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Wat. Sci. Tech. Vol. 36, No. 2–3, pp. 139–146, 1997.
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Printed in Great Britain.
0273–1223/97 $17·00 + 0·00

PII: S0273-1223(97)00380-6

AEROBIC AND ANAEROBIC BIODEGRADATION OF NITROGLYCERIN IN BATCH AND PACKED BED BIOREACTORS

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ABSTRACT

Glycerol trinitrate (GTN) is a contaminant commonly found in the waste streams of munitions manufacturing and pharmaceutical plants. It is a highly toxic substance harmful to humans and other living organisms. In this study, the bioconversion of GTN was examined under aerobic conditions using mixed bacterial cultures and *Phanerochaete chrysosporium*, and under strict anaerobiosis using anaerobic digester sludge. Batch reactor experiments indicated that activated sludge, *P. chrysosporium*, and anaerobic digester sludge can completely denitrate GTN via a mechanism which forms isomeric glycerol dinitrate (GDN), glycerol mononitrate (GMN) and a utilizable carbon source, most likely glycerol. The rate of bioconversion in the aerobic and the anaerobic systems was dependent upon the concentration of cosubstrate. Continuous flow experiments in immobilized bed reactors indicated that anaerobic degradation of GTN can achieve high destruction efficiencies of the parent compound (99.9%) and the intermediate metabolic nitrate ester compounds (GDN, GMN) at relatively low cosubstrate requirements compared to the aerobic reactors. © 1997 IAWQ. Published by Elsevier Science Ltd

KEYWORDS

Aerobic; anaerobic; batch reactor; biodegradation; nitroglycerin; packed bed reactor.

INTRODUCTION

Nitroglycerin ($C_3H_5(ONO_2)_3$) is a powerful explosive used in various gun and rocket propellant formulations. In addition to military applications, the physiological and therapeutic effects of nitroglycerin have been widely used for the treatment of blood pressure and heart diseases. It is a pale yellow viscous liquid with a vapor pressure of 0.00026 mm of Hg at 20°C and a solubility of 1.25 g/liter (The Merck Index, 1989). It can be readily adsorbed through the skin and lungs. Prolonged exposure to nitroglycerin may cause nausea, vomiting, cyanosis, palpitations of the heart, coma, cessation of breathing, and even death. Therefore, waste streams contaminated with nitroglycerin cannot be directly disposed in the environment without prior treatment.

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Process streams contaminated with nitroglycerin can be treated chemically with strong acid or digested using a base to form partially denitrated GTN, glycidol and glycidyl nitrate, other carbon compounds, nitrate and nitrite (Kaplan *et al.*, 1982). The post hydrolysis liquor is rich in BOD and nitrogen and may require further treatment prior to final disposal. Moreover, glycidol and glycidyl nitrate are found to be mutagenic and must be destroyed (Kaplan *et al.*, 1982). It is therefore critical to develop treatment methods which are capable of degrading GTN and its intermediates in an environmentally sound manner.

Biodegradation is one of the most promising treatment methods for the destruction of GTN and its metabolic byproducts namely glycerol 1,2-dinitrate (1,2-GDN), glycerol 1,3-dinitrate (1,3-GDN), glycerol 1-mononitrate (1-GMN) and glycerol 2-mononitrate (2-GMN) (Christodoulatos *et al.*, 1995). Wendt *et al.* (1978) have investigated the possibility of microbial degradation of nitroglycerin by activated sludge under aerobic conditions. Two isomers of GDN and one isomer of GMN were detected during their studies. Based on these results, they postulated a microbial aerobic degradation pathway where bioconversion proceeds via successive denitration of GTN to glycerol. Several other authors (Kaplan *et al.*, 1982; Ducrocq *et al.*, 1989; Servent *et al.*, 1992; Pesari and Grasso, 1993; White *et al.*, 1996) examined the biodegradation of GTN under aerobic and anoxic conditions using different bacterial and fungal species. However, complete mineralization of nitroglycerin has not been reported in any of these studies and a primary carbon source was required for denitration to commence. Since aerobic biodegradation does not occur in the absence of a cosubstrate, requiring a primary carbon source, GTN is believed to be an inhibitory nongrowth substrate (Pesari and Grasso, 1993). In addition, the intermediate biotransformation products 1,2-GDN and 2-GMN are found to be mutagenic (Ellis *et al.*, 1978) and their complete destruction is critical to the performance of any treatment method.

Although the aerobic biodegradation of GTN has been studied by several investigators, very little information exists in the literature on anaerobic degradation of this compound. Smets *et al.* (1995) examined the thermodynamic feasibility of GTN biodegradation, adopting the degradation pathway proposed by Wendt *et al.* (1978), and they concluded that complete degradation can be attained under both aerobic and anaerobic conditions without addition of external carbon and nitrogen sources. Experiments conducted in our laboratory (Christodoulatos *et al.*, 1995) in microcosm vials, suggested that GTN and its metabolites are amenable to microbial attack under strict anaerobiosis and that complete mineralization is possible.

This paper presents the results from aerobic and anaerobic experiments in completely mixed batch and in continuous immobilized bed bioreactors. The aerobic studies were performed with activated sludge and the fungus *P. chrysosporium*. The anaerobic studies were conducted with microorganisms from an anaerobic digester. The batch experiments demonstrated that the microorganisms tested were able to completely denitrate GTN but at different rates and cosubstrate requirements. Subsequently, three immobilized bed reactors were operated in a continuous mode. The packed bed reactor studies indicated that nitroglycerin and its intermediates can be effectively removed, provided the retention time is sufficiently high to allow for the desired destruction of the more persistent di- and mononitrates Glycerol dinitrate and glycerol mononitrate may escape treatment in conventional biological systems, such as activated sludge, under typical operating conditions. It would therefore be prudent, from a practical and economical perspective, to pretreat in-process streams prior to their discharge in the main treatment system. BOD is usually present in sufficient quantities and addition of cosubstrates would not be required in commercial applications of the system. The immobilized bed bioreactor technology, developed for the destruction of nitroglycerin, is especially suitable for relatively small volumes of wastewater generated from individual process streams and for compounds which form intermediates of low biodegradability relative to the parent molecule.

MATERIALS AND METHODS

Bacterial and fungal growth media were used as described elsewhere (Christodoulatos *et al.*, 1995; Pal, 1993). The mixed aerobic (activated sludge) and anaerobic (digester sludge) bacterial cultures were obtained from a nearby municipal wastewater treatment plant (Linden, NJ). *P. chrysosporium* was obtained from American Type Culture Collection (ATCC). Nitroglycerin and its biotransformation intermediates were quantified by high pressure liquid chromatography (HPLC) using a liquid chromatograph equipped with

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solvent pump, autosampler, diode array detector (Varian Instruments Co., Palo Alto, CA) and a Partisil 10 ODS-3, 4.6 x 250 mm (Whatman Inc., Clifton, NJ) column at room temperature. A mobile phase containing 70% acetonitrile and 30% deionized (DI) water at a flow rate of 1 ml/min was used and nitroglycerin was detected at 210 nm. The intermediates were detected at a wavelength of 205 nm using a mobile phase of 5% acetonitrile and 95% DI water at a flow rate of 1 ml/min.

Batch aerobic experiments with *P. chrysosporium* and activated sludge were carried out in 250 ml Erlenmeyer flasks. About 150 ml of growth media along with GTN and glucose were added to each flask. The flasks were inoculated with the respective cultures and incubated in a gyratory shaker at 30°C. The initial GTN concentration was 97 mg/l and glucose was added at an average rate of 55 mg/day to sustain the degradation process. All flasks were prepared in duplicate. Samples were withdrawn periodically for analysis of GTN and intermediates. Batch aerobic experiments with mixed bacterial culture were also carried out in a batch fermenter (New Brunswick Scientific, NJ) in order to minimize oxygen transfer limitations. The fermenter is equipped with temperature and dissolved oxygen controllers. About 2 liter of growth media along with GTN and glucose were placed in the fermenter and inoculated. The initial GTN concentration was 120 mg/l and glucose addition was maintained at 643 mg/day. The temperature was set to 30°C. Samples were withdrawn periodically for analysis of GTN and intermediate products. The batch anaerobic experiments were carried out in 160 ml serum vials. The growth media was purged with nitrogen and all vial manipulations were performed in a glove box in an oxygen free atmosphere. The vials were sealed using teflon coated septa and incubated in a gyratory shaker at 30°C, rotating at 200 rpm. The microcosms were amended with 1000 mg/l of glucose and the initial GTN concentration was 163 mg/l. The serum vials were prepared in duplicate. Samples were periodically withdrawn using a syringe and analyzed for GTN and intermediates.

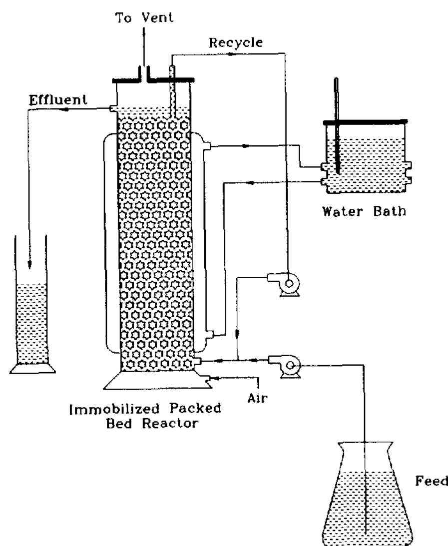

Figure 1. Schematic of the flow through packed bed experimental setup.

A schematic of the packed bed bioreactor, utilized in the continuous experiments, is shown in Figure 1. Three jacketed tubular bioreactors (Curtin Matheson Scientific, Inc., Houston, TX) with 2 inch internal diameter and an effective volume of 0.54 liter, were filled with 1/6 inch plastic flakes resulting in a porosity of about 0.69. The plastic flakes provided the surface area required for microbial attachment. Reactors 1 and 2 were inoculated with activated sludge and *P. chrysosporium* respectively. The third reactor was operated anaerobically. Peristaltic pumps were connected to the reactors to control the influent flow rate and the internal recycle. To ensure complete mixing, the reactor liquor was recirculated at an internal rate at least 10 times higher than the feed rate. All reactors were maintained at 30°C. Air was supplied from the bottom of

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the reactor. Initially only glucose was supplied to the reactors until sufficient biomass was accumulated on the packing. The reactors were operated under various retention times, and glucose-to-nitroglycerin ratios in the feed. The effluent was monitored daily for GTN, metabolic intermediates and glucose. Glucose was analyzed by the ortho-toluidine method (Zender, 1963).

RESULTS AND DISCUSSION

Bioconversion in batch reactors

The concentration-time profiles for GTN and the produced intermediates are shown in Figures 2, 3, and 4 for activated sludge, *P. chrysosporium*, and anaerobic cultures, respectively. Each point on these graphs represents the average value of duplicate experiments. The concentration of nitroglycerin in the activated sludge flasks dropped below the instrument detection limits (1 mg/l) at the 6th day. The concentration of the dinitrate isomers reached their maximum concentration after four days of incubation and were completely degraded after 23 days. The 1,3-GDN isomer accumulated at higher concentrations than those expected by random attack on the primary and secondary nitro groups of GTN indicating preferential cleavage of the secondary carbon. This regiospecificity at the secondary position was also observed during the second denitration step favoring production of 1-GMN. The preferential attack at the central carbon was also verified in separate experiments with 1,2-GDN where the ratio of 1-GMN to 2-GMN formed was about 2.5. High biomass concentration, due to continuous addition of glucose, resulted in insufficient dissolved oxygen which reduced biomass activity and degradation of the GMN isomers ceased. To overcome the oxygen transfer limitations associated with high biomass concentrations in shaker flasks, aerobic experiments were conducted in a chemostat with continuous aeration. The concentration history of combined glycerol nitrates (GTN+1,2-GDN+1,3-GDN+1-GMN+2-GMN) in the chemostat is depicted in Figure 2. Complete denitration was achieved after 42 days.

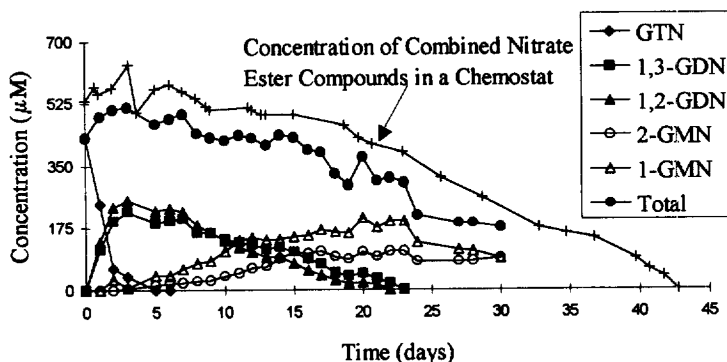

Figure 2. Bioconversion of GTN and intermediates in shaker flask and Bioflo reactor using activated sludge.

The bioconversion of GTN by *P. chrysosporium* followed a similar pathway, as illustrated in Figure 3. The rates of conversion appear to be lower although the external carbon source was supplied at approximately equal rates, 55 mg/d. GTN completely disappeared after 19 days and the GDN and GMN isomers were still present after 30 days of incubation. These concentration profiles suggest that in order to attain denitration efficiency comparable to that obtained by activated sludge longer reaction times or higher cosubstrate concentrations are required.

The anaerobic bioconversion history of GTN is shown in Figure 4. Only 1 g/l of glucose was initially added to this system. Complete denitration was achieved after 45 days of incubation. Complete disappearance of GTN occurred on the 7th day. The GDN isomers reached a maximum concentration at the 6th day. Complete degradation of 1,2-GDN and 1,3-GDN occurred after 18 and 27 days of incubation, respectively.

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The mononitrate isomers were completely denitrated on the 45th day. As in the case of aerobic degradation, production of 1,3-GDN and 1 GMN appears to be favored indicating enzymatic regiospecificity at the central nitro group of GTN.

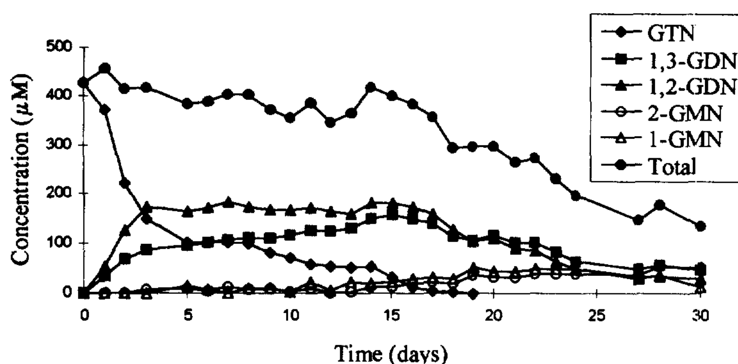

Figure 3. Bioconversion of GTN and intermediates by *P. chrysosporium* in a shaker flask.

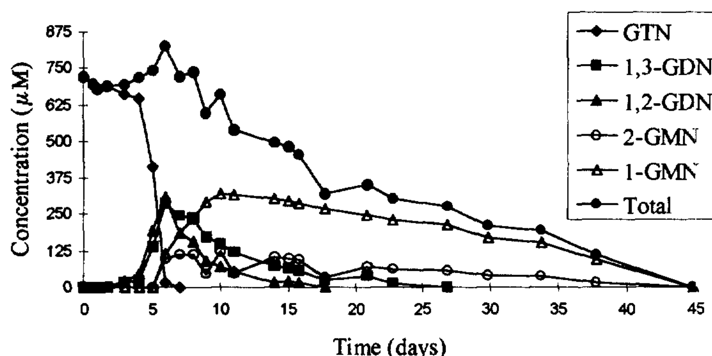

Figure 4. Anaerobic bioconversion of GTN and metabolites in a serum vial.

The results obtained from the batch reactor experiments indicated that GTN was converted simultaneously and concomitantly to isomers of glycerol dinitrate and glycerol mononitrate by all three cultures. More specifically, the first denitration produced isomeric 1,3-GDN and 1,2-GDN, the second denitration formed isomeric 1-GMN and 2-GMN which were subsequently converted most likely to glycerol (Meng *et al.*, 1995). This general degradation pathway appeared to be similar in both the aerobic and anaerobic degradation systems. Both the aerobic and anaerobic microorganisms exhibited substantial regiospecificity at the first and second denitration steps with preferential denitration of the central carbon which favored production of 1,3-GDN and 1-GMN. The rates of denitration were substantially lowered at each denitration step. The batch experiments demonstrated, for the first time, that complete denitration of nitroglycerin is possible in aerobic and anaerobic environments. The formed intermediates, which have higher solubility than the parent compound and potentially higher toxicity, are also biodegradable. The rate of GTN bioconversion was found to depend on the concentration of the cosubstrate and to be controlled by the last denitration step. Aerobic degradation occurs only in presence of a cosubstrate and it has substantially higher cosubstrate requirements than the anaerobic process where denitration continues even after the cosubstrate is depleted. The observed glucose requirement per mg of GTN for activated sludge, *P. chrysosporium* and digester sludge were 465, 407 and 6 mg respectively.

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Bioconversion in packed bed reactors

Five experimental runs were conducted using packed bed reactors operating in a continuous mode. Runs 1 and 2 correspond to the activated sludge and *P. chrysosporium* cultures, respectively and Runs 3, 4, and 5 were executed in the anaerobic system. Effluent concentration profiles obtained from Runs 1, 2 and 3 are shown in Figures 5, 6, and 7. The operating conditions, feed and effluent compositions for each run are summarized in Table 1. The aerobic reactors achieved steady state within 8 retention times whereas 5 retention times were required for the anaerobic reactor. The steady state effluent glucose concentration was below the method detection limit of 20 mg/l in all runs.

Comparing the results obtained from Runs 1, 2, and 3 it can be concluded that the *P. chrysosporium* reactor achieved the highest conversion efficiency with respect to GTN (99%) and the highest overall conversion (33%) with respect to total nitro-compounds (GTN + intermediates). Under identical glucose-to-GTN feed ratio and retention time, the activated sludge system exhibited 91% conversion with respect to GTN and only 1% overall conversion. The anaerobic reactor had a GTN conversion efficiency of 91% and an overall denitration of 25%, however, it was operated at a glucose-to-GTN feed ratio of half that of the aerobic systems. Glycerol 1,2-dinitrate and glycerol 1-mononitrate effluent concentrations in the aerobic reactors were significantly higher than the concentration of their corresponding isomers. However, in the anaerobic reactor both isomers appear in the effluent at approximately equal concentrations. The ability of the anaerobic culture to denitrate GTN at rates comparable to the aerobic systems, with substantially lower cosubstrate requirements, makes it more attractive for field applications. It was therefore decided to focus on the optimization of the anaerobic system.

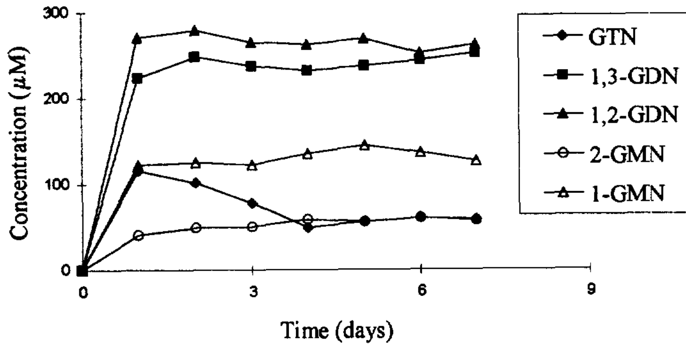

Figure 5. Effluent concentrations of GTN and metabolites in an activated sludge packed bed reactor (Run 1).

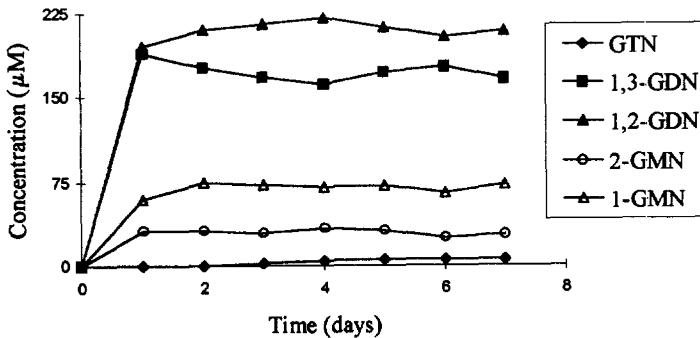

Figure 6. Effluent concentrations of GTN and metabolites in *P. chrysosporium* packed bed reactor (Run 2).

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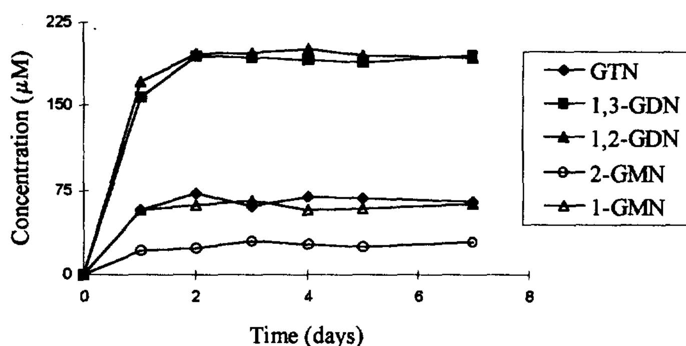

Figure 7. Effluent concentrations of GTN and metabolites in an anaerobic packed bed (Run 3).

Runs 4 and 5 were performed using the anaerobic reactor under identical retention times of 26.8 hours and different glucose-to-GTN feed ratios, in order to assess the effect of retention time and cosubstrate concentration on the performance of the process. The feed ratios for Runs 4 and 5 were 10.5 and 12.82 respectively. The bioconversion of GTN in Run 4 was greater than 99.9% with a corresponding overall nitro compound conversion of 63%. An increase of the glucose-to-GTN feed ratio of 25% in Run 5, keeping all other input parameters constant, resulted in a 12% increase in the overall denitration efficiency. This clearly demonstrates the influence of the cosubstrate concentration on process performance. In field applications, requirements of external carbon sources will be in general lower than the ones observed in this study because waste streams from munitions manufacturing and pharmaceutical facilities are usually rich in BOD.

Table 1. Operating conditions and denitration efficiencies for packed bed systems (Runs 1 through 5)

Run No.	Culture	Feed Composition		Retention Time (hours)	Effluent Composition	
		GTN (μM)	Glucose to GTN Ratio (mg/mg)		GTN (μM)	Total Nitro Compounds (μM)
1	Activated sludge	617	7.14	6.5	55 (91)*	610 (1)*
2	*P. chrysosporium*	617	7.14	6.5	5 (99)	410 (33)
3	Anaerobic sludge	722	3.07	13.5	65 (91)	540 (25)
4	Anaerobic sludge	343	10.25	26.8	< 5 (> 99.9)	120 (63)
5	Anaerobic sludge	343	12.82	26.8	< 5 (> 99.9)	78 (75)

* Numbers in parenthesis represent percent denitration

CONCLUSIONS

From the results obtained in this study the following conclusions can be stated:

1. Activated sludge, *P. chrysosporium* and anaerobic microorganisms can fully degrade GTN. Degradation proceeds by successive denitration of the GTN to form isomeric GDN and GMN which is converted to a utilizable carbon form most likely glycerol. This reaction scheme appears to be similar in fungal and bacterial cultures and in both aerobic and anaerobic environments.

2. In the presence of cosubstrates, both the aerobic and anaerobic microorganisms exhibited substantial regiospecificity at the first and second denitration steps with preferential cleavage of the central nitrate ester group. This favors production of glycerol 1,3-dinitrate and glycerol 1-mononitrate.

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3. The rates of conversion are reduced at each denitration step with the rate of 1-GMN being the lowest. This implies that design of biological treatment processes must be based on the rate of accumulation and disappearance of the mononitrate isomer.

4. The rates of conversion are significantly higher in the packed bed reactor for all three types of microorganisms tested. The anaerobic reactors exhibited higher conversion efficiency at lower cosubstrate-to-GTN ratios and are preferable, for field applications, to the aerobic system which has high cosubstrate requirements and additional capital and operating costs due to aeration.

5. The performance of the immobilized bed reactors can be improved by optimizing the retention time and the cosubstrate concentration to meet the desired overall denitration efficiency.

Finally, since the formed metabolites (GDN and GMN) are more soluble than the parent compound and mutagenic they must be destroyed if GTN is to be successfully treated. The performance of any GTN treatment method must therefore be assessed according to its ability to completely degrade the produced metabolites.

ACKNOWLEDGMENTS

This work was performed at the James C. Nicoll Jr. Laboratory at Stevens Institute of Technology for Picatinny Arsenal, Dover, New Jersey, and it was sponsored by the U.S. Army AMCCOM under contract No. DAAA21-93-C-1018.

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