OPEN ACCESS
Eurasian Journal of Analytical Chemistry
ISSN: 1306-3057
2017 12(Special Issue):641-652
DOI 10.12973/ejac.2017.00195a

Enhanced Aerobic Biodegradation of Soil Contaminated with Explosives (TNT and PETN) By Rhamnolipid

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Received 24 December 2014 ▪ Revised 27 January 2015 ▪ Accepted 13 March 2015

ABSTRACT

The aim of this study was to investigate the bioremediation of two explosives 2, 4, 6-trinitrotoluene (TNT) and Pentaerythritol Tetranitrate (PETN) in mixture by aerobic process. Microbial inocula were obtained from a textile wastewater treatment plant activated sludge. Addition of rhamnolipid surfactant (60 mg/l) increased the removal efficiencies of TNT and PETN from 53% and 57% to 98% and 91%, respectively. Explosives degradation reaction is expressed to be of first-order and the kinetic reaction parameters are calculated based on different initial concentrations of TNT and PETN. The first-order rate constants of the rhamnolipid amended experiments were at least 3 orders and 2.5 orders of magnitude higher for TNT and PETN, respectively, than those found for not amended rhamnolipid experiments. The metabolites pentaerythritoldinitrate,3-hydroxy-2,2-bis [(nitrooxy)methyl]propanal,and2,2-bis-[(nitrooxy)methyl]-propanedialfor PETN and2-amino-4, 6-dinitrotoluene and 4-amino-2, 6-dinitrotoluene for TNT were identified by LC-MS. Concomitant degradation of TNT and PETN resulted in decrease of environment pH. Inoculated bacteria have capability to use of explosive as source of nitrogen and energy. It seems that the addition of rhamnolipid showed great potential for treatment of explosives by textile activated sludge.

Keywords: TNT, PETN, rhamnolipid, textile activated sludge.

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INTRODUCTION

Contamination of soils, ground water, and air with hazardous and toxic chemicals, has become one of the main problems facing the industrialized world today. A great part of the contamination originated from industrial, and military activities [1]. Intensive military activities over the past century have occasioned in extensive contamination of soil and water with residues of explosives and related compounds [2]. Penetration of these hazardous and toxic chemicals to the soilbysurfacerunofforleachingintogroundwater,led to contamination of streams and aquifers [3]. Among such compounds, 2,4,6-trinitrotoluene (TNT) and Pentaerythritol Tetranitrate (PETN) are the predominant contaminant at ammunition plants, testing facilities and military zones [2][4].TNT is considered to be toxic for a wide range of aquatic species [5][6], terrestrial organisms [1] mammals [5] and human monocytes [6]. In addition, TNT has been stated to have a mutagenic and carcinogenic potential in studies with several organisms [7]. EPA classified TNT as class C(possible human carcinogen).PETN is classified as a munitions constituent of greatest concern because of its extensive use and potential environmental impact [4][6]. Furthermore, it was reported that short-term exposure may affect the cardiovascular system [4]**.** Due to the risks related to nitroenergetic compounds, considerable attentions have been made to implement effective remediation and decontamination approaches in explosive-contaminated sites [9][10][11]. Bioremediation is one remediation method appealing worldwide attention since it is usually a less expensive technique of destroying organic pollutants than traditional engineering based technologies [11].

Because of the low solubility of explosive compounds [2] however, bioavailability of these hydrophobic organic compounds (HOCs) to microorganisms, could be a limiting phase during the biodegradation process [12]. Addition of external agent such as surfactants to contaminated soil, at concentrations above their critical micelle concentration (CMC) values, can be a feasible approach to reducing the interfacial tension, enhancing the solubility and therefore, increasing their biodegradation [12][14]. The application of biosurfactants such as rhamnolipid has increasingly augmented during the past decade as potential candidates for many commercial applications in the petroleum, pharmaceuticals, biomedical, and food processing industries because of their biodegradability, lower toxicity and greater diversity than the available synthetic surfactants [14]. It seems that aerobic bioremediation has a better performance in degradation of explosives. under aerobic degradation, nitroaromatic compounds are used primarily as carbon, nitrogen and energy sources for bacteria and can be completely mineralized [11].

It was assumed that activated sludge contain nutrients and/or bacteria that could improve the biodegradation process. Previous studies [15][16] have shown usage of activated sludge as inoculant for removal of pollutant. In this study activated sludge from a textile waste water treatment plant was added as inoculant assuming that bacteria have been encountering previously with aromatic compounds present in wastewater so can be helpful in removal of explosive.

Successful application of rhamnolipid biosurfactant has been reported in removal of PAHs from contaminated wastewater [17].To our knowledge, available information about the effects of rhamnolipid addition on improved biodegradation of explosives contaminated soil are sparse. The present study therefore investigated the effect of rhamnolipid biosurfactant on concomitant removal of explosives (PETN, TNT) from the soil. Also kinetic studies on reductive degradation of TNT and PETN explosives in contaminated soil were investigated.

MATERIALS AND METHODS

2.1. Biosurfactant

The biosurfactant used in this study was a rhamnolipid (C32H58O13) obtained from the Genetic Engineering and Biotechnology Institute (Iran). It is an extra-cellular natural substance produced during precisely controlled fermentation processes using certain bacterial strains [18][19]. Its molecular weight was 650 g/mol.

2.2Soil preparation

Eight soil-pan (1 to 8) experiments were conducted. The clean natural soil was derived from Garden University of Isfahan. The physical and textural characteristics of these soils are given in Table 1. Soil samples were sieved through a 2-mm sieve to remove coarse fragments. Contaminated soil was then prepared by dissolving an appropriate quantity of TNT and PETN in water/acetonitrile solution and a known weight of soil was added with continuous mixing. The resultant mixture was placed in a ventilation hood to allow the complete evaporation of the solvent. The contaminated soil was stored at room temperature for 7 days.

Each pan experiment was prepared by placing 3000 g of contaminated soil in a square plastic pan (30 cm ×20 cm ×20cm in height). This mass of soil occupied the pans approximately two-thirds full. Since addition of amendment can improve soil management properties [18], wood chips was added to the contaminated soil. In this treatment, contaminated soil represented nearly 95% of the total soil mixture, while the wood chips amendment comprised the remaining 5% (dry-weight basis).

Aeration in pans carried out by forced aeration. Pan 1and 5 served as control. Pans 2, 3, 4, 6, 7 and 8 were designed to be biologically active treatments. Textile wastewater treatment plant activated sludge was added to pans 2, 3 and 4 and rhamnolipid at CMC of 60 mg/l plus sludge was added to the pans 6, 7 and 8. Pans 2 and 6, 3 and 7, 4 and 8 have explosive concentration of 50,100 and 200 mg/kg of PETN respectively and 200,500 and 1000 mg/kg of TNT respectively. Kinetic studies for each set of experiments were conducted. But results obtained from pans 4 and 8 were reported for biodegradation study (highest concentration).

Aeration was performed each day for 10 min. When the soils in the aerated pans(1 to 8) were dry enough to break apart, flooding with deionized water was done in the range of 4 to 6 d.

Table 1: Properties of soil samples used in this study

Parameter	Value (%)
Clay	16
Sand	34
Silt	46
Total carbon	4

2.3. Chemicals

Allchemicalsusedwereofanalyticalgrade;2, 4, 6-trinitrotoluene (TNT); pentaeritritholtetranitrate (PETN) were from Zarrinshahr Chemical Industries (Esfahan, Iran).All other chemicals were obtained from Sigma–Aldrich and Merck.

2.4. Sampling and chemical analyses

Samples of soil were taken periodically during the experiment for analyses of explosives. Sampling were done once every two weeks. the three grab samples was collect from the top 3 cm of soil in the pans during each sampling event. The explosives in soil samples were extracted in accordance with the US EPA Method 8330. Soil samples were air-dried; then 5 g (mixture of three grab samples) of soil was transferred to a clean glass vial and extracted with 20 ml of acetonitrile. The mixture was then centrifuged for 5 min at 3,000 rpm, followed by filtration with 0.22 µm Pall membrane. The prepared sample was analyzed for explosives (TNT, PETN) with high-performance liquid chromatography (HPLC). The HPLC system used was from Waters (Milford,

MA, USA), consisted of a Model 600E pump, fitted with a Rheodyne 7725i injector valve, a Model 486 UV programmable multi wavelength detector, a data module, a Model 600E system controller, Detector and a Nova-pak C18 guard column. The analytical column was an ODS2-Optimal column (25cm×4.6mm id, 5µm) from Capital HPLC (West Lothian, UK). A water-acetonitrile mixture (20:80, v/v) was used as the mobile phase at a flow rate of 1.0 mL/min. The injection volume was 20µL and the absorbance was measured at a wavelength of 210 nm.

2.5 Analysis of biodegradation products by LC–MS

TNT and PETN biodegradation products, were analyzed by LC–MS using a Shimadzu LCMS- 2010 EV (Japan) equipped with two pumps (LC-10 ADvp), controller (SCL- 10Avp), autoinjector (SIL-10ADvp) and a UV2000 UV/VIS detector. The analytes were separated on 250 mm 4.6 mm 5 µm C18Hypersil GOLD column (Thermo, Waltham, MA) by acetonitrile–water gradient elution (90:10, v/v), at a flow rate of 0.2 mL/min.

2.6 Soli bacteria density

Cell growth in pans was determined using the standard total plate count method [12]. At first, 1mL of soil sample was mixed with 9mL sterile phosphate buffer and stirred for 2min to detach the bacteria from soil matrix. Then, serial dilutions of the treated samples were performed in the range of 10−4–10−8and 0.1mL of each diluted sample was spread onto nutrient agar plates (as duplicate). The colonies on each plate were counted after 48 h of incubation at 30 ◦C and the average of the two measurements was reported as bacterial growth in colony forming units per milliliter of soil (CFU/mL-soil).

RESULT AND DISCUSSION

3.1. Kinetic studies

In order to determine the kinetics of the explosives degradation, different concentration of TNT and PETN were used.Fig. 1(a-d) shows biodegradation of explosives as a function of time.Analysis of the data showed that, explosives biodegradation followed a general exponential equation representative of first-order kinetics (Table 2). Rate constant k [d-1] is calculated from the slope of the line for ln [C0/C] vs. reaction time.

$dC/dt = - kt$(1)

$C = C0 \exp(- kt)$(2)

Where C0 = initial explosives concentration (mg/kg); k = rate constant (d-1);
t = degradation time (d).

Table.2: Reductive degradation of explosives with different initial concentrations. Experimental data was fit to the first-order kinetic equation

TNT Concentration(mg/kg)		With rhamnolipid			Without rhamnbolipid		
		200	500	1000	200	500	1000
Equation - Ln(C/C0)=Kt	R2	0.94	0.95	0.98	0.98	0.99	0.96
	K[d-1]	0.0122	0.0199	0.0237	0.0056	0.0059	0.0061
PETN Concentration(mg/kg)		50	100	200	50	100	200
Equation - Ln(C/C0)=Kt	R2	0.95	0.96	0.98	0.92	0.98	0.99
	K[d-1]	0.0094	0.0154	0.01773	0.0053	0.0057	0.006

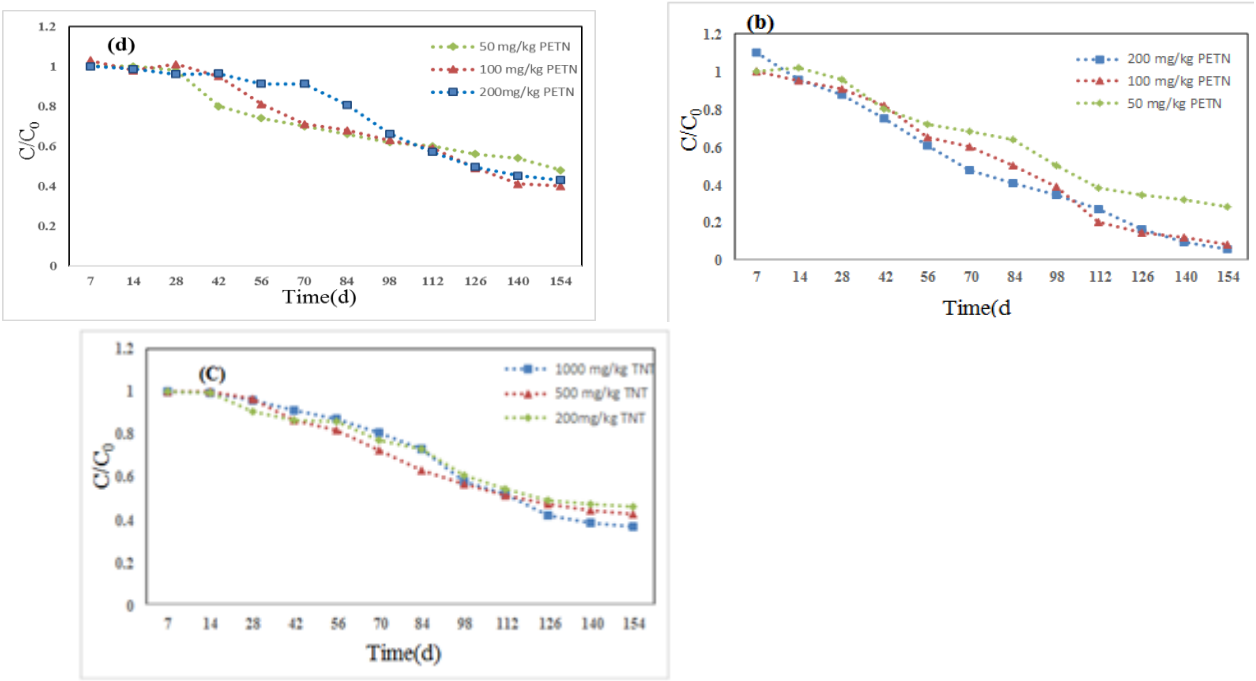

Fig.1: Disappearance of TNT and PETN with time by addition of rhamnolipid (a), (b) and without addition of rhamnolipid (c), (d)

In general, removal rate of explosives were higher in experiments that rhamnolipid was added. As table 2 shows the first-order rate constants of the rhamnolipid amended

experiments were at least 3 orders and 2.5 orders of magnitude higher for TNT and PETN, respectively, than those found for not amended rhamnolipid experiments. This can be due to addition of rhamnolipid which result in increasing the solubility of explosives and then enhancing the explosive biodegradation. Surfactants can increase the surface area of hydrophobic materials, so increasing their water solubility and subsequently increase the biodegradation of complex hydrocarbons [22].

3.2. Effect of rhamnolipid on the removal of TNT

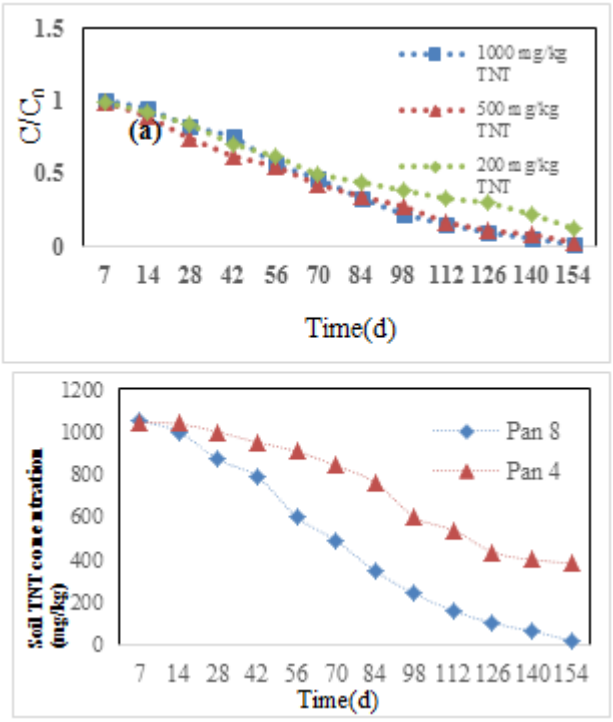

Fig.2: Effect of rhamnolipid on bioremediation of TNT. Pan 4 amended with sludge. Pan 8 amended with rhamnolipid plus sludge.

The concentrations of TNT are given in Fig.2. The soil-TNT concentration in pan 4 that received only wastewater sludge decreased gradually. As shown in fig.2 there is insignificant TNT removal during the first 4 weeks of experiment. This observation suggests that the bacteria need time to adjust new environment. Although living bacteria in sludge have been encountering previously with aromatic compounds present in wastewater, but additional time is needed to adjusting to new environment. Also insignificant TNT removal can be due to the low bacterial population at the first 4 weeks. After the 28 days, removal of TNT pursued with higher rate and shows a maximum TNT removal of 53% after 154 days. [15] reported that addition of sludge caused 32.6% elimination of TNT. Compared to result of the present work, lower removal of TNT in their experiment can be attributed to the fact that

their study conducted in anaerobic condition while present work done aerobic, since the bacteria have higher growth rate in aerobic condition, therefore significant increase in the bacterial population and thus higher removal rate can be expected in the present work. In this study textile wastewater treatment plant sludge was used as seeding agent for explosives degradation. This is based on the fact that aerobic sludge contain consortia of microorganisms that degraded aromatic compound [20].

Fig.2 also shows that in the pan 8 with both sludge and rhamnolipid biosurfactant, TNT was removed faster than that for pan 4. The soil-TNT concentration in this pan dropped from 1053 mg/kg to 20 mg/kg of soil on 154 day of the experiment (98% removal). Adding rhamnolipid not only provides micelles for higher solubilization of the TNT, but also accelerates emulsification, with a resulting increase in bioavailability for degradation. Referring to Fig. 2 it can be observed that 67 % TNT removal in pan 8 was achieved in 84 days whiles only 16% of TNT was removed in the same time for pan 4. Since TNT exhibit toxicity to microorganisms [11][21], a possible explanation for this higher removal rate is that rhamnolipids can act as agent which reduce the toxicity of TNT. Entrapment of TNT in rhamnolipid micelles, reduced the toxicity of TNT. This is in accordance with the results obtained by [22] who reported that toxicity of chlorophenols could be significantly reduced by the entrapment of chlorophenols in biosurfactant micelles which in turn resulted in improved microbial growth and higher biodegradation of chlorophenols.

3.3. Effect of rhamnolipid biosurfactant on the removal of PETN

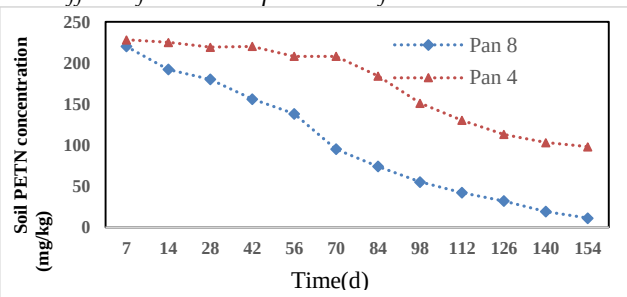

Fig.3: effect of rhamnolipid on bioremediation of PETN. Pan 4 amended with sludge. Pan 8 amended with rhamnolipid plus sludge.

Tests of PETN biodegradation with and without amendment of rhamnolipid (pan8 and 4 respectively), were shown in fig 3. In the absence of rhamnolipid, approximately 5% of the initial PETN removed by 84 days (7 weeks). After 154 d, the removal rate of PETN was slightly improved and reached 56%. In our study biodegradation of TNT and PETN was conducted in mixture. We found that the biodegradation of TNT and PETN in a mixture (pan4)does not occur simultaneously. Compare to TNT, degradation of PETN began with a lag phase of 84 days. It seems that PETN degradation rate was impacted by the presence of TNT. The following potential reasons can be proposed for the observed result: One might be competitive biodegradation in which TNT preferentially utilized by the bacteria. Result of other studies confirmed this hypothesis. For example [23] reported that in presence of BTEX, MTBE degradation inhibited due to the preferential utilization of BTEX compounds.. Also

insignificant PETN removal in pan 4 can be related to the low solubility of PETN. The low solubility of PETN(<40mg/L) [2], limit its ability to be transported into microbial cells and thus be biodegraded. Toxicity of TNT maybe another possible mechanism that describe the observed result. In the initial stages of biodegradation, TNT and its metabolite might be toxic for PETN degrading bacteria and cause a change in the microbial population. [24] showed that the growth of RDX degrading bacteria completely inhibited by TNT. Acclimation of indigenous bacteria (originated from textile wastewater treatment plant sludge) to PETN can be one explanation for increased removal of that from 85 to 154 days. This is in agreement with the result of [14] who reported that it was possible that 12 days incubation was enough for adapting of resting cells to the presence of hydrophobic substrate phenanthrene. Biodegradation of PETN was followed by addition of rhamnolipid in pan 8. As fig.3 showed at the end of 154 days PETN removal rate was 91%. Compare to pan 4 which no rhamnolipid was added, PETN removal in pan 8 was 1.6 -fold higher than that of the pan 4. This can be due to addition of rhamnolipid. Low solubility of PETN is the limiting factor for its mobilization and subsequent degradation [2],addition of rhamnolipid led to enhanced mobilization and increase the bioavailability. This is in agreement with the result obtained by [25] who reported that after 15 days the degradation efficiency of octane in soil increased significantly. The authors proposed that mobilization of octane molecules and consequent intensification in their bioavailability was the main reason of the observed differences.
Initial nitroaromatic transformations are catalyzed by nitroreductases which are related to the old yellow enzyme (OYE) of yeast [21]. Moreover, the OYE enzymes are able of denitrating some explosives such as PETN and glycerol trinitrate (GTN) and TNT by a mechanism which consist of a reductive denitration with the releaseofnitrite(26). We assume that the rhamnolipid enhanced nitroreductases activity which resulted in an increase in the amount of released nitrite. Nitrite analyses in this study confirmed our assumption. Compare with pan 4, nitrite releasing in pan 8 which rhamnolipid was added, showed higher amount (data not shown). similar results were found by [22]. They reported that rhamnolipid had a positive effect on diesel fuel biodegradation in cultures containing 4-chlorophenol (4-CP) or 2, 4-dichlorophenol, by dehydrogenase activity.

3.4. Microbial concentration

Bacterial plate counts were done several times during the experiment on soil samples taken from each of the pans. The bacterial population densities in the soils contained rhamnolipid (Pan 8) were consistently higher than those in the pan 4 and control pans (Table.3). Much higher densities were present in pan 8 compared to pan 4 and control during the experimental period specifying that rhamnolipid improves bacterial activity in explosive contaminated soil. With highlycontaminated soil, furthermore addition of organic material successfully dilutes the concentration of explosives and may prevent toxicity to the microbial [27]. The result obtained by the [18] show that in cultures that enriched with molasses, higher bacterial growth was observed. They conclude that molasses is the suitable source for bacteria compare to the other source. Although bacterial population in pan 8 was higher at first but it was decreased gradually. The drop in bacterial density very likely indicates that the rhamnolipid had been used up or environment condition have undergo change.

Table 3: Bacterial plate counts (colony forming units/g of soil)

Pan	Day 30	Day 60	Day 90	Day 120	Day 150
control	8×104	17×104	12×104	15×104	21×104
4	5×106	7×106	6×106	1.7×107	7×106
8	2.8×107	4.3×107	5×107	2.1×107	1.8×107

3.5. Metabolic pathway

Since microorganisms have the capability of producing many different types of enzymes, different metabolic pathways and mechanisms for explosives biodegradation have been suggested in recent years. In the present work, LC–MS analysis of explosives showed that two reduction metabolites, 2-amino-4, 6-dinitrotoluene and 4-amino-2, 6-dinitrotoluene were observed during TNT metabolism. The same results has been reported by several workers [11][28]. According to our results, proposed possible metabolic pathway for TNT biodegradation which was also reported by others [11][29] involved reduction of one nitro group to form a hydroxylamino group and subsequent reduction of the other nitro group to an amino group. in the presence of oxygen some of these intermediate products could be polymerize to form tetranitroazotoluene [30]. Furthermore, 4, 4′6, 6′ -tetranitro-2, 2′ –azoxytoluene was detected based on LC-MS analyses. The previous studies confirmed our result [11][31]. Identification of PETN degradation products was perused by the LC-MS analysis. Analysis of explosives showed the following metabolite during PETN metabolism: Pentaerythritol dinitrate, 3-hydroxy-2, 2 bis [(nitrooxy) methyl] propanal, 2, 2-bis [(nitro-oxy) methyl] propanedial and pentaerythritol respectively. This is in consistent with the result obtained by others [4][8] who reported that transformation of PETN by PETN reductase yield intermediates such as identified in our study. Based on the identified intermediate products it is proposed that PETN degradation in the aerobic condition follows a successive reductive degradation pathway with the release of NO2- in each denitration step. It is interesting to note that pentaerythritol mononitrate was not detected in the intermediate products. This result confirmed that obtained by [4] who, stated that the apparent absence of pentaerythritol mononitrate (PEMN) in the sequential denitration pathway may be due to the fact that the rate of PEMN to PE conversion was faster compare to its formation from PEDN and the low amount of PEMN, which resulting in the concentration being below the detection limit of HPLC analysis.

Since there is no addition of nitrogen source, our study conducted under nitrogen limited condition. Growth of degrading bacteria and successive degradation of explosives indicated that this degrading bacteria have ability to use of TNT and PETN as source of nitrogen. Result of other researchers [8][32] confirmed our result.

Measurement of pH in pan 8 showed that from the week of 12 pH of the environment decreased and became acidic. Relative decrease in bacterial population from week 12 in pan 8 as shown in table 3 can be related to acidic condition. It is possible that in acidic condition growth of TNT degrading bacteria inhibited to some extent. Result of other study showed the adverse effect of lower pH on bacterial growth [33].In spite of acidic condition and relative decrease in bacterial population, resultsshows continued degradationofTNT. This may be due to the PETN reductase enzyme activity. This finding is in agreement with the

result of previous studies [29][34] that reported PETN reductase enzyme have a capability to degrade TNT. Since PETN reductase activity is pH-dependent [35][36][37] it seems that acidic environment in our experiment have positive effect on degradation of explosives.

CONCLUSION

This study showed the removal of TNT and PETN from the soil in aerobic conditions. Application of rhamnolipid biosurfactant was effective in biodegradation of explosives. Activated sludge obtained from textile wastewater treatment plant was a good source of nutrient for degrading bacteria. TNT was degraded better in concomitant bioremediation of TNT and PETN by synergistic effect of PETN degrading bacteria and acidic pH was favored for removal of TNT. This study suggests that concomitant bioremediation of TNT and PETN could be useful in TNT contaminated area.

ACKNOWLEDGEMENT

The authors acknowledge all financial supports provided by Esfahan University of Medical Sciences. The authors declare that there is no conflict of interest.

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