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Treatment of Trinitrotoluene (TNT)-contaminated Wastewater in Constructed Wetland

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For my dear Family

List of publications relevant to this work

1. Xiang X. M., Machate T., Wang R., Schramm K. W., Yang F. L., Zhou J. T., and Kettrup A., 2001. Sucrose-enhanced TNT removal in constructed wetland. Proceedings of the 11th International Symposium on Environmental Pollution and its Impact on Life in the Mediterranean Region. Oct. 6th to 10th, 2001. Limassol, Cyprus. P. E 39.

2. Xiang X. M., Machate T., Hoffmann I., Behechti A., Schramm K.-W., Yang F. L., Kettrup A., and Zhou J. T.. Sucrose enhanced anaerobic removal of 2,4,6-trinitrotoluene (TNT) and studies on the acute aquatic toxicities of 2,4,6-TNT and some related monoamino metabolites. Manuscript, tentatively accepted by Chemosphere in 2001.

3. Hoffmann I., Xiang X. M., Schramm K.-W., Machate T., Kettrup A., 2000. Remediation of contaminated water containing TNT and related nitroaromatic compounds in constructed wetland plant. Poster 7th International FCK/TNO Conference on Contaminated Soil. Leipzig.

Abstract

A novel treatment process involving external carbon-enhanced phytoremediation of TNT-contaminated wastewater was developed in this study. External carbon sources such as sucrose and molasses were used to enhance the treatment of TNT contaminated wastewater in a free-water surface pilot-scale CW. The constructed wetland consists of four cascades, which contains five steel tanks filled with lava and planted with *Typha spp.* and *Scirpus lacustris*. The results showed that addition of appropriate cosubstrate to CW could increase the removal rate of TNT, reduce treatment facilities, save constructed areas and thus provide a cost-effective technology for wastewater treatment. The effects of other factors such as HRT (hydraulic retention time) and technical aeration on TNT transformation in a CW were also examined in order to optimize the operational conditions for the CW. The results indicated that TNT removal efficiencies increased with increasing of HRT and no significant effects of technical aeration on TNT removal was found.

^{14}C-labelled TNT was used in studies of the mechanism of TNT transformation in closed bottle cultures with addition of cosubstrate. No significant mineralization of TNT was found. 20-35% of the initial ^{14}C-activity was found in sludge, which was associated with biomass and 55 to 70% in the culture supernatants.

The predominant TNT transformation products detected in extracts of effluent were monoaminodinitrotoluenes (4A2,6-DNT and 2A4,6-DNT), diaminonitrotoluenes (2,4DA6-NT, and 2,6DA4-NT), dinitrotoluenes (2,4-DNT and 2,6-DNT), and nitrotoluenes (2-NT and 4-NT). Addition of cosubstrates such as sucrose and molasses could enhance both the removal of TNT and the further transformation of its intermediates in the CW.

Analysis of plant materials showed the presence of both the parent compound TNT and its reduction products, 4A2,6-DNT, 2A4,6-DNT, and 2-NT. Adsorption of TNT and its transformation products on to lava materials occurred. However, with low TNT influent concentrations, the adsorption of TNT and its transformation products was negligible.

Zusammenfassung

In der vorliegenden Arbeit wurde ein neuartiger Prozess zur Behandlung TNT-kontaminierter Abwässer auf der Basis einer Phytoremediation mit extern zugesetzten Kohlenstoffquellen entwickelt. Es wurden kommerziell erhältliche, wirtschaftlich günstige Kohlenstoffquellen, wie Sucrose und Molasse verwendet, um die Behandlung von TNT-kontaminiertem Abwasser in einer Pilot-Pflanzenkläranlage zu verbessern. Die Pflanzenkläranlage besteht aus fünf Stahlbecken, die in Form von vier Kaskaden angeordnet, mit Lava befüllt sowie mit *Typha spp*. bzw. *Scirpus lacustris* bepflanzt sind. Die Ergebnisse zeigten, dass die Zugabe von geeignetem Cosubstrat in die Pflanzenkläranlage die Eliminierungsrate von TNT deutlich erhöht. Dadurch können derartige Abwasserbehandlungsanlagen verkleinert und die für die Behandlung erforderliche Flächen reduziert werden. Deshalb stellt das Verfahren eine kostengünstige Technologie zur Abwasserbehandlung dar. Effekte anderer Einflussfaktoren auf die TNT-Transformation, wie die Verweilzeit und die Belüftung wurden ebenfalls geprüft, um die Betriebsbedingungen der Pflanzenkläranlage zu optimieren. Die Ergebnisse zeigten, dass die TNT Abbaurate stieg mit zunehmender Verweilzeit. Ausserdem wurde keine signifikante Änderung der Abbaurate bei Belüftung gefunden.

Um den Mechanismus der TNT-Transformation unter Zugabe von Cosubstrat zu studieren, wurde ^{14}C-TNT verwendet. Dabei wurde keine signifikante Mineralisierung von TNT beobachtet. 20-35 % der eingesetzten ^{14}C-Aktivität wurde in der Biomasse gefunden, die mit mikrobiellen Organismen assoziiert war, 55-70 % wurde im wässrigen Überstand ermittelt.

Die im Abflussextrakt detektierten TNT-Transformationsprodukte setzten sich vorwiegend aus Monoamino-Dinitrotoluenen (4A2,6-DNT und 2A4,6-DNT), Diamino-Nitrotoluenen (2,4DA6-NT und 2,6DA4-NT), Dinitrotoluenen (2,4-DNT und 2,6-DNT) und Nitrotoluenen (2-NT und 4-NT) zusammen. Die Zugabe von Cosubstraten förderte sowohl die TNT-Eliminierung als auch die weitere Transformation von TNT-Abbauprodukten.

Die Analyse des Pflanzenmaterials zeigte dass sowohl im Sprossteil wie auch in den Wurzeln neben dem Ausgangsstoffes TNT, die Reduktionsprodukte, 4A2,6-DNT, 2A4,6-DNT und 2-NT gefunden wurde. An Lavamaterial wurde in geringem Umfang eine Adsorption von TNT und seinen Abbauprodukten beobachtet.

List of abbreviation

ADNTs	Aminodinitrotoluenes
CW	Constructed wetland
DAD	Diode-array detector
DANTs	Diaminonitrotoluenes
DCM	Dichloromethane
DNTs	Dinitrotoluenes
DO	Dissolved oxygen
DOC	Dissolved organic carbon
EMME	Ethylene glycol monomethyl ether
GR	Gradient grade for analysis
HPLC	High performance liquid chromatography
LSC	Liquid scintillation counter
HRT	Hydraulic retention time
MPW	Millipore water
MSM	Mineral salt medium
NTs	Nitrotoluenes
SF	Surface flow
SMPW	Sterile MPW
SPE	Solid phase extraction
SSF	Sub-surface flow
TAT	Triaminotoluene
TLC	Thin layer chromatography
TNT	2,4,6-Trinitrotoluene
TOC	Total organic carbon
UV	Ultraviolet
VOCs	Volatile organic chemicals
4A2,6-DNT	4-amino-2,6-dinitrotoluene
2A4,6-DNT	2-amino-4,6-dinitrotoluene
2,4DA6-NT	2,4-diamino-6-nitrotoluene
2,6DA4-NT	2,6-diamino-4-nitrotoluene
2,4-DNT	2,4-dinitrotoluene
2,6-DNT	2,6-dinitrotoluene
2-NT	2-nitrotoluene
4-NT	4-nitrotoluene

Contents

1. Introduction

1.1 Constructed wetlands (CW)

Concepts

Wetlands, either natural or artificial (constructed), have substantial capacity for wastewater treatment or renovation (Venus, 1987). The use of constructed wetlands (CWs) for wastewater treatment has gradually developed over the past 20 years. At the International Conference on Wetland Systems for Water Pollution Control held in Vienna on the 19th of September 1996, CWs were defined as any setups that have been realized by human interference in order to treat wastewater and that are inhabited by plants (Makerere University, 1997). These systems are designed to mimic natural wetland systems, utilizing wetland plants, soil, and associated microorganisms to remove contaminants from wastewater effluents (EPA, 1993). Compared to conventional wastewater treatment technologies such as incineration, filtration, coagulation, and adsorption, constructed wetland treatment systems are cost-effective, easy to operate, provide secondary benefits (e.g. habitat enhancement) (Pinney, et al., 2000) and have strong potential for application in both developed and developing countries.

Classification and principles of the treatment process

Depending on the abundance of existing aquatic plants or macrophyte, CWs used for the treatment of wastewater can be divided into the following three systems:

- free floating systems
- rooted emergent systems
- submerging systems

The vast majority of the systems used in Europe are based on rooted emergent macrophyte systems. Within this group, different designs are again categorized according to surface (SF) or sub-surface (SSF) wastewater flow patterns (Haberl et al., 1995).

For different types of CWs, the principles of the treatment processes are almost the same. A

variety of physical, chemical and biological processes are involved in the removal of pollutants and pathogens from the vegetated systems (Bavor et al., 1995). These include sedimentation, precipitation and adsorption to substrate, assimilation or uptake by the plant tissue, and microbial activity.

Plants existing in CWs play an important role in the removal of pollutants. As the microorganisms do, plants play an important role in nature in sustaining and restoring environments. Plants can directly or indirectly take up, accumulate and metabolize various organic substances (McFarlane et al., 1990). Plant roots can foster the degradation of organic pollutants by releasing compounds capable of supporting growth of microorganisms, which are capable of biodegrading environmental pollutants (Walton and Anderson, 1990; Anderson et al., 1994; Shann, 1995; Jordhal et al., 1997). Since aquatic plants have natural mechanisms of pumping air via their root systems, their root areas provide oxygen-rich environments suitable for development of aerobic microfauna around them (Loveridge et al., 1995). The extensive root system of the weeds provide large surface areas for the growth of these microorganisms thus increasing their potential for decomposing organic matter. Greater microbial density and diversity can be found in the rhizosphere (Anderson et al., 1994). Thijs et al. (1994) has reported that microorganisms isolated from natural system rhizospheres actually show greater rates of xenobiotic degradation. In addition, aquatic macrophytes can also improve the hydraulic conductivity of the growth medium through their digging actions.

Microorganisms play a central role in the transformation of nutrients and removal of toxic organic compounds added to wetlands (Hoppe et al., 1988; Pitter and Chudoba, 1990; Savin and Amador, 1998). The metabolic activity of the microbiological community causes chemical degradation of organic molecules to inorganic compounds such as H_2O, CO_2, SO_4^{2-}, PO_4^{3-} and NH_3. Ammonia can further be removed through volatilization and nitrification/denitrification, and phosphorus is removed through adsorption and precipitation (van Oostrom and Russell, 1994; Tanner et al., 1999).

Application

Research over the last two decades has accumulated much quantitative information on the performance of CW systems. CWs are used for treating various types of wastewater, e.g. domestic wastewater (Cooper et al., 1997; Schreijer et al., 1997), acid mine drainage water (Kleinmann and Girts, 1987; Brodie et al., 1989; Howard et al., 1989; Wenerick et al., 1989) agricultural wastewaters (DuBowry and Reaves, 1994; Rivera et al., 1997), landfill leachate (Dombush, 1989; Trautmann et al., 1989; Staubitz et al., 1989), urban storm-water (EPA, 1993), and for purifying advanced treated wastewater effluents for recirculation to freshwater resources (Schwartz et al., 1994; Gschloessl et al., 1998). Machate et al., (1997) and Noll (1997) reported promising results of a pilot-scale CW for purifying polycyclic aromatic hydrocarbons (PAHs)-contaminated wastewater. CWs are also used for treating eutrophic lake waters (D'Angelo and Reddy, 1994), and for conservation of nature (Worrall et al, 1997). Wetlands have recently been suggested as alternatives for the treatment of nitrate-contaminated aquifers, denitrification of nitrified sewage effluents and irrigation return flow (Baker, 1998).

1.2 TNT and explosives

2,4,6-Trinitrotoluene (TNT) is a widely used explosive and propellant that has been in use for more than 130 years. Wilbrand discovered TNT in 1863 and in 1902, an important application of TNT in the German military industry was found (McConnel and Flinn, 1946). Other countries soon adopted it. During the World War I and II, millions of tons of TNT were produced and used mainly as an ingredient in binary explosives due to its safe production and stability (Zitting et al., 1982). It is estimated that, in Germany, about 60 former production sites of TNT exist and approximately 800,000 tons of TNT were produced during the World War II (Preuss and Haas, 1987).

TNT manufacturing involves stepwise nitration of toluene in a three-stage batch process or continuous process producing mono-, di-, and finally trinitrotoluene, respectively. The

undesired isomers plus residual dinitrated species are removed from the reaction mixture by treatment with aqueous sodium sulfite solution, which reacts with everything except the desired 2,4,6-isomer. These impurities are made water soluble in the selliting operating, producing a waste stream commonly referred to as "red water". The "red water" is a complex mixture of nitroaromatics and various inorganic salts. For further disposal, the "red water" was treated by evaporation-concentration and concentrated incineration. The remaining TNT is washed and cast molten onto a flaker belt for packing. Considerable amount of water is used to wash purified TNT, to clean equipment, and to clean the interior of the finishing plant buildings. Wastewater from the TNT finishing process is called "pink water". "Pink water" is a saturated TNT solution (up to 150 ppm TNT), which turns pink in sunlight due to photolysis of dissolved TNT to form complex dye-like molecules (Patterson et al., 1976; Tatyrek 1976). It is estimated that a single manufacturing plant can generate as much as 2000 m^3 of wastewater per day, containing TNT as well as other nitrocompounds (Pereira et al., 1979).

The major route of entry of munition compounds into surface water environment is through discharge of waste streams generated during their manufacturing and processing. The levels of their compounds in such wastewaters vary widely, depending on the intensity of the manufacturing operations and efficacy of the treatment technologies employed. For example, numerous surveys of effluent TNT levels at munitions plants have reported concentrations ranging from < 0.05 mg/L to near saturation in the worst cases (Ryon et al., 1984). During the manufacture of TNT, numerous nitroaromatic by-products are formed and enter the effluent as plant cleanup and scrubber wastes and as condensates from evaporative concentration of "red waters". Characterization of the steam-volatile trace organics of a representative TNT effluent led to the identification and quantitation of over 30 nitroaromatics (Spanggord et al., 1982).

Disposal of TNT and its degradation products from munitions manufacturing plants presents a potentially serious hazardous environmental problem. Soil contamination by munitions compounds has occurred at open-burning and incinerator sites, and also as a result of seepage

from operational sills, landfills and wastewater holding facilities. Montemagno (1991) has reported that subsurface soil at a TNT burning ground was contaminated at depths of about 3.9 – 4.6 m. The greatest concentrations of explosives have been found in soils at depths of 1.5 – 3.4 m. Soil surveys of most other munitions plants in the USA, indicated that crystalline TNT was frequently found in contaminated sites, though TNT was no longer manufactured in the United States (McGrath, 1995). In the field, TNT exists as chunks of weathered, tiny crystals embedded in the soil matrix, and as molecules adsorbed onto the soil surface. These large and small TNT crystals in the soil matrix serve as a continuous contamination source.

An important consequence of the contamination of soils by munitions compounds is the contamination of ground water. Explosives and their transformation products have leached from contaminated surfaces into subsurface aquifers. Aquifer pollution plumes continue to spread beyond military surface property boundaries and are threatening municipal water supplies (Shulman 1992; Jenkins et al., 1994). Montemagno (1991) reported that levels of TNT of more than 2 mg/L was found in shallow groundwater from the TNT burning ground site at Newport Army Ammunition Plant. Concerns about the environmental fate of TNT residues have intensified because pollution plumes also have negative impacts on agricultural irrigation wells, since irrigation and vegetation of contaminated plots could allow TNT, TNT metabolites, and plant-produced TNT intermediates to be introduced into the food chain (Harvey et al., 1990).

The toxicity of TNT and its degradation products is well documented. Previous research has shown inhibition of activities of gram-positive bacteria, fungi, actinomycetes, yeasts, and fresh-water algae as well as plant growth by TNT at concentrations between 2 and 50 mg/L (Klausmeier et al 1973, Smock et al. 1976, Won et al. 1976, Goerge et al. 1994). TNT has also been shown to affect the central nervous system, to cause dizziness, headache, fatigue and sleepiness, and liver damage and even death in cases of extreme exposure in workers at munition plants (von Oettingen, 1941). In addition, TNT has been found to be a frameshift

mutagen that accelerates the reversion rate of a frameshift tester *Salmonella typhimurium* (Ames, et al. 1973). Therefore there is a clear need to develop procedures for the remediation of water and soil contaminated with TNT.

Incineration is used as one of the techniques for remediation of TNT contaminated soil currently. This is a costly and energy-intensive process that destroys a lot of the organic portion of the soil leaving ash as the primary residue (Rieger and Knackmuss, 1995). Activated carbon adsorption is used for the treatment of "pink water". Removal of 99.5% of TNT has been reported (Preslan et al., 1993). However, this treatment method suffers a serious limitation in that the carbon can not be safely regenerated. Loaded carbon is disposed of by incineration after a single use (EPA 1976; Gordon and Hartley, 1992).

Apart from physical and chemical methods of decontamination, biological methods are hold promising as more effective and less costly treatment strategies. Many microorganisms appear well equipped to reduce the aromatic nitro group functionality (Haidour and Ramos, 1996, Bruns-NageL, et al., 1998, Toze and Zappia, 1999). Numerous common aerobic and anaerobic bacterial genera including *Pseudomonas, Desulfovibrio, Escherichia, Bacillus, Citrobacter, Enterobacter, Klebsiella, Veillonella,* and *Clostridium* have been reported to be capable of transferring TNT. Fungi (e.g. *Phanerochaete chrysosporium*) show promising ability for bioremediation of TNT contaminated soil and groundwater (Amerkhnova and Naumova, 1978; Fernando et al., 1990; Bumpus and Tatarko, 1994; Spain 1995; Collie and Donnelly, 1995; Martin et al., 1997). Toxic TNT can be degraded by microorganisms into various metabolic products, or finally into carbon dioxide, water, and energy (Bumpus and Tatarko, 1994; Bradley and Chapelle, 1995; Boopathy and Manning, 1999).

Phytoremediation of munitions such as 2,4,6-trinitrotoluene (TNT) was also investigated. Many studies have reported the fact that plants and microorganisms can take up and transform or degrade TNT both from water and soil (Boopathy, et al., 1994a; Hughes et al., 1997; Rivera,

et al., 1998; Kreslavski, et al., 1999). As demonstrated by Best et al. (1997a,b,c, 1998), the removal of TNT from water was enhanced in the presence of several submersed and emergent plants. CWs as a practical technology of phytoremediation has therefore great potential on the treatment of munitions contaminated wastewater.

1.3 Cometabolism and cosubstrate-enhanced TNT biodegradation

Cometabolism refers to the transformation of an organic compound by a microorganism that is unable to use the substrate or its constituent elements as a source of energy (Alexander 1967; Horvath R. S., 1972b). In this case, two types of reactions are possible. In one type of reaction, the cometabolized substrate is transformed in the presence of a second compound that supports growth. In the second, the substance is transformed in the absence of a second substrate (Horvath and Alexander 1970). Cometabolic transformations are of great environmental significance because of the tendency of the organic metabolic products to accumulate (Horvath, 1972a). Many microorganisms (such as *Pseudomonas sp., Micrococcus sp., Streptomyces aureofaciens*) have been demonstrated to have the capability to effect cometabolic degradation of organic pollutants through enrichment of biodegradable analogues of the pollutant to the microbial ecosystem. It has also been suggested that cometabolism may account for the degradation of many pesticides which do not sustain microbial growth (Horvath 1972a,b.).

It is generally accepted that TNT, like other nitroaromatic compounds, is relatively persistent to biological degradation, although it can be metabolized by specific microbes under appropriate conditions (Funk et al., 1993; Bradley and Chapelle, 1995; Crawford, 1995a). It remains controversial whether TNT can be used as sole C- or N-source, or not. One case where microbial grown on TNT as sole carbon source has been reported (Traxler et al., 1974). However most of the studies reported that the removal of TNT was very slow in cultures that received TNT as sole source of carbon and energy, and the requirement of supplementary nutrients for the metabolism of TNT was generally well-recognized (Nay et al., 1974; Naumova

et al., 1988; Montemagno, 1991; Boopathy et al., 1993b, 1994b; Roberts et al., 1996; Tharakan and Gordon, 1999). Externally readily degradable carbon sources like glucose, molasses, succinate, citric acid, yeast extract, and even surfactants have been used to enhance the biodegradation of TNT (Boopathy et al., 1994b; Boopathy and Manning, 1999; Tharakan and Gordon, 1999). It is likely that additional carbon sources will be required to maintain an active microbial population in the treatment system and the resulting increase in cell density and oxygen demand will likely promote reduction of the nitro moiety of TNT (Martin et al., 1997). In spite of the inherent difficulties of biological treatment, the cosubstrate-enhanced phytoremediation of TNT contaminated wastewater presents an attractive alternative to conventional treatment methods. From these observations, it is expected that the combination of phytoremediation and cometabolism could be more economical and effective if proper care were taken in designing and operating the treatment system.

Little was known about the combined potential of phytoremediation and cometabolism on removal of munitions waste till now. Further researches on the combined potential of CWs and cosubstrates on munition waste treatment as well as their separate potential are crucially needed before cosubstrate-enhanced phytoremediation can be successfully applied to remedy munition-contaminated soils and groundwater.

2. Objectives

Although CWs have been widely used for treatment of agricultural wastewater, landfill leachate and domestic sewage, their application on munition-contaminated groundwater is relatively new. In addition, very few studies on the application of CWs in treatment of toxic organic wastewaters have been done. Little is known about the combined potential of cosubstrate and phytoremediaton on wastewater treatment. This is the first attempt to use cosubstrates for wastewater purification in CWs. The conclusion to be drawn from this research will be applicable to phytoremediation of toxic organic wastewaters and is of special importance for broadening the application of CWs, which can provide a low-cost, low-impact technology instead of the traditional cost-intensive wastewater treatment plants.

The overall objective of this thesis is to investigate the efficiency and suitable operating conditions of CWs in treating TNT contaminated wastewater with addition of potential cosubstrates.

Sucrose and Molasses were selected as potential cosubstrates in this research. Sucrose was selected because it is one of the cheap and pure food productions that can be used as external carbon sources by microorganisms (Mehna et al., 1995; Schwarz et al., 1999). Molasses is an important by-product of sugar cane production, which contains about 50% sugar in form of sucrose, glucose and fructose and is rich in mineral elements such as calcium, potassium, sodium, iron, choline, and magnesium (Hemmerly, 1983). It is favorable for growth of microorganims and therefore selected as one of the cosubstrates for TNT removal from wastewater by CW.

In summary, the following objectives are addressed in this thesis:

I. Studies of the performance of CWs on the removal of TNT from wastewater in the absence of cosubstrate.

These investigations will provide basic information on capability of CW on TNT-contaminated wastewater treatment. The information obtained would help: to determine the effectiveness of CWs to decrease concentrations of TNT from inflow to outflow; to determine the appropriate CW design parameters for treatment of TNT contaminated groundwater; and to explain the role of growing plants on the depletion of TNT from water.

The topics to be covered include:
(i). The role of plants on the removal of TNT from wastewater.
(ii). The effect of technical aeration on the removal of TNT from wastewater
(iii). The effect of hydraulic retention time on the removal of TNT from wastewater.

II. Studies on the combined potential of cometabolism and phytoremediaion on TNT removal from wastewater in the CW.

The present study should aim more directly for the application of cosubstrate in CWs. The effectiveness of CW on TNT removal with the presence of cosubstrates will be examined in a pilot-scale CW. The present study will also provide necessary parameters, such as the type of cosubstrates and their optimal concentrations to be used in phytoremediation process.

The topics to be covered include:
(i). The effectiveness of sucrose as cosubstrate on the removal and transformation of TNT from wastewater in CW.
(ii). The effectiveness of molasses at different concentrations as cosubstrate on the removal and transformation of TNT from wastewater in CW.

3. Site description

Constructed Wetland site description

A pilot scale surface flow wetland, designed by the Umweltschutz Nord GmbH & Co., was constructed in the GSF-National Research Center for Environment and Health, Institute of Ecological Chemistry (Munich, Germany) in 1991. The constructed wetland system consists of four cascades. One of the cascades was designed for control leaving uncontaminated with any artificial wastewater. Two of them were used to perform the treatment of TNT-contaminated artificial wastewater in this study while the last one was not used in this study.

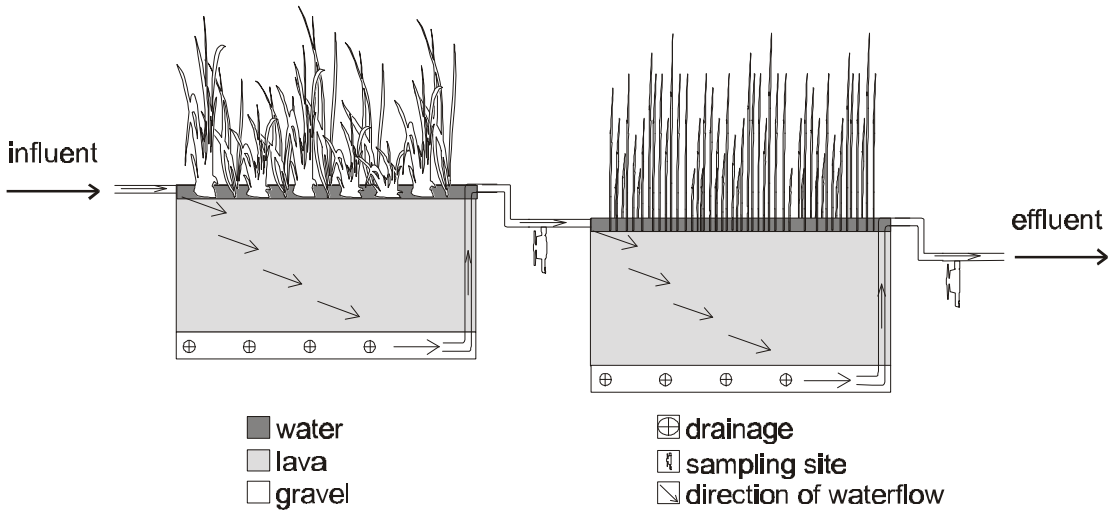


Fig. 1. Longitudinal view of the constructed wetland system

Each cascade consists of five steel tanks (2.5m L x 2.3m W x 1.2m H) connected in series, providing approximately 29 m^2 of surface area altogether. The first two tanks of each cascade were built on the same horizontal level, and the next three tanks, which were cascade-like, were built at a different horizontal level of 25 cm below. The water depth in each tank kept at

approximately 5 cm above the support medium surface. At the bottom of each tank, collector drains were installed to discharge the wastewater to the pond located near the outlet of each tank. The wastewater is pumped into the first tank and the treated water is collected in the collecting pond then flows into the next tanks by gravity (Fig. 1). This feature chosen has reduced hydraulic short-circuiting through the wetland.

All tanks were filled with lava materials (nominal diameter 2-8 mm, porosity 55%, filling height 1m), which served as the growing plant substrate. The first two tanks (except the first tank of Cascade 2) of each cascade were planted with cattails (*Typha spp*.) and the other three tanks were planted with bulrushes (*Scirpus lacustris*). The first tank of Cascade 2 was left unplanted, which was designed to serve as control with no growing plants. At the time of this study (June 1999-Oct. 2000), wetland vegetation was well established. The total view of the wetland plant is shown in Fig. 2. At the outlet of the CW an activated carbon filter was installed to retain residual nitroaromatic compounds from the wastewater.

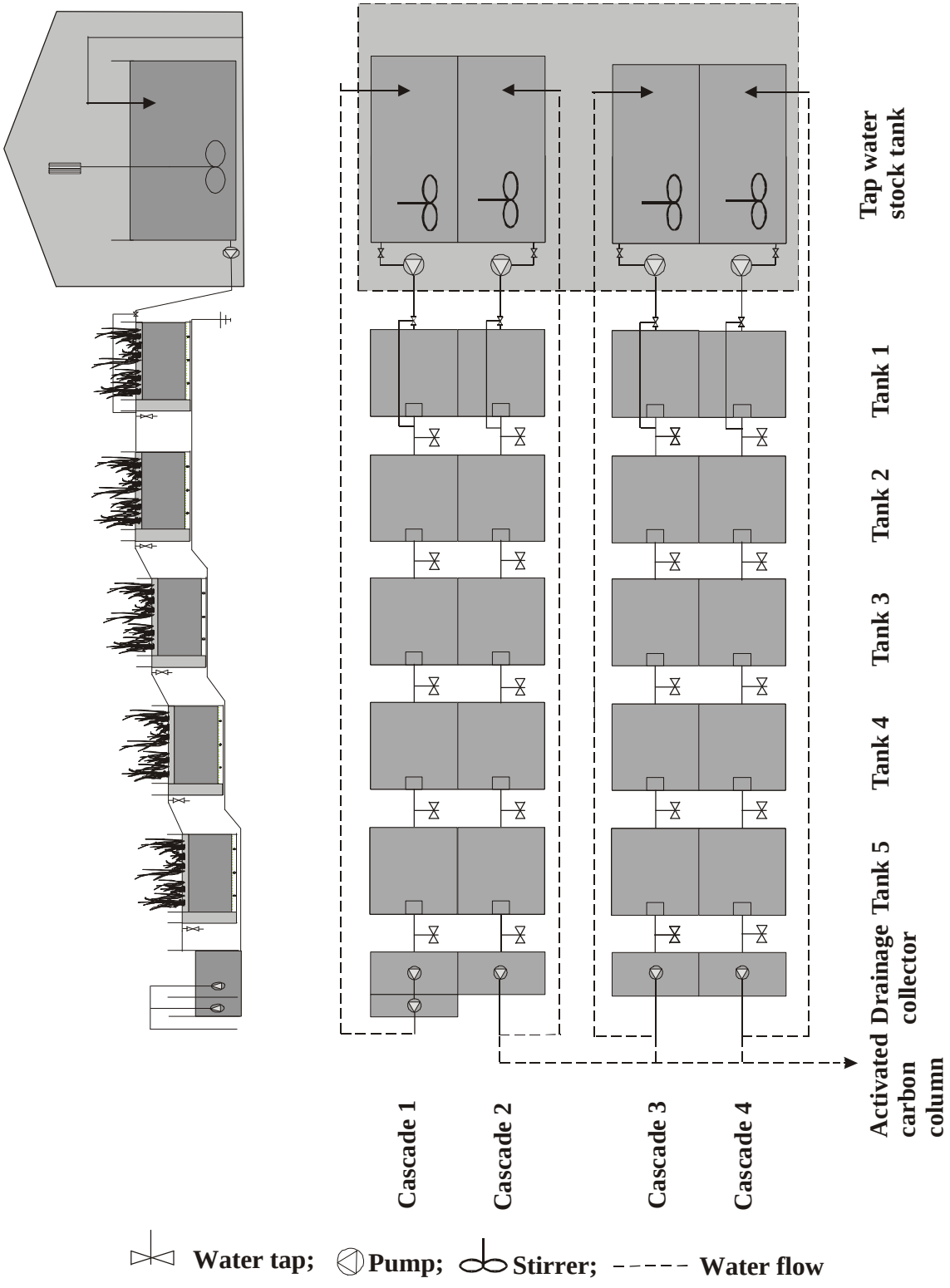


Fig.2. The over view of the constructed wetland

Preparation of artificial wastewater

Artificial wastewater was produced using a TNT wastewater generator as shown in Fig. 3. A steel column equipped with stainless screen at both ends and filled with solid TNT (approx. 20g, particle diameter approx. 3 mm), was continually fed with tap water at a flow rate of 50-100 ml/min. The resultant solution was directed into a mixing chamber where it was diluted contineously with tap water to a final TNT concentration of about 1.0-5.0 mg/L. This artificial wastewater was then pumped into the CW at designed flow rate of 2 L/min, 1 L/min, and 0.5 L/min, which achieved calculated hydraulic retention time (HRT) of 2.4 –9.6 days through each tank. Flow rate was measured weekly. Appropriate amount of TNT was added to the TNT-generator every day during the experimental period to compensate the reduced mass of TNT.

When it was needed for the experiments, stock solution of cosubstrates (10% (w/v) of sucrose; 0.0625%, 0.625% and 6.25% (w/v) of molasses) prepared in tap water was supplied using a peristalitic pump into the inlet of the CW. The resultant concentration of sucrose in the influent of the CW was 0.25 % (w/v), and the concentrations of molasses were 0.001%, 0.01% and 0.1% in the influent of the CW, respectively.

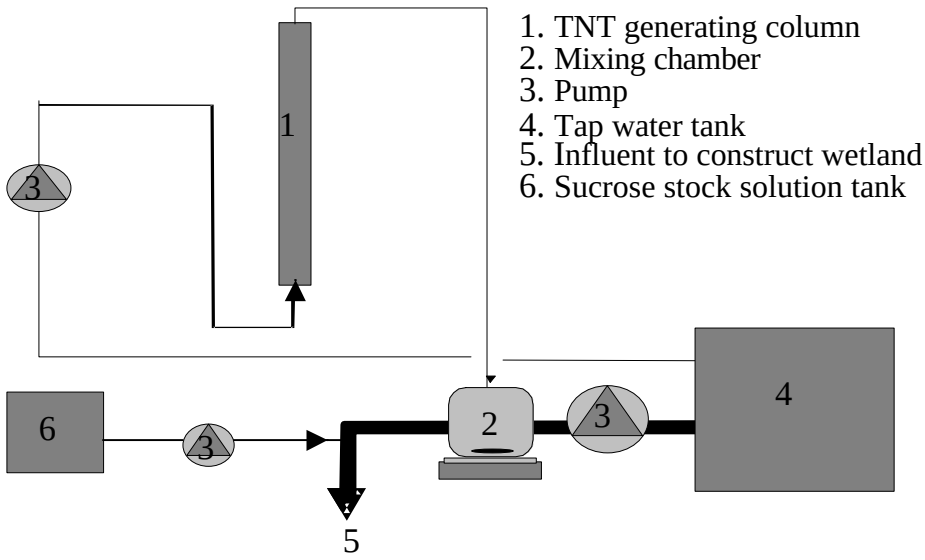


Fig. 3. Artificial TNT wastewater generator

4. Materials and methods

4.1 Materials

4.1.1 Chemicals and reagents

Chemicals: Methanol, toluene, and ethylene glycol monomethyl ether (EMME) were obtained from Merck KGaA (Darmstadt, Germany). Acetonitrile and dichloromethane (DCM) came from Riedel-deHaën (Seelze, Germany). All solvents were HPLC grade and used as purchased. Water used for HPLC analysis and for all laboratory experiments was prepared in a Millipor purifying system (Milli-Qplus Rios, Millipore, Molsheim, French) and abbreviated as MPW (Millipore water) in all following text. Sterile MPW (SMPW) was obtained by autoclaving MPW at 121^{o}C for 30 min.

Standard solutions: A multi standard solution of 1000 µg/ml each of nitrobenzene, 1,3-dinitrobenzene, 2,4-dinitrotoluene (2,4-DNT), 2,6-dinitrotoluene (2,6-DNT), 2,4,6-trinitrotoluene (TNT), hexogen (RDX, hexahydro-1,3,5-trinitro-1,3,5-triazine), 1,3,5-trinitrobenzene (TNB), octogen (HMX, 1,3,5,7-tetranitro-1,3,5,7-tetrazocan), 4-amino-2,6-dinitrotoluene (4A2,6-DNT), 2-amino-4,6-dinitrotoluene (2A4,6-DNT), 2-nitrotoluene (2-NT), 3-nitrotoluene (3-NT), 4-nitrotoluene (4-NT), and tetryl (N-methyl-N, 2,4,6-tetranitroaniline) was purchased from Dr. Ehrenstorfer GmbH (Augsburg, Germany). 2,4-diamino-6-nitrotoluene (2,4DA6-NT) of 1000 µg/mL in methanol with purity of 95% was supplied by Umweltschutz Nord Company, Germany. 2,6-diamino-4-nitrotoluene (2,6DA4-NT) of 100 µg/mL in methanol with purity of 99% was obtained from Dr. Ehrenstorfer, Germany. The standard solutions were used for identification and quantitative analysis of TNT and its metabolites by HPLC.

Radiolabelled ^{14}C-TNT ([ring-U-^{14}C]) with a purity of more than 95% and specific activity of

85.41 µCi/mg was purchased from the Institute of Isotopes Co., Ltd., Budapest (Hungary). Prior to use the purity of the ^{14}C-TNT was 98.35% as determined by TLC (thin layer chromatography). The non-radioactive TNT was supplied by Fraunhofer Institut fuer Chemische Technologie, ICT , Pfinztal, Germany.

Sucrose (commonly used household sugar) was purchased from REWE-HANDELSGRUPPE GmbH, (Koeln, Germany) and molasses from BayWa Cop., Munich, Germany.

Other chemicals, reagents and consumables: Reagents and consumables used for microbial culture medium preparation, measurement of ^{14}C-activity, total organic carbon (TOC) and for all other experiments are listed in tables 1, 2, and 3 with information on their quality, concentration, and the manufacturers.

Table 1. Mineral salt medium (MSM) for microbial culture medium[a]

Salts	Quality	Amount taken in 1 liter deionized water
Dibasic potassium phosphate (K_2HPO_4)	GR	0.8 g
Potassium dihydrogen phosphate (KH_2PO_4)	GR	0.2 g
Potassium nitrate (KNO_3)	GR	1.0 g
Magnesium sulfate heptahydrate ($MgSO_4 \cdot 7H_2O$)	GR	0.2 g
Calcium chloride dihydrate ($CaCl_2 \cdot 2H_2O$)	GR	0.1 g
Sodium chloride (NaCl)	GR	0.1 g
Iron (III) chloride hexahydrate ($FeCl_3 \cdot 6H_2O$)	GR	0.01 g
Trace elements for MSM	GR	1 ml of stock solution
pH 7.35		Adjusted using K_2HPO_4/ KH_2PO_4

[a] All the substances were obtained from MERCK, Darmstadt, Germany.

Table 2. Composition of trace elements for MSM[a]

Trace elements[b]	Quality	Amount contained in 1 liter stock solution
Iron (II) sulfate heptahydrate ($FeSO_4 \cdot 7H_2O$)	GR	200 mg
Cobalt (II) chloride hexahydrate ($CoCl_2 \cdot 6H_2O$)	GR	20 mg
Copper (II) chloride dihydrate ($CuCl_2 \cdot 2H_2O$)	GR	10 mg
Zinc sulfate heptahydrate ($ZnSO_4 \cdot 7H_2O$)	GR	10 mg
Manganese (II) chloride dihydrate ($MnCl_2 \cdot 4H_2O$)	GR	3 mg
Sodium molybdate dihydrate ($Na_2MoO_4 \cdot 2H_2O$)	GR	3 mg
Nickel chloride hexahydrate ($NiCl_2 \cdot 6H_2O$)	GR	6 mg
Ethylenedinitrilo tetraacetic acid disodium salt dihydrate (EDTA-Na $C_{10}H_{14}N_2Na_2O_8 \cdot 2H_2O$)	GR	500 mg
Boric acid (H_3BO_3)	GR	30 mg

[a] The stock trace element solution was stored in 2 ml aliquots in small vials at –30°C and warmed up to room temperature before use.

[b]The trace elements stock solution was purchased from MERCK, Darmstadt, Germany.

Table 3. Other chemicals, reagents and consumables

Chemicals, reagents and consumables	Quality	Manufacturers
For measurement of ^{14}C activity		
PERMABLEND® III (PPO 91% + bis-MSB 9%)		PACKARD Instrument, Meriden, USA
PERMAFLUOR® E^+ [(a)]		PACKARD BioScience B.V., Groningen, The Netherlands
CARBO-SORB® E (3-methoxypropylamine)		
ULTIMA GOLD™ (XR) [(b)]		
For measurement of TOC		
Hydrochloric acid 32%	GR	MERCK, Darmstadt, Germany
Concentrated phosphoric acid	GR	
Potassium hydrogen phthalate (KHP, $C_8H_5O_4K$)	GR	

Table 3. Other chemicals, reagents and consumables (Continued)

Chemicals, reagents and consumables	Manufacturers
Other consumable materials	
Activated charcoal	Fluka Chemika, Buchs, Schwitzerland
ET 250/4 Nucleosil 120-5 C_{18} column	Macherey-Nagel, Dueren, Germany
Chromabond HR-P cartridge	
Chromabond SA column	
0.2 µm filter membrane	Schleicher &Schuell, Dassel, Germany
0.45 µm filter membrane	
Filter paper (S&S 595)	
Disposable super - polyethylene vial^{TM}- 20 ml for LSC	PACKARD BioScience, Germany

(a) a blend of 1,2,4-trimethylbenzene (Pseudocumene) and propyleneglycol[mono] methylether with 2,5-diphenyloxazole[PPO] and 1,4-bis[2-methylstyryl]benzene [bis-MSB]).

(b) a blend of alkylnaphtalenes with scintillators PPO and bis-MSB and emulsifiers.

Cocktail-I for measuring ^{14}C-volatile organic chemicals (VOCs) consisted of 11 g of PERMABLEND®III dissolved in 1-liter toluene.

Cocktail-II for absorption and measurement of $^{14}CO_2$ was a mixture of CARBO-SORB® E and PERMAFLUOR®E^{+} in a ratio of 2:3 (v/v).

Equipment and instruments: Equipment and instruments used are given in this table (Table 4).

Table 4. Equipments and instruments

Equipments and instruments	Manufacturers
Freeze-drier (BETR 1-16)	Martin Christ, Germay
Peristaltic pump (MW-WM5)	Kremer & Kreiler, Munich, Germany
Ultrasonic water baths (SONOREX RK510S)	BANDELIN electronic, Berlin, Germany
Centrifuge (Heraeus cryofuge 8500 i)	Heraeus Sepatech, Germany
Vortex (VF2)	IKA-Labortechnik, Staufen i. Breisgau, Germany
Dismembrator (ULTRA-TURRAX)	IKA WERK, Staufen i. Br. Germany
Rotary evaporator (LABO ROTA S300)[(a)]	Resona Technics, Switzerland
Vacuum controller (vacu-Box PVK600)	Labor-Technik, Wagen, Switzerland
Autoclave (Model C)	Webeco, München, Germany
Liquid scintillation counter (TRI-CARB 1600TA)	PACKARD Instrument, Meriden, USA
Tri-Carb Sample Oxidizer (Model 307)	
Automatic TLC-Linear Analyzer (TRACEMASTER 20)	Laboratorium BERTHOLD, Wildbad, Germany
Pre-coated TLC plates (Silica gel 60 F_{254}, 0.25 mm)	MERCK, Darmstadt, Germany
Air compressor (Motor model SD 420, 400 mbar, 28 m^3/min)	Elektror KARL W. Mueller, Esslingen, Germany
Dosage pump (gamma/4-w)	Heidelberg, Germany
Rain gauge (nach Prof. Hellmann)	Kremer & Kreiler, Munich, Germany
EGA 133 electrode for pH	Sensortechnik Meinsberg GmbH, Meinsberg, Germany
Microprocessor Oximeter Oxi96	WTW Wissenschaftlich-technische Werkstaetten, Weilheim, Germany
Redox-potential meter (Inlab 501)	Wissenschaftlich-Technische Werkstaetten GmbH, Weilheim, Germany

[(a)] equipped with a vacuum controller (vacu-Box PVK600).

4.1.2 Source of inoculum for laboratory biodegradation studies

Biodegradation studies with radiolabelled ^{14}C-TNT were carried out in closed bottles using microorganisms obtained from the constructed wetland, situated at GSF National Research Center for Environment and Health, Munich, Germany, which was used formerly to purify TNT-contaminated wastewater. The microorganisms were extracted from Lava samples collected from the wetland plant. 0.5 kg lava was added to 1.0 L of autoclaved mineral salt medium (MSM) in a glass bottle. The mixture was shaken in a rotary shaker (100 rpm) at room temperature for 2h. The solution was allowed to settle overnight and the supernatant to be used as stock inoculum solution stored at 4 ^{o}C until use.

4.2 Experimental methods

4.2.1 Pilot-scale experiments in the CW

Pilot-scale experiments in a constructed wetland (CW) were conducted to examine the efficiency of using sucrose and molasses as cosubstrates and to study the effects of technical aeration on the transformation of TNT as well as the effects of growing helophytes on the removal of TNT. In a two-year study period, the following experiments were performed:

Evaluation of effects of sucrose as cosubstrate and effects of technical aeration on TNT transformation in the CW: Four experiments were conducted over a four-month study to evaluate the effects of sucrose as cosubstrate and a technical aeration of the wastewater on TNT transformation. All experiments were performed in sequence with the same cascade of the CW at different time. The detailed experimental design was listed in Table 5. The HRT as calculated by dividing the net water volume of the first tank by the flow rate was 2.4 days. Hence each experiment was run at least three to four weeks (i.e. 21 – 28 days) to achieve stable operation conditions. Water samples were drawn from each tank of the cascade twice a week. Technical

aeration was performed by means of air compressor (Elektror KARL W. Mueller, Esslingen, Germany).

Table 5. Details of the experiments designed for studies of effects of sucrose as cosubstrate and aeration on TNT transformation

Experiment[a]	Cosubstrate	Aeration	Operating cascade[b]	Running period
1	--	--	Cascade 2	June 16th-July 10th 1999
2	--	Aerated	Cascade 2	July 11th -Aug. 1st 1999
3	0.25% (w/v) sucrose	Aerated	Cascade 2	Aug. 2nd-Sept. 2nd 1999
4	0.25% (w/v) sucrose	--	Cascade 2	Sept. 3rd-Sept. 24th 1999

[a]Experiment 1 = Exp. 1; Experiment 2 = Exp. 2;
Experiment 3 = Exp. 3; Experiment 4 = Exp. 4.

[b]Operating cascade refers to the cascade of the CW used in this study.

Evaluation of the effects of hydraulic retention time (HRT) on performance of the CW: An experiment was conducted to examine the performance of CW on TNT removal using different HRTs. The experiment was performed in Cascade 3 for a period of three months (10th June – 13th Sept. 2000). During this operation period, neither additional aeration nor cosubstrate was supplied to Cascade 3. Three different influent flow rates of 2, 1, and 0.5 L/min were used, corresponding to calculated HRTs of 2.4, 4.8, and 9.6 days for each tank, respectively. The detailed experimental design was listed in Table 6 from Experiment 5 to Experiment 7.

Evaluation of the effects of growing helophytes on TNT transformation: The effect of growing helophytes on TNT transformation was evaluated by comparing simultaneous operation of a planted and an unplanted tank. Tank 1 of Cascade 3 was operated with helophytes whereas Tank 1 of Cascade 2 was left unplanted, which served as control. The results obtained from these two tanks were used to evaluate the effect of growing helophytes on TNT removal and

transformation in the CW. Considering that the existence of growing plants and plant decays in planted Tank 1 of Cascade 3 greatly reduced the irradiation of sunlight to the water surfaces of the tanks, the prevention of irradiation of sunlight to water surface of unplanted Tank 1 of Cascade 2 was carried out by covering the tank with a wood board. This experiment was conducted for a period of four months (June – Oct. 2000) at an influent flow rate of 2 L/min. The influences of growing helophytes were checked both under cosubstrate ammended and cosubstrate-free conditions. Molasses was used in this experiment as cosubstrate. Concentrations of molasses applied in these experiments were 0.001, 0.01, and 0.1% (w/v). The detailed experimental design was given in Table 6 from Experiment 1 to Experiment 7.

Table 6. Details of the experiments conducted in the CW in the year of 2000[a]

Experiment	Cosubstrate	Influent flow rate	Operating cascade[b]	Running period
1	--[d]	2 L/min	Cascade 2[c]	July 6th – Aug. 5th 2000
2	0.001% (w/v) Molasses	2 L/min	Cascade 2[c]	Aug. 5th – Aug. 26th 2000
3	0.01% (w/v) Molasses	2 L/min	Cascade 2[c]	Aug. 27th-Sept. 18th 2000
4	0. 1% (w/v) Molasses	2 L/min	Cascade 2[c]	Sept. 19th - Oct. 6th 2000
5	--	2 L/min	Cascade 3	June 10th - July 6th 2000
6	--	1 L/min	Cascade 3	July 7th - Aug. 6th 2000
7	--	0.5 L/min	Cascade 3	Aug. 7th – Sept. 13th 2000

[a]In this table the details of the experimental design were listed for the studies of a) Evaluation of the effects of hydraulic retention time (HRT) on performance of CW; b) Evaluation of the effects of growing helophytes on TNT transformation; and c) Screening of the optimal molasses concentration for TNT removal.

[b]Operating cascade refers to the cascade of the CW used in the study.

[c]The first tank of Cascade 2 was left unplanted and covered with a wood board during this study.

[d]No addition of cosubstrates.

Screening of the optimal molasses concentration for TNT removal: Three concentrations of molasses of 0.001, 0.01, and 0.1% (w/v) were selected for this experiment. This experiment was conducted with Cascade 2 during Aug. – Oct. 2000 at an influent flow rate of 2 L/min. The detailed experimental design was listed in Table 6 from Experiment 2 to Experiment 4.

System monitoring. At every sampling time (twice a week), *in situ* physical and chemical parameters of effluent from each tank and the free standing surface water of each tank were measured. Redox potential measurements were performed using a platinum electrode connected with an Ag/AgCl reference system in KCl 3 mol/L solution (Wissenschaftlich-Technische Werkstaetten GmbH, Weilheim, Germany). pH was measured using an EGA 133 electrode (Sensortechnik Meinsberg GmbH, Meinsberg, Germany). Water temperature and dissolved oxygen (DO) measurements were performed using a Microprocessor Oximeter Oxi96 with an oxygen electrode WTW EO96 (WTW Wissenschaftlich-technische Werkstaetten, Weilheim, Germany). The amount of precipitation was measured daily during the experimental period using a standard rain gauge (Kremer & Kreiler, Munich, Germany). Ambient air temperature was recorded daily by means of a thermometer. The main meteorological conditions of the experimental area are presented in Table 7.

Table 7. Monthly meteorological conditions of field experimental site[a].

Month	Temperature (^{o}C)	Precipitation (mm)
Year 1999		
June	16.9	58.8
July	20.7	72.6
August	20.0	76.0
September + October	16.7	70.6
Year 2000		
June	18.3	34.0
July	18.1	93.8
August	20.6	75.7
September + October	14.7	154.9

4.2.2 Laboratory-scale experiments using radiolabelled ^{14}C-TNT

Radiolabelled ^{14}C-TNT was applied to investigate the fate of TNT in a bacterial consortium in the presence and absence of sucrose as cosubstrate by means of a trap system shown in Fig. 4. The system consisted of an air inlet filter (1), a 250-ml narrow-mouthed glass culture bottle (3), 3 glass absorption traps linked in series, an activated charcoal tube (7) to protect the pump from radioactive compounds and liquid contamination, an air-flow control valve (8), a vacuum pump and a timer. The culture bottle was wrapped with aluminium foil to prevent photodegradation of TNT and was connected to the traps through a syringe needle (5) entering into the rubber cap of the culture bottle. Another syringe needle (2) in the cap served as an air inlet to the culture bottle. A sterile 0.2 µm membrane filter (1) was installed in the air inlet needle to prevent bacteria entering the culture bottle. The pump controlled by a timer aerated the culture medium 15-min per hour during exposure period.

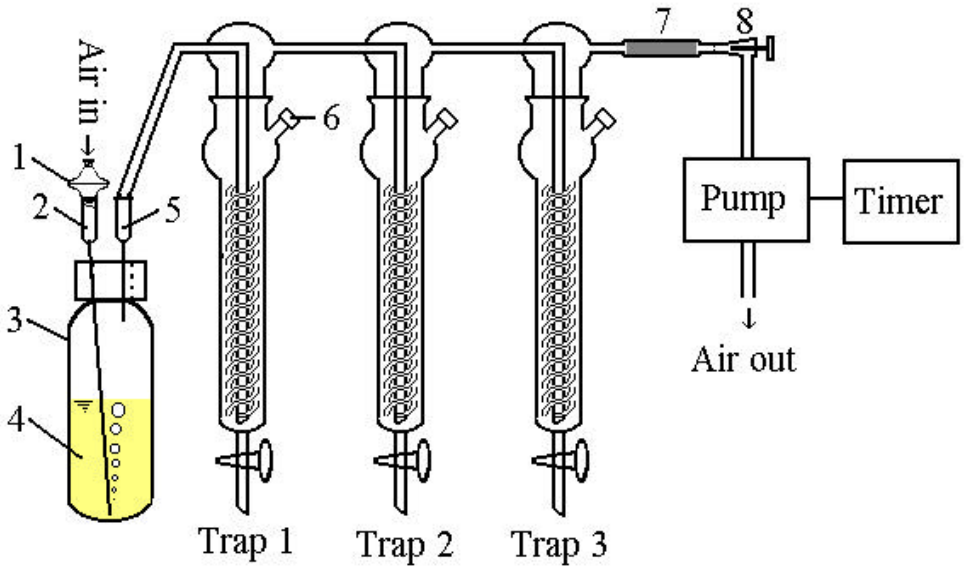


Fig. 4. Trap system for radiolabelled laboratory experiments.

1- 0.2 um sterile membrane filter, 2- air inlet (syringe needle), 3- incubation bottle, 4- culture, 5- air outlet, 6- glass stopper with teflon liner, 7- activated charcoal, 8- control valve.

The vacuum pump sucked air through the filter (1), passed through culture medium and left the culture bottle through air outlet (5). Finally the airflow was directed to a system consisting of three traps connected in series. The first trap (Trap 1) was filled with 10 ml EMME (ethylene glycol monomethyl ether), a selective absorption medium for volatile organic compounds (VOCs as well as ^{14}VOCs) removed from the culture medium, i.e. parent ^{14}C-TNT and non-radiolabelled TNT and its volatile metabolites. Trap 2 and Trap 3 were containing 15 ml Cocktail-II (CARBO-SORB® E + PERMAFLUOR® E^{+} in a ratio of 2:3 (v/v)) each. Cocktail II was implemented to absorb CO_2 as well as $^{14}CO_2$ produced by ^{14}C-TNT mineralization. At the outlet of Trap 3 the airflow was prompted to pass a filter of activated charcoal (7) to remove any residual radioactivity and vapor of absorption media.

Preparation of culture medium and inoculum: Mineral salts medium (MSM) was prepared as listed in Tables 1 and 2 and was autoclaved at 121 ^{o}C for 30 min. Inoculum was prepared by lava extraction, as described in 4.1.2.

Preparation of test culture: The test culture of ^{14}C-TNT was prepared in a 250-ml culture bottle (as shown in Fig. 4. (3)) containing a known volume of ^{14}C-TNT, MSM and inoculumn. ^{14}C-TNT stock solution was prepared by evaporating 1.5 ml dichloromethane (DCM) solution containing 0.35 mCi/ml of ^{14}C-TNT to dryness then adding 250 ml aqueous solution of non-radioactive TNT with concentration of 100 mg/L to a dry 250 ml flask. The solution was then agitated in an ultrasonic bath for 10 min and was stirred until all ^{14}C-TNT completely dissolved. The resulting ^{14}C-TNT stock solution (100 mg/L, 4.66x10^{6} dpm/ml) was then used in the radiolabelled experiments of TNT transformation. Two initial ^{14}C-TNT concentrations of 10 and 50 mg/L were prepared by diluting appropriate volumes of ^{14}C-TNT stock solution with MSM. Appropriate volume of 1% (w/v) sucrose stock solution was added to the test culture to obtain designed sucrose concentration of 0.1% (w/v). Prior to adding to the test culture, all the stock solutions of TNT and sucrose were filtered with a sterile 0.2 µm membrane filter to remove bacteria. The autoclaved culture spiked with ^{14}C-TNT was used as blank to check if

there was any loss of radioactivity during the experiment. All other cultures were inoculated with 15 ml inoculum. Cultures containing no sucrose were also prepared as controls to measure the transformation of TNT in absence of cosubstrate. In total, four treatments were conducted, namely blank- treatment (Treatment 1, with initial concentration of TNT of 10 mg/L, no sucrose, sterile condition), control-treatment (Treatment 2, with initial concentration of TNT of 10 mg/L, no sucrose, inoculated), enriched-treatment 1 (Treatment 3, with initial concentration of TNT of 10 mg/L, with sucrose, inoculated), and enriched-treatment 2 (Treatment 4, with initial concentration of TNT of 50 mg/L, with sucrose, inoculated). Duplicates were prepared for each treatment. All preparations were conducted under aseptic conditions. The detailed experimental treatment design of the radiolabelled experiments is summarized in Table 8. The experiments were set up using a trap system as shown in Fig. 4.

Table 8. Detailed experimental treatment design of the radiolabelled experiments

Treatment	Experimental conditions
1	TNT 10 mg/L, no C-source, sterile
2	TNT 10 mg/L, no C-source, inoculated
3	TNT 10 mg/L, with addition of 0.1% (w/v) of sucrose, inoculated
4	TNT 50 mg/L, with addition of 0.1% (w/v) of sucrose, inoculated

Determination of TNT transformation: The cultures were incubated at room temperature (20 - 25 ^{o}C) for 28 days and were protected from light (i.e. wrapped with aluminum foil). Samples were drawn from trap solutions at 48-h intervals during the incubation period. Trap 1 solution (^{14}C-VOC-absorbed EMME) was drained in a 20-ml LSC vial. The trap was rinsed with 10 ml Cocktail-I (11 g PERMABLEND® dissolved in 1 liter toluene), which was added to the vial containing Trap 1 solution. The vial was then counted for ^{14}C-VOC on LSC (liquid scintillation counter). After removal of trap solution, Trap 1 was refilled with 10 ml EMME. The $^{14}CO_2$-absorbing Cocktail-II in Trap 2 and Trap 3 were collected each in 20-ml LSC vials. Each

trap was rinsed with 5 ml Cocktail-II and the rinse solution was combined to the respective sample vial. The vials were also counted for $^{14}CO_2$ on LSC. Trap 2 and Trap 3 were also refilled with 15 ml of Cocktail II. At the end of the test period, the remaining culture medium was centrifuged and the residual ^{14}C-activity in the pellet and supernatant was determined. The pellet was combusted in a Tri-Carb Sample Oxidizer (Model 307, USA) and the $^{14}CO_2$ formed by combustion was absorbed in 15 ml ULTIMA GOLD^{TM}. $^{14}CO_2$-radioactivity of the solution was determined on LSC, representing the total ^{14}C-compounds bound to the biomass. The supernatant was extracted as described in Section 4.4.1 and analyzed with HPLC.

4.3 Sampling of the pilot-scale study in the CW

Water sampling: During the experimental period, 1 liter of water samples of influent as well as effluent were collected in brown 1-liter "Schott" bottles twice a week at the inlet and outlet of each tank of the operated cascade. Water samples were transported to the laboratory immediately for the solid phase extraction.

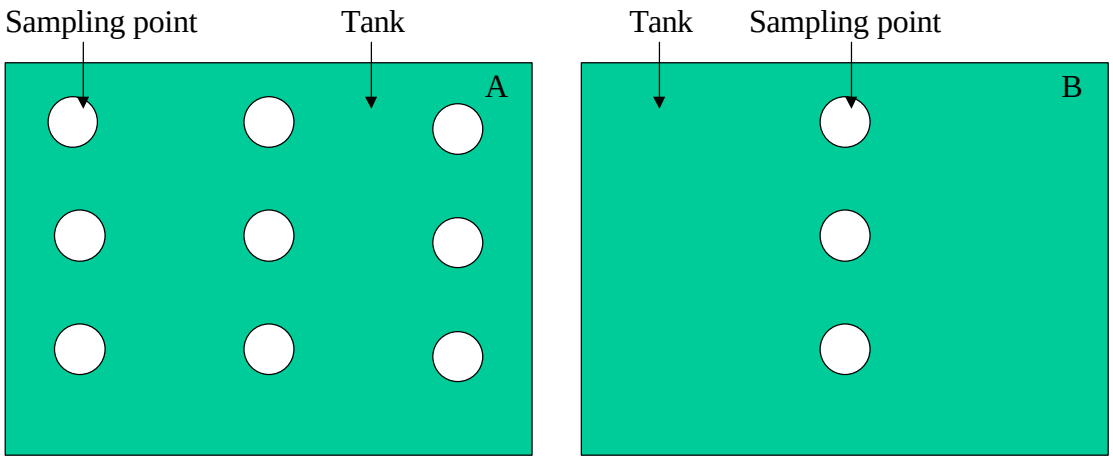


Fig. 5. A: Distribution of plant sampling points;
B: Distribution of lava sampling points.

Plant sampling: Plant tissues were collected once every year after the completion of the experiments. Shoots and roots were collected from the first two tanks of each cascade of the CW. As shown in Fig. 5 (A), plant samples were taken from 9 points in each tank and all the plant samples collected from one tank were combined together. Stainless steel scissors were used to cut the aboveground portion of plants (namely shoots). Lava was excavated to get subsurface plant materials (namely roots). All shoots and roots materials were washed first with tap water then with MPW to remove surface-associated media. The plant materials were then placed into plastic bags and immediately frozen at –80°C. The samples were later warmed up to room temperature before further processing, extraction, and analysis in the laboratory.

Lava sampling: Lava samples were taken from inlet, outlet and at center area of each tank at three different depths of 10, 50, and 90 cm, respectively, once every year after the completion of the experiments. The sampling points are shown in Fig. 5 (B). Lava samples were obtained by slowly drilling a spiral-shaped borer (Ø 5 cm) into a stainless steel pipe (Ø 6.5 cm) inserted into the lava media. Lava samples (about 200 g aliquot) were stored in closed glass bottles at –30 °C.

4.4 Analytical methods

4.4.1 Preparation, extraction, and analysis of samples

Aqueous samples: Solid phase extraction cartridges (SPE, Chromabond HR-P cartridge from Macherey-Nagel, Dueren, Germany) were used for extraction of TNT and its metabolites from aqueous samples. The cartridges were placed on a Visiprep solid-phase extraction manifold and preconditioned twice with each 3-ml methanol and then washed with 3ml-MPW. 1000-ml aliquotes of all water samples from the CW and 100-ml aliquotes of water samples from radiolabelled laboratory experiments were acidified to pH<2 with hydrochloric acid and drawn through the cartridges at a flow rate of approx. 10 ml/min. After drying in ambient air under

vacuum for 1 min, the cartridges were eluted three times with 1 ml methanol-acetonitrile mixture (1:1, v/v) at about 2 ml/min. The resulting extracts (about 3 ml) were evaporated to approximately 0.5 ml under a gentle stream of nitrogen at room temperature. Extracts were then adjusted to 1 ml with methanol in 1 ml flasks and analyzed by high-performance liquid chromatograph (HPLC).

Lava samples: Water content was determined by drying 10 grams of wet lava material at 100 ^{o}C to a constant weight and was found to lie in the range of 10-25% (w/w). Three replicates of each 10 grams of wet lava were separated from residual plant and root materials and were extracted with 10 ml of acetonitrile in a ultrasonic bath (Bandelin RK 510S) for 1 hour. The extraction tubes were stored in the refrigerator at about 4^{o}C for at least 12 hours to precipitate suspended particles. 5 ml supernatants of each sample were transferred to a 10-ml tube and were evaporated to approximately 0.5 ml in a gentle stream of nitrogen at room temperature. Residual extract was adjusted to 1 ml with methanol in 1 ml flasks and subjected to high-performance liquid chromatograph (HPLC) analysis.

Plant materials: Plant tissues (emerse plant material and hairy root samples) were rinsed with tap water followed by Millipor water to remove surface-associated media. 10 grams fresh weights of the plant tissues were cut into small pieces (less than 1 cm). The cut up samples were frozen dried in a freeze-drier (BETR 1-16, Martin Christ, Germany) to constant weights (approximately 24 h). 400 mg aliquots of the freeze-dried plant tissues were mixed with 30 ml of acetonitrile and minced with a dismembrator (IKA WERK, Staufen i. Br., Germany). The minced plant material was then extracted with acetonitrile for 8 h in an ultrasonic bath (Bandelin RK 510S). The temperature was kept below 30 ^{o}C using an ice bath. After ultrasonification, the mixtures of plant materials and solvents were centrifuged in a centrifuge (Heraeus cryofuge 8500 I, Germany) at 3500 rpm for 35 minutes. The supernatants were removed and evaporated with a rotary evaporator (LABO ROTA S300, Switzerland) at room temperature to 4 ml. The resulting 4 ml deep green extracts were then passed through

Chromabond® SA columns (Macherey-Nagel, Dueren, Germany) to remove chlorophyll. The columns were previously filled with 0.5 g dry Na_2SO_4 and washed with methanol and conditioned with acetonitrile. The columns were rinsed with 1 ml of acetonitrile to remove residual adsorbed analytes. The chlorophyll-free eluates were evaporated to approximately 0.5 ml under a gentle stream of nitrogen at room temperature, and then adjusted to 1 ml with acetonitrile fro HPLC analysis. Each plant sample was extracted and analyzed in triplicate.

4.4.2 HPLC analysis

HPLC conditions for analysis of TNT and its metabolites: The analysis of TNT and its metabolites was performed with a Perkin Elmer High-Performance liquid chromatograph equipped with 200LC solvent pumps, a Perkin Elmer diode array detector 235C (wavelength set at 255 nm), a temperature-controlled column compartment set at 18 ^oC, and an ISS 200 auto sampler. An ET 250/4 Nucleosil 120-5 C_{18} column from Macherey-Nagel (Dueren, Germany) was used for separation of TNT and its metabolites. A water-methanol gradient mobile phase with a flow rate of 0.8 ml/min was used. The detailed information about the gradient mobile phase is listed in Table 9.

Table 9. Detailed information on the gradient mobile phase used for HPLC analysis of TNT and its metabolites

Step	Time (min)	CH_3OH (%)	H_2O (%)
0	15.0	41.0	59.0
1	3.0	41.0	59.0
2	14.0	53.0	47.0
3	4.0	53.0	47.0
4	3.0	70.0	30.0
5	10.0	70.0	30.0
6	5.0	100.0	0.0
7	15.0	100.0	0.0
8	5.0	41.0	59.0

Peak identification was based on comparison of retention times and UV spectra with authentic standards. Spectra were acquired in the wavelength range of 190-390 nm. Quantification was performed using an external standard curve for each compound ($r^2 > 0.99$). Integration was performed using Turbochrom Navigator version 4.0 (available from Perkin Elmer).

Calibration of HPLC: Calibration curves were prepared for the following compounds: 2,4,6-TNT, 4A2,6-DNT 2A4,6-DNT 2,4-DNT, 2,6-DNT, 2-NT, 4-NT, 2,4DA-6NT, and 2,6DA-4NT. Mixture of high purity stock standards of TNT and the other compounds (with a concentration of 1000 µg/ml) listed above, except 2,4DA-6NT (with a concentration of 1000 µg/mL) and 2,6DA-4NT (with a concentration of 100 µg/mL) were used for quantitative and qualitative analysis. Standard calibration curves were performed in a concentration range of 0.25 - 50.0 µg/ml by triplicate injection of each concentration level. Calibration of 2,4DA-6NT and 2,6DA-4NT was performed with the same HPLC but in a concentration range of 1.25 - 50 mg/l and three injections were made for each concentration level. The calibration curves (r^2=0.99) were used to calculate the concentrations of TNT and its metabolites.

HPLC conditions for analysis of ^{14}C-TNT and its metabolites: The analysis of ^{14}C-TNT and its metabolites was performed on a HPLC (MERCK-HITACHI, Darmstadt, Germany) equipped with an isotope detector (Ramona 2000, Raytest, Sprockhoevel, Germany), a variable wavelength UV monitor (655A-22) set at 255 nm, an auto sampler (655A-40), a temperature-controlled column compartment set at 15 ^{o}C, and an ET 250/4 Nucleosil 120-5 C_{18} column from Macherey-Nagel. Water-methanol gradient mobile phase and eluent flow were the same as used for analysis of TNT and its metabolites (listed in Table 9). Compounds were identified by comparison of retention times of sample peaks with non-radioactive external standards.

HPLC calibration for analysis of ^{14}C-TNT and its metabolites: Calibration was performed with appropriate dilutions (methanol) of a ^{14}C-TNT standard (0.25 mCi/ml as determined by LSC) in

an activity range of $4.8x10^5 – 1.25x10^8$ dpm, whose radioactivity was measured by a liquid scintillation counter. Each radioactivity level was performed as triplicate. A 5 µg/mL nonradioactive external standard mixture containing TNT and its metabolites was injected to the HPLC before every measurement to obtain chromatograph for comparing of retention times of the corresponding radioactive TNT and its metabolites. The calibration curve (Fig. 6) was used to calculate radioactivity.

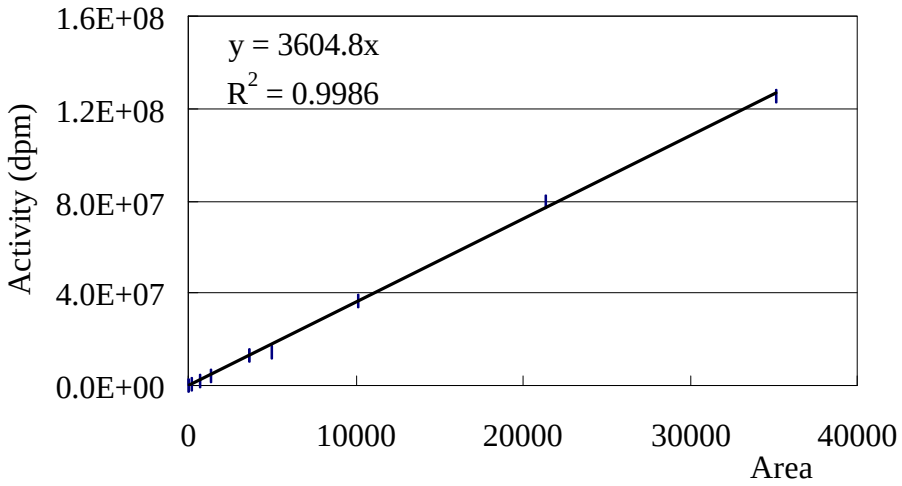


Fig. 6 Calibration curve of HPLC for the measurement of radioactivity.

4.4.3 Chemical analysis

Total organic carbon (TOC): TOC measurements were performed using a 5050 Shimadzu TOC analyzer. Samples of 5 ml of the aqueous phase were filtered through 0.45 µm membrane filters to remove solid particles, and then acidified with 0.025M hydrochloric acid. The filtrate was homogenized in a vortex mixer before analysis in the TOC analyzer.

5. Results and discussion

5.1 Influence of hydraulic retention time on TNT removal in the CW

Experiments were carried out in Cascade 3 during the period between June and Sept. 2000 to examine the performance of the CW on TNT removal under natural conditions. TNT removal efficiencies were examined by use of three different hydraulic loading rates (Q = 2, 1, 0.5 L/min). The calculated hydraulic retention times (HRT) of each tank were 2.4, 4.8, and 9.6 days, respectively, under these three different hydraulic loading rates. The highest and lowest air temperatures and the amount of precipitation during the experimental period are listed in Table 10.

Table 10. The air temperatures and the amounts of precipitation recorded during the experimental period.

Influent loading rates	Lowest temperature (℃)	Highest temperature (℃)	Precipitation (mm)
Q = 2 L/min	11.9	26.6	56.3
Q = 1 L/min	12.4	22.9	84.1
Q = 0.5 L/min	13.3	26.6	114.6

The lowest air temperatures were from 11.9 to 13.3℃, and the highest from 22.9 to 26.6℃. The amounts of precipitation were 56.3, 84.1 and 114.6 mm during the experimental period corresponding to influent loading rates of 2, 1 and 0.5 L/min, respectively.

The influent TNT concentrations used in each experiment are shown in Fig. 7. It is evident that the influent TNT concentration depended on the amount of solid TNT contained in the column (depicted in Fig. 3, Section 3). Due to the consumption of TNT in the CW, the influent TNT concentration decreased daily with time until new solid TNT was refilled.

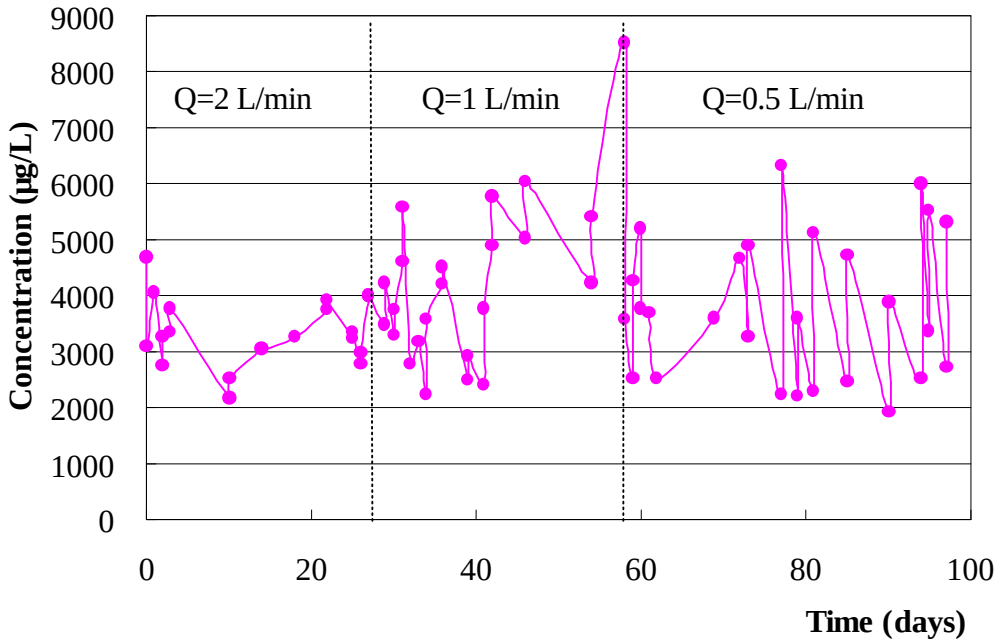


Fig. 7. Influent TNT concentrations in the CW.

To determine influent TNT concentration, two samples were taken every day before and after TNT refilling to the column. The average concentrations of these two samples, which represented the lowest and highest influent TNT concentrations of TNT, were taken as the influent TNT concentration. The determined average influent concentrations of TNT were 3217, 4009, and 3946 µg/L, for influent loading rates of 2, 1, and 0.5 L/min, respectively.

5.1.1. Monitoring of TNT removal at different hydraulic loading rates

Table 11 shows the mean inflow and outflow concentrations and percentage removals of TNT in each tank for the 3 influent flow rates studied. Obviously, with decreasing influent flow rates, TNT removal efficiency increased. As illustrated in Table 11 TNT removal efficiency in Tank 1 at a flow rate of 0.5 L/min was more than that at a flow rate of 2 L/min. The removal efficiency in Tank 1 at a flow rate of 1 L/min, however, was lower than that at a flow rate of 2 L/min. This

may be because of the high influent TNT concentration. The influent TNT concentration at a flow rate of 1 L/min was nearly 25% higher than that obtained in the experiment with a 2-L/min-flow rate. Nevertheless, after passage through the first two tanks, TNT was nearly completely removed from the wastewater at all three influent flow rates of 2 L/min, 1 L/min and 0.5 L/min.

Table 11. The mean inflow and outflow concentrations and removal percentages and removal rates of TNT in the CW.

	Q = 2 L/min	Q = 1 L/min	Q = 0.5 L/min
Influent (µg/L)	3217	4009	3946
Influent load rate[a] (g TNT/m^2, d)	1.612	1.004	0.494
Tank 1			
Outflow (µg/L)	632.9	1355	523.6
Removal (%)	80.3	66.2	86.7
Removal rate[b] (g TNT/(m^2, d))	1.295	0.665	0.429
Tank 2			
Outflow (µg/L)	4.8	4.4	0.1
Removal (%)	99.2	99.7	100
Removal rate (g TNT/(m^2, d))	0.315	0.338	0.0655
Tank + Tank 2			
Removal (%)	99.9	99.9	100
Removal load (g TNT/(m^2, d))	1.609	1.003	0.494

[a]Influent loading rate = C_i*Q/A

[b]Remocal rate = (C_i-C_o)*Q/A

In which: C_i = Influent concentration of TNT

C_o = Effluent concentration of TNT

Q = Flow rate

A = Area of each tank, 5.75 m^2.

Table 11 also shows the influent loading rates and removal rates of TNT in the CW. As the influent loading rates of TNT increased, TNT-removal rates increased. For example, after passage through the first tank, the highest removal rate was 1.295 g TNT/(m^2, d), which was found at the highest influent flow rate of 2 L/min. This observation indicates that influent flow rates, which are higher than 2 L/min, may also be applied to this CW. However, because of the technical limitation in this study, influent flow rates higher than 2 L/min were not achievable. Therefore, the performance of this CW at higher influent loading rates was not examined.

5.1.2 Analysis of extractable TNT transformation products in effluent in CW

HPLC analysis showed that the main extractable metabolites of TNT from effluent were aminodinitrotoluenes (ADNTs) and diaminonitrotoluenes (DANTs). Some other metabolites of TNT such as dinitrotoluene (DNT) and nitrotoluene (NT) were also detected, however in very low concentrations. Fig. 8 shows the concentrations of TNT metabolites detected from effluents in Tanks 1, 2, 3 and 4 in this study.

It was found that the sum of the concentrations of metabolites detected in the effluents varied with the influent flow rates. In the effluents of the first tank, metabolite levels increased with increasing influent flow rates. The sum of metabolite concentrations detected in the effluent in Tank 1 were 341, 530 and 635 µg/L, at Q = 2, 1, and 0.5 L/min, respectively, the highest concentration of metabolite level being found at Q = 0.5 L/min. In the effluent in Tank 2, the sum of metabolite concentrations detected were 197.9, 250.5 and 14.6 µg/L, at Q = 2, 1, and 0.5 L/min, respectively. The lowest metabolite concentration level was found at Q =0.5 L/min, being only 6-7% of the metabolite concentrations found at Q = 2 and 1 L/min. The higher removal efficiency (100%) of TNT and the lower metabolite concentration level found in Tank 2 at Q = 0.5 L/min suggest that TNT and its metabolites were more completely removed at this flow rate than at other influent flow rates. It is obvious that the slow influent flow rate prolonged the wastewater-root zone and wastewater-biomass contact, which improved the removal and transformation of TNT and its intermediates.

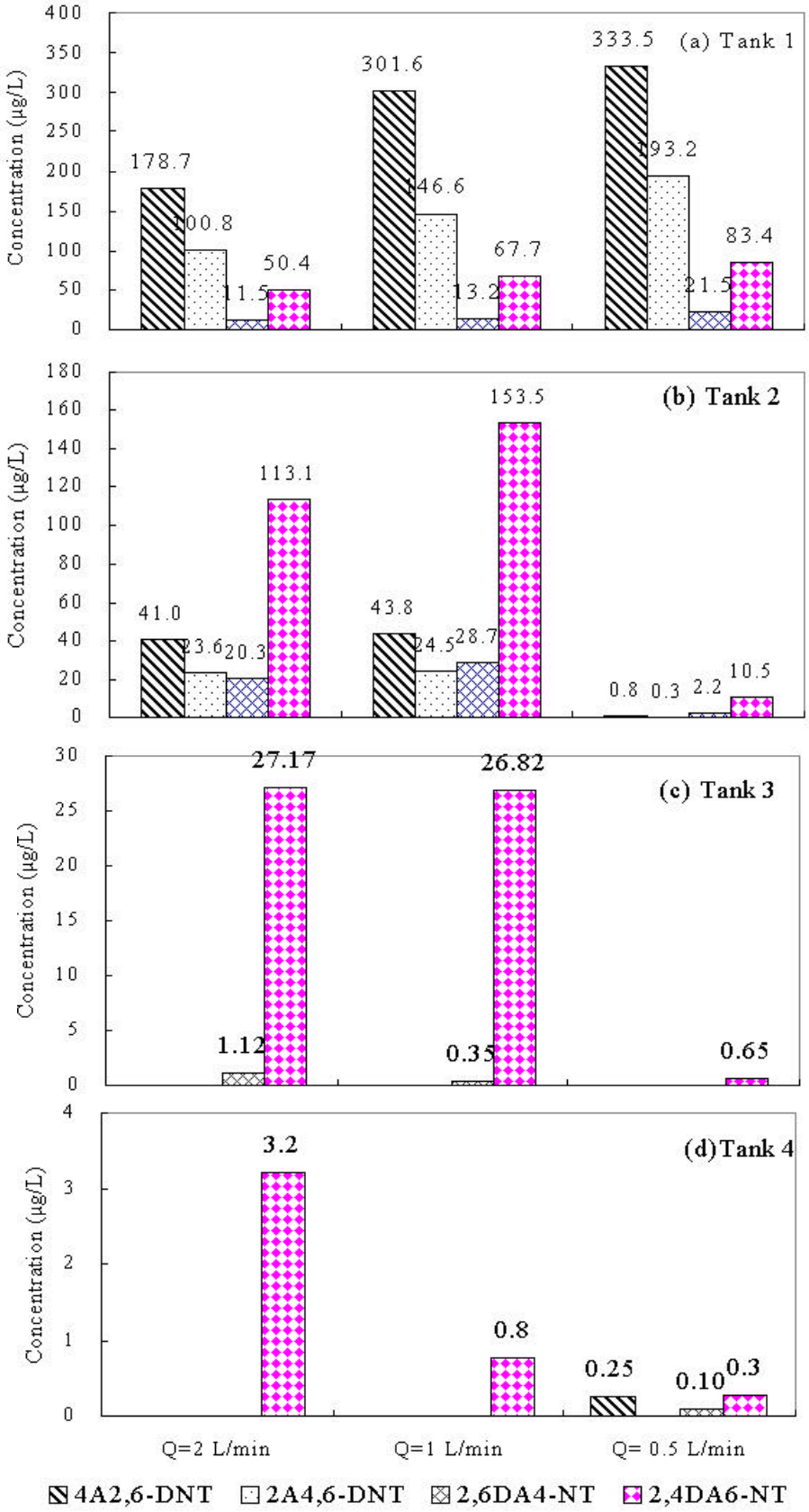


Fig. 8. Concentrations of TNT transformation products extracted from effluents in Tanks 1, 2, 3 and 4.

During passage through the resting three tanks after leaving Tank 1, the concentrations of TNT and its metabolites decreased greatly as shown in Fig. 8 (a) to (d). After passing through five tanks, neither TNT nor its known metabolites could be detected from the effluent. This finding indicates that not only parent TNT but also transformation products formed in Tank 1 were removed from wastewater by passing through the proceeding tanks.

Considering the concentrations of the metabolites in Tank 1 and Tank 2, with the disappearance of ADNTs, a general increase in concentrations of DANTs was observed. At the influent flow rate of 2 L/min, the concentrations of 2,4DA6-NT and 2,6DA4-NT increased from 11.5 to 20.3 µg/L and from 50.4 to 113.1 µg/L, respectively, whereas the concentrations of 4A2,6-DNT and 2A4,6-DNT decreased from 178.7 to 41.0 µg/L and from 100.8 mg/L to 23.6 µg/L, respectively. A similar result was found at an influent flow rate of 1 L/min, indicating a consecutive reduction of aromatic nitro-groups of monoaminodinitrotoluenes to form diaminonitrotoluenes

The formation of 4A2,6-DNT and 2A4,6-DNT was of particular interest. As shown in Fig. 8 (a), 4A2,6-DNT and 2A4,6-DNT were found as absolute major metabolites in the effluent of Tank 1. The concentrations of 4A2,6-DNT found at various influent rates were approximately 1.5 to 2 times higher than that of 2A4,6-DNT. These data indicate that the *para* nitro group of TNT was more readily reduced than its *ortho* nitro group. This observation coincides with findings of other studies (McCormick et al., 1976).

To elucidate the mechanisms of TNT transformation in CW, the concentrations of 2,4DA6-NT and 2,6DA4-NT have to be considered in detail. Without any exception, the concentrations of 2,4DA6-NT were much higher than those of 2,6DA4-NT as shown in Fig. 8 (a) to (d). This result is consistent with the results obtained for aminodinitrotoluenes, in which 4A2,6-DNT were present at higher concentrations than 2A4,6-DNT. Kalafut et al., (1998) reported that under anaerobic conditions 4A2,6-DNT was biologically reduced mainly to 2,4DA6-NT and 2A4,6-DNT mainly to 2,6DA4-NT, which supports the observations made in our present study.

5.1.3 Redox-potential, pH and DO concentration in effluent in the CW

Parameters measured during this study such as dissolved oxygen (DO), redox-potential and pH are listed in Table 12. The measured concentrations of DO in the effluent pond were relatively low, lying between 0.24 and 1.53 mg/L. The measured concentrations of DO in the surface water of each Tank were however relatively high, lying between 2.85 and 5.50 mg/L. This observation suggests a DO concentration change from aerobic to anaerobic or anoxic condition with tank depth, where anoxic conditions were created in the deep zones of the tanks. It was also found that the DO concentrations in the effluent of each tank slightly decreased with decreasing influent flow rates. For example, DO values in effluent of Tank 1 were 1.53, 1.20, and 0.70 mg/L at Q = 2, 1, and 0.5 L/min, respectively. A similar observation was made with the variations of DO concentrations in effluent of the following tanks.

To explain this observation, it is necessary to consider the potential sources of the dissolved oxygen in the CW as well as the possible reasons for its exhaustions. Three factors may contribute to the concentration of dissolved oxygen in the wastewater: (1) the dissolved oxygen originally existing in the influent wastewater at the start of the experiments; (ii) recovery of oxygen from air to water surface; (iii) oxygen which was pumped into the wastewater by pant-root systems. Two main factors could cause the observed exhaustion of DO: (i) bio-chemical transformation process of TNT and its intermediate products; (ii) microbial degradation of organic compounds which were released from plant roots and plant decay processes. The low DO concentrations at low influent flow rates could due to the relatively more complete bio-chemical transformation of TNT and its intermediate products at lower influent flow rates and also due to the microbial degradation of organic compounds released from plant roots and plant decays. In the mean time, because of the low influent flow rates, the total amount of DO in wastewater decreased.

Table 12. Determined effluent parameters in each tank during the study

		DO in effluent (mg/L)	DO in surface water (mg/L)	Redox-potential in effluent (mV)	pH in effluent
Tank 1					
Q= 2 L/min	mean[a]	1.53	5.08	44.00	7.20
	S.D.[b]	0.41	0.58	18.51	0.08
Q= 1 L/min	mean	1.20	5.16	41.29	7.20
	S.D.	0.58	1.45	21.39	0.10
Q= 0.5 L/min	mean	0.70	4.46	-81.33	7.18
	S.D.	0.50	2.63	64.64	0.06
Tank 2					
Q= 2 L/min	mean	1.05	5.00	-163.50	7.10
	S.D.	0.54	1.15	23.18	0.03
Q= 1 L/min	mean	0.60	5.50	-253.71	7.09
	S.D.	0.31	2.61	57.43	0.04
Q= 0.5 L/min	mean	0.34	3.37	-300.00	7.12
	S.D.	0.36	1.87	49.22	0.14
Tank 3					
Q= 2 L/min	mean	1.08	3.70	-170.83	7.00
	S.D.	0.42	1.28	20.19	0.05
Q= 1 L/min	mean	0.86	5.73	-237.00	7.01
	S.D.	0.40	1.32	55.63	0.05
Q= 0.5 L/min	mean	0.24	2.92	-264.22	6.89
	S.D.	0.31	2.38	82.97	0.41
Tank 4					
Q= 2 L/min	mean	1.07	3.00	-163.67	6.94
	S.D.	0.51	0.91	21.57	0.09
Q= 1 L/min	mean	0.67	3.84	-237.57	6.98
	S.D.	0.16	1.66	56.92	0.06
Q= 0.5 L/min	mean	0.28	2.85	-268.75	7.06
	S.D.	0.36	1.51	66.87	0.21

[a]mean = average results of at least four weeks' work (n=7-10); S.D. = standard deviation.

The observed pH variation in the effluent was not remarkable. Only a very slight decrease in pH was found during the passage of the effluent through the tanks. This is assumed to be a result of the utilization of organic matter released from plants by microorganisms under anoxic conditions, during which organic acids were formed (Zhang et al., 1989; He, 1998).

A great drop in redox-potential in effluents of each tank was observed as influent flow rates decreased. As the influent flow rate decreased from 2 to 0.5 L/min, the effluent redox-potential decreased from 44 to –81.33 mV in Tank 1 and from –163.50 to –300.00 mV in Tank 2, respectively. A similar observation was found in Tanks 3 and 4. In addition, the redox-potential varied during effluent passage through the tanks. The lowest redox-potential was found in the effluent in Tank 2, the highest in Tank 1, whereas effluent redox-potentials of Tanks 3 and 4 were higher and were nearly on the same level. The great drop in redox-potential suggests relatively complete removal and transformation of TNT and its intermediates. Lowest redox-potential was observed, where most TNT and its intermediates were removed or transformed. This result was consistent with those reported in an earlier study (Roberts, et al., 1996). In the study of Roberts et al., (1996), the addition of glucose to statically incubated soil reactors allowed for the fastest reduction in redox-potential and produced cultures with the lowest redox potential (-400 mV), where the removal of TNT and its metabolic intermediates occurred very rapidly.

5.2 TNT removal at different operating conditions in the CW

Since the removal efficiency of TNT from contaminated wastewater under natural conditions, as shown in the previous section (Section 5.1), was not as satisfactory in the first tank of the cascade, further efforts were made to optimize the performance of the first treatment stage. For this purpose, the removal of TNT from contaminated wastewater was tested in a series of experiments using an additional carbon source and a technical aeration device. This study was carried out in the summer of 1999 (June to Sept.). During the whole experimental period, the water temperature varied from 16 to 20 °C and the average water temperatures recorded during Exps. 1, 2, 3 and 4 were 17.3, 18.1, 18.6 and 17.6 °C, respectively. The average temperature therefore remained mainly constant during the study.

The influent concentrations of TNT in each experiment in the CW are shown in Fig. 9.

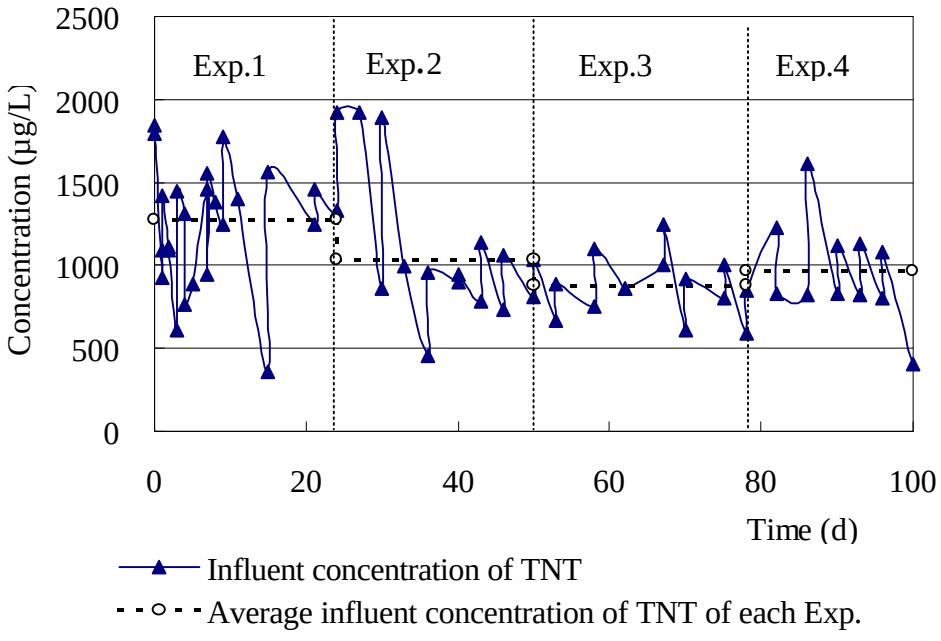


Fig. 9. Influent concentrations of TNT during the study in the CW.

As discussed in Section 5.1, the influent TNT concentration varied diurnally because of the consumption of solid TNT contained in the column (depicted in Fig. 3, Section 3). The average influent concentrations of TNT were 1278, 1034, 875, and 970 µg/L for Exps. 1, 2, 3, and 4, respectively.

5.2.1 Effects of cosubstrate on TNT removal in the CW

Fig. 10 shows the influent and effluent TNT concentrations in the CW under four different experimental conditions. The removal of TNT increased in all the experiments amended with sucrose (Exp. 3 and Exp. 4) as compared with that in the sucrose-free treatments (Exp.1 and Exp. 2). The most significant decrease in TNT concentrations was achieved in the first tank in presence of sucrose. The removal efficiencies were 99.8% in Exps.3 and 4. In contrast, in the sucrose-free treatments, only 91% of the initial TNT was removed in the first tank. Similar observations with TNT removal efficiency were found in Tanks 2 and 3. Even at TNT concentration level of about 1.0 µg/L, the effectiveness of sucrose as external carbon source was indicated. Lower TNT effluent concentration levels were found in the experiments amended with sucrose than in the sucrose-free treatments (as shown in Fig. 10).

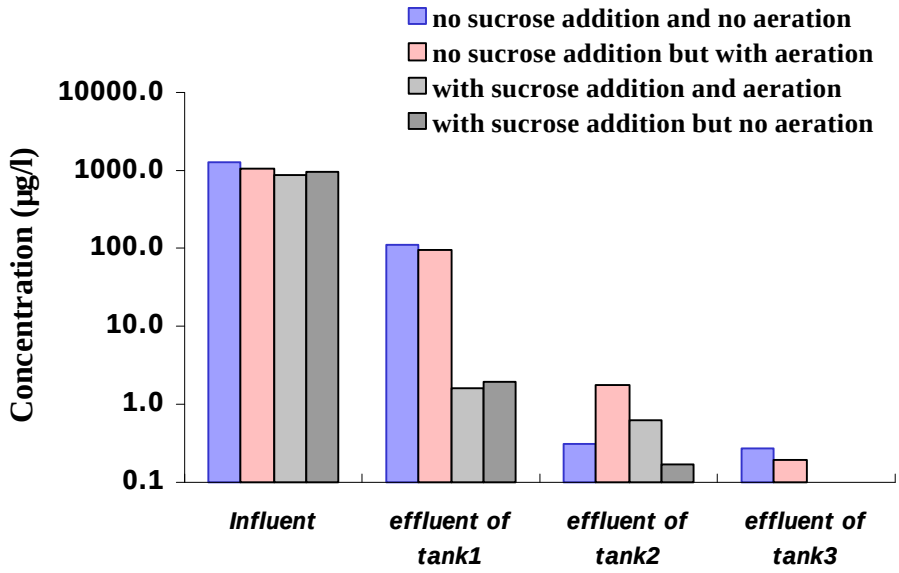


Fig. 10. TNT concentrations in influent and effluent of Tanks 1, 2, and 3 in the CW. (n=5-7)

The concentrations of TNT found in effluent of Tank 4 were below the detection limit (0.25μg/l). Therefore, no data were available for comparison of the effects of external carbon on TNT removal in Tank 4. In the experiments amended with sucrose, the effluent TNT concentration was as low as 1.5-2.0 μg/L after passage through the first tank. To achieve the same effluent TNT concentration level, two tanks were required in the sucrose-free experiments. In view of practical applications, addition of external carbon source such as sucrose can reduce treatment facilities and save the constructed areas, providing a cost-effective technology.

Table 13. Influent loading rates and removal rates of TNT in the first tank of the CW[a].

Experiment	Influent loading rate of TNT[b] g TNT/(m^{-2}, d)	Removal rate of TNT[c] g TNT/(m^{-2}, d)	Removal efficiency of TNT
Exp. 1	0.640	0.586	91%
Exp. 2	0.518	0.469	91%
Exp. 3	0.438	0.437	99.8%
Exp. 4	0.486	0.485	99.8%

[a]Experimental design was described in Table 5 in Section 4.2.1.

[b]Influent loading rate = C_i*Q/A

[c]Removal rate = (C_i-C_o)*Q/A

In which: C_i = Influent concentration of TNT

C_o = Effluent concentration of TNT

Q = Influent flow rate

A = Area of each tank (5.75 m^2)

Table 13 summarized the removal rates and removal efficiencies of TNT in the first tank of the CW. From this table, we found that the removal rates of TNT in sucrose-enriched experiments (Exps. 3 and 4) were slightly lower than in sucrose-free treatments (Exps. 1 and 2). This maybe resulted from the relatively lower influent loading rates of TNT in Exps. 3 and 4. In order to

make the results more comparable and more conceivable, we tried to obtain the operation conditions as the same as possible for all experiments, including the same influent loading rates of TNT. However, for a pilot-scale study, it is less possible to keep the operation conditions exactly the same for every experiment. Nevertheless, the positive effect of sucrose on TNT removal in the CW was confirmed by two factors, (i). the removal efficiencies of Tank 1 in sucrose-enriched experiments were almost 100% and (ii). the left TNT concentration levels in effluent of Tank 1 in the sucrose-enriched experiments were as low as 2 µg/L, which were nearly one sixtieth to one fiftieth of those in the sucrose-free experiments.

5.2.2 Redox-potential, pH and DO concentration in influent and effluent of the CW

Table 14 shows the experimental results of Tank 1 during the four experiments. Since most of TNT was removed in Tank 1, macro parameters of influent and effluent in Tank 1 are emphasized and are listed in this table.

Table 14. The results obtained from Tank 1 during the four experiments[a].

	Exp.1		Exp.2		Exp.3		Exp.4	
	I[b]	E[b]	I	E	I	E	I	E
TOC (mg/L)	19.8	6.4	5.7	9.1	1098.2	780.8	1045.3	920.3
Removal of TOC (mg/L)[c]		13.4		-3.4		317.4		125.0
DO (mg /L)[d]	9.4	0.6	9.3	5.9	6.9	1.0	3.1	0.1
Redox-potential (mV)[e]	195.3	80.7	195.3	201.6	-26.3	-217.4	-110.8	-264.0
pH[f]	7.4	7.2	7.4	7.2	7.3	5.6	7.0	5.0

[a]Average results of at least three weeks' work (n = 5 -7)

[b]I = influent; E = effluent.

[c]Removal of TOC = influent TOC - efluent TOC.

[d]DO influent = DO in surface water of Tank 1.

[e]Redox-potential influent = Redox-potential of surface water of Tank 1.

[f]pH influent = pH of surface water of Tank 1.

For this experimental set up TOC was determined, to get information on the consumption of the carbon source. The results in Table 14 show the variations of TOC, DO, Redox-potential, and pH in various experiments. The addition of sucrose provided high concentrations of influent TOC (more than 1000 mg/L), which increased the consumption of DO and resulted in the decrease of DO both in surface water and in effluent of Tank 1. For example, in the experiments without sucrose addition, the concentrations of DO in surface and effluent waters were 9.4 and 0.6 mg/L, and 9.3 and 5.9 mg/L, under conditions with and without aeration, respectively. Whereas in the experiments which included use of the sucrose as cosubstrate, the DO in surface and effluent waters were only 6.9 and 1.0 mg/L (with aeration), and 3.1 and 0.1 mg/L (without aeration). Low DO (less than 1.0 mg/L) in effluent indicated that this wetland operation system was a combined aerobic and anoxic system (except in Exp. 2). Since there was no additional external carbon source and the system was aerated, effluent DO of Exp. 2 was more than 5.0 mg/L, which indicated an aerobic operation system.

The redox-potential of effluent from Tank 1 decreased greatly with the removal of TOC. In the sucrose-free experiments, redox-potentials of surface water and effluent of Tank 1 were positive, while in the experiments amended with sucrose, they were negative, e. g. less than –200 mV in the effluent water. This observation suggests that the removal of TOC enhanced the decrease in redox-potential of the system and further more, more TNT was removed under these conditions. This result is consistent with an earlier study (Roberts, et al., 1996), which reported favorable TNT degradation under lower redox-potentials.

The effluent pHs in Tank 1, in the experiments supplied with sucrose was lower than those in Tank 1 in sucrose-free experiments. The effluent pH's were 5.6 and 5.0 in Exps. 3 and 4, respectively, in contrast to 7.2 in Exps. 1 and 2. This was probably due to the formation of organic acids as a result of the utilization of sucrose by microorganisms under anoxic or anaerobic conditions (Zhang et al., 1989; He, 1998).

5.2.3 Effects of aeration on TNT removal in the CW

Comparing the results shown in Fig. 10, no significant differences in TNT removal was observed between the experiments with and without aeration. In the experiments, which were unamended with sucrose, the removal efficiencies of TNT in Tank 1 were almost the same under the conditions of aeration or nonaeration (91.5% and 90.8%, respectively). The same observation was found in the experiments supplemented with sucrose as cosubstrate, where TNT removal efficiency was 99.8% under the conditions of aeration and nonaeration.

Considering the sucrose-amended experiments, TOC drop was found to be twice as much in the experiment provided with aeration to that without aeration (Table 14). This may be explained by the favourble degradation of sucrose under aerobic conditions. The differences for other parameters such as pH, DO, redox-potential were not evident. The slight increase in TOC in Exp. 2 was assumed to be a consequence of dissolved organic carbon (DOC) leaching from wetland plant materials (Pinney, et al., 2000).

Because of technical limitation in this study, no typical aerobic condition was created (except Exp. 2). As found in Exp. 3, effluent DO in Tank 1 was approximately 1.0 mg/L, even though aeration was provided. The input of oxygen seemed to be limited by concurrent consumption of the carbon source. Results obtained from this study indicate that much more important for an extensive removal of TNT in the CW is an external carbon source, whereas aeration does not play an important role.

5.2.4 Analysis of TNT and its transformation products in effluent of the CW

HPLC analysis results showed that the main extractable metabolites of TNT phytoremediation in CW in this study were aminodinitrotoluenes (ADNTs, 4A2,6-DNT and 2A4,6-DNT) and

diaminonitrotoluenes (DANTs, 2,4DA6-NT and 2,6DA4-NT). Dinitrotoluenes (DNT, 2,6-DNT and 2,4-DNT) and nitrotoluenes (2-NT and 4-NT) were also detected in water samples in the four experiments, however their concentrations were very low, as shown in Fig. 11 (A) and (B).

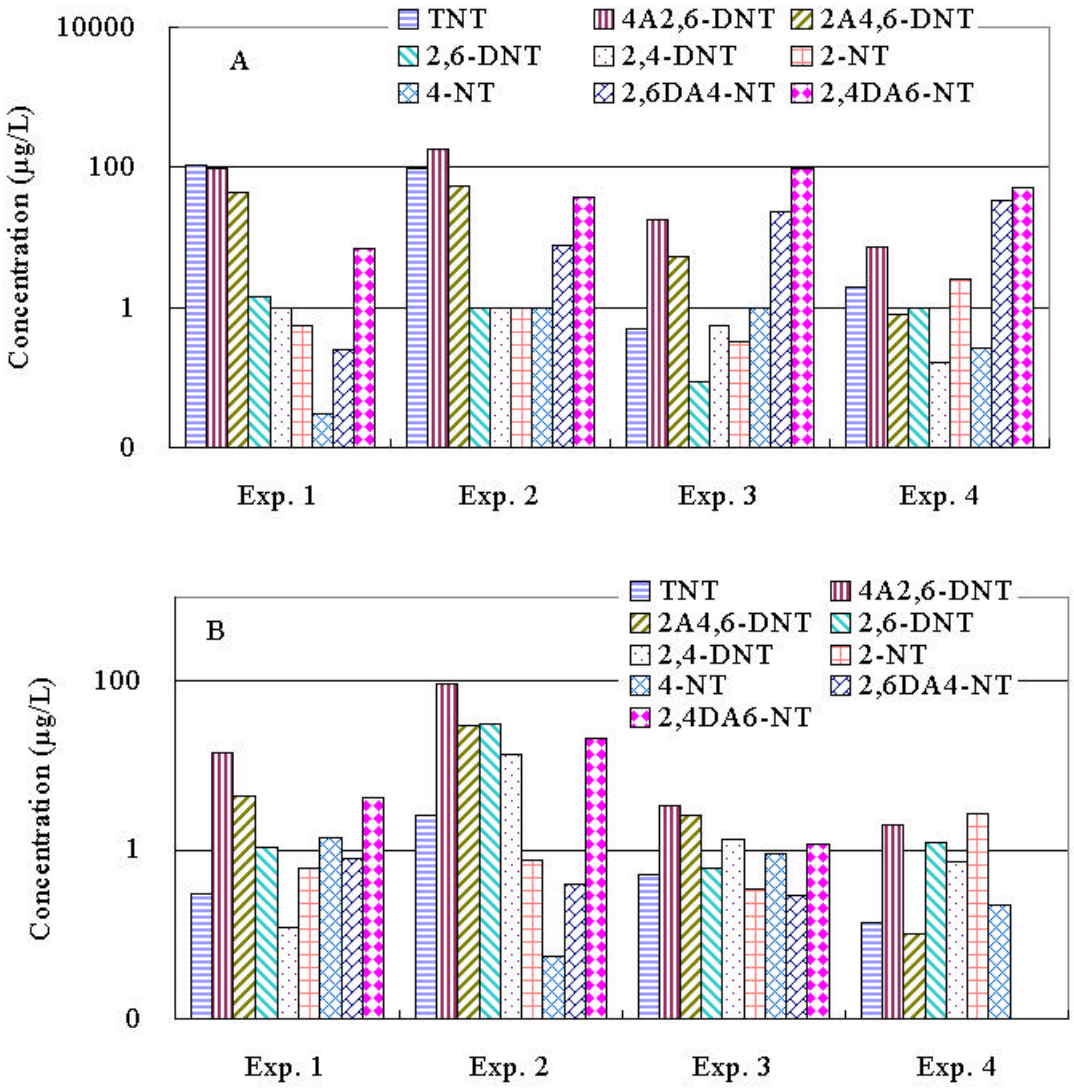


Fig. 11. Concentrations of TNT and its transformation products detected in effluents in Tank 1 (A) and Tank 2 (B).

ADNT transformation products have actually been found in all the previous experiments involving TNT bio- or phytoremediation (Boopathy, *et. al.*, 1993a; Martin, et al., 1997; Labidi et al., 2001). The bio-reduction of TNT to DANTs has been observed in aerobic as well as in anaerobic environments (McCormick, et al, 1976; Naumova et al., 1988; Schackmann and Mueller, 1991; Preuss and Rieger, 1995). Boopathy et al. (1993 b) reported that a sulfate-reducing bacterium could reduce TNT and reductively deaminate it to toluene.

Our present study was conducted in a CW, in which TNT was removed and/or metabolized was accomplished through a complex process involving physical, chemical, biological and photochemical interactions. Therefore, it was difficult to confirm if all the TNT metabolites detected in the effluent of CW were resulted from microbial transformation of TNT. Nevertheless, microbial transformation of TNT occurred in the CW was indirectly confirmed in the radiolabelled laboratory experiments using inocula obtained from the CW (discussed in Section 5.4.2).

Table 15 shows the sum of concentrations of TNT transformation products detected in effluent in Tanks 1 and 2. Comparing the concentrations of TNT transformation products detected in Tanks 1 and 2 under different experimental conditions, we found that more TNT transformation products were detected in the experiments with aeration, as shown in Table 15. In the sucrose-free experiments (Exp. 1 and Exp. 2), the metabolites concentrations detected in aerated experiments were 1.8 to 7 times higher than in the experiments without aeration. In the experiments amended with sucrose (Exp. 3 and Exp. 4), the metabolites level detected in experiment aerated was about 1.5 times that of in the experiment without aeration. Although aeration has no significant effect on the removal of TNT in the CW, more metabolites were detected in the experiments which were aerated. This observation suggests that aeration slightly reduced the further degradation or transformation of the products formed from parent TNT in the CW.

Table 15. Sum of the concentrations of TNT transformation products detected in the effluent in Tanks 1 and 2 in the CW. (µg/L).

Experiment	Transformation products in the effluent in Tank 1 (µg/L)	Transformation products in the effluent in Tank 2 (µg/L)
Exp. 1 (no aeration, no sucrose addition)	148.6	27.3
Exp. 2 (with aeration, no sucrose addition)	277.8	190.0
Exp. 3 (with aeration, with sucrose addition)	138.9	11.5
Exp. 4 (no aeration, with sucrose addition)	97.5	7.7

From Table 15, we also observed that TNT transformation products were present at higher concentrations in sucrose-free experiments than in experiments which were amended with sucrose. A comparison was made between Exp. 1 and Exp. 4 and between Exp. 2 and Exp. 3. From these comparisons we observed that in the effluent in Tank 1, the metabolite concentration levels in sucrose-free experiments were 1.5 to 2 times as high as those in experiments which were amended with sucrose. Similarly, in the effluent in Tank 2 the metabolite concentration levels in sucrose-free experiments were 3.5 to 17 times of that in experiments done with addition of sucrose. These results indicated that not only the conversion of TNT, but also the further conversions of its intermediates (such as 4A2,6-DNT and 2A4,6-DNT) were promoted by addition of sucrose.

Figs. 12 & 13 show the concentrations of 4A2,6-DNT, 2A4,6-DNT, 2,4DA6-NT and 2,6DA4-NT detected in the effluents in Tanks 1 and 2 under different experimental conditions. In the Tank 1 effluent (Figs. 12 (A)& 13 (A)), under sucrose-free conditions, ADNTs were predominant TNT transformation products with a concentration (sum of 4A2,6-DNT and 2A4,6-DNT) of 139.5 µg/L in Exp. 1 and 234.1 µg/L in Exp. 2, which accounted for 93.9 and 84.3%, respectively, of all the metabolites detected. However, the concentrations of ADNTs detected in Exp.3 and Exp. 4 (sucrose amended experiments) were only 17.8 and 9.1 µg/L, which accounted for only 12.8 and 9.3%, respectively, of all the metabolites detected. The

decline in ADNT concentrations was accompanied with an increase in concentrations of DANTs. The concentrations of DANTs (sum of 2,4DA6-NT and 2,6DA4-NT) accounted for 4.7% and 15.7% of the total TNT metabolites detected in Exps. 1 and 2, respectively, and increased to 83.0 and 84.0% in Exps. 3 and 4, respectively. Some earlier studies have reported further reductions of ADNTs to DANTs under anaerobic, anoxic as well as aerobic conditions (McCormic et al., 1976; Naumova et al., 1989; Hwang et al., 2000 (a)). Based on these findings and on our observation in this study, we assume that ADNTs were further reduced to DANTs in the experiments, which were amended with sucrose, and the further transformation of ADNTs to DANTs was greatly stimulated with the addition of the cosubstrate.

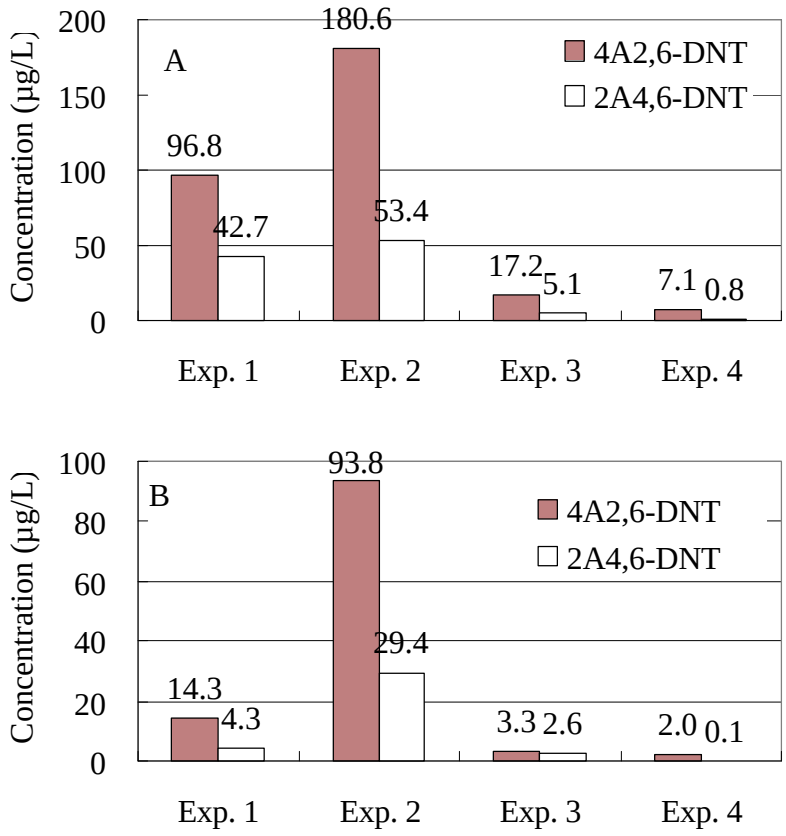


Fig. 12. Concentrations of 4A2,6-DNT and 2A4,6-DNT detected in the effluents in Tank 1 (A) and Tank 2 (B)

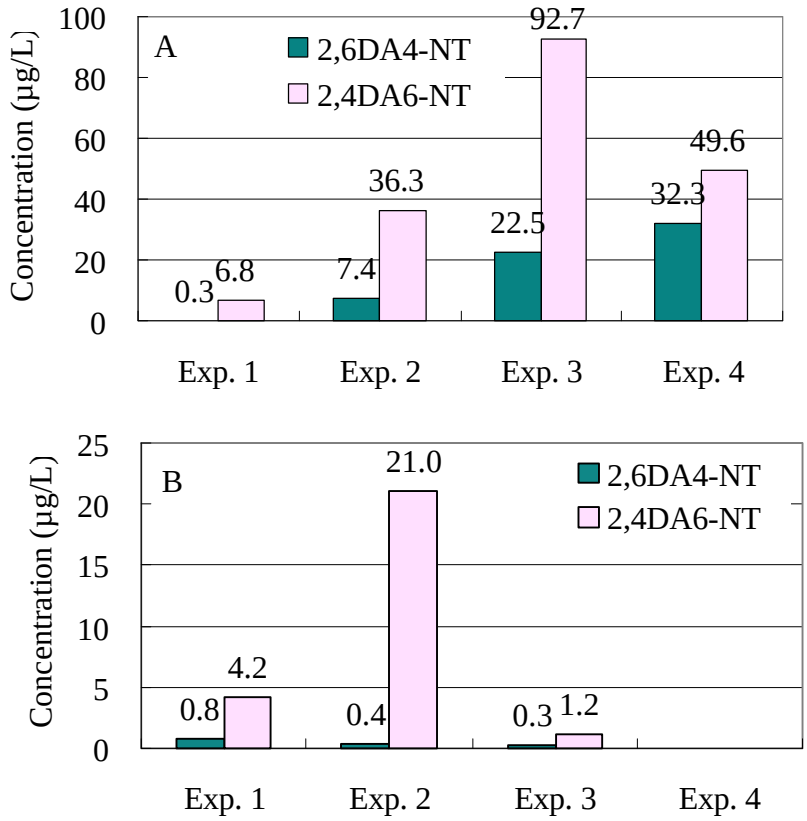


Fig. 13. Concentrations of 2,6DA4-NT and 2,4DA6-NT detected in the effluents in Tank 1 (A) and Tank 2 (B).

Data shown in Figs. 12 & 13 illustrate that, of the two ADNT isomers, 4A2,6-DNT was always present at higher concentrations than 2A4,6-DNT. Similarly, of the two DANT isomers, 2,4DA6-NT was always found at higher concentrations than 2,6DA4-NT in all the four experiments in this study. This is consistent with our earlier studies (Section 5.1.2), in which a preference for reduction of TNT at the *para* position was found. The reactivity of the nitro groups appears to depend not only on other substituents but also on the position of the nitro groups relative to these substituents. The *para* nitro groups were more readily reduced than the *ortho* groups of mononitrotoluenes (McCormick et al., 1976). The same was found in the study of Hoffsommer et al. (1978), which reported that microbiological degradation of TNT in wastewater produced 4A2,6-DNT and 2A4,6-DNT at a ratio of 1.0:0.12.

5.2.5 TNT and its transformation products detected in lava and in plant materials taken from the CW

At the end of this study, plant samples were taken from Tank 1 and Tank 2. As shown in Table 16, TNT and its transformation products were found in the extracts of stem and root materials taken from the first two tanks. As it has been reported in earlier studies, plants are capable of degrading TNT and the products of transformation are partially taken up by the plants (Goerge, et al., 1994; Hughes, et al., 1997). The identifiable transformation product extracted from plant materials in the CW in this study was 4A2,6-DNT.

Table 16. Concentrations of TNT and its metabolites in stem and root materials[a].

Sample		TNT	4A2,6-DNT
Stem of Tank 1	mean	0.97	1.83
	S. D.[b]	0.14	0.35
Stem of Tank 2	mean	0.84	1.28
	S. D.	0.05	0.23
Root of Tank 1	mean	0.27	0.82
	S. D.	0.09	0.42
Root of Tank 2	mean	< D.L.[c]	0.26
	S. D.	--	0.09

[a]Average concentrations (n=3-5) in µg/g dry weight

[b]S. D. = Standard deviation

[c]< D. L. = the concentration was below detective limit (0.25 µg/g) of this study.

The concentrations of TNT and its metabolites in stem materials were much higher than in root materials. This result was in accordance with the research result of Larson, et al., (1999), in which the highest concentration levels of TNT were found in the plant partials above the water surface other than in root tissues directly exposed to TNT contaminated wastewater. The low

concentrations of TNT and its metabolites in the root materials suggested that either the rate of removal of TNT or its metabolites from these tissues was high or that the adsorption of TNT and its metabolites in these tissues from the wastewater was slow. The presence of ADNT in plant tissues suggests transformation of parent TNT either by plant metabolism or by microbial activity.

The analysis of lava samples from CW showed that the concentrations of TNT and its metabolites were below detection limits of this study. This suggests that adsorption of TNT and its metabolites by lava was negligible under the conditions of this study.

5.3 Effects of Molasses on TNT removal and transformation in the CW

In the earlier experiments (Section 5.2), which were amended with sucrose, the favorable impact of a cosubstrate on TNT removal in the CW was demonstrated. Since sucrose (commonly available as household sugar) is too expensive for large-scale use, a similar but cheap product was tested. In this study, the performance of molasses as cosubstrate was examined. The main constituents of molasses are listed in Table 17.

Table 17. Composition of U. S. Sugar's heavy mill run cane molasses [a]

Component	Content[b]	Component	Content
Crude protein	6.3%	Manganesse	5 ppm
Total sugar	49.9%	Zinc	8 ppm
Calcium	0.8%	Biotin	3 ppm
Potassium	4.2%	Calcium pantothenate	60 ppm
Chloride	3.1%	Pyridoxine	4 ppm
Magnesium	0.27%	Choline	700 ppm
Sodium	0.03%	Riboflavin	2.5 ppm
Copper	14 ppm	Thiamine	1.8 ppm
Iron	130 ppm		

[a]Data supplied by United States Sugar Corporation, Molasses & Liquid Feeds Division. (Florida, USA).

[b]Content based on the wet weight.

Molasses is a by-product of sugar production. The quality and characteristics of molasses are dependent upon the concentration and degree of processing. Molasses can be used both as animal feed and as an industrial input for production of yeast, citric acid, lysine production, as well as distilled spirits. By far, the use of molasses as a livestock feed is very wide spreading throughout Europe, Japan and United States of America. About 50% of sugar in form of sucrose, glucose and fructose are contained in molasses. In addition, the mineral elements such as calcium, potassium, sodium, iron, and magnesium found in molasses are also necessary for the growth of microorganisms.

Three different concentrations of molasses (0.001, 0.01, and 0.1% (w/v)) were used in this study. One experiment that was not supplemented with molasses served as control. The study was performed at Cascade 2 of the CW at an influent flow rate of 2 L/min (HRT of 2.4 days per tank). The first tank was left unplanted and covered with a wood board in order to be able to compare the performance of a CW containing hydrophytes. The average influent concentrations of TNT were between 3557 and 5580 µg/L, as shown in Fig. 14.

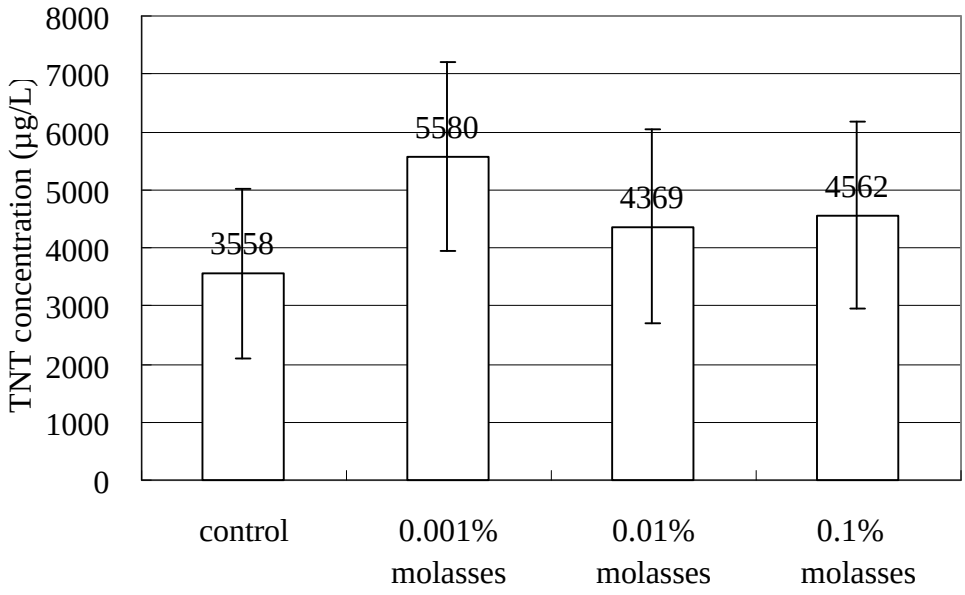


Fig. 14. Influent TNT concentrations of CW with different concentrations of molasses supplied

The large error bars for the influent concentrations of TNT are due to the diurnal variation in concentration of TNT. As discussed in the previous section (Section 5.1), the average concentrations of two samples which were taken before and after the refill of TNT and represented the lowest and highest concentrations of TNT, were taken as the influent concentrations of TNT.

5.3.1 Effects of molasses on TNT removal efficiency in the CW

TNT removal efficiencies were compared in experiments amended with different concentrations of molasses as well as in a molasses-free experiment. Although TNT was removed in all experiments, the removal rates were not the same for all experiments. Fig. 15 shows TNT-removal efficiencies obtained in different experiments and the TNT removal rates in different tanks are shown in Table 18.

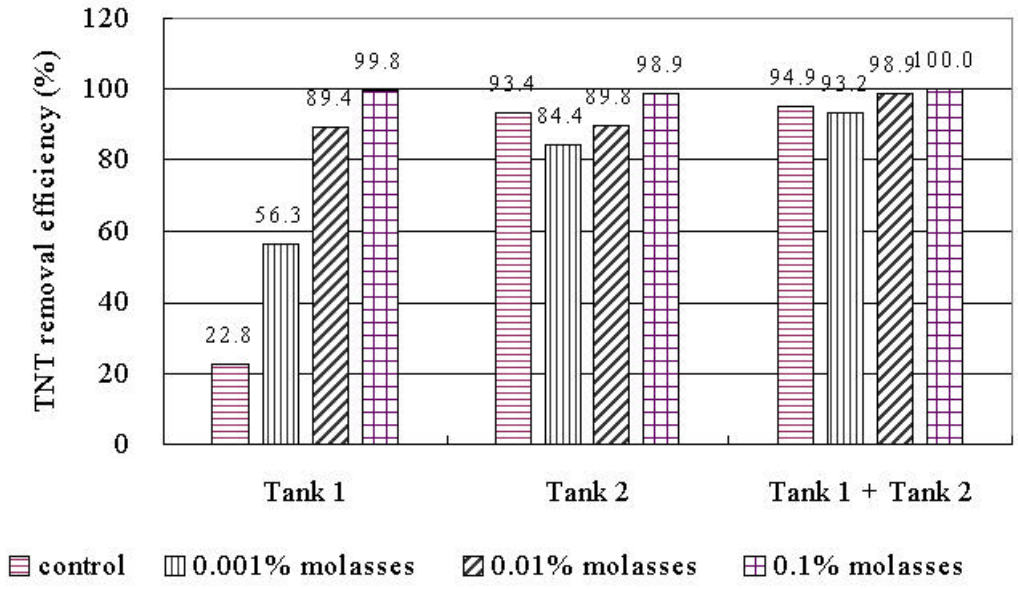


Fig. 15. TNT removal efficiencies in molasses-amended experiments and in control experiment.

Considering TNT-removal efficiencies in the first Tank, 56-99.8% of TNT was removed in the experiments amended with molasses, whereas only 22.8% of the initial TNT was removed in the control experiment. It was also found that TNT removal efficiencies increased with increasing molasses concentrations. This result proved that supplementing of molasses as cosubstrate greatly improved the removal efficiency and removal rate of TNT in the CW.

With regard to TNT-removal efficiencies in Tank 2, differences in experiments which were amended with different concentrations of molasses as well as in molasses-free experiment were no longer evident. Percentage removal rates were found to be: 84.4%, 89.8%, and 98.9% in the experiments amended with 0.001%, 0.01%, and 0.1% (w/v) molasses, respectively, and 93.4% of TNT was removed in the control experiment. The relatively lower TNT- removal efficiencies in the experiments amended with 0.001% and 0.01% of molasses maybe due to the lower inlet TNT concentrations to Tank 2. Most of the TNT was removed in Tank 1 and few TNT was left to Tank 2. However, the high TNT-removal efficiency obtained in Tank 2 in the control experiment suggests that the CW performed well without addition of cosubstrate, but more experimental tanks were needed to achieve the same performance as those in experiments which were amended with molasses.

After passage through two tanks, 98.9 and 100% of initial TNT, respectively, were removed in the experiments amended with 0.01 and 0.1% molasses. However, only 93.2% of the initial TNT was removed in the experiment amended with 0.001% of molasses, which is even lower than the 94.9% removal in the control experiment. This maybe resulted from the relatively higher influent concentration (5590 µg/L) in the experiment amended with 0.001% of molasses. Comparing the removal rates of TNT (listed in Table 18), it is clear that molasses performed well even at a low concentration (0.001% (w/v)). TNT removal rate in the experiment amended with 0.001% molasses was 1.302 g TNT/(m^2, d), which is much higher than that obtained in the control experiment, i.e. 0.846 g TNT/(m^2, d).

Table 18. Influent loading rates and removal rates of TNT in the first two tanks of the CW.

Experiment	Influent loading rates of the first tank[a] g TNT/(m^2, d)	Removal rates of the first tank[b] g TNT/(m^2, d)	Removal rate of the first two tanks g TNT/(m^2, d)
Control experiment	1.782	0.406	0.846
0.001% molasses	2.795	1.574	1.302
0.01% molasses	2.188	1.956	1.082
0.1% molasses	2.285	2.043	1.143

[a]Influent loading rate = C_i*Q/A

[b]Remocal rate = (C_i-C_o)*Q/A

In which: C_i = Influent concentration of TNT

C_o = Effluent concentration of TNT

Q = Influent flow rate

A = Area of each tank (5.75 m^2)

In terms of TNT-removal efficiencies and removal rates obtained in this study, addition of molasses stimulated the removal of TNT in the CW. Although CW (unamended with molasses) also had the capability to remove TNT from wastewater, more experimental tanks were needed to achieve the same effluent TNT level. As discussed in the previous section (Section 5.2.1), the addition of cosubstate can reduce the need of experimental facilities. Considering economic feasibility and removal efficiency of the first tank as well as removal rate of TNT in the first tank of the CW, 0.01% (w/v) of molasses was considered as the optimal concentration of molasses supplied to the CW.

5.3.2 Effects of molasses on TNT transformation in the CW

5.3.2.1 Analysis of TNT and its transformation products in Tank 1 in the CW

The major TNT intermediates identified in this study were aminodinitrotoluenes (4A2,6-DNT, and 2A4,6-DNT), diaminonitrotoluenes (2,4DA6-NT and 2,6DA4-NT), and dinitrotoluenes (2,4-DNT and 2,6-DNT). Nitrotoluenes (2-NT, and 4-NT) were also detected at low concentrations. Fig. 16 shows the concentrations of TNT transformation products detected in effluents in Tank 1 in experiments which were amended with different concentrations of molasses and in control experiment. More TNT transformation products (based on the sum of concentrations of all intermediates detected) were found in the molasses-enriched experiments compared to those found in the control experiment, as shown in Table 19. This observation suggests that the transformation of TNT was stimulated by addition of molasses as cosubstrate. But the highest concentration level of TNT transformation products were found neither in the experiment enriched with the highest concentration of molasses (0.1%), nor in the experiment enriched with the lowest concentration of molasses (0.001%), but rather in the experiment amend with 0.01% molasses (data shown in Table 19). Possible explanations for this observation include (i). by increasing the concentration of molasses, the TNT removal rate increased and thus, more transformation products were detected. In this case, therefore, more TNT transformation products were detected in the experiment amended with 0.01% molasses than in experiment amended with 0.001% molasses, (ii). by increasing the concentration of molasses, not only the parent compound TNT but also its transformation products were further transformed and/or removed from the wastewater, thus leading to a decrease in concentrations of TNT transformation products. This explains why less TNT transformation products were detected in the experiment amended with 0.1% molasses than in the experiment amended with 0.01% molasses. As a consequence, if the concentration of molasses was lower than 0.01% (w/v), the concentrations of TNT transformation products detected in effluent increased at a rate equal to the increase in molasses concentration. If, however, the concentration of molasses

was higher than 0.01%, then the concentrations of TNT transformation products detected in effluent decreased at a rate equal to the increase in molasses concentration.

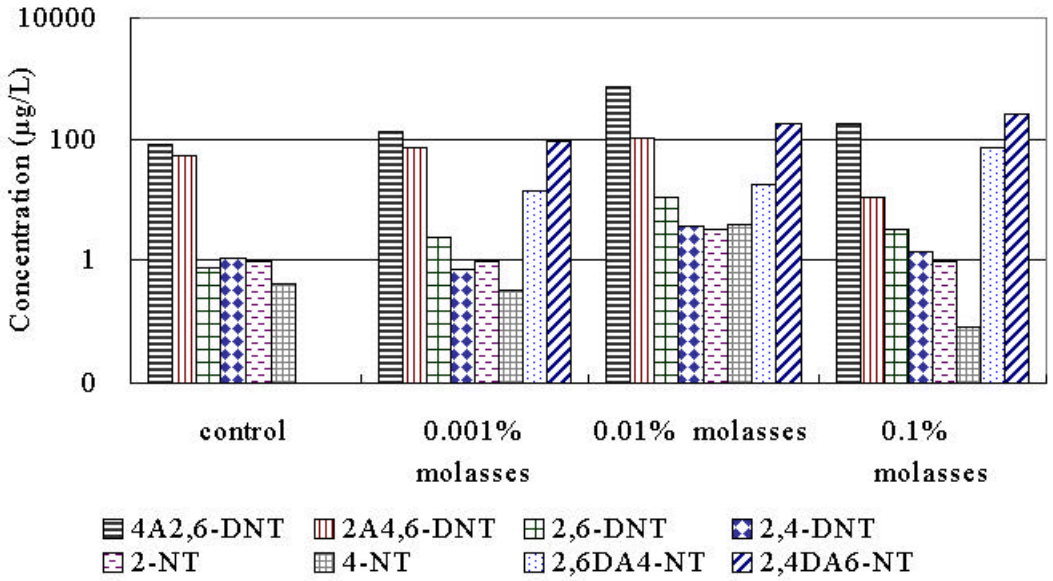


Fig. 16. Concentrations of TNT and its transformation products detected in effluents in Tank 1 in the molasses-amended experiments and in control experiment

As discussed in Section 5.3.4, 4A2,6-DNT and 2A4,6-DNT were found as main transformation products in almost all experiments which agrees with reports of previous studies (Boopathy, *et. al.*, 1993a; Martin, et al., 1997; Labidi, et al., 2001).The highest 4A2,6-DNT and 2A4,6-DNT concentrations were detected in the experiment amended with 0.01% molasses, i.e. 738 and 106 µg/L, respectively, as shown in Fig. 17. The lowest concentration of these products was detected in the control experiment. The variation of the concentrations of aminodinitrotoluenes obtained in different experiments indicates that addition of molasses promoted the formation and conversion of these metabolites. In the experiment amended with 0.01% molasses, the high concentrations of aminodinitroluenes indicate that their formation was stimulated. In the experiment amended with 0.1% molasses, the concentrations of aminodinitroluenes dropped,

which indicates that the conversion of aminodinitroluenes was improved with the addition of high concentrations of molasses.

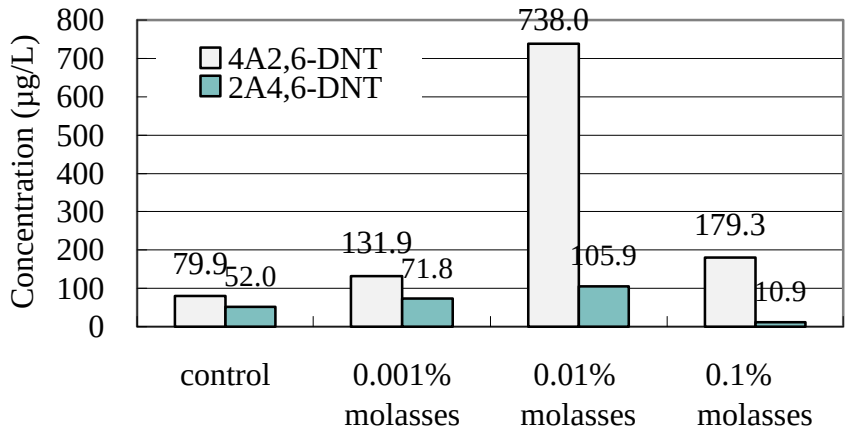
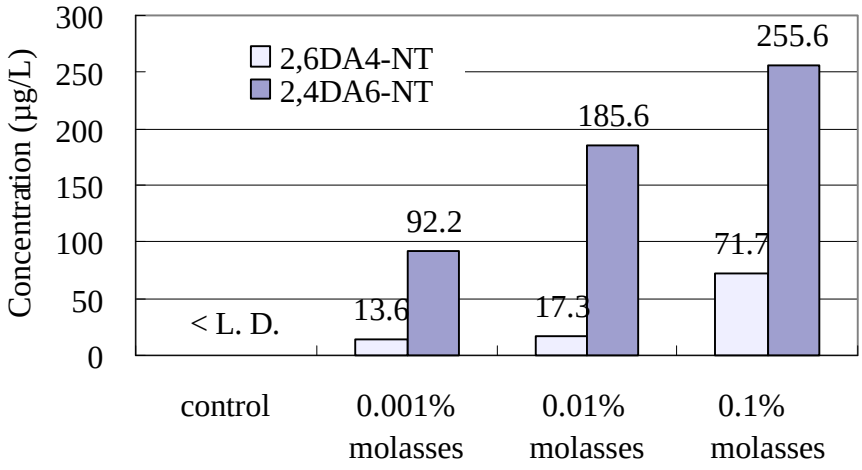


Fig. 17. The concentrations of aminodinitrotoluenes detected in effluents in Tank 1 in experiments amended with molasses and in control experiment.

It has to be noted that, in all experiments, the concentrations of 4A2,6-DNT were much higher than those of 2A4,6-DNT. This tendency was consistent with that found in other studies (Section 5.2.1 and 5.2.2), in which 4A2,6-DNT was always present at higher concentrations than 2A4,6-DNT. A possible explanation for this observation was discussed in Section 5.2.4.

It was also found that the concentrations of diaminonitrotoluenes increased with increasing concentrations of molasses. As shown in Fig. 18, the concentrations of 2,6DA4-NT and 2,4DA6-NT increased from 13.6 to 71.7 µg/L and from 92.2 to 255.6 µg/L, respectively, in the experiments which were amended with molasses. In the control experiment, the concentrations of diaminonitrotoluenes detected were below detection limit of this study (less than 0.25 µg/L). This result indicates that the formation of diaminonitrotoluenes was promoted by addition of molasses. The higher the concentration of the molasses was added, the higher the concentration of diaminonitrotoluenes was produced.



Note: < L. D. = below detection limit.

Fig. 18. The concentrations of diaminonitrotoluenes detected in effluents in Tank 1 in experiments amended with molasses and in control experiment

Dinitrotoluenes (2,6-DNT and 2,4-DNT) and nitrotoluenes (2-NT, 4-NT) were also found in the effluents in Tank 1 at very low concentrations (less than 10 µg/L) as shown in Fig. 19. The highest concentrations of these compounds were detected in the experiment which was amended with 0.01% (w/v) molasses.

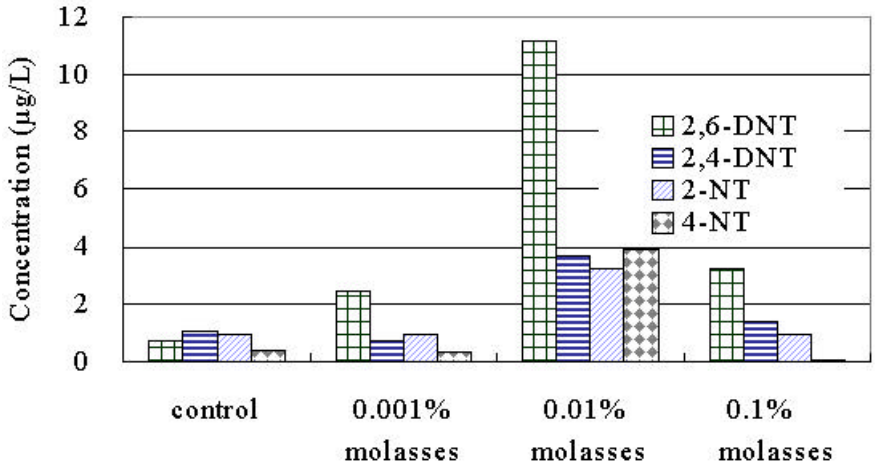


Fig. 19. The concentrations of dinitrotoluenes and nitrotoluenes detected in effluents in Tank 1 in experiments amended with molasses and in control experiment

The concentrations of aminodinitrotoluenes, diaminonitrotoluenes, dinitrotoluenes as well as nitrotoluenes are summarized in Table 19. The results from this table show that, with the exception of the experiment that was amended with 0.1% molasses, the concentrations of TNT metabolites varied in following order: aminodinitrotoluenes > diaminotoluenes > dinitrotoluenes > nitrotoluenes. In the experiment that was amended with 0.1% (w/v) molasses, the concentrations of diaminonitrotoluenes were higher than those of aminotoluenes. It is assumed that the transformation of aminonitrotoluenes to diaminonitrotoluenes was faster than the further transformation of diaminonitrotoluenes in the experiment that was amended with 0.1% molasses. This result indicates that addition of molasses did actually increase the further transformation of TNT intermediates, especially at high molasses concentrations.

Table 19. Concentrations of TNT transformation products detected in effluents in Tank 1 in the CW.

Experiment	Aminonitrotoluenes µg/L	Diaminonitrotoluenes µg/L	Dinitrotoluenes µg/L	Nitrotoluenes µg/L	Sum[b] µg/L
Control	131.9	< L. D.[a]	1.8	1.4	135.1
0.001% of molasses	203.7	105.8	3.2	1.3	313.9
0.01% of molasses	843.8	202.9	14.9	7.2	1068.9
0.1% of molasses	190.2	327.3	4.6	1.1	523.2

[a]<L.D. = below detection limit.

[b]Sum = sum of the concentrations of TNT transformation products.

Interestingly, we observed that the amount of TNT transformation products detected in effluents in Tank 1 was much less than that of the removed TNT. Table 20 shows percentages of removed TNT and metabolites detected in effluents in Tank 1. In Tank 1, 22.8, 56.3, 89.4, and 99.8% of the initial TNT were removed in the control experiment and in the experiments that were amended with 0.001%, 0.01%, and 0.1% (w/v) of molasses, respectively. However, the

amount of TNT transformation products present in all the experiments accounted for only 19.3, 12.2, 32.7, and 14.8% of the removed TNT, respectively. Obviously, the differences between the amount of TNT removed and the percentages of TNT transformation products detected increased with the increase of concentrations of molasses added as shown in Table 20.

Table 20. TNT removal efficiencies and the amounts of TNT transformation products detected in the effluents in Tank 1 with addition of different concentrations of molasses.

Transformation products[a]	Control	0. 001% of molasses	0. 01% of molasses	0.1% of molasses
4A2,6-DNT (%)	11.36	4.84	21.78	4.54
2A4,6-DNT (%)	7.39	2.63	3.12	0.28
2,6-DNT (%)	0.12	0.10	0.36	0.09
2,4-DNT (%)	0.16	0.03	0.12	0.04
2,6DA4-NT (%)	0.0	0.59	0.60	2.14
2,4DA6-NT (%)	0.0	3.98	6.46	7.63
Sum of TNT transformation products (%)	19.31	12.23	32.74	14.76
TNT removal efficiency (%)	22.8	56.3	89.4	99.8

[a]TNT transformation products are presented as percentages of removed TNT in different experiments.

Several possibilities have to be considered to explain these differences between the amounts of TNT removed and the amounts of TNT transformation products. Firstly, some of the TNT and its metabolites were absorbed on lava. We analyzed the lava samples at the end of the experiment and did find TNT and its metabolites in these materials. The results are shown in the next section (Section 5.3.4.1). Secondly, based on the results reported by others (Boopathy et al. 1993 b; Hwang et al., 2000 (a)), we suspected that some TNT transformation products like triaminotoluene (TAT) or toluene, were the likely product formed, though we did not detected

TAT or toluene in the effluents in Tank 1. Because triaminotoluene is unstable (Preuss et al., 1993; Ederer et al., 1997; Krumholz et al., 1997), its detection in the effluent can be difficult. As for toluene, we did not develop the analytical method for it in this study. It is also possible that unknown TNT transformation products were formed. The HPLC analyses of the effluents under different experimental conditions showed that at least two unknown intermediate products were present in effluents in experiments that were amended with molasses. The retention times of the unknown peaks were 10.40 minutes and the other one 17.88 minutes, respectively. No unknown intermediates were found in the control experiment.

5.3.2.2 Analysis of TNT and its transformation products in Tank 2 in the CW

HPLC analysis of the extracts of the effluents in Tank 2 showed that the same TNT transformation products as those detected in effluents in Tank 1 were found as shown in Fig. 20.

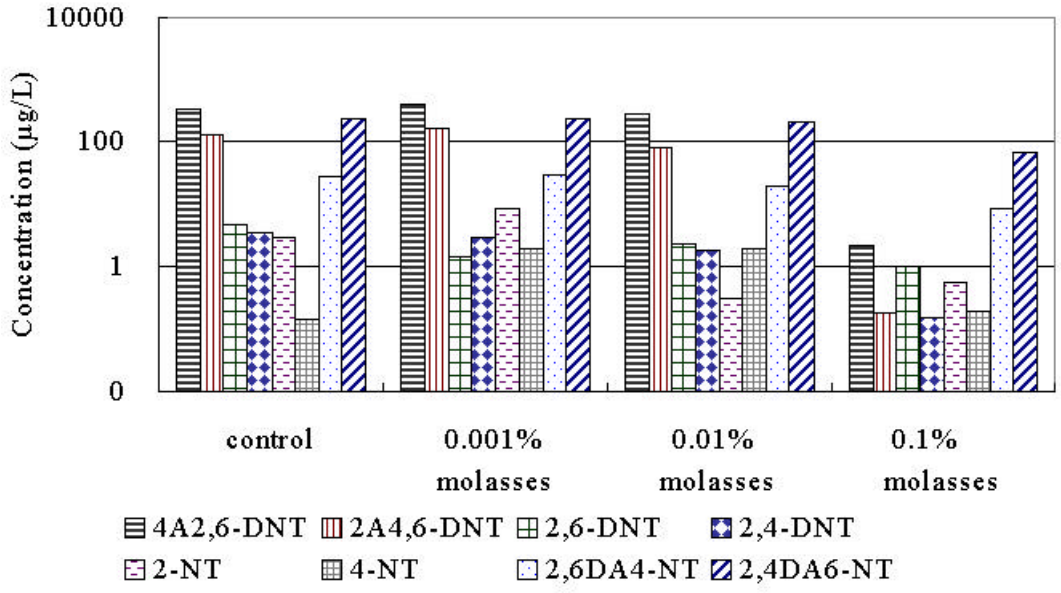


Fig. 20. The concentrations of TNT transformation products detected in effluents in Tank 2 in molasses-amended experiments and in control experiment.

Table 21. Concentrations of TNT transformation products detected in effluents in Tank 2 in the CW.

Experiment	Aminonitrotoluenes µg/L	Diaminonitrotoluenes µg/L	Dinitrotoluenes µg/L	Nitrotoluenes µg/L	Sum[a] µg/L
Control	469.8	265.1	8.1	3.0	746
0.001% of molasses	564.2	266.2	4.3	10.0	844.7
0.01% of molasses	361.0	229.5	4.0	2.2	596.7
0.1% of molasses	2.3	74.7	0.2	0.7	77.9

[a]Sum = sum of the concentrations of TNT transformation products.

The concentrations of aminodinitrotoluenes, diaminonitrotoluenes, dinitrotoluenes and nitrotoluenes are summarized in Table 21. Comparing metabolite concentration levels (based on the sum of the concentrations of all TNT metabolites) detected in the effluents in Tank 2 to those detected in Tank 1, it was observed that the metabolite concentration levels in Tank 2 increased in both the control experiment as well as in the experiment that was amended with 0.001% molasses, data are shown in Table 21. The concentration level of all metabolites detected in Tank 2 was 5.5 times higher than in Tank 1 in the control experiment. Whereas in the experiment amended with 0.001% (w/v) of molasses, the concentration level of all metabolites detected in Tank 2 was 2.7 times that of in Tank 1. To explain the increase in the concentrations of all metabolites detected in effluents of Tank 2, it is necessary to consider the removal efficiencies of Tank 1. In these two experiments, the TNT-removal efficiencies in the first tank were relatively low, only 22.8 and 56.3%, respectively. The rest of the TNT was continuously removed to Tank 2 and transformed in Tank 2, which resulted in the increasing of metabolite concentration levels in Tank 2. In contrast to these two experimental results, the experiments that were amended with 0.01 and 0.1% molasses achieved higher TNT-removal efficiencies in the first tank, i.e. 89.4 and 99.8%, respectively. Correspondingly, the concentration levels of all metabolites detected in Tank 2 decreased from 1068.9 (in Tank 1) to 597.0 µg/L (in Tank 2) in the experiment added with of 0.01% (w/v) of molasses and from 523.2 (in Tank 1) to 78.1 µg/L

(in Tank 2) in the experiment added with of 0.1% (w/v) of molasses, respectively. These results suggest that the performance of CW can be improved either by increasing experimental facilities or by supplying enough cosubstrate. For example, addition of 0.01% and 0.1% of molasses contributed to the most transformation of TNT in the first tank, which makes it possible to make economic saving with limited experimental facilities.

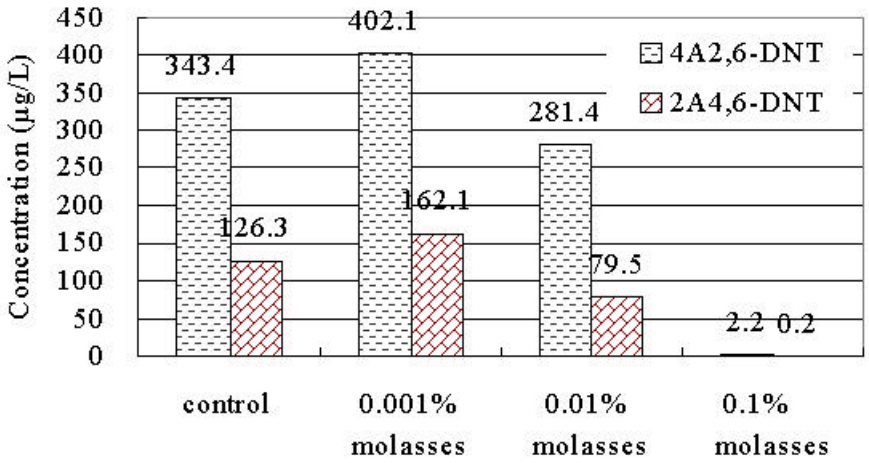


Fig. 21. Concentrations of aminodinitrotoluenes detected in effluents in Tank 2 in experiments amended with molasses and in control experiment

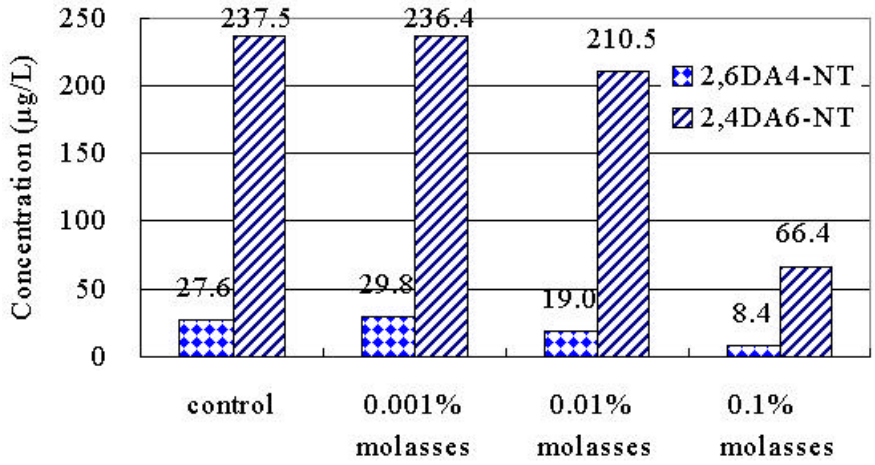


Fig. 22. Concentrations of diaminonitrotoluenes detected in effluents in Tank 2 in experiments amended with molasses and in control experiment

Figs 21 & 22 show the concentrations of aminodinitrotoluenes and diaminonitrotoluenes. Considering only the aminodinitrotoluene isomers, the concentrations of 4A2,6-DNT were approximately 2.5-3.5 times as that of 2A4,6-DNT detected in all the experiments that were amended with molasses as well as in the control experiment. Similarly, the concentration ratios of 2,4DA6-NT to 2,6DA4-NT in all these experiments were between 7.9-11. The high concentrations of 4A2,6-DNT and 2,4DA6-NT indicate that the nitro group of TNT in the *para* position is much more easily reducible than that in the *ortho* position, as discussed in the precious Section 5.1.2.

5.3.3 Dissolved oxygen (DO), pH, and redox-potential in the effluents in Tanks 1 and 2.

Figs. 23 (A), (B), (C) & 24 (A), (B), (C) show the variations of pH, redox-potential, and DO in effluents in Tanks 1 and 2. Considering the variations of these parameters in effluents in Tank 1, it was found that with the exception of the experiment which was amended with 0.1% molasses, the pHs of the effluents in all experiments were within neutral range (7.15-7.25). In the experiment that was amended with 0.1% molasses as cosubstrate, the effluent pH was 6.28, indicating a slight acidification of the wastewater. This may be resulted from the fermentation of molasses under the anaerobic or anoxic conditions. This observation was consistent with the observed variation of DO concentrations in the effluents in Tank 1. In the experiment which was amended with 0.1% molasses, the average effluent DO concentration was only 0.4 mg/L, indicating an anoxic condition in the bottom layer of Tank 1. Obviously, addition of cosubstrate led to a drop in the DO concentration. With increasing concentrations of molasses from 0.0% (control experiment) to 0.1%, DO concentrations decreased from 4.68 mg/L (in the control experiment, in which no molasses was added) to 0.40 mg/L (in the experiment which was amended with 0.1% molasses). Boopathy et al. (1993b) have reported that the activities of the anaerobes depend on the availability of other carbon sources and electron acceptors. In the present study, the activities of these anaerobes were increased by addition of different

concentrations of cosubstrate. As a consequence, more TNT and its metabolites were removed or/and transformed.

Similarly, due to the exhaustion of DO resulting from oxidative utilization of molasses by aerobic or anaerobic heterotrophs, the redox-potential of the effluent decreased from 193 mV in the control experiment to –224 mV in the experiment which was amended with 0.1% molasses, which indicated that anaerobic or anoxic conditions were achieved in the bottom layer of this CW. As Robert et al. (1996) reported, TNT reduction is more favored with low redox-potential. In our present study, the addition of 0.1% (w/v) molasses to the CW allowed for the fastest TNT removal in Tank 1 and produced the Tank 1 with the lowest redox potentials (-224mV). In addition, due to the observation that not only TNT but also its transformation products were further removed from the CW in the experiments supplemented with molasses, it was assumed that not only TNT but also its intermediate metabolites were transformed favorably under conditions of low redox-potential.

With regard to the variations of parameters in the effluents in Tank 2, the average pH in effluents in the experiment which was amended with 0.1% molasses was 6.61, and were between 7.11 and 7.40 in other experiments. Data were shown in Fig. 24 (A). Comparing the DO concentrations and values of redox-potential in the effluents in Tanks 2 to 1, it was found that the average DO concentration was lower in the effluents in Tank 2 than that in Tank 1. The same result was found for the redox-potential. In the effluents in Tank 2, the redox-potentials were lower than –200 mV even in the control experiment. It is likely that this low redox-potential, which indicated anaerobic or anoxic conditions, led to the higher removal of TNT even in the control experiment (93.4% of initial TNT were removed in Tank 2).

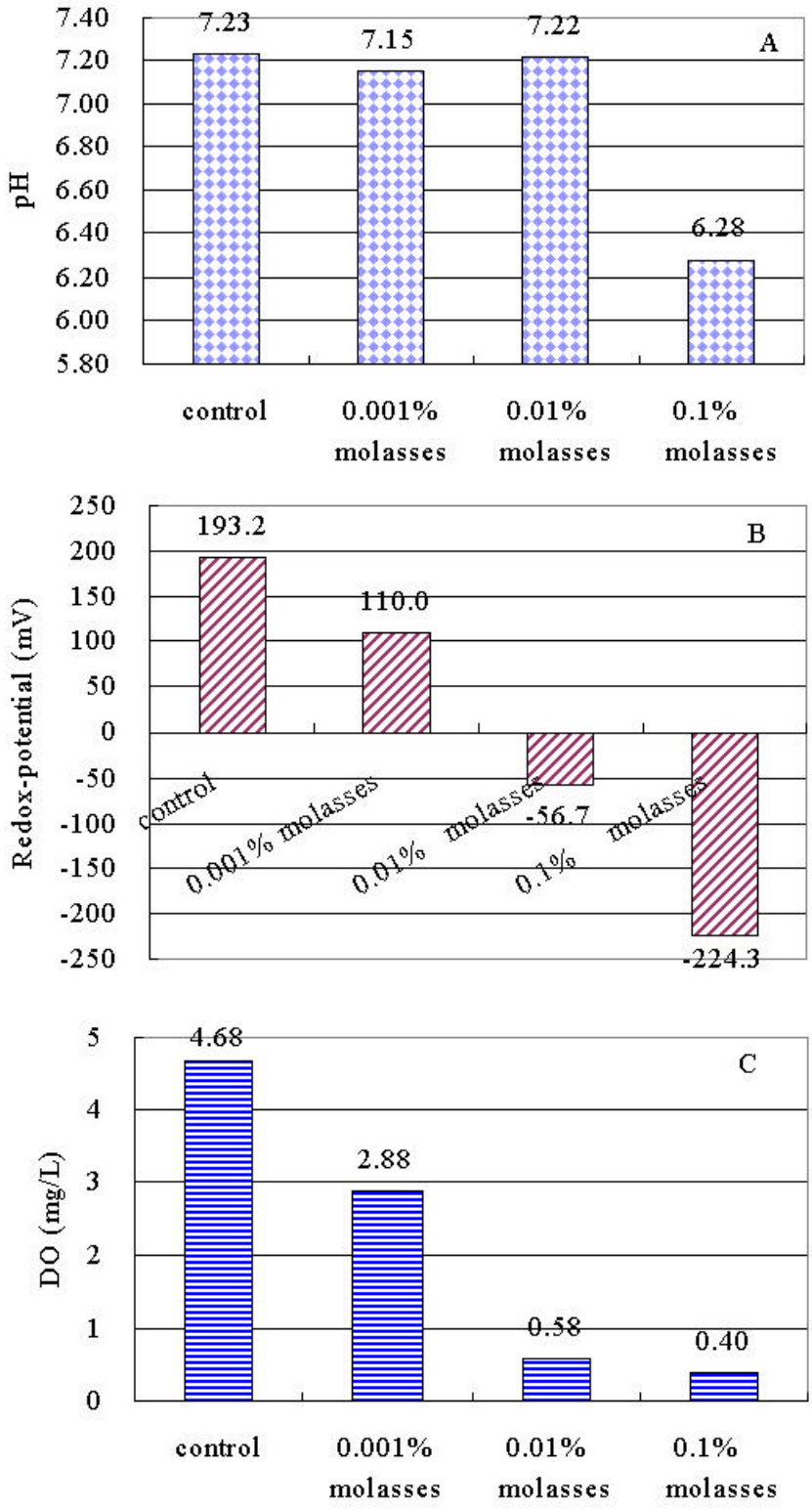


Fig. 23. pH (A), redox-potential (B) and DO concentration (C) in the effluents in Tank 1 in experiments amended with molasses and in control experiment.

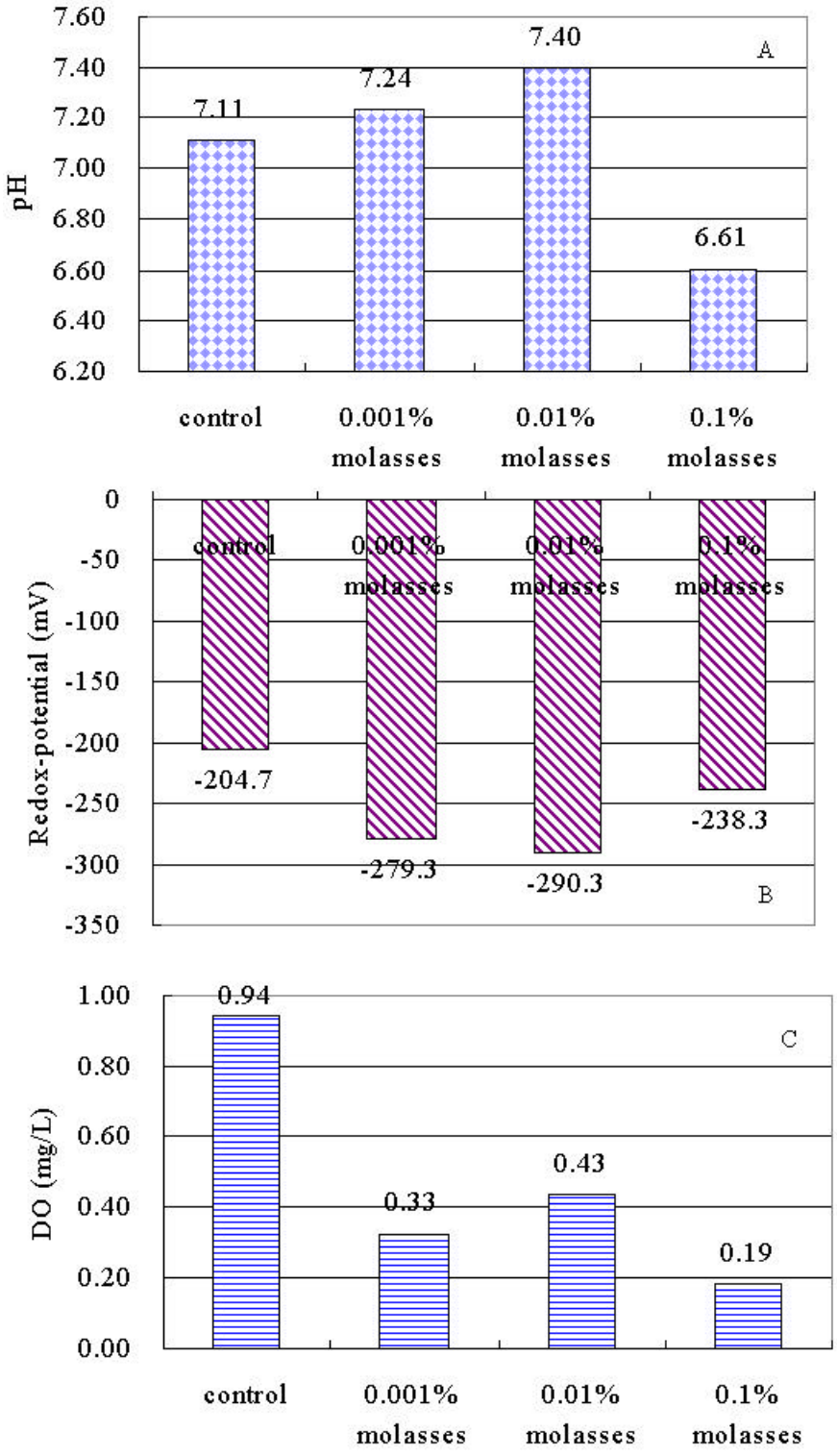


Fig. 24. pH (A), redox-potential (B) and DO concentration (C) in the effluents in Tank 2 in experiments amended with molasses and in control experiment

5.3.4 TNT and its transformation products extracted from lava and plant materials (stems and roots) taken form the CW.

Samples of lava and plant materials were taken at the end of these experiments, namely after all the four experiments were finished. They were extracted and analyzed by HPLC in triplicate and the results obtained are presented in the following sections.

5.3.4.1 Analysis of TNT and its transformation products extracted from lava taken from the CW.

HPLC analysis of lava samples from Tank 1 and Tank 2 showed that, in addition to TNT, at least five transformation products were present in Tank 1 and two transformation products in Tank 2, and their concentrations were shown in Figs. 25 & 26. TNT was found only in Tank 1 at Position 1, nearest to the inlet of the wastewater. TNT concentrations were 0.15 and 0.06 µg/(g lava) at 10 cm and 50 cm depths, respectively. At the other sampling positions in Tank 1 and Tank 2, the concentrations of TNT in lava were below detection limit (0.05µg/(g lava)).

With regard to TNT transformation products detected in lava extracts, 4A2,6-DNT, 2A4,6-DNT, 2,6-DNT, 2,4-DNT, 2,4DA6-NT were found in Tank 1, and 4A2,6-DNT and 2,6-DNT in Tank 2. Generally, with increasing depths of sampling positions, the concentrations of TNT and its transformation products (based on the sum of their concentrations) decreased, as shown in Table. 20. More metabolites (based on the sum of their concentrations) were detected in the upper layer of lava, for example at a depth of 10 cm. In Tank 2, the transformation products of TNT were found only at a depth of 10 cm. However, their concentrations were below detective limit at other depths.

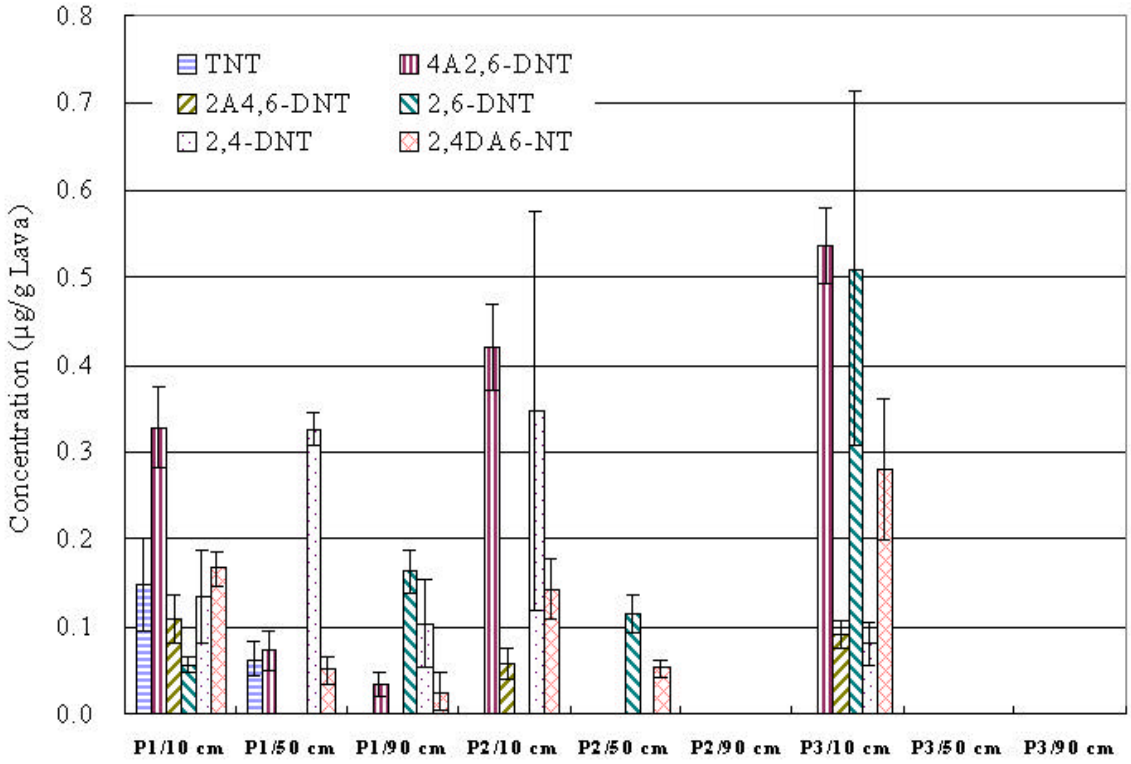


Fig. 25. Concentrations of TNT and its transformation products detected in lava extracts in Tank 1. *P1/10 cm = Sample taken at Position 1, at a depth of 10 cm; P2/50 cm = Sample taken at Position 2, at a depth of 50 cm; P3/90 cm = Sample taken at Position 3, at a depth of 90 cm.

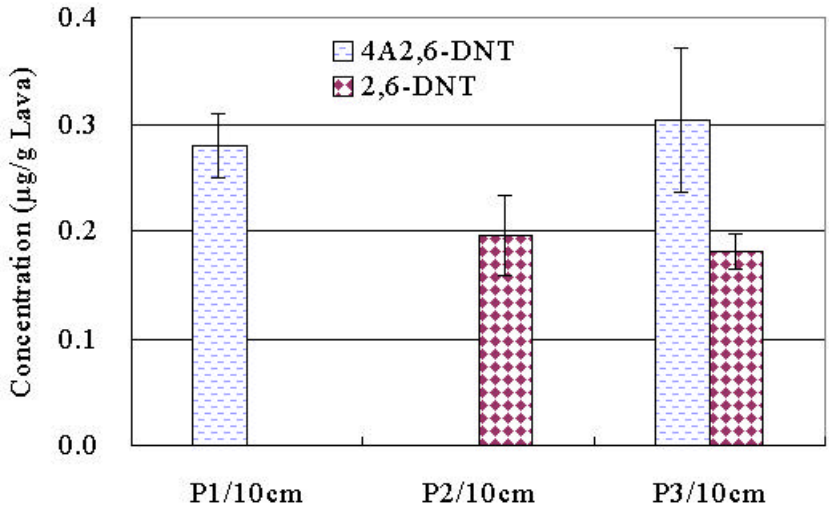


Fig. 26. Concentrations of TNT transformation products detected in lava extracts in Tank 2. *P1/10 cm = Sample taken at Position 1, at a depth of 10 cm; P2/10 cm = Sample taken at Position 2, at a depth of 10 cm; P3/10 cm = Sample taken at Position 3, at a depth of 10 cm.

Table 22. The sum of concentrations of TNT and its metabolites detected in lava samples from Tanks 1 and 2[a].

Samples of Tank 1	Sum (μg/(g lava))	Samples of Tank 2	Sum (μg/(g lava))
P1/10 cm[b]	0.94	P1/10 cm	0.28
P1/50 cm	0.51	P1/50 cm	< L. D.
P1/90 cm	0.33	P1/90 cm	< L. D.
P2/10 cm	0.97	P2/10 cm	0.20
P2/50 cm	0.17	P2/50 cm	< L. D.
P2/90 cm	< L. D.	P2/90 cm	< L. D.
P3/10 cm	1.50	P3/10 cm	0.48
P3/50 cm	< L. D.	P3/50 cm	< L. D.
P3/90 cm	< L. D.	P3/90 cm	< L. D.

[a]Data shown were average of triplicates.

[b]P1/10cm = Sample taken at Position 1, depth of 10 cm; P2/50 cm = Sample taken at Position 2, depth of 50 cm; P3/90 cm = Sample taken at Position 3, depth of 90 cm.

Comparing the concentrations of TNT and its transformation products in Tank 1 to those in Tank 2, we found that fewer metabolites (based on the sum of their concentrations) were present in Tank 2 than in Tank 1. This observation confirmed that more TNT and its transformation products were absorbed by lava in those tanks where more TNT exposed. Although no lava samples were collected from other tanks, i.e. from Tanks 3, 4, and 5, it can be deduced that fewer and fewer transformation products of TNT would be present Tanks 3, 4, and 5.

5.3.4.2 Analysis of TNT and its transformation products extracted from plant materials taken from the CW.

Table 23 shows the concentrations of TNT and its transformation products extracted from plant materials (stems and roots). Because Tank 1 was left unplanted in this study, stem samples were taken from Tank 2 and Tank 3, whereas root samples were taken only from Tank 2. The data given in Table 23 indicate that plants can take up and transform TNT (Goerge et al., 1994; Pavlostathis et al., 1998).

Table 23. Concentrations of TNT and its transformation products detected from the extracts of plant materials (stems and roots).[a]

Sample		TNT	4A2,6-DNT	2A4,6-DNT	2-NT	Sum[b]
Stems in Tank2	mean[d]	3.31	1.96	<L. D.[c]	1.23	6.50
	S. D.[e]	0.30	0.24	--	0.17	--
Stems in Tank3	mean	<L. D.	1.08	<L. D.	1.96	3.04
	S. D.	--	0.04	--	0.05	--
Roots in Tank 2	mean	0.24	3.13	0.92	<L. D.	4.29
	S. D.	0.05	0.11	0.08	--	--

[a]Concentration in µg/(g plant).

[b]Sum = Sum of the concentrations of TNT and its transformation products extracted from plant materials.

[c]<L. D. = below detection limit.

[d]mean = average (n=3).

[e]S. D. = standard deviation, n=3.

TNT was extracted in concentrations of 3.31, and 0.24 µg/(g plant) from stem and root materials in Tank 2, respectively. The TNT concentration in stem materials in Tank 3 was below detection limit of the HPLC. From stem materials, 4A2,6-DNT and 2-NT were the detected

TNT transformation products, whereas from the root materials 4A2,6-DNT and 2A4,6-DNT were the detected TNT transformation products. Comparing the TNT concentrations obtained from extracts of stem and root materials, more TNT was found in stem materials than in root materials. This observation was consistent with the results discussed in the previous Section 5.2.5.

Although the concentrations of TNT transformation products were lower in stem materials than in root materials, it cannot be concluded that TNT was more metabolized in roots than in stems. To confirm this, mass balance experiments using radiolabelled ^{14}C-TNT would be necessary.

The analysis of lava and plant material confirmed that TNT and its transformation products were adsorbed to the lava matrix and that plants can take up and/or transform TNT and/or its transformation products. The presence of transformation products of TNT in the plant materials suggests that the parent TNT was transformed by plant as well as by microbial activity.

5.4 Aerobic transformation of ^{14}C-labelled 2,4,6-trinitrotoluene in closed bottle-experiments

Results from the experiments conducted in the CW show that significant percentages of TNT were removed from the CW both in the presence and in the absence of cosubstrate. But as discussed in Section 5.3.2.1, it was found that balancing of TNT degradation in CW yielded a mass balance gap between TNT and its products. In addition, the fate of transformed TNT remained unclear. This study was conducted to estimate the fate of ^{14}C-labelled 2,4,6-trinitrotoluene in aerobic bioreactors both in the presence and in the absence of sucrose. For this purpose incubation tests were started with TNT solutions incubated with aqueous extracts from the CW containing a bacterial microflora. Experiments were carried out at concentration levels of 10 mg/L and 50 mg/L. The TNT-concentration of 50 mg/L was used to assess the bioremediation potential of higher contaminant levels. Since it has been reported that TNT, at concentrations in excess of 50 mg/L, is inhibitory to gram-positive bacteria, actinomycetes, yeasts, and fungi (Klausmeier et al., 1973). The detailed experimental treatment design of the radiolabelled experiments is listed in Table 8.

5.4.1 Radioactivity distribution in the test system

During the aerobic treatment period (28 days of incubation), samples of culture solution, VOCs and CO_2 were taken on every sampling date to determine the extent of TNT degradation by measuring their radioactivity. Less than 0.1% of the radioactivity added to the system was released in form of $^{14}CO_2$ from all inoculated treatments. In the control treatments (sterile treatments), no $^{14}CO_2$ was detected. The emission of VOCs was also very low, less than 0.6% of the added radioactivity in all treatments. The low percentages of $^{14}CO_2$ and VOCs indicate that no significant TNT mineralization occurred and that the production of VOCs was negligible. Similar observations have been reported by other researchers (Carpenter, et al., 1978; Best et al., 1999 (a)).

Most of the radioactivity was found in the pellets and the supernatants. After termination of the incubation, culture solutions mixed with sludge were centrifuged and separated to give supernatants and pellets. Pellet samples were combusted on a sample oxidizer and the released ^{14}C-activities were measured in a Liquid Scintillation Counter (LSC). The determined radioactivities represented the total ^{14}C-compounds sorbed on sludge. The amounts of radioactivity in the supernatants were also measured in the LSC. In the inoculated treatments, approximately 20-30% of the radioactivity was found in the pellets and 50-70% was found in supernatants. However, in the blank treatments (sterile), about 95% of the initially supplied radioactivity were found in the supernatants.

A mass balance of ^{14}C-radioactivity in the test system (Fig. 4) was achieved by measuring ^{14}C-activity in all components within the systems. Results, expressed as percentages of recovered ^{14}C-radioactivity, are presented in Table 24. Satisfactory mass recoveries were obtained, showing that the radio reactor systems were suitable for the present study. The total recoveries of ^{14}C-activity ranged from 90% to 94% of the ^{14}C-activity applied in the test system.

Table 24. Distribution of ^{14}C in the various components of the test systems[a]

Treatment	VOC	CO_2	Pellet	Supernatant	Recovery rate
1	0.16	--[b]	--	94.6	95
2	0.52	0.05	22.8	70.8	94
3	0.44	0.10	25.1	66.4	92
4	0.18	0.13	33.7	55.7	90

[a]Data shown are averages of duplicate and they are presented as percentages of ^{14}C-radioactivity applied to the test system.

[b]Below detection limit.

5.4.2 Transformation products of TNT in the cultural solutions (supernatants)

At the end of the incubation period of 28 days, culture solutions were centrifuged and the resulting supernatants passed through solid phase extraction cartridges as described in Section 4.4.1. TNT and its metabolites were extracted with methanol-acetonitrile mixture (1:1 v/v) and then analyzed by HPLC to quantify the amounts of residual TNT and the metabolites produced during microbial transformation processes. The results of the analysis are summarized in Table 25.

Table 25. The percentages of TNT and its metabolites in extracts of culture solutions as determined by HPLC[a]

Extracts	Treatment 1	Treatment 2	Treatment 3	Treatment 4
2,6DA4-NT	--[b]	0.11	0.72	0.27
2,4DA6-NT	--	0.26	6.7	4.2
TNT	90.2	40.1	8.6	15.1
4A2,6-DNT	--	10.6	8.3	4.4
2A4,6-DNT	--	4.1	0.35	1.3
2,6-DNT	--	0.15	0.13	--
2,4-DNT	--	0.07	0.14	--
2-NT	--	0.12	0.15	0.08
4-NT	--	0.06	0.21	0.03
Total	90.2	55.6	25.3	25.4

[a]Data shown are averages of duplicate and they are presented as percentages of initial amount of ^{14}C-radioactivity applied to the test system.

[b]Below detection limit.

Obviously, the total percentage levels of radiolabelled compounds detected in the culture extracts were lower than the total concentrations of ^{14}C-radioactivity detected in the

supernatants from each treatment (comparing Tables 24 with 25). This may be explained by the fact that some produced compounds of TNT were not identified by the present analytical methods and that there was also lost during the extraction process. With the exception of Treatment 1 (sterile treatment), all other extracts showed a very broad peak during the first 4 min of HPLC elution, possibly hiding some very polar metabolites of TNT. These unidentified polar compounds accounted for most of the ^{14}C-radioactivity, for example, they accounted for 9.6%, 38%, and 25% of the total ^{14}C-radioactivity supplied to each treatment, in Treatments 2, 3, and 4, respectively.

TNT was found to be reduced by microorganisms to various metabolites, including 2,6DA4-NT, 2,4DA6-NT, 4A2,6-DNT, 2A4,6-DNT, 2,6-DNT, 2,4-DNT, 2-NT, and 4-NT, which were detected in extracts of the culture solutions. This result confirmed the results obtained in earlier experiments, in which the same compounds were detected as the transformation products of TNT (see Section 5.1, 5.2 and 5.3). No TNT transformation products were found in the sterile treatment, which indicates that the metabolites found in the other treatments (Treatments 2, 3, and 4) were produced as a result of TNT microbial transformation, since the only difference between the sterile treatment and other treatments was the inoculation situation.

5.4.3 Influences of cosubstrate on TNT microbial transformation

Comparing the results (shown in Table 25) obtained from Treatments 2, 3, and 4, we found that the average concentration level of TNT in the culture solutions in Treatment 2 was the highest (40.1%) at the end of incubation time, which reflects the lowest TNT removal efficiency of 59.9%. However, in Treatment 3, in which 0.1% (w/v) of sucrose was supplied as cosubstrate, TNT average concentration level detected in the culture solutions was 8.6%, which suggests that TNT removal by microorganisms was favored by addition of cosubstrate. This suggestion is also supported by the results obtained in Treatment 4. In the sucrose-enriched Treatment 4, most of the TNT was removed from the culture solutions and only 15.1% residual TNT was

detected, although original TNT concentration was very high (50 mg/L). This observation also suggests that under proper inoculation and organic medium conditions, TNT can also be metabolized by microorganisms, even at high initial concentrations.

Of all the metabolites detected in inoculated treatments, 4A2,6-DNT and 2,4DA6-NT were the major metabolites. But their concentrations in different treatments were largely varying. In the sucrose-enriched Treatments 3 and 4, 4A2,6-DNT accounted for 8.3% and 4.4% of the total initial ^{14}C-radioactivity supplied to the test system, respectively, which were lower than that in sucrose-free Treatment 2, in which 4A2,6-DNT accounted for 10.6% of the total initial ^{14}C-radioactivity. The amounts of 2,4DA6-NT in the sucrose-enriched treatments (Treatments 3 and 4) were much higher than those in sucrose-free treatment (Treatment 2), i. e. 16 to 25 times higher. This observation suggests that the addition of sucrose increased the further reduction of aminodinitrotoluenes, which were produced from TNT microbial-reduction. The amount of unidentified polar metabolites increased with the addition of sucrose, from 9.6% (in Treatment 2) to 38% (in Treatment 3) and 25% (in Treatment 4), respectively. It is assumed that these unidentified polar metabolites maybe the further transformation products of aminotrotoluenes, whose reductions were stimulated by addition of cosubstrate.

The amounts of radiolabelled residues level found in sludge increased with the addition of sucrose. Because no sludge from all the treatments was extracted in this study, the ^{14}C-components in sludge could not be identified. Some researchers have reported that the radioactive carbon presented in biomass is mainly associated with lipid and protein components of the microbial organisms (Carpenter et al., 1978; Vanderberg et al., 1995) or TNT metabolites, which are irreversibly bound (unextractable) in soil (Hundal et al., 1997; Achtnich et al., 1999). The increase of radioactivity in sludge may result from the increasing growth of microorganisms and enhanced TNT-transformation by addition of sucrose.

5.4.5 Sub-summary

Addition of sucrose as cosubstrate increased the removal of TNT and enhanced the formation of TNT-reduction products. As no TNT metabolites were found in the sterile treatment, the transformation products found in the inoculated treatments were confirmed as microbial TNT-transformation metabolites. No significant mineralization of TNT was found in all the treatments (both sucrose-enriched and sucrose-free). Comparing the amounts of radioactivities found in sludge and culture solutions (supernatants), the amount of ^{14}C-VOCs was negligible. Results from this study clearly indicated that most of the radioactivity remained in cultural solutions and sludge, for example, 89% to 95% of the initially added ^{14}C-radioactivity were found in culture solutions and sludge.

Most of the TNT metabolites found were aminodinitrotoluenes and diaminonitrotoluenes. The addition of cosubstrate enhanced the further transformation of aminodinitrotoluenes to diaminonitrotoluenes. Unidentified polar metabolites were found in all inoculated treatments, and their concentration increased with the addition of cosubstrate. Further study should be carried out for the identification of these unidentified polar metabolites.

6. General discussion

In our present study, after passing through four or five tanks of the CW, 100% of the initial applied TNT was removed from wastewater both in experiments which were amended with cosubstrates and in cosubstrate-free experiments. Although plants are the most obvious components of the wetland ecosystem, TNT removal and transformation were accomplished through an integrated combination of biological, physical, chemical as well as photochemical interactions between the plants, the substrate and the inherent microbial community (Hwang et al. 2000b). The possible processes involved in TNT elimination are depicted in Fig. 27. Results obtained from analysis of plant and lava materials show that the absorption and/or uptake of TNT and its reduced products by plants as well as its adsorption of TNT and its metabolites by lava materials occurred (Section 5.3.4). The results obtained in the laboratory-scale radiolabelled TNT transformation experiments indicate that microbial transformation of TNT contributed most in the TNT removal (Section 5.4). Discussion of the pathways of TNT microbial transformation was emphasized.

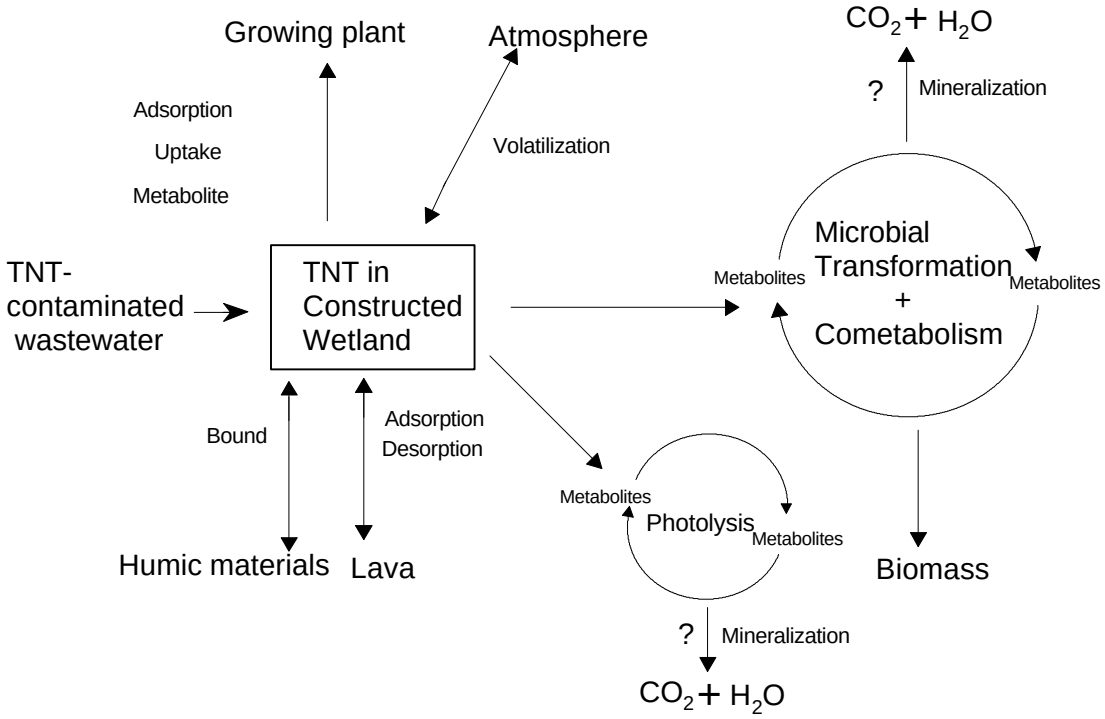


Fig. 27. Processes involved in TNT removal and transformation in the CW.

6.1 TNT microbial transformation pathway

Microbial transformation of TNT by aerobic, anaerobic, or through combined pathways has been well demonstrated (Won et al, 1974; McCormick et al., 1976; Carpenter et al., 1978; Kaplan and Kaplan, 1982; Naumova et al., 1989; Boopathy et al., 1993 a, b; Funk, et al, 1993; Roberts et al., 1996; Drzyzga et al., 1998; Vorbeck et al., 1998; Hawari et al, 1998). Most investigators have reported microbial transformation of TNT via reductive pathways under aerobic and anaerobic conditions (Won et al, 1974; Kaplan and Kaplan, 1982; Funk, et al, 1993). Under both conditions, the *para* nitro group of TNT is typically reduced through nitroso and hydroxylamino intermediates to form aminodinitrotoluenes (Won et al, 1974; McCormick et al., 1976; Carpenter et al., 1978; Kaplan and Kaplan, 1982; Boopathy et al., 1993 a, b; Funk, et al, 1993). Many studies have also reported the detection of diaminonitrotoluene isomers as products of TNT metabolism (Naumova et al., 1989; Hawari et al., 1998). There are only fewer reports on the reductive elimination of TNT nitro groups, and two cases have been reported. Duque et al. (1993) reported the denitration of TNT to DNT and NT in the presence of *Pseudomonas* hybrid strain. Martin et al., (1997) also reported the transformtion of TNT to 2,4-DNT by *P. Savastanoi* under nitrogen-limiting conditions. In our study, not only these nitro group reduction products but also those products formed as a result of the elimination of TNT nitro groups were detected as metabolites of TNT transformation both in the CW as well as in the radiolabelled laboratory experiments. The detected metabolites were the aminodinitrotoluene isomers (4A2,6-DNT, 2A4,6-DNT), the diaminonitrotoluene isomers (2,4DA6-NT, 2,6DA4-NT), the dinitrotoluene isomers (2,4-DNT, 2,6-DNT) and the nitrotoluene isomers (2-NT, 4-NT).

The nitro group of TNT exists as a resonance hybrid. Because the oxygen atoms are more electronegative than the nitrogen atom, the polarization of the nitrogen-oxygen bond causes the nitrogen atom to carry a partial positive charge and to serve as an electrophile (Jiang et al., 1990). Therefore, the most common reaction of the nitro groups in biological systems is the

reduction of nitro groups, which involves the reduction of the nitro groups to the amine or/and diamine products. As found in our study, most of the initial products of TNT transformation were 4A2,6-DNT, 2A4,6-DNT, 2,4DA6-NT and 2,6DA4-NT. High concentrations of aminodinitrotoluenes were found than those of diaminonitrotoluenes in the cosubstrate-free experiments, whereas, in the cosubstrate enriched experients, TNT removal was enhanced and the levels of diaminonitrotoluene concentrations increased greatly as shown in Figs. 11 (A) & 16. It was supposed that first, the reduction of one nitro group occurred, leading to the formation of isomeric aminodinitrotoluenes. The isomeric aminodinitrotoluenes were further reduced to diaminonitrotoluenes, and especially under cosubstate addition, the formation of diaminotoluene was enhanced.

As discussed in Sections 5.2.4 & 5.3.2.1, in the presence of cosubstrate, the formation of diaminonitrotoluenes was enhanced. This may result from the fact that addition of carbon may result in an increase in cell density and oxygen demand, which are favored by formation of DANTs from ADNTs. Rieger and Knackmuss (1995) have reported that the ease of reduction of the aromatic nitro group depends on the nature of the other substituents in the ring and on the reducing potential of the environment. Electron-withdrawing groups activate the molecule for reduction of the nitro groups, whereas electron-donating groups make the ring more susceptible to electrophilic attack. In the case of the nitrotoluenes, the probability of reduction increases, and the probability of electrophilic attack decreases as the number of nitro groups increases. Therefore, the reduction of one nitro group of TNT is very rapid under a variety of conditions, including those prevalent in growing cultures of aerobic bacteria. In contrast, reduction of aminodinitrotoluenes requires a lower redox potential, and reduction of diaminonitrotoluene requires a redox potential below –200 mV (Funk et al., 1993), because the electron-donating properties of the amino groups lower the electron deficiency of the molecule.

Based on the data obtained in our study and on the results of many other workers (Boopathy et al., 1993 a, b; Duque et al., 1993; Naumova et al., 1989; Drzyzga et al., 1998), we proposed the pathways of TNT microbial transformation as follows:

1. TNT transformation pathway A: TNT is reduced stepwise to amino-, diamino- and triamino-compounds and, under strict anaerobic conditions, to toluene. Toluene is obtained in the final step by complete reductive deamination of 2,4,6-triaminotoluene. This pathway was proposed by Boopathy et al., (1993a), Vorbeck et al, (1998) and Hawari et al., (1998).

2. TNT transformation pathway B: the three nitro groups on TNT are removed as nitrite ions. In this reaction, removal of nitro group involves the formation of a hydride-Meisenheimer complex. Vorbeck et al., (1998) have reported that a hydride-Meisenheimer complex is the initial metabolite in aerobic TNT bioconversion. The main metabolic product, toluene, is recovered after all the three nitro groups on TNT are removed. This pathway is supported by the studies of Duque et al., (1993), in which TNT was found to be transformed to ADNT, DANTs, DNTs and NT (2-NT) by two *Pseudomonas* hybrid strains and finally metabolized to toluene. The proposed pathways of TNT bioconversion are depicted in Fig. 28 (a) and (b).

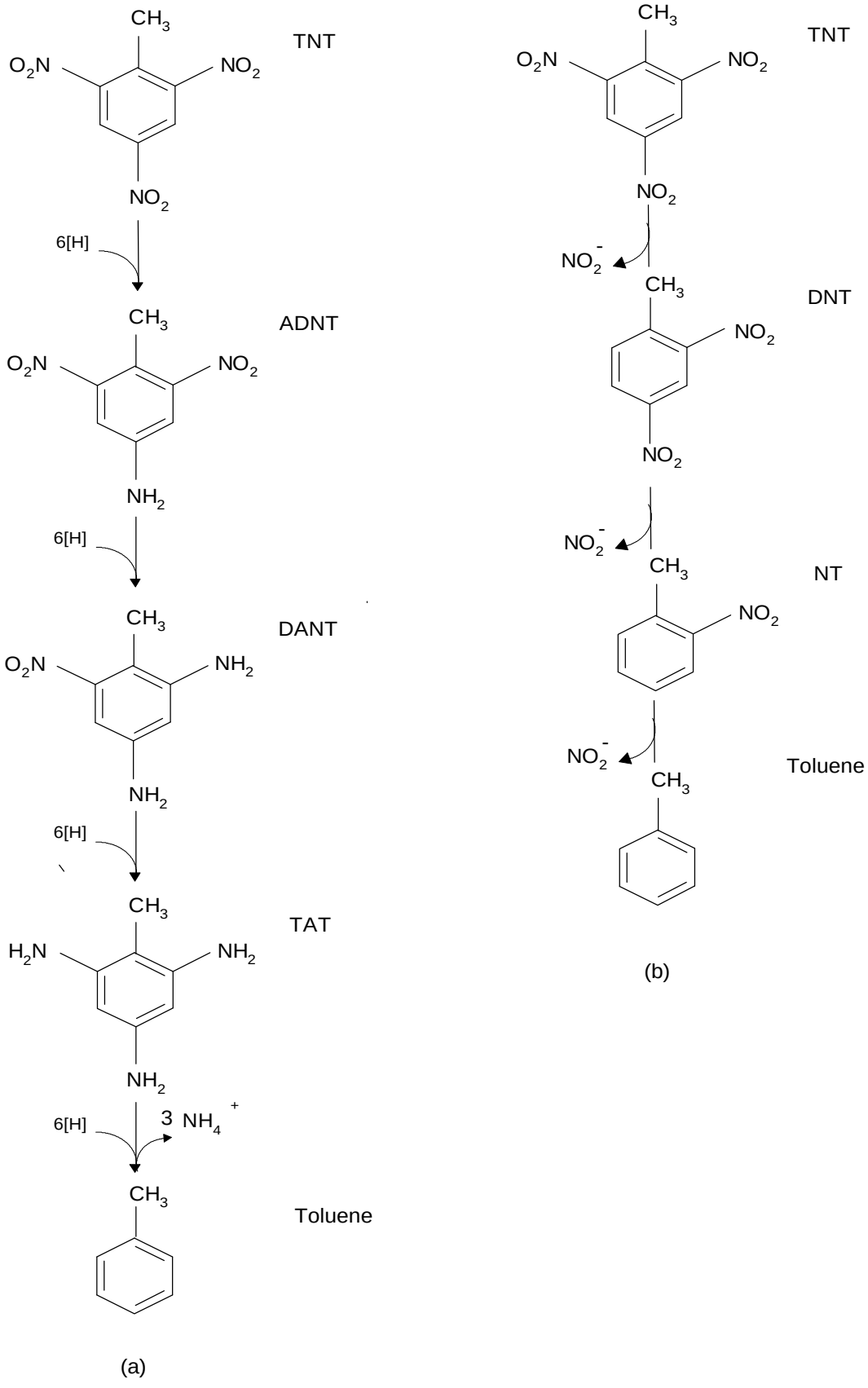


Fig. 28. Proposed pathways of TNT biotransformation (only one isomer is presented).

Shelley et al., (1996) have estimated the Gibbs free energy change for TNT biodegradation pathways A and B, to be -350.4 kcal/mol and -35.1 kcal/mol. The pathway A, which possesses the larger overall negative Gibbs free energy change is considered as the more probable pathway because it is assumed that the microorganisms can extract more energy from this process.

Hawari et al. (1998) have reported that TNT disappeared rapidly in an anaerobic system and its amine metabolites 4A2,6-DNT, 2A4,6-DNT, 2,4DA6-NT and 2,6DA4-NT were formed in a stepwise reduction. Preuss and Rieger (1995) reported that the reduction of 2,4DA6-NT is the rate-limiting step in the overall reduction processes of TNT.

In our study, we have found 4A2,6-DNT, 2A4,6-DNT, 2,4DA6-NT, and 2,6DA4-NT, which may be products of TNT transformation through pathway A, although we have not found any TAT (triaminotoluene) in our experiments. Because TAT is strongly bond to humic acids and clay minerals and is unstable (Preuss et al., 1993; Ederer et al., 1997; Krumholz et al., 1997; Daun et al., 1998), its detection is problematic. The concentration levels of 4A2,6-DNT detected in all our experiments were higher than those of 2A4,6-DNT, and the concentration levels of 2,4DA6-NT were higher than those 2,6DA4-NT, which suggest that NO_2 group favoring reduction takes place at the *para*- position instead at the *ortho*- position.

TNT transformation pathway B also possesses negative Gibbs free energy, however, this pathway has had only modest success in TNT biodegradation experiments, probably due to its smaller overall Gibbs free energy change (-35.1 kcal/mol). Martin et al., (1997) have observed the release of NO_2^- from TNT in medium containing *Pseudomonas* species, *P. Savastanoi,* and 2,4-DNT was identified as a TNT metabolite. Duque et al., (1993) also indicated that the two *Pseudomonas* hybrid strains utilize TNT transformation pathway B and transform TNT to DNT and NT. In addition, these bacteria which were used in the studies of Matin et al., (1997) and Duque et al., (1993) can also degrade TNT using TNT transformation pathway A, which

is supported by the reported high concentrations of ADNTs and DANTs. The metabolites such as DNTs and NTs found in our experiments confirmed the TNT transformation pathway B. In our study, we have also detected 2,6-DNT, 2,4-DNT, 2-NT, and 4-NT as TNT metabolites (see Sections 5.1, 5.2 and 5.3). Based on the fact that not only ADNTs and DANTs, but also DNTs and NTs were detected as TNT metabolites in our experiments, we prefer to assume that TNT microbial transformation process in CW as well as in our radiolabelled laboratory experiments occurred through a combination of pathways A and B.

Mineralization of TNT by microorganisms remains, till now, controversial. Many investigators have reported that no significant mineralization of TNT occurred. (Carpenter et al., 1978; Boopathy et al., 1994a, 1994b; Breitung et al., 1996). However, few studies have reported that TNT mineralization occurred to some extent. Michels and Gottschalk (1994) have reported 30% of TNT mineralization in the presence of white rot fungus *phanerochaete chrysosporium*. In our laboratory radiolabelled experiments, less than 0.1% of the radioactivity added to the system was present in the metabolically produced $^{14}CO_2$. The low $^{14}CO_2$ production indicates limited capacity of the microbial organisms in this CW to directly utilize TNT as C-source. TNT is a known inhibitor of microbial growth (Middlebrook et al., 1993; Crawford 1995b) and its recalcitrance to complete biodegradation is due, in part, to the three nitro groups that reduce the electron density on the benzene ring and impede oxidative attack on the ring structure by electrophilic oxygenases (Bruhn et al., 1987; Thiele et al., 1988; Rieger and knackmuss 1995). Therefore, mineralization of TNT was very limited in our experiments. Nevertheless, mineralization of TNT remains the preferred aim of biological treatment processes, and results from many earlier studies have shown promising possibilities (Michels and Gottschalk, 1994; Hodgson et al., 2000; Rho et al., 2001). 12.3% and 15.3% mineralization of the TNT in *P. chrysosporium* cultures enriched with TNT have been demonstrated in their studies. Further studies on optimization of TNT mineralization should be carried out for the development of a TNT remediation technology.

6.2 Cometabolic transformation of TNT in the CW

One of the objectives of this study was to examine the effects of sucrose and molasses as cosubstrates on TNT removal and transformation in the CW. Results obtained from this study indicate that addition of both sucrose and molasses could stimulate the removal and transformation of TNT. As discussed in Sections 5.2.1 and 5.3.1, addition of sucrose and molasses increased the TNT removal efficiency from 91 to 99.8% and from 22.8 to 99.8%, respectively. Similarly, TNT removal rate was also increased approximately 5 times (from 0.406 to 2.043 g TNT/(m^2, d), see Table 18) in the tank without growing plants by addition of molasses.

In our study, the involvement of co-metabolism was not clearly demonstrated. Because of the fact that TNT decrease was also found in the control experiments (in which no cosubstrates were supplied) both in our laboratory radiolabelled experiments as well as in the pilot-scale study in the CW. In the pilot-scale study in the CW, it was not possible to separate the influences of factors such as photolysis, phytolysis, microbial cometabolism of TNT induced by the exudates released by plants and by microbial degradation of TNT. Any decrease in TNT concentrations and any transformation of TNT in the CW were attributed to the comprehensive effects of these factors (Hwang et al., 2000b). Therefore, it is more practical to verify the cometabolism of TNT in a laboratory-scale experiment. However, in our laboratory radiolabelled TNT transformation experiments, we used the natural microbial populations obtained from the CW and no extra separations or pre-culture of bacteria were done. The suspended solution extracted from lava, which was collected from the CW, contained not only the needed inocula, but also some organic compounds released by plants, although the concentration levels of these compounds maybe relatively low. We used this suspended solution for inoculation in the radiolabelled experiments in the treatments enriched with sucrose as well as in the sucrose-free treatments.

Nevertheless, the fact that addition of sucrose or molasses increased the removal and transformation of TNT was confirmed in our study. The positive effects of cosubstrates on TNT removal and transformation were confirmed, not only because the removal rates of TNT increased, but also because the concentration levels of the transformation products were changed. In the experiments which were enriched with cosubstrates, more diaminonitrotoluene isomers were detected, as shown in Figs. 17 & 18 and Table 14. This result suggests that the further transformation of aminodinitrotoluenes as TNT transformation products was also stimulated by addition of cosubstrates.

As described in Section 1.3, it is well recognized that external carbon source for TNT transformation and/or degradation by microorganisms was required. In many earlier studies, the authors suggested that TNT cannot be used as the sole C-source and is metabolized only by cometabolism (Osmon and Klausmeier, 1972; Amerkhanova and Naumova 1978; Preuss et al., 1993). Hoffsommer et al., (1978) in their pilot-plant scale study, noted that although TNT is toxic to microorganisms, it might be biologically transformed on a continual basis by a relatively simple process in the presence of supplemental nutrient. It is likely that additional carbon sources will be required to maintain an active microbial population and the resulting increase in cell density and oxygen demand will likely promote nitro moiety reduction. In our pilot-scale study, with the addition of cosubstrates, the concentrations DO and redox-potentials in effluents in the CW decreased greatly and more TNT was removed as already discussed in Sections 5.2.2 and 5.3.3.

From the different cosubstrates studies that were undertaken, the relatively inexpensive molasses was found to produce better results in terms of TNT removal. With addition of 0.01% (w/v) of molasses, 89% of TNT was removed after passing through one tank. Considering economic feasibility and TNT removal efficiency of the first tank, 0.01% (w/v) of molasses was considered as the optimal concentration level supplied to the CW. The cost of molasses is significantly less than that of sucrose, which makes a large-scale TNT biotreatment-system using molasses as cosubstrate economically feasible.

6.3 The contribution of growing plants in TNT removal in the CW

The contribution of growing plants in the CW in TNT removal was evaluated in our study. Comparing the results from Sections 5.1, 5.2 and 5.3 (data shown in Table 26), it was observed that the existence of growing plants in the CW enhanced the removal of TNT from wastewater. Under natural conditions (no addition of cosubstrate and no technical aeration), TNT removal efficiency in planted Tank 1 was 3.5 times higher than in unplanted Tank 1. Similarly, the removal rate of TNT in planted Tank 1 was about 3 times of that in unplanted Tank 1. However, it must be noted that the higher removal efficiency and removal rate obtained in planted Tank 1 resulted not only from the phydegradation by growing plants but also from the photodegradation of TNT (Hwang et al., 2000b). Although the existence of growing plants and plant decays in planted Tank 1 of Cascade 3 reduced the irradiation of sunlight, the water surface was not totally in darkness. Therefore, photodegradation of TNT might occur. However, covering of the unplanted Tank 1 of Cascade 2 made the water surface totally in darkness and no photodegradation of TNT was possible. Nevertheless, The contribution of plants in TNT removal and transformation in the CW was supported by both the high removal rate obtained in planted Tank 1 and the detection of TNT and its metabolites in plant materials.

Considering the cosubstrate-enriched experiments, because of the different influent loads of TNT and the two different cosubstrates used, a reasonable comparison of the results from the planted and the unplanted tanks was difficult to obtain. Nevertheless, we observed that removal efficiency of TNT in the unplanted Tank 1 was as high as that obtained in the planted Tank 1. This observation suggests that the addition of a cosubstrate can enhance the removal of TNT, and the addition of molasses as cosubstrate performed as well as the growing plants as far as the removal efficiency of TNT from wastewater was concerned.

Table 26. The summary of results obtained from the planted as well as the unplanted Tank 1 in the CW.

	Influent Load (g TNT/m^2,d)	Removal Rate (g TNT/m^2,d)	Removal efficiency (%)
Planted Tank 1			
Cosubstrate-free Experiment	1.612	1.295	80.3
Experiment added with sucrose (0.25% (w/v))	0.486	0.485	99.8
Unplanted Tank 1			
Cosubstrate-free Experiment	1.782	0.406	22.8
Experiment added with molasses (0.001% (w/v))	2.795	1.574	56.3
Experiment added with molasses (0.01% (w/v))	2.188	1.956	89.4
Experiment added with molasses (0.1% (w/v))	2.285	2.043	99.8

Many studies have shown that plants can support TNT transformation and that TNT transformation products can also be partially taken up by the plants (Goerge et al., 1994; Hughes et al., 1997; Pavlostathis et al., 1998). Best et al., (1999a) also observed that TNT disappeared completely from groundwater incubated with growing plants, to a less extent with substrates and a least extent in controls without growing plants. Analysis of the plant materials exposed to TNT in our present studies indicates the existence of TNT and its metabolites in the stems and roots (see Section 5.3.4.2). How can plants enhance the removal of hazardous organic substances? It would be very interesting and of great significance to answer this question. We tried to elucidate the effects of plants on the removal of TNT or other organic compounds in the following ways:

(1). Plants may take up, metabolize and/or accumulate some hazardous organic substances (Pan and Dong, 1995).

In the studies of Best et al., (1999b), they used radiolabelled TNT to test the effects of growing plants on TNT removal and found that 24-79% of the labelled compounds were recovered in submerged plants, which indicated that at least 24-79% of the TNT removal was attributed to growing plants. In another experiment, Sens et al., (1998) reported that approximately 59% of the initial ^{14}C-TNT added to the culture solutions was adsorbed and taken up by plants, of which 43% was found in the cytoplasm and 57% in the cell wall. In our present study, we extracted the plant materials exposed to TNT and found, not only the parent compound TNT, but also several metabolites of TNT such as 4A2,6-DNT, 2A4,6-DNT, and 2-NT in the stem and root materials (data shown in Tables 15& 21).

(2). Plants can support the growth of microbial communities at their root zones by rhizodeposition and root exudation. Therefore, to evaluate the impact of plants on bioremediation, not only the plants but also the microbial communities at their root zones have to be considered.

Experimental evidence suggests that organic contaminants often disappear more quickly from planted soils than from soils without vegetation (Walton and Anderson 1990; Cunningham et al., 1995). Many researchers believe that increased degradation of xenobiotic compounds may result from cometabolic processes enhanced by organic substances originating from roots (Walton and Anderson, 1990; Anderson et al., 1994; Jordhal et al., 1997). Plant decay and root exudation are two means by which substrate is provided to the rhizosphere. Root exudates include sugars, amino acids, organic acids, fatty acids, sterols, growth factors, nucleotides, flavanones, enzymes, and numerous other compounds (Pan and Dong, 1995). Some of these compounds released in the processes of root exudation are required for microbial metabolism and may favor the growth of specific microbial species or contain

enzymes, which facilitate degradation of xenobiotic contaminants (Anderson et al., 1993; Fletcher and Hegde, 1995; Schnoor et al., 1995; Newman 1995). In many studies, higher microbial population density and diversity was found in the rhizosphere of soils, and microorganisms isolated from natural system rhizospheres actually show greater rates of xenobiotic degradation. (Anderson et al. 1993; Lee and Banks 1993; Anderson et al., 1994; Thijs et al. 1994).

(3). The microorganisms at the root zone, which contribute in the remediation of organic pollutants, also have positive effects on plant growth.

It is well known that rhizobia can help to perform the process of N_2-fixation (Pan and Dong, 1995), which are necessary for plant growth. Labidi et al., (2001) reported that rhizobia were also able to transform TNT in liquid cultures using glucose and glutamate as C-source to hydroxylaminodinitrotoluenes, aminodinitrotoluenes, and diaminonitrotoluene. The additional of the ready-degraded organic compounds may maintain an active microbial population and result in increase in cell density (Martin et al., 1997) at the root zones, which may improve the growth of plants. In this case, addition of sucrose or molasses may provide better growth

of plants. Although we did not evaluate the growth of plants in the CW during our study, we observed that plants in the tanks that were amended with sucrose or molasses were higher and stronger than those in the cosubstrate-free tanks. In this point, using of cosubstrates-enhanced phytoremediation technology for wastewater treatment provides an ecological and economical water pollution control method.

7. Summary

The pilot-scale constructed wetland used in this study was successful in removing and biodegrading TNT from contaminated wastewater. With influent concentrations of TNT between 1200 to 4000 µg/L, 91% to 66% of TNT were removed from the wastewater after passage through the first tank of the CW, providing TNT removal rates between 0.4 to 1.3 g TNT/(m^2, d). After passage through three or four tanks, the concentrations of TNT and its extractable metabolites were below detection limit. These results were obtained under natural CW operation conditions, i.e. without addition of cosubstrates and no technical aeration. The results from this study confirmed that constructed wetlands could be used to purify wastewater in a cheaper and more environmentally acceptable manner.

The contribution of growing plants to TNT removal in the CW was evident. TNT removal efficiency was higher in the planted tank than in the unplanted tank. Growing plants enhanced the removal and transformation of TNT not only because of their ability to take up and/or transform TNT and/or its transformation products, but also because of their ability to support the growth of microbial communities at their root zones by root exudation and plant decay. Analysis of growing plant tissues showed the presence of both the parent compound TNT and its reduction products such as 4A2,6-DNT, 2A4,6-DNT, and 2-NT.

The adsorption of TNT and its metabolites in lava materials was demonstrated. In analysis of lava extracts, TNT, 4A2,6-DNT, 2A4,6-DNT, 2,6-DNT, 2,4-DNT, and 2,4DA6-NT were detected. Most of TNT and its extractable metabolites were found in the upper layer of lava and at positions near the wastewater inlet. However, with low influent TNT concentrations, the adsorption of TNT and its metabolites by lava was negligible.

Addition of cosubstrates such as sucrose and molasses enhanced the phytotransformation of TNT. The removal of TNT in the CW was always faster with addition of cosubstrates. The

results showed that not only the transformation of TNT but also the further transformation of its intermediates was stimulated by addition of cosubstates. Molasses is significantly cheaper than sucrose, which will make a large-scale TNT biotreatment-system economically feasible. Considering economic feasibility and TNT removal efficiencies of the first tank, 0.01% (w/v) of molasses was considered as the optimal concentration level of cosubstrate for the remediation of TNT-contaminated wastewater in the CW. The results demonstrated that addition of cosubstrates to the CW made it possible to save half of the treatment facilities and of the construction areas, and thus save the cost of investments. This is of great significance to developing countries and those countries where large free-land are not available for construction of wastewater treatment plants.

Radiolabelled ^{14}C-TNT experiments conducted in closed bottle cultures indicated that no significant amount of TNT was mineralized. Low TNT mineralization rates indicated that reduction is the primary route of TNT microbial transformation. Approximately 20-35% of the initial applied ^{14}C-activity was found in the sludge, which was associated with microbial organisms and 55 to 70% in culture supernatants. The results obtained from these experiments also indicated that the native microbial organisms presented in the pilot-scale CW were capable of transforming TNT at both low influent TNT concentration (10 mg/L) and high influent TNT concentration (50 mg/L). Inoculated experiments resulted in a much higher TNT removal and formation of metabolites as compared with sterile experiments. This result indicated that TNT was removed due to biological activity and that abiotic factors had only a minor influence. No evident inhibition to microbial transformation of TNT in culture bottles due to high concentrations of TNT was observed during the 28-day experimental period.

Phyto-transformation of TNT in CW via nitro group reduction or elimination in situ is supported by the detected high concentrations of aminodinitrotoluenes, diaminonitrotoluenes, dinitrotoluenes and relatively low concentrations of nitrotoluenes. The major TNT transformation products detected in extracts of effluent in the CW were 4A2,6-DNT,

2A4,6-DNT, 2,4DA6-NT, 2,6DA4-NT, 2,4-DNT, and 2,6-DNT. Nitrotoluenes (2-NT, and 4-NT) were also detected but at very low concentrations.

Lava was able to support the growth of wetland plants and associated ecosystem at least for 3 or 4 months continuously. This study gives an indication that the use of lava should be further investigated in full-scale CW projects. During the entire experimental period, no evidence of clogging was observed, suggesting a good physical-chemical property of lava as a substrate in CWs.

The results of this study have provided a better understanding of TNT transformation in a constructed wetland. Data obtained in this study can be used as a guide for future full-scale treatment of TNT-contaminated wastewater as well as for other wastewater treatments. To provide practical wastewater treatment technologies nowadays, the following factors have to be considered: (i) provision of a high removal efficiency; (ii) cost-effectiveness; (iii) savings on constructed areas; (iv) avoidance of generation of secondary pollution; and (v) provision of socially acceptable elegant and viable technology. Cosubstrate-enhanced phytoremediation of wastewater is one of the practical technologies which meet most of the requirements mentioned above and has a strong potential in water pollution control both in developed and in developing countries.

8. Outlook

Plant-microbe-based phytoremediation of TNT-contaminated wastewater offers an alternative cost-effective treatment technology. Phytoremediation of organic pollutants is a very complicated process, which involves a variety of physical, chemical and biological processes. The present study provides some understanding of this process. However further studies involved in the following suggested areas are necessary.

Little is known about the mechanisms of plant uptake and metabolism of TNT. Since TNT and its metabolites can enter the food chain via growing plants (Goerge et al., 1994), and given that TNT and its metabolites are toxic, mutagenic and cancerogenic, the processes by which TNT transformation takes place with presences of plants should be studied further under laboratory as well as field conditions in order to provide an accurate risk assessment. Investigations using radiolabelled ^{14}C-TNT can provide a better understanding of this subject.

Investigations are also needed for understanding of the long-term performance of CW in TNT removal. The results obtained from the present study demonstrate a promising performance of CW in TNT removal. The present study was carried out only during the summer time (June to October) of each year. Therefore, the performance of the CW over the whole year period, especially in winter, is subject to further investigation,

To provide a better understanding of TNT microbial transformation processes, identification of unknown transformation products of TNT is of significance. This is of particular interest with regard to the formation of triaminonitrotolouene (TAT) and its polymerization and binding onto the soil matrix. Further studies involving bio-mineralization of TNT is needed, since mineralization of TNT remains the optimal remediation strategy.

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Annex

Annex-1 List of Figures

Annex-2. List of Tables

Annex-3

Curriculum vitae

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