Toxicity assessment and biodegradation potential of water-soluble sludge containing 2,4,6-trinitrotoluene

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ABSTRACT

The water-soluble phase of trinitrotoluene-containing sludge (SLP) was characterized with regard to trinitrotoluene (TNT) concentration, ecotoxicity, and a model biodegradation experiment as evaluation criteria for further development of appropriate treatment technologies. SLP contained 67.8 mg TNT/l. The results of germination and root-elongation tests indicated that SLP had a species-specific phytotoxic effect. The results of a 21 day degradation experiment demonstrated TNT conversion to 4-amino-2,6-DNT and 2-amino-4,6-DNT, with a simultaneous reduction in the total concentration of nitroaromatics. Addition of inoculum stimulated the TNT degradation process. The presence of the sludge solid phase inhibited microbial activity. Measurement of microbial enzyme activity was used to assess changes in the microbial community during the biodegradation process.

Key words | biodegradation, enzyme activity, indigenous microorganisms, phytotoxicity, solubility, TNT

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ABBREVIATIONS

CLE	cabbage leaf extract
DHA	dehydrogenase
FDA	fluorescein diacetate
SLP	sludge liquid phase
SSP	sludge solid phase
TNT	2,4,6-trinitrotoluene
NACs	nitroaromatic compounds

INTRODUCTION

Explosive compounds enter the environment as a result of demilitarization activities, production of ammunition and explosives, open detonation and burning of explosives at army depots, evaluation facilities, artillery ranges and ordnance disposal sites (Nehrenheim & Odlare 2010). One of the most widely used explosives is 2,4,6-trinitrotoluene (TNT). Nitroaromatic compounds (NACs) of this type do not occur naturally and most microorganisms are therefore not adapted to degrade and mineralize these substances. The symmetry around the toluene molecule generates a very stable structure that is extremely resistant to microbial degradation (Ribé *et al.* 2010). There is an urgent need for a cost-effective method for removal of explosive compounds in water and sludge.

Technological approaches for decontamination of TNT- and other NAC-containing wastewaters can be divided into physico-chemical and biological methods, or combinations of these. Physico-chemical treatment of red water via sorption by pine bark (Chusova *et al.* 2012; Nehrenheim *et al.* 2011), activated polystyrene microspheres (Meng *et al.* 2012), activated coke from lignite (Zhang *et al.* 2011), vacuum distillation (Zhao *et al.* 2010), oxygen excess at 550 °C (Chang *et al.* 2013), and catalytic degradation using iron nanocatalysts and iron oxide bentonite nanocomposite (Badawi *et al.* 2012) are among the

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methods that have been reported for efficient decontamination of NAC-containing wastewaters.

The incredible versatility inherent in microbial metabolism has meant that explosives have become part of the biogeochemical cycle (Singh *et al.* 2012). Knowledge of the microbial dynamics that drive TNT biodegradation is limited, particularly in native aquifer sediments where TNT poses a threat to water resources (Hoffsommer *et al.* 1978; Harrison & Vane 2010; Fahrenfeld *et al.* 2013).

The use of slurry and packed-bed bioreactors (Shen *et al.* 2009; Wang *et al.* 2010; Mulla *et al.* 2013), and constructed wetlands (Best *et al.* 1997, 1999; Sikora *et al.* 1998) have been shown to be effective for NAC-containing wastewater treatment. A mixed microbial population in digested sewage culture degraded 110 mg TNT/l under strict anaerobic conditions in 6 days (Kwon 2000).

Extracellular enzymes, e.g. from white rot fungi, have been shown to be effective degraders of TNT. However, high production cost inhibits the widespread use of extracellular enzymes for remediation (Rugabber & Talley 2006).

Ammonium nitrate and chloride as nitrogen sources and molasses as a carbon source were found to be efficient amendments for TNT biodegradation (Yasin *et al.* 2008). Molasses have been reported as an efficient carbon source for the co-metabolism of explosives in many studies (Rodgers & Bunce 2001; Gerth *et al.* 2003; Clark & Boopathy 2007; Lamichhane *et al.* 2012). Crude plant extracts (e.g. spinach and parrotfeather) and cabbage leaf extract are effective amendments for the NAC biodegradation process (Medina *et al.* 2004; Muter *et al.* 2008, 2012).

Biodegradation of explosives waste is influenced by temperature, oxygen supply, nutrient supply, pH, the availability of the contaminant to the microorganism, the concentration of the waste, and the presence of substances toxic to the microorganisms (e.g. mercury) (US Army Environmental Center 2000; Wang *et al.* 2004). Conditions must therefore be carefully controlled in order to gain optimal results in treatment of explosives waste.

The aim of the present study was to study microbiological and ecotoxicological development during TNT degradation using three different media. The specific aim was to characterize the water-soluble fraction of a demilitarization factory waste sludge and its biodegradation potential, by assessing TNT concentration, toxicity, and microbial activity before and after treatment.

METHODS

Characterization of SLP physico-chemical properties

TNT-containing sludge was kindly provided by the former munition utilization plant Nammo Vingåkersverken (Sweden). The concentrations of TNT and its metabolites in the sludge solid phase (SSP) and its water-soluble phase (SLP) were measured using a HPLC-DAD (high-performance liquid chromatography diode array detector) system in gradient mode using the Phenomenex Synergi Hydro 250 × 4.6 mm column (4 μm particles). Calibration was performed using reference standards (Dr. Ehrenstorfer, Germany).

Ecotoxicological study

Biotests on seed germination and root elongation were performed in triplicate according to EPA 712-C-96-154 guidelines (EPA 1996) on seeds of cress (*Lepidium sativum*), wheat (*Triticum* sp.), rye (*Secale cereale*), barley (*Hordeum vulgare*), oat (*Avena sativa*), clover (*Trifolium* sp.), alfalfa (*Medicago sativa*), soya bean (*Glycine max*), and radish (*Raphanus sativus*). An SLP dilution factor of 0.5 (100%; 50%; 25%; 12.5%; 0%) was used in the tests.

Microbiological study

The total bacteria counts in SSP and SLP were determined by colony counts from triplicate samples after incubation on Tryptone Glucose Yeast Extract Agar (Sifin, Germany) at +28 °C for 72 h.

Biochemical study

Enzyme activities of microorganisms in samples were tested as follows. Dehydrogenase (DHA) activity was determined by reduction of 2-*p*-iodo-3-nitrophenyl-5-phenyltetrazolium chloride (INT) to iodonitrophenylformazan. Fifty microlitres of a mixture containing 40 mg INT, 1 ml 1% glucose, and 20 ml 0.25 M TRIS was added to a 50 μl sample and incubated at +28 °C for 48 h. Three hundred microlitres of extraction solution (ethanol and dimethylformamide, 1:1) was then added to the mixture, vortexed and centrifuged at 5,000 rpm after 30 min incubation at ambient temperature. Optical density was measured at 485 nm. Urease activity was determined by the colorimetric method according to NH_3-N formation in urea-amended 0.2 M phosphate

buffer pH 7.1 (after 48 h incubation at +28 °C) (Kandeler & Gerber 1988). Concentration of NH_4^+-N was measured with Nessler reagent by optical density at 425 nm. Fluorescein diacetate (FDA) hydrolysis activity was determined by hydrolysis of FDA in 0.06 M phosphate buffer pH 7.6 for 60 min at +37 °C (Chen *et al.* 1988). Optical density was measured at 490 nm.

Batch experiment

Three variants with different combinations of sludge and inoculum were prepared in duplicate 250 ml Duran bottles (Table 1). The bottles were mixed on a shaking table for 21 days at 100 rpm at ambient temperature. The liquid phase was made up with sterile distilled water and M8* stock solution containing 60 g/l $Na_2HPO_4{\cdot}12H_2O$, 30 g/l KH_2PO_4, and 5 g/l NaCl. Cabbage leaf extract (CLE) and molasses were used as amendments. CLE and inoculum (cultivated from the sludge liquid phase) were prepared as described by Muter *et al.* (2008). Sugar beet molasses contained 42% of reducing sugars. After the enrichment procedure (48 h, 28 °C), an inoculum was made up with a cell concentration of 1.0×10^4 CFU/ml using bacteria isolated from sludge on TGA medium. The composition of the enrichment medium for the set L (excluding sludge) is shown in Table 1.

RESULTS AND DISCUSSION

Physico-chemical characterization of the sludge liquid phase

TNT concentration in air-dried sludge was around 30%. The water-soluble phase of the tested sludge contained 67.8 mg TNT/l and no other NACs, i.e. TNT degradation products were found. Current literature values of TNT solubility in water vary widely between 100 and 200 mg/L at room temperature. Solubility in water plays an important role in toxicity and biodegradability of chemicals. Under natural conditions, NACs have only limited bioavailability due to their low solubility in water. Sludge composition may influence TNT solubility, and the presence of heavy metals reduces TNT solubility (Nehrenheim *et al.* 2011). The concentration of explosives in groundwater is affected by the size and surface area of individual explosive particles, the degree of weathering, and the rate of dissolution (Lynch *et al.* 2002; Speitel *et al.* 2002). In addition, if two sparingly soluble compounds are present, neither can dissolve to the full extent of its reported aqueous solubility (Clausen *et al.* 2006). This means that the low TNT concentration in the water phase could be due to adsorption on suspended solids or low dissolution rate. Temperature and pH also significantly affect TNT solubility (Lynch *et al.* 2001; Phelan & Barnett 2001). The pH of the SLP varied between 6.3 and 6.9 during the 21 day incubation, which should be considered modest regarding the effect on TNT solubility but can significantly affect conversion of TNT (Qiao *et al.* 2010). The redox potential was in the range 55.0–65.0 mV.

Ecotoxicological assessment of the sludge liquid phase

Effluents from TNT production have been reported to be toxic to different test organisms, e.g. daphnia, bacteria (Ribeiro *et al.* 2012). TNT leakage to water, its fate and toxic effect in the environment are important research areas, particularly in the context of marine ammunition dumping sites (Ek *et al.* 2007). One of the most important characteristics of any toxic compound is its bioavailability. Physico-chemical factors of compounds in contaminated water result in toxic effects on biota.

A comparative study of SLP toxicity was performed with a battery of monocot and dicot plants using seed germination and primary root-elongation tests as the main criteria to evaluate toxic effects of TNT under the studied conditions. Inhibition of seed germination by SLP was found to be species-specific; 50% SLP (33.9 mg/l) inhibited germination of cress salad by 50%, but did not influence

Table 1 | The experimental setup with different substrate/inoculum combinations

Set	Sludge liquid phase, ml	Sludge solid phase, g moist w	Water, ml	M8* stock, ml	Molasses, 30%(w/v), ml	CLE, ml	Inoculum, ml
L[a]	124	0	38	20	8	10	0
LI[b]	124	0	18	20	8	10	20
LIS[c]	124	5	13	20	8	10	20

[a]L – sludge liquid (water) phase.
[b]LI – sludge liquid (water) phase + inoculum.
[c]LIS – sludge liquid (water) phase + inoculum + sludge solid phase.

germination of rape and wheat seeds. However, undiluted SLP inhibited germination of all the tested plants in a species-specific manner (results not shown). Among the tested plants, cress, rye, barley, and radish showed the highest sensitivity to the SLP in the root-elongation test (results not shown). Oat, clover and alfalfa were more resistant to the presence of SLP. SLP actually stimulated primary root development in soya bean. SLP exposure thus results in different responses in different plant species.

Large numbers of culturable bacteria were detected in both SSP and SLP, i.e. 2.3×10^6 cfu/g and 5.3×10^3 cfu/ml respectively.

TNT degradation study

A biodegradation study was performed as indicated in Table 1. In this experiment, SLP was amended with mineral salt composition M8*, CLE and molasses. These amendments were previously shown to be efficient for TNT biodegradation (Boopathy *et al.* 1998; Gerth *et al.* 2003; Limane *et al.* 2009). Addition of molasses provided approximately 0.5% reducing sugars (mostly sucrose). Non-diluted CLE also contained reducing sugars (mostly glucose and fructose). A comparative study showed that the concentration of reducing sugars in the CLE prepared from different cultivars was usually in the range 10–25 g/100 ml, while the total nitrogen and carbon was in the range 0.2–1.0 and 0.5–1.3 (vol.%) respectively (Grube *et al.* 2008). This study compared sets with different combinations of sludge liquid phase (L), solid phase (which is water-soluble to a limited extent) (LIS) and inoculum originating from the same sludge (LI, LIS). As shown in Figure 1, two TNT biodegradation products were formed during incubation, 4-Am-2,6-DNT and 2-Am-4,6-DNT. These compounds are known to be the typical products of microbial biodegradation of TNT (McCormick *et al.* 1976; Hoffsommer *et al.* 1978; Schackmann & Müller 1991; Alvarez *et al.* 1995; Esteve-Nunez *et al.* 2001). Addition of inoculum stimulated the TNT degradation process. Thus, after 21 days incubation the total concentration of NACs in media with inoculum was 3.7 mg/l, compared to 12.4 mg/l in media without inoculum (Figure 1). The presence of SSP resulted in an increase in water-soluble NACs in the medium within 4 days of incubation. TNT degradation via conversion to other NACs enabled additional solubilization of TNT. Thus the total concentration of TNT degradation products after 21 days incubation in the presence of SSP was considerably higher compared to the sets without SSP (Figure 1). This may indicate more intensive microbial activity in the presence of SSP. These findings will be examined in greater detail in follow-up studies.

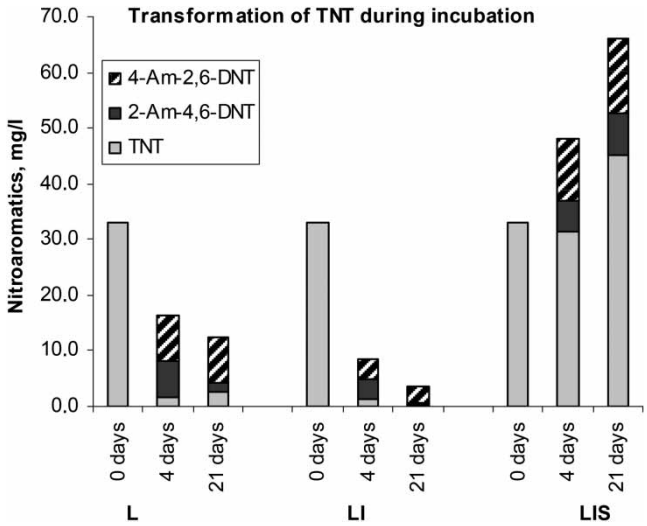

Figure 1 | Degradation of TNT to two recognized degradation metabolites in the sludge liquid phase. Description of the sets is shown in Table 1. L – sludge liquid (water) phase, LI – sludge liquid (water) phase + inoculum; LIS – sludge liquid (water) phase + inoculum + sludge solid phase, 4-Am-2,6-DNT – 4-amino-2,6-dinitrotiluene, 2-Am-4,6-DNT – 2-Amino-4,6-dinitrotiluene.

Microbial activity in the sludge liquid phase with different amendments

DHA activity typically occurs in all intact viable microbial cells. DHA activity therefore correlates to the presence of viable microorganisms and their oxidative capability. In this study, a comparison of DHA activity dynamics showed a wide variation, although activity had a tendency to increase with incubation time in all three sets tested (Figure 2(a)). After 21 days incubation the DHA activity was highest in set L, i.e. without inoculum. FDA hydrolysis activity was also higher in set L compared to sets LI and LIS (Figure 2(b)). Addition of SSP resulted in total inhibition of FDA hydrolysis activity on the 21st day of the experiment (Figure 2(b)). Measurement of FDA hydrolysis has been suggested as an appropriate method in integrated bioecosystem studies because the ubiquitous lipase, protease, and esterase enzymes are involved in the hydrolysis of FDA (Green *et al.* 2006). Determination of urease activity is based on the formation of ammonium ions in the presence of urea as a substrate. The highest NH_4^+ concentrations were found at the beginning of the experiment in all tested sets. NH_4^+ concentration was lower on the eighth day, and subsequently increased again (Figure 2(c)). In this study,

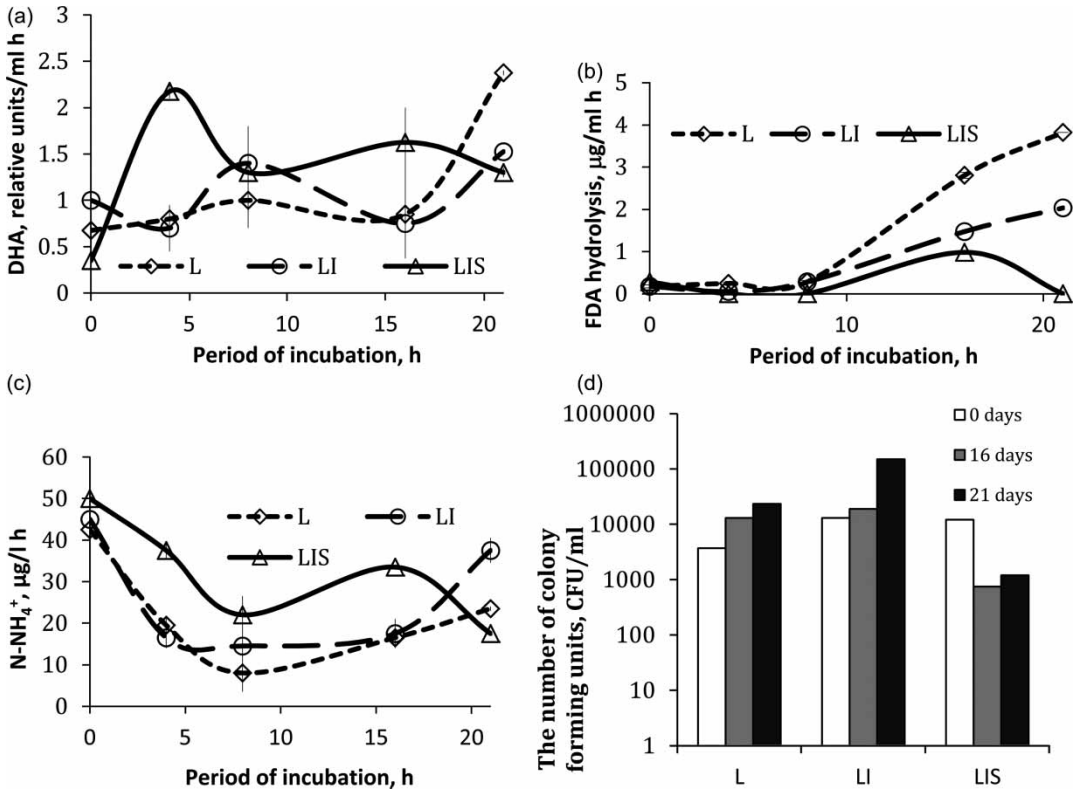

Figure 2 | (a), (b), (c) Changes of the microbial enzyme activity analysed as DHA, FDA hydrolysis, and urease activity during 21 days incubation with SLP. (d) Number of colony forming units in the SLP during biodegradation. L – sludge liquid (water) phase, LI – sludge liquid (water) phase + inoculum; LIS – sludge liquid (water) phase + inoculum + sludge solid phase. Error bars represent the standard deviation at 5% level of significance.

NH_4^+ concentration was influenced by other factors in addition to urease activity. Biotransformation of TNT may increase NH_4^+ concentration via microbial conversion (Martin *et al.* 1997; Esteve-Nunez *et al.* 2001). However, controls lacking urease did not show detectable concentrations of NH_4^+ in the medium.

The number of culturable microorganisms increased in the set with inoculum (LI) from 3.3×10^3 to 2.4×10^5 cfu/ml after 21 days incubation. In turn, addition of SSP to incubation media resulted in a slight decrease in the total count of culturable microorganisms (Figure 2(d)). As shown in Figure 1, addition of inoculum to liquid medium stimulated TNT degradation. Hence, a decrease of TNT concentration in the SLP can be mostly explained by microbial activity. At the same time, DHA and FDA hydrolysis activity in the set with inoculum was lower than in the set without inoculum after 21 days incubation. These changes in the medium are most likely due to enhanced (i) TNT biotransformation and biodegradation, (ii) consumption of nutrients, and (iii) accumulation of metabolites, leading to a decrease in microbial enzyme activity in the set with inoculum.

CONCLUSIONS

TNT-containing sludge and its water-soluble fraction were characterized using different approaches. The main conclusions are as follows:

- The toxicity study revealed a species-specific toxic effect of SLP on higher plants. Cress salad was found to be one of the more sensitive plants in this study.
- Use of indigenous bacteria as inoculum stimulated TNT degradation. Further identification and characterization of bacteria may be an important strategic tool in the context of TNT biodegradation in water.
- Counts of colony forming units as well as DHA and FDA hydrolysis activity dynamics indicated an increase in microbial activity with time in SLP. However, the presence of SSP in liquid medium resulted in strong inhibition of microbial activity on the 21st day of incubation.
- Composition of the liquid medium used in this study was found to be appropriate for TNT-degrading activity of indigenous microorganisms. Further comparative study

is needed to optimize the TNT-containing wastewater treatment process with emphasis on its cost efficiency.

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