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Summary

Most biospheric nitroaromatic compounds (NACs) are industrial chemicals, such as explosives, dyes, polyurethane foams, herbicides, insecticides, and solvents, that often are recalcitrant to biological treatments and persist in the environment, constituting a hazard due to their toxicity and mutagenicity. In the present study, we evaluated perspectives for bioremediation and phytoremediation strategies at a nitroaromatic-contaminated site. Our research focussed mainly on 2,4-DNT and TNT.

Two consortia, isolated by selective enrichment from a soil sample from a TNT-contaminated site, metabolised 2,4-DNT as their sole nitrogen source without accumulating one or more detectable intermediates. Though originating from the same sample, the optimised consortia had no common members, so that selective enrichment could not be repeated. Consortium 1 had four bacterial species and consortium 3 contained six, but both had two members that could collectively degrade 2,4-DNT. *Variovorax paradoxus* VM685 (consortium 1) and *Pseudomonas* sp. VM908 (consortium 3) initiated the catabolism of 2,4-DNT by an oxidation step, thereby releasing nitrite and forming 4-methyl-5-nitrocatechol (MNC). Both strains contained a homologue to the *dntAa* gene encoding 2,4-DNT dioxygenase. They subsequently metabolised MNC to 2-hydroxy-5-methylquinone (HMQ) and nitrite, indicative of DntB or MNC monooxygenase activity. A second consortium member, *Pseudomonas marginalis* VM683 (consortium 1) or *P. aeruginosa* VM903, *Sphingomonas* sp. VM904, *Stenotrophomonas maltophilia* VM905 or *P. viridiflava* VM907 (consortium 3), was indispensable for efficient growth on 2,4-DNT and for the metabolisation of the intermediates MNC and HMQ. We have limited knowledge about the interactions in this degradative step.

However, a gene homologous to the *dntD* gene of *Burkholderia* sp. strain DNT that catalyses ring fission was demonstrated by DNA-DNA hybridisation in the second member strains.

Attempts were unsuccessful at selective enrichment on other nitroaromatic compounds as the sole nitrogen source (i.e., TNT, 2,6-DNT, 2-NT, and 4-NT) with soil and water samples from this industrial site. To select a consortium for further applications, we then compared the ability of each consortium to break down TNT. Consortium 3 performed the best, towards a fast and complete TNT transformation, decrease in genotoxicity and high co-metabolic activity, even when an excessive amount of TNT was added. Therefore, this consortium was chosen for remediations of TNT-contaminated soil and water (Chapter 7, Chapter 8).

In plant-systems, TNT may be transformed in the rhizosphere by membrane-bound plant enzymes, exudated plant enzymes or the prevailing microflora, and may bind irreversibly to roots by amino or hydroxylamino functional groups and are further chemically transformed (Burken *et al*., 2000). These findings suggested the possibility of using a process of rhizofiltration for remediation, which was further investigated in the sunflower *Helianthus annuus.* Sampling hydroponic media initially containing 5-, 10-, and 20-mg TNT/L showed that 98.1, 97.5, and 76.2%, respectively, of TNT was removed after 21 days of culture. With an initial concentration of 20 mg/L TNT in the medium, the pseudo first-order rate constant for elimination was lower, indicating a toxic threshold value between 10- and 20-mg TNT/L for sunflowers. The responses of *Helianthus annuus* to TNT were evident primarily in the roots, and consisted of discoloration and growth restriction. Radiolabeled TNT, at 10 mg/L, was used to evaluate the fate of the compound in the plant. Of the total plant-associated ^{14}C accumulated finally 92.6% in the root, 5.3% in the stem and 2.1% in the leaves, indicating that translocation to the aerial parts of the plant was inefficient. Temporal sampling of plant tissues revealed that an increasing percentage of the activity became irreversibly bound to the plant's structural material, suggesting its sequestration. We also assessed the impact of

multiple consecutive TNT additions to the plant-system; the results suggested that *Helianthus annuus* “tolerates” TNT concentrations up to 10 mg/L.

We next examined the effect of TNT-contaminated soils on germination and early development of the sunflower seedling. The concentration of TNT was not reflected in percent germination: however, seedling growth was retarded at a concentration of 34 mg TNT/kg soil and above. In addition, the uptake of the contaminants by the plants and the dissipation of NACs in the rhizosphere were evaluated. Nitroaromatic compounds were detected only in root tissues. After a month’s exposure to contaminated soil, there was no significant degradation of these compounds by the sunflower’s rhizosphere.

We attempted to enhance the rate of immobilisation of nitroaromatic compounds in the soil in an aerobic slurry process by biostimulation or bioaugmentation (inoculating consortium 3) that would be advantageous in a large-scale reactor. While addition of consortium 3 considerably decreased the time needed for remediation, questions still remain about the frequency of reinoculation of the consortium, the addition of a surfactant, and/or the supply of extra nutrients to remove 100% of the TNT. Detailed studies are required to determine the remaining toxicity.

We investigated furthermore the feasibility of phytotreatment of TNT-contaminated water using the common reed, *Phragmites australis,* in a system based on a horizontal subsurface-flow constructed wetland. In addition, we examined the efficacy of inoculating the system with the bacterial consortium 3 to enhance biodegradation. Several inherent problems became evident, among which the biologic activity in the influent reservoirs, problems with plant growth, and the poor survival or death of the inoculum. Further research is required on their physical, chemical, and biological causes before this technology can be successfully established.

Samenvatting

De meeste nitroaromatische componenten in de biosfeer zijn van industriële oorsprong zoals explosieven, kleurstoffen, polyurethaan schuimen, herbiciden, insecticiden en solventen. Deze componenten zijn over het algemeen zeer moeilijk biologisch afbreekbaar waardoor ze aanwezig blijven in de biosfeer en een risico vormen voor de menselijke gezondheid die te wijten is aan toxische en mutagene effecten. In deze studie werden de perspectieven voor bioremediatie en fytoremediatie strategieën op nitroaromaat gecontamineerde bodem en water geëvalueerd. Het onderzoek concentreerde zich voornamelijk op de componenten 2,4-DNT en TNT.

Twee bacteriële consortia, geïsoleerd door selectieve aanrijking van een bodemstaal afkomstig van een TNT-gecontamineerde site, kunnen 2,4-DNT als hun enige stikstofbron gebruiken zonder de accumulatie van één of meerdere detecteerbare intermediairen. Hoewel geïsoleerd uit hetzelfde bodemstaal hadden de geoptimaliseerde consortia geen gemeenschappelijke stam, hetgeen erop wijst dat selective aanrijking niet altijd herhaald kan worden. Consortium 1 en consortium 3 bestaan respectievelijk uit vier en zes bacteriële stammen, maar in beide gevallen waren slechts twee specifieke stammen noodzakelijk om 2,4-DNT afbraak te realiseren. *Variovorax paradoxus* VM685 (consortium 1) and *Pseudomonas* sp. VM908 (consortium 3) initiëren 2,4-DNT catabolisme door een oxidatieve stap waarbij nitriet vrijkomt en 4-methyl-5-nitrocatechol (MNC) gevormd wordt. Beide stammen bevatten een gen homoloog aan het *dntAa* gen dat codeert voor 2,4-DNT dioxygenase in *Burkholderia* sp. DNT (Suen *et al*., 1996). Vervolgens metaboliseren zij MNC tot 2-hydroxy-5-methylquinone (HMQ) en nitriet, kenmerkend voor DntB of MNC monooxygenase activiteit. Een tweede

species van het consortium, *Pseudomonas marginalis* VM683 (voor consortium 1) of *P. aeruginosa* VM903, *Sphingomonas* sp. VM904, *Stenotrophomonas maltophilia* VM905 of *P. viridiflava* VM907 (voor consortium 3), bleek essentieel te zijn voor een efficiënte groei van het consortium op 2,4-DNT en de metabolisatie van de intermediairen MNC en HMQ. Informatie omtrent de interacties in deze stap van de afbraakcyclus ontbreekt echter. Een gen homoloog aan het *dntD* gen van *Burkholderia* sp. DNT (Haigler *et al*., 1999), verantwoordelijk voor ring fissie, werd aangetoond door DNA-DNA hybridisatie in de vermelde tweede reeks aan stammen.

Selectieve aanrijkingen op andere nitroaromatische componenten (TNT, 2,6-DNT, 2-NT, 4-NT) als enige stikstofbron werden uitgevoerd met bodem- en waterstalen van de industriële site, echter zonder succes. Daarop werden beide consortia 1 en 3 getest op hun TNT degradatie capaciteiten. Consortium 3 gaf de beste resultaten naar snelle en complete TNT transformaties, genotoxiciteitsverlaging en vertoonde een hoge co-metabolische activiteit zelfs wanneer TNT in overmaat werd toegevoegd. Daarom werd dit consortium gekozen om mee verder te werken in haalbaarheidsstudies voor de remediatie van TNT-gecontamineerde bodem en grondwater (Hoofdstuk 7, Hoofdstuk 8).

In plant-systemen kan TNT getransformeerd worden in de rhizosfeer door extracellulaire of membraan-gebonden plantenzymen of door de aanwezige microflora, hetgeen resulteert in de vorming van amino of hydroxylamino functionele groepen. Deze kunnen irreversiebel binden aan het worteloppervlak en verder chemische transformaties ondergaan (Burken *et al*., 2000). Deze bevindingen wijzen op het mogelijk gebruik van een rhizofiltratie-proces voor de eliminatie van TNT, hetgeen verder onderzocht werd gebruik makende van *Helianthus annuus* (zonnebloem). Staalnamen van hydroponische media toonden een TNT verwijdering van 98.1, 97.5 en 76.2% na 21 dagen van plantenkweek wanneer respectievelijk 5, 10 en 20 mg TNT/L werd toegevoegd. Een initiële TNT concentratie van 20 mg/L resulteerde in een lagere pseudo eerste-orde snelheidsconstante voor de eliminatie van TNT in het medium, hetgeen wijst op

een toxische grenswaarde voor *Helianthus annuus* tussen 10 en 20 mg TNT/L. Biologische responsen van *Helianthus annuus* op TNT werden voornamelijk waargenomen ter hoogte van de wortel en bestonden uit verkleuring en groeireductie. Radiogelabelde TNT werd gebruikt om de bestemming van de component in *Helianthus annuus* te evalueren aan een initiële concentratie van 10 mg/L. Van de totaal plant-geassocieerde ^{14}C accumuleerde finaal 92.6% in de wortel, 5.3% in de stengel en slechts 2.1% in de bladeren, hetgeen duidt op een inefficiënte translocatie naar de bovengrondse delen van de plant. Staalnamen van plantenweefsel in functie van de tijd toonden aan dat een toenemend percentage van de activiteit irreversiebel gebonden werd, hetgeen duidt op sequestratie. Verder werd de impact van meerdere opeenvolgende TNT addities aan het plant-systeem geëvalueerd. De resultaten suggereren dat *Helianthus annuus* TNT-concentraties tot 10 mg/L "tolereert". Ook werd het effect van TNT op de kieming en ontwikkeling van *Helianthus annuus* bepaald op gecontamineerde bodems. Er werd geen negatief effect van TNT in de bodem waargenomen op de kieming van de zaden, de groei van zaailingen werd echter beïnvloed vanaf een concentratie van 34 mg TNT/kg bodem. Bovendien werd de opname van de contaminanten door de planten en de verwijdering van nitroaromatische componenten (NACen) in de rhizosphere geëvalueerd. NACen werden uitsluitend gedetecteerd in wortelweefsels. Significante rhizodegradatie werd niet geobserveerd na 4 weken blootstelling van de bodem aan *Helianthus annuus.*

Er werd getracht de snelheid van immobilisatie van NACen in de bodem te verhogen in een aërobe slurry aan de hand van biostimulatie of bioaugmentatie (inoculatie van consortium 3) hetgeen voordelig is in een reactor op grote schaal. De test toonde aan dat het toevoegen van consortium 3 de periode die nodig is voor remediatie aanzienlijk kan verminderen. Op vragen betreffende de frequentie van reïnoculatie van het consortium, het toevoegen van een surfactant en/of het toevoegen van extra nutriënten om een 100% TNT verwijdering te bekomen moet nog een antwoord gevonden worden. Verder zijn gedetailleerde studies vereist omtrent het bepalen van de resterende toxiciteit.

Een haalbaarheidsstudie voor de behandeling van TNT gecontamineerd water werd uitgevoerd met *Phragmites australis* (riet) in een systeem gebaseerd op een horizontaal vloeirietveld. Verder werd het effect van inoculatie van consortium 3 om de biodegradatie te versterken onderzocht. Verschillende problemen eigen aan het systeem kwamen echter opzetten, waaronder biologische activiteit in de influent reservoirs, plant groei problemen en overlevingsproblemen van het geïnoculeerde consortium. Mogelijke fysische, chemische en biologische oorzaken worden aangehaald. Verder onderzoek is nodig voor het succesvol opstarten.

CHAPTER 1
INTRODUCTION

Part of this chapter has been published by Snellinx et al. (2002).
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1.1 PHYTOREMEDIATION

1.1.1 General Introduction

Phytoremediation is a technology that uses green plants and their associated microorganisms to remediate soil, sediment, surfacewater and groundwater contaminated with heavy metals, metalloids, radionuclides, organics and nutrients. As all remediation techniques, phytoremediation either removes the contaminant from the polluted matrix in a process called decontamination, or sequester it into the matrix via stabilisation (Cunningham *et al.*, 1996).

Processes involved in phytodecontamination are phytoextraction, phytodegradation, phytovolatilisation, rhizofiltration and rhizodegradation. Phytoextraction describes the case where a contaminant is taken up by the plant and is stored in a recoverable form into the harvestable parts of aboveground shoots. The process often occurs with heavy metals (Pb, Cd, Zn, Ni, Cu) (Baker and Brooks, 1989; Reeves and Baker, 2000), but may be important for certain organic chemicals (e.g. DDT, RDX) that are resistant to plant metabolism (McMullin, 1993; Harvey *et al.*, 1991). In the case of phytodegradation, contaminants are metabolised in plant tissues following uptake. Phytodegradation has been studied extensively to understand the fate of herbicides in crop plants

(Hatzios and Penner, 1982; Hatzios, 1991). Phytovolatilisation is the release of contaminants from plant tissues into the atmosphere after uptake. The process has been observed for contaminants such as trichloroethylene (Orchard *et al*., 2000), chlorobenzene (Baeder-Bederski *et al*., 1999), selenium (Terry and Zayed, 1998) and mercury (Rugh *et al*., 1998; Heaton *et al*., 1998). In addition, volatilisation can occur from the soil surface by the activity of plant-associated microorganisms as been observed for selenium (De Souza *et al*., 1999). Mechanisms of removal by rhizofiltration might include surface sorption, biological processes (including intracellular uptake and vacuolar deposition) and root-mediated precipitation (probably involves the release of root exudates) (Salt *et al*., 1995). Rhizofiltration may be important for the removal of heavy metals (Pb, Cd, Zn, Ni, Cu) (Dushenkov *et al*., 1995; Salt *et al*., 1997) and radionuclides (U, Cs, Sr) (Cooney, 1996; Richman, 1996) from polluted effluents, surfacewater and groundwater. In rhizodegradation, microorganisms in the rhizosphere and/or enzymes excreted by plant roots transform the contaminant. Rhizodegradation is for instance responsible for enhanced removal of petroleum hydrocarbons, PAH, BTEX and TCE from soil (Carman *et al*., 1998; Jordahl *et al*., 1997; Newman *et al*., 1999; Suominen *et al*., 2000).

Phytostabilisation occurs where pollutants precipitate from the solution or are absorped or entrapped in either plant tissue or the soil matrix (Cunningham *et al*., 1995). Processes include incorporation of hydrophobic organics into lignin and soil humus, absorption and precipitation of toxic metals in soil particles, and upward flux of water and contaminants in the soil (Cunningham *et al*., 1996). These processes reduce bioavailibility and off-site migration (i.e. erosion, leaching, and wind transport).

The specific interactions of a pollutant with soil, water, and plants will vary depending on the chemical properties of the contaminant, the physiological properties of the introduced plant species, and the contaminated medium. Collectively, these properties will determine whether a contaminant is subject to phytoextraction, phytodegradation, phytovolatilisation, rhizodegradation, or phyto-

Table 1.1 Typical plants used in various phytoremediation applications (Dietz and Schnoor, 2001)

Remediation Technique	Media	Contaminants	Plant
Phytoextraction	Soil, sediment	Metals (Pb, Cd, Zn, Ni, Cu)	Indian mustard (*Brassica juncea*) *Thlaspi caerulescens*
Rhizofiltration	Groundwater, wastewater through constructed wetlands	Metals (Pb, Cd, Zn, Ni, Cu); radionuclides, hydrophobic organics	Sunflower (*Helianthus* spp.) Wetland plants: Emergents (bulrush, cattail, coontail, pond weed, arrowroot) Submergents (algae, stonewort, parrot feather, *Hydrilla* spp.)
Phytodegradation	Soil, groundwater, land application of wastewater	Herbicides; chlorinated aliphatics (e.g. TCE); aromatics (e.g. BTEX); ammunition wastes (TNT, RDX, HMX, perchlorate); nutrients (nitrate, ammonium, phosphate)	Phreatophytic trees (Salix family, including poplar, willow and cottonwood) Grasses (rye, fescue, Bermuda grass, sorghum, switchgrass, reed, canary grass) Legumes (clover, alfalfa, cowpeas)
Phytovolatilisation	Soil, sediment	Selenium, arsenic, mercury, volatile organic compounds (e.g. MTBE)	*Brassica juncea* Wetland plants Phreatophytic trees for groundwater capture
Rhizodegradation	Soil, sediment	Biodegradable organics (BTEX, TPH, PAHs, PCBs, pesticides)	Grasses with fibrous roots (Bermuda, fescue, rye) Phenolics releasers (mulberry, apple, osage orange) Phreatophytic trees
Phytostabilisation	Soil	Metals (Pb, Cd, Zn, As, Cu, Cr, Se, U); hydrophobic organics that are not biodegradable	Phreatophytic trees for hydraulic control Grasses with fibrous roots for erosion control

BTEX, benzene, toluene, ethylbenzene, and total xylene; HMX, octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine; MTBE, methyl-tert-butyl ether; PAHs, polycyclic aromatic hydrocarbons; PCB, polychlorinated biphenyls; RDX, hexahydro-1,3,5-trinitro-1,3,5-triazine; TCE, trichloroethylene; TPH, total petroleum hydrocarbons.

stabilisation. In each case the process of phytoremediation begins with contaminant transport towards the plant root. Typical plants used in various phytoremediation applications are shown in Table 1.1.

Compared to more conventional techniques, using a diverse set of thermal, chemical, and physical methods, including excavation, incineration and soil washing, plants are ideal for environmental cleanup. Potential benefits of phytoremediation are low capital cost, minimal ongoing operating costs (solar-energy driven and passive technique), easy implementation, non-intrusive, and high public acceptance (aesthetically pleasing). However, clean-up rates may prove slower than conventional methods and it may be difficult to establish the vegetation because of toxicity or intolerance to site conditions. Phytoremediation is most effective at sites with shallow (< 5 m depth) and moderate levels of contamination. However, “pump and tree” may treat deep groundwater contaminants. Often, phytoremediation is used as a final polishing step, after other clean-up technologies have been used (Schnoor *et al.*, 1995). The recent US-EPA report (EPA 2000) lists 166 ongoing field projects, many of them for research or demonstration. The main pollutants treated are petroleum, heavy metals and chlorinated solvents.

Phytoremediation as a strategy for the removal of heavy metals, metalloids and radionuclides from the environment, will not be discussed in further detail. Rhizodegradation and phytodegradation, the two basic phytoremediation strategies for organic contaminants, are reviewed below.

1.1.2 Phytoremediation of Organic Contaminants

1.1.2.1 Rhizodegradation (Phytoremediation ex Planta)

1.1.2.1.1 Plant-Associated Bioremediation

The excretion of soluble root exudates (photosynthates such as sugars, phenolics, organic acids, alcohols, and proteins) by plants and the decay of fine-root biomass

build up the organic carbon in the root-soil interface or rhizosphere. As a consequence, the region near the plant root is defined by increased microbial growth and activity (Anderson *et al*., 1994; Cunningham *et al*., 1996). Bacterial community densities in the rhizosphere are a 10- to 1000-fold greater than in bulk soil (Paul and Clark, 1989; Anderson *et al*., 1993), depending on different factors including soil type and plant species. Important groups of culturable plant-associated rhizosphere bacteria are *Burkholderia* (*B. cepacia*), *Pseudomonas* (*P. fluorescens, P. putida, P. aeruginosa*), *Ralstonia, Bacillus, Arthrobacter, Azotobacter, Azospirillum, Acetobacter, Serratia, Rhizobium* and *Agrobacterium.*

A major benefit of the rhizosphere may be the presence of degrader organisms at high cell numbers, thereby shortening the acclimation period for biodegradation of specific contaminants. In addition, diverse species of heterotrophic microorganisms are brought together in the rhizosphere, which may enhance stepwise transformation of xenobiotics by consortia, or provide an environment that is conductive to genetic exchange and gene rearrangements. In some instances, cometabolism of compounds is enhanced. Cometabolism is the process by which a compound, that cannot support microbial growth, can be modified or degraded when another growth-supporting substrate is present. Furthermore, structural analogous of various xenobiotics are found in root exudates, cell wall components and lysates. These compounds may fortuitously select for microorganisms that metabolise or cometabolise the xenobiotics in question. Furthermore, transpiration of water by plants may influence the transport of water-soluble compounds by increasing their mass flow to the root surface, where they subsequently can be degraded by the rhizosphere microflora. However, the rhizosphere may as well have little or no effect on the degradation of xenobiotics. For instance, if a degrader population is absent from the soil or is noncompetitive in the rhizosphere, or if the degrader organisms can grow independently on the substrate such that the presence of the substrate is superfluous. Anderson and Coats (1995), Crowley *et al.* (1997) and Siciliano and Germida (1998a) provided valuable review information about the potential role of the rhizosphere in bioremediation of herbicides (e.g. propanil, 2,4-

D, atrazine) and industrial chemicals (e.g. PAHs, PCBs, chlorinated phenols). Some examples will be presented. Sandmann and Loos (1984) found very high rhizosphere/bulk soil ratios of 2,4-D-degrading organisms in sugarcane (*Saccharum officinarium*) rhizosphere soil. Batch experiments by Burken and Schnoor (1996) with ^{14}C-atrazine and hybrid poplar trees showed a stimulated microbial degradation by root exudates. Similar, a study by Carman *et al*. (1998) revealed microbial-enhancing processes in the rhizosphere of willow trees that stimulated biodegradation of diesel range organics. Siciliano and Germida (1998b) investigated the effect of *Pseudomonas* inoculants, that promote phytoremediation of 2-chlorobenzoic acid (2CBA), on the rhizosphere community of Dahurian wild rye (*Elymus dauricus*) and meadow brome (*Bromus biebersteinii*). Inoculating Dahurian wild rye had little effect on the root-surface microbial community, whereas the converse was true for meadow brome. This result indicates that the mechanism by which bacterial inoculants promote phytoremediation differs between plants. Moreover, a wide range of plant species was screened for the production of polyphenolic compounds capable of supporting PCB-degrading bacteria. Fletcher and Hedge (1995) identified mulberry (*Morus rubra* L.) as a possible plant species well suited to remediate PCB-contaminanted soil. Since not all plants produce and release the same types of phenolics, it would be expected that some plants may preferentially harbour PCB-degrading bacteria in their rhizosphere (Wenzel *et al*., 1999).

It is clear that plant-associated bioremediation can enhance contaminant attenuation in the environment, and thus it should be considered in the evaluation of phytoremediation. However, a prediction of the rhizosphere effect for a specific compound requires knowledge of its chemical characteristics, as well as its likely degradation pathway, and an understanding of the influence of plant root growth on the microorganisms that are involved in the degradation process.

1.1.2.1.2 Plant-Derived Degradative Enzymatic Effects

In addition to secreting organic compounds that support the growth and activities of microorganisms in the rhizosphere, plant roots also release a number of degradative enzymes into soil and sediments. These enzymes include laccases, dehalogenases, nitroreductases, nitrilases and peroxidases (Schnoor *et al*., 1995). The action of peroxidases on pollutants often results in the polymerisation of the pollutant either onto the root surface or onto the soil humic fraction. Subsequently, these polymeric complexes, referred to as bound residues, are no longer subject to biological processes. Cunningham *et al*. (1996) speculated that oxido-reductases (including peroxidases) are capable of metabolising nitroaromatic compounds. Dehalogenase helps to degrade chlorinated solvents such as trichloroethylene (TCE) to chloride ion, carbon dioxide and water (Carreira and Wolfe, 1996). Schnoor *et al*. (1995) demonstrated furthermore that nitroreductase and laccase enzymes catalyse the degradation of TNT and triaminotoluene (TAT) respectively. Finally, nitrilase was found to degrade 4-chlorobenzonitrile (Masunaga *et al*., 1995).

Planting selected species (identified via immunoassays for the presence of specific enzymes) could be used to accelerate cleanup levels. However, still little is known about the contribution of plant-associated enzymes in the degradation of soil contaminants. This subject requires detailed examination.

1.1.2.2 Phytodegradation (Phytoremediation in Planta)

1.1.2.2.1 Bioavailability and Sorption to Roots

Successful phytodegradation requires organic contaminants to be biologically available for absorption to plant or plant-associated microbial systems. The bioavailability of organic contaminants is primarily under the control of environmental soil factors such as soil type (determines clay and organic matter content), pH, cation exchange capacity and moisture content, which can be modified by plant species in their rhizosphere. Assuming constant plant and

environmental characteristics, lipophilicity is the most important property of a chemical, which determines bioavailability, direct root uptake and translocation within the plant (Briggs *et al*., 1982). Lipophilicity will be discussed later in the text.

Bioavailability of organics in soils appear to be a primarily restriction for effective phytoremediation of organic pollutants (Cunningham *et al*., 1996; Schnoor *et al*., 1995; Salt *et al*., 1998). The use of synthetic surfactants (e.g. triton X-100, SDS), biosurfactants (e.g. rhamnolipids) or cyclodextrins, could be considered in increasing bioavailability of organics (Miller, 1995; Nivas *et al*., 1996).

Lipophilicity is related to the partition coefficient of the pollutant between 1-octanol and water (K_{ow}). When chemical contaminants in soil water or groundwater come into contact with roots, they may sorb for instance to hemicellulose in the cell walls or to the lipid bilayer of plant membranes. Such sorption should be relatively reversible for efficient uptake to occur. Hydrophobic organic chemicals ($\log K_{ow} > 3$) are strongly bound to the surface of roots and soils and will not be substantially translocated within the plant. Examples include *m*-xylene, ethylbenzene, pentachlorophenol and 1,2,4-trichlorobenzene. These compounds are candidates for phytostabilisation. Chemicals, such as 1,4-dioxane, that are considered to be very water-soluble ($\log K_{ow} < 0.5$), are not sufficiently sorbed to roots and soil, and are a serious risk for groundwater contaminantion. These compounds are not appropriate targets for phytoremediation. For moderately hydrophobic compounds ($\log K_{ow}$ between 0.5 and 3) the removal mechanism is at its most efficient. Examples include BTEX compounds (benzene, toluene, ethylbenzene and xylenes), polychlorinated biphenyls (PCBs), trichloroethylene (TCE) and ammunition wastes (RDX, nitroglycerin) (Schnoor *et al*., 1995; Burken and Schnoor, 1998).

The efficiency of sorption of a chemical onto roots via the aqueous phase can be expressed by its root concentration factor (RCF) defined as (Briggs *et al*., 1982):

RCF = (concentration in roots)/(concentration in external solution)

As mentioned before, hydrophobicity is an important factor controlling sorption. The following relationships between RCF and log K_{ow} were established respectively by Briggs *et al*. (1982), using crop species (barley), and by Burken and Schnoor (1998), using hybrid poplar trees:

$\log (RCF - 0.82) = 0.77 \log K_{ow} - 1.52$ (Briggs *et al*., 1982)

$\log (RCF - 3.0) = 0.65 \log K_{ow} - 1.57$ (Burken and Schnoor, 1998)

It is clear that, the greater the hydrophobicity (log K_{ow}) of a chemical, the greater the tendency to partition onto roots. However, some contaminants, especially these with amino (-NH_2) and hydroxy (-OH) functional groups, are transformed rapidly at the root surface by extracellular enzymes or by membrane-bound enzymes. These chemicals and their metabolites bind irreversibly to roots and are further chemically transformed. As an example, pentachlorophenol (log K_{ow} = 5.04) and 1,2,4-trichlorobenzene (log K_{ow} = 4.25) are highly sorbed to root tissues because of their hydrophobicity (RCF > 10), but aniline (log K_{ow} = 0.90) and phenol (log K_{ow} = 1.45) bind tightly to roots because of specific sorption and enzymatic transformation. Other examples include the reduction and transformation of nitroaromatic explosive compounds such as TNT (log K_{ow} = 1.98). Nitroaromatics may bind tightly to roots and be transformed as the nitro group is reduced to an amino or hydroxylamino group. In conclusion, chemicals that react biochemically at the root-water interface do not follow the above relationship (Dietz and Schnoor, 2001).

1.1.2.2.2 Uptake and Translocation

Nutrients and metal cations (Ca^{2+}, Mg^{2+}, K^{+}, and Na^{+}) are taken up by active transport mechanisms. Organic chemicals can be taken up by plants via diffusion (passive uptake) through cell walls and membranes. Direct uptake of organics is again most efficient for moderately hydrophobic chemicals ($\log K_{ow} = 0.5 – 3.0$).

The transpiration rate is the key variable that determines the translocation efficiency within a plant and depends besides plant species characteristics (e.g. plant type, leaf area) also on growth conditions such as soil moisture, nutrients, temperature, wind conditions and relative humidity (Schnoor *et al.*, 1995; Cunningham *et al.*, 1996). For rooted vascular plants, with contaminants that do not biochemically react at the root-water interface, the translocation efficiency is described by its transpiration stream concentration factor (TSCF), defined as (Briggs *et al.*, 1982):

TSCF = (concentration in transpiration stream)/(concentration in external solution)

Briggs *et al*. (1982) and Burken and Schnoor (1998) determined the following relationship between TSCF en log K_{ow} for respectively crop species and hybrid poplar trees:

$TSCF = 0.756 \exp-[(\log K_{ow} – 2.50)^2/2.58]$ (Briggs *et al.*, 1982)
$TSCF = 0.784 \exp-[(\log K_{ow} – 1.78)^2/2.44]$ (Burken and Schnoor, 1998)

Both these relationships predict an optimal uptake for moderately hydrophobic compounds. In general, plants take up organic chemicals via diffusion. Most lipophilic pollutants ($\log K_{ow} > 3.0$) do not pass beyond the root surface due to the high proportion of lipids on the root surface (Trapp *et al.*, 1990; Paterson and Mackay, 1994; Simonich and Hites, 1995). Thus it is unlikely that plants will

accumulate significant amounts of lipophilic contaminants. However, there are few exceptions, suggesting that not all plants use a passive uptake process for lipophilic compounds (Hulster *et al*., 1994). In addition, Aitchison *et al*. (2000) showed that the very water-soluble compound 1,4-dioxane, with a log K_{ow} value of –0.27, was rapidly taken up and translocated by hybrid poplar cuttings, suggesting uptake via hydrogen bonding with water molecules into the transpiration stream.

Quantitative models for terrestrial plants, such as the RCF and the TSCF, may help to predict plant uptake rates, assaying treatment times for phytoremediation technologies. Models have not been developed for aquatic plants where diffusion and partitioning are thought to play a larger role in uptake.

1.1.2.2.3 Enzymatic Transformations

Following root uptake, organic compounds may be translocated to other plant tissues (Cataldo *et al*., 1987; Fellows *et al*., 1996; Schroll *et al*., 1994) and subsequently volatilised, partially or completely degraded (Goel *et al*., 1997; Newman *et al*., 1997; Schnoor *et al*., 1995; Orchard *et al*., 2000), or transformed to less toxic compounds and sequestered in vacuoles or bound to insoluble cellular structures, such as lignin (Fletcher and Hedge, 1995). Most organic xenobiotics appear to undergo some degree of transformation in plant cells, however, few chemicals appear to be fully mineralised by plants to H_2O and CO_2, and where this occurs (e.g. TCE), it only represents a small percentage of the total parent compound (Newman *et al*., 1997). This property puts plants at a relative disadvantage compared with bacteria in degrading organic pollutants. The reason for this is that plants are not heterotrophs and therefore do not have an extensive array of catabolic pathways for use of organic compounds as carbon or nitrogen source. The metabolism of xenobiotics involved in phytodegradation is generally considered to be a detoxification process, remarkably similar to those processes occurring in mammalian livers, hence the “green liver” concept is often used to

describe phytodegradation (Sandermann, 1994). Once an organic chemical is taken up and translocated, it is metabolised in three phases: transformation, conjugation, and sequestration.

Phase I consists of transformation reactions which introduce functional groups (-OH, -NH_2, -SH) into various xenobiotic compounds as a result of a reduction, oxidation, or hydrolysis (Komossa *et al*., 1995). These functional groups are amenable to subsequent conjugation reactions. Oxidation is the most frequently observed reaction type in the metabolism of pesticides. Reduction reactions are less common, but have been found for certain nitroaromatic compounds. Hydrolytic reactions may occur with carboxylic acid ester xenobiotics, organophosphorus compounds, carbamates, and anilines (Komossa *et al*., 1995). Phytotransformation has been studied most with pesticides in crop plants (Nebert and Gonzales, 1987; Bolwell *et al*., 1994; Komossa *et al*., 1995). Plant enzymes that typically catalyse phase I reactions are P450 monooxygenase and carboxylesterases (Komossa *et al*., 1995). However, introducing functional groups onto substrates do not always result in products with decreased phytotoxicity, and as most of the mentioned reactions are reversible, true detoxification is only reached by conjugating enzymes.

Phase II is characterised by conjugation of the transformed chemicals with biomolecules of plant origin such as glutathione, sugars and amino acids, resulting in soluble, polar compounds (soluble conjugates) (Marrs, 1996). The conjugate formation plays a key role in the metabolism of xenobiotic compounds by plants because it leads to a reduction in toxicity to the plant (Coleman *et al*., 1997; Trapp, 1995). Several enzymes are known to catalyse these reactions, of which *O*- and *N*-glucosyltransferases, malonyltransferases, and glutathione-S-transferases (GST) are the most studied (Coleman *et al*., 1997; Sandermann *et al*., 1991; Picket and Lu, 1989; Marrs, 1996; Pflugmacher *et al*., 2000). Detoxification of herbicides is attributed to conjugation with glutathione catalysed by GST (Lamoureux *et al*., 1991).

Phase III, sequestration, involves storage of conjugates in the vacuole (Coleman *et al*., 1997), where they are no longer subject to biological processes, and

incorporation in the cell wall, where they are characterised as bound (i.e. nonextractable) residues (Sandermann, 1987). Phase III enzymes, ATP-dependent membrane pumps, recognise and transfer conjugates across membranes for sequestration in the vacuole or storage in the apoplast. Many aromatic or heteroaromatic compounds are known to be covalently bound to lignin or other cell wall components such as hemicellulose.

Through an improved understanding of these processes, the prospects for the successful exploitation of detoxification metabolism in plants appear good. Plant metabolic engineering could be a novel way to lead to optimised plant characteristics in the remediation of organic compounds. Already genetic engineering of crops with plant or bacterial genes has produced transgenic plants that are resistant to herbicides (Dale, 1995).

1.2 BIOLOGICAL REMEDIATION OF TNT

1.2.1 General Introduction

Nitroaromatics form an important group of recalcitrant xenobiotics. Only few aromatic compounds, bearing one nitro group as a substituent on the aromatic ring, are produced as secondary metabolites by microorganisms. The majority of nitroaromatic compounds in the biosphere are industrial chemicals such as explosives, dyes, polyurethane foams, herbicides, insecticides and solvents. These compounds are generally recalcitrant to biological treatment and remain in the biosphere, where they constitute a source of pollution due to both toxic and mutagenic effects on humans, fish, algae and microorganisms (Won *et al.*, 1976; Verschueren, 1977; Tan *et al.*, 1992). However, few microorganisms have been described as being able to use nitroaromatic compounds as nitrogen and/or carbon source (Table 1.2).

Table 1.2 Microorganisms reported to use nitrotoluenes as nitrogen and/or carbon source

Compound	Condition/Microorganism	Metabolism	Reference
2-NT	**Aerobic**		
	Pseudomonas sp. strain JS42	C-, N-source	Haigler *et al*., 1994
3-NT	**Aerobic**		
	Pseudomonas putida	C-, N-source	Ali-Sadat *et al*., 1995
4-NT	**Aerobic**		
	Mycobacterium sp. strain HL4-NT-1	C-, N-source	Spiess *et al.*, 1998
	Pseudomonas fluorescens	C-, N-source	Michan *et al.*, 1997
	Pseudomonas sp. 4NT	C-, N-source	Haigler and Spain, 1993
	Pseudomonas sp. TW3	C-, N-source	Rhys-Williams *et al*., 1993
2,4-DNT	**Aerobic**		
	Burkholderia cepacia R34	C-, N-source	Johnson *et al.*, 2000
	Burkholderia sp. DNT	C-, N-source	Spanggord *et al.*, 1991
	Pseudomonas sp. clone A	N-source	Duque *et al.*, 1993
	Pseudomonas sp. ST53	N-source	Taghavi, pers. comm.
	Two identified consortia	N-source	Snellinx *et al.*, submitted
2,6-DNT	**Aerobic**		
	Burkholderia cepacia strain JS850	C-, N-source	Nishino *et al.*, 2000
	Hydrogenophaga palleronii strain JS863	C-, N-source	Nishino *et al.*, 2000
	Pseudomonas sp. clone A	N-source	Duque *et al.*, 1993
TNT	**Aerobic**		
	Enterobacter cloacae PB2	N-source	French *et al.*, 1998
	Pseudomonas sp. clone A	N-source	Duque *et al.*, 1993
	Pseudomonas sp. clone A (TOL)	C-, N-source	Duque *et al.*, 1993
	Pseudomonas sp. ST53	N-source	Jones *et al*., 1995
	Serratia marcensens	N-source	Taghavi, pers. comm.
	Stenotrophomonas maltophilia M91-3	C-, N-source	Montpas *et al*., 1997; Oh and Kim, 1998
	Anaerobic		
	Desulfovibrio sp. strain B	N-source	Boopathy and Kulpa, 1992
	Desulfovibrio sp.	N-source	Preuss *et al*., 1993
	Pseudomonas sp. strain JLR11	N-source	Esteve-Nunez and Ramos, 1998

The best-known nitroaromatic compound is the explosive TNT (2,4,6-trinitrotoluene). The highest amount of TNT was produced at the end of WWII, when the world production was estimated on 150 k ton per month (Zeman, personal communication). Though most of the munitions waste sites date back from the past, many are still a serious environmental hazard. Sites contaminated with TNT range from ammunition plants and testing facilities to military zones and battlefields. Although this contamination is known to be widespread, little information is available in literature documenting these sites and their degree of contamination. A determined high number of contaminated sites are located in Germany and the United States. In the near surrounding of former ammunition plants, very high levels of pollution can be found, concentrated into rather small areas. At military sites, levels of pollution are rather low and diffuse, due to incomplete burning of TNT during explosion. Because TNT is manufactured by nitration of toluene, TNT contamination is often accompanied with impurities of dinitrotoluenes (2,4-DNT, 2,6-DNT and 3,4-DNT), and nitrotoluenes (2-NT, 3-NT and 4-NT). Amino derivatives, resulting from biotic and abiotic reduction reactions, are more of a problem than the TNT parent material. They are more stable in the environment and more mobile, posing a potential threat to drinking water supplies, human health and the environment (Hartter, 1985). An example of a contaminated groundwater sample found at the former site of an ammunition production facility is given in Table 1.3. Its chemical composition provides a nice overview of the complexity of the historical pollution found at former TNT production and handling sites.

At present, the only proven technology for the elimination of TNT and its metabolites from contaminated soil is incineration. Incineration is a prohibitively costly, destructive process and results in the release of undesirable greenhouse-effect gases such as CO_2 and NO_x. In addition, the ash from incineration must be treated as hazardous waste. Therefore bio- and phytoremediation represent important alternative approaches. Several observations have suggested a variety of

biological techniques for treating TNT contaminated soil. This includes composting, the use of the white-rot fungus *Phanerochaete chrysosporium* and bioslurry systems.

The best current available technology for treating explosives-containing water is activated carbon adsorption. Although effective, the treatment is expensive due to the high carbon requirements and the associated cost of regeneration or incineration. Other physicochemical treatments include chemical reduction (Agrawal and Tratnyek, 1996; Arienzo, 2000a, 2000b; Emmrich, 1999; Larson *et al*., 1996), photocatalytic degradation (Schmelling *et al*., 1996), and ozonolysis (Lang *et al*., 1998; Spanggord *et al*., 2000). Alternative biological approaches include activated sludge systems, the use of the fungus *Phanerochaete chrysosporium* and the use of plants in wetland systems.

Table 1.3 Concentrations of explosives and related compounds in a wastewater sample

Compound	Concentration (µg/L)
2-nitrotoluene	2560
3-nitrotoluene	175
4-nitrotoluene	854
2,4-dinitrotoluene	201
2,6-dinitrotoluene	136
3,4-dinitrotoluene	9
2,4,6-trinitrotoluene	228
2-amino-4,6-dinitrotoluene	92
4-amino-2,6-dinitrotoluene	144
Hexogen (RDX)	59
2,4-DNT-sulfonic acid-3	177
2,4-DNT-sulfonic acid-5	98
Sum aromatic amines	480

1.2.2 Metabolism by Microorganisms

1.2.2.1 Aerobic Metabolism

Electrophilic attack by oxygenases is unknown for trinitro compounds such as TNT, due to the electron-withdrawing character of the nitro groups and consequently the electron deficiency of the aromatic ring. Instead, nitro groups or the aromatic nucleus are reduced. Reduction of nitro groups on aromatic rings is

widely distributed among living organisms and proceeds via nitroso and hydroxylamino intermediates (Rieger and Knackmuss, 1995). The nitro group with the greatest negative charge localisation is likely to be the most readily protonated. In TNT, the *para* position is much more easily reduced than the *ortho* nitro groups (Barrows *et al*., 1996). These reactions are favoured under microaerophilic and even under more under anaerobic conditions.

Pathways for aerobic metabolism of TNT in bacterial species is shown in Figure 1.1. As mentioned, in most cases aerobic metabolism of TNT by microorganisms involves the reduction of one or more nitro groups on the aromatic ring. This results in the accumulation of aminodinitrotoluenes (2-ADNT, 4-ADNT) or diaminonitrotoluenes (2,4-DANT). In general, these resist further oxidative microbial degradation. Only few studies report aerobic metabolism of these amino compounds by microorganisms (Naumova *et al*., 1988; Gilcrease and Murphy, 1995; Kalafut *et al*., 1998; Vanderberg *et al*., 1995). However, little details are known.

In addition, under aerobic conditions tetranitroazoxytoluenes (2,2'-azoxy, 4,4'-azoxy, 2',4-azoxy and 2,4'-azoxy), resulting from the condensation of partially reduced intermediates (hydroxylamino- and nitrosodinitrotoluenes), have been found (McCormick *et al.*, 1976; Kaplan and Kaplan, 1982; Duque *et al.*, 1993; Haïdour and Ramos, 1996; Vasilyeva *et al.*, 2000; Wang *et al.*, 2000). These azoxy compounds are again highly recalcitrant to bacterial degradation.

Another reductive degradation mechanism by aerobic organisms has been described: the nucleophilic attack by a hydride ion, originating from a reduced pyrimidine nucleotide, to form a hydride-Meisenheimer complex (Vorbeck *et al.*, 1994). Duque *et al.* (1993) reported the isolation of *Pseudomonas* sp. clone A that was capable of aerobic growth with TNT as the sole nitrogen source. The elimination of the first two nitro groups on the ring was suggested to take place via the production of a hydride-Meisenheimer complex. Haïdour and Ramos (1996) showed that this complex was *in vitro* transformed into 2,4-DNT and an unidentified compound. However, the formation of such an hydride complex could

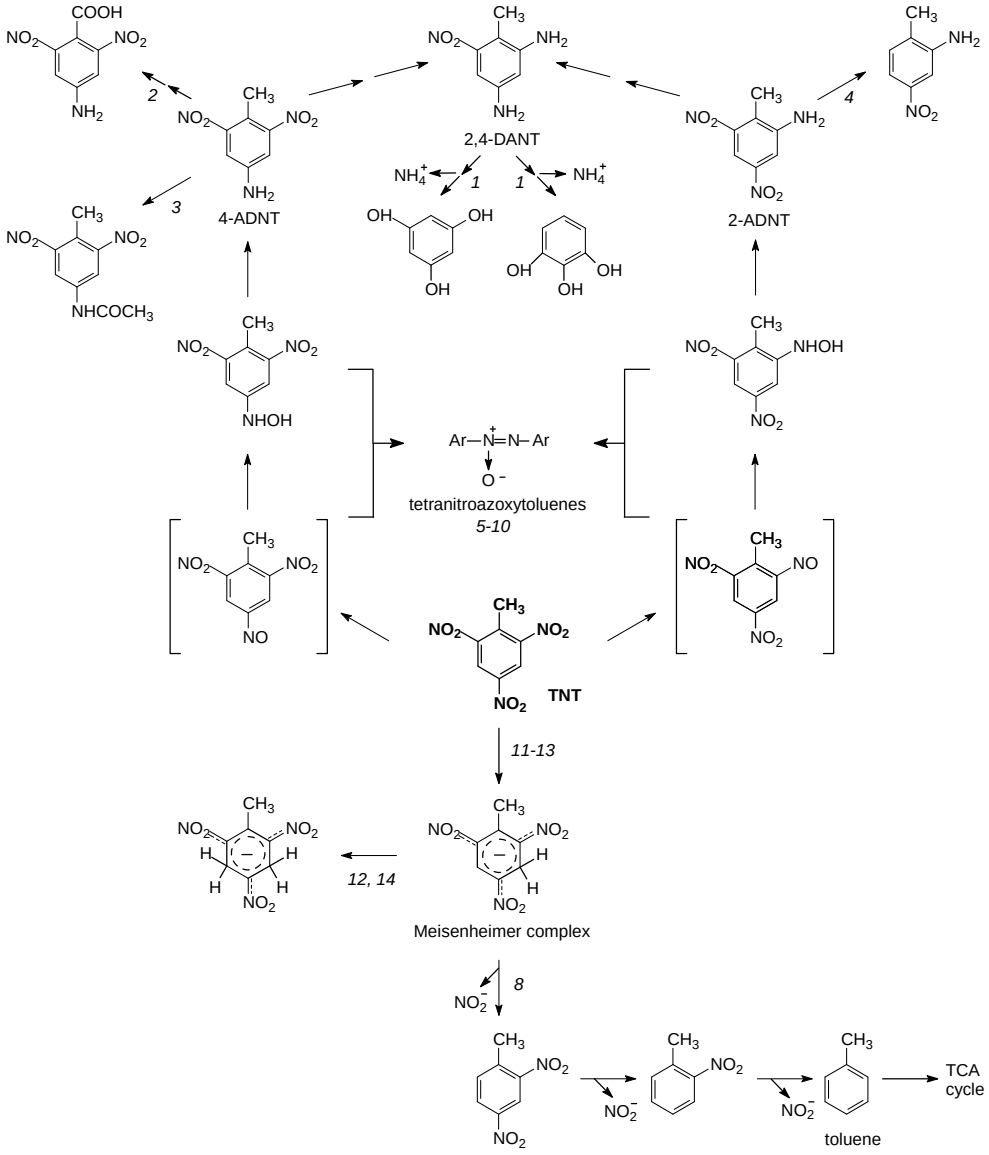

Figure 1.1 Pathways for the aerobic metabolism of TNT by bacteria (Adapted from Esteve-Nunez *et al*., 2000)
1, Naumova *et al*., 1988; 2, Vanderberg *et al*., 1995; 3, Gilcrease and Murphy, 1995; 4, Kalafut *et al*., 1998; 5, McCormick *et al*., 1976; 6, Kaplan and Kaplan, 1982; 7, Duque *et al*., 1993; 8, Haïdour and Ramos, 1996; 9, Vasilyeva *et al*., 2000; 10, Wang *et al*., 2000; 11, French *et al*., 1998; 12, Pak *et al*., 2000; 13, Vorbeck *et al*., 1994; 14, Vorbeck *et al*., 1998.

not be identified by Vorbeck *et al.* (1998) in the presence of *Pseudomonas* sp. clone A. The presence of an H^--TNT complex was successfully demonstrated when *Rhodococcus erythropolis* HL PM-1 or *Mycobacterium* strain HL 4-NT-1 were exposed to TNT (Vorbeck *et al.*, 1994). Unlike the H^--picric acid complex, the corresponding TNT complex undergoes neither nitrite elimination nor rearomatisation to 2,4-DNT or 2,6-DNT. Rather than eliminating a nitrite ion, the establishment of a protonated dihydride-Meisenheimer complex of TNT ($2H^-$-TNT) was identified in the presence of both strains (Vorbeck *et al.*, 1998). Successive transfer of two hydride ions instead of one is observed also with picric-acid (Lenke and Knackmuss, 1992). This route of dihydride complex formation is unproductive for the catabolism of both TNT and picric acid in *Rhodococcus* spp. The pentaerythritol tetranitrate (PETN) degrading strain *Enterobacter cloacae* PB2 was found to grow in mineral medium with TNT as the sole nitrogen source (French *et al.* 1998), causing the formation of H^--TNT and $2H^-$-TNT. 2,4-DNT, 2,6-DNT, and 2-NT were not detected. Recently, Pak *et al*. (2000) reported the purification of an NADH-dependent flavoprotein oxidoreductase (XenB) from *Pseudomonas fluorescens* I-C. XenB catalysed the reduction of TNT either by hydride addition to the aromatic ring and the concomitant formation of a dihydride Meisenheimer complex, or by nitro group reductions resulting in the accumulation of 2-hydroxylamino-4,6-dinitrotoluene and 4-hydroxylamino-2,6-dinitrotoluene. However, elimination of a nitro group from TNT is required to decrease the electrophilic nature of the molecule and allow a dioxygenases attack to complete the mineralisation. Several species are known to mineralise 2,4-DNT or 2,6-DNT (Spanggord *et al*., 1991; Nishino *et al*., 2000).

Recently, *Pseudomonas* sp. ST53 (Taghavi, unpublished results) was identified. The strain efficiently used 2,4-DNT and TNT as N-source under nitrogen-limiting conditions. Complete removal of 2,4-DNT and TNT was observed at concentrations up to 100 ppm, without the accumulation of partial reduced TNT derivatives. Significant mineralisation of ^{14}C-TNT was also observed. The mechanism by which this strain metabolises TNT and 2,4-DNT is presently under

investigation.

The white rot fungus *Phanerochaete chrysosporium* has been studied intensively for its ability to mineralise TNT. Mineralisation of ^{14}C-TNT (10-20%) has been demonstrated in both soil and liquid cultures (Fernando *et al.*, 1990; Fernando and Aust, 1991; Bumpus and Tatarko, 1994; Hawari *et al.*, 1999). As in other organisms, the initial steps in the fungal degradation of TNT involve the reduction of nitro groups, likely through a membrane-associated redox system (Stahl and Aust, 1993a; Rieble *et al.*, 1994). In a subsequent oxidative step, the reduction products of TNT are transformed mainly due to extracellular ligninolytic enzymes such as lignin peroxidases (LiP) and manganese peroxidases (MnP). In non-ligninolytic conditions, the ADNT metabolites were slowly reduced to DANTs and the concentration of azoxytetranitrotoluenes increased. When cultures became ligninolytic, the reduced aromatic compounds and azoxytetranitrotoluenes disappeared at the onset of manganese-dependent peroxidases (MnP), but mineralisation was not observed until lignin peroxidases were produced by the fungus (Stahl and Aust, 1993b). However, the catabolic potential of *P. chrysosporium* for TNT mineralisation is limited because of TNT toxicity caused by hydroxylaminodinitrotoluenes. In liquid systems, mineralisation was shown to be inhibited at TNT concentrations higher than 15 mg/L (66 µM) by Spiker *et al.* (1992) or 20 mg/L (88 µM) by Michels and Gottschalk (1994). This prevents the fungus from being a good candidate for bioremediation of TNT-contaminated sites. More recently, other wood white rot fungi and litter decay fungi have been investigated for their ability to metabolise TNT. Scheibner and colleagues (1997a, 1997b, 1998) demonstrated that cell-free preparations of MnP from the white rot basidiomycete *Nematoloma frowardii* and the litter-decaying basidiomycete *Stropharia rugosoannulata* were able to mineralise a mixture of reduction products from TNT. Van Aken *et al.* (1997, 1999) showed that a concentrated preparation of MnP from the white rot fungus *Phlebia radiata* was able to completely degrade TNT, with 22% mineralisation, and 2-ADNT, with 76% mineralisation. In

addition, several fungal species have been reported to tolerate high TNT concentrations. *Cladosporium resinae* and *Cunninghamella echinulata* var. *elegans* transform TNT to reduced products, including azoxytetranitrotoluenes, but no mineralisation was detected (Bayman and Radkar, 1997). The authors consequently proposed a two-step process in which TNT is initially reduced by a fungus such as *C. resinae* and then mineralised by *P. chrysosporium.* However, a disadvantage of this system is that these fungi are native to wood rather than soil and may not compete well with native soil fungi in field remediation situations (Bayman and Radkar, 1997).

Data suggest that partial reduced metabolites (aminodinitrotoluenes, diaminonitrotoluenes), and tetranitroazoxytoluenes are the principal end products of TNT degradation under aerobic conditions. Because these compounds are very recalcitrant, they are a serious obstacle to bioremediation processes. Cleavage of the aromatic nucleus of TNT has not been demonstrated by defined bacteria, or it occurs at very low efficiency. Most promising results towards mineralisation were obtained with fungal systems. However, further knowledge on the TNT degradation pathways of these organisms is required before their potential can be fully exploited for the remediation of TNT contaminated soil and water.

1.2.2.2 Anaerobic Metabolism

The reduction of nitro substituents by anaerobic microorganisms has been described for a number of nitroaromatic compounds including TNT. The electron-donating character of amino substituents weakens the electron deficiency originally present in TNT. Therefore reaction velocities decrease with the number of nitro groups being converted into amino groups. As a consequence, complete conversion of TNT to 2,4,6-triaminotoluene (TAT) requires strict anaerobic conditions ($E_h \leq$ -200 mV).

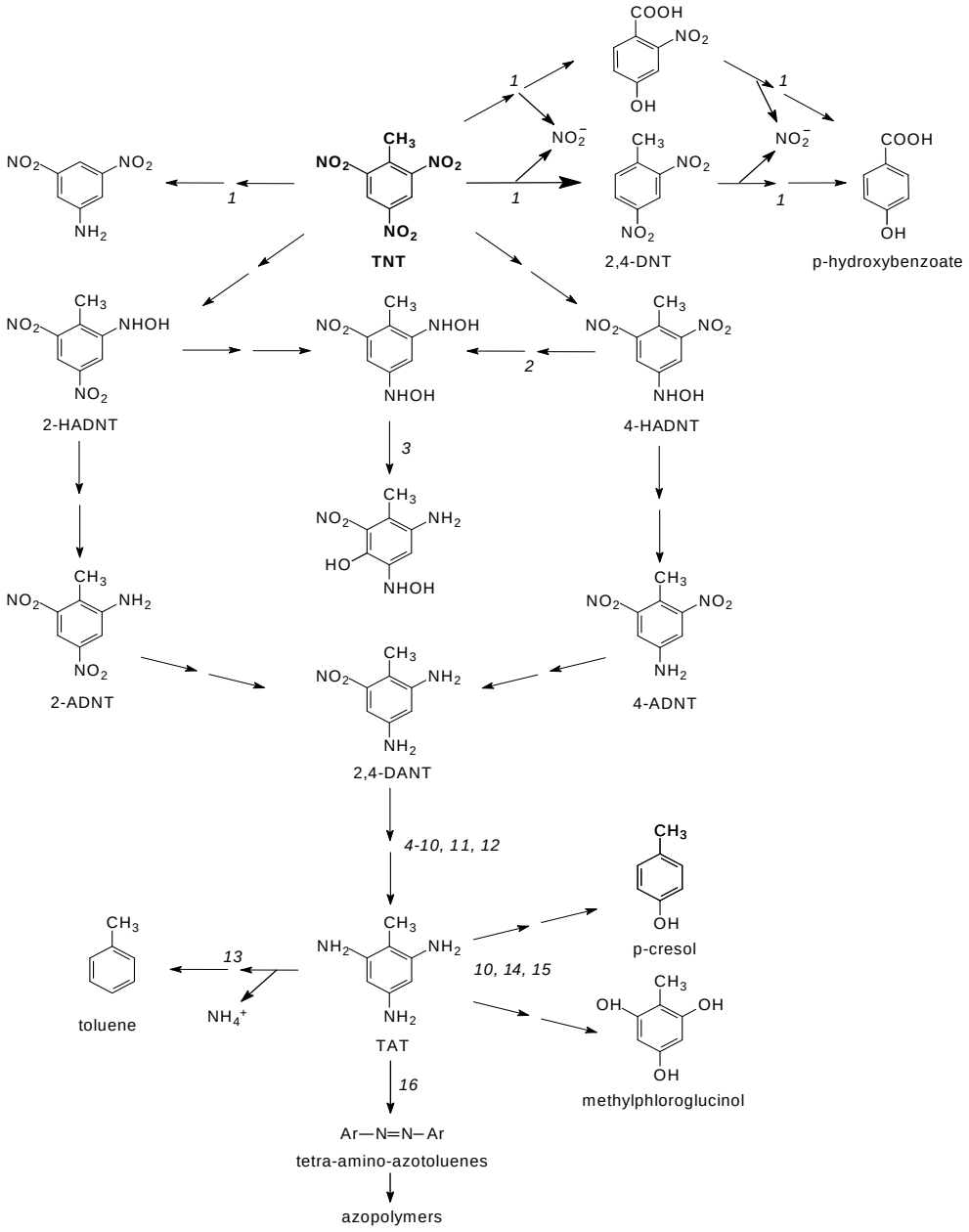

Figure 1.2 Mechanisms for anaerobic metabolism of TNT by bacteria (Adapted from Esteve-Nunez *et al*., 2000)
1, Esteve-Nunez *et al*., 1998; 2, Hughes *et al*., 1998a; 3, Hughes *et al*., 1998b; 4, Khan *et al*., 1997; 5, Drzyzga *et al*., 1998; 6, Ederer *et al*., 1997; 7, Funk *et al*., 1993; 8, Hawari *et al*., 1999; 9, Lewis *et al*., 1997; 10, Lewis *et al*., 1996; 11, McCormick *et al*., 1976; 12, Preuss *et al*., 1993; 13, Boopathy and Kulpa, 1992; 14, Regan and Crawford, 1994; 15, Funk *et al*., 1993; 16, Hawari *et al*., 1998.

Pathways for anaerobic TNT metabolism in bacteria are shown in Figure 1.2. McCormick *et al.* (1976) first described the sequential reduction of the nitrogroups of TNT by a *Veillonella alkalescens* strain. The ultimate fate of TNT through nitro-reductive routes is TAT (Preuss and Rieger, 1995), an unstable, electron rich intermediate. The ability to rapidly reduce TNT to TAT seems widely distributed among *Clostridium* spp. and *Desulfovibrio* spp. (Funk *et al.*, 1993; Ederer *et al.*, 1997; Khan *et al.*, 1997; Boopathy and Kulpa, 1992; Preuss *et al*., 1993). Several reports describe cometabolic TNT transformation by *Clostridium* strains isolated from a long-term bioreactor fed with explosives (Funk *et al*., 1993; Lewis *et al*., 1996; Regan and Crawford, 1994). In these cultures TAT was detected together with *p*-cresol (4-methylphenol) and methylphloroglucinol. However, a recent study by Shen *et al.* (2000) suggested that *p*-cresol was not a metabolite of the anaerobic degradation of TNT, but an intermediate in an amino acid degradation pathway that was shown to be inhibited by TNT and its metabolites. This would explain why *p*-cresol accumulated. Lewis *et al.* (1996) reported that anaerobic treatment of TNT with *Clostridium bifermentans* LJP-1 produced in addition to TAT a small amount of phenolic compounds from TAT hydrolysis. Hughes *et al*. (1998a,b), detected the formation of 2-amino-4-hydroxylamino-5-hydroxyl-6-nitrotoluene and 2-hydroxylamino-4-amino-5-hydroxyl-6-nitrotoluene in cell extracts from *Clostridium* strains. Boopathy *et al.* (1993) found that TNT was used as a sole nitrogen source by the sulphate-reducing *Desulfovibrio* sp. strain B. Toluene was produced after transforming the substrate into TAT. A *Desulfovibrio* strain was isolated by Preuss *et al*. (1993), which could use TNT as the sole nitrogen source and pyruvate as the carbon and energy source. The strain catalysed the reduction of TNT to TAT. However no reductively deamination reaction was found, and no toluene was detected. Hawari *et al.* (1998) suggested two distinctive biotic cycles that where taking place during TNT biodegradation with anaerobic sludge. The first cycle is responsible for the reduction of TNT to TAT, the second leads to conversion of TAT into its azo-derivatives (tetra-amino-azotoluenes) at pH 7.2, which further react to produce azo-polymers. In the presence of oxygen and/or

under acid conditions (pH ≤ 3), TAT undergoes slow hydrolyses to the corresponding phenolics (hydroxyldiaminotoluenes, dihydroxylaminotoluenes, trihydroxyltoluenes). Eventually these phenolic products disappeared to produce unknown polymers. None of the transformation cycles explained by Hawari *et al.* (1998) were accompanied by mineralisation. As TAT is an electron-rich compound, it should be readily oxidised by aerobic microorganisms. But in the presence of O_2, particularly when heavy metal ions (e.g. Mn^{2+}, Ni^{2+}) are present, TAT undergoes rapid auto-oxidation and polymerisation to generate dark polymers. These abiotic reactions prevent productive metabolism of TAT by aerobic microorganisms.

Recently, under anoxic conditions with glucose as cosubstrate and TNT as the sole nitrogen source, *Pseudomonas* sp. JLR11 showed some pathway intermediates in the productive removal of nitro groups from TNT (Esteve-Nunez and Ramos, 1998; Esteve-Nunez *et al.*, 2000). Mass balances revealed that about 85% of the total N-TNT content was incorporated as cell biomass. The productive initial attack on TNT seemed to involve nitrite release, since nitrite accumulated transitory with TNT consumption. Respiration by *Pseudomonas* sp. strain JLR11 can be potentially useful for the biotreatment of TNT in polluted water and soils, particularly in phytorhizodegradation, in which bacterial cells are transported to the deepest root zones, which are poor in oxygen.

Since degradation of TAT by individual bacterial strains is slow and unproductive, the elimination of TNT and its metabolites via anaerobic pathways to yield harmless products, preferably CO_2, H_2O and NH_4^+, cannot yet be efficiently used for bioremediation of polluted sites.

1.2.3 Metabolism by Plants

For the remediation of explosives, phytoremediation is still in its early stage of development. The ability of plants to take up and transform TNT has been shown in several studies: under hydroponic conditions (Palazzo & Leggett, 1986; Harvey

et al., 1990; Görge *et al.*, 1994; Schneider *et al.*, 1996; Larson, 1997; Best *et al.*, 1997; Hughes *et al.*, 1997; Vanderford *et al.*, 1997; Rivera *et al.*, 1998; Pavlostathis *et al.*, 1998), and after addition of TNT to soil (Scheidemann *et al.*, 1998; Thompson *et al.*, 1998a).

To differentiate between the contributions of plants and microorganisms during TNT disappearance, axenic plant tissues were used. Hughes *et al.* (1997) provided mass balances that confirmed the ability of plants to take up and transform TNT. Different plant systems (*Catharanthus roseus* hairy root cultures, axenic *Myriophyllum* plants, and native *Myriophyllum*) were exposed to uniformly labeled ^{14}C-TNT, and the fate of ^{14}C was evaluated. TNT was completely transformed in all systems containing viable plant tissue and mineralisation did not occur. Products of metabolism were characterised as aminodinitrotoluenes, unidentified ^{14}C-labeled soluble products, unidentified extractable plant-associated ^{14}C products, and bound residues. Later studies revealed that the bound fraction increased with time, with a commensurate decrease in levels of extractable plant-associated metabolites (Vanderford *et al.*, 1997).

The following sections provide the current state of understanding of the metabolism of TNT by plants. The sections are organised as stages in the green liver model presented previously. The proposed pathways of TNT metabolism in plants are shown in Figure 1.3.

1.2.3.1 Transformation

Based on the structure of TNT, two initial transformation processes are possible. First is the reduction of one or more nitro groups, yielding hydroxylamino and amino groups. Second is oxidation of either the methyl group or the ring directly.

1.2.3.1.1 Reduction Reactions

TNT transformation by plants has been accompanied by the appearance of low levels of 2-ADNT and 4-ADNT. Diaminonitrotoluenes are seldom detected,

although 2,4-DANT has been observed in hybrid poplars (Thompson *et al*., 1998a). The transformation of TNT into TAT by plants has only been proposed (Rivera *et al.*, 1998). However, it is doubtful that TAT would be produced and accumulates in plant cells. As discussed by Rieger and Knackmuss (1995), TAT formation requires a strong reducing potential ($E_h \leq$ -200 mV), which is not present in plant cells. Two studies have reported the formation of azoxy compounds in the aquatic species *Myriophyllum* (Bhadra *et al*., 1999a; Pavlostathis *et al*., 1998).

1.2.3.1.2 Oxidation Reactions

In contrast to the reductive pathway, the activity of oxidative pathway(s) as part of the plant's degradation route for TNT remains largely to be investigated. Oxidative metabolism of TNT has only been reported by Bhadra *et al.* (1999a). Six metabolites were isolated and characterised in an aquatic phytoremediation system of *Myriophyllum aquaticum.* The compounds identified included 2-amino-4,6-dinitrobenzoic acid, 2,4-dinitro-6-hydroxybenzyl alcohol, 2-N-acetoxyamino-4,6-dinitrobenzaldehyde, and 2,4-dinitro-6-hydroxytoluene. The detection of these compounds provides clear evidence of initial oxidative transformation of TNT by plants via oxidation of the methyl group or through aromatic hydroxylation. Oxidation of the methyl group of TNT can also result from light-catalysed reactions (Gorontzy *et al*., 1994). Care was taken in the plant studies cited above to eliminate photooxidation. The oxidation products appear to follow formation of the hydroxylamino compounds. When *M. aquaticum* was exposed to either 2-ADNT or 4-ADNT, none of the oxidation products was detected. In addition, the presence of trinitrobenzene (TNB) in a cell-free extract prepared from a suspension culture of *Populus simonii* was determined after 3 hours of incubation at 28°C with TNT (Nepovím *et al.*, 1999; Vanek and Schwitzguébel, 2000). Again, care was taken that the oxidation of the methyl group was not caused by light.

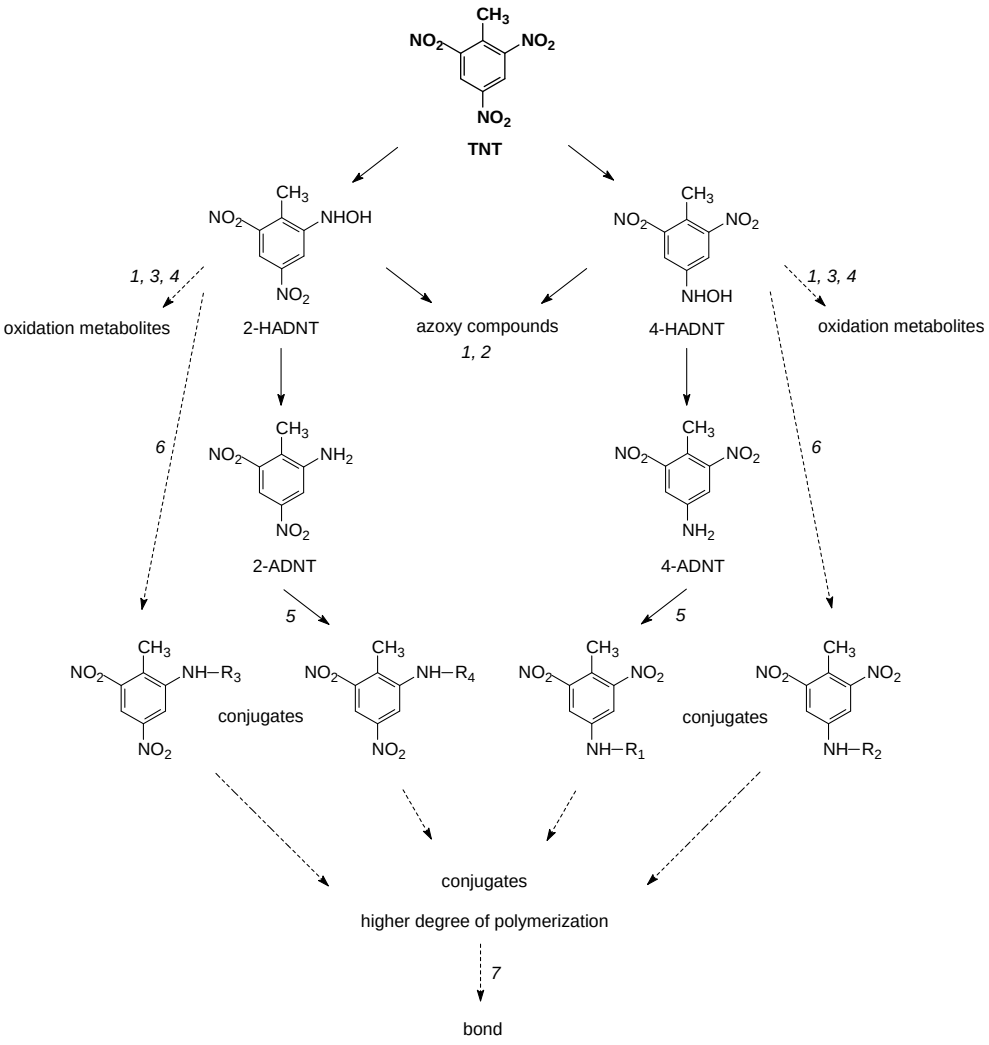

Figure 1.3 Proposed pathways of TNT metabolism in plants (Burken *et al*., 2000). R_1, R_2, R_3 and R_4 are six carbon sugars
1, Bhadra *et al*., 1999a; 2, Pavlostathis *et al*., 1998; 3, Nepovím *et al*., 1999; 4, Vanek and Schwitzguébel, 2000; 5, Bhadra *et al*., 1999b; 6, Wayment *et al*., 1999; 7, Sens *et al*., 1999.

1.2.3.2 Conjugation

Harvey *et al*. (1991) first suggested that acid hydrolysable conjugates could be formed during the metabolism of TNT in plants. Since that report, several TNT-derived conjugates have been isolated (Bhadra *et al*., 1999b). Four TNT-derived conjugates were isolated from *Catharanthus roseus* hairy root cultures. Two have UV-Visible spectroscopic characteristics similar to 2-ADNT, the others have UV-Visible spectroscopic characteristics similar to that of 4-ADNT. From the mass spectral evidence, it is likely that at least a six-carbon unit from the plant intracellular milieu was involved in conjugate formation. A study by Wayment *et al*. (1999) suggested that hydroxylamino intermediates were involved in conjugation processes. When hairy roots were exposed to either 2-ADNT or 4-ADNT, only one identifiable conjugate was detected in addition to several unique unidentified conjugates.

1.2.3.3 Sequestration

In some cases, sequestration has only been characterised as a decrease in extractability of conjugates over time (Vanderford, 1997). However, Sens *et al*. (1999) examined the incorporation of ^{14}C from ^{14}C-TNT into different cell wall fractions of wheat and beans. Most of the ^{14}C in the cell wall was covalently bound in the lignin fraction and not in the pectin, cellulose, or protein fractions.

Generally, bound residues are considered to be non-toxic or lesser toxic than parent compounds or intermediates. However, little is known about the toxicity of these plant tissues.

1.2.3.4 Enzymes Involved

There is increasing evidence that plant roots release a number of degradative enzymes into the soil and sediments. Schnoor *et al*. (1995) demonstrated that nitroreductase and laccase enzymes catalyse the degradation of TNT and TAT respectively. Nitroreductase is capable of sequentially reducing TNT to TAT. TAT

is an unstable molecule that autooxidises readily in aerobic conditions, resulting in dark polymers that prevent efficient metabolisation. However, many plants contain the enzyme laccase, which catalyses ringcleavage. Subsequently the broken ring structures are incorporated into new plant material or organic detritus that becomes a part of sediment organic matter. An assay for the plant enzyme nitroreductase has been developed. Levels of this enzyme in plant species appear to correlate with the ability to degrade explosives (Medina and McCutcheon, 1996). More recently, transgenic plants that express microbial degradative enzymes for TNT bioremediation have been developed (French *et al*., 1999). Transgenic tabacco plants that carry the *onr* gene, which encodes the pentaerythritol tetranitrate reductase from *Enterobacter cloacae* (French and Hedge, 1995; French *et al*., 1998), showed detectable expression of the reductase enzyme in leaf and root tissue. Transgenic plants expressing nitroreductase show a striking increase in ability to tolerate, take up, and detoxify TNT as compared to wild-type plants. Work performed by Hannink *et al.* (2001) suggests that expression of nitroreductase in plants suitable for phytoremediation could facilitate the effective cleanup of sites contaminated with high levels of explosives.

However, still little is known about the contribution of plant-associated enzymes in the degradation of nitroaromatic compounds. This subject requires further detailed examination.

1.2.3.5 Rhizodegradation

Plant-bacterium combinations to remediate TNT contaminated soil are being developed with a *Pseudomonas* strain, which is able to transform TNT to aminodinitrotoluenes and diaminonitrotoluenes. Inoculation of meadow bromegrass (*Bromus erectus*) with this strain led to a reduction in soil TNT levels (Siciliano *et al*., 2000).

Table 1.4 Representative aquatic and terrestrial species tested in laboratory studies with TNT (Burken *et al*., 2000)

Wetland Species	Reference
Myriophyllum aquaticum (Parrot feather)	Best *et al*. 1997; Hughes *et al*., 1997; Vanderford *et al*., 1997; Rivera *et al*., 1998; Bhadra *et al*., 1999a
Myriophyllum spicaticum (Eurasian water milfoil)	Best *et al*. 1997; Hughes *et al*., 1997; Pavlostathis *et al*., 1998
Scriptus validus (Bulrush)	Rivera *et al*., 1998
Cannaceae sp. (Canna)	Rivera *et al*., 1998
Elodea canadensis (Elodea)	Rivera *et al*., 1998
Salviniaceae sp. (Fern)	Rivera *et al*., 1998
Bamusa pynaea (Bamboo)	Rivera *et al*., 1998
Sagittaria (Arrowhead)	Best *et al*. 1997; Rivera *et al*., 1998
Egeria densa (Egeria)	Best *et al*. 1997
Vallisneria americana (Vallisineria)	Best *et al*. 1997
Potamogeton crispus (Curlyleaf pondweed)	Best *et al*. 1997
P. pectinatus (Sago pondweed)	Best *et al*. 1997
P. nodosus (American pondweed)	Best *et al*. 1997
Heteranthera dubia (Water star-grass)	Best *et al*. 1997
Eleocharis parvula (Dwarf spikerush)	Best *et al*. 1997
E. obtusa (Blunt spikerush)	Best *et al*. 1997
Ceratophyllum demersum (Coontail)	Best *et al*. 1997
Alisma subcodatum (Water-plantain)	Best *et al*. 1997
Carex vulpinoidea (Fox sedge)	Best *et al*. 1997
Scirpus cyperinus (Wool-grass)	Best *et al*. 1997
Phalaris arundinacea (Reed canary grass)	Best *et al*. 1997
Typha angustifolia (Narrowleaf cat-tail)	Best *et al*. 1997
Nitella (Stonewort)	Wolfe *et al*., 1994
Terrestrial Species	**Reference**
Phaseolus vulgaris (Bush bean)	Harvey *et al*., 1991; Scheidemann *et al*., 1998
Medicago sativa (Alfalfa)	Görge *et al*., 1994; Scheidemann *et al*., 1998
Allium schoenoprasum (Allium)	Görge *et al*., 1994
Populus sp. *deltoides* × *nigra* (Hybrid poplar)	Thompson *et al*., 1998a, 1998b
Catharanthus roseus (Periwinkle)	Hughes *et al*., 1997; Bhadra *et al*., 1999b
Lupinus angusifolius	Scheidemann *et al*., 1998
Trifolium repens (Clover)	Scheidemann *et al*., 1998
Phacelia seicea	Scheidemann *et al*., 1998
Triticum aestivum (Wheat)	Scheidemann *et al*., 1998; Sens *et al*., 1999
Aleopecurus pratensis	Scheidemann *et al*., 1998
Bromus inermis	Scheidemann *et al*., 1998
Festuca rubra (Red fescue)	Scheidemann *et al*., 1998
Lolium perenne	Scheidemann *et al*., 1998
Phleum sp.	Scheidemann *et al*., 1998

1.2.3.6 Other Considerations

The ability to remove TNT seems to be ubiquitous across a wide range of aquatic and terrestrial species. Representative species are listed in Table 1.4.

Disappearance of TNT is known to be dependent on plant biomass concentration (Pavlostathis *et al.*, 1998). Under conditions of low TNT/plant biomass ratios, a pseudo-first order assumption is appropriate to characterise disappearance of TNT in the medium. Under the proposed conditions, TNT will disappear to below detection limits. At high initial concentrations, TNT remains in the medium, indicating interference with plant activity (Burken *et al.*, 2000). Lower TNT disappearance rates were observed in plants that experience light deprivation. Furthermore, addition of azide, a metabolic inhibitor, to a reactor containing *Myriophyllum aquaticum* plants, did not result in removal of TNT from the solution. Again these findings link TNT removal to plant metabolism, which seems to be an active plant process (Pavlostathis *et al.*, 1998).

Studies on terrestrial plants revealed that 2-ADNT and 4-ADNT along with low concentrations of TNT accumulate in root tissues and were translocated to a lesser extent into stems and leaves. Root concentrations were up to 20 and 30 times higher than shoot concentrations (Görge *et al.*, 1994). From predictive relationships developed by Briggs *et al.* (1982) and by Burken and Schnoor (1998), TNT should be more mobile and reach the stem and leaves. The lack of mobility and the inability to isolate TNT from root tissues indicate that TNT is rapidly transformed in the root tissues.

1.3 INTERACTION OF TNT AND ITS METABOLITES WITH SOIL

TNT and its metabolites interact with soil components by different mechanisms. The chemical structure of the sorbate, the nature of the sorbing agents, and the environmental or incubation conditions determine whether physical or van der Waals interactions, hydrogen boundings, or ionic or covalent bounds are the

predominant sorption mechanisms. Clay minerals (montmorillonite) and soil organic matter (humic acids) are known as the major sorbents in soil. Adsorption of TNT, 2-ADNT, and 4-ADNT to montmorillonite is fully reversible, whereas the adsorption of 2,4-DANT is only partially reversible. These observations indicate that the amino groups bind to the polyanion surfaces of the clay minerals (Daun *et al*., 1998). The interaction of 2-HADNT, 4-HADNT and TAT is more important. A strong interaction of hydroxylaminodinitrotoluenes became obvious when TNT was cometabolically reduced during fed batch fermentation of glucose in the presence of montmorillonite (Daun *et al*., 1998). Sorption of TAT to montmorillonite is very fast and irreversible. In natural soil diverse binding mechanisms of TAT exist. These range from ionic interaction of the TAT monomer with the polyanion structures of clay minerals and humic substances (interlamellar adsorption) up to very complex O_2-dependant reactions that give rise to the formation of polymers (humification). This latter process in particular is highly irreversible (Hundal *et al.*, 1997; Daun *et al.*, 1998; Drzyzga *et al.*, 1998; Achtnich *et al.*, 1999). This irreversible binding does not require the participation of oxygen, but the process becomes considerably enhanced under aerobic conditions.

The interaction of TNT and its metabolites with the soil organic matter due to covalent binding of amino groups to humic substances seems to be more important than sorption to clay minerals. TAT became irreversibly bound to humic acids much faster than TNT, 4-ADNT, and 2,4-DANT (Daun *et al*., 1998). Though TNT and 2,6-DANT bind slowly with humic acids (Li *et al*., 1997), 2-HADNT and 4-HADNT interact rapidly with these soil compounds (Daun *et al*., 1998). Sorption of 2,4-DANT and TAT occurs through binding of the amino groups, but the mechanism of interaction of hydroxylaminodinitrotoluenes with humic acids is unclear. Cometabolic reduction of TNT in the presence of humic acids leads to a complete disappearance of TNT and its metabolites. Because hydroxylaminodinitrotoluenes are often initial TNT metabolites in various

microbial systems under anaerobic and aerobic conditions, they appear to play a crucial role in binding processes (Lenke *et al*., 2000).

In addition to cometabolic reductions, abiotic reductions of the nitrogroups can occur in sediments, soils, and aquifers (Haderlein and Schwarzenbach, 1995; Haderlein *et al*., 2000). Numerous potential electron donors are present in natural systems (e.g. reduced iron species, reduced sulphur species, and natural organic matter).

On one hand, binding reduces the availability of TNT and its metabolites for microbial degradation and mineralisation, but on the other hand, these processes open new possibilities of soil remediation through cometabolically induced immobilisation. Several biological treatment systems (compost systems, slurry systems) have been developed, differing in the duration and intensity of the anaerobic and/or aerobic incubation, in the amount and type of external carbon sources added, and the degree of water saturation (Isbister *et al*., 1984; Kaplan and Kaplan, 1982; Pennington *et al*., 1994; Roberts *et al*., 1996; Lenke *et al*., 1998). However, further studies are necessary to assess the sustainability of the immobilisation.

1.4 TOXICITY OF TNT AND ITS METABOLITES

As mentioned before, the reduction of nitro groups on aromatic rings is widely distributed among living organisms. The nitroso and hydroxylamino groups, responsible for the toxicity of nitroaromatic compounds, react with biological molecules and cause mutagenesis and carcinogenesis (Banerjee *et al*., 1999; Berthe-Corti *et al*., 1998; Brooks *et al*., 1997; Gong *et al*., 1999; Honeycutt *et al*., 1996). Complete reduction of the nitro group to an amino group seems to decrease the mutagenic effect of the compound (Cash, 1998; George *et al*., 2001; Tan *et al*., 1992). The toxicity and mutagenicity of TNT and its intermediates to animals and especially mammals (including humans) is of major concern for the acceptance of

any treatment technology. The basic question: “Are the degradation products less toxic than the parental contaminant?” has still not been completely answered, this due of the limited data available on the toxicity of the many TNT derived degradation products. TNT and its best studied degradation products HADNTs, ADNTs, and DANTs were evaluated with the Ames test: the results showed the mutagenic properties of TNT and ADNTs (Tan *et al.*, 1992; Spanggord *et al.*, 1982). Azoxytetranitrotoluenes caused a higher rate of mutations than does TNT in a *Salmonella* microsuspension bioassay (George *et al.*, 2001). Cytotoxicity was evaluated using the human lymphoblastic cell lines V79 and TK6. For both cell lines, trinitrobenzene (TNB) and TAT were more toxic than TNT. On the contrary, the primary TNT metabolites (ADNTs and DANTs) were less cytotoxic than TNT. TNB, TNT and most of the primary TNT metabolites were also found to be mutagenic in the *Salmonella* fluctuation test with the exception of 4-ADNT and 2,4-DANT. On the other hand none of the tested compounds were mutagenic for the V79 mammalian cells with or without S9 metabolic activation (Lachance *et al*., 1999).

Toxicity of TNT to plants has been evaluated as well. The level of phytotoxicity depends on several factors, including plant species, stage of growth, and environmental factors. TNT was found to be toxic within the range of 1 to 30 mg/L in hydroponic systems (Palazzo and Leggett, 1986; Pavlostathis *et al*., 1998; Thompson *et al*., 1998b; Peterson *et al*., 1998). Plants grown in soil have a higher threshold to TNT levels. *Datura innoxia* and *Lycopersicon peruvianum* were able to grow in soils contaminated with 750 mg TNT/kg. *Phaseolus vulgaris* tolerated soil TNT concentrations up to 500 mg/kg (Scheidemann *et al*., 1998). The reason that toxicity is lower in soil systems than in hydroponic systems is most likely a decrease in the bioavailability of TNT in soil, which subsequently limits exposure of the plants to TNT (Burken *et al*., 2000).

1.5 TREATMENT TECHNOLOGIES FOR TNT CONTAMINATED SOIL AND WATER

1.5.1 Solid Phase Treatment Systems

1.5.1.1 Composting

To start the composting process, degradable organic matter (such as manure, straw, wood chips, alfalfa and vegetable wastes) is mixed with maximum 30% contaminated soil and an appropriate amount of moisture. The process utilises native aerobic mesophilic and thermophilic microorganisms to break down organic substances. Self-heating is caused by a mass proliferation of the microorganisms. Finally, composting results in products stable for storage or application to land without adverse environmental effects (Haug, 1993). Aeration of the system is necessary because composting is primarily an aerobic process. Both windrow composting (Williams *et al*., 1992; Funk *et al.*, 1996) and anaerobic/aerobic composting (Breitung *et al.*, 1996; Bruns-Nagel *et al*., 1997; Bruns-Nagel *et al*.; 1998) seem to be highly effective to bioremediate soil contaminated with the explosives TNT, RDX, and HMX.

Williams *et al.* (1992) studied composting as a method for removing explosives from contaminated soil and concluded that TNT, RDX and HMX can be removed effectively. For the three explosives, the relative rates of biotransformation were TNT > RDX >> HMX (Griest *et al.*, 1993). Laboratory studies indicate that while significant portions of RDX and HMX were mineralised during composting (Doyle and Isbister, 1982), TNT was not mineralised, but biotransformed and incorporated into humic substances (Kaplan and Kaplan, 1982; Griest *et al.*, 1993; Pennington *et al.*, 1995). Bruns-Nagel *et al.* (1997) tested different composting systems for the bioremediation of TNT-contaminated soil. A two-step (anaerobic/aerobic) composting system with low amounts of co-substrate was found to be most effective for bioremediation of soil containing more than 1,000 mg TNT/ kg dry soil. However, low levels of explosives and metabolites, bacterial mutagenicity and

toxicity, and leachable toxicity remained.

Although such systems can reduce the explosive content in soils and sediments, no information is available on the organisms or mechanisms involved. Particular concern has been expressed about composting, which does not necessarily degrade contaminants completely and may leave products whose ultimate fate is unknown.

1.5.1.2 Soil Pile – Two Stage

A two-stage soil (TOSS) process was developed by Waste Management, Inc. (WMI), Cincinnati, OH. The TOSS process was designed to degrade and immobilise explosive compounds through a sequence of reducing (anaerobic) and oxidising (aerobic) treatment stages (Green *et al.*, 1998). A pilot-scale demonstration was exhibited on soil contaminated with TNT, RDX and HMX (Green, 1999). The process was able to degrade TNT, RDX, and HMX to levels which would allow the reuse of the remediated soil as landfill cover. The combined concentration of TNT and amino intermediates was reduced by more than 99,5%. RDX was not appreciably degraded until the combined concentration of TNT and its reduction intermediates was less than the concentration of RDX.

1.5.1.3 Soil Pile – Fungal-Based Remediation

Fungal-based soil remediation is based on the pollutant-degrading abilities of wood decay fungi and consists of amending the contaminated soil with a fungal inoculum. The inoculum consists of a pure culture of the selected fungus grown on a lignocellulosic substrate (e.g. cottonseed hulls, sawdust, straw). Aeration of the pile controls the temperature via removal of metabolic heat and ensures an adequate supply of oxygen to the fungus. EarthFax Engineering, Logan, VT conducted a treatability study on TNT contaminated soil to evaluate the comparative abilities of several different species of white rot fungi (e.g. *Trametes versicolor, Phanerochaete chrysosporium*) to decrease the TNT concentrations. All the fungi were able to cause rapid and extensive reduction of TNT in the soils.

However, there were significant differences among the organisms used in rates and extents of treatment (Jerger and Woodhull, 2000).

1.5.2 Reactor Treatment Systems

Reactors are filled with a mixture of soil and water to which cosubstrates (e.g. starch, glucose, sucrose, or molasses) and nutrients can be added as necessary. These systems are designed to optimise mass transfer of nutrients and electron acceptors by using mechanical mixing and/or aeration. Cosubstrates play two main roles in anoxic processes: oxygen removal by growing aerobes, and electron donation for nitro group reduction of TNT. Slurry reactors can target mineralisation of the explosive or irreversible binding of TNT metabolites to the soil matrix.

1.5.2.1 Anaerobic Slurry Reactors

Funk *et al.* (1993, 1995) demonstrated the efficiency of an anaerobic treatment system for the bioremediation of explosive contaminated soil (primarily TNT) in open bulk containers of soil, phosphate buffer and potato starch. The potato starch served as a readily degradable carbon source, which allowed the rapid establishment of anaerobiosis. Although mass balances and biochemical mechanisms of conversion of TNT are largely lacking, to date, this is the only commercially available system specifically developed for biologically remediating nitroaromatic contaminated soils (SABRE™, developed, patented and licensed by the University of Idaho). Strains of the genus *Clostridium*, nitroaromatic-degrading anaerobes, are expected to play a major role in this process. Spores of these strains can be useful as inoculants for bioremediation (Sembries and Crawford, 1997).

1.5.2.2 Sequential Anaerobic-Aerobic Slurry Reactors

To eliminate the hydroxytoluenes or aminotoluenes remaining in the anaerobic bioreactors following the disappearance of TNT, second-stage aerobic reactors have been proposed to hasten the removal of those intermediates that are more

rapidly degraded under aerobic conditions. Upon aeration, TAT bound to soil undergoes an oxidative polymerisation, which immobilises the chemical. However, further research is required to demonstrate permanent immobilisation and to investigate whether any residual toxicity remains. Anaerobic treatment of originally contaminated soil was carried out in a laboratory slurry reactor (Lenke *et al.*, 1998). While fermenting glucose to ethanol, acetate, and propionate, the anaerobic bacteria completely reduced TNT to TAT, which led to complete and irreversible binding of the reduced products to the soil. The fermentation products were eliminated in a subsequent aerobic treatment. TNT was not mineralised. This process was tested on a pilot scale (Terranox® reactor) (Knackmuss, 1997; Lenke *et al.*, 1998). Ecotoxicological tests performed with aquatic systems (luminescent bacteria, daphnids, algae) and terrestrial systems (respiring bacteria, nitrifying bacteria, cress plants, earth worms) showed that no residual toxicity could be detected in the remediated soil.

1.5.2.3 Aerobic/Anoxic Slurry Reactors

An aerobic/anoxic soil slurry reactor studied by Boopathy *et al.* (1998) successfully removed TNT, RDX, HMX, and other contaminants present in the soil. Molasses was selected as carbon source. Radio-labelled TNT incubated with reactor biomass showed that after 14 days 23% of ^{14}C-TNT was mineralised, 27% converted to biomass, and 8% adsorbed onto the soil. The rest of the ^{14}C-TNT was accounted for as metabolites, including a ring cleavage product identified as 2,3-butanediol. Improvement to the reactor system by adding a food-grade surfactant (Tween 80) was tested (Boopathy and Manning, 1999). Adding both Tween 80 and molasses greatly enhanced the degradation rate and might be cost effective in large-scale cleanup operations.

1.5.2.4 Fluidized Bed Reactor (FBR)

A pilot-scale field demonstration was conducted to collect reliable cost and

performance data for an aerobic, biological FBR system that treats groundwater contaminated with nitrotoluenes (Spain *et al.*, 1999). The FBR was inoculated with a mixed culture of bacteria that had been acclimated to a mixture of mono- and dinitrotoluenes. The groundwater contaminants were TNT, 2,4-DNT, 2,6-DNT, 2-NT, 3-NT, and 4-NT. A range of loading rates and a variety of operating conditions were used to evaluate the FBR performance. It can be concluded that the FBR process can effectively remove mono- and dinitrotoluenes from contaminated groundwater with an aerobic treatment process. However, the removal of TNT was poor.

1.5.2.5 Fungus-Based Reactor System

Sublette *et al.* (1992) successfully developed a cost effective treatment system for the remediation of TNT in wastewater from ammunition plants; this by the use of *Phanerochaete chrysosporium* immobilised on a rotating biological contactor. More than 90% removal of TNT from simulated waste water, containing 120-150 mg TNT/L, was observed within a residence time of about 24 hours. Bumpus and Tatarko (1994) showed that a 2-liter fixed film silicone membrane bioreactor was able to degrade 30 mg TNT/L to less than 0.06 mg/L within 96 hours. Stahl and Aust (1995) proposed a two stage reactor system. The first stage can be a non-ligninolytic stirred reactor (absence of ligninocellulose nutrient sources such as sawdust and corn cobs) containing large amounts of fungus that would rapidly reduce TNT to amino metabolites. The reduced metabolites would then be completely degraded in a second stage ligninolytic reactor (fixed-film or air-lift designs). However, systems for the remediation of TNT-contaminated water and soil by *P. chrysosporium* are still mainly in their developmental stage.

1.5.3 Phytotreatment Systems

Work on phytoremediation of explosives has dealt almost exclusively with wetlands. A demonstration was initiated to test the feasibility of treating

contaminated groundwater with constructed wetlands (Richman, 1996). Two wetland types were compared, including a lagoon and a gravel-bed system. The lagoons were planted with sago pond weed (*Potamogeton pectinatus*), water stargrass (*Heteranthera dubia*), elodea (*Elodea canadensis*) and parrot feather (*Myriophyllum aquaticum*). The gravel-bed wetlands were planted with canarygrass (*Phalaris arundinacea*), woolgrass (*Scirpus cyperinus*), sweetflag (*Acorus calamus*) and parrot feather. In both the lagoon and gravel-bed wetlands TNT was removed below detection limit. With the gravel-bed wetland RDX and HMX were also removed from the groundwater, whereas no efficient removal of these compounds was observed with the lagoon wetland. Rivera *et al.* (1998) conducted continuous flow phyto-reactor studies. In their studies, up to 100% of the TNT was removed, but mineralisation of the compound was extremely low. The end-point levels of ADNT and DANT in the effluent were attributed to the influent concentration and the hydraulic retention time, but supplementary batch studies confirmed that ADNT and DANT were phytodegraded as well. Preliminary batch studies were also conducted on the degradation of RDX and HMX. These batch studies indicated that removal of RDX was slower than that observed for TNT. A study with HMX indicated that the removal rates were reasonable, but required a lag phase. It is speculated that reduction products of TNT, RDX and HMX decreased also due to biotransformations by microbes (Rivera *et al.*, 1998; Best *et al.*, 1999). In addition, Best *et al.* (1999) demonstrated in aquatic and wetland plant treatments photolysis of TNT. In contrast to TNT, RDX was mineralised to some extent, but photolysis was not observed, indicating that different mechanisms are involved in the degradation of both compounds. In summary of the results, screening of wetland species revealed plants with the capability of removing TNT and RDX from aqueous systems. Plants with the greatest potential for use in wetland systems were the submerged plants coontail (*Ceratophyllum demersum*) and American pondweed (*Potamogeton nodosus*) and the emergent plants common arrowhead (*Arrowhead*), reed canary grass (*Phalaris arundinacea*), and fox sedge (*Carex vulpinoidea*).

Thompson *et al*. (1998a, 1998b, 1999) evaluated the potential use of poplar trees (*Populus* sp. *deltoides* × *nigra*) to remediate TNT and RDX contamination at the Iowa Army Ammunition Plant. From this case, the authors concluded that the potential use of terrestrial vegetation was restricted by competition with soil sorption, and therefore, it would take too long to reach cleanup goals. In this particular case, the use of terrestrial plants appeared to be limited to stabilisation of residual contamination.

1.6 CONCLUSIONS

In order to define optimal remediation strategies for TNT contaminated sites, the metabolic activities of bacteria, fungi and plants should be considered as complementary systems capable of detoxifying or mineralising these xenobiotics. Another important consideration in developing a remediation strategy is the short- and long-term fate and potential toxicity of the metabolic end products.

The optimal remediation strategy for nitroaromatic compounds depends on many site-specific factors. Composting and the use of reactor systems lend themselves to treating soils contaminated with high levels of explosives (e.g. at former ammunition production facilities, where areas with a high contamination level are common). Compared to composting systems, bioreactors have the major advantage of a short treatment time, but the disadvantage of being more labour intensive and more expensive. Phytoremediation, although not widely used now, has the potential to become an important strategy for the remediation of soil and water contaminated with explosives. It is best suited where contaminant levels are low (e.g. at military sites where pollution is rather diffuse) and where larger contaminated surfaces or volumes have to be treated. In addition, phytoremediation can be used as a polishing method after other remediation treatments, such as composting or bioslurry, have taken place. This *in situ* treatment method has the advantage of lower treatment costs, but has the disadvantage of a considerable

longer treatment time. In order to improve the cost-efficiency, phytoremediation of organic xenobiotics could be combined with bio-energy production. This requires, however, detailed knowledge on the fate of the contaminants in the plants as well as the development of efficient treatment methods for the contaminated biomass that minimise the spreading of the contaminants into the environment during post harvest treatment.

CHAPTER 2
AIM AND OUTLINE OF THE THESIS

The world production of nitroaromatic compounds, such as di- and trinitrotoluene, is estimated to be more than 10^4 tons per year. These chemicals have multiple applications, such as raw materials for the synthesis of polyurethane foams, herbicides, insecticides and industrial explosives.

Nitroaromatic compounds are extremely toxic for living organisms. Toxicity not only results from their nature, but is also a consequence of their metabolism. Many organisms are able to reduce the nitro groups to highly reactive nitroso forms, which react with proteins and nucleic acids, leading to the loss of essential functions and mutagenesis. However, a number of microorganisms have been described as being able to deal efficiently with specific nitroaromatic compounds. Some of the nitroaromatic compounds, including picric acid, 2,4-DNT, 2,6-DNT, mononitrotoluenes, dinitrocresol, nitrobenzoates, and nitrophenols, can serve as growth substrates for specialized microorganisms and do not accumulate in the environment, this unless the assimilative capacity of the ecosystem is exceeded. Therefore, bioremediation is a valid alternative when it comes to cleaning soil and groundwater polluted with these compounds. Other nitroaromatic compounds, including the explosives TNT, RDX and HMX, can, due to their electrophilic character, not serve microorganisms as growth substrates in the form of carbon or nitrogen source, and often persist for extended periods in the environment. However, they can be transformed and detoxified by cometabolic processes, but to date their complete biomineralisation has never been observed. Composting, reactor systems and phytoremediation have emerged as promising alternative technologies for remediation of explosives-contaminated soil and water.

A high number of explosives contaminated sites is located in Germany and the United States. Though the extent of contamination in Belgium is either undetermined or not available to the public, the problem is significant. Examples of explosives contaminated brownfields in the Flemish part include PRB in Balen and Kaullile. FN in Zutendaal is a company, which still processes ammunition. Explosive contamination has also been found on military training sites such as in Leopoldsburg and Houthalen-Helchteren, or on military airfields such as in Brogel, Zutendaal and Brustem.

The present study focussed more specifically on the explosive TNT and 2,4-DNT (a precursor of the TNT production), both important pollutants present at the examined brownfield. The major objectives of this project were: (1) to evaluate the bacterial degradation potential present in the indigenous microflora of a former ammunition production site; (2) to examine whether biostimulation or bioaugmentation were valuable alternatives to improve the bioremedation of the TNT contaminated soil; and (3) to design and evaluate an efficient phytoremediation-based strategy for the removal of TNT and its metabolites primarily from contaminated water and to a lesser extent from low contaminated soil.

Chapter 3 describes the successful isolation and characterisation of 2,4-DNT degrading consortia. The genetic/biochemical basis of 2,4-DNT degradation under aerobic conditions was explored, and this resulted in determining the most probable degradation pathways. In addition, their degradation characteristic towards other nitroaromatic compounds, especially TNT, was examined.
In **Chapter 4**, *Helianthus annuus* (sunflower) was evaluated for its potential use in a rhizofiltration process involving TNT. Using this strategy we were able to remove TNT efficiently from hydroponic cultures.

Chapter 5 investigated the fate of TNT in *Helianthus annuus* hydroponic systems using [ring-^{14}C]TNT, in order to obtain an improved understanding of the plant's metabolism. Based on the experimental data, a mass balance was determined. In addition, the sustainability of the plant system to multiple consecutive TNT additions was investigated.

Chapter 6 gives an introduction to the potential use of *Helianthus annuus* in a rhizodegradation process to remediate TNT contaminated soil. Using this concept we observed no positive effect on the NAC concentration in the rhizosphere within the four weeks of cultivation.

In **Chapter 7**, a preliminary slurry test was performed to examine the feasibility of a reactor system for the biological remediation of a highly TNT contaminated soil. The effect of biostimulation and bioaugmentation (by inoculating consortium 3, isolated in Chapter 3) was evaluated. Bioaugmentation emerged as most promising strategy.

Chapter 8 explores the feasibility of a wetland-based system with *Phragmites australis* (common reed) as plant species towards TNT removal from contaminated water. Furthermore, the effect of introducing consortium 3 was evaluated. However, we were confronted with several physical, chemical and biological problems inherent to the system, which caused poor plant growth and poor survival of the inoculated consortium.

Chapter 9 provides a summarising discussion and perspectives.

CHAPTER 3
ISOLATION AND CHARACTERISATION OF MICROBIAL CONSORTIA ABLE TO DEGRADE 2,4-DNT BY INTERSPECIES METABOLISM

Part of this work is submitted for publication

3.1 INTRODUCTION

As a precursor of toluene diisocyanate, 2,4-dinitrotoluene (2,4-DNT) is an important industrial intermediate in the production of polyurethane foams. Furthermore, dinitrotoluenes, but in particular 2,4-DNT, are precursors of the explosive 2,4,6-trinitrotoluene (TNT). In the past, waste streams resulting from these industrial processes were released in the environment. Because of its toxicity and carcinogenic properties, the U.S. EPA lists 2,4-DNT as a priority pollutant (Keith and Telliard, 1979). Therefore, its removal is of significant environmental concern.

Burkholderia sp. strain DNT was the first 2,4-DNT mineralising strain isolated and has been extensively studied at the biochemical and genetic level regarding aerobic 2,4-DNT mineralisation (Spanggord *et al*., 1991). Up to now, all other isolates that grow on 2,4-DNT use the same degradation pathway as strain DNT (Nishino *et al*., 2000). The pathway starts with the di-oxygenation of 2,4-DNT, which results in the formation of 4-methyl-5-nitrocatechol (MNC) and the release of nitrite (Figure 3.1). Then, mono-oxygenation of MNC yields the second nitrite group and 2-hydroxy-5-methylquinone, which is subsequently reduced to 2,4,5-

trihydroxytoluene prior to ring cleavage (Suen and Spain, 1993). The *dntA, -B,* and *-D* genes, encoding DNT di-oxygenase, MNC mono-oxygenase, and THT oxygenase respectively, have been cloned and characterised (Suen *et al.*, 1996; Haigler *et al.*, 1996; Haigler *et al.*, 1999).

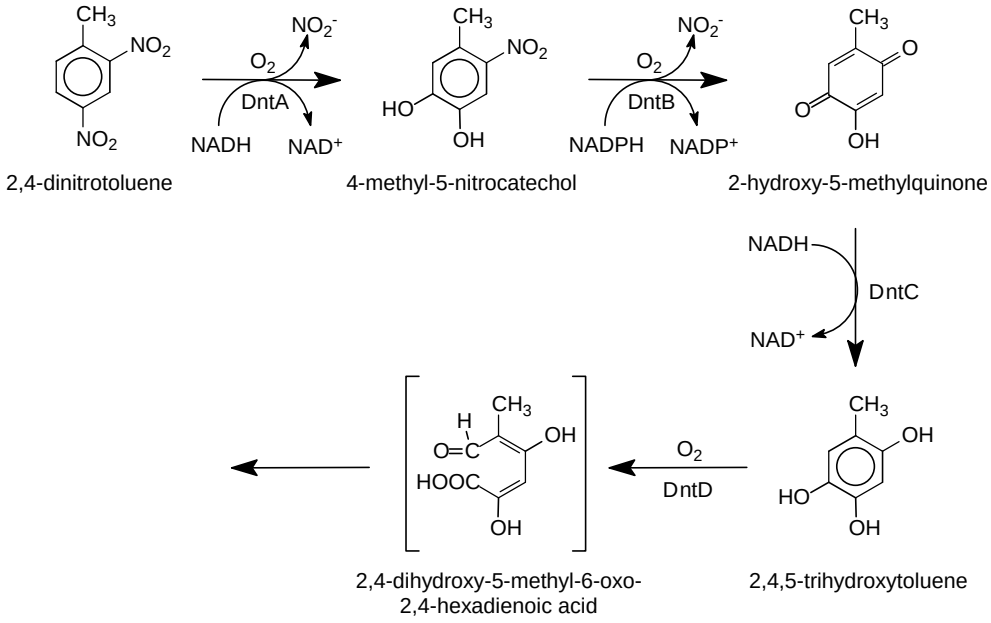

Figure 3.1 Proposed pathway for the degradation of 2,4-DNT by *Burkholderia* sp. strain DNT. DntA, 2,4-DNT dioxygenase; DntB, MNC monooxygenase; DntC, HMQ reductase; DntD, TNT oxygenase (based on Suen and Spain, 1993).

The initial objective of this research was the isolation, characterisation and identification of bacterial strains, able to aerobically mineralise nitroaromatic compounds (NACs) including TNT, 2,4-DNT, 2,6-DNT, 2-NT and 4-NT. Soil and water samples from a former ammunition production site were used as source for NAC degraders in selective enrichment studies. Attempts to isolate strains were only successful when 2,4-DNT was added as nitrogen source. In contrast with other studies, which isolated single strains that grow on 2,4-DNT, 2,4-DNT degrading consortia were isolated. As such, the present study was directed to explore how a

microbial community can function as an integrated unit in the degradation of this compound. In addition, degradation characteristics of the consortia towards other NACs, especially TNT, was examined.

3.2 MATERIALS AND METHODS

Chemicals. 2,4-Dinitrotoluene (2,4-DNT), 2,6-dinitrotoluene (2,6-DNT), 2-nitrotoluene (2-NT), 4-nitrotoluene (4-NT), 2-amino-4-nitrotoluene (2-A-4-NT), 2-amino-6-nitrotoluene (2-A-6-NT), 4-amino-2-nitrotoluene (4-A-2-NT) were purchased from Sigma-Aldrich (Germany), 2-amino-4,6-dinitrotoluene (2-ADNT) and 4-amino-2,6-dinitrotoluene (4-ADNT) from Supelco (PA). The purity of the chemicals was 95% to 99%. 4-Methyl-5-nitrocatechol (MNC) and 2,4,5-trihydroxytoluene (THT) were kindly provided by S. F. Nishino, AFRL/MLQR, Tyndall AFB, FL. The THT sample contained 2-hydroxy-5-methylquinone (HMQ) due to spontaneous conversion of THT to the quinone. 2,4,6-Trinitrotoluene (TNT) was kindly provided by J. C. Libouton, Nobel Explosifs, Centre de Recherches, Châtelet, Belgium.

Culture media. The composition of the minimal medium, used to isolate nitroaromatic-degrading microorganisms, was described previously (Ramos *et al*., 1996). However, the phosphate/NaCl stock solution (10 × concentrated, pH 6.8) was slightly modified: $Na_2HPO_4 \bullet 2H_2O$, 40 g/L; KH_2PO_4, 30 g/L; NaCl, 5 g/L. When 2,4-DNT was added as the sole source of nitrogen, different carbon sources were added simultaneous, i.e. fructose, gluconate, glucose, lactate, and succinate (3 mM of each). A selective minimal medium contained 275 µM (50 mg/L) 2,4-DNT either as a source of nitrogen and/or carbon. Rich medium 869 (pH 7.0) was prepared as described previously (Mergeay *et al*., 1985). Solid media were prepared with 15 g Difco agar per litre.

Enrichment of 2,4-DNT degrading cultures. Soil and water samples from an ammunition brownfield were inoculated separately into selective medium with 2,4-

DNT as nitrogen source. A first enrichment experiment occurred with freshly collected samples, a second enrichment experiment with a soil sample that had been stored at –80°C. Storage at –80°C occurred in a solution of glycerol (15%) and NaCl (0.85%) in a ratio of 1.5 ml solution per g soil. After centrifuging and removal of the supernatant, the sample was used as inoculum for enrichment. Nitroaromatic contents of the samples used as inoculum are shown in Table 3.1. One gram soil samples or 10 ml water samples were used as inoculum into 100 ml selective minimal medium in which 2,4-DNT was present as the sole source of nitrogen. Incubation occurred at 28°C while shaking at 150 rpm. Cultures were monitored for growth, and transferred to fresh medium at appropriate intervals ($OD_{660} \geq 0.25$). Initially, the dilution-factor was 10, but was gradually increased to 50.

Isolation and identification of bacteria. Individual strains were isolated on morphological basis from the adapted consortia by plating on solid media, including 1/10 869 and selective medium containing 2,4-DNT as the sole nitrogen source. After purification of the isolates, REP-PCR was performed as described by De Bruyn (1992) to identify similar strains, using the Goldstar Red DNA Polymerase kit (Eurogentec, Belgium). Strains were characterised by standard methods for phenotypic characterisation (Basic bacteriological tests, FAME-analysis, Biolog GN, API20NE) and by 16S rDNA gene sequence analysis on genomic DNA. 16S rDNA gene sequences were determined using the Thermo Sequenase Fluorescent Labelled Primer Cycle Sequencing kit (Amersham Pharmacia, UK). The obtained gene sequences were compared with published sequences from the GenBank database (http://www.ncbi.nlm.nih.gov/blast).

Reconstitution and combinations of consortium constituents. A reconstituted consortium was obtained by adding 0.5 ml of pure cultures of the consortium constituents into 100 ml selective medium. The pure cultures were obtained after overnight growth on 869 liquid medium, washed twice, and brought to an optical density of 1.0 in $MgSO_4$ (10 mM). A reconstituted consortium was transferred twice (1/50 dilution) to fresh selective medium before use in an experiment.

Furthermore, for both consortia, all possible permutations of two and more consortium members were made.

Enzyme activity. Microplate assays for the detection of DNT dioxygenase, and MNC monooxygenase, based on concomitant colour changes in the medium were performed as described by Suen and Spain (1993). THT oxygenase activity was detected by suspending cells in microplate wells containing 100 µl phosphate buffer (pH 6.8) with 100 µM 4-methylcatechol, a structural analog for THT (Suen and Spain, 1993). The conversion of the medium from colourless to yellow was indicative for THT oxygenase activity. This alternative was chosen, as dissolving THT from the received sample already gave a pink coloration to the buffer, indicating oxidation from THT to HMQ. Nitrite release was measured as described by Daniels *et al*. (1994), when DNT dioxygenase and MNC monooxygenase were expressed.

High Performance Liquid Chromatography (HPLC). Soil samples and water samples were extracted and prepared for analyses as described by EPA method 8330A (1998). To follow nitroaromatic compound degradation in liquid cultures, 500 µL liquid culture samples were supplemented with 500 µL methanol, mixed thoroughly, and filtered through a 0.45 µm PTFE filter. HPLC analyses were performed on an Alltima C_{18} reverse-phase column, 250 mm × 4.6 mm, 5 µm particle size (Alltech, IL). The mobile phase was 55:45 methanol-water and detection occurred at 254 nm with an UV-VIS detector (Model L-4250, Hitachi, Japan). The flow rate was 1 ml/min and the injections loop volumes 20 µL. Products were identified by comparison with authentic standards. Under these conditions, retention times of 2,4-DNT, 4-A-2-NT, 2-A-4-NT and MNC were 16.99, 9.42, 8.44, and 6.53 min respectively.

Gas Chromatography – Mass Spectrometry (GC-MS). For sample preparation, 20 ml liquid culture samples were extracted three times with 10 ml of diethylether. The extracts were pooled and dried by adding 3 g Na_2SO_4. After decanting, the organic phase was evaporated completely. Hydroxylated compounds were derivatised overnight at 80°C with 100 µl BSTFA (containing 1% TMCS).

Injection and identification occurred onto a GC-MS Voyager system (Interscience, Netherlands) with a Zebron ZB-5 capillary column, 25 m × 0.25 mm × 0.25 µm Df (Bester, Netherlands).

Electrospray Ionization Tandem Mass Spectrometry Collision-Induced Dissociation (ESI-MS/MS-CID). As described previously by Yinon *et al*. (1997).

DNA-DNA hybridisation. Genomic DNA of the test strains was digested using *Eco*RI as restriction enzyme (Life Technologies, CA). After Southern blotting, DNA-DNA hybridisation was applied using the probes *dntAa* and *dntD* from the 2,4-DNT degrading genes. The gene probes were obtained by PCR on genomic DNA of *Burkholderia* sp. strain DNT using the primerset 646F (5'-AAC TGG TAG TAG AAC CCC TC-3') and 1687R (5'-GGG GTT CAC TCA TGG CTT GG-3') for probe *dntAa*, and 81F (5'-CCA TGT CCG TTG CAG ACA TT-3') and 1040R (5'-GCT ACT GGG TTC AGT TGT GC-3') for probe *dntD*. Probes were labelled using the *Gene Images* random prime labelling module RPN 3540 (Amersham Life Science, UK). Detection was performed using the *Gene Images* CDP-*Star* detection module RPN 3510 (Amersham Life Science, UK). The membrane was washed twice with a mixture of 2× SSC and 0.1% SDS, at room temperature during 15 minutes, and twice with a mixture of 0.5× SSC and 0.1% SDS, at 60°C during 15 minutes.

Polymerase Chain Reaction (PCR) amplification. A Platinum *Taq* DNA Polymerase kit (Life Technologies, CA) was used to perform PCR. The PCR thermal profile, conducted in a reaction volume of 100 µl (5 µl PCR Buffer minus Mg (10×), 1 µl dNTP (2.5 mM each), 1.5 µl $MgCl_2$ (50 mM), 1 µl of each primer (1 µg/µl), 0.5 µl Platinum *Taq* DNA Polymerase (5U/µl), 89 µl autoclaved, distilled water, and 1 µl template DNA (100 ng/µl)), consisted of an initial denaturation step at 94°C for 2 min, 35 cycles of 30 sec denaturation at 94°C, primer annealing for 30 sec at 55°C and extension for 1 min at 72°C and finally an extension step of 8 min at 72°C.

Denaturing Gradient Gel Electrophoresis (DGGE) profiling. PCR amplification of 16S rDNA fragments was performed with the following eubacterial primer set: GC-63F (5'-CAG GCC TAA CAC ATG CAA GTC-3') and 518R (5'-ATT ACC GCG GCT GCT GG-3') (El-Fantroussi *et al*., 1999). DGGE was performed on an Ingeny-phorU system (Ingeny, Netherlands) in an 8% (w/v) polyacrylamide gel (acrylamide/bisacrylamide 37,5:1) with a denaturing gradient ranging from 35% to 65%. The electrophoresis was run at 60°C for 15 hours at 120 V. Afterwards, the gel was fixed in 0.5% acetic acid and stained in TAE buffer with 1% sybr green I solution (BioWhittaker Molecular Applications, ME).

Table 3.1 Nitroaromatic concentrations in the soil and water samples used as inoculum for selective enrichment under aerobic conditions

Sample (*)	TNT	4-ADNT	2-ADNT	2,4-DNT	2,6-DNT
S1 (1)	3046.3	11.2	28.7	51.6	43.4
W1 (1)	9.8	0.5	1.9	ND	ND
W2 (2)	10.0	0.6	2.2	ND	ND
S17-2 (3)	173.7	4.6	7.9	ND	ND
S72 (4)	ND	ND	ND	ND	ND
S163 (5)	27130.2	131.8	286.5	ND	ND

S, soil sample, concentration in mg/kg dry matter; W, water sample, concentration in mg/L; ND, not detected (< 0.01 mg/L). The water samples were taken in former, non-covered pits where the groundwater welled.
*, Sampling place: codes 1,2,3 were taken at the front site of the TNT manufacturing plant; code 1, distance ≤ 5 m; code 2, r ≈ 15 m; code 3, r ≈ 22 m. Code 4, a distant spot at the site; code 5, at a TNT store.

3.3 RESULTS

3.3.1 Isolation of 2,4-DNT degrading consortia

Four soil and two water samples (listed in Table 3.1) from a former ammunition production site were used to inoculate minimal medium with 2,4-DNT as sole nitrogen source for enrichment of 2,4-DNT degrading cultures. After incubation for 7 days, all cultures showed good growth ($OD_{660} \geq 0.50$), resulting from traces of nitrogen from the original samples. The culture fluids coloured orange-brown to red, which indicated incomplete degradation of the prevailing nitroaromatic

compounds. After three transfers, two enrichment cultures still showed significant growth ($OD_{660} \geq 0.25$). These cultures, which originated from the samples S1 and W1, grew very slowly. For both, almost 14 days were necessary to reach an optical density of 0.25. In addition, the culture fluids coloured orange-like, indicating the accumulation of degradation products. After further transfers over a period of 5 months, the culture time reduced to three to four days. The medium from the culture that originated from S1 brightened in contrast with the medium from the culture, which originated from W1 that stabilised at an orange stage. Upon repeated transfers, these cultures showed stable DGGE patterns and identical 2,4-DNT degrading properties. In the scope of this study, these optimised cultures originating from S1 and W1 are designated as consortium 1 and consortium 2 respectively. A second enrichment was started eight months later with sample S1, which had been the source of enrichment of consortium 1. A third consortium developed. REP-PCR and DGGE revealed initially similar strains but later on this consortium diverged from consortium 1. Plating of this optimised consortium, consortium 3, revealed the presence of six distinct strains.

The growth and degradation characteristics of the three consortia on 2,4-DNT as sole nitrogen source are presented in Figure 3.2. In consortium 1, growth was accompanied by gradual 2,4-DNT disappearance, without the detectable accumulation of metabolic end products (Figure 3.2A). However, during the first period of growth (from 25 to 48 hours), the culture medium coloured pink. This colour faded away during the second period of growth (from 48 to 73 hours), indicating the transient accumulation of a metabolite. Gas Chromatography-mass spectrometry (GC-MS) analysis in Electron Impact-mode, indicated the presence of 2-hydroxy-5-methylquinone (HMQ) based on its retention time, molecular weight and characteristic fragment ions (in respect to measured relative intensities compared with the HMQ-standard). Consortium 2 showed, compared to consortium 1, a faster 2,4-DNT degradation that was accompanied by a stoichio-

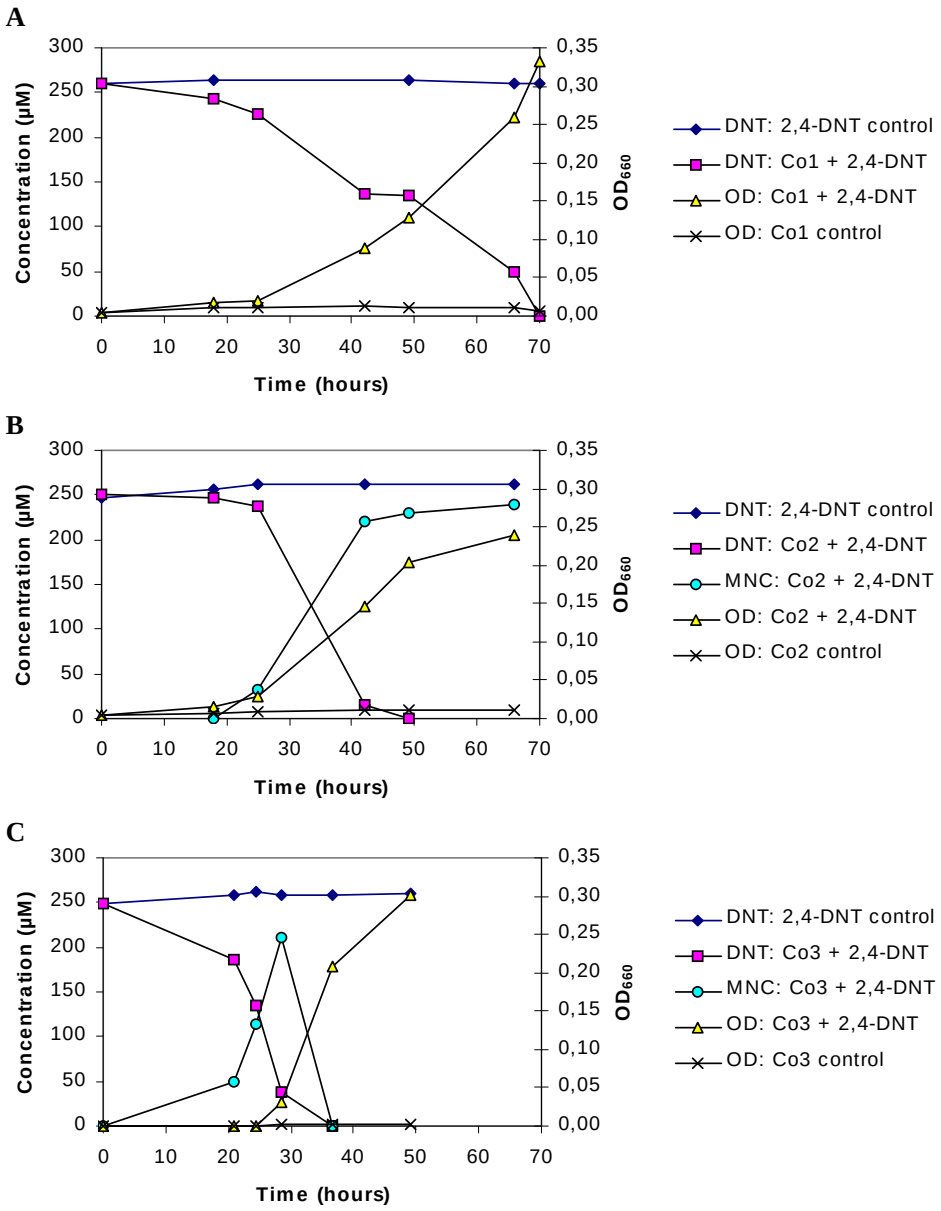

Figure 3.2 Growth and 2,4-DNT degradation characteristics under nitrogen-limiting conditions. A, consortium 1; B, consortium 2; and, C, consortium 3. The increase in optical density was negligible in the absence of 2,4-DNT, and the concentration of 2,4-DNT remained around 250 µM in the control samples (data not shown).

metric accumulation of an orange metabolite (Figure 3.2B). The compound was identified by HPLC and GC-MS as 4-methyl-5-nitrocatechol (MNC). As for consortium 2, MNC was observed in the culture fluid of consortium 3 (Figure 3.2C), but with the difference that its accumulation was transient. The partial degradation of 2,4-DNT to MNC was found to be the rate-limiting step, as further removal of MNC was accomplished within a few hours. Furthermore, the decline in the MNC concentration was accompanied by an increase in growth (at 29 hours). None of the consortia was able to grow in nitrogen-free medium and no accumulation of nitrite was observed. Additional tests showed that the maximum optical density was linear related to the initial concentration of 2,4-DNT unto a concentration of 439 µM or 80 mg/L (Consortium 1: OD_{660} = 0.0034 × concentration (mg/L), r^2 = 0.998; Consortium 3: OD_{660} = 0.0042 × concentration (mg/L), r^2 = 0.997). These findings demonstrated that all three consortia used nitrite released from 2,4-DNT as nitrogen source.

3.3.2 Characterisation of the 2,4-DNT-degrading consortia

Table 3.2 Identification of the isolates from consortium 1 and consortium 3

Strain code	Biochemical identification* (LMG)	Genetical identification (Blast)
Consortium 1		
VM683	*Pseudomonas marginalis*	*Pseudomonas marginalis* (96%)
VM684	*Clavibacter michiganense*	*Microbacterium* sp. (96%)
VM685	not identified	*Variovorax paradoxus* (95%)
VM686	*Alcaligenes* sp.	*Alcaligenes* sp. (98%)
Consortium 3		
VM903	*Pseudomonas aeruginosa*	*Pseudomonas aeruginosa* (99%)
VM904	*Sphingomonas* sp.	*Sphingomonas* sp. (97%)
VM905	*Stenotrophomonas maltophilia*	*Stenotrophomonas maltophilia* (98%)
VM906	*Serratia* sp.	*Serratia proteamaculans* (98%)
VM907	*Pseudomonas fluorescens* group	*Pseudomonas viridiflava* (97%)
VM908	*Janthinobacterium lividum*	*Pseudomonas* sp. (98%)

*, Biochemical identification included basic bacteriological tests (Gram-staining), FAME-analysis, Biolog GN, and API20NE. For genetical identification, the 16S rRNA gene was amplified, cloned, sequenced and the sequence was compared against published sequences from GenBank.

Because of the incomplete degradation of 2,4-DNT by consortium 2, the work was further focussed on consortium 1 and consortium 3. Four distinct strains were isolated from consortium 1, whereas six strains from consortium 3. The results of their identification are shown in Table 3.2. Remarkable was that, though started from "the same" soil sample, consortium 1 and consortium 3 consists of different members with no single common strain. Morphological, physiological and phenotypic characterisations of the strains are presented in Table 3.3.

The reconstituted consortia 1 and 3, consisting of four and six strains respectively, showed degradation characteristics similar to the original consortia, indicating that the key-strains in the degradation of 2,4-DNT were isolated (Figure 3.3). In addition, DGGE separation patterns of the individual strains, the original consortia and the reconstituted consortia were compared after growth on 2,4-DNT (Figure 3.4). Both consortium 1 (lane 5) and reconstituted consortium 1 (lane 6) showed a comparable pattern and the presence of *Pseudomonas marginalis* VM683 and *Variovorax paradoxus* VM685 was demonstrated in both. The absence of *Microbacterium* sp. VM684 and *Alcaligenes* sp. VM686 in the patterns of the consortia can be due to a low abundance of the strains in the consortia, a lower number of 16S rDNA copies and/or preferential amplification during the PCR reaction. The two latter hypotheses are not unlikely as demonstrated in lane 7, which is a result of PCR on a DNA-mixture of the participating strains, made by mixing equal amounts of equal DNA concentrations (100 ng/µl). Concerning consortium 3, the presence of *Pseudomonas aeruginosa* VM903 and/or *Pseudomonas viridiflava* VM907 and *Pseudomonas* sp. VM908 is clear in both the original consortium 3 (lane 15) and the reconstituted consortium 3 (lane 16). A weak or no signal was observed for *Sphingomonas* sp. VM904, *Stenotrophomonas maltophilia* VM905, and *Serratia proteamaculans* VM906, for which the same hypotheses can be posed as for *Microbacterium* sp. VM684 and *Alcaligenes* sp. VM686.

Table 3.3 Characterisation of the isolated strains

Morphological characterisation

	Gram stain	Colony morphology on 869
VM683	-	Beige, large, siderophore-producing (medium coloured blue-green)
VM684	+	Yellow, round
VM685	-	Vivid yellow, small, round, dry, aggregated cells
VM686	-	White-beige, large
VM903	-	Beige, large, siderophore-producing (medium coloured blue-green)
VM904	-	Vivid yellow, relative small
VM905	-	Light yellow, small
VM906	-	Beige, large, smooth, medium coloured darker
VM907	-	Beige, smooth
VM908	-	Beige, small, relative transparent, slow growing (needs 2 days to push on 869)

Gram stain: +, positive; -, negative; 869, rich medium (Mergeay *et al*., 1985); The strains are listed by their VM-numbers, their complete identifications are: *Pseudomonas marginalis* VM683, *Microbacterium* sp. VM684, *Variovorax paradoxus* VM685, *Alcaligenes* sp. VM686, *Pseudomonas aeruginosa* VM903, *Sphingomonas* sp. VM904, *Stenotrophomonas maltophilia* VM905, *Serratia proteamaculans* VM906, *Pseudomonas viridiflava* VM907, *Pseudomonas* sp. VM908.

Physiological and phenotypic characterisation

VM-n°:	**683**	**684**	**685**	**686**	**903**	**904**	**905**	**906**	**907**	**908**
Physiological characterisation: growth on different carbon sources										
Azel	+	-	-	+	+	-	-	-	-	+-
Fruct	+	-	-	-	+	+	-	+	+	-
Gl	+	-	-	-	+	+	+-	+	+	-
Gluc	+	-	+	+	+	-	-	+	+	-
Lact	+	-	+	+	+	-	+-	+	-	+-
Succ	+	-	+	+	+	+	-	+	+	+-
Tol	-	-	-	-	-	-	-	-	-	-
Mix	+	-	+	+	+	+	+-	+	+	+-
Contr	-	-	-	-	-	-	-	-	-	-
Phenotypic characterisation: resistance to heavy metals										
Cd 0.8	-	-	-	+	+	-	-	+	-	-
Cd 1	-	-	-	+-	+	-	-	-	-	-
Cu 1	-	-	-	-	-	+-	-	-	-	-
Cu 2	-	-	-	-	-	-	-	-	-	-
Ni 1	-	-	-	+-	-	-	-	-	-	-
Ni 2	-	-	-	-	-	-	-	-	-	-
Pb 0.5	+	-	+	+	+	+	+-	+	+	-
Pb 1	+	-	-	+	+	+-	-	+	+	-
Zn 1	+	-	-	+	+	+	-	+	-	-
Zn 2	-	-	-	+	+	+-	-	+-	-	-

Physiological and phenotypic characterisation occurred on 284 minimal medium (Mergeay *et al*., 1985). Carbon sources: azel, azelate; fruct, fructose; gl, glucose; gluc, gluconate; lact, lactate; succ, succinate; tol, toluene; mix, azel + fruct + gl + gluc + lact + succ; contr, without additional carbon source. The tested heavy metal concentration is noted behind its symbol and expressed in mM. In the presence of Pb, the phosphate buffer is replaced by MOPS (3-morpholinopropane sulfonic acid) to prevent complexation and precipitation. Mark: +, growth; +-, restricted growth; -, no growth.

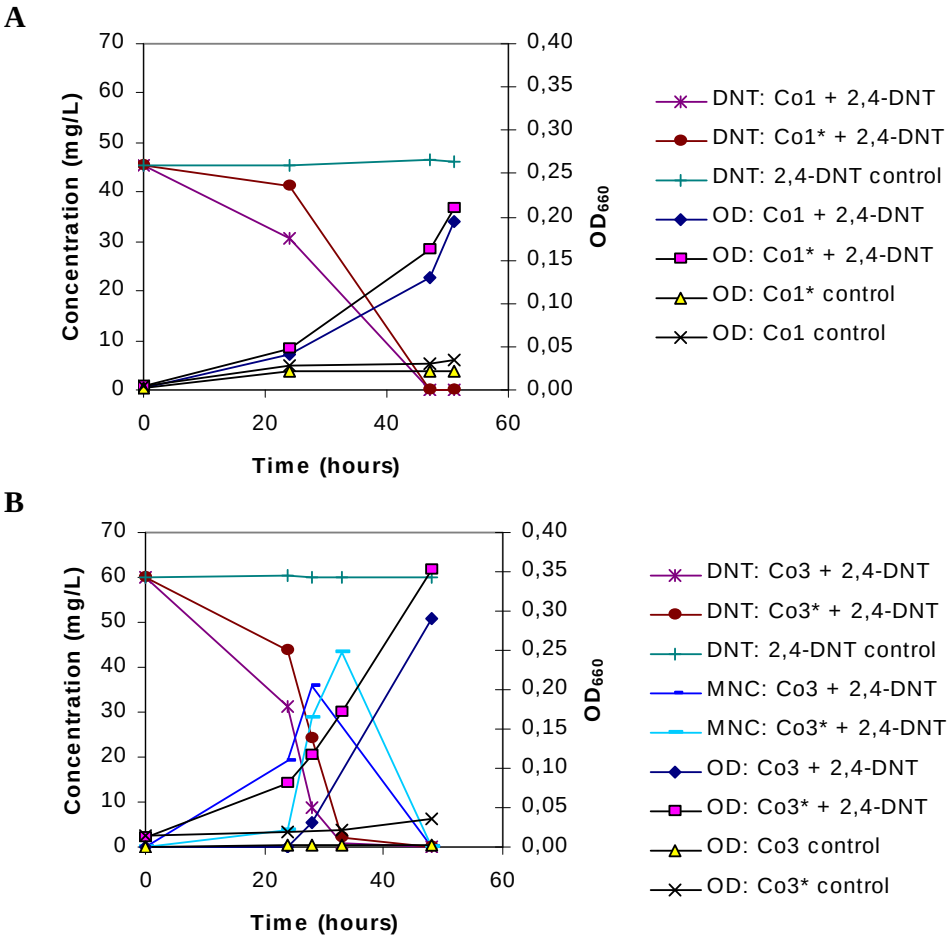

Figure 3.3 Growth and 2,4-DNT degradation characteristics under nitrogen limiting conditions. A, consortium 1 (Co1) versus reconstituted consortium 1 (Co1*); B, consortium 3 (Co3) versus reconstituted consortium 3 (Co3*).

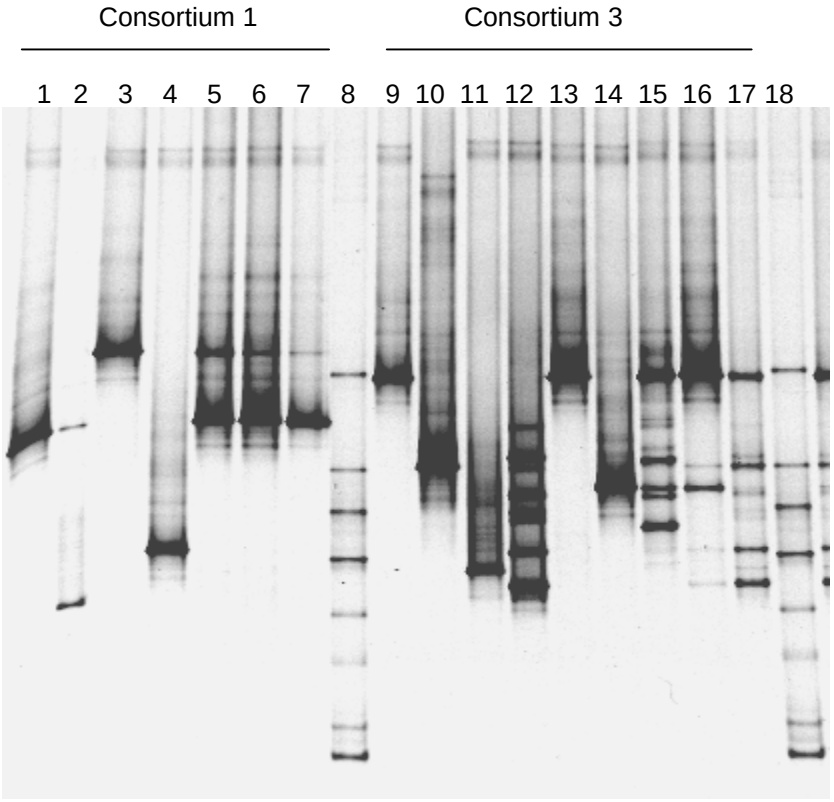

Figure 3.4 DGGE fingerprint characterising consortium 1 and 2. 1, *Pseudomonas marginalis* VM0683; 2, *Microbacterium* sp. VM0684; 3, *Variovorax paradoxus* VM0683; 4, *Alcaligenes* sp. VM0684; 5, consortium 1; 6, reconstituted consortium 1; 7, PCR on DNA-mix of participating strains; 8, marker; 9, *Pseudomonas aeruginosa* VM0903; 10, *Sphingomonas* sp. VM0904; 11, *Stenotrophomonas maltophilia* VM0905; 12, *Serratia proteamaculans* VM0906; 13, *Pseudomonas viridiflava* VM0907; 14, *Pseudomonas* sp. VM0908; 15, consortium 3; 16, reconstituted consortium 3; 17, PCR on DNA-mix of participating strains; 18, marker.

3.3.3 Co-operation of strains in the degradation of 2,4-DNT

Two types of co-operation among the organisms of consortium 1 were observed. The first became clear on selective medium with 2,4-DNT as the sole nitrogen source. When the isolates were streaked separately on the same plate, *Variovorax paradoxus* VM685 generated a brown-pink pigment, which diffused into the agar. Growth of *Pseudomonas marginalis* VM683 was only observed in close proximity of *Variovorax paradoxus* VM685 while the colour faded away. In addition, the

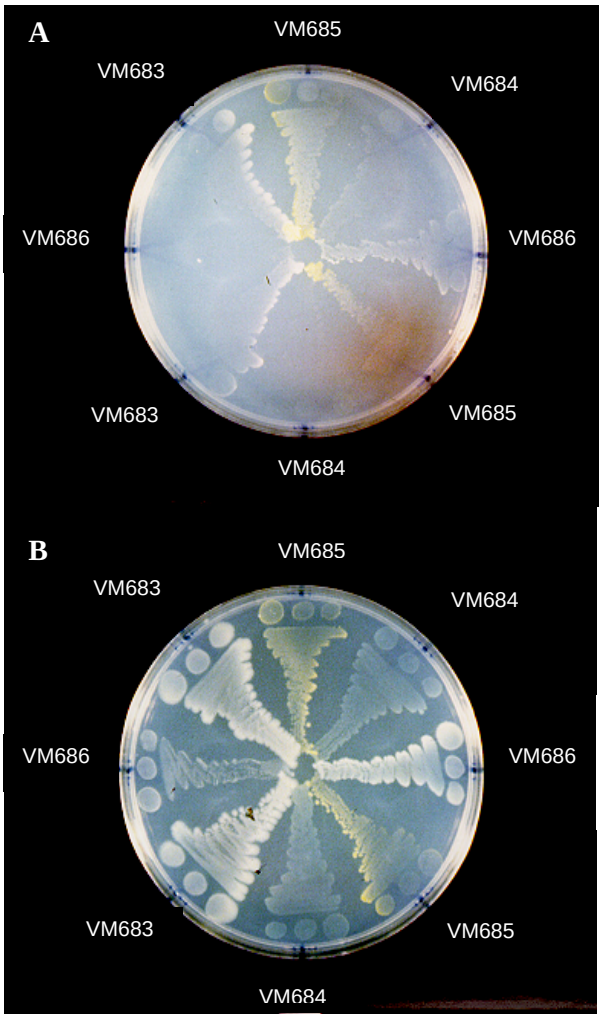

Figure 3.5 Individual strains, isolated from consortium 1, streaked out in combination (A) on selective medium with 2,4-DNT as nitrogen source and (B) on a complete minimal medium with NH_4Cl as nitrogen source. List of strains: *Pseudomonas marginalis* VM683*; Microbacterium* sp. VM684; *Variovorax paradoxus* VM685; *Alcaligenes* sp. VM686.

fading of the colour went together with growth of *Variovorax paradoxus* VM685 in close proximity of *Pseudomonas marginalis* VM683 (Figure 3.5, A). The results show that *Variovorax paradoxus* VM685 degrades 2,4-DNT only partially and excretes one or more intermediates. In a next step, the presence of *Pseudomonas marginalis* VM683 is indispensable to continue the degradation and to support growth of *Variovorax paradoxus* VM685. The second type of co-operation was

observed when testing combinations of *Alcaligenes* sp. VM686 plus *Variovorax paradoxus* VM685, *Microbacterium* sp. VM684 plus *Pseudomonas marginalis* VM683, and *Microbacterium* sp. VM684 plus *Alcaligenes* sp. VM686. Growth of the first strain for each combination was slightly stimulated in close proximity of the latter strains on selective as well as on non-selective medium in which NH_4Cl was added as nitrogen source. Co-operation is most probably based on the transfer of one or more micronutrients, e.g. vitamins, by the latter (Figure 3.5, A, B). In contrast, growth inhibition of *Alcaligenes* sp. VM686 by *Pseudomonas marginalis* VM683 was found on both selective and non-selective minimal media. This might indicate that growth inhibition of *Alcaligenes* sp. VM686 is caused by the excretion of a secondary metabolite (e.g. an antibiotic) by *Pseudomonas marginalis* VM683.
In addition, the relative abundance of the strains during growth on 2,4-DNT was investigated for consortium 1. At different time intervals, the optical density was determined and the colony forming units were counted after plating on 1/10 869 (Figure 3.6). Colonies from *Pseudomonas marginalis* VM683 and *Alcaligenes* sp. VM686 were morphologically difficult to distinguish and therefore counted together.

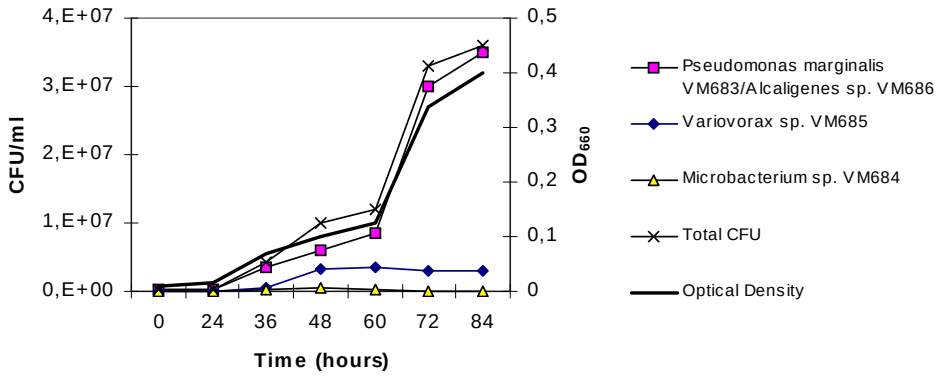

Figure 3.6 Colony Forming Units (CFU) of the individual strains and growth of consortium 1 in nitrogen limiting conditions

A culture had the following composition at the end of the first growth phase: *P. marginalis* VM683 plus *Alcaligenes* sp. VM686 70%, *Variovorax paradoxus* VM685 28% and *Microbacterium* sp. VM684 2%, changing respectively to 93%, 7% and 0.007% at the end of the second growth phase. Though the abundance of *Variovorax paradoxus* VM685 is underestimated, due to the characteristic of the cells to form aggregates, these results indicate the importance of this particular strain during the first growth phase. Similar experiments with the members of consortium 3 were not performed. However, shifts in abundance after reconstitution of consortium 3 were evaluated using DGGE (Figure 3.7).

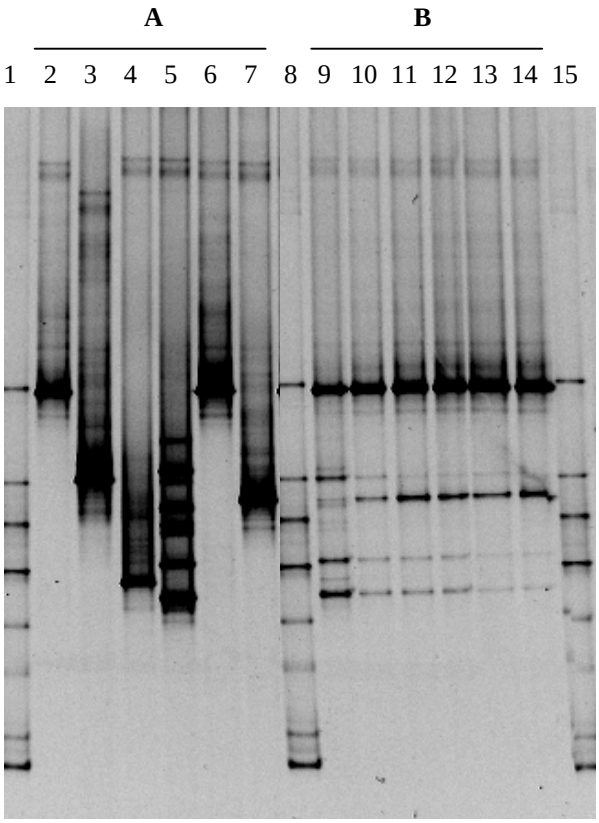

Figure 3.7 (A) Individual strains, (B) Shifts in abundance after reconstruction of consortium 3. 1, marker; 2, *Pseudomonas aeruginosa* VM0903; 3, *Sphingomonas* sp. VM0904; 4, *Stenotrophomonas maltophilia* VM0905; 5, *Serratia proteamaculans* VM0906; 6, *Pseudomonas viridiflava* VM0907; 7, *Pseudomonas* sp. VM0908; 8, marker; 9, starting material; 10, consortium after first transfer; 11, consortium 3 after second transfer; etc.; 15, marker.

Initially equal amounts of pure cultures were added together. Prior to 1/50 dilution into fresh selective medium, a culture sample was taken and genomic DNA

extracted. The importance of *Pseudomonas aeruginosa* VM903 and/or *Pseudomonas viridiflava* VM907 and *Pseudomonas* sp. VM908 in the degradation of 2,4-DNT was demonstrated.

The complementary action of strains in the 2,4-DNT degradation has been further examined in liquid cultures. Therefore, reconstitutions with all possible combinations of two or three members of the respective consortia were made and showed that for both consortia two main members were required to observe degradation beyond the colour intermediates (MNC and HMQ) and growth. In consortium 1, total degradation of 2,4-DNT could be described as an interaction between *Variovorax paradoxus* VM685 and *Pseudomonas marginalis* VM683 (Figure 3.8). Within four days, the 2,4-DNT supply was depleted and the increase in growth slowed down. Other combinations of members from consortium 1, resulted in some transformation of 2,4-DNT to its reduced intermediates (2-A-4-NT and 4-A-2-NT), while no growth was observed. A three-member consortium was only successful when *Variovorax paradoxus* VM685 and *Pseudomonas marginalis* VM683 were present. These results indicate that the other isolates are not important for the major catabolic activity of the consortium.

In consortium 3, various efficient two-member combinations could be made to generate 2,4-DNT degradation, but the presence of *Pseudomonas* sp. VM908 was absolutely necessary. The best results were achieved in combination with *Sphingomonas sp.* VM904 or *Pseudomonas viridiflava* VM907 (Figure 3.9). After two days of incubation, no aromatic compounds remained in the culture medium. In combination with *Pseudomonas aeruginosa* VM903 or *Stenotrophomonas maltophilia* VM905, complete degradation of 2,4-DNT took three days. Degradation was not efficient in combination with *Serratia proteamaculans* VM906. After three days, still 63% of the initial 2,4-DNT concentration remained in the medium.

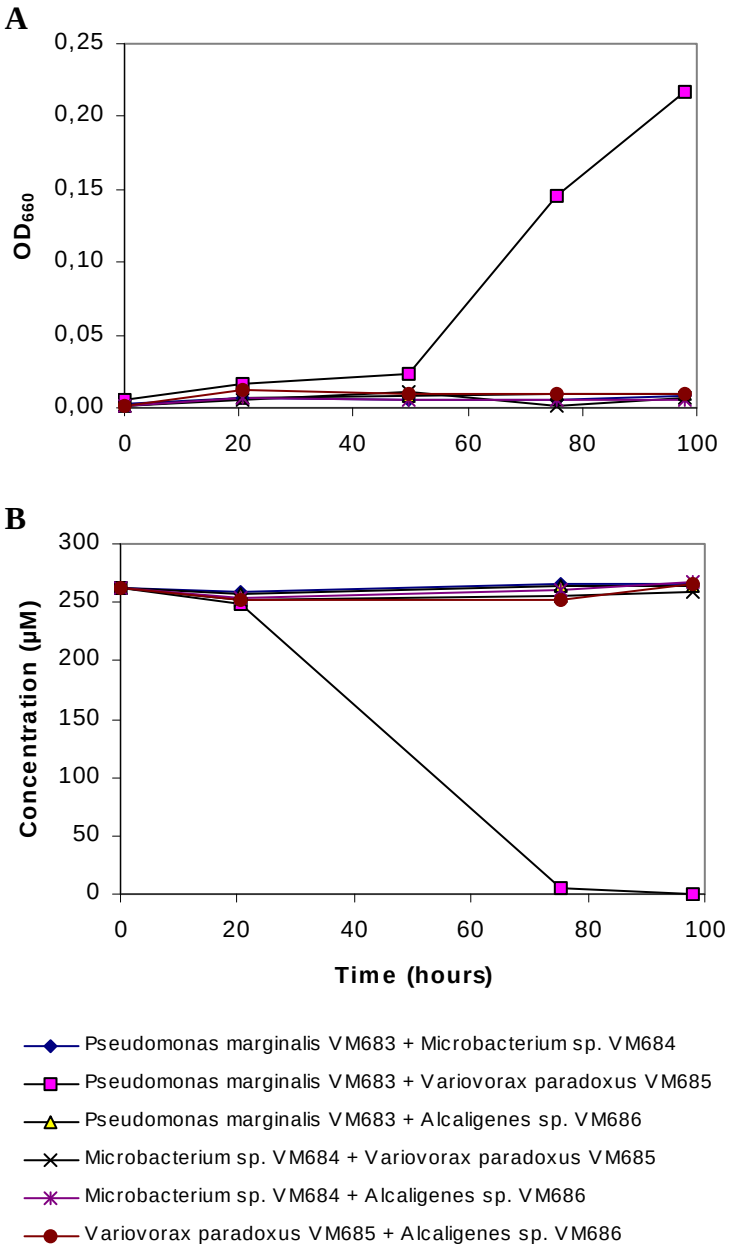

Figure 3.8 (A) Growth and (B) 2,4-DNT-degradation of two-member co-cultures from consortium 1 under nitrogen-limiting conditions

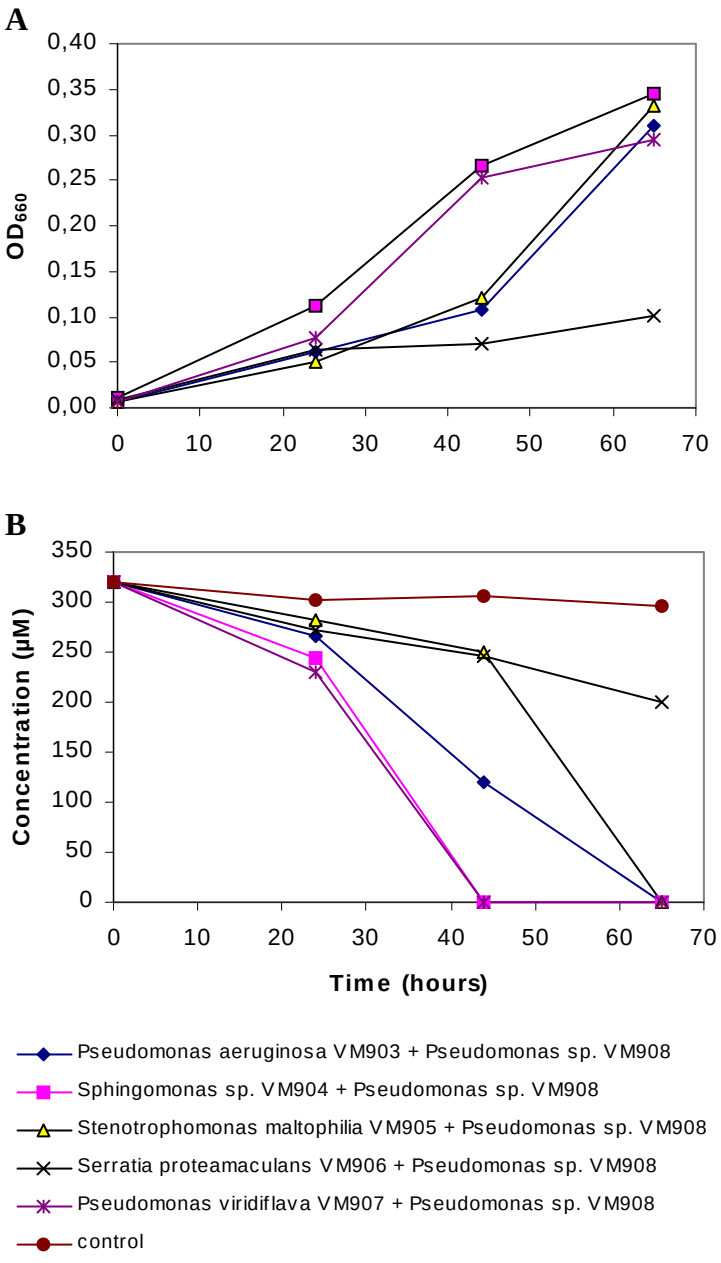

Figure 3.9 (A) Growth and (B) 2,4-DNT-degradation of two-member co-cultures from consortium 3 under nitrogen-limiting conditions.

An important finding was that none of the individual strains from consortium 1 and 3 was able to grow on 2,4-DNT as the sole nitrogen source. However, transformations did occur: *Pseudomonas* sp. VM908 from consortium 3 and *Variovorax paradoxus* VM685 from consortium 1, accumulated the intermediate MNC in the medium (though this compound was never observed in consortium 1) and nitrite was detected, indicating that they initiate the attack on 2,4-DNT. In contrast, the other pure cultures showed reduction reactions leading to the formation of 4-A-2-NT and 2-A-4-NT. This result indicates that both consortium 1 and 3 use the same initial oxidative step in their 2,4-DNT degradation pathway. The identification of MNC and HMQ as degradation intermediates of 2,4-DNT in consortium 1 and 3 suggested the presence of the same oxidative pathway for 2,4-DNT as in *Burkholderia* sp. strain DNT (Suen and Spain, 1993; Figure 3.1). In order to examine this hypothesis and in addition the role of the different members in the 2,4-DNT degradation, enzyme activity tests and DNA-DNA hybridisation were performed on the individual members. The results are shown in Tables 3.4 and 3.5.

Table 3.4 Hybridisation with probes from the oxidative 2,4-DNT-degradation pathway by *Burkholderia* sp. strain DNT

Strain	***dntAa***	***dntD***
Consortium 1		
Pseudomonas marginalis VM683	-	+
Microbacterium sp. VM684	-	-
Variovorax sp. VM685	+	-
Alcaligenes sp. VM686	-	(+)
Consortium 3		
Pseudomonas aeruginosa VM903	-	+
Sphingomonas sp. VM904	-	+
Stenotrophomonas maltophilia VM905	-	+
Serratia sp. VM906	-	-
Pseudomonas viridiflava VM907	-	+
Pseudomonas sp. VM908	+	(+)
Burkholderia sp. strain DNT	+	+

Identification hybridisation signal: +, strong; (+), weak; (-) lacking.

Table 3.5 Enzyme activity

Strain	2,4-DNT DO (DntA)	MNC MO (DntB)	HMQ R (DntC)	THT O* (DntD)
Consortium 1				
Pseudomonas marginalis VM683	-	-	-	-
Microbacterium sp. VM684	-	-	-	-
Variovorax sp. VM685	+	+	-	-
Alcaligenes sp. VM686	-	-	-	-
Consortium 3				
Pseudomonas aeruginosa VM903	-	-	-	-
Sphingomonas sp. VM904	-	-	-	-
Stenotrophomonas maltophilia VM905	-	-	-	-
Serratia sp. VM906	-	-	-	-
Pseudomonas viridiflava VM907	-	-	-	-
Janthinobacterium lividum VM908	+	+	-	-
Burkholderia sp. strain DNT	+	+	+	+

2,4-DNT DO, 2,4-dinitrotoluene dioxygenase; MNC MO, 4-methyl-5-nitrocatechol monooxygenase; HMQ R, 2-hydroxy-5-methylquinone reductase; THT O, 2,4,5-trihydroxytoluene oxygenase. The corresponding gene product is added between brackets. *, THT oxygenase activity tested determined with 4-methylcatechol as substrate.

Only *Variovorax paradoxus* VM685 and *Pseudomonas* sp. VM908 exhibited 2,4-DNT dioxygenase enzyme activity, as indicated by concomitant colour changes and the detection of nitrite. In addition, only these two strains hybridized with the *dntAa* probe. We never succeeded to amplify the *dntB* fragment in strain DNT to use further as probe, despite choosing different primers as well as PCR conditions. However, MNC mono-oxygenase activity was demonstrated in the presence of both *Variovorax paradoxus* VM685 and *Pseudomonas* sp. VM908. MNC oxidised to HMQ and nitrite was detected. There are no indications about which strains are capable to further reduce HMQ to THT. All enzyme activity tests were negative and as the HMQ reductase gene (*dntC*) has not been sequenced, hybridisation could not be performed. However, the presence of THT was demonstrated by GC-MS in the culture media of both consortium 1 and consortium 3. A DNA fragment containing the *dntD* gene, which is required for the oxidation of THT into 2,4-dihydroxy-5-methyl-6-oxo-2,4-hexadienoic acid, was used as probe for the detection of a homologous gene in our consortia. *Pseudomonas marginalis*

VM683, *Pseudomonas aeruginosa* VM903, *Sphingomonas* sp. VM904, *Stenotrophomonas maltophilia* VM905 and *Pseudomonas viridiflava* VM907 showed a strong hybridisation signal. This is consistent with our previous observations, where strain VM683 in combination with strain VM685 and the strains VM903, VM904, VM905, or VM907 in combination with VM908 showed efficient 2,4-DNT degradation. *Pseudomonas* sp. VM908 and *Alcaligenes* sp. VM686 showed a weak hybridisation signal. No signals were obtained for *Serratia proteamaculans* VM0906, *Microbacterium* sp. VM0684 and *Variovorax paradoxus* VM0685. Possible THT oxygenase activity was further tested with 4-methylcatechol as substrate. THT oxygenase is known to show substrate specificity towards 4-methylcatechol in *Burkholderia* sp. strain DNT (Haigler *et al*., 1999). However, our results were negative. To provide extra evidence for ring cleavage, consortium 1 and 3 were tested for their ability to metabolise 2,4-DNT under carbon limiting conditions. The compound was supplemented at 550 µM (100 mg/L). Both consortia completely degraded 2,4-DNT within 7 days. Transformations to 4-A-2-NT and 2-A-4-NT were found, but the sum of both aminonitrotoluenes was less than 1 % of the initial 2,4-DNT concentration. Other metabolites were not detected and growth was observed during the exposure time (OD_{660} about 0.050 compared to the control without 2,4-DNT, which had an OD_{660} about 0.003 after 8 days).

3.3.4 Metabolism of other nitroaromatic compounds under nitrogen limiting conditions

For both 2,4-DNT-grown consortia, no transformation was observed for 2-, 4-NT, 2-A-4-NT, 2-A-6-NT, 4-A-2-NT and 2,6-DNT. All substrates were provided at 50 mg/L. Within 96 hours consortium 1 transformed TNT partially (59 %) into 2-ADNT (2 %), 4-ADNT (5 %) and several unidentified metabolites. During these 96 hours of exposure, the culture became progressively more red-brown. The causative compound(s) has (have) not been identified. Within 96 hours, consortium

3 transformed TNT completely into ADNTs (21 %) and unidentified metabolites. Unlike consortium 1, the medium did not discoloured. When TNT was added in excess to a culture of consortium 3, a five-fold increase in optical density was observed within 48 hours, which was not related to growth (CFU and protein concentration of the consortium were similar or even slightly lower after treatment with TNT compared to the control). HPLC and ESI-MS/MS-CID analyses indicated the presence of four azoxy isomers (binuclear compounds which arise from the condensation of partially reduced intermediates of TNT) in cell pellet extractions with acetonitrile. Four different peaks with a retention time of respectively 43.47, 47.71, 50.25 and 61.22 minutes showed all to contain a highly apolar molecul with mass 405 (MW_{azoxy} = 406). Unfortunately standards of azoxy compounds are not commercial available. Cell-pellet extracts of the individual strains cultured under the same growth conditions revealed that both *Serratia* sp. VM906 and *Pseudomonas viridiflava* VM907 could be responsible for this characteristic of consortium 3.

In addition, the *umu*-test (Oda *et al*., 1985), a bacterial genotoxicity and toxicity test for the screening of chemicals, demonstrated that consortium 3 was able to decrease the genotoxicity of TNT-contaminated water (10 mg/L) more than consortium 1. Toxicity was not detected before and after treatment.

3.4 DISCUSSION

Efforts to isolate 2,4-DNT mineralising cultures were only successful when a detectable 2,4-DNT contamination was present in the starting material, as for the isolation of consortium 1 and consortium 3 who originated from sample S1 (Table 1). Enrichment of sample W1 resulted in the isolation of consortium 2. Under nitrogen limiting conditions, this consortium accumulated MNC, an intermediate in the degradation of 2,4-DNT.

All three consortia originated from samples taken in or close to the 2,4-DNT hot spot (S1, hot spot; W1, at 3 m distance), where the exposure must have been in favour to select for specific degraders. This observation is consistent with previous reports in which 2,4-DNT-mineralising strains could only be isolated from 2,4-DNT-contaminated sites (Spanggord *et al*., 1991; Johnson *et al*., 2000; Nishino *et al*., 2000). Similar findings were illustrated for some agriculture chemicals, such as the herbicides atrazine and linuron (De Souza *et al*., 1998; El-Fantroussi, 2000).

An interesting finding was that addition of *Pseudomonas marginalis* VM683, isolated from consortium 1, to consortium 2 resulted in complete disappearance of the intermediate MNC (results not shown). This result suggests the lack of genes in consortium 2 to continue the degradation or the absence of crucial interacting strains. Examples of synergism between two or more members include interspecies transfer of growth factors (Dangmann *et al*., 1996) and biosurfactant production (Arino *et al*., 1998). Pelz *et al*. (1999) demonstrated a metabolite overflow to other members of a 4-chlorosalycilate degrading-community. As a result of this sharing, toxic metabolites did not attain significant levels.

Despite the fact that both consortia 1 and 3 originated from the same soil sample, no common strain was isolated. However, in an early stage of the second enrichment, which resulted in the isolation of consortium 3, two strains were identified that had the same REP-PCR patterns as observed for strains present in consortium 1. This result indicates that on obtained selective enrichment cannot always be repeated. Complex biotic and abiotic interactions (in this study including the storage of the soil sample at –80°C) determine the adaptive responses, evolution and final structure of a community. Competition among strains can change during the enrichment as a result of horizontal gene transfer, mutations and small differences in selective pressure.

The fact that the intermediates MNC, HMQ and THT were detected in the culture media of the consortia 1 and 3, indicates that the same pathway is used as in *Burkholderia* sp. strain DNT. This is not unexpected as to date all strains that grow on 2,4-DNT appear to use the same pathway as strain DNT (Nishino *et al*., 2000).

Analogous results were obtained from bacteria having the capacity to degrade the herbicide atrazine. All atrazine-mineralising pure or mixed cultures have shown to contain genes homologous to *atzA, -B,* and *–C* from *Pseudomonas* sp. strain ADP. These results suggest a recent evolutionary origin and distribution of the degrading genes (De Souza *et al*., 1998).

The genetic and biochemical data demonstrate that *Variovorax paradoxus* VM685 (consortium 1) and *Pseudomonas* sp. VM908 (consortium 3) initiate 2,4-DNT catabolism of the consortia by carrying out reactions similar to the first two metabolic steps observed for *Burkholderia* sp. DNT: 2,4-DNT is converted to HMQ via MNC, with release of both nitrite groups. However, the conversion of MNC requires the interaction of other species from the consortia, as this compound accumulates in the absence of other members. A plausible explanation could be that MNC mono-oxygenase is target for feedback inhibition by HMQ. Elimination of HMQ by the interacting bacteria promotes the equilibrium of the reaction towards the quinone. However, as nitrite was released, but growth was not observed for *Variovorax paradoxus* VM685 or *Pseudomonas* sp. VM908 when they were individually inoculated under selective conditions, intervention of other strains could also be at the point of e.g. transferring micronutrients. MNC was never detected when consortium 1 or its functional two-member derivative consortium was inoculated in selective conditions. This in contrast with consortium 3, where MNC accumulated until 2,4-DNT was almost completely depleted. This result suggests an additional inhibition of 2,4-DNT on MNC mono-oxygenase in consortium 3.

The results of *dntD* hybridisation, and the observation that both consortia grow on 2,4-DNT as carbon source, suggest that *Pseudomonas marginalis* VM683 (consortium 1), *Pseudomonas aeruginosa* VM903, *Sphingomonas sp.* VM904, *Stenotrophomonas maltophilia* VM905 or *Pseudomonas viridiflava* VM907 (consortium 3) are essential to complete the mineralisation of 2,4-DNT. However, the catabolic step resulting in ring fission could not be confirmed by an enzyme activity test using 4-methylcatechol as substrate, suggesting there is similarity

present in the ring fission enzymes despite a difference in substrate specificity towards 4-methylcatechol.

In consortium 1 and 3, although catabolism of 2,4-DNT by specific two-member derivative consortia was revealed, both consortia were found to consist of more than two members. A similar observation was made by Lappin *et al.* (1985) who selected a five-member mecoprop-degrading consortium in preference to several equally capable two-member derivative consortia. This mecoprop-degrading community failed to reduce its species number, indicating metabolic cross-feeding. In our consortia an interspecies metabolic interaction can be proposed as well. The nitrite released by *Variovorax paradoxus* VM685 or *Pseudomonas* sp. VM908 allows nitrogen assimilation by the other consortia members. The presence of *Pseudomonas marginalis* VM683, *Pseudomonas aeruginosa* VM903, *Sphingomonas sp.* VM904, *Stenotrophomonas maltophilia* VM905 or *Pseudomonas viridiflava* VM907 allowed further assimilation of the aromatic compound. The surplus value to the consortium of the other strains (*Microbacterium* sp. VM684, *Alcaligenes* sp. VM686, *Serratia* sp. VM906) which seem dispensable for the 2,4-DNT degradation but which presence persist, can e.g. be based on the transfer of micronutrients (e.g. vitamins, amino acids) or complexing agents for heavy metals. This hypothesis was not further investigated in our consortia. However, Timmis *et al.* (2001) concluded that the diversity of interacting species and the presence of redundant species in a community favours stability and ensures effective resource utilisation under changing environmental conditions.

Many reports have described the metabolism of TNT by microorganisms, but in most cases metabolism involved the reduction of one or more nitro groups on the aromatic ring and the accumulation of aminonitrotoluenes (Rieger and Knackmuss, 1995; Esteve-Nunez *et al*., 2001). In addition, under aerobic conditions azoxy compounds resulting from the condensation of hydroxylamino and nitroso dinitrotoluenes have been found (McCormick *et al*., 1976; Duque *et al*., 1993; Haïdour and Ramos, 1996; Vasilyeva *et al*., 2000; Wang *et al*., 2000). Such

partially reduced nitrotoluenes and azoxy compounds are highly recalcitrant to bacterial degradation. Consortium 1 generated ADNTs as major by-products. In the presence of consortium 3 ADNTs as well as azoxy binuclear compounds were abundant. Metabolism of TNT by consortium 3 was fast and complete and the remaining genotoxicity of the contaminated medium was found to be lower compared to the remaining genotoxicity after treatment with consortium 1. Therefore consortium 3 was selected for application in further experiments with TNT (Chapter 7, Chapter 8).

CHAPTER 4
THE USE OF *HELIANTHUS ANNUUS* (SUNFLOWER) TO REMOVE TNT FROM AQUEOUS SOLUTIONS

4.1 INTRODUCTION

In a preliminary test, we assessed the toxic effect of TNT-contaminated water on germinated *Phaseolus vulgaris* plants (bushbean) in hydroponic cultures. The water sample was taken from a well at an ammunition contaminated site, and contained in addition to 10 mg TNT/L, 0.6 mg 4-ADNT/L and 2.2 mg 2-ADNT/L (Table 3.1, sample W2). Its colour was orange-brown, indicating the presence of photodegradation products and partially reduced forms of TNT. Compounds giving rise to the colour include azoxytoluenes, trinitrobenzonitrile and isoanthranil (Tom Hess, pers. comm.). After 14 days of plant treatment, their roots were affected. Visible symptoms to the roots consisted of an orange-brown discoloration and growth inhibition. Phytotoxic effects of nitroaromatic compounds were previously observed by other groups (Palazzo and Leggett, 1986; Görge *et al.*, 1994; Scheidemann *et al.*, 1998). Rinsing of the roots with H_2O_{dest} could not remove the coloured compounds, indicating a strong adsorption to the root surface. Nitroaromatic explosive compounds may be transformed by root tissues or by the prevailing microflora and bind irreversibly to roots by their amino or hydroxylamino functional groups (Hughes *et al.*, 1997; Thompson *et al.*, 1998a). These findings suggest the possible use of a rhizofiltration process, which includes root surface sorption as a removal mechanism. The ability of *Helianthus annuus* (sunflower) roots to accumulate heavy metals and radionuclides in a rhizofiltration process has already been exploited (Bing, 1996; Cooney, 1996; Richman, 1996). *Helianthus annuus* plants develop a dense root system in hydroponics, yield high

biomass and have a high water use. In addition, this species can be easily and homogeneously cultivated.

On the findings mentioned above, *Helianthus annuus* was selected as model plant in our experiments. The purpose of the study was to assess the toxicity, uptake and metabolism of TNT by this plant grown in hydroponic cultures containing TNT levels of 5, 10, and 20 mg/L. Root tissues were extracted to qualify and quantify extractable concentrations of nitroaromatic compounds (NACs).

4.2 MATERIALS AND METHODS

Chemicals. Described previously in Chapter 3.

Plant cultivation. *Helianthus annuus* L. var. *annuus* (sunflower) seeds were obtained from Aveve (Belgium). Seeds were pre-germinated for four nights at 21°C between rock wool (Grodan, Netherlands) moistened with tap water. Ten seedlings were placed on one polystyrene foam raft with their roots pending in a 3 L PP container. Compressed air was used to obtain good aeration and prevent microaerophilic conditions in the water containers. To develop root biomass, the plants were pre-grown for 14 days in a non-contaminated Hoagland solution (Epstein, 1972) under greenhouse conditions (± 20°C, ± 65% RV) with additional AgroSon T and AQI lamps (photoperiod: 16h of light, 8h of darkness).

Uptake and transformation studies. After two weeks of plant cultivation under standard conditions, a fresh nutrient solution was artificially contaminated with TNT in concentrations of 5, 10, and 20 mg/L respectively. TNT amendment occurred by solving the crystals in H_2O_{dest} (used to dilute the Hoagland stock solutions) at 80°C. The 3 L volume in the containers was adjusted daily by weighing. To control the salt concentration, H_2O_{dest} and half-strength Hoagland nutrient solution were added in turn. Triplicate plant samples were exposed to the

initial TNT concentrations mentioned above. Three plant-free containers containing 5, 10, and 20 mg TNT/L respectively were used as medium control.

Sampling of hydroponic solution. To follow TNT removal from the nutrient solution, triplicate sampling was performed weekly. Therefore, 500 µL aqueous samples were supplemented with 500 µL methanol, mixed thoroughly, and filtered through a 0.45 µm PTFE filter prior to HPLC analysis.

Harvesting of plants. After 21 days of treatment, plants were harvested and the roots from plants grown on the same polystyrene foam raft were harvested together. The roots were rinsed in 100 ml acetic acid (1%, pH 2.6) to remove surface-associated nutrients and NACs by shaking for 15 min. Subsequently, plant roots were dried with paper tissue, weighed and frozen at –80°C. The rinsing solution was analysed by HPLC.

Plant tissue extraction. Frozen plant roots were lyophilised until no ice remained in the sample (approximately 48h). Freeze dried samples were homogenised in a mortar to small pieces, using a pestle and liquid nitrogen. The extraction and purification were performed in triplicates as described by Larson *et al*. (1999).

HPLC analysis. Instruments and running conditions are described in Chapter 3.

Data analysis. The kinetics of TNT removal can be described by a second order rate expression, since the rate of TNT removal is known to be dependent on the TNT concentration and the root biomass concentration: $-(dC/dt) = KPC$ (1), where C = TNT concentration (mg/L), t = time (days), P = wet root concentration (g/L), and K = second-order TNT disappearance rate constant (L/g day). By assuming the plant biomass concentration constant throughout the incubation period, equation 1 reduces to $-(dC/dt) = kC$ (2), where $k = KP$ = first-order rate constant (per day) for a given plant concentration. This k is also often described as a pseudo first-order rate constant and the kinetics, which obey equation 2, pseudo first-order kinetics. Integration of equation 2 leads to $\ln(C/C_0) = -kt$ (3). This equation was used to determine the value of k based on TNT concentration data over the incubation period (Pavlostathis *et al*., 1998).

4.3 RESULTS

4.3.1 Visual observations

Root growth was most affected by the presence of TNT. Visible symptoms of injury to roots consisted of stunted growth and discoloration, which were in proportion to the initial TNT concentration (Figure 4.1). At 20 mg TNT/L, growth of young roots was very strongly reduced and swollen. However, no significant differences in yield (on fresh weight basis as well as on dry weight basis) were observed. Dry weights (g) of roots of 10 plants were 4.04 (± 0.09), 3.99 (± 0.08), 4.21 (± 0.12), and 4.22 (± 0.10) at 0, 5, 10, and 20 mg TNT/L respectively after 21 days of treatment. Shoot length was not significantly affected to a concentration of 20 mg/L (data not shown). In the third week of growth, a light chlorosis was observed in young leaves of plants growing on 10 and 20 mg TNT/L. The nutrient solutions, with or without plants, turned first pink and gradually orange-brown during the experimental period. As observed for the roots, the discoloration was in proportion to the initial TNT concentration.

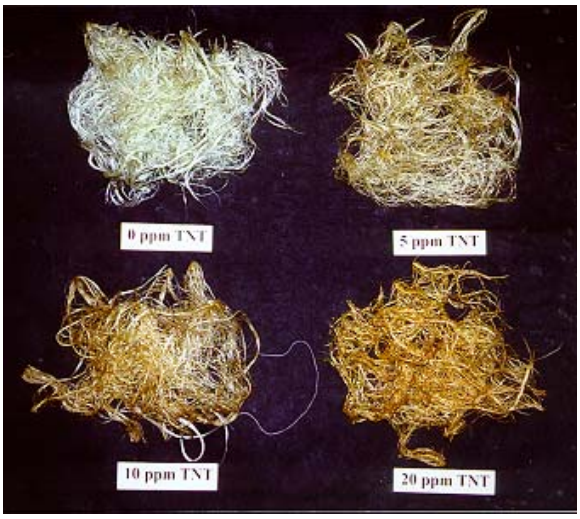

Figure 4.1 Roots from *Helianthus annuus* plants after 21 days of exposure to TNT. The root masses at every concentration originate from 10 plants. The initial TNT concentrations are presented in ppm (mg/L).

4.3.2 Analysis of the hydroponic solutions

Results from the sampling of the hydroponic media are presented in Table 4.1.

Table 4.1 Temporal TNT concentrations in hydroponic cultures with or without *Helianthus annuus* plants

Concentration mg TNT/L	Condition	Time (days) 0	7	14	21
5	**Plants**	5.71 ± 0.50	2.57 ± 0.42	0.78 ± 0.11	0.11 ± 0.07
	Control	6.00	5.50	4.82	4.82
10	**Plants**	10.93 ± 0.12	5.67 ± 0.31	1.56 ± 0.48	0.28 ± 0.03
	Control	11.04	9.99	9.58	8.81
20	**Plants**	21.57 ± 0.51	15.63 ± 1.09	9.26 ± 2.55	5.13 ± 0.55
	Control	22.35	20.24	20.04	18.51

Results are presented in mg TNT/L. Values of plant hydroponics are the average of triplicates ± their respective standard deviation. The control indicates plant-free medium.

Analysis of these media shows a TNT removal of 98, 97, and 76% after 21 days of plant culture when 5, 10, and 20 mg TNT/L were added respectively. TNT disappearance was found to follow a pseudo first-order kinetic. The k and r^2 values are presented in Table 4.2.

Table 4.2 Elimination constants in hydroponic cultures *Helianthus annuus* exposed to TNT

Initial TNT concentration (mg/L)	First-order rate constant (k, per day)	Correlation coefficient r^2
5	0.17	0.95
10	0.16	0.95
20	0.06	0.98

At 5 and 10 mg/L initial TNT contamination, k values were similar, thus independent of the TNT concentration. At an initial TNT concentration of 20 mg/L, this removal rate constant was much lower. Nevertheless, these plants eliminated a larger mass of TNT over time as compared to those plants exposed to lower initial TNT concentrations. Containers with an initial TNT concentration of 20 mg/L showed an average reduction of 16.44 mg/L over the 21 days of incubation, whereas those with an initial TNT concentration of 5 and 10 mg/L had an average decrease of 5.60 and 10.65 mg TNT/L respectively.

In the hydroponic solution, the only TNT transformation products detected were 4-ADNT and 2-ADNT. Other compounds either had limited UV-Visible absorption and/or eluted in the polar solvent front (retention times < 5 min). These ADNTs were not detected in the solutions at the beginning of the experiment. However, as the TNT concentration decreased during the incubation period, ADNTs accumulated and accounted finally for 7, 8 and 11% of the initially added TNT under the corresponding 5, 10, and 20 mg TNT/L contamination conditions.

Plant-free controls were used to obtain information about any adsorptive losses and photolytic breakdown of TNT. A slight decrease of the original TNT concentrations was observed, without the accumulation of other detectable NACs. Furthermore, the fraction that "disappeared" seemed to be independent of the initial concentration, as finally about 20% disappeared from all the controls.

4.3.3 Analysis of the root rinsing solutions

After shaking of the roots for 15 min in an acetic acid solution (1%), the rinsing solution was analysed by HPLC. Under the performed HPLC conditions, four compounds were detected, including TNT, 4-ADNT and 2-ADNT (Table 4.3). The major peak belonged to an unknown polar compound with a retention time of 5.70 min. Addition of acetic acid (1%) to TNT and ADNT standards did not show an extra peak beyond the polar solvent front, indicating that the unknown compound is not the result of a reaction between one of these nitroaromatics and acetic acid. No further attempts were made to identify the unknown compound. The shaking of the roots with the 1% acetic acid solution caused some lysis of the cells, as the root appeared somewhat "slimy" after the treatment. This eventually could have caused some leakage of TNT and its metabolites from the cells. Rinse off with the acetic acid solution or simply with H_2O_{dest} is suggested for future experiments.

Table 4.3 Analysis of the acetic acid solutions used to rinse *Helianthus annuus* roots after 21 days of exposure to TNT

	Initial TNT concentration (mg/L)			
Compound	**0**	**5**	**10**	**20**
	µg/g dry weight (*)			
Unknown	N.D.	6954327 ± 818065	8834231 ± 1537555	9443091 ± 1029561
TNT	N.D.	N.D.	N.D.	15.56 ± 2.31
4-ADNT	N.D.	10.46 ± 4.08	13.89 ± 3.39	12.03 ± 1.34
2-ADNT	N.D.	N.D.	5.91 ± 0.17	13.99 ± 6.63

*, The value of the unknown compound is presented in area counts/g dry weight by lack of a standard. N.D., not detected. The results are presented as mean values of three replicates ± their respective standard deviation.

4.3.4 Analysis of root tissues

After 21 days of incubation, plant root tissues were extracted and analysed. The only detected NACs were 4-ADNT and 2-ADNT in similar amounts (values were not significantly different although 4-ADNT concentrations seem slightly higher), and a limited small quantity of the parent compound TNT (Table 4.4). Though total extractable nitroaromatic concentrations increased with increasing TNT contamination, at 5 and 10 mg/L initial TNT contamination, the fractions of ADNT and TNT were similar and accounted for about 97 and 3% respectively. At 20 mg/L, extractable ADNT and TNT fractions were 78 and 22% respectively. Thus, in the range of the TNT contamination tested (5 to 20 mg/L), ADNT root concentrations levelled off, however, a strong increase in TNT root concentrations was observed.

Table 4.4 Analysis of NACs in *Helianthus annuus* root tissues after 21 days of exposure to TNT

	Initial TNT concentration (mg/L)			
Compound	**0**	**5**	**10**	**20**
	µg/g dry weight			
TNT	N.D.	0.94 ± 0.29	3.52 ± 1.16	16.19 ± 2.57
4-ADNT	N.D.	6.62 ± 1.10	19.34 ± 1.67	26.49 ± 3.23
2-ADNT	N.D.	5.30 ± 0.66	17.80 ± 2.02	23.86 ± 2.51

N.D., not detected. The results are presented as mean values of three replicates ± their respective standard deviation.

4.4 DISCUSSION

Visible symptoms of TNT toxicity were mainly observed at the level of the roots of *Helianthus annuus*. Though visible observation indicated a stronger reduction of root growth with increasing initial TNT concentrations, no significant difference in biomass was observed compared to the control. This probably was the result of the production of a shorter but swollen root system. The symptoms of injury (root discoloration and root growth restriction) are typical biological responses to TNT (Palazzo and Leggett, 1986; Peterson *et al*., 1996; Peterson *et al*., 1998; Won *et al*., 1976). Seedlings of *Panicum virgatum* (switchgrass), *Bromus inermis* (smooth bromegrass) and *Festuca arundinacea* (tall fescue), developed a swollen meristematic region of the primary root, indicating a disrupted cell division (Peterson *et al*., 1996; Peterson *et al*., 1998). In the present study, the observed abnormal, swollen tissue can be explained by similar disrupted cell divisions. However, the above mentioned authors reported a lower plant yield than the control in the presence of TNT, which we did not.

At all tested concentrations, the presence of plants resulted in an efficient decrease of TNT in the medium over the experimental period (Table 4.1). Based on the available experimental data we concluded that between 5 and 10 mg/L initial TNT contamination, the rate constants could be considered similar (Table 4.2). An initial TNT concentration of 20 mg/L resulted in a lower rate constant for the elimination of TNT in the medium, indicating a toxic threshold value for *Helianthus annuus* between 10 and 20 mg TNT/L. The toxicity of explosives has been evaluated in screening studies (Palazzo and Leggett, 1998; Pavlostathis *et al*., 1998; Peterson *et al*., 1998; Thompson *et al*., 1998b), and TNT is found to be toxic to plants within the range of 1 to 30 mg/L in hydroponic systems.

The TNT disappearance from the solutions was found to follow a pseudo first-order kinetic. In previous reports with whole plants (Pavlostathis *et al*., 1998) and with cell cultures (Wayment *et al.*, 1999) pseudo first-order kinetics were described. Pavlostathis *et al*. (1998) illustrated that a pseudo first-order assumption

is appropriate at low TNT/biomass ratios, but not at higher TNT concentrations (> 50 mg/L).

In addition, the disappearance of TNT in the plant-containing hydroponic solutions resulted in the formation of low concentrations of 2-ADNT and 4-ADNT, which were absent in the control solutions. The presence of ADNTs suggests transformation of the parent TNT molecule either by *Helianthus annuus* roots or their associated microflora. Various studies reported the formation of traces of ADNTs in the media (always less than 20% of the initially added TNT) (Best *et al*., 1999; Bhadra *et al*., 1999a, 1999b; Görge *et al*., 1994; Harvey *et al*., 1990; Hughes *et al*., 1997; Larson *et al*., 1999; Palazzo and Leggett, 1986; Thompson *et al*., 1998a; Vanderford *et al*., 1997; Wayment *et al*., 1999).

Polypropylene, polystyrene, and glass showed limited adsorption capacities of TNT and ADNTs (data not shown). Therefore, photodecomposition is suggested to be the major cause of decrease of TNT in the controls (without plants). TNT solutions were sporadically exposed to light during replenishment of the nutrient solution and sampling. The solution turned first pink and gradually slightly orange-brown. Görge *et al*. (1994) reported that TNT solutions of more than 10 mg/L, exposed to growth chamber lights, turned pink to brown, and observed an average decrease of 33% of the total TNT amount in the control solutions after 28 days. Best *et al*. (1999) found a decrease in TNT levels in groundwater controls of 22% in darkness and 40% in light, showing a significant effect of photolysis.

In extractions of *Helianthus annuus* roots very little of the initially added TNT was recovered (Table 4.4). This can be attributed to phytodegradation, i.e. NACs remain within the plant in an unextractable form and/or exist in a form undetectable by the HPLC method employed. TNT, 2-ADNT and 4-ADNT were the only NACs. In addition, the concentration of ADNTs in root tissues was much higher than the concentration of TNT in root tissues. This is consistent with previous reports on TNT transformation within the plant (Görge *et al*., 1994; Harvey *et al*., 1990; Hughes *et al*., 1997; Larson *et al*., 1999; Palazzo and Leggett, 1986; Scheideman *et al*., 1998; Thompson *et al*., 1998a; Vanderford *et al*., 1997).

In our study, 2-ADNT and 4-ADNT concentrations were not significantly different. 4-ADNT is usually the most prevalent compound reported by the authors mentioned above. However, Görge *et al.* (1994) found equal concentrations of 2-ADNT and 4-ADNT in root tissues of *Medicago sativa* (alfalfa).

Extractable NACs from the *Helianthus annuus* roots significantly increased with increasing TNT concentrations, indicating that the uptake of NACs in the plants was related to the initial TNT contamination. However, the ADNT concentration did not keep pace with the increasing TNT content in the roots but levelled off, which suggest a decrease in metabolic activity within the plant, probably due to phytotoxicity.

The sum of extractable nitroaromatic compounds (TNT, 2-ADNT and 4-ADNT) in root tissues accounted for 0.40, 0.74, and 0.56% of the added TNT at 5, 10, and 20 mg/L respectively, whereas 9, 11, and 38% of the added TNT remained in the medium and 0.37, 0.36, and 0.35% was found in the root rinsing solution. Taking into account the 20% disappearance from the plant free-controls, 70.23, 67.90, and 41.09% of the added TNT was lacking in the mass balance of the 5, 10, and 20 mg/L hydroponics respectively. As the percentage lacking in the mass balance decreased with increasing TNT concentration, and thus increasing toxicity, proves again that TNT removal is an active process in plants. Unfortunately, the contribution of the unidentified compound in the rinsing solution (Table 4.3) to the mass balance is unknown. Nitroaromatic explosive compounds are reported to bind irreversible (covalent) to the root surface by their amino or hydroxylamino functional group (Hughes *et al*., 1997; Thompson *et al*., 1998a). However, it is not unlikely that the acetic acid could have removed sorbed compounds that are otherwise difficult to extract (Hughes, pers. comm.). Furthermore, incorporation of TNT into different cell wall fractions of the root (unextractable TNT fraction) and translocation to aerial parts should be examined to determine a complete mass balance. The final fate of TNT using [ring-^{14}C]TNT is evaluated in the experiments described in Chapter 5. Up to date neither significant mineralisation nor the

production of volatile organic transformation products is observed, and therefore we did not concentrate on these processes.

CHAPTER 5
TEMPORAL MASS BALANCE OF TNT AND ITS METABOLITES IN *HELIANTHUS ANNUUS* (SUNFLOWER) HYDROPONIC SYSTEMS

5.1 INTRODUCTION

The intrinsic capacity of plants to take up and transform xenobiotic chemicals has been demonstrated with numerous organic compounds, including herbicides (Hatzios and Penner, 1982; Hatzios, 1991; Sandermann, 1994; Schnoor *et al*., 1995) and explosives (Hughes *et al*., 1997; Vanderford *et al*., 1997; Bhadra *et al*., 1999). Critical to any remediation process is an understanding of the metabolic fate of transformation products in order to elucidate the mechanisms of detoxification. The green liver model is often used to describe the fate and disposition of xenobiotic compounds within plants (Rivera *et al*., 1998). During detoxification, xenobiotic compounds are metabolised in three stages: transformation, conjugation, and sequestration. More detailed information on the subject can be found in Chapter 1.

The ability of *Helianthus annuus* (sunflower) to take up and transform TNT has been demonstrated in Chapter 4. In the present chapter, the fate of TNT in *Helianthus annuus* hydroponic systems was investigated using [ring-^{14}C]TNT. The first objectives of this study were to investigate the time course of uptake, the fate of [ring-^{14}C]TNT within the plant anatomical structures, and the time course of product incorporation into plant biomass. Subsequently, the sustainability of the plant system to multiple consecutive TNT additions was examined.

5.2 MATERIALS AND METHODS

Chemicals. 2,4,6-Trinitrotoluene was received from J. C. Libouton, Nobel Explosifs, Centre de Recherches, Châtelet, Belgium. [Ring-^{14}C]-2,4,6-trinitrotoluene was provided by Chem Syn Science Laboratories (KS, USA), with a specific activity of 21.58 mCi/mmol and a purity of 98%. An internal standard kit for liquid scintillation couting (capsules of ^{14}C-O) was purchased from Wallac (Finland).

Plant cultivation. Culture of *Helianthus annuus* was previously described in Chapter 4. However, compressed air was provided by the use of a perforated PVC tubing, which was fixed with silicon sealant onto the bottom of the container. The work was performed in a non-conditioned, restricted greenhouse (Studie Centrum voor Kernenergie, Mol, Belgium). Additional light was provided by AgroSon T lamps (photoperiod: 16h of light, 8h of darkness).

Uptake and transformation studies. Nutrient solutions of two-week old hydroponic cultures of sunflower were supplemented with TNT by adding 10000 Bq [ring-^{14}C]TNT/L as well as 10 mg unlabeled TNT/L. This concentration was chosen because it is a high but still realistic TNT concentration that can occur in ammunition contaminated groundwater (Table 3.1). A stock solution of [ring-^{14}C]TNT for culture amendment was prepared in ethanol (120000 Bq/ml). A 0.6% stock solution of cold TNT was also prepared in ethanol. *Helianthus annuus* plants (10 plants/container) were grown on five 3-L containers, of which three were employed for the study of TNT uptake and transformation, and two as plant control. An additional single container served as medium control. After 3, 7, and 21 days of incubation, systems were dismantled. Plant roots, stems, and leaves were harvested separately and plant roots were rinsed with distilled water. Plant tissues were weighed, frozen, lyophilised for 48 hours, and powdered using a

laboratory ball mill. Mixed tissue samples of 10 plants originating from single containers, were obtained.

In addition, two-week old hydroponic cultures of *Helianthus annuus* were used for the study of adsorption and tolerance: four cultures were supplemented with TNT, and one served as plant control. Two medium controls (plant-free) were also prepared, one with 0.1% sodium azide (metabolic inhibitor) and one without. Addition of sodium azide to one of our plant systems caused death of the plants within 20 minutes, as they "fainted" completely. Nutrient solutions were contaminated by adding [ring-^{14}C]TNT that would yield about 53333 Bq/L as well as 10 mg/L unlabeled TNT. A stock solution of 925000 Bq/ml [ring-^{14}C]TNT was prepared in ethanol. At day 7 and 14, the prevailing ^{14}C activities in the media and the corresponding TNT concentrations were brought back to their initial values (without replacing the nutrient solutions). Harvesting of plants at day 3, 7, 14, and 22 and sample preparation were performed as described earlier this chapter.

Analytical instruments and methods. ^{14}C was quantified with a Tri-Carb 1600TR Liquid Scintillation Analyser (LSA) (Canberra Packard). For the analysis of TNT uptake and transformation, one-ml samples (day 0, 1, 2, 3, and 4) or 2 ml samples (day 7, 10, 15, and 21) of the nutrient solution were added to disposable scintillation vials containing 19 or 18 ml of Insta-Gel plus (Packard) respectively. Samples were taken in triplicates. Counting times were 15 min at day 0 and 1, 30 min at day 2 and 3, 60 min at day 4 and 7, and 90 min at day 10, 15, and 21. A modification in the method was applied when measuring ^{14}C activity in the nutrient solutions of the second study, where multiple consecutive additions were performed. One-ml samples were added to the scintillation vials containing 19 ml of Insta-Gel plus (Packard). Counting times were 10 min. The internal standard showed an efficiency of the LSA of 92.9% (± 0.1%).

The fraction of ^{14}C associated with plant matter was determined by combusting plant samples with a Biological Material Oxidiser (BMO) (Beckman) and collecting $^{14}CO_2$. Therefore, a predetermined weight of plant sample was combusted at 900°C for 4 min under a stream of oxygen. The $^{14}CO_2$ that evolved

was trapped in 10 ml Carbo Max plus (Packard). The trap was rinsed twice with 5 ml of a methanol-toluene mixture (75% MeOH, 25% toluene). The Carbo Max and the rinsing solution were pooled and ^{14}C quantified with the LSA. LSA counting times were 20 min. The internal standard indicated an efficiency of the BMO of 94.3% (± 2.8%).

Mass Balance Analysis. Medium samples were taken for ^{14}C analysis. Total plant-associated ^{14}C was determined by oxidation of 0.150 g freeze-dried plant samples (in duplicate). The amount of extractable ^{14}C associated with plant tissue was evaluated using a modification of the method described by Bhadra *et al*. (1999a). Duplicate 0.150 g tissue samples were extracted three times, each time for 5 hours, with 10 ml methanol in a water-bath sonicator. The temperature was controlled below the flash point of methanol (56°C) with ice. The extract was separated from the solid residue by centrifugation for 15 min at 13000 rpm. Following three extractions of each sample replicate, the extracts were pooled, evaporated, and combusted in the BMO. Non-extractable ^{14}C that was bound to plant biomass was determined by combusting the residue of the previously extracted tissue samples. The sum of extractable and non-extractable ^{14}C was related to the corresponding total plant-associated ^{14}C in order to determine the loss of activity during extraction. $^{14}CO_2$ was not trapped during plant treatment. However, mineralisation and subsequent losses by evapotranspiration through the plant stomata are unlikely to occur, as to date significant mineralisation has never been observed.

5.3 RESULTS

5.3.1 Impact of a single TNT addition to the plant system

According to the protocol described above, three plant samples were exposed to a TNT concentration of 10 mg/L. One plant-free control, containing only the TNT solutions, was used to determine biotic and/or abiotic losses. Two controls containing *Helianthus annuus* plants in a TNT-free nutrient solution were used to

determine toxic effects of TNT to plant health and to determine plant background ^{14}C-values.

5.3.1.1 Phytotoxic effects

The biological response of *Helianthus annuus* to TNT was different from the one in Chapter 4, where no significant effect on root yield was observed after 21 days. In the present study, root growth (expressed as dry weight) was most affected, followed by the leaves (Table 5.1). After 21 days, root and leave dry weights decreased with 38.7% and 25.3% respectively when grown in the presence of TNT. Stem yield was not affected. Total plant biomass was 15.2% lower compared to the control. Visible symptoms of injury to roots consisted, as in the experiment described in Chapter 4, of discoloration and growth restriction. However, the development of strongly reduced and swollen new roots was less pronounced. New leaves of treated plants were smaller, and showed chlorosis. Stem morphology was the least affected.

Table 5.1 Plant dry weights of *Helianthus annuus* grown in 10 mg TNT/L for 3, 7, and 21 days

	^{14}C-TNT			Plant control	
Compartment	**Day 3**	**Day 7**	**Day 21**	**Day 3**	**Day 21**
Leave	2.33	2.83	15.12	2.45	20.23
Stem	1.90	2.45	19.82	1.97	18.92
Root	0.71	0.93	4.60	0.74	7.50
Total	4.95	6.21	39.54	5.16	46.65

A single addition of TNT was applied on day 0. Dry weights are expressed in g.

5.3.1.2 Nutrient solution analyses

The hydroponic solutions were sampled and analysed in time (Figure 5.1). As for the study in Chapter 4, a first-order kinetic for ^{14}C-TNT disappearance in plant systems dependent on medium concentration with time was demonstrated. In the present study, a rate constant k of 0.20 per day (r^2 = 0.97) was found, which seems to indicate a more efficient nitroaromatic compound removal compared to the

study in Chapter 4, where a constant *k* of 0.16 per day (r^2 = 0.95) was found at 10 mg TNT/L. In addition, a first-order rate constant of 0.05 per day (r^2 = 0.91) was found in the plant-free control, whereas a *k*-value of 0.01 per day (r^2 = 0.97) was found in the study described in Chapter 4. However, the very low recovery of nitroaromatic compounds from the plant-free control in this study (44.9%) compared to the experiment in Chapter 4 (80.8% at 10 mg/L), already indicates an effect due to a modification in experimental set-up. An adsorption test on the materials used in the present experiment, demonstrated a significant contribution of ^{14}C adsorption by the PVC tubing and the silicon sealant (data not shown).

At the end of the exposure time, 97.1% of the initially added ^{14}C disappeared in the plant system, which is comparable to the 97.5% disappearance of added TNT in Chapter 4 at 10 mg/L. However, a comparison between both studies can not exactly be made. First, the contribution of adsorption in the present study could not be assessed. Desorption processes from materials might be bigger in the plant systems compared to the plant-free controls. And second, ^{14}C concentrations (this Chapter) are compared to HPLC detectable NAC concentrations (Chapter 4).

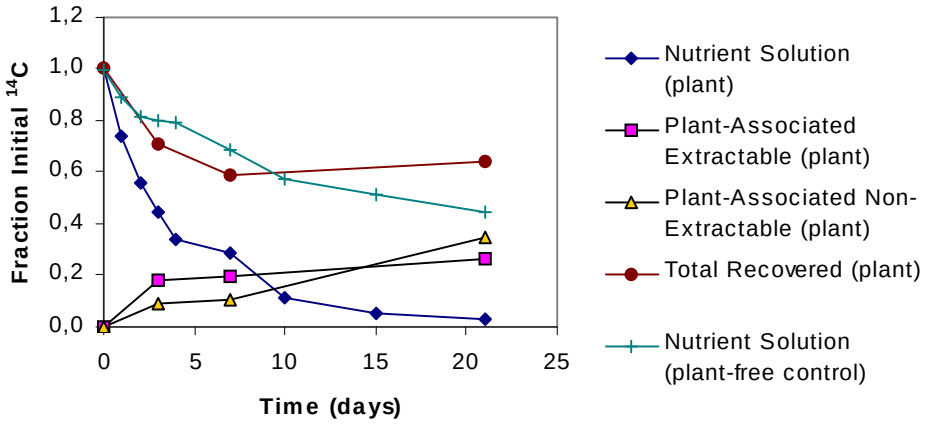

Figure 5.1 Raw mass balance of ^{14}C in hydroponic systems of sunflower over a period of 21 days. The initial TNT concentration was 10 mg/L.

5.3.1.3 Plant tissue extractions

One of three plant systems exposed to TNT was dismantled at day 3, 7, and 21 of incubation, and tissue extractions were performed. The study provided no information on the identification of TNT-products, they were only characterised as bound or extractable. Results are presented in Table 5.2.

Table 5.2 Percent recovery of initial ^{14}C added in plant anatomical structures of *Helianthus annuus* and aquatic medium

Compartment		Day 3 % of added	Day 7 % of added	Day 21 % of added
Leave	**B**	0.31	0.58	0.63
	E	0.50	0.75	0.70
Stem	**B**	1.43	1.83	2.84
	E	0.42	0.41	0.45
Root	**B**	7.25	8.11	31.92
	E	16.96	18.79	26.01
Total Plant	**B**	8.99	10.52	35.39
	E	17.88	19.95	27.16
Medium		44.22 ± 5.71	28.34 ± 12.22	2.92
Total		71.09	58.81	65.47

Initial concentrations were 10 mg TNT/L and 10000Bq [ring-^{14}C]TNT/L. B, bound fraction; E, extractable fraction. Values of plant-associated ^{14}C are the average of two extractions of a tissue mixture originating from 10 plants. Medium values are the average of triplicate samples (± standard concentration) from three, two, and one container(s) at day 3, 7, and 21 respectively.

Leave ^{14}C concentrations were very low, and showed a minor increase in the bound and extractable fraction over the time investigated. Stems showed no significant difference in extractable ^{14}C between 3 and 21 days, however, there was a gradual rise in the bound fraction. For roots, the bound as well as the extractable fraction strongly increased during the experimental period. Quantitatively, most of the ^{14}C was always recovered in the roots of the plants. Following 3, 7, and 21 days of exposure, 90.3%, 88.5%, and 92.6% of the total plant associated ^{14}C accumulated in the root tissues, whereas about 6.7%, 7.2%, and 5.3% was recovered in the stem, and 3.0%, 4.3%, and 2.1% was found in leaf tissues respectively. The extractable plant-associated fractions accounted for the majority of the products at day 3 and 7, and comprised respectively 66.8% and 65.3% of the total ^{14}C present in the plant.

Analysis at day 21 revealed an increase in the bound fraction. At that time, the extractable fraction was as much as 43.5% of the plant-associated ^{14}C, whereas the bound fraction accounted for 56.5%, compared to 33.2% and 34.7% at day 3 and 7 respectively. A comparison with the results of tissue extraction in Chapter 4 can not be made, as the NACs (TNT, 4-ADNT, 2-ADNT) in the extractions of this study could not be identified and quantified by HPLC analysis. However, the extremely low fraction of detected extractable NACs in Chapter 4 (0.7% of the initially added TNT after 21 days) compared to the fraction in this chapter (43.5% of the initially added TNT after 21 days), suggest a major presence of conjugates and other TNT-transformation products (e.g. oxidation metabolites) in the extracts. These latter metabolites could not be detected by applied the HPLC method. In addition, leakage of NACs out of the cells due to shaking for 15 min in an acetic acid solution could have removed most extractable compounds in the experiment performed in Chapter 4.

5.3.1.4 Mass balance

The plant-associated ^{14}C accounted for 26.9%, 30.5%, and 62.6% of the initially added ^{14}C at day 3, 7, and 21 respectively. The percentage of ^{14}C recovered from the plant nutrient samples amounted for 44.2%, 28.3% and 2.9% of the initially added ^{14}C, respectively. Taking into account the 5.8%, 8.2%, and 10.8% loss of activity during tissue extractions (data not shown) at day 3, 7, and 21 respectively, would yield a mass balance of 76.9%, 67.0%, and 76.3% at day 3, 7, and 21 respectively. However, to make the complete mass balance, losses due to adsorption should be included. Due to possible bigger desorption processes present in the plant systems, these losses could not be assessed based on sorption values in the plant-free systems.

5.3.2 Impact of multiple consecutive TNT additions to the plant system

Subsequently, four plant systems were exposed to TNT for three weeks, however, every seventh day, concentrations were brought back to the initial concentration. Two plant-free controls, one containing only TNT and the other in addition sodium azide, were used to make a distinction between biotic and abiotic losses. One plant control contained *Helianthus annuus* in a TNT-free nutrient solution.

5.3.2.1 Phytotoxic effects

The phytotoxic effects observed were similar as described in the former experiment: leave chlorosis (young leaves), and a general growth inhibition. In addition, the margin of young leaves curled downwards, and some older leaves showed necrosis. However, during the experiment plant growth continued (Table 5.3), indicating sunflower "tolerated" TNT concentrations to 10 mg/L.

Table 5.3 Plant dry weights of *Helianthus annuus* grown in 10 mg TNT/L for 3, 7, 14 and 22 days

	^{14}C-TNT				Plant control
Compartment	**Day 3**	**Day 7**	**Day 14**	**Day 22**	**Day 22**
Leave	1.13	1.58	3.38	8.37	11.87
Stem	1.13	2.01	5.03	12.06	19.07
Root	0.31	0.37	1.17	2.65	3.11
Total	2.57	3.96	9.58	23.08	34.05

Addition of TNT occurred at day 0, 7, and 14. Dry weights are expressed in g.

5.3.2.2 Nutrient solution analyses

Results from the temporal sampling of media are presented in Figure 5.2. Three sequential TNT and [ring-^{14}C]TNT additions were made, each to their initial concentration. The plant systems achieved a higher extent of TNT removal each consecutive 7-days, as in order 53.1%, 65.0%, and 73.0% of the initial ^{14}C disappeared. In addition, the *k*-values of the first-order TNT disappearance kinetics observed in the plant systems were 0.12 (r^2 = 0.95), 0.14 (r^2 = 0.98) and 0.18 (r^2 = 0.83) per day over the three consecutive additions. Comparing the first *k*-value of

this experiment (0.12 per day at the start) to the *k*-value of the experiment mentioned before in this chapter (0.20 per day, paragraph 5.3.1.2), demonstrates a less efficient TNT-removal in the present experiment. This can be explained by a lower root biomass (Table 5.1 versus Table 5.3). The lower growth rate is probably caused by seasonal differences in temperature, light intensity, light quality, and humidity, as the first study was performed in late summer (September 2000; mean outdoor temperature: 16°C; 125 h of sunshine) and the present in spring (April 2001; mean outdoor temperature: 8°C; 95 h of sunshine).

One plant-free control, containing natrium azide in addition to the TNT solutions, was used to determine any potential mineralisation loss by native microorganisms. Figure 5.2 shows that both plant-free controls decreased slightly and parallel to each other, indicating a loss exclusively due to adsorption. Average losses were 9.2%, 13.9%, 9.3%, and 10.3% at day 3, 7, 14, and 22 respectively.

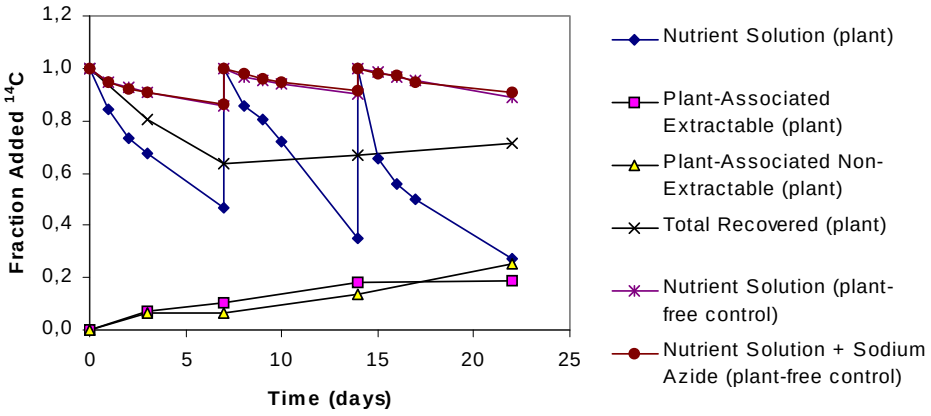

Figure 5.2 Raw mass balance over three consecutive additions in hydroponic cultures of sunflower. The maximum TNT concentration was 10 mg/L.

5.3.2.3 Plant tissue extractions

Concerning distribution of TNT throughout plants, similar conclusions can be drawn as for the previous study: the majority of TNT-metabolites are (1) mainly

located at the root level of the plants, and are (2) increasingly characterised as bound residues, indicating sequestration. Results from leaf- and stem-associated ^{14}C (both extractable and non-extractable values) at day 3 and 7 showed that a similar fraction of the added TNT was transported to the aerial parts as observed in the first study (Table 5.4 versus Table 5.2). However, total root-associated ^{14}C values were 2.1 to 2.4 times smaller than the first study. Linking these results to plant dry weights (Table 5.1 versus Table 5.3), demonstrate relative higher transport rates of ^{14}C from the roots to the shoots in the second study. The uptake and transport rate of a specific contaminant is controlled by the transpiration flow, which is, for a given plant species, influenced by external factors such as temperature, light intensity, and air humidity. It might be possible that the stomata, controlling respiration, were more closed in September (first study) as protection against the lower air humidity.

Table 5.4 Percent recovery of added ^{14}C in plant anatomical structures of *Helianthus annuus* and aquatic medium

Compartment		Day 3 % of added	Day 7 % of added	Day 14 % of added	Day 22 % of added
Leave	**B**	0.35	0.53	0.59	0.89
	E	0.45	0.65	0.63	0.95
Stem	**B**	1.89	1.87	2.47	3.80
	E	0.55	0.67	1.05	0.56
Root	**B**	4.04	4.00	10.89	20.90
	E	5.91	8.9.	16.23	17.24
Total Plant	**B**	6.28	6.40	13.95	25.59
	E	6.92	10.28	17.91	18.75
Medium		67.38 ± 1.41	46.94 ± 2.11	34.96 ± 2.42	27.24
Total		80.58	63.62	66.82	71.58

Added were 10, 10, 16, and 23 mg TNT/L and 53333, 53333, 85333, and 122666 Bq [ring-^{14}C]TNT/L at 3, 7, 14, and 22 days. B, bound fraction; E, extractable fraction; n.a. not available. Values of plant-associated ^{14}C are the average of two extractions of a tissue mixture originating from 10 plants. Medium values are the average of triplicate samples (± standard concentration) from four, three, two, and one container(s) at day 3, 7, 14 and 22 respectively.

A linear correlation was found between the root bound ^{14}C and the root dry weight (Figure 5.3), suggesting that throughout the experiment additional adsorption and

sequestration occurred mainly at and in newly formed root tissue. Growing roots vary both anatomically and physiologically along their longitudinal axes. In general, there is a tendency for the rate of metabolic activity per unit root length to decline as the distance from the apex increases (Marschner, 1995).

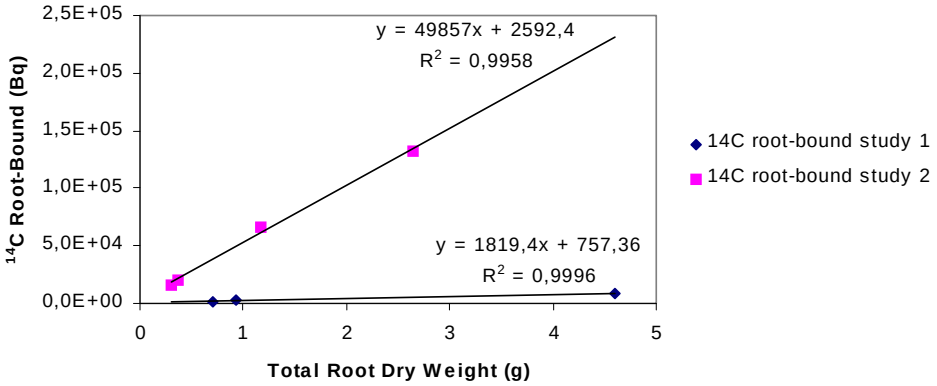

Figure 5.3 Relationship between the bound (non-extractable) activity in the root and the total root dry weight. Data were collected on day 3, 7, and 21 of incubation in study 1 (September) and on day 3, 7, 14, and 22 in study 2 (April). The equations and r^2-values of the linear correlations are displayed on the chart.

5.3.2.4 Mass balance

An attempt was made to provide a mass balance. Plant-associated ^{14}C accounted for 13.2%, 16.7%, 31.9%, and 44.3% of the added ^{14}C at respectively day 3, 7, 14 and 22, whereas 67.4%, 46.9%, 35.0%, and 27.4% of the added ^{14}C remained in the medium. Taking into account the the 13.5%, 23.8%, 5.0%, and 8.7% loss of total activity during extraction at the corresponding days, brings the mass balance in the plant systems to 94.1%, 87.4%, 71.9%, and 80.4% respectively. Again, adsorptive losses could not be determined.

5.4 DISCUSSION

Expressed by the percentage of [ring-^{14}C]TNT taken up by the plant, roots contained finally 86.6% to 92.6% of the plant-associated TNT, stems 5.3% to 9.8% and leaves only 2.1% to 4.2%. The presence of TNT in stems and leaves indicates that there occurs transport of TNT and/or metabolites from the roots , which is consistent with the model of contaminant uptake and transport in terrestrial plants (Trapp and McFarlane, 1995). However, roots accumulated by far the greatest amount of ^{14}C, indicating that TNT and its metabolites were not efficiently translocated from the roots to the shoots. The localisation of nitrotoluenes or radiolabel primarily in the root tissue has been reported in several studies (Görge *et al*., 1994; Harvey *et al*., 1990; Thompson *et al*., 1998a; Vanderford *et al*., 1997). Root concentrations in *Allium schoenoprasum* (allium) and *Medicago sativa* (alfalfa) were up to 20 and 30 times higher than shoot concentrations when exposed to 5 mg TNT/L for 28 days (Görge *et al*., 1994). Roots from *Phaseolus vulgaris* (bushbean) plants exposed for 1 and 7 days to 10 mg TNT/L contained approximately 10 times the amount of ^{14}C measured in shoot tissues (Harvey *et al*., 1990). Thompson *et al*. (1998a) found that for poplar cuttings (*Populus* sp. *deltoides* × *nigra*, DN34) up to 75% of the explosive taken up remained in the root tissue after 42 days of exposure to 30 mg TNT/L.

In addition, an increasing percentage of the original compound became tightly bound to the plant structural material over time (Table 5.1, Table 5.2). Studies of ^{14}C binding in *Myriophyllum aquaticum* (parrot feather) by Vanderford *et al*. (1997) demonstrated that after 5 days of incubation, the plant-associated fraction became increasingly resistant to extraction with methanol. Additional studies focused on ^{14}C distribution in axenic root cultures of *Catharanthus roseus* (periwinkle) during the initial stages of TNT metabolism (Bhadra *et al*., 1999a). Plant tissues converted the TNT taken up rapidly to an unidentified intracellular extractable fraction, which was subsequently converted to an intracellular bound residue. Thompson *et al*. (1998a) found that the majority of TNT was not

extractable from plant tissues of poplar cuttings. During the detoxification of TNT, the compound is metabolized in three stages: transformation, conjugation and sequestration (Burken *et al*., 2000; Dietz and Schnoor, 2001). Sequestration of TNT-derived molecules is established by the incorporation of conjugates into biopolymers (e.g. lignin) where they are characterized as bound (i.e. non-extractable) residues (Sens *et al*., 1999).

The hypothesis posed in the results that the majority of NACs in the extracts do not consist of TNT, 4-ADNT and 2-ADNT, is not unlikely. Bhadra *et al*. (1999a) examined the formation of fate products of TNT beyond its aminated reduction products in axenic root cultures of *Catharanthus roseus*. Mass balance analysis indicated that 75 h after TNT amendment, extractable conjugates comprised 22% of the initial TNT radiolabel, bound residues comprised 29%, 2-ADNT was 4% and the rest remained unidentified. In addition, the same group investigated TNT transformation processes in aquatic systems of *Myriophyllum aquaticum*. Mass balance analysis 12 days after TNT application revealed that oxidation metabolites comprised 35.9% of the initial radiolabel, conjugates 2%, binuclear metabolites another 5.6%, whereas TNT and ADNTs accounted for 10% and 7.6% respectively. Bound residues comprised 33%, and 6% remained unidentified (Bhadra *et al*., 1999b).

Mass balances (adsorptive losses excluded) were performed in our experiments. The results show after 21 days an adsorptive loss of about 35.1 to 55.1% of ^{14}C-activity in the plant-free control and about 19.6 to 23.7% in the plant systems. The lower medium concentration of NACs in the plant systems might result in more desorption of the compounds from the materials (PVC, silicon sealant), which might explain lower adsorptive losses in the plant systems. Special attention should be paid to the sorption characteristics of materials used in an experiment, especially when working with (moderate) hydrophobic compounds.

Root and leaf dry weights reduced respectively 38.7% and 25.3% in the first experiment, and 14.8% and 29.5% in the second experiment when exposed to TNT. Stem yield was not affected in the first experiment, but decreased 36.8% in the

second. The total plant yields were 15.2% and 32.2% lower than the controls in experiment one and two respectively, indicating an extra growth inhibition (2-fold) in the latter experiment due to general higher solution concentrations resulting in higher toxicity. The effect of TNT (0, 5, 10 and 20 mg/L) on *Cyperus esculentus* (yellow nutsedge) was assessed by Palazzo and Leggett (1986). After 42 days of exposure, total plant yields were 54% to 74% lower in the presence of TNT compared to the control. A TNT-contaminated effluent also reduced growth of *Festuca arundinacea* (tall fescue) plus *Lolium perenne* (ryegrass) and *Medicago sativa* (alfalfa) plus *Dactylis glomoerata* (orchardgrass) plus *Lolium perenne* (Palazzo and Leggett, 1983). The higher yield of the tall fescue-ryegrass mixture than the alfalfa-orchardgrass-ryegrass mixture indicated higher tolerance to TNT of the former plant combination. The phytotoxicity threshold for TNT and its degradation products has shown to be dependent on plant species, the duration of the toxic pressure, and is influenced by climatological conditions (e.g. temperature, light quantity and quality influence factors such as metabolic activity and transpiration).

CHAPTER 6
PHYTOREMEDIATION OF TNT CONTAMINATED SOIL

6.1 INTRODUCTION

Uptake and transformation of nitroaromatic compounds (NACs) by microorganisms, fungi, or plants can contribute to the natural attenuation of the compounds in the subsurface and provide the basis for various technologies to remediate contaminated sites. Natural attenuation also comprises NAC-interactions with soil matrix constituents. Clay minerals (e.g. montmorillonite) and the soil organic matter (mainly humic substances) are known as the major sorbents in soil. Binding studies with humic acids and clay minerals indicate that an irreversible binding of certain reduction products of TNT, such as the isomeric hydroxylaminodinitrotoluenes or TAT, with soil components is inevitable (Haderlein *et al*., 1996; Weissmahr *et al*., 1998; Parris, 1980; Thorn *et al.*, 1996). However, it is clear that abiotic and biotic processes that are relevant to the fate of NACs are often interconnected and occur simultaneously. For instance, slow desorption of NACs from the solid to the aqueous phase may limit the bioavailability and thus the rate of biodegradation in the subsurface. Conversely, microbial activity creates an anoxic environment that favours various abiotic reduction processes of NACs. However, still little is known about the subsurface interactions, making soil a complex matrix to work with.

Research in the area of rhizodegradation has been performed for insecticides (Hsu and Bartha, 1979), herbicides (Sandman and Loos, 1984; Lappin *et al.*, 1985), trichloroethylene (Walton and Anderson, 1990), pentachlorophenol (Ferro *et al*., 1994), and PAH (Aprill and Sims, 1990; Reilley *et al*., 1996). Low concentrations

of TNT in the rhizosphere of *Phaseolus vulgaris* (bushbean) and high concentrations of TNT in the bulk soil were reported by Klunk *et al.* (1996). Scheidemann *et al.* (1998) demonstrated that four of the six tested cultivars of *Triticum aestivum* (wheat) were able to significantly reduce the TNT concentration in the rhizosphere soil. In all cases dissipation of the compounds tested was enhanced in the rhizosphere due to enhanced biodegradation by microorganisms, plant-derived degradative enzymes, or uptake into the plant. Biological processes that lead to a complete mineralisation of TNT are not yet available. Research indicates that if plants can be established on TNT-contaminated soil, the rhizosphere can accelerate reductive transformation of TNT and promote bound-residue formation (Kreslavski *et al.*, 1999).

The response of a plant species to munitions compounds such as TNT is needed to determine its phytoremediation potential. Our objective was to determine the effects of TNT on germination and early seedling development of *Helianthus annuus* (sunflower) on field soils. In addition, uptake of the contaminants by the plants and the dissipation of NACs in the rhizosphere were evaluated.

6.2 MATERIALS AND METHODS

Soil conditioning. The contaminated soil was obtained from a former ammunition production site (Belgium) and contained 3476 mg TNT/kg. The soil type was sand (95.5%) with 1.22% humus and a pH of 7.55. To obtain a concentration gradient of 0, 10, 50, 100, 250, 500 and 1000 mg TNT/kg, this soil was mixed with a prepared standard soil with the same characteristics. The standard soil was a mixture of 18% soil from Bilzen (Belgium), which contained 4.17% humus and had a pH of 7.15, and 82% sand from the Teut (Zonhoven, Belgium), which contained 0.61% humus and showed a pH of 5.60. To augment the pH of the standard soil, 0.1% of seaweed lime was supplemented. In addition, 10% compost (on wet weight basis) was added

to the mixture of contaminated soil and standard soil in order to promote plant growth in the relative poor sand soil. Of each concentration, nine polyethylene pots were filled with 500 g soil mixture. The soil mixture was left to rest for 11 weeks at field capacity (moistened with distilled water) under controlled greenhouse conditions (± 20°C, ± 65% RV, AgroSon T and AQI lamps, photoperiod: 16h of light, 8h of darkness). Final soil parameters after 11 weeks of "aging" are listed in Table 6.1.

Table 6.1 Parameters of the experimental soils

	CEC meq/100g d.wt	**pH (H_2O)**	**pH (KCl)**	**OMC % humus**	**Cond. µS/cm**
Soil Bilzen	11.3	7.15	6.99	4.17	389
Sand Teut	1.1	5.60	4.40	0.61	64.3
SAS	3.6	7.55	7.54	2.43	228
0-ppm**	4.0	6.86	6.08	5.22	333
10-ppm*	3.9	6.60	6.21	4.96	373
50-ppm*	3.7	6.57	6.06	5.22	401
100-ppm*	4.2	6.68	6.19	5.57	362
250-ppm*	3.8	6.85	6.27	2.26	360
500-ppm*	3.7	6.98	6.50	2.52	478
1000-ppm*	3.9	7.25	6.84	2.83	422
Compost	65.6	5.01	4.67	31.30	696

Table 6.1 (continue)

	Heavy Metals - mg/kg d.wt.				
	Cd	**Cu**	**Ni**	**Pb**	**Zn**
Soil Bilzen	< 2.0	5.7	< 5.0	24	65.7
Sand Teut	< 2.0	1.6	< 5.0	11	12.0
SAS	5.8	25.9	8.9	364	404.0
0-ppm**	< 2.0	3.1	< 5.0	< 10	29.6
10-ppm*	< 2.0	3.4	< 5.0	< 10	34.1
50-ppm*	< 2.0	3.1	< 5.0	< 10	38.1
100-ppm*	< 2.0	3.4	< 5.0	17	41.4
250-ppm*	< 2.0	4.1	< 5.0	28	59.9
500-ppm*	2.2	5.9	< 5.0	55	93.3
1000-ppm*	2.4	9.9	< 5.0	110	161.5
Compost	< 1.0	12.1	< 5.0	13	29.0

SAS, soil ammunition site; ppm, mg/kg d.wt.; **, standard soil; *, specific mixture of standard soil and soil from the ammunition site; CEC, cation exchange capacity; OMC, organic matter content; cond, conductivity; d.wt., dry weight. All analyses were performed in triplicates and mean values are represented, except for the heavy metal analyses (single analysis).

Plant cultivation. In six pots of each TNT concentration, five *Helianthus annuus* L. var. *annuus* seeds (Aveve, Belgium) were planted. Three pots of each TNT concentration were used as plant-free controls. The seeds germinated and developed under the same greenhouse conditions as described above during four weeks. The soils were moistened with distilled water.

Soil sampling and harvesting of plants. After four weeks, the control soils (kept plant-free) and rhizosphere soils (sticking to the roots) were sampled. Plant roots and shoots were harvested. Roots were rinsed with distilled water and dried with a paper tissue. Both plant parts were weighed. Plant parts from replications were combined (e.g. the roots of all samples grown in soil with 43 mg TNT/kg were put together, etc.), and triplicate subsamples from each composite were analysed by HPLC with UV-Visible detection.

HPLC. Soil samples were prepared and analysed as described by EPA method 8330 (1994), plant tissues were prepared and analysed as previously described in Chapter 4.

6.3 RESULTS

6.3.1 Germination and seedling growth

Germination typically began four days after initiation. The effect of the soil's TNT concentration was not reflected in germination percentage, as finally 98% of all *Helianthus annuus* seeds germinated, independent of the TNT concentrations. Root and shoot appearance at a concentration of 6.17 mg TNT/kg soil or less appeared to be similar to that observed for the control plants (grown in uncontaminated soil). Root and shoot growth rates were slightly affected at a concentration of 43 mg TNT/kg soil. Higher NACs concentrations substantially delayed seedling development. Results of root and shoot lengths after 4 weeks of exposure are presented in Figure 6.1.

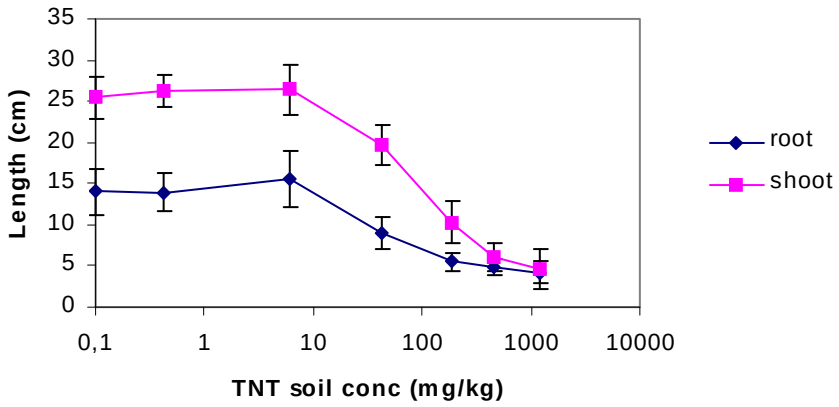

Figure 6.1 Length of *Helianthus annuus* roots and shoots grown in various TNT contaminated soils for 4 weeks. The values are the average of 30 plants.

In addition, the root morphology of plants exposed to 43 mg TNT/kg soil or more was affected (Figure 6.2).

Figure 6.2 Root morphology of *Helianthus annuus* after 4 weeks of exposure to TNT contaminated soil. Soil TNT concentrations are indicated at the bottom.

Primary roots were swollen with stunted secondary roots at 43 mg TNT/kg. Starting at 186 mg TNT/kg soil, secondary roots were expressed as nodules and roots coloured brownish. At a concentration of 186 mg TNT/kg soil or more, some chlorotic spots on the leaves were observed.

6.3.2 Uptake of NAC in plants

No nitroaromatic compounds were detected in the leaves, revealing that TNT and its metabolites were not efficiently translocated from the roots to the shoots. Table 6.2 shows the results of root tissue extractions. Unto a concentration of 43 mg TNT/kg soil, the main NACs detected in roots were 2-ADNT and 4-ADNT. Only small quantities of the parent compound TNT were observed in the root samples at these low TNT concentrations. At a concentration of 186 mg TNT/kg soil, the total ADNT content in the plant still exceeded the quantity of TNT. Higher TNT soil concentration resulted in higher plant-associated TNT concentrations compared to plant-associated ADNT concentrations. In addition, the relative accumulation in plant material was found to be higher at lower soil concentrations. 2,4-DNT and 2,6-DNT were not detected.

Table 6.2 Nitroaromatic compounds extracted from the roots of *Helianthus annuus*, cultivated for 4 weeks in TNT contaminated soil

	Soil TNT Concentration (mg/kg)						
	0	**0.43**	**6.17**	**43**	**186**	**451**	**1227**
TNT	0,00	0,26	1,84	5,16	23,71	52,12	101,35
4-A-2,6-DNT	0,00	0,41	2,01	9,21	16,93	23,39	26,09
2-A-4,6-DNT	0,00	0,24	4,63	10,49	19,20	22,27	26,96
NAC_{root}	0,00	0,91	8,48	24,86	59,84	97,78	154,40
NAC_{root}/NAC_{soil}	0,00	0,58	0,67	0,41	0,29	0,21	0,12

Results are presented in µg/g d.wt. Values of TNT, 4-A-2,6-DNT and 2-A-4,6-DNT are the average of three extractions of a tissue mixture originating from 15-30 plants. NAC_{root}, sum TNT, 4-A-2,6-DNT and 2-A-4,6-DNT; NAC_{soil}, sum TNT, 4-A-2,6-DNT and 2-A-4,6-DNT in control soil (plant-free).

6.3.3 Rhizodegradation

Table 6.3 shows that the growth of *Helianthus annuus* during 4 weeks did not result in a significant rhizodegradation of the nitroaromatic compounds as compared to the untreated control soil. Furthermore, it is clear that the aimed concentration array of TNT and the real concentration array are different. This can be due to heterogeneity of TNT in the starting soil from the ammunition site, irreversible interactions with soil components (clay minerals, soil organic matter) after mixing with the standard soil and compost, and/or leaching from the flowerpots. Leaching likely occurred as a red colour was observed on the bottom of the boxes in which the pots were placed. The discolouration, occurred after exposure to light for soil concentrations ≥ 186 mg TNT/kg.

Table 6.3 Nitroaromatic compounds extracted from the soil (plant-free controls) or rhizosphere soil of *Helianthus annuus* after 4 weeks of exposure

	Aimed TNT concentration (mg/kg)						
	0	**10**	**50**	**100**	**250**	**500**	**1000**
TNT							
Control (plant-free)	0,00	0,43	6,17	42,80	186,47	450,70	1227,1
	(0,00)	(0,50)	(0,26)	(17,81)	(8,54)	(65,28)	(153,67)
Plants	0,00	1,05	6,16	15,86	196,72	371,99	1071,66
	(0,00)	(0,69)	(1,98)	(1,29)	(89,16)	(95,47)	(124,77)
4A2,6DNT							
Control (plant-free)	0,00	0,69	3,00	8,50	11,37	12,44	10,78
	(0,00)	(1,04)	(0,19)	(1,39)	(0,52)	(1,27)	(0,49)
Plants	0,00	0,50	2,61	9,35	12,42	12,24	12,35
	(0,00)	(0,43)	(0,29)	(1,52)	(1,18)	(0,90)	(4,18)
2A4,6DNT							
Control (plant-free)	0,00	0,44	3,41	8,13	9,86	10,21	9,56
	(0,00)	(0,93)	(0,23)	(0,90)	(0,70)	(0,74)	(0,11)
Plants	0,00	0,37	3,19	10,14	11,44	10,96	9,14
	(0,00)	(0,57)	(0,05)	(1,36)	(0,60)	(0,36)	(2,16)

Results represent the average of three replicates and or indicated in mg/kg. The standard deviation is mentioned between brackets.

6.4 DISCUSSION

Germination and seedling development has been reported in detail for some grass species. Exposure to concentrations of 30 mg TNT/L or greater consistently reduced germination of *Festuca arundincea* (tall fescue) on nutrient-free agar (Peterson *et al*., 1996). A study by the same group showed that TNT did not affect *Panicum virgatum* (switchgrass) germination within the range tested of 0–60 mg TNT/L, but *Bromus inermus* (smooth bromegrass) germination reduced significantly at 30 mg TNT/L or greater (Peterson *et al*., 1998). These findings were confimed by Krishnan *et al*. (2000), whose research indicated that *Festuca arundincea* and *Bromus inermus* could germinate and grow in soils with concentrations less than 31 and 24 mg TNT/L soil solution, respectively. TNT did not affect the germination capacity of *Helianthus annuus* (sunflower) to a concentration of 1227 mg total extractable TNT/kg soil. Unfortunately the water extractable concentrations (bioavailable fractions) were not determined in the present study, which makes it impossible to compare with these available data from literature. Seed morphology was indicated by Krishnan *et al*. (2000) as an important factor to determine the germination capacity of plant species in a contaminated environment. They concluded that the seed coat of *Panicum virgatum*, which is harder than the one from *Bromus inermus*, likely restricted TNT penetration into the seed. Furthermore, *Bromus inermus* seed also retains a lemma and palea, which may mechanically restrict chemical movement to the embryo. Analogous, it can be suggested that the very thick seed coat of *Helianthus annuus* physicochemically limits TNT penetration. In addition, the large cotyledonous contain much reserves and consequently the embryo is less dependent on soil nutrients in the first phase of its development. These findings might explain the germination capacity independent of the soil TNT concentration. However, as the radicle begins to emerge from the seed, it is exposed to the available TNT and amino derivatives in the soil and they may affect cell growth and cell divisions. Seedling growth of *Helianthus annuus* was significantly affected at a concentration

of 43 mg TNT/kg soil or more. The non-linear response of seedling growth to the TNT concentration reflects the importance of concentration on the toxicity response. This is typical of seedling growth and biological responses to toxic compounds (Tipton, 1984; Bewley and Black, 1994; Hodgson and Levi, 1994; Peterson *et al.*, 1998).

Phytotoxic effects of TNT such as delayed seedling development, reduced secondary root growth, chlorosis of leaves, reduction in root and shoot length and discoloration of roots were previously observed (Görge *et al.*, 1994; Mueller *et al.*, 1993; Palazzo and Leggett, 1986; Peterson *et al*., 1996). Swelling of the meristematic regions and the absence of secondary roots at high TNT concentrations as we observed for *Helianthus annuus* are also consistent with earlier reports of disrupted cell division in biological systems (Won *et al*., 1976; Peterson *et al*., 1996, Peterson *et al*., 1998) and has also been observed in experiments described in Chapter 4 and Chapter 5.

As known, plants take up nitroaromatic compounds from the soil. A positive and linear correlation was found between soil contamination and the concentration of extractable NACs in the roots of *Phaseolus vulgaris* (bushbean) in the tested range of 0 to 500 mg TNT/kg (Scheidemann *et al*., 1998). Schneider *et al*. (1996) demonstrated a correlation between the concentration of NACs in plant roots of six vegetables and the logarithm of the measured concentrations in soil, indicating a more effective uptake via the plant root at lower concentrations in the soil. In the present study, a specific correlation was not observed. However, the relative accumulation of the nitroaromatic compounds in plant material was also higher at lower soil concentrations.

Unto a concentration of 43 mg TNT/kg soil, the main NACs detected in roots were 2-ADNT and 4-ADNT, while only small quantities of TNT were found. Although TNT was the main contaminant in soil, ADNTs dominated in the plants. These results are in agreement with those from Görge *et al*. (1994), Klunk *et al*. (1996), Hughes *et al*. (1997), Scheidemann *et al*. (1998), and Schneider *et al*. (1996). Obviously before toxicity takes over, an extensive metabolism to ADNTs takes

place in the rhizosphere before uptake into the plant or in the plant itself after TNT-uptake. DNTs were not detected in our study. To date, only from the roots of *Phaseolus vulgaris* cultivated in soil, low quantities of 2,4-DNT and 2,6-DNT were extracted (Görge *et al*., 1994; Koss *et al*., 1989; Scheidemann *et al*., 1998). This indicates that the removal of nitro groups is not a common TNT metabolising pathway occurring in plants.

Scheidemann *et al*. (1998) cultivated eleven species (five dicotyledonous, six monocotyledonous species) for eight weeks in soil contaminated with 10, 100, and 500 mg TNT/kg. Concentrations of NACs in plant roots cultivated in soil with 10 mg TNT/kg soil ranged from 0.63 µg/g dry weight (*Phacelia seicea*) to 2.72 µg/g dry weight (*Medicago sativa*). At 100 mg TNT/kg soil total NAC values ranged from 14.9 µg/g dry weight (*Lupinus angustifolius*) to 98.6 µg/g dry weight (*Triticum aestivum*). Only *Phaseolus vulgaris* was able to grow in the presence of 500 mg TNT/kg soil with very high levels of NACs in the roots (460 µg/g d.wt.). Compared to these values, our results show a relative high NAC content (8.48 µg/g d.wt.) at 6.17 mg TNT/kg soil. There are obvious species dependent differences in the level of NACs in the plant roots.

Uptake and/or sequestration of nitroaromatics by plants reducing the level of extractable nitroaromatics in the rhizosphere soil have been observed. Klunk *et al*. (1996) found a reduction of TNT in the rhizosphere of *Phaseolus vulgaris*, cultivated for 11 to 12 weeks in a soil contaminated with 440 mg TNT/kg soil. In addition, Scheidemann *et al*. (1998) showed that specific *Triticum aestivum* cultivars were able to reduce significantly the TNT concentration in the rhizosphere soil. Analyses were performed after eight weeks of cultivation in 100 mg TNT/kg soil. Within the four weeks of cultivation of *Helianthus annuus*, no positive degradative effect on the NAC concentration in the rhizosphere was observed. This might indicate that the duration of the greenhouse study was to short or that *Helianthus annuus* is no suitable candidate for rhizodegradation purposes on soil. Unlike its fast and extended root growth on hydroponics (Chapter 4 and Chapter 5), root production was significantly restricted by the soil matrix in

these four weeks, which resulted in a limit rhizosphere activity. However, even in the long term grasses may be more suitable because of their extensive root systems particularly in soil matrices.

CHAPTER 7
A PRELIMINARY STUDY ON BIOREMEDIATION OF TNT-CONTAMINATED SOIL IN AEROBIC SLURRY CONDITIONS

7.1 INTRODUCTION

The present approach to remediate TNT contaminated soil is incineration, a very costly and soil destructive process. Although it has been tried to develop bioremediation strategies, explosives such as TNT, RDX and HMX do not serve as growth substrates for bacteria, making them a difficult target for complete biodegradation. However, they can be transformed and detoxified by cometabolism. The most common biological transformation of TNT is the reduction of its nitro groups. Binding studies with humic acids and clay minerals indicate that certain reduction products, such as the isomeric hydroxylamino-dinitrotoluenes (under aerobic and anaerobic conditions) and TAT (under anaerobic conditions), show irreversible binding with soil components. Therefore, the reduction of TNT opens the possibility of soil remediation through cometabolically induced immobilisation. Several cost-effective strategies are now available for cometabolic treatment of nitroaromatic explosives in contaminated soil: composting (Pennington *et al*., 1995; Williams *et al*., 1992), land farming (Widrig *et al*., 1997), and slurry reactors (Boopathy *et al*., 1998; Boopathy and Manning, 1999; Funk *et al*., 1993; Funk *et al*., 1995; Lenke *et al*., 1998). These different biological treatment systems mostly differ in the duration and intensity of the aerobic or anaerobic incubation, in the amount and type of external carbon (C) sources added, and the degree of water saturation (Lenke *et al*., 2000). More information on biological treatment processes currently employed in the field is presented in Chapter 1.

In the present preliminary test, biostimulation and bioaugmentation were evaluated as strategies to improve bioremediation of TNT-contaminated soil in an aerobic slurry process. Is it really necessary to add bacteria to bioremediation systems, or can bacteria that are indigenous to the matrix get the job done?

7.2 MATERIALS AND METHODS

Soil slurry conditions. TNT-degradation in a 20% soil slurry was followed in batteries of Kimax tubes (15 ml), which contained 1.2 g of a moist (18.75% soil humidity), sieved ($\leq$ 1 mm^2), non-sterile TNT-contaminated soil (1179 mg TNT/kg; 70 mg ADNT/kg). To evaluate bioaugmentation, 5 ml of a culture from consortium 3 [2.09×10^8 ($\pm$ 3.54×10^7) CFU/g soil] was added. The inoculated consortium 3 was pre-grown on minimal medium with 2,4-DNT as the sole N-source (Chapter 3), centrifugated and resuspended in fresh minimal medium with a mixture of C-sources. No extra N-source was provided to the soil slurry. Biostimulation was evaluated by a battery without the inoculation of extra bacteria but with addition of 5 ml minimal medium with additional C-sources. It was chosen to work in a battery (i.e. an array of tubes under the same conditions) to maintain the homogeneity of the slurry (constant soil-water ratio) and to facilitate sampling. All tubes were incubated aerobically in the dark at 28°C and at 200 rpm. Duplicate tubes were taken daily. The experiment continued for 4 days. Both the water phase and the soil phase were sampled. Sample preparation for analysis and HPLC conditions are described in Chapter 3.

Soil characteristics. 0.3% clay; 4.2% loam; 95.5% sand; pH_{KCl} 5.85; pH_{H2O} 7.06; 221 µS/cm conductivity; 2.66% humus; 9.38 meq/100g dry weight cation exchange capacity; heavy metals (mg/kg dry weight): 8.7 Cd, 44.9 Cu, 23.1 Ni, 283 Pb, 600 Zn.

7.3 RESULTS

7.3.1 Water-phase analysis

HPLC analyses were employed to monitor the elimination of TNT in the slurry system. Results of water-phase TNT concentrations are presented in Figure 7.1. After four days of incubation, only 21% of the maximum TNT concentration in the water-phase (maximum solubility about 18 mg/L) was detected when consortium 3 was present. Until 72 hours, a first-order rate disappearance (Chapter 4) was observed for TNT (k = 0.020, r^2 = 0.982), indicating that the TNT disappearance is dependent on the available TNT concentration in the water-phase. After 72 hours, a steady-state value of about 4 mg/L seemed to establish between desorption from the soil and biodegradation. No decline in the TNT concentration (18.01 mg/L) was found in the uninoculated slurry. 4-ADNT and 2-ADNT were not detected in any system.

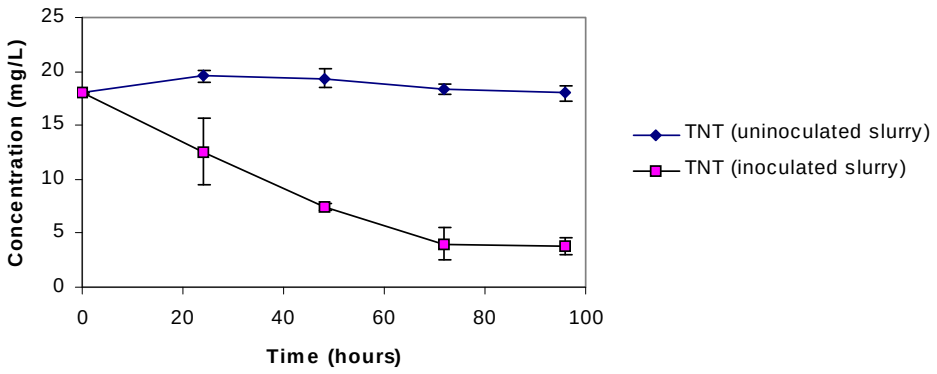

Figure 7.1 Water-phase TNT concentrations in an aerobic soil slurry with and without inoculation of consortium 3. ADNTs were not detected. Error bars indicate the standard deviation of duplicates.

7.3.2 Soil-phase analysis

Soil analyses show a TNT disappearance of 287 mg TNT/kg (24%) after 96 hours of incubation with consortium 3 (Figure 7.2). Corresponding to the observations in the water-phase, elimination processes were biggest within the first 72 hours. A decrease of 128 mg TNT/kg (11%) was observed in the uninoculated slurry after 96 hours. These results indicate a 157 mg/kg (13%) TNT removal attributed to consortium 3 when inoculated. ADNT concentrations in the uninoculated and inoculated slurry increased respectively from 70 to 127 mg ADNT/kg and 70 to 113 mg ADNT/kg, however, comparing both conditions, concentrations did not differ significantly.

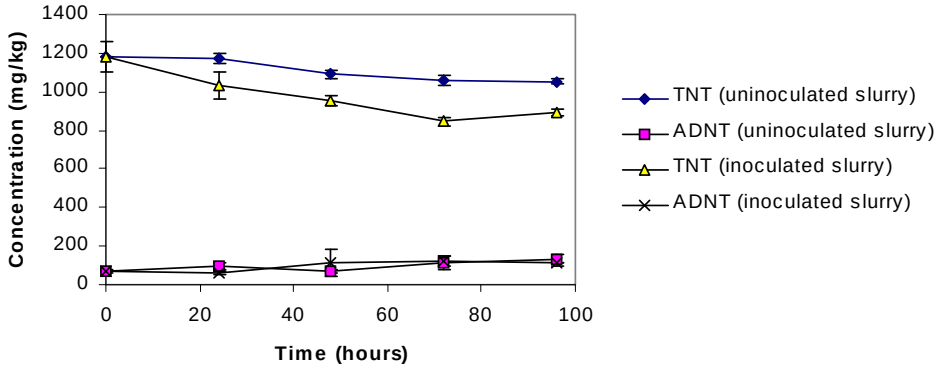

Figure 7.2 Soil-phase NAC concentrations in an aerobic soil slurry with and without inoculation of consortium 3. Error bars indicate the standard deviation of duplicates.

7.4 DISCUSSION

A comparison between both inoculated and uninoculated systems suggests that for this particular soil bioaugmentation might be the best choice. However, successful biodegradation requires organic contaminants to be biologically available, which in general includes that they are dissolved in the soil water-phase. The specific inoculum's activity was found to decline in the water-phase and levelled off at

about 4 mg TNT/L, indicating that the activity might be limited by a too low available TNT concentration. The incomplete removal of low concentrations of a pollutant is a common disadvantage associated with inoculation, because other compounds might preferentially be used during metabolisation (Edgehill, 1999). The low solubility of TNT (about 18 mg/L in the present study) and the low rate of desorption of TNT from the soil are important factors controlling this degradation rate. If desorption is the limiting factor, than the bioavailability can be augmented by the addition of a surfactant. A study by Boopathy and Manning (1999) showed that water-soluble TNT increased almost linearly with the increase in Tween 80 concentration. In the absence of Tween 80, water-soluble TNT was at a minimum of 50 mg/L. In contrast, at 5% Tween 80, water-soluble TNT increased to 620 mg/L. They selected the 3% Tween 80 concentration for further experiments, not to overload the system with surfactant. The addition of this surfactant to their reactor system reduced the treatment time by 10 days (from 45 to 35 days). Furthermore, TNT is cometabolised and consequently cannot provide energy to our inoculum. Therefore depletion of a suitable C-source might also be the cause for the observed decline in the consortium's activity, while depletion of other essential nutrients cannot be excluded either. Addition of Tween 80 can have another advantage besides its surfactant characteristics. Because it primarily contains four long-chain fatty acids, including palmitic acid, oleic acid, stearic acid, and linoleic acid, it can also serve as C-source for soil microorganisms and will therefore not remain in the slurry after treatment (Boopathy and Manning, 1996). However, Boopathy and Manning (1999) found that a reactor enriched with surfactant and molasses performed better than reactors with either molasses or surfactant alone. Molasses is a relative inexpensive C-source, with a very complex composition (which contains besides carbon also nitrogen, phosphorus, vitamins, and minerals), making it favourable to the growth of many types of bacteria. In addition to a low available TNT concentration and a depletion of nutrients, the presence of additional chemicals (e.g. heavy metals) or the persistent toxicity of NAC can weaken metabolic activity in time. Reinoculation of the bacterial consortium at 72 hours

may promote degradation again. If the hypotheses of a depletion of nutrients or an exhausted microflora hold true, a rise in the water-phase TNT concentration can be expected after the pit at the end of the experiment. Unfortunately, the experiment was stopped too early.

Finally 157 mg TNT/kg soil disappeared extra from the slurry system inoculated with consortium 3. An experiment with radiolabeled TNT should show the fate of the "disappeared" compound, which is expected to become partially immobilised to the soil (clay, loam and humus fraction), converted to biomass, and transformed into undetectable metabolites. Therefore, toxicity and mutagenicity tests are needed after treatment to ascertain remediation. A minor mineralisation can-not be excluded but is very unlikely to occur (Spain *et al*., 2000; Chapter 3).

A decrease of 128 mg TNT/kg soil was observed in the uninoculated slurry system, though the TNT concentration in the water-phase never decreased. This result indicate very low metabolic activity of indigenous organisms in the starting soil. Most environments contain microorganisms capable of degrading almost any pollutant, however, the number of organisms with the desired trait may be extremely small and therefore there may take a long time before noticeable disappearance of the pollutant occurs (Edgehill, 1999). The present study shows that the addition of exogenous microorganisms will be necessary to shorten the time period for remediation considerably. The use of bioaugmentation has been mainly demonstrated for pesticides, oil, or specific hydrocarbon constituents of oil (Barles *et al*., 1979; Crawford and Mohn, 1985; Edgehill and Finn, 1983; Mishra *et al*., 2001; Pignatello *et al*., 1983; Shirkot and Gupta, 1985).

In conclusion, this preliminary study indicates that a slurry reactor, inoculated with consortium 3, has the potential to enhance the rate of remediation of TNT contaminated soil, which is advantageous in a large-scale reactor as a decreased residence time increases output and consequently reduces cleanup costs. Research during a longer period will make clear if frequent reinoculation of the consortium, the addition of a surfactant and/or the supply of extra nutrients (e.g. carbon, nitrogen, phosphorus, vitamins, or minerals) will be necessary to achieve 100%

TNT removal. Furthermore, detailed studies are needed to determine if biodegradation will transform, degrade, or immobilise the TNT to chemicals that are no longer hazardous.

CHAPTER 8
FEASIBILITY STUDY FOR THE PHYTOTREATMENT OF TNT USING A WETLAND-BASED SYSTEM

8.1 INTRODUCTION

The application of phytoremediation using constructed wetlands provides a relatively simple, inexpensive, and robust solution for the treatment of wastewater. Compared to other treatment options constructed wetlands usually need less operation and maintenance. Additional benefits include their tolerance against fluctuations of flow, the facility of water reuse and recycling, the provision of habitat for many wetland organisms, and the more aesthetic appearance of a natural system compared to technical treatment options. Wetland treatment systems are effective for the removal of organic matter, nitrogen, and phosphorus as main constituents of municipal and industrial wastewater. In addition, pollutants like heavy metals, specific organic compounds, and pathogens can be eliminated. Experience on organic pollutants exists with wastewater from petroleum industry, different food processing industries, pulp and paper wastewater, and waters containing surfactants, pesticides, and phenol. The major removal mechanisms for common wastewater treatment are sorption and microbial degradation by fermentation, and aerobic and anaerobic respiration. The most important contribution of the macrophytes (large aquatic plants) to the treatment process is of physical kind (e.g. plants affect pH and pO_2, and roots provide surface area for microbes that mediate most of the pollutant transformations). However, for pollutants like heavy metals and organic pollutants the selection of suitable plant species can play a major role in enhancing treatment efficiency (Kadlec and Night, 1996; Langergraber and Haberl, 2001).

Perennial grasses, such as *Phragmites australis*, are of particular interest in constructed wetlands due to their extended root systems, high biomass production, high water use, resistance to be flattened, adaptability to a wide range of environments (e.g. alkine, slightly salty, and different climatic conditions), rapid establishment, and tolerance to many xenobiotics (e.g. heavy metals, specific organic compounds) (Aprill and Sims, 1990; Anderson *et al.*, 1993; Massacci *et al*., 2001). The extended root system is of particular interest, as sorption to the root surface was found to be an important removal mechanism of TNT (Chapter 4). A disadvantage is that *Phragmites australis* is a rampant plant, the main reason why this plant is often omitted in wetland treatments in the USA.

Phytoremediation of TNT and RDX in groundwater using constructed wetlands is a potential remediation technology (Medina and McCutcheon, 1996). The disappearance of TNT and RDX from water was found to occur more rapidly in the presence of selected submersed and emergent plants than without plants. The disappearance of RDX was partly attributed to plant-stimulated activity of microorganisms inherent to the explosives-contaminated water (Schnoor *et al*., 1995; Best *et al*., 1997). Degradation of TNT by freshwater sediments proved to be mediated by enzymes of aquatic plant origin (Van Beelen and Burris, 1995).
The Milan Army Ammunition Plant located near Milan (TN) was selected as a demonstration site for an *ex situ* application of phytoremediation to remove munition contaminations from groundwater using constructed wetlands (Hoagland, 1996). The demonstration involved the comparison of two wetland types, a lagoon system with submersed plants (*Elodea canadensis* (elodea), *Potamogeton pectinatus* (sago pondweed), and *Heteranthera dubia* (water stargrass)) and a subsurface flow gravel-bed with emergent plants (*Myriophyllum aquaticum* (parrot feather), *Acorus calamus* (sweet flag), *Phalaris arundinacea* (reed canary grass), and *Scirpus cyperinus* (wool grass)). Plants were determined based on metabolic (e.g. nitroreductase production) and ecological (e.g. perenniality, high productivity, high leaf and root surface areas) characteristics. Average influent concentrations

into the wetland systems were 1.28, 1.74 and 0.10 mg/L for TNT, RDX and HMX, respectively. The gravel-bed efficiently removed TNT, RDX and HMX, as demonstrated by effluent concentrations below the detection level. The lagoon system performed similarly well in removing TNT from the water but was less efficient in removing RDX and HMX. At the Iowa Army Ammunition Plant in Middletown (IA), studies were conducted to assess the potential for using phytoremediation with wetlands to treat explosives contamination (TNT, RDX) in surfacewater and groundwater *in situ* (Burken *et al.*, 2000). A full-scale lagoon treatment system was evaluated. Soil excavation pits were not backfilled but converted into constructed wetlands. Sediment was obtained from a nearby stump lake to introduce species such as *Ceratophyllum demersum* (coontail), *Potamogeton nodosus* (pondweed) and *Sagittaria latifolia* (arrowhead). It was believed that these species were accomplishing remediation by endogenous enzymes (laccase, dioxygenases and nitroreductase). The wetlands infiltrated with groundwater contaminated with TNT (0.7 mg/L) and RDX (13 mg/L), showed effective removal for both TNT and RDX. Another project was conducted at the Volunteer Army Ammunition Plant in Chattanooga (TN) using three submerged species (*Elodea canadensis* (elodea), *Ceratophyllum demersum* (coontail), and *Potamogeton nodosus* (pondweed)) and one emergent species (*Typha angustifolia* (narrowleaf cattail)) (U.S. Department of Defense-Supported Projects, 1999-2000). The different plant species were planted in sediment and held in aboveground growth tanks. The study showed that the submerged plants failed to survive in groundwater containing 2.7 mg TNT/L and 95 mg/L of other nitrotoluenes. While *Typha angustifolia* did survive and grow and seemed to enhance disappearance of the explosives, rates of degradation of the contaminants were more dependent upon the presence of ultraviolet light from sunlight than the presence of plants. In conclusion, the effectiveness of phytoremediation at this site was unclear.

Phytoremediation of water contaminated with explosives is a promising technology, however, questions still exist regarding the final fate of contaminants and the long term ecological impact of potential metabolites. Implementation of

phytoremediation to treat RDX and TNT contaminated water in full-scale systems is ahead of lab studies and scientific knowledge. Full- and pilot-scale systems can not be predicted by laboratory studies, even though the same species, sediments, and waters are used. In full-scale systems the combined effect of photolysis, plant, microbial and sediment interaction appear to remove explosives at rates in excess of what existing laboratory studies predict. *Ex situ* studies would allow better understanding and characterisation of the integrated wetland systems. Such understanding will allow improved engineering and utilisation of plants to treat nitroaromatic and explosive compounds.

The aim of the present investigation was to evaluate the efficiency of wetlands, using *Phragmites australis* (common reed) in removing TNT and ADNT from contaminated groundwater, and the effect of inoculating the system with a bacterial consortium. Consortium 3 is able to transform TNT efficiently to amino derivatives and azoxy compounds and lowers the toxicity level. This should result in reduced plant stress and in enhanced removal efficiencies of the nitroaromatic compounds by the plants. The community compositions in the systems were monitored by PCR/DGGE in order to evaluate the survival of the inoculum and shifts in populations during operation. The experimental set up consisted of a plant system on mesocosm-scale, which simulated field conditions that would occur during remediation of TNT-contaminated water in horizontal subsurface-flow constructed wetlands. A continuous flow process was used to feed surfacewater originating from an ammunition production site.

8.2 MATERIALS AND METHODS

Experimental design. The design was based on the construction of horizontal subsurface-flow (SSF) wetlands. Three beds were prepared for the treatment of TNT-contaminated water (Figure 8.1). Two beds contained 25 *Phragmites austra-*

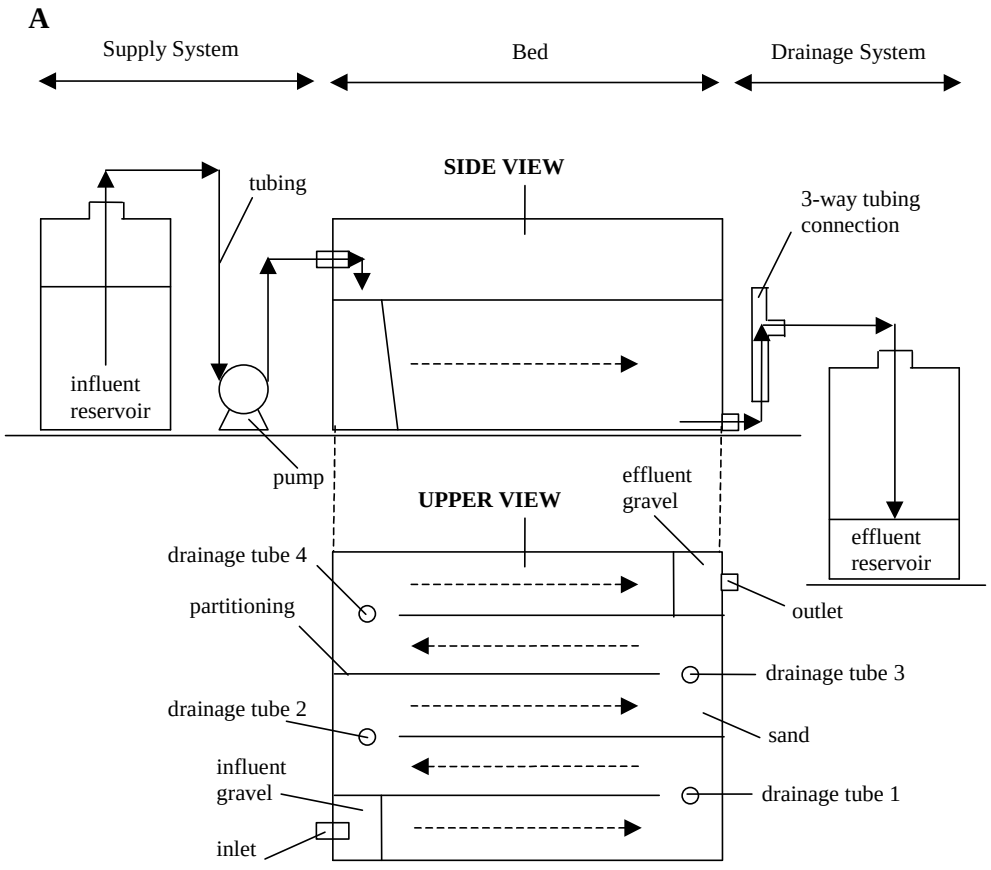

Figure 8.1 (A) Schematic of a bed system, (B) Photograph from the experimental set-up (13th of June 2001).

lis plants, the third contained no plants and was used as a control. One of the beds planted with *Phragmites australis* was inoculated with consortium 3 (Chapter 3). The size of the beds (PVC) was 800 × 600 × 400 mm^3 (L × W × H). The dimension of the partitionings (PVC) was 700 × 3 × 400 mm^3. 96 L (800 × 600 × 200 mm^3) substrate was used, of which 2 × 2.5 L gravel (5 to 30 mm, 50% porosity) and 91 L sand (1 to 2 mm, 40% porosity). Pond textile separated both substrates. Four drainage pipes (PE) were put into each bed for water sampling. The influent was pumped (Watson Marlow, England) from a reservoir (25 L, PE-HD) to the inlet of a bed through a Viton tubing (Rubber BV, Nederland). At the outlet of the system, the effluent was drained away to a reservoir by the use of a 3-way tubing connection (glass, home-made). The study on TNT-contaminated surfacewater was conducted from the 13th of August 2001 to the 29th of October 2001 in a green house tunnel to mimic ambient conditions and to prevent overflow of the beds by heavy rainfall. For 8 weeks (from the 13th of June to the 13th of August), Hoagland nutrient solution (Epstein, 1972) flew through the beds, in order to establish the plants (pre-phase). Each bed had a hydraulic capacity of 39 L and a hydraulic retention time of 14 days. However, after five weeks, a gradient in shoot length, shoot colour and root development was observed: near the inlet plants were slightly taller and greener and roots were more extended. An analysis of the effluent solutions showed no shortens in macro-nutrients (N, P, K). Analyses occurred by the use of Spectroquant® photometric test kits (Merck, Germany). Yellowing of leaves is also typical for iron deficiency. Therefore, one extra dose of the Hoagland Fe-EDTA solution (23 ml/39 L) was distributed equally over the surface of the beds, which was repeated 2 days later. No positive effect was observed within 7 days, and consequently the beds were poured with a 0.4% solution of Green Granule® (Asef, Belgium) once a week for two weeks. A slight improve was observed. Diluted surfacewater from an ammunition site was fed to the beds starting from the 13th of August. For the first two weeks, a mixture of 50% surfacewater (without the extra addition of TNT) and 50% Hoagland nutrient

solution (2 times concentrated, resulting in a final concentration of 1 in the mixture) was fed to the beds in order to make a more transitional change to the plants. However, due to the poor plant growth, it was decided to keep the ½ dilution factor of the surfacewater during the experiment. For the subsequent nine weeks, in which 4.5 bed volumes passed the beds, TNT was spiked into the reservoirs to an influent concentration of 10 mg/L or 44 µM.

Weekly, water-balances were made by weighing the effluent reservoirs, presuming that per week 20 L was pumped into each system.

The isolation of consortium 3 by enrichment of indigenous bacteria from a TNT contaminated soil sample, was described in Chapter 3. The surfacewater sample used in this present study originates from the same site, at about 4 m from the location of which the soil sample was taken. Consortium 3, consisting of *Pseudomonas aeruginosa* VM903, *Sphingomonas* sp. VM904, *Stenotrophomonas maltophilia* VM905, *Serratia proteamaculans* VM906, *Pseudomonas viridiflava* VM907 and *Pseudomonas* sp. VM908, was used to inoculate the first reed bed. The consortium is capable of decreasing the toxicity of the surfacewater. The EC_{50} value determined by the Vitotox® test (Verschaeve *et al*., 1999) increased from 1.55% (controle) to 26.13% (incubated for 2 days at 28°C with 4.2×10^8 cells/ml). To prepare the inoculum, consortium 3 was grown in 1-liter of a minimal salt medium with additional carbon sources and 2,4-DNT (100 mg/L) as sole nitrogen source as described in Chapter 3. In the pre-phase (8 weeks before the start of the experiment), weekly, one reed bed was inoculated with consortium 3, at a density of 7.5×10^6 cells/ml surfacewater or 2.1×10^6 cells/g substrate. Inoculation occurred by pouring down a full-grown culture of the consortium equally over the bed. The consortium was additionally inoculated on day 6 and 42 of the experiment.

Phragmites australis (common reed) was chosen as test species. A density of 50 plants/m^2 was applied to establish a dense root biomass within 2 months (13^{th} of June to 13^{th} of August).

Surfacewater from a soil excavation pit at an ammunition production site was used to feed the beds. The water characteristics are summarised in Table 8.1. However, the TNT present at first (Table 3.1, sample W2) was found to be completely photodegraded at the time of sampling (1st of August) for this experiment. Therefore, samples were spiked with a TNT stock solution for the continuous-flow experiment. The experiment was conducted for 77 days, in which 5.5 bed volumes passed through the systems. During the first 14 days, no TNT was spiked. The 500 L sample used to feed the beds was stored in reservoirs (25 L, PE-HD) at a temperature of 8°C (cellar).

Table 8.1 Characteristics of the surfacewater used for the continuous-flow experiment

Characteristic	**Data**	
Colour	red	
pH	7.39	
Conductivity	369 µS/cm	
Ammonium-N	< 0.20 mg/L	
Nitrate-N	< 0.5 mg/L	
Nitrite-N	< 0.010 mg/L	
Phosphate-P	< 0.5 mg/L	
BOD	< 5 mgO_2/L	
Nitroaromatic Compounds	**TNT**	**0 mg/L**
	4-A-2,6-DNT	0.164 mg/L
	2-A-4,6-DNT	0.428 mg/L
Heavy metals	Cd	9.4 µg/L
	Cu	< 5 µg/L
	Pd	47 µg/L
	Ni	< 5 µg/L
	Zn	559 µg/L

Initially, influent and effluent water samples were collected frequently from the reservoirs and, in addition, intensive sampling of water at interior locations in the reed beds occurred. Interior water samples were collected at equal distances between the inlet and outlet at 6 locations: one in the gravel at the inlet, four in subsequent drainage tubes, and one in the gravel at the outlet. However, data collected for four weeks demonstrated that no nitroaromatic compounds were detected further than drainage tube 1, therefore further sampling occurred up to and

including drainage tube 2. Water samples were analysed for nitroaromatic compounds via HPLC as described in Chapter 3.

In addition, one g of sand substrate or root tissue was sampled in duplicate and stored at –80°C for total genome DNA extractions. Storage of the samples occurred at –80 °C in a solution of glycerol (15%) and NaCl (0.85%) in a ratio of 1.5 ml solution per g matter.

DNA extraction. Performed as described previously by El-Fantroussi *et al.* (1997).

PCR/DGGE. To monitor the survival of the introduced bacterial strains and in addition the community dynamics of the indigenous bacteria in all three beds, PCR/DGGE was applied to the extracted bacterial DNA. The applied method has been described in Chapter 3. Community profiles were compared by the computer program Bionumerics (Eichner *et al.*, 1999; El-Fantroussi *et al.*, 1999). The cosine coefficient, based on densitometric curves of the band patterns, was used to compare DGGE profiles.

8.3 RESULTS

8.3.1 Influent Reservoir

Once a week, the reservoirs were replenished. At the start of the experiment (after the pre-phase), the reservoirs were filled with a mixture of surfacewater, Hoagland nutrient solution and, starting at day 14, a TNT solution in ethanol. However, on day 21, a strong odour and brownish, slimy foam were observed in the reservoirs. Because a fresh mixture was added on day 21, a sample from the reservoirs was taken four days later and showed complete disappearance of TNT. It was decided to rinse the complete supply system with hypochlorid (30%) and separate the surfacewater spiked with TNT from the nutrient solution by adding an extra reservoir on day 28. The development of an odour and foam stopped. Both the contaminated water and the nutrient solution united at the inlet of the reed bed. Two to three times a week a sample was taken from the reservoirs and analysed for

TNT, 4-ADNT and 2-ADNT. As an example, results from the first reservoir are illustrated in Figure 8.2. An arrow indicates the days that the reservoir was replenished. Still a decrease in the TNT concentration was observed though much slower.

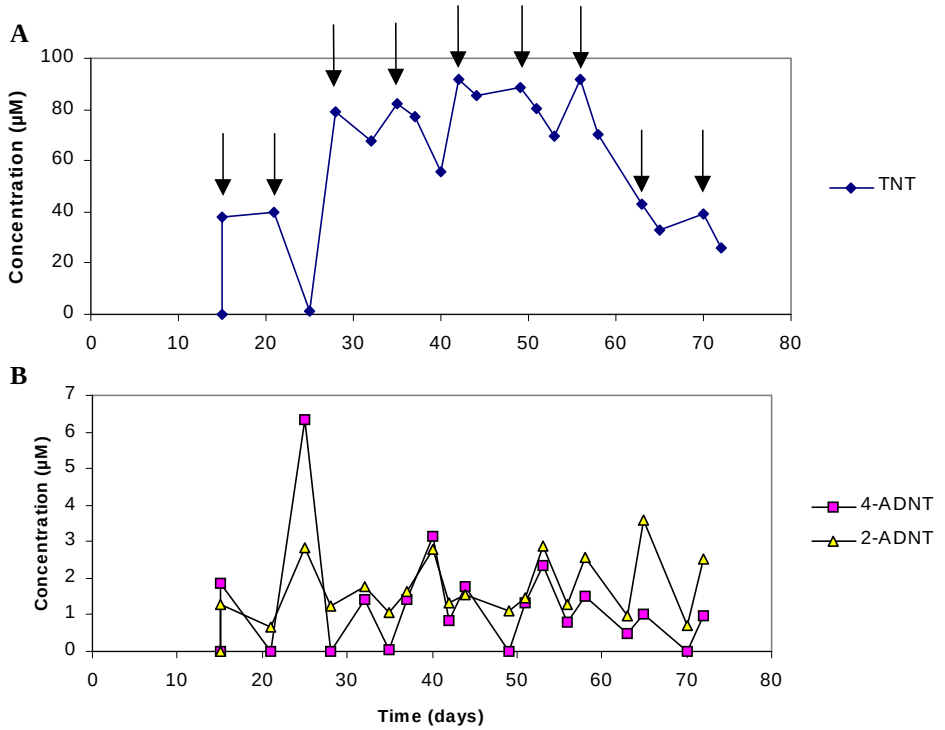

Figure 8.2 (A) TNT, (B) 4-ADNT and 2-ADNT concentrations in the influent reservoir from reed bed 1. The arrows indicate the days that the reservoir was replenished. The influent feed was always set on 44 µM TNT (10 mg/L). From day 28 to day 63, the contaminated surfacewater was ½ diluted with Hoagland nutrient solution at the inlet, leading to a TNT concentration of 88 µM (20 mg/L) in the surfacewater reservoir.

For a period of 30 days (between day 28 and 58), TNT concentrations in the reservoir fluctuated between 55 and 91 µM. From day 63, the Hoagland nutrient solution supply stopped, which implied that the TNT concentration in the influent reservoir was brought back to 44 µM. The two other reservoirs (belonging to bed 2 and bed 3) showed similar results. Standard deviations on the values of the 3 beds

fluctuated between 0.16 and 8.64 µM. The decrease in TNT coincided with a small increase in 2-ADNT and 4-ADNT, however, not in proportion to the TNT that disappeared. Standard deviations on 2-ADNT were between 0.06 and 1.87 µM, and on 4-ADNT between 0.12 and 1.20 µM. The presence of DANT and TAT was not examined.

8.3.2 Beds

8.3.2.1 Plants

Along the experiment, a gradient in plant morphology became more outspoken. Near the inlet, shoots were taller and greener, roots were better developed and penetrated deeper into the tray. In addition, plants in the reed bed, which was not inoculated with consortium 3, grew better. Figure 8.3 clearly illustrates that more water evaporated in this plant system. Furthermore, when digging into the substrate of both reed beds, a mire odour was observed and the colour of the sand was grey to black as a consequence of biofilm production, indicating bacterial activity under anaerobic conditions. These observations were less pronounced in the control without plants. However, in the control, a dense algae layer developed on top of the substrates and in the three-way tubing connection, which at day 49 of the experiment blocked the drainage. Consequently, the supplies remained in the bed and the water level raised. The three-way tubing connection was cleaned and the bed was covered with black, non-transparent plastic. The transpiration decreased and the presence of algae diminished.

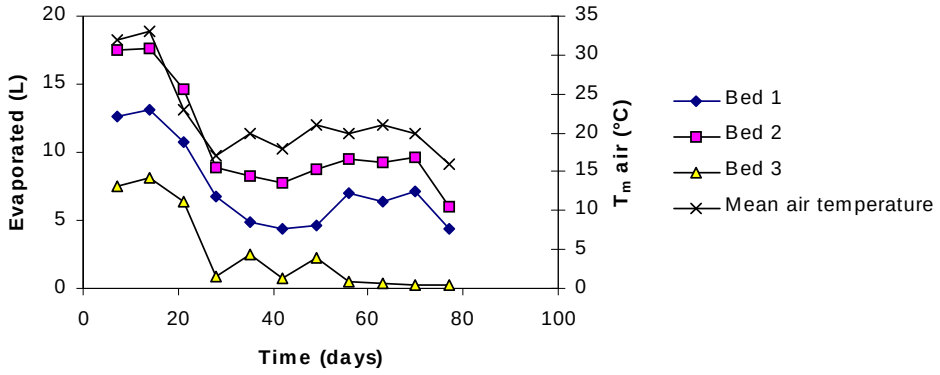

Figure 8.3 Weekly water-balance in the three beds and weekly mean air temperature (T_m) at 11h30 in the tunnel greenhouse

8.3.2.2 Nitroaromatics concentrations

Samples taken at the level of the influent gravel showed the presence of TNT, 4-ADNT and 2-ADNT, however, concentrations were low (< 13 µM) compared to the aimed concentration of 44 µM TNT. Results from the first reed bed are shown in Figure 8.4. Results from the two other beds were similar (standard deviation for TNT between 0.14 and 1.86 µM, for 2-ADNT between 0.17 and 2.34 µM, and for 4-ADNT between 0.23 and 1.99 µM).

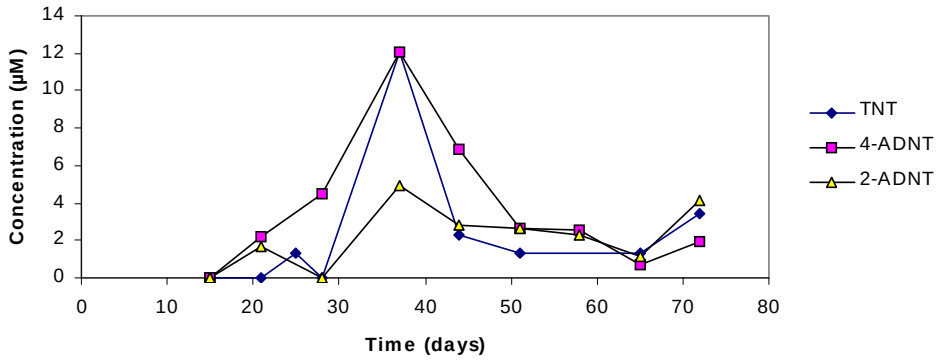

Figure 8.4 Nitroaromatic compound concentrations in the influent gravel of reed bed 1

In addition, a slimy, brownish biofilm was observed on top of the influent gravel column. At day 63, it was decided to stop the nutrient supply in order to stop biofilm development. This might explain the increase of nitroaromatic compounds at the end in Figure 8.4. The first drainage tube of the beds showed only the temporary presence of 4-ADNT at a low concentration (Figure 8.5). No effect of the inoculation of consortium 3 was demonstrated. In addition to 4-ADNT, 2-ADNT was detected in the first drainage tube of the control. No nitroaromatic compounds were detected in samples taken from the second drainage tubes.

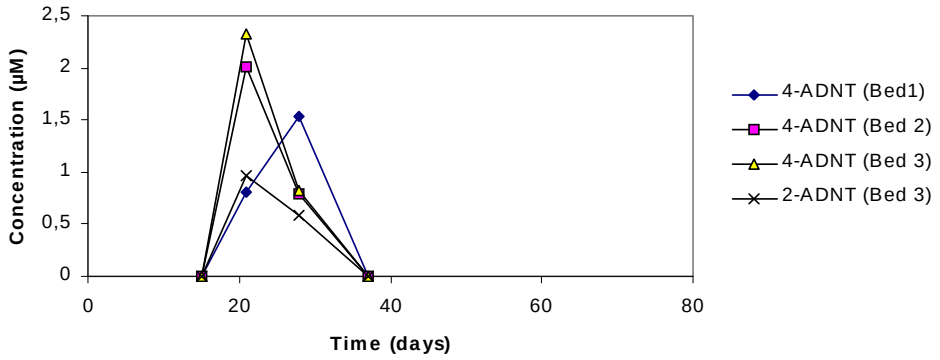

Figure 8.5 ADNT concentrations in the first drainage tube of all three beds. Nitroaromatic compounds were not detected after 37 days. 2-ADNT was not detected in the presence of plants (bed 1 and bed 2).

8.3.2.3 Community dynamics

To evaluate the eubacterial community composition, sand samples were taken in all three beds. At first in the reed beds, sand samples were taken between the roots and assumed to be rhizosphere samples. Starting from day 56, root samples were taken for bacterial DNA extraction. Initially, sampling occurred halfway the bed (between drainage tube 2 and 3), but as plants were gradually loosening more strength in the centre compared to the plants near the inlet, from day 63 on samples were taken near the inlet. PCR/DGGE results are shown in Figure 8.6. Profiles

from the centre clustered separately from those at the inlet, with the exception of the sample taken in reed bed 1 on day 21.

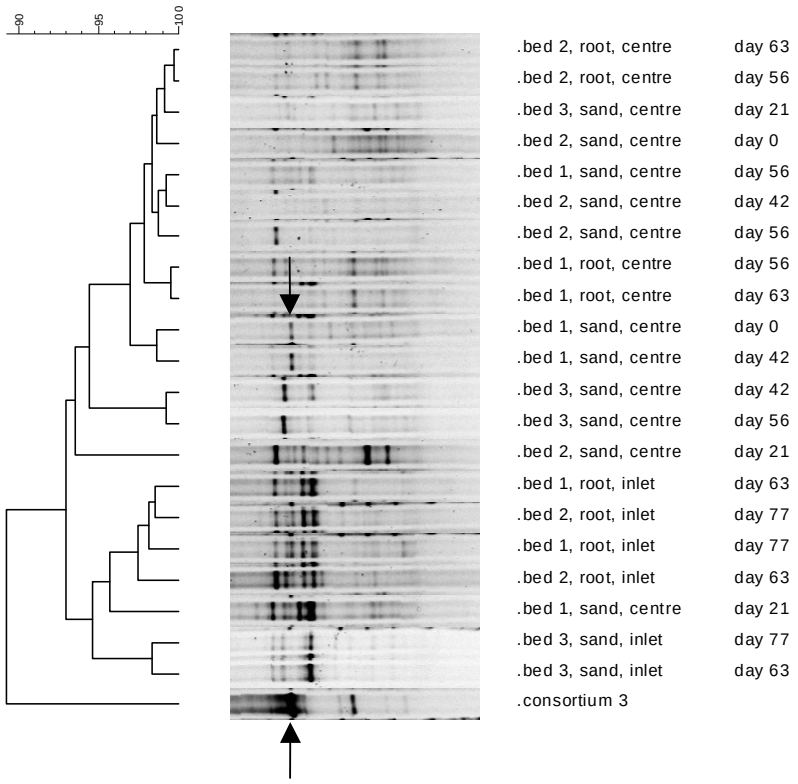

Figure 8.6 DGGE analyses of PCR amplified 16S rDNA fragments. The cosine coefficient was used to compare the DGGE profiles. DNA extractions were performed in duplicates and PCR/DGGE demonstrated that the DNA extraction was reproducible (duplicates not shown). Bed 1, *Phragmites australis* + consortium 3; bed 2, *Phragmites australis*; bed 3, control without plants and without consortium 3.

Though samples at the inlet and at the centre were taken at different times, the results from the samples taken at day 63 suggest that communities found in the centre differ significantly from those at the inlet. In addition, a weaker fingerprint was obtained from samples taken in the centre compared to those from the inlet. Another observation was that the microbial communities present at the inlet were similar for the three beds, with the highest similarity between the rhizosphere

samples of the reed beds. According to the applied pattern analysis, there is no similarity found in any sample with consortium 3. Though it should be mentioned that in the samples taken in bed 1 at day 0 (three days after inoculation) and day 42 (immediately after an additional inoculation), the first band of the consortium is visible (as indicated by an arrow). It is clear that survival of the consortium was poor. No conclusions can be made regarding shifts in community dynamics, since to little analogous samples were taken.

8.4 DISCUSSION

The observation of foam and odour in the influent reservoir at day 21 is characteristic for biological activity under anaerobic conditions. Foam and odour indicate the development of gases, which might have included among others NH_3, N_2, and H_2S resulting from fermentation and anaerobic respiration. The original "cocktail" in the reservoir consisted of sufficient macro- and micronutrients, ethanol (final concentration 0.1%) was present as carbon substrate, and the whole was incubated under static conditions at mid-summer temperatures. Aerobic heterotrophs, present naturally in the surfacewater, quickly consumed the oxygen from the static cultures, creating anaerobic conditions. In the second phase, when TNT spiked surfacewater and nutrients were separated (starting at day 28), anaerobic conditions in the surfacewater were presumably created much slower and TNT reductions in the influent reservoir did not occur rapidly. Under both aerobic and anaerobic conditions, the initial step in the metabolisation of TNT is typically a reduction of a nitro constituent to an amino group. The next reduction produces diaminonitrotoluene isomers. The reduction of the third nitro group occurs only under strictly anaerobic conditions (Preuss and Rieger, 1995). The nitro group can be easily reduced, either chemically (abiotic oxidation-reduction reactions) in the presence of reducing agents (e.g. reduced iron and sulfur species), cometabolically by a variety of metabolic enzymes (e.g. NAD(P)H-dependent reductases and H_2-

hydrogenase-ferrodoxin) or spontaneously by reduced electron carriers (e.g. $FADH_2$) in the absence of the enzyme (Haderlein and Schwarzenbach, 1995; Preuss and Rieger, 1995). Two genera have been extensively studied because of their anoxic metabolism of TNT: *Clostridium* (nitrate-reducing conditions) and *Desulfovibrio* (sulfate-reducing conditions). An overview is presented by Esteve-Nunez *et al*. (2001).

Plant growth was already affected in the pre-phase before contaminated groundwater entered the systems. Most of the existing constructed and natural wetland treatment systems previously had or currently have plant growth problems and some experience is needed (Kadlec and Knight, 1996). The plant growth observations in the present study are likely a combination of several of physical, chemical, or biological factors, which might include flood stress (function of the dissolved oxygen regime in the water), micro-nutrient stress (limitations of Fe, Mg, Mo, etc.), dissolved oxygen (DO) stress (caused by e.g. ammonia loading, smothering by sludge), pathogens (plant disease), and the weather (e.g. high root temperatures, excessive evapotranspiration). It is clear that the DO concentration is a key factor in plant growth and survival. Though hydrophytic plants may be adapted to survive and grow in specific flooded conditions, e.g. by the development of aerenchymous plant tissues or lenticels, these plants may not be able to grow or survive during several months in stagnant or “dirty” water conditions. Sludge reduces oxygen diffusion and increases reducing conditions because of the presence of increased metabolic activity. Atmospheric reaeration tends to restore DO to its saturated value. However, with high loading of biological oxygen demand (BOD) and total ammonia, the atmospheric diffusion rate is often insufficient to fully offset the biological utilisation of oxygen in the water column, and resulting DO levels are typically close to zero even near the water surface. In conclusion, the majority of the saturated bed is anaerobic under most wastewater design loadings (Kadlec and Knight, 1996). However, augmenting the flow rate or lowering the oxygen demand can augment flood tolerance. Another possible solution in our systems would be lowering the water level to promote root

penetration and DO input. Furthermore, a vertical flow constructed wetland instead of a horizontal flow can overcome problems of flood, oxygen and nutrient stress. In these systems, water is fed intermittently. The water infiltrates into the substrate, than gradually drains down vertically and is collected by a drainage network at the base. Until the next feeding, oxygen re-enters the system. Stressors, which are more difficult to observe, are a variety of plant pathogens including rusts, fungi, viruses, and bacteria. These organisms are not considered to be especially virulent in any of the plant species planted in most constructed wetlands, but are common residents in wetland plant populations and add additional stress to plants already under stress from other factors. Inoculation of consortium 3 to reed bed 1 might be the reason for the delay in plant growth compared to reed bed 2. Inoculation of bacteria might result in extra oxygen consumption, and consequently even stronger anaerobic conditions, creating enhanced stress. Furthermore, it was found that *Pseudomonas viridiflava* is listed as a prevalent phytopathogenic bacterium, however, nothing is known about the contribution of the strain in the consortium and its survival and activity in the reed bed. This result indicates the importance of ascertaining the identity of the target bacterial strains. In general, for bioremediation, survival of the constituent isolates of a bacterial consortium is as important as any other factor (Ramos *et al*., 1991; Thiem *et al*., 1994). To facilitate detection, a bacterium can be modified by chromosomal integration of marker genes, resulting in resistance to a particular antibiotic or heavy metal, or show fluorescence characteristics. Subsequently, survival can be followed by selective plating or biolumuniscence detection. Recent developments in molecular techniques as DNA:DNA hybridisation, PCR and DGGE have made it easier to identify the introduced strains and assess their survival (Di Giovanni *et al*., 1999; Guo *et al*., 1997; Huertas *et al*., 1998; Thiem *et al*., 1994; Versalovic *et al*., 1991; Weidmann-Al-Ahmad *et al*., 1994).

Temporal nitroaromatics concentrations were evaluated in the systems. Biological activity might contribute to the low concentrations of nitroaromatics already detected in the influent gravel, as a biofilm clearly developed on the influent

gravel. In addition, abiotic processes might play as well, but losses due to adsorption on the viton tubing, gravel and PVC tray were neglectable (data sorption test not shown). The disappearance of detectable NAC from day 37, suggests an adaptation phase for the biological activity.

In general, the composition of microbial communities is linked at the redox situation and other hydrogeochemical parameters (Röling *et al*., 2000). This might explain the heterogeneity in the bacterial community from the centre and the inlet. However, on day 21 there was a similarity found between both communities. It should be mentioned that on day 14 the beds were poured again with 10L of a 0.4% green granule solution, which diminished the stressors mentioned above and disturbed the established gradient in the bed, which might explain the observed result. The weak fingerprint from communities in the centre can be due to a lower cell density or PCR inhibition by toxic compounds. Higher concentrations of toxic compounds (toxins excreted by plants, heavy metals, nitroaromatic compounds) due to evapotranspiration and the passage along more roots can influence both survival and PCR reactions. Comparing the community composition from the inlet, it is obvious that the same endogenous bacteria are present in the three systems, with additional strains belonging to the rhizosphere.

The inoculated consortium 3 declined to a density below detection limit, because the organisms failed to survive some initial biotic and abiotic stress. Much of the research that has been done on inoculation of bacteria in soil and water to enhance biodegradation has centred on pesticides, oil, or specific hydrocarbon constituents of oil (Barles *et al*., 1979; Crawford and Mohn, 1985; Edgehill and Finn, 1983; Mishra *et al*., 2001; Pignatello *et al*., 1983; Shirkot and Gupta, 1985). Many factors affect survival and activity of introduced microorganisms, including inoculum density, nutrient levels, salinity, pH, temperature, presence of toxic chemicals, competition with indigenous microorganisms, predation by protozoa and even the inoculation protocol (Acea and Alexander, 1988; England *et al*., 1993; Heijnen *et al*., 1992; Jackman *et al*., 1992; Kästner *et al*., 1998; Recorbet *et al*., 1992; Swindoll *et al*., 1988; Vandenhove *et al*., 1991; Wessendorf and Lingens, 1989). In

the presence of an organic nutrient that one bacterial species but few other members of the microbial community can use, the bacterium carrying out the transformation presumably is able to multiply. However, up to date, bacterial mineralisation of TNT can be excluded. Introducing consortium 3 might have the only advantage of lowering plant toxicity fast by cometabolism of TNT, and in addition, the transformations are advantageous to initiate plant metabolism through adsorption and absorption.

We concluded that we were confronted with several physical, chemical and biological problems inherent to the system, which caused poor plant growth and poor survival of the inoculated consortium. Consequently, from our results it is not clear whether *Phragmites australis* (common reed) has the potential to strongly enhance the elimination of TNT and ADNTs from contaminated water in a well-established wetland system. However, total ADNT concentrations were slightly lower in our plant-systems (Figure 8.5) compared to the control (Figure 8.6), which might indicate a positive effect contributed to the plants. There are no indications that inoculation with the bacterial consortium 3 would result in enhanced biodegradation in a well-established wetland, as the inoculum was found not to survive in our system. Consequently, as to be expected, the efficiency of both reed beds (with and without inoculation) was similar towards nitroaromatic compounds removal. However, plant growth was stronger inhibited when consortium 3 was inoculated, indicating the presence of an extra stress factor to the plants. Further detailed research is required for successful establishment.

CHAPTER 9
GENERAL DISCUSSION

9.1 BIOREMEDIATION OF NITROAROMATIC COMPOUNDS

Selective enrichment of cultivable indigenous bacteria from a heavily contaminated brownfield revealed the potential for metabolizing 2,4-DNT at the site. An attempt to isolate degrading strains by selective enrichment on other NACs was unsuccessful, suggesting that these particular strains did not have the ability to degrade these compounds, that they are not cultivable, or that the enrichment procedure was faulty. However, two 2,4-DNT-metabolizing consortia, consortium 1 and consortium 3 with four and six members, respectively, were isolated from a 2,4-DNT hot spot. Both consortia used 2,4-DNT as their sole nitrogen source without accumulating detectable degradation intermediates. Our results are consistent with previous publications in which NAC-metabolizing bacteria were isolated by selective enrichment only from sites contaminated with the nitro compounds of interest, and not from nearby uncontaminated sites (Johnson *et al.*, 2000; Nishino *et al.*, 2000; Spain 1995; Spanggord *et al.*, 1991). These observations indicate that such strains are not widely distributed in ecosystems that have not been exposed to the nitro compounds. Selection pressure caused by historical contamination seems to be important for soils to become enriched with nitroaromatic-degrading bacteria. Although microorganisms generate, as secondary metabolites, a few aromatic compounds bearing one nitro group as a substituent (chloroamphenicol, nitropyoluteorin, oxypyrrolnitrin, and phidolopin), most NACs in the environment are caused by human activities (e.g., solvents, explosives, and precursors for pesticides, dyes, polymers, and pharmaceuticals). This probably explains why NACs are relatively refractory to biological degradation and why these xenobiotic compounds are not easily incorporated into ongoing biological

cycles. Due to the strong electron-withdrawing character of the nitro groups, the aromatic ring of these compounds is electron deficient. Several NACs, such as NB, 2-NT, 4-NT, 2,4-DNT, and nitrobenzoates, are mineralized relatively slowly by aerobic microorganisms through their initial oxygenation. TNT is more recalcitrant than mono- and dinitro-toluenes mainly because of the symmetric arrangement of the nitro groups on the aromatic ring so limiting attack by the classic dioxygenase enzymes involved in the microbial metabolism of aromatic compounds (Rieger and Knackmuss, 1995).

Though both our consortia originated from the same sample of soil, the optimised consortia had no common strain, indicating that an obtained selective enrichment cannot always be repeated. However, the culture media of both consortia included the intermediates MNC, HMQ, and THT, demonstrating that together their members constitute the same metabolic pathway as evidenced in *Burkholderia* sp. strain DNT (Suen and Spain, 1993). This finding was not unexpected as, to date, all strains that grow on 2,4-DNT appear to follow the same pathway as strain DNT (Nishino *et al*., 2000). These results suggest a recent evolutionary origin and distribution of the degrading genes (De Souza *et al*., 1998). How the DNT-degrading phenotype evolved or was distributed at contaminated sites is a still-unfolding story. Studies suggest that the initial dioxygenase evolved once from naphthalene dioxygenase or from a common ancestor and then spread horizontally through the bacterial communities at DNT-contaminated sites (Suen *et al*., 1996; Johnson and Spain, 1997; Nishino *et al*., 2000). However, it is unknown how these genes became distributed at sites as geographically far apart as the United States, Canada, Scotland, and Germany.

In contrast with other studies, which isolated single strains that grow on 2,4-DNT as the sole source of carbon, nitrogen, and energy, we isolated 2,4-DNT-degrading consortia. This result suggests that, so far, the prevailing conditions at our site probably did not naturally select a single organism able to mineralize the

compound. However, the interaction between microorganisms with divers degradative properties might be an important starting point in the evolution of novel catabolic strains. A single strain might evolve by appropriately recruiting catabolic functions by gene transfer, mutation, or alteration of gene expression. On the other hand, mixed cultures containing metabolically cooperating genes might be stably maintained anyway. Pelz *et al.* (1999) examined a 4-chlorosalicylate-degrading consortium that seems to be stable because each member plays a crucial role in it, either by providing carbon skeletons to the others, or by scavenging toxic metabolites that would inhibit the primary degrader if they were to accumulate. Seemingly, the metabolic and physiological weakness of the primary degraders may be effectively compensated for by recruiting other organisms with appropriate complementary physiology to build a robust consortial unit that can extract the maximal metabolic benefit from the nutritional opportunity. The herbicides linuron and atrazine are other examples of xenobiotic compounds on which both consortia and pure strains were isolated. Several research groups have separated bacterial consortia that can mineralise linuron (Cullington and Walker, 1999; El-Fantroussi *et al*., 1999; El-Fantroussi, 2000; Roberts *et al*., 1998; Roberts *et al*., 1993). Very recently, a *Variovorax paradoxus* WDL1 was isolated, encoding the total degradation of linuron (W. Dejonghe, pers. comm.). Interdependent catabolism of atrazine to CO_2 was reported by, for instance, Alvey and Crowly (1996), Assaf and Turco, (1994) and De Souza *et al*. (1998), whereas pure cultures of bacteria were described by Bouquard *et al*. (1997), Mandelbaum *et al*. (1995), Moscinski *et al*. (1996), Radosevich *et al*. (1995), and Yanze-Kontchou and Gschwind (1994).

Genetic- and biochemical-data demonstrated that in both consortium 1 and 3 the 2,4-DNT mineralization occurred by specific two-member derivative consortia, though both consisted of more than two strains. Lappin *et al* (1985) similarly selected a five-member mecoprop- (herbicide) degrading consortium in preference to several equally capable two-member derivative consortia, indicating the

existence of metabolic cross-feeding. In addition, De Souza *et al*. (1998) examined an atrazine-catabolizing bacterial consortium containing four or more members, of which two members, *Clavibacter michiganese* ATZ1 and *Pseudomonas* sp. CN1, collectively mineralised atrazine. However, the consortium collectively metabolised atrazine faster than did *C. michiganese* ATZ1 and *Pseudomonas* sp. CN1 in co-culture. Only the consortium, but neither strain individually or the two-member co-culture, could grown on ethylamine (a fission product) as the sole C- and N-source. In addition, the consortium grew more rapidly than did *C. michiganese* alone on isopropylamine (fission product). These data suggested that the occurrence of low steady-state concentrations of alkylamines might selectively maintain the stable consortium. The *Variovorax paradoxus* WDL1, mentioned above, was isolated from a five-member linuron-degrading consortium. Though this pure strain mineralised the compound, degradation was stimulated by synergistic interactions within the consortium (W. Dejonghe, pers. comm.). Timmis *et al*. (2001) concluded that the diversity of interacting species in a community along with the presence of redundant species favors stability and ensures effective utilization of resources under changing environmental conditions.

Neither consortium 1 nor 3 transformed the compounds 2-NT, 4-NT, 2-A-4-NT, 4-A-2-NT, 2,6-DNT, and 2-A-6-NT; however, they did transform TNT but this was not accompagnied by growth. Cells of *Burkholderia* sp. strain DNT grown in DNT transformed 2,3-DNT, 2,6-DNT, 3,4-DNT, and TNT more slowly than 2,4-DNT. No type of mononitrotoluenen was transformed. None of these nitrotoluenes tested could serve as growth substrate for this particular strain (Spanggord *et al*., 1991). Despite of a similar degradation pathway as found in strain DNT, we did not observe 2,6-DNT transformations for our consortia. In *Burkholderia* sp. strain DNT 2,4-DNT dioxygenase catalysed the reaction very slowly and only at low 2,6-DNT concentrations. There are no transformation data available for the other reported 2,4-DNT-degrading strains. However, most isolates grow on a very narrow range of nitroaromatic compounds, typically degrading only the compound

on which they were isolated (Parales, 2000). Consortium 1 transformed TNT partially into 2-A-4,6-DNT, 4-A-2,6-DNT, and unidentified metabolites. This most likely indicates to an aspecific reduction mechanism. Consortium 3 transformed TNT quickly and completely into its amino derivatives, azoxy isomers and unknown compounds. As discussed earlier, the strong electron-deficiency of the aromatic ring of trinitro-compounds, such as TNT and picric acid, prevents electrophilic attack by the oxygenases of aerobic organisms. Instead, nitro groups or the aromatic nuclei are reduced (Rieger and Knackmuss, 1995; Lenke *et al*., 2000; Esteve-Nunez *et al*., 2001). These transformations appear to be occurring co-metabolically and are widely distributed among living organisms. The formation of azoxytetranitrotoluenes indicates the condensation of early reduction products, such as the hydroxylamino or nitroso derivatives of TNT. From the current status of knowledge, mineralization and productive breakdown of TNT still is problematic, the major hurdle being the formation of toxic dead-end metabolites. A way out is through microbial-induced immobilization of the compounds in soil (Lenke *et al*., 2000). Several alternative strategies (e.g., reactor systems, windrow composting, fungal-based remediation, land farming) now are available for co-metabolic treatment of contaminated soil (Jerger and Woodhull, 2000). In general, these procedures involve the complete reduction of the polynitroaromatic compounds and their subsequent binding to organic matter under oxic conditions. However, a better understanding of the mechanisms and kinetics of sorption is required for soil remediation. Furthermore, current research in the field focuses on discovering and developing strategies that would allow TNT to serve as the growth substrate for microorganisms. A *Pseudomonas* strain was isolated that apparently utilized TNT as its sole nitrogen source. The introduction of the TOL plasmid into this strain gave in a hybrid organism that reportedly grows on TNT (Duque *et al*., 1993; Ramos *et al*., 1995). At high initial cell densities, slow growth and nitrite release was observed. However, there was no evidence of sustained growth and degradation. In addition, amino and azoxy compounds were formed, indicating that degradation was inefficient and that nitro groups were being reduced. Overall,

early attempts to engineer nitroarene pathways did not create robust strains useful for bioremediation. Nevertheless, catabolic pathways might evolve under selective conditions. This already was observed for atrazine, the most widely used *s*-triazine herbicide. Atrazine has been employed only over the last 40 years and previously was considered to be non-degradable by the majority of soil bacteria. During the first 35 years of its use, bacterial catabolism of atrazine was proposed to occur largely via N-dealkylation reactions, resulting in the accumulation of aminotriazine compounds (Assaf and Turco, 1994; Behki *et al*., 1993; Bekhi and Khan, 1986; Erickson and Lee, 1989; Giardina *et al*., 1982). More recently, consortia and pure cultures of bacteria were described that break down atrazine to CO_2 (Bouquard *et al*., 1997; Mandelbaum *et al*., 1995; Moscinski *et al*., 1996; Yanze-Kontchou and Gschwind, 1994).

Isolation of our consortia was impelling because a potential for 2,4-DNT degradation was demonstrated to be present at the site. Stimulation via oxygen and/or nutrient amendment, or augmentation via inoculation of enriched bacteria and nutrients might have demonstrated their value as *in situ* environmental remediation strategies. However, questions still remain about the regulation of the degradative pathways and the factors that control efficient biodegradation. Furthermore, most contaminated environments contain combinations of nitroaromatic compounds, which complicate bioremediation efforts. By using genetic engineering or an augmentation approach (inoculating other nitroaromatic-degrading bacteria), it should be possible to combine pathways for breaking down several nitroaromatic compounds. An insight into these aspects will be important in developing effective bioremediation systems.

In our preliminary test presented in Chapter 7, we attempted to enhance the rate of immobilization of TNT in a controlled aerobic slurry process either by stimulating the indigenous bacteria (*ex situ* biostimulation) or by inoculating consortium 3 (*ex situ* bioaugmentation). The results showed that the augmented slurry system has the potential to effectively treat concentrations of high explosives in soil. Research

over a longer period should clarify whether frequent re-inoculation of the consortium, the addition of a surfactant, and/or the supply of extra nutrients (e.g., carbon, nitrogen, phosphorus, vitamins, or minerals) will be necessary to achieve remove 100% of the TNT. Furthermore, detailed studies are needed to determine if biodegradation will transform, degrade, or immobilise the TNT into chemicals that no longer are hazardous.

9.2 PHYTOREMEDIATION OF NITROAROMATIC COMPOUNDS

In this part of the study, we evaluated the feasibility of some techniques of phytoremediation, including rhizofiltration, rhizodegradation, and phytodegradation, to remove TNT and its metabolites primarily from contaminated water, and, to a lesser extent, from lowly contaminated soil.

The ability of the sunflower, *Helianthus annuus*, to take up and subsequently transform and sequester TNT and its metabolites was demonstrated (Chapter 4, Chapter 5). Furthermore, contaminants, such as TNT, are rapidly reduced at the root surface by extracellular enzymes, plant membrane-bound enzymes, or by the rhizosphere microflora. These metabolites, especially amine and hydroxy functional groups bind irreversibly to roots and are further chemically transformed (Hughes *et al.*, 1997). Consequently, we expected inefficient translocation of TNT and its metabolites from the sunflower's roots. Our study showed that the roots contained 86.6 to 92.6% of the plant-associated ^{14}C after 21 days of exposure to ^{14}C-TNT on hydroponics; 47.1 to 51.0 % was non-extractable. The latter might include the fraction sorbed on the roots' surfaces and the fraction sequestered in the root after uptake; however, a distinction cannot be made. These findings are consistent with previous reports (Görge *et al.*, 1994; Harvey *et al.*, 1990; Thompson *et al.*, 1998a; Vanderford *et al.*, 1997). In addition, an increasing percentage of the original compound taken up does became tightly bound to the

plant's structural material over time. Studies by Vanderford *et al*. (1997) and Bhadra *et al*. (1999a) revealed that the bound fraction increased with time, with a commensurate decrease in the levels of extractable plant-associated metabolites. The metabolism of foreign compounds in plant systems is generally considered to be a detoxification process. During the detoxification of TNT, the compound is metabolized in three stages: transformation, conjugation, and sequestration (Burken *et al*., 2000; Dietz and Schnoor, 2001). Sequestration of TNT-derived molecules is established by the incorporation of conjugates into biopolymers (e.g., lignin) where they are characterized as bound (i.e., non-extractable) residues (Sens *et al*., 1999).

A rhizofiltration process using *Helianthus annuus* (sunflower) already exploited for heavy metals and radionuclides (Bing, 1996; Cooney, 1996; Richman, 1996), showed considerable promise for TNT and its metabolites. Removal of 98-, 97-, and 76-% TNT was found after 21 days of plant culture in 5-, 10-, and 20-mg TNT/L, respectively. In addition, the plant was demonstrated to "tolerate" easily concentrations up to 10 mg TNT/L. However, the sunflower is an annual plant, and culturing it needs more operation and maintenance in the field compared to grasses, for instance. Therefore, we chose to work with the common reed, *Phragmites australis* because of its easy establishment and its ecological attributes (e.g., perenniality, high productivity, high leaf- and root-surface areas) mesocosmic wetlandsystems. In addition, we examined the effect of inoculating a system with the bacterial consortium 3 to enhance biodegradation. Compared to consortium 1, metabolism of TNT by consortium 3 was fast and complete and the remaining genotoxicity after treatment of TNT-contaminated water with this particular consortium was lower. Therefore, we expected that introducing consortium 3 might have the advantage of lowering plant toxicity. In addition, the partial reductions of TNT to HADNTs and ADNTs are beneficial in initiating plant metabolism through adsorption at the root's surface and uptake. Therefore, elimination of TNT and its derivatives by plants was expected to occur faster in the presence of TNT-degrading bacteria. However, problems with plant growth and bacterial survival

emerged even before the contaminants entered the system. The reduced plant growth probably reflected a combination of several stressful factors, of which lack of dissolved oxygen (DO) seemed to be the most important as the reed beds were completely saturated with water and flow rates were low. A possible solution in our systems would be to lower the water level to promote DO input. Furthermore, a vertical-flow-constructed wetland instead of one with horizontal flow can overcome problems of oxygen. In these systems, water is fed intermittently. It infiltrates into the substrate, than gradually drains down vertically and is collected by a drainage network at the base. Until the next watering, oxygen enters the system. Furthermore, the inoculated consortium 3 declined to a density below detection limit. The organisms failed to survive some initial biotic- and abiotic-stresses that need further examination. In addition, it is important to facilitate the detection of the target bacterial strain(s), for instance by integrating marker genes into the chromosome. Subsequently, survival can be followed by selective plating or the detection of bioluminescence, which is less labor-intensive or is a valuable addition to DNA:DNA hybridisation, PCR, and DGGE. From our results it is unclear whether *Phragmites australis* has the potential to strongly enhance the elimination of TNT and ADNTs from contaminated water in a well-established wetland system. However, total ADNT concentrations were slightly lower in our plantsystems compared to the control, which might indicate a positive contribution from the plants. There are no indications that inoculating with bacterial consortium 3 would enhance biodegradation in a well-established wetland, as the inoculum did not survive in our system. However, plant growth was more strongly inhibited when consortium 3 was inoculated, indicating that an extra stress factor was imposed on the plants. Further detailed research undoubtedly is required; nevertheless, in general, the potential for removing explosives from natural wetland systems has been demonstrated (Medina and McCutcheon, 1996; Hoagland, 1996).

Work on phytoremediation of explosives has dealt almost exclusively with wetlands. Terrestrial systems appear to be largely constrained by the bioavailability

of the compounds, and the use of terrestrial plants appears to be limited to stabilizing the residual contamination and would be too slow when active removal and degradation are the goals in cleaning a highly contaminated soil (Dietz and Schnoor, 2001). In our study,the rhizosphere of the sunflower, *Helianthus annuus* had not degraded the explosives after 1 month of exposure to contaminated soil. However, Klunk *et al* (1996) and Scheidemann *et al* (1998) observed lower levels of extractable nitroaromatics in rhizosphere soil after 12 and 8 weeks respectively. It may be that the duration of our greenhouse study was to short, or that *Helianthus annuus* is not a suitable candidate for rhizodegradation of soil. Screening plants for the desired characteristics will be an important step (i.e., an extended root system, the release of degradative enzymes such as nitroreductase and laccase, and a high toxicity threshold). Here, perennial grasses might be of particular interest due to their extensive root systems, adaptability to a wide range of environments, rapid establishment, and tolerance to many xenobiotics (April and Sims, 1990; Anderson *et al*., 1993). In addition, expanding the metabolic functions of rhizospheric bacteria to degrade pollutants may prove to be a useful strategy for bioremediation. Experiments by Brazil *et al*. (1995) demonstrated that a rhizosphere-adapted *Pseudomonas fluorescens* could be genetically manipulated to metabolise PCB without affecting its ecological competence. To date, the most promising candidate for our purpose would be *Pseudomonas* sp. clone A (TOL), a hybrid organism that was reported to significantly mineralize TNT (Duque *et al*., 1993; Ramos *et al*., 1995). Bacteria, such as *Pseudomonas* spp., are common in rhizosphere soils (Campell, 1985; Vancura and Kunc, 1989; Nakas and Hagedorn, 1990). Though this particular clone A strain was isolated from soil, it was demonstrated to be a good colonizer of grasses (J. L. Ramos, pers. comm.). Efficient colonization by the introduced bacteria also will be vital. We expect that a bacterial inoculant having the ability to use toxic compounds, such as TNT, should have a competitive advantage over soil bacteria lacking this ability. Gunner *et al* (1966) first demonstrated the effect of specific compounds on the selection of rhizospheric microflora, showing that the dominant species in the rhizosphere of bean plants

sprayed with an organophosphate insecticide were those able to use the insecticide metabolites as nutrient source.

Overall, in addition to characterizing phytocatalytic pathways (i.e., identifying intermediates, reactions, enzymes, and genes), it will be important to gain insight into the combined effects of photolysis, and the interactions between plants, microbes, and the sediment soil. Research also needs to address questions about the toxicity of bound residues and unidentified metabolic products that may accumulate in plant tissues. The biological endpoint of contaminants is of great concern.

9.3 CONCLUSION

The optimal remediation strategy for nitroaromatic compounds depends on many site-specific factors. Composting and using reactor systems lend themselves to treating soils contaminated with high levels of explosives, such as those at former ammunition-production facilities, where areas with a high level of contamination are common. Compared to composting systems, bioreactors have the major advantage of a short treatment time, but the disadvantage of being more labor-intensive and more expensive. Although phytoremediation has not yet been widely used, it has the potential to become an important strategy for remediating soil and water lightly contaminated with explosives (e.g., at military sites where pollution is rather diffuse and where largecontaminated surfaces or volumes have to be treated). In addition, phytoremediation can be used as a finishing method after using other remediation treatments. This *in situ* treatment method has the advantage of costing less, but the disadvantage of requiring a considerably longer time for treatment.

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