as raw materials to produce fertilizers (Mamun*et al.,* (2012).The large amount of algal sludge represent a potential source of fuel and recovered nitrogen and phosphorus fertilizer (Mulbry*et al.,* 2005).It can also be used for biodiesel production (Ahmad *et al.,* 2013;Aguoru and Okibe, 2015). Chisti, (2007) estimated that microalgae can produce 5000-20,000 gallons of oil per acre per year.

5.3 Proximate Analysis

The proximate analyses of the effluent and control sample are represented in Table 4.2. This gives the different biochemical composition of the *Chlorella vulgaris* cultured in the effluent and control media. The composition of protein obtained in this work was low compared to the work of Ahmad *et al*., (2013) who reported 38.56% protein content as against 11.67% and 9.68% for effluent and control respectively. The carbonhydrate content of 23.25% and 23.14% for the effluent and control respectively were also low compared to the 38.56% content obtained in their work.The lipid content obtained from

algae cultured in the effluent was 8.28% while that of the control was 10.36%. The low lipid content could be as a result of low aeration of sample medium and the absence of a carbon source. It was reported that lipid content could be enhanced by light intensity and aeration as highest lipid content of 18.32% was obtained with natural sunlight, while aerated system gave 11.2% against 6.17% lipid content in the unaerated system (Agwa and Abu, 2014).

5.4 GC-MS of Extracted Oil

The fatty acid profile was analyzed by GC-MS and it was found that the oil contained both saturated and unsaturated fatty acid with similar composition. Resultof the effluent showed 49.33% unsaturated fatty acid (Linoleic acid and Oleic acid) and 30.64% saturated fatty acid (Palmitic acid and Stearic acid) while the control had 50.9% unsaturated fatty acid (Linoleic acid and Oleic acid) and 17.5% saturated fatty acids (Palmitic acid and Stearic acid). This result agrees with Chinnasamy*et al*., (2010) that observed similar fatty acid profile in crude algal oil using GC. Gouveia and Oliveira, (2009) reported that microalgal lipids of *Chlorella vulgaris, Scenedesmusobliquus, Scenedesmus maxima, Nannochloropsisoleabundans* were mainly composed of unsaturated fatty acid (50-65%). Ahmad*et al*., (2013) reported similar fatty acid profile with 77.85% unsaturated fatty acid and 21.5% saturated fatty acid while Chattip*et al*., (2012) reported fatty acid profile with 65.3% unsaturated fatty acid and 34.7% saturated with same algae specie. Microalgae oil contained large number of unsaturated fatty acids which could be used as a source of Omega-3 and Omega-6 fatty acid. These fatty acids are of importance to health as it has the potential to reduce risk of diseases i.e. heart disease, cancer and autoimmune disorder (Willett, 2007)

5.5 FT-IR of Extracted Oil

Appendix 7 shows the IR bands of the extracted oil. The major bands are the C-H absorption band at 2868cm^{-1}characteristic of the symmetric and asymmetric stretching vibration of aliphatic CH_2, strong C-Hof symmetric and asymmetric stretching vibration of aliphatic CH_3at 2936cm^{-1} and weak bending vibration of CH_2 and CH_3 at 1457.27cm^{-1}, O-H stretch of carboxylic acids in the region of 3450.77cm^{-1}, carbonyl stretch of esters at 1734cm^{-1} andcarbonhydrate radical from triglyceride at 1029.06cm^{-1}characteristic of microalgae lipids.This result agreed with (Praba*et al*., 2014) on the assigned bands for microalgae lipids. It is also comparable with the FT-IR of edible vegetable oil (corn oil) with ester carbonyl functional group of triglyceride at 1746cm^{-1}, symmetric and asymmetric stretch of CH_2 and CH_3 at 2854cm^{-1} and 2962cm^{-1} respectively as reported by Tegou*et al*., (2006).Nazima and Adeel, (2013) reported C-H stretch at 2965cm^{-1}, carbonhydrate radical from triglyceride at 1160cm^{-1} for edible oils.

CHAPTER SIX

SUMMARY, CONCLUSION and RECOMMENDATION

6.1 Summary and Conclusion

The remediation of pharmaceutical effluent was carried out at 20% effluent dilution and the control experiment was performed using the 20% dilution of Bold Basal medium. Herbal pharmaceutical effluent collected from NIPRD was inoculated with *Chlorella vulgaris* after been allowed to attain lag phase from Bold Basal medium. The growth of the algae was monitored by dry weight determination every 24hours. The physicochemical analysis was carried out every 5days for 30days and results showed satisfactory treatment quality when compared with WHO standard for drinking water and USEPA standard for effluent discharge. Biomass was produced from the algae and result of the proximate composition suggests that *Chlorella vulgaris* can be harnessed in the agricultural sector as feeds and the lipids can also be used as biofuel source for transportation.

Based on the result of this study:

It can be concluded that *Chlorella vulgaris* can be used to remediate herbal pharmaceutical wastewater. However, their performance varied depending on the culture environment and the concentration of the effluent. The remediation was done at 20% as 40%-100% did not favour algal growth with time. The Maximum biomass was obtained on day 15 for effluent (22.21mg/l) and day 21 for control (26.37mg/l). The biomass harvested can be used as feeds and raw material for biofuel as seen from the proximate analysis and fatty acid profile respectively.

6.2 Recommendation

- *Chlorella vulgaris* should be cultured in photo bioreactors using wastewater as growth medium. This will likely optimize yield and hence create opportunity for its use as feed for biofuel production.

- Studies should be carried out on the remediation of this wastewater using *Chlorella vulgaris* in the presence of growth promoting bacteria. This will likely enhance remediation at higher concentration and better biomass and biochemical composition yields.

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APPENDICE

Appendix 1

Mean and standard deviation of the physicochemical parameters for the effluent sample with treatment days.

Parameters	0	5	10	15	20	25	30
pH	6.6±0.1	6.9±0.1	7.3±0.1	8.4±0.1	7.6±0.1	7.5±0.1	7.1±0.1
DO_2	0.92±0.02	3.73±0.03	8.55±0.05	10.38±0.02	9.79±0.02	9.27±0.02	9.24±0.02
BOD	460±5	91.6±0.2	35.01±0.92	0±0	0±0	0±0	0±0
COD	3720±120	3000±60	1600±20	480±6	64±0.2	320±4	320±6
Sulphate	30±2	24±2	19±1	10±1	10±1	0±0	0±0
Phosphate	5.85±0.01	4.1±0.01	2.82±0.02	1.88±0.02	0.66±0.01	0.52±0.02	0.46±0.02
Nitrate	7.2±0.02	5.12±0.02	3.16±0.06	2±0.82	1.62±0.02	1.52±0.02	1±0.02
Iron	0.961±0.001	0.522±0.002	0.415±0.00	0.25±0.01	0.16±0.02	0.13±0.03	0.13±0.01
Zinc	0.424±0.002	0.309±0.003	0.194±0.00	0.15±0.00	0.088±0.00	0.048±0.02	0.009±0.00
Copper	0.054±0.001	0.035±0.0002	0.025±0.00	0.02±0.00	0.015±0.00	0.012±0.00	0.01±0.00
Cadmium	0.004±0.003	0.0035±.0002	0.003±0.00	0.0028±0.00	0.0025±0.00	0.002±0.00	0.002±0.00
Nickel	ND	ND	ND	ND	ND	ND	ND
Lead	0.047±0.0004	0.032±.0002	0.021±0.00	0.018±0.00	0.007±0.00	0.0055±0.00	0.002±0.00

All units are in mg/L except pH

Appendix 2

Mean and standard deviation values for the physicochemical parameters of the control sample with treatment days

Parameters	0	5	10	15	20	25	30
pH	7.1±0.0	8.2±0.0	8.2±0.0	8.5±0.0	9.0±0.0	7.4±0.0	7.2±0.0
DO_2	9.12±0.14	12.29±0.31	12.46±0.13	12.64±0.39	11.45±1.43	10.38±0.56	10.10±0.12
BOD	72.15±3.14	35.00±0.60	27±2	21.33±1.53	15.68±0.08	10.2±0.52	4.53±0.06
COD	40±5	34.67±3.06	24.33±1.16	18.67±1.53	14.07±1.21	16.97±0.40	16.63±1.15
Sulphate	31.3±1.53	28.67±1.53	23.67±0.58	17.33±1.16	12±1	9.67±0.58	4±1.73
Phosphate	20.87±0.06	4.21±0.01	3.16±0.01	2.60±0.01	2.24±0.04	1.09±0.01	0.54±0.006
Nitrate	0.5±0.1	0±0.0	0±0.0	0±0.0	0±0.0	0±0.0	0±0.0
Iron	1.79±0.02	0.46±0.01	0.24±0.01	0.11±0.01	0.11±0.01	0.06±0.00	0.053±0.01
Zinc	4.873±0.00	1.445±0.00	0.416±0.00	0.184±0.00	2.968±0.00	0.416±0.00	0.184±0.00
Copper	0.034±0.00	0.034±0.00	0±.0.0	0±0.0	0±0.0	0±0.0	0±0.0
Cadmium	ND	ND	ND	ND	ND	ND	ND
Nickel	ND	ND	ND	ND	ND	ND	ND
Lead	ND	ND	ND	ND	ND	ND	ND

All units are in mg/L except pH

Appendix 3

ANOVA results for effluent sample

		Sum of Squares	df	Mean Square	F	Sig.
pH	Between Groups	6.051	6	1.009	100.857	.000
	Within Groups	.140	14	.010		
	Total	6.191	20			
DO2	Between Groups	234.768	6	39.128	50721.407	.000
	Within Groups	.011	14	.001		
	Total	234.778	20			
BOD	Between Groups	516172.512	6	86028.752	23263.230	.000
	Within Groups	51.773	14	3.698		
	Total	516224.285	20			
COD	Between Groups	32762324.846	6	5460387.474	2067.429	.000
	Within Groups	36976.080	14	2641.149		
	Total	32799300.926	20			
Phosphate	Between Groups	76.583	6	12.764	47024.789	.000
	Within Groups	.004	14	.000		
	Total	76.587	20			
Nitrate	Between Groups	93.600	6	15.600	161.061	.000
	Within Groups	1.356	14	.097		
	Total	94.956	20			
Sulphate	Between Groups	2404.286	6	400.714	200.357	.000
	Within Groups	28.000	14	2.000		
	Total	2432.286	20			
Iron	Between Groups	1.644	6	.274	1271.135	.000
	Within Groups	.003	14	.000		
	Total	1.647	20			
Zinc	Between Groups	.397	6	.066	15894.366	.000
	Within Groups	.000	14	.000		
	Total	.397	20			
Cadmium	Between Groups	.004	6	.001	11045.218	.000
	Within Groups	.000	14	.000		
	Total	.004	20			
Lead	Between Groups	.005	6	.001	38.138	.000
	Within Groups	.000	14	.000		
	Total	.005	20			
Copper	Between Groups	.053	6	.009	45068.650	.000
	Within Groups	.000	14	.000		
	Total	.053	20			

All units are in mg/L except pH

Appendix 4

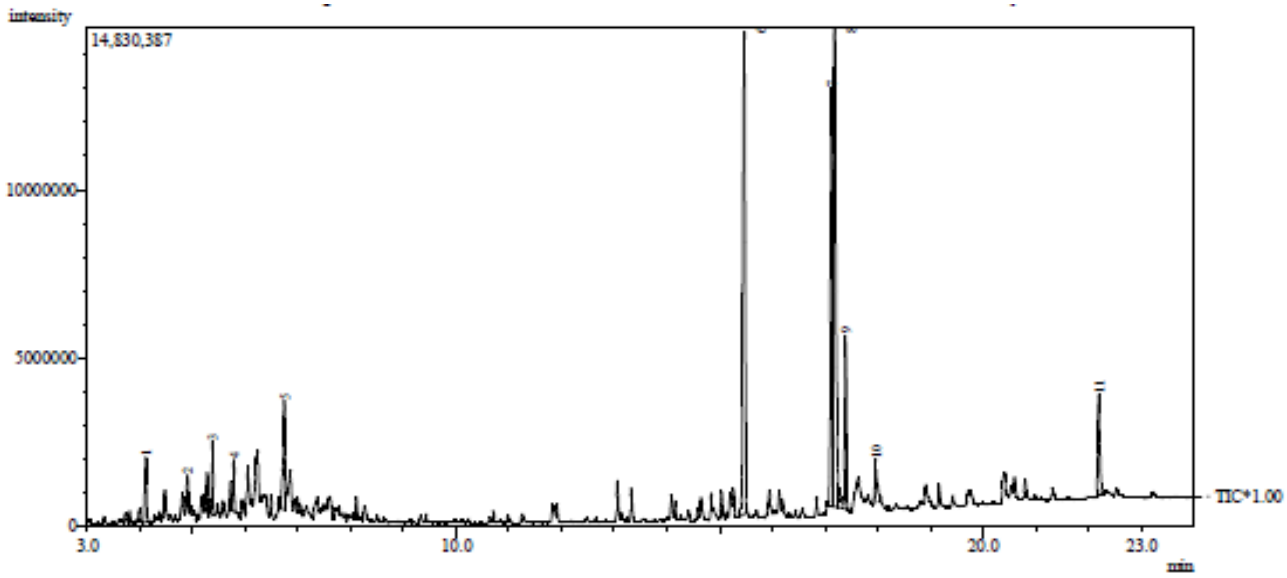
ANOVA results for control sample

		Sum of Squares	df	Mean Square	F	Sig.
pH	Between Groups	9.351	6	1.559	.	.
	Within Groups	.000	14	.000		
	Total	9.351	20			
DO2	Between Groups	34.688	6	5.781	12.575	.000
	Within Groups	6.437	14	.460		
	Total	41.125	20			
BOD	Between Groups	9145.125	6	1524.188	634.510	.000
	Within Groups	33.630	14	2.402		
	Total	9178.755	20			
COD	Between Groups	1799.172	6	299.862	52.621	.000
	Within Groups	79.780	14	5.699		
	Total	1878.952	20			
Phosphate	Between Groups	912.892	6	152.149	71319.656	.000
	Within Groups	.030	14	.002		
	Total	912.921	20			
Nitrate	Between Groups	.643	6	.107	75.000	.000
	Within Groups	.020	14	.001		
	Total	.663	20			
Sulphate	Between Groups	1876.476	6	312.746	205.240	.000
	Within Groups	21.333	14	1.524		
	Total	1897.810	20			
Iron	Between Groups	7.115	6	1.186	10827.768	.000
	Within Groups	.002	14	.000		
	Total	7.117	20			
Zinc	Between Groups	58.047	6	9.674	1991806.147	.000
	Within Groups	.000	14	.000		
	Total	58.047	20			
Cadmium	Between Groups	.000	6	.000	.	.
	Within Groups	.000	14	.000		
	Total	.000	20			
Lead	Between Groups	.000	6	.000	.	.
	Within Groups	.000	14	.000		
	Total	.000	20			
Copper	Between Groups	.005	6	.001	2890.000	.000
	Within Groups	.000	14	.000		
	Total	.005	20			

All units are in mg/L except pH

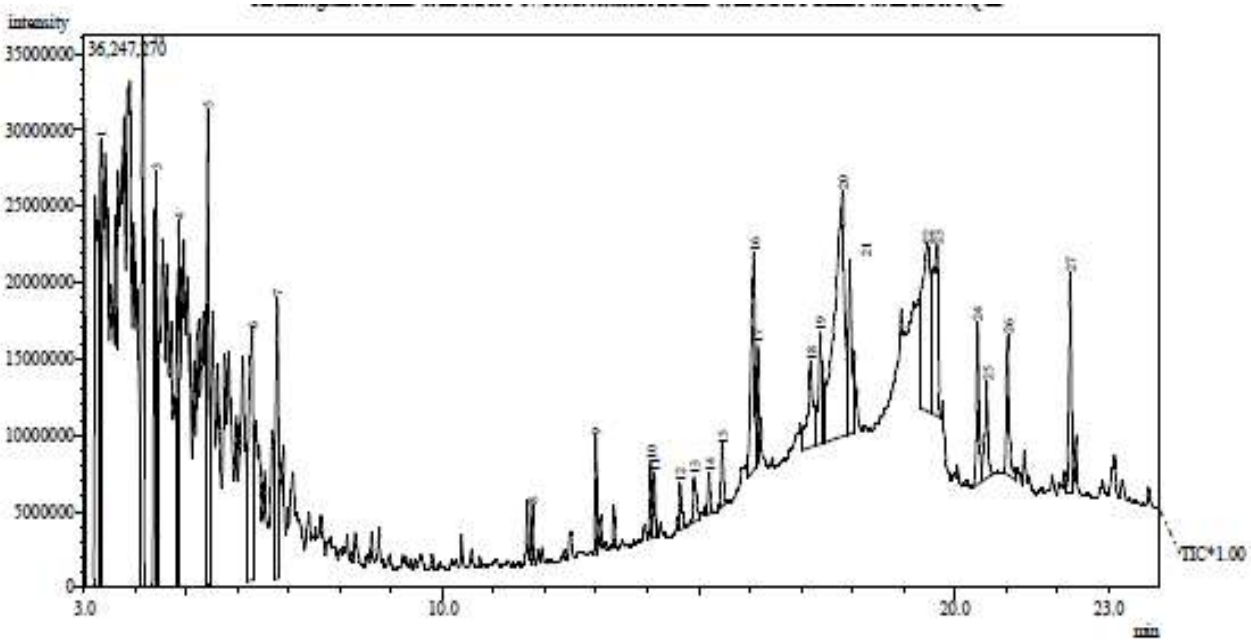
Appendix 5

GC-MS of extracted oil from effluent sample



Appendix 6

GC-MS of extracted oil from control sample



APPENDIX 7

FT-IR of Extracted Oil

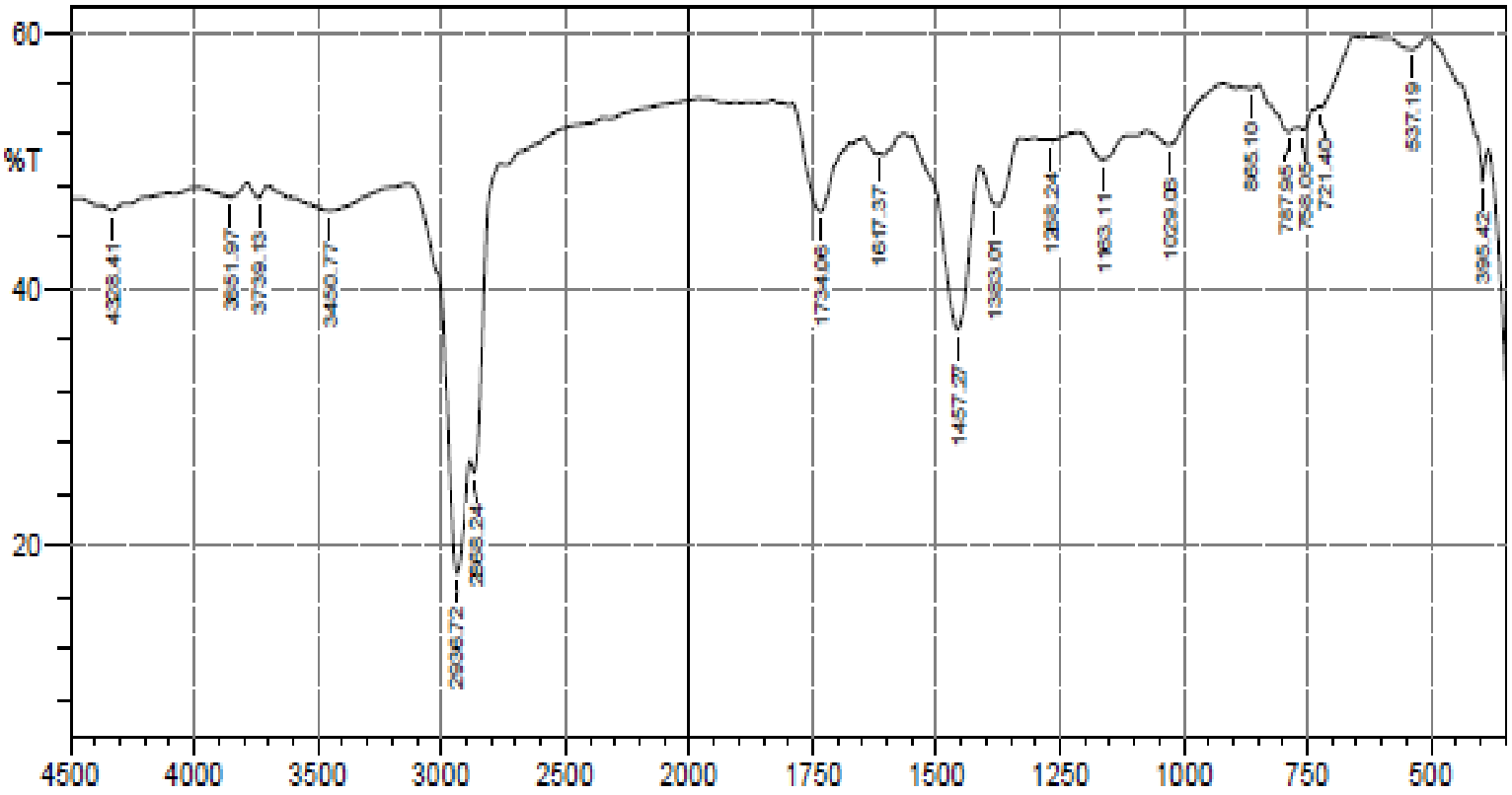


Figure 4.15: FT-1R Spectrum of algae oil

APPENDIX 8

Percentage removal efficiency in control sample with treatment days

Parameters	0	5	10	15	20	25	30
pH	0	15.49	15.49	19.72	26.76	4.23	1.408
DO_2	0	34.76	36.62	38.6	25.55	13.82	10.75
BOD	0	51.49	62.58	70.44	78.27	85.86	93.72
COD	0	13.33	39.18	53.33	64.83	57.58	58.43
Sulphate	0	8.4	24.38	44.63	61.66	69.11	87.22
Phosphate	0	57.71	84.85	87.54	89.27	94.78	97.41
Nitrate	0	100	100	100	100	100	100
Iron	0	74.3	86.59	93.85	93.85	96.65	97.04
Zinc	0	70.35	91.46	96.22	39.09	91.46	96.22
Copper	0	0	100	100	100	100	100

All units are in mg/L except pH

APPENDIX 9

Percentage removal efficiency in effluent sample with treatment days

Parameters	0	5	10	15	20	25	30
pH	0	4.54	10.61	27.27	15.15	13.64	7.58
DO_2	0	305.4	829.3	1028.7	964.1	907.6	904.4
BOD	0	80.9	92.39	100	100	100	100
COD	0	19.36	56.99	87.10	98.28	91.40	91.40
Sulphate	0	20	36.67	66.67	66.67	100	100
Phosphate	0	29.91	51.8	67.86	88.71	91.11	92.14
Nitrate	0	28.89	56.11	72.22	77.5	78.89	86.11
Iron	0	45.68	56.62	73.99	83.35	86.47	86.47
Zinc	0	27.12	54.25	64.62	79.25	88.68	97.88
Copper	0	35.19	53.7	62.96	72.22	77.78	81.48
Cadmium	0	12.5	25	30	37.5	50	50
Nickel	ND	ND	ND	ND	ND	ND	ND
Lead	0	31.91	53.32	61.7	85.7	88.3	95.74

ND means not detected. All units are in mg/L except pH