Biodegradation and toxicity of melamine at high activated sludge concentrations in a membrane bioreactor
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ABSTRACT
Melamine is recalcitrant and toxic to bacteria in conventional activated sludge systems. In this study, we investigated the degradation and toxicity of melamine in a membrane bioreactor (MBR) system operated at high activated sludge concentrations (∼8.5 g TSS/L). Melamine was dosed at 3 mg/L for about 100 days. The average melamine removal efficiency in the MBR system was 20 ± 11%. Meanwhile, batch studies showed the acclimated sludge from the MBR had higher removal efficiencies after the depletion of readily biodegradable substrate (acetate) while non-acclimated sludge did not remove any melamine. As acclimated sludge had removal efficiencies ranging from 33 ± 6% (by 1.7 g TSS/L biomass) to 41 ± 10% (by 8.5 g TSS/L biomass), microbial specialists with unique hydrolytic enzymes in the acclimated sludge were likely responsible for melamine degradation. Since bacteria prefer to use readily biodegradable substrates for growth in the MBR, the population of microbial specialists capable of degrading melamine or the capability of cometabolism appeared not to increase with an increase in biomass concentration. Nevertheless, because of high sludge concentrations and thus low mass ratio of toxic melamine to biomass in the MBR, the long-term melamine exposure did not affect MBR activated sludge performance.

Key words | biodegradation, cometabolism, melamine, membrane bioreactor, sludge adaption

INTRODUCTION
Microbial adaptations contribute to the fitness and plasticity of microorganisms in response to environmental stress and chemical exposure. There are several interrelated adaptation mechanisms including (i) selective enrichment of microorganisms, (ii) induction and/or depression of specific enzymes, and (iii) genetic changes resulting in new metabolic capabilities (Leahy & Colwell 1990; Rittmann & McCarty 2001). Hence, activated sludge acclimation or adaptation generally improves degradation rates of recalcitrant organic compounds in the environment. A selection and concentration of specialized bacteria during acclimation improve biodegradation rates of synthetic organic chemicals such as nitrobenzoate and chlorophenol (Hu et al. 2005a, 2005b).

Membrane bioreactor (MBR) systems are excellent in solid–liquid separation and allow for higher biomass concentrations than conventional activated sludge (CAS) systems resulting in better effluent quality (Metcalf & Eddy 2003; Ersu et al. 2010). The biomass concentration in MBR systems can be 10 times that of CAS systems (Galil & Jacob 2009), resulting in more efficient pollutant removal (Fleischer et al. 2005; Monclús et al. 2010). Additional benefits can be achieved through MBR operation, such as shorter hydraulic retention times (HRTs) and higher volumetric loading rates, and longer solids retention times (SRTs) and thus less sludge production (Metcalf & Eddy 2003; Bhatta et al. 2004). Although membrane fouling is an operational problem (Charcosset 2006), MBR is increasingly used in wastewater treatment for wastewater reuse (Yoon et al. 2004; Rosenberger et al. 2006; Iversen et al. 2009; Juang et al. 2010; Mutamim et al. 2013).

For treating organic compounds with low biodegradability, the mixed liquor suspended solids (MLSS) must be high enough to improve biodegradation by enabling the development of slow-growing microbial populations (Mutamim et al. 2013). Highly diverse bacterial consortium with more slowly growing microorganisms can be cultivated in MBR systems to improve biodegradation of these compounds (Boonmorat et al. 2014). Hence, at high biomass concentrations, MBR may create more opportunities for the
degradation of slowly biodegradable or recalcitrant compounds including melamine. In CAS systems, however, melamine which is known as a nitrogen-rich (67% nitrogen by mass) heterocyclic aromatic compound that has fatal effects on living organisms is toxic to bacteria and its biodegradation is very limited even after a long-term sludge adaptation (Xu et al. 2013). Melamine could enter the municipal wastewater at a concentration of about 3 mg/L considering some industrial wastewater streams containing melamine and its derivatives at a concentration of about 30 mg/L and the fact of dilution with other wastewater prior to entering the wastewater treatment plant (WWTP) (Xu et al. 2013). It is believed that the competent biomass fraction (Hu et al. 2005a) or the number of bacterial specialists capable of degrading melamine was too low to degrade melamine in wastewater. The main objective of this study was to determine the effect of long-term sludge adaptation on the biodegradation of melamine at higher biomass concentrations (i.e. 5 × biomass concentration in the CAS) using MBR technology. Furthermore, the effect of long-term input of melamine on MBR activated sludge performance was evaluated.

MATERIALS AND METHODS

MBR setup and operation

A submerged MBR equipped with a ZeeWeed hollow fiber membrane module (GE Water & Process Technologies, Trevose, PA) was used in the study (supporting information, Figure S1, available with the online version of this paper). The membrane module was made of polyvinylidene fluoride (PVDF) with a nominal pore size of 0.1 μm and a total effective surface area of 0.047 m². The MBR, operated in a Modified Ludzack-Ettinger (MLE) process, had anoxic (first, 2.4 L) and aerobic chambers (4.8 L) with a total effective volume of 7.2 L. There was a glass baffle installed to separate the anoxic and aerobic chambers and recirculation from the aerobic chamber to the anoxic chamber at a flow rate equal to the influent flow rate. The upper and lower water level sensors (Cole-Palmer, Vernon Hills, IL, USA) were applied to maintain a relatively constant mixed liquor volume in the MLE-MBR. The volume difference between the upper and lower water level was less than 5% of the total mixed liquor volume in the MLE-MBR. As the water level reaches the upper limit because of continuous feeding, the upper level sensor triggers the operation of a permeate pump. When the water level reaches the lower limit, the lower level sensor assures pump shut-down. In this study, a suction peristaltic pump after the membrane module was used for permeate collection. An online digital pressure gauge (Cole-Palmer) was installed to measure the transmembrane pressure (TMP). The speed of the permeate pump was set at a permeate flux higher than the influent flow rate so that the permeate pump was intermittently turned on and off by the upper and lower water level sensors, respectively, to keep the total mixed liquor volume relatively constant. An air pump supplied compressed air to the built-in orifices at the bottom of the membrane module at a constant flow rate of 6 L/min to provide aeration and control membrane fouling. The dissolved oxygen was maintained at 4–6 mg/L in the aerobic chamber and <0.5 mg/L in the anoxic chamber, respectively.

At an HRT of 1 d, the MBR was fed continuously with synthetic wastewater containing nonfat dry milk powder at a chemical oxygen demand (COD) concentration of approximately 500 mg/L. The detailed composition of synthetic wastewater has been described elsewhere (Xu et al. 2013). The seed sludge was taken from the aerobic basin at the Columbia WWTP (Columbia, MO, USA), which has a treatment capacity of 75.7 × 10³ m³ per day. During the start-up period, there was no sludge wasted until the biomass concentration reached 8.5 g total suspended solids (TSS)/L. Afterward, sludge was wasted daily with the target SRT of approximately 150 days in order to maintain relatively constant, high biomass concentrations in the MLE-MBR.

During the operating period, the TMP and permeate flux of the MBR were closely monitored. When the TMP increased dramatically in a short period of time or the TMP level exceeded the predefined value (44.5 kPa), as suggested by the manufacturer, the membrane module was taken out of the MBR for physical cleaning. The membrane module was rinsed with tap water for about 30 min before it was submerged again in the MBR.

Effect of long-term melamine dosing on bioreactor performance

Melamine (99%) was purchased from Acros Organics (Morris Plains, NJ, USA). Starting from day 85, a melamine stock solution with a concentration of 122.8 mg/L was fed continuously for about 100 d at a flow rate of 0.172 L/d to reach a nominal influent concentration of 3 mg/L (Xu et al. 2013). The change in the HRT due to melamine addition was negligible because the flow rate of melamine stock was much lower than that of the influent (6.9 L/d). Wastewater effluent from the MBR system was collected and analyzed.
for melamine, NH$_4^+$-N, NO$_3^-$-N, NO$_2^-$-N, and COD following the standard methods (APHA, AWWA & WEF 2005).

**Batch study on melamine adsorption and degradation**

To differentiate the role of adsorption from biodegradation, batch studies were conducted by using both live cells and dead cells. After collected from Columbia WWTP and concentrated at a final biomass concentration of about 8.5 g TSS/L, half of the sludge was killed by heating at 80 °C for 20 min (Hu et al. 2009b). Then aliquots (5 mL) of the melamine stock solution (1,000 mg/L) were added to the sludge samples at a final melamine concentration of 10 mg/L. The mixed liquor in each beaker was magnetically mixed at 350 rpm at 25 ± 1 °C under aerobic and anoxic conditions. An aeration pump was used to supply air in addition to mixing under aerobic conditions. Excess sodium nitrate was added to the mixed liquor under anoxic conditions. At predetermined times (4, 12, 16, 24, 36, 54, and 72 h), aliquots (5 mL) of the mixed liquor were collected for melamine analysis. After 10 min of sedimentation, the supernatant was passed through a 0.45 μm nylon syringe filter and the filtrate was stored at 4 °C before analysis.

Batch studies were also conducted to compare the melamine degradation behavior (with a initial melamine concentration of 10 mg/L) by the acclimated activated sludge and unacclimated sludge, respectively, in the presence of primary organic substrate (acetate). Each study was conducted in a 250 mL flask with 50 mL headspace. Each flask covered with a cotton stopper was placed on a platform shaker running at 200 rpm to ensure complete mixing of the mixed liquor. The acclimated sludge samples were collected from the aeration chamber of the MBR system after 100 d of continuous melamine dosing. The sludge samples with biomass concentrations of 8.5 g TSS/L reached a melamine-to-biomass ratio of 1.2 mg/g TSS and the sludge samples with biomass concentration of 1.7 g TSS/L (diluted from 8.5 g TSS/L sludge) reached a melamine-to-biomass ratio of 6 mg/g TSS, respectively. All of the samples were washed with distilled water three times to remove the residual carbon and nutrients before they were re-suspended in the medium that had the same recipe as the feed solution except the use of acetate as a readily biodegradable substrate (~400 mg COD/L) instead of milk powder and removal of ammonia.

The medium containing ammonia was also used in the low concentration of acclimated sludge systems (1.7 g TSS/L), in order to determine the effect of ammonia (20 mg/L) as a readily biodegradable nitrogen source on melamine degradation. Finally, the experiment was repeated at different melamine concentrations (2.5, 3, 5, 10 and 20 mg/L) using acetate as the primary organic substrate at the acclimated sludge concentration of 8.5 g TSS/L. Correspondingly, the respective melamine-to-biomass ratios (in mg/g TSS) were about 0.3, 0.35, 0.6, 1.2 and 2.4. The activated sludge taken from the Columbia WWTP served as a control (unacclimated sludge).

**Effect of melamine on nitrifying community structure and microbial activities**

To evaluate the effect of melamine on MBR activated sludge process operation, activated sludge samples in the MLE-MBR were collected before and after melamine dosing for deoxyribonucleic acid (DNA) extraction and nitrifying community structure analysis using terminal restriction fragment length polymorphism (T-RFLP) (Xu et al. 2014).

To determine the change in heterotrophic and autotrophic microbial activities, aliquots of mixed liquor were periodically taken from the aeration chamber to determine the specific oxygen uptake rate (SOUR) of activated sludge in the MBR, with detailed procedures described in supporting information (SI).

**Chemical and statistical analysis**

The melamine concentration was determined following our previous study (Xu et al. 2013). Briefly, high-pressure liquid chromatography (HPLC) coupled with a Zorbax SB-C8 column with a dimension of 4.6 mm ID × 250 mm was used for the analysis. For each 5 μL HPLC injection, a mobile phase consisting of 15% acetonitrile and 85% buffer solution (10 mM citric acid + 10 mM sodium octane-sulphonate at pH 3) with a flow rate of 1 mL/min was provided. Acetic acid in the batch studies was measured by HPLC with ultraviolet detection at 210 nm. The HPLC injection volume was 10 μL, and the mobile phase used was 0.1% o-phosphoric acid circulated at 0.8 mL/min at room temperature (Yang et al. 2012). Wastewater influent and effluent samples were collected twice a week for COD, ammonium-N, nitrite-N, and nitrate-N measurements following standard methods (APHA, AWWA & WEF 2005). One-way analysis of variance (ANOVA) analysis was conducted to assess the significance of the differences among groups, with p-values less than 0.05 indicating statistical significance.
RESULTS AND DISCUSSION

Biodegradation of melamine in the MBR system

As the activated sludge concentration in the MBR became stabilized at approximately 8.5 g TSS/L (SI, Figure S2, available with the online version of this paper) (Phase I from day 56 on), melamine was dosed continuously starting from day 85 (Phase II). As can be seen in Figure 1, the average influent melamine concentration over the Phase II period was 3.0 ± 0.2 mg/L, while the average permeate or effluent melamine concentrations from the MBR were 2.4 ± 0.3 mg/L (removal efficiency = 20 ± 11%). There was a significant difference in the melamine concentration between the influent and effluent samples ($p < 0.001$). Melamine removal through adsorption was not observed even at high biomass concentrations (~8.5 g TSS/L) (Figure 2). Hence, melamine can be partially removed by the activated sludge in the MBR via biodegradation. However, the removal efficiency of melamine in the MBR was similar to that in CAS systems with sludge concentrations of about 1.7 g TSS/L, which is 14–20% (Xu et al. 2013). The result suggests that high biomass concentration (8.5 g TSS/L) in the MBR system does not improve melamine degradation.

A series of batch experiments were therefore conducted to determine the effect of sludge acclimation on melamine degradation using acetate as a primary organic substrate. As shown in Figure 3, there was no removal of melamine by unacclimated activated sludge (1.7 g TSS/L) during 7 d of incubation. For comparison, acclimated sludge had removal efficiencies ranging from 33 ± 6% (by 1.7 g TSS/L biomass) to 41 ± 10% (by 8.5 g TSS/L biomass). There was
significant difference of the removal efficiencies ($p < 0.001$) between the acclimated sludge at different sludge concentrations. Meanwhile, HPLC results showed that acetate was depleted in the first day (data not shown), which could drive the bacterial specialists to degrade melamine through cometabolism under stress conditions. By comparing the removal efficiencies in the first day with that over the rest of the days of cultivation, which were $0.1\%$ vs $35 \pm 6\%$ in the presence of $1.7$ g TSS/L biomass and $12 \pm 4\%$ vs $35 \pm 7\%$ in the presence of $8.5$ g TSS/L biomass, the removal efficiencies after the depletion of acetate were significantly higher ($p$-values $< 0.03$). In fact, the significant increase in degradation started on day 4 which was probably due to the activated sludge starting under endogenous respiration. We also added extra ammonia as a preferred nitrogen source to repeat the experiment in acclimated activated sludge (1.7 g TSS/L) system, but did not observe much difference in melamine biodegradation efficiency ($35 \pm 8\%$) compared to the system without ammonia, since melamine was fully oxidized in the first day (data not shown). For comparison, in the continuous flow MBR, where the readily biodegradable organic substrate was fed continuously, the removal of melamine was fairly limited (Figure 1). The results demonstrate the role of long-term sludge acclimation in melamine biodegradation and the impact of the readily biodegradable carbon source on melamine biodegradation. Microbial adaptation mechanisms such as selective enrichment of microorganisms, induction and/or depression of specific enzymes, and genetic changes resulting in new metabolic capabilities could occur after the long-term dose of melamine. As acetate was used up resulting in an immediate decrease in polyhydroxyalkanoates (PHAs) storage, some microorganisms might develop such adaptation mechanisms to degrade melamine under less favorable growth conditions (Çığın et al. 2011). Similar observation were also reported for the degradation of other recalcitrant compounds (Li et al. 2001).

The results also suggest that the total activated sludge concentration plays a minimum role in melamine biodegradation. Instead, the microbial specialists capable of degrading melamine through cometabolism or fortuitous degradation are likely responsible, but their population appeared not to proportionally increase as the acclimated sludge concentration increased from 1.7 g TSS/L to 8.5 g TSS/L. Another batch experiment with fixed biomass concentration of 8.5 g TSS/L was conducted to investigate the effect of initial melamine concentration on biodegradation. Regardless of initial melamine concentration (i.e. 2.5, 5, 10 and 20 mg/L), or the melamine to biomass ratio correspondingly (0.5, 0.6, 1.2 and 2.4 mg/g TSS), similar amounts (0.18–0.2 mg melamine in a total of 200 mL wastewater) were removed in one week (Figure 4), with the removal efficiencies ranging from 5% (at initially 20 mg/L melamine) to 41% (at initially 2.5 mg/L melamine). This result further suggests the degradation of melamine is not involved in regular cell metabolism, but is more related to cometabolism or fortuitous degradation by some special enzymes (e.g. housekeeping enzymes) (Gold et al. 2000). These enzymes could be already possessed by the microorganisms but with less specific requirements for substrates.

**Activated sludge process performance before and after melamine exposure**

During the start-up period (days 0 to 55), biomass concentrations in the MBR gradually increased from about 5 to 8.5 g TSS/L because there was no sludge wasted. Thereafter, at a target SRT with regular sludge wasting, the biomass concentrations became stable between 7.6 and 8.5 g TSS/L (days 56–84, Phase I) before melamine dosing began (days 85–185, Phase II). At an influent COD concentration of $511 \pm 76$ mg/L, the average effluent COD concentrations during Phase I and Phase II were $17 \pm 5$ mg/L and $9 \pm 5$ mg/L, respectively (Figure 5), with the average COD removal efficiencies of 97% and 99%, respectively. The continuous melamine dosing did not negatively affect COD removal, as opposed to the findings from the CAS system (Xu et al. 2013). Meanwhile, there were no significant differences in effluent NH$_4^+$-N ($p = 0.4$), NO$_2^-$-N ($p = 0.8$) or NO$_3^-$-N ($p = 0.3$) concentrations between Phase I and Phase II. The effluent NH$_4^+$-N concentrations during Phase I and Phase II were $0.2 \pm 0.1$ mg/L and $0.1 \pm 0.2$ mg/L, respectively.
with removal efficiencies of 99% and 100%, respectively (Figure 6(a)), indicating almost complete nitrification regardless of melamine dosing. Correspondingly, the effluent NO₂⁻-N concentrations during Phase I and Phase II were 0.3 ± 0.3 mg/L and 0.2 ± 0.2 mg/L, respectively (Figure 6(b)), and the effluent NO₃⁻-N concentrations were 11.2 ± 5.4 mg/L and 15.2 ± 4.4 mg/L, respectively (Figure 6(c)). Therefore, unlike the results from the CAS systems where the presence of melamine (3 mg/L) inhibited activated sludge growth and resulted in poor effluent water quality (Xu et al. 2016), the continuous melamine dosing (3 mg/L) in the MBR with higher biomass concentration (5 times higher than that in CAS) had no impact on COD and nitrogen removal.

The MBR water quality data were also consistent with the SOUR measurements, which indicated normal heterotrophic and autotrophic activities before and after melamine dosing (SI, Figure S3, available with the online version of this paper). The average heterotrophic SOUR values of the sludge before and after melamine dosing were 18.4 ± 1.4 mg O₂/g TSS/h and 18.0 ± 1.1 mg O₂/g TSS/h, respectively. There was no significant difference (p = 0.49) in the heterotrophic SOUR values before and after melamine dosing. Similarly, the autotrophic SOUR values before and after melamine dosing were 14.8 ± 0.7 and 13.8 ± 1.5 mg O₂/g TSS/h, respectively. There was no significant difference in autotrophic SOUR values before and after melamine dosing (p = 0.1).

**Bacterial community structure before and after melamine dosing**

The T-RFLP analysis specifically targeting sensitive bacteria such as ammonia-oxidizing bacteria (AOB) and nitrite-oxidizing bacteria (NOB) in activated sludge shows the nitrifying bacterial community structure in the MBR system before and after melamine dosing. The peak heights in Figure 7(a) and 7(b) represent the relative abundance of each species in the MBR system (Luna et al. 2004; Luna...
et al. 2006). About one week before melamine dosing, *Nitrosomonas* (Figure 7(a)) was the dominant genus of AOB while *Nitrospira* (Figure 7(b)) was dominant among NOB. One month after melamine dosing, similar levels of AOB and NOB in the MBR were detected. This result suggests the long-term exposure to low concentration of melamine did not affect nitrifying bacterial structure in the MBR system with higher sludge concentrations, consistent with the effluent water quality (Figure 6) and nitrifying bacterial activity (SI, Figure S3) results before and after melamine dosing.

**Implications of melamine biodegradation in MBR operated at high activated sludge concentrations**

Biodegradation of melamine starts with hydrolysis (Figure S4, available with the online version of this paper) (Dodge et al. 2012), with the rate described in the following (Rittmann & McCarty 2001):

\[ r_{\text{hyd}} = -k_{\text{hyd}}S_p \]

where \( r_{\text{hyd}} \) is the rate of accumulation of substrate due to hydrolysis, \( S_p \) is the concentration of the substrate and \( k_{\text{hyd}} \) is the first-order hydrolysis rate coefficient which is proportional to the concentration of hydrolytic enzymes. The theory suggests \( k_{\text{hyd}} \) is related to the activated biomass concentration in many cases (Rittmann & McCarty 2001). It is believed that the competent biomass, a specific population of the microbial community that has capability of degrading melamine through cometabolism or fortuitous degradation, is responsible for melamine biodegradation (Cook & Huetter 1981; Takagi et al. 2012). Acclimation of the entire microbial community to melamine in an MBR system operated at long SRTs may allow a better selection of microorganisms containing enzymes and pathways or
development of new catabolic pathways for the removal of recalcitrant organic chemicals such as melamine (Boonnorat et al. 2014). However, the result of no improvement in melamine degradation by increasing biomass concentration to about 8.5 g TSS/L in the continuous-flow MBR suggests that as long as the readily biodegradable substrate (e.g. milk) is available, activated sludge would not actively perform cometabolism. Because the bacterial growth and sludge concentration increase are solely dependent on the readily biodegradable substrate (not melamine), and because the degradation of melamine starts with hydrolysis, which requires a special enzyme or a series of enzymes, the melamine biodegradation efficiencies appeared to reach the limit (at about 20%) in both MBR and CAS systems fed continuously with the readily biodegradable substrate. Gene expression and/or hydrolytic enzymes involved in melamine degradation may be repressed at low concentrations (i.e. 5 mg/L) because there is no need to reduce toxicity (at a biomass concentration of 8.5 g TSS/L) or use the pollutants as carbon and/or energy sources in the presence of readily biodegradable substrates. Although high activated sludge concentration did not help improve melamine degradation, melamine had no detrimental effect on the MBR activated sludge process performance, as the low mass ratio of melamine to activated sludge could reduce the toxicity of melamine to microorganisms.

CONCLUSIONS

The degradation and toxicity of melamine were investigated in MBR activated sludge process operated at a high biomass concentration (8.5 g TSS/L). Although sludge acclimation improved melamine degradation in batch studies, there was no improvement of melamine degradation in the continuous-flow MBR even after a long period of sludge acclimation (100 d), as melamine could be only removed through cometabolism or fortuitous degradation. With continuous feeding of wastewater containing readily biodegradable substrates, the population of microbial specialists with unique enzymes capable of degrading melamine appeared not to increase proportionally with an increase in the biomass concentration to 8.5 g TSS/L in MBR operation. Nevertheless, compared to its detrimental effect on CAS performance, melamine had no impact on MBR activated sludge process performance due to the lower ratio of melamine to biomass in the MBR.

REFERENCES


