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# Biodegradation of explosives mixture in soil under different water-content conditions

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This paper is dedicated to the memory of Prof. Ronit Nativ who passed away on 31 October 2006.

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ABSTRACT

Soil redox potential plays a key role in the rates and pathways of explosives degradation, and is highly influenced by water content and microbial activity. Soil redox potential can vary significantly both temporally and spatially in micro-sites. In this study, when soil water content increased, the redox potential decreased, and there was significant enhancement in the biodegradation of a mixture of three explosives. Whereas TNT degradation occurred under both aerobic and anaerobic conditions, RDX and HMX degradation occurred only when water content conditions resulted in a prolonged period of negative redox potential. Moreover, under unsaturated conditions, which are more representative of real environmental conditions, the low redox potential, even when measured for temporary periods, was sufficient to facilitate anaerobic degradation. Our results clearly indicate a negative influence of TNT on the biodegradation of RDX and HMX, but this effect was less pronounced than that found in previous slurry batch experiments: this can be explained by a masking effect of the soil in the canisters. Fully or partially saturated soils can promote the existence of micro-niches that differ considerably in their explosives concentration, microbial community and redox conditions.



## 1. Introduction

In many explosive-contaminated sites, the soil is contaminated with mixtures of explosives, most commonly 2,4,6-trinitrotoluene (TNT), hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) and octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX) [1,2] (Fig. 1). Biodegradation of these compounds appears to be an effective means of remediating contaminated soil and water. Redox conditions highly influence the efficiency of explosives biodegradation in the sub-surface, as well as dictate the degradation pathway and the accumulated degradation products.

TNT can be biodegraded both aerobically and anaerobically. In both cases, the initial products formed are the reduced amino derivatives 4-amino-2,6-dinitrotoluene (4-Am-2,6-DNT) and 2-amino-4,6-dinitrotoluene (2-Am-4,6-DNT) [1]. Further degradation to the most reduced product, triaminotoluene (TAT), requires highly negative redox potential values—below −200 mV—and therefore TAT is found only in reduced environments [3]. Condensation of the partly reduced amino intermediates may occur, leading to the formation of azoxy intermediates [3,4].

The biodegradation of RDX occurs under both aerobic and anaerobic conditions but the anaerobic process is significantly faster [5]. Aerobically, it has been found that microbial cleavage of one of the N—$NO_2$ bonds produces unstable intermediates, and is followed by rapid cleavage of the triazine ring [1]. Anaerobically, sequential reduction of the nitro groups to produce mono-, di- and trinitroso derivatives (MNX, DNX and TNX, respectively) is most frequently observed. The nitroso derivatives may be further transformed to produce the unstable hydroxylamine derivatives, leading to ring cleavage [6]. This pathway was described for RDX incubation with municipal sludge under measured $E_h$ values of −250 to −300 mV [7].

HMX mostly undergoes anaerobic biodegradation through the reduction of nitro groups to form the corresponding nitroso derivatives, or alternatively via direct ring cleavage [8].

The soil's redox potential plays a key role in the degradation pathway of explosives, as well as in the rate of degradation [1,5,8–10]. Thus, to predict the fate of explosives in the environment, knowledge of the redox conditions is required. Explosives degradation experiments in which redox potential was measured have involved experiments in soil slurries that do not represent natural conditions [9,11].

Soil redox potential is known to be highly influenced by both water content and microbial respiration. Molecular oxygen acts as

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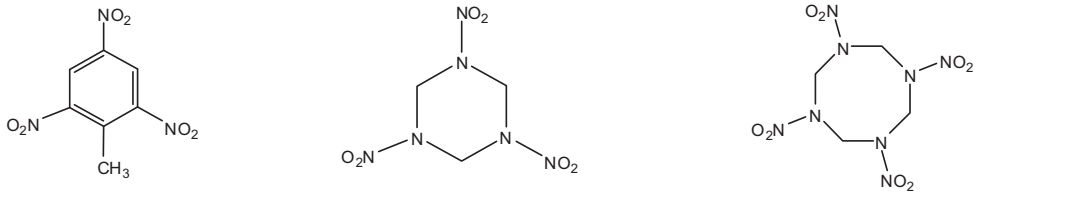


2,4,6-trinitrotoluene (TNT) | Hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) | Octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX)

**Fig. 1.** The molecular structures of TNT, RDX and HMX.

the preferred electron acceptor but once it is consumed, the microbial activity switches to fermentation and anaerobic respiration [12]. The soil water potential and pore structure determine the rate at which oxygen is replenished in soil since the diffusion coefficient of oxygen is lower in water than in gas [13]. It has been shown that soil redox potential can be very dynamic, varying both temporally and spatially by several orders of magnitude, in micro-sites [12]. Since under natural conditions soils are normally unsaturated, the question is which of the pathways that are detected in the laboratory can also occur in the field, and under what water contents. We hypothesized that anoxic conditions might develop in the unsaturated zones that are sufficient for the anaerobic degradation pathways to occur in the field. This is reinforced, for example, by the detection of RDX's anaerobic nitroso derivatives along unsaturated soil profiles [14].

The degree of saturation can affect the extent at which one compound affects the degradation of another, as observed in slurry experiments [2]. It is possible that lower saturation will decrease the availability of inhibiting compounds.

To fill in some of these gaps in our knowledge of explosives biodegradation under unsaturated conditions we explored whether anaerobic conditions might exist in unsaturated soils with different water content and whether these conditions would be sufficient for anaerobic biodegradation of the explosives. We further determined whether the inhibitory effect of TNT and its degradation products on RDX and HMX degradation occurs in unsaturated soils, similar to the reported observation for saturated conditions [2].

## 2. Experimental

### 2.1. Chemicals

TNT, RDX and HMX (>95% purity) provided by the Israeli Military Industry were used for the biodegradation experiment. Analytical standards for HPLC analysis of RDX, 2-Am-4,6-DNT and 4-Am-2,6-DNT were purchased from Supelco (Bellefonte, PA). Analytical standards for 4-nitro-2,4-diazabutanal (NDAB), MNX, DNX, and TNX were from SRI International (Menlo Park, CA). TNT and HMX standards for HPLC analysis were also prepared from solid powder (>95% purity). Methanol and acetone were HPLC-grade, and all other chemicals were reagent-grade.

### 2.2. Biodegradation of explosives mixtures under different water-content conditions

Batch experiments were conducted to characterize the biodegradation of three explosives—TNT, RDX and HMX in a mixture, under different conditions of water content: dry (1%, w/w), moderate (7%, w/w) and saturated (19%, w/w). Since intensive fermentation and gas production occurred in the saturated soil treatment on the first 2 days, water leaked from the canisters, and the above water content was determined after the leakage. The soil for all treatments (94% sand, 1% silt and 5% clay on a dry weight basis) was excavated 10 cm below the soil surface of an infiltration pond which has been used for over 20 years to dispose of untreated wastewater from explosives-manufacturing plants [14]. Soil (200 kg) was sieved to exclude coarse materials and mixed with 25 L of nitrogen-free sterile mineral solution [15] containing molasses (5 g/L as carbon), which served as the external carbon source. The soil was then air-dried for 4 days, and then sieved again and mixed with explosives powder. The soil was then mixed with distilled water to obtain the desired water content and packed to completely fill 1-L stainless-steel canisters. The canisters with moderate water content were rotated twice a day (180° each time) to avoid water drainage and to maintain homogeneous water-content conditions. Three replicates of each water-content treatment were taken at every sampling point (once a week), and the concentrations of the explosives and water content in the samples were monitored for the three treatments (in triplicate, 3–5 subsamples from each canister) every week. The replicate canisters from the final sampling point (i.e. the three replicates of each water content that were sampled at the end of the experiment) contained Ag/AgCl electrodes (Cole-Parmer® ORP electrode, in-line, double-junction, Vernon Hills, IL) which enabled continuous monitoring of the redox potential throughout the entire experiment (a period of 20 weeks). Note that the Ag/AgCl electrode readings represent only the immediate environment of the electrode and that the redox potential may vary, as mentioned above, both temporally and spatially in micro-sites [12].

In the first experiment, the different treatments were sampled once a week, for a total period of 20 weeks. This first experiment focused on the degradation of the three explosives, i.e. in the presence of TNT. Then, a second experiment was carried out, this time focusing solely on RDX and HMX degradation. The second experiment was performed under moderate and saturated water contents only (7% and 16% (w/w), respectively), in the absence of TNT. The treatments in the second experiment were sampled every 1–2 weeks, for a total period of 20–24 weeks. In the second experiment, the influence of TNT on the biodegradation of RDX and HMX was also examined. Therefore, although the soil for this experiment was contaminated only with RDX and HMX, each treatment had additional control canisters which included TNT as well. The TNT-containing canisters were sampled every 2–4 weeks.

The initial concentration was calculated from concentrations determined in five replicate soil extracts (Table 1), and the

**Table 1**
Initial concentrations of explosives.

| Explosive compound | First experiment (mg/kg of dry soil) | Second experiment (mg/kg of dry soil) | |
|---|---|---|---|
| | | Without TNT | With TNT |
| TNT | 440 ± 63 | | 406 ± 94 |
| RDX | 343 ± 20 | 317 ± 84 | 274 ± 22 |
| HMX | 352 ± 32 | 268 ± 24 | 222 ± 27 |

uncertainties (±values) represent one standard deviation of the replicate measurements.

### 2.3. Effect of explosives concentrations and soil water content on the microbial population

In order to follow shifts in microbial populations, DNA was extracted from 0.65 g of soil from several samples of the three treatments of the first biodegradation experiment (Section 2.2) with Power Soil DNA Kit (Mo Bio, Carlsbad, CA). Polymerase chain reaction (PCR) followed by denaturing gradient gel electrophoresis (DGGE) was preformed as describe previously [2,16]. Major bands from the gel were carefully excised, DNA was extracted from gel slices and cloned in the plasmid pTZ57R/T using the InsTAclone™ PCR Cloning Kit (Fermentas, Hanover, MD). DNA sequencing was performed by Macrogen Inc. (Seoul City, Korea).

### 2.4. Effect of TNT on soil toxicity

Soil toxicity was examined in several soil samples obtained from different samples of the wet and moderate water-content treatments of the second experiment (Section 2.2). Toxicity assay was conducted using biosensor (CheckLight Qiryat-Tivo'n, Israel), a microbial sensor that is engineered to produce light in response to environmental toxic effects [17].

Soil (1.5 g) from several replicates of the wet treatment of the second experiment was suspended in 3 mL double-distilled water and shaken on a rotary shaker (150 rpm) for 1 h. The suspended sediment was allowed to settle and the supernatant was centrifuged to further reduce suspended solids. The toxicity of this solution was tested using PCB-TOX (ToxScreen3) that contains the luminescent bacterium *Photobacterium leiognathi* according the manufacturer's instructions for microliter plate assay (CheckLight Ltd.). The plates were incubated and read by Tecan Infinite M200 micro-plate reader (Männedorf, Switzerland). The toxicity was expressed as $IC_{50}$ ($IC_{50}$ representing the percentage sample dilution causing a twofold decrease in luminescence, while lower $IC_{50}$ values signify higher toxicity), where $IC_{50}$ values of the different samples were calculated using an Excel spread sheet provided by the toxicity kit manufacturer.

### 2.5. Analytical methods

To determine explosives concentrations, soil samples from the different experiments were extracted in methanol using Accelerated Solvent Extraction (ASE-200, Dionex Corporation, Sunnyvale, CA) according to the method of Ronen et al. [20]. Concentrations of the compounds TNT, 2-Am-4,6-DNT, 4-Am-2,6-DNT, RDX, MNX, DNX, TNX and HMX were analyzed by HPLC (Agilent 1100 series, Agilent Technologies, Inc., Santa Clara, CA) according to EPA method 8330 [18]. The detection limit for the above compounds in the soil extracts was 0.05 mg/L.

Water content in the soil samples was determined gravimetrically in three to five replicate soil samples from each canister by comparing a sample weight relative to its weight after drying for 24 h at 105 °C.

## 3. Results

### 3.1. Biodegradation of explosives mixture under different water-content conditions

In both experiments, the increase in water content resulted in an increase in degradation extent. In the first experiment, under gravimetric water content (GWC) of 1%, we observed some disappearance of TNT but no disappearance of RDX or HMX (Fig. 2a).

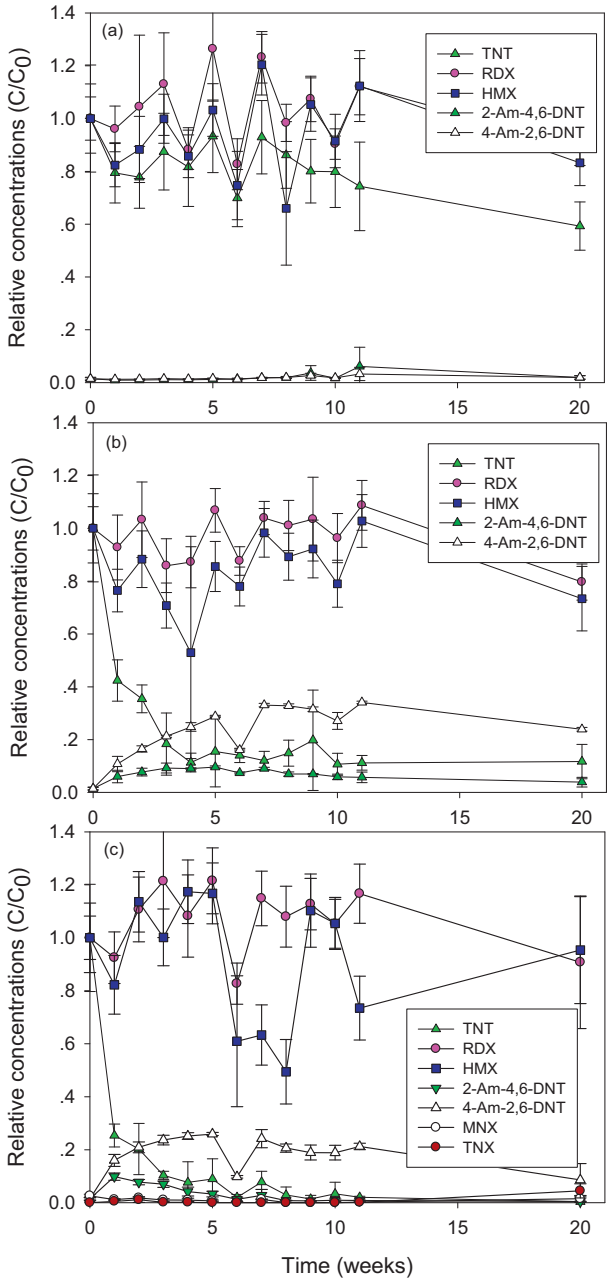


**Fig. 2.** Biodegradation of TNT, RDX and HMX under: (a) dry conditions (1% GWC); (b) moderate water-content conditions (7% GWC) and (c) high water-content conditions (19% GWC). Data points of representative experiments are the means of triplicates ± SD.

Under 7% GWC, although TNT was degraded, no degradation of RDX or HMX was observed (Fig. 2b). In the saturated treatment of the first experiment, almost full disappearance (>98%) of TNT was observed after 11 weeks (Fig. 2c). RDX degradation was delayed and started only after 20 weeks, as indicated by the slight accumulation of the anaerobic nitroso products. An average of ca. 7% of the initial RDX amount has recovered as nitroso derivates at this sampling point (a total of 107 μmol/kg). These degradation products appeared when TNT had almost completely disappeared from the soil and the concentration of the TNT amino intermediates was ca. 10 ± 7% of the initial TNT concentration. At the end of the incubation period, the HMX concentration did not differ from its initial concentration indicating that biodegradation of HMX did not occur in any of the treatments.

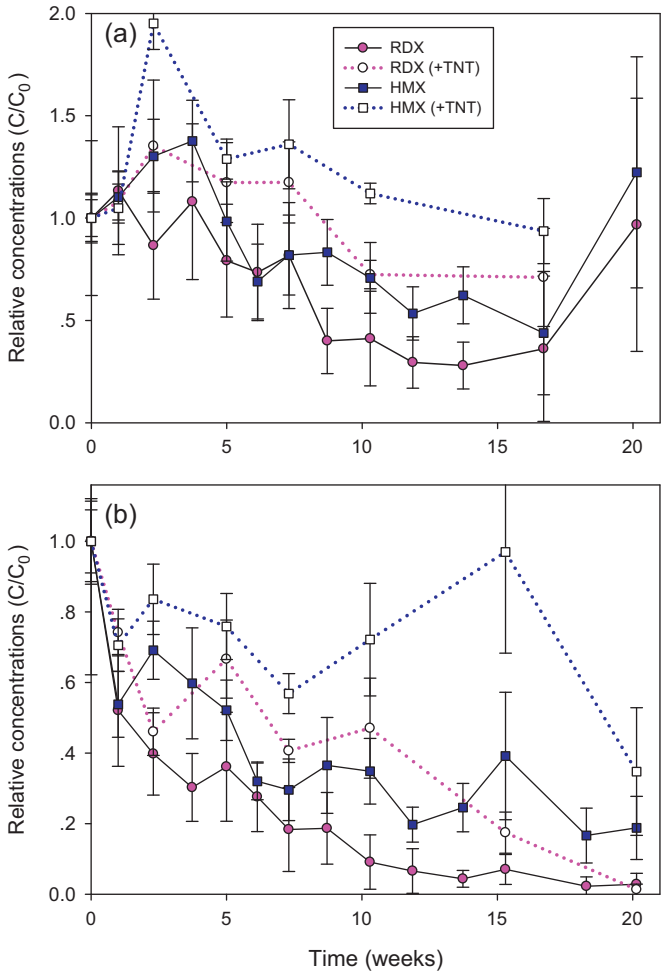


**Fig. 3.** Biodegradation of RDX and HMX in the presence and absence of TNT under: (a) moderate water-content conditions (7%, w/w) and (b) high water-content conditions (16%, w/w). Data points of representative experiments are the means of triplicates ± SD.

In the second experiment in the absence of TNT, degradation of RDX and HMX was not expected to be retarded and it was therefore possible to study their behavior under different water contents. The biodegradation rates of RDX and HMX (Fig. 3a and b) were found to be faster with increasing water content. Although retardation in RDX and HMX degradation was observed in the TNT-containing canisters, complete degradation inhibition was not observed, in contrast to the stronger inhibitory effect detected in the presence of TNT in the first experiment.

In the last sample of the 7% water-content treatment, extremely high variation in degradation extents was observed between the different canisters: whereas 75% and 43% degradation of RDX and HMX, respectively, were found in one of the canisters, no degradation of RDX and HMX was observed in the other two replicates (Fig. 3a and b).

### 3.2. Redox potential during biodegradation of explosives under different water-content conditions

All treatments presented a general trend of decreasing redox potential with increasing water content. Nevertheless, the temporal trends of the different replicates and treatments were very different. For example, in the 7% GWC treatment of the first experiment, three different patterns of redox potential were observed for each of the replicates (Fig. 4b). This pattern was also observed in 7% GWC treatment of the second experiment (Fig. 5a).

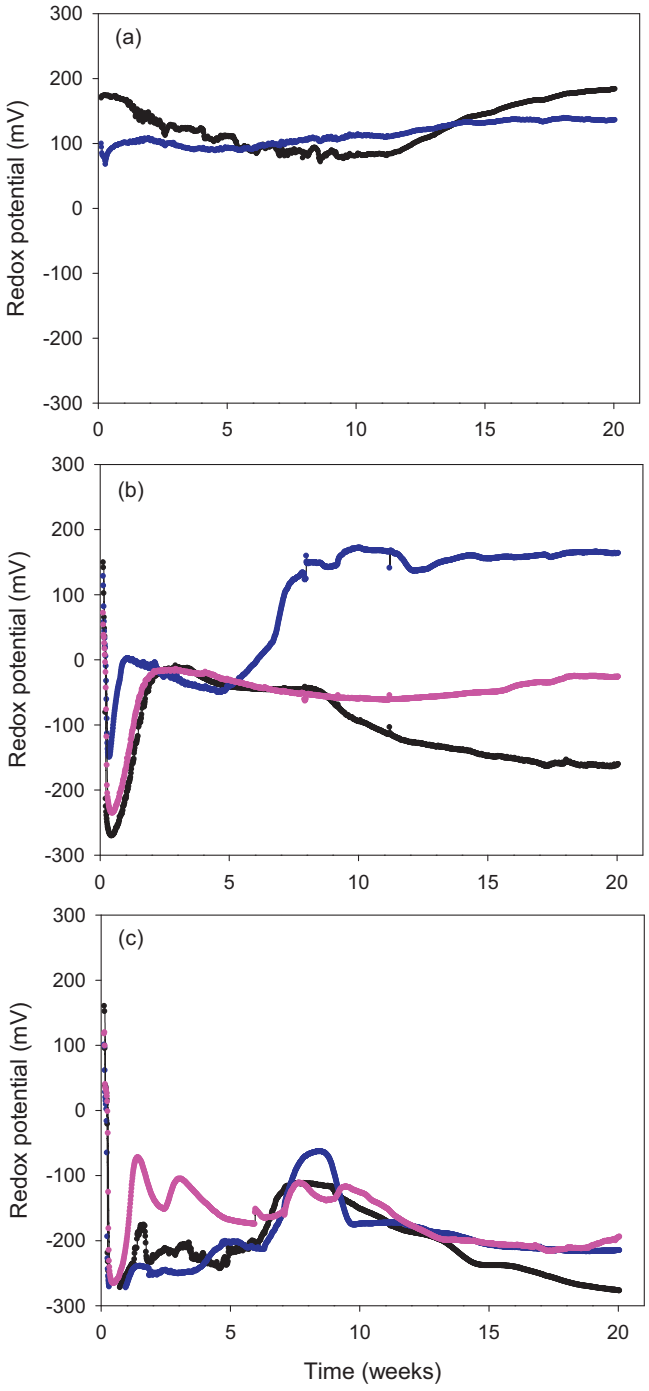


**Fig. 4.** Redox potential (first experiment) under: (a) dry conditions (1% GWC); (b) moderate water-content conditions (7% GWC) and (c) high water-content conditions (19% GWC).

In the high water-content treatment (19% GWC) of the first experiment, significantly lower redox-potential values were observed (Fig. 4c): a significant decrease (~170 mV) in redox potential was observed on the first 4 days (Fig. 4c). This rapid decrease was followed by an increase in redox potential to a range between −240 and −80 mV. Finally, anaerobic redox potential was measured in this treatment, with final values of −195, −215 and −277 mV. In the high water-content treatment of the second experiment (16% GWC), patterns similar to the first experiment were observed, with final values below −360 mV (Fig. 5b).

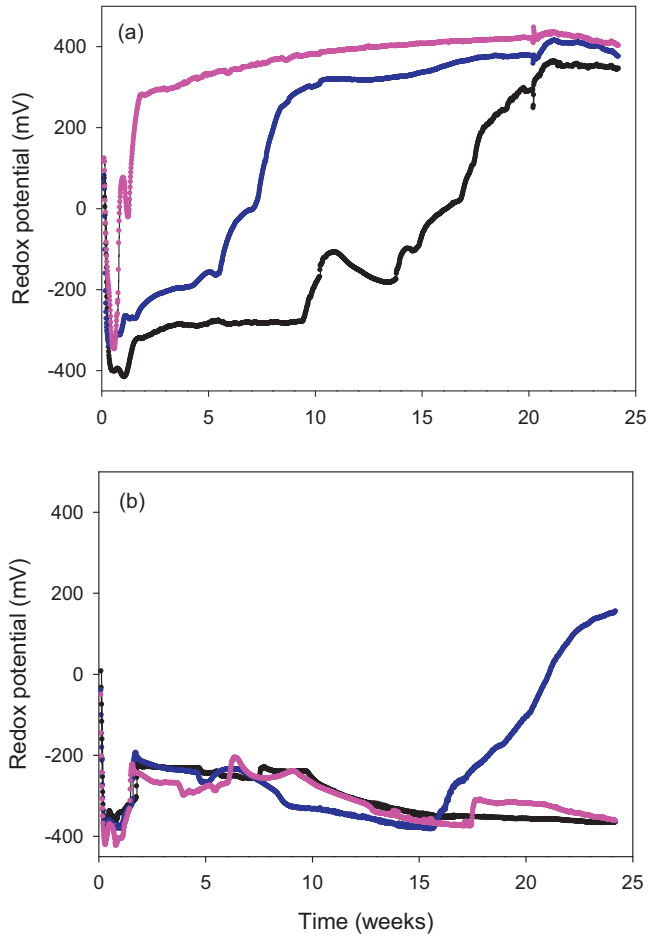


**Fig. 5.** Redox potential (second experiment) under: (a) moderate water-content conditions (7% GWC) and (b) high water-content conditions (16% GWC).

### 3.3. Effect of explosives concentrations and soil water content on the microbial population

PCR-DGGE analysis of samples from the first experiments was performed to examine the effect of soil water content and explosives concentration on the natural microbial population. In the first experiment, two bands showed up in all except the dry treatment, where they became very weak after the first week, probably due to lack of activity under these conditions (Fig. 6). These bands exhibited low sequence similarity to the 16S rRNA gene of uncultured *Bacteroidetes* and uncultured *Sphingobacteria*, respectively (marked with arrows 1 and 2 in Fig. 6). Another strong band, which exhibited 96% sequence similarity with the 16S rRNA gene of *Sporolactobacillus kofuensis*, appeared after 11 weeks in the wet treatment (marked with a circle in Fig. 6). This was the last sample before RDX degradation began (9 weeks later). Overall, none of these sequences was associated with a known explosives degrader.

The composition of the microbial population after 20 weeks of the wet treatment was significantly different from that of the initial population, with several new bands. Since in the soil samples taken after 20 weeks, RDX-degradation products (i.e. MNX, DNX and TNX) appeared for the first time, these bands might represent the microbial population capable of RDX degradation. Similarity analysis of the different lanes using Equity 1 1D software (BioRad) provided some interesting results (Fig. 7). Three main groups were clustered: M1, W1, M6 and M11 from the first period of the experiment (where M represents the moderate and W the wet treatments, and the integer is the elapsed time in weeks). The "week 0" sample was also included in this group and generally, the groups in this cluster were not much affected by the water content or the incubation time. The second group of M20, M6 and W11 appeared to indeed be influenced by both water content and time. The last group consisted of the three replicates of the W20 samples, with the D1 (dry) sample distantly clustering with this group.

**Table 2**
Soil toxicity during the incubation period under the different treatments (means of duplicates ± SD).

| Treatment | Week | $IC_{50}$ (%) |
|---|---|---|
| Wet | 1 | 35.4 ± 9.76 |
| Wet | 10 | 27.8 ± 1.27 |
| Wet | 20 | 24.25 ± 0.35 |
| Wet + TNT | 1 | 6.64 ± 1.98 |
| Wet + TNT | 10 | 29.6 ± 1.84 |
| Wet + TNT | 20 | 7.65 ± 0.78 |
| Moderate | 1 | 32.4 ± 1.7 |
| Moderate | 10 | 34.1 ± 1.56 |
| Moderate | 20 | 29.6 ± 0.14 |
| Moderate + TNT | 1 | 8.1 ± 0.85 |
| Moderate + TNT | 10 | 23.5 ± 0.71 |
| Moderate + TNT | 17 | 7.95 ± 1.63 |

### 3.4. Effect of TNT on soil toxicity

Toxicity was tested using the luminescent bacterium *P. leiognathi* (CheckLight Ltd.). In the absence of TNT and presence of RDX and HMX, soil toxicity increased slightly with time in the wet treatment ($IC_{50}$ decreased systematically from 35.4 to 24.25%), while no significant difference in toxicity was observed during the experimental period in the moderate water treatment ($IC_{50}$ remained between 29.6 and 34.1%) (Table 2).

In the presence of TNT, soil toxicity was significantly higher after 1 week in comparison to its toxicity in the absence of TNT ($IC_{50}$ of 6.64% in comparison to 35.4% in the wet treatment and $IC_{50}$ of 8.1% in comparison to 32.4% in the moderate water-content treatment). In the soils containing TNT, toxicity decreased in the samples taken after 10 weeks and increased again after 10 additional weeks.

The toxicity effect was observed for each compound separately (data not shown) and therefore we could not separate the toxicity effect of each compound in the explosives mixture and consequently could not calculate the $IC_{50}$ concentrations for each explosive compound.

## 4. Discussion

We assessed the effect of water content and redox potential on the biodegradation of a mixture of explosives in soil samples from the unsaturated zone. Results revealed a link between water content, redox potential and the rate and extent of explosives biodegradation. As expected, when soil water content increased, the redox potential decreased, and significant enhancement in the biodegradation of all three explosives was observed (Figs. 2 and 3). This implies that although TNT and RDX can be degraded aerobically, this degradation pathway is not as rapid as anaerobic degradation. It was also observed that the low redox potential, even when measured for temporary periods, was sufficient to facilitate anaerobic degradation throughout the 20 weeks of incubation.

The interplay between water content, redox potential and biodegradation rate can be demonstrated by comparing the different treatments: under dry conditions, only slight degradation of TNT and no biodegradation of RDX and HMX were observed during the 20 weeks of the experiment due to the low water content. Although aerobic biodegradation (positive redox potential) of both TNT and RDX is known [19,20], it require higher soil water content [20]. Moreover, a recent study has shown that the enzyme

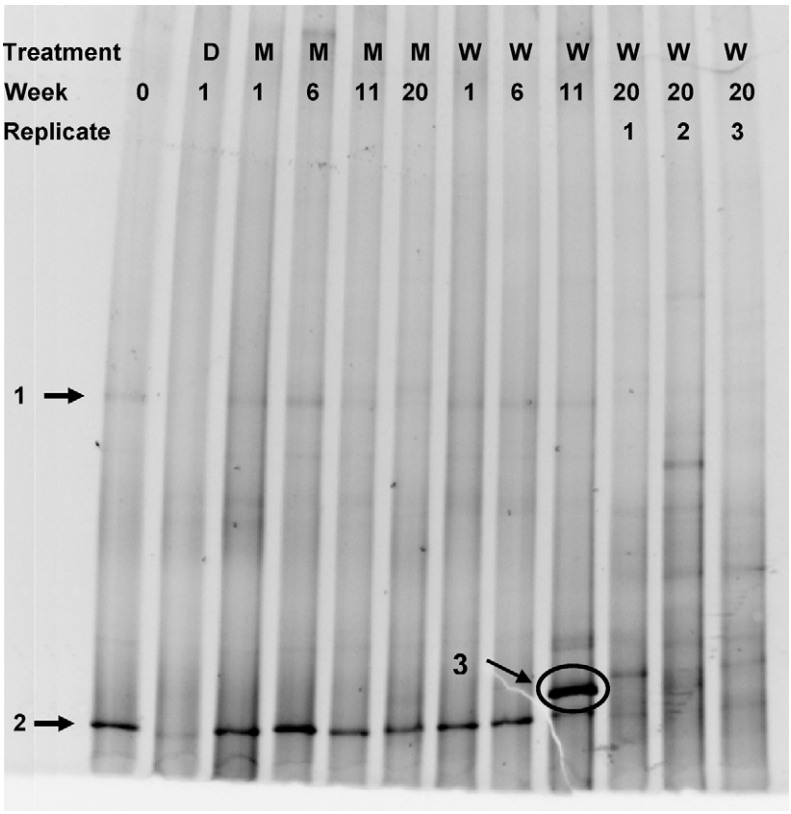


**Fig. 6.** PCR-DGGE analysis of the microbial population under different explosives concentrations and soil water-content conditions.

xenobiotic reductase, involved in the biotransformation of different explosives by *Pseudomonas fluorescens* I-C, is sensitive to high oxygen tension [21].

The redox trends in all replicates of 7% GWC presented transient formation of anaerobic conditions, reaching low negative redox potentials, albeit for differing durations (Fig. 4). In the first experiment, in which a mixture of all three explosives was tested, we observed partial degradation of TNT and no degradation of RDX or HMX, and the redox potential remained positive during most of the experimental period. In the second experiment, in which only RDX and HMX were included, partial degradation of these explosive compounds indeed occurred (Fig. 3), while anaerobic conditions were developed for different periods (Fig. 5a). The formation of temporary anaerobic conditions in unsaturated soils presumably occurs in soil micro-sites [12]. The transient conditions and high variability between replicates are unique to the heterogeneous moderate water content, and are not observed under dry or saturation conditions, which are expected to be more homogeneous. Such anaerobic micro-sites are less abundant under dry conditions, whereas under saturation the entire soil canisters are expected to be anaerobic. Clearly the soil bulk redox potential, which is measured by the $Ag/AgCl_2$ electrode (with diameter of 0.5 cm), cannot represent the local conditions within a microbial biofilm (with a thickness of only a few micrometers) that is attached to the sediment under unsaturated conditions. It can be assumed that biodegradation of RDX and HMX at moderate water content occurred mostly in these anaerobic micro-sites, rather than in aerobic micro-sites, since anaerobic biodegradation was seen to be more enhanced. The fact that anaerobic micro-sites are more active is further supported by the observed accumulation of RDX nitroso derivatives, which appear only during anaerobic degradation, and the complete absence of NDAB, an aerobic RDX derivate which is considered to be a relatively persistent product [22]. However, it should be noted that we cannot exclude the formation of some aerobic degradation product (NDAB) and its further transformation. The trend in the explosives degradation with time did not show changes correlated to the fluctuations in the bulk redox potential, which may further support the assumption that the bulk redox potential does not represent the actual redox conditions in separate micro-niches.

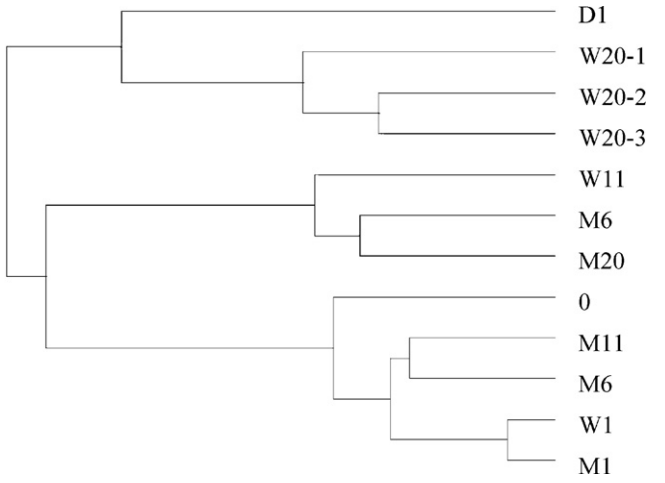


**Fig. 7.** Dendogram describing the similarity between the different microbial populations at different time points during the experiment as calculated from the DGGE analysis (Fig. 6). D, M, and W represent the dry, moderate and wet treatments, respectively, and the integer represents the elapsed time in weeks.

The conclusion that explosives degradation is enhanced with decreasing redox potential is reinforced by the results of the last samples from the moderate GWC treatment of the second

experiment, in which the $Ag/AgCl_2$ electrodes provided continuous monitoring of the redox potential in the canisters throughout the entire experimental period. The three replicates of the last sampling point showed three different redox patterns in the three replicate canisters, which were correlated to degradation extents: the greatest degradation of RDX and HMX (75% and 43%, respectively) was found in the canister in which the redox potential remained below −300 mV for over 9 weeks. In the other two replicate canisters, on the other hand, in which the anaerobic period was much shorter and the redox potential was higher, no degradation of RDX or HMX was observed. Therefore, we suggest that redox potential measurements in unsaturated soil can provide only a rough indication of the efficiency of reductive biodegradation processes. It should be noted that although the three canisters displayed different redox patterns while having similar water contents, it does not imply that the water content and redox potential are actually not correlated. The correlation between the water content and the redox potential is reinforced by the comparison of the moderate water composition to dry and saturated conditions. Whereas the dry and saturated soils set a clear trend between these two extreme situations, the observations from the moderate water content treatment is in correlation to this trend. Thus the high variability between the three replicates of moderate water content may be the result of the high variability between the three canisters.

At high water content in the absence of TNT, nearly full degradation of RDX and HMX (up to 98.3% and 90.5%, respectively) was detected by the end of the second experiment. Anaerobic redox conditions were measured in two of the three replicates of this treatment throughout the entire experimental period, with final redox values below −360 mV. The exceptional third replicate presented an increase in redox potential after approximately 15 weeks, reaching a final value of 155 mV. Nevertheless, the extent of RDX and HMX degradation in this canister was similar to that in the other two. It is suggested that the low redox potential observed early on in this canister was sufficient to promote anaerobic degradation, and that these conditions remained within micro-niches in the canister but were not represented by the high, bulk redox potential.

The general observation of more enhanced RDX degradation under anaerobic conditions is in agreement with previous studies. Price et al. [11] observed relative stability of RDX under oxidizing and moderately reducing conditions, and instability of RDX under highly reducing conditions (−150 mV), which was accompanied by the appearance of nitroso derivatives. Those derivatives were detected at only very low concentrations under oxidizing or moderately reducing conditions. Ringelberg et al. [9] showed that the rate of RDX loss is significantly greater when the soil is saturated, coinciding with a gradual increase in the anaerobicity of the system and the formation of nitroso intermediates, followed by their disappearance from the system. In contrast, no nitroso intermediates were detected in the unsaturated microcosms. The general observation of more enhanced RDX degradation with increasing water content is in agreement with previous studies with soil from the same site. Ronen et al. [20] calculated the RDX half life in carbon-amended saturated soil to be 6 days, while in carbon-amended unsaturated soil (0.1 bar metric potential) it was 21 days.

It should be noted that the moderate water content may represent the real saturation status of soil in the environment. Saturated, or close to saturated water-content conditions may be less abundant, but may still be observed, e.g. in the vicinity of low conductive soil layers. In both cases, the total biodegradation of explosives can be facilitated, and will be enhanced with the increase in water content (where anaerobic biodegradation dominates). This is supported by field observations on a subsurface soil profile from the same site that showed enriched $\delta^{15}N$ values of RDX (indicating greater degradation extents) in the vicinity of clayey layers, where higher water contents are observed [14]. This enrichment was also correlated to an increase in the detected nitroso derivatives of RDX, which suggested that anaerobic RDX biodegradation in these sampling points was dominant.

Similar to previous experiments under saturated conditions, this study shows that TNT and its metabolite inhibit the biodegradation of RDX and HMX. Our results clearly indicate a negative influence of TNT on the biodegradation of RDX and HMX by indigenous soil microorganisms. The presence of TNT in the soil decreased RDX and HMX degradation rate during the experimental period (Fig. 3). Nevertheless, the inhibitory effect in this study was not as pronounced as that observed in uniform slurries [2], which presented stronger inhibition of RDX and HMX degradation in the presence of TNT and its intermediate tetranitroazoxytoluene. This inhibition was explained by a probable cytotoxic effect on the RDX- and HMX-degrading microbial population, as well as direct inhibition of enzymes involved in RDX and HMX degradation [21,23]. Luminescent bacterium *P. leiognathi* toxicity tests performed in our current work indicated that soil toxicity significantly increases in the presence of TNT and decreases after its degradation (after 10 weeks) (Table 2). The second increase in soil toxicity 10 weeks later can be explained by the possible formation of the TNT intermediate tetranitroazoxytoluene, which has been found to be more toxic than TNT itself and to cause a higher rate of mutations [3].

The fact that the effect of TNT on the degradation of RDX and HMX in this study was less pronounced can be explained by a masking effect of the soil in the canisters. In contrast to homogeneous slurry experiments, fully or partially saturated soils can promote the existence of micro-niches that differ considerably in their explosives concentration, microbial community and redox conditions. Thus, biodegradation of RDX and HMX may proceed in micro-niches in which TNT and its toxic degradation product do not exist (either because they have already been fully degraded in these niches, or because they were completely absent in the first place).

Changes in microbial populations were evident upon analysis of the DGGE gel. The strong bands (marked 1 and 2 in Fig. 6) were most similar to the 16S rRNA gene of uncultured *Bacteroidetes* (JN695872) and to the 16S rRNA gene (96%) of the uncultured *Sphingobacteria* (EF520602). These are freshwater sediment organisms with no known relation to explosives degradation. The unique band in the wet treatment after 11 weeks was most similar (96%) to the *S. kofuensis* 16S rRNA gene (AJ634661.1), a lactic acid bacteria present in the soil but with no known ability to degrade explosives [24]. Nevertheless, it appears from Fig. 7 that the population changes with time. The complete divergence of samples after 20 weeks in the wet treatment from the rest of the samples showed that overall, water content was the most important factor in the evolution of microbial populations during the experiment. The inability to detect 16S rRNA gene sequences belonging to known explosives degraders prevented us from assessing the effects of explosives concentration on the microbial population. A functional marker for genes involved in TNT, RDX and HMX degradation would be useful in future work.

## 5. Conclusions

We found that the soil's indigenous microbial population can degrade TNT, RDX and HMX in a mixture, under conditions that reflect the natural conditions of the contaminated vadose zone. The degradation was shown to be strongly affected by soil water content and consequently, redox conditions. While the degradation of TNT occurred, in these experiments, under both aerobic and anaerobic conditions, the degradation of RDX and HMX occurred only when water-content conditions resulted in a prolonged period of

negative redox potential. These conditions usually developed under saturation, but could also develop temporarily in soil micro-sites under moderate water content and thus promote RDX and HMX degradation.

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