Microbial adaptation to biodegrade toxic organic micro-pollutants in membrane bioreactor using different sludge sources

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Abstract

Biodegradation of toxic organic micro-pollutants in municipal solid waste (MSW) leachate by membrane bioreactor (MBR) was investigated. The MBR systems were seeded with different sludge sources, one was from a pilot-scale MBR system treating MSW leachate and the other was from an activated sludge sewage treatment plant. The biodegradation of BPA, 2,6-DTBP, BHT, DEP, DBP and DEHP, DCP and BBzP, by sludge from both reactors were found improved with time. However, enhanced biodegradation of micro-pollutants was observed in MBR operated under long sludge age condition. Bacterial population analyses determined by PCR-DGGE revealed the development of phenol and phthalate degrading bacteria consortium in MBR sludge during its operation.

Keywords

Bacterial community, membrane bioreactor, microbial adaptation, micro-pollutants, PCR-DGGE

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1. Introduction

Phenols and phthalates are the micro-pollutants commonly found in municipal solid waste leachate. In landfill site, these chemical compounds are derived from wasted materials such as plastic, coating materials and cosmetics industries (Slack *et al.*, 2005). Recently, several national and international organizations such as World Health Organization, United States Environmental Protection Agency, European Union International Programme on Chemical Safety (IPCS) and China National Environmental Monitoring Center classified these compounds as a priority environmental pollutants and as endocrine disrupting compounds (Huang *et al.*, 2010). In addition, phthalates have been reported to affect biochemical process in humans including effect on reproduction, sperm damage, early onset of puberty in female, anomalies of reproductive tract, infertility, adverse outcomes of pregnancy, to neurodevelopment and allergies (Mankidy *et al.*, 2013).

Membrane bioreactor (MBR) is reported as an effective wastewater treatment system and it is a good option for the treatment of municipal solid waste leachate that contains micro-pollutants or toxic organic compounds. In MBR, biological treatment takes place under unique environmental condition in which microorganisms are maintained at much higher mixed liquor suspended solids (MLSS) concentration when compared to other conventional biological treatment processes and the MBR can also improve the removal of micro-pollutants or toxic organic compounds via adsorption onto sludge, biodegradation and membrane filtration (Boonyaroj *et al.*, 2012, Wijekoon *et al.*, 2013). Nevertheless, the removals of micro-pollutants depends on their molecular properties (Tadkaew *et al.*, 2011) and environmental conditions such as pH and temperature (Tadkaew *et al.*, 2010, Hai *et al.*, 2011). To improve the biodegradation of some recalcitrant organic compounds, MBR system provides an opportunity to operate under long sludge age condition through an operation without sludge wastage. This environmental condition makes the system capable of cultivating slow growing microorganisms, resulting in highly diverse bacterial consortium in the system and thus enhancing the biodegradation of those compounds. Even though, solid retention time (SRT) has been considered as an important operating criteria for the removal of micro-pollutants, its effect on the microbial adaptation and population change during the treatment of wastewater containing toxic organic micro-pollutants in long term is not well known. Therefore, this research was carried out to study effect of sludge source to

biodegrade those compounds in the MBR systems. Batch experiments were also carried out using microbial sludge obtained from laboratory scale MBR of this study to determine the maximum micro-pollutants biodegradation capacities. Microbial population shift in response to the performance of MBR systems was studied using polymerase chain reaction - denaturing gradient gel electrophoresis (PCR-DGGE) technique during long term operation of the MBR systems.

2. Methods

2.1. Membrane bioreactor set-up and operation

The experiment was conducted with two laboratory scale MBR units made of acrylic plastic with 0.30 m in width, 0.10 m in length x 0.50 m height (0.30 m water depth) dimension and working volume of 9 L. A polyvinylidene difluoride (PVDF) hollow-fiber microfiltration (MF) membrane module (Sterapore SADF0790M, Mitsubishi Rayon, Japan) with nominal pore size of 0.4 μm and effective membrane surface area of 0.07 m^2 was used for solid-liquid separation in the reactor. Membrane permeate was intermittently withdrawn by suction pumps in a 5-minute on/1-minute off cycle to maintain constant hydraulic retention time (HRT) in the MBR at 24 hours. The reactor was operated under room temperature condition (25-30°C) where pH of mixed liquor was 7.0-8.0 and dissolved oxygen (DO) in the reactor was maintained at 5-6 mg/l.

Leachate used in the experiment was prepared by mixing fresh and stabilized leachate obtained from a municipal solid waste landfill site in Thailand and that biochemical oxygen demand (BOD) and chemical oxygen demand (COD) were maintained constant at about 3,000 and 6,500 mg/l. The concentrations of toxic organic micro-pollutants including bisphenol A (BPA), 2,6-di-tert-butylphenol (2,6-DTBP), 2,6-di-tert-4-methyl-butylphenol (BHT), ethyl phthalate (DEP), butyl phthalate (DBP), bis (2-ethylhexyl) phthalate (DEHP), 2-chlorophenol (DCP) and benzyl butyl phthalate (BBzP) were adjusted equally at 1,000 $\mu\text{g/l}$ and supplied into the MBR systems. The characteristics of leachate used in this study are shown in Table 1.

Insert Table 1

In order to investigate the removals of toxic organic micro-pollutants in different sludge sources of MBR systems, two sets of MBR system were operated in parallel. The first reactor (MBR 1) was initially seeded with microbial sludge obtained from a pilot-scale MBR installed at a solid waste disposal site where leachate was obtained. The pilot-scale MBR had been operated continuously for about 2 years without sludge wastage condition and this sludge is named 'acclimatized sludge'. After initial seeding of sludge, the operation of laboratory scale MBR 1 was also performed under no sludge wastage condition (for maintain its source condition) except for sampling purpose (approx. 100 ml every 7 days) so actual sludge age in this reactor was increasing with the operation days until the end of experiment (day 550). The other reactor (MBR 2) utilized seed sludge from an activated sludge sewage treatment plant which is operating at a SRT of 15 days (non-acclimatized sludge). After the start-up, this reactor (MBR 2) was operated at a constant SRT of 15 days which for maintain its source condition by wasting excess sludge daily from the reactor. These experimental set-up was employed to ensure that microorganisms presented in the MBR systems had been originally cultivated at the same HRT (24 h) but different SRT (infinite and 15 days respectively) as they are operated in this study.

All water quality parameters in the influent (leachate) and treated water (membrane permeate) were analyzed every 7 days. The analyzed parameters included pH, BOD, COD, ammonia nitrogen, total kjeldahl nitrogen (TKN), total nitrogen (TN) and total phosphorus (TP). The biomass concentrations in the reactor were determined in term of MLSS and mixed liquor volatile suspended solids (MLVSS). The removal of toxic organic micro-pollutants during the reactor operation was studied by analyzing their concentrations in influent and effluent and adsorbed amount on biomass (sludge) samples every 7 days over the entire experimental period (550 days).

2.2 Determination of micro-pollutant biodegradation in batch experiments

Batch experiments were performed to investigate the maximum removal capacities of toxic organic micro-pollutants by MBR sludge in each reactor at the beginning (day 0) and towards the end of the experiment (day 519). In order to differentiate the removals of studied compounds via adsorption and biodegradation mechanisms, batch experiments were

performed using active (untreated) and inactivated MBR sludge following the procedures described in our previous study (Boonyaroj *et al.*, 2012). Sludge was tested for their capacity in removing eight compounds presented in municipal solid waste leachate. Batch experiments were performed under aeration at DO level of 4-5 mg/l. In the batch experiment, sludge samples having MLSS of 3.0 g/l were spiked with BPA, 2,6-DTBP, BHT, DEP, DBP, DEHP, DCP and BBzP at an initial concentration of 3,000 µg/l and their concentrations were determined every 2 hours during a 24 h experiment which was equal to the HRT of the MBR (reactor experiment). The initial concentration of those compounds was selected based on our preliminary experiments to ensure that the tested compounds remained after 24 hours during batch experiment so that maximum removal capacities by sludge in batch experiment could be obtained. These batch experiments were all conducted in triplicate.

For sludge inactivation, the MBR sludge was repeatedly autoclaved at 121°C for 15 minutes three times in order to completely terminate microbial activities. The removals of toxic organic micro-pollutants by active sludge were due to both adsorption and biodegradation mechanisms whereas the removal by inactivated sludge was only due to adsorption of compounds onto sludge particles. Maximum biodegradation capacities of micro-pollutants by sludge were expressed in terms of the percentages of micro-pollutants being removed via biodegradation during 24 hours of batch experiment. The removals of micro-pollutants via biodegradation was derived from the amount of micro-pollutants removed by active sludge was subtracted by the amount of micro-pollutants removed by inactivated sludge.

The percentage of micro-pollutants biodegraded during batch experiment and first order rate constants for the removals of each micro-pollutant in batch experiment by MBR 1 and MBR 2 sludge were compared at day 0 and day 519 to investigate the effect of sludge source on the micro-pollutant biodegradation capacities of MBR sludge.

2.3. Water qualities and toxic organic micro-pollutants analyses

Chemical characteristics of leachate and treated water were regularly monitored using the analytical procedures described in Standard Methods for the Examination of Water and Wastewater (APHA, 2005).

The concentrations of toxic organic micro-pollutants in water sample were determined by solid phase extractions (SPE). To determine their concentrations in soluble form, the water samples obtained after separation of suspended solids by GF/C filtration were used. The VertipakTM-C18 tubes or SPE tubes were used for extraction of toxic organic compounds. Firstly, SPE tubes were clean with 10 ml dichloromethane: methanol (1:9 v/v), followed by 10 ml pure water and 50 ml filtrated water sample. The SPE tubes were left for drying with clean air and eluted with 5 ml of dichloromethane: methanol (1:9 v/v). The solvent volume of soluble extracted sample was reduced to 2 ml by nitrogen evaporator and concentrator (ChanoVap, Amani, Thailand). To determine the micro-pollutants in solid bounded form, sludge samples obtained on GF/C filter were added to 20 ml dichloromethane: methanol (1:9 v/v) and mixed for 1 hour followed by sonication for 15 min. The solvent samples (5 ml) were then pumped into C18-SPE column and the collected samples were concentrated to 2 ml by evaporator and concentrator.

Gas chromatography with mass spectrometry (Shimadzu, GCMS-QP2010 plus) was used for determination of the concentrations of toxic organic micro-pollutants. The device was equipped with a 30 m RTX-35MS capillary, 0.25 mm I.D. with thickness of 0.5 μ m. Helium was used as carrier gas. Sample 1 μ l was injected automatically. Three different GC-MS programs were set for the analyses of eight toxic organic micro-pollutants targeted in this study. The first program for BPA analysis having the operating condition as follows oven temperature was set at 60°C and then increased to 150°C at a rate of 7°C/min with holding time of 5 minutes. The instrument condition was set at electron impact of 70 eV, ion source temperature of 200°C and interface temperature of 250°C. The second program for BHT, 2,6-DTBP and DCP analyses was set at oven temperature of 60°C, increased to 100°C with holding time of 2 minutes and then further increased to 150°C at a rate of 3.5°C/minute with holding time of 5 minutes. The instrument condition was set at electron impact at 70 eV, ion source temperature at 150°C and interface temperature at 250°C. The third program for DEP, DBP, DEHP and BBzP analyses was set at oven temperature of 60°C, increased to 100°C with holding time of 5 minutes, then increased from 100°C to 150°C at a rate of 10°C/minute and holding time of 5 minutes, followed by an increase from 150°C to 195°C at a rate of 3°C/minute with holding time of 5 minutes, increased from 195°C to 205°C at a rate of 1°C/minute with holding time of 10 minutes and finally increased from 205°C to 250°C at a rate of 10°C/minute with holding time of 15 minutes. The instrument condition was set at

electron impact of 70 eV, ion source temperature of 200°C and interface temperature of 270°C. The peak chromatogram were referenced with Wiley library MS-spectra. The lower detection limit for the analyses was 1.0 µg/l and the analyzed results were compared with blank samples using MilliQ water (molecular grade pure water). The same analytical procedures were applied to internal standards of known concentrations (3,000 µg/l) of all eight toxic organic micro-pollutants. The recovery percentages obtained from using internal standards were varied between 86.5% and 97.2%.

2.4. Bacteria community analyses

PCR-DGGE technique was used for the identification of major bacteria species and their population changes during the operation of MBR. Mixed liquor sludge samples are collected from both reactors at day 0 (start-up), day 104, day 207, day 310, day 412 and day 519 respectively. Collected samples were refrigerated at -80°C until analysis. Genomic DNAs from bacteria are extracted by the PrepEase[®] Tissue and Cell DNA Spinkit (USB Corporation, USA) in accordance with the manufacturer's specifications. This PCR was performed using 338GC-F (5'-ACTCCTACGGGAGGCAGC-3') and 518R (5'-ATTACCGCGGCTGCTGG-3') as universal bacteria primer for bacteria community screening in samples. The combination of these primer had generated a PCR fragment about 180-190 bp which was suitable for subsequent DGGE analysis.

The PCR amplification of genes in this study was performed according to the following condition; 50 µl of PCR reaction contained 1 µl of genomic DNA template, 1x PCR buffer, 3 mM MgCl₂, 200 µM deoxynucleoside triphosphates (dNTPs), 10 pmol of each primer, and 1 U of *Taq* DNA polymerase (Qiagen, Germany). The PCR was conducted in a Perkin-Elmer GeneAmp PCR System 9700 (Applied Biosystem, USA). The thermal program was set as followed; initial denaturation at 95°C for 10 minutes, followed by 30 cycles of denaturation at 95°C for 50 seconds, annealing at 60°C for 30 seconds and extension at 72°C for 50 seconds. Finally, the extension step was performed at 72°C for 7 minutes. The size and amount of PCR products were visualized on a 1.2% (w/v) agarose gel electrophoresis staining with ethidium bromide and estimated for DNA quantity using a NanoDrop 1000 spectrophotometer (thermo scientific, USA).

DGGE analyses of 16s DNA amplified PCR products were using the DGGE-2000 system apparatus (CBS Scientific Company, Del Mar, CA). They were performed with different ranges of denaturants for PCR products amplification. The polyacrylamide gel concentration of MBR1 samples were applied on 9% (w/v) polyacrylamide gel in range of 30-70% denaturing gel gradient. The MBR 2 samples were applied on 8% (w/v) polyacrylamide gels with a ranged of 40-60% denaturing gel gradient. The eighty percent of denaturant is corresponded to 5.6 M of urea and 32% (v/v) formamide in 1x TAE. Electrophoresis of both DGGE analysis was performed for 16 h at a constant voltage of 80 V. The temperature was set at a constant temperature of 60°C. The polyacrylamide gel was stained with SYBR Gold nucleic acid gel stain (Invitrogen, USA) for 30 min before visualized on a UV trans-illumination and photographs were capture using Biovision CN 1000/26 M (Vilber Lourmat, France). DGGE profiles were observed from obtained band light intensities at different locations. The selected bands were excised from the polyacrylamide gel and re-suspended in 20 µl of MilliQ water and stored overnight at 4°C. The eluted DNA were used as the templates for re-amplification. The DNA sequences were manually identified using the online BLAST software on the NCBI website (<http://www.ncbi.nlm.nih.gov/>) and aligned with the GenBank reference.

3. Results and discussion

3.1. Treatment performance and removals of toxic organic micro-pollutants of different sludge source in MBR under long term operation

In this experiment, we continuously operated the membrane bioreactors (MBR 1 and MBR 2) for a period of 550 days and monitored the qualities of influent/effluent water and toxic organic micro-pollutants concentrations. The influent and effluent water qualities in Table 1 from both MBR systems. The average BOD, COD, and TN concentrations in fed leachate were 3074, 6469 and 205 mg/l. After the treatment, effluent from MBR1 contained average BOD, COD and TN concentrations of 5.0, 131 and 30.6 mg/l corresponding to the removals of 99.8%, 98.0% and 85.1% respectively. In MBR 2, the removals of BOD, COD and TN were 99.5%, 96.4% and 83.7% yielding average effluent concentrations of 16, 234 and 33.5 mg/l respectively. Meanwhile, the removals of TP were equal (84.6%) in both reactors. For

this study, the removals of nutrients (N, P) were mainly due to their uptake for microbial growth as both reactors were operated under aerobic condition.

Figure 1 shows the time courses of influent and effluent COD and MLSS concentrations in MBR 1 and MBR 2. During the start-up period (day 0 - day 110), MLSS concentrations in MBR 1 were gradually increasing from about 5 g/l to 16 g/l in response to organic loading to the reactor as it was operated under no sludge wastage condition. Then, MLSS concentrations reached a plateau and became stable between 16 - 18 g/l during the steady operation period (day 110 - day 550). For MBR 2, MLSS increased to about 12 g/l during start-up period and maintained at 10 - 14 g/l during steady operation. MLSS concentrations in MBR 2 operated at SRT of 15 days were found to be about 25% lower than those observed in MBR 1.

Insert Figure 1

The influent, in reactor (sludge) and effluent concentrations of toxic organic micro-pollutants in MBR system during steady operation (day 110 - 550) are shown in Figure 2. The influent concentrations of all compounds were varied at about 1,000 µg/l and then after treatment the effluent concentrations were found less than 10 µg/l - only 2,6 DTBP, BHT, DEP, DBP, DEHP and BBzP were observed at a low noticeable level. However, from this treatment, it was shown that most of those compounds found at early stage of operation were disappeared from the effluent after 400 days of operation. For BPA, 2,6 DTBP, DEP and DCP, the removals were mainly contributed from their biodegradation as they were not accumulating onto sludge solids. For BHT, DBP, DEHP and BBzP, their accumulations onto sludge solids were observed, but among them, only DEHP was found at higher level.

Insert Figure 2

For MBR 2, as shown in Figure 3, the concentrations level of all toxic organic micro-pollutants were found below 10 µg/l - except those of DEHP which were between 10 - 20 µg/l. In most cases, the effluent concentrations were regularly detected during the whole experimental period. Accumulation onto sludge solids were observed for the same compounds as MBR 1 but they were detected at lower level. The concentrations detected on

sludge solids also leveled off during most of the operation period. Hence, it was suggesting that there was a balance between inflow of toxic organic micro-pollutants, biodegradation in the reactor and their outflow via effluent and sludge discharges.

Insert Figure 3

Table 2 shows average concentrations of toxic organic micro-pollutants in the influent, MLSS and effluent of MBR 1 and MBR 2. The concentrations in waste sludge was also considered in MBR 2. Average removal efficiencies of toxic organic micro-pollutants in MBR 1 were 99.8% for BPA, 99.9% for 2,6-DTBP, 99.3% for BHT, 99.8% for DCP, 99.7% for DEP, 99.5% for DBP, 99.1% for DEHP and 99.4% for BBzP. In MBR 2, the removal efficiencies were 99.7% for BPA, 98.8% for 2,6-DTBP, 99.0% for BHT, 99.4% for DCP, 99.5% for DEP, 99.5% for DBP, 98.1% for DEHP and 99.1% for BBzP. Among them, the removal of DEHP was found lowest and most of their removals were due to adsorption onto sludge solids. In MBR 1, 75.7% of DEHP were adsorbed onto MLSS which was kept inside the reactor. In MBR 2, 26.0% of DEHP was kept inside the reactor and 29.2% were discharge together with excess sludge.

Insert Table 2

It was found that most of toxic organic micro-pollutants, except DEHP, were mainly removed by biodegradation mechanism as the contribution from adsorption onto sludge solids in their removal was less than 10%. One of the key factors affecting their removals was their chemical properties. The compounds with higher octanol-water partition coefficient (K_{ow}) or hydrophobic in nature tend to adsorb onto sludge solids and accumulated in reactors or discharge with excess sludge whereas other compounds with lower K_{ow} were removed in aqueous by biodegradation. Considering the properties of micro-pollutants other than DEHP in this study, their log K_{ow} values were ranged from 3.32 to 5.10 (Table 3). Meanwhile, DEHP which is relatively hydrophobic compound (log K_{ow} = 7.50) and mainly found associated with sludge solids was removed through adsorption in the MBR. In general for compounds with log K_{ow} < 2.5, the adsorption to activated sludge was not contribute significantly to removal of the organic micro-pollutants via excess sludge withdrawal.

Between log K_{ow} of 2.5 to 4, moderate adsorption is expected whereas values higher than 4.0 are synonym to high adsorption potential.

Insert Table 3

3.2. Biodegradation of toxic organic micro-pollutants by MBR sludge

The biodegradation of toxic organic micro-pollutants was studied in batch experiments using MBR sludge at day 0 and day 519 of operation. Table 3 shows percentages of toxic organic micro-pollutant removals via biodegradation and their first order removal rate constant in MBR 1 and MBR 2. On day 0, MBR 1 sludge had high biodegradation efficiencies in range between 79% and 87%. The highest removals were from those compounds having lower log K_{ow} values whereas the lowest removal was BBzP. In case of MBR 2 sludge, the biodegradation percentages were 61-71% - lower values were those of DEHP and BBzP and highest value was DCP. Significant higher biodegradation percentages were found in case of MBR 1 sludge which obtained from pilot-scale MBR system operated at solid waste disposal site. The difference in biodegradation percentages between both sludge was mainly due to acclimatization of sludge to leachate characteristics and toxic organic micro-pollutants.

From the batch experiment, it was found that micro-pollutant were degraded well by sludge of two reactors. In comparison, the batch experiment of acclimatized-sludge from MBR 1 or sludge that were familiar with toxic compounds for over 500 days performed better with higher removal efficiencies than those of non-acclimatized sludge from MBR 2. However, the biodegradation of all type of sludge was found improved with time and they were highest at day 519. During the whole operation period, the biodegradation removals of BPA, 2,6-DTBP, BHT, DCP, DEP, DBP, DEHP and BBzP in MBR 1 were adaptively improved by 8-12%. For non-acclimatized sludge from MBR 2, the removal efficiencies was improved by 5-17%.

The rates of micro-pollutants removals were found to follow first-order kinetic expression as follow.

$$\ln (C_t/C_0) = -kt \quad (1)$$

where C_0 ($\mu\text{g/l}$) and C_t ($\mu\text{g/l}$) were the concentrations of toxic organic micro-pollutants in aqueous solution at initial time and time t (hours or h). k (h^{-1}) was first order rate constant for the removal of the micro-pollutants.

Considering the rate constant (k) of the micro-pollutant from the batch experiment mentioned above, it was found that removal rate of each toxic organic micro-pollutant by biodegradation mechanism was adaptively improved. The rate constant (k) of day 0 of acclimatized sludge from MBR 1 was ranged between $0.187\text{--}0.289 \text{ h}^{-1}$ and by day 519 was improved to $0.205\text{--}0.362 \text{ h}^{-1}$. As well as the batch experiment of non-acclimatized sludge from MBR 2, the rate constant (k) was ranged between $0.124\text{--}0.216 \text{ h}^{-1}$ and improved to $0.171\text{--}0.295 \text{ h}^{-1}$ by day 519.

From this batch experiment, the key factors that affected the removal efficiencies of toxic organic micro-pollutants were described as follows:

(1) *Hydrophobicity*: This was determined by the ' $\log K_{ow}$ ' of those toxic organic micro-pollutants and with low $\log K_{ow}$ were degraded well with biodegradation such as BPA and DCP

(2) *Molecular weight*. From our experiment, those compounds with low molecular weight were degraded well and better than those compounds with high molecular weight. For example, DEHP and BBzP which have high molecular weight and were degraded in lower rate with biodegradation compared to other compounds.

(3) *Chemical structure e.g. branch structure or aromatic ring*. From the experiment, it was shown that DEHP and BBzP with long branch chain and many aromatic rings were degraded later when compared to other compounds. Tadkaew *et al.* (2011) reported that functional group in chemical structure was one of the factors that affected the degradation rate. In our study, function group of all compounds contained electron donating group (EDG) which were considered to be easily degraded in the MBR system. The EDG group were including primary amine group, hydroxyl group, ether group, carbonyl group and alkyl group.

3.3 Bacteria community in MBR sludge

The PCR-DGGE technique was used to determine bacteria community in MBR sludge and their changes along the reactor operation. On day 0, nine bacterial strains were found in acclimatized sludge (MBR 1) including *Pseudomonas* sp. UW4, *Pseudomonas fluorescens* Pf0-1, *Pseudomonas syringae* B728a, *Burkholderia* sp. CCGE1002, *Nitrosomonas* sp. AL212, *Enterobacter* sp. OM1, *Burkholderia multivorans* ATCC17616, *Sphingobacterium* sp. 21, *Rhodopseudomonas palustris* CGA009. Meanwhile, seed sludge from non-acclimatized sludge (MBR 2) was found to contain seven bacterial strains including *Nitrobacter hambergensis* X14, *Pseudomonas syringae* B728a, *Pseudomonas fluorescens* Pf0-1, *Methylobacillus flagellatus* KT, *Agrobacterium fabrum* str C58, *Rhodospirillum centenum* SW, *Maribacter* sp. HTCC2170. Many researchers had suggested the presence of phenolic and phthalate degrading bacteria in acclimatized sludge on day 0 (Figure 4) and there was fewer effective bacteria to biodegrade such compounds in non-acclimatized sludge (Figure 5).

Insert Figure 4

Insert Figure 5

Along the operation of MBR 1 over 550 days, most of the bacteria population originally presented in seeded sludge was maintained within the system as well as those newly detected species along the operation. The classification of bacteria community included 3 phylums, i.e. Bacteroidetes, Proteobacteria and Firmicutes. The Bacteroidetes included 2 species, i.e. *Flavobacterium psychrophilum* JIP02/86, *Sphingomonas* sp. 21. Proteobacteria found in the reactor was identified in three classes, i.e. β - Proteobacteria, α - Proteobacteria and γ - Proteobacteria including 14 species as follows; *Burkholderia* sp. CCGE1002, *Burkholderia multivorans* ATCC17616, *Ralstonia pickettii* 12J, *Nitrosomonas* sp. AL212, *Dechloromonas aromatica* RCB, *Enterobacter* sp. OM1, *Klebsiella oxytoca* KCTC1686, *Klebsiella pneumonia* subsp. pneumonia MGH78578, *Pseudomonas* sp. UW4, *Pseudomonas fluorescens* Pf0-1, *Pseudomonas syringae* B728a, *Xanthomonas oryzae* pv. *oryzae* KACC10331, *Rhodopseudomonas palustris* CGA009 and *Agrobacterium* sp. H13-3. For phylum Firmicutes, *Bacillus subtilis* No.66 was found (Figure 4).

The DGGE profile revealed development of new bacteria population in MBR 1 over long term operation. *Dechloromonas aromatica* RCB that could biodegrade organic compounds with aromatic ring structure was detected on day 207. Meanwhile, *Bacillus subtilis* No.66 which had been reported on their phthalate degradation ability was first detected on day 310 together with *Klebsilla pneumoniae* subsp. MGH78578 which could degrade phenols as well as phthalates. On the later day, *Xantomonas oryzae* pv. *oryzae* KACC10331 which utilized phenols was found on day 412 and day 519. *Agrobacterium* sp. H13-3, *Klebsiella oxytoca* KCTC1686, *Ralstonia pickettii* 12J were found on day 519 which all of these species had been reported on their abilities to biodegrade toxic organic micro-pollutants.

The changes in bacteria community in MBR 2 are shown in Figure 5. There were 2 phylums and 19 strains. Phylum Bacteroidetes included *Maribacter* sp. HTCC2170 and *Sphingomonas wittichii* RW1. Phylum Proteobacteria were identified in 3 classes, i.e. β - Proteobacteria, α - Proteobacteria and γ - Proteobacteria including *Burkholderia* sp. CCGE1002, *Nitrosomonas eutropha* C91, *Nitrosomonas* sp. Is79A3, *Methylobacillus flagellatus* KT, *Methylovorus glucosetrophus* SIP3-4, *Klebsiella pneumonia* subsp. *pneumonia* MGH78578, *Pectobacterium wasabiae* WPP163, *Pseudomonas* sp. UW4, *Pseudomonas fluorescens* Pf0-1, *Pseudomonas syringae* B728a, *Pseudomonas putida* KT2440, *Xanthomonas albilineans* GPE PC73, *Rhodopseudomonas palustris* CGA009, *Nitrobacter hambergensis* X14, *Agrobacterium fabrum* str C58, *Rhodospirillum centenum* SW and *Rhodomicrobium vannielii* ATCC17100.

For MBR 2 bacterial population changes along the operation were also observed (Figure 5). Some bacteria which was originally presented in seeded sludge were found disappeared at some stage of operation. *Pseudomonas syringae* B728a, *Pseudomonas fluorescens* Pf0-1 disappeared after 104 days of operation whereas *Pseudomonas putida* KT2440 was later found on day 310-412 and disappeared on day 519. *Rhodopseudomonas palustris* CGA009 which is capable of degrading phenolic compounds was found on day 0 to day 412 but later disappeared on 519 days.

The DGGE profile helped confirmed that micro-pollutants removal efficiency was depending on characteristics of bacteria community in each sludge. The acclimatized sludge in MBR 1

were degraded well with bacteria that were familiar with toxic organic micro-pollutants. It contained a lot of bacteria species that available in nature to help degrade phenol and phthalate. However, bacteria species in both MBR 1 and MBR 2 had adaptively improved during reactor operation by time after they become familiar with those micro-pollutants.

In addition, phenol and phthalate compounds contain aromatic ring in their chemical structure. The biodegradation of phenols and phthalates have 'catechol' and 'protocatechuate' as intermediate compounds. It was reported that enzyme responsible for phenol degradation was 'phenol hydroxylase' (Chen *et al.*, 2003). This enzyme is responsible for converting phenol to catechol, and the reaction is an initial and rate-limiting step of phenol degradation. Proteobacteria, especially the genus *Pseudomonas* including *Pseudomonas* sp., *Pseudomonas putida*, *Pseudomonas fluorescens*, *Pseudomonas syringae* has been reported to produce phenol hydroxylase (Zhang *et al.*, 2004). The catechol is an intermediate compounds in phenol degradation could be degraded by 'catechol dioxygenase' enzyme that produced from *Acinetobacter*, *Klebsiella*, *Bacillus*, *Ralstonia* and *Pseudomonas* (Kalogeris *et al.*, 2006; Jiang *et al.*, 2004).

For phthalate degradation, enzyme responsible for hydrolysis of ester bond is 'esterase' and *Bacillus subtilis* (Peerzada *et al.*, 2006), *Pseudomonas fluorescens* and *Pseudomonas putida* were reported to produce this enzyme (Rehdorf *et al.*, 2012; Shaw *et al.*, 2006). In addition, 'phthalate dioxygenase' is an enzyme responsible for key step in phthalate degradation (Batie *et al.*, 1987; Stanislauskiene *et al.*, 2011). The degradation of phthalates by phthalate dioxygenase, oxygen is the main importance factor of degradation pathway under aerobic condition and there are some reports mentioning bacterial strains capable to produce phthalate dioxygenase as *Burkholderia* sp., *Burkholderia multivorans*, *Ralstonia picketti* and *Pseudomonas putida*.

Moreover, 'protocatechuate dioxygenase' is also the key enzyme for breaking aromatic ring of protocatechuate and *Pseudomonas* sp. has been reported to produce protocatechuate dioxygenase (Yun *et al.*, 2004). The products of phenols and phthalates degradation include pyruvate, oxaloacetate, succinate and acetyl Co A (Zhang *et al.*, 2004; Vamsee *et al.*, 2008) which are completely transformed in the 'krebs cycle'.

4. Conclusions

Toxic organic micro-pollutants removals in MBR seeded with acclimatized sludge from leachate treatment system and non-acclimatized sludge from activated sludge sewage treatment plant were investigated. The removals of BPA, 2,6-DTBP, BHT, DEP, DBP and DEHP, DCP and BBzP, in both reactors were higher than 98% but their biodegradation in MBR seeded with acclimatized sludge. The biodegradation capacities of sludge were also improved by time. PCR-DGGE analyses revealed development of phenolic and phthalate degrading bacteria along with improved micro-pollutants biodegradation. The biodegradation of micro-pollutants by acclimatized and non-acclimatized sludge was improved with time because of microbial adaptation in the systems.

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Table 1
Leachate characteristic and treatment performance of MBR

Parameters	Unit	Influent	Effluent MBR 1	Effluent MBR 2
BOD	mg/l	3,074 ± 234	5.0 ± 10 (99.8 %)	16 ± 9 (99.5 %)
COD	mg/l	6,469 ± 473	131 ± 97 (98.0 %)	234 ± 32 (96.4 %)
TOC	mg/l	1,856 ± 110	29 ± 53 (98.5 %)	38 ± 42 (98.0 %)
TN	mg/l	205 ± 29	30.6 ± 48 (85.1 %)	33.5 ± 41 (83.7 %)
TKN	mg/l	109 ± 40	18.0 ± 28 (83.5 %)	20.7 ± 14 (81.0 %)
NH ₃ -N	mg/l	51 ± 15	0.7 ± 0.5 (98.6 %)	0.7 ± 0.3 (98.6 %)
TP	mg/l	14 ± 3	2.2 ± 0.4 (84.6 %)	2.2 ± 0.6 (84.6 %)
Remark: No. of sample = 47				

Table 2
Micro-pollutant concentrations in influent, MLSS, waste sludge and effluent of MBR

Compounds	MBR 1			MBR 2			
	Inf.	Concentration ($\mu\text{g/l}$) [†]		Inf.	Concentration ($\mu\text{g/l}$) [†]		Eff.
		MLSS	Eff.		MLSS	waste sludge	
BPA	1,015 (114) 100 %	0 (0) 0 %	2 (2) 0.2 %	1,015 (114) 100 %	0 (0) 0 %	0 (0) 0 %	3 (2) 0.30 %
2,6-DTBP	963 (163) 100 %	0 (0) 0 %	11 (4) 0.1 %	963 (163) 100 %	0 (0) 0 %	0 (0) 0 %	12 (5) 1.2 %
BHT	1,006 (86) 100 %	58 (17) 5.8 %	7 (3) 0.7 %	1,006 (86) 100 %	25 (7) 3.0 %	16 (8) 2.0 %	10 (3) 1.0 %
DCP	887 (152) 100%	0 (0) 0 %	2 (3) 0.2 %	887 (152) 100 %	0 (0) 0 %	0 (0) 0 %	5 (5) 0.6 %
DEP	958 (74) 100 %	0 (0) 0 %	3 (2) 0.3 %	958 (74) 100 %	0 (0) 0 %	0 (0) 0 %	5 (3) 0.5 %
DBP	1,087 (120) 100 %	103 (54) 9.5 %	5 (2) 0.5 %	1,087 (120) 100 %	25 (8) 2.30 %	21 (11) 1.9 %	5 (3) 0.5 %
DEHP	804 (182) 100 %	609 (126) 75.7 %	7 (4) 0.9 %	804 (182) 100 %	209 (73) 26.0 %	235 (92) 29.2 %	15 (4) 1.9 %
BBzP	1,003 (95) 100 %	71 (50) 7.1 %	6 (4) 0.6 %	1,003 (95) 100 %	33 (14) 3.3 %	24 (27) 2.4 %	9 (8) 0.9 %

[†] Average value (SD), No. of samples = 58

Table 3

Biodegradation capacities of micro-pollutants of MBR sludge at day 0 and day 519.

Micro-pollutants	Molecular weight (g/mol)	log K _{ow}	Biodegradation			
			D 0 [†]		D 519 [†]	
			% Removal	<i>k</i> (h ⁻¹)	% Removal	<i>k</i> (h ⁻¹)
BPA 	228.29	3.32	87 ¹ , 70 ²	0.289, 0.216	98, 81	0.318, 0.264
2,6-DTBP 	206.33	4.92	85, 63	0.178, 0.154	96, 73	0.211, 0.175
BHT 	220.35	5.10	87, 65	0.208, 0.134	95, 76	0.245, 0.177
DCP 	128.56	2.27	87, 71	0.224, 0.231	98, 80	0.362, 0.295
DEP 	222.24	2.39	83, 69	0.196, 0.144	95, 80	0.247, 0.203
DBP 	278.34	4.61	85, 63	0.193, 0.130	95, 73	0.256, 0.214
DEHP 	390.56	7.54	85, 61	0.179, 0.154	95, 72	0.221, 0.193
BBzP 	312.36	4.77	79, 61	0.187, 0.124	90, 66	0.205, 0.171

[†] from triplicate experimentThe values show % removal and k of ¹MBR 1 and ²MBR 2 sludge

Fig. 1. Influent BOD, COD and MLSS concentrations in MBR1 and MBR2

Fig. 2. Variations of micro-pollutants concentrations in MBR 1 (a) BPA (b) 2,6 DTBP (c) BHT (d) DEP (e) DBP (f) DEHP (g) DCP and (h) BBzP $\cdots \bigcirc \cdots$ influent
█ effluent $\cdots \triangle \cdots$ adsorption onto sludge

Fig. 3. Variation of micro-pollutant concentrations in MBR 2 (a) BPA (b) 2,6 DTBP (c) BHT (d) DEP (e) DBP (f) DEHP (g) DCP and (h) BBzP
 ---○--- influent
 ■ effluent
 ---△--- adsorption onto sludge

Fig. 4. DGGE profile of PCR amplification of MBR 1 samples on day 0, 104, 207, 310, 412 and 519.

Fig. 5. DGGE profile of PCR amplification of MBR 2 samples on day 0, 104, 207, 310, 412 and 519