Journal of Hazardous Materials 322 (2017) 445–453

Contents lists available at ScienceDirect

Journal of Hazardous Materials

journal homepage: www.elsevier.com/locate/jhazmat

The behavior of melamine in biological wastewater treatment system

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HIGHLIGHTS

- The removed of melamine (MA) was mainly performed by activated sludge adsorption instead of biodegradation.
- High concentration of MA could not easily removal and had adverse impacts on biological wastewater treatment.
- MA inhibited the enzyme activities of NOR, NR, NIR and PPX, which were closely relevant to nitrogen and phosphorus removal.
- High MA concentrations promoted the metabolism of glycogen, thereby providing the advantage for the growth of GAOs.

ARTICLE INFO

Article history:
Received 8 June 2016
Received in revised form 13 October 2016
Accepted 16 October 2016
Available online 17 October 2016

Keywords:
Melamine
Biological nitrogen and phosphorus removal
Activated sludge
Toxicity

ABSTRACT

Melamine (MA) is a significant raw material for industry and home furnishing, and an intermediate for pharmacy. However it is also a hazardous material when being added to food as a protein substitute due to the high nitrogen content. In this study, the behavior of MA in activated sludge was investigated. Experiments showed that MA was removed during biological wastewater treatment process, and the removal was mainly achieved by activated sludge adsorption instead of biodegradation. Low levels of MA (0.001–0.10 mg/L) in wastewater had negligible influence on the performance of activated sludge, but high levels of MA deteriorated biological nitrogen and phosphorus removal. The presence of MA (1.00 and 5.00 mg/L) decreased total nitrogen removal efficiency from 94.15% to 79.47% and 68.04%, respectively. The corresponding concentration of effluent phosphorus increased from 0.11 to 1.45 and 2.06 mg/L, respectively. It was also observed that MA inhibited the enzyme activities of nitrite oxidoreductase, nitrate reductase, nitrite reductase and exopolyphosphatase, which were closely relevant to nitrogen and phosphorus removal. Further investigation showed that the presence of high MA concentrations promoted the consumption and synthesis of glycogen, thereby providing the advantage for the growth of glycogen accumulating organisms.

1. Introduction

Melamine (MA, 1,3,5-triazine-2,4,6-triamine or 2,4,6-triamine-1,3,5-triazine, $C_3N_3(NH_2)_3$), which is a heterocyclic aromatic compound and a type of industrial material with high nitrogen content (66.67% by mass), widely exists in the environment. It is a significant raw material for the synthesis of melamine formaldehyde resin, such as manufacture of laminates, plastics, coatings, commercial filters, adhesives, dishware, and kitchenware. MA can be also used as water-reducer, formaldehyde detergent, and a chemical intermediate for pharmaceutical manufacturing. In addition, MA and MA salts (such as cyanurate, phosphate, pyrophosphate, polyphosphate, borate, phthalate and oxalate) are extensively used in production and daily life as environmental friendly flame retardants [1,2]. The extensive application of MA inevitably causes its entrance into the environment, especially the water environment. According to the survey on MA level in rivers from 1986 to 1994 in Japan, the concentration of MA ranged from ≤0.0001 to 0.0076 mg/L[3]. Ono et al. reported that wastewater steam from a MA factory had a total solids percentage of 1.80%,

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http://dx.doi.org/10.1016/j.jhazmat.2016.10.036
0304-3894/

yielded MA accounted for 1.27% of the total solids [4]. Qin et al. found that the concentration of MA from different MA factories ranged from 22 to 100 μg/L, while levels of 0.167, 1.974, 6.253 and 226.766 mg/L were detected in four other samples. They also measured MA from irrigation water samples, and the highest concentration of 0.198 mg/L was found [5].

MA was widely known by the public due to the relevant unpleasant social events occurred in recent years. It could not be distinguished with protein by Kjeldahl method, thus MA had been illegally adulterated in food to give the illusion of an increase of the apparent protein content [6]. Therefore, MA was detected in the products such as pet foods, powdered infant formula, and hydrated lime nitrogen granular fertilizer [7–9]. Histopathological alterations of tilapia were evident in the liver, gills and kidney in turn, and the severity of lesions associated with the subjected dosage of melamine (10 g/kg feed) [10]. Feed contaminated with MA (250, 500 and 1000 mg/kg) for rainbow trout could cause lipid peroxidation. Fish muscle residues of MA exhibited a dose-response relationship [11]. Mice test (MA, 30, 140 and 700 mg/(kg d)) found that MA could disrupt the blood-testis barrier and cause testicular toxicity [12], and it had ability to increase sperm abnormality rate and DNA damage [13]. The combination of MA and cyanuric acid was responsible for acute renal failure in cats [14]. Monkeys' gavage with MA (700 mg/(kg d)) caused the clinical signs including turbid and whitish urine, urine crystals, red blood cell changes, increased serum alanine aminotransferase, nephrotoxicity, pericarditis and hematopoiesis increasing [15]. According to the carcinogenic potential of MA for humans and animals, MA was classified as a category III carcinogen (the carcinogenicity to humans has not yet to be classified).

Many studies have been carried out to examine the toxicities of MA to organisms, such as fish, rats, cats, and monkeys, and their toxic effects on these species have been reported. As the final defense line prior to entering into the aquatic environment, wastewater treatment plant (WWTP) with activated sludge has been reported to remove many pollutants via aggregation, settling, precipitation, biosorption, degradation, or other processes. The main function of activated sludge is to achieve nitrogen and phosphorus removal from wastewater via a series of biochemical processes, such as nitrification, denitrification, phosphorus anaerobic release and aerobic/anoxic uptake [16]. MA entered into in wastewater treatment process via drainage may also bring risks to these biochemical processes.

Up to now, the behavior of MA in biological wastewater treatment system was rarely reported. Thus, the aim of this work was to evaluate the mass balance of MA in wastewater treatment process and to investigate the potential effect of MA on biological nitrogen and phosphorus removal in a long-term exposure period in an anaerobic/aerobic/anoxic sequencing batch reactor (SBR). This study could provide a theoretical reference for stringent regulation on MA production, marketing, application and ultimate disposal, and give a reminder for water environment security management.

2. Materials and methods

2.1. Synthetic wastewater and seed sludge

The seed sludge was obtained from the secondary sedimentation tank outlet of a WWTP in Changsha, China. The synthetic wastewater consisted of CH_3COONa, NH_4Cl, KH_2PO_4, $MgSO_4$ and $CaCl_2$ to get the average initial concentrations of chemical oxygen demand (COD_{Cr}) 300–350 mg/L, NH_4^+-N 35 mg/L, PO_4^{3-}-P 10 mg/L, and pH 7.00 –7.20. The initial pH was adjusted by 1 M HCl and 1 M $NaHCO_3$, respectively.

Table 1
The dosages of MA in each SBR.

	SBR Reactors					
	SBR1 (Control)	SBR2	SBR3	SBR4	SBR5	SBR6
MA concentration (mg/L)	0	0.001	0.01	0.10	1.00	5.00

2.2. Sequencing batch reactor operation

The study was conducted in a SBR with a working volume of 18 L. The SBR operated 3 cycles daily, and each cycle consisted of a 90 min anaerobic period, a 150 min aerobic period, and a 120 min anoxic period, followed by 55 min settling, 5 min decanting and 60 min idle periods. In the decanting period, 12 L of the supernatant was discharged from the SBR, and 12 L synthetic wastewater was pumped into the reactor during the first 2 min of the anaerobic period. At the beginning of the anaerobic period, the initial pH in the SBR was adjusted to 7.00 ± 0.05 by adding either 1 M HCl or 1 M $NaHCO_3$. The sludge retention time was maintained at approximately 15 d by withdrawing 1.2 L of the sludge mixtures once per day at the end of the anoxic phase before settling.

2.3. Exposure experiments of MA in SBR system

The adaption stage of activated sludge was lasted for 120 days to achieve stable operation. Afterwards, the mixture in the SBR was divided evenly into 6 parts and transferred into 6 identical reactors, with a working volume of 3L each. The 6 reactors were operated the same as parent SBR. These 6 reactors received wastewaters containing 0, 0.001, 0.01, 0.10, 1.0 or 5.0 mg/L of MA, which were prepared by adding relevant volumes of MA stock solution to wastewater (Table 1). The exposure experiments were continuously operated for 90 d.

2.4. Analytical methods

The analysis of COD, NH_4^+-N, NO_3^--N, NO_2^--N, PO_4^{3-}-P, suspended solid (SS) and volatile suspended solid (VSS) were conducted in accordance with the Standard Methods [17,18]. Sulfuric acid-anthrone colorimetry was used for glycogen detection [19,20]. The measurements of ammonia monooxygenase (AMO), nitrite oxidoreductase (NOR), nitrate reductase (NR), nitrite reductase (NIR), exopolyphosphatase (PPX), and polyphosphate kinase (PPK) activities were referred to the methods of others (detailed in Supplementary material) [16,19,21]. The Scanning electron microscope (SEM) analysis was used to detect the surface morphology of active sludge after long-term exposed to MA by the FEI Quanta 200 SEM at 20 kV (detailed in Supplementary material) [16,19]. All experiments were performed in triplicate.

2.5. PHA analysis by GC

Poly-3-hydroxybutyrate (PHB), poly-3-hydroxyvalerate (PHV), and poly-3-hydroxy-2- methylvalerate (PH2MV) were measured by gas chromatography (GC, Shimadzu 2010C) with a HP-5 column (30 m length × 0.32 mm id × 0.25 μm film) (Supplementary material) [19,21,22]. The total polyhydroxyalkanoates (PHA) was calculated as the sum of measured PHB, PHV, and PH2MV.

2.6. Melamine extraction and HPLC analysis

MA (HPLC, ≥98%) was purchased from Shanghai Yuanye biological technology Co.Ltd. (Shanghai, China). Extraction of MA in sample was based upon solid phase extraction (SPE) by poly-Sery

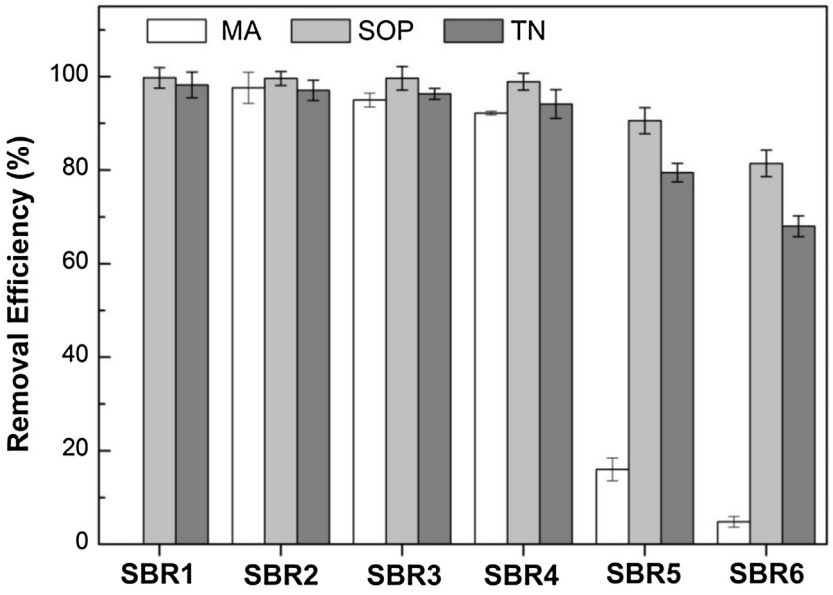

Fig. 1. The removal efficiencies of MA, SOP and TN in the reactors with different MA concentrations during stable operation.

MCX column. Before extraction, the SPE columns were preprocessed with 3 mL methanol and 5 mL ultrapure water in turn. Then 500 mL water sample was loaded at the same rate after filtration via 0.45 μm membrane. The SPE column was leached by 3 mL ultrapure water and 3 mL methanol respectively, eluted with 6 mL 5% amonium methanol. The flow velocities in SPE extraction process were not greater than 1 mL/min. Then the elution was collected and evaporated to dryness by a gentle nitrogen stream at 50 °C with Pressure Blowing Concentrator (NDK200, Shanghai Haozhuang Instruments Co., Ltd.). The residues were redissolved in 1 mL mobile phase and mixed by vortex mixer for 1 min. The concentrated solution was filtrated via 0.22 μm organic phase membrane before detection [5,23].

The sludge samples were prepared for HPLC using following steps. The lyophilized sludge sample was put into plastic centrifuge tube. Each tube was added with 10 mL 5% amonium methanol and then mixed by vortex mixer for 1 min. After being sonicated in a sonic-bath at 50 °C for 5 min, samples were centrifuged at 4000 r/min for 5 min. The supernatant was transferred to a tube. Repeated the above steps to process the residue at the bottom, the two extracted supernatants was merged. Then took 10 mL extracting solution and evaporated to dryness by a gentle nitrogen stream at 50 °C. The residues were redissolved in 1 mL mobile phase and vortex mixed for 1 min. The liquid filtered through a 0.22 μm organic phase membrane into a 2 mL autosampler vial for detection [5,24].

MA was detected by An Agilent 1100 series HPLC system with a 1100 series automatic injector. The system consisted of a quaternary pump, a degasser, a column oven, and a diode array detector. The mobile phase was composed of 15% acetonitrile and 85% buffer solution (10 mM citric acid + 10 mM sodium 1-octanesulfonate at pH 3) at a flow rate of 1 mL/min. Test conditions: C 18 (4.6 × 75 mm, 3.5 μm particle, Agilent) column, column temperature of 40 °C, the injection volume was 20 μL, detected at 240 nm. The limit of detection (LOD) was 0.05 μg/L. All experiments were performed in triplicate.

The mass balance of MA was calculated via the follow Eq. (1).

$$MA_{B,t}(\mu g/g) = \frac{(MA_{In}\text{-}MA_{L,t}) \times V\text{-}(MA_{S,t}\text{-}MA_{bv}) \times MLSS}{MLSS} \quad (1)$$

In which MAIn (mg/L) was the initial concentration of MA in the beginning of a cycle, V (L) was the working volume of SBR, MLSS (g/L) was the mixed liquor suspended solids, MAbv was the background value of MA in activated sludge at the beginning of a cycle, MAL,t (mg/L) and MAS,t (μg/g) were the concentrations of MA in liquid and solid phase respectively at point-in-time. MAB,t (μg/g) was the possible biodegradation amount of MA at the same sampling time.

2.7. Measurement of intermediate products

Ammeline, ammelide and cyanuric acid were the intermediate products of MA degradation. They were detected by SPE process and GC/MS (QP2010). The ENVI-Carb column (3 mL) had been preconditioned with 3 mL dichloromethane, 4 mL methanol and 3 mL 1% trichloroacetic acid in turn. Then 100 mL water sample was loaded at the same rate after filtration via 0.45 μm membrane. After loading, the column washed by 3 mL ultrapure water, then eluted by 5 mL methanol. The elution was collected with graduated centrifuge tube and evaporated to dryness by a gentle nitrogen stream at 50 °C with Pressure Blowing Concentrator. The flow velocities in SPE extraction process were not greater than 1 mL/min. The dry sample was redissolved with 400 μL pyridine and 200 μL derivatization reagent by vortex-mixing for 1 min. The solution was derived at 70 °C for 45 min, and then detected by GC–MS after cooling (detailed in Supplementary material) [25].

3. Result and discussion

3.1. The behavior of MA in SBR systems

The removal efficiencies of MA during stable operation in different reactors were shown in Fig. 1. By comparing the MA concentration in influent and effluent, the removal efficiencies of MA were above 90% in the systems with MA levels of 0.001, 0.01 and 0.10 mg/L. When MA level increased to 1.00 and 5.00 mg/L, the average effluent MA was 0.84 ± 0.03 mg/L and 4.76 ± 0.07 mg/L, respectively. The removed MA can be either adsorbed by activated sludge or decomposed by microorganisms during the process of wastewater treatment. The mass balance of MA in liquid and solid phase during the stable operation of the biological wastewater

Table 2
Mass balance of MA during the stable operation of wastewater treatment systems.

	Influentmg/L	Effluent mg/L	The decrease in liquid phase (DL)mg	The increase in solid phase (IS)mg	IS/DL%
SBR1	0	–[a]	–[a]	–[a]	–[a]
SBR2	0.001	0.279 ± 0.071 ($\times 10^{-3}$)	3.387 ± 0.180 ($\times 10^{-3}$)	2.990 ± 0.002 ($\times 10^{-3}$)	98.502 ± 5.543
SBR3	0.01	0.500 ± 0.180 ($\times 10^{-3}$)	28.488 ± 1.961 ($\times 10^{-3}$)	28.500 ± 0.526 ($\times 10^{-3}$)	97.108 ± 4.896
SBR4	0.10	7.797 ± 0.460 ($\times 10^{-3}$)	0.254 ± 0.015	0.277 ± 0.001	96.982 ± 0.644
SBR5	1.00	0.840 ± 0.030	0.478 ± 0.028	0.480 ± 0.050	99.945 ± 4.566
SBR6	5.00	4.763 ± 0.068	0.555 ± 0.013	0.570 ± 0.204	96.887 ± 4.330

[a] not detected.

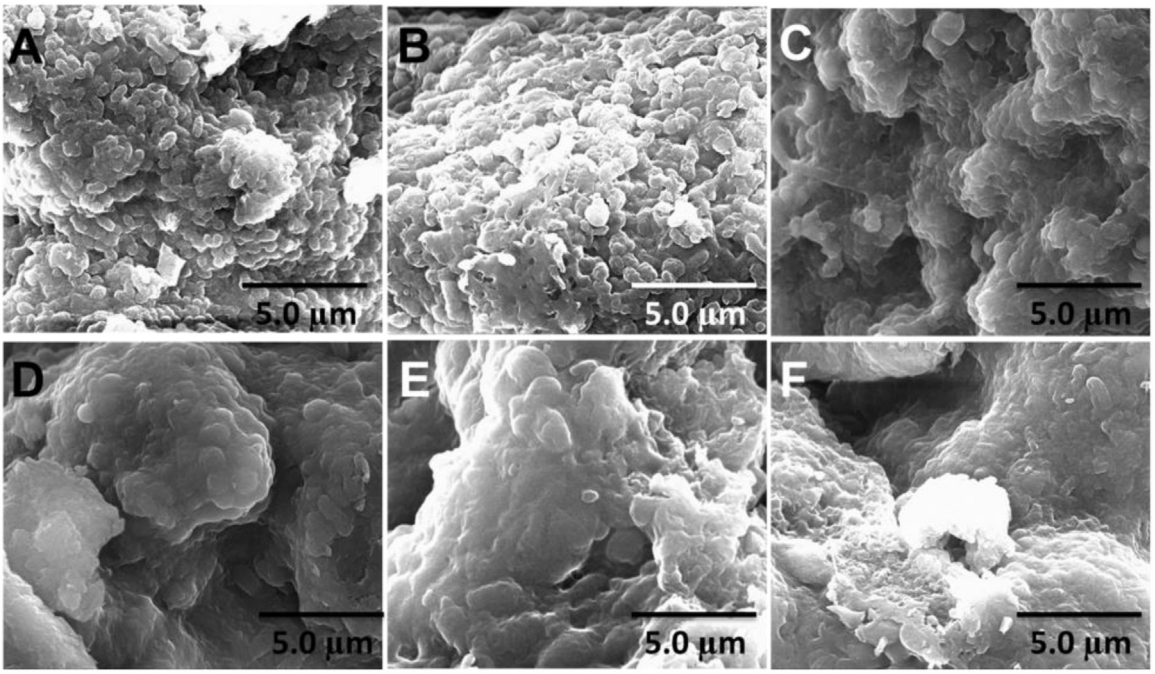

Fig. 2. SEM of activated sludge taken from the 6 reactors with different concentrations of MA in stable operation. SBR1 (A), SBR2 (B), SBR3 (C), SBR4 (D), SBR5 (E), and SBR6 (F).

treatment systems was calculated to investigate the loss of MA in liquid phase. Table 2 is designed to reveal that the reduced portions of MA in the liquid phase were mainly transferred to the solid phase. In addition, the intermediate products of MA degradation such as ammeline, ammelide and cyanuric acid were not detected in either solid or liquid phases. It can be concluded that the influent MA was primarily removed via biomass adsorption instead of biodegradation. Compared with the low effluent MA level in the reactors fed with 0.001, 0.01 and 0.10 mg/L of MA, the concentration of effluent MA in the high MA level reactors was much higher than that in the lower ones, resulted in lower removal efficiency (Fig. 1). However, the MA level detected in the activated sludge in the latter reactors was higher than that in the former reactors, suggesting that the adsorption of MA in the latters was saturated. The results further suggested the activated sludge in SBR system had a certain adsorption for MA, with the threshold of MA between 0.10 mg/L and 1.00 mg/L. High loading of MA could not be easily removed in the biological nitrogen and phosphorus removal system. The facts with high MA levels (1.00 and 5.00 mg/L) were consistent with the results obtained by Xu et al. who investigated the effect of MA on two types of continuous flow reactors with 3.0 mg/L, and suggested that MA is not removed by biodegradation or adsorption either [26].

Fig. 1 shows the effect of MA on the removal of phosphorus and nitrogen. It can be seen that the impact of MA on both phosphorus and nitrogen removal was dosage-dependent. The average soluble *ortho*-phosphorus (SOP) removal efficiencies in SBR2, SBR3 and SBR4 (all above 98%) were roughly the same as that in the control test ($99.25 \pm 0.71\%$). However, lower SOP removal efficiency was achieved in both SBR5 ($90.57 \pm 2.80\%$) and SBR6 ($81.43 \pm 2.84\%$). The result indicated that phosphorus removal in systems at low MA loading were not significantly affected, but the removal rates decreased in SBR5 and SBR6 with high MA levels. The average total nitrogen (TN) removal was also varied at different concentrations of MA. The average removal efficiency of TN in SBR1 ($97.07 \pm 2.15\%$), SBR2 ($94.31 \pm 1.15\%$) and SBR3 ($90.15 \pm 3.07\%$) were all maintained at $\geq$90%, suggesting that MA in low concentration ($\leq$0.10 mg/L) bring no measurable influence on TN removal. However the removal efficiency of TN reduced to $79.47 \pm 1.98\%$ when the dosage of MA increased to 1.00 mg/L, and further increase of MA to 5.00 mg/L caused a decrease of TN removal rate to $68.04 \pm 2.22\%$.

SEM could characterize the activated sludge in micro-view, and the morphology, size and community structures of microorganisms could be distinctly observed [16,19,27,28]. The comparison of different SEM photos could provide the difference of activated sludge samples in the size and morphology of microorganisms and the cell integrity. As seen in Fig. 2, a large number of coccus-shaped cells were observed in activated sludge samples of control and low MA exposed systems (i.e., 0.001, 0.01 and 0.10 mg/L). With the increasing dosage of MA, there was a difference in particle size of the activated sludge. The size of coccus-shaped cells increased, and the cellular outline became ambiguous, suggesting that the surfaces of cells were a little damaged by high concentrations of MA.

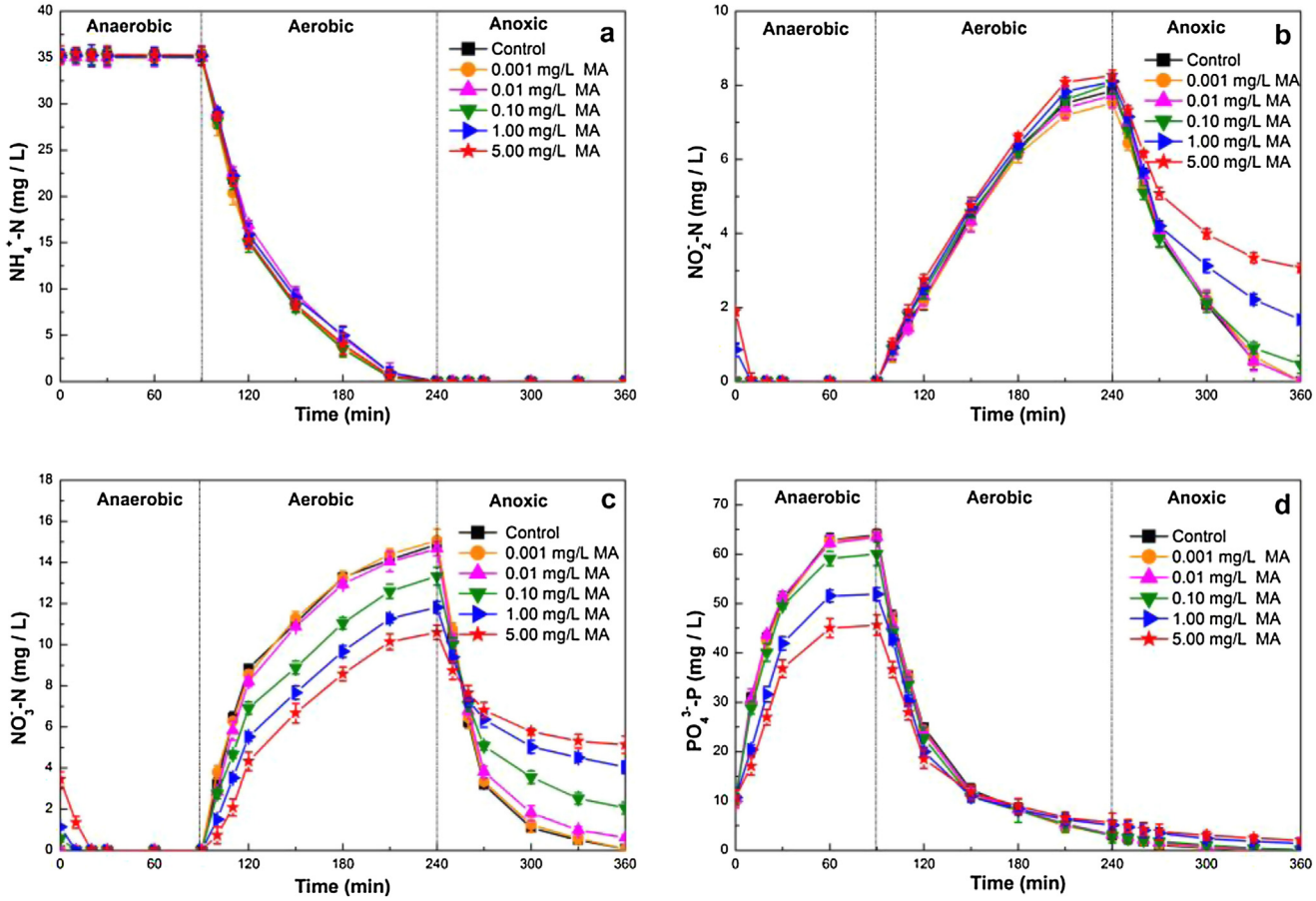

Fig. 3. Effects of MA on the variation of (a) NH_4^+-N, (b) PO_4^{3-}-P, (c) NO_2^--N and (d) NO_3^--N during a single cycle at different MA concentrations. (*Error bars represent standard deviations of triplicate measurements.)

In the literatures, exposure of most of contaminants was found to bring adverse influences in biological wastewater treatment. Likewise, it was observed that, long-term exposure of MA at low concentrations did not cause significant impacts, but high levels of MA resulted in inhibitory influences on biological nitrogen and phosphorus removal. In the following text the mechanisms of MA influencing on nitrogen and phosphorus removal were investigated.

3.2. Mechanisms of melamine affecting nitrogen and phosphorus removal

Fig. 3 shows the cycle transformations of nitrogen and phosphorus at the different MA concentrations. As seen in Fig. 3(a), the average removal efficiency of NH_4^+-N was almost 100% at any MA concentration investigated in this study. It is known that NH_4^+-N is oxidized to NO_2^--N and NO_3^--N under aerobic condition by ammonia oxidizing bacteria (AOB) and nitrite oxidizing bacteria (NOB) [29,30], then nitrate and nitrite were reduced ultimately to N_2, NO, or N_2O via denitrification under the anoxic condition. It can be seen that the low loading of MA (0.001–0.10 mg/L) in SBRs had negligible effects on denitrification. However, when the concentrations of MA increased to 1.00 and 5.00 mg/L, the activities of NOB with high MA exposure were lower than other reactors, which led to the higher NO_2^--N and lower NO_3^--N. At the end of aerobic period, the concentration of NO_3^--N in SBR5 and SBR6 were 11.82 ± 0.28 mg/L and 10.59 ± 0.34 mg/L, respectively. At the end of anoxic period, the NO_2^--N were 1.67 ± 0.10 mg/L and 3.07 ± 0.12 mg/L, respectively, while 4.05 ± 0.31 mg/L and 5.13 ± 0.42 mg/L of NO_3^--N were respectively determined in SBR5 and SBR6, suggesting that denitrification process was inhibited by high levels of MA.

Biological phosphorus removal depends on the alternative phosphate release and excessive phosphate uptake, respectively. Fig. 3(d) shows that in the anaerobic period, the concentrations of SOP were 51.89 ± 1.31 and 45.61 ± 2.04 mg/L when active sludge were exposed in 1.00 and 5.00 mg/L concentrations of MA, respectively. Both of them were lower than that in the control test (63.86 ± 1.18 mg/L). The result indicated that the process of phosphorus release was inhibited by high levels of MA. NO_2^--N and NO_3^--N were not completely consumed at the end of anoxic phase, which would enter into the anaerobic phase of next cycle. Thus denitrification process would happen and might affect the process of phosphorus release in the anaerobic period. Previous studies reported that the nitrate could affect biological phosphorus removal [31–33]. Under anaerobic period, both denitrification and phosphorus release needed organic substrate as the electron donor. It seems that there was a competition for available carbon source between denitrifiers and PAOs in anaerobic condition because of the denitrification and phosphorus release. The denitrification process is prone to select organic substrate as the electron donor, and the presence of nitrate had obvious negative impact on anaerobic phosphorus release. The phenomenon could be interpreted as that when nitrate was completely denitrified, more polyphosphate would be hydrolyzed to provide ATP and PAOs had more opportunity to utilize VFAs to synthesis PHA [33–35].

The comparison of SBR5 and SBR6 showed that denitrification and phosphorus removal proceeded simultaneously when nitrate existed at the beginning of anaerobic condition. The results indi-

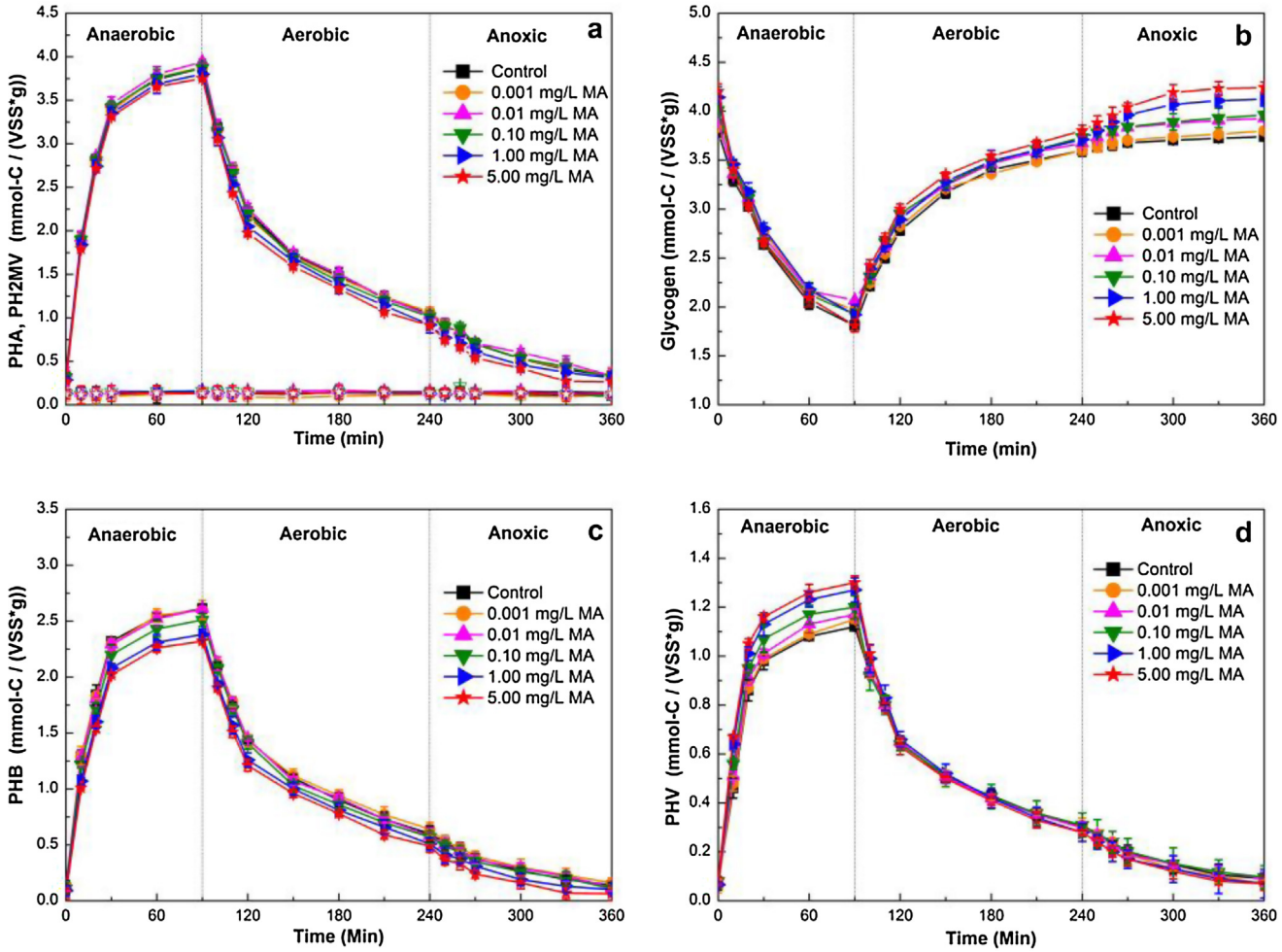

Fig. 4. Effects of MA on the variation of (a) PHA (solid) and PH2MV (open), (b) glycogen, (c) PHB and (d) PHV during a single cycle at different MA concentrations. (*Error bars represent standard deviations of triplicate measurements.)

cated that denitrifiers did not have an absolute priority over PAOs in the competition for electron donor, though the denitrification process would produce more ATP than polyphosphate cleavage under anaerobic period. Previous study pointed out phosphorus release would stop when the concentration of nitrate was higher than 0.5 mg/L [32], while in our study phosphorus release still happened in the presence of nitrate (3.45 ± 0.37 mg/L) in SBR6.

In the aerobic phase, biological aerobic phosphorus uptake is greater than the anaerobic phosphorus release, leading to the net phosphorus removal. The SOP concentration in SBR1 to SBR4 decreased to 3.04 ± 0.79, 3.24 ± 1.44, 3.21 ± 0.55 and 2.92 ± 1.40 mg/L at the end of aerobic phase, respectively. In high dosages of MA (SBR5 and SBR6), with the unsatisfactory anaerobic phosphorus release, the rates of phosphorus uptake were lower than other reactors, and the concentrations SOP dropped down to 5.08 ± 1.07 and 6.17 ± 1.85 mg/L, respectively.

In the anoxic phase, the concentrations of PO_4^{3-}-P in 5 MA addition reactors were respectively 0.02 ± 0.19, 0.02 ± 0.01, 0.11 ± 0.08, 1.45 ± 0.24 and 2.06 ± 0.23 mg/L, the concentrations in SBR5 and SBR6 were higher than other reactors (0.01 ± 0.02 mg/L in the control test). All the facts indicated that high levels of MA had a negative effect on phosphorus release and uptake. In the next text, the mechanism of how MA affects nitrogen and phosphorus removal was discussed.

3.3. Effect of melamine on nitrogen and phosphorus transformation and metabolism

It is well known that the biological phosphorus removal process is realized by phosphate release and excessive uptake in alternative anaerobic/aerobic (or anoxic) phases, which is related to the transformations of intermediates including intracellular polymer PHA and glycogen [19,36]. Anaerobically, the carbon source (volatile fatty acids, VFAs) can be transformed to polymers and stored in the cells as PHA, meanwhile intracellular polyphosphate is degraded to produce energy and release orthophosphate, and glycogen is converted to pyruvate and produced reducing equivalents (nicotinamide-adenine dinucleotide, NADH) [37–39]. Subsequently an opposite observation appears in the aerobic (or anoxic) zone. PAOs consume PHA, absorb phosphate, and simultaneously recover polyphosphate and glycogen [37]. Fig. 4 shows the variation of PHA (PHB, PHV and PH2MV) and glycogen in a cycle. As shown in Fig. 4(a), the variety of PH2MV was almost the same as the control test in the whole process no matter what the concentration of MA is. At low concentrations of MA, PHB and PHA had no significant variation, but the synthesis and decomposition of PHV and glycogen had an inconspicuous increase.

When the activated sludge were exposed to 1.00 mg/L and 5.00 mg/L of MA, the variations of PHA and glycogen were signif-

Table 3
Activities of the enzymes related to nitrogen and phosphorus removal in the different MA concentrations.

	AMO[a]	NOR[a]	NR[a]	NIR[a]	PPX[b]	PPK[c]
SBR1	0.025 ± 0.004	0.078 ± 0.002	0.053 ± 0.003	0.253 ± 0.005	0.024 ± 0.005	0.267 ± 0.004
SBR2	0.028 ± 0.003	0.077 ± 0.004	0.051 ± 0.002	0.252 ± 0.003	0.025 ± 0.003	0.265 ± 0.002
SBR3	0.027 ± 0.002	0.075 ± 0.003	0.050 ± 0.004	0.249 ± 0.004	0.023 ± 0.004	0.267 ± 0.003
SBR4	0.025 ± 0.003	0.072 ± 0.003	0.047 ± 0.003	0.247 ± 0.004	0.022 ± 0.003	0.266 ± 0.005
SBR5	0.026 ± 0.002	0.066 ± 0.005	0.041 ± 0.003	0.240 ± 0.002	0.017 ± 0.003	0.263 ± 0.003
SBR6	0.024 ± 0.003	0.057 ± 0.004	0.033 ± 0.004	0.235 ± 0.003	0.013 ± 0.004	0.259 ± 0.002

[a] The unit is μmol nitrite/(min mg protein).
[b] The unit is μmol *p*-nitrophenol/(min mg protein).
[c] The unit is μmol NADPH/(min mg protein).

icant in both aerobic and anoxic conditions. In Fig. 4(c) and (d), PHB synthesis rate was slow and even stopped at the beginning of anaerobic period, which was likely due to the competition of VFAs between denitrifiers and PAOs. However, the consumption of glycogen and the synthesis of PHV still proceeded at the same time. The inhibition of PHB synthesis did not cause an obvious decrease of PHA due to the increase in PHV. Subsequently in the aerobic phase, with the increasing concentration of MA, the amount of PHV increased. The rate of PHV consumption and glycogen regeneration were both higher than other reactors. Thus, the results showed that the activated sludge exposed to high MA concentrations could still utilize the synthesis PHA to take up phosphate and the recovery of glycogen could be improved under the condition of high dosage of MA. In the final anoxic condition, compared with the control test, the decrease of PHB almost stopped, but PHV was still consumed and glycogen was recovered in the presence of MA with high concentrations at high level. Consequently, in the whole process of a cycle, there were no significant influences when the dosages of MA were low. When the activated sludge was exposed to the high MA concentration, the biological nitrogen and phosphorus removal decreased in phosphorus release and PHB synthesis, but increase in PHV and glycogen synthesis and consumption.

Poly-phosphate, PHA and glycogen were three important storages in biological phosphorus removal. PHA synthesized to store energy under anaerobic phase and consumed for phosphorus uptake. Glycogen is regarded as the primary source of energy and a branched polyglucose carbon storage reserve which is synthesized via gluconeogenesis and degraded via glycolysis (thereby providing reducing equivalents) [40,41]. Furthermore, the transformations of PHA and glycogen in biological phosphorus removal system were dominated by PAOs and glycogen accumulating organisms (GAOs). GAOs are defined as the phenotype of organisms that consume glycogen (the only energy source) and take up VFAs to convert to PHA without phosphate release under anaerobic condition, and regenerate glycogen without accumulating polyphosphate under aerobic condition [42]. Therefore, the synthesis and degradation of glycogen is mainly associated with the activities of GAOs, and a higher transformation of glycogen is relevant to a higher metabolism of GAOs [19,43]. In the biological nitrogen and phosphorus removal system, GAOs were the presence of organisms that can potentially compete with PAOs for taking up VFAs without phosphorus removal [44–46]. PHAs is comprised of PHB (from 2 acetyl-CoA molecules), PHV (from one acetyl-CoA and one propionyl-CoA), and PH2MV (from 2 propionyl-CoA). There was also another major difference between PAOs and GAOs on PHA synthesis. When fed with acetate, PAOs took up acetate and converted to acetyl-CoA, mainly produced PHB but little PHV production. Glycogen metabolic mechanism under anaerobic condition could generate both acetyl-CoA (ED or EMP pathway) and propionyl-CoA (succinate-propionate pathway) [36,43,45]. GAOs could product approximately 75% PHB and 25% PHV (C-mmol basis), that is to say GAOs tended to product more PHV than PAOs [47,48].

According to the results observed from SBR5 and SBR6, the presence of nitrate preferentially utilized organic substrate for denitrification and led to the slow response of phosphorus release and PHB synthesis. Meanwhile, the high activity of GAOs enhanced glycogen consumption and PHV production. PAOs could use synthesis PHA to uptake phosphate aerobically even the rates were a little lower than the other 4 reactors. In the final anoxic condition, denitrification process was inhibited, thus there was a significant increase of glycogen in SBR5 and SBR6. As a result, high MA concentrations (1.00 and 5.00 mg/L) loading to the cyclic process could inhibit the activity of NOB in aerobic phase and denitrifiers in anoxic phase, accordingly affecting the anaerobic phosphorus release by PAOs in next cycle. As time goes by, the phosphorus removal capability of PAOs became worse gradually. GAOs prefer to consume VFAs than PAOs and turned into the dominant species.

Bacteria realized nitrogen and phosphorus removal from wastewater through the function of enzymes. The activity of enzymes was relevant to the system efficiency. The reactions of NH_4^+-N to NO_2^--N and NO_2^--N to NO_3^--N were closely related to AMO and NOR, respectively. NR and NIR were the key enzymes in denitrification which was respectively involved in the reaction of NO_3^--N to NO_2^--N and NO_2^--N to N_2. PPX and PPK were directly related to polyphosphate synthesis and decomposition. Table 3 shows the activities of six key enzymes in SBRs with the different concentrations of MA. There was no measurable impact on the key enzymes in the low dosage of MA (0.001–0.10 mg/L). Compared with the control test, the activity of PPX decreased in SBR5 and SBR6. As for the key enzymes relevant to nitrogen removal, no significant effect of MA on the specific activity of AMO was found. However, the higher the MA concentrations, the lower the activities of NOR, NR and NIR are. The activities of nitrogen metabolism enzymes measured were in agreement with the high concentrations of NO_2^--N and NO_3^--N in the effluent and the low efficiency of TN. It can be seen that the higher concentrations of MA could inhibit biological nitrogen removal because of the low activities of the key enzymes such as NOR, NR and NIR.

4. Conclusion

In SBR system, trace of MA (0.001–0.10 mg/L) in wastewater had negligible influence on the operation of SBR system. MA removed was mainly performed by activated sludge adsorption instead of biodegradation. However, SBR systems exposed to high levels of MA would be affected. The presence of 1.00 and 5.00 mg/L of MA decreased TN and SOP removal efficiencies and could not be easily removed but discharged along with effluent. It was found that the surfaces of cells were damaged by MA exposure at the high concentrations investigated because of the increasingly blurred cellular outline. Moreover, MA inhibited the enzyme activities of NOR, NR, NIR and PPX. It was also observed that the presence of high MA concentrations promoted the consumption and synthesis of glycogen, and provided the advantage for the growth of GAOs. This study

demonstrated a macro phenomenon that MA could be absorbed by the activated sludge. In the future, we will carry out in-depth study to analyze the absorption and desorption of MA, and estimate the influence on wastewater and sludge treatment systems.

Acknowledgment

This study was financially supported by the project of National Natural Science Foundation of China (NSFC) (Nos. 51278175, 51378188, and 51478170).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jhazmat.2016.10.036.

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