3.0 MATERIALS AND METHODS

3.1 OUTLINE OF THE STUDY

The origin of the leachate used in the study was collected after the existing treatment processes at the Air Hitam Sanitary Landfill where the raw leachate has undergone aerobic treatment before being discharged to the river (Figure 12). For one batch of the study, the leachate was obtained from the Ampang Jajar Landfill, Penang.

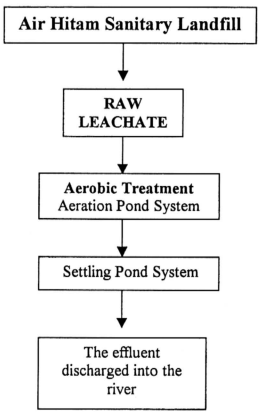


Figure 12: Leachate treatment in Air Hitam Sanitary Landfill.

3.2 CHARACTERISATION OF TREATED LEACHATE (TL)

The leachate used in the study has undergone aerobic treatment (retention time of 15 to 30 days) in ponds, where mechanical aeration is provided for oxidation of the leachate. This treated leachate was collected once a month and in the laboratory, it was kept in the cold room at 4 °C. Analyses were done to characterize every batch of leachate collected. Pollution parameters such as Chemical Oxygen Demand (COD), ammoniacal-nitrogen (NH_4-N), Phosphate (PO_4), Total Suspended Solids (TSS), pH, and temperature were determined. The analytical methods for the pollution parameter are described in sections 3.5.6 – 3.5.9. Analysis of heavy metal concentrations in TL was carried out in Batch I and VIII. The leachate samples were sent to the Centre for Environmental Technologies (CETEC) Laboratory for the heavy metal analysis. Twelve heavy metals including zinc (Zn), arsenic (As), cadmium (Cd), copper (Cu), chromium (Cr), lead (Pb), potassium (K), magnesium (Mg), iron (Fe), manganese (Mn), cobalt (Co) and nickel (Ni) were tested using the Atomic Absorbtion Spectrophotometer (AAS).

3.3 SCREENING OF MICROALGAE FOR GROWTH IN TREATED LEACHATE (TL)

3.3.1 Stock culture maintenance.

The microalgal cultures on agar slants (2% w/v) were maintained in sterile Bold's Basal Medium (BBM) (Nichols & Bold, 1965). The slant were placed on illuminated shelves, incubated at 25 °C under 12:12 h light-dark cycle with irradiance of 40 – 60 $\mu mol.s^{-1}.m^{-2}$. Subculturing was done every two months.

3.3.2 Preparation of inoculum for flask cultures

A loopful of each culture was taken from the slant and dispersed into 100 mL sterile BBM solution in 250 mL conical flasks. The flasks were shaken at 150 rpm in an incubator shaker (B.Braun), under illumination of 40 – 60 $\mu mols^{-1}m^{-2}$ with 12h : 12h, light : dark cycle and temperature at 25 ± 1°C for five days or when the optical density at 620 nm (OD_{620}) reached 0.2, as measured using a spectrophotometer (Shimadzu UV- 160A). The exponential phase culture was then ready to be used as innoculum. Ten percent innoculum was used in all experiments.

3.3.3 Innoculum for pond studies.

The inoculum for the HRAP was generated from cultures grown in plastic aquarium, which were rinsed with chlorox (5% hypochlorite solution) before use. For each of the five microalgal strains used, 1 L of exponential phase cultures (OD_{620}=0.2) was inoculated into the aquarium containing 2 L BBM and 3 L tap water. The cultures were aerated and incubated at room temperature for four days with an irradiation at 40 – 60 $\mu mol.s^{-1}.m^{-2}$ under 12h : 12h light : dark cycle. Aeration was provided by bubbling air through silicon tubes at the bottom of the tanks. A total of 40 L of a mixture of equal volume of the five strains microalgae, were used as inoculum for the HRAP.

3.3.4 Selection of microalgae for bioremediation of landfill leachate

Eleven species of microalgae representing two divisions of microalgae (Chlorophyta and Cyanophyta) were used. The microalgae were obtained from the University of Malaya Algal Culture Collection (UMACC) at the Institute of Postgraduate Studies, University of Malaya. The microalgae tested were:

Ankistrodesmus convolutus (UMACC 101), *Chlorella vulgaris* (UMACC 078), *Chlorella vulgaris* (UMACC 001), *Scenedesmus quadricauda* (UMACC 039), *Mougeotia* sp. (UMACC 069), *Ulothrix* sp. (UMACC 071), *Synechococcus* sp. (UMACC 075), *Chlorococcum oviforme* (UMACC 110), *Euglena* sp. (UMACC 058), *Oocystis polymorpha* (UMACC 153) and *Ankistrodesmus arcuatus* (UMACC 170).

The microalgae were subjected to a preliminary toxicity test to seven heavy metals, namely Cadmium (Cd), Cobalt (Co), Chromium (Cr), Cuprum (Cu), Ferum (Fe), Manganese (Mn) and Zinc (Zn). The toxicity tests were conducted with cultures of the microalgae in their exponential growth phase. The cultures were exposed to a range of 1 to 400 mg L^{-1} of the heavy metals and the endpoint of the test was based on percentage mortality of the cultures as indicated by chlorophyll *a* reduction.

Of the eleven microalgae tested, six species namely, *Ankistrodesmus convolutus* (UMACC 101), *Chlorella vulgaris* (UMACC 078), *Scenedesmus* sp. (UMACC 039), *Chlorococcum* sp. (UMACC 110), *Oocystis polymorpha* (UMACC 153) and *Ankistrodesmus arcuatus* (UMACC 170) were selected for toxicity testing with $CdCl_2$. These microalgae were used in 96 h single heavy metal exposure toxicity tests. This was done by incubating the microalgae in a range of metal concentrations in Bold's Basal Medium (BBM) in multiwell plates and the 96 h IC_{50} values were determined.

3.3.5 Preliminary Toxicity test

A preliminary test series was carried out for three reasons: (1) to conduct a series of screening experiments to select the species with high tolerance or sensitivity to heavy metals, (2) to select a species for detailed toxicity testing and (3) to select a species for the bioaccumulation study. All eleven algal species as mention in section 3.3.4 were subjected to this test. Seven metal species, Cd, Co, Cr, Cu, Fe, Mn and Zn, in a range of concentrations were tested individually for each microalgal species. Static Exposure System test is used with the duration of test at 96 h.

3.3.5.1 Preparation of test solutions

A series of concentrations (0.1, 1, 10, 100 and 400 mgL^{-1}) of Cd, Co, Cr, Cu, Fe, Mn and Zn including the control were prepared according to Table 6. A quantity of 2.0 µL of Cd, Co, Cr, Cu, Fe, Mn and Zn was pipetted and transferred into multiwell plates.

3.3.5.2 Test procedure

The multiwell plates, were incubated at 25 $\pm$ 1 ^{0}C under cool-white fluorescence light, 42 $\mu mol.photon.m^{-2}.s^{-1}$ and 12:12 light : dark cycle. The end point measurement for this test was based on colour of culture where (green colour indicated microalgal growth while brown or colourless indicated death of cells). Intensity of colour was used for growth comparison in response to the different heavy metals in the multiwell plates. The incubation period was 96 h. The species with highest tolerance for the metals was selected for toxicity testing.

Table 6 : Test solution for preliminary toxicity test.

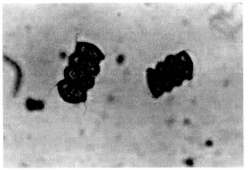
A	B	C	D	E
Test Concentration (mgL^{-1})	Stock Solution (mL)	Algae Sample (mL)	BBM or (mL)	Distilled Deionised Water (mL)
500	500	100	400	500
400	400	100	400	600
300	300	100	600	700
200	200	100	700	800
175	175	100	725	825
100	100	100	800	900
[Total volume (V) = 1000 mL]				
80	80	10	10	20
60	60	10	30	40
50	50	10	40	50
40	40	10	50	60
37.5	37.5	10	52.5	62.5
30	30	10	60	70
20	20	10	70	80
25	25	10	65	75
12.5	12.5	10	77.5	87.5
10	10	10	80	90
[Total volume (V) = 100 mL]				
1	1	1	8	9
0.1	0.1	1	8	9
0	0	1	9	10
[Total volume (V) = 10 mL]				

3.3.6 Laboratory studies on screening of microalgae for growth in Treated Leachate (TL)

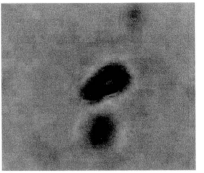
Three batches of TL were collected at different places and used in the laboratory studies for growth of microalgae in TL. Batch I was collected from Ampang Jajar Landfill on 15 July 1999 and in Batch II and III, the TL were collected from Air Hitam Sanitary Landfill on 2 June 2000 and 15 August 2000.

Five microalgae, *Scenedesmus quadricauda* UMACC 039 (Figure 13a), *Euglena* sp. UMACC 058 (Figure 13b), *Chlorella vulgaris* UMACC 011 (Figure 13c), *Ankistrodesmus convolutus* UMACC 101 (Figure 13d) and *Chlorococcum oviforme* UMACC 110 (Figure 13e), were used for growth studies in the treated leachate. The 100 mL cultures in 250 mL conical flasks with 10% inoculum were grown in Bold Basal's medium (BBM) containing various dilutions of treated leachate. The composition of BBM is shown in Appendix 5. Solution containing 0%, 25%, 50%, 75% and 100%, TL were made with Dilution Water (DW) (Appendix 6) (ASTM, 1993). For Batch I, 0% TL (composition of DW) was taken as control. For Batches II and III, two controls (0% TL and BBM) were used. All treatments were carried out in triplicate. The cultures were incubated at 42 $\mu molm^{-2}s^{-1}$ irradiance with 12h : 12h light : dark cycle and temperature of 27 $\pm$ 1°C. Initial pH of the growth medium was adjusted to 7.0 prior to inoculation. The pH during the experimental period was not controlled. Growth was monitored daily by cell count using a Improved Double-Neubauer Haemacytometer. The specific growth rate, μ was determined by the following relationship (Guillart, 1973).

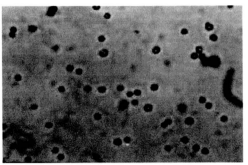
$$\text{Specific growth rate, } \mu\ (d^{-1}) = \frac{\ln N_2 - \ln N_1)}{t_2 - t_1}$$



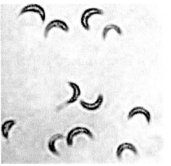
a) *Scenedesmus quadricauda* (UMACC 039)



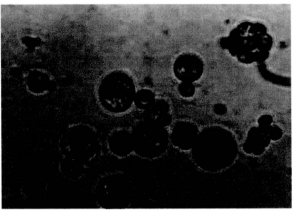
b) *Euglena* sp. (UMACC 058)



c) *Chlorella vulgaris* (UMACC 011)



d) *Ankistrodesmus convolutus* (UMACC 101)



e) *Chlorococcum oviforme* (UMACC 110)

Figure 13: Microalgae species used in the study

Where ; N_1 = the initial cell density at exponential phase

N_2 = the final cell density at exponential phase

t_1 = the beginning of the selected time interval

t_2 = the end of the selected time interval

Chlorophyll *a* content and dry weight of harvested microalgae on the final day were determined. The Inhibition Concentration (IC_{50}) was calculated based on day four and day eight results. Percentage inhibition is based on % inhibition of algal growth in treated leachate compared with growth in control medium (0% TL in Batch I; BBM in batches II & III). The IC_{50} value was determined using the ICPIN program (Norberg-King, 1993)

3.4 HIGH RATE ALGAL POND (HRAP)

3.4.1 HRAP design

Two high rate algal ponds (Figure 14) were constructed at the Block C roof top of the Institute Postgraduate Studies, University of Malaya. The ponds are single loop raceway mixed by paddle wheels type (Figure 15), measuring 1m x 0.5m x 0.3 m. A paddle wheel was installed at the center of one side of the loop to mix the pond culture. The paddle wheel was operated using a bonfiogli-gear motor attached with a variable speed gearbox (9-55 rpm). Transparent corrugated acrylic roof were fixed at the height of about 1.5m above the ponds to shelter the ponds from rain. Total volume for each pond was 40 L while the surface area of each pond was 0.71 m^2. Culture depth was 0.15 m and the flow rate was 15 cms^{-1}.

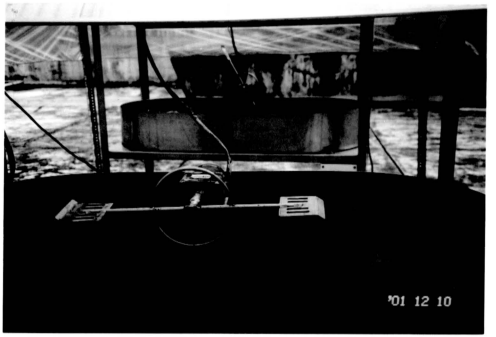


Figure 14: High Rate Algal Pond

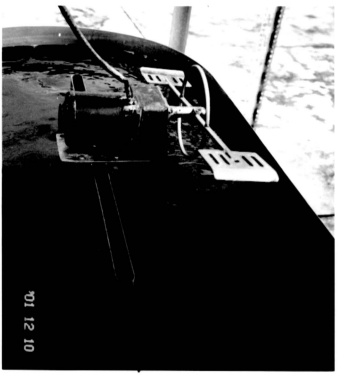


Figure 15: Paddlewheel

3.4.3 Semidiurnal studies

Semidiurnal studies were carried out for experiment I and II. Samples of the culture were taken every hour from 0700h to 1900h on selected days. Physical parameters like irradiance, temperature, dissolved oxygen and pH were monitored. Optical density (OD_{620nm}) and chlorophyll *a* content were determined to indicate the growth of the pond culture.

3.5 ANALYTICAL METHODS

3.5.1 Determination of pH, Temperature, Dissolved Oxygen and Irradiance

These parameters were obtained via instrumental measurement. pH was measured using a pH meter (Mettler Toledo 320). A YSI dissolved oxygen probe was used to detect the dissolved oxygen concentration (mgL^{-1}) and temperature (°C) of the samples. Irradiance (k Lux) was determined by a Panlux Electronic 2, Lux and Iluminance Meter. The unit for irradiance was converted to $\mu mol.s^{-1}.m^{-2}$ through the following equation :

$$10\ 000\ lux = 134.5\ \mu mol.s^{-1}.m^{-2}$$

3.5.2 Determination of Optical Density.

The optical density of the sample was measured in a 10 cm cuvette at wavelength 620 nm using a Shimadzu UV- 160A Spectrophotometer. Distilled water was used as the blank.

3.5.3 Determination of chlorophyll *a* (chl. *a*) concentration

The chl *a* concentration was determined using the spectrophotometric method (APHA, 1998). 10 ml of algal culture collected on a filter paper was mashed

and mixed homogenously with 10 ml of 100% acetone in a screw-cap centrifuge tube. The tubes were kept overnight at 4 °C in the dark before centrifugation at 3000 rpm, for 10 minutes. The optical density of the supernant at 630, 645 and 665nm was measured. The chl *a* concentration was calculated through the following equation;

$$\text{Chl. a } (mgm^{-3}) = \frac{\text{Ca X acetone volume (ml)}}{\text{Algal culture volume (L)}}$$

Where $Ca = 11.6 (OD_{665}) - 1.31(OD_{645}) - 0.14 (OD_{630})$

3.5.4 Algal cell count

The algal cell were counted using an Improved Double-Neubauer Haemacytometer. Appropriate dilution and homogenization of the algal cultures was performed prior to counting, to limit the cell number to below 100.

3.5.5 Determination of Algal Dry Weight (DW)

A known volume of algal culture was filtered through a preweighed dried 0.45 µm glass fiber cellulose filter. The filters were dried at 100 °C in an oven for 24 hours, cooled in a desicator filled with silica gel and weighed. The algal dry weight is determined through the following equation :

$$DW (mg\ L^{-1}) = \frac{\text{[weight of filters + dried algal biomass]-[weight of blank filters] (mg)]}}{\text{Volume of algal culture (L)}}$$

3.5.6 Ammoniacal Nitrogen (NH_4 –N) Assay

The NH_4 –N content of the sample was determined by the modified phenate method (APHA, 1998), Principally, indophenol and intensely blue compound is formed by the reaction of ammonia, hypochlorite and phenol catalysed by a manganous salt. 50 mL samples are measured into 100 ml stoppered flask. A series of standard ammonia solution were prepared by spiking the standard ammonia solution (1.2 μg/ml) into distilled water to make up a final volume of 50 ml. Two ml of phenol - alcohol solution, two ml of sodium nitroperusside solution and five ml of oxidizing solution (mixture of sodium hypochlorite : alkaline citrate solution in 1:4 propotion) was mixed thoroughly. 50 ml distilled water was treated as blank. The blue colour of the indophenol was allowed to develop at room temperature, for at least one hour. Absorbance was read at 640 nm. A standard curve was plotted, as reference for the unknown samples.

3.5.7 Phosphate (PO_4) Assay

Orthophosphate is referred to phosphate that responds to colorimetric tests without prior hydrolysis or oxidative digestion of the sample (APHA, 1998). The assay employed was the ascorbic acid method, based on the molybdenum blue colour development from phosphomolybdic acid. 10 ml of freshly prepared mixed reagent (a mixture of 100 mL ammonium molybdate, 250 mL sulphuric acid, 100 mL ascorbic acid and 50 mL potassium antimonyl tartrate solutions in sequence) was added to 100 ml of appropriately diluted sample and mixed at once. After 10 minutes and within two hours, the absorbance of the solution at a wavelength of 885 nm was recorded. 100 ml of distilled water was treated as the absorbance blank. Reference solutions containing a certain amount of phosphate

(PO_4^{3-}) were prepared by diluting the standard solution (1000 μg PO_4L^{-1}). A standard curve was plotted, as reference for the unknown samples.

3.5.8 Determination of Chemical Oxygen Demand (COD)

20 ml sample or a suitable diluted sample was pippetted into 250 ml round bottom flask. 0.4 g Mercury (II) sulphate ($Hg SO_4$) was then added, followed by 10 mL potassium dichromate ($K_2 Cr_7O_4$) 0.1 N. Slowly, 30 ml concentrated sulphuric acid (conc. $H_2 SO_4$) was then added. The mixture will turn to greenish orange. The solution was mixed well and refluxed for at least two hours. 100 mL distilled water added after the sample was cooled. A few drops of ferrous-1 :10-phenanthroline indicator solution was added before titration. The flask was swirled and titrated against ferrous sulphate (Fe_2SO_4) solution (0.1N) until the colour was changed from blue-green to reddish brown (end point). A blank determination was carried out. The amount of oxidisable organic matter, measured as oxygen equivalent, is proportional to the $K_2 Cr_7O_4$ used. The COD was calculated based on the formula:

$$COD\ (mgL^{-1}) = \frac{(A - B)\ C\ X\ 8000}{\text{Sample volume (mL)}}$$

Where; A = Amount of $Fe_2 SO_4$ (0.1N) (mL) used for blank

B = Amount of $Fe_2 SO_4$ (0.1N) (mL) used for sample

C = normality of $Fe_2 SO_4$ (0.1N)

Based on COD value, the carbon (C) content can be calculated using the formula given by Edwards *et al.*, (1980):

$$\text{Carbon (C) in } mgL^{-1} = COD\ (mgL^{-1})\ X\ \frac{12}{32}$$

3.5.9 Determination of Total Suspended Solids (TSS)

A known volume of homogeneous sample was measured and filtered through a preweighted dried 0.45μm pore size glass-fiber filter membrane, using a Milipore water suction vacuum pump. The filters were then dried in an oven at 100 °C overnight, cooled in a desicator and the final weight of the filter paper and residue were recorded. The TSS was calculated based on the following relationship,

$$\text{Suspended solids } (mgL^{-1}) = \frac{1000\ (a - b)}{c}$$

Where a = weight of filter membrane + residue (g)

b = weight of filter (g)

c = sample volume (L)

3.6 METEOROLOGICAL DATA

Meteorological data was obtained from the Malaysian Meteorological Services, Petaling Jaya Selangor. The data included,the total rainfall, number of rain days, mean air temperature and mean solar radiation.

3.7 STATISTICAL ANALYSES

The data were analyses using the Student t-test, Simple Correlation and One-way Analyses of Variance (ANOVA). T-test was used to assess the difference between the means of the environmental parameter of the two treatment ponds and to assess the difference between the means of the parameter of the two treatment ponds at the different loading rates.

The ANOVA was only conducted for the abiotic data. The one-way analyses of variance was used to assess the difference in environmental parameters of the two treatment pond and the difference in environmental parameters between different loading rate.

Simple correlation analyses was used to assess the concurrent association between different treatment pond. All statistical analyses were performed using the statistical software Statistica Version 5.5.