Using *Chlorella vulgaris* to Decrease the Environmental Effect of Garbage Dump Leachates

Sarunporn Thongpinyochai

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of

Master of Science in Technology and Environmental Management

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I hereby certify that this work has not been accepted in substance for any other degree, and is not being currently submitted in candidature for any degree.

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Abstract

Waste treatment in Phuket produces two sources of leachate. First, the Landfill Leachate and Secondly, the Garbage Pit Leachate. The objectives of this study were: 1) measure *Chlorella vulgaris* growth in Landfill and Garbage Pit Leachate, 2) measure the effectiveness of *Chlorella* to decrease the physico-chemical parameters NH_3-N, NO_3^--N, Total-P, BOD and COD 3) measure heavy metal removal from leachates. In the case of the Landfill Leachate - the minimum inoculum of *Chlorella* biomass which can grow in the Landfill Leachate was Chlorophyll *a* 0.259 µg/ml (A750 nm = 0.075) and *Chlorella* grew well in Landfill Leachate diluted 30 % with tapwater (in light 24 hours). In Garbage Pit Leachate – the minimum inoculum of *Chlorella* biomass in the Garbage Pit Leachate was Chlorophyll *a* = 0.92 µg/ml (A750 nm = 0.19) and 20% dilutions of Garbage Pit Leachate were tolerated by *Chlorella* and required continuous aeration by shaking to grow. *Chlorella* was tested for its ability to decrease NH_3-N, NO_3^--N, Total-P, BOD and COD, expressed as percentage (%) removal, compared to initial values. *Chlorella* grew well in Landfill Leachate diluted 30 % with tapwater: % removal of NH_3-N (53.91%), NO_3^--N (31.74%), Total-P (65.77%), BOD (52.78%) and COD (51.05%). In the Garbage Pit Leachate *Chlorella* grew well in 20% with tapwater: Percent removal of NH_3-N (41.5%), NO_3^--N (32.4%), Total-P (55.1%), BOD (49.2%) and COD (50.8%). 3). The Landfill Leachate heavy metal levels were already below legal limits the results showed that *Chlorella* grew in 100% of Landfill Leachate and Cr and Ni, already low in landfill leachate, were further decreased by 70% and 66% respectively. The Garbage Pit Leachate was very toxic and exceeded legal limits for Cr and Zn. In 20% dilution of Garbage Pit Leachate, Cr and Zn were decreased by 33% and 89.7% respectively to legal levels. *Chlorella* inoculations with a biomass of 1.17 µg/ml Chlorophyll *a* (A750 nm = 0.202) removed 90% of the Zn. PAM fluorometry experiments confirmed the higher toxicity of Garbage Pit Leachate.

Keywords: Leachate, *Chlorella vulgaris*, Bioremediation, Heavy metals, NH_3 and NO_3^--N, BOD, COD.

ชื่อวิทยานิพนธ์ การใช้สาหร่าย *Chlorella vulgaris* บำบัดน้ำชะขยะจากเตาเผาขยะจังหวัดภูเก็ต
ชื่อผู้แต่ง นางสาวศรัณย์พร ทองภิญโญชัย
สาขาวิชา การจัดการสิ่งแวดล้อม คณะเทคโนโลยีและสิ่งแวดล้อม
ปีการศึกษา 2556

บทคัดย่อ

การกำจัดขยะของจังหวัดภูเก็ต ทำให้เกิดน้ำชะขยะ 2 แบบ น้ำชะขยะจากบ่อฝังกลบ และน้ำชะขยะจากเตาเผาขยะ โดยวัตถุประสงค์ในการศึกษา 1) ศึกษาการเจริญเติบโตของ *Chlorella vulgaris* ในน้ำชะขยะ 2) เพื่อใช้สาหร่าย *Chlorella vulgaris* มาบำบัด ให้ค่า แอมโมเนีย ไนเตรท ฟอสฟอรัส BOD และ COD ลดลงจากเดิม 3) ติดตามปริมาณและการดูดซับโลหะหนัก ผลการศึกษา 1) การเจริญเติบโตของ *Chlorella vulgaris* ในน้ำชะขยะพบว่า ปริมาณ Chlorophyll *a* ของ *Chlorella vulgaris* ค่าน้อยที่สุดที่สามารถเจริญเติบโตได้ในน้ำชะขยะ 100% จากบ่อฝังกลบ และน้ำชะขยะจากเตาเผาขยะ มีค่า 0.259 µg/l ; (A_{750} = 0.075) และ 0.92 µg/ml Chlorophyll a (A_{750}= 0.19) ตามลำดับ น้ำชะขยะจากบ่อฝังกลบ เมื่อนำ *Chlorella* เลี้ยงพบว่า *Chlorella* เจริญเติบโตได้ดีที่สุด ในน้ำชะขยะบ่อฝังกลบ 30% (เจือจางกับน้ำประปา) ในสภาวะให้แสง (24 ชม.) ส่วนน้ำชะขยะจากเตาเผามีความเป็นพิษสูง *Chlorella* เจริญเติบโตได้ดีที่สุดในน้ำชะขยะเตาเผา 20 % (เจือจางกับน้ำประปา) ในสภาวะให้แสง (24 ชม.) โดยใช้ shaker 2) ผลการศึกษาหลังจากที่เลี้ยงสาหร่าย *Chlorella vulgaris* ในน้ำชะขยะบ่อฝังกลบ และน้ำชะขยะจากเตาเผาขยะ น้ำชะขยะจากบ่อฝังกลบ พบว่าความเข้มข้น 30% ทำให้ค่าคุณสมบัติต่างๆของน้ำชะขยะบ่อฝังกลบ ลดลงจากเดิม ดังนี้ ค่าแอมโมเนีย (53.91%), ไนเตรท (31.74%), ฟอสฟอรัส (65.77%) ค่า COD (51.05%) และค่า BOD (52.78%) และน้ำชะขยะจากเตาเผาขยะพบว่าความเข้มข้น 20% ทำให้ค่าคุณสมบัติต่างๆของน้ำชะขยะ ลดลงจากก่อนเข้าระบบค่าแอมโมเนีย (41.5%) ไนเตรท (32.4%) ฟอสฟอรัส (55.1%) ค่า COD (50.8%) และค่า BOD (49.2%) 3) ผลการติดตามปริมาณและ การดูดซับโลหะหนัก พบว่า ในน้ำชะขยะบ่อฝังกลบปริมาณโลหะหนักที่พบมีค่าต่ำกว่าค่ามาตรฐานน้ำทิ้ง และจากการทดลองนำ *Chlorella vulgaris* เลี้ยงในน้ำชะขยะจากบ่อฝังกลบที่ความเข้มข้น 100% ตรวจสอบค่าโลหะหนัก พบว่า สามารถดูดซับโครเมียมได้ 70% และ นิกเกิล 66% แต่น้ำชะขยะจากเตาเผาขยะ ปริมาณโลหะหนักที่พบ พบในปริมาณที่สูงเกินค่ามาตรฐานน้ำทิ้ง ได้แก่ สังกะสี และโครเมียม จากการทดลองนำ *Chlorella vulgaris* เลี้ยงในน้ำชะขยะจากเตาเผาขยะที่ความเข้มข้น 20% *Chlorella vulgaris* ดูดซับสังกะสีได้ถึง 89.7% และ โครเมียม 33% ปริมาณ Chlorophyll *a* ของ *Chlorella* = 1.17 µg/ml ; (A_{750} = 0.202) สามารถดูดซับโลหะหนักในน้ำชะขยะจากเตาเผาที่ความเข้มข้น 20% สามารถดูดซับสังกะสีได้ถึง 90% ปริมาณ Chlorophyll *a* ที่ 1.17 µg/ml สามารถดูดซับ สังกะสี ได้ดีเท่าๆ กับการใช้ *Chlorella* ที่มีค่า Chlorophyll *a* ในปริมาณที่สูงกว่า

คำสำคัญ: Leachate, *Chlorella vulgaris*, Bioremediation, Heavy metals, BOD

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CHAPTER 1

Introduction

1.1 Statement of the Problem

Garbage dump leachate is a worldwide problem; typically, they have high salinities, high ammonia content and typically high but variable levels of heavy metals (Cameron and Kock, 1980; Bull *et al*., 1983; Chueng *et al*., 1993; Devare and Bahadir, 1994; Xu *et al*., 2006). Lin *et al*. (2007) studied the leachate pond in Li Keng Landfill, Guangzhou, China about ammonia -nitrogen tolerance in microalgae grown in leachate physico-chemically similar to the leachate in Phuket with a very high ammonia-N and COD. Few studies have been made on leachates under tropical conditions. Phuket, Thailand there are lots of tourist attractions with a large number of hotels, restaurants, tourists and workers in the tourist industry and hence very large amounts of garbage is produced. Nowadays the garbage situation is getting to a crisis as it has increased from 429 to 526 tons per day. It rises every year (กัญญมล, 2548). The garbage from everywhere in Phuket is dumped in landfills near the incinerator after incineration. Generally the garbage is stockpiled for a couple of days before incineration. The landfill site is situated on Saphanhin Klongkogpee, Sakdidate Road which is near the sea shore and the city. Limited space is available. The incinerator in Phuket has been operating for more than 10 years and currently the garbage incinerator has two feed-heads which can receive about 530 tons of domestic garbage a day. The incinerator is very efficient but the leachate from the burnt garbage it is problem. There is also a leachate that arises from the stockpile awaiting incineration. In detail there are two main parts of the garbage management process. First, the incineration phase is run under a private corporation. The second phase is a water quality improvement (sewage treatment) plant in Phuket under Phuket Municipal control. There are two main sources of leachate. The first source is from breakdown of the garbage awaiting incineration in the holding bays of the incinerator and leakage from the garbage truck when it is dumped into the holding bays. This leachate arises directly from decomposition of raw garbage. The second source is from the ash of the garbage after burning is placed in a landfill which has a sandwich arrangement of layers of garbage and burnt garbage (ash)

then the leachate moves slowly down through all layers of the garbage (Fig 1.1). Hospitals in Phuket have separate incineration facilities and their ash is also dumped in the landfill. The content of their ash waste is well documented (Soadsing, 1999). Leachate in the landfill passes through ash and garbage layers picking up liquefied organic compounds, toxic organic compounds and heavy metals along the way. The landfill leachate is collected from the bottom of the landfill. Therefore, there are two types of leachate to deal with: the direct garbage leachate from the holding bays of the incinerator and the leachate from the landfill. Currently all leachate is fed into the water quality improvement plant mix along with the domestic sewage from the municipality. The BOD of the Garbage Pit Leachate is much too high for safe disposal in rivers or for recycling by spreading on agricultural land. The BOD load from the Garbage Pit Leachate has become a pressing problem ever since the incinerator had to be expanded to two input feed-heads.

The other major problem with the leachate arises from its potentially high heavy metal content. In the landfill leachate, the water leaching through the layers of garbage and garbage ash mobilize and dissolve the heavy metals. Early stages of the present study showed that the landfill leachate had relatively low heavy metal content but the Garbage Pit Leachate had very high levels of heavy metals, in particular zinc and chromium (Thongpinyochai and Ritchie, 2013). The zinc and chromium levels far exceeded environmentally permitted levels in the case of the Garbage Pit Leachate but did not in the case of the landfill leachate. The leachate from the holding bays of the incinerator also has very high BOD and COD but this waste stream has not been well documented in previous studied.

Several methods are currently being used for the removal of heavy metal ions from aqueous wastes. One of the alternative methods to remove heavy metals and nutrients in leachate is using some form of bioremediation. In the present study, growth of microalgae and nutrient absorption by microalgae from leachate was used to attempt to reduce the toxic effects of leachate.

Microalgae are widely employed as a tertiary treatment process to remove nitrogen and phosphorus from wastewater (de-Bashan and Bashan, 2010) since they require nitrogen, phosphorus, CO_2, and light for their autotrophic metabolic growth (de-Bashan and Bashan, 2004). Many species of microalgae have been used as a bioremediation agent when combined with wastewater treatment, but the most commonly used species are various species and

strains of *Chlorella* such as *Chlorella pyrenoidosa* (Cheung and Wong, 1981; Tam and Wong, 1989, 1990), and *Chlorella vulgaris* (Lau *et al*., 1995, 1998).

1.2 Objectives

1.2.1 To investigate and record heavy metal contamination of leachates from the Garbage Pit Leachate and Landfill Leachate. The separate documentation of the leachate from the holding bays of the Garbage Pit Leachate is important.

1.2.2 To investigate using green algae (*Chlorella vulgaris*) to reduce BOD in leachate before feeding the effluent into the recycling process.

1.2.3 To investigate heavy metal reduction in leachate by using bioassay based on *Chlorella vulgaris.*

1.2.4 It was expected that *Chlorella vulgaris* would also efficiently remove ammonia-N from leachate. Ammonia in leachate is a major contributor to its toxicity but ammonia also increases the solubility of heavy metals and also removal of ammonia has two beneficial effects – lessens the eutrophication hazard of the effluent and lessens the solubility of heavy metals.

1.3 Scope

1.3.1 Leachate samples were taken every month over a period of 6 months covering parts of both the tropical wet season and the dry season.

1.3.2. Location: Phuket Municipality Wastewater Treatment situated on Saphanhin Klongkogpee, Sakdidate Road, Phuket Province, Thailand.

1.4 Expected Outcomes

1.4.1 It is the policy of the Phuket Municipality to reduce the environmental impact of the wastewater treatment plant and reduce BOD, COD and toxic heavy metal content of effluent from the treatment plant.

1.4.2 Potential timesaving in water treatment processes. Saved time means shorter retention time and smaller holding ponds. This would help in preventing overflows of the system.

1.4.3 Some room is available at the waste treatment site for new ponds to deal specifically with an algal based treatment of leachate.

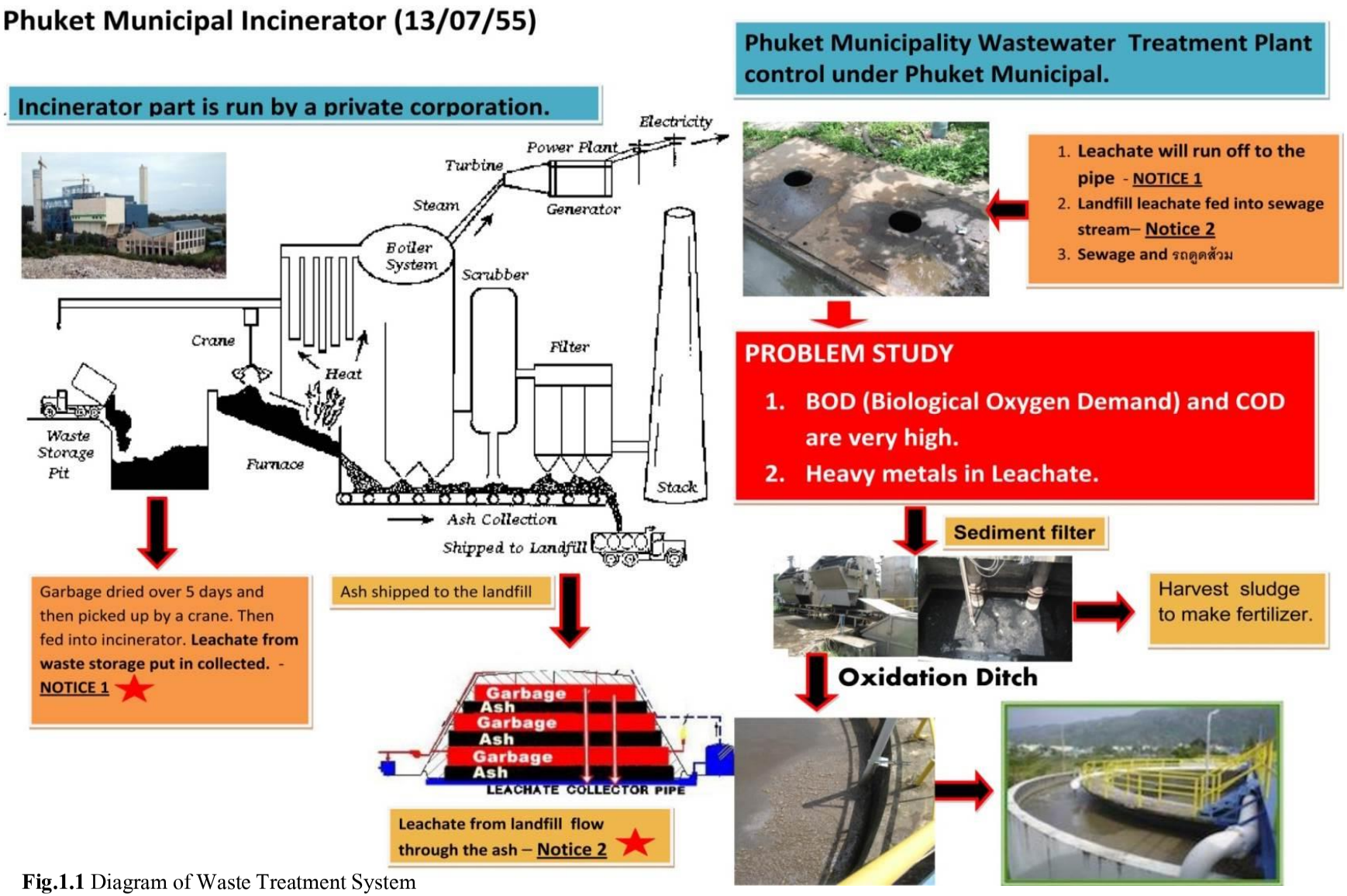

Fig.1.1 Diagram of Waste Treatment System

CHAPTER 2

Literature Review

2.1 The Leachate

Leachate is the liquid percolation of precipitation that drains through and out of the waste in the landfill. Its composition varies widely depending on waste type and the age of the waste (Cameron and Kock, 1980; Christensen *et al*., 1994; Lema *et al*., 1998; Lu *et al*., 1985; Pohland and Harper, 1985). During the formation of leachate, organic and inorganic compounds are transferred from waste to the liquid medium (Bohdziewicz *et al*., 2008) and pose a hazard to the receiving water bodies. Production of landfill leachate begins with introducing moist waste into a disposal area and continues for several decades following the landfill closure. Leachate contains high organic matter and ammonium nitrogen which varies from site to site and its composition also depends upon the landfill age, the quality and quantity of waste, biological and chemical processes that took place during disposal, rainfall density, average temperature and water percolation rate through the waste in the landfill (Mahmoudkhani *et al*., 2010). Typically, the leachate can be characterized into three major groups: mainly organic matter, inorganic matter and xenobiotic organic compounds (Lee *et al*., 2010). Several other approaches to dealing with leachate will also be reviewed. Briefly, to some extent leachate can be recirculated to the same landfill or treated by different methods: biological aerobic and anaerobic methods and or nitrification–denitrification to remove organic matter and ammonium nitrogen (Maranon *et al*., 2008).

2.2 Chemical composition and toxicity of leachate

The leachate composition may depend on such factors as organic content, solubility, sewage sludge content, etc. (Pohland and Kang, 1975; Cameron and Kock, 1980; Silva *et al*., 2004; Wilde *et al*., 2006). It is important to note that most work has been done on leachate in temperate

climates: in the present study emphasis has been put on the relatively few studies done in tropical locations such as Silva *et al.* (2004), Wilde *et al.* (2006) and Lin *et al*., 2007). The main parameters of leachate components assessed from the literature are shown in Table 2.1 (Ministry of Housing and Local Government. 1961; U.S. Department of Health. 1968).

Table 2.1 Leachate composition – representative values

Component (mg/l)	**Range found in typical leachate * (mg/l)**
BOD (5 days)	1,000 – 30,000
Kjedahl – N as N	2-600
Ammonia as N	150-600
Nitrate + nitrite as N	0-5
Phosphate as P	5
Suspended solids	100-500
Total dissolved solids	2,000 – 15,000
Sulphate	200-500
Iron	50-500
Chromium	0-1
Zinc	0-30
Copper	0-5
Nickel	0-1.0
pH	5.6-7.6

* U.S. Department of Health, 1968: Ministry of Housing and Local Government, 1961

If the landfill leachate is received by the municipal sewerage system, it carries a high BOD (biological oxygen demand) loading, nitrogen content (mainly ammonia), suspended solids and

generally has a high heavy metal content. Bull *et al*. (1983) and Devare and Bahadir (1994) found that leachate effluent had very high organic content and has environmental effects similar to raw sewage because of its very high BOD and COD. Leachate is a worldwide problem; typically containing high but variable levels of heavy metals. The variability of leachate is important, for example in the present study it was expected that the landfill leachate would have very high levels of heavy metals but it was found that the heavy metal content was relatively low (Thongpinyochai and Ritchie, 2013) and within the permitted levels in Thailand (Thailand Government Pollution Control Department: www.pcd.go.th/info_serv/en_reg_std_water04.html). They have divided effluent standard into 12 types such as Industrial Effluent Standards, Water Characteristics Discharged into Irrigation System and The control of sewerage systems. However industrial effluent standards and water characteristics discharged into irrigation system have the limits on heavy metals, the control of guidelines sewerage system does not have a heavy metals limit (Table 2.2).

Table 2.2 Effluent standard in Thailand (Pollution Control Department, Thailand)

Parameters	**Industrial Effluent Standards**	**Water Characteristics Discharged into Irrigation System**	**The control of sewerage systems**
pH value	5.5 – 9	6.5 - 8.5	5.5-9.0
Conductivity	-	2,000 µMole/cm	-
Total Dissolved Solids	Not more than 3,000 mg/l depending on receiving water or type of industry under consideration of PCC but not exceed 5,000 mg/l Not more than 5,000 mg/l exceed TDS of receiving water having salinity of more than 2,000 mg/l or TDS of sea if discharge to sea	1,300 mg/l	-

Table 2.2 (cont.)

Parameters	Industrial Effluent Standards	Water Characteristics Discharged into Irrigation System	The control of sewerage systems
Suspended solids (SS)	Not more than 50 mg/l depending on receiving water or type of industry or wastewater treatment system under consideration of PCC but not exceed 150 mg/l	30 mg/l	30 mg/l
Temperature	Not more than 40°C	-	-
. Color and Odor not objectionable	Not specified	Not objectionable	-
Sulphide as H_2S	Not more than 1.0 mg/l	1 mg/l	-
Permanganate (PV)	-	6 mg/l	-
Cyanide as HCN	Not more than 0.2 mg/l	0.2 mg/l	-
Fat, Oil & Grease (FOG)	Not more than 5.0 mg/l depending of receiving water or type of industry under consideration of PCC but not exceed 15.0 mg/l	5.0 mg/l	5.0 mg/l
Formaldehyde	Not more than 1.0 mg/l	1.0 mg/l	-
Free Chlorine	Not more than 1.0 mg/l	1.0 mg/l	-
Phenols	Not more than 1.0 mg/l	1.0 mg/l	-

Table 2.2 (cont.)

Parameters	Industrial Effluent Standards	Water Characteristics Discharged into Irrigation System	The control of sewerage systems
Pesticides	Not detectable	None	-
Biochemical Oxygen Demand (BOD)	Not more than 20 mg/l depending on receiving water or type of industry under consideration of PCC but not exceed 60 mg/l	20 mg/l	20 mg/l
Total Phosphorus	-	-	5 mg/l
Chemical Oxygen Demand (COD)	Not more than 120 mg/l depending on receiving water of type of industry under consideration of PCC but not exceed 400 mg/l	-	-
Heavy metals			
1. Zinc (Zn)	Not more than 5.0 mg/l	5.0 mg/l	-
2. Chromium (Hexavalent)	Not more than 0.25 mg/l	0.3 mg/l	-
3. Chromium (Trivalent)	Not more than 0.75 mg/l	-	-
4. Arsenic (As)	-	0.25 mg/l	-

Table 2.2 (cont.)

Parameters	Industrial Effluent Standards	Water Characteristics Discharged into Irrigation System	The control of sewerage systems
5. Copper (Cu)	Not more than 2.0 mg/l	1 mg/l	-
6. Cadmium (Cd)	Not more than 0.03 mg/l	0.03 mg/l	-
7. Barium (Ba)	Not more than 1.0 mg/l	1 mg/l	-
8. Lead (Pb)	Not more than 0.2 mg/l	0.1 mg/l	-
9. Nickel (Ni)	Not more than 1.0 mg/l	0.2 mg/l	-
10. Manganese (Mn)	Not more than 5.0 mg/l	0.5 mg/l	-
11. Selenium (Se)	Not more than 0.02 mg/l	0.02 mg/l	-
12. Mercury (Hg)	Not more than 0.005 mg/l	0.005 mg/l	-

If the BOD level is too high then the water could be at risk for further contamination, interfering with the wastewater treatment process and affecting the end product of the sewage plant. There are several factors that can contribute to high BOD levels: ammonia, nitrites, nitrates and phosphates present in the wastewater. High levels of ammonia and phosphate are the cause of eutrophication which is the response of aquatic ecosystems to the addition of artificial or natural nutrient substances, such as ammonia, nitrates and phosphates, derived from fertilizers or sewage (Falkowski and Raven, 2007). “Algal blooms” or great increases in phytoplankton in a water body as a response to increased levels of nutrients can cause considerable environmental problems including fish kills, making water unsuitable for domestic water supply and livestock poisonings. Negative environmental effects include hypoxia, the depletion of oxygen in the water, which results in “fish kills” and death of other animal populations. It also interferes with fish migration and reproduction.

2.3 Landfill System Design in Phuket

The Landfills in Phuket are a Bioreactor Landfill system established in 2537 BE (1994) with an area of 192,000 square meters (19.2 ha). It is situated at Saphanhin Klongkogpee, Sakdidate Road, Phuket. It is a sandwich system with alternating layer of incinerator ash and garbage. It has a compressed density of 0.7 tons per cubic meter. Each layer is 2.50 metres thick which includes the height of the top ash layer of 0.60 metres. Trash was embedded in the sanitation system composed of three elements; ash from the incinerator and garbage in alternating layers and a soil capping layer when the trash pyramid is completed. The sandwich arrangement is shown in Fig 1.1. Leachate is drained from the bottom of the landfill and there is a groundsheet to prevent leachate entering the groundwater.

2.3.1 Gas Venting of the Landfill

Methane accumulation in landfills is potentially dangerous. The embedded area has a system of perforated pipes installed to remove methane. The collected gas is flamed or used as a gas fuel in the incinerator or in the sewage plant. Gas is drained by drilling holes through the landfill and inserting perforated pipes. Exploratory holes are drilled to locate pockets of methane.

2.3.2 Drainage System

Runoff from the landfill is managed by placing a water drainage pipe around the landfill area to collect the drainage. This drainage is included in the leachate.

2.3.3 Leachate Collection

Leachate is collected by a system of waste water system pipes, which are placed under the floor of the trash layer to collect waste water. This leachate is fed into the wastewater treatment stream at the sewage plant. Phuket has a tropical monsoon climate and so runoff from the landfill and

leachate volumes are high in the wet season (May to October) and much lower in the dry season (November to April). This study includes work on samples collected during both the wet and dry seasons.

2.4 Heavy metals in leachate

Heavy metals are generally defined as those that appear low in the periodic table with atomic weights greater than iron or relative density greater than 5g.ml^{-1}; over thirty metallic elements fit this definition. Nickel, Copper and Zinc are essential trace elements for plants but most elements described as heavy metals are not essential for plants (Atwell *et al*., 1999). Many of these elements are abundant within the Earth's crust with consequent tolerance adaptations within both plant and animal metabolisms. Only a few heavy elements are essential nutrients for example Nickel, Copper and Zinc as well as Iron and are referred to as trace elements. Most of the elements classified as heavy metals are toxic because they act as toxic analogues of essential elements and in themselves have no known biological function. Although iodine is essential for animals it is generally not essential for land plants but is known to be essential for some marine algae. Other heavy metals are found typically at low concentrations and are highly toxic but it is important to know that the redox state of a heavy metal is an important determinant of their toxicity (for example, cadmium, chromium and mercury) and also their solubility for example Fe^{2+} is soluble but Fe^{3+} is not. In the presence of oxygen Fe^{2+} is rapidly converted to Fe^{3+} which forms insoluble oxides and hydroxides. The release of metals into the environment as a result of natural processes and anthropogenic activities occurs continuously in the biosphere.

The effects of heavy metals on *Chlorella* species has been well studied (Cd, Cu, Hg, Zn, Pb, Rosko and Rachlin, 1977; Cu, Zn, Wilde *et al*., 2006; Co, Cu, Zn, Afkar *et al*., (2010). Cozens (1995) and Wilde *et al.* (2006) have used *Chlorella vulgaris* and *Chlorella* sp.as a bioindicator of trace metal contamination, in particular copper (Cu) and nickel (Ni). Louries et al. (2010) using several algal species found that *Chlorella* sp. and *Pseudokirchneriella subcapitata* showed the best ability in absorption of metals. Sorption of Co, Zn, Ni, and Cd from solutions with high levels of both heavy metals and calcium increased by almost 50% when a source of tannic acid (bark) was added to the

culture medium. Low pH (pH 3.0) had no influence on metal sorption from solutions with high metal content. Leachate usually, but not always, contains enough heavy metals to make it a hazardous material (cf. Thongpinyochai and Ritchie, 2013). Cameron and Kock (1980) working in Vancouver, Canada concluded that about 94% of the toxicity of leachates could be attributed to ammonia, pH, copper content and recalcitrant organic materials (tannins). Most of the toxicity was actually due to the ammonia content and high ammonia has the additional effect of increasing the solubility and hence toxicity of the heavy metals (ammonia mobilization). High levels of ammonia are usually found in landfill leachate (Bull *et al*., 1983; Ritchie *et al*., 2001; Lin *et al*., 2007), and stripping (venting to the atmosphere) can be successful in removing this pollutant, which increases wastewater toxicity because it is not only toxic in itself but it increases the solubility of many heavy metals (Cameron and Kock, 1980; Marttinen *et al*., 2002). Silva *et al*. (2004) also found that ammonia stripping after coagulation and flocculation was effective in reducing toxicity.

2.4.1 Bioremediation monitoring

Bioassay methods are a convenient way of monitoring the toxicity of leachates (Chueng *et al*., 1993; Devare and Bahadir, 1994) and can be used to monitor the effectiveness of bioremediation experiments. Reports on the use of microalgae in wastewater treatment are readily available (Bull *et al*., 1983; Przytocka-Jusiak *et al*., 1984; Tam and Wong, 1989, 1990; Craig and Keith, 1995; Lau *et al*., 1995; Craggs *et al*., 1997; Zimmo *et al*., 2004; Lin *et al*., 2007). Some algal strains are sensitive to environmental contaminants and can be used to assess the toxicity of landfill leachate (Clément *et al*., 1996; Baun *et al*., 1999).

2.4.1.1 Bioassay using *Chlorella* sp.

2.4.1.1.1 *Chlorella* sp. Taxonomy

Chlorella is a non-motile highly tolerant unicellular fresh water alga that is easy to grow in fully defined media (Stein, 1973; Rai *et al*., 1996); it is both acid-tolerant and alkaline-tolerant and tolerant of pH changes (Cozens, 1995). *Chlorella vulgaris* was first described by Beijerinck in the 1890s (Oh-Hama and Miyachi, 1993) but different *Chlorella* species are difficult to identify. According to taxonomical grouping based on morphology and physiological properties, it belongs to genus *Chlorella*, family Oocystaceae, order Chlorococcales, class Chlorophyceae, division Chlorophyta of the kingdom Plantae. At present (21 Dec 2013) only *Chlorella variabilis* is completely sequenced (KEGG, 2013).

2.4.1.1.2 Cell wall properties

The ion exchange properties of cell wall - The cell wall has binding sites for metal cations in many algae (Ritchie and Larkum, 1982a, b; Cho *et al*., 1994; Wehrheim and Wettern, 1994). The cation exchange properties of cell walls of algae are understood and are the result of the Donnan system set up by the fixed negative charges of cell wall components such as pectins and sulphonated pectin like compounds (Ritchie and Larkum, 1982a, b). Helfferich (1962) found cation-exchangers have the following properties:

(a) They adsorbed more cations than anions from their environment due to the presence of fixed anions (a Donnan system).

(b) Cell wall heavy metal is bound by a cation exchange mechanism. Thus most of the heavy metal absorbed by algae are bound to the cell was and is not actually taken up by the cells into the cytoplasm.

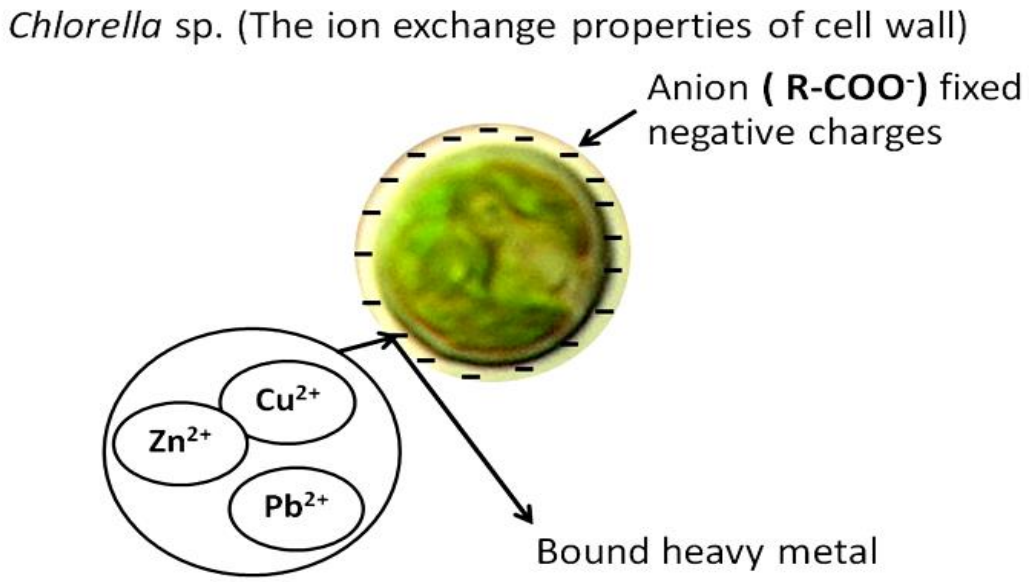

Fig 2.1 Cell wall properties of *Chlorella* sp.

2.4.1.1.3 Ion uptake

Ion uptake by *Chlorella pyrenoidosa* was extensively studied by Barber (Barber, 1968a, Shieh and Barber, 1971) who was careful to take the cation-binding properties of the cell walls of the cells into account. The non-vacuolate cells are 5 μm or larger in size and measurements of the electrical potential across the plasma membrane of the cells were made. The cells have a negative membrane potential about -100 mV. The electrochemical potential measurements show the existence of active influxes of potassium and chloride and efflux of sodium. Barber suggested that the ionic relations of *Chlorella* sp. were explicable in terms of exchange mechanisms across the cell membrane but at that time the role of the proton exctrusion ATPase in maintaining the membrane potential of plant cells was not known (Atwell *et al*., 1999). Ion transport is often light independent and is closely interrelated with changes in membrane potential. By inserting electrodes directly into individual *Chlorella* sp. cell, Barber (1968a) was able to demonstrate that the equilibrium potentials for K^+, Na^+ and Cl^- were all light dependent. Concomitant with the light-stimulated uptake of K^+ or Na^+ in

Hydrodictyon africanum (a giant-celled green algae) there is a stimulation of Cl^- uptake (Raven, 1968a, 1969b). The action spectrum for the responsible Na-K-Cl pumps coincides with photosystem II and led Raven to conclude that ATP was the energy source (Raven, 1969a). The light-stimulated portions of alkali-ion effluxes share several important characteristics with the respective influxes (Barber, 1968a, Raven, 1968a). The light-dependent uptake of HCO_3^- at high pH may depend on a specific, ATP-independent HCO_3^- pump coupled to photosystem I activity (Raven, 1968b).

2.4.1.1.4 pH tolerance

Cook (1965) and Stengel (1970) showed that there was a pH dependent effect on the growth of *Chlorella* of the dissociation rates and ionic state of polar inorganic and organic compounds on the availability of many algal nutrients such as CO_2, iron and organic acids. Furthermore, Hegewald (1972) and Bentrup (1971) showed that the pH affects the electrical charge of cell wall surface because the pKa (dissociation constant) of the pectins of the cell wall of *Chlorella* is about 3 and there are also effects on ion transport systems at the plasmalemma, and on the associated membrane potential (Barber 1968a). Kessler (1967a) compared the acid tolerance of numerous *Chlorella* strains and has demonstrated species-specific differences which appear to be genetically determined. *Chlorella saccharophilla*, with a lower pH limit of 2, shows the greatest acid tolerance. The pH may also affect the state and mobility of heavy metals. Also Kanasawa and Kanazawa (1969) observed an increase of Cu^{2+} toxicity in *Chlorella* sp. with decrease in pH. The acid/base properties of the cell walls is the fundamental reason why binding of heavy metals to the cell walls of algae is pH dependent (Ritchie and Larkum 1982a, b; Wilde *et al*., 2006).

pH tolerance of *Chlorella* sp. has been widely studied in physiological and biochemical experiments as well as in leachate treatments because of its effects on the growth rate and nutrient uptake efficiency. Many researchers have reported on growing *Chlorella vulgaris* in landfill leachate and other wastewater effluents because it is a standard benchmark species to use. González *et al*. (1997) found that *Chlorella* can use ammonium ion and nitrate as nitrogen sources and phosphorus from the wastewater. In a similar study, Jinsoo *et al*. (2010) used *Chlorella* to remove

nitrogen in the form of ammonia and ammonium ion (NH_3/NH_4^+) from wastewater. They concluded that *Chlorella vulgaris* has the potential to remove nitrogen at a reasonable uptake rate from wastewater while being cultivated using wastewater effluent and using bicarbonate ion as a source of inorganic carbon. Where cells are using bicarbonate as an inorganic carbon source this leads to an alkalinisation of the medium, since the pKa of ammonia is 9.4 that means that under alkaline conditions NH_4^+ is converted to NH_3 which is volatile and tends to be lost to the atmosphere. Chueng *et al*. (1993) used *C. vulgaris* and *C. pyrenoidosa* to show that there was a significantly enhanced growth of *Chlorella* in diluted leachate (pH 4.5 to 5.5). They concluded that the *Chlorella* species were using organic substances in the leachate (hence growing photoheterotrophically or mixotrophically) and were found to be tolerant to toxic substances that may have been present in the leachate. This is important. *Chlorella* sp. growing in media with high BOD are generally growing photoheterotrophically – that is using light as a source of energy to rearrange and metabolise organic carbon compounds they take up from their environment rather than using the ATP and NADPH produced from the light reactions of photosynthesis to fix CO_2.

2.5 Photorespiration

Photorespiration is defined as a light dependent O_2 uptake and CO_2 release that occurs in photosynthetic tissues. Its overall effect is to reduce the total fixation of carbon by photosynthesis and occurs under conditions of high irradiance and high oxygen levels. Marrett (1973) and Ne (1974) found many algae have been shown to produce glycolate and to posses enzymes of the glycolate pathway. Typically algae secrete glycolate (C_2) and glycolate is a significant component of dissolved organic carbon in many bodies of water. The inhibition of photosynthesis by O_2 or the Warburg effect was first shown in algae by Warburg (1920) and attributed to photorespiratory activity by Turner (1962) and Bows (1972). Warburg's description (Warburg 1920) of O_2 inhibition of photosynthesis by *Chlorella* has been referred to as the Warburg O_2 effect.

The key enzyme of the Calvin Cycle is RUBISCO. Using CO_2 as the substrate Ribulosebisphosphate (RuBP) is converted into two phosphoglycerate (PGA) molecules which are fed in

to the Calvin Cycle (Atwell *et al*., 1999). In the presence of high O_2 RUBISCO acts as an oxygenase and converts RuBP into one PGA and one phosphoglyollate. Photorespiration can be explained metabolically by glycolate biosynthesis in the chloroplast followed by its metabolism in peroxisomes and mitochondria. These reactions occurring in unicellular green algae are similar to those in higher plants with a few modifications, particularly in the enzymatic oxidation and fate of glycolate. In many algae glycolate is simply secreted rather than being fully metabolized by the photorespiratory pathway. Photorespiration differs from mitochondrial respiration in that it does not occur in the dark, does not conserve energy as ATP, and does not utilize substrate of the tricarboxylic acid cycle. The final result of the photorespiratory pathway is to convert glycolate into CO_2 with consumption of oxygen. Photorespiration is basically caused by the dual function of RUBISCO as a carboxylase enzyme and as an oxygenase enzyme. In *Chlorella* and many other algae the oxygenase activity is minimized by creating a high internal concentration of CO_2. These mechanisms are called carbon concentrating mechanisms (Giordano *et al*., 2005).

2.5.1 Light and algal growth

The relationships between light intensity and the rates of photosynthesis and photoautotrophic growth typically show a rectangular hyperbolic function with an inhibition of growth occurring at supersaturating light intensities. At very high light intensities photoinhibition sets in. A model curve called the Waiting-in-Line function can be used to model both the saturating behavior of photosynthesis as light is increased and the photoinhibitory effects of very high light (Ritchie 2008b). A similar situation applies to algae suspensions growing in light and dark cycle (Sorokin and Krauss, 1962). Sorokin *et al*. (1962), Setlik *et al*. (1969) found that the shape of light photosynthesis curves and of light growth curves is markedly affected by temperature. Watt (1969) found that extracellular release of photosynthetic carbon products also varies with light intensity and seems to reach its maximum at light intensities which inhibit photosynthesis. The release of extracellular carbon is also high at very low light intensities (Watt, 1966, 1969, Watt and Fogg, 1966). This is consistent with the

oxygenase/carboxylase dual metabolism of RUBISCO (Atwell *et al.* 1999; Giordano *et al*., 2005) because at low light intensities the carbon concentrating mechanism in not fully activated.

Karlander and Krauss (1966) found that some *Chlorella* sp. strains cannot grow heterotrophically but are able to utilize organic substrate in light (photoheterotrophy) well below the compensation point of photoautotrophic photosynthesis. It is not clear how oxygen concentrations affect photoheterotrophy. It has not been properly investigated.

2.6 Photosynthesis measurements

2.6.1 Using PAM (Pulse Amplitude Modulation Fluorometry)

Fluorometric methods are a very convenient way to measure photosynthesis of algae such as in *Chlorella* (Ritchie, 2008; Ritchie and Larkum, 2013; Seatae, Bunthawin and Ritchie, 2013). Light saturation curve measurements on the algae were made using a Junior PAM portable chlorophyll fluorometer (Gademann Instruments GmbH, Wurzburg, Germany) fitted with a 1.5 mm diameter optic fibre and a blue diode light source. The Junior PAM uses a magnetic clamp to hold specimens about 1 mm from the end of the light pipe. PAM parameters (effective quantum yield, rETR, NPQ) were calculated using the WINCONTROL software (2.133/03.00) using standard settings for rapid light curves (Heinz Walz GmbH, Effeltrich, Germany) (Genty *et al*., 1989). The default absorptance factor of 0.84 and the default value of 0.5 for estimated absorption of light by PSI and PSII were used on the Junior PAM to calculate the relative Electron Transport Rate or rETR (Ritchie, 2008b). On the standard settings for a rapid light curve, sets of PAM light curve measurements took about 88s to complete with 10 s between actinic flashes of light and each flash of light was 0.8 s duration. The flashes were in order of increasing intensity as nine graded irradiance increments from a nominal zero irradiance. The protocols used for using the Junior-PAM in the present study are described in Saetae *et al*. (2013).

A junior PAM can be used to measure photosynthesis of algae by simply making an "artificial leaf" by filtering algae onto a glass fibre disk. Replicate samples of algal cells (usually 3 or 5 ml cell suspensions) were filtered onto Whatman GF-C glass fibre filters (Whatman International,

Maidstone, England, UK) in a Millipore filtration apparatus for 25 mm filters then dark treated in a Petri dish with disks of filter paper impregnated with seawater, for at least 10 min. The absorptance of the algae-impregnated disc was measured using a Blue-RAT machine (Reflectance/Absorbance/transmission) as described by Ritchie and Runcie (2013). Using the actual absorptance value the actual ETR rather than relative ETR can be calculated. Only one light saturation experiment is run on each filter to avoid confounding effects of multiple experimental treatments. The inside diameter of the Millipore filtration apparatus is 15.9 mm and so the disks of algae adhering to the glass-fibre filter had a surface area of 198.6×10^{-6} m^2. The algal-impregnated disks provide highly reproducible material for experiments. Care needs to be taken to avoid the algae-impregnated disks drying out.

The great advantage of using a PAM machine to measure photosynthesis is that they are able to collect very large amounts of data very quickly compared to oxygen electrode, infrared gas analyzers (IRGA) and $^{14}CO_2$ techniques (Walker, 1990).

Figure 2.2 JUNIOR-PAM Teaching Chlorophyll Fluorometer**.**

2.6.2 Chlorophyll Measurement

After photosynthetic electron transport determinations, chlorophyll was extracted from the glass fiber disks using ethanol (99.5% ethanol neutralised with magnesium carbonate) and chlorophylls determined as described previously (Ritchie, 2006, 2008a). It was difficult to extract chlorophyll from *Chlorella* on glass fibers disks or as pellets in ethanol unless the cells were heated in alcohol in a water bath at about 80 ^{o}C for about 3 minutes. Care needs to be taken to expose extracts to minimum light during extraction and storage. The alcohol extracts were made up to 3 ml and stored at -20 ^{o}C as described previously (Ritchie, 2006; Ritchie, 2008a). Extracts were stored in the dark in a freezer at -20 ^{o}C before spectrophotometric assay for as short a time as practicable. Generally chlorophyll assays were made within a few hours of extraction or the next day but heat-treated chlorophyll extracts (chlorophyllase is readily deactivated by heating) appear to be stable and could be stored at -20 ^{o}C indefinitely.

Chlorophylls were determined from spectrophotometric readings made using a Shimadzu UV-1601 UV-visible spectrophotometer using quartz glass or plastic cuvettes as described previously (Ritchie, 2006; Ritchie, 2008a). Replicate disks from the same batch of cells generally varied by less than ± 2% in chlorophyll *a* content.

2.6.2.1 Calculation of rETR on a Chlorophyll Basis

The Walz software calculates ETR on a surface area basis (the surface area of the object illuminated by the beam of blue light) as mol $^{(e-)}$ m^{-2} s^{-1}: this can be converted to mol (O_2) m^{-2} s^{-1} assuming 4e-/O_2. The diameter of the glass fibre disks of algae and their chlorophyll content were both known and so mg of chlorophyll per square metre could be calculated. Relative ETR (rETR) in mol $^{(e-)}$ m^{-2} s^{-1} was converted to mol $^{(e-)}$ mg Chl $\underline{a}^{-1}$ h^{-1} using the chlorophyll assays (as mg Chl $\underline{a}$ m^{-2}). Since the chlorophyll a content per unit volume of the cultures was also known, gross photosynthesis could also be expressed as per unit volume of culture (mol $^{(e-)}$ m^{-3} h^{-1}). If the actual absorptance of the algae on the glass

fibre disk is known it is possible to convert the rETR (based on the standard assumption that the Absorptance is 0.84) into actual ETR. A simple device called a Reflectance Absorptance Transmission (RAT) machine has been developed to routinely measure absorptance (Abt) instead of just using a default value of 0.84 (Ritchie and Runcie 2013).

2.6.2.2 Light Curve Fitting

Light curves were fitted to the Waiting-in-Line equation (Ritchie, 2008b; Ritchie, 2012, Ritchie and Larkum, 2013). A form of the equation that is easy to fit using non-linear least squares methods where good initial guesses of P_g, max and E_{opt} are required (Ritchie, 2012) is,

$$P_g = \frac{P_{g,max} \cdot E}{E_{opt}} \cdot e^{1-E/Eopt} \qquad \text{Equation 1}$$

where, P_g is gross photosynthesis measured as rETR, O_2 evolution or CO_2 uptake,

$P_{g,\,max}$ is the maximum gross photosynthesis,

E_{opt} is the optimal irradiance,

E is the Irradiance (μmol m^{-2} s^{-1} 400 – 700 nm PPFD).

2.7 Dark respiration

A PAM machine is a convenient way to measure gross photosynthesis of plants and algae however; it provides no information on respiration. Respiration is best measured using oxygen electrode methods (Walker, 1990). Algae species which lack photosynthetic pigments are necessarily heterotrophic. It is also known that some algal derive energy from the oxidation of inorganic compounds. Gibbs (1962) and Danforth (1967) found that energy production in the dark involves glycolysis, the

pentose-phosphate cycle, the tricarboxylic acid (TCA) cycle and the metabolism of glycolate in the dark is different to photorespiration. Kandler *et al.* (1961) showed that glycolysis and the pentose phosphate cycle was functional under both autotrophic and heterotrophic conditions in *Chlorella ellipsoidea.* Hutner and Provasoli (1951) found the effect of pH on cellular respiration of algae and the oxidation of organic acids by acetate flagellates precede most quickly at an acid pH. Under acid conditions the organism is more permeable to the undissociated acids than to the charged anions and so do not need to use energy to take it up. However, a drawback to this is that under acid conditions cells can be easily killed by excessive concentrations of organic acids because the cell cannot control uptake of the uncharged acid and cannot excrete it easily. The ammonia has the effect of short-circuiting the intracellular pH regulation mechanism leading to cell death.

2.8 Statistics

Simple statistical methods will be used in the present study. All measurements were done in at least 6 replicates and are quoted as means and ±Standard Errors. ANOVA was used to identify statistically different treatment means. Standard curves were fitted using non-linear least squares methods. Most analyses were made using Microsoft Excel. The standard statistical textbook used as a reference was Zar (2010).

CHAPTER 3

Material and Method

3.1 Collecting Leachate

Leachate samples were taken every month over a period of 6 months covering parts of both the tropical wet season (June, July, October) and the dry season (March, April, November).

Two types of leachate samples were collected (a) Leachate from the base of the landfill (LF) (Fig.1.1) and (b) Leachate from the Garbage Pit Leachate (GPL). This was a direct garbage leachate.

3.2 Physico-chemical Properties of Leachate Sample

The leachates were settled to remove particulate matter, and the supernatant used for the experiments. Characteristics of leachate such as pH, Ammonia-N, Nitrate – N, Total Phosphorus, BOD, COD were measured (APHA 1998- Appendix I). Heavy metals were measured using Inductively Coupled Plasma Optical Emission Spectrometry at the PSU Scientific Equipment Centre (SEC). Photosynthetic measurements were made using a Pulse Amplitude Modulation Fluorometry (PAM) machine (Junior PAM). Six replicates were used routinely in the present study.The Garbage Pit Leachate was quickly found to be very toxic (Thongpinyochai and Ritchie 2013) and also had very high levels of heavy metals, phosphate and nitrogen content. Samples had to be greatly diluted to get measurements on scale. No single dilution factor was suitable for running all the analyses. A suitable dilution factor had to be separately determined for each measured parameter.

3.3 *Chlorella vulgaris* culture conditions and growth measurement

3.3.1 Culture: *Chlorella vulgaris*

Chlorella vulgaris was cultured with BG-11 in flasks for exponential growth phase for 7 days in a temperature range 25-27 ^{o}C under 24 hours light using cool-white fluorescent lamps with light intensities = 200 μmol (quanta) m^{-2} s^{-1} (PAR,400-700 nm). Cultures were grown either statically, grown on a shaker [WiseShake (SHO-2D)] or agitated by magnetic stirrers. The cultures grew well in freshwater or seawater and so the salinity of the leachates used in the present study would not have been limiting for the growth of *Chlorella* simply because of their salinity. The *Chlorella vulgaris* strain used was from the Coastal Fisheries Research and Development Station (Phuket).

3.3.2 Growth measurement

Absorbance (optical density, OD) of cell cultures was measured at 750 nm because at this wavelength the decrease in OD is due to scattering by the cells and not due to absorption by pigments and Chlorophyll *a* was used to monitor the number of *Chlorella* cells present. 5 ml samples were centrifuged at 5,000 rpm for 5 minutes. After centrifugation, the liquid was decanted off as much as possible. The pellet was then mobilised by vortexing before adding 3 ml of ethanol. The 3 ml of ethanol extract then left in the refrigerator for about 1 hr. The samples were then centrifuged at 5,000 rpm for 5 minute and the supernatant were used for chlorophyll determination using a spectrophotometer (Shimadzu UV-1601) at 649 and 665 nm using $A_{750\ nm}$ as the absorbance blank, and the equations of Ritchie (2006) were used to estimate Chlorophyll *a* [Chl a (μg/ml) = $11.867 \times (A_{665\ nm}-A_{750\ nm}) - 5.201 \times (A_{649\ nm}-A_{750\ nm})$].

3.4 Heavy Metal: Basic use of bioremediation/bioassay methods

Exponential growth rate constant or doubling time (t_2) was calculated using a non-linear least squares fit to the exponential growth equation compared between different experimental conditions. Experiments to measure the doubling time of *Chlorella vulgaris* were set up. $A_{750\ nm}$ and Chlorophyll *a* of cultures were measured 2 times per day in the morning and in the afternoon and the results plotted. Six replicates were used.

Relationship the absorbance A750 and Chlorophyll *a*

X: axis ⟶ Chlorophyll *a* of *Chlorella vulgaris*

Y: axis ⟶ Absorbances 750 nm used to follow growth.

3.4.1.1 Doubling time calculation

Doubling time curves were fitted to the Exponential Growth equation.

$$A_t = A_0 e^{kt}$$

where, A_t is the absorbance ($A_{750\ nm}$) at time (t)

A_0 is the calculated absorbance ($A_{750\ nm}$) at t = 0

k is the exponential constant and the doubling time (t_2) = -Ln(2)/k

3.4.2 The effect of different concentrations of leachate on algal growth.

The growth rate in different leachate concentrations was measured using a protocol similar to ISO 8692:2012 which is a standard algal growth bioassay.

Independent factor – leachate concentration

Control

- *Chlorella vulgaris.* Used a standard inoculation: known volume of cells with a measured $A_{750\ nm}$.

- 24 hours light, 24 – 27 °C.
- 9 days experimental period, amount of growth was measured each day rather than simply at the end of the 9 day incubation.
- There are ISO standard protocols for such tests specifically using *Chlorella* sp. as a model organism. In this study I followed the growth of the algae each day whereas the ISO protocols usually only measure growth after a specific length of time rather than look at the time course of growth of the alga.

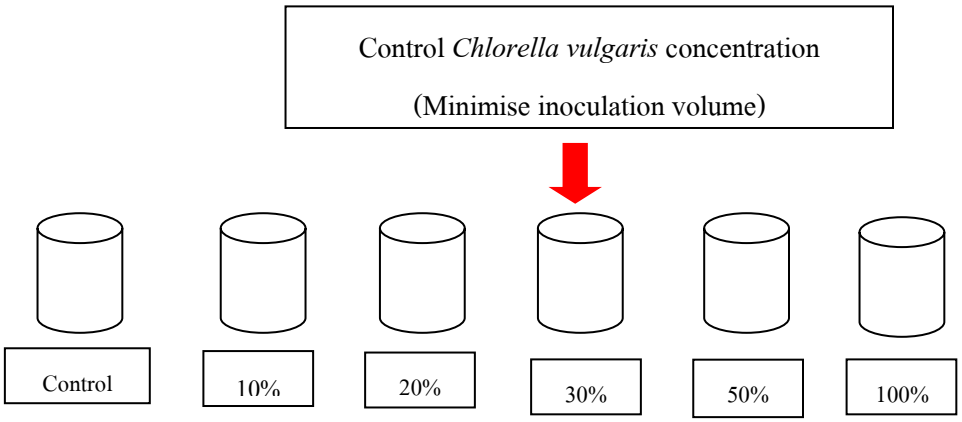

Fig 3.1 The effect of different concentrations of leachate on algal growth

Growth experiment of *Chlorella vulgaris* in the leachate was set up in a culture room with 24 hours under fluorescence light and 25 °C of culture room. However, the toxicity of leachate from incinerator and landfill leachate were different and growth was different under non-shaking and shaking conditions.

3.4.2.1 Phase 1: Growth in the light in a non-shaking condition.

The incinerator and landfill leachate incubation experiments were set up with 5 concentrations of leachate and a zero control: 0 (control), 10, 20, 30, 50, 100% (leachate diluted) with distilled water. *Chlorella* cells grown up in BG-11 were first centrifuged and the supernatant discarded

and the cells resuspended in distilled water. The cultures were then inoculated with *Chlorella vulgaris* cell suspension with a biomass of Chlorophyll *a* = 0.92 µg/ml or in terms of absorbance A_{750} = 0.19 and incubated for 9 days. Cultures were stirred each day before taking samples. Growth of *Chlorella vulgaris* was followed by measuring Chlorophyll *a* as an unambiguous measurement of live *Chlorella* cells. The leachate was coloured and so absorbance measurements at 750 nm (A_{750}) were not suitable for estimating growth rates and cells counts were also not practical because the leachate had a lot of particulate matter making it difficult to properly count the cells. Three replicate samples were taken at each sampling time in each leachate concentration and also in the control.

3.4.2.2 Phase 2: Growth in light on a shaker.

Incubations were also run on *Chlorella* grown on a shaker at 150 rpm. The alga grew much better on a shaker than in static culture. *Chlorella vulgaris* was set up in leachate at 5 different dilutions of leachate 10, 20, 30, 50, 100% (leachate diluted) with a distilled water *Chlorella* in distilled water acted as the control. Cultures were inoculated with *Chlorella vulgaris* cells prepared as described above (3.4.2.1) and incubated for 9 days. Growth of *Chlorella vulgaris* was followed each day using Chlorophyll *a* measurements. Three replicate samples were taken at each sampling time in each leachate concentration and also in the control.

Fig 3.2 Growth experiment with light and shaking condition

3.4.3 Determination of optimal leachate concentration for growth

Experiments were set up on to find out the most suitable minimal algal concentration for inoculation.

3.4.3.1 Experiments were set up with the optimum amount of leachate for growth and a range of *Chlorella* inoculums were tested for growth.

The range of *Chlorella* 10 ml inoculums used had Chlorophyll *a* densities of 0.75 , 0.85, 0.93, 2.2, 2.99, 4.39, 5.58 µg/ml or in terms of absorbance at 750 nm (A_{750}) 0.1, 0.115, 0.19, 0.295, 0.4, 0.75.

3.4.3.2 Inoculation *Chlorella vulgaris* was varied over a range of Chlorophyll *a* biomasses and the growth of *Chlorella vulgaris* measured over time.

The optimal leachate inoculum was found to be 20% based on the result from experiment 3.4.2.1 and3.4.2.2.

The determination of minimum -*Chlorella vulgaris*,cell concentration in terms of Chlorophyll *a* which efficiently removes metals.

Control

- Use optimal leachate concentration (20% Garbage Pit Leachate 100 ml)
- Used 24 hours light
- 9 days experimental period
- Growth was run on a shaker

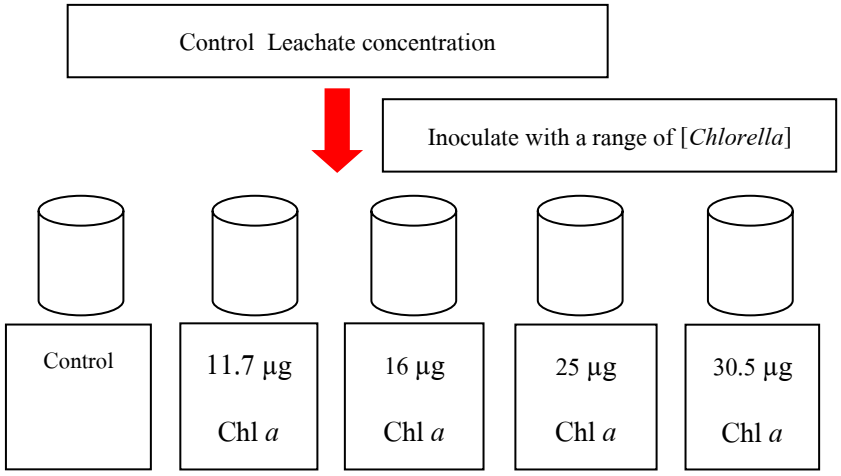

Fig 3.3 Inoculation *Chlorella vulgaris* was varied over a range of Chlorophyll *a* biomasses

3.4.4 Setting the optimal experiment show in Fig 3.3 leachate and algae concentration.

3.4.5 Measurements of heavy metal content of cultures to determine how much heavy metal was removed by the *Chlorella*.

Heavy metals, ammonia-N, nitrate –N, Total-Phosphorus BOD and COD were also measured on the supernatant of the experimental cultures before inoculation with *Chlorella* and at the end of the *Chlorella* incubations.

3.4.6 The effect of pH on heavy metal absorption and used of lime (CaO)

3.4.6.1 Experiment treated with Lime with shaking providing well aerated conditions.

Lime ($CaO/Ca(OH)_2$) were added to Garbage Pit Leachate to increase its pH 10g of Lime per Garbage Pit Leachate 1 liter was added and aerated overnight.

3.4.6.2 Experiment pH varied pH 2- pH 7

pH is known to have large effects on both growth of *Chlorella* sp. and absorption of heavy metals and their toxicity. *Chlorella vulgaris* was grown in leachate at a range of different pH to determine the optimal pH for growth and absorption of metals. This type of experiment was done with appropriate buffers to maintain pH. HEPES buffer was used. The use of acetate as a buffer was not successful.

3.4.7 Using PAM (Pulse Amplitude Modulation Fluorometry) to monitor toxicity on the *Chlorella*

Light saturation curve measurements on the algae were made using a Junior PAM portable chlorophyll fluorometer (Gademann Instruments GmbH, Wurzburg, Germany) fitted with a 1.5 mm diameter optic fibre and a blue diode light source. The Junior PAM uses a magnetic clamp to hold specimens about 1 mm from the end of the light pipe. PAM parameters (effective quantum yield, rETR, NPQ) were calculated using the WINCONTROL software (2.133/03.00) using standard settings for rapid light curves (Heinz Walz GmbH, Effeltrich, Germany) (Genty *et al*., 1989). The default absorptance factor of 0.84 and the default value of 0.5 for estimated absorption of light by PSI and PSII were used on the Junior PAM to calculate the relative Electron Transport Rate or rETR (Ritchie, 2008b). On the standard settings for a rapid light curve, sets of PAM light curve measurements took about 88s to complete with 10 s between actinic flashes of light and each flash of light was 0.8 s duration. The flashes were in order of increasing intensity as nine graded irradiance increments from a nominal zero irradiance. The protocols used for using the Junior-PAM in the present study are described in Saetae *et al.* (2013).

A junior PAM can be used to measure photosynthesis of algae by simply making an "artificial leaf" by filtering algae onto a glass fibre disk. Replicate samples of algal cells (usually 3 or 5 ml cell suspensions) were filtered onto Whatman GF-C glass fibre filters (Whatman International, Maidstone, England, UK) in a Millipore apparatus for 25 mm filters then dark treated in a Petri dish with disks of filter paper impregnated with water (or diluted leachate), for at least 10 min. The absorptance of the algae-impregnated disc was measured using a Blue-RAT machine (Reflectance/Absorbance/transmission) as described by Ritchie and Runcie (2013). Using the actual absorptance value the actual ETR rather than relative ETR can be calculated. Only one light saturation experiment is run on each filter to avoid confounding effects of multiple experimental treatments. The inside diameter of the Millipore filtration apparatus was 15.9 mm and so the disks of algae adhering to the glass-fibre filter have a surface area of 198.6×10^{-6} m^2. The algal-impregnated disks provide highly reproducible material for experiments. Care needs to be taken to avoid the algae-impregnated disks drying out.

The advantage of PAM methods for monitoring toxicity is that results can be measured very quickly and non-destructively (White and Critchley, 1999; Ritchie 2006, Ritchie 2008b; Seatae *et al*., 2012). Similar results as found in the growth experiments can be obtained in a day or two, rather than in periods of weeks. The other advantage of PAM methods is that rapid responses to heavy metal can be monitored. Short-term and long-term effects of metals can be very different. PAM experiments were set up to check the toxicity of leachate which effect to *Chlorella vulgaris* in the short term. Two experiments were set up.

3.4.7.1 Preliminary measurements showed that the Garbage Pit Leachate had very high concentrations of Zn (Thongpinyochai and Ritchie 2013). The toxicity of Zn on *Chlorella* was tested as $ZnSO_4$.

Materials and method:

1) Experiments were set up with media containing a range of $ZnSO_4$ concentrations over a range covering the range of [Zn] found in the leachates 0, 10, 30, 50, 100 µM

2) Two hundred ml of *Chlorella vulgaris* culture grown in BG-11 was centrifuged at 5000 rpm then the pellet was discarded and resuspended again in fresh BG-11 and divided into 7 bottles of 20 ml.

3) The necessary amount of 10 mM stock of $ZnSO_4$ was added to bring the concentration of Zn to the desired concentration. The cells were then incubated for 1 hour under fluorescence light.

4) A millipore filtration apparatus was used to filter 3 ml of cells onto 4 glass fibre disks and placed in a petri dish with a layer of moist filter paper and placed in the dark before being used in a PAM determination of photosynthesis using standard protocols described below.

5) It was shown that $ZnSO_4$ was not very toxic at the normal pH of BG-11 (pH8). The experiment was repeated at pH 5 to test for toxicity of Zinc under acid pH condition as found in Garbage Pit Leachate.

3.4.7.2 The leachate experiment with *Chlorella vulgaris*

Material and method:

1) The leachate was centrifuge at 5000 rpm. 10 minutes and a series of dilutions with distilled water set up: 100%, 50%, 30%, 20% and 10% and a zero control. Each experimental medium had a volume of 20 ml.

2) Six equal volumes of *Chlorella vulgaris* culture were centrifuged and the supernatants discarded. The pellets were then resuspended by vortexing and resuspened in the experimental media. Each cell suspension in the range of leachate concentrations was incubated for 1 hour under fluorescence light.

3) Used a milliopore filtration apparatus to filter 3 ml of cells onto 4 replicate glass fibre disks. The disks were placed in petri disks with a moist filter paper in the dark for 10 minutes before photosynthesis was measured using the PAM machine to investigate the effects of Garbage Pit Leachate on ETR and Yield of *Chlorella vulgaris* inoculations.

3.5 Effectiveness of *Chlorella* sp. in cleaning leachate.

The primary criteria used in this study were % removal of Total- P, N, COD and BOD and how much metals were removed. Chlorophyll *a* of incubated cultures was monitored as a measure of how well the *Chlorella* grew in the various experimental treatments. For metal assay, the supernatant of the cultures and the sediment were (cells + precipitates) measured.

3.6 Chlorophyll Measurement

After photosynthetic electron transport determinations for PAM experiments, chlorophyll was extracted from the glass fiber disks using ethanol (99.5% ethanol neutralised with

magnesium carbonate) and chlorophylls determined as described previously (Ritchie, 2006, 2008a). Chlorophyll measurements on cell suspensions were done on 5 ml sample centrifuged at 5000 rpm for 5 minutes, the supernatant was discarded and the pellet was resuspended by vortexing before adding 3 ml of neutralized ethanol. Resuspending the pellets is important otherwise the added ethanol will not extract Chlorophyll efficiently.

It was difficult to extract chlorophyll from *Chlorella* on glass fibers disks or as pellets in ethanol unless the cells were heated in alcohol in a water bath at about 80 ^{o}C for about 3 minutes. Care needs to be taken to expose extracts to minimum light during extraction and storage. The alcohol extracts were made up to 3 ml and stored at -20 ^{o}C as described previously (Ritchie, 2006; Ritchie, 2008a). Extracts were stored in the dark in a freezer at -20 ^{o}C before spectrophotometric assay for as short a time as practicable. Generally chlorophyll assays were made within a few hours of extraction or the next day but heat-treated chlorophyll extracts (chlorophyllase is readily deactivated by heating) appear to be stable and could be stored at -20 ^{o}C indefinitely.

Chlorophylls were determined from spectrophotometric readings made using a Shimadzu UV-Vis-1601 UV-visible spectrophotometer using quartz glass or plastic cuvettes as described previously (Ritchie, 2006; Ritchie, 2008a). In PAM experiments replicate disks from the same batch of cells generally varied by less than ± 2% in chlorophyll *a* content. For routine chlorophyll *a* determinations on centrifuged cell suspensions replicates normally varied by no more than ± 5%.

3.7 Calculation of rETR on a Chlorophyll Basis

The Walz software calculates ETR on a surface area basis (the surface area of the object illuminated by the beam of blue light) as mol $^{(e-)}$ m^{-2} s^{-1}: this can be converted to mol (O_2) m^{-2} s^{-1} assuming $4e^-/O_2$. The diameter of the glass fibre disks of algae and their chlorophyll content were both known and so mg of chlorophyll per square metre could be calculated. Relative ETR (rETR) in mol $^{(e-)}$ m^{-2} s^{-1} was converted to mol$^{(e-)}$ mg Chl a^{-1} h^{-1} using the chlorophyll assays (as mg Chl *a* m^{-2}) and finally calculated as mol O_2 mg Chl a^{-1} h^{-1} based on ($4e^-/O_2$). If the actual absorptance of the algae on the glass fibre disk is known it is possible to convert the rETR (based on the standard assumption that the

Absorptance is 0.84, Björkman and Demmig 1987) into actual ETR. A simple device called a Reflectance Absorptance Transmission (RAT) machine has been developed to routinely measure ETR (Ritchie and Runcie 2013). In most cases it was found that the actual absorptance was very close to 0.84 and so in general ETR was taken as equivalent to rETR. No absorptance correction was needed because the absorptance of *Chlorella* on disks is very similar to the default values of 0.84.

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3.7.1 Light Curve Fitting

Light curves were fitted to the Waiting-in-Line equation (Ritchie 2008b; Ritchie 2012, Ritchie and Larkum 2013). A form of the equation that is easy to fit using non-linear least squares methods where good initial guesses of $P_{g,\,max}$ and E_{opt} are required (Ritchie 2012) is,

$$P_g = \frac{P_{g,max} \,.\, E}{E_{opt}} \,.\, e^{\,1-E/Eopt} \qquad \text{Equation 1}$$

where, P_g is gross photosynthesis measured as rETR, O_2 evolution or CO_2 uptake,

$P_{g,\,max}$ is the maximum gross photosynthesis,

E_{opt} is the optimal irradiance,

E is the Irradiance (μmol m^{-2} s^{-1} 400 – 700 nm PPFD).

3.8 Statistics

Simple statistical methods were used in the present study. All measurements were done in at least 4 replicates and means and the ±95% confidence limits calculated ($t_{0.05,\,two\text{-}tailed}$). ANOVA tests were used to identify statistically different treatment means. Standard curves were fitted using non-linear least squares methods. Most analyses were made using Microsoft Excel using Zar (2010) as the standard statistical reference text.

CHAPTER 4

Results and Discussion

4.1 Physico-chemical properties of leachate sample

Leachates from two sources were studied first from the holding bay in the incinerator complex (Fig. 1.1) and second from the collecting pond of the landfill site (Fig. 1.1). The leachate from the Garbage Pit Leachate was found to be very toxic and highly contaminated with heavy metals such as Cr and Zn which were both well above legal limits (ref Thai regulations in lit review). The leachate was acid (pH around 4.59-5.22), with high BOD (50-100 g O_2/l), a high COD level (3,000-9,000 mg /l), very high NH_3 concentration (763–2045 mg/l), but with a relatively low level of nitrate-N (14-260 mg /l) and total phosphorus – P (60-270mg/l). The landfill leachate was not above legal limits in heavy metals. The leachate was neutral-basic (pH around 7-8), with lower BOD (60-405 mg/l), a lower COD level (32-160mg/l), NH_3 concentration (170–256 mg/l), relatively low level of nitrate-N (13.6- 48.86 mg/l) and total phosphorus – P (5.57-36.63 mg/l) (Table 4.1).

Table 4.1 Physico-chemical properties of leachate sample

Parameters (mg/l)	Year 2013											
	Mar		April		June		July		Oct		Nov	
	GPL	LF	GPL	LF	GPL	LF	GPL	LF	GPL	LF	GPL	LF
Salinity	0.02	0.01	0.02	0.02	0.01	0.02	0.02	0.02	0.01	0	0	0
pH	4.56	7.3	4.59	8.53	5	8	5.22	8.15	4.58	7.75	5.03	7.84
Ammonia - N	1256.79	234.91	2045.60	197.76	1508.58	217.71	1785.47	256.50	1057.90	218.80	763.8	205.75
Nitrate – N	43.95	17.61	176.79	126.63	267.03	22.25	46.86	14.86	45.98	13.60	50.61	10.35
Total Phosphorus	69.67	5.57	406.25	36.33	290.90	9.16	409.95	18.64	78.91	17.10	146.47	25.97
COD	6,336	86.40	9,088	704	32,640	2,624	3,648	164.26	3,968	32	9,312	128
BOD	65,500	69.28	90,000	70	90,085.7	207	126,750	1,121.25	52,500	405	67000	970

Table 4.1 (cont.)

Heavy Metals (µg/l)	Mar		April		June		July		Oct		Nov	
	GPL	LF	GPL	LF	GPL	LF	GPL	LF	GPL	LF	GPL	LF
Lead (Pb)	20	< 10	20	< 10	< 13	< 13	< 3	< 3	< 12	< 12	< 10	< 1
Chromium (Cr)	150	40	470	20	180	< 2	40	< 1	230	< 10	220	70
Zinc (Zn)	430	< 2	17,300	< 2	1,660	< 4	1,150	< 2	5,580	< 2	7,830	< 4
Copper (Cu)	20	< 1	50	< 1	7	12	< 1	< 1	90	< 2	80	< 1
Nickel (Ni)	590	40	380	30	480	9	170	< 2	330	< 3	320	< 3

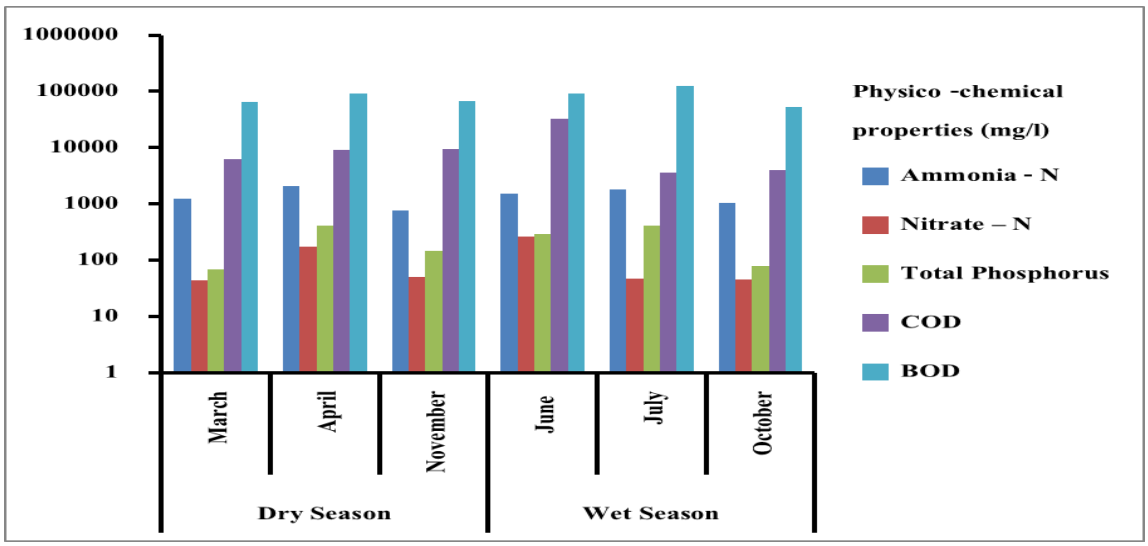

Fig 4.1 Physico-chemical properties of Garbage Pit Leachate in Dry and Wet Season

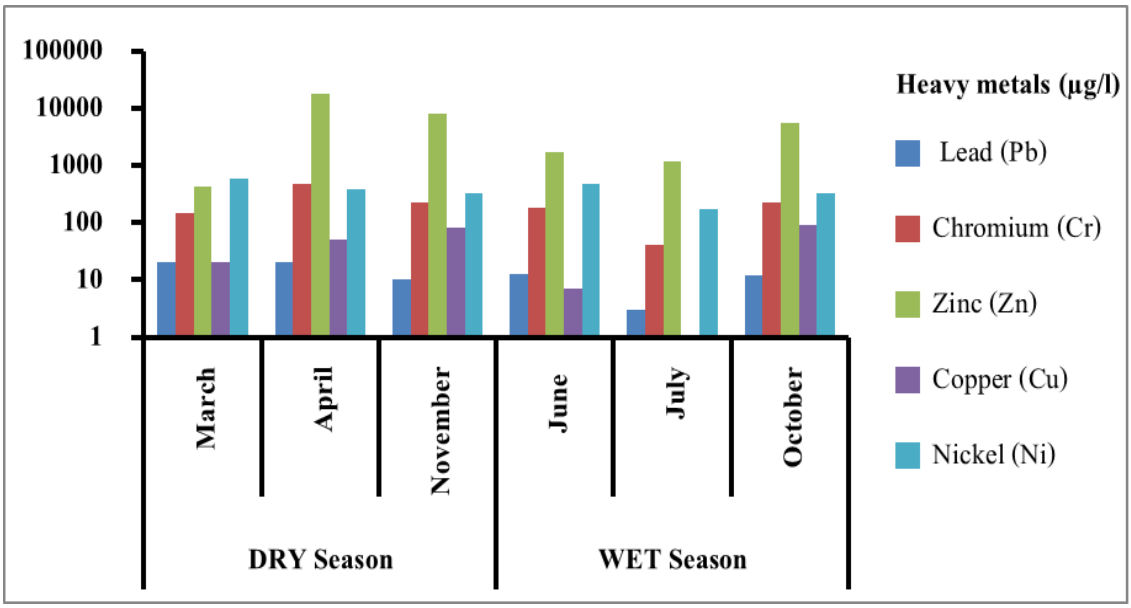

Fig 4.2 The heavy metals of Garbage Pit Leachate in Dry and Wet Season.

Notice : Fig 4.1, 4.2: Y axis is logarithmic base 10

All physico-chemical parameters of Garbage Pit Leachate were very high: ammonia-N (763 – 2045 mg l^{-1}, high COD level (3,000-9,000 mg l^{-1}) and high BOD (50-100gO_2l^{-1}). In Wet and Dry Season (Fig 4.1 and 4.2) the Garbage Pit Leachate is the leakage from piles of garbage awaiting incineration and is under cover so it does not receive any rainwater. The leachates are therefore quite different in how they are generated and so their physico-chemical properties would be expected to be different. Leachate quality was similar to Lin *et al*. (2007) who studied the leachate pond in the Li Keng Landfill, Guangzhou, China however the pH was considerably different. The leachate of the Phuket incinerator had a low pH of about 5.5 but the leachate studied by Lin *et al*. (2007) had a pH of about 7.8. The landfill leachate studied by Lin *et al*. (2007) was from a landfill filled with domestic garbage and its metal content was lower than found for the Phuket Garbage Pit Leachate which comes from garbage that is only a few days old and awaiting incineration.

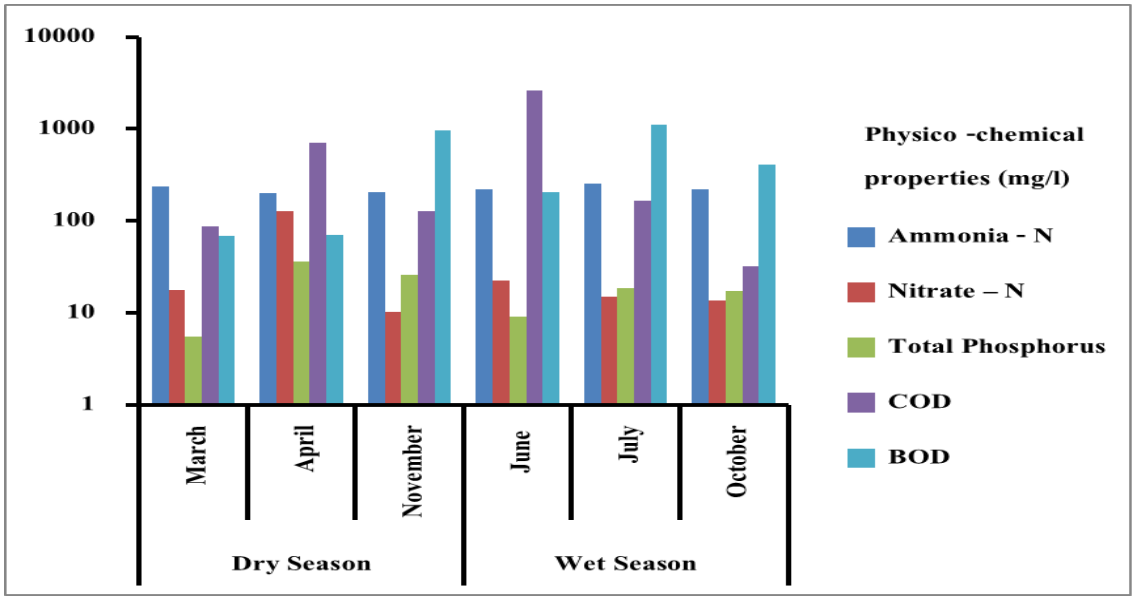

Fig 4.3 Physico-chemical properties of Landfill Leachate in Dry and Wet Season

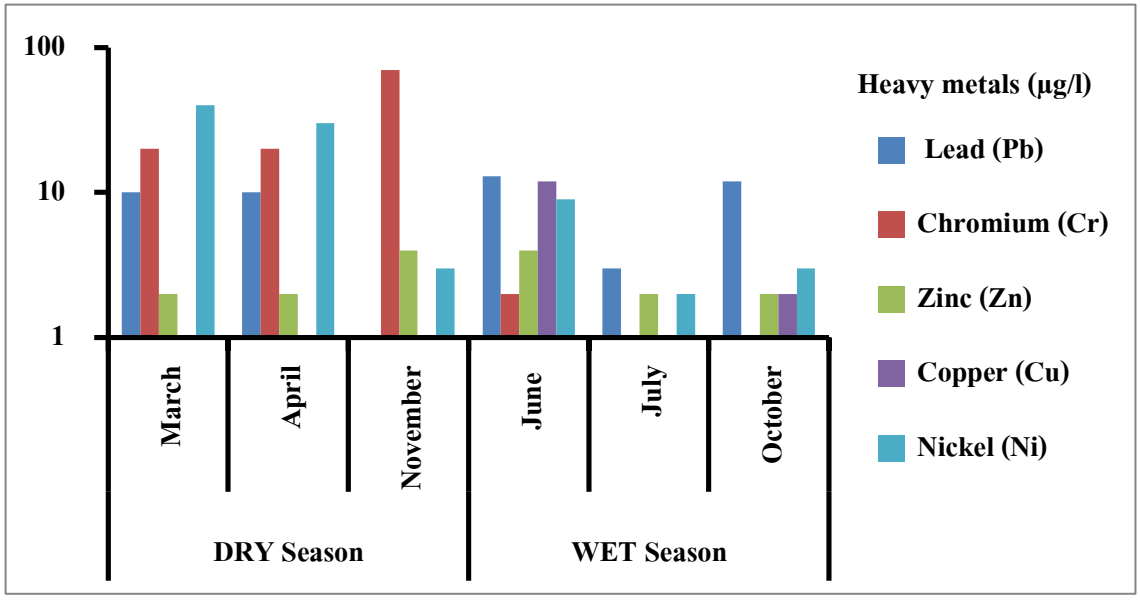

Fig 4.4 The heavy metals of Landfill Leachate in Dry and Wet Season.

Notice : Fig 4.3, 4.4 : Y axis is logarithmic base 10

The physico-chemical analyses of Landfill Leachate in Dry and Wet season (Fig 4.3 and 4.4) show that the heavy metals such as Chromium are higher in the dry season than in the wet season but nevertheless are still below the Thai standards legal limit. The source of landfill leachate is primarily rainwater percolating through the landfill site (and hence would be expected to be different in both volume and composition in the wet and dry season). Pohland and Kang (1975) and Chain and De Walle (1975) have shown that the chemical composition of landfill leachate depends on factors such as the fill material (organic content, degradability, solubility), geological conditions and the age of the landfill. Bull *et al*., (1983) states that the composition of leachate cannot be predicted accurately but quoted the typical ranges of composition derived from local experience (suburban Sydney, Australia). The Effluent standard in Thailand (Pollution Control Department, Thailand) has published data for the typical composition of leachates from UNEP (2005) has published data on typical leachates found from municipal solid waste landfills in developing countries. Phuket has characteristics of both a developed and a developing economy because it has large tourist areas with a western lifestyle.

Table 4.2 Comparison of physico-chemical properties of leachate with the Effluent standard in Thailand (Pollution Control Department, Thailand), UNEP (2005) compared to findings for Phuket landfill and Garbage Pit Leachate.

Component (mg/L)	Characteristics of leachate generated from decomposition of municipal solid wastes in developing countries (UNEP, 2005). (mg/l)	Effluent standard in Thailand (Pollution Control Department, Thailand)	Phuket-Landfill leachate (mg/l)	Phuket-Garbage Pit Leachate (mg/l)
BOD (5 days)	20 – 40,000	< 60	60-405	52,500 - 126,750
COD	500 - 60,000	< 400	32-160	3,000-9,000
Ammonia-N	30 - 3,000	-	170– 256	763 – 2045
Nitrate –N	0.1 - 50	-	13.6- 48.86	14- 260
Phosphate as P	0.1 - 30	-	5.57-36.63	60-270
pH	4.5-9	5.5-9	7-8	4.59-5.22

Table 4.2 (cont.)

Heavy metals (µg/l)	Characteristics of leachate generated from decomposition of municipal solid wastes in developing countries (UNEP, 2005). (µg/l)	Effluent standard in Thailand (Pollution Control Department, Thailand) (µg/l)	Phuket-Landfill leachate (µg/l)	Phuket-Garbage Pit Leachate (µg/l)
-Chromium	Not available	< 1,000	< 1 - 20	40 – 470
-Zinc	30 – 120,000	< 5,000	< 2	430 – 17,300
-Copper	4 - 1,400	< 2,000	< 1	20 – 90
-Nickle	Not available	< 1,000	< 10 - 40	170 – 590
-Lead	8-1,020	< 200	< 13	< 3- 20

The physico-chemical composition of landfill leachate in Phuket was comparable to the range of values found in Effluent standards in Thailand and typical of values found in developing countries (Pollution Control Department, Thailand and UNEP, 2005).Table 4.2 shows the physico–chemical properties of leachate from the Phuket landfill and the Phuket Garbage Pit Leachate compared with Thai and International standards values. The analyses for Phuket shown in Table 4.2 are analyses based on 6 separate collection trips spread over 6 months that included collections made in both the wet and dry season (Table 4.1 and Figs 4.1, 4.2, 4.3, 4.4). The BOD level of Phuket Landfill and Phuket-Garbage Pit Leachate were both higher than the Thai standard, however BOD level of Phuket Landfill was in the range of international standards. The COD levels were on the lower side of values found internationally but the COD of Phuket Garbage Pit Leachate was well over the Thai standard. Levels of metals were very low by both Thai and International standards with the notable exception of zinc in the Garbage Pit Leachate which sometimes exceeded both Thai and international standards. Phuket does not have a large amount of heavy industry. Ammonia levels of the Phuket landfill are high but the Garbage Pit Leachate has extremely high incinerator BOD (5 days) levels and very high COD. Nitrate levels in both the Phuket landfill leachate and the Garbage Pit Leachate were much higher than UNEP (2005) Standard. The BOD

of the Garbage Pit Leachate is exceptionally high. In combination with the very high ammonia content this results in BOD > COD. The Garbage Pit Leachate would have had many ammonia oxidizing bacteria which resulted in BOD > COD as a result of microbial oxidation of the ammonia (ultimately to NO_3) (section 5-13-APHA, 1998, Attiogbe *et al*., 2007). This interpretation is supported by the high observed Nitrate-N. Total Phosphorus of the Garbage Pit Leachate was also exceptionally high. The levels of heavy metals in the Phuket landfill leachate were generally low by international standards but the levels of heavy metals in the Phuket Garbage Pit Leachate were high by international standards in particular Zinc.

Heavy Metal: Basic use of bioremediation methods

4.2 *Chlorella vulgaris* standard growth

4.2.1 Standard curve for blank (medium) solution. (In Triplicate)

Exponential growth rate constant or doubling time (t_2) were both calculated and compared between different experimental conditions. A *Chlorella* culture was set up to measure doubling time of *Chlorella vulgaris* under optimum conditions in BG-11 medium. Growth was followed as absorbance at 750 nm (A_{750}).

X: axis ⟶Period (hours)

Y: axis⟶Absorbance 750 nm

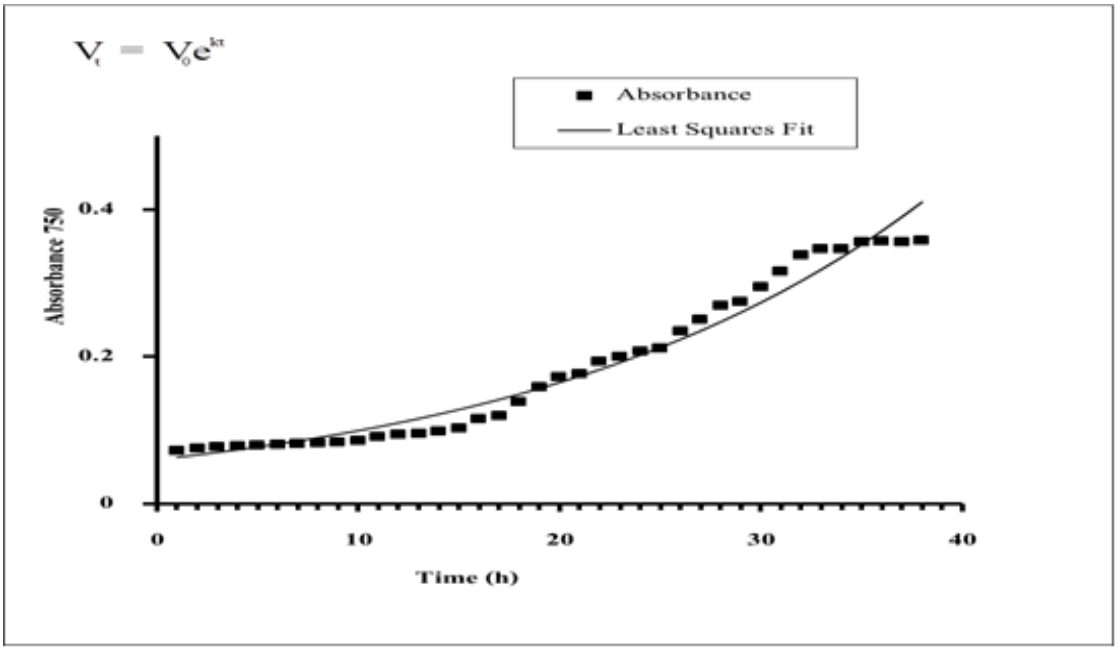

Fig 4.5 *Chlorella vulgaris* growth curve apparent doubling time.

The growth curve shown in Fig. 4.5 is a typical growth curve for a microbe. There is a lag-phase, a log phase and a stationary phase. The Fig. 4.5 *Chlorella vulgaris* growth curve apparent doubling time for the log phase was: $k = 0.0506 \pm 0.00357\ h^{-1}$ ($n = 38$, ±95% conf. lim.), $t_2 = 13.7 \pm 0.96$ h with a correlation $r = 0.984$.

4.2.2 Standard relationship between Absorbances 750 nm and Chlorophyll *a*

This experiment was set up to measure the relationship between $A_{750\ nm}$ and biomass as measured by Chl *a* of *Chlorella vulgaris*. Using the A_{750}/Chl *a* relationship the amount of *Chlorella* used as a starting inoculum for experiments could be calculated.

X: axis ⟶Absorbances 750 nm used to measure the amount of cells present.

Y: axis ⟶Chlorophyll *a* measured as described by Ritchie (2006).

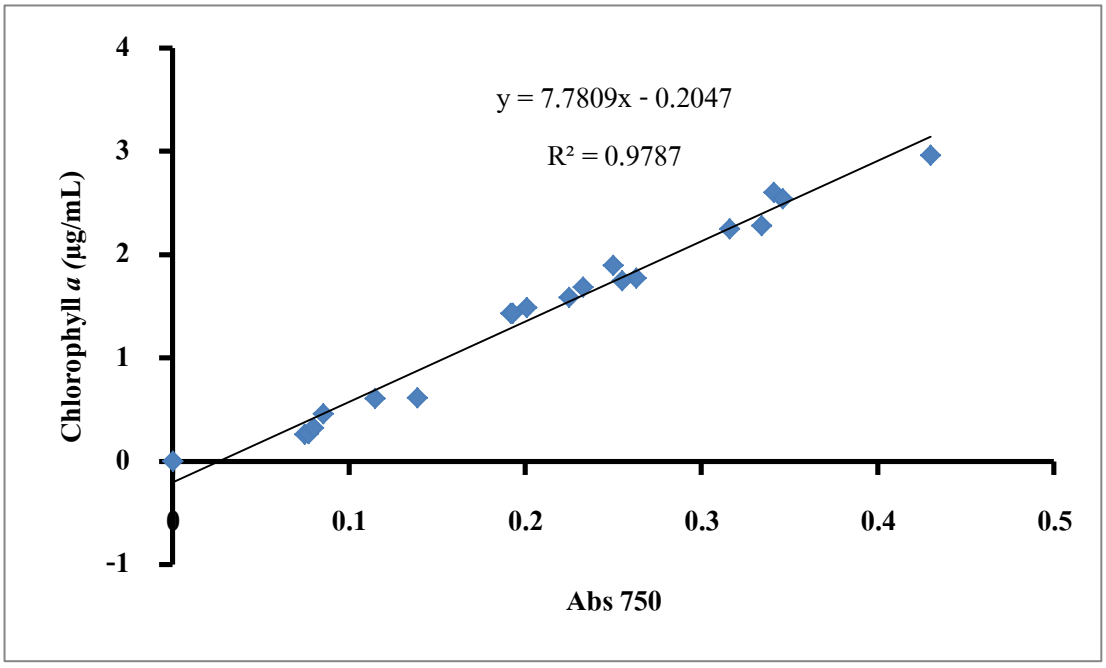

Fig. 4.6 Chlorophyll *a* content of culture vs. Absorbance of culture

4.3 Determine minimum inoculation of *Chlorella vulgaris* for growth in leachate in terms of Chlorophyll *a*

Chlorophyll *a* of *Chlorella vulgaris* was determined at the start of the experiment as described above (Fig. 4.1 and 4.2). Experiments were set up with inoculations of 0.259, 0.32, 0.92, 1.485 and 2.543 µg/ml Chlorophyll *a* and absorbances (A_{750}) of 0.075, 0.08, 0.19, 0.201 and 0.346 and cultivated in 100% the landfill leachate and Garbage Pit Leachate (the leachate of March) for 7 days in the light.

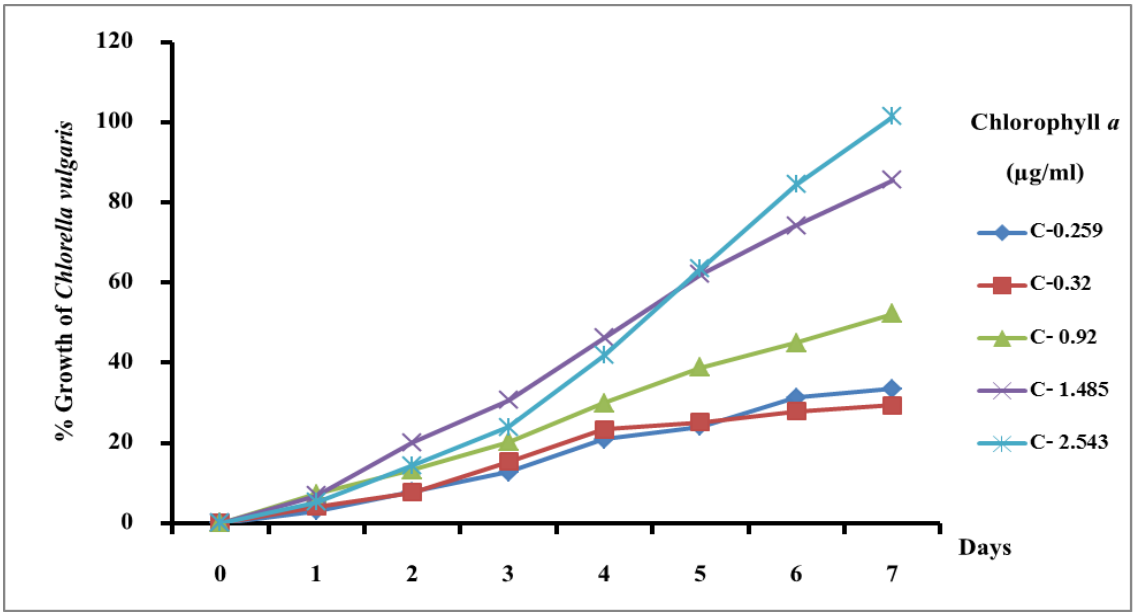

Fig 4.7 Effect of inoculation volume on the establishment of *Chlorella* in landfill leachate.

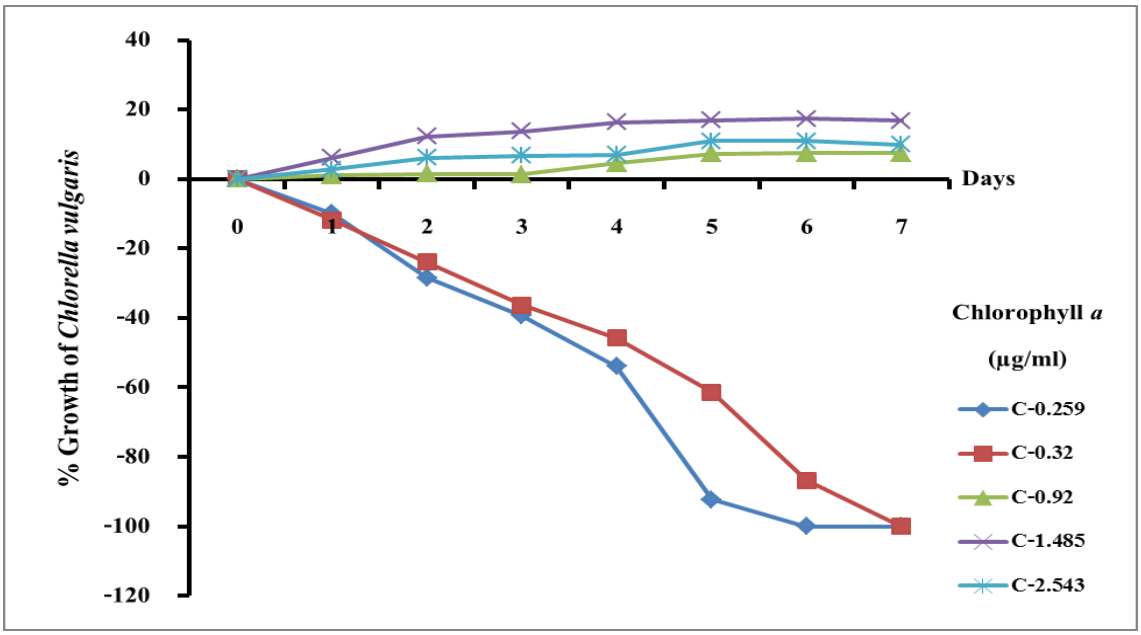

Fig. 4.8 Effect of Inoculation volume on the establishment of *Chlorella* in Garbage Pit Leachate

Notice: In Figs 4.7 and 4.8 the SE errorbars of the calculated means (n = 3) are less than ±2% and

so do not show on the graphs.

Fig. 4.7 shows that *Chlorella* established itself very well in the landfill leachate and grew exponentially even with the minimum inoculum. The results showed in the landfill leachate a *Chlorella* inoculum as low as 0.259 µg/ml Chlorophyll a (A_{750} = 0.075) grew well in the landfill leachate. Fig. 4.8 shows that the Garbage Pit Leachate was highly toxic. Low-level inoculations of *Chlorella* slowly died off. The heavier inoculations did not die but only grew marginally. Only the heaviest inoculation showed substantial growth (0.92 µg/ml Chlorophyll *a* ($A_{750\ nm}$ = 0.19). From these experiments it was concluded that a heavy inoculation of *Chlorella* was needed for the alga to grow in the Garbage Pit Leachate. We used the same biomass of Chlorophyll *a* (0.92 µg/ml) to cultivate in the landfill leachate and Garbage Pit Leachate.

4.4 The effect of different concentrations and conditions of leachate on algal growth.

4.4.1 Phase 1: Growth in light under non-shaking conditions.

4.4.1.1 Growth experiments on landfill leachate

The growth curves of *Chlorella vulgaris* in 10%, 30%, 50% and 100% (undiluted) landfill leachate solutions and incubation experimental landfill diluted with tap water (10%, 30%, 50% and 100%) were set up and inoculated with 10 ml of actively growing *Chlorella* culture (Chlorophyll *a* 0.9 µg/ml, Abs_{750} = 0.19; Chl *a* calculated from the result of Fig 4.7 and Fig 4.8). Growth was followed over a 9 day experimental period. *Chlorella* grew in all the dilutions of landfill leachate (in terms of Chlorophyll *a*). The *Chlorella* grew the best in media containing 30% landfill leachate (Analysed using two way ANOVA with replication). The growth experiment was run on each monthly sample (over 6 months) with 3 replicates per concentration (the data points shown in Fig. 4.9 are means ± SE error bars from the 6 months data. The detailed data is in the Appendices III).

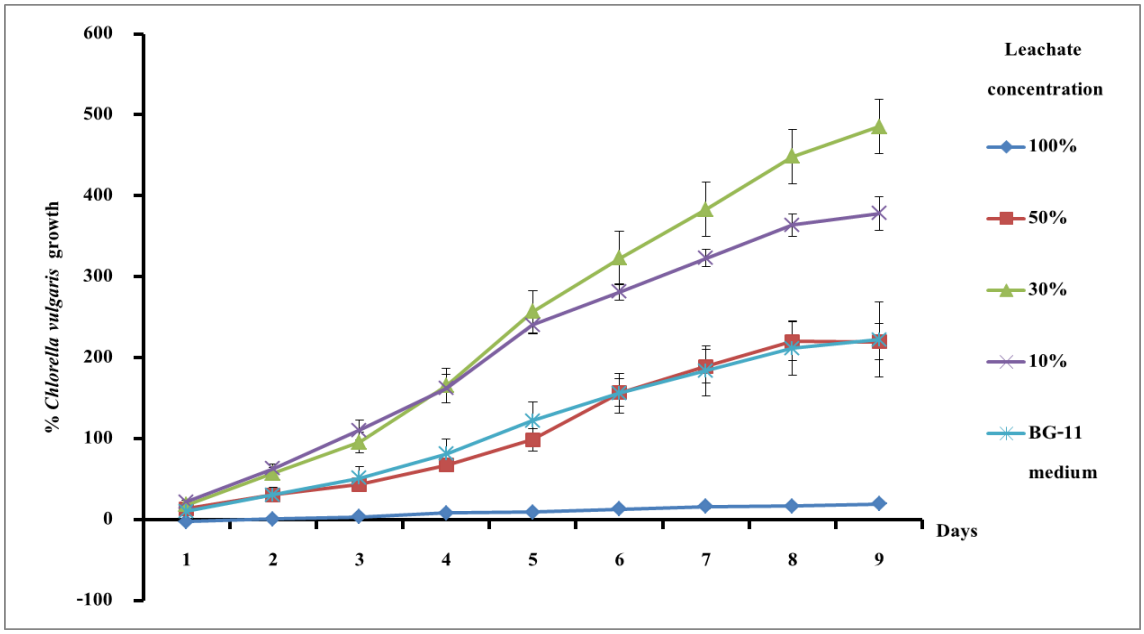

Fig 4.9 Percent growth of *Chlorella* in different concentration of landfill leachate

Chlorella grew the best in landfill leachate diluted 30% but grew almost as well in 10% leachate. *Chlorella* survived but did not grow very well in 100% leachate. The pH of the leachate was about pH 7 to 8 (Table 4.1). The leachate has high ammonia-N (Table 4.1) and this might account for the poor growth in 100% leachate. Che Sa (2011) used landfill leachate diluted with seawater (5%-10% diluted) for culturing microalgae. *Chlorella* and *Nannochloropsis* were able to grow in seawater diluted leachate but most of the microalgae tested would not grow in the diluted leachate. Cultures were grown under 12:12 hours (Dark: Light) but the experiments were run under 24 hours light. Seawater also has a pH of about 8.1 but metals tend to precipitate out of seawater because of its high salinity and dissolved bicarbonate. Thus the growth rates they found would be expected to be different (Fig 4.9).

4.4.1.1.1 Measurement of Total-P, ammonia-N, nitrate-N, BOD and COD on supernatant of experimental cultures at the beginning and end of incubations.

Percent removal of Ammonia-N, Nitrate-N, Total Phosphorus, COD and BOD were measured in cultures of *Chlorella* grown in leachate diluted to 30% with tapwater over a 9 day period. Controls were diluted leachate with no added *Chlorella*. Ammonia-N,Nitrate-N, Total Phosphorus, COD and BOD were measured at end of the incubation experiment (t = 9 d) and the leachate used was assayed at the beginning of the experiment (t = 0 d). The percent removal rates from the diluted leachate (compared to the leachate properties at t = 0) were: ammonia–N (53.91±0.75%), nitrate-N (31.74±3.49%), total phosphorus (65.77±2.60%), COD (51.05±1.17%) and BOD were removed (52.78 ±1.38%). Percent removal rate of ammonia-N was relatively a high compared to the low rate of removal of nitrate-N because ammonia is the preferred N-source over nitrate and *Chlorella* will use up the ammonia before turning to nitrate as a N-source because nitrate has to be first converted to nitrite and then to ammonia before it can be assimilated by Glutamine synthetase in *Chlorella* (Glutamate + NH_3+ATP ⟶ Glutamine + ADP + Pi).

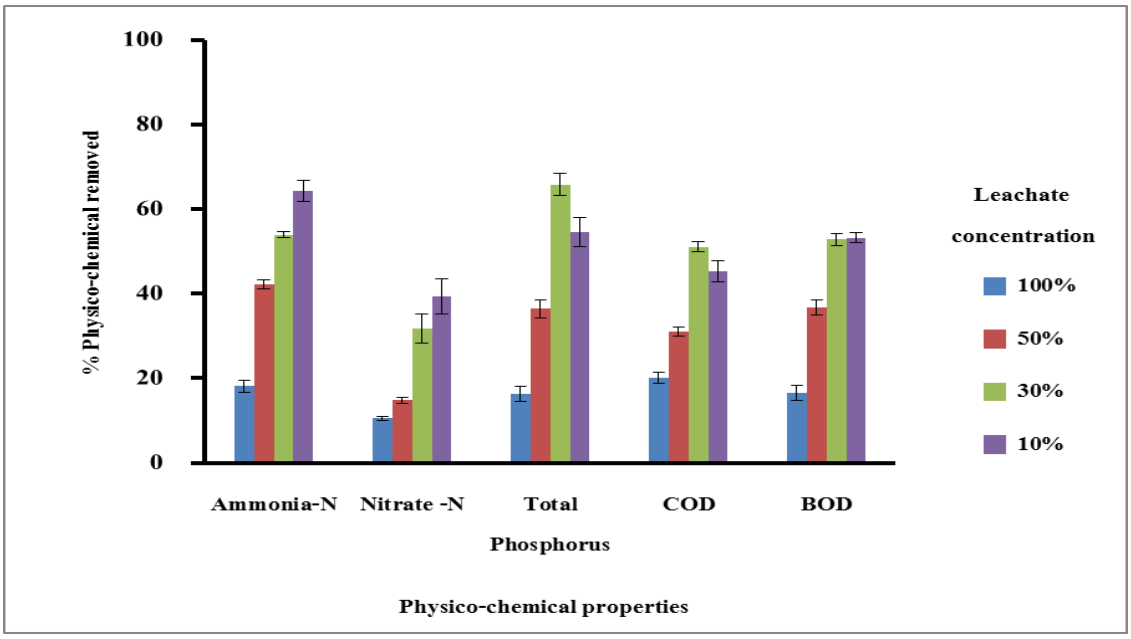

Fig4.10 Percent of nutrients removed from the landfill leachate after 9 days incubation.

Fig. 4.10 shows that % removal of ammonia-N, nitrate-N, total-P, COD and BOD were all extremely poor in 100% leachate, intermediate for leachate diluted 50% with tapwater and highest for leachate diluted to 10 and 30% using tapwater.

Significant % removal effects were tested for using the Tukey Test interval (p 0.05) as described by Zar (2010). Ammonia –N removal at all different concentrations of landfill leachate was significant at the ±95% confidence level. Nitrate – N % removal by 100% and 50% landfill leachate were not significantly different and % removal was low (about 12% removal), in both the 10% and 30% landfill leachate about 35% was removed. Total-P percent removal was significant at all different concentrations of landfill leachate at the ±95% level. COD and BOD percent removal by 30% and 10% landfill leachate was both about 53% but removal of BOD and COD in 50% landfill leachate was about 30% and significantly different to the removal rates in 50 and 100% landfill leachate. Removal of both COD and BOD in 100% leachate was very low (about 16%) and was significantly lower than removal at all other dilutions of landfill leachate. (The ANOVA and Tukey test results are on the data disk).

4.4.1.1.2 Measurements of heavy metal content of cultures to determine how much heavy metal was removed by the *Chlorella.*

The *Chlorella* grew in every concentration of landfill leachate even though in 100% undiluted leachate the *Chlorella* survived but did not grow (Fig. 4.9). The heavy metals in landfill leachate before treatment were under the legal limit but nevertheless the alga grew very poorly in undiluted leachate. However, the sample was sent after treatment in 100% of leachate (because it was the maximum of leachate in which *Chlorella* grew) to determine the removal of heavy metals.

Table 4.3 Percent removed of heavy metal from landfill leachate by *Chlorella*

Heavy metals	**Landfill leachate**		
(µg/l)	**Before treated**	**After treated**	**% Removed**
Copper (Cu)	< 1	< 1	-
Chromium (Cr)	20	6	70
Lead (Pb)	< 10	< 10	-
Nickel (Ni)	30	10	66
Zinc (Zn)	< 2	< 2	-

Table 4.2 shows percent removed heavy metal after incubation *Chlorella* in 100% of landfill leachate 70% of Chromium and 66% of Nickel were removed. Copper, Lead and Zinc were not detectable in the landfill before or after inoculation. From the results shown in Figs 4.9 and 4.10 and Table 4.2 it is reasonable to conclude that either the toxicity of the heavy metals is at extremely low concentrations (1 ppm) or that the toxicity of the leachate is not a function of its heavy metal content but some other pollutant. Heavy metal content of the landfill leachate was consistently low (Table 4.1) but nevertheless unless it was diluted with tapwater to about 30% *Chlorella* would not grow very well. Previous studies have shown that much of the toxicity of landfill leachates is due to the very high ammonia content (Bull *et al*., 1983; Ritchie *et al*., 2001; Silva *et al*., 2004; Lin *et al*., 2007). Ammonia toxicity is also commonplace in sewage treatment plants (Adamsson *et al*., 1998). It is known that ammonia exacerbates metal toxicity because it increases the solubility of metals. Ammonia toxicity can be reversed by ammonia stripping (alkalinisation then aeration to evaporate the NH_3 into the atmosphere) (Silva *et al*., 2004) or by biological removal (Adamsson *et al*., 1998).

The *Chlorella vulgaris* growth in landfill leachate Phase1: *Chlorella* in light under non-shaking conditions grew the best in 30% of landfill leachate. Compared to measurements made at the beginning of the incubation, the overall rates of removal based on all six replicate runs of the

experiment were (mean % ± SE%): Ammonia-N 53.91 ± 0.75%, Nitrate-N 31.74 ± 3.49%, Total-P 65.77 ± 2.6%, COD 51.05 ± 1.17% and BOD 52.78 ± 1.38%. The heavy metal content of the landfill leachate was consistently low but nevertheless unless it needed to be diluted with tapwater to about 30% for good growth of *Chlorella* to occur. *Chlorella* grew but not very well in 100% landfill leachate but under such conditions removed 70% of the Chromium and 66% of the Nickel.

4.4.1.2 Growth experiments on Garbage Pit Leachate

The Garbage Pit Leachate was much more toxic than the landfill leachate (Thongpinyochai and Ritchie 2013). Experiments to test the toxicity of Garbage Pit Leachate followed a similar protocol to those described above for experiments with landfill leachate. *Chlorella* would not grow in 100%, 50% or 30% leachate and slowly died off over the course of the experiment (Fig. 4.11). *Chlorella* grew well in the undiluted tapwater and grew marginally in Garbage Pit Leachate diluted to 20% and 10% in tapwater (20% leachate grew +11% and 10% leachate grew+9%). Fig. 4.12 shows the growth data in more detail without including the control shown in Fig. 4.11. The data was analysed using two-way ANOVA with replication. The experiment was repeated each month over 6 months and each growth experiment was run in 3 replicates per concentration.

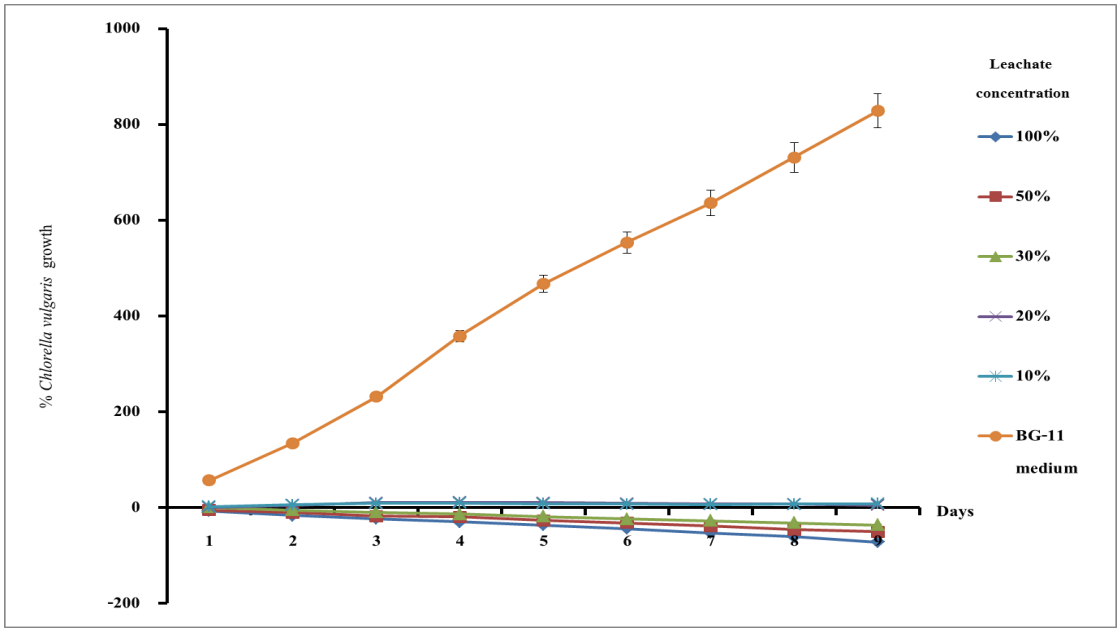

Fig 4.11 Percent growth of *Chlorella* in different concentrations of Garbage Pit Leachate (treatment control with no leachate). The garbage pit leachate is very toxic.

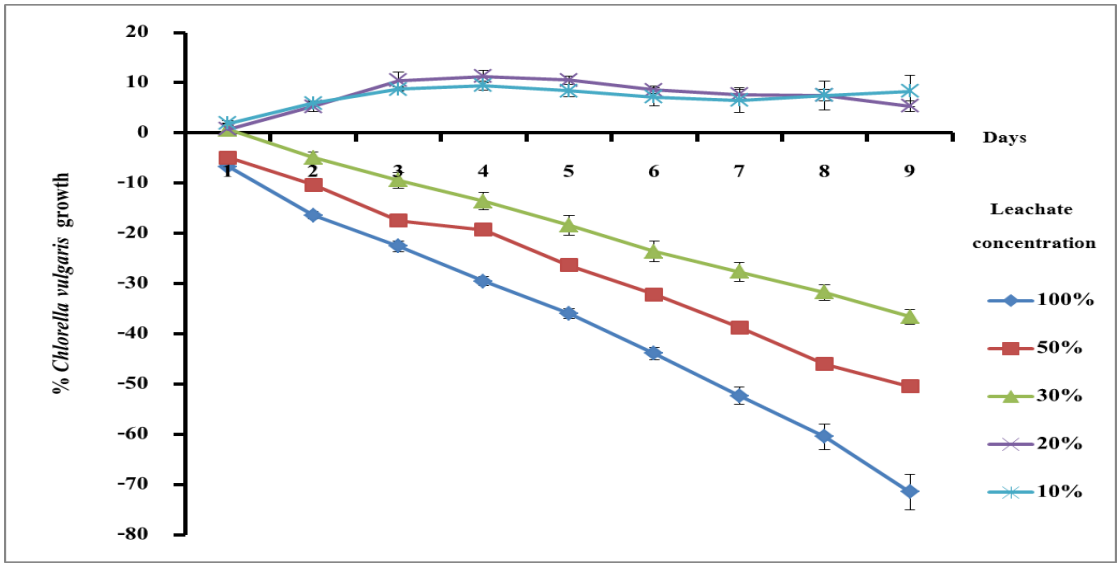

Fig 4.12 Percent growth of *Chlorella* in different concentration of Garbage Pit Leachate (treatment) showing more detail on the effects of various dilutions of added Garbage Pit Leachate shown in Fig. 4.11.

4.4.1.2.1 Measurement of ammonia-N, nitrate-N, Total-P, BOD and COD on supernatant of experimental cultures at the beginning and end of incubations.

Garbage Pit Leachate–Percent removal of Total-P, ammonia-N, BOD and COD by *Chlorella* cultures were measured after a 9 day incubation in a range of dilutions of Garbage Pit Leachate. Percent removal of Total- P, ammonia-N, BOD and COD were calculated using dilutions of leachate that were not inoculated with *Chlorella*. The Total-P, ammonia-N, BOD and COD were also measured in the Garbage Pit Leachate at the start of the experiment. The 20% Garbage Pit Leachate diluted with tapwater showed the highest % removal rates than the other concentrations of leachate (Fig 4.13). The percent removed compared to the control blank were: ammonia–N (24.67 ± 1.45%), nitrate-N (20.17 ± 1.90%), total phosphorus (27.32±0.96%), COD (25.21 ± 1.50%) and BOD (24.6 ± 1.22%). Removal of the pollutants from the Garbage Pit Leachate was much poorer than in the case of the similar incubation experiments run on landfill leachate (Fig. 4.10). The experiment was repeated on monthly collections of leachate over six months and each experiment was run in 3 replicates.

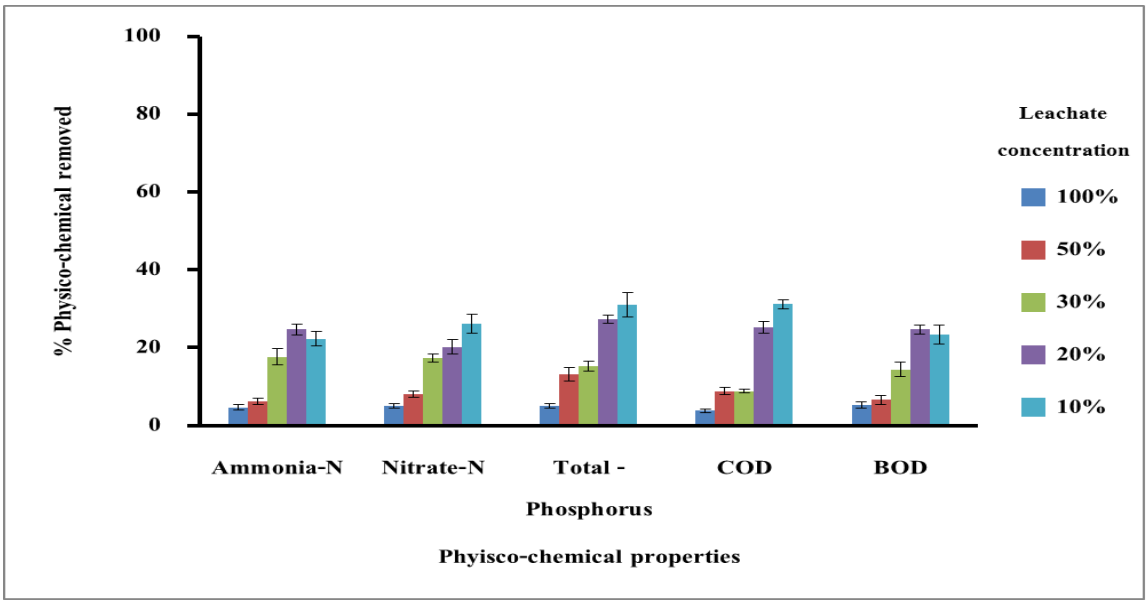

Fig 4.13 Percent removal of nutrients by *Chlorella* from dilutions of Garbage Pit Leachate after 9 days incubation.

In the case of the Garbage Pit Leachate all the physico-chemical were high compared to the landfill leachate (Table 4.1 and 4.2) and growth of *Chlorella* was very poor in dilutions of Garbage Pit Leachate (Fig. 4.9 vs. Fig. 4.11). The Garbage Pit Leachate was so inhibitory that positive growth was only recorded in the lowest dilutions of leachate (10% and 20%, Figs 4.11 and 4.12). BOD, COD and ammonia-N were all very high in the Garbage Pit Leachate (Table 4.1) and the heavy metals Chromium (Cr) and Zinc (Zn) were both well above legal limits.

Significant percent removal effects were tested for using the Tukey Test interval (p 0.05) as described by Zar (2010). Ammonia –N removal by 100% and 50% Garbage Pit Leachate were not significantly different and % removal was low (about 5% removal); in both the 10% and 20% Garbage Pit Leachate about 23% was removed. Nitrate – N % removal by 100% and 50% Garbage Pit Leachate were not significantly different and % removal was low (about 6% removal); in both the 30% and 20% Garbage Pit Leachate about 18% was removed. Total-P % removal by 50% and 30% Garbage Pit Leachate were not significantly different and % removal was low (about 14% removal), in both the 10% and 20% Garbage Pit Leachate about 29% was removed. COD % removal was significant by 10% and 20% Garbage Pit Leachate at the ±95% level; in both the 50% and 30% Garbage Pit Leachate about 8% was removed. BOD % removal was significant by 100%, 50% and 30% Garbage Pit Leachate at the ±95% level; in both the 20% and 10% Garbage Pit Leachate were not significantly different, about 24% was removed (The ANOVA and Tukey test results are on the data disk).

4.4.1.2.2 Measurements of heavy metal content of cultures to determine how much heavy metal was removed by the *Chlorella*

The Garbage Pit Leachate was very toxic, with high ammonia and low pH (4.9-5.22). These factors were thought to be the important factors that affect the growth of *Chlorella* in leachate. Dilutions of Garbage Pit Leachate inoculated with *Chlorella* and grown without shaking for 9 days were sent for analysis for heavy metal content (Table 4.3).

Table4.4 The heavy metal result from Garbage Pit Leachate by *Chlorella* incubated without shaking.

Heavy metals (µg/l)	Dilutions of Leachate									
	100%		50%		30%		20%		10%	
	Before	After	Before	After	Before	After	Before	After	Before	After
Copper (Cu)	30	30	3	7	< 1	< 1	< 1	< 1	< 1	< 1
Chromium (Cr)	22	21	11	12	60	60	30	40	< 1	< 1
Lead (Pb)	< 10	< 10	< 10	< 10	< 10	< 10	< 10	< 10	< 10	< 10
Nickel (Ni)	350	320	170	180	100	100	70	120	100	100
Zinc (Zn)	7,930	7,290	4,230	4,300	2,640	2,640	1,600	2710	270	370

The results of heavy metals analysis (Table 4.4) shows that *Chlorella* was not successful in removing heavy metals at any of the dilutions of Garbage Pit Leachate tested. This can be attributed to the lack of growth of the *Chlorella* resulting in no binding up of the toxic metals in insoluble form (Roscko and Rachlin, 1977). This is consistent with the very high toxicity shown in Figs 4.11 and 4.12 and the poor removal of ammonia-N, nitrate-N, Total-P and BOD and COD (Fig. 4.13). In the dilution treatments where there was some growth of *Chlorella* (10% and 20% Garbage Pit Leachate diluted with tap water) there was some increase in available Zn probably due to the breakdown of organic matter. *Chlorella* slowly died in 100%, 50% and 30% Garbage Pit Leachate (Fig. 4.11). The pH of Garbage Pit Leachate started at pH 5.05 and the end of incubation pH was 4.9. This pH change is small but Starodub *et al.* (1987) and Rai *et al.* (1993) found increasing metal toxicity with decreasing pH to be due to the predominance of the free metal ion at low pH.

The *Chlorella vulgaris* growth in Garbage Pit Leachate Phase1: *Chlorella* in light under non-shaking conditions grew the best in 20% of Garbage Pit Leachate. Compared to measurements made at the beginning of the incubation, the overall rates of removal based on all six replicate runs of the experiment were (mean % ± SE%): Ammonia-N 24.67 ± 1.45%, Nitrate-N 20.17 ± 1.90%, Total-P 27.32 ± 0.96%, COD 25.21 ± 1.50% and BOD 24.65 ± 1.22%. Heavy metals: *Chlorella vulgaris* was not successful in removing heavy metals at any of dilutions of Garbage Pit Leachate tested (Table 4.4).

4.4.2 Phase 2: Growth in light under shaking, well aerated, conditions

Growth experiment –*Chlorella* was grown over a 9 day cultivation period with shaking on an orbital shaker in a range of diluted Garbage Pit Leachate: 100%, 50%, 30%, 20%, 10% leachate diluted with tapwater. Growth in 100% BG-11 medium was used as the blank control. Fig. 4.14 shows that growth in undiluted leachate was very poor and the alga died off with time. Positive growth occurred in 20% and 10% leachate (Analysed with ANOVA two ways with 5-fold replication) with another concentration. Fig. 4.15 shows more detail of the response of the *Chlorella* to various concentrations of leachate. In terms of Chlorophyll *a*, the alga was able to grow continuously over the course of the experiment in 10 and 20% Garbage Pit Leachate but at all higher concentrations gradually died off with time. Growth of *Chlorella* in Garbage Pit Leachate was much better under well-aerated shaking conditions (Figs 4.14 and 4.15) than cultures kept under not shaking conditions (Figs 4.11 and 4.12) nevertheless Garbage Pit Leachate at any of the dilutions tested was severely inhibitory compared to the blank control grown in tapwater. Comparison with the results for the landfill leachate experiments clearly show that the Garbage Pit Leachate was much more highly toxic.

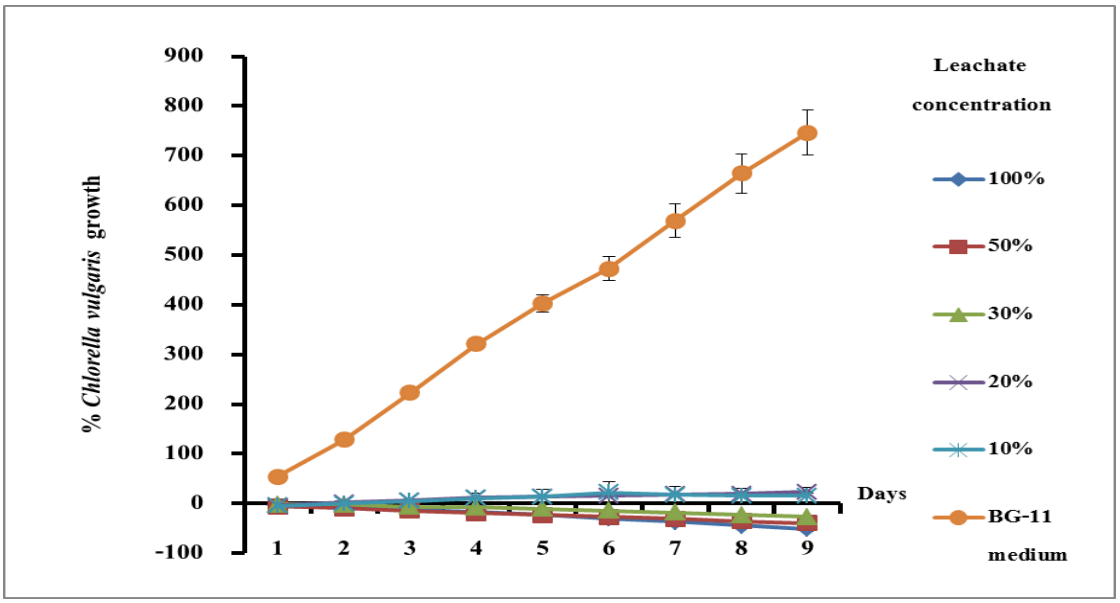

Fig 4.14 Effect diluted Garbage Pit Leachates on growth of *Chlorella* under shaken conditions (Treatments and control – BG-11)

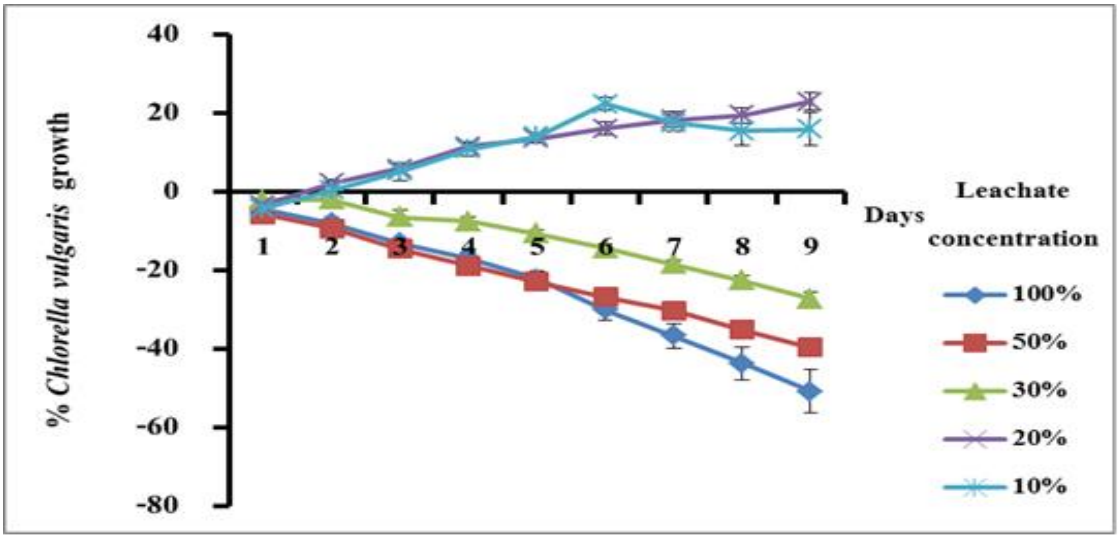

Fig 4.15 More details on the effect of diluted Garbage Pit Leachates on the growth of *Chlorella* in light and shaken conditions. Aeration only slightly reduces the toxicity of Garbage Pit Leachate.

4.4.2.1 Measurements of Ammonia-N, Nitrate-N, Total-P and BOD and COD of diluted Garbage Pit Leachates inoculated with *Chlorella* and incubated for up to 9 days.

Removal of pollutants from the Garbage Pit Leachate by *Chlorella* was better under aerated and shaken conditions than in the case of static cultures (Fig. 4.16). During the 9 day cultivation period, the 20% Garbage Pit Leachate diluted with tapwater had the highest % removal the initial condition (Garbage Pit Leachate at t = 0): from control of ammonia-N (41.5 ± 1.22%), nitrate-N (32.4 ± 1.45%), Total Phosphorus (55.1 ± 2.56%), BOD (49.2 ± 1.74%) and COD (50.8 ± 3.20%). Similar results were found for the 10% Garbage Pit Leachate experiment. There was no improvement in these parameters in the case of 100% Garbage Pit Leachate inoculated with *Chlorella*. The results seem to correlate very well with the *Chlorella* growth measurements shown in Figs 4.11, 4.12 and 4.14 and 4.15: the better the *Chlorella* survived and grew the greater the improvement in the ammonia-N, nitrate-N, Total-P, BOD and COD. Aeration by shaking had a very positive effect on the ability of *Chlorella* to both survive in diluted Garbage Pit Leachate and to remove ammonia-N, nitrate-N, Total-P, BOD and COD.

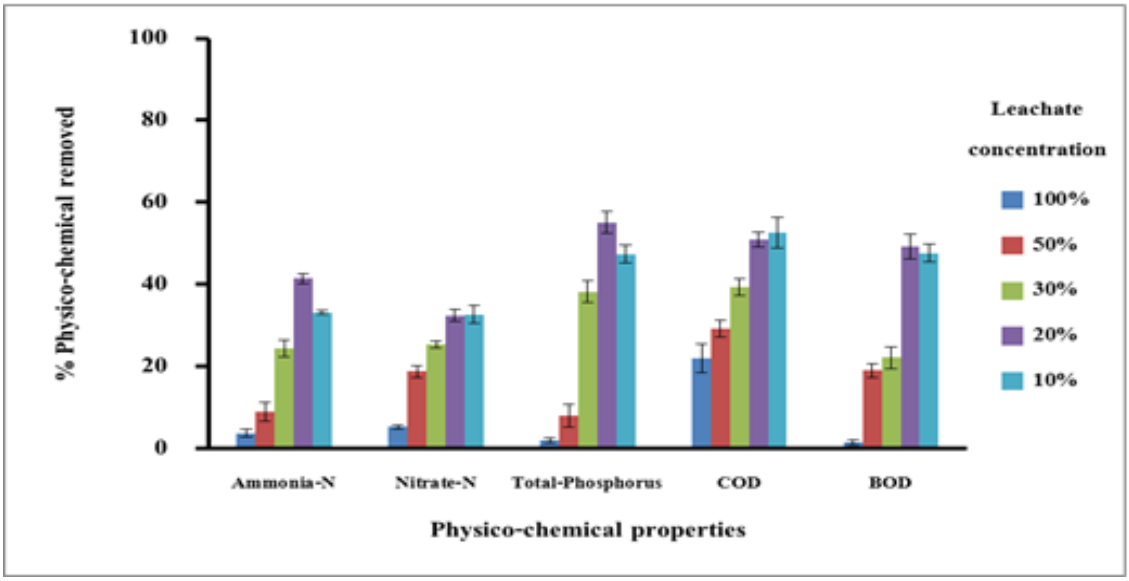

Fig 4.16 Percent of nutrients removed from the Garbage Pit Leachate after 9 days incubation in the light under shaken conditions.

Significant percent removal effects were tested for using the Tukey Test interval (p 0.05) as described by Zar (2010). Ammonia –N removal was significant by 10%, 20% and 30% Garbage Pit Leachate at the ±95% level; in both the 100% and 50% Garbage Pit Leachate were not significantly different and only about 5% was removed. Nitrate – N percent removal was significant by 100%, 50% and 30% Garbage Pit Leachate at the ±95% level; in both the 10% and 20% Garbage Pit Leachate were not significantly different from each other and about 32% was removed. Total-P percent removal by 100% and 50% Garbage Pit Leachate were not significant different and % removal was low (about 4.5% removal); in both the 10% and 20% Garbage Pit Leachate about 51% was removed. COD and BOD percent removal were significant by 100% Garbage Pit Leachate at the ±95% level, 50% and 30% Garbage Pit Leachate were not significant different from each other and about 20% was removed; in both the 10% and 20% Garbage Pit Leachate were not significant different to one another and about 48% was removed. (The ANOVA and Tukey test results are on the data disk).

4.4.2.2 Measurements of heavy metal removal from *Chlorella* cultures grown in diluted Garbage Pit Leachate to determine how much heavy metal was removed by the *Chlorella* under well-aerated conditions.

The Garbage Pit Leachate was very toxic, high ammonia and low pH (4.9-5.22) would have worsened the effects of heavy metals. Heavy metals were measured in *Chlorella* cultures grown in various dilutions of Garbage Pit Leachate diluted with tapwater as described above for similar experiments on cells grown under shaking conditions (4.4.1.2.2) but in this case were incubated on a shaker to ensure efficient aeration and stirring. *Chlorella* in diluted Garbage Pit Leachates were grown on the shaker for 9 days. Metals were measured at the beginning and end of the incubation experiments for each dilution condition.

Table4.5 The heavy metal result from aerated Garbage Pit Leachate by *Chlorella* incubated with light and shaking condition.

Heavy metals (µg/l)	Dilutions of Leachate									
	100%		50%		30%		20%		10%	
	Before	After	Before	After	Before	After	Before	After	Before	After
Copper (Cu)	90	3	4	10	< 1	< 1	< 1	< 1	< 1	< 1
Chromium (Cr)	230	130	100	100	50	20	30	20	6	70
Lead (Pb)	< 12	< 12	< 10	< 10	< 10	< 10	< 10	< 10	< 10	< 10
Nickel (Ni)	330	220	180	180	90	70	60	60	20	< 3
Zinc (Zn)	5,580	3,540	2,900	2,900	1,640	690	1,170	120	670	< 4

Table 4.6 Percent removal heavy metals from Garbage Pit Leachate compared to the controls (in the light and under shaking conditions)

Heavy metals	Dilution of leachate				
	100%	50%	30%	20%	10%
Copper (Cu)	96.7	-	Not detected	Not detected	Not detected
Chromium (Cr)	43.5	0	60	33.3	-
Lead (Pb)	Not detected	Not detected	Not detected	Not detected	Not detected
Nickel (Ni)	33.3	0	22.2	0	85
Zinc (Zn)	36.6	0	41.0	89.7	99.4

Remarks:-blank dashes mean no removal and heavy metal after treatment more than before

treated.

: 0 (zero) means no removal.

: Not detected means the heavy metal was so low it was not detectable

Chlorella grown in 20% Garbage Pit Leachate diluted with tapwater removed 89.7% of the Zn and 33.3% of the Chromium. The errors in these standard chemical analyses by the ISO-certified laboratory in Hat Yai would be about ± 2% relative error. *Chlorella* in 30% leachate removed 41% of the Zinc and 22% of the Nickel and 60% of the Chromium. *Chlorella* in 10% Garbage Pit Leachate removed nearly all the Zinc (99.4%) and most of the Nickel (85%). During experiment the pH in 30%, 20%, 10% leachate increased on average from pH 5.5 to pH 7.56. This would have made metals less soluble. pH increase and heavy metal decrease was also found by Wild *et al*. (2006) who observed that surface- bound metal concentrations increased when pH was varied over the range 6.0 – 8.0 and growth of *Chlorella* sp. improved as the pH increased. This is what you would expect for cation binding to fixed negative changes in the cell walls of the alga as the pH increased (Ritchie and Larkum 1982). Parent and Campbell (1994) and Franklin *et al*. (2000) proposed that the apparent protective effect of increasing pH on metal toxicity may be due to reduced competition between H^+ and metal biding site at the surface of the cell membrane of the alga. Crist *et al.* (1988) and Macfie *et al*. (1994) showed that as the pH increased from 4 to 7 there was an increase in the number of negative charge sites on the cell wall surface. This is what would be predicted from the cation exchanger properties of cells walls, (Ritchie and Larkum, 1982) and the Proton concentration may also alter plasma membrane permeability to metals, thereby affecting metal binding and uptake. Many transport systems for metal ions are secondary active transport mechanisms using the proton motive force (electrochemical potential for protons), given a relatively constant membrane potential and intracellular pH (pH_i) an increase in external pH tends to decrease the proton motive force and hence metal uptake would be expected to decrease as outside pH increased.(Ritchie RJ, 1998) The major effect though of external pH is to decrease the solubility of metals because most heavy metal hydroxides are extremely insoluble (Atwell *et al*., 1999).

The *Chlorella vulgaris* growth in Garbage Pit Leachate Phase2: *Chlorella* in light under shaking conditions grew the best in 20% of Garbage Pit Leachate. Compared to measurements made at the beginning of the incubation, the overall rates of removal based on all six replicate runs of the experiment were (mean % ± SE%): Ammonia-N 41.5 ± 1.22%, Nitrate-N 32.4 ± 1.45%, Total-P 55.1 ± 2.56%, COD 50.81 ± 1.74% and BOD 49.2 ± 3.02%. Heavy metals: *Chlorella* grown in 20% Garbage Pit Leachate with tap water removed Zinc 89.7% and Chromium 33%. (Relative errors about ± 2%).

4.5 Determination of the optimal Garbage Pit Leachate concentration for growth under well aerated conditions.

The experiment was set up based on the protocol used for Experiment 4.3 to determine the minimum inoculation of *Chlorella* for growth in Garbage Pit Leachate in terms of Chlorophyll *a*. Experiments were run with 3 replicates and the cultures were grown under shaking conditions. The highest concentration of leachate which supported actual growth was Garbage Pit Leachate diluted to 20% using tapwater (Fig 4.14 and 4.15). Various levels of inoculum were tried to optimize growth. Inoculation with *Chlorella vulgaris* was varied over a range of Chlorophyll *a* biomasses: 3.01µg/ml (A_{750}= 0.35), 2.5µg/ml (A_{750}= 0.316), 1.6µg/ml (A_{750}= 0.246), 1.17µg/ml (A_{750}= 0.202).

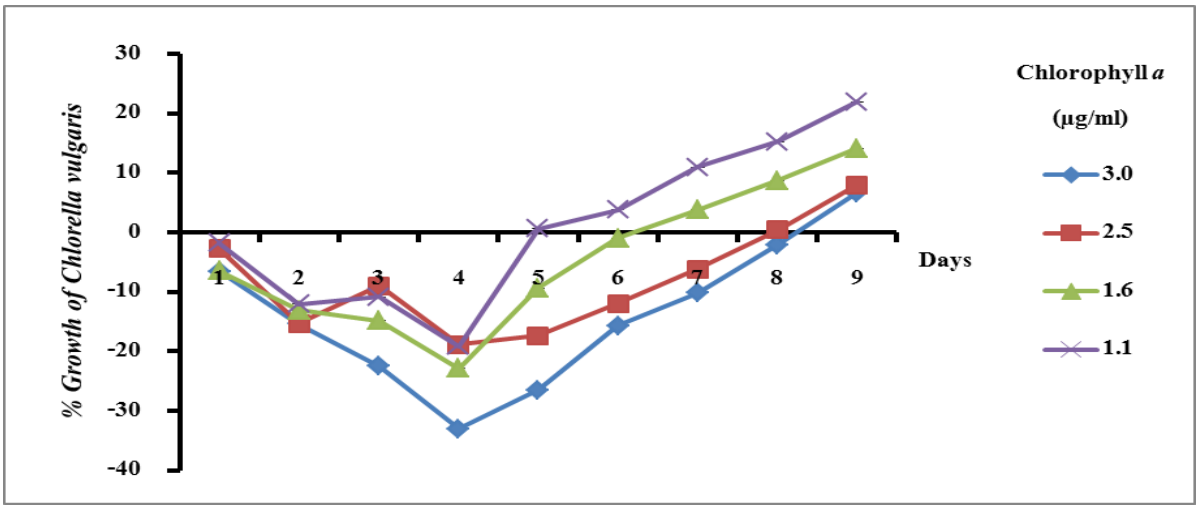

Fig 4.17 Percent growth of *Chlorella* in 20% Garbage Pit Leachate diluted with tapwater.

The results show that the *Chlorella* slowly decreased on the 1st, 2nd, 3 rd days and slowly started increasing on the 4 th day (Fig 4.17). This effect had not been noticeable in the previous experiment where the *Chlorella* inoculum had been held constant and the dilution of the Garbage Pit Leachate had been varied (Fig. 4.14 and 4.15). One possible reason is that this experiment where the *Chlorella* inoculum was varied was done on Garbage Pit Leachate that was not freshly collected: it had been kept at 4 °C in the lab for 5 days before the experiment. The four different biomasses of Chlorophyll *a* showed the same pattern of initial die-off for the first few days and then a resumption of growth. The rate of increase in *Chlorella* when it resumed growth seemed to be independent of the size of the initial inoculum of cells. During the first 3 days of the experiment the pH was about pH 5.5 but after 4 days when the alga started growing again the pH had drifted up to pH 7.3 on day 6 and had climbed to pH 8.2 by the end of the experiment (Fig. 4.18).

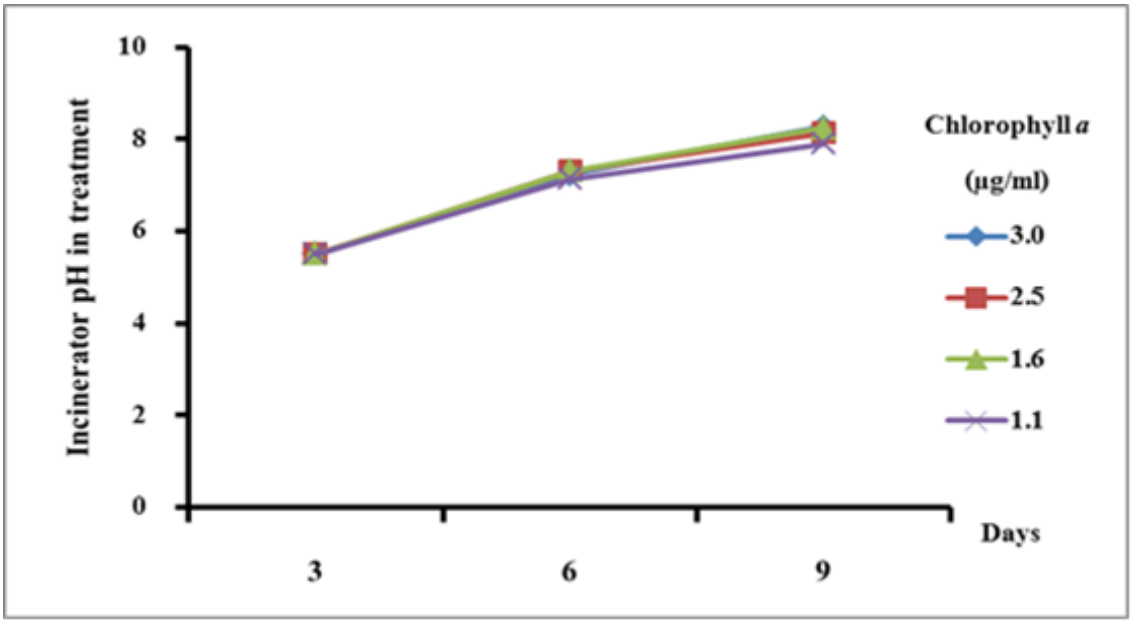

Fig 4.18 pH level of Garbage Pit Leachate in treatment.

4.5.1 Measurement of removal of Ammonia-N, Nitrate-N, Total - P, BOD and COD in the supernatant of experimental cultures at the beginning and end of incubations in Garbage Pit Leachate diluted to 20% with tapwater but with different levels of inoculation with *Chlorella*.

The removal of ammonia-N, nitrate-N, total-P and BOD and COD were measured using the same batch of Garbage Pit Leachate as was used for the growth determination described in Fig. 4.17. The initial concentrations of ammonia-N, nitrate-N, total-P, BOD and COD in the leachate at the start of the experiment were used as the control. During the 9 day cultivation period, the different biomasses of Chlorophyll *a* in 20% leachate all had similar effects on ammonia-N, nitrate-N, Total-P, BOD and COD (Fig.4.19). About 50% of ammonia-N was removed but only about 20% of nitrate-N. The heaviest inoculum of *Chlorella* removed the most ammonia-N and had started to use some of the nitrate-N after 9 days incubation. A large proportion of the Total –P was removed (≈ 80%) and both BOD and COD were reduced by about 60%.

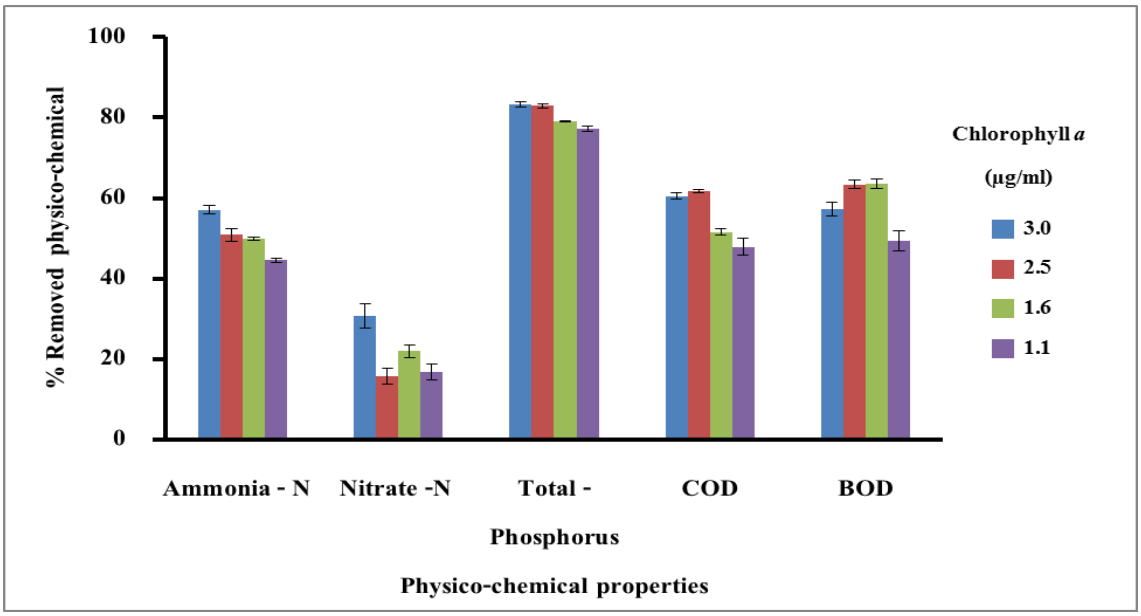

Fig 4.19 Percent removal of nutrients from Garbage Pit Leachate with different inoculating

biomasses of Chlorophyll *a* of *Chlorella vulgaris*

Significant percent removal effects were tested for using the Tukey Test interval (p 0.05) as described by Zar (2010). Ammonia –N removal using all the inoculation biomasses of Chlorophyll *a* (*Chlorella vulgaris*) were significant compared to the control. Nitrate – N % removal Chlorophyll *a* biomasses 3.0, 2.5 and 1.1 µg/ml were not significantly different and about 18% was removed. Total-P % removal by Chlorophyll *a* biomasses 3.0 and 2.5 µg/ml were not significantly different and % removal was high (about 82% removal). COD % removal were not significantly different by 3.0 and 2.5 µg/ml of Chlorophyll *a* with both removing about 60%; in both 1.6 and 1.1 µg/ml of Chlorophyll *a* about 49% was removed. BOD % removal were not significantly different by Chlorophyll *a* biomasses 3.0, 2.5 and 1.6 µg/ml with all three removing about 61%. (The ANOVA and Tukey test results are on the data disk).

4.5.2 Measurements of heavy metal removal from *Chlorella* cultures grown in diluted Garbage Pit Leachate to determine a minimum biomass to remove the heavy metal under well-aerated conditions.

Table 4.7 The heavy metal removal of heavy metals from 20% Garbage Pit Leachate with varying levels of *Chlorella* inoculation.

Heavy metals (µg/l)	Concentration of Chlorophyll *a* (µg/ml)				
	Before treated	3.0	2.5	1.6	1.1
Copper (Cu)	1	< 1	< 1	< 1	< 1
Chromium (Cr)	40	10	8	8	9
Lead (Pb)	< 1	< 1	< 1	< 1	< 1
Nickel (Ni)	60	50	50	40	40
Zinc (Zn)	1850	90	130	10	80

Table 4.8 Percent removal heavy metals from 20% Garbage Pit Leachate with varying levels of *Chlorella* inoculation

Heavy metals	Concentration of Chlorophyll *a* (µg/ml)			
	3.05	2.5	1.6	1.17
Chromium (Cr)	75	80	80	97.75
Copper (Cu)	Not detected	Not detected	Not detected	Not detected
Lead (Pb)	Not detected	Not detected	Not detected	Not detected
Nickel (Ni)	16.66	16.66	33.33	33.33
Zinc (Zn)	95.13	92.97	94.59	95.67

Remarks: Not detected means the heavy metal was so low it was not detectable before or after incubation

Fig. 4.20 shows the percentage removal of heavy metals from 20% Garbage Pit Leachate incubated with different levels of initial inoculum of *Chlorella*. The initial concentrations of heavy metal in the leachate at the start of the experiment were used as the control. The same batch of cells was used for the determinations as used for the growth measurements and nutrient removal determination experiments described above. Fig. 4.20 and Table 4.7 shows that Zinc was very efficiently removed (90% or more). Nearly all the Chromium was also removed. No significant removal of Nickel was achieved. There was little evidence for a systematic effect of the starting concentration of *Chlorella* cells upon the eventual removal of heavy metals after incubation for 9 days on the shaker.

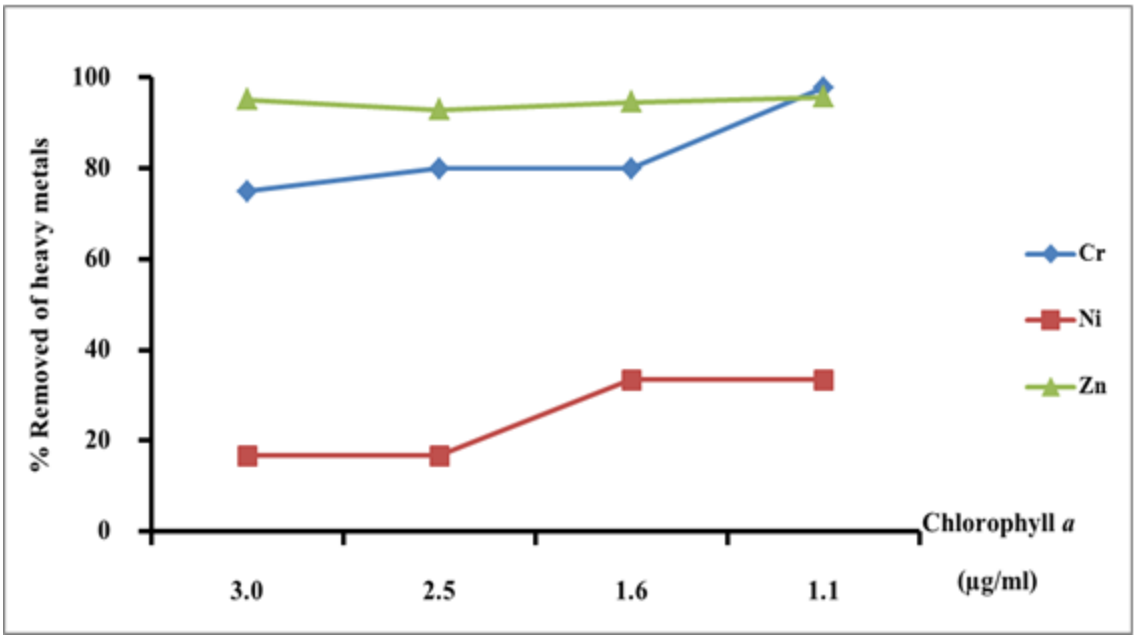

Fig. 4.20 Percent removal of heavy metals from 20% Garbage Pit Leachate with varying levels of *Chlorella* inoculation

High levels of removal of ammonia-N, total-P, BOD and COD (Fig 4.19) and Zinc and Chromium (Fig. 4.20) can be achieved on Garbage Pit Leachate provided it is diluted to about 20% of its original concentration and is incubated with *Chlorella* under shaking, well aerated conditions. Heavy inoculation with *Chlorella* is not necessary: *Chlorella* cultures with Chlorophyll *a* 1.17 µg/ml (A_{750}= 0.202) performed just as well as higher inoculums.

4.6 pH effects on removal of heavy metal.

Growth determinations and measurements of nutrient and heavy metal removal pointed to a crucial role of pH in the toxicity of the leachates, in particular the toxicity of the Garbage Pit Leachate. Lime ($CaO/Ca(OH)_2$) is routinely used to adjust the pH of treated sewage and leachates and

Chlorella is known to be highly tolerant of alkaline pH. Treatment with lime was expected to decrease the toxicity of Garbage Pit Leachate. This was tested experimentally.

4.6.1 Treatment with lime with shaking well-aerated conditions.

The experiment was set up to compare the Garbage Pit Leachate and incinerator treated with lime. The pH of the raw Garbage Pit Leachate was pH 4.5 – 5.5. Raw undiluted Garbage Pit Leachate was highly toxic to *Chlorella* which only showed positive growth in media containing only 20% or 10% leachate diluted with tapwater (Fig. 4.14 and Fig 4.15). Large amounts of lime were needed to be added to leachate in order to increase its pH. 10 g/l of Lime was added and the leachate aerated overnight. The pH was found to be pH 7.03 the next day. *Chlorella* culture (Chlorophyll *a* = 1.17µg/ml; ABS_{750}= 0.202) was added to 100%, 50%, 20% and 10% Garbage Pit Leachate diluted with tapwater and to lime-treated Garbage Pit Leachate diluted over the same range. Cultures were grown over a 9 day cultivation period with shaking on an orbital shaker.

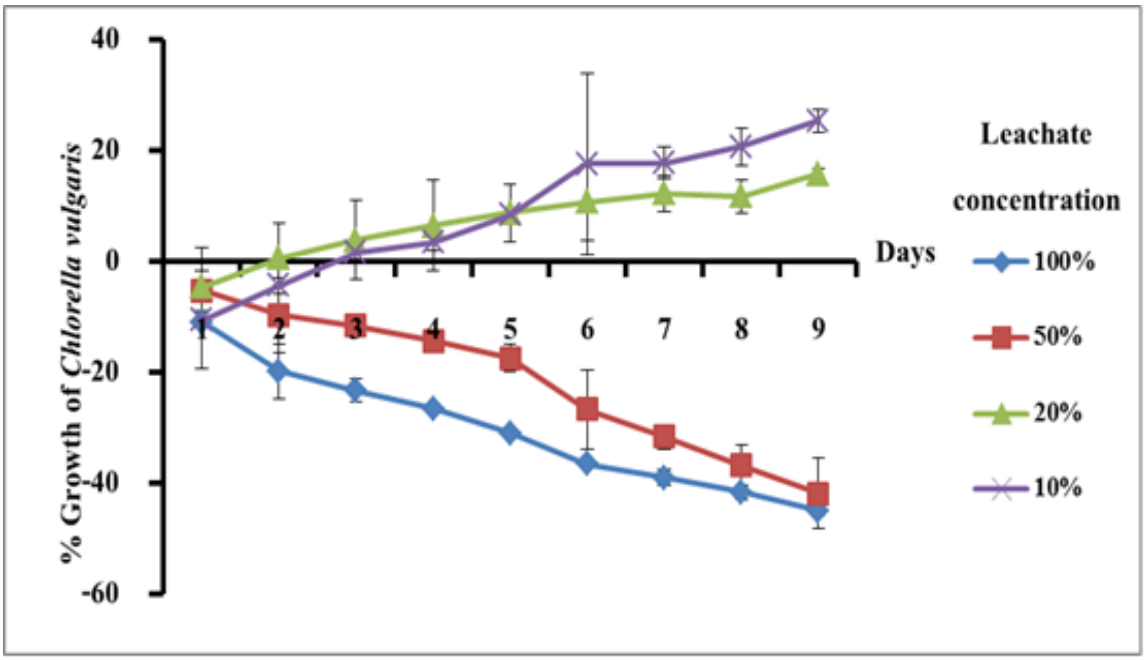

Fig 4.21 Percent growth of *Chlorella vulgaris* in the Garbage Pit Leachate (Control experiment Garbage Pit Leachate 20% undiluted with tapwater without Lime).

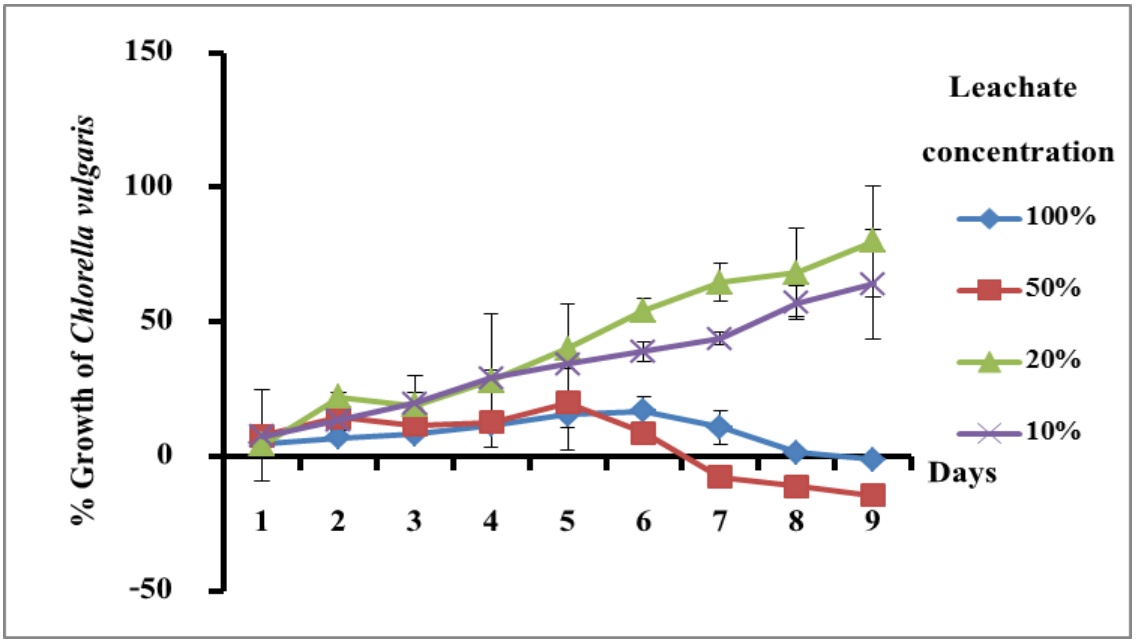

Fig4.22 Percent growth of *Chlorella vulgaris* in the Garbage Pit Leachate treated with lime

Fig 4.21 and Fig 4.22 compare the growth of *Chlorella* under shaking conditions in diluted Garbage Pit Leachate and diluted Garbage Pit Leachate treated with lime. The control experiment shows the severe toxicity of Garbage Pit Leachate found before in previous experiments (Fig 4.14 and 4.15 and Fig. 4.17). In contrast, *Chlorella* grew well in 20% diluted Garbage Pit Leachate that had been treated with lime (Fig. 4.22), however 50% and 100% Garbage Pit Leachate was still toxic to *Chlorella* even after being treated with lime. However, this toxic effect in 50% and 100% Garbage Pit Leachate was not apparent until the cultures had been incubated for several days. Treatment with lime reduced the toxicity of Garbage Pit Leachate but did not eliminate it. If the toxicity of Garbage Pit Leachate was entirely simply a result of high ammonia concentration and high levels of heavy metal then lime treatment should have largely de-toxified the leachate: the high pH would have converted NH_4^+ to volatile NH_3 which would have been lost by evaporation and the alkaline pH would have precipitated

most heavy metals as hydroxides (Crofts, 1967; Greene, 1984). Later in the study some other evidence was found which supported the proposition that high NH_3 and heavy metals concentrations were not only major reasons for the high toxicity of Garbage Pit Leachate. Short term toxicity experiments based on fluorometric PAM technology confirmed the toxicity of Garbage Pit Leachate but a toxicity experiment on *Chlorella* using $ZnSO_4$ showed that Zn by itself was not very toxic.

4.6.1.1 Measurement of removal of Ammonia-N, Nitrate-N, Total - P, BOD and COD in the supernatant of experimental cultures at the beginning and end of incubations in Garbage Pit Leachate diluted with tapwater and Garbage Pit Leachate diluted with tapwater treated with lime.

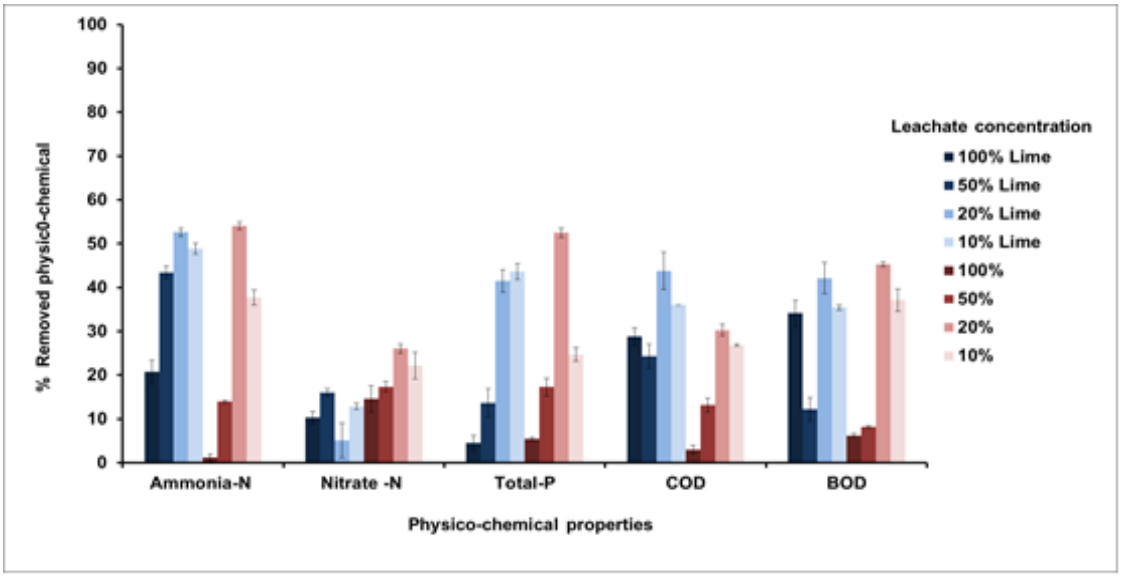

Fig4.23 Percent removal of nutrients from Garbage Pit Leachate with different concentration diluted with tapwater and Garbage Pit Leachate diluted with tapwater treated with lime.

Fig 4.23 shows that the Lime experiment had an important effect on Ammonia-N, treatments treated with lime (100% Lime, 50% Lime, 20% Lime and 10% Lime) have much higher % removal than the experiments not treated with lime (100%, 50%, 20% and 10%). Total-P percent

removal by 20% and 10% treated with lime were not significantly different with about 42% removed in both cases. COD percent removal in the lime experiment had a higher percent removal than the experiments not treated with lime. The results for BOD are not very clear: low dosages of lime did not give consistent results, but high dosages of lime gave results similar to the experiments not treated with lime and so addition of lime did not have a significant effect at 50% and 100% lime.

4.6.1.2 Measurements of heavy metal removal from *Chlorella* cultures grown in diluted Garbage Pit Leachate treated with lime under well-aerated conditions.

Chlorella was grown in diluted Garbage Pit Leachate on the shaker for 9 days. Metals were measured at the beginning and end of the incubation experiments for each dilution condition following protocols used in similar previous experiments (Section 4.5.2 and Table 4.6)

Table 4.9 The heavy metal result from aerated Garbage Pit Leachate treated without lime (grown in the light and under shaking conditions)

Heavy metal (µg/ml)	Raw leachate 100%	Dilutions of Leachate						
		GPL 100% after	GPL 50%		GPL 20%		GPL 10%	
			Before	after	Before	After	Before	after
Chromium (Cr)	180	150	90	70	36	6	10	2
Copper (Cu)	7	6	3.5	3	< 1	< 1	< 1	< 1
Nickel (Ni)	480	350	240	190	96	70	40	10
Lead (Pb)	< 13	< 13	< 13	< 13	< 13	< 13	< 13	< 13
Zinc (Zn)	1660	1060	830	570	330	8	160	4

Table 4.10 Percent removal heavy metals from Garbage Pit Leachate treated with lime compared to the controls (grown in the light and shaking conditions)

Heavy metals (µg/ml)	Dilutions of Leachate							
	100%		50%		20%		10%	
	Lime	No Lime	Lime	No Lime	Lime	No Lime	Lime	No Lime
Chromium (Cr)	33.33	16.67	44.44	22.22	72.22	83.33	80	80
Copper (Cu)	14.28	0	-	14.28	0	0	0	0
Nickel (Ni)	4.16	36.14	58.26	27.08	6.25	27.08	25	97.5
Zinc (Zn)	99.75	36.14	97.59	31.32	93.93	97.5	97.5	97.5

Remarks : the blank **(-)** mean not removed and heavy metal after treated more than before treated

: 0 means no removal

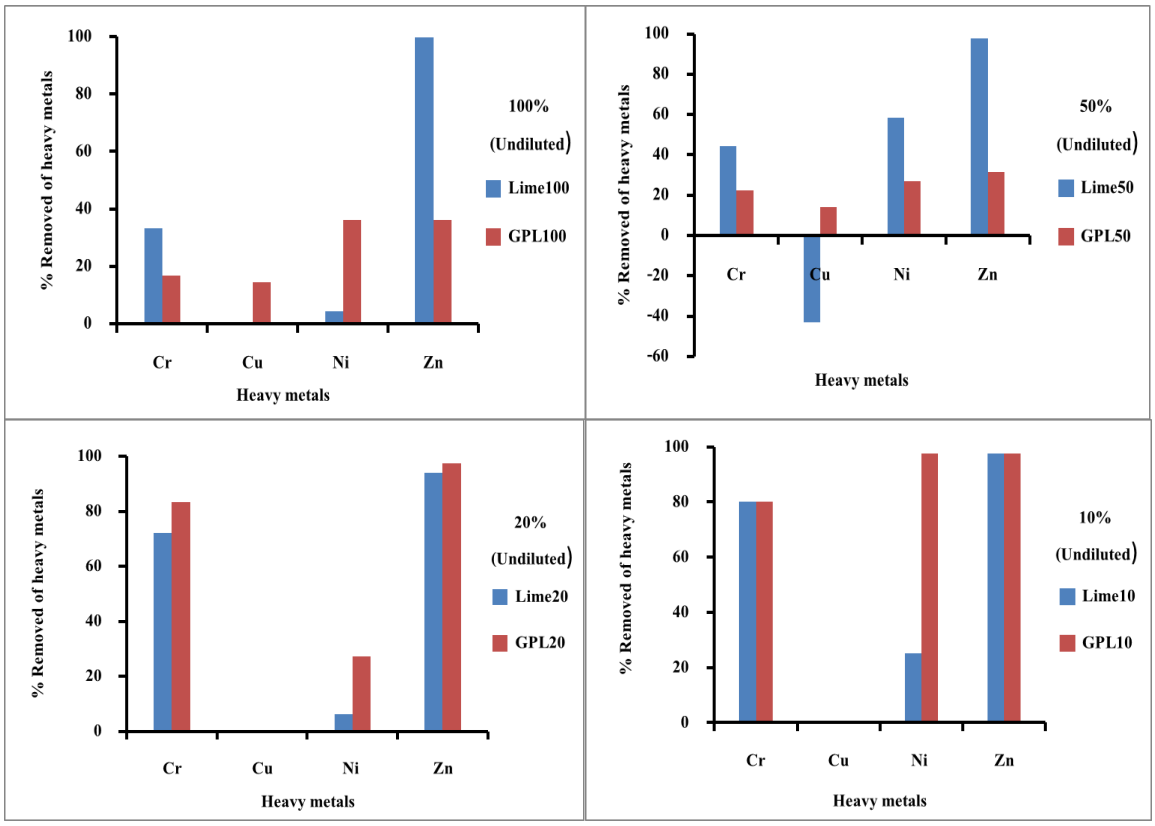

Fig 4.24 Comparison of percent removal of heavy metals between Garbage Pit Leachate and Garbage Pit Leachate treat with lime leachate treated with lime in different dilutions of leachate (100%, 50%, 20%, and 10%)

Fig 4.19 shows that in the 100% in Garbage Pit Leachate treated with lime the *Chlorella* can remove Zinc 99.75% compared with Garbage Pit Leachate not treated with lime where only 36.14% of the Zinc was removed. Heavy metals were equally well removed with or without treatment with lime in the case of 10% and 20% diluted Garbage Pit Leachate. The wastewater treatment plant attempted to detoxify the Garbage Pit Leachate with lime to precipitate the heavy metals and to remove ammonia, however, they did not use enough lime because the holding pond for Garbage Pit Leachate was still acid. Lime treatment is ineffective if the pH is not moved to alkaline conditions. Zinc removal was very high in 100% (undiluted) Garbage Pit Leachate provided the pH is alkaline enough but using too little lime is

ineffective. In the case of 20% and 10% diluted leachate the % removal of Zinc was similar. From the results it can be concluded that pH in leachate is the important factor of *Chlorella* growth and its ability to effectively remove heavy metals but Garbage Pit Leachate is nevertheless highly toxic for reasons other than its pH and heavy-metal content.

The experimental lime treatment with shaking to achieve well-aerated conditions: *Chlorella vulgaris* grew well in 20% diluted Garbage Pit Leachate (Fig 4.22) and percent removal of Ammonia-N, COD and Total-P treatments treated with lime (100% Lime, 50% Lime, 20% Lime and 10% Lime) had much higher % removal than in the experiments not treated with lime (100%, 50%, 20% and 10%). Heavy metal removal in the 100% in Garbage Pit Leachate treated with lime and inoculated with *Chlorella* was 99.75% for Zinc. In comparison, Garbage Pit Leachate inoculated with *Chlorella* but not treated with lime only 36.14% of the Zinc was removed. Heavy metals were equally well removed with or without treatment with lime in the case of 10% and 20% diluted Garbage Pit Leachate.

4.6.2 Effect of a range of pH on growth of *Chlorella* in 20% dilution of Garbage Pit Leachate varied pH from pH 2 – pH 7

The experiment were set up in the 20% (undiluted) Garbage Pit Leachate and inoculated with *Chlorella* (Chlorophyll *a* = 1.17 µg/ml, A_{750} = 0.202) and incubated in the light in the light with no shaking for 7 days adjusted to a range of pH from pH 2 to pH 7. pH was checked daily but the drift in the pH each day was small, there was very little drift in the pH. Fig. 4.25 shows that there was no positive growth of any of the cultures even at pH 7. This result is consistent with the previous experiments on diluted Garbage Pit Leachate where the cultures were not shaken (Section 4.4.1.2, Figs 4.11 and 4.12).

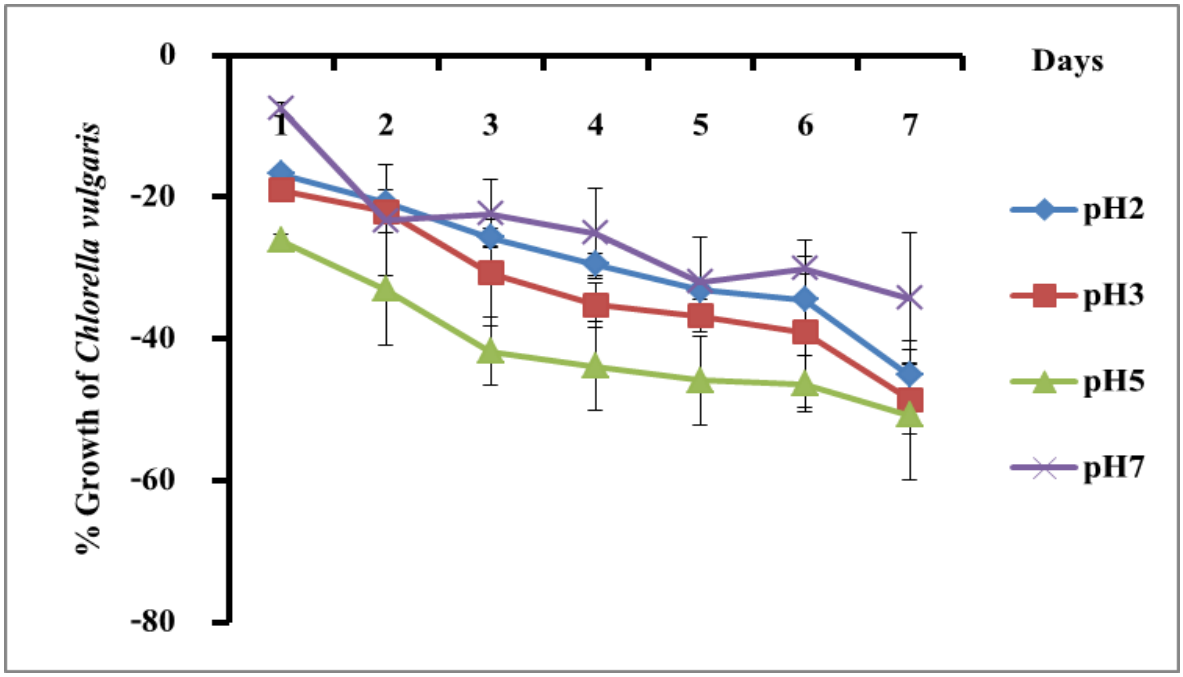

Fig 4.25 Percent growth of *Chlorella vulgaris* Garbage Pit Leachate varied pH

4.6.2.1 Measurements of heavy metal removal from Chlorella cultures grown in 20% undiluted Garbage Pit Leachate with pH varied from pH 2 to pH 7 for 7 days.

Heavy metal removal was measured in the cultures using the protocol described previously. The initial heavy metal measurements (t = 0) were used as the controls to determine % removal of metals after a 7 day incubation. Table 4.11 shows the heavy metal measurements before and after 7 day incubation. Table 4.12 shows the calculated percentage removal.

Table 4.11 The heavy metal result for incubations under varied pH (incubated in the light without shaking)

Heavy metals (μg/ml)	Raw leachate 20% (undilted)	pH varied							
		pH 2		pH 3		pH 5		pH 7	
		Before	After	Before	After	Before	After	Before	After
Chromium (Cr)	30	50	50	40	30	20	20	20	20
Copper (Cu)	< 1	1	1	< 1	< 1	< 1	< 1	< 1	< 1
Lead (Pb)	< 10	< 10	< 10	< 10	< 10	< 10	< 10	< 10	< 10
Nickel(Ni)	60	60	60	70	70	70	70	50	50
Zinc (Zn)	1,170	1,740	170	1,490	1,450	620	440	490	220

Table 4.12 Percent removed of heavy metal result pH varied experiment (light with none shaken conditions)

Heavy metals	pH varied			
	pH 2	pH 3	pH 5	pH 7
Chromium (Cr)	0	25	0	0
Copper (Cu)	0	0	0	0
Lead (Pb)	0	0	0	0
Nickel (Ni)	0	0	0	0
Zinc (Zn)	2.29	2.68	29.03	55.10

Table 4.11 and 4.12 show that *Chlorella* removed very little heavy metal under the conditions tested and there was little effect of pH. This is consistent with the negative growth of *Chlorella* in the Garbage Pit Leachate shown in Fig. 4.25. There was no significant removal of Chromium, Copper, Lead or Nickel at pH 2, 3, 5 or 7. There was a small degree of removal of Zinc at pH 2 and 3 but there was some removal of Zinc at pH 5 (29%) and pH 7 (55%). Zn ions are much less soluble in wastewater at pH 7 than Copper, Lead or Nickel (Greene 1984).

There was not enough time to repeat the above pH experiments using cultures grown on a shaker to provide adequate aeration. From the results of experiments on aerated cultures grown in diluted Garbage Pit Leachate (Sections 4.4.2.1, 4.4.2.2, 4.5) it would be predicted that both growth and heavy metal removal would be much better under well-aerated conditions than the results of the pH experiments under static culture conditions.

4.7 Using PAM (Pulse Amplitude Modulation Fluroometry) for monitoring toxicity.

PAM methods offer the opportunity to measure the toxicity of wastewater in very quick short term experiments. The toxicity of landfill and Garbage Pit Leachate was expected to be easy to detect using PAM methods. *Chlorella* was grown in BG-11 and cells were centrifuged (5000 rpm, 10 minutes) and resuspended in a range dilutions of landfill and Garbage Pit Leachates diluted with tap water. Cells were incubated in the various dilutions of leachate for 1 hrs in the light. 5mls of cells in 4 replicates were filtered onto glass fibre disks and placed in the dark for at least 10 min before doing rapid light curves on the algal cells impregnated onto the glass fibre disks (Ritchie 2008, Ritchie and Larkum 2013, Seatae *et al*., 2013). Hence, these PAM experiments measured the very short-term effects of leachates. At the end of the PAM measurements the chlorophyll *a* content of the cells impregnated onto the glass fibre disks were determined as described by Ritchie (2008) and Seatae *et al*. (2013) using the ethanol solvent equations developed by Ritchie (2006).

Rapid light curves were performed on the algal disks using routine methods as described by Seatae *et al*. (2013) using the Walz standard software. Maximum Yield was calculated by

fitting Yeild vs. irradiance to an exponential decay curve and calculating the yield at zero irradiance (Ritchie and Buthawin 2010a, b, Ritchie 2012, Seatae *et al*. 2013). ETR (Electron Transport Rate) calculated using the Walz software and using a standard absorptance of 0.84 for *Chlorella* (Ritchie and Runcie 2014). Maximum Yield and maximum ETR were determined as described above using the standard rapid light curve protocol. ETR as mol m^{-2} s^{-1} was converted to mol mg $Chla^{-1}$ h^{-1} using the measured chlorophyll *a* content of the algal disks determined as mg Chl a/m^2 (Ritchie 2008). ETR (as mol mg Chl a^{-1} h^{-1}) was plotted against irradiance and the maximum photosynthesis (Pmax) was determined by fitting the waiting-in-Line curve to the data as described in the methods.

4.7.1 PAM experiment using leachate inoculated with *Chlorella vulgaris*

4.7.1.1 Landfill leachate

Chlorella was grown in BG-11 and cells were centrifuged (5000 rpm, 10 minutes) and resuspended in a range of both landfill leachate diluted to 10, 20, 30, 50 and 100% leachate. The cells were incubated for 1 hr before measuring photosynthesis using the PAM machine.

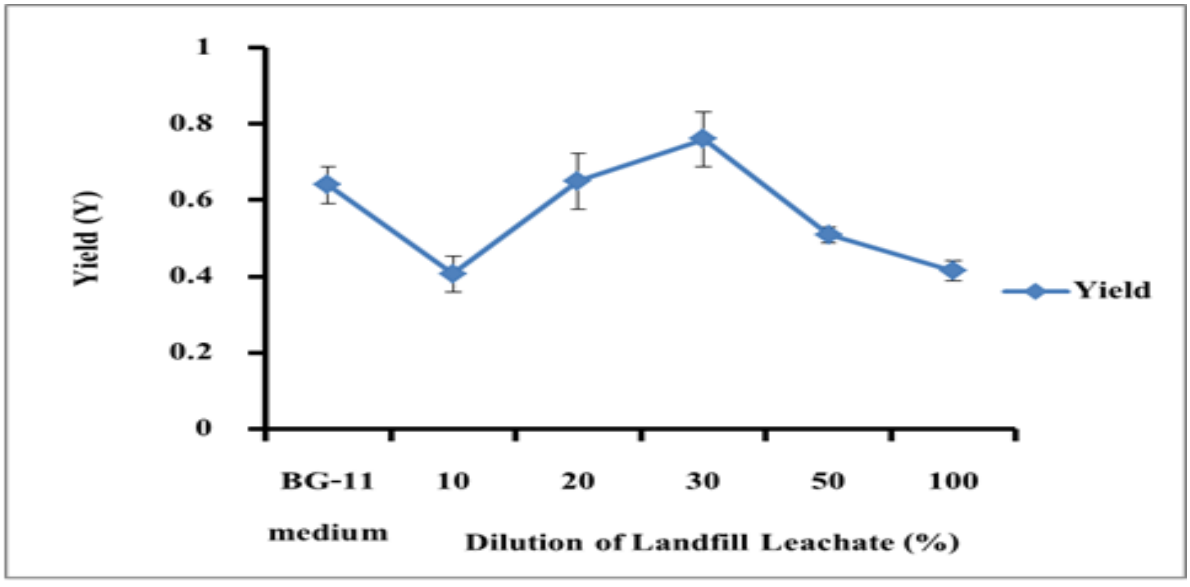

Fig. 4.26 Yield of *Chlorella* incubated in landfill leachate

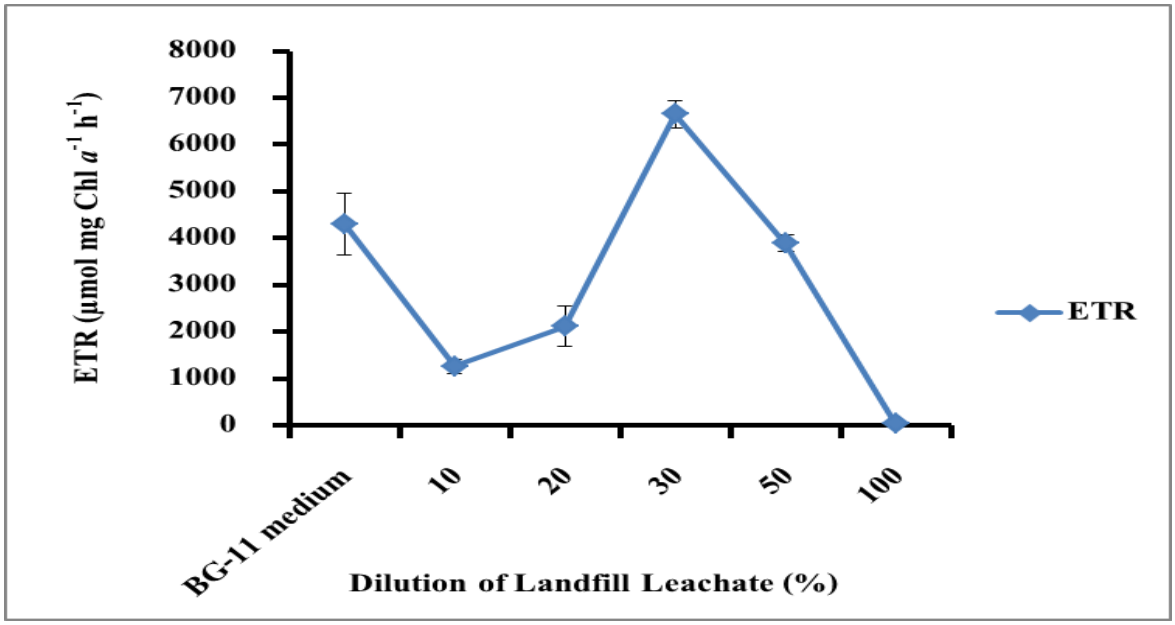

Fig 4.27 ETR (µmol mg Chla^{-1} h^{-1}) of *Chlorella* in dilution of landfill leachate

4.7.1.2 Garbage Pit Leachate

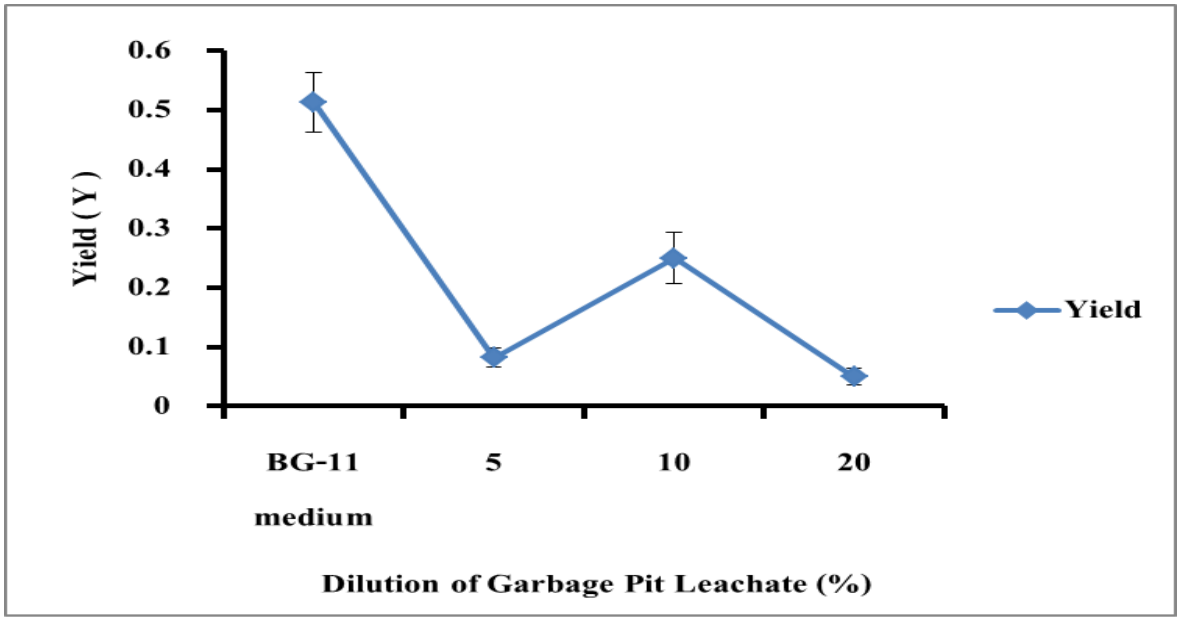

Fig4.28 Yield of *Chlorella* grown in dilutions of Garbage Pit Leachate

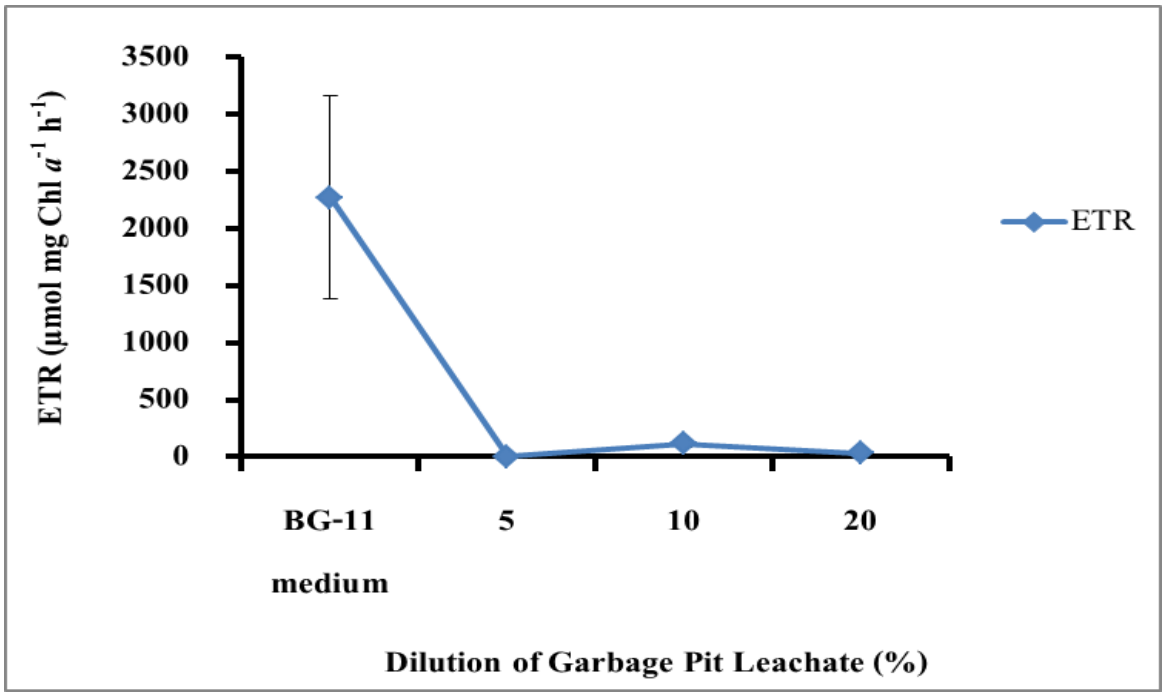

Fig 4.29 ETR (µmol mg Chla^{-1} h^{-1}) of *Chlorella* in dilution of Garbage Pit Leachate. Shows that the toxicity of Garbage Pit Leachate is immediate and not a long term effect.

The PAM experiments on diluted landfill and Garbage Pit Leachates clearly show that Garbage Pit Leachate is highly toxic and its toxicity is apparent even after an incubation of 1 hr. This shows that the toxicity of both types of leachate is apparent even in a short-term experiment of only 1 hr exposure to the leachate.

4.7.2 Zinc Toxicity

Preliminary measurements showed that Garbage Pit Leachates had very high concentrations of Zn (Thongpinyochai and Ritchie 2013) and it was thought that it was likely that it was a major source of the toxicity of the leachate, particularly the Garbage Pit Leachate. The toxicity of Zn

on *Chlorella* was tested as $ZnSO_4$ at concentrations comparable to those found in leachate (Table 4.1). The PAM experiments above (Fig 4.28 to 4.29) show that the toxicity of landfill and Garbage Pit Leachate was detectable easily using PAM methods after an incubation time of only 1 hr. This shows the advantage of PAM experiments to measure toxicity over growth experiments which require several days. The other not unimportant result from the PAM experiments was the finding that the toxicity effects were essentially immediate and not a slow-acting toxicity.

4.7.2.1 Zinc Toxicity in BG-11 medium pH 8

Chlorella was grown in BG-11 and cells were centrifuged (5000 rpm, 10 minutes) and resuspended in BG-11 with a range of Zn concentrations from zero to 200 µM (13.74 ppm or mg/l). Table 4.1 shows that Garbage Pit Leachate reached 7.83 ppm Zn. Cells were incubated in the various Zinc concentrations for 1 h in the light. 5 mls of cells in 4 replicates were filtered onto glass fibre disks and placed in the dark for at least 10 min before doing rapid light curves on the algal cells impregnated onto the glass fibre disks (Ritchie 2008, Ritchie and Larkum 2013, Seatae *et al*., 2013). Hence, this PAM experiment measured the very short-term effects of Zinc. At the end of the PAM measurements the chlorophyll *a* content of the cells impregnated onto the glass fibre disks were determined as described by Ritchie (2008) and Seatae *et al.* (2013) using the ethanol solvent equations developed by Ritchie (2006).

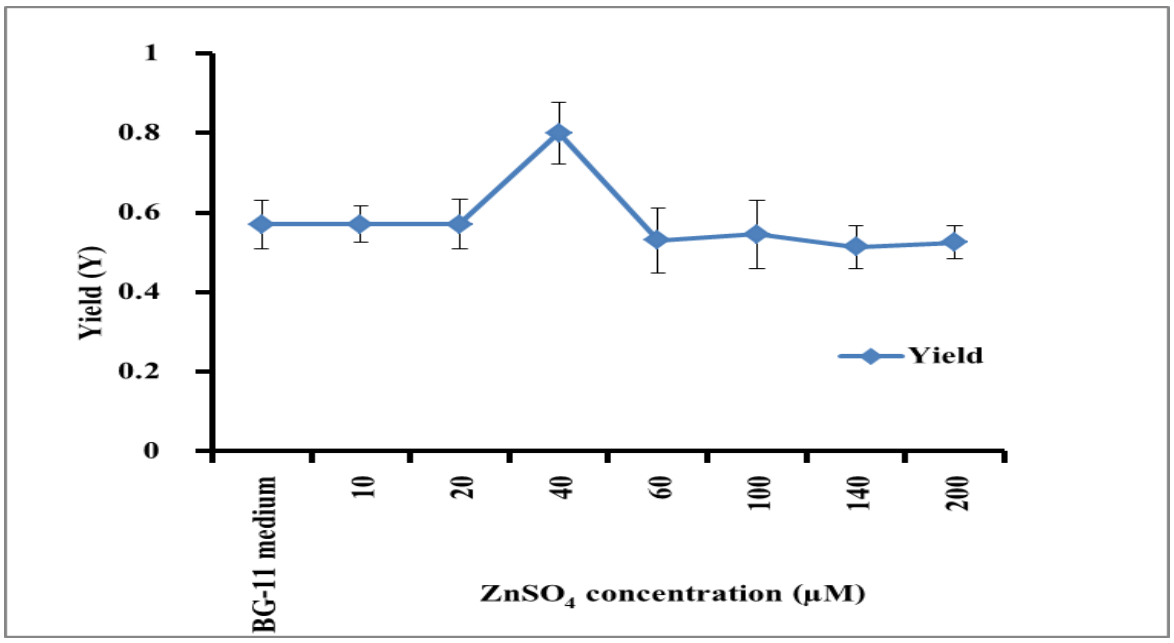

Fig 4.30 PAM Yield (max) of *Chlorella* incubated in different concentrations of $ZnSO_4$ (µM) in BG-11 medium at pH 8

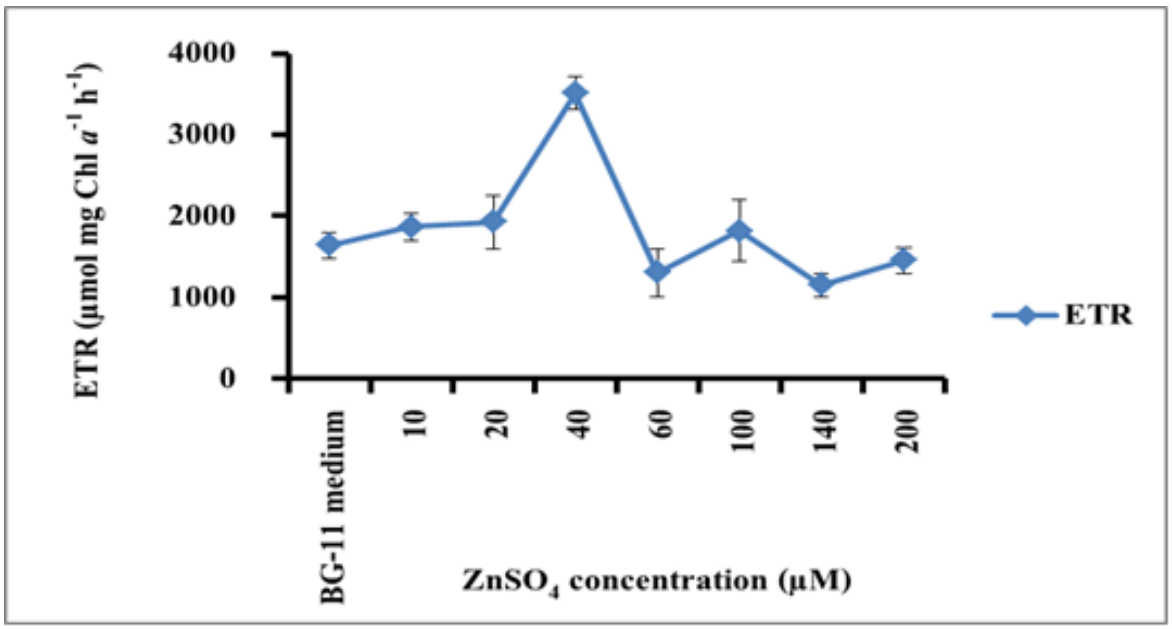

Fig 4.31 ETR (max) (µmol mg Chla^{-1} h^{-1}) of *Chlorella* in different concentration of $ZnSO_4$ (µM) at pH 8.

Figs 4.30 and 4.31 show that the Zinc sulphate experiment shows very little apparent toxicity of Zn to *Chlorella* in the short-term. This is a quite different result to what would have been expected if Zn was the main reason for the toxicity of the Garbage Pit Leachate. There was little or no obvious toxicity. One reason for this anomaly was that the experiment was run in BG-11 at pH 8 where Zn is almost insoluble (Greene, 1984). An experiment was therefore run under acid conditions similar to those found in Garbage Pit Leachate which has a pH of about 5.

4.7.2.2 PAM experiment using different concentrations of Zinc under acid pH.

Chlorella cells grown in BG-11 were centrifuged and resuspended twice in BG-11 medium with the pH adjusted to pH 5.13. The cells were divided into six 20 ml volumes. One acted as the control and 10, 30, 50 and 100 µM Zinc was added to the other volumes. Cells were incubated for 1 hr then filtered onto glass fibre disks and rapid light curves were measured on the discs after keeping the disks in the dark for at least 10 minutes.

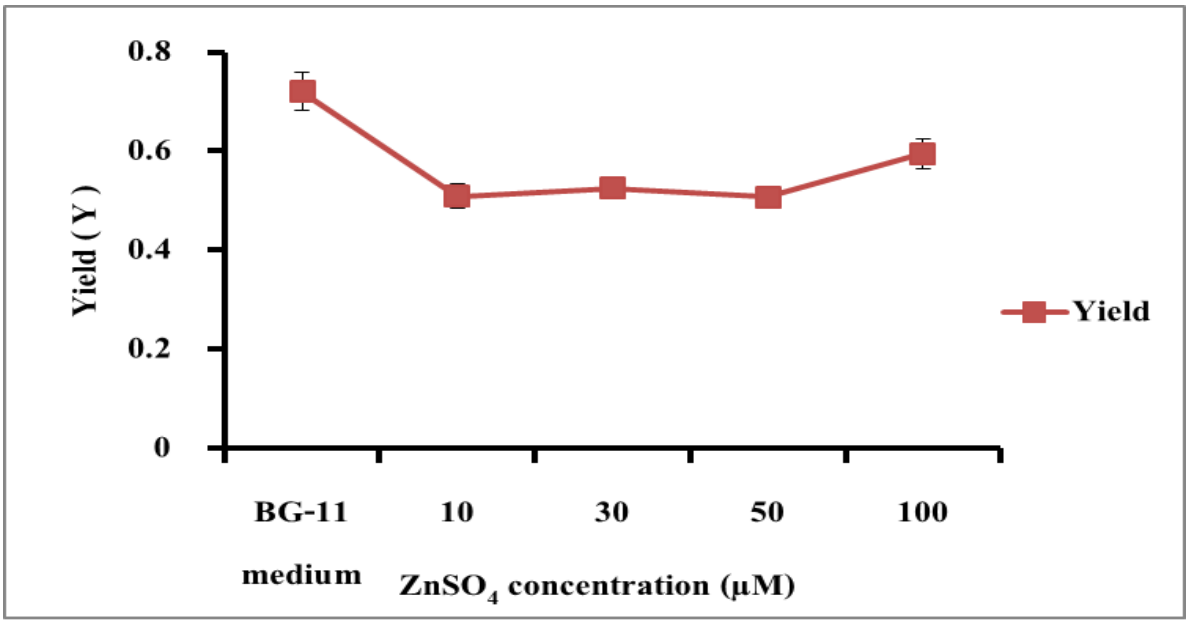

Fig. 4.32 Effects of Zn on *Chlorella* incubated in pH 5.13 on Yield

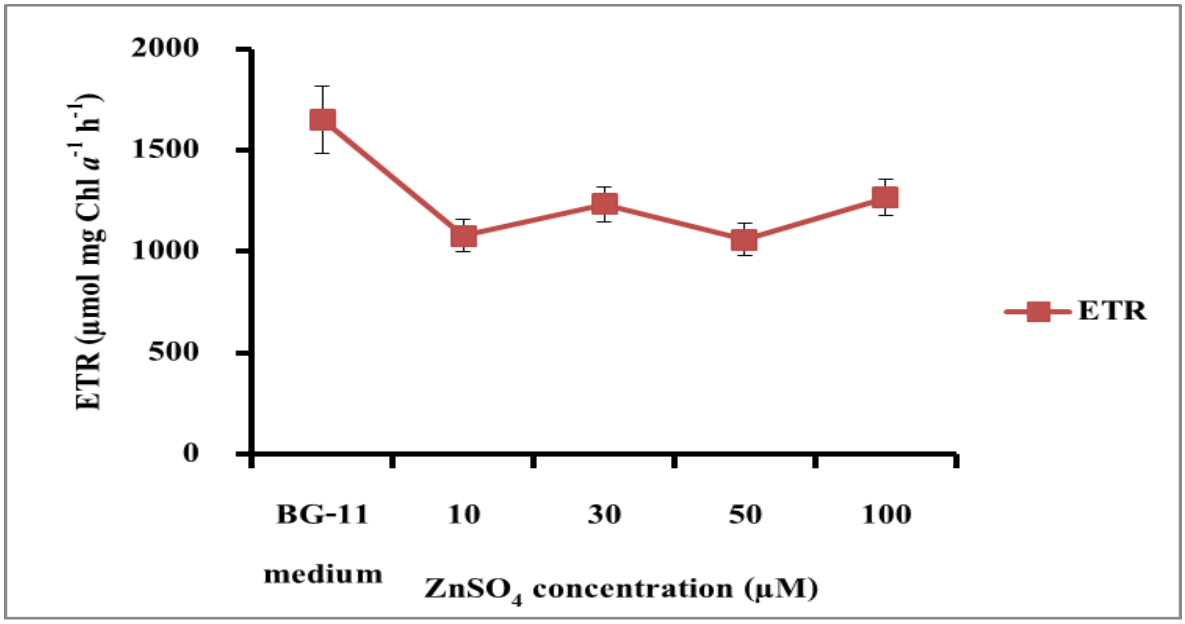

Fig 4.33 Effects of Zn on *Chlorella* incubated in pH 5.13 on ETR

Figs 4.32 and 4.33 show that Zinc alone was not very toxic to *Chlorella* at the concentrations found in Garbage Pit Leachate at pH 5.13. It was also not very toxic at pH 8 and so it can be concluded that although Zinc has an inhibitory effect on *Chlorella* Zinc alone cannot account for the very high toxicity of Garbage Pit Leachate.

4.8 Overall Conclusions about the high toxicity of Garbage Pit Leachate

This study has consistently found that the Garbage Pit Leachate was highly toxic in both growth experiments and in PAM fluorometry experiments. Based on the very high amounts of Zinc in Garbage Pit Leachate and the positive effects of lime on removing Zinc and decreasing the toxicity of leachate it was thought that illegally high levels of Zinc were the primary cause of the toxicity of the leachate. However, the PAM experiments on photosynthesis of *Chlorella* in various concentrations of

Zinc at pH 8 and pH 5 do not support the contention that Zinc is the primary cause of the toxicity of Garbage Pit Leachate although it may be a contributing factor.

CHAPTER 5

Conclusion

5.1 Conclusion

The garbage dumped in Phuket, Thailand has increased every year and efforts are needed to decrease the volumes of garbage reaching landfills. In Phuket Municipality, there are two main parts of the garbage management process: the incinerator (with its garbage pit temporary storage of garbage) and the water treatment plant. There are two leachate streams to be dealt with: the landfill leachate and the Garbage Pit Leachate from the garbage holding bay of the incinerator. Currently both leachates are fed into the water quality improvement plant along with the domestic sewage from the municipality. The landfill leachate was shown to be of relatively low toxicity in the present study (passes environmental guidelines for both BOD and heavy metals) but the Garbage Pit Leachate is extremely toxic (very high BOD and illegal levels of Zinc and Chromium). Currently the landfill leachate is fed into the sewage stream and after treatment the treated sewage in the holding pond is used for watering parklands in Phuket and excess is disposed of in a canal. This appears to be an adequate policy for the landfill leachate. This study shows that bioremediation of landfill leachate is possible using *Chlorella* but the leachate needs to be diluted about to 30% for *Chlorella* to grow reliably and growth was not dependent on the level of aeration. *Chlorella* did remove heavy metals and reduced the BOD.

The Garbage Pit Leachate presents major problems. The BOD load from the Garbage Pit Leachate has become a pressing problem ever since the incinerator had to be expanded to two input feed-heads. The present study has shown that the Garbage Pit Leachate is heavily contaminated with Zinc and Chromium and has very high BOD and very high levels of ammonia as well. The Garbage Pit Leachate was so toxic it was difficult to get *Chlorella* to grow even in heavily diluted Garbage Pit Leachate. *Chlorella* would only grow reliably in 20% dilutions of Garbage Pit Leachate and required constant aeration in order to grow. There is a deadly combination of toxic effects of the Garbage Pit Leachate. The Garbage Pit Leachate is acid (pH around 4.59-5.22), this high acidity solubilises heavy

metals and so increases their toxicity. There are very high levels of heavy metal levels especially Zinc (0.43 – 17.3 mg/l), high BOD (50-100 g O_2/l) and a high COD level (3,000-9,000 mg /l). In addition there are high levels of ammonia-N (763 – 2045 mg/l) which increases the solubility and toxicity of heavy metals and high levels of nitrate-N (14- 260 mg /l) which also increases the solubility of heavy metals. The Total-P levels were 60 – 270 mg/l. Except for the phosphate content all of these parameters are above legal limits: in contrast the leachate was not above legal limits in heavy metals. The landfill leachate was neutral-basic (pH around 7-8), with lower BOD (60-405 mg/l), COD level (32-160mg/l), NH_3 concentration (170– 256 mg/l), nitrate-N (13.6- 48.86 mg/l) and Total–P (5.57-36.63 mg/l).

Chlorella vulgaris was a satisfactory bioremediation agent for the landfill leachate provided the leachate was diluted to 20%. The minimum inoculum of *Chlorella* biomass on a Chlorophyll *a* basis which can grow in the landfill leachate was 0.259 µg/ml Chlorophyll *a* (A_{750} = 0.075). Much higher inoculums were necessary for the diluted Garbage Pit Leachate. In the Garbage Pit Leachate an inoculum of 0.92 µg/ml Chlorophyll *a* (A_{750}= 0.19) was needed. *Chlorella* can grow (or more accurately survive) in 100% landfill leachate but grows well in leachate diluted to 30% with tapwater over a 9 day period: percent removal of Ammonia-N (53.91±1.35%), Nitrate-N (31.74±0.47%) ,Total-P (65.77±1.67%) ,COD (51.05±1.27%) and BOD (52.78±1.78%). The percent removal of heavy metals in 100% (undiluted leachate) were already very low in landfill leachate but *Chlorella* removed 70% of Chromium and 66% of Nickel but the level of Zinc in the landfill leachate was already below the detection limits even before treatment with *Chlorella*.

It was much more difficult to get *Chlorella* to grow in Garbage Pit Leachate. Garbage Pit Leachate needed to be diluted to 20% in order for *Chlorella* to grow reliably but heavier inoculations were needed than in the case of the landfill leachate (biomass of Chlorophyll *a* = 0.92 µg/ml, $A_{750\ nm}$= 0.19). *Chlorella* would only grow in Garbage Pit Leachate diluted to 20% in tapwater if it was well aerated (shaker). The percent removal of pollutants were: ammonia-N (41.5 ± 1.22%), nitrate-N (32.4 ± 1.45%), Total Phosphorus (55.1 ± 2.56%), COD (50.8 ± 1.74%) and BOD (49.2 ± 3.02%). The results of heavy metals analysis shows *Chlorella* grown in 20% Garbage Pit Leachate diluted with tapwater removed 90% of the Zinc and 33% of the Chromium. *Chlorella* in 10% Garbage Pit Leachate and grown on a shaker removed nearly all the Zinc (99.4%) and most of the Nickel (85%). The biomass

of Chlorophyll *a* 1.17 µg/ml ($A_{750\ nm}$= 0.202) removed 90% of the Zinc and performed just as well as higher inoculums. The proper pH condition for the best growth of *Chlorella* was pH 7 – 8. Pretreatment with lime successfully removes nearly all the heavy metals and does not inhibit growth of *Chlorella* and so pretreatment with adequate lime to precipitate metals followed by bioremediation using *Chlorella* is a viable proposition because *Chlorella* is highly tolerant of high pH.

PAM experiment were run to rapidly check the toxicity of leachates and dilutions of leachates only 1 hour after exposure to the leachate. It was found that 30% landfill leachate diluted with tapwater gave the highest Electron transport Rate (ETR) which was also the dilution which gave the best growth of *Chlorella* in diluted landfill leachate. The ETR was near zero in 100% landfill leachate. ETR of *Chlorella* incubated in Garbage Pit Leachate was zero in all the dilutions of leachate tested (20% to 100%) leachate diluted with tapwater. PAM experiments on *Chlorella* incubated in BG-11 containing $ZnSO_4$ at concentrations similar to those found in Garbage Pit Leachate showed that Zn concentrations were not very inhibitory at pH 5 or pH 8. This shows that Zn toxicity was not complete explanation of the toxicity of Garbage Pit Leachate.

5.2 Recommendations

Logistics and funding restricted the scope of the present study and more complete collections of data would facilitate better statistical analysis. Sampling protocols need to be more rigorous than used in the present study to achieve more homogeneous and representative samples. The results of this study show however that the landfill leachate has a relatively low toxicity but the Garbage Pit Leachate is highly toxic and is a hazardous material.

The PAM experiments did show that the toxicity of the leachates is readily measurable using *Chlorella* as a test organism and are much quicker than the growth experiments. The results confirmed that the toxicity of the leachates was an immediate effect and not a slow cumulative effect.

The X-Ray spectrometry is probably cheaper and easier to use for the heavy metal analysis and is likely to be accurate enough for the study of leachates. It also has the advantage of measuring nearly all elements that are of environmental concern and so elements that might appear to be

of concern will show up in X-ray analysis, for example Thorium and cadmium were not measured in the present study. Usually X-ray spectrometry can be used for all elements with atomic weights heavier than Sodium or Magnesium depending on the machine.

The results of the lime experiments were very promising to develop a cheap method of improving the leachates wastes, in particular the Garbage Pit Leachate. The present study showed that the levels of lime currently routinely used by the treatment plant are too low to effectively remove heavy metals. Studies are needed to develop the optimum lime treatment with a proper level of replication.

Experiment showed that Zn toxicity could not fully account for the toxicity of Garbage Pit Leachate. There are other factors which are the main contributors to the toxicity of Garbage Pit Leachate. Since Garbage Pit leachate was still very toxic even after lime treatment it is likely that its toxicity is not entirely due to heavy metals because lime treatment should have precipitated heavy metals at alkaline pH.

The experimental protocols used in the present study were as simple as possible to allow for their practical usage at the treatment plant. In particular ordinary domestic tapwater was used as the diluent for the Landfill and Garbage Pit Leachates. However, the domestic water supply of Phuket is routinely chlorinated. Other studies have shown in the laboratory that the Phuket domestic water supply sometimes has enough chlorine to kill larval fish. Routine removal of chlorine using thiosulphate might have improved the results of some of the experiments in this study. However, Saetae *et al* (2013) showed that *Chlorella* is tolerant of much higher levels of chlorine than fish or invertebrate larvae and so the use of dechlorinated tapwater probably would not have changed the general conclusions of the present study.

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Appendices

Appendices I

Standard Method Mainly based on APHA, 1998

1. The determination of pH

Standard pH electrode was used to measure pH (Lutron WA-20117SD). The pH electrode was calibrated with pH 4 and pH 7.2 standard buffers.

2. BOD 5 day test for Biological Oxygen Demand (BOD) method (APHA 1998)

2.1 Apparatus

a. Incubation bottles: Standard BOD bottles (300 ml) with ground-glass stopper and flared mouth were used. Bottles were cleaned with detergent, rinsed thoroughly, and drained before use. As a precaution against drawing air into the dilution bottle during incubation a water seal was used. The satisfactory water seal routine was to slightly overfill the flared mouth of the special BOD bottles.

BOD bottles were incubated in a standard incubator at a thermostatically controlled at 20 ± 1 ^{o}C. Light was excluded to prevent the possibility of photosynthetic production of DO.

2.2 Reagents

Reagents were prepared in advance but discarded if there is any sign of precipitation or biological growth in the stock bottles.

a. Phosphate buffer solution: 8.5 g KH_2PO_4, 21.75 g K_2HPO_4, 33.4 g $Na_2HPO_4 \cdot 7H_2O$, and 1.7g NH_4Cl were dissolved in about 500 ml distilled water and diluted to 1 L. The pH was checked and found to be the expected pH 7.2 without further adjustment.

b. Magnesium sulfate solution: 22.5 g $MgSO_4 \cdot 7H_2O$ was dissolved in distilled water and diluted to 1 L.

c. Calcium chloride solution: 27.5 g $CaCl_2$ was dissolved in distilled water and diluted to 1 L.

d. Ferric chloride solution: 0.25 g $FeCl_3 \cdot 6H_2O$ was dissolved in distilled water and diluted to 1 L.

e. Ammonium chloride solution: 1.15 g NH_4Cl was dissolved in about 500 ml distilled water, adjusted pH to 7.2 with NaOH solution, and then diluted to 1 L. This solution contained 0.3 mg N/ml.

f. Manganese sulfate solution: 480 g of Manganese sulfate tetrahydrate ($MnSO_4.4H_2O$) was dissolved in distilled water made up to a volume of 1000 ml. Sufficiently pure Manganese sulfate solution should show no reaction when added to the potassium iodide solution. No reaction was observed.

g. Alkali – Iodide – Azide solution: 500 g Sodium hydroxide (NaOH), 135 g Sodium iodide (NaI) were first dissolved in about 900 ml distilled water. 10 g of Sodium azide (NaN_3) was then first dissolved in 40 ml of distilled water and then added to the Alkali – Iodide solution and the total volume adjusted to 1000 ml with distilled water.

h. 0.025 N of Standard sodium thiosulfate titrant: 6.205 g of Sodium thiosulfate pentahydrate ($Na_2S_2O_3.5H_2O$) and 0.4 g of Sodium hydroxide (NaOH) was dissolved in distilled water and adjusted to 1000 ml.

2.3 Procedure

Analytical method for saturated O_2

a. 1ml of Magnesium sulfate solution, Calcium chloride solution, Ferric chloride solution, and Phosphate buffer solution were added per 1 liter of water.

b. The solution was equilibrated to the atmosphere by aeration for about 1 – 2 hrs.

c. The water was then poured into BOD bottles and capped using the special BOD bottle gas plugs. Oxygen was measured after preparation for the Day (0) DO_0 measurements and the other bottles were incubated at $20\pm3\ ^{O}C$ for 5 days. Dissolved oxygen was determined on the maturity date (DO_5, Dissolved oxygen after 5 day standard incubation) and compared to the dissolved O_2 recorded for the DO_0 bottles.

Determination of dissolved oxygen (DO)

a. 1 ml of Manganese Sulfate Solution and 1 ml of Alkali - Iodide-Azide solution were added to the BOD bottles, taking care not to add bubbles of air to the water in the BOD bottle. The BOD bottle cap is then carefully replaced and the content mixed carefully 15 times. - The brown/white precipitate was allowed to settle.

b. 1 ml of 1 M H_2SO_4 was then added avoiding any loss of the oxidised floc. The cap was replaced and the bottle shaken back and forth until the floc redissolved. I_2 is released from the floc in the presence of H_2SO_4 in equimolar amounts for the amount of oxygen dissolved in the water.

c. 99 ml of water was poured out of the BOD bottle.

d. The Iodine present was titrated with 0.025 N sodium thiosulfate standard solutions until the solution was light yellow. Starch solution was then added and the titration was continued until the dark color disappears. The volume of sodium thiosulfate titrant is recorded. One mole of thiosulphate titrates one mole of I_2. Therefore, since the thiosulphate solution is 25 mM, 1 ml of thiosulphate titrant $\equiv$ 25 μmoles of O_2. Since the molecular weight of O_2 is 32 then 1 ml of titrant is equivalent to 0.8 mg O_2.

Calculation of BOD value

$BOD\ (mg/L) = DO_0 - DO_5$

DO_0 = The DO measurement at Day 0

DO_5 = Average DO of the sample titration after incubation for 5 days

3. Chemical Oxygen Demand (COD) - Closed Reflux, Titrimetric Method

3.1 Apparatus

a. Digestion vessels: Borosilicate culture tubes were used, 16 x 100 mm, 20 x 150 mm, 25 x 150 mm, with TFE-lined screw caps.

b. Block heater (150 ± 2 ^{o}C), with holes to accommodate digestion vessels was used.

3.2 Reagents

a. Standard potassium dichromate digestion solution, 0.01667 M. 4.903 g $K_2Cr_2O_7$ was added to about 500 ml distilled water, primary standard grade, previously dried at 150 ^{o}C for 2 h, 167 ml conc H_2SO_4, and 33.3 g $HgSO_4$ were then carefully added. The solution was dissolved and then cooled to room temperature, and diluted to 1000 ml.

b. Concentrated Sulfuric acid reagent.

c. Ferroin indicator solution: 1.485 g 1,10-phenanthroline monohydrate and 695 mg $FeSO_4 \cdot 7H_2O$ was dissolved in distilled water and diluted to 100 ml.

d. Standard ferrous ammonium sulfate titrant (FAS), approximately 0.10M: 39.2 g $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$ was dissolved in distilled water. 20 ml conc H_2SO_4 was added and cooled then diluted to 1000 ml.

$$\text{Molarity of FAS solution} = \frac{\text{Volume 0.01667M } K_2Cr_2O_7 \text{ solution titrated, ml} \times 0.1000}{\text{Volume FAS used in titration, ml}}$$

3.3 Procedure

Culture tubes and caps were washed with 20% H_2SO_4 before first use to prevent contamination. Proper sample and reagent volumes are shown in Table 3.1 for. The H_2SO_4 titrant needs to be measured to ± 0.1 ml. Samples are placed in culture tubes and the digestion solution added. The sulfuric acid reagent was carefully run down the inside of vessel so an acid layer is formed under the sample-digestion solution layer. Tubes are then tightly capped and inverted several times to mix completely.

Tubes were placed in block digester preheated to 150 ^{o}C and refluxed for 2 h behind a protective shield. After incubation the tubes were cooled to room temperature by turning off the heater then placed in a test tube rack. Some mercuric sulfate might precipitate out but this does not affect the analysis. 0.05 to 0.1 ml (1 or 2 drops) of ferroin indicator were added and stirred rapidly on a magnetic stirrer while titrating with standardized 0.1M FAS. The end of point was a sharp color change from blue-green to reddish brown, although the blue-green colour may reappear within a few minutes after the completion of the titration. A blank containing the reagents and a volume of distilled water equal to that of sample was refluxed and titrated in the same manner as the samples. The blank gives a titration higher than the samples.

Calculation COD

$$\text{COD as mg } O_2/L = \frac{(A - B) \times M \times 8000}{\text{ml sample}}$$

Where : A = ml FAS used for blank

B = ml FAS used for sample

M = molarity of FAS

8000 = milliequivalent weight of oxygen x 1000 ml/l (4 mol $e^- \equiv$ 1 mol O_2)

Table 3.1 Sample and reagent quantities for various digestion vessels.

Digestion Vessel	Sample (ml)	Digestion Solution (ml)	Sulfuric Acid Reagent (ml)	Total Final Volume (ml)
Culture tubes:				
16 x 100 mm	2.5	1.5	3.5	7.5
20 x 150 mm	5.0	3.0	7.0	15.0
25 x 150 mm	10.0	6.0	14.0	30.0

4. Ammonia-N (Solozano, 1969)

4.1 Apparatus

a. Spectrophotometer (Shimadzu UV-Vis 1601) for measuring absorbance at 640 nm.

4.2 Reagents

a. Phenol-alcohol solution. 10 g of reagent grade phenol dissolved in in 100 ml of 95% v/v ethyl alcohol.

b. Sodium nitroprusside 0.5%. 1 g of sodium nitroprusside dissolved in 200 ml of water. The solution is stored in an amber bottle for not more than one month.

c. Alkaline solution. 100 g of trisodium citrate and 5 g of sodium hydroxide dissolved in 500 ml of water.

d. Sodium hypochlorite solution. A solution of commercial hypochlorite (e.g., Chlorox) which should be at least 1.5 N was used. The solution slowly decomposes and its strength should be checked periodically.

e. Oxidizing solution. 100 ml of sodium citrate solution and 25 ml of hypo chlorite solution was mixed and used the same day.

4.3 Procedure

For natural waters and food extracts, the procedure consisted of the successive addition of 2 ml of phenol solution, 2 ml of sodium nitroprusside solution, and 5 ml of oxidizing reagent to 50 ml of a sample, mixing thoroughly after each addition. The color was allowed to develop at room temperature (22-27 ^{o}C) for 1 hr and the absorbance recorded at 640 nm in the spectrophotometer with a 10 mm length cuvette. All the glassware used must be cleaned by washing initially with warm dilute hydrochloric acid and rinsing thoroughly with distilled water. Sodium nitroprusside at a concentration of 0.5% was sufficient to catalyze the reaction and produce a stable and low blank. The absorbance of samples is unchanged for at least 24 hr. A higher concentration of nitroprusside produces a high and unstable blank which increases with time. The addition of reagents as indicated above gave the best results. At high pH values, the reaction was faster but a slight blue coloration (not due to the presence of ammonium compounds but associated with the presence of nitroprusside) is suppressed when working with distilled water. At the pH used in the current technique (10.4 in distilled water), the blue color from the nitroprusside is formed equally in both samples and blanks and the development time for the color was not unreasonably long. Hydrogen sulphide which interferes with the Solozano assay was not present in the sample used in the present study. The sensitivity of the method is identical in freshwater and seawater so that either can be used for calibration; distilled water was used for blanks in the present study.

Calculation

A standard curve was prepared for ammonia concentrations zero to 5 mg NH_3 using water as the blank in the dual-beam spectrophotometer. (Fig A- 1 – Apendix II)

5. Nitrate – N (Colorimetric, Brucine)

5.1 Apparatus

a. Spectrophotometer (Shimadzu UV-Vis 1601-SHO-2D) for measuring absorbance at 410 nm.

b. Test tube wire racks to hold sample tubes.

d. Water bath suitable for use at 100°C. This bath contained a stirring mechanism so that all tubes were incubated at the same temperature. Incubation temperature is critical in the Brucine nitrate assay.

e. Water bath suitable for use at 10-15°C.

5.2 Reagents

a. Distilled water free of nitrite and nitrate was used in the preparation of all reagents and standards.

b. Sodium chloride solution (30%): 300 g NaCl dissolved in distilled water and diluted to 1 L.

c. Sulfuric acid solution: 500 ml conc. H_2SO_4 was carefully added to 125 ml distilled water with mixing to avoid overheating. It was kept in a tightly stoppered bottle to prevent absorption of atmospheric moisture.

d. Brucine-sulfanilic acid

Reagent: 1 g brucine sulfate $[(C_{23}H_{26}N_2O_4)_2 \cdot H_2SO_4 \cdot 7H_2O]$ and 0.1 g sulfanilic acid ($NH_2C_6H_4SO_3H \cdot H_2O$) was dissolved in 70 ml hot distilled water. 3 ml conc. HCl was then added, cooled, mixed and diluted to 100 ml with distilled water. The brucine solution was stored in a dark bottle at 5 °C. This solution is stable for several months; the pink color that develops slowly does not effect its usefulness. Brucine is dangerous, the bottle was marked: CAUTION: Brucine Sulfate is toxic; take care to avoid ingestion.

e. Potassium nitrate stock solution: 1.0 ml = 0.1 mg NO_3-N. 0.7218 g anhydrous potassium nitrate (KNO_3) was dissolved in distilled water and diluted to 1 liter in a volumetric flask. The solution is stable for at least 6 months.

f. Potassium nitrate standard solution: 1.0 ml = 0.001 mg NO_3-N. 10.0 ml of the stock solution (e) was diluted to 1 liter in a volumetric flask. The standard solution should be prepared fresh weekly.

g. Acetic acid (1 + 3): 1 volume glacial acetic acid (CH_3COOH) was diluted with 3 volumes of distilled water.

h. Sodium hydroxide (lN): 40 g of NaOH was dissolved in distilled water. After cooling it was diluted to 1 l.

5.3 Procedure

a. The pH of the samples was adjusted to approximately pH 7 with acetic acid (2-g) or sodium hydroxide (2-h).

b. The required number of sample tubes were set up in a test tube rack to handle reagent blank, standards and samples. Space tubes needed to be evenly throughout the rack to allow for even flow of bath water between the tubes and hence achieve uniform heating of all tubes.

c. The samples were not strongly coloured and so it was not necessary to correct for color or dissolved organic matter which would cause color on heating. In situations where colour is significant it is necessary to run a set of duplicate samples must be run to which all reagents except the brucine-sulfanilic acid are added.

d. 10.0 ml of standards and samples or an aliquot of the samples were diluted to 10.0 ml and pipetted into the sample tubes.

e. The samples used in the present study were not highly saline and so NaCl did not need to be added to the reagent mixes. The contents of each tube was mixed by swirling and placed in a rack in a cold water bath (0 - 10°C).

f. 10.0 ml of sulfuric acid solution (2-c) was pipetted into into each tube and mixed by swirling. Tubes were allowed to come to thermal equilibrium in the cold bath. A uniform starting temperature is critical.

g. 0.5 ml brucine-sulfanilic acid reagent (2-d) was added to each tube (except the interference control tubes, if required) and carefully mixed by swirling, then placed the rack of tubes in the 100°C water bath for exactly 25 minutes.

CAUTION: The uniformity of incubation temperature was critical. Immersion of the tube rack into the bath should not decrease the temperature of the bath more than 1 to 2°C. crowding of test tubes in the hot water bath has to be avoided. The rack of tubes was removed from the hot water bath and immersed in the cold water bath and allowed to reach thermal equilibrium (20-25°C).

i. Absorbances were read against the reagent blank at 410 nm using a 1 cm or longer cell.

Calculation

a. A standard curve was prepared by plotting the absorbance of standards run by the above procedure against mg NO_3-N/l. (FigA-2 – Apendix II)

b. Where colour blanks have to be used the absorbance of the sample without the brucine-sulfanilic reagent is subtracted from the absorbance of the sample containing brucine-sulfanilic acid and determine mg NO_3-N/l. The dilution factor for the samples needs to be taken into account.

6. Total Phosphorus – Ascorbic Acid Method (Boyd and Tucker, 1992)

6.1 Apparatus

a. A Shimadzu double beam spectrophotomter (UV-Vis 1601) was used and absorbance measured a 880 nm.

b. Acid-washed glassware: Used acid-washed glassware for determining low concentrations of phosphorus. Phosphate contamination is common because of its absorption on glass surfaces. Commercial detergents containing phosphate were avoided in cleaning test tubes. All glassware was washed with hot dilute HCl (10%) and rinsed well with distilled water.

6.2 Reagents

a. Sulfuric acid, H_2SO_4, 5N: 70 ml conc H_2SO_4 was diluted to 500 ml with distilled water.

b. Potassium antimonyl tartrate solution: 1.3715 g $K(SbO)C_4H_4O_6 \cdot ½H_2O$ was dissolved in 400 ml distilled water in a 500-ml volumetric flask and diluted to volume. The solution was stored in a glass-stoppered bottle.

c. Ammonium molybdate solution: 20 g $(NH_4)6Mo_7O_{24} \cdot 4H_2O$ was dissolved in 500 ml distilled water. Stored in a glass-stoppered bottle.

d. Ascorbic acid, 0.1 M: 1.76 g ascorbic acid was dissolved in 100 ml distilled water. The solution was stable for about 1 week at 4 ^{o}C.

e. Combined reagent: The above reagents were mixed in the following proportions for 100 ml of the combined reagent: 50 ml *5N* H_2SO_4, 5 ml potassium antimonyl tartrate solution,

15 ml ammonium molybdate solution, and 30 ml ascorbic acid solution. The order of addition is critical. The solution was mixed after addition of each reagent. If turbidity formed in the combined reagent, the mixture was shaken and left stand few minutes until turbidity disappeared before proceeding. The reagent was stable for 4 h.

f. Potassium persulfate ($K_2S_2O_8$), 10 g was dissolved in 200 ml of distilled water. This solution is not stable and so was prepared daily.

6.3 Procedure

a. 50 ml of a whole sample or an appropriate amount of sample was diluted to 50 ml with distilled water.

b. 1 drop phenolphthalein indicator was added. If a red color developed, sulfuric acid solution was added until the color just disappeared.

c. 1 ml of sulfuric acid solution was then added and 0.4 g of ammonium persulfate (or 10 ml).

d. Acidified samples were autoclaved gently for 30 minutes 120 ^{o}C. This treatment should hydrolyse all organic P compounds present and solubilize all inorganic phosphate.

e. After cooling, add 1 drop of phenolphthalein was added and the solution neutralized to a faint pink color with 1 N sodium hydroxide.

f. The neutralized digestion solution was made up to 100 ml with distilled water. 25 ml was taken for P determination.

g. 4 ml of the combined reagent was added to the sample.

h. At least 10 minutes (but not more than 30 minutes) was allowed for color development and measure absorbance at 880 nm.

Calculation

A standard curve was prepared by plotting the absorbance of standards run by the above procedure against mg NO_3-N/l. (FigA-3 – Apendix II)

Appendices II

Standard Curves

1. Ammonia – N Standard Curve

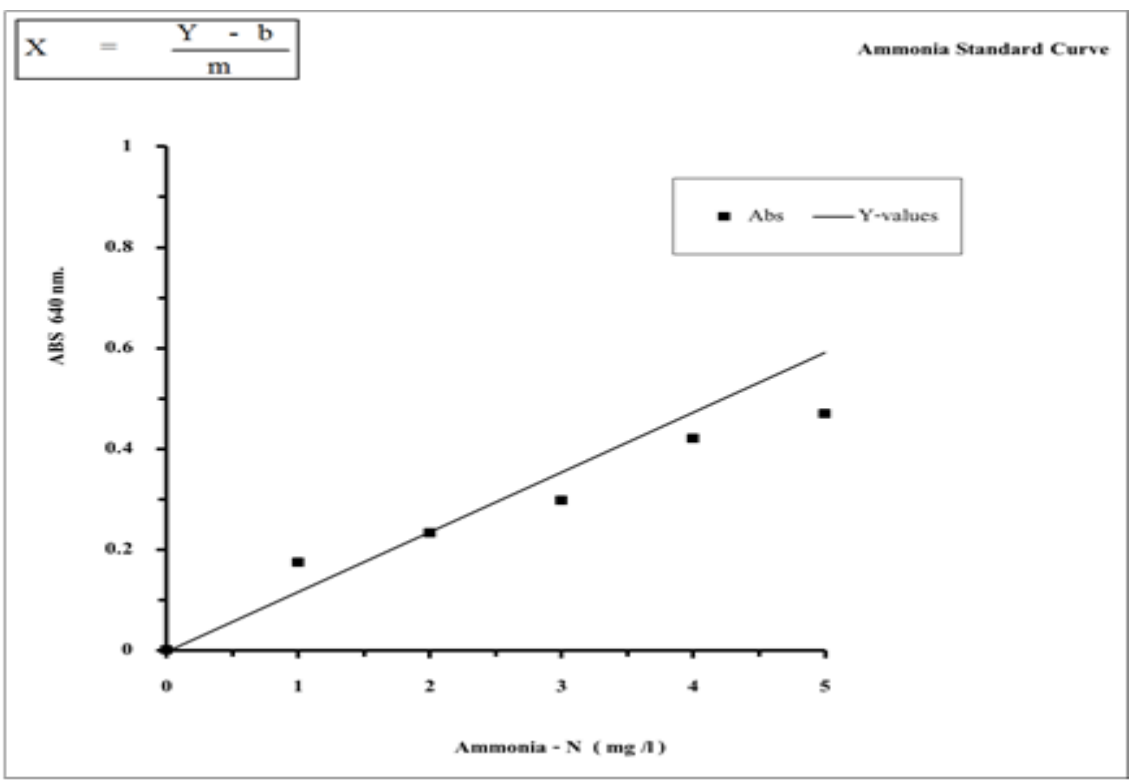

Fig A- 1 - Ammonia – N standard curve: r = 0.98

2. Nitrate – N Standard Curve

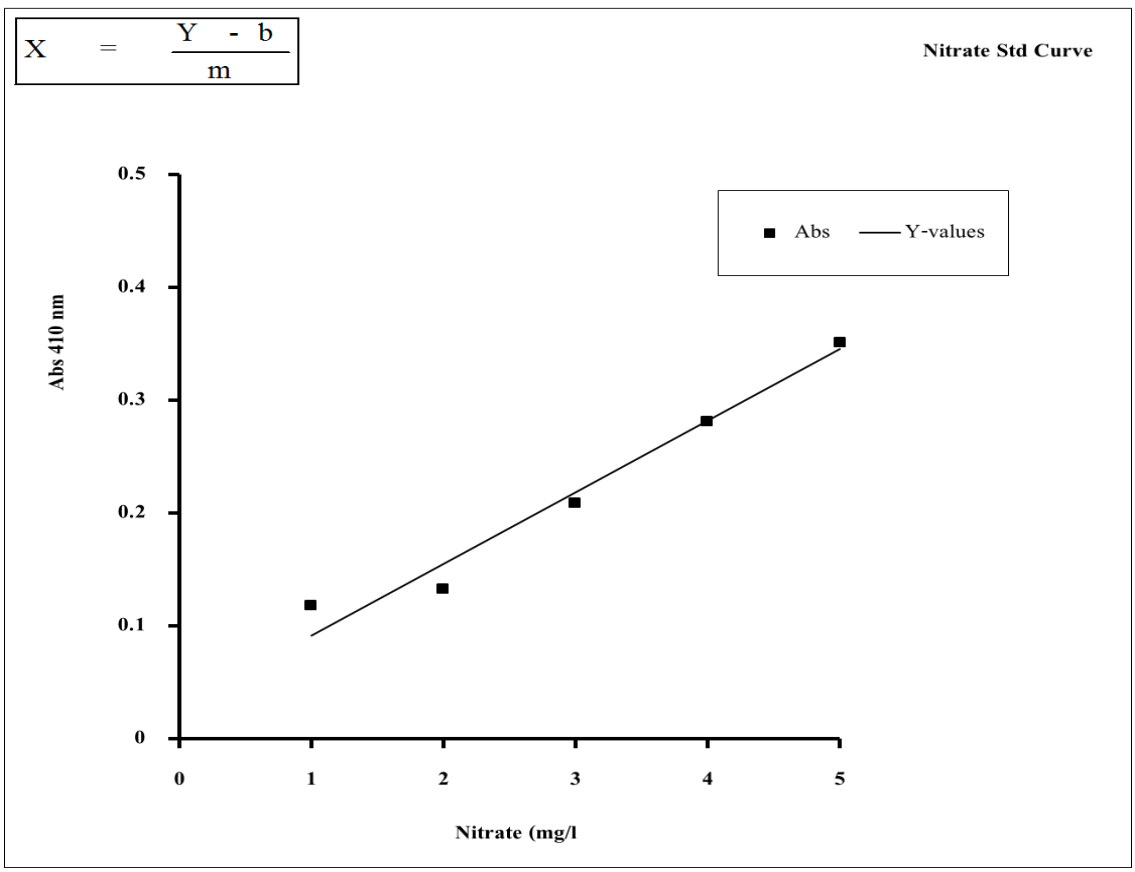

Fig A - 2 Nitrate -N (Colorimetric, Brucine) : r = 0.99

3. Total Phosphorus Standard Curve

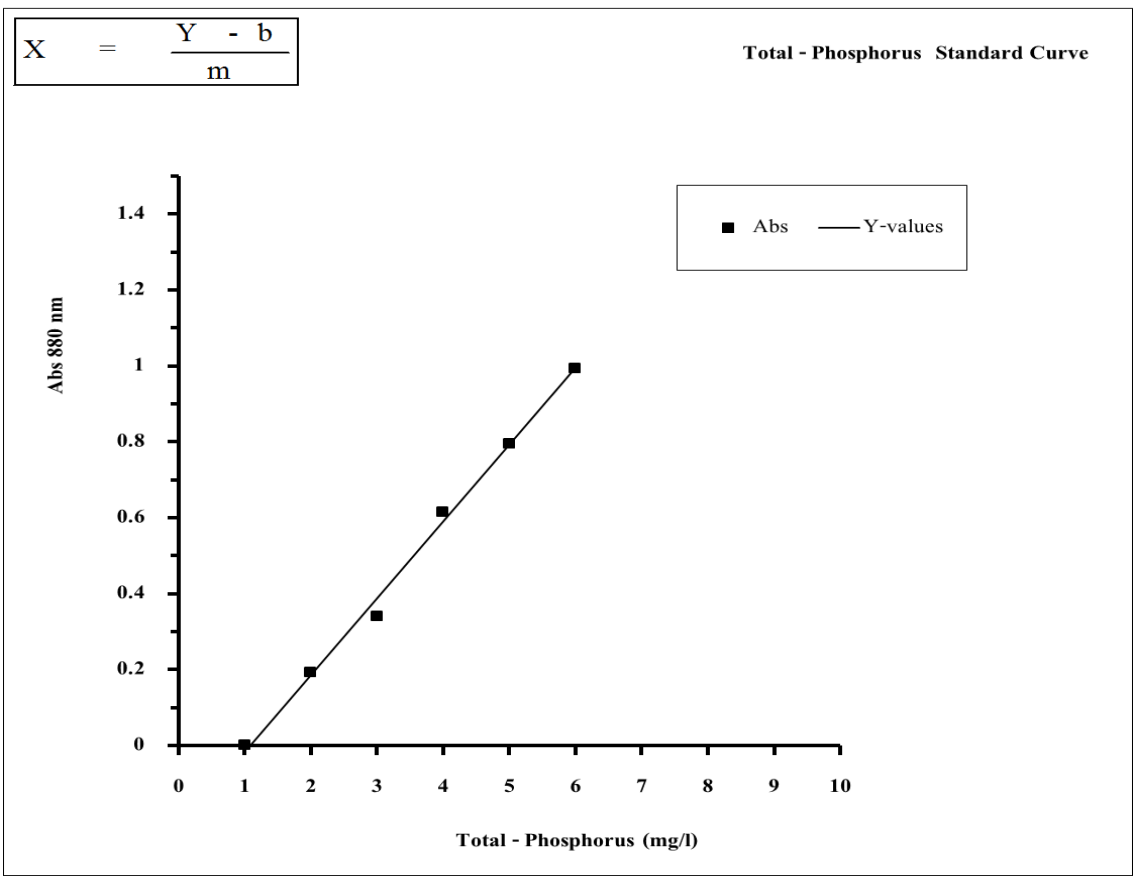

Fig A - 3 Total Phosphorus –Standard curve: r = 0.99

Appendices III

The Growth Experiment of *Chlorella vulgaris* in leachate

1. Phase 1: Growth in light under non-shaking conditions.

1.1 Growth experiment on Landfill Leachate

1.1.1 Experiment on March

1.1.1.1. *Chlorella vulgaris* growth in term of Chlorophyll *a*

Table III -1 *Chlorella vulgaris* growth in term of Chlorophyll *a*

Landfill leachate concentration	**Initial of Chlorophyll *a* = 0.9 µg/ml Abs_{750}= 0.19**									
	Inoculation Period (Days)									
	0	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
100%	0.345	0.333	0.347	0.359	0.368	0.389	0.392	0.415	0.422	0.425
	0.355	0.35	0.368	0.389	0.394	0.39	0.41	0.425	0.43	0.426
	0.365	0.342	0.372	0.377	0.387	0.395	0.43	0.415	0.433	0.467
50%	0.364	0.434	0.566	0.693	0.789	0.998	1.234	1.424	1.688	1.601
	0.354	0.398	0.435	0.514	0.632	0.887	1.15	1.323	1.589	1.612
	0.367	0.423	0.599	0.622	0.785	1.06	1.11	1.456	1.67	1.688
30%	0.38	0.42	0.666	0.892	1.33	1.78	2.09	2.22	2.45	2.65
	0.323	0.456	0.713	0.911	1.43	1.89	2.33	2.45	2.56	2.76
	0.346	0.467	0.892	1.14	1.523	1.754	2.43	2.57	2.689	2.893
10%	0.321	0.476	0.789	1.034	1.35	1.46	1.589	1.632	1.542	1.768
	0.39	0.522	0.8	1.23	1.578	1.644	1.52	1.69	1.7	1.943
	0.38	0.45	0.679	0.98	1.42	1.72	1.69	1.734	1.82	1.88
BG-11 Medium	0.32	0.65	0.98	1.32	1.68	2.22	2.55	3.05	3.5	4.122
	0.34	0.56	0.87	1.22	1.74	2.43	2.76	3.24	3.65	4.02
	0.323	0.68	0.92	1.43	1.89	2.25	2.65	3.09	3.45	3.75

Table III -2 The average of Chlorophyll *a*

Landfill leachate concentration	Inoculation Period (Days)									
	0	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
100%	0.36	0.34	0.36	0.38	0.38	0.39	0.41	0.42	0.43	0.44
50%	0.36	0.42	0.53	0.61	0.74	0.98	1.16	1.40	1.65	1.63
30%	0.35	0.45	0.76	0.98	1.43	1.81	2.28	2.41	2.57	2.77
10%	0.36	0.48	0.76	1.08	1.45	1.61	1.60	1.69	1.69	1.86
BG-11 medium	0.33	0.63	0.92	1.32	1.77	2.30	2.65	3.13	3.53	3.96

1.1.1.2. Percent growth of *Chlorella vulgaris*

Calculation percent growth

% Growth of *Chlorella vulgaris* = [Chlorophyll *a* T_n X 100 / Chlorophyll T_0] -100

where, Chlorophyll *a* T_0 = Chlorophyll *a* initial (Day 0)

Chlorophyll a T_n = Chlorophyll a initial (Day n)

Table III-3 Percent growth of *Chlorella vulgaris* in term Chlorophyll *a*

Landfill leachate concentration	Inoculation Period (Days)								
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
100%	-3.76	2.07	5.63	7.89	10.23	15.68	17.84	20.66	23.76
50%	15.67	47.47	68.57	103.32	171.43	222.03	287.37	355.94	351.71
30%	28.03	116.49	180.55	308.29	417.06	553.00	590.18	633.94	691.52
10%	32.72	107.88	197.34	298.53	342.16	339.87	363.43	363.98	412.47
BG-11 medium	92.27	181.79	303.87	440.18	601.93	709.77	854.22	978.33	1109.77

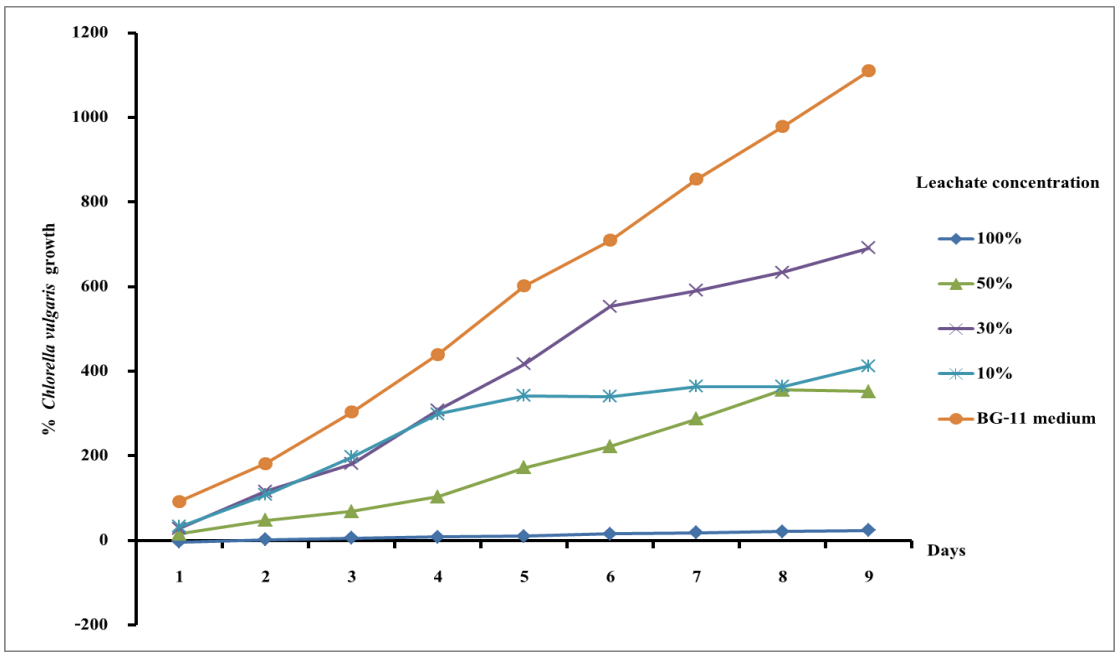

Fig A – 4 Percent growth of *Chlorella vulgaris* in different concentration of landfill leachate on March.

1.1.2 Experiment on April

1.1.2.1. *Chlorella vulgaris* growth in term of Chlorophyll *a*

Table III-4 *Chlorella vulgaris* growth in term of Chlorophyll *a*

Landfill leachate concentration	Initial of Chlorophyll *a* = 0.9 µg/ml Abs_{750}= 0.19									
	Inoculation Period (Days)									
	0	1	2	3	4	5	6	7	8	9
100%	0.32	0.32	0.33	0.33	0.36	0.39	0.39	0.39	0.40	0.41
	0.31	0.30	0.32	0.32	0.34	0.36	0.37	0.38	0.39	0.39
	0.35	0.31	0.32	0.34	0.37	0.37	0.39	0.40	0.37	0.38
50%	0.30	0.32	0.34	0.35	0.37	0.38	1.01	1.16	1.34	1.53
	0.33	0.35	0.35	0.37	0.38	0.39	1.04	1.21	1.35	1.43
	0.30	0.31	0.33	0.38	0.39	0.40	1.12	1.25	1.39	1.46

Table III-4 (cont.)

Landfill leachate concentration	Initial of Chlorophyll *a* = 0.9 µg/ml Abs_{750}= 0.19									
	Inoculation Period (Days)									
	0	1	2	3	4	5	6	7	8	9
	0.29	0.30	0.33	0.37	0.40	1.32	1.65	1.93	2.31	2.42
30%	0.32	0.34	0.36	0.39	1.02	1.28	1.54	1.78	2.11	2.34
	0.30	0.32	0.35	0.37	0.39	1.45	1.78	2.01	2.43	2.74
	0.31	0.32	0.35	0.38	0.40	1.23	1.37	1.54	1.74	1.98
10%	0.30	0.32	0.34	0.36	0.39	1.03	1.35	1.69	1.87	2.01
	0.31	0.33	0.37	0.37	0.39	1.13	1.43	1.61	1.85	2.13
	0.30	0.43	0.65	0.88	1.09	1.33	1.55	1.76	2.11	2.43
BG-11 Medium	0.31	0.57	0.84	1.12	1.33	1.50	1.74	1.99	2.30	2.33
	0.32	0.54	0.75	0.98	1.22	1.54	1.65	1.87	2.10	2.34

Table III -5 The average of Chlorophyll *a*

Landfill leachate concentration	Inoculation Period (Days)									
	0	1	2	3	4	5	6	7	8	9
100%	0.33	0.31	0.32	0.33	0.35	0.37	0.38	0.39	0.39	0.39
50%	0.31	0.33	0.34	0.36	0.38	0.39	1.06	1.21	1.36	1.47
30%	0.30	0.32	0.35	0.38	0.60	1.35	1.66	1.91	2.28	2.50
10%	0.31	0.32	0.35	0.37	0.39	1.13	1.38	1.61	1.82	2.04
BG-11 medium	0.31	0.51	0.75	0.99	1.21	1.46	1.65	1.87	2.17	2.37

1.1.2.2. Percent growth of *Chlorella vulgaris*

Table III-6 Percent growth of *Chlorella vulgaris* in term Chlorophyll *a*

Landfill leachate concentration	Inoculation Period (Days)								
	1	2	3	4	5	6	7	8	9
100%	-5.01	-1.63	1.23	7.87	13.48	16.55	19.20	18.18	20.84
50%	5.50	10.24	17.89	22.20	26.40	241.59	290.09	339.66	376.29
30%	6.03	13.60	23.46	98.57	344.08	444.96	527.19	651.10	722.59
10%	5.32	14.66	19.33	27.36	268.08	350.60	425.52	492.83	564.50
BG-11 medium	65.06	140.09	219.40	290.14	368.38	429.47	502.36	597.75	660.99

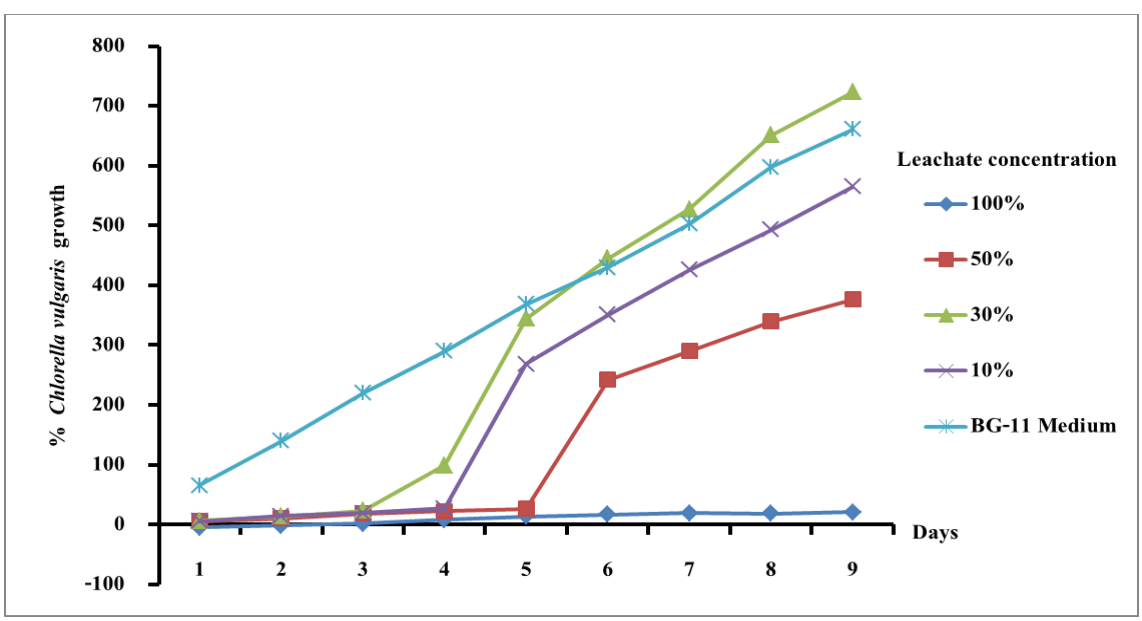

Fig A – 5 Percent growth of *Chlorella vulgaris* in different concentration of landfill leachate on April

1.1.3 Experiment on June

1.1.3.1. *Chlorella vulgaris* growth in term of Chlorophyll *a*

Table III -7 *Chlorella vulgaris* growth in term of Chlorophyll *a*

Landfill leachate concentration	**Initial of Chlorophyll *a* = 0.9 µg/ml Abs_{750}= 0.19**									
	Inoculation Period (Days)									
	0	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
	0.30	0.31	0.32	0.31	0.32	0.32	0.32	0.33	0.33	0.33
100%	0.29	0.30	0.31	0.32	0.32	0.34	0.32	0.33	0.34	0.37
	0.30	0.31	0.31	0.33	0.32	0.32	0.33	0.33	0.34	0.37
	0.30	0.36	0.39	0.43	0.49	0.53	0.58	0.61	0.66	0.67
50%	0.30	0.35	0.38	0.40	0.46	0.51	0.54	0.59	0.64	0.68
	0.31	0.35	0.40	0.46	0.50	0.52	0.58	0.60	0.65	0.67
	0.30	0.35	0.40	0.44	0.49	0.67	0.72	0.89	1.01	1.21
30%	0.30	0.37	0.42	0.49	0.53	0.78	0.97	1.30	1.40	0.17
	0.31	0.36	0.41	0.47	0.65	0.89	1.09	1.45	1.80	2.01
	0.33	0.43	0.54	0.66	0.78	1.02	1.25	1.23	1.26	1.32
10%	0.32	0.45	0.51	0.62	0.74	1.13	1.24	1.22	1.32	1.20
	0.29	0.40	0.49	0.54	0.73	1.09	1.18	1.24	1.43	1.65
	0.45	0.68	0.98	1.23	1.55	1.87	2.03	2.40	2.89	3.34
BG-11 Medium	0.38	0.77	1.22	1.43	1.56	1.67	2.34	2.36	2.65	3.09
	0.37	0.54	0.87	1.42	1.75	1.93	2.11	2.23	2.40	2.87

Table III -8 The average of Chlorophyll *a*

Landfill leachate concentration	Inoculation Period (Days)									
	0	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
100%	0.30	0.31	0.32	0.32	0.32	0.33	0.32	0.33	0.33	0.36
50%	0.30	0.35	0.39	0.43	0.48	0.52	0.57	0.60	0.65	0.67
30%	0.30	0.36	0.41	0.47	0.56	0.78	0.93	1.21	1.40	1.13
10%	0.31	0.43	0.51	0.61	0.75	1.08	1.22	1.23	1.34	1.39
BG-11 medium	0.40	0.66	1.02	1.36	1.62	1.82	2.16	2.33	2.65	3.10

1.1.3.2. Percent growth of *Chlorella vulgaris*

Table III-9 Percent growth of *Chlorella vulgaris* in term Chlorophyll *a*

Landfill leachate concentration	Inoculation Period (Days)								
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
100%	3.04	6.64	7.21	8.11	10.25	9.68	11.71	12.73	20.38
50%	14.79	27.60	41.40	57.83	71.30	86.75	97.48	113.47	119.93
30%	17.11	35.42	53.07	82.89	156.58	205.15	299.12	361.62	271.16
10%	35.81	63.03	93.11	139.19	243.22	288.77	290.89	324.79	341.74
BG-11 medium	65.97	156.05	240.28	305.34	356.21	440.45	482.99	562.22	675.65

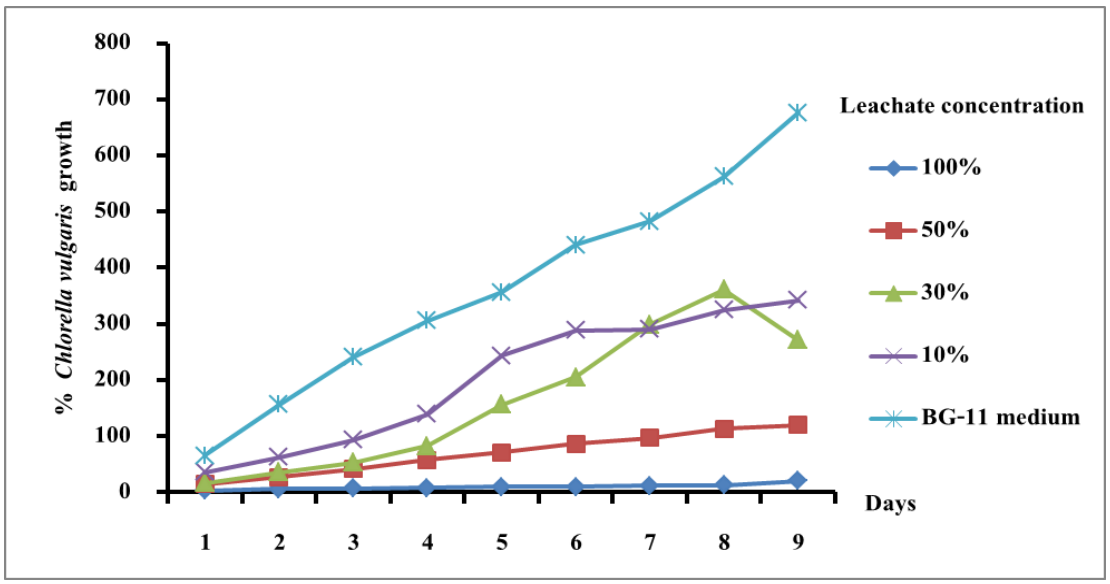

Fig A – 6 Percent growth of *Chlorella vulgaris* in different concentration of landfill leachate on June

1.1.4 Experiment on July

1.1.4.1. *Chlorella vulgaris* growth in term of Chlorophyll *a*

Table III-10 *Chlorella vulgaris* growth in term of Chlorophyll *a*

Landfill leachate concentration	Initial of Chlorophyll *a* = 0.9 µg/ml Abs_{750}= 0.19									
	Inoculation Period (Days)									
	0	1	2	3	4	5	6	7	8	9
	0.42	0.42	0.41	0.43	0.44	0.43	0.46	0.48	0.52	0.52
100%	0.43	0.41	0.40	0.42	0.43	0.44	0.45	0.48	0.50	0.51
	0.39	0.37	0.39	0.41	0.42	0.42	0.44	0.49	0.51	0.53
	0.36	0.39	0.44	0.49	0.52	0.57	0.62	0.75	0.78	0.81
50%	0.34	0.40	0.42	0.48	0.53	0.58	0.63	0.67	0.69	0.73
	0.38	0.44	0.47	0.49	0.51	0.56	0.62	0.65	0.69	0.73
	0.34	0.41	0.51	0.59	0.62	0.66	0.71	0.79	0.91	1.10
30%	0.31	0.42	0.53	0.58	0.61	0.67	0.73	0.81	1.00	1.45
	0.32	0.46	0.51	0.56	0.60	0.65	0.71	0.90	1.13	1.56

Table III-10 (cont.)

Landfill leachate concentration	Initial of Chlorophyll *a* = 0.9 µg/ml Abs_{750}= 0.19									
	Inoculation Period (Days)									
	0	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
10%	0.31	0.40	0.46	0.53	0.62	0.84	0.99	1.20	1.25	1.20
	0.31	0.39	0.45	0.59	0.62	0.79	0.87	1.03	1.23	1.34
	0.32	0.41	0.49	0.61	0.65	0.76	0.89	1.11	1.34	1.36
BG-11 Medium	0.36	0.64	1.00	1.34	1.68	1.92	2.45	2.78	3.20	5.65
	0.37	0.77	1.12	1.20	1.56	1.87	2.22	2.98	3.09	4.23
	0.36	0.67	0.98	1.45	1.64	1.93	2.23	2.65	3.45	4.78

Table III -11 The average of Chlorophyll a

Landfill leachate concentration	Inoculation Period (Days)									
	0	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
100%	0.42	0.40	0.40	0.42	0.43	0.43	0.45	0.48	0.51	0.52
50%	0.36	0.41	0.44	0.49	0.52	0.57	0.63	0.69	0.72	0.76
30%	0.32	0.43	0.52	0.58	0.61	0.66	0.72	0.83	1.01	1.37
10%	0.31	0.40	0.46	0.58	0.63	0.80	0.92	1.11	1.27	1.30
BG-11 medium	0.36	0.69	1.03	1.33	1.63	1.91	2.30	2.80	3.25	4.89

1.1.4.2. Percent growth of *Chlorella vulgaris*

Table III-12 Percent growth of *Chlorella vulgaris* in term Chlorophyll *a*

Landfill leachate concentration	Inoculation Period (Days)								
	1	2	3	4	5	6	7	8	9
100%	-3.78	-3.94	1.85	4.18	3.61	9.24	16.14	22.81	25.38
50%	13.38	23.05	35.32	45.72	58.46	74.54	92.38	100.46	111.34
30%	32.17	60.02	77.70	88.18	102.57	121.07	157.35	212.44	322.40
10%	27.15	47.61	83.78	101.38	153.13	191.62	254.19	305.09	313.57
BG-11 medium	90.83	184.04	266.06	347.71	424.77	533.03	671.56	793.58	1244.95

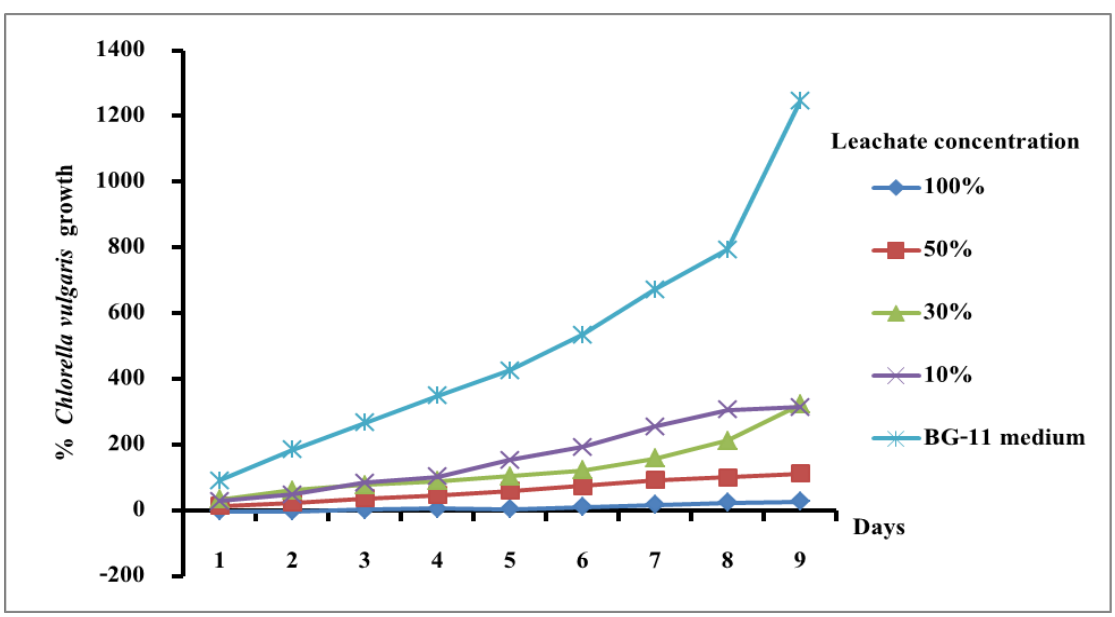

Fig A – 7 Percent growth of *Chlorella vulgaris* in different concentration of landfill leachate on July

1.1.5 Experiment on October

1.1.5.1. *Chlorella vulgaris* growth in term of Chlorophyll *a*

Table III -13 *Chlorella vulgaris* growth in term of Chlorophyll *a*

Landfill leachate concentration	**Initial of Chlorophyll *a* = 0.9 µg/ml Abs_{750}= 0.19**									
	Inoculation Period (Days)									
	0	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
	0.39	0.34	0.26	0.25	0.34	0.28	0.28	0.28	0.28	0.30
100%	0.39	0.34	0.48	0.46	0.44	0.39	0.37	0.36	0.35	0.34
	0.38	0.36	0.39	0.36	0.40	0.41	0.44	0.45	0.47	0.47
	0.40	0.51	0.59	0.72	0.89	1.13	1.35	1.62	1.82	1.51
50%	0.38	0.49	0.65	0.71	1.06	1.34	1.45	1.58	1.62	1.48
	0.37	0.50	0.66	0.67	0.94	1.33	1.55	1.68	1.77	1.51
	0.44	0.44	0.74	1.17	1.80	1.96	2.15	2.46	2.63	2.76
30%	0.36	0.44	0.69	0.98	1.55	2.00	2.34	2.56	2.68	2.28
	0.36	0.48	0.87	1.32	1.84	2.17	2.45	2.66	2.74	2.70
	0.38	0.54	0.73	1.07	1.32	1.36	1.47	1.55	1.56	0.94
10%	0.45	0.41	0.80	1.28	1.75	1.49	1.56	1.51	1.49	1.07
	0.46	0.41	1.01	1.56	1.79	1.59	1.59	1.66	1.71	1.84
	0.32	0.54	0.73	1.12	1.40	1.76	2.23	2.76	3.20	3.46
BG-11 Medium	0.33	0.53	0.82	1.09	1.53	1.87	2.30	2.80	3.13	3.50
	0.31	0.52	0.81	1.20	1.65	1.98	2.45	2.98	3.45	3.80

Table III -14 The average of Chlorophyll *a*

Landfill leachate concentration	Inoculation Period (Days)									
	0	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
100%	0.38	0.35	0.37	0.36	0.39	0.36	0.36	0.36	0.37	0.37
50%	0.38	0.50	0.63	0.70	0.96	1.27	1.45	1.63	1.74	1.50
30%	0.39	0.45	0.76	1.16	1.73	2.04	2.31	2.56	2.68	2.58
10%	0.43	0.45	0.85	1.30	1.62	1.48	1.54	1.57	1.59	1.28
BG-11 medium	0.32	0.53	0.79	1.14	1.53	1.87	2.33	2.85	3.26	3.59

1.1.5.2. Percent growth of *Chlorella vulgaris*

Table III-15 Percent growth of *Chlorella vulgaris* in term Chlorophyll *a*

Landfill leachate concentration	Inoculation Period (Days)								
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
100%	-9.54	-2.69	-6.24	1.65	-6.76	-5.90	-5.29	-4.94	-3.82
50%	29.22	65.22	81.65	151.48	230.17	277.39	324.00	352.96	291.48
30%	17.11	97.16	198.62	346.26	426.83	496.30	560.36	592.09	565.35
10%	4.91	97.82	204.36	278.96	246.06	259.55	267.65	270.77	200.00
BG-11 medium	65.01	145.24	253.00	374.12	480.75	622.57	784.06	912.42	1013.87

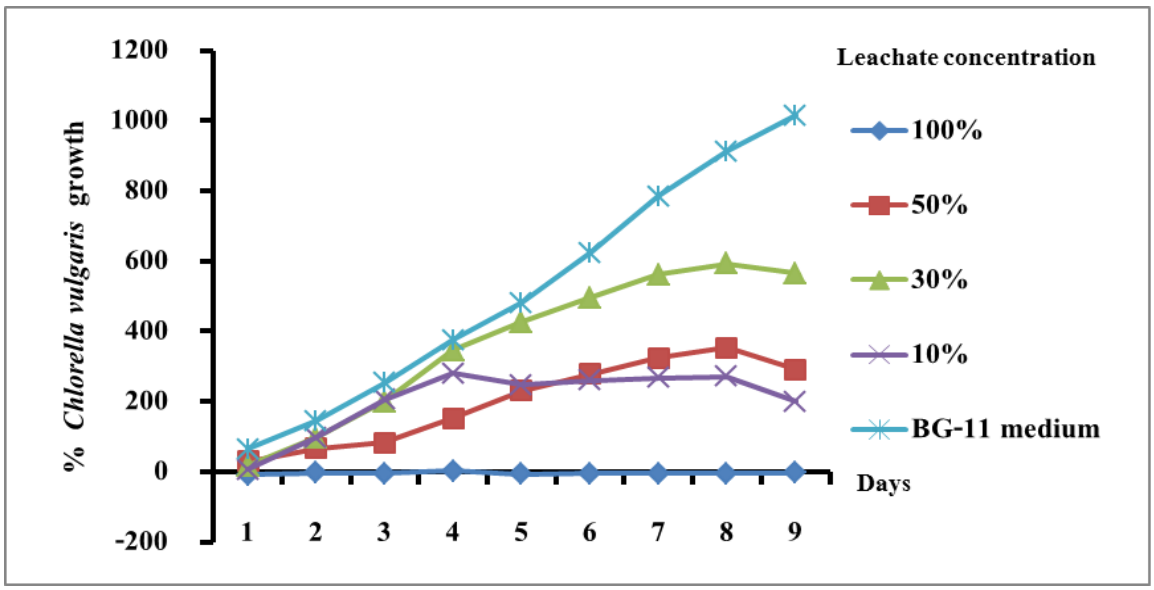

Fig A – 8 Percent growth of *Chlorella vulgaris* in different concentration of landfill leachate on October

1.1.6 Experiment on November

1.1.6.1. *Chlorella vulgaris* growth in term of Chlorophyll *a*

Table III -16 *Chlorella vulgaris* growth in term of Chlorophyll *a*

Landfill leachate concentration	Initial of Chlorophyll *a* = 0.9 µg/ml Abs_{750}= 0.19									
	Inoculation Period (Days)									
	0	1	2	3	4	5	6	7	8	9
	0.33	0.35	0.35	0.37	0.38	0.40	0.42	0.45	0.40	0.40
100%	0.29	0.30	0.31	0.33	0.35	0.36	0.40	0.42	0.42	0.41
	0.32	0.32	0.33	0.34	0.37	0.40	0.42	0.40	0.42	0.40
	0.29	0.29	0.31	0.34	0.36	0.39	0.41	0.42	0.47	0.51
50%	0.30	0.31	0.32	0.35	0.37	0.40	0.40	0.42	0.46	0.50
	0.31	0.32	0.34	0.35	0.38	0.43	0.44	0.47	0.49	0.50
	0.31	0.31	0.35	0.42	0.52	0.68	0.83	1.01	1.40	1.67
30%	0.29	0.31	0.36	0.39	0.43	0.49	0.54	0.61	0.71	0.93
	0.32	0.37	0.41	0.47	0.59	0.61	0.62	0.81	1.02	1.45

Table III -16 (cont.)

Landfill leachate concentration	Initial of Chlorophyll *a* = 0.9 µg/ml Abs_{750}= 0.19									
	Inoculation Period (Days)									
	0	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
10%	0.30	0.42	0.54	0.62	0.87	1.02	1.11	1.45	1.65	2.01
	0.29	0.32	0.36	0.41	0.64	0.97	1.21	1.53	1.78	2.22
	0.31	0.38	0.41	0.45	0.52	0.61	0.89	0.94	1.30	1.50
BG-11 Medium	0.33	0.38	0.42	0.54	0.73	0.92	1.30	1.62	1.89	2.15
	0.35	0.39	0.45	0.55	0.69	0.98	1.40	1.74	2.09	2.43
	0.31	0.37	0.45	0.56	0.75	1.02	1.34	1.56	1.89	2.13

Table III -17 The average of Chlorophyll *a*

Landfill leachate concentration	Inoculation Period (Days)									
	0	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
100%	0.32	0.32	0.33	0.35	0.37	0.39	0.41	0.43	0.41	0.40
50%	0.30	0.31	0.33	0.35	0.37	0.41	0.42	0.44	0.47	0.50
30%	0.31	0.33	0.37	0.43	0.51	0.59	0.67	0.81	1.04	1.35
10%	0.30	0.37	0.44	0.49	0.68	0.87	1.07	1.31	1.58	1.61
BG-11 medium	0.33	0.38	0.44	0.55	0.72	0.97	1.35	1.64	1.96	2.24

1.1.6.2. Percent growth of *Chlorella vulgaris*

Table III-18 Percent growth of *Chlorella vulgaris* in term Chlorophyll *a*

Landfill leachate concentration	Inoculation Period (Days)								
	1	2	3	4	5	6	7	8	9
100%	2.22	4.76	10.15	17.55	22.94	31.40	35.20	31.50	28.12
50%	2.66	8.19	15.28	22.48	34.77	39.20	44.96	57.25	67.66
30%	7.70	20.61	39.05	66.81	92.84	116.70	163.99	239.59	339.26
10%	24.44	45.56	64.67	125.67	188.67	256.67	335.56	425.56	436.67
BG-11 medium	13.98	32.60	66.00	118.31	193.76	306.44	394.97	490.54	575.05

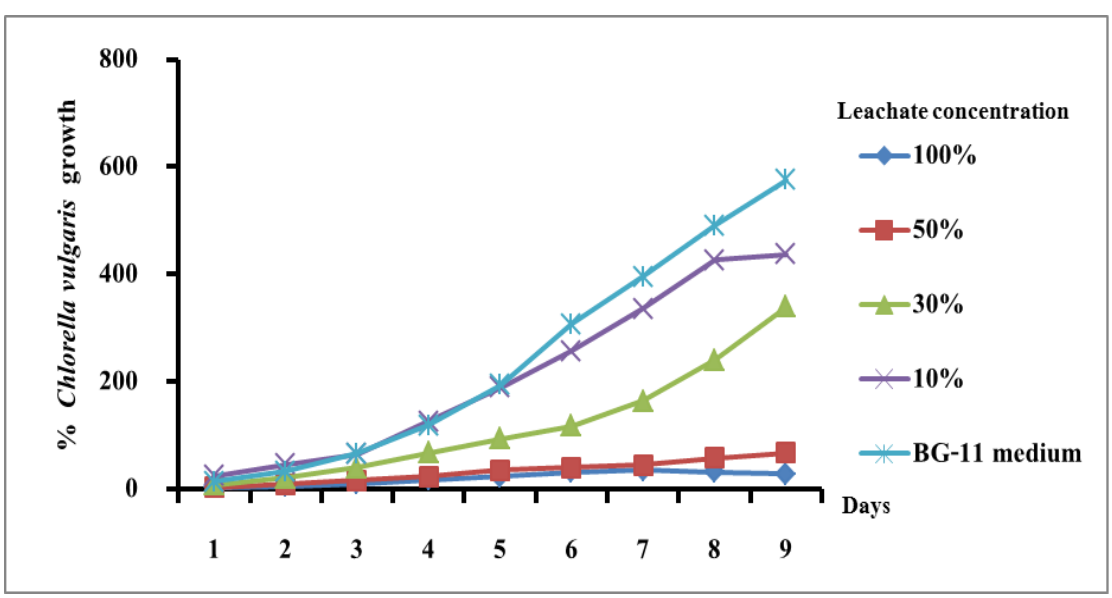

Fig A – 9 Percent growth of *Chlorella vulgaris* in different concentration of landfill leachate on November

1.2 Growth experiment on Garbage Pit Leachate

1.2.1 Experiment on March

1.2.1.1. *Chlorella vulgaris* growth in term of Chlorophyll *a*

Table III-19 *Chlorella vulgaris* growth in term of Chlorophyll *a*

Garbage Pit Leachate concentration	Initial of Chlorophyll *a* = 0.9 µg/ml Abs_{750}= 0.19									
	Inoculation Period (Days)									
	0	1	2	3	4	5	6	7	8	9
	0.36	0.33	0.28	0.26	0.23	0.23	0.19	0.19	0.18	0.16
100%	0.34	0.31	0.25	0.23	0.20	0.19	0.18	0.17	0.16	0.14
	0.31	0.30	0.26	0.24	0.22	0.20	0.19	0.14	0.14	0.13
	0.32	0.30	0.25	0.21	0.20	0.18	0.17	0.16	0.14	0.15
50%	0.30	0.28	0.26	0.24	0.25	0.21	0.19	0.18	0.12	0.14
	0.32	0.29	0.27	0.22	0.22	0.20	0.19	0.17	0.14	0.15
	0.29	0.31	0.32	0.29	0.27	0.22	0.20	0.20	0.18	0.16
30%	0.31	0.32	0.29	0.24	0.22	0.21	0.20	0.18	0.17	0.15
	0.31	0.30	0.25	0.24	0.20	0.19	0.18	0.17	0.16	0.16
	0.29	0.32	0.35	0.36	0.36	0.33	0.32	0.34	0.35	0.36
20%	0.30	0.33	0.34	0.36	0.37	0.36	0.33	0.34	0.32	0.32
	0.31	0.32	0.34	0.39	0.39	0.35	0.35	0.32	0.32	0.32
	0.30	0.32	0.35	0.35	0.37	0.38	0.35	0.33	0.31	0.30
10%	0.31	0.32	0.34	0.36	0.38	0.35	0.31	0.29	0.26	0.25
	0.32	0.30	0.32	0.34	0.35	0.32	0.30	0.29	0.28	0.29
	0.34	0.63	0.88	1.34	1.79	2.33	2.65	3.01	3.43	3.98
BG-11 Medium	0.31	0.54	0.98	1.25	1.89	2.43	2.84	3.12	3.60	4.12
	0.32	0.62	0.95	1.42	1.86	2.40	2.94	3.35	3.76	4.20

Table III -20 The average of Chlorophyll *a*

Garbage Pit Leachate concentration	Inoculation Period (Days)									
	0	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
100%	0.34	0.31	0.26	0.24	0.22	0.21	0.19	0.17	0.16	0.14
50%	0.31	0.29	0.26	0.22	0.22	0.20	0.18	0.17	0.13	0.14
30%	0.31	0.31	0.29	0.25	0.23	0.21	0.19	0.18	0.17	0.16
20%	0.30	0.32	0.34	0.37	0.37	0.35	0.33	0.33	0.33	0.33
10%	0.31	0.31	0.33	0.35	0.37	0.35	0.32	0.30	0.28	0.28
BG-11 medium	0.33	0.60	0.93	1.34	1.85	2.39	2.81	3.16	3.60	4.10

1.2.1.2. Percent growth of *Chlorella vulgaris*

Table III -21 Percent growth of *Chlorella vulgaris* in term Chlorophyll *a*

Garbage Pit Leachate concentration	Inoculation Period (Days)								
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
100%	-7.02	-22.26	-29.28	-35.31	-38.87	-45.00	-50.84	-53.02	-57.17
50%	-7.05	-16.99	-28.95	-28.95	-37.07	-41.88	-46.58	-57.05	-53.95
30%	1.97	-6.66	-17.03	-25.44	-32.42	-37.01	-40.07	-44.32	-48.36
20%	7.33	13.56	23.44	23.78	16.33	10.00	11.22	10.00	9.78
10%	1.62	7.97	12.72	18.00	12.72	2.91	-1.94	-8.62	-9.05
BG-11 medium	83.40	186.99	311.27	467.62	633.61	763.73	871.31	1005.53	1160.25

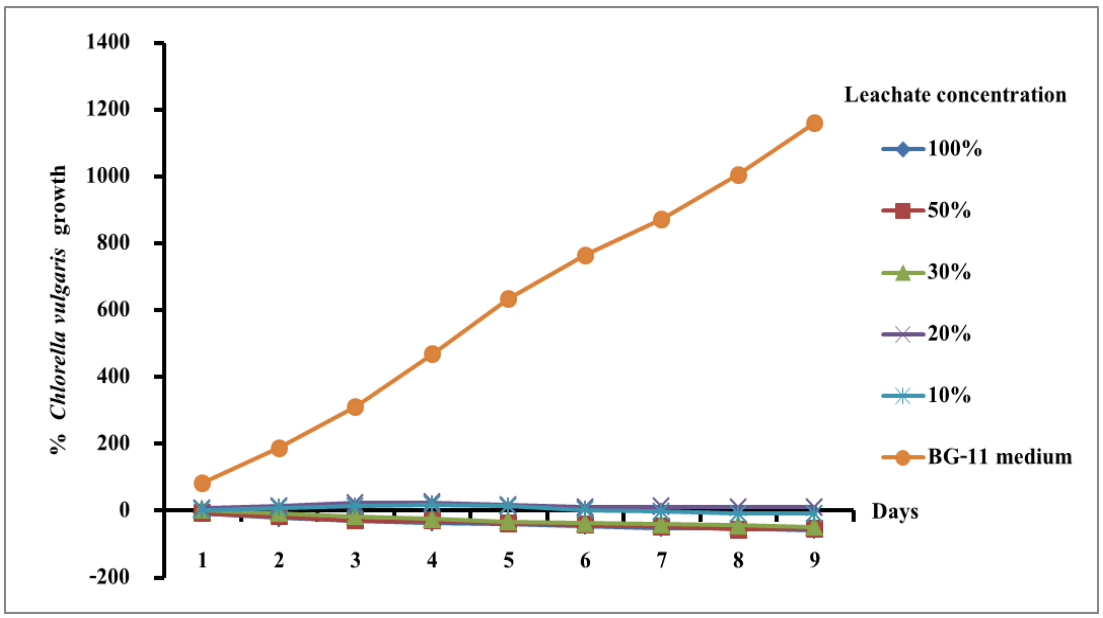

Fig A – 10 Percent growth of *Chlorella vulgaris* in different concentration of Garbage Pit Leachate on March with control (*Chlorella vulgaris* grew in BG-11 medium)

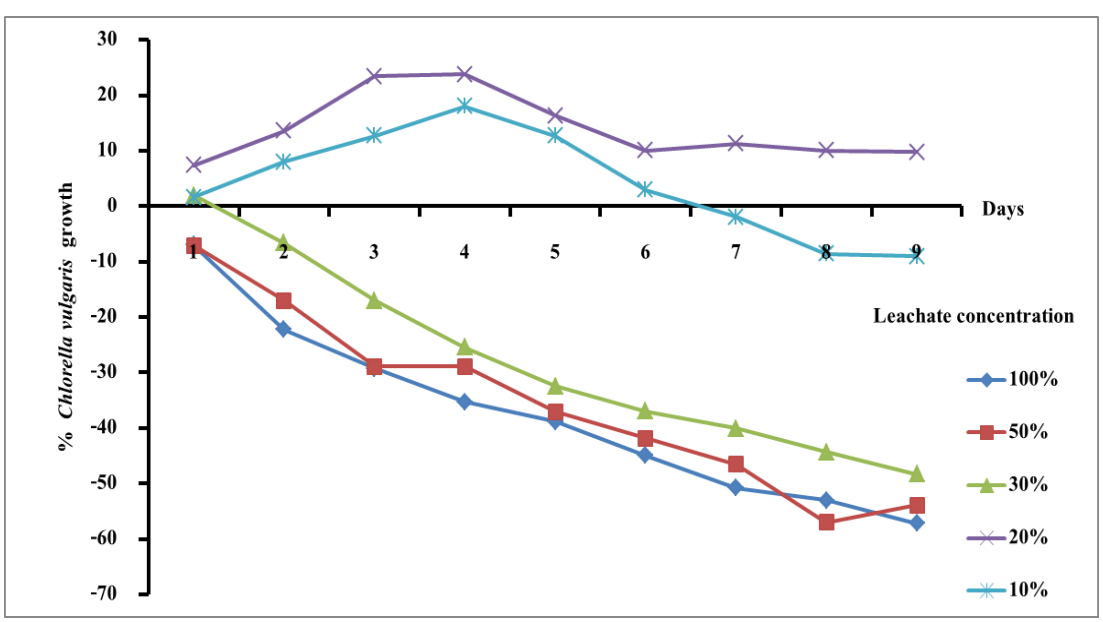

Fig A – 11 Percent growth of *Chlorella vulgaris* in different concentration of Garbage Pit Leachate on March without control (*Chlorella vulgaris* grew in BG-11 medium)

1.2.2 Experiment on April

1.2.2.1. *Chlorella vulgaris* growth in term of Chlorophyll *a*

Table III-22 *Chlorella vulgaris* growth in term of Chlorophyll *a*

Garbage Pit Leachate concentration	Initial of Chlorophyll *a* = 0.9 µg/ml Abs_{750}= 0.19									
	Inoculation Period (Days)									
	0	1	2	3	4	5	6	7	8	9
	0.30	0.29	0.27	0.25	0.21	0.20	0.19	0.16	0.14	0.11
100%	0.32	0.30	0.29	0.25	0.23	0.20	0.19	0.16	0.14	0.11
	0.30	0.29	0.27	0.24	0.22	0.20	0.18	0.15	0.12	0.10
	0.29	0.27	0.26	0.24	0.24	0.23	0.21	0.20	0.19	0.17
50%	0.29	0.28	0.25	0.23	0.22	0.20	0.19	0.18	0.17	0.15
	0.28	0.25	0.23	0.21	0.21	0.20	0.20	0.18	0.16	0.13
	0.30	0.31	0.32	0.30	0.28	0.27	0.24	0.22	0.20	0.17
30%	0.29	0.30	0.29	0.28	0.27	0.26	0.24	0.21	0.20	0.20
	0.29	0.29	0.28	0.27	0.24	0.23	0.21	0.20	0.18	0.15
	0.31	0.30	0.31	0.32	0.33	0.36	0.34	0.34	0.35	0.33
20%	0.33	0.31	0.32	0.33	0.35	0.37	0.37	0.36	0.37	0.36
	0.30	0.30	0.31	0.32	0.33	0.35	0.36	0.37	0.37	0.38
	0.30	0.29	0.30	0.30	0.32	0.32	0.33	0.35	0.37	0.36
10%	0.27	0.29	0.31	0.31	0.32	0.34	0.34	0.33	0.33	0.35
	0.29	0.30	0.32	0.32	0.31	0.32	0.32	0.32	0.33	0.36
	0.30	0.40	0.86	1.09	1.44	1.65	1.97	2.23	2.54	2.77
BG-11 Medium	0.31	0.65	0.88	1.10	1.42	1.84	2.13	2.45	2.64	2.87
	0.31	0.66	0.76	0.92	1.30	1.67	1.90	2.20	2.44	2.66

Table III -23 The average of Chlorophyll *a*

Garbage Pit Leachate concentration	Inoculation Period (Days)									
	0	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
100%	0.31	0.29	0.28	0.25	0.22	0.20	0.18	0.16	0.13	0.11
50%	0.29	0.27	0.25	0.23	0.22	0.21	0.20	0.19	0.17	0.15
30%	0.29	0.30	0.30	0.28	0.26	0.25	0.23	0.21	0.19	0.17
20%	0.31	0.31	0.32	0.32	0.34	0.36	0.36	0.36	0.36	0.35
10%	0.29	0.29	0.31	0.31	0.32	0.33	0.33	0.33	0.34	0.36
BG-11 medium	0.31	0.57	0.83	1.04	1.39	1.72	2.00	2.29	2.54	2.77

1.2.1.2. % growth of *Chlorella vulgaris*

Table III -24 Percent growth of *Chlorella vulgaris* in term Chlorophyll *a*

Garbage Pit Leachate concentration	Inoculation Period (Days)								
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
100%	-5.09	-10.29	-18.74	-27.63	-35.43	-40.09	-49.40	-56.34	-65.22
50%	-7.33	-13.60	-20.35	-22.44	-26.63	-29.30	-34.77	-40.23	-46.40
30%	2.15	1.36	-3.74	-10.09	-14.97	-21.66	-28.46	-34.24	-40.93
20%	-2.34	0.96	2.13	7.66	13.51	14.47	13.62	15.85	12.77
10%	2.45	8.98	9.33	11.55	14.47	15.17	16.22	20.30	24.39
BG-11 medium	86.48	172.63	239.15	353.65	462.70	554.31	650.27	730.97	805.13

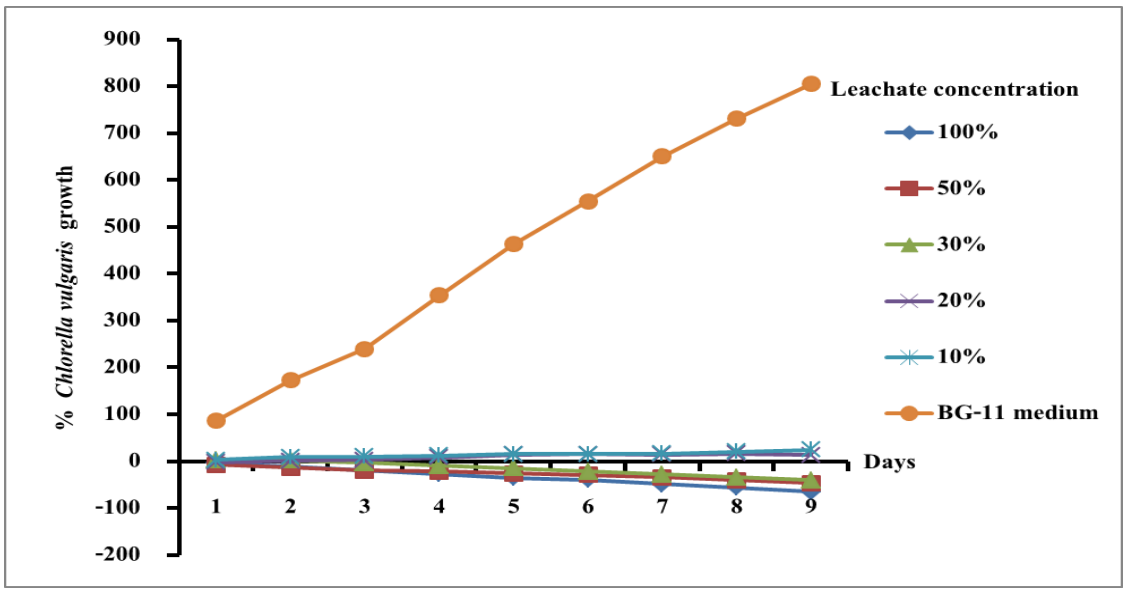

Fig A – 12 Percent growth of *Chlorella vulgaris* in different concentration of Garbage Pit Leachate on April with control (*Chlorella vulgaris* grew in BG-11 medium)

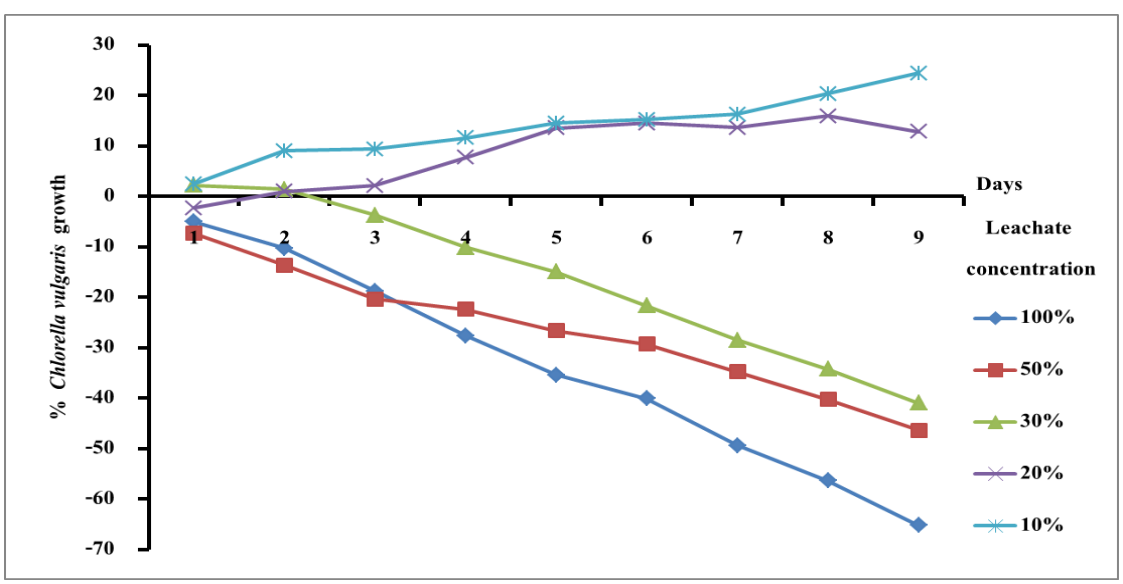

Fig A – 13 Percent growth of *Chlorella vulgaris* in different concentration of Garbage Pit Leachate on April without control (*Chlorella vulgaris* grew in BG-11 medium)

1.2.3 Experiment on June

1.2.3.1. *Chlorella vulgaris* growth in term of Chlorophyll *a*

Table III-25 *Chlorella vulgaris* growth in term of Chlorophyll *a*

Garbage Pit Leachate concentration	Initial of Chlorophyll *a* = 0.9 µg/ml Abs_{750}= 0.19									
	Inoculation Period (Days)									
	0	1	2	3	4	5	6	7	8	9
	0.30	0.29	0.27	0.25	0.22	0.20	0.18	0.15	0.12	0.09
100%	0.29	0.28	0.25	0.26	0.24	0.20	0.18	0.14	0.14	0.11
	0.29	0.28	0.26	0.24	0.23	0.22	0.19	0.15	0.11	0.09
	0.30	0.31	0.30	0.28	0.27	0.26	0.23	0.20	0.17	0.15
50%	0.29	0.29	0.28	0.26	0.25	0.23	0.21	0.19	0.18	0.17
	0.29	0.28	0.28	0.27	0.26	0.24	0.20	0.19	0.16	0.14
	0.31	0.29	0.29	0.27	0.26	0.24	0.22	0.20	0.20	0.19
30%	0.28	0.27	0.27	0.25	0.24	0.24	0.20	0.21	0.19	0.17
	0.29	0.28	0.27	0.26	0.24	0.23	0.22	0.19	0.18	0.17
	0.30	0.28	0.30	0.31	0.31	0.32	0.30	0.31	0.30	0.29
20%	0.29	0.28	0.29	0.30	0.31	0.31	0.29	0.28	0.28	0.27
	0.30	0.29	0.30	0.30	0.33	0.33	0.31	0.30	0.29	0.28
	0.30	0.31	0.32	0.32	0.34	0.34	0.36	0.35	0.36	0.36
10%	0.29	0.30	0.31	0.32	0.32	0.33	0.35	0.36	0.36	0.36
	0.28	0.29	0.30	0.31	0.32	0.33	0.34	0.34	0.35	0.36
	0.28	0.40	0.50	1.06	1.56	1.87	1.92	2.21	2.43	2.89
BG-11 Medium	0.30	0.36	0.48	0.97	1.43	1.99	2.24	2.45	2.87	3.01
	0.29	0.36	0.47	0.94	1.39	1.65	1.87	2.11	2.34	2.65

Table III -26 The average of Chlorophyll *a*

Garbage Pit Leachate concentration	Inoculation Period (Days)									
	0	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
100%	0.29	0.28	0.26	0.25	0.23	0.21	0.18	0.15	0.12	0.10
50%	0.29	0.29	0.28	0.27	0.26	0.24	0.22	0.20	0.17	0.15
30%	0.29	0.28	0.28	0.26	0.25	0.24	0.21	0.20	0.19	0.17
20%	0.30	0.28	0.30	0.31	0.32	0.32	0.30	0.30	0.29	0.28
10%	0.29	0.30	0.31	0.32	0.32	0.33	0.35	0.35	0.36	0.36
BG-11 medium	0.29	0.37	0.48	0.99	1.46	1.84	2.01	2.26	2.55	2.85

1.2.1.2. Percent growth of *Chlorella vulgaris*

Table III -27 Percent growth of *Chlorella vulgaris* in term Chlorophyll *a*

Garbage Pit Leachate concentration	Inoculation Period (Days)								
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
100%	-3.42	-11.53	-14.50	-21.80	-29.57	-38.36	-48.97	-57.99	-67.47
50%	-0.11	-3.40	-9.40	-12.00	-17.89	-26.95	-33.75	-42.70	-48.02
30%	-3.99	-5.70	-11.52	-15.51	-19.27	-26.57	-31.58	-35.92	-40.82
20%	-3.72	0.68	3.61	7.90	8.35	1.92	0.34	-2.48	-5.98
10%	3.46	6.57	9.33	12.21	14.86	19.47	21.08	23.73	25.00
BG-11 medium	29.55	67.67	244.03	406.95	538.47	598.73	684.47	785.28	890.73

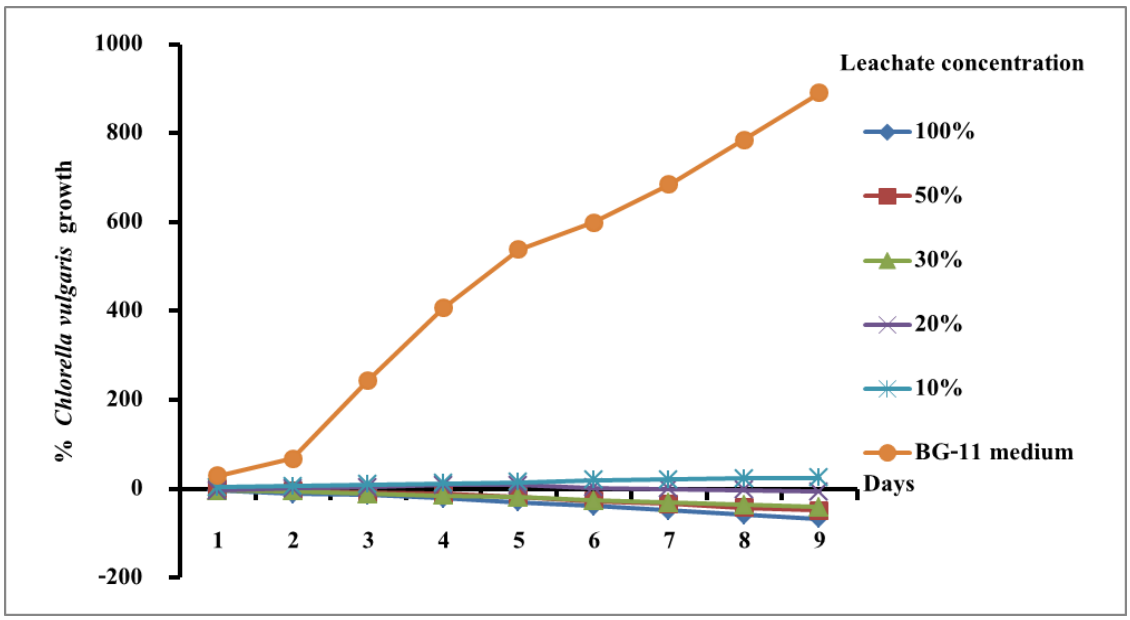

Fig A – 14 Percent growth of *Chlorella vulgaris* in different concentration of Garbage Pit Leachate on June with control (*Chlorella vulgaris* grew in BG-11 medium)

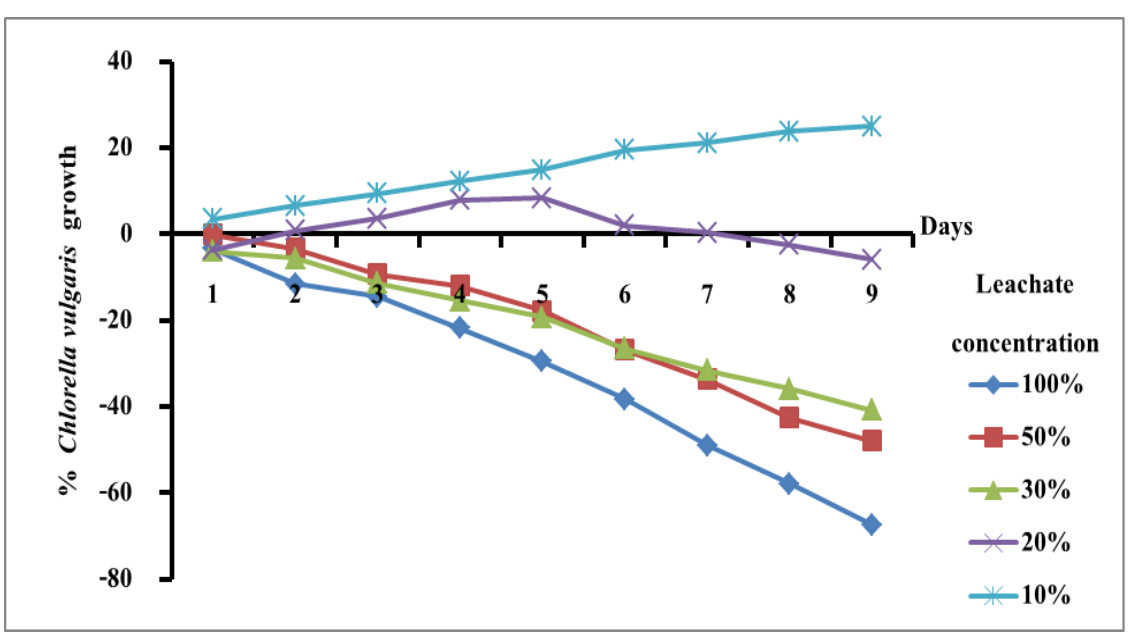

Fig A – 15 Percent growth of *Chlorella vulgaris* in different concentration of Garbage Pit Leachate on June without control (*Chlorella vulgaris* grew in BG-11 medium)

1.2.4 Experiment on July

1.2.4.1. *Chlorella vulgaris* growth in term of Chlorophyll *a*

Table III-28 *Chlorella vulgaris* growth in term of Chlorophyll *a*

Garbage Pit Leachate concentration	Initial of Chlorophyll *a* = 0.9 µg/ml Abs_{750}= 0.19									
	Inoculation Period (Days)									
	0	1	2	3	4	5	6	7	8	9
	0.29	0.25	0.24	0.22	0.21	0.19	0.16	0.11	0.05	0.00
100%	0.30	0.29	0.25	0.23	0.20	0.19	0.12	0.09	0.02	0.00
	0.30	0.25	0.24	0.23	0.21	0.17	0.13	0.11	0.09	0.00
	0.31	0.30	0.31	0.30	0.28	0.28	0.25	0.24	0.20	0.19
50%	0.33	0.31	0.32	0.30	0.29	0.26	0.23	0.20	0.19	0.18
	0.36	0.33	0.33	0.31	0.30	0.28	0.27	0.22	0.19	0.16
	0.32	0.30	0.33	0.33	0.31	0.30	0.29	0.28	0.25	0.23
30%	0.29	0.28	0.29	0.30	0.31	0.29	0.28	0.25	0.21	0.19
	0.31	0.31	0.32	0.32	0.31	0.30	0.27	0.25	0.23	0.20
	0.29	0.27	0.29	0.29	0.30	0.29	0.30	0.30	0.30	0.30
20%	0.27	0.26	0.27	0.28	0.28	0.28	0.29	0.28	0.29	0.30
	0.30	0.30	0.31	0.31	0.32	0.32	0.32	0.32	0.31	0.31
	0.28	0.27	0.28	0.29	0.30	0.31	0.33	0.35	0.37	0.36
10%	0.31	0.30	0.31	0.33	0.32	0.34	0.35	0.36	0.38	0.38
	0.29	0.28	0.29	0.30	0.31	0.32	0.33	0.36	0.34	0.34
	0.35	0.53	0.75	1.02	1.34	1.44	1.67	1.84	2.01	2.40
BG-11 Medium	0.38	0.65	0.88	0.95	1.23	1.54	1.45	1.67	1.89	2.03
	0.32	0.43	0.65	0.87	1.34	1.30	1.54	1.56	1.65	1.89

Table III -29 The average of Chlorophyll *a*

Garbage Pit Leachate concentration	Inoculation Period (Days)									
	0	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
100%	0.30	0.26	0.24	0.23	0.21	0.18	0.14	0.10	0.05	0.00
50%	0.33	0.31	0.32	0.30	0.29	0.27	0.25	0.22	0.20	0.18
30%	0.31	0.30	0.31	0.32	0.31	0.30	0.28	0.26	0.23	0.21
20%	0.29	0.27	0.29	0.29	0.30	0.30	0.30	0.30	0.30	0.31
10%	0.29	0.28	0.29	0.31	0.31	0.32	0.34	0.36	0.36	0.36
BG-11 medium	0.35	0.54	0.76	0.95	1.30	1.43	1.55	1.69	1.85	2.11

1.2.4.2. Percent growth of *Chlorella vulgaris*

Table III -30 Percent growth of *Chlorella vulgaris* in term Chlorophyll *a*

Garbage Pit Leachate concentration	Inoculation Period (Days)								
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
100%	-11.39	-17.36	-22.21	-29.20	-38.90	-53.44	-65.61	-81.96	-100.00
50%	-5.41	-4.11	-8.72	-13.23	-18.74	-24.55	-33.27	-41.18	-47.39
30%	-3.58	1.63	3.47	1.08	-3.36	-8.57	-14.75	-23.86	-32.43
20%	-3.97	0.93	2.68	4.32	4.55	5.72	4.90	5.72	7.00
10%	-3.53	0.23	5.23	6.14	10.69	15.13	21.27	23.66	22.18
BG-11 medium	54.36	118.60	172.29	274.88	310.35	346.79	386.10	432.12	505.94

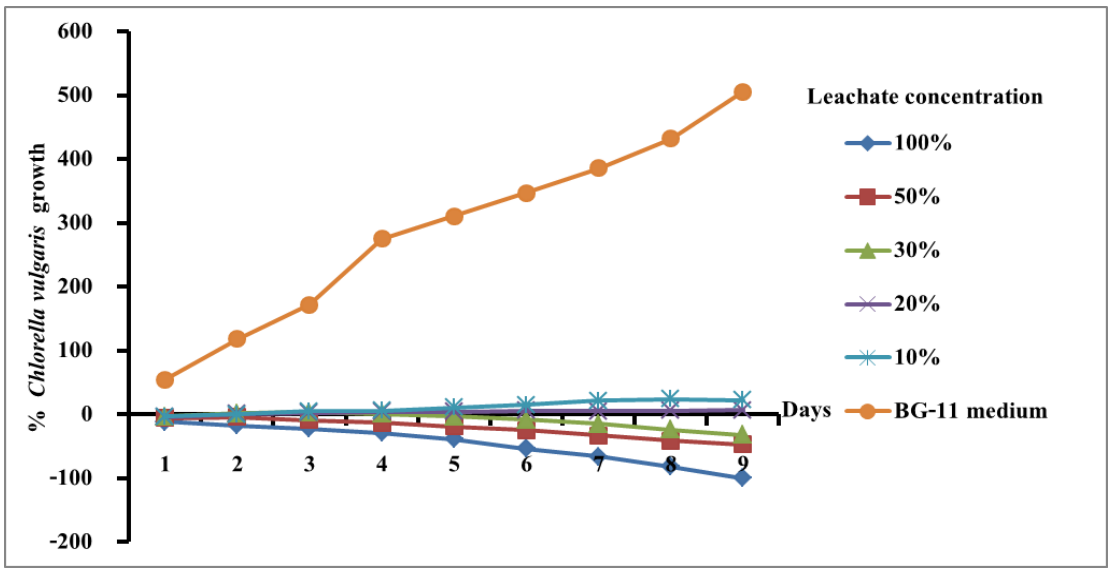

Fig A – 16 Percent growth of *Chlorella vulgaris* in different concentration of Garbage Pit Leachate on July with control (*Chlorella vulgaris* grew in BG-11 medium)

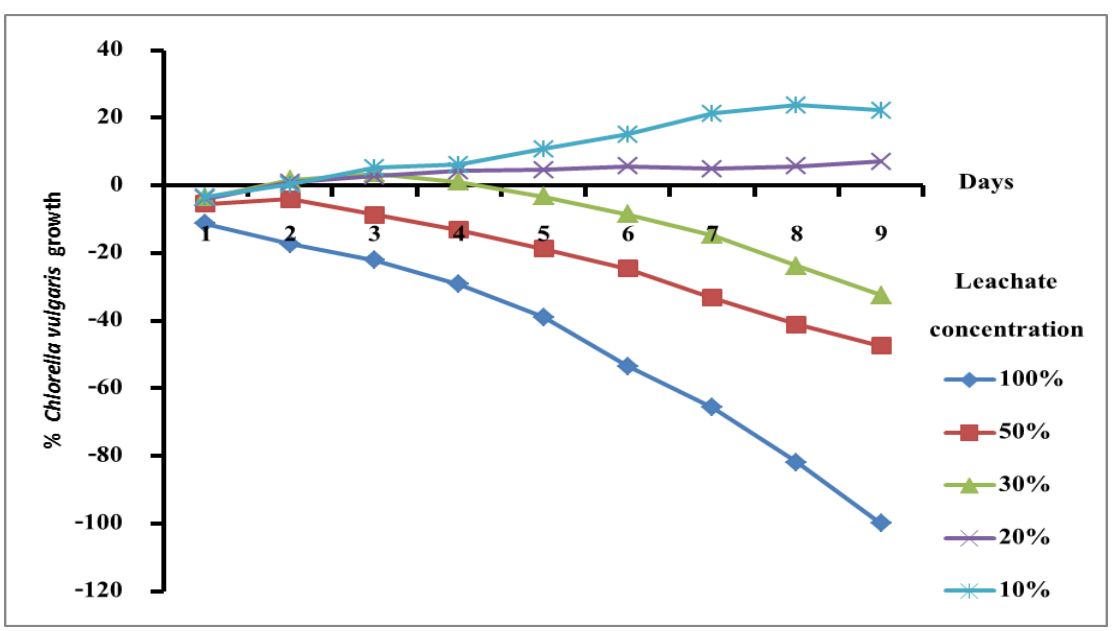

Fig A – 17 Percent growth of *Chlorella vulgaris* in different concentration of Garbage Pit Leachate on July without control (*Chlorella vulgaris* grew in BG-11 medium)

1.2.5 Experiment on October

1.2.5.1. *Chlorella vulgaris* growth in term of Chlorophyll *a*

Table III-31 *Chlorella vulgaris* growth in term of Chlorophyll *a*

Garbage Pit Leachate concentration	Initial of Chlorophyll *a* = 0.9 µg/ml Abs_{750}= 0.19									
	Inoculation Period (Days)									
	0	1	2	3	4	5	6	7	8	9
	0.28	0.24	0.22	0.18	0.17	0.16	0.13	0.10	0.09	0.05
100%	0.30	0.28	0.25	0.21	0.20	0.18	0.15	0.13	0.09	0.00
	0.29	0.26	0.22	0.20	0.19	0.15	0.13	0.09	0.05	0.00
	0.30	0.29	0.27	0.25	0.24	0.21	0.19	0.16	0.13	0.12
50%	0.31	0.29	0.28	0.27	0.23	0.22	0.20	0.19	0.17	0.13
	0.29	0.28	0.27	0.24	0.21	0.20	0.20	0.17	0.16	0.12
	0.36	0.32	0.31	0.29	0.27	0.24	0.21	0.19	0.17	0.16
30%	0.29	0.28	0.25	0.23	0.21	0.20	0.18	0.17	0.15	0.12
	0.32	0.30	0.28	0.25	0.24	0.21	0.17	0.15	0.14	0.12
	0.31	0.30	0.31	0.32	0.32	0.32	0.32	0.30	0.31	0.31
20%	0.31	0.30	0.31	0.33	0.31	0.32	0.32	0.31	0.31	0.31
	0.29	0.29	0.31	0.33	0.33	0.33	0.33	0.31	0.31	0.31
	0.28	0.29	0.30	0.30	0.29	0.29	0.27	0.27	0.26	0.25
10%	0.32	0.32	0.32	0.32	0.32	0.29	0.27	0.25	0.25	0.23
	0.29	0.31	0.31	0.33	0.32	0.31	0.29	0.28	0.25	0.25
	0.31	0.43	0.66	0.98	1.34	1.68	1.98	2.25	2.65	2.84
BG-11 Medium	0.33	0.45	0.82	1.09	1.56	1.77	2.10	2.34	2.45	2.65
	0.35	0.44	0.71	1.11	1.54	1.86	2.09	2.11	2.54	2.84

Table III -32 The average of Chlorophyll *a*

Garbage Pit Leachate concentration	Inoculation Period (Days)									
	0	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
100%	0.29	0.26	0.23	0.20	0.19	0.16	0.14	0.11	0.08	0.02
50%	0.30	0.29	0.27	0.25	0.23	0.21	0.20	0.17	0.15	0.12
30%	0.32	0.30	0.28	0.26	0.24	0.22	0.19	0.17	0.15	0.13
20%	0.30	0.30	0.31	0.33	0.32	0.32	0.33	0.31	0.31	0.31
10%	0.30	0.30	0.31	0.32	0.31	0.30	0.28	0.27	0.25	0.24
BG-11 medium	0.33	0.44	0.73	1.06	1.48	1.77	2.06	2.23	2.55	2.78

1.2.5.2. Percent growth of *Chlorella vulgaris*

Table III -33 Percent growth of *Chlorella vulgaris* in term Chlorophyll *a*

Garbage Pit Leachate concentration	Inoculation Period (Days)								
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
100%	-10.13	-19.91	-31.65	-35.44	-44.07	-51.55	-62.72	-74.11	-94.25
50%	-4.45	-10.34	-15.24	-24.25	-28.81	-34.82	-42.49	-49.72	-58.51
30%	-6.75	-12.98	-19.52	-25.34	-32.71	-42.16	-47.14	-52.02	-58.57
20%	-1.88	2.98	8.07	6.19	6.41	7.73	2.10	2.54	3.31
10%	2.49	5.08	7.34	5.20	0.00	-5.42	-10.17	-14.80	-17.51
BG-11 medium	33.23	121.99	222.70	349.85	437.99	525.13	578.82	674.06	743.97

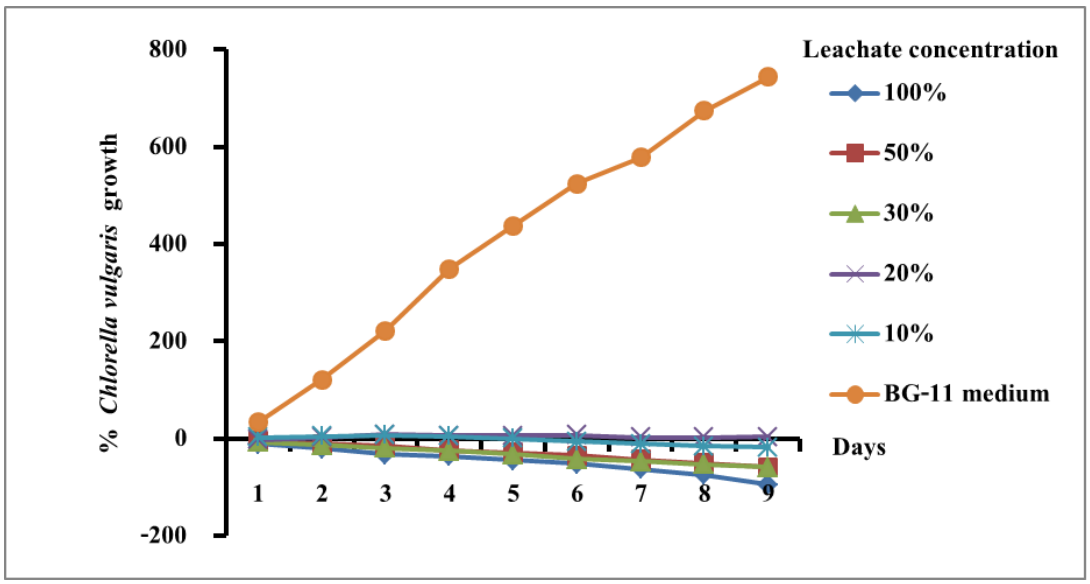

Fig A – 18 Percent growth of *Chlorella vulgaris* in different concentration of Garbage Pit Leachate on October with control (*Chlorella vulgaris* grew in BG-11 medium)

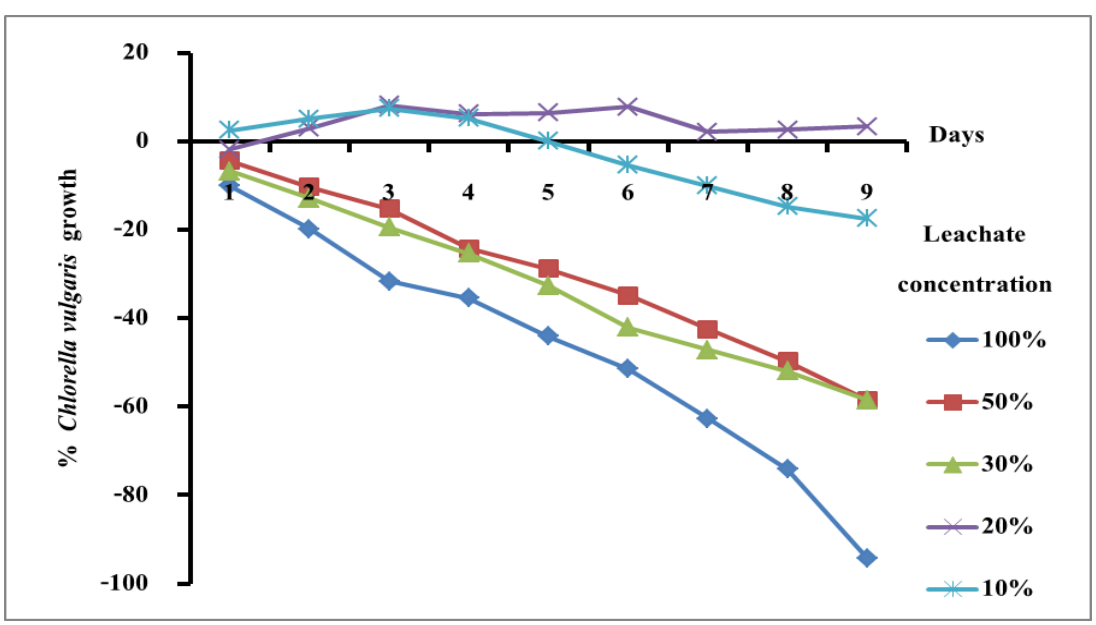

Fig A – 19 Percent growth of *Chlorella vulgaris* in different concentration of Garbage Pit Leachate on October without control (*Chlorella vulgaris* grew in BG-11 medium)

1.2.6 Experiment on November

1.2.6.1. *Chlorella vulgaris* growth in term of Chlorophyll *a*

Table III-34 *Chlorella vulgaris* growth in term of Chlorophyll *a*

Garbage Pit Leachate concentration	Initial of Chlorophyll *a* = 0.9 µg/ml Abs_{750}= 0.19									
	Inoculation Period (Days)									
	0	1	2	3	4	5	6	7	8	9
	0.29	0.28	0.25	0.24	0.23	0.23	0.19	0.19	0.19	0.17
100%	0.29	0.28	0.25	0.24	0.21	0.20	0.21	0.19	0.18	0.17
	0.31	0.31	0.24	0.24	0.21	0.20	0.18	0.19	0.17	0.16
	0.30	0.29	0.26	0.22	0.20	0.20	0.19	0.19	0.16	0.16
50%	0.30	0.28	0.25	0.24	0.31	0.22	0.18	0.16	0.18	0.17
	0.29	0.28	0.25	0.23	0.24	0.21	0.20	0.17	0.15	0.14
	0.29	0.38	0.25	0.25	0.21	0.20	0.19	0.18	0.19	0.16
30%	0.29	0.30	0.27	0.23	0.23	0.21	0.20	0.18	0.18	0.17
	0.30	0.33	0.24	0.23	0.23	0.20	0.18	0.18	0.17	0.16
	0.30	0.34	0.35	0.36	0.35	0.34	0.32	0.33	0.33	0.32
20%	0.31	0.36	0.35	0.38	0.37	0.35	0.36	0.35	0.35	0.33
	0.30	0.29	0.32	0.37	0.35	0.34	0.33	0.36	0.35	0.31
	0.27	0.32	0.32	0.31	0.30	0.29	0.28	0.27	0.30	0.31
10%	0.32	0.32	0.32	0.33	0.31	0.29	0.29	0.29	0.31	0.30
	0.31	0.30	0.31	0.32	0.32	0.29	0.28	0.27	0.29	0.33
	0.31	0.43	0.66	0.83	1.15	1.56	1.87	2.12	2.45	2.93
BG-11 Medium	0.31	0.53	0.87	1.04	1.35	1.78	2.10	2.56	2.76	3.01
	0.32	0.51	0.76	0.97	1.22	1.57	1.98	2.34	2.89	3.14

Table III -35 The average of Chlorophyll *a*

Garbage Pit Leachate concentration	Inoculation Period (Days)									
	0	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
100%	0.30	0.29	0.25	0.24	0.22	0.21	0.19	0.19	0.18	0.16
50%	0.29	0.28	0.25	0.23	0.25	0.21	0.19	0.17	0.16	0.15
30%	0.29	0.34	0.25	0.23	0.22	0.20	0.19	0.18	0.18	0.16
20%	0.30	0.33	0.34	0.37	0.35	0.34	0.34	0.34	0.34	0.32
10%	0.30	0.31	0.32	0.32	0.31	0.29	0.28	0.28	0.30	0.31
BG-11 medium	0.31	0.49	0.76	0.95	1.24	1.64	1.98	2.34	2.70	3.03

1.2.6.2. % growth of *Chlorella vulgaris*

Table III -36 Percent growth of *Chlorella vulgaris* in term Chlorophyll *a*

Garbage Pit Leachate concentration	Inoculation Period (Days)								
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
100%	-3.26	-16.85	-19.10	-27.53	-28.76	-34.83	-36.40	-39.55	-44.72
50%	-4.98	-13.48	-21.86	-14.84	-28.54	-35.45	-41.34	-45.30	-48.47
30%	14.20	-13.75	-20.68	-23.86	-30.91	-35.23	-38.64	-40.00	-44.09
20%	9.17	12.38	22.54	17.57	13.92	11.71	13.70	13.15	5.08
10%	4.83	6.63	8.20	3.71	-2.25	-4.49	-7.30	0.56	4.94
BG-11 medium	56.14	142.27	200.85	294.07	420.13	530.30	643.64	758.05	861.86

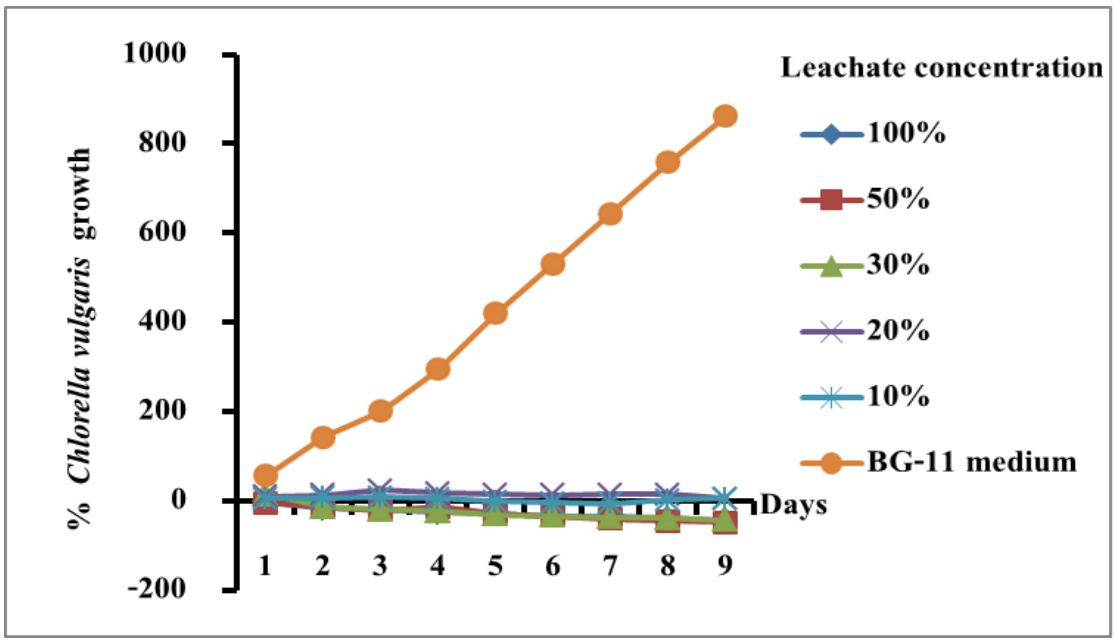

Fig A – 20 Percent growth of *Chlorella vulgaris* in different concentration of Garbage Pit Leachate on November with control (*Chlorella vulgaris* grew in BG-11 medium)

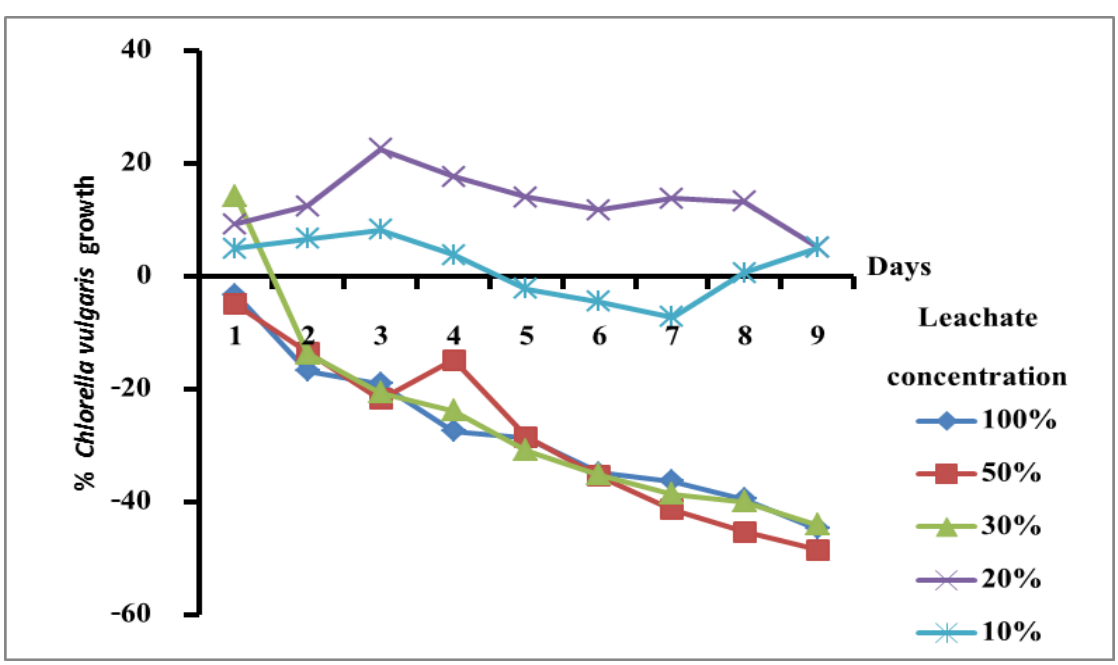

Fig A – 21 Percent growth of *Chlorella vulgaris* in different concentration of Garbage Pit Leachate on November without control (*Chlorella vulgaris* grew in BG-11 medium)

2. Phase 2: Growth in light under shaking, well aerated, condition: Garbage Pit Leachate

2.1 Experiment on April

2.1.1 *Chlorella vulgaris* growth in term of Chlorophyll *a*

Table III-37 *Chlorella vulgaris* growth in term of Chlorophyll *a*

Garbage Pit Leachate concentration	Initial of Chlorophyll *a* = 0.9 µg/ml Abs_{750}= 0.19									
	Inoculation Period (Days)									
	0	1	2	3	4	5	6	7	8	9
	0.32	0.30	0.30	0.27	0.26	0.24	0.23	0.21	0.20	0.19
100%	0.32	0.30	0.29	0.28	0.27	0.24	0.25	0.23	0.22	0.22
	0.30	0.30	0.29	0.28	0.25	0.24	0.22	0.20	0.20	0.19
	0.35	0.35	0.33	0.31	0.29	0.24	0.26	0.24	0.22	0.22
50%	0.40	0.39	0.37	0.33	0.31	0.29	0.28	0.25	0.24	0.22
	0.40	0.39	0.36	0.31	0.29	0.28	0.28	0.27	0.26	0.23
	0.37	0.36	0.37	0.37	0.35	0.32	0.31	0.29	0.29	0.28
30%	0.34	0.35	0.34	0.32	0.31	0.29	0.28	0.28	0.26	0.25
	0.38	0.35	0.35	0.35	0.34	0.32	0.31	0.30	0.29	0.27
	0.32	0.32	0.34	0.37	0.39	0.40	0.42	0.44	0.35	0.37
20%	0.37	0.38	0.39	0.40	0.42	0.42	0.45	0.50	0.51	0.53
	0.34	0.36	0.36	0.39	0.40	0.42	0.44	0.44	0.48	0.50
	0.40	0.41	0.42	0.44	0.46	0.48	0.49	0.50	0.49	0.48
10%	0.36	0.37	0.38	0.40	0.41	0.42	0.44	0.43	0.46	0.47
	0.37	0.36	0.39	0.40	0.40	0.41	0.43	0.43	0.42	0.43
	0.30	0.32	0.50	0.89	1.30	1.64	1.88	2.43	2.66	3.01
BG-11 Medium	0.29	0.32	0.54	0.99	1.40	1.77	1.98	2.30	2.64	2.91
	0.29	0.33	0.47	0.83	1.20	1.68	1.84	2.20	2.76	3.12

Table III -38 The average of Chlorophyll *a*

Garbage Pit Leachate concentration	Inoculation Period (Days)									
	0	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
100%	0.31	0.30	0.29	0.28	0.26	0.24	0.24	0.21	0.21	0.20
50%	0.38	0.37	0.35	0.32	0.29	0.27	0.27	0.25	0.24	0.22
30%	0.36	0.35	0.35	0.34	0.33	0.31	0.30	0.29	0.28	0.27
20%	0.34	0.35	0.36	0.39	0.40	0.41	0.44	0.46	0.45	0.47
10%	0.37	0.38	0.40	0.41	0.43	0.44	0.45	0.46	0.46	0.46
BG-11 medium	0.29	0.32	0.50	0.90	1.30	1.70	1.90	2.31	2.69	3.01

2.1.2 Percent growth of *Chlorella vulgaris*

Table III -39 Percent growth of *Chlorella vulgaris* in term Chlorophyll *a*

Garbage Pit Leachate concentration	Inoculation Period (Days)								
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
100%	-4.06	-6.52	-11.65	-17.41	-22.22	-24.47	-31.41	-33.97	-36.54
50%	-2.44	-8.53	-17.23	-23.32	-29.50	-29.77	-33.86	-37.95	-41.78
30%	-2.12	-2.40	-4.79	-8.66	-14.38	-16.87	-20.28	-23.32	-26.27
20%	1.95	4.77	12.66	18.01	20.84	27.17	34.57	30.48	36.03
10%	0.98	6.41	9.70	13.52	16.73	21.26	21.80	22.33	22.95
BG-11 medium	10.35	71.79	208.30	343.69	479.07	548.46	688.40	816.95	928.44

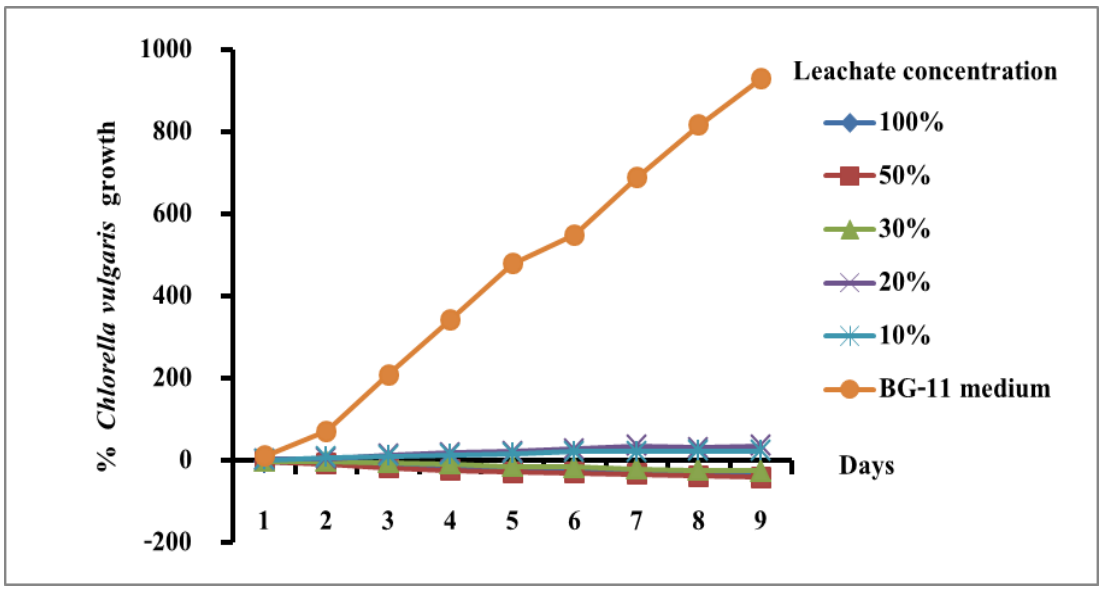

Fig A – 22 Percent growth of *Chlorella vulgaris* in different concentration of Garbage Pit Leachate, aerated condition on April with control (*Chlorella vulgaris* grew in BG-11 medium)

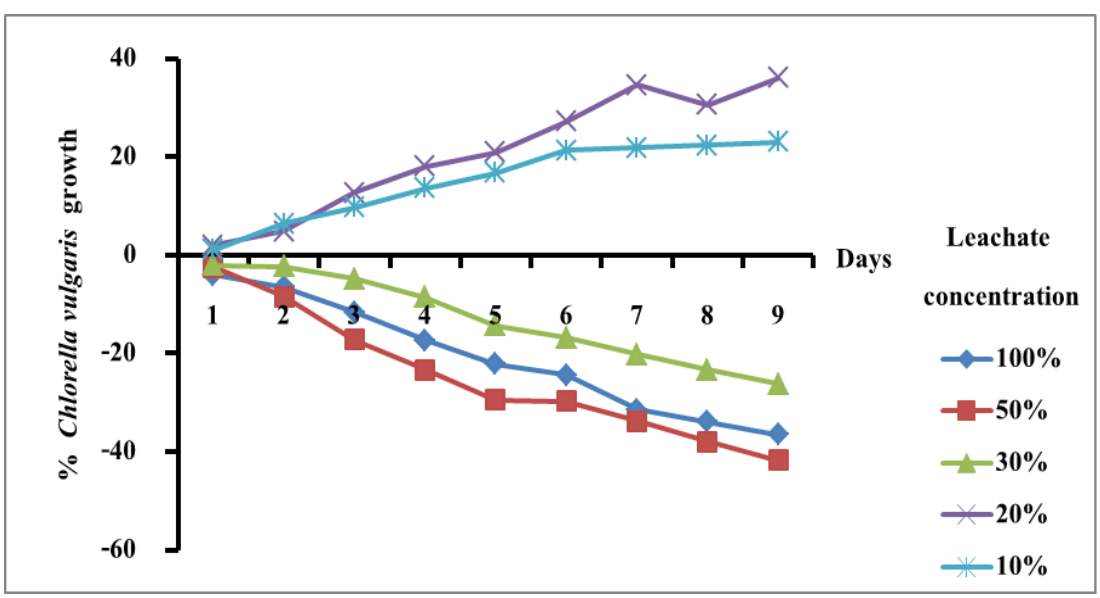

Fig A – 23 Percent growth of *Chlorella vulgaris* in different concentration of Garbage Pit Leachate, aerated condition on April without control (*Chlorella vulgaris* grew in BG-11 medium)

2.2 Experiment on June

2.2.1 *Chlorella vulgaris* growth in term of Chlorophyll *a*

Table III-40 *Chlorella vulgaris* growth in term of Chlorophyll *a*

Garbage Pit Leachate concentration	Initial of Chlorophyll *a* = 0.9 µg/ml Abs_{750}= 0.19									
	Inoculation Period (Days)									
	0	1	2	3	4	5	6	7	8	9
	0.30	0.29	0.29	0.28	0.26	0.24	0.23	0.21	0.22	0.20
100%	0.30	0.30	0.28	0.28	0.25	0.23	0.22	0.21	0.20	0.18
	0.32	0.31	0.29	0.29	0.28	0.26	0.25	0.24	0.23	0.22
	0.31	0.31	0.30	0.30	0.27	0.27	0.26	0.25	0.24	0.24
50%	0.28	0.29	0.28	0.27	0.26	0.24	0.22	0.22	0.21	0.20
	0.30	0.30	0.29	0.28	0.27	0.27	0.24	0.23	0.23	0.23
	0.29	0.30	0.30	0.30	0.29	0.29	0.28	0.26	0.25	0.25
30%	0.33	0.32	0.34	0.32	0.30	0.30	0.28	0.27	0.27	0.26
	0.30	0.31	0.32	0.31	0.30	0.29	0.28	0.28	0.27	0.26
	0.31	0.31	0.32	0.33	0.36	0.36	0.36	0.37	0.38	0.39
20%	0.32	0.32	0.32	0.34	0.37	0.38	0.38	0.36	0.37	0.41
	0.33	0.30	0.32	0.35	0.35	0.37	0.37	0.38	0.38	0.39
	0.29	0.31	0.34	0.35	0.37	0.37	0.38	0.38	0.40	0.40
10%	0.31	0.32	0.34	0.34	0.36	0.36	0.39	0.38	0.39	0.41
	0.31	0.31	0.33	0.34	0.37	0.37	0.38	0.41	0.40	0.41
	0.30	0.41	0.65	0.83	1.02	1.21	1.32	1.44	1.64	1.82
BG-11 Medium	0.32	0.62	0.86	1.02	1.21	1.34	1.44	1.65	1.78	1.80
	0.31	0.54	0.73	0.98	1.09	1.23	1.33	1.44	1.78	1.90

Table III -41 The average of Chlorophyll *a*

Garbage Pit Leachate concentration	Inoculation Period (Days)									
	0	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
100%	0.31	0.30	0.29	0.28	0.26	0.25	0.24	0.22	0.21	0.20
50%	0.30	0.30	0.29	0.28	0.27	0.26	0.24	0.24	0.23	0.22
30%	0.31	0.31	0.32	0.31	0.30	0.29	0.28	0.27	0.26	0.26
20%	0.32	0.31	0.32	0.34	0.36	0.37	0.37	0.37	0.38	0.40
10%	0.30	0.32	0.33	0.35	0.37	0.37	0.38	0.39	0.40	0.41
BG-11 medium	0.31	0.53	0.75	0.94	1.11	1.26	1.36	1.51	1.73	1.84

2.1.2 Percent growth of *Chlorella vulgaris*

Table III -42 Percent growth of *Chlorella vulgaris* in term Chlorophyll *a*

Garbage Pit Leachate concentration	Inoculation Period (Days)								
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
100%	-2.49	-6.59	-8.65	-14.59	-20.32	-23.57	-28.22	-30.38	-35.14
50%	-0.44	-3.55	-5.99	-11.65	-14.21	-20.31	-21.75	-24.20	-26.53
30%	0.65	3.35	1.08	-3.90	-5.63	-8.98	-12.12	-15.04	-16.45
20%	-3.93	-0.72	4.86	11.38	14.06	15.10	14.89	16.34	23.58
10%	4.30	10.04	14.35	20.86	21.63	26.49	29.25	31.02	34.55
BG-11 medium	69.54	141.12	204.63	257.37	306.89	340.26	387.62	459.74	494.19

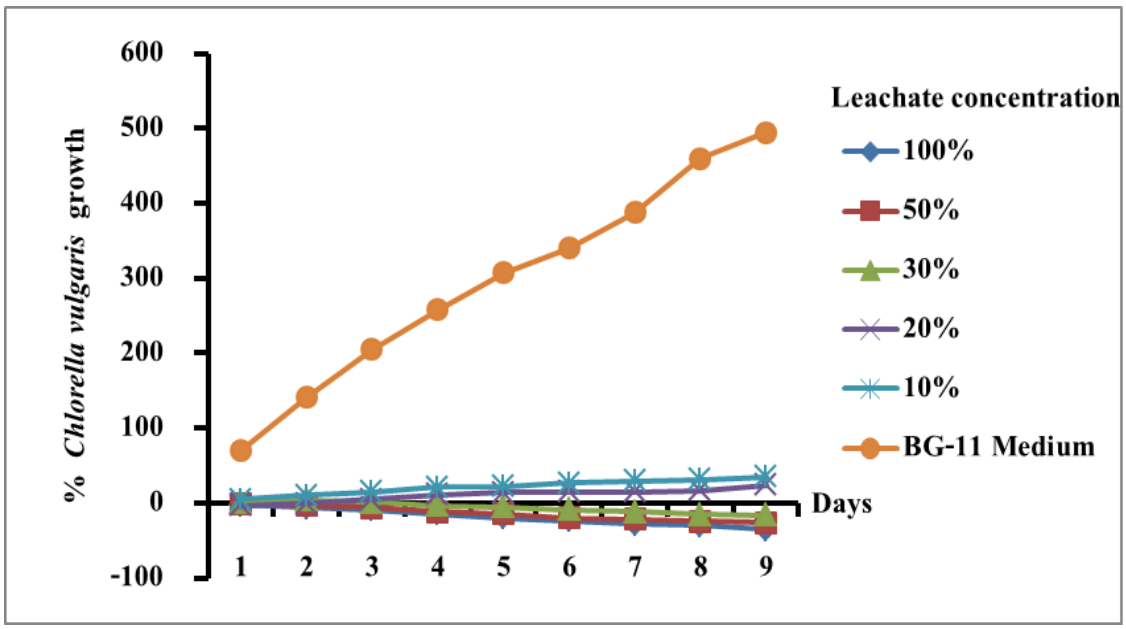

Fig A – 24 Percent growth of *Chlorella vulgaris* in different concentration of Garbage Pit Leachate, aerated condition on June with control (*Chlorella vulgaris* grew in BG-11 medium)

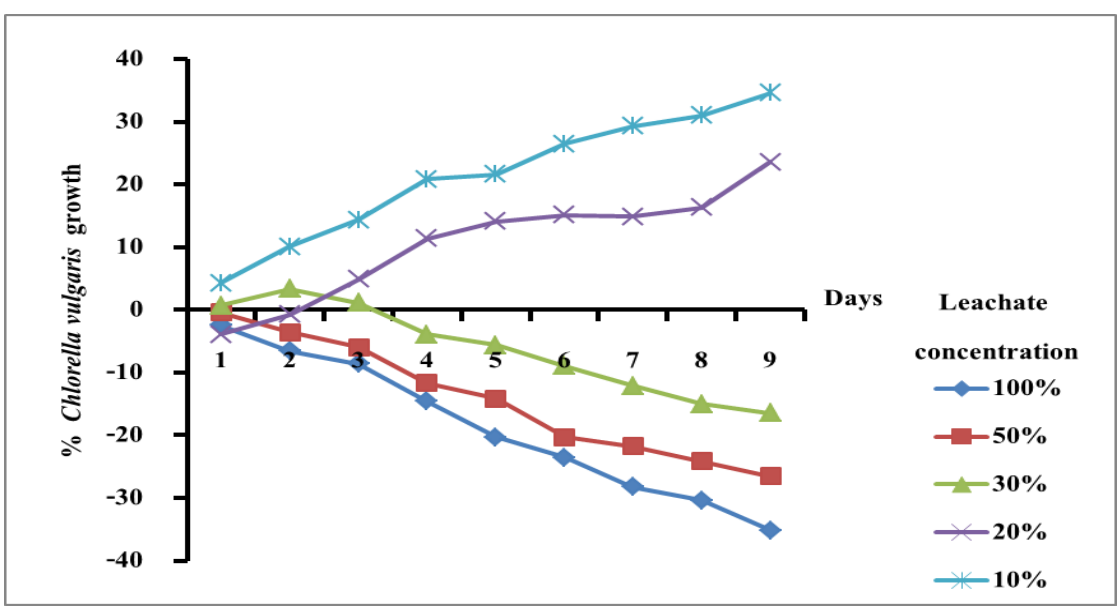

Fig A – 25 Percent growth of *Chlorella vulgaris* in different concentration of Garbage Pit Leachate, aerated condition on June without control (*Chlorella vulgaris* grew in BG-11 medium)

2.4 Experiment on October

2.4.1 *Chlorella vulgaris* growth in term of Chlorophyll *a*

Table III-43 *Chlorella vulgaris* growth in term of Chlorophyll *a*

Garbage Pit Leachate concentration	Initial of Chlorophyll *a* = 0.9 µg/ml Abs_{750}= 0.19									
	Inoculation Period (Days)									
	0	1	2	3	4	5	6	7	8	9
100%	0.34	0.37	0.34	0.30	0.30	0.30	0.27	0.24	0.21	0.20
	0.35	0.39	0.37	0.31	0.30	0.29	0.27	0.25	0.22	0.19
	0.35	0.37	0.36	0.31	0.30	0.30	0.25	0.24	0.22	0.21
50%	0.41	0.38	0.31	0.31	0.30	0.29	0.28	0.26	0.24	0.24
	0.40	0.36	0.37	0.30	0.30	0.30	0.29	0.29	0.26	0.25
	0.40	0.35	0.34	0.32	0.30	0.29	0.29	0.28	0.25	0.22
30%	0.37	0.37	0.36	0.32	0.32	0.33	0.32	0.31	0.31	0.30
	0.40	0.37	0.37	0.30	0.32	0.32	0.32	0.31	0.31	0.29
	0.37	0.36	0.36	0.30	0.37	0.34	0.33	0.32	0.30	0.30
20%	0.35	0.32	0.38	0.35	0.41	0.41	0.44	0.46	0.47	0.47
	0.38	0.38	0.39	0.40	0.41	0.42	0.43	0.44	0.46	0.47
	0.37	0.35	0.41	0.41	0.47	0.45	0.43	0.44	0.47	0.48
10%	0.41	0.33	0.34	0.37	0.40	0.47	0.57	0.47	0.34	0.33
	0.44	0.28	0.32	0.32	0.40	0.43	0.55	0.39	0.34	0.33
	0.36	0.31	0.32	0.35	0.40	0.39	0.51	0.35	0.33	0.30
BG-11 Medium	0.31	0.53	0.75	1.03	1.40	1.76	2.09	2.43	2.97	3.02
	0.33	0.47	0.87	1.23	1.56	1.84	2.13	2.53	2.86	3.20
	0.32	0.51	0.75	1.11	1.43	1.69	1.94	2.31	2.64	3.08

Table III -44 The average of Chlorophyll *a*

Garbage Pit Leachate concentration	Inoculation Period (Days)									
	0	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
100%	0.35	0.37	0.35	0.31	0.30	0.30	0.26	0.24	0.21	0.20
50%	0.40	0.36	0.34	0.31	0.30	0.29	0.29	0.28	0.25	0.23
30%	0.38	0.37	0.36	0.31	0.34	0.33	0.32	0.31	0.31	0.30
20%	0.37	0.35	0.39	0.39	0.43	0.43	0.43	0.45	0.46	0.47
10%	0.40	0.31	0.33	0.35	0.40	0.43	0.54	0.40	0.33	0.32
BG-11 medium	0.32	0.50	0.79	1.12	1.46	1.76	2.05	2.42	2.82	3.10

2.4.2 % growth of *Chlorella vulgaris*

Table III -45 Percent growth of *Chlorella vulgaris* in term Chlorophyll *a*

Garbage Pit Leachate concentration	Inoculation Period (Days)								
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
100%	7.89	2.21	-10.59	-13.76	-14.73	-24.35	-29.45	-38.02	-42.25
50%	-10.73	-15.69	-23.37	-25.85	-27.58	-29.15	-31.05	-37.57	-42.11
30%	-3.24	-4.99	-19.51	-11.55	-13.47	-15.57	-18.02	-19.95	-22.40
20%	-3.74	7.85	6.39	17.35	16.71	18.54	22.01	27.03	28.95
10%	-23.86	-18.95	-13.13	-0.17	8.23	35.00	0.08	-16.71	-20.37
BG-11 medium	56.24	144.48	247.78	352.84	445.92	535.71	650.26	774.10	859.75

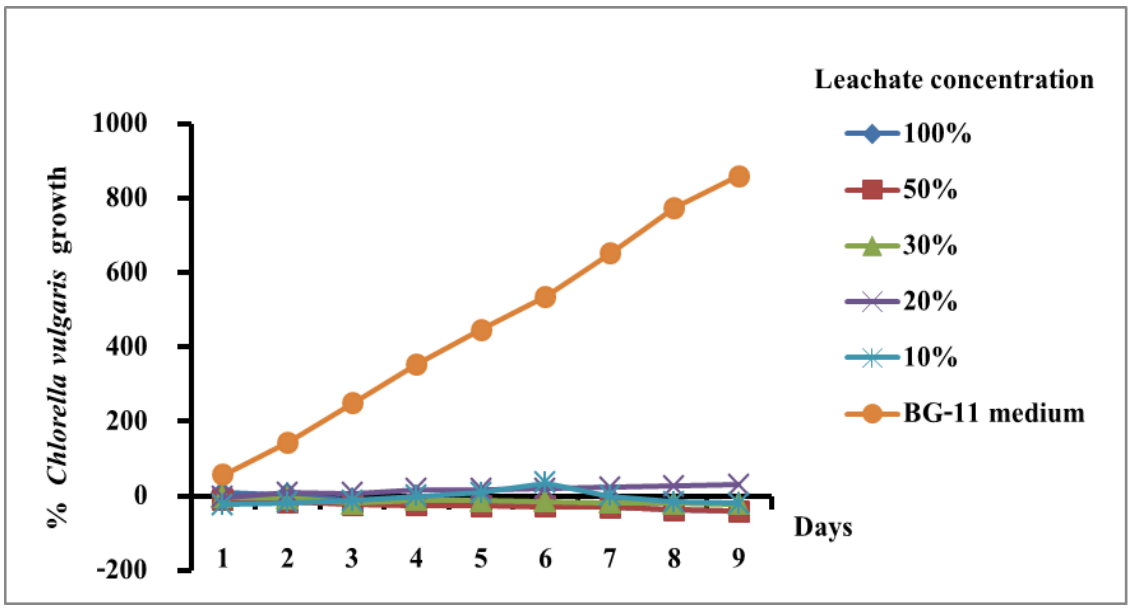

Fig A – 28 Percent growth of *Chlorella vulgaris* in different concentration of Garbage Pit Leachate, aerated condition on October with control (*Chlorella vulgaris* grew in BG-11 medium)

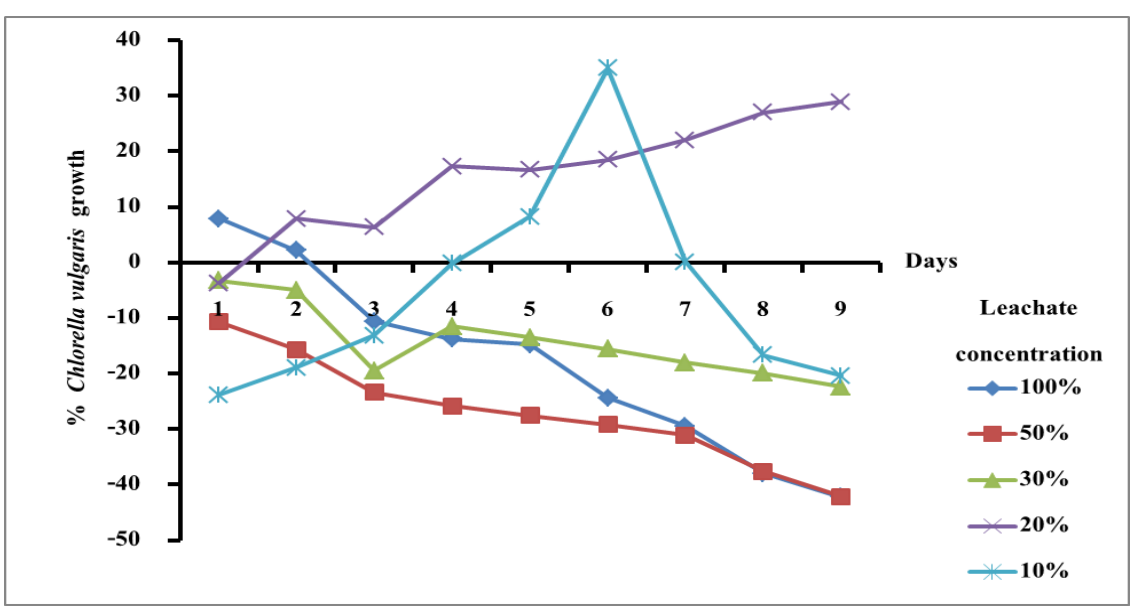

Fig A – 29 Percent growth of *Chlorella vulgaris* in different concentration of Garbage Pit Leachate, aerated condition on October without control (*Chlorella vulgaris* grew in BG-11 medium)

2.5 Experiment on November

2.5.1 *Chlorella vulgaris* growth in term of Chlorophyll *a*

Table III-46 *Chlorella vulgaris* growth in term of Chlorophyll *a*

Garbage Pit Leachate concentration	Initial of Chlorophyll *a* = 0.9 µg/ml Abs_{750}= 0.19									
	Inoculation Period (Days)									
	0	1	2	3	4	5	6	7	8	9
100%	0.31	0.28	0.29	0.30	0.30	0.29	0.26	0.24	0.22	0.187
	0.37	0.31	0.31	0.30	0.31	0.30	0.27	0.24	0.235	0.22
	0.33	0.30	0.30	0.29	0.30	0.28	0.24	0.23	0.209	0.194
50%	0.41	0.36	0.34	0.32	0.30	0.29	0.27	0.28	0.265	0.243
	0.38	0.35	0.34	0.34	0.33	0.31	0.28	0.27	0.253	0.234
	0.34	0.30	0.28	0.27	0.26	0.24	0.24	0.22	0.216	0.198
30%	0.40	0.40	0.37	0.35	0.33	0.34	0.32	0.30	0.284	0.26
	0.41	0.40	0.38	0.36	0.34	0.32	0.30	0.28	0.284	0.23
	0.39	0.38	0.37	0.34	0.35	0.33	0.32	0.29	0.263	0.25
20%	0.33	0.31	0.32	0.34	0.35	0.37	0.38	0.39	0.401	0.412
	0.35	0.33	0.36	0.38	0.39	0.40	0.41	0.40	0.405	0.409
	0.32	0.29	0.31	0.32	0.34	0.35	0.36	0.36	0.377	0.379
10%	0.33	0.32	0.33	0.35	0.36	0.36	0.35	0.36	0.364	0.381
	0.29	0.30	0.31	0.33	0.35	0.35	0.35	0.36	0.361	0.371
	0.29	0.29	0.31	0.31	0.33	0.33	0.33	0.33	0.335	0.342
BG-11 Medium	0.31	0.54	0.76	1.23	1.54	1.78	2.13	2.54	2.84	3.21
	0.33	0.64	0.85	1.35	1.65	1.89	2.22	2.75	3.09	3.4
	0.32	0.55	0.95	1.11	1.42	1.87	2.34	2.76	3.11	3.46

Table III -47 The average of Chlorophyll *a*

Garbage Pit Leachate concentration	Inoculation Period (Days)									
	0	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
100%	0.34	0.30	0.30	0.30	0.30	0.29	0.25	0.24	0.22	0.20
50%	0.38	0.34	0.32	0.31	0.30	0.28	0.26	0.26	0.24	0.23
30%	0.40	0.39	0.37	0.35	0.34	0.33	0.31	0.29	0.28	0.25
20%	0.33	0.31	0.33	0.35	0.36	0.37	0.38	0.39	0.39	0.40
10%	0.30	0.31	0.31	0.33	0.34	0.34	0.35	0.35	0.35	0.36
BG-11 medium	0.32	0.58	0.85	1.23	1.54	1.85	2.23	2.68	3.01	3.36

2.5.2 Percent growth of *Chlorella vulgaris*

Table III -48 Percent growth of *Chlorella vulgaris* in term Chlorophyll *a*

Garbage Pit Leachate concentration	Inoculation Period (Days)								
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
100%	-11.57	-11.47	-12.36	-9.69	-14.84	-24.43	-28.78	-34.32	-40.55
50%	-9.78	-14.40	-17.07	-20.53	-24.80	-29.78	-31.38	-34.76	-40.00
30%	-2.08	-7.24	-12.48	-14.81	-16.72	-21.71	-27.12	-30.87	-38.44
20%	-6.87	-1.59	3.29	6.77	11.35	13.94	15.54	17.83	19.52
10%	0.99	4.08	9.37	13.56	13.34	14.22	15.66	16.87	20.62
BG-11 medium	78.86	163.94	282.28	377.72	474.09	593.26	734.20	836.79	943.52

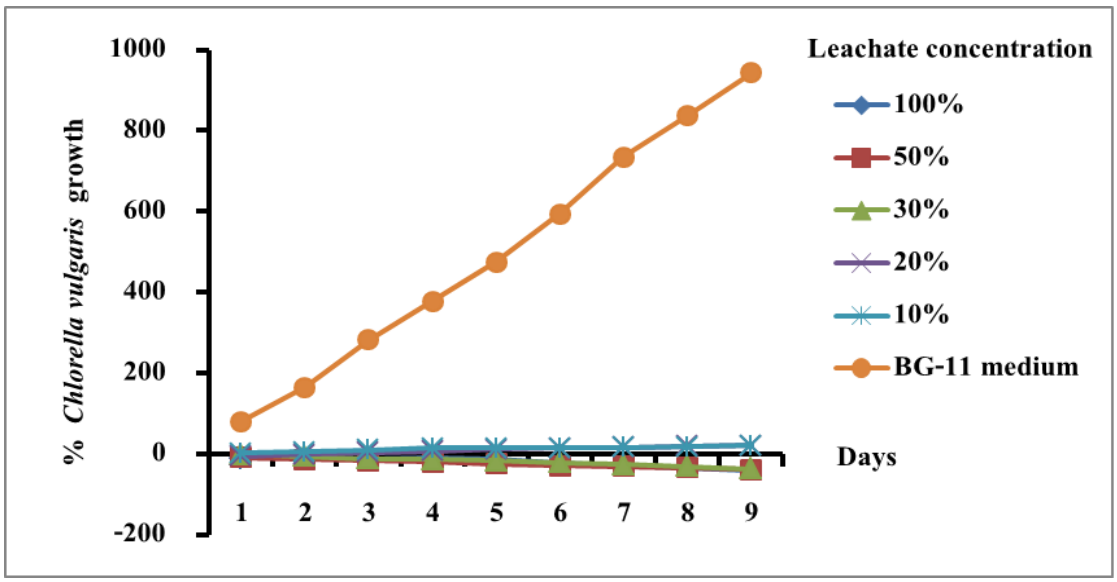

Fig A – 30 Percent growth of *Chlorella vulgaris* in different concentration of Garbage Pit Leachate, aerated condition on November with control (*Chlorella vulgaris* grew in BG-11 medium)

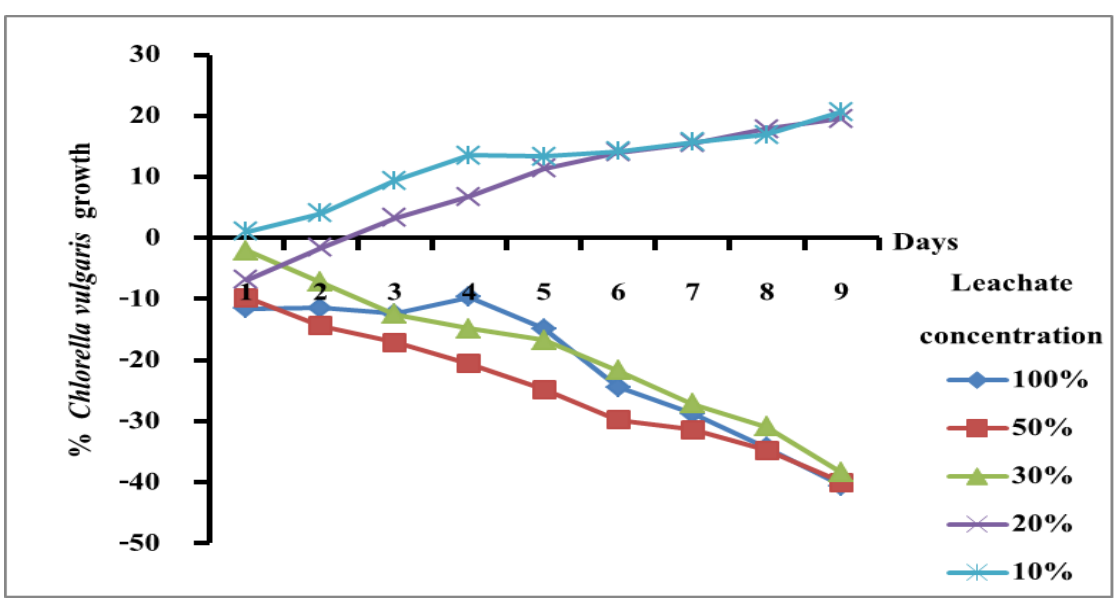

Fig A – 31 Percent growth of *Chlorella vulgaris* in different concentration of Garbage Pit Leachate, aerated condition on November without control (*Chlorella vulgaris* grew in BG-11 medium)

Vitae

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List of Publication and Proceeding

Thongpinyochai S and Ritchie RJ (2013). Bioremediation of Landfill and Garbage Pit Leachates using Growth of the Green Alga *Chlorella vulgaris.* The International Conference on Waste Management and Environment (ICWME) 2013, Institute of Biological Sciences, Faculty of Science, University of Malaya, 50603 Kuala Lumpur, Malaysia. 26 – 27th August 2013, University of Malaya, Kuala Lumpur, p 41.