Nitrogen mass balance and microbial analysis of constructed wetlands treating municipal landfill leachate

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Abstract Experiments were conducted to investigate the feasibility of applying constructed wetlands (CW) to treat a sanitary landfill leachate containing high nitrogen (TN) and bacterial contents. Under the tropical conditions (temperature of about 30 °C), the CW units operating at a hydraulic retention time (HRT) of 8 days yielded the best treatment efficiencies with BOD_5 removal of 91%, TN removal of 96%, total and fecal coliforms (TC and FC) removal of more than 99%. Cadmium removal in the in the SFCW bed was found to be 99.7%. Mass balance analysis, based on TN contents of the plant biomass and dissolved oxygen (DO) and oxidation - reduction potential (ORP) values, suggested that 88% of the input TN were uptaken by the plant biomass. Fluorescence *in situ* hybridization (FISH) results revealed the predominance of bacteria including the heterotrophic and autotrophic bacteria responsible for BOD_5 removal. Nitrifying bacteria was not found to be present in the SSFCW beds.

Keywords Bacterial species and fluorescence *in situ* hybridization (FISH); landfill leachate; nitrogen mass balance; subsurface-flow constructed wetland (SSFCW)

Water Science & Technology **Vol 54 No 11–12 pp 147–154** © IWA Publishing 2006

Abbreviations

SSFCW	Subsurface-flow constructed wetland
FISH	Fluorescence in situ hybridization
TC	Total coliform bacteria
FC	Fecal coliform bacteria
TN	Total nitrogen
D	day

Introduction

Landfill leachate is wastewater emanating from sanitary landfills treating a variety of municipal and industrial solid wastes. Due to the anaerobic conditions and long retention time prevailing in sanitary landfills, landfill leachate normally contains high concentrations of organic matter, nutrients, pathogens and heavy metals which, if not properly collected and treated, can cause serious pollution to nearby surface and groundwater sources. The presence of heavy metals at high concentrations in landfill leachate usually causes toxic effects to microbes, making it difficult for them to be treated biologically. Although several physical, chemical and biological processes can be employed to treat landfill leachate, for tropical developing countries they can be expensive in construction and operation and require high-skilled labor in operation. On the other hand, where land is available at low-cost, natural systems, such as waste stabilization ponds and constructed wetlands are attractive alternatives for landfill leachate treatment. Because constructed wetland (CW) are known to be effective in removing nitrogen from wastewaters through nitrification/denitrification, plant uptake and volatilization

doi: 10.2166/wst.2006.847

(Kootatep and Polprasert, 1997), it would be of interest to employ FISH technology to determine the presence of nitrifying and denitrifying bacteria present in the CW beds and identify the predominant species of bacteria growing in the CW beds.

The objectives of this research were to: (1) investigate the feasibility of applying a CW system to treat landfill leachate containing high TN and bacterial contents; (2) analyze mass balance of TN removal in CW beds; and (3) identify the presence and major bacterial species including the nitrifying and denitrifying bacteria in the CW beds.

Materials and methods

The pilot-scale experiments on SSFCW treatment of landfill leachate were conducted under ambient conditions with an average temperature of about 30 °C. Sanitary landfill leachate samples, collected from the Pathumthani municipal landfill site, located about 30 kilometres north-east of Bangkok city, Thailand, had characteristics as shown in Table 1.

Experimental set up and operating conditions of CW

To avoid odors problems, CW units were operated in subsurface flow mode in which all the influent wastewater was made to flow through the CW beds with no wastewater flowing above the CW beds. This type of CW is called subsurface-flow CW or SSFCW.

Two pilot-scale SSFCW units, made of reinforced concrete, have been built at the Asian Institute of Technology, Thailand, each with dimensions of $0.5 \times 4.0 \times 0.5$ m (width × length × depth), and a bed slope of 1%. The support media of these units consisted of large gravel (2–3 cm), medium gravel (1–2 cm) and sand (0.1 cm) at depths 15, 20, and 15 cm, respectively. The cattails (*Typha angustifolia* L.) were planted at a density of 40 no./m^2 (Figure 1A). The campus domestic wastewater was fed continuously to the SSFCW units to acclimatize the soil microbes and to support growth of the cattail plants.

After the cattails were fully grown to an average height 3 m, the SSFCW units were continuously fed with the campus wastewater at an initial organic loading rate (OLR) of 100 kg BOD_5/ha/d until a steady state condition, based on relatively constant BOD_5 concentrations in the effluent of at least 3 times the hydraulic retention time (HRT), was reached. After that, the landfill leachate diluted with tap water but spiked with Cd to a concentration of 1000 μg/l (to control SSFCW influent characteristics) was fed to the experimental SSFCW unit, while the campus wastewater was still fed to the control SSFCW unit. The effects of HRT on the treatment performance of SSFCW were studied by varying the HRT at 1, 3, 5 and 8 d. Harvesting of the cattail plants was done once in

Table 1 Characteristics of landfill leachate used in the SSFCW experiments*

Parameters	Unit	Range	Mean value
pH	–	7.7–8.1	7.9
Conductivity	mS	8.1–8.5	8.3
Salinity	ppt	4.7–4.9	4.8
Total solids	mg /l	550–9800	5175
SS	mg /l	300–7100	3700
COD	mg /l	400–3330	1865
BOD	mg /l	130–730	430
TKN	mg /l	140–312	226
Total phosphorus	mg /l	15.0–19.4	17.7
Fecal coliforms	no./100 ml	200–1600	985
Apparent color	ADMI unit	300–600	450
Cd	μg /l	0.13–2.33	2.46
Mn	μg/l	2500–5370	3935

*Data taken from Nov 11 2003 to Dec 7, 2004

Figure 1 A: Subsurface-flow constructed wetland (SSFCW), B: Harvesting of *Typha angustifolia l.* plants grown in SSFCW units

every 3 months by cutting the plant stems over about 50% of the bed areas (Figure 1B). The harvested plants were then analyzed for biomass production, TN and Cd contents. All the physical, chemical and biological parameters of the wastewater and plant biomass samples were analyzed according to the methods described in the *Standard Methods* (*Standard Methods for the Examination of Water and Wastewater*, 20th ed., 1998).

Fluorescence *in situ* hybridization (FISH) analysis

The FISH method involves application of oligonucleotide probes to permeablized whole microbial cells and, specifically, hybridized the cells to their complementary target sequence in the ribosomes. In this study, only samples of the cattail roots and soil of the SSFCW units operating at HRT of 8 days were collected to determine the presence of nitrifying bacteria. The samplings, done randomly at 3 locations in each of the SSFCW units, were conducted 3 times in steady state conditions. The collected samples were put in sterile plastic plastic bags and transported to the laboratory for FISH analysis.

Sample preparation. About 20 g of the collected samples was put in 20 ml of 0.85% NaCl solution, centrifuged at 150 rpm for 60 min to extract the bacterial cells from the cattail roots and soil, and again centrifuged at 3000 rpm for 10 min to separate the bacterial cells from other contaminants.

Cell fixation. The supernatant from the above was again centrifuged at 700 rpm for 3 min to homogenize the bacterial cells and make them settle. To fix the bacteria cells, the settled cells had 1 ml of paraformaldehyde added, followed by washe with 1 ml phosphate-buffered saline (PBS), the fixed bacteria cells were then stored in a freezer ($-20\,^{\circ}C$) for the hybridization step.

Hybridization. The fixed cells were spotted on a glass slide, dried, dehydrated with ethanol and dried again. After fixation, a hybridization buffer solution containing the specific probe was added to the fixed cell slide and the slide was put in the hybridization chamber and incubated for 1 hour at $46\,^{\circ}C$. As the possible biochemical reactions occurring in the SSFCW beds include nitrification, denitrification and anoxic-ammonia oxidation (Anammox), probes corresponding to these bacterial groups were employed, as shown in Table 6. In addition, EUB 338 I, II and III probes were also employed to identify the general bacterial groups present in the SSFCW beds.

Sample washing and staining. After hybridization, the fixed cell slide was washed with a buffer solution to remove the unbound probe, then stained with 4,6-diamido-2-phenylindol (DAPI). An anti-fade reagent was added to the fixed cell slide prior to viewing with a fluorescent microscope (Axioskop 40 Fl, Zeiss, Germany).

Results and discussion

Both the experimental and control SSFCW units were observed to function well with healthy growth of cattail plants in the SSFCW beds. The treatment performance of the 2 SSFCW units operating at the various HRT is shown in Table 2. At steady-state conditions, the SSFCW units operating at HRTs of 5 and 8 d had about 90% of organic (BOD_5) removal, with the effluent BOD_5 concentrations being less than 20 mg/l, suitable for reuse in agriculture or discharge to nearby water sources (Thailand Water Quality Standard, 1996). The % COD removal in the experimental SFCW unit was slightly lower than those in the control unit which was probably due the presence of non-biodegradable organic compounds in the landfill leachate. The total phosphorus (TP) and Cd removal in the SSFCW was rather effective, probably through precipitation and adsorption, resulting in effluent TP and Cd concentrations of less than 1 mg/l and 1 μg/l, respectively, when operating at HRTs 5 and 8 d. However, the experimental SSFCW unit was not effective in removing color, resulting in an effluent color of 50-80 ADMI – an additional polishing unit would be required to further remove the color. Total N (TN) removal in the SSFCW units ranged from 78 to 96%. The bacteria indicators were reduced about 98–99%. The FC concentrations of 1000/100 ml or less are recommended as a guideline for reuse in unrestricted irrigation or aquaculture (Polprasert, 1996). The experimental data shown in Table 2 indicated the significance of HRT on treatment performance of the SSFCW. The HRTs of 1 and 3 d were found to be too short for the SSFCW units to effectively treat the landfill leachate.

The mechanisms responsible for BOD_5 and COD reduction were probably the bacterial degradation in which oxygen photosynthetically produced by the cattail leaves was transferred to the root zones for the bacteria growing in the SSFCW beds to biodegrade the organic compounds. The DO and oxidation–reduction potential (ORP) shown in Table 3 indicate the prevalence of anoxic conditions in the SSFCW beds which should enhance DO transportation from the cattail leaves and atmosphere to the root zones. TN removal of more than 90% in the SSFCW beds could be due to plant uptake, nitrification–denitrification activities and volatilization Jing *et al.* (2001) and Lin *et al.* (2001) also found efficient N removal of CW systems treating a polluted river water and aquaculture wastewater, respectively. Details of TN mass balance are presented in the following section. The removal of TC and FC was due primarily to natural die-off because of fewer nutrients and DO and other unfavorable conditions prevailed in the SSFCW beds. Because of relatively long HRT, Cd removal could be due to precipitation, absorption and plant uptake, resulting in rather low Cd concentrations in the effluent samples, especially at the HRTs of 5 and 8 d. The SSFCW effluent characteristics of the units operating at HRT of 5 and 8 days met the Thailand Water Quality Standard (1996) for discharged and agricultural reuse (Polprasert, 1996).

Biomass production and mass balance in SSFCW units

Harvesting of the cattail plants was conducted once every 3 months by cutting the plant stems at about 50 cm above the SSFCW beds. About 50% of the cattails plants were harvested each time to allow for the SSFCW beds to maintain the treatment efficiencies. As shown in Table 4, because the influent of the experimental unit had higher N contents than the control SSFCW unit (Table 2), the cattail (or biomass) growth in the experimental

Table 2 Treatment performance of SSFCW units at various HRTs

Description	Experimental unit				Control unit			
	HRT(d)				HRT(d)			
	1	3	5	8	1	3	5	8
BOD (inf), mg/l	110	110	130	130	60	25	20	45
BOD (eff), mg/l	45	40	20	10	15	6	2	2
%BOD removal	59.4	61.2	84.9	91.0	75.4	77.0	86.7	89.1
COD (inf), mg/l	460	315	250	385	100	50	20	60
COD (eff), mg/l	376	160	80	70	44	19	2.3	4.1
%COD removal	18.6	49.0	69.2	81.6	56.0	62.7	86.7	93.0
TP (inf), mg/l	3.61	3.67	3.48	3.46	2.04	1.93	2.00	1.96
TP (eff), mg/l	0.78	0.62	0.34	0.22	1.33	0.54	0.22	0.05
%TP removal	78.5	83.0	90.2	93.6	35.0	71.5	88.6	97.5
TN (inf), mg/l	13.33	13.74	12.03	25.70	0.74	0.64	0.80	1.07
TN (eff), mg/l	2.96	2.31	1.15	1.11	0.43	0.15	0.10	1.11
%TN removal	77.79	83.17	90.45	95.69	42.45	76.83	87.06	89.81
NO_3^- (inf), mg/l	0.22	0.25	0.23	0.21	ND	ND	ND	ND
NO_3^- (eff), mg/l	ND	ND	ND	ND	ND	ND	ND	ND
% NO_3^- removal	–	–	–	–	–	–	–	–
NO_2^- (inf), mg/l	0.04	0.51	0.51	0.49	ND	ND	ND	ND
NO_2^- (eff), mg/l	ND	ND	ND	ND	ND	ND	ND	ND
% NO_2^- removal	–	–	–	–	–	–	–	–
TC (inf)	1674	1233	1330	19763	465,057	4884	65,703	75,727
TC (eff)	461	123	75	68	56,086	287	410	5552
%TC removal	72.5	90.1	94.3	99.7	87.9	94.1	99.4	92.7
FC (inf)	677	275	208	241	442,200	2364	46,206	63,490
FC (eff)	247	32	11	<2	55,086	287	313	1807
%FC removal	63.5	88.5	94.5	99.2	87.5	87.9	99.3	97.2
Cd (inf), μg/l	1003	1003	1003	1003	0.1	0.1	0.1	0.1
Cd (eff), μg/l	18.16	4.87	0.68	0.27	ND	ND	ND	ND
%Cd removal	81.8	95.1	99.3	99.7	~100	~100	~100	~100

Remark: TC and FC unit in no./100 ml; Inf - influent; eff - effluent; ND-non-detectable

Table 3 Dissolved oxygen (DO) and oxidation–reduction potential (ORP)

Description	Experimental unit				Control unit			
	HRT(d)				HRT(d)			
	1	3	5	8	1	3	5	8
DO (inf), mg/l	1	1	1	2	0	2	1	2
DO (eff), mg/l	1	2	1	1	1	2	2	2
DO (SFCW), mg/l	0	0	0	0	0	0	0	0
ORP (inf), mV	−140	−180	−142	−164	−77	−147	−132	−125
ORP (eff), mV	−122	−113	−113	−67	−77	−102	−122	−86

Table 4 Biomass production in the SSFCW units

	Experimental unit	Control unit
Biomass growth g/m^2/d	0.13	0.07
N, % content	4.25	3.94
P, % content	0.95	0.99
Cd, mg/kg (dry weight)	4.52	0.32

unit was found to be 0.13 g/m^2/d as compared to 0.07 g/m^2/d for the control unit. However, the % N and P contents of the cattail plants in both units were similar. Because of the higher Cd content in the experimental SSFCW influent (Table 2), due to plant uptake, the Cd content in the cattail plants of the experimental unit was accordingly higher than that of the control unit.

Analysis of TN mass balance was done for the experimental unit operating at an HRT of 8 d. As shown in Table 5, the TN input, output and the uptake by the cattail biomass were found to be 0.89, 0.03 and 0.79 g/d, respectively, indicating that about 88% of the TN input was uptaken by plant biomass. The wet weights of the harvested cattails from each harvest of the experimental and control SSFCW units were 10.5 and 6.5 kg, respectively. The unaccounted TN was about 8% which could be due to other microbial reactions, adsorption and volatilization occurring in the SSFCW beds.

The 88% of TN plant uptake was higher than those previously reported by Kootatep and Polprasert (1997) of 43% for free-water surface CW and by Breen (1990) of 55% for vertical flow CW units. However, Rogers *et al.* (1991) reported a high N uptake rate of 85% by *Schoenoplectus validus* plants growing in vertical flow CW. Reddy and Debusk (1987) reported a range of plant uptake rates of 16–75% for various types of wetlands. The high TN plant uptake rate as found in this study was probably due to the high biomass growth (Table 4) and subsurface-flow characteristics of the influent wastewater which enhanced effective TN uptake by the cattail plants in the experimental SSFCW unit. Frequent harvesting of the cattail plants, once in 3 months, could contribute to the high plant biomass productivity.

FISH results

The FISH results shown in Table 6 indicated the absence of the nitrifying bacteria: *Nitrosomonas*, *Nitrobacter* and *Beta-proteobacterial* probably because the DO concentration was 0 mg/l in the SSFCW beds (Table 3), hence the nitrifying bacteria could not grow. The negative ranges of ORP values found in the SSFCW beds would not be favorable for the nitrification process. The non-detectable NO_3^--N concentrations in the SSFCW effluents confirmed the absence of nitrifying bacteria in the SSFCW beds.

Table 5 TN mass balance in SSFCW unit

HRT d	Influent			Effluent		
	Conc. mg/l	Flow l/d	Input g/d	Conc. mg/l	Flow l/d	Out put g/d
8	25.70	34.70	0.89	1.11	26.51	0.03

	Cattail biomass					Unaccounted TN
	H_2O %	Wet wt kg per harvest	Dry wt kg per harvest	TN content %	TN uptake g/d	g/d
	84	10.50	1.68	4.25	0.79	0.07

Table 6 FISH analysis for bacteria identification in SFCW beds

Probe name	Target bacteria	Sequence	Results %
EUB338 mix (I,II and III)	Most bacteria, *Plantomycetales, Verrucomicrobiales*	GCT GCC TCC CGT AGG AGT	49
Amx820	Anammox	AAA ACC CCT CTA CTT AGT GCCC	NF
NEU	*Nitrosomanas*	CCC CTC TGC TGC ACT CTA	NF
Nsm156	*Nitrosomonas*	TAT TAG CAC ATC TTT CGAT	NF
NIT3	*Nitrobacter*	CCT GTG CTC CAT GCT CCG	NF
Ntspa662	genus *Nitrospira*	GGA ATT CCG CGC TCC TCT	NF
Ntspa1026	*Nitrospira moscoviensis*	AGC ACG CTG GTA TTG CTA	NF
NSO1225	*Betaproteobacterial* Ammonia-oxidizing bacteria	CGC CAT TGT ATT ACG TGT GA	NF
DSV407	Some sulfate-reducing bacteria of the *Desuifovibionaceae*	CCG AAG GCC TTC TTC CCT	NF
EURY499	*Methanosarina, Methanosaeta, Methanomicrobiales* groups	CGG TCT TGC CCG GCC CT	NF

Remark: NF- not found

The anammox bacteria require NO_2^- as electron accepter to oxidize NH_4-N to become gaseous N_2. Because of the absence of nitrifying bacteria (as reported above) and the low NO_3^- and NO_2^- concentrations (Table 2), the FISH results did not find the presence of anammox bacteria and nitrifying bacteria (genus *Nitrospira, Nitrospira moscoviensis*) in the SSFCW beds. The FISH results suggested that denitrification and anammox were not the major reactions responsible for TN removal in the SSFCW units, which corresponded well to the TN mass balance analysis (Table 5), which showed cattail plant uptake to the major mechanisms for TN removal.

The EUB 338 mix probes revealed the presence of about 49% of most bacteria, *Plantomycetales* and *Verrucomicrobiales* which include the heterotrophic and autotrophic bacteria. The remaining 51% could be those of inactivated bacterial cells and *Archaea*. Because there were 85–91% BOD_5 removal in the SSFCW units operating at an HRT of 5–8 days, the bacteria responsible for these BOD_5 removal are hypothesized to be in the group identified by the EUB 338 mix probes.

Conclusions

Based on the experimental results obtained, the following conclusions are made.

1. The SSFCWs were found to be effective in treating the leachate wastewater, resulting in a treated effluent suitable for reuse in agriculture or discharge to the nearby environment.
2. Due to the high TN contents in the leachate, the cattail biomass production was found to be 0.13 $g/m^2/d$ in the experimental unit as compared to 0.07 $g/m^2/d$ in the control

unit. Similarly, due to the high Cd content in the leachate, the Cd content of the cattail biomass in the experimental unit was found to be 4.52 mg/kg (dry wt), which was about 10 times higher than that of the control unit.

3. Based on mass balance analysis at HRT of 8 d, the cattail plants were found to uptake about 88% of the TN input, while the 8% unaccounted values were postulated to be due to some microbial reactions, adsorption and volatilization.
4. The FISH analysis did not reveal the presence of nitrifying and anammox bacteria. The presences of 49% of most bacteria are hypothesized to include heterotrophic and autotrophic bacteria responsible for BOD_5 removal.
5. The overall experimental results demonstrated the feasibility of applying SSFCW units to treat landfill leachate wastewaters.

Acknowledgements

Grateful acknowledgement is expressed to the Royal Thai Government for providing scholarships for the first author to study at AIT. The authors thank Prof. Ajit P. Annachhatre for his support of FISH probes and their chemicals.

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