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

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Abstract

Waste treatment in Phuket, Thailand produces two sources of leachate. The Landfill Leachate (LF) has low BOD (60-405 mg O_2/L) and heavy metal levels are below legal limits. Garbage awaiting incineration, Garbage Pit Leachate (GPL), also produces a second leachate with a very high BOD (50-100 g O_2/L) and NH_3-N (763-2,045 mg/L). Zn and Cr exceed standards but Cu and Pb are low. *Chlorella vulgaris* was tested for its ability to decrease NH_3-N, NO_3--N, Total-P, BOD and COD and to remove and/or immobilize heavy metal at varying dilutions of leachate. The objectives of this study on *Chlorella* bioremediation were: 1) measure *Chlorella* 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, COD 3) measure heavy metal removal from leachates. The minimum inoculum of *Chlorella* biomass which can grow in the LF was Chlorophyll a 0.259 μg/mL (A_{750}=0.075). *Chlorella* significantly lowered its BOD and COD: Cr and Ni, already low in landfill leachate, were decreased by 70% and 66%. *Chlorella* grew well in LF diluted 30 % with tapwater: % removal of NH_3-N (53.91%), BOD (52.78%) and COD (51.05%). The GPL was very toxic: only 10-20% dilutions of GPL were tolerated by *Chlorella* and cultures required continuous aeration by shaking to grow. The minimum necessary inoculum of *Chlorella* biomass in the GPL was Chlorophyll a=0.92 μg/mL (A_{750}=0.19). In 20% dilution of GPL, Cr and Zn were decreased by 33% and 90% respectively to legal levels. % removal of NH_3-N (41.5%), NO_3--N (32.4%), Total-P (55.1%), BOD (49.2%) and COD (50.8%). *Chlorella* inoculations with a biomass of 1.17 μg/mL Chlorophyll a (A_{750}=0.202) removed 90% of the Zn.

Keywords: *Chlorella vulgaris*; Bioremediation; Heavy metals; Landfill Leachate, Garbage Leachate

Abbreviations

A_{750}: Absorbance at 750 nm; A_{649} nm: Absorbance at 649 nm; BOD: Biochemical Oxygen Demand; Chl a: Chlorophyll a (μg/mL); COD: Chemical Oxygen Demand (mg/L); Cr: Chromium; GPL: Garbage Pit Leachate; LF: Landfill; OD: Optical Density; Total-P: Total Phosphorus; t_0: Initial Time (h); Zn: Zinc

Introduction

Garbage dump leachate is a worldwide problem; typically, they have high salinities, high ammonia content and typically high but variable levels of heavy metals [1]. Lin et al. [2] studied ammonia-nitrogen tolerance in microalgae grown in a leachate physico-chemically similar to the leachate in Phuket with a very high ammonia-N and COD from a leachate pond at the Li Keng Landfill, Guangzhou, China. Few studies have been made on leachates under tropical conditions. In 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. 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 leachate from these holding bays which will be referred to as Garbage Pit Leachate (GPL) in the present study. 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 after dumping in the landfill creates a second leachate stream which will be referred to as landfill leachate (LF).

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. The Garbage Pit Leachate (GPL) arises directly from decomposition of raw garbage. The Landfill leachate (LF) arises from the landfill which has a sandwich arrangement of layers of garbage and burnt garbage (ash). The leachate moves slowly down through all layers of the garbage (Figure 1) and is collected at the base of the landfill. 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. Leachate in the landfill passes through ash and garbage layers picking up dissolved organic compounds, toxic organic compounds and heavy metals along the way. There are two types of leachate to deal with: the direct garbage leachate from the holding bays of the incinerator (GPL) and the leachate from the landfill (LF). Currently all leachate is fed into the water quality improvement plant mixed along with the domestic sewage from the municipality. The BOD of the GPL is much too high for safe disposal in rivers or for recycling by spreading on agricultural land. The BOD load from the GPL has become a pressing problem ever since the incinerator had to

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be expanded to two input feed-heads because it overloads the sewage treatment plant and is very toxic.

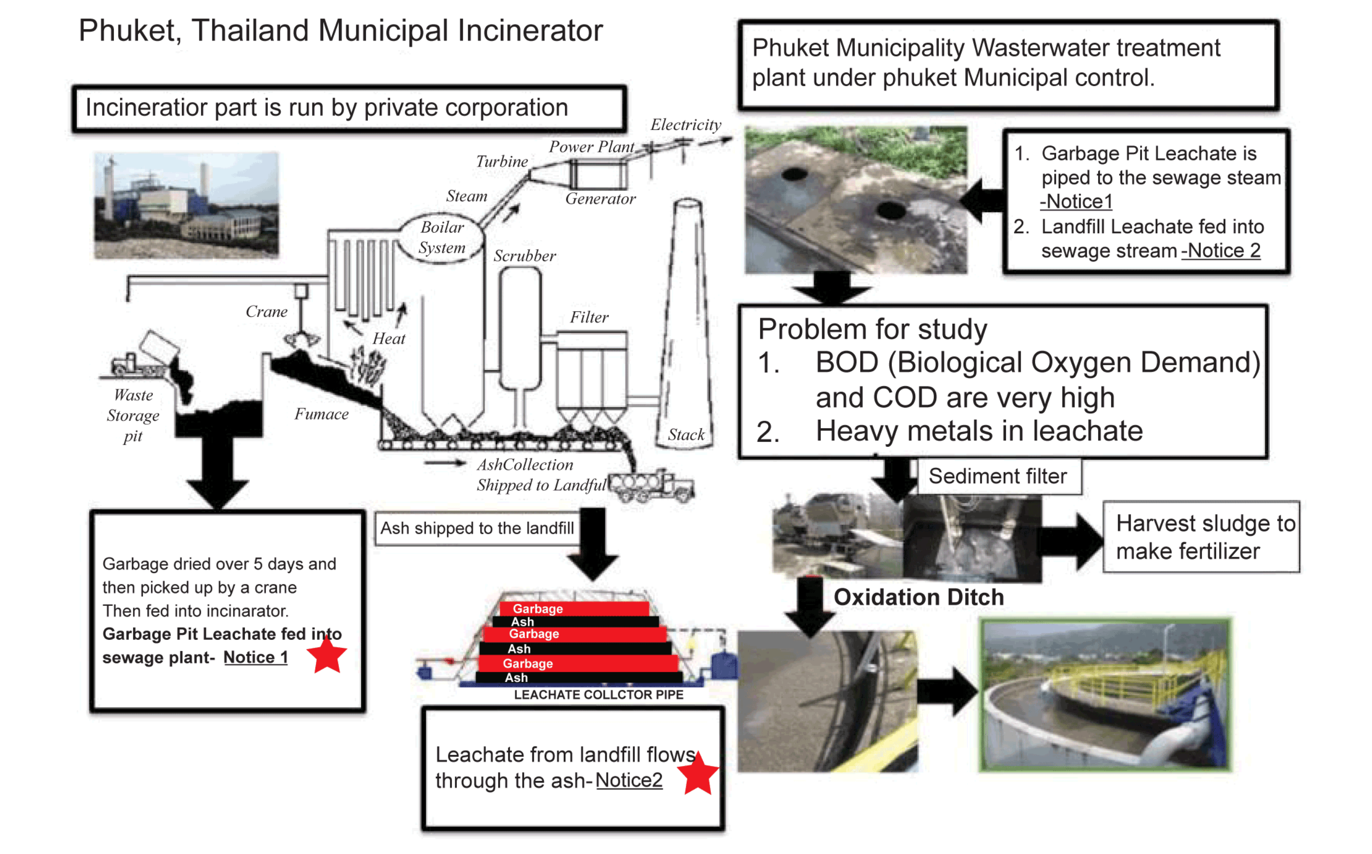

Figure 1: Waste Treatment system in Phuket

The other major problem with the leachate arises from its potentially high heavy metal content. In the LF, the water leaching through the layers of garbage and garbage ash would be expected to mobilize sand dissolve the heavy metals. Early stages of the present study showed that the LF had relatively low heavy metal content but the GPL had very high levels of heavy metals, in particular Zinc and Chromium [3]. The Zinc and Chromium levels far exceeded environmentally permitted levels in the case of the GPL but did not in the case of the LF. The leachate from the holding bays of the incinerator also has very high BOD and COD [3].

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 since they require nitrogen, phosphorus, CO_2, and light for their photoautotrophic metabolic growth. Many species of microalgae have been used as a bioremediation agent when combined with wastewater treatment, but the most commonly used are various species and strains of *Chlorella* such as *Chlorella pyrenoidosa*, and *Chlorella vulgaris*. *Chlorella* is a very hardy alga able to tolerate variable salinities, a wide range of pH and is not hypersensitive to metal poisoning. *Chlorella* species predominate in the sewage ponds at the Saphanhin Klongkopee sewage plant and also in prawn farm ponds in the region. *Chlorella* is able to use many organic compounds photoheterotrophically and so can use many organic compounds in wastewater and leachates [4].

Our study focused on 3 major topics 1) To investigate and record heavy metal contamination of leachates from the GPL and LF, 2) To investigate using green algae (*Chlorella vulgaris*) to reduce BOD in the leachates before feeding the effluent into the recycling process, 3) To investigate heavy metal reduction in leachates by using bioassay based on *Chlorella vulgaris*.

Materials and Methods

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) and (b) Leachate from the Garbage Pit (GPL).

Physico-chemical properties of leachate sample

The leachates were settled to remove solid matter, and the supernatant used for the experiments. Characteristics of leachate such as pH, ammonia-N, nitrate-N, total phosphorus, BOD, COD were measured using industry standard methods. Heavy metals were measured using Inductively Coupled Plasma Optical Emission Spectrometry at the PSU Scientific Equipment Centre, Hat Yai (SEC).

Chlorella vulgaris culture conditions and growth measurement

Culture-*Chlorella* vulgaris: *Chlorella vulgaris* was cultured with BG-11 in 250 mL and 150 mL flasks for exponential growth phase for 7 days in a temperature range 25 - 27°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 at 120 rpm [WiseShake (SHO-2D)] or agitated by magnetic stirrers. The *Chlorella vulgaris* strain used was from the Coastal Fisheries Research and Development Station (Phuket, Thailand).

Growth measurement: Absorbance (optical density, OD) of cell cultures was measured at 750 nm. For chlorophyll determinations, 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 mobilized by vortexing before adding 3 mL of ethanol. The 3 mL of ethanol extract was then left in the refrigerator for about 1 hr, then centrifuged at 5,000 rpm for 5 minutes and the supernatant was used for chlorophyll determination using a spectrophotometer (Shimadzu UV-1601) at 649 and 665 nm using A_{750} nm as the absorbance blank. The equations of Ritchie [5] were used to estimate Chlorophyll a [Chl a (μg/mL)=11.867 × (A_{665} nm-A_{750} nm) – 5.201 × (A_{649} nm-A_{750} nm)].

Heavy metal and nutrient removal: basic use of bioremediation/bioassay methods

The minimum *Chlorella* inoculation required to successfully start cultures in 100% – GPL and LF raw leachate: The range of *Chlorella* 10 mL inoculums in 100 mL total volume had Chl 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} nm) 0.1, 0.115, 0.19, 0.295, 0.4, 0.75. Only minimal inoculations were usually needed to start cultures in LF but heavy inoculations were needed to establish *Chlorella* in GPL.

The effect of different concentrations of leachate on algal growth: *Chlorella vulgaris* was grown at a different concentration of LF and GPL. Leachate samples for experiment were centrifuged at 5,000 rpm. All experiments were set up in temperature range 25-27°C under 24 hours light using cool-white fluorescent lamps with light intensities=200 μmol (quanta) m-2 s-1 (PAR,400-700 nm). Experiment set up in two sets (1) LF diluted with tap water to following concentrations: 10%, 30%, 50%, 100% (undiluted leachate) and (2) GPL diluted with tap water to following concentrations: 10%, 20%, 30%, 50% and 100% (undiluted leachate) with two conditions: shaking [120 rpm-WiseShake (SHO-2D)] and non-shaking. The non-shaken cultures were given a manual gentle stir each day.

The optimum biomasses of *Chlorella* in terms Chl a to remove heavy metals:GPL was very toxic and had to be diluted to 20% GPL/80% tap water for *Chlorella* to grow. Tap water was used as the diluent because in realistic field situations deionised water or distilled water would not be used. The optimal GPL inoculum was 20% of GPL and 80% of tap water based on the results from experiments however *Chlorella* would grow successfully even in undiluted LF and besides the heavy metals in LF did not exceed the environmental standard values. For the determination of minimum *Chlorella vulgaris* cell concentrations which efficiently removes metals, cell densities of 11.7, 16.0, 25.0, 30.5 μg Chl a /mL were used as starting inocula.

Statistical analysis

Simple statistical methods were used in the present study. All measurements were done in at least 4 replicates and values are quoted as means ± standard errors (SE). 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 [6] as the standard statistical reference text.

Results

Physico-chemical properties of leachate samples

The leachate from the GPL 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). 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 (60-270 mg/L). The LF was not above legal limits in heavy metals. The LF was neutral-basic (pH around 7-8), with lower BOD (60-405 mg/L), a lower COD level (32-160 mg/L), NH_3 concentration (170–256 mg/L), relatively low level of nitrate-N (13.6-48.86 mg/L) and total phosphorus (5.57-36.63 mg/L) (Table 1). The physico-chemical analyses of LF in Dry and Wet season (Figure 2a and 2b) 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 LF 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). The garbage pit was designed to be fully covered and flood-proof to prevent rainwater making it more difficult to incinerate the garbage (Figure 1). The physico-chemical properties of GPL in the Dry and Wet seasons are not different because GPL comes from the leakage from piles of garbage awaiting incineration kept under cover so that it does not receive any rainwater (Figure 3a and 3b).

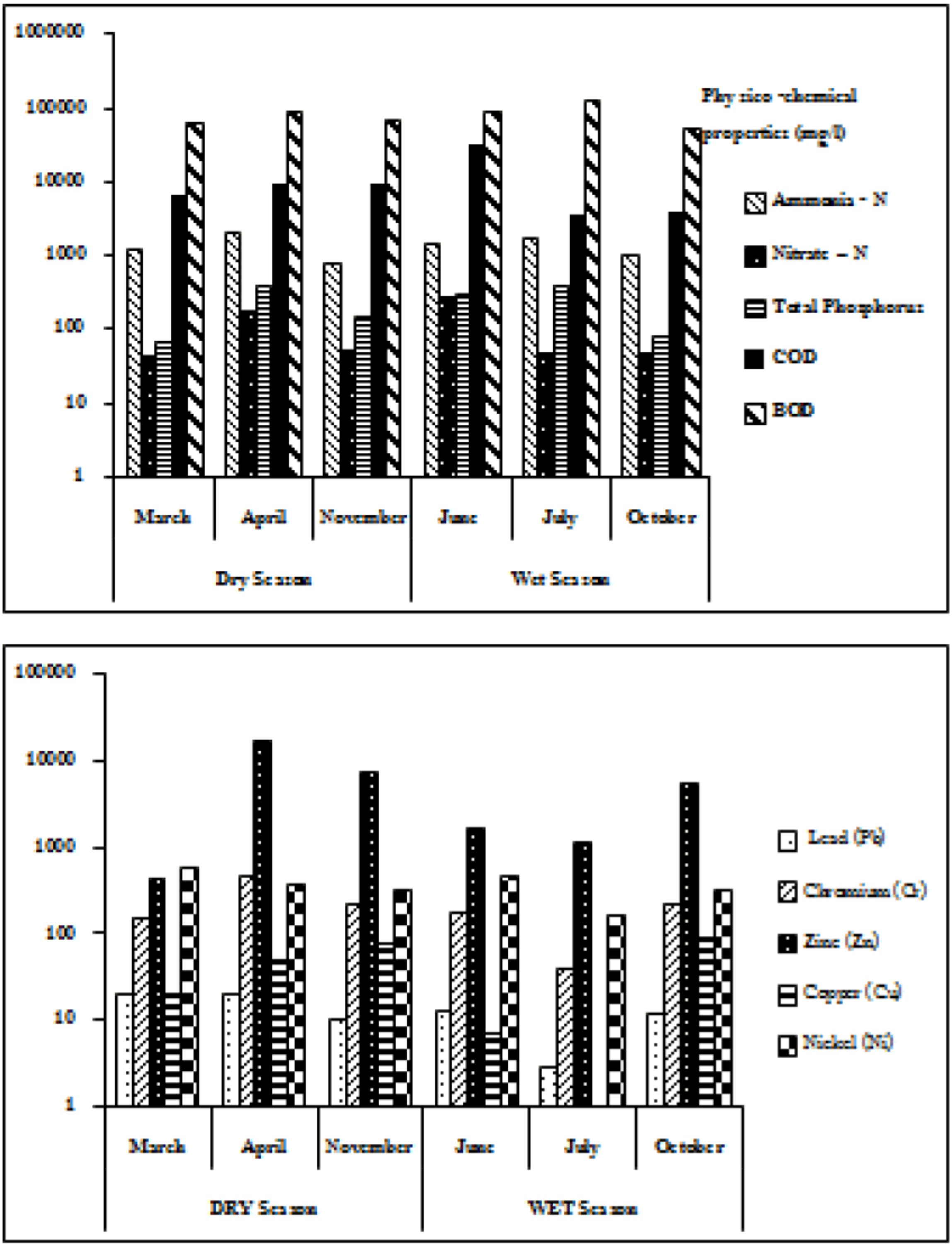

Figure 2: Physico-chemical properties (a) and heavy metals (b) of GPL in Dry and Wet Season

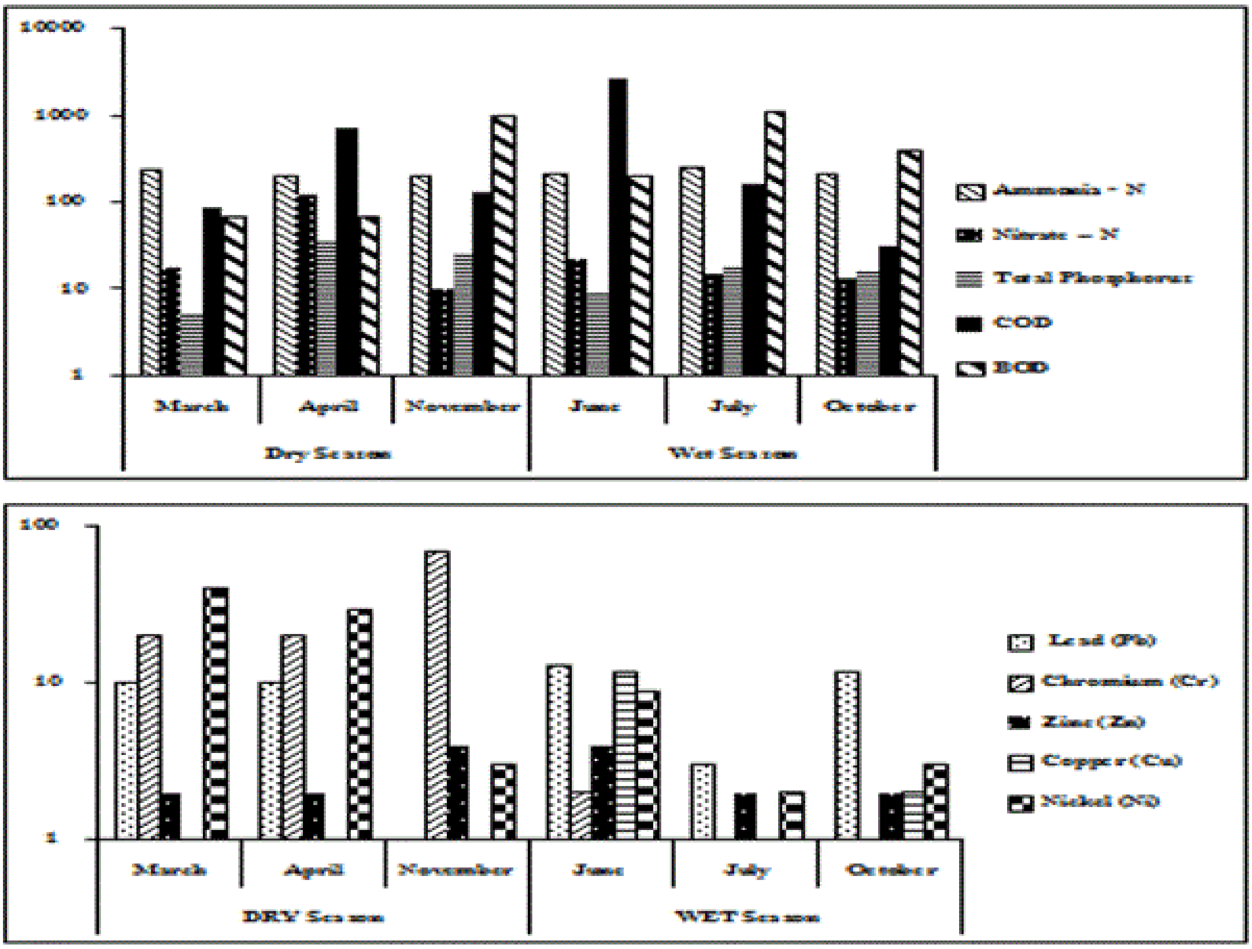

Figure 3: Physico-chemical properties (a) and heavy metals (b) of LF in Dry and Wet Season

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	2045.6	197.76	1508.58	217.71	1785.47	256.5	1057.9	218.8	763.8	205.75
Nitrate – N	43.95	17.61	176.79	126.63	267.03	22.25	46.86	14.86	45.98	13.6	50.61	10.35
Total Phosphorus	69.67	5.57	406.25	36.33	290.9	9.16	409.95	18.64	78.91	17.1	146.47	25.97
COD	6,336	86.4	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	90,085.70	207	126,750	1,121.25	52,500	405	67000	970
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

Table 1: Physico-chemical properties of leachate samples

Chlorella vulgaris standard growth

Standard curve for blank (medium) solution (in triplicate): Exponential growth rate constant (k) and doubling time (t2) 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} nm). For *Chlorella vulgaris* the apparent doubling time for the log phase was: k=0.0506 ± 0.00357 h-1 (n=38, ±95% conf. lim.), t2=13.7 ± 0.96 h (±95% conf. lim.) with a correlation r=0.984.

Standard relationship between absorbances 750 nm and Chlorophylla: The relationship between A_{750} nm and biomass as measured by Chl a (µg) of *Chlorella vulgaris* was determined spectrophotometrically. For *Chlorella vulgaris* the equation y=7.7809x - 0.2047 (n=18, ±95% conf. lim.), with a correlation r=0.9787 can be used to describe the relationship between A_{750} nm (x) and µg of Chl a (y).

Standard relationship between Absorbances 750 nm and cell number: The relationship between A_{750} nm and cell number count by the hemocytometer method was determined. Using the A_{750}/cell number relationship, the amount of *Chlorella* used as a starting inoculum for experiments could be calculated. In the range of absorbances measured (up to A_{750}=1.4) the absorbance was directly proportional to cell numbers. For *Chlorella vulgaris* cells in log phase an A_{750} of 0.00738 ± 0.00060 was equivalent to a cell density of 106 cells/mL (n=23, ±95% conf. lim.), Pearson's r=0.9354.

Heavy metal and nutrient removal: Basic use of bioremediation/bioassay methods

The minimum *Chlorella* in terms of Chl a growth in 100% raw leachate: Chl a of *Chlorella vulgaris* was determined at the start of the experiment as described above. Experiments were set up with starter *Chlorella* inoculations with absorbances (A_{750} nm) of 0.075, 0.08, 0.19, 0.201 and 0.346 (equivalent to 0.259, 0.32, 0.92, 1.485 and 2.543 µg/mL Chl a respectively) and cultivated in 100% LF or GPL for 7 days in the light.

Chlorella established itself very well in the LF and grew exponentially even with the minimum inoculum (Figure 4a). The results showed that in LF a *Chlorella* inoculum as low as 0.259 µg/mL Chl a (A_{750} nm=0.075) grew well. GPL on the other hand was highly toxic (Figure 4b). Low-level inoculations of *Chlorella* slowly died off (0.259 and 0.32 µg/mL Chl a). The heavier inoculations did not die but only grew marginally. Only the heaviest inoculation showed substantial growth (2.543 µg/mL Chl a). The minimum inoculation which survived and grew in GPL was 0.92 µg/mL. It was concluded that a heavy inoculation of *Chlorella* was needed for the alga to grow in the GPL. We used the same biomass of Chl a (0.92 µg/mL) to cultivate *Chlorella* in the LF and GPL.

The effect of different concentrations of leachate on algal growth

Growth experiments on LF: The growth curves of *Chlorella vulgaris* in 10%, 30%, 50% and 100% (undiluted) LF solutions and diluted with tap water (90%, 70%, 50% and 0%) were set up and inoculated with 10 mL of actively growing *Chlorella* culture (Chl a 0.9 µg/mL, Abs750 nm=0.19). Tap water was used as the diluent because in realistic field situations deionised water or distilled water would not be used.

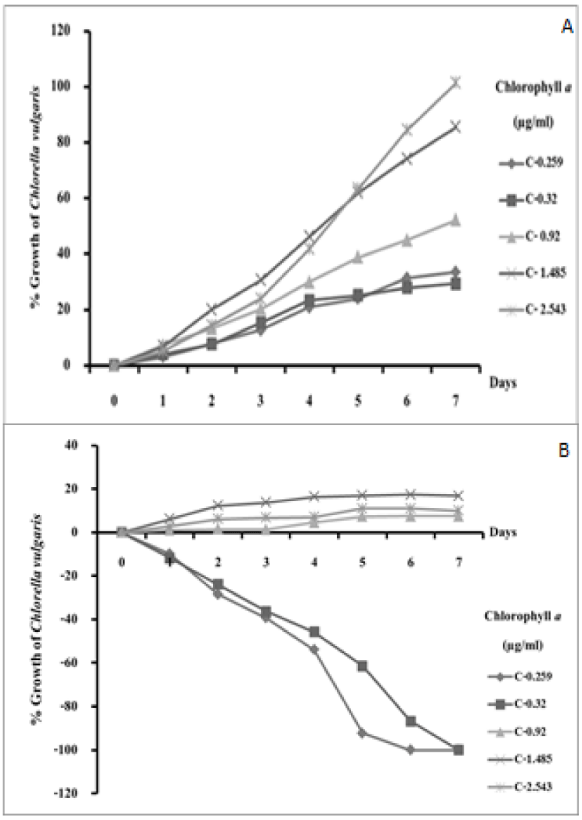

Figure 4: Effect of inoculation volume on the establishment of *Chlorella* in LF (a) and GPL (b). The SE errorbars of the calculated means (n=3) are less than ±2% and do not show on the graphs.

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Growth was followed over a 9 day experimental period. *Chlorella* grew in all the dilutions of LF (in terms of Chl a). The *Chlorella* grew the best in media containing 30% LF (Analyzed using two ways 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 Figure 5a are means ± SE error bars from the 6 months of data).

Growth experiment on GPL (not shaken but under light conditions): The GPL was much more toxic than the LF [3]. Experiments to test the toxicity of GPL followed a similar protocol to those described above for experiments with LF. *Chlorella* would not grow in 100%, 50% or 30% leachate and slowly died off over the course of the experiment (Figure 5b). *Chlorella* grew well in the undiluted tap water and grew marginally in GPL diluted to 20% and 10% in tap water (20% leachate grew +11% and 10% leachate grew +9%). The data was analyzed 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. The data points shown in Figure 5b are means ± SE error bars from the 6 months of data.

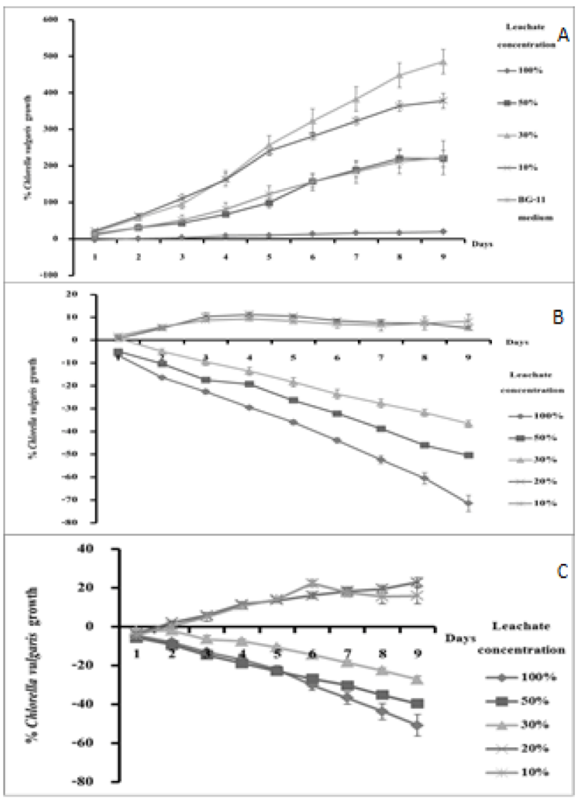

Figure 5: Percentage of nutrients removed by *Chlorella* from the landfill leachate (LF) after 9 days incubation (A), removal of from dilutions of Garbage Pit Leachate after 9 days incubation in static culture (B) and removal from the Garbage Pit Leachate after 9 days incubation in the light under shaken conditions (C).

Growth experiment on GPL (well aerated conditions): *Chlorella* was grown over a 9 day cultivation period with shaking on an orbital shaker (120 rpm) in a range of diluted GPL: 100%, 50%, 30%, 20%, 10% leachate diluted with tap water. Growth in 100% BG-11 medium was used as the blank control. Growth in undiluted leachate was very poor and the alga died off with time (Figure 5c) even though some marginal growth might have occurred during the early stages of the incubation. Positive growth occurred in 20% and 10% leachate (Analyzed with two way ANOVA with 5-fold replication). In terms of Chl a, the alga was able to grow continuously over the course of the experiment in 10 and 20% GPL but at all higher concentrations gradually died off with time. Growth of *Chlorella* in GPL was much better under well-aerated shaking conditions (Figure 5c) than cultures kept under non shaking conditions (Figure 5b), Nevertheless GPL at any of the dilutions tested was severely inhibitory compared to the blank control grown in tap water. Comparison with the results for the LF experiments clearly shows that the GPL was much more highly toxic. The data points shown in Figure 5b and c are means ± SE error bars from the 6 months of data based on 5 replicates. Some error bars are smaller than the symbols used for the mean values.

Nutrient removal by *Chlorella vulgaris*

Nutrient removal on Landfill Leachate: Percentage removal of ammonia-N, nitrate-N, total phosphorus, COD and BOD were measured in cultures of *Chlorella* grown in LF leachate diluted with tap water over a 9 day period (Figure 6a). 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%). The experiment was repeated on monthly collections of leachate over 6 months and each experiment was run in 3 replicates. The data points shown in Figure 6a are means ±SE error bars.

Nutrient removal on garbage pit Leachate (no shaking and under light conditions): GPL-Percentage removal of ammonia-N, total-phosphorus BOD and COD by *Chlorella* cultures were measured after 9 days incubation in a range of dilutions of GPL. Percentage removal of ammonia-N, Total-P, BOD and COD were calculated using dilutions of leachate that were not inoculated with *Chlorella*. The ammonia-N, total-phosphorus, BOD and COD were also measured in the GPL at the start of the experiment. The 20% GPL diluted with tap water showed the highest percent removal rates than the other concentrations of leachate (Figure 6b). The percentage 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 GPL was much poorer than in the case of the similar incubation experiments run on LF (Figure 6a-6c). The experiment was repeated on monthly collections of leachate over 6 months and each experiment was run in 3 replicates (Figure 6b). The data points shown in Figure 6b are means ± SE error bars.

Nutrient removal on garbage pit Leachate (well aerated conditions): Removal of pollutants from the GPL by *Chlorella* was better under aerated and shaken conditions. During the 9 day cultivation period, the 20% GPL diluted with tap water had the highest percentage removal compared to the initial condition (GPL at t=0): percentage removal compared to the t=0 control were: 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%) (Figure 6b and 6c). The data points shown in Figure 6b and 6c are means ± SE error bars based on 5 replicates.

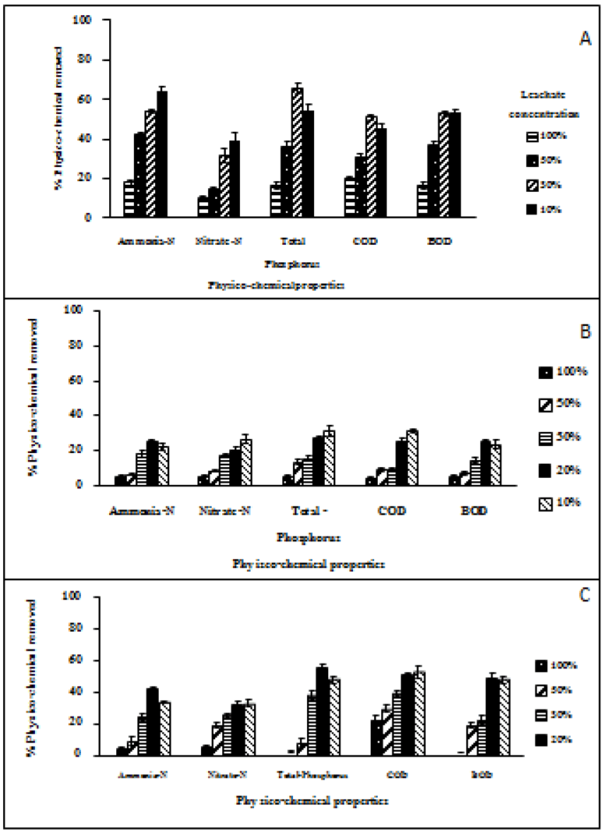

Figure 6: Percent growth of *Chlorella* in different concentration of landfill leachate (a) and in 20% GPL under unshaken (b) and shaken (c) conditions in the light.

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

Landfill Leachate (LF): The *Chlorella* survived but did not grow well in 100% undiluted leachate but grew substantially in all the dilutions of leachate (Figure 5a). The heavy metals in LF before treatment were under the legal limit but nevertheless the alga grew very poorly in undiluted leachate. Despite not growing well in undiluted leachate, removal of heavy metals by *Chlorella* incubated 100% of leachate was measured to determine the removal of heavy metals. Substantial amounts of metals might have been removed by binding to the *Chlorella* cells even though the cells might did not have grown well. As we anticipated from the cation-exchanger properties of cell walls of plant cells. Table 2 shows that despite the cells not growing in 100% LF the *Chlorella* cells nevertheless removed 70% of Chromium and 66% of Nickel by simple adsorption. Copper, Lead and Zinc were not detectable in the LF before or after inoculation.

Heavy metals (µg/L)	Landfill leachate		
	Before treatment	After treatment	% Removal
Copper (Cu)	<1	<1	–
Chromium (Cr)	20	6	70
Lead (Pb)	<10	<10	–
Nickel (Ni)	30	10	66
Zinc (Zn)	<2	<2	–

Table 2: Percent removal of heavy metal from Leachates by *Chlorella*

Garbage Pit Leachate (GPL) (non-shaking condition): The results of heavy metals analysis (Table 3) shows that *Chlorella* was not successful in removing heavy metals at any of the dilutions of GPL 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 [7] or that the GPL was so overloaded with Zn, Cr and Nickel that binding of these metals to the cellular material was not significant because the cells failed to grow well.

Garbage Pit Leachate (well aerated conditions): *Chlorella* grown in 20% GPL diluted with tap water 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% GPL removed nearly all the Zinc (99.4%) and most of the Nickel (85%).

The optimum biomass of *Chlorella* in terms Chl a to remove heavy metals

An experiment was set up based on the protocol used for Experiment 3.4.3 to determine the minimum inoculation of *Chlorella* for growth in GPL in terms of Chl 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 GPL diluted to 20% using 80% tap water diluent (Figure 5c). Various levels of inoculum were tried to optimize growth. Inoculation with *Chlorella vulgaris* was varied over a range of Chl a biomasses: 3.01 µg/mL (A_{750}nm= 0.35), 2.5µg/mL (A_{750}nm= 0.316), 1.6µg/mL (A_{750}nm= 0.246), 1.17µg/mL (A_{750}nm= 0.202) (Table 6). The percentage removal of heavy metals from 20% GPL incubated with different levels of initial inoculum of *Chlorella* is also shown in Table 6. 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. Zinc was very efficiently removed (90% or more) (Table 6). Nearly all the Chromium was also removed (>80%) but 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.

Heavy metals (µg/L)	Percent of Leachate in Incubation Medium									
	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

Table 3: Heavy metal removal from GPL by *Chlorella* incubated without shaking

Discussion

Physico-chemical of leachates

Chain and De Walle [8] have shown that the chemical composition of LF depends on factors such as the fill material (organic content, degradability, solubility), geological conditions and the age of the landfill. Bull et al., [1] 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 and UNEP [9] has published data on typical leachates found from municipal solid waste landfills in developing countries.

The physico-chemical composition of LF in Phuket was comparable to the range of values found in Effluent standards in Thailand and typical of values found in developing countries [9]. The physico-chemical properties of leachate from the LF and the GPL compared with Thai and International standards values are provided as a Supplementary table. The analyses for Phuket are analyses based on 6 separate collection trips spread over 6 months that included collections made in both the wet and dry season (Table 1 and Figures 2, 3 and 5). The BOD level of LF and GPL were both higher than the Thai standard, however BOD level of LF was in the range of international standards. The COD levels were on the lower side of values found internationally but the COD of Phuket GPL 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 GPL which sometimes exceeded both Thai and international standards. Phuket does not have a large amount of heavy industry. The source of so much Zn in GPL from a non-industrial city is not clear. Ammonia levels of the Phuket LF are high but the GPL has extremely high BOD (5 days) levels and very high COD. Nitrate levels in both the Phuket LF and the GPL were much higher than . The BOD of the GPL is exceptionally high. In combination with the very high ammonia content this results in BOD > COD. The GPL 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) [10]. This interpretation is supported by the high observed nitrate-N. Total phosphorus of the GPL was also exceptionally high. The levels of heavy metals in the Phuket LF were generally low by international standards but the levels of heavy metals in the Phuket GPL were high by international standards in particular Zinc.

Chlorella vulgaris growth in leachate

Landfill leachate (LF): *Chlorella* grew the best in LF 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. The leachate has high ammonia-N (Table 1) and this might account for the poor growth in 100% leachate. CheSa [11] used LF 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.

Garbage Pit Leachate (GPL): The GPL was much more toxic than the LF [3]. *Chlorella* would not grow in 100%, 50% or 30% leachate and slowly died off over the course of the experiment in light under non-shaking conditions (Figure 5b). *Chlorella* grew well in the undiluted tap water and grew marginally in GPL diluted to 20% and 10% in tap water (20% leachate grew +11% and 10% leachate grew +9%). Other growth experiments were set up in the light with shaking conditions to maintain aeration. *Chlorella* was grown over a 9 day cultivation period with shaking on an orbital shaker in a range of diluted GPL: 100%, 50%, 30%, 20%, 10% leachate diluted with tap water. Growth in 100% BG-11 medium was used as the blank control. Growth in undiluted GPL was very poor and the alga died off with time (Figure 5b). Positive growth occurred in 10 and 20% leachate (Analyzed with ANOVA two ways with 5-fold replication). In terms of Chl a, the alga was able to grow continuously over the course of the experiment in aerated 10 and 20% GPL but at all higher concentrations gradually died off with time. Growth of *Chlorella* in GPL was much better under well-aerated shaking conditions (Figure 5c) than cultures kept under non shaking conditions (Figure 5b), nevertheless GPL at any of the dilutions tested was severely inhibitory compared to the blank control grown in tap water. Comparison with the results for the LF experiments clearly shows that the GPL was much more highly toxic.

Heavy metals

Landfill leachate: Table 2 shows percent removed heavy metal after incubation *Chlorella* in 100% of LF 70% of the Chromium and 66% of the Nickel was removed. Copper, Lead and Zinc were not detectable in the LF before or after inoculation. From the results shown in Figures

5a and 6a and Table 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 LF was consistently low (Table 1) but nevertheless unless it was diluted with tap water to about 30% *Chlorella* would not grow very well. Previous studies have shown that much of the toxicity of LF is due to the very high ammonia content [1,2,12,13]. Ammonia induced toxicity is commonplace in sewage treatment plants [14]. 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) [13] or by biological removal [14].

Garbage Pit Leachate

• *Chlorella* **incubated without shaking:** The results of heavy metals analysis (Table 3) shows that *Chlorella* was not successful in removing heavy metals at any of the dilutions of GPL 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 [7]. This is consistent with the very high toxicity shown in Figure 5b and 5c and the poor removal of ammonia-N, nitrate-N, total-phosphorus and BOD and COD (Figure 6b and 6c). In the dilution treatments where there was some growth of *Chlorella* (10% and 20% GPL 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% GPL. The pH of GPL started at pH 5.05 and the end of incubation pH was 4.9. This pH change is small but Starodub et al. [15] and Rai et al. [16] found increasing metal toxicity with decreasing pH to be due to the predominance of the free metal ion at low pH [16].

The *Chlorella vulgaris* growth in GPL (non-shaking conditions): *Chlorella* in light under non-shaking conditions grew the best in 20% of GPL. 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-phosphorus 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 GPL tested (Table 3).

• *Chlorella* **incubated under aerated conditions:** *Chlorella* grown in 20% GPL diluted with tap water removed 89.7% of the Zn and 33.3% of the Chromium (Table 4 and 5). 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% GPL removed nearly all the Zinc (99.4%) and most of the Nickel (85%). During the course of the 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 as the pH increased and soluble heavy metal would decrease. It was also observed that surface-bound metal concentrations increased when pH was varied over the range pH 6.0 – 8.0 and growth of *Chlorella* sp. improved as the pH increased. This can be explained in terms of cation binding to fixed negative changes in the cell walls of the alga increasing as the pH increased. Parent and Campbell [17] and Franklin et al. [18] 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. [19] and Macfie et al. [20] 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 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 (pHi) 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 [21]. The major effect though of external pH is to decrease the solubility of metals because most heavy metal hydroxides are extremely insoluble [22].

Heavy metals (µg/L)	Percent of Leachate in Incubation Medium									
	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: Heavy metal removal from aerated GPL by *Chlorella* incubated in the light and under shaking conditions

Feasibility of bioremediation

Bioremediation of LF using an algal pond to receive LF diluted with treated sewage plant effluent or river water appears feasible as a means of locking up toxins in the sediment. The GPL appears to be too toxic for bioremediation using simple algal pondages. Current practice of feeding it into the sewage steam appears to be the most practical disposal method. Monitoring of GPL, and dilution with sewage plant effluent before passing into the sewage treatment stream when necessary, is needed to protect the microbial flora of the floc tanks from the toxicity of GPL.

Conclusion

The GPL was very toxic *Chlorella* grew in 20% and required constant aeration in order to grow.

The LP was not toxic and *Chlorella* grew in diluted LF. The BOD and ammonia-N were very high. The heavy metal in GPL exceeded the legal limit. *Chlorella* grown in 20% GPL diluted with tap water removed 41.5 of the ammonia-N, 49.2% of the BOD, 90% of the Zinc (90%) and 33% of the Chromium.

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	89.7	99.4

Table 5: Percent removal heavy metals from GPL compared to the controls (in the light and under shaking conditions)

Heavy metals (µg/L)	Concentration of Chlorophyll a (µg/mL)				
	Before treatment	1.1	1.6	2.5	3
Copper (Cu)	1	<1	<1	<1	<1
Chromium (Cr)	40	9	8	8	10
Lead (Pb)	<1	<1	<1	<1	<1
Nickel (Ni)	60	40	40	50	50
Zinc (Zn)	1850	80	10	130	90
Percentage removal of heavy metals					
Copper (Cu)		97.75	80	80	75
Chromium (Cr)		Not detected	Not detected	Not detected	Not detected
Lead (Pb)		Not detected	Not detected	Not detected	Not detected
Nickel (Ni)		33.33	33.33	16.66	16.66
Zinc (Zn)		95.67	94.59	92.97	95.13

Table 6: Removal of heavy metals from 20% GPL with varying levels of *Chlorella* inoculation

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