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Comparison of Nitroaromatic Compounds Metabolites Types by Sequencing Performance of Anaerobic-Aerobic and Aerobic-Anaerobic Processes

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ABSTRACT

Introduction: Xenobiotic contamination is a global concern**.** Nitroaromatic compounds enter the environment through ammunition, ordnance disposal, burning the outdoors, and leakage of ammunition**.** Thirty percent of explosives enter the environment without any change, which can cause pollution of soil, water, and health concerns. So, effective remediation of the contaminated area is necessary.

Materials and Methods: Soil bioreactors consisted of plastic pans placed in larger pans. Explosives were analyzed using a High-Performance Liquid Chromatography (HPLC) system, Model 486 UV detector, and a Nova Pak C18 guard column. LC-MS detected intermediates on an RP18 analytical column equipped with a C18 guard column.

Results: Rhamnolipid significantly affected 2,4,6-trinitrotoluene (TNT) and Pentaerythritol tetranitrate (PETN) biodegradation rates with the help of indigenous bioaugmentation. The mentioned condition was also effective on the maximum bacterial growth in various nitroaromatics (S0) concentrations, in which the top change occurred. The specific growth rate was foremost in a setup containing microbial inoculated and biosurfactant (0.19) responding to 800 mg/kg TNT and 150 mg/kg PETN. The maximum bacterial enumeration of sludge and biosurfactant were 4.8×10^8 and 4.1×10^8 CFU/g, respectively. The aerobic-anaerobic sequence could be able to produce less harmful metabolites. In an aerobic-anaerobic sequence process, using the anaerobic process could help complete the azo compounds degradation in the aerobic stage.

Conclusion: Aerobic-anaerobic condition is suitable for bioremediation contaminated explosive sites and achieving complete mineralization. Generally, this proposed method is possible for in situ bioremediation**.**

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Introduction

Nitroaromatics are released into the environment by human activities. These compounds have at least one nitro group attached to aromatic rings [1,2,3]. Production, transportation, and disposal or destruction of army weapons can cause contamination of the environment [4, 5]. These substances are carcinogenic and were categorized as

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priority pollutants by the U.S Environmental Protection Agency [4]. Explosive can enter the environment via ammunition usage, ordnance disposal, burning the outdoors, and leakage of ammunition [6,7]. Thirty percent of explosives enter the environment without any change, causing health concerns. Therefore, effective remediation of contaminated sites is necessary. TNT and PETN are explosives that have broad applications. TNT has mutagenicity effects and is categorized as C human carcinogen [8,9]. PETN is a powerful explosive usually used in detonators [10]. Explosives are classified as xenobiotics and have challenged microorganisms [11]. The environmental fate of explosive residues and intermediates has intensified.

Explosive diffusion negatively affects the ecosystem and is introduced into the food chain [12]. Some traditional methods were used for explosive waste disposals, such as dumping in the sea, special landfilling [13], and incineration [14]. Landfilling has groundwater and soil contamination potential, while incineration causes air, water, and soil contamination. Currently, incineration is the most effective and widely used alternative remediation, but this method is expensive because of its costs related to soil excavation, transport, and energy for incineration. Recently, many new biological processes have been developed [15, 16]. Bioremediation is an efficient, relatively cost-effective, and environmentally friendly method. These methods include soil slurry reactors, composting, bioventing, land farming, and phytoremediation. Land farming is performed in the solid phase, and soil is aerated periodically by mechanical turning [17, 18]. Composting and land farming methods have high similarities except for the organic substrates added as a carbon source to stimulate bacterial growth in composting process and cause higher degradation rates [19]. Compared to the other methods, biological processes are more cost-effective and significantly reduce soil toxicity.

Moreover, microorganisms can utilize xenobiotics as a carbon and nitrogen source for metabolism and growth [20]. Biosurfactants can help release hydrocarbons absorbed from the soil tissue by solubilizing or emulsifying them and increase the concentrations of hydrocarbons in the liquid phase, leading to an increase in mass transfer rate. The advantage of biosurfactants over chemical surfactants is low toxicity, higher biodegradation, higher environmental compatibility, and their ability to synthesize from renewable sources. Other studies have shown that adding rhamnolipid significantly affects the development of biodegradation of petrochemical chemical compounds in wastewater. In addition, biosurfactants can be used in biological reduction; the performance of this process will be more economical and eco-friendly [15]. Some bacterial species (such as *Pseudomonas, Desulfovibrio, Escherichia, Enterobacter*) and Fungi have been reported that can degrade TNT [10]. Due to the low bioavailability, various methods have been examined to increase the biological availability of these compounds. Thavasi et al. used a Lactobacillus biosurfactant to accelerate crude oil biodegradation and found that biosurfactant was capable of promoting oil biodegradation [21]. A study by Zhuang et al. on PETN anaerobic biodegradability by DARAMEND as co-substrate showed that DARAMEND has an essential role in PETN biodegradability [20]. Moreover, moisture is critical, so insufficient humidity can postpone the removal of explosives [24].

This study aims to compare metabolite diversities (TNT and PETN) in aerobic-anaerobic and anaerobic-aerobic as inverse sequences at a pilot scale. The biosurfactant role and specific growth rate of microorganisms were also investigated.

Material and Methods

Laboratory experiments

Soil bioreactors consisted of a plastic pan (30 × 20 × 15 cm) placed in a larger pan.

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Figure 1 reveals that the bottom of the smaller pan was perforated with 2-mm-diameter holes that the space was 8 cm, allowing for drainage. The fluid leachate was recycled to the surface soil of the pans. To improve the soil porosity, screened sawdust was mixed with the soil (in a 1:1 ratio). In all anaerobic reactors, 4 kg of contaminated soil was added, and water content was controlled once a week (to maintain the water level relatively 3 cm above the surface of the soil). The setup condition for aerobic pans was the same (4 Kg soil) [25]. The control pans consisted of contaminated soil only.

During the aerobic phase, the air was automatically injected into the soil by button diffusers twice a day for 60 minutes. The PETN and other contaminant concentrations, bacterial growth rate, and intermediate diversities were monitored periodically in all reactors. In one setup, a Rhamnolipid biosurfactant was added to stimulate biological activity and improve the solubility of TNT and PETN. Two pilot setups were designed and prepared. The first was anaerobic-aerobic, and then aerobic-anaerobic sequences that operated simultaneously. The operation of the reactors was approximately 150 days (anaerobic phase: 100 days and then in the aerated reactor: 50 days). Moreover, 500 mL of the mixed activated sludge from the municipal wastewater treatment plant was added for the aerobic bioremediation start-up. *Rhamnolipid* produced through *P.aeruginusa* strains was purchased from the National Institute for Genetic Engineering and Biotechnology, IRA Research Institute of Chemistry and Chemical Engineering. The used rhamnolipid concentration was 60 mg/l that was calculated by the CMC method on laboratory scale [26].

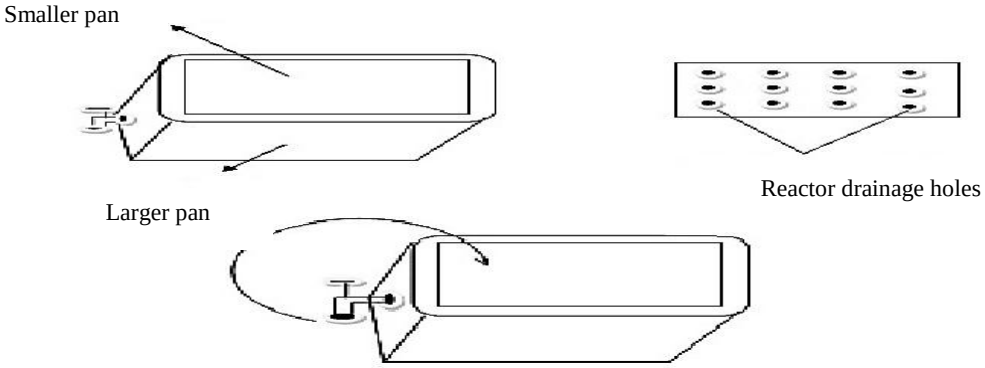

Figure 1: A schematic of aerobic and anaerobic pans

Explosive and intermediates analyses

TNT and PETN were analyzed using an HPLC, Model 486 UV detector, and a Nova Pak C_{18} guard column. The analytical column was an ODS_2 optimal column (25cm × 4.6 mm id, 5µm) from capital HPLC. Explosives sampling and analyzing were done by 8330 B EPA methods [18].

Sample intermediate analysis was performed on an RP18 column (2× 250 mm, dp 5µm) equipped with a C18 guard column (dp 5µm). The mobile phase included water and acetonitrile (20:80), with a flow rate of 200µl/min [27].

Heterotrophic plate count analysis

To monitor bacterial enumeration, soil samples were collected from different parts of pans and homogenized. One gram of each sample was added to 9 mL of sterile deionized water and mixed using a vortex for 5 min; each sample was serially diluted as required. Then 0.1 mL of each sample was inoculated on a nutrient agar plate and incubated at 37 ± 2°C for five days. The number of colony-forming units was reported as CFU/g soil[18].

Results

Effect of biosurfactant on microbial growth rate

Figures 2 and 3 show the specific microbial growth rate (µ) in different stages of biodegradation conditions.

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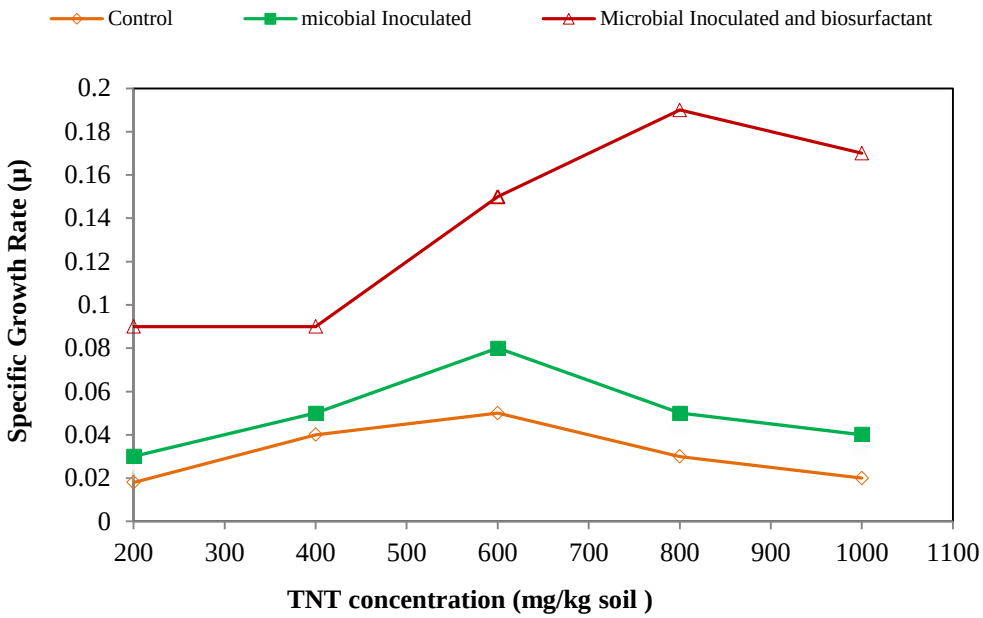

Figure 2: Specific microbial growth rate (μ) for 200, 400, 600, 800, and 1000 mg/kg of TNT in aerobic soil

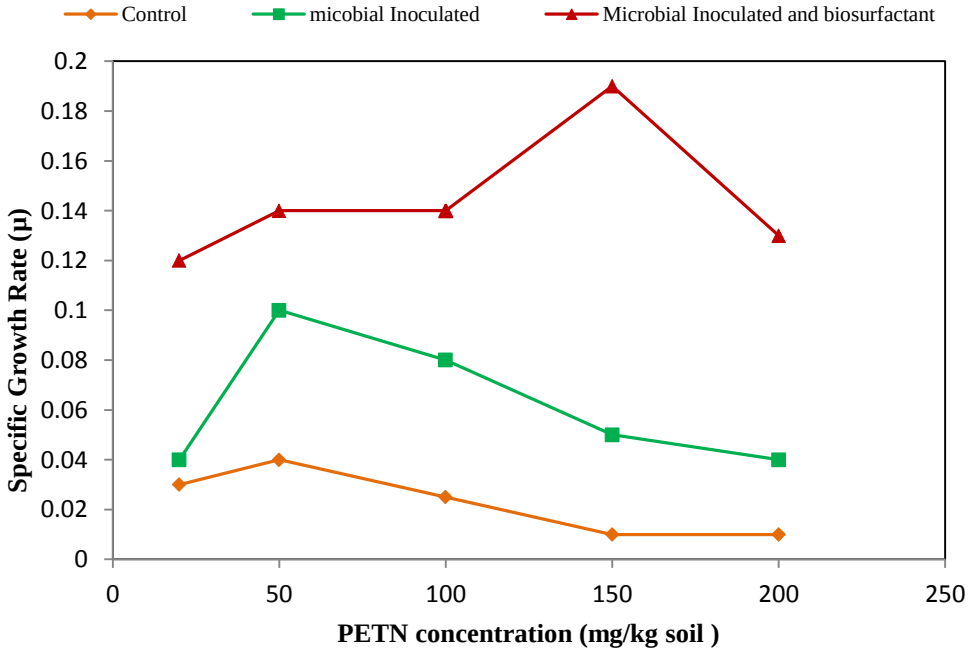

Figure 3: Specific microbial growth rate (μ) for 20, 50, 100, 150, and 200 mg/kg PETN in aerobic soil

The results show that an increase in the concentration of PETN and TNT led to an increase in specific growth rates, while it had a negative effect on higher pollutant concentrations.

Biosurfactant with the help of bioaugmentation (microbial inoculation) led to changes in the condition of microbial growth rate variations (μ). On the other hand, the results showed that rhamnolipid and microbial bioaugmentation could affect the maximum microbial growth rate. The heterotrophic plate count (HPC) results are shown in Tables 1 and 2.

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Table 1: Heterotrophic plate count at the end of the process in Anaerobic-Aerobic sequence of explosive bioremediation (CFU/g soil) *****

Time (month)	anaerobic		aerobic		Control
	sludge	sludge + biosurfactant	sludge	sludge + biosurfactant	
1	-	-	2×10^8	2.5×10^8	1.5×10^5
2	1.9×10^8	2.4×10^8	2×10^8	2.4×10^8	1.8×10^5
3	2×10^8	2.4×10^8	2.2×10^8	2.8×10^8	1.8×10^5
4	2.5×10^8	2.7×10^8	2.8×10^8	3×10^8	1.4×10^6
5	3.8×10^8	4×10^8	3.9×10^8	4.1×10^8	1.4×10^6

* Initially TNT and PETN concentrations of each reactor were 1000 and 200 mg/l, respectively.
As shown, over time, the bacteria population increased. Rhamnolipid assisted with bioaugmentation are two critical factors affecting microbial population growth.

Table 2: Heterotrophic plate count at the end of process in aerobic-anaerobic sequene of explosive bioremediation (CFU/g soil) *****

Time (month)	anaerobic		aerobic		Control
	Sludge	Sludge + biosurfactant	Sludge	Sludge + biosurfactant	
1	1.7×10^8	2.3×10^8	-	-	1.5×10^5
2	2×10^8	2.4×10^8	-	-	1.6×10^5
3	2.2×10^8	2.8×10^8	-	-	1.6×10^5
4	-	-	3.1×10^8	4.5×10^8	1.2×10^6
5	-	-	3.1×10^8	4.8×10^8	1.2×10^6

* Initially TNT and PETN concentrations of each reactor were 1000 and 200 mg/l, respectively.

Biosurfactants could increase the solubility and bioavailability of explosives, which subsequently increases the amount of carbon and nitrogen and results in the growth of heterotrophic bacteria. Also, sludge could act as a good seeding microorganism source, so that complete enumeration related to sludge and biosurfactant were 4.8×10^8 and 4.1×10^8 CFU/g, respectively.

Anaerobic- aerobic and aerobic-anaerobic metabolites of nitroaromatic bioremediation

The effect of mutagenicity and carcinogenicity of TNT and its metabolites result in performing effective removal techniques to remediate contaminated soil. Intermediates of TNT and PETN biodegradation can be seen in Figure 4. The LC-MS mass spectrum graph shows the mass-to-charge ratio (m/z) and can be used to identify the metabolites produced. Figure 4 shows the metabolites of biodegradation of TNT and PETN during anaerobic bioremediation.

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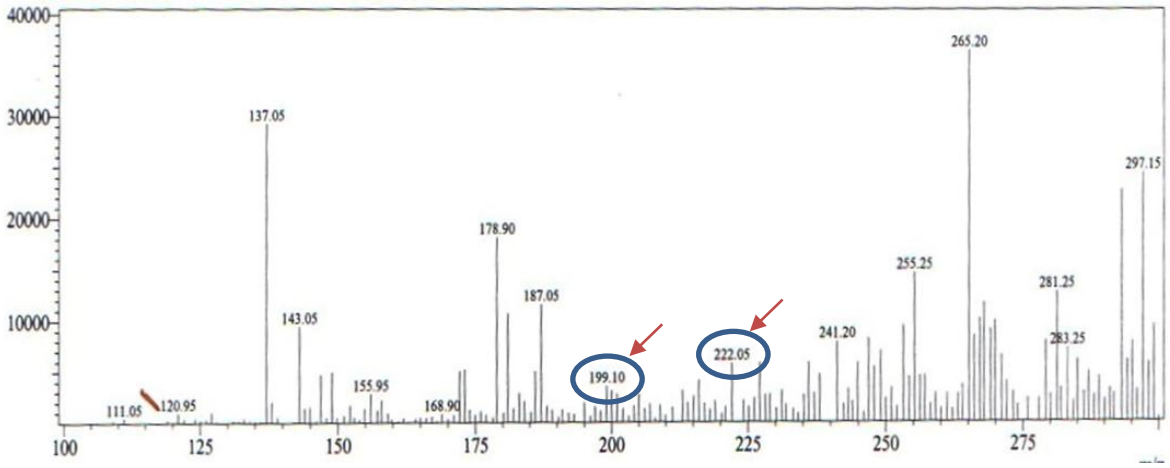

Figure 4: TNT and PETN metabolites detected by LC-MS

Metabolites that most have been detected in anaerobic biotransformation of TNT, were 2-ADNT and 4-ADNT, which were finally reduced to 2,4-DANT. Also, nitro benzoic acid and 2,4,6-triaminotoluene (TAT) are produced alone as intermediates under anaerobic conditions (Figure 4). Table 3 shows the metabolites of TNT and PETN in anaerobic-aerobic sequences process.

Table 3: Metabolites of anaerobic-aerobic explosive bioremediation

PETN (pentaerythritol tetranitrate)	TNT (2,4,6-trinitrotoluene)
Pentaerythritol dinitrate	NODNT (nitrosodinitrotoluene)
3-hydroxy-2,2-bis[(nitrooxy)methyl]propanal.	HADNT (hyroxylaminodinitrotoluenes)
PEMN (mononitrate pentaerythritol)	ADNT (aminodinitrotoluenes) DANT (diaminonitrotoluenes) 4-hydroxytoluene

Table 3 and Figure 4 show that metabolites in the anaerobic-aerobic process include the molecules with the chemical formulas $C_5H_{10}N_2O_8$, $C_5H_8N_2O_8$, and $C_5H_6N_2O_8$. The mentioned metabolites are Pentaerythritol dinitrate, 3-Hydroxy-2,2 Base (Nitroxymethyl) Propanol, and 2,2 Base (Nitroxymethyl) Propanedial. The mass-to-charge ratio (m/z) values of the metabolites listed are 226, 224, and 222, respectively. 4-Hydroxytoluene was the final product that was detected in the anaerobic-anaerobic sequence process. In the present study, TAT was not observed as a metabolite of the anaerobic-aerobic process due to its high reactivity and the irreversible binding to humic material in the soil. Therefore, after the reaction with the humic material, only a tiny amount of it remains accessible and becomes 4-Hydroxytoluene in process performance. Mono, Di, and Trinitrotoluene compounds are formed in the anaerobic process. In the aerobic phase, the concentration of these compounds is deficient, and it is due to the formation of covalent bonds of aromatic compounds with humic compounds in the soil [28, 29]. Aerobic-anaerobic metabolites are shown in Table 4.

Table 4: Metabolites of aerobic- anaerobic explosive bioremediation

Pentaerythritol tetranitrate (PETN)	TNT (2,4,6-trinitrotoluene)
Pentaerythritol dinitrate	complex (H--TNT) Meisenheimer - Hydride
Pentaerythritol dinitrate	Dihydride -Meisenheimer complex (2H--TNT)
Pentaerythritol	Diarylamines

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Table 4 shows that in the aerobic-anaerobic process, biochemical metabolites include pentaerythritol dinitrate, 3-hydroxy-2,2 base (nitroxymethyl) propanol, and 2,2 base (nitroxymethyl) propanedial, pentaerythritol mono-nitrate, and pentaerythritol. The responsible values for the metabolites mass-to-charge ratio (m/z) are 226, 224, 222, 196 and 136, respectively.

Discussion

This study aimed to compare produced metabolites of nitroaromatic bioremediation. The results showed that bacterial inoculation and biosurfactant could effectively improve biodegradation rates (Figures 2, 3). Bacteria enumeration also confirmed recent findings (Tables 1, 2). Boopathy et al. used surfactant Tween 80 to increase the solubility and bioavailability of explosives. The results showed that the usage of Tween 80 at limited concentration increased the amount of biodegradation, while at higher concentrations reduced its efficiency. The reason for the efficiency limitation could be related to surfactant toxicity affecting the microorganism population. In the present study, rhamnolipid concentration (60 mg/l) has no toxic effect on the microorganism population, which could be related to its structure. Results of previous studies have shown that using biosurfactants is more acceptable than surfactants due to synthetic surfactants. Moreover, the toxicity of biosurfactants is much lower than chemical surfactants [30, 31].

TNT resistance to biological degradation is due to the electron-withdrawing character of three nitro groups. As a result, the central ring encounters a lack of electrons and creates steric constraints. Therefore, the aromatic ring is resistant to remediation processes[32]. Metabolites most detected in anaerobic biotransformation of TNT were 2-ADNT and 4-ADNT, which were finally reduced to 2,4-DANT and TAT. Hawari et al. reported that amino derivatives were further transformed into triaminotoluene (TAT) under strictly anaerobic conditions at less than -200 mV redox potential [32]. Moreover, PETN was reduced to pentaerhytritol trinitrate (PETriN), which was reduced to dinitrate (PEDiN), mononitrate (PEMN), and pentaerhytritol (Table 3). Zhaung et al. (2012) reported that PETN was reduced to PETriN, PEDiN, PEMN, and Pentaerythritol under anaerobic conditions by denitrifying bacteria [21]. Increasing metabolite concentrations in the soil confirmed the induction processes related to the acclimation of indigenous bacteria and the enzyme induction process. One of the produced metabolites associated with the anaerobic-aerobic process was NODNT. This compound was introduced by adding $2e^- + 2H^+$ and losing a water molecule from the TNT molecule. The next metabolite was HADNT produced by adding $2e^- + 2H^+$. The biological process continued, and water molecules were lost regularly. The ADNT and then DANT, and eventually 4-Hydroxytoluene were produced [28-29].

Intermediates in the anaerobic-aerobic process were produced by reducing one or two nitric groups and forming ADNT and DANT metabolites in different stages of biotransformation. Nitro (NO_2^-) groups were converted to nitroso (-NO) and hydroxylamino (-NHOH) and eventually to the amino group (NH_2^-), respectively [33]. The Bernstein et al. study showed that TAT (Triaminotoluene) was detected as a result of an intermediate analysis[34]. One of the essential pathways for the biological transformation of TNT is the direct reduction of the aromatic ring by adding ionic hydride and forming a variety of complexes, known as Meisenheimer and adding a hydride ion to TNT structure. In the next stage, hydride ion was added to complex composition and Dihydride –Meisenheimer was formed. A survey of produced metabolites in aerobic- the anaerobic process has shown that detected metabolites are usually highly active and can spontaneously oxidize in the conversion of TNT nitro group to amino compounds, such as Hydroxylamino and Nitroso compounds (Table 4). In the final stage, diarylamine compounds are produced due to reaction with the other existing amino compounds and the liberation of nitrite ions.

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The results of a study by Christoff et al. showed that Hydroxylamino and Nitroso compounds from aerobic biodegradation had higher toxicity for plants and animals than the final anaerobic metabolites [35]. Anaerobic or aerobic process leads to the effective elimination of explosives and their carcinogenic metabolites[28,34,36]. More than 99% removal of TNT and its metabolites were observed in the biological reactor in the form of aerobic - anaerobic sequences. The results of aerobic-anaerobic sequencing studies showed that TNT was completely degraded, and simple organic acids and carbon compounds, such as CO_2 were produced as the final products [37]. In aerobic conditions, the production of active compounds of Hydroxylamino leads to azo or azoxy compounds. The reaction of these compounds with the other metabolites led to dimerization or polymerization and production of compounds with higher toxicity. Anaerobic metabolism is accomplished in two steps. First, TNT is converted to amino products. It should be considered that the products of this phase are similar to the primary aerobic products. In the second step, the anaerobic process begins with the decomposition of non-aromatic products; this stage is followed by the removal of the third nitro group [38].

The PETN biodegradable metabolites are produced from multi-stage denitrification. These metabolites are shown in Tables 3 and 4. In the study of Binks et al., PETN is biodegraded by Enterobacter cloacae PB2 microorganism under aerobic conditions. The main identified metabolites were similar to the present study [9]. The results showed PETN degradation metabolites under anaerobic-aerobic and aerobic-anaerobic conditions include NO_2 release at each reduction stage. One of the PETN biodegradation metabolites is pentaerythritol mono-nitrate (PEMN). This product was not detected in the anaerobic-aerobic step, which could be related to the lack of completion of the denitrification process. This compound was not observed in the study by zwang et al., conducted with iron granules [20]. In anaerobic conditions, PETN was biotransformed to pentaerythritol metabolite (Figure 4), while redox potential was -350 mV in this condition. It can be interpreted that the formation of this compound reflects the completion of reduction conditions by donating the nitro group [22].

Conclusion

Using biosurfactants with the help of indigenous bacteria inoculation significantly affected PETN removal efficiency. In other words, microbial inoculation led to an increase in the active microbial consortium, and also, biosurfactants could improve the solubility and bioavailability of the explosives. Moreover, rhamnolipid and microbial bioaugmentation could effectively maximize microbial growth separately and simultaneously with different concentrations of TNT and PETN (S0), in which the top microbial growth has occurred. The results of anaerobic-aerobic and aerobic-anaerobic sequencing processes were closely similar, but the most important difference was related to intermediate and final metabolites. According to metabolites type, the aerobic-anaerobic sequence could produce less harmful metabolites for the environment and health. Dangerous intermediate compounds, such as Azoxy and Azo may be produced in aerobic-anaerobic sequence processes. Therefore, it cannot be predicted whether or not this sequence is better than the anaerobic-aerobic sequence. In an aerobic-anaerobic sequence process, the anaerobic process could help remove produced amino acids from the aerobic stage. Given that in an aerobic-anaerobic sequence, the reactions were more accomplished, and its final product was pentaerythritol. It seems that aerobic-anaerobic condition was suitable for the biodegradation process and capable of nitro aromatic mineralization.

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Conflict of interest

The authors declare that there is no conflict of interest.

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