Journal of Hazardous Materials 179 (2010) 526–532

Contents lists available at ScienceDirect

Journal of Hazardous Materials

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

Performance of mesophilic anaerobic granules for removal of octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX) from aqueous solution

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ARTICLE INFO

Article history:
Received 11 November 2009
Received in revised form 10 February 2010
Accepted 6 March 2010
Available online 12 March 2010

Keywords:
Octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine
(HMX)
Degradation
Mesophilic anaerobic granules
Metabolic inhibitor
Enrichment culture

ABSTRACT

The performance of mesophilic anaerobic granules to degrade octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX) was investigated under various conditions. The results of batch experiments showed that anaerobic granules were capable of removing HMX from aqueous solution with high efficiency. Both biotic and abiotic mechanisms contributed to the removal of HMX by anaerobic granules under mesophilic conditions. Adsorption appeared to play a significant role in the abiotic process. Furthermore, HMX could be biodegraded by anaerobic granules as the sole substrate. After 16 days of incubation, 99.04% and 96.42% of total HMX could be removed by 1 g VSS/L acclimated and unacclimated granules, respectively. Vancomycin, an inhibitor of acetogenic bacteria, caused a significant inhibition of HMX biotransformation, while 2-bromoethanesulfonic acid, an inhibitor of methanogenic bacteria, only resulted in a slight decrease of metabolic activity. The presence of the glucose, as a suitable electron donor and carbon source, was found to enhance the degradation of HMX by anaerobic granules. Our study showed that sulfate had little adverse effects on biotransformation of HMX by anaerobic granules. However, nitrate had significant inhibitory effect on the extent of HMX removal especially in the initial period. This study offered good prospects of using high-rate anaerobic technology in the treatment of munition wastewater.

1. Introduction

Octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX) is of considerable industrial importance as the main raw material in the manufacturing of explosives and propellants [1]. As a heterocyclic compound with eight-membered ring, HMX shows higher stability and detonation power than other conventional explosives, such as 2,4,6-trinitrotoluene (TNT) and hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX). Large amounts of HMX waste from manufacturing, testing, and demilitarizing processes have resulted in widespread contamination of the environment [2]. The toxicity caused by such energetic chemical has been well documented in different aquatic and terrestrial species [3]. HMX has adverse effects on the central nervous system, and has been classified as a class D carcinogen by U.S. EPA [4]. Recently, concerns over HMX contamination have increased because this explosive has been produced and used in great quantities during the past decades and presented a significant risk for environment and human health. There is an urgent need for effective and safe treatment strategies for such toxic compound.

Biological removal of explosives has been proven feasible in many laboratory studies. Although HMX and RDX have similar chemical structures, HMX, with the unique heterocyclic ring and stable crown conformation, has shown more resistance to biological and chemical degradation than RDX [5]. In several previous studies, biodegradation of explosives has been demonstrated under aerobic conditions [5,6]. But these compounds have often been shown to be more recalcitrant under oxygen-enriched conditions compared to oxygen-limited environments [7]. As a more prominent alternative, biodegradation of HMX under anaerobic condition has been extensively reported [7–10]. The likely mechanism of HMX biotransformation was proposed to be similar to the sequential reduction process of RDX and TNT [11]. Although many researches on HMX elimination have been reported before, particularly on aspects relating to degradation pathways in pure bacterial cultures, investigations on anaerobic HMX removal in bioreactor systems are relatively limited in scope.

Anaerobic granules are widely used in various high-rate anaerobic treatment systems, such as upflow anaerobic sludge blanket (UASB) and expanded granular sludge bed (EGSB) reactors. These aggregates may degrade organic pollutants through synergetic

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doi:10.1016/j.jhazmat.2010.03.035

Table 1
Batch experimental conditions.

Batch	Sludge type	Sludge concentration	Additional substrate
HMX as sole substrate	Acclimated and unacclimated	1 and 2 g VSS/L	/
Effect of metabolic inhibitors	Acclimated	1 g VSS/L	0.17 mM Vancomycin and 50 mM BESA
Effect of additional electron donor	Acclimated	1 g VSS/L	1.25 mM Glucose
Effect of additional electron acceptors	Acclimated	1 g VSS/L	2 mM Na_2SO_4 and 3 mM $NaNO_3$

interactions among a variety of bacterial groups. Meanwhile, the layered granular structure can also protect anaerobic bacteria from being exposed directly to inhibitory and toxic compounds. High-rate anaerobic reactors, based on the well settleability and high activity of anaerobic granules, have many advantages over other conventional processes [12]. Previous studies have demonstrated that high-rate anaerobic systems were effective for the treatment of some polycyclic and heterocyclic aromatic hydrocarbons [13]. However, despite this prevalence and concern in the literature, few studies have focused on the high-rate anaerobic treatment technology of HMX pollutant. With large amounts of contaminated water, high-rate reactor treatment would be advantageous for field-scale cleanup of contaminated water and soil.

In this paper, we investigated the performance of anaerobic granules for HMX degradation under mesophilic conditions. Batch tests were conducted to determine the contributions of both physicochemical and biological processes involved in HMX removal. We studied the ability of anaerobic granules to degrade HMX under various conditions, so that the data can be used to develop anaerobic granule-based bioreactor technology, and further achieve complete removal of explosive compounds from wastewater.

2. Materials and methods

2.1. Biomass sources

The anaerobic granules were taken from a mesophilic UASB reactor treating food wastewater. The reactors had been in operation for 6 years with an organic loading rate of 5 kg COD/(m^3·d). The sludge aggregates were black and almost spherical, with the diameters between 0.45 and 2 mm. The sludge sources were elutriated to remove the fine particles and residual carbon source before use in the batch experiments.

2.2. Medium and growth conditions

Anaerobic granules were grown in serum bottles under strictly anaerobic conditions. The basal medium used for the study consisted of the following components (mM): NH_4Cl (1.2), K_2HPO_4 (0.2), $MgSO_4{\cdot}7H_2O$ (0.05), $CaCl_2{\cdot}2H_2O$ (0.05) and 1 mL trace elements, with composition modified from the descriptions of dos Santos [14], containing per litre (mM): H_3BO_3 (0.8), $FeCl_3{\cdot}6H_2O$ (1.1), $ZnCl_2$ (0.3), $MnCl_2{\cdot}4H_2O$ (0.5), $CuCl_2{\cdot}2H_2O$ (0.03), $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$ (0.02), $AlCl_3{\cdot}6H_2O$ (0.03), $CoCl_2{\cdot}6H_2O$ (1.7), $NiCl_2{\cdot}6H_2O$ (0.05), $Na_2SeO_3{\cdot}5H_2O$ (0.11), EDTA (1.6) and HCl 36% (0.02), buffered with 70 mM $NaHCO_3$ at a pH of around 7. The anaerobic granules were acclimatized with gradual increasing of HMX concentration for six months at 35 °C under anaerobic condition. The pH in the reactor was adjusted to 6.8–7.2 using $NaHCO_3$.

2.3. Abiotic removal experiments

In order to estimate the contribution of abiotic mechanisms to the removal of HMX, batch assays were performed under sterilized conditions to avoid the influence of HMX biodegradation. Since acclimation process may cause potential changes in physical and chemical properties of biomass [15], abiotic tests were conducted with acclimated and unacclimated granules at different concentrations. Heat-treated form of anaerobic granules was prepared by autoclaving the sludge at 120 °C and 110 kPa for 30 min. HMX was added by injecting a small volume of acetone stock solution, which contained HMX, into the empty 250 mL serum bottles. The organic solvent was removed by evaporation prior to the addition of the aqueous medium. Autoclaved acclimated and unacclimated anaerobic granules, at concentration of 1 and 2 g volatile suspended solids (VSS) per liter, were weighted into the bottles respectively, and then 100 mL of basal medium as described above was added. The serum bottles were closed with butyl rubber stoppers, flushed with high-purity nitrogen and incubated in a rotary shaker (120 rpm) in the dark at 35 °C. The initial concentration of HMX in the system was 16.2 μM. Blank controls were incubated without sludge for determining abiotic HMX conversions. The HMX concentration in solution was detected after 16 days. For the purpose of measuring HMX adsorbed by anaerobic granules, the wet granules were taken out and crushed by pushing them through a syringe and needle with descending diameter under anaerobic conditions, as described by Sipma et al. [16]. Residual HMX concentrations in granules for subsequent analysis were determined using U.S. EPA Method 8330 [17]. Additionally, batch adsorption experiments were also studied by varying the dose of granular sludge from 0.5 to 5 g VSS/L on an initial HMX concentration of 16.2 μM for a contact time of 24 h.

2.4. Biodegradation batch studies

Batch biodegradation experiments were performed under anaerobic conditions, with the same procedures as described above. Granules were added to 250 mL anaerobic serum bottles. The initial concentration of HMX in the solution was 16.2 μM for all the experiments. The medium composition was similar to that in growth culture, except $MgSO_4{\cdot}7H_2O$ was omitted in the assays to study the effect of electron acceptors. Four series of tests were conducted and the batch experimental conditions are summarized in Table 1. Autoclaved controls were used to determine the removal of HMX under abiotic conditions. Batch tests were performed in triplicate for each experimental condition to ensure the reproducibility of the results. Additionally, in the series for detecting gas end products with HMX as the sole substrate, nitrogen-contained compounds were omitted from the medium. The anaerobic culture bottles were flushed with helium to remove oxygen. HMX unamended and autoclaved controls were used to compare the results.

2.5. Analytical methods

HMX was analyzed using high-performance liquid chromatography (HPLC). The HPLC instrument, a Varian 210 HPLC system (CA, USA), was equipped with two solvent pumps and a C-18 column, 250 mm in length and 4.6 mm in internal diameter. A mobile phase consisting of methanol/water (50:50, v/v) was used at a flow rate of 1.0 mL/min. The explosive compounds were continuously monitored with a Model 320 programmable multi-wavelength ultraviolet detector set at 254 nm. Samples were collected periodically via syringe and needle. All samples were filtered through sterile, 0.2-μm-pore-size PTFE filters before injection. Gas products

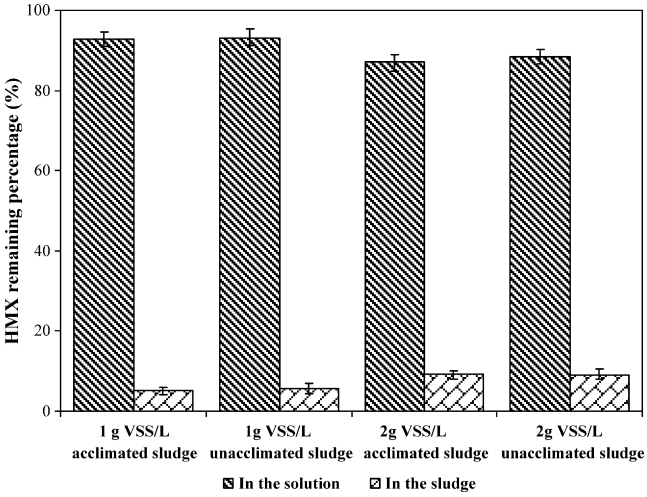

Fig. 1. Removal of HMX by anaerobic granules under different abiotic conditions.

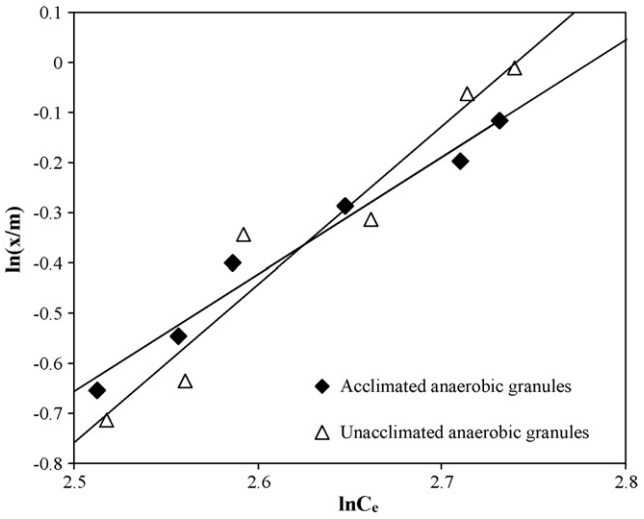

Fig. 2. Adsorption isotherms for HMX by acclimated and unacclimted anaerobic granules.

were also analyzed for N_2, N_2O, CH_4 and CO_2 by Agilent 6890 GC system coupled to thermal conductivity detector as described in detail [18,19]. Identification of the gas was confirmed by comparison with standard reference. VSS was analyzed according to APHA standard methods [20].

2.6. Chemicals

HMX was obtained from the Xi'an Modern Chemistry Research Institute (Xi'an, China) and had a purity of 99% or greater. Octahydro-1-nitroso-3,5,7-trinitro-1,3,5,7-tetrazocine (mononitroso-HMX) was obtained from SRI International (CA, USA). HPLC grade methanol was obtained from Fisher Scientific Co. (NJ, USA). Vancomycin and 2-bromo-ethanesulfonic acid (BESA) were obtained from Sigma Chemical Company (MO, USA). All other chemicals used were of reagent grade quality or higher.

3. Results and discussion

3.1. Abiotic removal of HMX with anaerobic granules

The remaining percentages of HMX at various abiotic conditions are illustrated in Fig. 1. In the aqueous solution, the HMX remaining percentages with 1 g VSS/L acclimated and unacclimated granules were 92.76% and 93.15%, respectively. In the corresponding sludge samples, the HMX remaining percentages were 5.06% and 5.79% for acclimated and unacclimated granules. It was obvious that the main part of remaining HMX was in solution, rather than in sludge. Furthermore, when the sludge concentrations were 2 g VSS/L, the remaining HMX in solution decreased in the tests with both kinds of granules, while HMX remaining percentages in sludge increased to 9.28% and 8.97% for acclimated and unacclimated granules, respectively. These results showed that autoclaved anaerobic granules could cause slight removal of HMX. No significant differences of remaining HMX were obtained between the series with acclimated and unacclimated granules. Additionally, a negligible amount of HMX loss was detected in sludge free experiments.

Physicochemical mechanisms, such as adsorption, hydrolysis, and oxidation, often have important effects on the removal of pollutants in wastewater treatment system. In our study, the decrease of HMX was observed under various sterile conditions, indicating abiotic removal may contribute to the deduction of HMX in system. Furthermore, although different distribution patterns of HMX were observed in these experiments, the total amounts of HMX in aqueous solution and in sludge were all above 96% of initial HMX concentration for each trial (Fig. 1). Therefore, it is assumed that the main mechanism for abiotic HMX removal is adsorption and chemical reduction is negligible. Additionally, the removal of HMX caused by alkaline hydrolysis reaction could be omitted within well-operating anaerobic reactors of near neutral pH.

In a previous study, it was reported that HMX decreased to 76% of its initial concentration after 115 days incubation with autoclaved marine sediment [10]. But in another study, organic adherence on livestock manure only resulted in a loss of approximately 4.8% of HMX over a 24-h period [21]. This difference of adsorption abilities is mainly due to different actual conditions of test systems. It is known that the anaerobic granules possess the potential for adsorption of aromatic compounds and heavy metals [13]. Anaerobic granules usually have weaker adsorption capacity as compared to that of flocculent sludge. This is because of the fact that granular sludge has less specific surface area and more compact structure where many potential adsorption places have been already occupied by the links of bacteria and inorganic particles. Moreover, it was shown in present study that there was little difference in HMX adsorption behaviors between acclimated and unacclimated granules, although acclimation process may cause potential changes in physical and chemical properties of biomass [15]. The information will be important for deciding the role of abiotic removal in suitable wastewater treatment process.

The adsorption studies were also conducted at a fixed initial concentration of HMX with varying sludge dose. The equilibrium data have been fitted into the linearized Freundlich adsorption isotherm, which is of the form:

$$\ln\left(\frac{x}{m}\right) = \ln K_f + \frac{1}{n}\ln C_e$$

where x is the amount of HMX adsorbed on the granular sludge (μmol), m is the weight of the granular sludge used (g VSS), C_e is the equilibrium concentration in aqueous solution (μM) and K_f and n are constants incorporating all factors affecting the adsorption process such as adsorption capacity and intensity, respectively. The result shows that the equilibrium data of HMX can be simulated by Freundlich isotherm quite well, with correlation coefficient more than 0.9 (Fig. 2). Freundlich constants K_f and n of acclimated sludge were found to be 1.51 μmol/kg VSS and 0.43, respectively. As for the unacclimated sludge, Freundlich constants K_f and n of were 0.18 μmol/kg VSS and 0.32, respectively. The results illuminated that sorption capacity of HMX on anaerobic granular sludge is limited. Under a constant temperature, the n values increased

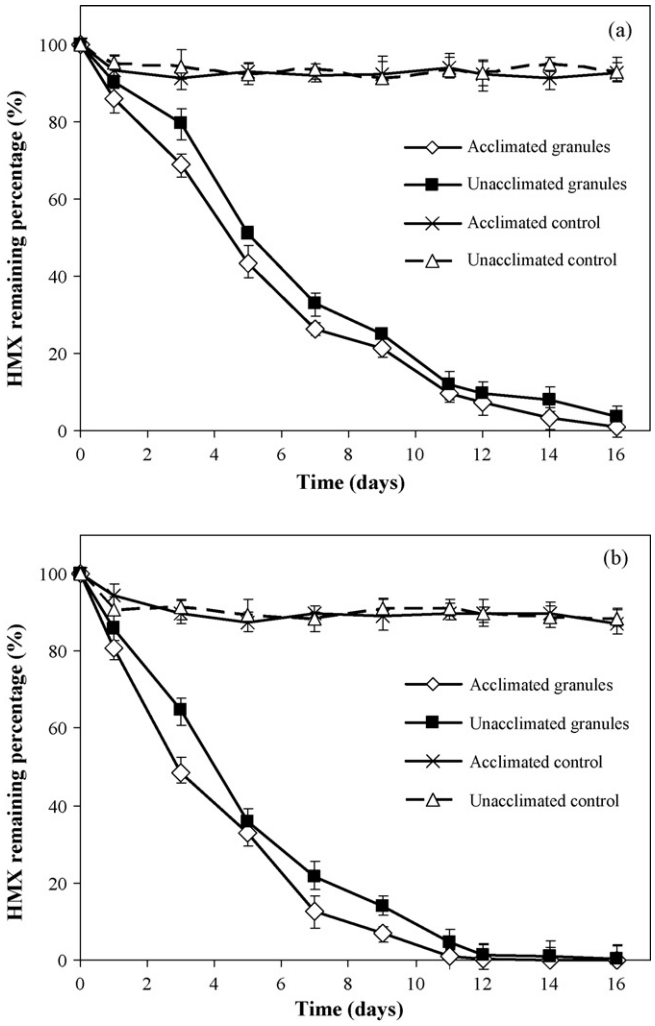

Fig. 3. HMX degradation by (a) 1 g VSS/L and (b) 2 g VSS/L anaerobic granules with HMX as the sole substrate.

with increasing adsorption energy, which implied that the larger the *n* value, the stronger the adsorption intensity [22]. The sorption capacity constants of unacclimated sludge are slightly less than those of acclimated sludge. The adsorption of HMX on acclimated granules was believed to be more favorable than that on unacclimated granules.

3.2. Degradation of HMX as the sole substrate

Anaerobic granules were evaluated for their ability to degrade HMX as a sole substrate. The effects of biomass concentration on HMX biodegradation were also investigated using acclimated and unacclimated granules at concentrations of 1 and 2 g VSS/L, respectively (Fig. 3). In Fig. 3(a), variations in HMX concentrations are presented for the vials containing 1 g VSS/L anaerobic granules. When HMX with an initial concentration of 16.2 μM was added to the bottle with acclimated cultures, about 99.04% of HMX was removed from the aqueous solution after 16 days of incubation. HMX was apparently eliminated without a lag period. Complete degradation of HMX could also be achieved with unacclimated cultures. However, the HMX removal rate was slightly lower than that carried out with acclimated granules, particularly in the beginning of the process. The final remaining percentage of HMX in the series with 1 g VSS/L unacclimated granules was 3.58%. The results showed that improved degradation could be obtained, if the biomass was subjected to a long acclimatization period before the tests. Similar trends were observed for unacclimated and acclimated granules at 2 g VSS/L, as shown in Fig. 3(b). When different sludge concentrations were used, more rapid removal of HMX was observed in the tests with higher biomass concentration. HMX in experiments containing 2 g VSS/L acclimated cultures was completely depleted within only 12 days. The degradation of HMX was enhanced by increasing the sludge concentrations for both acclimated and unacclimated incubations. Biodegradation of HMX with anaerobic granular sludge produced an array of products. Mononitroso-HMX was identified by comparing its HPLC retention time to authentic standard. No ^{15}N and ^{14}C-labeled HMX was available at the time of the present experiment, but the presence of end products was confirmed by comparing their GC retention times with reference standards. The result showed neither N_2O nor N_2 was observed in HMX unamended and autoclaved controls with helium. The formation of N_2O and N_2 during the degradation of HMX was considered to be mainly biological because the gas only appeared in the tests that contained HMX and live sludge. Furthermore, CH_4 and CO_2 were detected in the live series with and without HMX. No significant decrease in HMX was observed in the autoclaved controls after more than 16 days, indicating that the HMX removal in the living sludge was biologically mediated.

Many munition compounds are recalcitrant to biodegradation, especially for those heterocyclic explosives with firm carbon-nitrogen bond and nitro substituent. The biotransformation fate of TNT and RDX under anaerobic conditions has been investigated by a number of researchers. In contrast, less research has been performed on the anaerobic degradation of HMX. Our study showed anaerobic granules could degrade HMX and use it as the carbon source. Even after correcting the calculated abiotic reaction, the biotic removal efficiency appeared to be more than 89% with HMX as the sole substrate (Fig. 3). Compared with the previous application of sequencing batch reactors (SBR) [23], the anaerobic granules from UASB showed better performance in removing HMX from aqueous solution. Most of the previous work was mainly to enhance the activity of one specific group of microorganisms. Nevertheless, the different microbial consortia within anaerobic granules can present more suitable degradation ability than pure-culture isolation for the treatment of complex wastewater. A previous study suggested that the soil bacteria needed about 20 days acclimation period to metabolize HMX in contaminated soil [24]. But in our study, it was shown biodegradation of HMX could be carried out by granules without prior acclimation, although acclimation may slightly improve the degradation rate (Fig. 3). HMX at the concentration of 16.2 μM has no toxic effect on metabolic activity of granular sludge. Furthermore, the result indicates that HMX can act as potential electron acceptor in this process, as evidenced by the removal of HMX and the transient appearance of nitroso groups. Under aerobic conditions, it is difficult to obtain a high HMX removal efficiency because oxygen, as a more effective electron acceptor, could compete with HMX and lead to less transformation of HMX. When HMX was the sole substrate under anaerobic conditions, however, better degradation was obtained because HMX was generally the sole terminal electron acceptor in the system. Additionally, the biomass dosage is an important parameter because this determines the degradation capacity of anaerobic granules for a given initial HMX concentration. The ability to retain high amounts of biomass could allow anaerobic granular system to achieve higher HMX removal rates than reported for other nongranular systems.

The removal of HMX was accompanied with reductive conversion of the nitro to nitroso group. Such initial enzymatic reactions could destabilize the ring structure and lead to ring collapses to generate small aliphatic metabolites. These products may in turn be utilized by the various microorganisms in anaerobic granular sludge. Also, some of direct ring-cleavage intermediates are

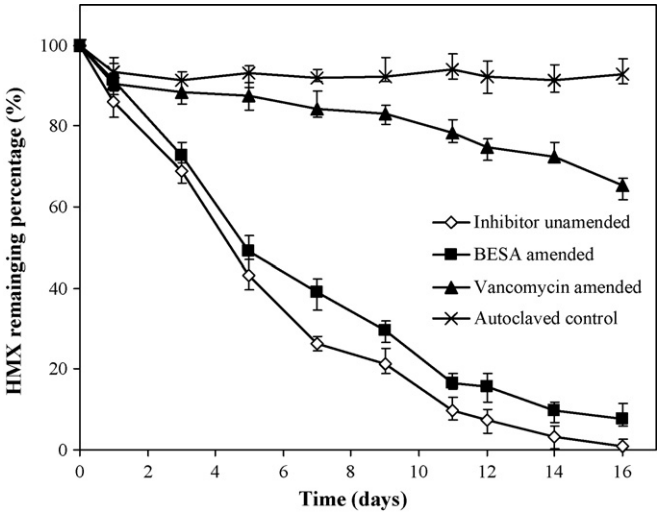

Fig. 4. Effect of different metabolic inhibitors on HMX biodegradation by anaerobic granules.

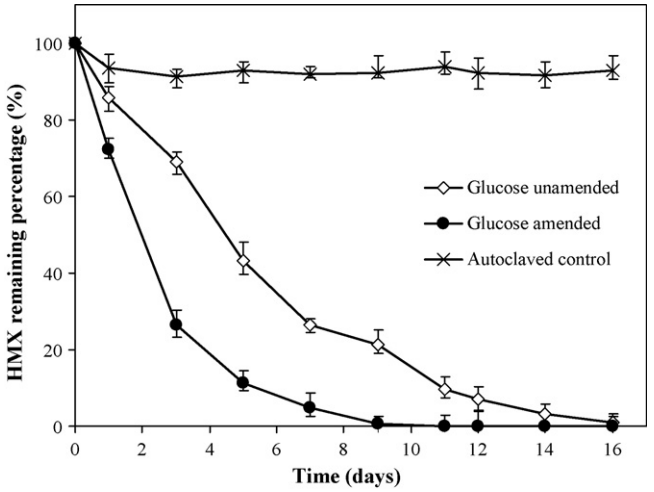

Fig. 5. Effect of additional electron donor on HMX biodegradation by anaerobic granules.

thought to be unstable and spontaneously decompose in water [11]. Favorable mineralization of HMX might be achieved in the system with anaerobic granules. We have no solid evidence on the possible direct ring-cleavage pathway since similar end products may also be generated through sequence reduction pathway. Also the sludge used in the present study is expected to contain a complex microbial community that can provide a wide range of enzymes, which might lead to more than one formation route during HMX incubation with anaerobic granular sludge.

3.3. *Effect of metabolic inhibitors on HMX biodegradation*

Batch experiments were conducted to study the roles of acetogens and methanogens in anaerobic granules. Typical removal patterns obtained in these tests are presented in Fig. 4, which describes changes of HMX in the environments with different metabolic inhibitors. Vancomycin, an inhibitor used to inhibit acetogenic bacteria [25], almost completely inhibited the transformation of HMX. After 16 days of incubation, the addition of 0.17 mM vancomycin to the vials resulted in degradation of only 34.67% of total HMX as compared to 99.04% in the absence of vancomycin. The degradation process started with an apparent lag phase in the presence of vancomycin. Meanwhile, the role of methanogens in the degradation of HMX was also evaluated by addition of BESA, a potent methanogenic inhibitor [26]. In contrast, the addition of BESA caused slight inhibition in the treatment of HMX. During the course of the 16-day incubation period, HMX was depleted to 7.58% of its initial concentration under the conditions with BESA, while the HMX concentration in vancomycin amended series reached 65.33% of its initial concentration at the same time.

Anaerobic sludge consists of various groups of bacteria which may be involved in the multistep degradation process, including hydrolysis, acidogenic fermentation, acetogenesis, and methanogenesis. Particularly, anaerobic granules have high biomass content, which is enriched with acetogenic and methanogenic bacteria. The study about their roles during the metabolic process would help to understand the fate of HMX in anaerobic granules. Degradation of HMX was significantly inhibited in sludge amended with vancomycin, a specific inhibitor of cell wall synthesis in Gram-positive eubacteria [25]. As a matter of fact, our results reveal that acetogenic bacteria play a major role in the degradation of HMX by anaerobic granules. Adrian and Arnett [7] proposed that acetogens may be responsible for the explosives transformation in a methanogenic enrichment culture amended with ethanol. However, acetogens are unlikely to be the only group contributing to HMX metabolism within anaerobic granules, because the presence of vancomycin did not appear to completely inhibit HMX elimination. We can hypothesize that the non-acetogenic bacteria are involved in HMX biotransformation.

Furthermore, the degradation of HMX was also slightly inhibited in the presence of BESA, an inhibitor for methanogenesis in the biodegradation process. The results presented in this study are in contrast to previous studies by Beller [26] who reported that the addition of BESA did not affect RDX degradation. In anaerobic granules, methanogenic bacteria may convert hydrogen, carbon dioxide and acetic acid to methane. If methanogenesis is inhibited, numerous other fermentation products may be formed and accumulated. The high volatile fatty acid accumulation could eventually lead to retardation of the treatment process in the high-rate anaerobic reactors [27]. There is no proof that methanogenic bacteria have the direct role in the cleavage of HMX, but data presented here show that inhibition of methanogenic bacteria might result in inactivation of the continuous digestion process. Therefore, the performance of methanogenic bacteria will also influence the overall anaerobic metabolism of HMX. Although both of acetogenic and methanogenic bacteria can have important impacts on the HMX biodegradation, none of the above individual species in the anaerobic granules is capable of completely degrading HMX. Different mechanisms may play a role in the removal of HMX by granular sludge. The metabolic products of some microbes could provide energy and carbon for other strains to meet their energy requirements. A consortium of various bacteria in anaerobic granular sludge could facilitate the complete degradation of HMX through competitive and synergetic interactions.

3.4. *Effect of additional electron donor*

In this study, glucose, a highly degradable carbon source, was evaluated for its ability to stimulate the transformation of HMX in anaerobic granules. Fig. 5 illustrates the batch test results using HMX as feed and glucose as the additional electron donor. It was found that a glucose supplement of 1.25 mM apparently promoted biodegradation of HMX. Removal of HMX appeared to occur rapidly and without a lag. In the absence of additional electron donor, the sludge decreased HMX to 6.99 μM, 43.17% of its initial concentration after 5 days. But when glucose was present in growth medium, the sediment decreased HMX concentration to 1.82 μM, 11.24% of

its initial concentration after 5 days, indicating that supplementation of glucose significantly improved HMX removal. Finally, it took about 9 days to completely remove the HMX with glucose supplement compared to 16 days without glucose supplement. The presence of the glucose cosubstrate, although not a prerequisite, enhanced and improved HMX degradation. In the present case, glucose was the suitable electron donor to sustain the remediation of HMX.

The presence of a suitable electron donor is necessary to stimulate the anaerobic biodegradation of organic compounds. The results of our batch tests showed that the rate of HMX degradation was enhanced significantly by the addition of glucose (Fig. 5). Such enhancement effect could be due to several reasons. On one hand, the metabolism of glucose by the microorganisms in granules could generate low-potential electrons, similar to what has been found for the degradation of RDX [28]. The addition of an organic electron donor could help to create the anaerobic and reducing conditions for the development of anaerobes. During the anaerobic digestion, the production of hydrogen, formate, ethanol, propionate, or acetate could serve as the source of reducing equivalents and electron donors required for the biodegradation of explosives [26,28]. On the other hand, this result suggests that anaerobic granules can use glucose as carbon and energy source. The supplemental substrate in aquatic solution could also activate enzymes that degrade HMX and support the microbial growth that the lower solubility of HMX cannot sustain. Glucose can support the HMX reduction with high biotransformation efficiency, indicating that glucose is a suitable cosubstrate for HMX degradation in anaerobic granules. Similar results were also observed when suitable substrates were added to enhance the biotransformation of RDX and HMX in the explosive-contaminated soil [7].

Supplementing additional substrate may be a promising approach to enhance the microbial degradation process, particularly for the oligotrophic nature of many munition-contaminated environments. Additionally, biodegradation of RDX and HMX is often attributed to cometabolism in the presence of a primary carbon source [23]. However, the stimulatory process found in our experiments is not be simply explained by the cometabolic effect because anaerobic granules could also degrade HMX completely without the addition of glucose. The results presented herein suggest that suitable alternative substrates have a very significant effect in enhancing the reductive biotransformation of explosives. The stimulation of microbial degradation with additional electron donors is also the feasible remediation strategy for anaerobic bioreactor operation.

3.5. Effect of additional electron acceptors

The sulfate and nitrate may play important roles as electron acceptors and influence the anaerobic digestion process. At the concentration of 2 mM, the presence of sulfate had little adverse effect on the amount of HMX transformed (Fig. 6). For example, in the tests without sulfate addition, almost 56.83% of HMX was transformed after 5 days; when 2 mM sulfate was added, 52.32% of HMX was transformed. However, the observed degradation pattern of HMX under nitrate-enriched conditions was in turn different from those observed under sulfate-enriched conditions. As shown in Fig. 6, for the incubation supplied with 3 mM nitrate, enriched mixed cultures only removed 31.01% of 16.2 μM of HMX within the first 5 days. With the nitrate-enriched operating mode, it was also observed to obtain a high final concentration of 8.93% of initial HMX after 16 days. The extent of HMX removal was significantly less in nitrate-amended culture than in nitrate-unamended culture.

Since different electron acceptors, such as sulfate and nitrate, are often present in the munition wastewater environments, it is important to study the effects of different electron acceptors

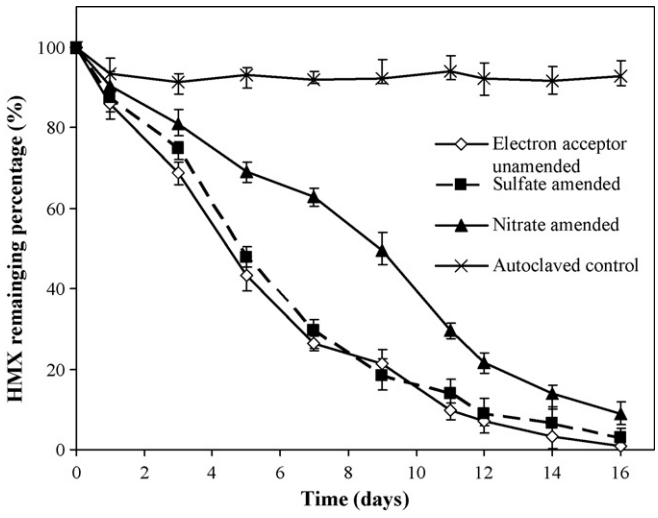

Fig. 6. Effect of additional electron acceptors on HMX biodegradation by anaerobic granules.

on biodegradation of explosive compounds. There have been few studies in which degradation of HMX by mixed cultures under different electron acceptor conditions has been examined. In a previous study, TNT degradation by anaerobic soil bacterial consortium could be enhanced by the addition of sulfate and nitrate as electron acceptors, while TNT removal completely ceased in the absence of additional sulfate and nitrate even after 60 days of incubation [29]. However, our study showed sulfate had slightly negative effects on biotransformation of HMX, while nitrate had significant inhibitory effect on the extent of RDX removal especially in the initial period. Inhibitive effects were also observed for RDX biotransformation in the presence of nitrate [26]. Although the removal process was inhibited in the presence of sulfate and nitrate, the complete degradation of HMX could still be achieved after a period of incubation in our study. These results suggest that the anaerobic granules contain significant numbers of nitrate reducing and sulfate-reducing bacteria. Sulfate and nitrate may compete with HMX, as an electron acceptor, for the reducing equivalents available, thus causing an adverse effect on the HMX degradation process. Nitrate is an energetically much more favorable electron acceptor than sulfate [30]. Moreover, anaerobic granules are a mixed microbial system with various groups of anaerobes. Many sulfate-reducing bacteria could also use nitrate rather than sulfate as the terminal electron acceptor [31]. Therefore, nitrate makes a relatively higher contribution to the inhibition effects than sulfate. These results corroborate the earlier findings that the removal of HMX under sulfate-reducing condition is higher than that under nitrate reducing condition [32]. Furthermore, in this study we have also demonstrated that metabolism of HMX was not affected in the absence of sulfate and nitrate. Hence, this eliminates the need for sulfate and nitrate additions for HMX metabolism in a high-rate treatment system using anaerobic granules.

4. Conclusion

The results obtained in this study show that the anaerobic granules could remove HMX with high efficiency. Both biotic and abiotic mechanisms may contribute to the remediation of HMX by anaerobic granules under mesophilic conditions. Anaerobic granules are capable of degrading HMX and using it as the sole substrate. Furthermore, the experiment using inhibitors indicates acetogenic bacteria could play a major role in the remediation, while non-acetogenic bacteria are also involved in this process. HMX degradation can be enhanced significantly by the addition of glu-

cose, which not only provide primary electron donor in microbial respiration, but also supply the high maintenance requirements of the anaerobic microorganisms. However, our study showed sulfate had little adverse effects on biotransformation of HMX, while nitrate had significant inhibitory effect on the extent of RDX removal especially in the initial period. Therefore, the present study will be useful in understanding the mechanisms about how anaerobic granules can be deployed to effectively treat HMX and other challenging explosive compounds. The results can potentially be applied to the design and optimization of high-rate anaerobic systems for the elimination of HMX in industrial effluents and contaminated groundwater.

Acknowledgements

This research was supported by the Major State Basic Research Development Program of MOST (2006CB403307 and 2005CB724200). We are also grateful for anonymous reviewers for their helpful suggestions and advices.

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