

Kinetic parameters and nitrate, nitrite changes in bioremediation of Toxic Pentaerythritol Tetranitrate (PETN) contaminated soil

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Abstract

Background: Cleanup of areas contaminated by explosives is a public health concern. Some explosives can be carcinogenic in humans. Pentaerythritol Tetranitrate (PETN), a powerful explosive with very low water solubility, can be easily transported to ground waters.

Objective: This study was conducted to determine the removal efficiencies of PETN from soil by bioremediation, and obtain kinetic parameters of biological process.

Methods: This experimental study was conducted at the Environmental Health Engineering Lab (Isfahan University of Medical Sciences, Isfahan, Iran) in 2015-2016. In the present work, bioremediation of the explosive-polluted soils by PETN in anaerobic-aerobic landfarming method was performed. The influence of seeding and biosurfactant addition on bioremediation was also evaluated. The data were analyzed using Microsoft Excel software.

Results: The results show that, as the initial concentration of PETN increased, the lag phase was increased and the specific growth rate was increased up to 0.1/day in concentration of 50 mg/kg, and then it was decreased to 0.04/day. Subsequent decreases in specific growth rate can cause substrate inhibition. Seeding causes decrease in lag phase significantly. Biosurfactant addition had little to no impact on the length of lag phase, but biosurfactant plus seeding can increase the growth rate to 0.2/day, however, inhibitory effect of the initial concentration was started in very high concentration of PETN (150 mg/kg).

Conclusion: Biosurfactant addition and seeding together have an impressive effect on biodegradation of PETN, furthermore seeding can enhance active microbial consortium and biosurfactant can improve the poor aqueous solubility of PETN, therefore making the substrate more accessible.

Keywords: PETN, Soil, Bioremediation, Nitrate, Nitrite, Kinetic parameters

1. Introduction

Cleanup of areas contaminated by explosives is a public health concern; therefore, considerable efforts have been invested into finding economical remediation technologies (1). Toxic and mutagenic effects of some explosives to

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various organisms ranging from microorganisms and humans have been described, even at low concentration (2). For example, TNT as a possible human carcinogenic (class C) has been classified by the United States Environmental Protection Agency (EPA) (3). Several methods, such as composting, chemical oxidation, adsorption and incineration have been developed for treatment of explosive contaminated sites (4, 5). Economical and environmental limitations and high costs of such treatment cause recent researches to have more focus on biotreatment processes which are efficient, cost effective and environmental friendly (6). Pentaerythritol Tetranitrate (PETN), is a powerful explosive usually used in detonators, and is also used as a coronary vasodilator treatment (7). It is highly hydrophobic and has very low water solubility, thus, sorbs weakly to organic materials in the soil and sediment and therefore, can be readily transported to ground waters. PETN have not been detected as naturally occurring compounds, and introduction to the environment by industrial production and usage constitutes considered true xenobiotic challenges to microorganisms (8). Biological treatment processes have been intensively investigated for remediating explosive contaminated soil (9). The biological processes used for explosive remediation differ in the aerobic or anaerobic amount, type of external carbon sources added, and degree of water saturation (10). In order to achieve a more complete incorporation of metabolized nitroaromatic in soil, combined anaerobic-aerobic composting was proposed. Several reports deal with anaerobic or anaerobic-aerobic incubations of TNT-contaminated soil (11). Injecting of microorganism into the polluted soil is a strategy to increase the biodegradation rates. Several studies confirm that increasing the initial biomass level, may enhance the biodegradation rate and improve the treatment process by reducing the time needed for adaptation (12). Another factor associated with the bioavailability of pollutants is low water solubility that often corresponds to inhibition of the degradation rate (13). Surface active compounds or surfactant can be used to increase the bioavailability of poorly accessible compounds, thus helping to overcome the diffusion-related mass transfer limitations (14). Recently, microbial base surface active compounds have gained much scientific attention, since these compounds have biodegradability and lower toxicity compared to their synthetic counterparts (15). Rhamnolipids are among the most investigated and well-described group of compounds and are potential agents for enhancing the efficiency of in situ bioremediation attempts in polluted terrestrial environments (16). Because biological transformation causes a higher percent of explosive to convert to nitro-intermediate, obviously, it is not incompetent to assess remediation efficiency of the polluted soil through monitoring residual concentration of PETN; but, the chemical oxidation demand (COD) is more suitable to reflect the removal efficiency of all nitro-compound pollutants as an integrated index. A 2013 study by Baoping Xin showed that total organic carbon (TOC) can reflect the removal efficiency of TNT and the intermediate of bioremediation (17). It is necessary to evaluate the relationship between the specific growth rate and the concentration of substrate to describe the biodegradation conditions. Kinetic study is necessary for improvement of the process control of contaminated soil bioremediation and wastewater treatment processes. Investigations on the kinetic model for the degradation of compounds such as phenol and TNT have been published (18, 19). In the present works, bioremediation of the explosive-polluted soils by PETN in anaerobic-aerobic landfarming method was performed. The influence of seeding and biosurfactant addition on bioremediation was also evaluated. For evaluating the remediation efficiency of PETN polluted soils, studies of three aspects were conducted, 1) removal efficiencies of PETN concentration from soil under different conditions. 2) Removal efficiencies of the organic compound from water leachate of soil based on COD analysis; 3) measurement of nitrate and nitrite medium as a result of bacterial transformation of PETN. 4) Obtain kinetic parameters of bioremediation.

2. Material and Methods

This experimental study was conducted at the Environmental Health Engineering lab, Isfahan University of Medical Sciences, Isfahan, Iran in 2015 – 2016.

2.1. PETN-contaminated soil

For polluted soil, PETN dissolved in acetone and transferred into soil and was evenly distributed to obtain a final PETN concentration of 200 mg/kg in soil matrix.

Garden soil containing 5.5% organic matter was used for bioremediation experiment. The PETN concentration was 200 mg/kg soil. Rhamnolipids were purchased from the National Institute of Genetic Engineering and Biotechnology of Iran.

2.2. Laboratory batch experiment

For aerobic and anaerobic soil bioremediation, six soil-pan experiments were conducted. Each pan was prepared by placing 3000 g of contaminated soil in a square polyethylene with aluminum foil as surface coatings for aerobic and 4000 g for anaerobic. This mass of soil filled the pans approximately two-thirds full. For Improvement of soil porosity, 40 g sown dust was mixed thoroughly into 1000 g of the soil. A total of 4 sets of treatments were

conducted; set-1, were control samples with no amendment added; set-2 contained 3000 g of contaminated soil plus 200 g of activated sludge; set-3 contained 3000 g of contaminated soil plus biosurfactant; set-4 contained 3000 g of contaminated soil plus biosurfactant and 200 g activates sludge as a source of microbes. The bottoms of the pans were perforated to allow drainage of fluids during and after flooding phases, via 1.5 mm diameter holes spaced 2 cm apart in a square grid. Each pan was placed inside a slightly larger glass pan to provide nutrient solutions and sampling of drainage water for COD and PETN concentrations. The anaerobic pan continuously flooded with deionized water to maintain anaerobic conditions in the pan. During the aerobic phase, air was introduced into the soil slurry system. Air was supplied twice daily for 20 minutes, through a diffuser. During sets 3 and 4, rhamnolipid biosurfactants were added to stimulate biological activity and improve the solubility of PETN. The duration of the anaerobic phase was 80 days. This was followed by an aerobic phase of 20 days in a different pan, by transferring the leachate of the anaerobic pan. At the start of the aerobic phase, 500 mL of the mixed bacterial culture obtained from activated sludge was added. PETN-contaminated soil spiked and was treated anaerobically by percolation for 80 days with tap water. After 80 days, the water was drained off and reactor was flushed with air, and the soil was treated aerobically for 20 days.

2.3. Chemical analyses

PETN was supplied by a local explosives producer in Isfahan city. Other chemicals were of analytical grade, and were obtained from Sigma-Aldrich and Merck. Nitrate and ammonia concentrations were analyzed by colorimetric methods using Hach water analyses reagent kits (20). Analysis of PETN transformation was carried out using a Waters HPLC system (Milford, USA) equipped with a UV detector. Water–methanol–acetonitrile mixture (40:50:10, v/v/v) was used as the mobile phase at a flow rate of 0.8 ml/min. The injection volume was 20 μ L and the absorbance was measured at a wavelength of 210 nm. The detection limit was 0.1 mg/L. For the LC/MS analysis, a Waters 2790 LC system was coupled to a Quattro Micro mass spectrometer (Waters, Milford, MA, USA). PETN metabolite was measured by LC-MS. Biological kinetics calculated by a method used by Admassu and et al 1998, and biological processes data, was analyzed and shown by Microsoft Excel software (21).

3. Results

Combination of anaerobic and aerobic treatment for explosive bioremediation used in this study is similar to previous studies that used this combination for 2, 4, 6-Trinitrotoluene biotransformation (22). Analysis of the soil mixture showed that the anaerobic treatment with rhamnolipid biosurfactant (80 days) caused an almost 74% transformation of PETN and 30 days' aeration of the reactor led to an elimination of most of the remaining PETN (almost 98%). It should be mentioned that a 24% PETN removal was observed in the control experiments (without biosurfactant). Kinetic study is necessary for improvement of the process control of contaminated soil bioremediation. In this study, some of the biological kinetic parameter in reactor that has an important role in efficiency and rate of bioremediation was investigated. Biological kinetic parameter in this study investigated for aerobic reactor after the anaerobic. The specific growth rates (μ) at five initial concentrations of PETN (20, 50, 100, 150, 200 mg/kg) were determined in the exponential growth phase from the slope of linear semi logarithmic plots of bacterial growth (cfu/ml) against time. Figure 1 shows a plot of bacterial growth (cfu/ml) against time for a sample taken from the aerobic pan, with initial concentration 20 mg/kg PETN that is seeding with activated sludge. The specific growth rate (μ) with other initial PETN concentrations was performing from 20 mg/kg to 200 mg/kg in a different pan pattern. The influence of PETN concentration on the duration of the lag phase and on the specific growth rates is shown in Figure 2 for pans seeding with activated sludge. The results show that, as the initial concentration of PETN increased, the lag phase was increased and the specific growth rate was increased up to 0.1/day in concentration of 50 mg/kg, then it was decreased to 0.04/day. The influence of seeding and biosurfactant on the duration of the lag phase and on the specific growth rates is shown in different initial concentrations of PETN (Figures 3 and 4). It is notable that seeding causes decrease in lag phase significantly. Figure 4 shows the positive effects of seeding and biosurfactant on the specific growth rates. Increases in initial PETN concentration decreased the specific grow rate in natural soil. Although the concentration of PETN in culture media increased, but organism populations cannot show effective response and causes initial concentration inhibitory effect. Seeding causes increase in specific grow rate to 0.1/day. Maximum growth rate observed in 50 mg/kg PETN that is higher than natural soil. SEM images of the Micellar aggregates in the soil sample with 60 mg/kg soil (CMC) rhamnolipid plus sludge siding, shows in Figure 5. The results in Table1 show that nitrate and nitrite in the natural soil sample does not change significantly. This result is in consonant with the PETN removal efficiency. On the other hand, nitrite in the sample with biosurfactant and seeding has initial increase and late decreases, and nitrate decrease in first and increases subsequently.

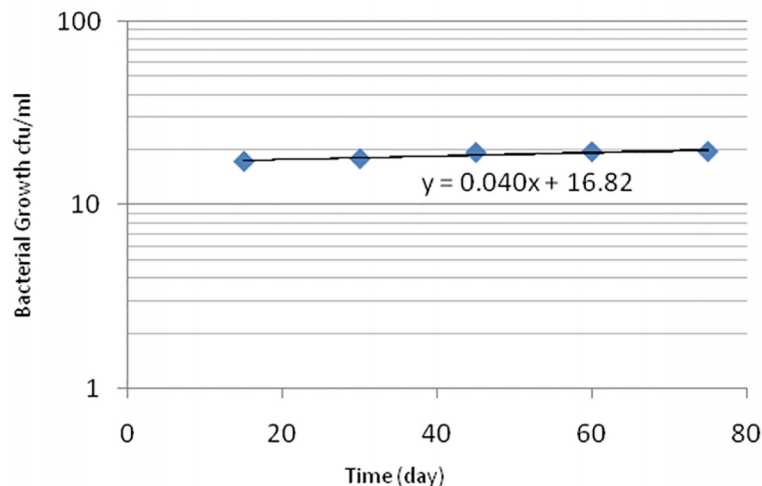


Figure 1. Plot of bacterial growth (cfu/ml) against time for sample taken from aerobic pan with initial concentration 20 mg/kg soil PETN that has seeding with activated sludge.

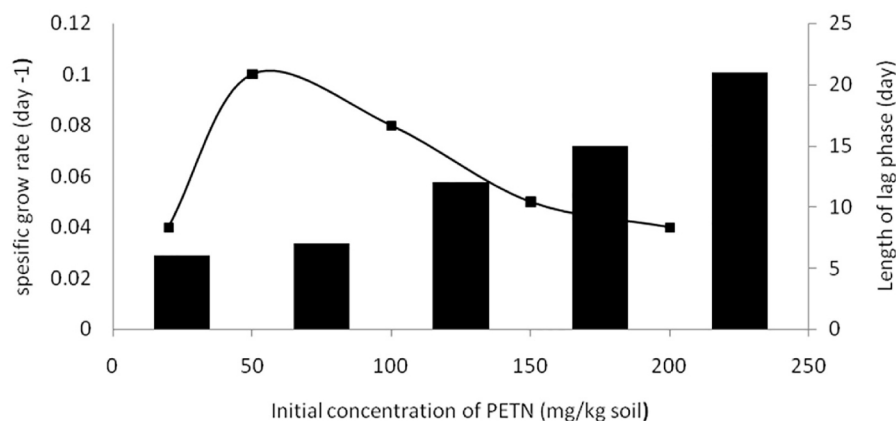


Figure 2. The influence of PETN concentration on the duration of the lag phase and on the specific growth rates for pan that has seeding with activated sludge.

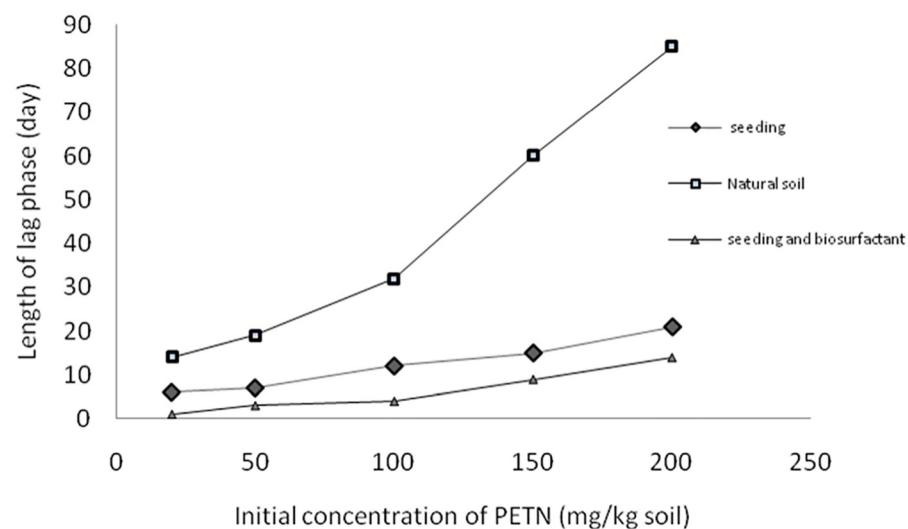


Figure 3. The influence of PETN concentration on the duration of the lag phase in pan content natural soil, soil with seeding and soil with seeding plus biosurfactant.

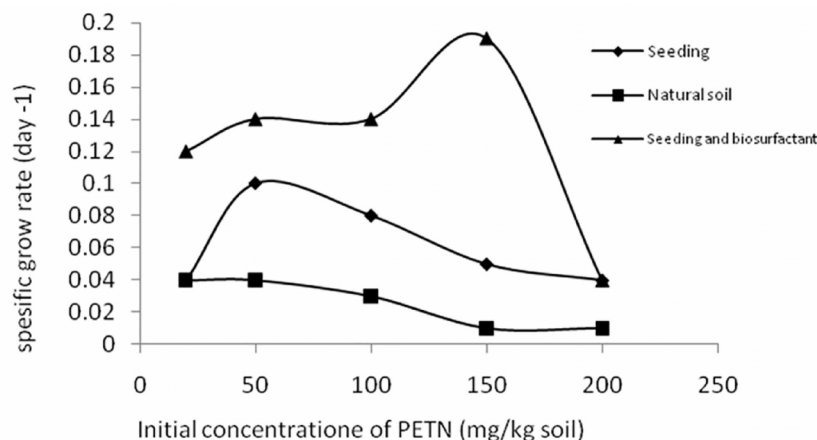


Figure 4. The influence of PETN concentration on the specific growth rate for pan content natural soil, soil with seeding and soil with seeding plus biosurfactant.

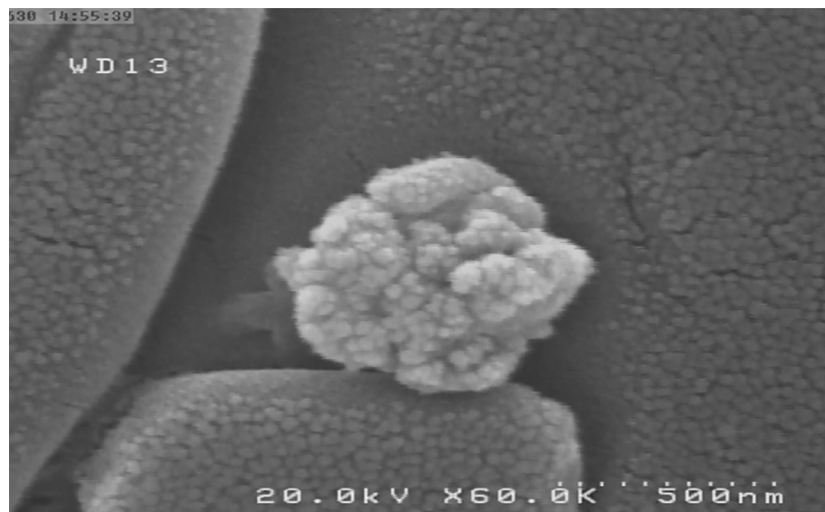


Figure 5. SEM images of the Micellar aggregates in soil sample with 60 mg/kg soil (CMC) rhamnolipid plus sludge siding.

Table 1. Change in nitrate and nitrite concentration over the bioremediation in natural soil and soil with seeding plus biosurfactant.

Time (week)	No ₂ (g/kg soil)		No ₃ (mg/kg soil)	
	Natural soil Sample	Sample with seeding and biosurfactant	Natural soil Sample	Sample with seeding and biosurfactant
1	2	6	85	85
4	8	10	82	51
8	14	74	68	30
12	14	41	65	92
15	15	2	66	112

4. Discussion

The results show that, as the initial concentration of PETN increased, the lag phase was increased and the specific growth rate was increased up to 0.1/day in concentration of 50 mg/kg, then it was decreased to 0.04/day. Specific growth rate 0.04/day is the maximum growth rate. Subsequent decreases in specific growth rate can be caused by substrate inhibition. The results of previous studies in TNT show that substrate inhibition effect may occur at TNT concentrations higher than 20 mg/L in aquatic environments (23). The results show that seeding causes decrease in lag phase significantly. Several studies confirm that increasing the initial biomass level may enhance the

biodegradation rate, and improve the treatment process by reducing the time needed for adaptation. Several studies proved that the inoculation with selected microorganisms in order to achieve an enhanced bioremediation of hydrocarbon contaminated soil, may be a valid and efficient strategy (24). Increasing the initial biomass concentration can cause increase of activated organism populations for bioremediation, thus with increases in initial PETN concentration, length of lag phase does not change significantly. Other studies used this strategy for biodegradation of high concentration petroleum hydrocarbon oil (14,000 mg/kg) (25). Despite seeding, biosurfactant addition had little to no impact on the length of lag phase. It can be due to organisms living in different habitats needing adaptations to building enzymes and use the substrate. Biosurfactant effect such as increased solubility also seems unbeneficial for this. Biosurfactant plus seeding, can increase the growth rate to 0.2/day, on the other hand, inhibitory effect of the initial concentration was started in very high concentration of PETN (150 mg/kg). Biosurfactant addition and seeding together, have an impressive effect on biodegradation of PETN. On the one hand, seeding can enhance active microbial consortium, and biosurfactant can improve the poor aqueous solubility of PETN, which therefore makes the substrate more accessible. Actually, seeding and biosurfactant act as complementary. Combination of bioaugmentation and biosurfactant addition applied for diesel-oil, contaminated soil bioremediation and caused significant improvement of bioremediation efficiency (26). Enhancement of xenobiotic compound solubility is one of the main mechanisms that surfactants can improve the bioavailability of hydrophobic organic pollutants. This so-called 'solubilisation' is caused by the presence of micelles (figure 5). Formation of micelles is a specific characteristic of surfactants that consist of small aggregates of surfactant molecules. In the center of the micelles, hydrophobic compounds will dissolve, whereas more hydrophilic molecules may be present in the core and the shell of the micelles. The results of older studies show that in many cases, transport of micellar hydrocarbon to the aqueous phase can be very rapid (27). Applications of rhamnolipid biosurfactant for improvement of xenobiotic bioremediation examined in other studies such as, Zhang and Miller (1992) for octadecane (28), Beal and Betts (2000) for hexadecane (29), Noordman et al. (2002) for hexadecane (30), Al-Awadhi et al. (1994) for oil-contaminated desert sands (31), Rahman et al. (2003) for n-alkanes in petroleum sludge (32), and Park et al., (1990) for polycyclic or poly nuclear aromatic hydrocarbons (PAHs) (33). The result in Table1 show that nitrite in sample with biosurfactant and seeding, has initial increase and late decreases and nitrate decrease at first and increase subsequently. This nitrite increase can result from multistage nitro group release from PETN structure. Then, some of the nitrate oxidized to nitrite and the nitrite concentration increased in last stages of bioremediation significantly. These changes in nitrate and nitrite can be correlated with the sequential reductive denitration processes that are bases for PETN biodegradation and some demineralization evidence. PETN underwent sequential reductive denitration processes, releasing nitrite in each denitration step, which was subsequently reduced to ammonia (34). On the other hand, release of NO₂ group from nitroaromatic such as TNT, a positive evidence of demineralization has previously been reported. Sequential reductive denitration processes are a basis for PETN and TNT biodegradation, thus nitrite and nitrate release can be considered as PETN demineralization evidence. As PETN degraded, the denitrated metabolites consisting of PETriN, PEDN and PEMN are generated. This metabolite is the same as others mentioned in previous studies. The presence of PETriN, PEDN, PEMN and the potential presence of pentaerythritol can be evidence that three or four nitro groups are sequentially removed from PETN via biological reactions (35). As in PETN degradation, the removal of the three intermediates in the presence or absences of rhamnolipid in anaerobic and aerobic were different. In anaerobic treatment, in the tab with biosurfactant, PETriN, PEDN, PEMN are detected but in the tab without biosurfactant only PETriN was detected. These results indicate that the tab with biosurfactant denitration process is more completed than the tab without biosurfactant. Although, after the end of anaerobic phase with biosurfactant shows higher removal of PETN, the denitration metabolites (PEDN, PEMN) were still found. Only during the aerobic phase did the denitration metabolites disappear completely. These results can be due to humification, and bounding the denitration metabolites to the soil. In this study, it was not possible to access the radiolabel explosive material. Research on radiolabel explosives in bioremediation can help to determine the ultimate fate of intermediate and final compounds.

5. Conclusions

As a result of this study, it is concluded that, Biosurfactant plus seeding can increase constant growth rates. On the other hand, inhibitory effect of the initial concentration was started in very high concentration of PETN. Biosurfactant addition and seeding together have an impressive effect on biodegradation of PETN, and seeding can enhance active microbial consortium. Biosurfactant can improve the poor aqueous solubility of PETN, therefore making the substrate more accessible. Actually, seeding and biosurfactant act as complementary. Change in nitrate and nitrite concentration in soil samples can be correlated with the sequential reductive denitration processes that are bases for PETN biodegradation and some demineralization evidence.

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Conflict of Interest:

There is no conflict of interest to be declared.

Authors' contributions:

All authors contributed to this project and article equally. All authors read and approved the final manuscript.

References:

- 1) Lewis TA, Newcombe DA, Crawford RL. Bioremediation of soils contaminated with explosives. *J Environ Manage.* 2004; 70(4): 291-307. PMID: 15016438.
- 2) Ayoub K, van Hullebusch ED, Cassir M, Bermond A. Application of advanced oxidation processes for TNT removal: a review. *J Hazard Mater.* 2010; 178(1): 10-28. doi: 10.1016/j.jhazmat.2010.02.042. PMID: 20347218.
- 3) Gandia - Herrero F, Lorenz A, Larson T, Graham IA, Bowles DJ, Rylott EL, et al. Detoxification of the explosive 2, 4, 6 - trinitrotoluene in *Arabidopsis*: discovery of bifunctional O - and C - glucosyltransferases. *The Plant J.* 2008; 56(6): 963-74. doi: 10.1111/j.1365-313X.2008.03653.x. PMID: 18702669.
- 4) Mercimek HA, Dincer S, Guzeldag G, Ozsavli A, Matyar F, Arkut A, et al. Degradation of 2,4,6-trinitrotoluene by *P. aeruginosa* and characterization of some metabolites. *Braz J Microbiol.* 2015; 46(1): 103-11. doi: 10.1590/S1517-838246120140026. PMID: 26221094. PMCID: PMC4512054.
- 5) Barreto-Rodrigues M, Silva FT, Paiva TC. Characterization of wastewater from the Brazilian TNT industry. *J Hazard Mater.* 2009; 164(1): 385-8. doi: 10.1016/j.jhazmat.2008.07.152. PMID: 18818021.
- 6) Fu D, Zhang Y, Lv F, Chu PK, Shang J. Removal of organic materials from TNT red water by Bamboo Charcoal adsorption. *Chemical engineering journal.* 2012; 193: 39-49. doi: 10.1016/j.cej.2012.03.039.
- 7) Binks PR, French CE, Nicklin S, Bruce NC. Degradation of pentaerythritol tetranitrate by *Enterobacter cloacae* PB2. *Applied and Environmental Microbiology.* 1996; 62(4): 1214-9. PMID: 8919782. PMCID: PMC167887.
- 8) White GF, Snape JR. Microbial cleavage of nitrate esters: defusing the environment. *Microbiology.* 1993; 139(9): 1947-57. doi: 10.1099/00221287-139-9-1947. PMID: 8245825.
- 9) Esteve-Núñez A, Caballero A, Ramos JL. Biological degradation of 2,4,6-trinitrotoluene. *Microbiol Mol Biol Rev.* 2001; 65(3): 335-52. doi: 10.1128/MMBR.65.3.335-352.2001. PMID: 11527999, PMCID: PMC99030.
- 10) Knicker H, Achtnich C, Lenke H. Solid-state nitrogen-15 nuclear magnetic resonance analysis of biologically reduced 2,4,6-trinitrotoluene in a soil slurry remediation. *J Environ Qual.* 2001; 30(2): 403-10. PMID: 11285900.
- 11) Funk SB, Roberts DJ, Crawford DL, Crawford RL. Initial-phase optimization for bioremediation of munition compound-contaminated soils. *Appl Environ Microbiol.* 1993; 59(7): 2171-7. PMID: 8357251, PMCID: PMC182253.
- 12) Szulc A, Ambrożewicz D, Sydow M, Ławniczak Ł, Piotrowska-Cyplik A, Marecik R, et al. The influence of bioaugmentation and biosurfactant addition on bioremediation efficiency of diesel-oil contaminated soil: Feasibility during field studies. *J Environ Manage.* 2014; 132: 121-8. doi: 10.1016/j.jenvman.2013.11.006. PMID: 24291585.
- 13) Harms H, Bosma TN. Mass transfer limitation of microbial growth and pollutant degradation. *Journal of Industrial Microbiology and Biotechnology.* 1997; 18(2-3): 97-105. doi: 10.1038/sj.jim.2900259.
- 14) Makkar RS, Rockne KJ. Comparison of synthetic surfactants and biosurfactants in enhancing biodegradation of polycyclic aromatic hydrocarbons. *Environ Toxicol Chem.* 2003; 22(10): 2280-92. PMID: 14551990.
- 15) Bordoloi NK, Konwar BK. Bacterial biosurfactant in enhancing solubility and metabolism of petroleum hydrocarbons. *J Hazard Mater.* 2009; 170(1): 495-505. doi: 10.1016/j.jhazmat.2009.04.136. PMID: 19619942.

- 16) Chrzanowski Ł, Ławniczak Ł, Czaczyk K. Why do microorganisms produce rhamnolipids?. *World J Microbiol Biotechnol.* 2012; 28(2): 401-19. doi: 10.1007/s11274-011-0854-8. PMID: 22347773, PMCID: PMC3270259.
- 17) Xin B, Shen M, Aslam H, Wu F. Remediation of explosive-polluted soil in slurry phase by aerobic biostimulation. In *Journal of Physics: Conference Series.* IOP Publishing. 2013; 439(1): 012047.
- 18) Pawlowsky U, Howell JA. Mixed culture biooxidation of phenol. I. Determination of kinetic parameters. *Biotechnology and Bioengineering.* 1973; 15(5): 889-96. doi: 10.1002/bit.260150506.
- 19) Admassu W, Sethuraman AV, Crawford R, Korus RA. Growth kinetics of *Clostridium bifermentans* and its ability to degrade TNT using an inexpensive alternative medium. *Bioremediation Journal.* 1998; 2(1):17-28. doi: 10.1080/10889869891214187.
- 20) Ormazá-González FI, Villalba-Flor AP. The measurement of nitrite, nitrate and phosphate with test kits and standard procedures: A comparison. *Water Research.* 1994; 28(10): 2223-8. doi: 10.1016/0043-1354(94)90035-3.
- 21) Der Yang R, Humphrey AE. Dynamic and steady state studies of phenol biodegradation in pure and mixed cultures. *Biotechnology and Bioengineering.* 1975; 17(8): 1211-35. doi: 10.1002/bit.260170809.
- 22) Bruns-Nagel D, Drzyzga O, Steinbach K, Schmidt TC, Von Loew E, Gorontzy T, et al. Anaerobic/aerobic composting of 2,4,6-trinitrotoluene-contaminated soil in a reactor system. *Environmental science & technology.* 1998; 32(11): 1676-9. doi: 10.1021/es970757z.
- 23) Park C, Kim TH, Kim S, Lee J, Kim SW. Biokinetic parameter estimation for degradation of 2,4,6-trinitrotoluene (TNT) with *Pseudomonas putida* KP-T201. *J Biosci Bioeng.* 2002; 94(1):57-61. PMID: 16233270.
- 24) Mishra S, Jyot J, Kuhad RC, Lal B. Evaluation of inoculum addition to stimulate in situ bioremediation of oily-sludge-contaminated soil. *Appl Environ Microbiol.* 2001; 67(4): 1675-81. doi: 10.1128/AEM.67.4.1675-1681.2001. PMID: 11282620, PMCID: PMC92784.
- 25) Liu PW, Chang TC, Whang LM, Kao CH, Pan PT, Cheng SS. Bioremediation of petroleum hydrocarbon contaminated soil: effects of strategies and microbial community shift. *International Biodeterioration & Biodegradation.* 2011; 65(8): 1119-27. doi: 10.1016/j.ibiod.2011.09.002.
- 26) Lin TC, Pan PT, Young CC, Chang JS, Chang TC, Cheng SS. Evaluation of the optimal strategy for ex situ bioremediation of diesel oil-contaminated soil. *Environ Sci Pollut Res Int.* 2011; 18(9): 1487-96. doi: 10.1007/s11356-011-0485-5. PMID: 21538227.
- 27) Volkering F, Breure AM, van Andel JG, Rulkens WH. Influence of nonionic surfactants on bioavailability and biodegradation of polycyclic aromatic hydrocarbons. *Appl Environ Microbiol.* 1995; 61(5): 1699-705. PMID: 16535016, PMCID: PMC1388434.
- 28) Zhang YI, Miller RM. Enhanced octadecane dispersion and biodegradation by a *Pseudomonas* rhamnolipid surfactant (biosurfactant). *Appl Environ Microbiol.* 1992; 58(10): 3276-82. PMID: 1444363, PMCID: PMC183091.
- 29) Beal R, Betts WB. Role of rhamnolipid biosurfactants in the uptake and mineralization of hexadecane in *Pseudomonas aeruginosa*. *J Appl Microbiol.* 2000; 89(1): 158-68. PMID: 10945793.
- 30) Noordman WH, Wachter JH, De Boer GJ, Janssen DB. The enhancement by surfactants of hexadecane degradation by *Pseudomonas aeruginosa* varies with substrate availability. *J Biotechnol.* 2002; 94(2): 195-212. PMID: 11796172.
- 31) Al-Awadhi N, Williamson KJ, Isok JD. Remediation of Kuwait's oil-contaminated soils. *Hydrocarbon contaminated soils and ground water.* 1993; 3: 9-21.
- 32) Rahman KS, Rahman TJ, Kourkoutas Y, Petsas I, Marchant R, Banat IM. Enhanced bioremediation of n-alkane in petroleum sludge using bacterial consortium amended with rhamnolipid and micronutrients. *Bioresour Technol.* 2003; 90(2): 159-68. doi: 10.1016/S0960-8524(03)00114-7. PMID: 12895559.
- 33) Park KS, Sims RC, Dupont RR. Transformation of PAHs in soil systems. *Journal of Environmental Engineering.* 1990; 116(3): 632-40. doi: 10.1061/ASCE.0733-9372116:3(632).
- 34) Zhuang L, Gui L, Gillham RW. Biodegradation of pentaerythritol tetranitrate (PETN) by anaerobic consortia from a contaminated site. *Chemosphere.* 2012; 89(7): 810-6. doi: 10.1016/j.chemosphere.2012.04.062. PMID: 22647196.
- 35) Zhuang L, Gui L, Gillham RW. Degradation of pentaerythritol tetranitrate (PETN) by granular iron. *Environ Sci Technol.* 2008; 42(12): 4534-9. doi: 10.1021/es7029703. PMID: 18605582.