ANAEROBIC DEGRADATION OF 2,4,6- TRINITROTOLUENE (TNT): MOLECULAR ANALYSIS OF ACTIVE DEGRADERS AND METABOLIC PATHWAYS

by

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ABSTRACT OF THE DISSERTATION

Anaerobic Degradation of 2,4,6- Trinitrotoluene (TNT): Molecular Analysis of Active Degraders and Metabolic Pathways

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Nitroaromatic compounds have been historically used as dyes, explosives and pesticides. The disposal of these products has caused widespread contamination of both soil and groundwater. 2,4,6-trinitrotoluene (TNT) has been known as the worst of these contaminants because of the mutagenic properties of the compound, its persistence in the environment, and the wide range of sites that are contaminated with TNT. It is not only harmful to humans but it is also harmful to organisms in the lower trophic levels of ecosystems, and can affect the primary production of phytoplankton in the oceans.

The following series of experiments looks to determine ways that 2,4,6-trinitrotoluene could be biologically degraded in contaminated anaerobic environments. The experiments use dilution culturing, molecular techniques, and chemical analysis. Cultures were made from 3 different geographical sites (Arthur Kill, Norfolk Harbor, and an unexploded ordnance site in Hawaii) under both sulfidogenic and methanogenic conditions. The experiments demonstrate that there are bacteria present in the environment that could degrade TNT and the use of stable-isotope probing (SIP) in dilution cultures allowed the bacteria that are able to initially degrade TNT to be identified. The stable-isotope fed dilution cultures produced samples that were used for

chemical analysis to determine pieces of the biological degradation pathway. These experiments determined that 1) there are bacteria that are able to utilize both the carbon and/or nitrogen present in TNT for growth and 2) toluene, methylphloroglucinol, benzoate or a benzoate derivative, and a cresol are all metabolites of anaerobic TNT degradation.

Preface

There is limited information regarding the microorganisms responsible for anaerobic 2,4,6-trinitrotoluene degradation and mechanism by which it is metabolized. Both sulfate reducing and methanogenic enrichment cultures from various locales were established and examined to address these questions. Molecular methods and classical culturing techniques were used in attempts to characterize microbes capable of anaerobic 2,4,6-trinitrotoluene metabolism. Bacteria actively degrading 2,4,6-trinitrotoluene and the anaerobic pathway utilized by these microorganisms are identified and described herein.

Dedication

This work is dedicated to my amazing family and friends, especially Mom and Joe -it would not have been possible without all of your love, support, patience, and understanding.

The things that will destroy us are: politics without principle; pleasure without conscience; wealth without work; knowledge without character; business without morality; science without humanity; and worship without sacrifice.

– Mahatma Gandhi

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CHAPTER 1:

Introduction

1.1 Environmental Contamination

Nitroaromatic compounds have been used as dyes, pesticides, and explosives historically. The manufacturing, use, and disposal of these products has caused widespread contamination of both soil and groundwater (8, 9, 15). 2,4,6-trinitrotoluene (TNT) has been known as the worst of these contaminants because of the mutagenic properties of the compound, its persistence in the environment, and the wide range of sites that are contaminated with TNT (9). The highest concentrations of TNT found have been as high as 600,000 to 700,000 mg/kg in both soils and sediments at military installations in the United States and Europe, and in the United States alone, the contaminated sites extend over 1 million cubic yards of material (6, 12).

TNT is not only harmful to humans, but it is also harmful to organisms in the lower trophic levels of ecosystems, and can affect the primary production of phytoplankton in the oceans. The amount of TNT present in the environment made it a xenobiotic chemical of concern. TNT also has a relatively high water solubility making both contaminated waters and soils a priority (8, 15). The compound is mobile and water soluble in both surface and groundwater and as aquatic ecosystems are contaminated with TNT the water will take on a pinkish hue, causing these sites to be known as “pink water” sites (12).

Typically, the sites that are contaminated with TNT are industrial plants where TNT was manufactured, military installations, munitions storage areas, and areas where many unexploded ordnances (UXOs) are present (3, 8, 9, 15, 42). Depending upon the areas where the contamination is present, the TNT in the system will be degraded by different

means. Under aerobic conditions it is difficult to fully reduce TNT to 2,4,6-triaminotoluene (TAT) due to its rapid polymerization and binding to organic sediments in oxic conditions. The complete reduction to TAT occurs primarily under anaerobic conditions, however there are some cases where aerobic bacteria are able to degrade TNT when co-ammended with other nitrogen and carbon sources (27). In the fully reduced form, TAT, becomes less likely to be toxic because there are no nitro groups present, eliminating its overall nitrogenation (28).

The means of reduction of TNT anaerobically are: (1) using the nitro groups present as electron acceptors for respiration, (2) the use of Type I nitroreductase to reduce TNT, (3) the nitrogen present in the TNT molecule can also be used as the sole nitrogen source for bacteria such as various *Clostridia* and *Desulfovibrio* species (8, 36). Whether or not oxygen is present, the TNT in a system can be reduced abiotically, by the reduced nitrogen groups covalently bonding to the different organic matter present in the soils and sediments in a given system (8, 15). Additionally, it has been shown that different species of *E. coli* have the enzymes necessary to degrade TNT, by breaking down the compound and use the nitrogen and carbon present (11).

While the reduction of TNT to TAT is an important process in reducing the environmental toxicity of the molecule biotically or abiotically, previously no studies have shown that there are bacteria that are able to grow on TNT as a sole carbon source or as the bacteria's nitrogen and carbon source. If there are bacteria that can use TNT as a carbon source it will most likely be degraded by an anaerobic strain of bacteria since they are present under conditions in which TNT can be completely reduced and mineralized. It has been shown that TAT could possibly be both a carbon or a nitrogen

source under anaerobic conditions as well (8, 15). Illustrating that TNT can be mineralized to CO_2 by a bacterium, or consortium of bacteria, will provide a possible novel means of remediation and monitoring for TNT.

TNT is an important environmental pollutant because of its effect on entire food webs and ecosystems, from the primary producers through the higher trophic levels. The toxicological impacts of TNT include blood, liver, immune system, and reproductive health problems in wildlife and humans exposed, and decreased diversity in microorganism populations (21, 24, 25). This adds to the need to find a means to remediate contaminated sites.

1.2 Chemical Structure of 2,4,6-Trinitrotoluene (TNT)

TNT is a symmetrical nitroaromatic compound with nitro groups present on a carbon ring at the 2, 4, and 6 positions and a methyl group present in the 1 position. Due to the presence of multiple nitro groups on the aromatic ring the molecule is highly electron deficient and can be reduced both biotically and abiotically (8, 15, 18). More specifically the partial positive charge present on the nitrogen atom and the atoms high electronegativity makes it easily reducible (7, 8). Under aerobic conditions there is typically a reduction of the nitro groups to hydroxylamino groups and finally to amino groups. While under anaerobic conditions, the TNT is reduced sequentially to 2,4,6-Triaminotoluene (TAT) (8, 33, 41). See Figure 1.

The resistance of TNT to complete mineralization is because it is extremely easy for the reduction of the nitro groups to occur forming intermediates that are not as chemically favorable for reduction. Due to their chemical instability, these intermediates can also

bind covalently to organic matter present in the soil. All of the TNT intermediates, including the dinitroaminotoluenes, diaminonitrotoluenes, and TAT can bind to the soil and become unavailable to microorganisms or self-polymerize into chains that are also not typically available for biological degradation (8, 15). The binding and self-polymerization occurs more rapidly and more often under oxic conditions and is less likely to occur under anoxic conditions. These processes are the main reasons that the biological degradation of TNT will most likely occur under anaerobic conditions.

Isotopic studies of TNT degradation performed have shown that when TNT is present in soils and sediments the TNT will not be readily extracted from the organic matter (15, 41). The mineralization of TNT, especially under aerobic conditions, is minimal because of the fact that the soil and sediments adsorb and/or covalently bond many of the metabolites (41). In aerobic systems, there will not be complete mineralization of TNT because its fully reduced form, TAT, is highly reactive under oxic conditions and is subject to autoxidation, polymerization, and sorption to soil particles rendering it unavailable to the microorganisms present in the system (33). One of the difficulties in studying the degradation of TNT, whether it is biotic or abiotic, is that the metabolites of TNT are highly reactive with soil and sediment under both aerobic and anaerobic conditions (43).

1.3 2,4,6-Trinitrotoluene (TNT) Toxicity

The toxicity of TNT is specific to the organism that is exposed to the compound, the amount of TNT it is exposed to, and the route of exposure. In mammals the effects of TNT generally include central nervous system toxicity in the form of seizures,

hepatotoxicity, and immune system dysfunction (21, 24, 25). These effects differ from those present in invertebrates where reproduction is effected at lower doses in chronic dosing experiments (6, 23, 24, 34, 38). At higher concentrations TNT is lethal to the invertebrates tested. Microorganisms, such as bacteria and phytoplankton, experience toxic effects that are detrimental to primary productivity and alter the community structure in soils, sediments, and aquatic ecosystems (12, 27). Because TNT and its metabolites are toxic and represent an environmental risk to many organisms including mammals, fish, insects, and bacteria it is important to understand the mechanisms of TNT toxicity (24). The human health risk factors of TNT exposure primarily affect the employees of munitions factories and disposal sites. There have also been toxic effects of TNT and other nitroaromatic energetic compounds reported in the rank-and-file military personnel in both the United States and abroad (25).

It is necessary to understand the damage that occurs when different ecosystems are contaminated with TNT (12, 21). If the health of the ecosystem is damaged at lower trophic levels, the higher trophic levels will be also be negatively affected. This includes damage to the phytoplankton (primary producers) and also the total soil and sediment community structure.

TNT and its derivatives affect organisms differently based on exposure. The following is a brief synopsis of TNT exposure to humans and microorganism. Toxicity is determined based on dose concentration, route of exposure, length of exposure, damage to the target organ of TNT, and subsequent health problems that may arise.

Humans are typically exposed to TNT and its metabolites through occupational hazards as employees at munitions factories or disposal sites. The routes of exposure

consist of oral, inhalation, and skin absorption. There are also exposure risks to military personnel who handle bombs, grenades, and propellants that contain TNT. Measurable concentrations of TNT and dinitrotoluenes existed even in the restrooms of a munitions disposal factory, where the highest concentrations of TNT in the air were present in the water removal system and in the hand grenade intermediate storage area (25).

Microbial communities are impacted by TNT contamination by changing community structure, specifically lowering overall diversity, and reducing phytoplankton primary production (8, 27). It is difficult to the determine the overall effect of TNT contamination on specific soils and sediments because of the high levels of spatial variability naturally found in these environments, in addition to the physical characteristics not being uniform in contaminated zones.

To determine microbial toxicology, soil profiles are used to determine the diversity present in contaminated versus non-contaminated areas (27, 37). These assessments can be preformed using both denaturing gradient gel electrophoresis (DGGE) and/or terminal restriction fragment length polymorphism (T-RFLP). Both methods use the DNA present in a specific soil or sediment sample to give a profile of the community present. When the sites are contaminated with TNT, there is a reduction in the diversity of bacteria or phytoplankton present (27, 37). This could be due to the mutagenicity of TNT and its derivatives to the microorganisms. Lachance et al, 1999, found that the relative order of mutagenicity of TNT and its metabolites were trinitrobenzene > TNT = 2-aminodinitrotoluene > 2,6-diaminonitrotoluene >> 2,4-diaminonitrotoluene and 4-aminodinitrotoluene. While it is difficult to determine lethality, it was determined that all of these compounds were mutagenic to the microorganisms tested. It was also

hypothesized that this was true in the phytoplankton community causing a reduction in primary productivity when contaminated with TNT (8).

Because of complex community structure and the total number of members in complex microbial communities it is difficult to determine exact mechanisms of TNT toxicity and detrimental loads of TNT to the soil or sediment. The concentration of TNT that is seen by the microbial community cannot easily be determined based on the rapid degradation and transformation of TNT in the environment, in addition to the TNT molecules becoming trapped in pore water or bound to clay particles present.

1.4 Remediation Options for TNT

The aerobic degradation of TNT has not be as efficient as the anaerobic reduction due to the formation of many different intermediates that are not ideal for further degradation, mineralization, or the formation of intermediates that become not biologically available for consumption (8, 15, 33). Under aerobic conditions TNT cannot be completely mineralized, unless the pathway does not include TAT, because of the reasons discussed previously, TAT is a dead-end metabolite in TNT degradation under aerobic conditions, unless amended with co-substrates or additional electron acceptors (10, 11, 27, 33). The following body of work will focus on only the anaerobic degradation of TNT.

TNT and other nitroaromatic compounds can be metabolized by biological means. Plants, fungus, and bacteria have all shown the capability to transform nitroaromatic compounds (1, 5, 11, 35). The complete mineralization of these compounds to CO_2 is rare because of their highly reduced nature. When TAT is formed anaerobically, it is a more stable molecule than it is in aerobic conditions where it will immediately polymerize to itself or sorb to the organic matter present in soil (32, 39).

Previously, it has not been shown that there are bacteria that are able to grow on TNT as a sole carbon source or as the bacteria's nitrogen and carbon source. The compound could possibly be used as a carbon source by anaerobic bacteria since they are present under conditions in which TNT can be completely reduced and possibly mineralized. It has been shown that TAT can be both a carbon or nitrogen source under different conditions (8, 15). If it is discovered that TNT can be mineralized to CO_2 by a bacterium or consortium of bacteria it will provide a possible means of remediation and monitoring for TNT as an EPA priority contaminant.

1.4.1 Phytoremediation

To date, the mechanisms that have been approved for the treatment of soils contaminated with TNT and other nitroaromatic compounds have been physical-chemical; such as incineration, soil washing, or transformation using the Fenton oxidation reaction (22, 26). All of these methods are costly and do not completely destroy the compound to the point where the end product can be considered non-toxic. For these reasons, other mechanisms for the removal of TNT from contaminated sites are being investigated. One of the most popular methods is ex-situ composting (1, 20, 30, 32, 39). Other possible mechanisms that have been studied are ex-situ phytoremediation, in-situ phytoremediation, and possibly a combination of using a physical-chemical method such as soil washing followed by a secondary treatment of phytoremediation ex situ (5, 22, 26, 27, 29).

Phytoremediation is the process of utilizing plants to remove a contaminant from the soil or sediment of a specific site. More specifically phytoremediation utilizes green plants to clean up contaminated soil or water (5). The process is advocated because it is

inexpensive and more tolerant to contaminants than the use of microorganisms alone due to the ability of plants to release exudates that stimulate microbial activity and mineralization of some contaminants in the rhizosphere (5). For these reasons phytoremediation has attracted the interest of environmental engineers, scientists and regulators because of the effectiveness of the process and the cost efficiency (16). While most phytoremediation is strictly terrestrial and soil related there has been studies on using plants in a constructed wetland to remove TNT from the sediment present (5, 22). This opens the possibility of the use phytoremediation having broad applications.

Typical products of phytoremediation are aminodinitrotoluene (ADNT) and diaminonitrotoluene (DANT). The reduction of TNT to these compounds typically is due to the activity of nitroreductases present in the plant and in the root exudates of the plants (5, 16). The observation of low levels of TNT mineralization show that the primary means of TNT transformation in the soil is reduction of the nitro groups present on the aromatic ring to amino groups. At this point TNT is completely transformed to TAT and will most likely bind to any organic matter present (22). Mass recoveries of TNT from soils that have undergone phytoremediation show a lower concentration of TNT than in unplanted soil, in addition to lower concentrations of the TNT metabolites present in unplanted soils (5). These results can be seen in Table 1. The mass deficit seen in this table is most likely due to the fact that TNT and its metabolites have a strong tendency to sorb to the organic matter present in soil (5, 22, 29). In addition to identifying some of the metabolites present from the degradation of TNT, the use of phytoremediation has also retarded the migration of TNT in a contaminated soil (5). The retardation of a

relatively water-soluble compound in the soil is useful in bioremediation because it will reduce the total area of contamination.

One of the possible drawbacks to utilizing phytoremediation as a technique to remediate TNT contaminated sites, is that the TNT concentration at the sites may be high enough to cause toxic effects to the plants and inhibit their ability to perform remediation. Phytoremediation is an applicable choice for the remediation of TNT contaminated sites, however it is more effective when it is combined with bacterial degradation in the system (5, 22, 27, 29). In this symbiotic relationship, neither the plants nor the bacteria in a TNT contaminated system are able to function as well alone as they can together. The relationship could be due to the benefits found in the plant rhizosphere such as nutrients, water, alternating reduction potentials, high microbial diversity, the presence of biosurfactants, and protecting niches for sensitive microorganisms (22). It is apparent that the concentration of TNT present in systems will be reduced more quickly when bacteria are present, as opposed to systems where bacteria are not present in the rhizosphere. As demonstrated by Kreslavski et al, 1999 the TNT concentration was reduced more rapidly in a system where both plants and bacteria were present after 2 days of incubation. Chang et al, 2004 studied the dehydrogenase activity profiles in 2004, it was seen that there was a greater amount of microbiological activity when plants, specifically Indian mallow, were present. The higher levels of dehydrogenase activity is indicative of a higher amount of bacterial activity, even in the presence of TNT. These results are seen in Figure 2.

When bacteria were combined with cyanobacteria (*Anabaena*) similar effects were observed as seen in Figure 3 (29). The TNT disappearance in these cultures was slower

when only bacteria was present, in comparison to the mixed bacteria and *Anabaena* cultures. There was a high removal efficiency of TNT from this system, in upwards of 96%, without toxic effects to the cultures (29). The mixed cultures may have been more effective because of the photosynthetic capability of *Anabaena* and its ability to fix nitrogen. This could aid the bacteria present by producing alternate forms of nitrogen to be used than the nitrogen present in TNT. The Pavlostathis and Jackson, 2002 study further proves the notion that symbiosis between plants and bacteria in the remediation of TNT and other contaminants. Additionally, it illustrates the value of having a diverse microbiological population present to degrade contaminants.

1.4.2 Biological Remediation

In anaerobic systems TNT can be more efficiently removed from the system. Due to the fact that there is no oxygen present, fewer intermediates can be formed making the overall transformation or degradation of TNT much faster (7, 8). There are two main ways that TNT is anaerobically transformed or metabolized in the environment; either by respiration of the NO_2^- groups or by removal of the NO_2^- groups to be used as a terminal electron acceptor or as a nitrogen source (3, 7, 8, 11, 14, 15, 33). A Type I nitroreductase can be used in an anaerobic system because it is not oxygen sensitive and will reduce the nitro groups present on the TNT molecule. The reaction facilitated by Type I reductase is a 2 electron transfer completely reducing the NO_2^- (nitro) group to an NH_2 (amino) group (36). Isolates have been cultured that are able to transform TNT to monoaminonitrotoluenes, diaminonitrotoluenes, and possibly to triaminotoluene (8). Other organisms have been identified that are able to use these groups as a nitrogen source (8). See Table 2. In addition to the different organisms that are able to transform

and degrade TNT there are a number of different pathways, that have been both proposed and identified, in which this degradation or transformation occurs (2, 3, 8). See Figure 4.

Three different genera of bacteria are known to be readily able to metabolize TNT: *Clostridia* and *Desulfovibrio* (8, 33). The metabolism of TNT can generally be performed by *Clostridia*, in cell suspensions the bacteria are able to reduce the TNT present to TAT and some other metabolites that are not known (8). *E. coli* species have been determined to be able to enzymatically reduce TNT to TAT, and cleave the amino groups in order for the nitrogen to be used for growth when glucose is present as a co-substrate (8, 11). The nitrogen is released from the nitroarene ring by removal of nitrite or by the removal of ammonium after the reduction of a nitro group (8).

Nitroreductase enzymes are typically used to catalyze this reduction, however there are multiple other enzymes including aldehyde oxidase, dihydrophilic amide dehydrogenase, cytochrome b_5 reductase, diaphorases, hydrogenases, xanthine oxidase, and carbon monoxide dehydrogenase (8, 11, 17). These enzymes are present in a wide variety of bacteria and eukaryotes, leading to the initial metabolism of TNT occurring quickly in both laboratory and environmental settings.

In the *Desulfovibrio* genus there have been multiple strains isolated that are able to use TNT as a sole nitrogen source. *Desulfovibrio* sp. Strain B is able to use TNT as a nitrogen source and toluene has been shown to accumulate in the culture medium (2). Preuss et al. were able to isolate a *Desulfovibrio* culture using TNT as the sole nitrogen source with pyruvate as the carbon source present in the medium. Since both *Clostridia* and *Desulfovibrio* reduce TNT to TAT, the complete mineralization of TNT will require the removal of the amino groups from TAT. This transformation is only possible under

strictly anaerobic conditions (8, 15, 33). *E. coli* AB1157 is a wild type strain that can efficiently use TNT as a nitrogen source when glucose is present as a carbon source. This can occur if the *E. coli* cells are grown aerobically or anaerobically using nitroreductases or N-ethylamine reductase (11).

Although *Clostridia* and *Desulfovibrio* are very important organisms in the biotransformation and biodegradation of TNT they are not the only organisms able to partake in this degradation. *Pseudomonas* sp. Strain JLR11, isolated by Esteve-Nunez (2000), is able to use TNT as its sole nitrogen source by releasing nitrite from the aromatic ring, reducing it to ammonium, and incorporating the ammonium into carbon skeletons when glucose is present as a co-substrate. *Pseudomonas* sp. Strain JLR11 can also use TNT as a terminal electron acceptor in respiration (7, 8).

The transformation of TNT can occur under conditions where nitrate, sulfate, or carbon dioxide is present as an electron acceptor. See Table 3. If the TNT in the system is completely reduced to TAT and the nitro or amino groups are removed, the carbon skeleton will be able to be degraded. Hypothesized products from the elimination of the amino groups from TAT are: toluene, *p*-cresol, and methylphloroglucinol (8). It has been shown that toluene will accumulate in the media of some cultured isolates (2). All of the possible metabolites of TAT reduction are able to be utilized as a carbon energy source under anaerobically and are not to prevent complete mineralization.

1.5 Conclusions

The remediation of a site contaminated by TNT is a complicated task because the chemical properties of the molecule make it difficult to metabolize by bacteria and very likely to sorb to the organic matter present in the contaminated site. The best method for

the remediation of a TNT contaminated site will most likely be a combination of the aforementioned technologies. Neither composting, nor phytoremediation, nor bacterial metabolism will completely clean a contaminated site, but combining these technologies could prove to be successful. The combination of phytoremediation and bacterial metabolism to reduce the TNT concentration present in a system is a feasible mechanism. This is due to the fact that TNT concentrations are reduced more quickly when both plants and bacteria are present at a site and that TNT is not only sorbed to organic matter but is actually transformed. If a three-stage remediation process were possible, it may be the best method for the remediation of a TNT contaminated site. Beginning with composting, the TNT could be concentrated into a smaller amount of soil; this mature compost could then be planted, with Indian mallow for example, to utilize the benefits of phytoremediation. While bacteria would not need to be added to the system, the combination of plants and bacteria present in a non-sterilized soil could allow for the transformation of TNT in the system.

TNT is a pollutant of concern because of its persistence in the environment, its relatively high water solubility, and its toxicity to both humans and to organisms responsible for primary production. As more information about the organisms able to metabolize or degrade TNT and the metabolites generated in this process is discovered, making more informed decisions on site remediation will be possible. More studies need to be performed to determine the best and most cost effective option for the remediation of TNT contaminated sites. The following experiments will focus on the determination of microbial ability to utilize TNT, using a two pronged approach that includes novel molecular techniques to determine the active degrading population, in addition to more

classical techniques in metabolite determination from TNT degradation. They will also identify some metabolites present in anaerobic TNT degradation.

1.6 Figures

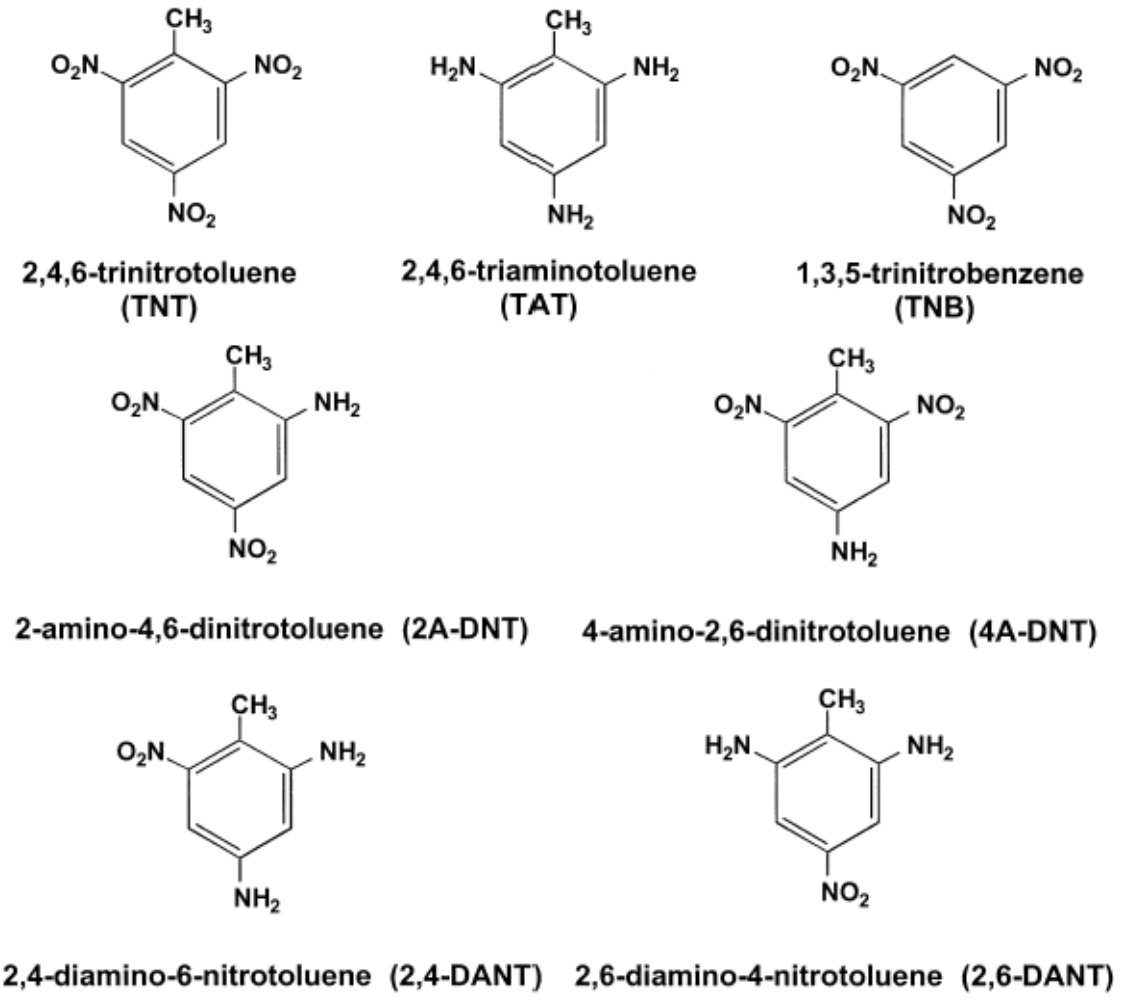

Fig 1. Illustration of the chemical structures of TNT, TAT, and some of its well known derivatives that will be important in the overall toxicity of TNT in the environment. Adapted from Lachannce et al, 1999.

Table 1. Illustrates the mass deficit of TNT, shows that more TNT is transformed or metabolized in planted soils than unplanted soils. Adapted from Chang et al, 2004.

Medium	***Contaminant***	***Mass in Planted Column (%)***	***Mass in Unplanted Column (%)***
Soil	TNT	23.2	48.1
	4ADNT	25.8	31.2
	2ADNT	5.8	6.1
Plant	TNT + ADNTs	0.2	-
	Mass Deficit	45.0	14.6

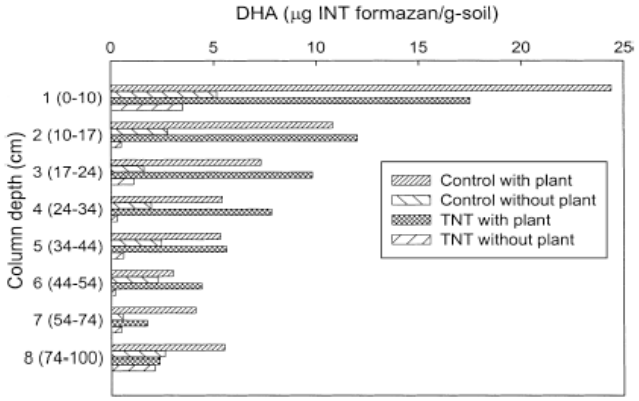

Fig. 2 Dehydrogenase activity profiles in each soil column after 50 days (from Chang et al, 2004).

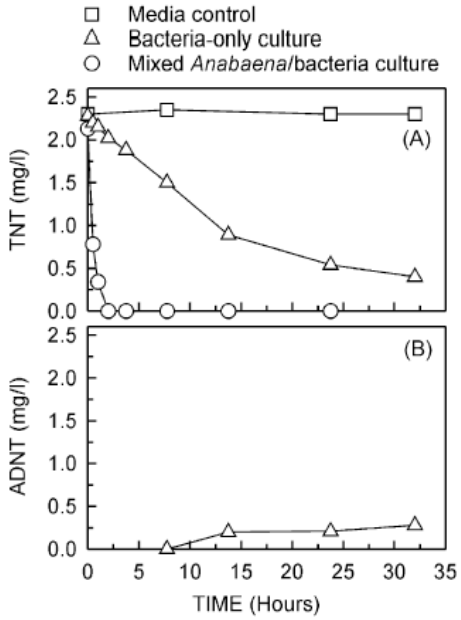

Fig. 3 TNT concentration in the media control, the bacteria-only culture and the mixed *Anabaena*/bacterial culture (A) as well at the metabolite concentration produced in the bacteria-only culture (B) in the incubation period.

Table 2. Microorganisms reported to anaerobically degrade or transform TNT. It also describes the mechanism used to reduce the TNT molecule and the metabolites that are produced. Adapted from Esteve-Nunez, et al 2001.

Microorganism	***Metabolism***
Clostridium acetobutylicum	Reduction of TNT to TAT
Clostridium bifermentans CYS-1	Degrades TNT to aliphatic polar compounds via 4ADNT and 2,4DANT
Clostridium bifermentans LIP-1	Transforms TNT into TAT and phenolic compounds
Clostridium paterianum	Reduction of TNT to TAT
Clostridium sardelii	Reduction of TNT to TAT
Clostridium sp.	Bamberger rearrangement of dihydroxylaminodinitrotoluene
Desulfovibrio sp. strain B	TNT as nitrogen source, toluene at putative intermediate
Desulfovibrio sp.	TNT as the sole nitrogen source, reduction of TNT to TAT
Desulfovibrio sp.	Transforms TNT into TAT and DANT; 42% of radioactivity from ^{14}C-TNT is associated with cell biomass
Escherichia coli	Reduction of TNT to TAT
Lactobacillus sp.	Reduction of TNT to TAT
Methanococcus sp. strain B	Reduction of TNT to DANT
Pseudomonas sp. strain JLR11	TNT as nitrogen source; TNT as final electron acceptor
Veillonella alkalescens	Reduction of TNT to TAT

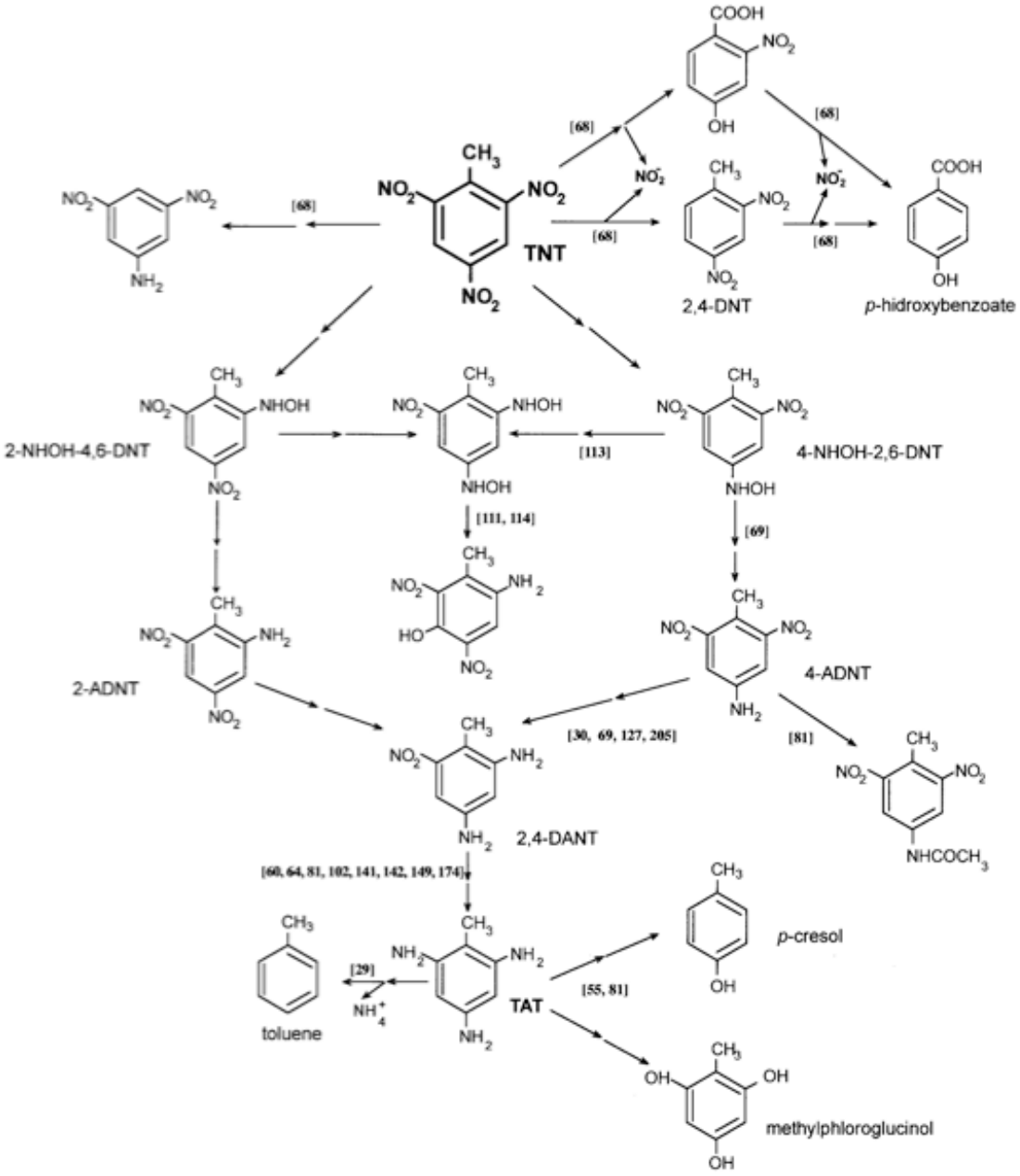

Fig. 4. Anaerobic TNT degradation and transformation pathways both known and hypothesized. From Esteve-Nunez et al, 2001.

Table. 3. Removal rates of TNT and bacterial growth rate as determined by protein analysis under different anaerobic growth conditions. The metabolites of the TNT removal are also shown. Adapted from Esteve-Nunez et al, 2001.

Growth Conditions	***Bacterial Growth Protein (mg/L)***	***% TNT Removal***	***Intermediates Produced***
Nitrate Reducing	85	82	2- and 4-aminodinitrotoluenes
Sulfate Reducing	18	30	2- and 4-aminodinitrotoluenes
Methanogenic	19	35	2- and 4-aminodinitrotoluenes
No specific electron acceptors	0	0	None

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CHAPTER 2

^{13}C-Carrier DNA Shortens the Incubation Time Needed to Detect Benzoate-Utilizing Denitrifying Bacteria by Stable-Isotope Probing (SIP)

ABSTRACT:

The active bacterial community able to utilize benzoate under denitrifying conditions was elucidated in two coastal sediments using stable-isotope probing (SIP) and *nosZ* gene amplification. The SIP method employed samples from Norfolk Harbor, Virginia, and a Long-Term Ecosystem Observatory (LEO-15) off the coast of Tuckerton, New Jersey. The SIP method was modified by use of archaeal carrier DNA in the density gradient separation. The carrier DNA significantly reduced the incubation time necessary to detect the ^{13}C-labeled bacterial DNA from weeks to hours in the coastal enrichments. No denitrifier DNA was found to contaminate the archaeal ^{13}C-carrier when [^{12}C]-benzoate was used as a substrate in the sediment enrichments. Shifts in the activity of the benzoate-utilizing denitrifying population could be detected throughout a 21-day incubation. These results suggest that temporal analysis using SIP can be used to illustrate the initial biodegrader(s) in a bacterial population and to document the cross-feeding microbial community.

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2.1 INTRODUCTION:

Many bacteria are able to degrade anthropogenic pollutants through metabolism or respiration in the environment (4, 28, 31). Although the ability of bacteria to degrade environmental contaminants is widely known, it is difficult to determine the bacteria that perform this metabolism. Since it is widely believed that <1% of the bacteria present in environmental samples can be cultured in the lab (6, 8), it is unlikely that the active degraders of any particular environmental contaminant will fall within the easily culturable portion of bacteria. Recently, stable-isotope probing (SIP) techniques have been used to elucidate the active population of bacteria among the total population of bacteria contributing to a particular metabolic pathway (16, 18, 20, 25, 30). The SIP method utilizes a ^{13}C-labeled substrate and PCR techniques to discern the members in a microbial community that incorporate the ^{13}C into their DNA. SIP capitalizes on the ability to separate DNA containing different carbon or nitrogen isotopic labels using cesium chloride (CsCl) gradient centrifugation (14, 20). In theory, the approach can ascertain the microorganism that initiates pollutant degradation if an appropriate time course can be utilized during a SIP experiment. However, the majority of SIP studies incubate for extended periods to generate DNA with sufficient label incorporation to visibly separate the DNA on a gradient (typically more than 20 days (16, 21, 22, 30). Within shorter time frames, there were difficulties utilizing SIP to its full capabilities (3, 15, 27). RNA-based SIP studies (5, 11, 12, 13, 17), on the other hand, have generally used shorter incubation periods. RNA is labeled faster than the genome since RNA is growth rate regulated in many bacteria (9, 10). However, there have been complications reported in RNA purification or separation using stable isotopes (5, 11, 22).

In this study, genetic signatures from denitrifying bacteria capable of benzoate utilization in coastal sediments were discerned using DNA-SIP and ^{13}C-carrier DNA for the physical separation of ^{13}C-DNA. The addition of an archaeal carrier DNA to the CsCl gradient allows for a significant reduction in incubation time. For example, the benzoate-utilizing denitrifying community present at these coastal sites that had incorporated the ^{13}C label could be detected within a 1-hour time frame. Furthermore, shifts in this benzoate-utilizing community could be observed over the course of a 21-day incubation. The study demonstrates that SIP methodology can be used on short timescales, reducing the possibility of stable-isotope transfer in alternate chemical forms (cross-feeding). This advance will also allow for the determination of active in situ microbial populations and may enable the mapping of microbial food webs where the metabolic intermediates are not known.

2.2 MATERIALS AND METHODS

Multiple tests were performed to determine if the addition of carrier DNA could be combined with SIP studies using both pure cultures and environmental enrichments. To test for cesium chloride gradient contamination of the ^{13}C-DNA band with ^{12}C-DNA, a pure culture of *Thauera aromatica* strain T1 (26) was grown on [^{12}C]-benzoate in a denitrifying medium, sparged with a 70%–30% mixture of N_2-CO_2, and amended with 100 μM NO_3. After the culture was grown for approximately 30 days, the cells were transferred 1:1 into fresh medium and amended with either 100 μM of uniformly ^{13}C-labeled benzoate or [^{12}C] benzoate as the sole carbon source. The culture was incubated

for approximately 30 days, and the DNA was extracted using a phenol-chloroform method (19, 23).

For determining natural populations of benzoate-utilizing denitrifying bacteria, sediments were enriched with benzoate and nitrate from a Long-Term Ecosystem Observatory (LEO-15) located off the coast of Tuckerton, New Jersey, representing a nonimpacted condition, while sediment from Norfolk Harbor in Virginia was considered a contaminated site. A 10% (vol/vol) sediment slurry was sparged with a 70%–30% mixture of N_2-CO_2 and amended with 100 μM NO_3 and 100 μM of uniformly ^{13}C-labeled benzoate, [^{12}C] benzoate, or no additional carbon source in 24 tubes (20-ml capacity). Triplicate tubes were sacrificed for each amendment at 1 h and 5-, 7-, 14-, and 21-day time points (three active tubes from each site with labeled substrate or unlabeled substrate). For each of the sampling points, approximately 400 μl of the sacrificed slurry was extracted using a modified phenol-chloroform extraction procedure, and the DNAs from the triplicate samples were pooled and resuspended in 100 μl of sterile deionized water (19, 23) Scala. The carrier DNA used for visualization of ^{13}C-DNA in this study was from *Halobacterium salinarium* grown in a ^{13}C-labeled ISOGRO powder growth medium (Isotec, Miamisburg, OH). The stable-isotope- enriched medium was prepared for halophilic bacteria (7) and grown aerobically at 25°C for approximately 20 days before the cells were harvested and DNA was extracted, as described above.

Approximately 300 ng of environmental-sample DNA and 300 ng of ^{13}C carrier DNA were added to a 500-μl CsCl density gradient (1 g/ml) containing 20 μg ethidium bromide and separated in a TLA 120 rotor on a Beckman Optima ultracentrifuge (Palo Alto, CA) at 225,000 *g* (29). After 16 to 24 h, the bands were visualized using UV light

and pulled from the gradient by first removing the ^{12}C-DNA band, changing the pipette tip, releasing a small air bubble above the height of the ^{12}C-DNA band, and proceeding to remove the ^{13}C-DNA band from the gradient. After band extraction, the genomic DNA was dialyzed using a 0.025-μm Millipore mixed cellulose ester dialysis filter (Bedford, MA) floating in a petri dish containing 10 mM Tris-HCl (pH 8.2).

For determination of the ^{13}C-assimilating denitrifying bacteria, terminal restriction fragment length polymorphism analysis (T-RFLP) of the nitrous oxide reductase (*nosZ*) gene was utilized (23). The primers used in the *nosZ* amplifi- cation were 752F (ACC GAY GGS ACC TAY GAY GG) and 1773R (ATR TCG ATC ARC TGB TCG TT), using a thermocycling program of 95°C for 5 min and then 35 cycles of 95°C for 30 seconds, 55°C for 30 seconds, and 72°C for 90 seconds, with a final extension at 72°C for 10 min. The forward primer was labeled with 6-carboxyfluorescien (6-FAM; Applied Biosystems) on the 5_ end. The amplicon provided by *nosZ* PCR amplification was then run on a 1% agarose gel and quantified. Fifteen nanograms of the PCR product was digested with MnlI endonuclease (New England Biolab, Beverly, MA). All digests were in 20-μl volumes for 6 h at 37°C. Precipitation of digested DNA was performed by adding 2 μl of 0.75 M sodium acetate solution and 0.3 μl glycogen (20 mg/ml) to the enzyme digest and precipitating with 37 μl of 95% ethanol. The precipitated DNA was washed with 70% ethanol and dried briefly. The dried DNA pellets were resuspended in 19.7 μl deionized formamide and 0.3 μl ROX 500 size standard (Applied Biosystems) for 15 min before analysis. T-RFLP fingerprinting was carried out on an ABI 310 genetic analyzer (Applied Biosystems, Foster City, CA) using Genescan software.

2.3 RESULTS

To test whether contamination between ^{12}C- and ^{13}C-DNA bands in a CsCl gradient could be observed, an experiment was designed using a benzoate-degrading bacterial strain, *Thauera aromatica* strain T1, that was either ^{12}C or partially ^{13}C labeled by growth on [^{12}C]benzoate or [^{13}C]benzoate after growth on [^{12}C]benzoate, as described above. The different T1 DNAs were mixed with ^{13}C-labeled "carrier" archaeal DNA and separated by ultracentrifugation, as described above. The ^{12}C- and ^{13}C-DNA bands were isolated, and the presence of *Thauera* DNA was tested by amplifying the 16S rRNA gene from each band using bacterium-specific primers (Fig. 1). Successful bacterium- specific amplification was observed in the ^{12}C band when either ^{12}C- or partially ^{13}C-labeled *Thauera* DNA was added to the gradients (lanes E and B). Likewise, a bacterial 16S rRNA gene product was detected in the ^{13}C band when partially ^{13}C-labeled *Thauera* DNA was added to the gradient (lane C). There was no bacterium-specific amplification product in the ^{13}C-DNA band when the *Thauera* DNA was labeled only with ^{12}C (lane F), suggesting that any ^{12}C contamination in the carrier DNA was below the PCR detection limit.

In addition to testing ^{13}C-DNA band contamination under pure culture conditions, control incubations using [^{12}C] benzoate amendments were performed on LEO-15 and Norfolk Harbor sediments on days 5 and 21 of the incubation. These ^{12}C-labeled samples were incubated and processed as the [^{13}C] benzoate-amended samples and *nosZ* amplifications were performed on the ^{13}C bands isolated from the cesium chloride gradients to identify the denitrifying bacteria (Fig. 2). No *nosZ* amplification could be

seen in the ^{13}C-DNA bands that were supplemented with [^{12}C] benzoate in the day 5 or 21 incubations from the LEO-15 or Norfolk Harbor sites (lanes C to F). However, when the environmental slurry was amended with [^{13}C] benzoate, a *nosZ* PCR product was detected in the ^{13}C labeled "carrier" band (lane G). This result illustrates that any bacterial ^{12}C-DNA from these environmental samples that may contaminate the ^{13}C band is below the *nosZ* PCR detection limit.

To assess whether short incubations and site-specific differences in benzoate denitrifying populations could be discerned using SIP, a time course of ^{13}C-labeled enrichment samples was characterized by T-RFLP analysis. The results are shown in Fig. 3. Both the non-impacted and the contaminated sites show distinct peaks at day 0 (60 min after substrate addition). The contaminated site, however, demonstrated a greater peak area at the 1-hour time point than the non-impacted site, implying that the bacterial community at Norfolk Harbor was primed for the anaerobic degradation of aromatic compounds from the chronic exposure of petroleum hydrocarbons. These results establish that ^{13}C-labeled DNA can be generated within short incubation times (hours) and that small amounts of ^{13}C-labeled DNA can be readily isolated with the use of carrier DNA.

For each sediment site, an increase in both peak number and peak area was observed during the first 2 weeks of the incubation (Fig. 4A). Both the LEO-15 and the Norfolk Harbor samples exhibited a five- to sixfold increase in the number of peaks by day 14. After the 2-week period, the community diversity of ^{13}C-labeled denitrifiers began to decrease. This increase in T-RFLP peaks during the SIP incubation implies the passing of ^{13}C-labeled metabolic intermediates or cellular constituents from the initial denitrifying degrader to another segment of the denitrifying community. (The use of *nosZ*

genes precludes the detection of non-denitrifying members of the bacterial community involved in benzoate degradation.) In addition to changes in peak number, the relative area of the different peaks changed over the course of the incubation (Fig. 4B). For the Norfolk Harbor sample, the terminal restriction fragment (TRF) at 122 base pairs showed an initially high peak area within an hour after [^{13}C] benzoate amendment that decreased during the course of the incubation. In the LEO-15 amendment, the TRF at 349 base pairs showed a similar pattern, with a slight lag in the 1-hour time point. This rapid incorporation of ^{13}C from benzoate suggests that these particular denitrifying microorganisms are the primary utilizers of benzoate in the enrichments. Conversely, the TRF at 427 base pairs from the Norfolk Harbor and the TRF at 211 base pairs from LEO-15 were not initially detected at the 1-hour or day 5 time points. However by the day 21 incubation, the peak area of these TRFs dominated the fingerprints, implying that these denitrifying bacteria are secondary utilizers of the carbon from benzoate. At both sites, the total area of TRFs from the primary utilizers decreased slowly over time, and the total area of the secondary utilizers increased over time.

2.4 DISCUSSION

In this report, we describe the metabolism of [^{13}C] benzoate under denitrifying conditions, using inocula from both a non-impacted and contaminated coastal sediment site. While the benzoate concentration present in the microcosms was not directly measured, the incorporation of the ^{13}C label into DNA demonstrated the metabolism of [^{13}C] benzoate during the incubation. Previous studies have shown that bacteria grown with nitrate as a terminal electron acceptor under anoxic conditions in the laboratory are

able to metabolize approximately 200 mg chemical oxygen demand per liter of benzoate in 48 to 50 h in the laboratory (1, 2). This benzoate degradation in hours in laboratory cultures is consistent with the incorporation of ^{13}C label into bacterial DNA in environmental enrichments within 60 min using carrier DNA. The addition of ^{13}C-labeled archaeal carrier DNA ensures that small amounts of ^{13}C-labeled bacterial DNA in the enrichment (below the visible-level detection limit) can still be removed from the gradient and amplified. Clearly, the use of carrier DNA greatly shortens the time necessary for performing a SIP study. While archaeal DNA was used in this study, any DNA in principle could be used as a carrier for SIP. The only requirements of the carrier DNA are that it does not contain the functional gene of interest (e.g., the *nosZ* gene in this study), or the target gene can be differentiated from the carrier gene (e.g., by the use of group PCR primers), and that the DNA is uniformly labeled with the stable isotope. For example, *Halobacterium* genomic DNA can be an ideal choice of carrier DNA in SIP studies looking at either bacterial or eukaryotic communities using rRNA target genes, since kingdom-specific PCR primers can prevent the amplification of the carrier ribosomal genes and select for the target community.

This ability to perform short-term SIP experiments is important for eliminating bottle effects and experimental artifacts. Although there are SIP experiments with incubations shorter than 20 days, there were no studies of DNA-based SIP utilizing PCR amplification and incubation times of 1 h prior to this report. One possible explanation for the detection of bacterial DNA in the archaeal carrier is contamination. For example, it has been reported that the buoyant density of DNA is affected by G+C content (22), and it is conceivable that ^{12}C-DNA with high G+C content may co-migrate with ^{13}C-

labeled DNA. However, in this study, there was no evidence of contamination in the ^{13}C-DNA band in both pure culture and environmental samples amended with the ^{12}C-labeled substrate, suggesting that the variable G+C content in natural samples will not impede environmental SIP studies using carrier DNA.

Furthermore, the use of a time course incubation study and the ability to use molecular techniques on the ^{13}C-labeled DNA have allowed for the detection of ^{13}C label incorporation into the DNA of what is possibly the initial benzoate degrader in the community. Previously, the use of long incubation times made the identity of the first microorganism to incorporate ^{13}C into its DNA difficult to distinguish because of the possibility of cross-feeding. While cross-feeding has been extensively mentioned as a shortcoming of the utilization of the SIP technique, it has never been illustrated (12, 21, 22, 24, 30). The use of a time course study with carrier DNA and short incubation times has allowed for the identification of cross-feeding microorganisms at both the Norfolk Harbor site and the LEO-15 site. In both cases, a small number of denitrifying bacteria with the ^{13}C label were detected first. The number of ^{13}C-labeled bacteria then increased over time. This finding implies that there are only a few organisms responsible for the degradation of an environmental pollutant (in this case benzoate), and there is a broader population able to utilize the ^{13}C-labeled molecules after the degradation.

Finally, the loss of ^{13}C-labeled T-RFLP peaks during the incubation suggests that the microorganisms bearing ^{13}C-DNA are being consumed by bactivorous protozoa in the enrichments, accelerating the remineralization of the benzoate during the incubation. Another possibility is that the microorganisms could be utilizing other carbon sources present in the sediment, causing a dilution of the ^{13}C label and lowering the number of

peaks seen from the T-RFLP fingerprint. Alternatively, the shifting of T-RFLP peaks could result from DNA turnover and the release of $^{13}CO_2$ during the extended incubation for many of the microorganisms. The increase and the shifting of T-RFLP peaks that are detectable in this anaerobic benzoate degradation experiment from day 0 to day 21 emphasize the need to perform a time course study to ascertain the true benzoate degraders active among the bacteria present in an environmental sample when using stable-isotope probing, as opposed to those gaining the ^{13}C label from cross-feeding. Clearly, the sample complexity within the enrichment can change over time, and the members of the community that are using the ^{13}C-labeled benzoate can also change. Furthermore, the microorganisms responsible for the initial ring cleavage of benzoate under anaerobic conditions can now be discerned by searching for the T-RFLP peaks at the earliest possible time point. In conclusion, the use of carrier DNA in SIP experiments can reduce incubation times and allow for a rapid assessment of the organisms present in a complex environmental sample that are able to utilize a labeled substrate. The incorporation of labeled benzoate into the DNA of organisms at both Norfolk Harbor and LEO-15 could be an important marker in the degradation abilities of the organisms present at these two sites. Since benzoate is a common intermediate in aromatic pollutant degradation (4, 31), the immediate incorporation of benzoate into the DNA of organisms at these sites could prove important in further degradation studies.

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2.6 FIGURES

FIG. 1. Agarose gel showing bacterial 16S rRNA gene amplification using pure-culture *Thauera aromatica* strain T1 that was either ^{12}C or ^{13}C labeled in combination with ^{13}C-labeled "carrier" archaeal DNA. Lanes: A) positive control, B) ^{13}C-fed ^{12}C-*T. aromatica* DNA band, C) ^{13}C-fed partially ^{13}C-*T. aromatica* DNA band, D) blank, E) ^{12}C-fed 12C-*T. aromatica* DNA band, F) ^{12}C-fed partially ^{13}C-*T. aromatica* DNA band.

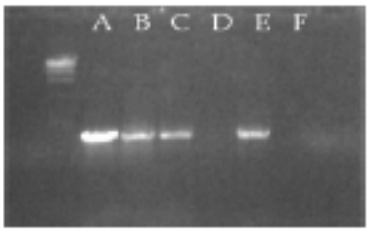


FIG. 2. Agarose gel showing *nosZ* gene amplification using carrier DNA in an environmental sample. Lanes: A) _ standard (125 ng), B) positive control, C) ^{13}C-DNA band fed [^{12}C]benzoate from LEO-15 incubation day 5, D) ^{13}C-DNA band fed [^{12}C]benzoate from LEO-15 incubation day 21, E) ^{13}C-DNA band fed [^{12}C]benzoate from Norfolk Harbor incubation day 5, F) ^{13}C-DNA band fed [^{12}C]benzoate from Norfolk Harbor incubation day 21, G) ^{13}C-DNA band fed [^{13}C]benzoate from Norfolk Harbor incubation day 5.

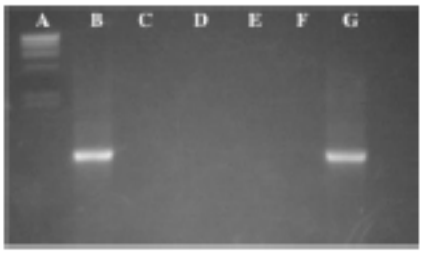


FIG. 3. Electropherogram illustrating different terminal restriction fragments (TRFs) over the time course from both environmental sites, Norfolk Harbor (A) and LEO-15 (B).

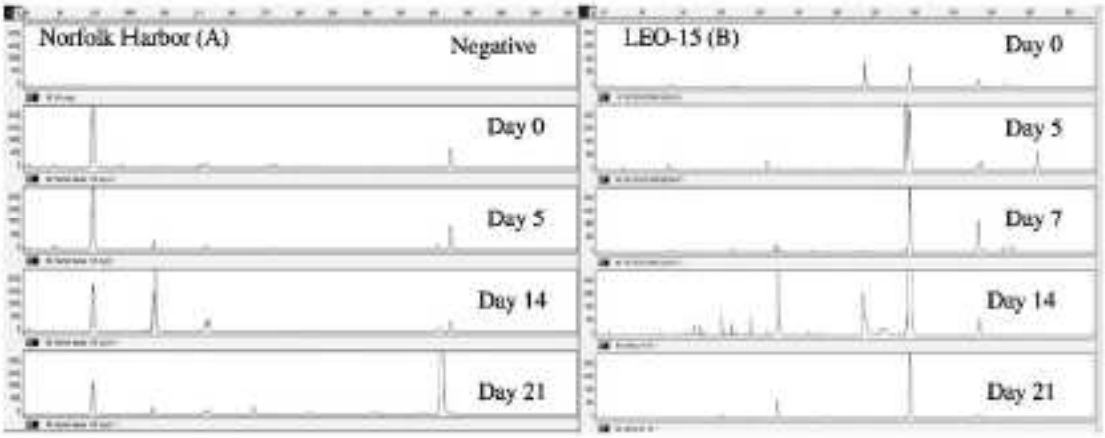
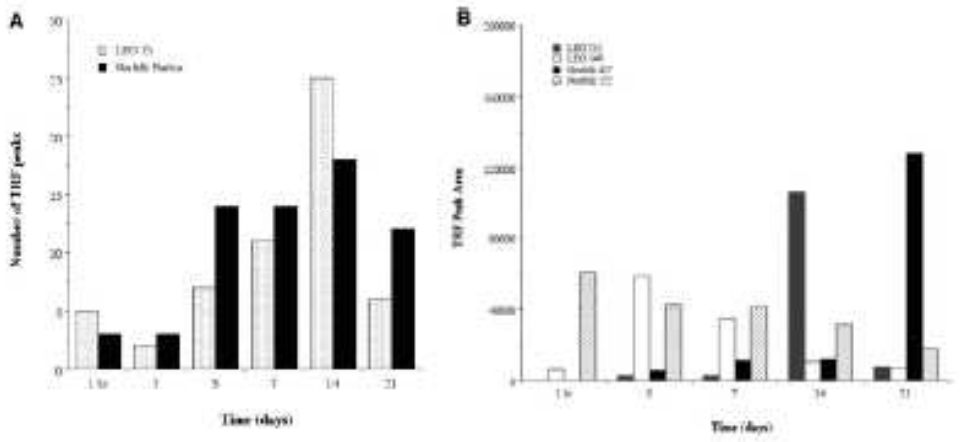


FIG. 4. (A) Change in peak numbers seen in the T-RFLP data during the time course of the SIP experiment. Data derived from the T-RFLP fingerprints are shown in Fig. 3. (B) The peak area of select TRFs was traced over time; for Norfolk Harbor, the TRFs were at 122 base pairs and at 427 base pairs, and for LEO-15, the TRFs at 349 base pairs and at 211 base pairs were presented. Data derived from the TRFLP fingerprints are shown in Fig. 3.



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CHAPTER 3

Detecting 2,4,6-Trinitotoluene Utilizing bacteria in Norfolk Harbor Sediments by ^{15}N and ^{13}C Stable Isotope Probing

ABSTRACT:

2, 4, 6- trinitrotoluene (TNT) is a major contaminant in a variety of Dept. of Defense sites and it is unclear whether bacteria can use this explosive for *in situ* growth. In order to determine if microbes in sulfidogenic sediments are capable of anaerobic TNT degradation, both uniformly labeled ^{15}N-TNT or ^{13}C-TNT were added separately to enrichment cultures from Norfolk Harbor, Virginia, USA. The sulfidogenic enrichment cultures were incubated for 2-28 (^{15}N) or for 35 days (^{13}C) and assayed by stable isotope probing to assess if ^{15}N or ^{13}C were incorporated into microbial genomic DNA using 16S rRNA gene analysis coupled to T-RFLP profiling. Three major peaks, measured in base pairs (bp), were detected in the ^{15}N-TNT cultures (60, 163, and 168 bp). One of these major peaks (60 bp) was also found in the ^{13}C-TNT cultures. A clone library of 16S rRNA genes indicated the 60 bp peak was distantly related to *Lysobacter taiwanensis* (93% identity over 400 bp). This study demonstrates that certain anaerobic bacteria are able to use TNT as both a nitrogen and carbon source for growth. The results also confirm that stable isotope probing, with either ^{15}N or ^{13}C labeled explosives, can ascertain the active members responsible for biodegradation from environmental samples.

3.1 INTRODUCTION:

The Department of Defense (DOD) has used millions of pounds of powerful chemicals and solvents and an equal amount of rounds of ammunition, missiles, and pyrotechnics on training ranges at its 1,700 installations over the years. The result of these military operations has been the inadvertent contamination of soil, sediments, and groundwater with unexploded ordinates (UXO's) at DOD facilities. It has been estimated that there are over 1 million cubic yards of material contaminated with 2,4,6-Trinitrotoluene (TNT) in the United States at concentrations as high as 600,000- 700,000 mg/kg (9). Large tracts of marine and estuarine sediments have also been impacted through the manufacturing, use, and/ or disposal of TNT. A promising remediation method would be *in situ* microbial biodegradation of these pollutants due to the large volume of contaminated soils/sediments. Questions remain, however, whether bacteria in an environmental setting can utilize TNT as a nitrogen or carbon source. Under aerobic conditions TNT, though partially metabolized appears to be largely unavailable to bacteria. On the other hand, TNT can be used by a variety of fungi as a carbon and nitrogen source in the presence of molecular oxygen (7). Under anaerobic conditions, specific strains of bacteria (*Clostridia*, *Desulfovibrio*, and *Pseudomonas* sp JLR11) have been reported to utilize TNT as a sole nitrogen source (6, 7). It is widely believed that nitroaromatic compounds cannot serve as growth substrates under anaerobic conditions *in situ* (10) and co-amendment strategies are suggested for stimulating TNT transformation to 2,4,6-triaminotoluene (TAT) (1). In addition to this biotic degradation, TNT can also be reduced abiotically to TAT under both oxic and anoxic environmental conditions. TAT is highly surface reactive, will polymerize with a variety of organic materials, and is

believed to biologically unavailable, giving the false impression of bioremediation when TNT concentrations diminish **(**for review, see Smets et al, 2007**)**. Given these difficulties, there is no direct evidence that TNT can be biodegraded *in situ* and there is little proof that anaerobic bacteria can utilize TNT as a sole carbon or nitrogen source in organic rich sediments.

The objective of this study was to determine if bacteria in Norfolk Harbor sediment are able to incorporate nitrogen (N) or carbon (C) from TNT into biomass. Sediment samples were amended with uniformly labeled ^{15}N-TNT and ^{13}C-TNT under sulfate reducing conditions to discern whether the microbial population could incorporate ^{15}N or ^{13}C labels into newly synthesized DNA (4, 5, 8, 15). The results indicate that a few microorganisms incorporate nitrogen and carbon into their genomes during short-term incubations (2 day). In contrast, numerous bacteria can incorporate the heavy labels during the 28-day incubation in the case of the ^{15}N-SIP and 35-day incubation in the case of the ^{13}C-SIP. Interestingly, one particular TRFLP peak (60 bp) was observed in both the ^{15}N and the ^{13}C incubations. A clone library was established from the ^{15}N-7 day incubation sample and screened for various TRFLP peaks of interest. An SSU gene from the 60 bp peak was identified, though attempts to identify the other major TRFLPs from the library were not successful in screening over 200 colonies. The SSU sequence from the 60 bp TRFLP peak indicated it is a gamma Proteobacteria related to *Lysobacter* sp. This finding clearly demonstrates that *anaerobic* bacteria can utilize the nitrogen and carbon in TNT for cell division and growth in the presence of organic rich sediments. Furthermore, this study illustrates the utility of activity measurements, like stable isotope

labeling, to discover novel microbial taxa with metabolic functions previously not described.

3.2 MATERIALS AND METHODS:

Experimental Design-

An inoculum of sediment from Norfolk Harbor, Virginia, USA, was added to a minimal salts media (10% sediment slurries), containing no additional nitrogen, with sulfate as the electron acceptor and TNT (100 μM) as the electron donor (adapted from Phelps and Young, 1999). The cultures were kept anaerobic in crimp sealed serum bottles with a 70%-30% mixture of N_2- CO_2 gases. There were three different treatments for this experiment: (1) inoculum in media with $^{14}N^{13}C$-TNT added, (2) inoculum in media with ^{15}N-TNT only added, and (3) inoculum in media with ^{13}C-TNT only added. The live cultures were incubated in triplicate, while the abiotic controls (autoclaved inoculum, media, electron donor, and electron acceptor) were incubated in duplicate for each treatment. The cultures were destructively sampled aseptically and anaerobically (1ml/ replicate) at 0, 2, 7, 14, 21, and 28 days for the ^{15}N-TNT amended cultures. The ^{13}C-TNT amended cultures were sampled at Day 35. The sample was centrifuged (Beckman, Palo Alto, California) for 5 minutes at 16,000 rpm, the liquid supernatant was discarded, and the solid pellet was frozen at –20˚C for molecular analysis.

DNA Extraction and Centrifugation-

The DNA was extracted in triplicate from the frozen sediment using a modified phenol-chlorform extraction procedure (13, 17). The DNA from the replicate samples

was pooled in 100 µL of sterile deionized water and carrier DNA was added. The carrier DNA was obtained by growing *Halobacterium salinarium* on uniformly labeled ^{13}C or ^{15}N-ISOGRO powder growth medium (Isotec, Miamisburg, OH) for approximately 20 days before the cells were harvested and the DNA extracted (8). For each 500 µl CsCl density gradient (1 g/ml), 300 ng of environmental sample DNA and 300 ng of heavy carrier DNA were added with 200 µg ethidium bromide. The gradients were established using a Beckman Optima ultracentrifuge with a TLA 120 rotor **(**Palo Alto, CA**)** at 225,000 x g for 24 hours. After 24 hours, the light and heavy DNA bands were visualized using ultra-violet light and sampled from the gradient as described in Gallagher et al, 2005. After band extraction, the genomic DNA was dialyzed for 45 min using 0.025 µm Millipore mixed cellulose ester dialysis filter (Bedford, MA) floating in a Petri dish containing 10 mM Tris (pH 8.2).

^{14}N and ^{12}C Controls

Control incubations (^{14}N or ^{12}C-TNT) were also established to ensure the SIP molecular results were not from un-labeled DNA appearing in the ^{15}N-carrier DNA band (8). At every time point, the DNA from the isotopically light TNT sample was run in a gradient with ^{15}N *H. salinarium* carrier DNA or ^{13}C *H. salinarium* carrier DNA. The heavy DNA band was collected and PCR was used to determine if there was measurable contamination from unlabeled DNA. The contamination control samples were processed in the same manner as the SIP study samples for terminal restriction fragment length polymorphism (T-RFLP) analysis for further assurance of contamination being below the limits of detection. These steps allowed for a verification of the active population capable

of utilizing TNT as a nitrogen source after 2 days of incubation and demonstrated there was no detectable contamination present in both the nitrogen and carbon SIP density gradients.

SSU Gene Analysis

The 16S rRNA gene was amplified using the 27F (AGAGTTTGATCMTGGCTCAG) and 1100R (GGGTTGCGCTCGTTG) primers, to determine the identity of bacteria able to assimilate the ^{13}C or ^{15}N label. The amplification conditions were: 94˚C for 5 min, followed by 94˚C for 0.5 min, 57˚C for 0.5min, and 72˚C for 1 min (25-30 cycles), with a final extension step of 72˚C for 7 min in a DNA thermocycler (model 2400, Perkin-Elmer, Foster City, CA). The forward primer was labeled with 6-carboxyfluorescien (6-FAM; Applied Biosystems). The amplicon provided by SSU gene PCR amplification was then run on a 1% agarose gel and quantified (13, 17). Fifteen (15) ng of positively amplified PCR product was digested with *MnlI* endonuclease (New England Biolab, Beverly, MA). All digests were in 20 µl volumes for 6 h at 37^{o}C. Precipitation of digested DNA was performed by adding 2 µl of 0.75 M sodium acetate solution with 10µg of glycogen and precipitating with 37 µl of 95% ethanol. The precipitated DNA was washed with 70% ethanol and dried briefly. The dried DNA pellets were re-suspended in 19.7 µl de-ionized formamide and 0.3 µl ROX 500 size standard (Applied Biosystems) for 15 minutes before analysis. Terminal restriction fragment length polymorphism (T-RFLP) fingerprinting was carried out on an ABI 310 genetic analyzer (Applied Biosystems, Foster City, CA) using Genescan software and an internal size standard. A non- fluorescent labeled primer was

used for cloning reaction amplification. Cloning and sequencing was performed by cloning 16S PCR products using the TOPO TA Cloning Kit (Invitrogen, Carlsbad, California). For sequence analysis, plasmids were purified by using the FlexiPrep Kit (Pharmacia, Piscataway, N.J.). Over 200 colonies were screened to determine clones of interest by 16S rRNA PCR, digestion with *Mnl1*, and T-RFLP analysis, as described above.

3.3 RESULTS:

DNA is composed of a lower number of nitrogen atoms than carbon. For every AT base pair there are 7 N and 20 C atoms and for every GC base pair there are 8 N and 19 C atoms. It has been reported that heavy N bands will migrate differently in cesium gradients with respect to heavy C (4, 5). We observed that the distance between the light and heavy band was 2.8 mm in the N-SIP gradients and 5.5 mm in the C-SIP gradients in good agreement with the lower N content in the DNA. This distance of separation remained constant for both pure culture gradients (data not shown) and environmental sample gradients (Figure 1).

Samples were incubated with light isotopes of nitrogen and the heavy isotope carrier band was tested for amplification to ensure there were no detectable levels of contamination during the SIP analysis. Since the heavy isotope band should only contain archaeal DNA, it should not amplify using bacterial specific primers. Conversely, the light band should always amplify using the 16S rRNA bacterial gene primers, since all samples contain isotopically light DNA regardless of which substrate is used in the incubation. In all ^{14}N control incubations, no amplification occurred in the heavy band

samples (lane 4 for day 2 or 28; Figure 2). These results demonstrate that no detectable DNA is present in the heavy-carrier DNA band after centrifugation as in Gallagher et al. 2005.

T-RFLP profiles were performed on the bacterial SSU amplicons to determine which microorganisms were able to utilize ^{15}N-TNT. Day 2 was the first time point where there was detectable 16S rRNA PCR product from the ^{15}N-DNA band. The time course of various T-RFLP fingerprints is presented in Fig. 3. Eight peaks (terminal restriction fragments or TRFs) are present at Days 2 and 7. Three of these peaks have much greater peak area compared with the other TRFs (60, 165, and 168 bp). Only one TRF can be seen throughout the entire incubation period (60 bp). The appearance of this TRF from Day 2 to Day 28 implies that this bacterium is able to utilize the ^{15}N-labeled TNT as a nitrogen source throughout the course of the experiment. However, it does not necessarily prove whether the bacterium is the primary reducer of the N in the aromatic ring or if it utilizing a downstream metabolite from cross-feeding.

To test whether a similar group of microorganisms can also obtain carbon from TNT for growth, ^{13}C TNT was added to identical Norfolk sediment enrichments and incubated for 35 days. The TRFLP fingerprint can be seen in Figure 4. After 35 days, fingerprints from the Norfolk Harbor bacterial population that contained the ^{13}C-label contained a total of 38 TRFs. When the T-RFLP electropherograms from Day 7 in the ^{15}N-TNT study and Day 35 from the ^{13}C-TNT study are placed side-by-side, the TRF present at 60 base pairs is discernable in both cultures. To gain a better understanding of the bacteria involved in N and C uptake from TNT, a clone library in *E. coli* with over 200 clones was screened to identify recombinant PCR product that matched the TRFs of

interest present from Day 2 and day 7. The 16S rRNA gene corresponding to the 60 bp TRF was identified in the clone library and sequenced. The SSU gene for the 60 bp TRF clone was related to *Lysobacter* and *Dokdonella* (see figure 5). The closest matches were to environmental clones obtained from a constructed wetland treating dichlorethenes in Germany (Accession # FM205963), a floating microbial mat above sulfidic waters in Movile Cave, Romania (EU662609), and from a deep well used for injection of radioactive waste in Siberia (AJ534675). All matches were >99% indicating a wide geographic distribution of this microorganism.

While there are prior reports of great difficulty in achieving distinct ^{15}N fractions with respect to genomic DNA GC content in cesium gradients (4, 5), our methods allow good recovery of uncontaminated DNA. One possible reason why this carrier SIP approach appears to be successful in generating uncontaminated heavy isotope bands is the practice of DNA quantification before addition to the gradient and the use of ethidium bromide for visualization. It has long been known that intercalating reagents can influence the density and the topology of DNA and they can have a profound influence on the apparent buoyant density in cesium gradients. Figure 6 illustrates replicate gradients containing the same mass of ^{12}C DNA that have been amended with increasing concentrations of ethidium bromide. As the visual evidence shows, the DNA can band in very different parts of the gradient depending on the DNA:ethidium bromide ratio. The prior reports of GC interference for ^{15}N-SIP experiments may result from the gradient being established in the absence of intercalating reagents. The addition of ethidium bromide appears to overwhelm the influence of GC content on buoyant density (mass and volume) by contributing to the DNA mass as well as changing the DNA volume

(topology). This finding is in agreement with the report by Buckley et al., 2007 where the intercalating reagent, bis benzimide, is used in the final purification step for preparing ^{15}N.

3.4 DISCUSSION:

TNT has been widely believed to be recalcitrant and not mineralized by bacteria in environmental samples due to its rapid transformation into amino derivatives and immobilization to soil particles (10). For example, a study of ^{15}N and ^{14}C labeled TNT in soil incubations lasting from 108 to 176 days found nearly a 70% conversion of TNT into the aminodinitrotoluene and diaminonitrotoluene metabolites (16-21%) with the bulk of the isotopic label associated with the non-extractable residue (50-30%) (21). In addition to the loss of label to the soil matrix, these researchers demonstrated the nitrogen and carbon from TNT underwent different fates during degradation in their bioreactors. In contrast, our report demonstrates both the nitrogen and the carbon from TNT can become incorporated into bacterial DNA under short incubation times, even in the presence of organic rich sediments containing a complex mixture of DON and DOC. Different TRFLP peaks were observed using ^{15}N or ^{13}C-TNT, supporting the concept of different bacteria using the heavy nitrogen and the heavy carbon in the incubations. Our data, however, also demonstrates a single bacterial signature that dominated the TRFLP profiles, incorporating both N and C into newly synthesized DNA. Overall, this incorporation of heavy label into DNA unequivocally demonstrates bacterial growth on TNT under sulfidogenic conditions in Norfolk Harbor sediments.

Our results are similar to a report of ^{15}N incorporation using the explosive, RDX, from samples collected at Picatinny Arsenal in New Jersey (16). Their study also utilized ^{14}N and ^{15}N substrates to test for incorporations into bacterial biomass. However, each microcosm was co-amended with cheese whey or yeast extract and initially provided with an aerobic headspace and allowed go anaerobic during a 25-day incubation. The results demonstrated difficulty in separating labeled from unlabeled DNA with respect to 16S rRNA genes. The researchers, however, were able to differentiate the fractions using a functional gene approach (cytochrome P450; *xplA* gene). Characterization of the SSU genes in the ^{15}N band from the Picatinny sample indicated α and γ Proteobacteria and *Actinobacteria*-like clones were present in the RDX enrichments. All sequences from the *xplA* library were related to *Rhodococcus* sp which were not detected in the SSU libraries. In contrast, our study using the archaeal carrier method did not yield amplifiable SSU genes in ^{15}N bands when using ^{14}N labeled substrate. The major TRFLP was related to *Lysobacter* sp. and the time frame necessary to detect signal was significantly reduced as has been described before (8).

Although this SIP approach shows incorporation of ^{15}N-label into DNA, the profiles also indicate loss of heavy label from the microcosm cultures over time. Initially, the ^{15}N-labeled microbial population increases in diversity and rRNA gene abundance represented by peak area from Day 2 to Day 14, as was seen previously for carbon incorporation from benzoate under denitrifying conditions in these sediments (8). However, the number of 15N-labeled TRF's then declines and nearly disappears completely by Day 28. This disappearance of the TRFs in the bacterial pool could result from (1) cross-feeding among the bacteria present that is diluting the label, or (2) the

labeled bacteria are being consumed by eukaryotic predators/viral lysis or (3) the ^{15}N label is being denitrified to N_2 gas and becoming biologically unavailable to the remainder of the bacterial population.

Finally, our finding of a *Lysobacter*-like bacteria that is capable of both nitrogen and carbon uptake from TNT into biomass is consistent with reports of phytoremediation of explosives (19, 20). *Lysobacter* species are found in soil, aquatic environments, and the rhizosphere. Furthermore, the group is known as a prodigious producer of enzymes or antibiotics. This may explain why the 60 bp peak is minor in the day 2 sample but then dominates the ^{15}N profiles after the *Lysobacter*-like population becomes established. It is conceivable the *Lysobacter*-like microorganism secretes an antibiotic and suppresses the other microorganisms under nitrogen starvation.

In conclusion, this data unequivocally demonstrates there are bacteria in estuarine sediments that are able to utilize TNT as a carbon and nitrogen source under anaerobic conditions. Furthermore, the stable isotope probing method in conjunction with heavy isotope carrier addition provides a rapid means of detecting those specific microorganisms growing in our microcosms. Using SIP in coordination with regular sampling and analysis of the metabolites present at sites contaminated with TNT, or other xenobiotics, could prove to be a cost effective and reliable method of site remediation. Alternatively, the SIP approach could be used in conjunction with compound specific C and N isotopic analysis to observe biological fractionation in situ (3). Further research will be necessary to develop methods for stimulating and monitoring the native bacterial populations capable of biodegradation.

3.5 ACKNOWLEDGEMENTS

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3.6 FIGURES:

Fig. 1 Cesium gradients demonstrating separation of light and heavy isotopes of carbon and nitrogen.

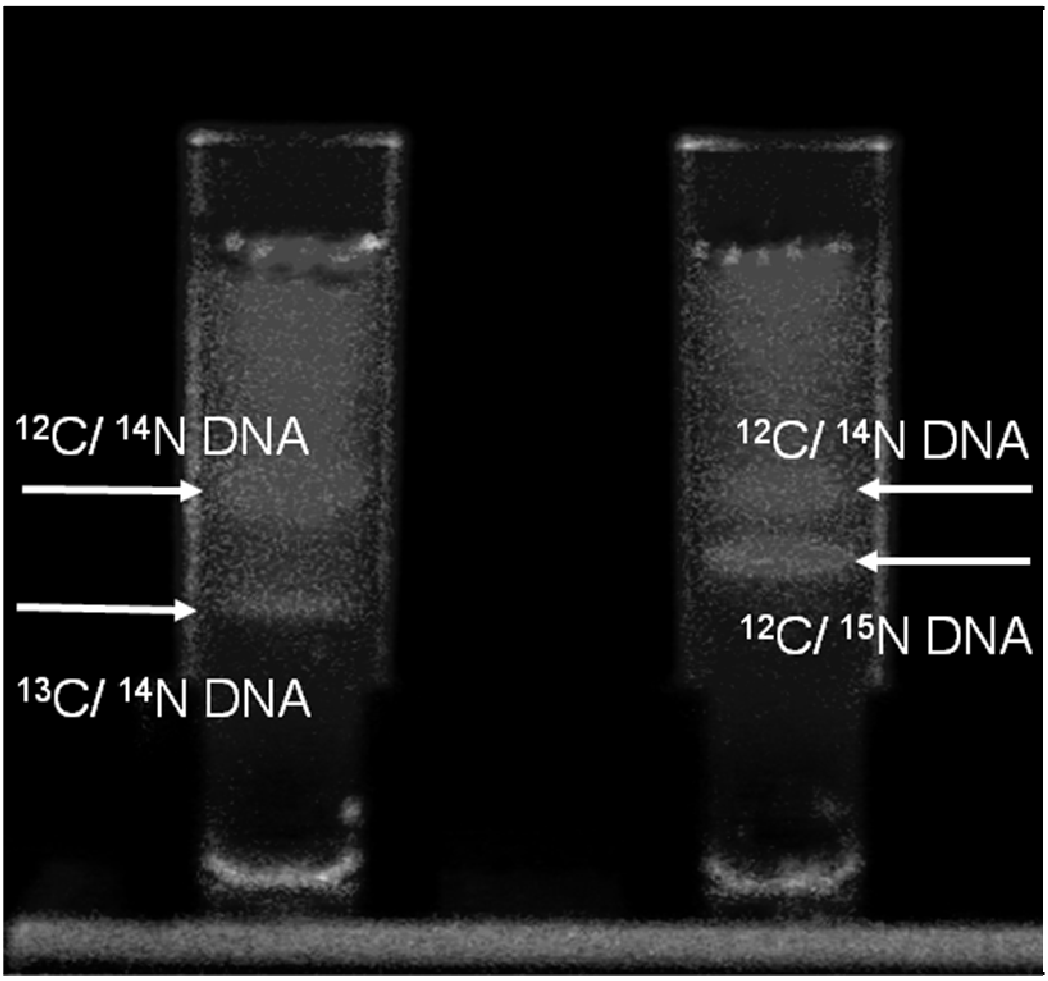


Fig. 2 λ: λ HindIII marker (125 ng/μL), Lane 1: 14N-DNA fed 15N-TNT with benzoate, Lane 2: 14N-DNA fed 15N-TNT with benzoate, Lane 3: 14N-DNA fed 14N-TNT with benzoate, Lane 4: 15N-DNA fed 14N-TNT with benzoate,, Lane 5: 14N-DNA fed 15N-TNT only, Lane 6: 15N-DNA fed 15N-TNT only

Illustrates no contamination in the 15N-DNA band from the 14N-DNA band when the enrichment was only fed 14N-TNT. The 14N-DNA does not get pulled down by the addition of 15N- *H. salinarium* carrier DNA to the CsCl gradient.

Fig. 3. Electropherograms showing the incorporation and subsequent loss of the ^{15}N-label from cultures fed ^{15}N-TNT only over time. The one terminal restriction fragment (TRF) that appears throughout the time course is at 60 base pairs.

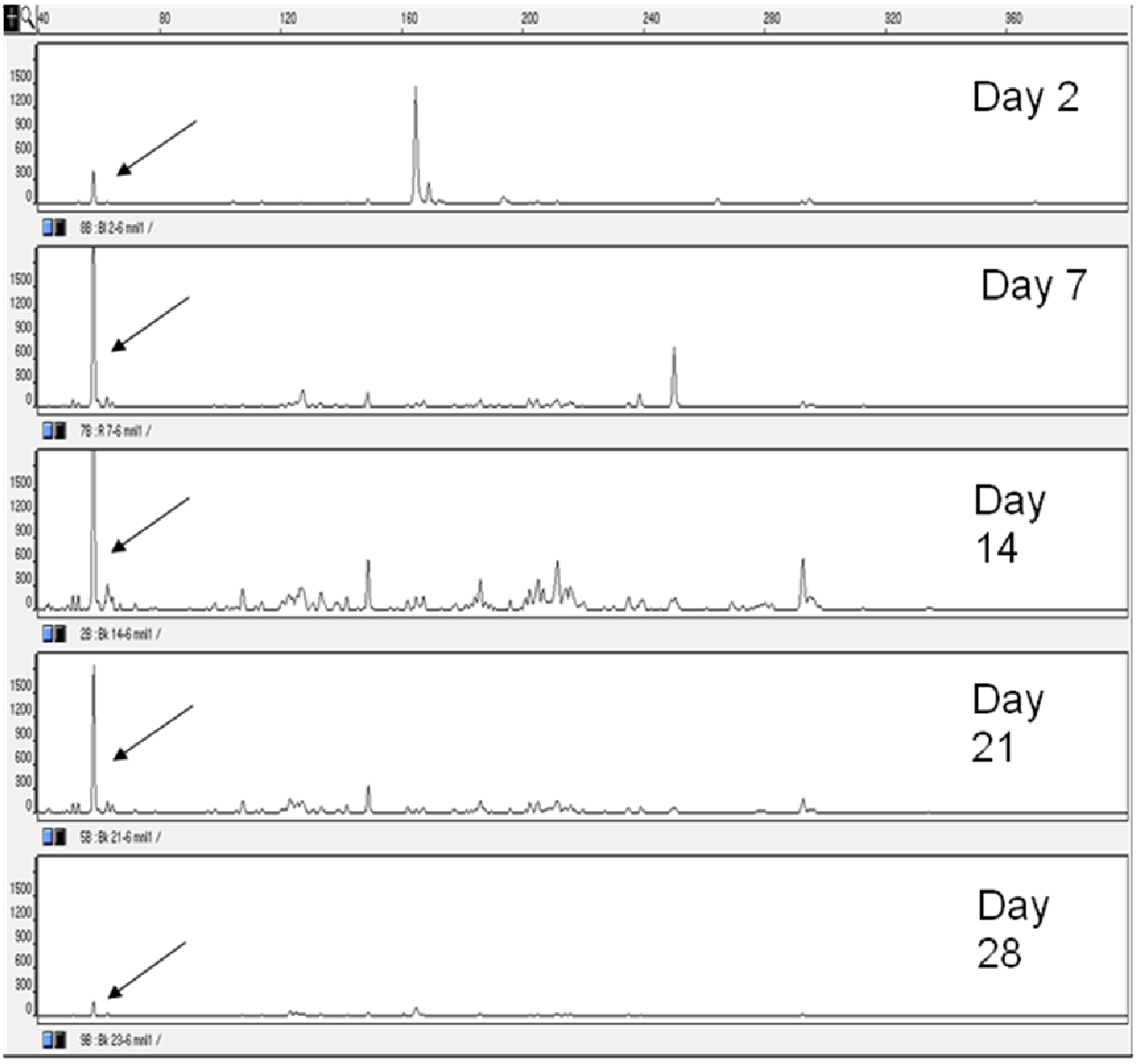


Fig. 4. Fig. 4 Eltectropherogram of ^{15}N and ^{13}C community in microcosms. The 60 bp TRFLP peak is indicated. This also suggests that the label is widely distributed in the bacterial community after 35 days.

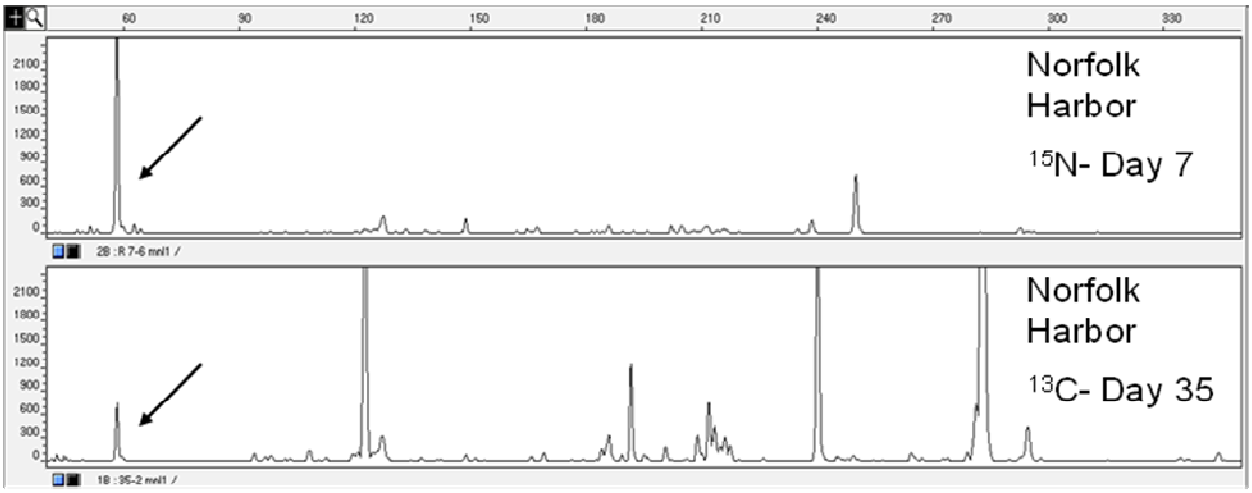


Fig. 5. Consensus maximum likelihood tree re-construction using known strains and closely related clones (based on 300 unambiguously aligned bp).

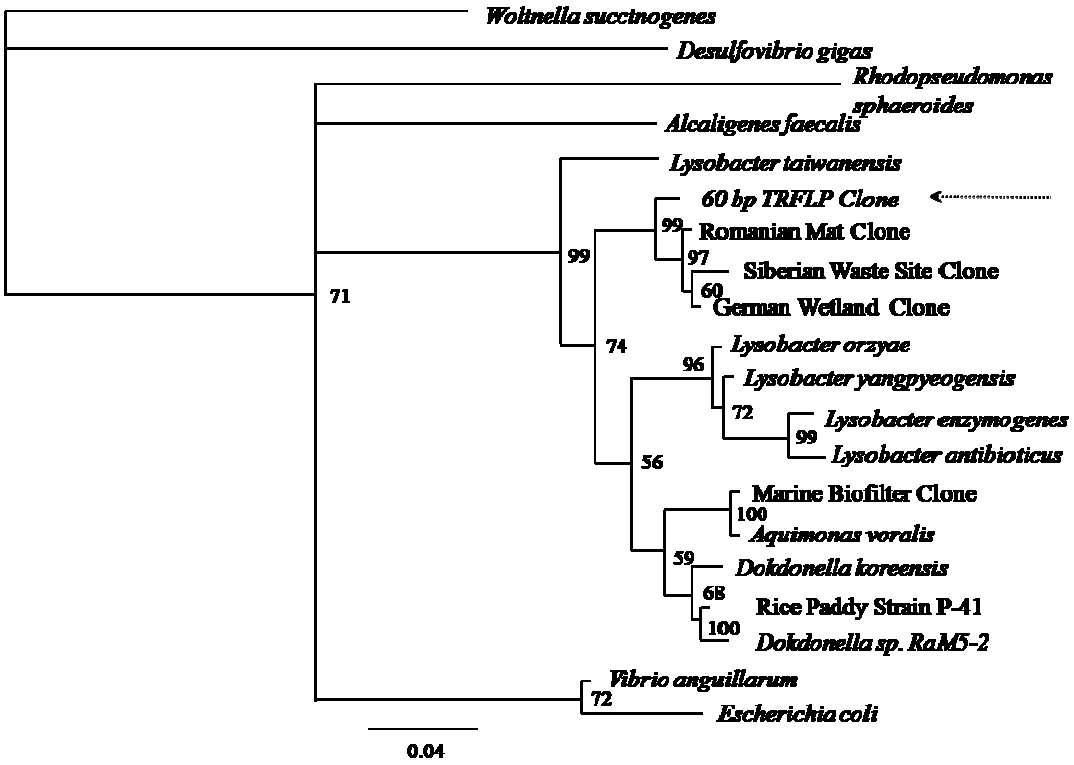
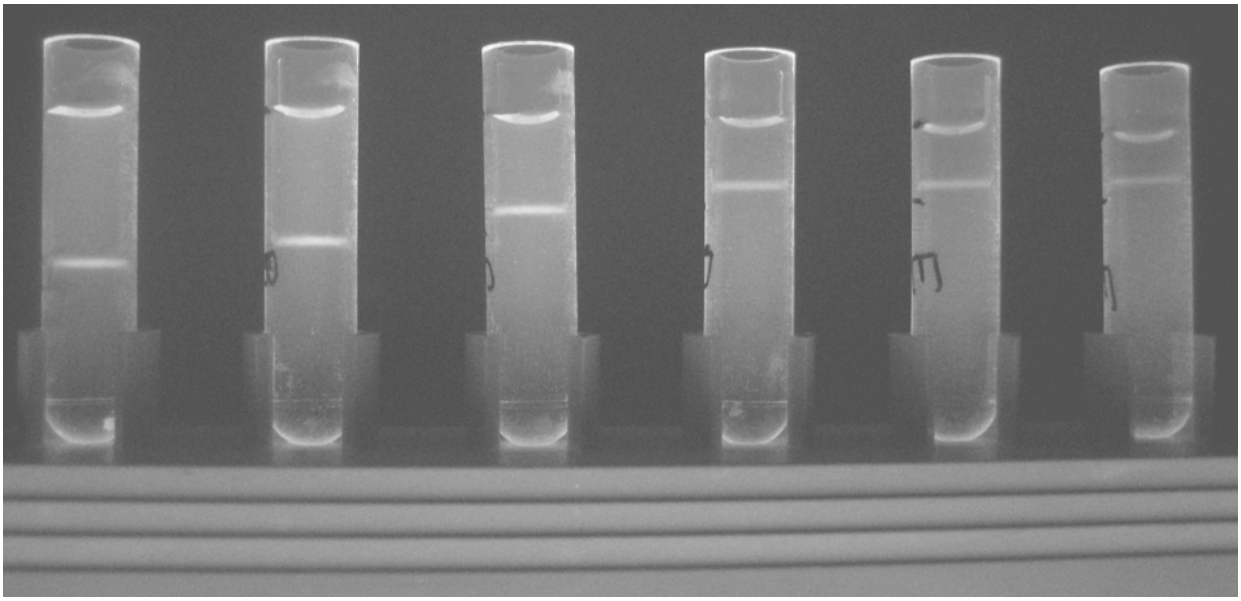


Fig. 6. Replicate CsCl gradients with the same DNA concentration and varying amounts of ethidium bromide. The higher the concentration of ethidium bromide that is added to the DNA CsCl gradients the lower the DNA band will fall in the gradient. This demonstrates that the addition of a DNA intercalator can change the buoyancy of DNA in a gradient and aid in clean separation for stable isotope probing. Bands are seen from highest concentration of ethidium bromide 8.0, 6.0, 4.0, 2.0, 1.0, 0.5 µls of a 1% ethidium bromide solution (concentrations match tubes from left to right).



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Chapter 4:

Identifying Bacteria Capable of ^{13}C-TNT Label Incorporation and Metabolites under Sulfate Reducing and Methanogenic Conditions

ABSTRACT:

TNT is a persistent environmental pollutant and under anaerobic conditions a degradation pathway has not yet been elucidated. The objective of this experiment was to identify the bacteria able of ^{13}C-TNT label incorporation and the metabolites of biotic TNT degradation under sulfate reducing and methanogenic conditions. Under both these conditions, at sites in the Arthur Kill (New York/New Jersey Harbor), Norfolk Harbor, and an unexploded ordnance (UXO) site in Hawaii, there were bacteria that were able to use TNT as a carbon source. The earliest utilizers were seen on Day 1 in Hawaii under sulfate reducing and methanogenic conditions, and by Day 3 from both Norfolk Harbor and Arthur Kill methanogenic and sulfate reducing conditions. The profiles of active bacteria varied slightly from site to site, with some peaks that represent bacteria being present universally in the cultures and others being unique to specific sites and/or conditions. The loss of TNT and the appearance of known metabolites were measured using a TNT specific HPLC methodology. Metabolites that were identified are toluene, methylphloroglucinol, a benzoate like compound, and a cresol. This set of experiments demonstrates that bacteria present under sulfate reducing and methanogenic conditions can incorporate the ^{13}C-label from uniformly labeled ^{13}C-TNT into their biomass when it is the sole exogenous carbon source, and identifies metabolites of TNT biological degradation.

4.1 INTRODUCTION:

2,4,6-Trinitrotoluene and other nitroaromatic compounds are known to be recalcitrant environmental pollutants. Unfortunately, large tracts of land and marine or estuarine sediments have been contaminated with nitroaromatics over the years (20). This contamination occurred through the manufacturing, use, and disposal of explosives in addition to other industrial uses for nitroaromatic compounds (14, 15, 20, 21, 38, 39). Rather than remediating these sites through removal and disposal, monitored natural attenuation (MNA) is a more cost-effective method, and less intrusive to the ecosystem in an impacted area. In order for MNA to be utilized more needs to be known about the identity of the organisms in the system that are able to degrade or utilize TNT and what metabolites are produced through this degradation. Currently, little is known about these organisms or the metabolic pathway of TNT degradation.

Using a combination of stable isotope probing (SIP) and labeled metabolite analysis in this experiment provided a clearer picture of the biological degradation possible under anoxic conditions. Distinguishing the active members of a community from those that are merely present is beneficial because it can describe the degradation capabilities present (8, 10, 18, 29, 30). Likewise, with the use of uniformly ^{13}C-labeled TNT, the metabolites of biotic TNT degradation can be identified with a greater degree of certainty. The biological pathway of TNT degradation under anaerobic conditions has not yet been determined and being able to distinguish between the biotic and abiotic metabolites will aid in elucidating the degradation pathway (15, 18, 30). By coupling

these experiments together we demonstrate that there is ^{13}C-TNT label incorporation concurrent with the appearance of ^{13}C-labeled TNT metabolites.

In previous stable isotope probing studies using both ^{13}C- and ^{15}N- labeled TNT it was demonstrated that there are anaerobic bacteria that can utilize the carbon and nitrogen present in TNT (Chapter 3). This study was designed to examine the diversity of anaerobic microorganisms capable of TNT utilization in the presence of two different terminal electron acceptors over a time course of 42 days. The purpose of looking at a variety of sites was not only to determine if there are differences in the population that are able to degrade TNT geographically, but also to determine if degradation can occur under different sediment conditions. If similar bacteria are present across various geographic sites, it would suggest that there are a wide variety of bacteria that are able to utilize TNT and the capability is more universal than previously thought. Additionally, varied sites and conditions could show differences in TNT metabolites with different sediment types and reducing conditions. One of the added benefits was monitoring the appearance and disappearance of the metabolites and bacteria able to incorporate the ^{13}C-label from TNT over time.

This set of experiments demonstrates that bacteria present under sulfate reducing and methanogenic conditions can incorporate the ^{13}C-label from uniformly labeled ^{13}C-TNT into their biomass when it is the sole exogenous carbon source, and identifies some metabolites of TNT biological degradation. There were a wide variety of bacteria present that were able to incorporate the ^{13}C-label from TNT, some were universal across all 3 sites analyzed and others were unique to the experimental sites. The metabolites that

were detected have been previously proposed though not proven and support a pathway for TNT mineralization under anaerobic conditions.

4.2 MATERIALS AND METHODS:

Experimental Design

Innocula of sediment from Arthur Kill in the NY/NJ Harbor, Norfolk Harbor, Virginia, USA, and an unexploded ordnance (UXO) site in the ocean off the coast of Hawaii were added to a minimal salts media (10% sediment slurries), with sulfate or carbon dioxide as the electron acceptor and TNT (100μM) as the electron donor (adapted from Phelps and Young, 1999). The cultures were kept anaerobic in crimp sealed serum bottles with a 70%-30% mixture of N_2- CO_2 gases.

There were a combination of 6 different conditions for this experiment; innocula from each of the 3 sediments mentioned above under both methanogenic and sulfate reducing conditions. The live cultures were incubated in triplicate, while the abiotic controls (autoclaved innocula, media, electron donor, and electron acceptor) were incubated in duplicate for each treatment. Over the course of the serial dilutions, the cultures were fed every 28-35 days. Samples were taken on Days 0, 7, 14, 21, and 28 to determine the level of TNT loss and metabolite production. When no TNT was left in the system it was amended with 100μM ^{12}C-TNT. This process took place for approximately 3 years prior to the stable-isotope probing experiment and allowed the cultures to be serially diluted until <1% sediment remained in the cultures. The serial dilution process eliminated bacteria present that were unable to utilize TNT and its intermediates as carbon sources. In order to ensure that the ^{12}C-TNT present in the cultures was fully utilized and would not dilute the heavy isotope label, the cultures were starved for 60

days prior to being amended with the uniformly labeled ^{13}C-TNT. The ^{13}C-TNT amended cultures were sampled at days 0, 1, 3, 7, 10, 14, 21, 28, 35, and 42. The sample was centrifuged (Beckman, Palo Alto, California) for 5 minutes at 16,000 rpm, the liquid supernatant was the liquid supernatant was placed in a acetone cleaned 20 ml Hungate tube and frozen prior to extraction, and the solid pellet was frozen at –20˚C for molecular analysis.

DNA Extraction and Centrifugation for SIP-

The DNA was extracted in triplicate from the frozen sediment using a modified phenol-chlorform extraction procedure (27, 32). The DNA from the replicate samples was pooled in 100 µL of sterile deionized water and carrier DNA was added. The carrier DNA was obtained by growing *Halobacterium salinarium* on uniformly labeled ^{13} C-ISOGRO powder growth medium (Isotec, Miamisburg, OH) for approximately 20 days before the cells were harvested and the DNA extracted (18). For each 500 µl CsCl density gradient (1 g/ml), 300 ng of environmental sample DNA, 300 ng of heavy carrier DNA, and 10 ng *E. coli* ^{12}C internal standard DNA were added with 200 µg ethidium bromide. The gradients were established using a Beckman Optima ultracentrifuge with a TLA 120 rotor (Palo Alto, CA) at 225,000 x g for 24 hours. After 24 hours, the light and heavy DNA bands were visualized using ultra-violet light and sampled from the gradient as described in Gallagher et al, 2005. After band extraction, the genomic DNA was dialyzed for 45 min using 0.025 µm Millipore mixed cellulose ester dialysis filter (Bedford, MA) floating in a Petri dish containing 10 mM Tris (pH 8.2).

^{12}C- Label and *E. coli* Quality Control Measures for SIP-

Control incubations (^{12}C-TNT) were also established to ensure the SIP molecular results were not from un-labeled DNA appearing in the ^{13}C-carrier DNA band. The contamination control samples were processed in the same manner as the SIP study samples for terminal restriction fragment length polymorphism (T-RFLP) analysis for further assurance of contamination being below the limits of detection (18). Additionally, the ^{12}C and ^{13}C band DNA was tested for the presence of internal standard DNA using the 16S *E. coli* specific primer set ECA 75F and ECR 619R. PCR was carried out using the method described by Sabat et al. The presence of *E. coli* DNA in ^{13}C bands served as an indicator of incomplete separation of labeled and unlabeled DNA (34). If no amplicon was seen on a 1% agarose gel stained with ethidium bromide, contamination was considered below limits of detection.

Terminal Restriction Fragment Length Polymorphism (T-RFLP) for SIP-

The 16S rRNA gene was amplified using the 27F (AGAGTTTGATCMTGGCTCAG) and 1100R (GGGTTGCGCTCGTTG) primers, to determine the identity of bacteria able to assimilate the ^{13}C label. The amplification conditions were: 94˚C for 5 min, followed by 94˚C for 0.5 min, 57˚C for 0.5 min, and 72˚C for 1 min (25-30 cycles), with a final extension step of 72˚C for 7 min in a DNA thermocycler (model 2400, Perkin-Elmer, Foster City, CA). The forward primer was labeled with 6-carboxyfluorescien (6-FAM; Applied Biosystems). The amplicon provided by SSU gene PCR amplification was then run on a 1% agarose gel and quantified (27, 32). Fifteen (15) ng of positively amplified PCR product was digested with *MnlI* endonuclease (New England Biolab, Beverly, MA). All digests were in 20 µl volumes for 6 h at 37^{o}C. Precipitation of digested DNA was performed by adding 2 µl of

0.75 M sodium acetate solution with 10µg of glycogen and precipitating with 37 µl of 95% ethanol. The precipitated DNA was washed with 70% ethanol and dried briefly. The dried DNA pellets were re-suspended in 19.7 µl de-ionized formamide and 0.3 µl ROX 500 size standard (Applied Biosystems) for 15 minutes before analysis. Terminal restriction fragment length polymorphism (T-RFLP) fingerprinting was carried out on an ABI 310 genetic analyzer (Applied Biosystems, Foster City, CA) using Genescan software and an internal size standard.

Clonal Library and Sequencing for SIP-

A non- fluorescent labeled primer was used for cloning reaction amplification. Cloning and sequencing was performed by cloning 16S PCR products using the TOPO TA Cloning Kit (Invitrogen, Carlsbad, California). For sequence analysis, plasmids were purified by using the FlexiPrep Kit (Pharmacia, Piscataway, N.J.). Over 200 colonies were screened to determine clones of interest by 16S rRNA PCR, digestion with *Mnl1*, and T-RFLP analysis, as described above. Clones of interest were identified and sequenced using 16S rRNA amplicons were processed using a Qiagen PCR purification kit (Valencia, CA USA). Sequencing samples were run on an ABI 3100 Genetic Analyzer (Foster City, CA USA). Resulting sequences were analyzed using the Blastn algorithm (2).

HPLC Extraction and Analysis for Metabolites-

Samples from the enrichment cultures were collected using anoxic and aseptic handling techniques. These samples were then spun in 1.5 ml Eppendorf tubes for 5 minutes at 16,00 rpm in table top centrifuge (Beckman, Palo Alto, California). After spinning the samples the supernatant was removed and added to 2 ml glass HPLC vials

with silicone septum in the caps. Samples were run on a Beckman HPLC with photo diode array. The HPLC was run with a solvent mix of phosphate-buffered water (pH 3.2), Solvent A, and methanol, Solvent B, with a flow rate of 1 ml/min. The gradient used was a 32 minute run starting at 80% A: 20% B, changing at 10% intervals every 2 minutes, to 80% B: 20% A. There was a hold at the starting conditions for 15 minutes at the end of each run. The PDA was used in conjunction with the HPLC to determine the UV spectrum of each biotic metabolite present from 260 to 420nm. Standards of TNT, 2,4,6-Triaminotoluene (TAT), toluene, *p*-cresol, *m*-cresol, *o*-cresol, benzoate and phenol were run to determine retention times of the possible and known metabolites.

LC/MS Extraction for Metabolites-

The LC/MS extraction procedure used a volume: volume liquid extraction with ethyl acetate as the organic solvent. Equal volumes of culture media and ethyl acetate were added to acetone washed glassware and the samples were shaken for 3 mins continuously with glass stoppers. The samples were then allowed to sit for 10 mins to separate. The organic layer was removed and set aside, an equal volume of ethyl acetate was added again, and the samples were shaken. The process was repeated for a total of 3 extractions. The samples were dried completely under a stream of argon gas in the fume hood. The samples were then resuspended in ~1 ml of acetonitrile for analysis on the LCMS. Samples were extracted at 14, 21, and 28 days for active samples and only at 28 days for the sterile controls.

LC/MS Analysis for Metabolites-

Metabolites are separated and analyzed with an LC/MS system including the Waters W616 pump and W600S controller, W717plus auto-sampler, and Waters W996

photodiode array (PDA) detector. UV data are collected and analyzed with the Waters Millennium® v. 3.2 software. After the 996 PDA detector the eluent flow is guided to a Varian 1200L (Varian Inc., Palo Alto, CA) triple quadrupole mass detector with electrospray ionization interface (ESI), operated in either positive, or negative ionization mode. The voltage is adjusted to -4.5 kV, heated capillary temperature was 280 ^{o}C, and sheath gas was air for the negative mode, and electrospray voltage is adjusted to 5 kV and sheath gas was nitrogen for the positive ionization mode; the mass detector was used in scanning mode from 65 to 1500 atomic mass units (amu). Data from the Varian 1200L mass detector was collected, compiled and analyzed using Varian's MS Workstation, v. 6.41, SP2. Substances were separated on a Phenomenex® Luna C-8 reverse phase column, size 250 x 4.6 mm, particle size 5 µm, pore size 100 Å, equipped with a Phenomenex® SecurityGuard™ pre-column. The mobile phase consisted of 2 components: Solvent A (0.5% ACS grade acetic acid in double distilled de-ionized water, pH 3-3.5), and Solvent B (100% Acetonitrile). The mobile phase flow was adjusted at 0.5 ml/min, and a gradient mode was used for all analyses. The initial conditions of the gradient were 85% A and 15% B; for 30 minutes the proportion reached 5% A and 95% B which was kept for the next 3 minutes; from minute 38 to minute 41 the gradient went back to initial conditions. A 10 minute equilibration interval was included between subsequent injections.

4.3 RESULTS:

Multiple bacteria were able to utilize TNT as a carbon source from all sites and conditions. Bacterial biomass containing the ^{13}C label appeared after 1 day of incubation

at the Hawaii UXO site and after 3 days of incubation in both the Norfolk Harbor and Arthur Kill sites, while the reducing conditions present did not seem to affect how quickly the bacteria were able to degrade the TNT. There were degraders present under both sulfate reducing and methanogenic conditions that were related are phylogenetically diverse. The metabolites produced from TNT degradation consisted of the compounds present in the proposed pathways, through dinitroaminotoluenes to diaminonitrotoluenes, and to TAT. Following TAT, there were multiple metabolites seen using both HPLC and LC/MS analysis, including toluene and methylphloroglucinol. The molecular data collected will be presented first, followed by the metabolic data.

SIP Results

To maintain quality control in all of the SIP samples an internal *E. coli* standard was run in all of the density separation gradients tested as illustrated in Singleton et al. The DNA samples for all 3 sites and both conditions in each site were not contaminated by ^{12}C-labeled DNA migrating to the ^{13}C-labeled DNA band. Based on no visible amplicon when using *E. coli* specific primers it can be assumed that any contamination that may have been present between the ^{12}C and ^{13}C bands in the CsCl separation gradient was below the limits of detection and will not affect the results.

There were a wide range of bacteria represented by the peaks with initial ^{13}C-label incorporation. The innoculum from Arthur Kill contained a total of 5 terminal restriction fragments (TRFs) that appeared after the 3 day incubation under methanogenic conditions and 3 TRFs that appeared under sulfate reducing conditions. Figure 1 shows the T-RFLP profiles for the bacteria that were able to incorporate the ^{13}C-label from TNT into their biomass after 3 days of incubation under methanogenic conditions and Figure 2

shows the profiles for incorporation under sulfate reducing conditions. The 58 bp peak that most closely associates with *Lysobacter taiwanese* (93%) is present under both sulfate reducing and methanogenic conditions suggesting that it is able to degrade TNT under both reducing conditions. Aside from the *Lysobacter taiwanese* there were no bacteria present that could incorporate the ^{13}C-label after 3 days that were present under both of the reducing conditions.

Additionally, Figures 1 and 2 show a time course that depicts the ^{13}C-label entering into a broader segment of the bacterial population represented by an increase in the total number of TRFs after Day 3. The label is lost or decreases in the community illustrated by the fewer incorporation peaks present at Day 42 under methanogenic conditions. The methanogenic cultures show a prevalent ^{13}C-label in Days 3 and 21, at 58, 120, 165, and 283 bps, but it begins to decrease by Day 28. When the time course reaches Day 42 there is only one peak with ^{13}C-label incorporation at 283 bps. Under sulfate reducing conditions, there were fewer incorporation peaks over time. On Day 3, there were peaks at both 58 bps and 115 bps. On Day 28 these were still present but in a different proportion, in addition to a peak at 248 bps. The label had completely disappeared by Day 42.

The initial degraders under methanogenic conditions using Norfolk Harbor sediment include, *Shewanella aquimarina* strain S5 (94%) most closely matched the 120 bp peak. This is also the first appearance of a peak related to *Thalassospira sp.* KMT005 know to associate with seagrass tissue, which matched 89% to the 165bp peak. The low relationship percentage means that the peak at 165 bps is not a bacteria previously entered into the GenBank database, however the closest known match is *Thalassospira*.

The peak related to *Thalassospira sp.* KMT005 also appears in the Norfolk Harbor inocculum under sulfate reducing conditions. Lastly, there was a TRF at 283 bps that most closely matched *Propionibacterium acnes* (97%). Under sulfate reducing conditions in the Norfolk Harbor innoculum the peaks representing the initial degraders included, a *Lysobacter taiwanese* peak appeared at 58bps and one related to *E. coli* sp. K12, matching at 95%, appeared at 115 bps. After screening over 200 clones the clone with a TRF of 182 was not found. Figure 3 shows the incorporation peaks from Norfolk Harbor sediments under methanogenic conditions and Figure 4 shows incorporation peaks from sulfate reducing conditions. The peak most closely related to *E. coli* sp. K12 was suspected to be a lab contaminant; however, this organism's DNA did not amplify with the *E. coli* primers used to test internal standard contamination.

Norfolk Harbor methanogenic cultures were able to incorporate the ^{13}C-label after 3 days of incubation. The predominant peaks were at 58, 240, and 283 bps. All of these peaks remained in the Day 21 sample with the addition of a peak at 165 bps. Under sulfate reducing conditions, Day 3 had the most diverse incorporation of the ^{13}C-label and it diminished from Days 28 to 42. The peak at 115 bps is the dominant peak that appears throughout the time course.

In the innoculum from the Hawaii UXO site there were a total 6 TRFs that appeared as the initial degraders after 1 day of incubation, 3 TRFs under methanogenic conditions and 3 TRFs under sulfate reducing conditions. Figure 5 shows the T-RFLP profiles that incorporated the ^{13}C-label from TNT into their biomass after only 1 day of incubation under methanogenic conditions and Figure 6 shows T-RFLP profiles under sulfate reducing conditions. The 58 bp peak closely related to *Lysobacter taiwanese* was

present under sulfate reducing conditions but did not appear under methanogenic conditions. Both the 115 bp peak that is most closely related to *E. coli* sp. K12, matching at 95%, and the 242 bp peak most closely related to *Shewanella algae* MAS2762, appeared under both sulfate reducing and methanogenic conditions. Under methanogenic conditions, the 208 bp peak most closely matched *Pseudomonas* sp. PDA that is involved with perchlorate respiration.

Over time, the Hawaii methanogenic cultures show little change in the diversity of the labeled population, as seen in Figure 5. The sulfate reducing cultures also show little change, although the label moves more quickly thought the community in these cultures as seen in Figure 6.

Table 1 summarizes the first detectable incorporation of the ^{13}C-label from TNT into the biomass of the active bacteria present, the T-RFLP peaks present in the active populations and the clonal identification of the peaks when possible. If no clonal match is reported it is because after screening over 200 colonies there were no matches to that specific fragment in the clonal libraries.

HPLC Results

Arthur Kill and Norfolk Harbor cultures were able to rapidly degrade TNT in 7 days. Under methanogenic conditions the Arthur Kill cultures degraded 41.84% of the TNT present in the cultures, while the sulfate reducing cultures degraded 77.52% of the TNT available in 7 days. Norfolk Harbor methanogenic and sulfate reducing cultures degraded 65.09% and 73.04% of the TNT available, respectively, within 7 days. All of the loss data has a margin of error of +/- 2.7%. The Hawaii degrading cultures were not able to rapidly utilize the TNT in 7 days but they showed similar TNT loss in 14 days

(data not shown). This illustrates that while TNT may abiotically bind to sediment present in these cultures, it remains bioavailable (4, 5, 9, 14). Being able to determine the degradation of TNT, even with some abiotic binding, was a key step in to be able to determine the loss and metabolites present.

The HPLC with a photo-diode array was used to monitor the decrease of TNT and the appearance of metabolites. Based on this information there were other peaks that were ruled out as abiotic by-products or media components by the abiotic controls (see Fig. 7). In the chromatograms from the biologically active cultures there are 5 peaks that appear in the enrichments actively degrading TNT that are not present in the sterile controls. These metabolite peaks appear at retention times of 7.36, 8.0, 11.60, 12.75, and 14.85 min. Of these 5 peaks, one has been determined to be toluene by UV spectral analysis and toluene spiked samples, and a cresol compound and a benzoate-like compound, either benzoate or hydroxybenzoate, are also present as metabolites. These metabolites are described in Table 2. The metabolite peak that appears at 11.6 min matches the standard of *p*- cresol. The metabolite that is unknown at Day 7 is at a retention time of 8.0 min and decreases by Day 14, when an unknown metabolite appears at a retention time of 7.36 min.

In Table 2, metabolites that have been "confirmed" by HPLC or LC/MS were further analyzed. For HPLC confirmation, the experimental sample was spiked with a known concentration of a standard to determine if the chromatogram peak increased in area respective to the concentration of the presumptive metabolite. Toluene was confirmed in this manner as a metabolite under methanogenic conditions for all 3 geographic sample sites.

LC/MS Results

To ensure that there would not be matrix interference by the culture media, samples of sterile culture media spiked with either ^{12}C-TNT or ^{13}C-TNT were extracted and analyzed on LC/MS to optimize analysis of the ^{13}C-TNT degradation samples. These controls are seen in Fig. 8 and show that it is possible to determine the presence of TNT and its metabolites in culture media after the extraction process. The mass shift between the ^{12}C-TNT and the uniformly labeled ^{13}C-TNT can also been seen in the spectra from the TNT standard run samples.

The LC/MS analysis showed large quantities of the proposed metabolites in the active samples and not in the sterile controls. Table 3 shows the molecular weight of known ^{13}C-labeled metabolites. Table 4 lists the metabolites based on molecular weight that were found under both methanogenic and sulfate reducing conditions at specific time points. Most of these compounds have been previously suggested to be metabolites for TNT degradation (2, 9, 10, 13) though not confirmed. The proposed compounds are methylpholorglucinol (140 g/mol), diamino- and dinitro- toluenes (174 and 204 g/mol), and TAT (144 g/mol). See Fig. 9 for the proposed biological metabolites of TAT degradation with their associated molecular weights assuming a uniform ^{13}C-label. The pathways shown are the most likely, considering the identified metabolites downstream from TAT (10, 17). The unidentified metabolites have molecular weights of 163, 182, 325 g/mol. The one unknown compound that is seen with a higher molecular weight than TNT (234 g/mol) may be a product of polymerization of the downstream metabolites.

4.4 DISCUSSION:

This study was able to show that there are a variety of bacteria present under both sulfate reducing and methanogenic conditions that can utilize TNT as a carbon source and identified some of the metabolites from anaerobic TNT degradation. Prior to this study, TNT was known to be able to be used as a nitrogen source without an additional carbon source under sulfate reducing conditions (10, 11, 15), however no utilization was known to occur under methanogenic conditions. Additionally, there were proposed pathways for degradation that the identified metabolites support (15, 24).

^{13}C-TNT Label Incorporation into DNA

Using SIP showed bacteria were not only present in the TNT degrading cultures, but they were actively degrading TNT. The rapid uptake of the carbon from TNT into biomass could not have been seen without the use of uniformly ^{13}C-labeled TNT in order to separate the ^{12}C- and ^{13}C- DNA . In this case, SIP allowed this population to be viewed after 24-hours in Hawaii sediments and after 72-hours in both Arthur Kill and Norfolk Harbor sediments. This is an exceptionally fast incorporation of a ^{13}C-label into biomass when the labeled substrate is a complex environmental contaminant.

The finding of a *Lysobacter*-like bacterium that is capable of both nitrogen and carbon uptake from TNT into biomass is consistent with reports of phytoremediation of explosives (37, 38). *Lysobacter* species are found in soil, aquatic environments, and the rhizosphere. In addition to *Lysobacter*, a peak related to *Thalassospira sp.* KMT005 was also found and this genus is known to associate with plants and plant tissue, especially sea grasses. Previous studies show TNT phytoremediation is more successful with

bacteria present in the soil, finding bacteria known to associate with plant tissue would lend validity to these claim (20, 37, 38).

Both *Shewanella algae* and *E. coli* sp. K12 are related to bacteria that are able to degrade contaminants. *Shewanella algae* is a marine bacterium and facultative anaerobe that is able to use metal cations as terminal electron acceptors. *E. coli* sp. K12 was the *E. coli* strain most closely related to the bacterium found in all sites and under both methanogenic and sulfate reducing conditions. *E. coli* has been reported to have the ability to enzymatically free the nitrogen present on TNT for use as a nitrogen source. This evidence suggests that *E. coli* may be able to utilize the carbon present in TNT as well (11, 19). *Sulfurimonas dinitrificans* DSM 125 is a known sulfur-oxidizer, with many genes that could facilitate growth in the spatially and temporally heterogeneous sediment habitat from where *Sulfurimonas denitrificans* was originally isolated. It is also known to have several genes that are predicted to encode heavy metal efflux transporters. *S. denitrificans* DSM 125 has many regulatory protein-encoding genes necessary to prevent and respond to oxidative stress (33). Lastly, a peak closely related to *Pseudomonas sp.*PDA was also found. *Pseudomonas sp.*PDA is a bacterium known to have the capability for perchlorate respiration and also has the ability to degrade perchlorate (23).

A peak that was a 97% match to the bacterium *Propionibacterium acnes* was found under methanogenic conditions in both Arthur Kill and Norfolk Harbor. It is not a bacteria known for plant association or associated with contaminant degradation, this bacteria was found in breast milk of healthy women and known to cause acne (25). *Shewanella aquamarina* strain S5, is slightly halophilic. It was isolated from the Yellow

Sea in Korea, it is a gram negative, motile, non-spore forming bacteria (40). While the Yoon et al study did not show that *Shewanella aquamarina* is capable of degrading contaminants, the ability to degrade TNT is possible (40).

The previous SIP study using TNT (Chapter 3) showed that the bacterium matching the 58 bp peak, most closely related to *Lysobacter taiwanese* (97%), was able to use not only the carbon present but also the nitrogen. In addition to bacteria that are closely related to those associated with plants and plant tissues, this study showed that there are bacteria that are related to degraders of environmental contaminants. This broadens the range of bacteria to be known TNT degraders and also shows that they are present in a wide variety of environments, both geographically, in different sediments, and under different reducing conditions. This is seen by the crossover of bacteria found in each site and under different conditions. The diagram, shown in Figure 10, best illustrates where there is overlap of the bacteria most closely associated with the T-RFLP peaks among and between the sites.

Time course TRFLP electropherograms show that the population capable of label incorporation does not change much over the course of the study. Using serial dilution coupled to amending the cultures with TNT as the sole exogenous carbon source, most likely eliminated a large portion of the community unable to utilize TNT or its intermediates. The lack of label dispersion suggests that while bacteria are able to incorporate the ^{13}C-label from TNT, the label does not migrate easily into the rest of the community or there are very few other bacteria present in the community. This could mean that cross-feeding, predation and viral lysis are limited in the TNT degradation pathway. Or, the linkage between the bacteria able to degrade TNT in these cultures

could be efficient to the point of excluding other bacteria from having access to the more labile intermediates in the metabolic pathway.

^{13}C-TNT Metabolites

Using a combination of HPLC and LC/MS analysis some of the metabolites that are produced during degradation were identified. These metabolites confirm proposed anaerobic metabolites and support the proposed degradation pathways for TNT under both methanogenic and sulfate reducing conditions (15, 24). While many TNT metabolites were transient and appeared and disappeared quickly in the degrading cultures, key metabolites were measureable using either HPLC or LC/MS. The confirmation of previously proposed metabolites and the identification of new metabolites further downstream from 2,4,6-triaminotoluene (TAT) suggests that TNT can be degraded anaerobically and used as a carbon source. It is a possibility that TNT can be completely mineralized biotically because the downstream metabolites are compounds known to be degraded under anaerobic conditions. Toluene, methylphloroglucinol, and *o*, *m*, and *p*-cresol have all proven to be mineralized under anaerobic conditions (3, 7, 9, 12, 13, 16, 17, 24, 36) .

Labeled diamino-, dinitro-, and triamino- toluene were seen in all active and sterile control samples. The concentrations were higher in the active samples, most likely due to the additive effects of biotic degradation over abiotic degradation. Methylphloroglucinol was seen in Hawaii UXO and Norfolk Harbor samples but was not present in any of the Arthur Kill samples analyzed. Additionally, the unknown metabolites with molecular weights of 182 and 325 were seen in Arthur Kill and Norfolk Harbor samples but were not detected in the Hawaii UXO site. The identification of TAT, methylphloroglucinol,

phenol, toluene, benzoate or hydroxybenzoate, and a cresol agree with the pathway described by Esteve-Nunez et al, 2001.

While incorporation of the ^{13}C-TNT was not explicitly linked to the production of metabolites in the time course of the experiments there is promise in using molecular techniques to link the metabolites present and the active degraders of TNT in the pathway. The appearance of multiple peaks after 3 years of serial dilution shows that there are a group of bacteria involved in the degradation of TNT, and they are phylogenetically diverse. The ability of a wider variety of bacteria, not only those associated with plants and plant tissues, to incorporate the ^{13}C-label present in TNT as part of the bacterial biomass is an expansion on bacteria previously identified as TNT degraders. The fact that there were peaks that represented bacteria found universally in the samples, in addition to those unique to specific sites, suggests the ability to degrade TNT under anaerobic conditions is wide spread in marine and estuarine environments.

These universally appearing bacteria could be utilizing TNT as an electron acceptor as described in Esteve-Nunez et al, 2001, this respiration would allow for them to appear under both methanogenic and sulfate reducing conditions. The peak most closely matching *Lysobacter taiwanese*, typically appears early on in the time course and could be producing the metabolites seen early in the degradation, such as TAT, or diaminonitrotoluenes or dinitroaminotoluenes. This is also true for the 165 bp peak that is closely related to *E. coli* sp. K12. While, the 283 bp peak that typically appears later in the time courses, most closely related to *Propionibacterium acnes*, could be associated with the production of some of the down-stream metabolites, its appearance most closely matches the appearance of toluene in the cultures. As seen in Table 5. Downstream

metabolites consisting of TAT, benzoate, and cresols could also be related to the appearance of the 242 bp peak most closely related to *Shewanella algae* MAS2762 based on the data collected in Table 5.

These experiments were able to identify ^{13}C-labeled compounds that are biologically derived metabolites of TNT. It can be assumed the labeled metabolites are produced from the bacteria incorporating the ^{13}C-label from TNT and this could allow for these compounds to be used as biomarkers for monitored natural attenuation. Determining label incorporation, showing TNT can be used as a carbon source, identifying biological metabolites, and finding both the incorporation and the metabolites present from the innocula and under both conditions from all 3 sites demonstrate that TNT can be degraded biologically under anaerobic conditions. These findings can be used to better inform remediation strategies at anaerobic TNT impacted sites.

4.5 ACKNOWLEDGEMENTS

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4.6 FIGURES:

Figure 1. TRFLP electropherograms of Arthur Kill methanogenic cultures with ^{13}C-TNT label incorporation.

Figure 2. TRFLP electropherograms of Arthur Kill sulfate reducing cultures with ^{13}C-TNT label incorporation.

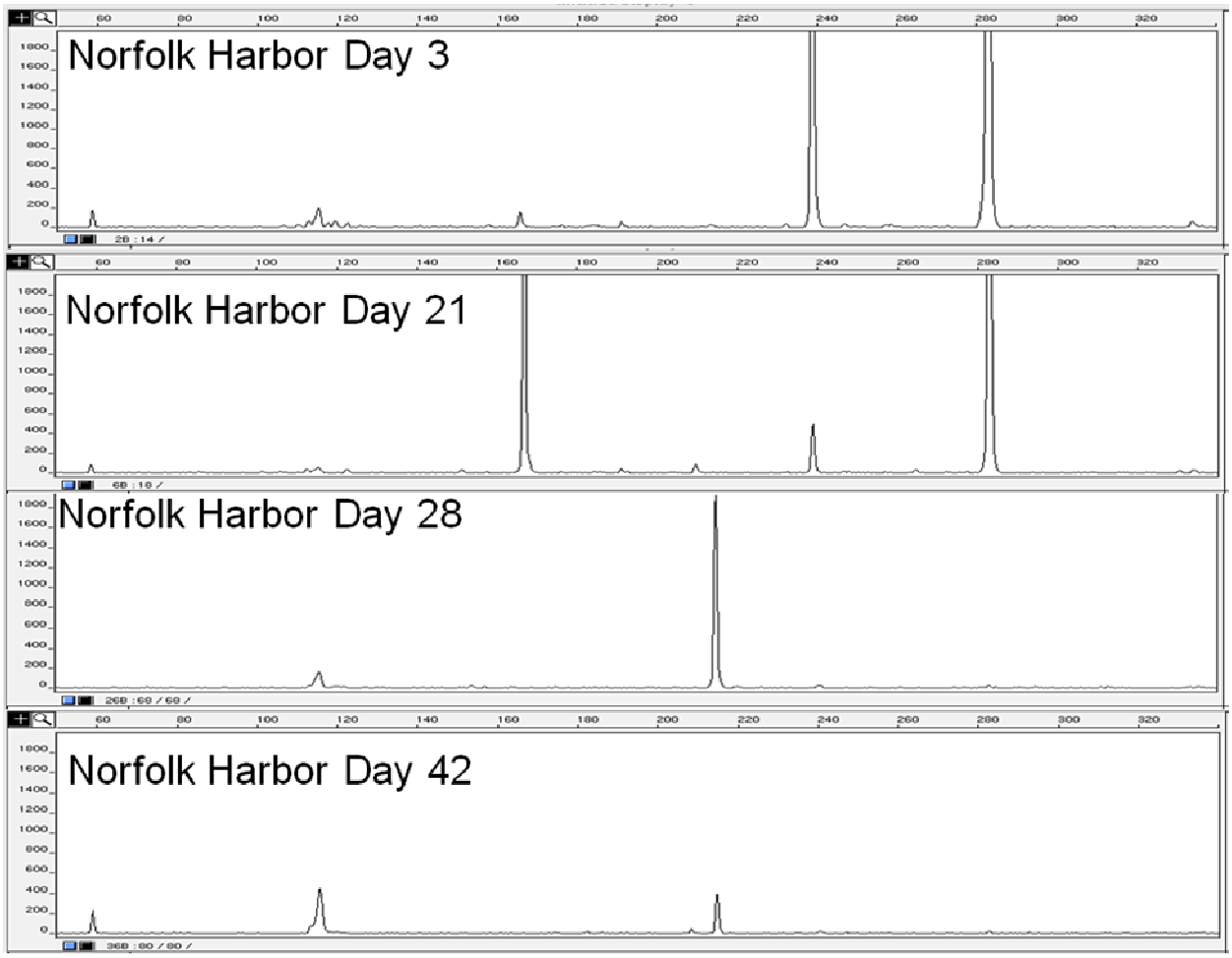


Figure 3. TRFLP electropherograms of Norfolk Harbor methanogenic cultures with ^{13}C-TNT label incorporation.

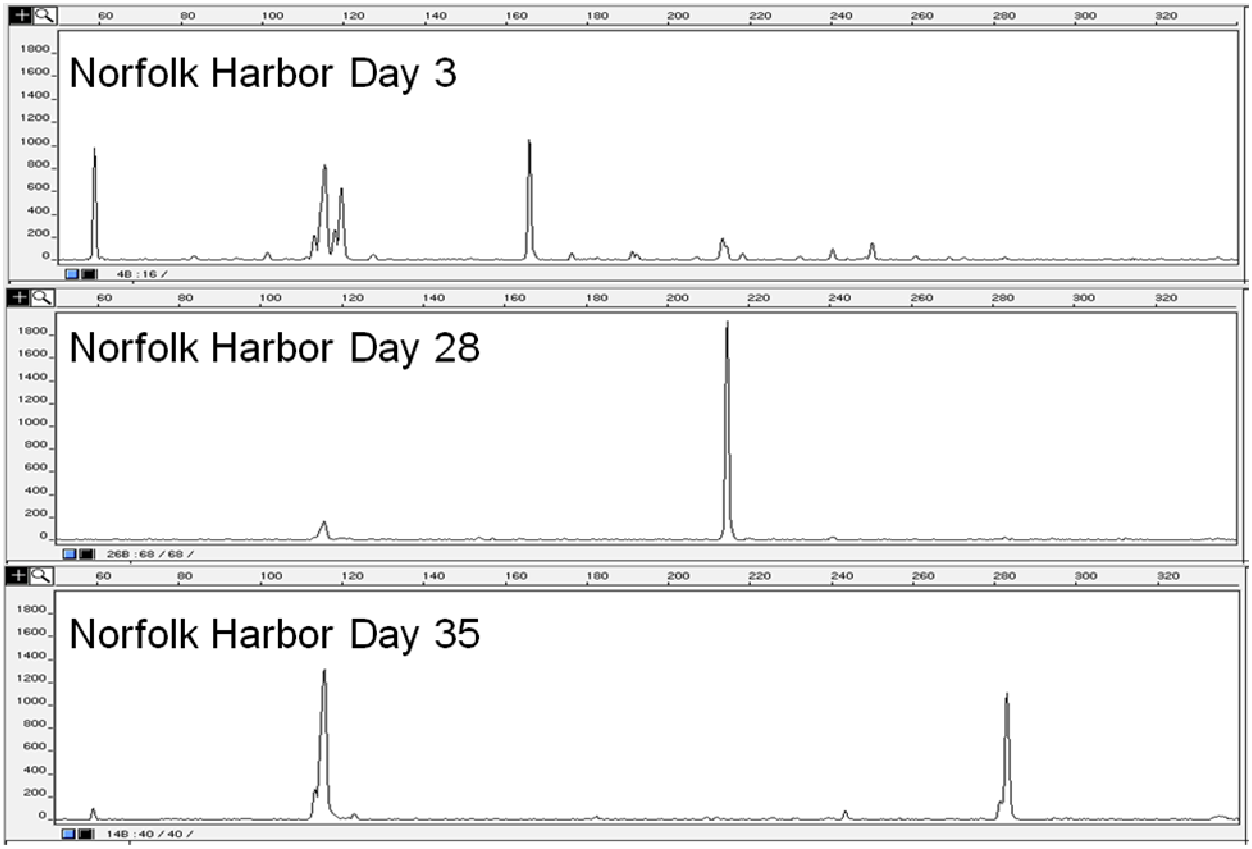


Figure 4. TRFLP electropherograms of Norfolk Harbor sulfate reducing cultures with ^{13}C-TNT label incorporation.

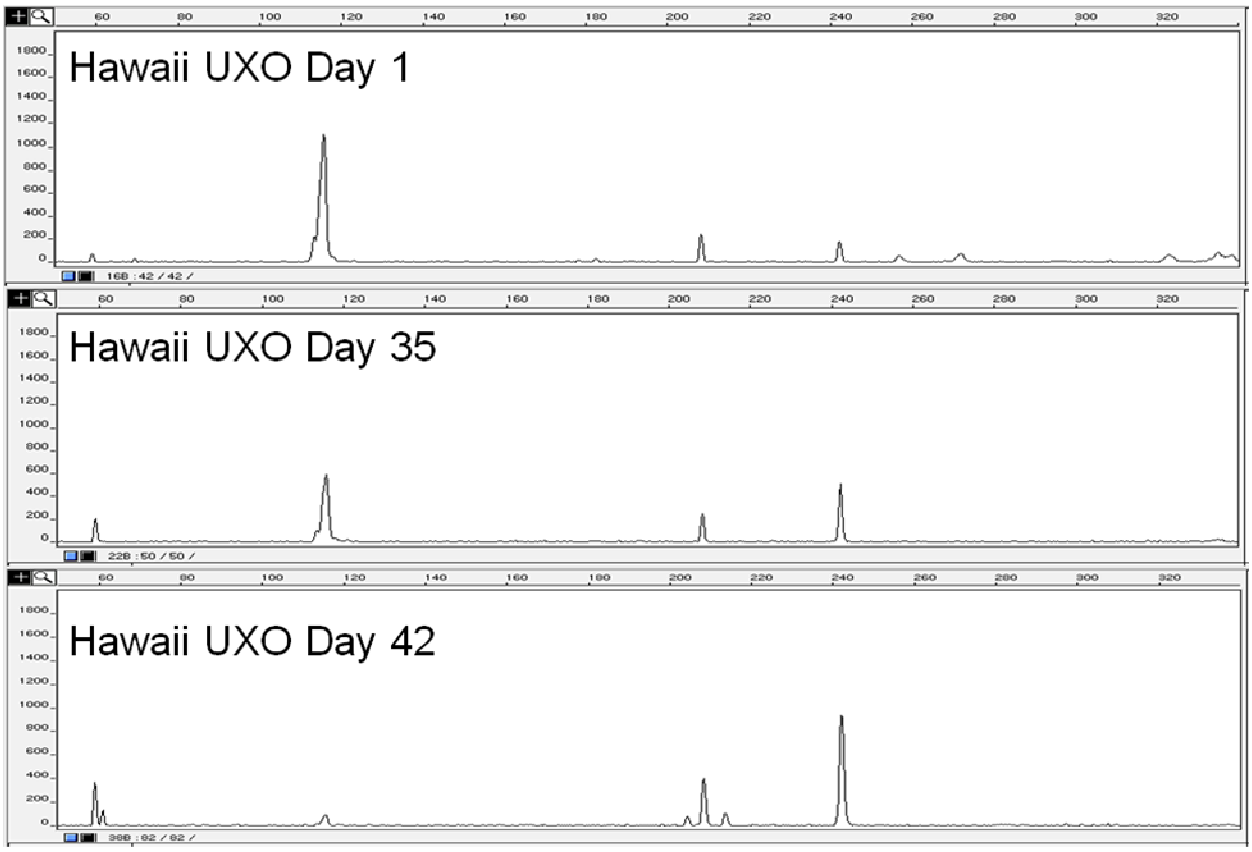


Figure 5. TRFLP electropherograms of Hawaii UXO methanogenic cultures with ^{13}C-TNT label incorporation.

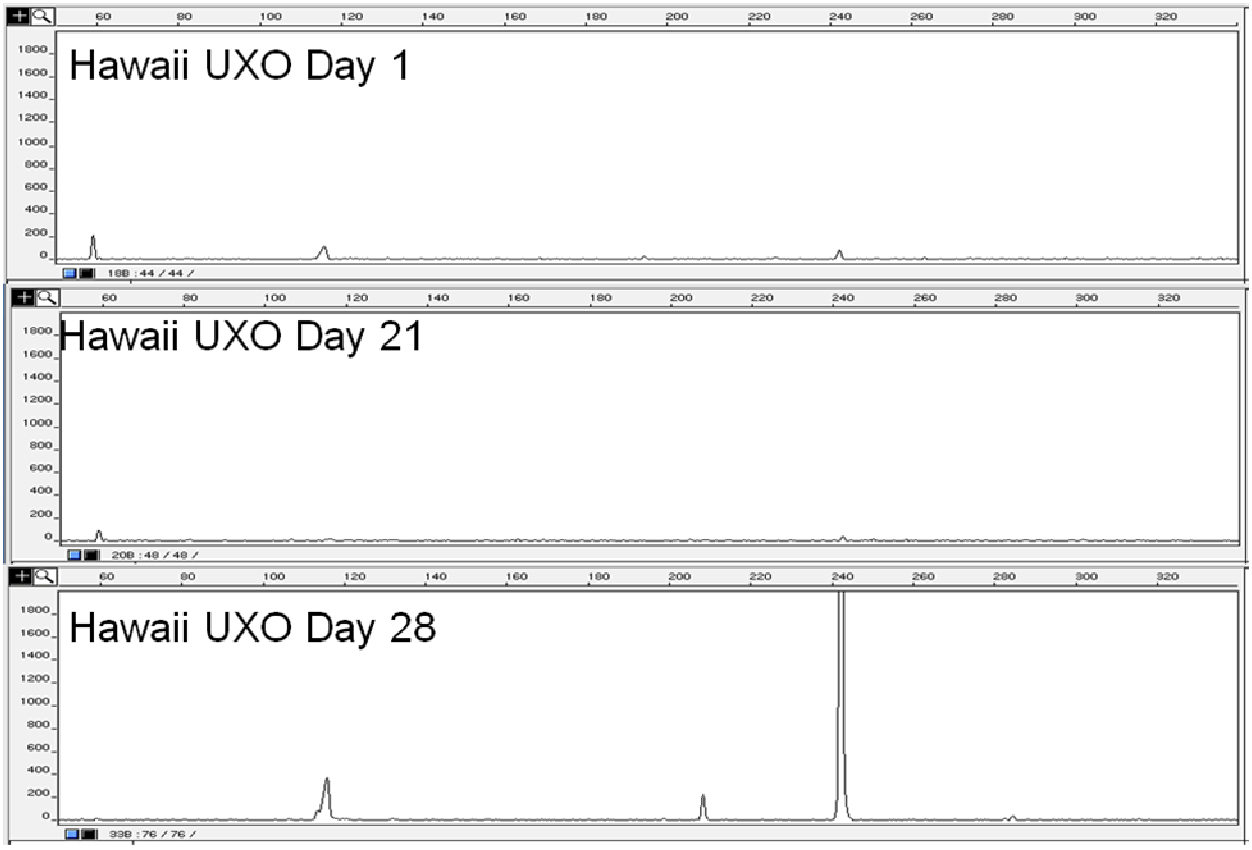


Figure 6. TRFLP electropherograms of Hawaii sulfate reducing cultures with ^{13}C-TNT label incorporation.

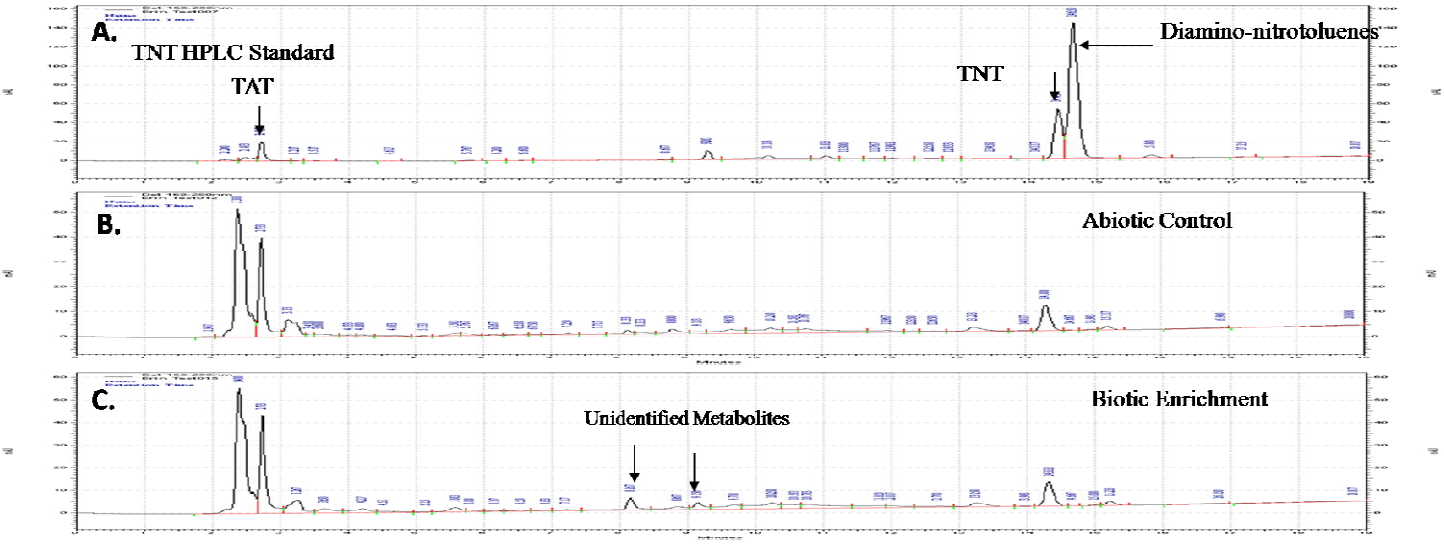
Table 1. Illustrates the bacteria present under different culture sites and conditions that are able to utilize the ^{13}C present in TNT as a carbon source.

Experimental Site and Condition	**First Appearance of 13C-label in Biomass**	**T-RFLP Peaks Present**	**Most Closely Identified Clones**	**Acession Number**
Arthur Kill Methanogenic	Day 3	58, 120, 165, 248, 283	58- *Lysobacter taiwanese* (93%) 120-*Shewanella aquamarina* strain S5 (94%) 165- *Thalassospira sp.* KMT005 (89%) known to associate with seagrass tissue 283- *Propionibacterium acnes* (97%)	FM205963 FJ589034 EU287937 AM157438
Arthur Kill Sulfate Reducing	Day 3	58, 115	58- *Lysobacter taiwanese* (93%) 115- *E. coli sp.*K12 (95%)	FM205963 CP000948
Hawaii Methanogenic	Day 1	115, 208, 242	115- *E. coli sp.*K12 (95%) 208- *Pseudomonas sp.*PDA known for perchlorate respiration 242- *Shewanella algae* MAS2762 (97%)	CP000948 AF323492 GQ372876
Hawaii Sulfate Reducing	Day 1	58, 115, 242	58- *Lysobacter taiwanese* (93%) 115- *E. coli sp.*K12 (95%) 242- *Shewanella algae* MAS2762 (97%)	FM205963 CP000948 GQ372876
Norfolk Harbor Methanogenic	Day 3	58, 240, 283	58- *Lysobacter taiwanese* (93%) 240- *Sulfurimonas denitrificans* DSM 125 283- *Propionibacterium acnes* (97%)	FM205963 CP000153 AM157438
Norfolk Harbor Sulfate Reducing	Day 3	58, 115, 165, 213	58- *Lysobacter taiwanese* (93%) 115- *E. coli sp.*K12 (95%) 165- *Thalassospira sp.* KMT005 (89%) known to associate with seagrass tissue	FM205963 CP000948 EU287937

Table 2. Shows the metabolites that were seen under the different reducing conditions, from the three sites that were used to establish the enrichment cultures.

Site	***Electron Acceptor***	***Metabolites Detected (HPLC)***	***Confirmed by HPLC or LCMS***
Norfolk Harbor	CO_2^-	Toluene	Yes- HPLC
Norfolk Harbor	SO_4^{2-}	Benzoate, *p*-cresol	
Arthur Kill	CO_2^-	Toluene	Yes-HPLC
Arthur Kill	SO_4^{2-}	Benzoate, *p*-cresol	
Hawaii UXO	CO_2^-	Toluene	Yes-HPLC
Hawaii UXO	SO_4^{2-}	Benzoate, *p*-cresol	

Figure 7. HPLC chromatograms illustrating TNT degradation and the metabolites identified from degradation. A. TNT standards that are used at the beginning of each HPLC run to determine the retention times of TNT, TAT, and the diamino-nitrotoluene intermediates. B. Abiotic control chromatogram. C. Biotic chromatogram (see the Black arrows pointing to novel biotic metabolites).



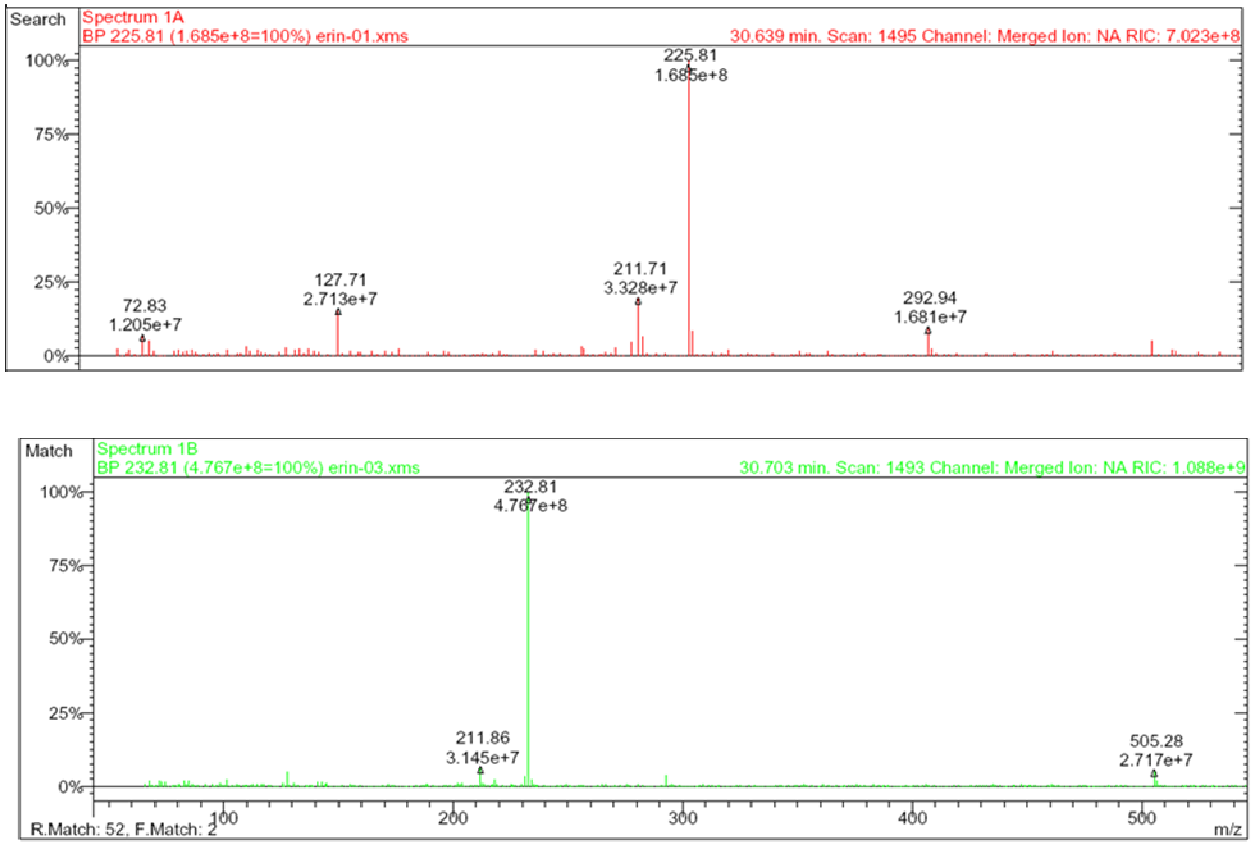


Figure 8. ^{12}C and ^{13}C-TNT LCMS standards. The top spectrum is of ^{12}C-TNT and the bottom is of uniformly labeled ^{13}C-TNT.

Table 3. Lists the molecular weights of ^{13}C-labeled TNT and its possible metabolites, also ^{13}C-labeled. Unknown metabolites found were at molecular wegiths of 163, 182 (odd number of nitrogens present), and 325.

Compound	*Molecular Weight (g/mol)*	*LC/MS Mass*
2,4,6-Trinitrotoluene (TNT)	234	233
2,4,6-Triaminotoluene (TAT)	144	143
Diaminonitrotoluene	174	173
Dinitroaminotoluene	204	203
Toluene	99	98
Methylphloroglucinol	140	139
Hydroxybenzoate	175	174
Phenol	100	99
o, *m*, or *p*-cresol	115	114

Table 4. The ^{13}C-labled TNT metabolites found in Arthur Kill, Norfolk Harbor, and Hawaii enrichment cultures using the LC/MS. This table includes the known metabolites in addition to those that are not known, but present in the active and not sterile cultures. ^{13}C-labeled dinitro-, diamino-, and triamino- toluene were present in all samples.

Site	*Time Point*	*Reducing Condition*	*Molecular Weights Detected*
Arthur Kill	14	Methanogenic	143, 163, 182, 325
		Sulfate Reducing	163, 182, 325
	21	Methanogenic	143, 325
		Sulfate Reducing	143, 182, 325
	28	Methanogenic	143, 182
		Sulfate Reducing	143
Hawaii	14	Methanogenic	139, 143, 163
		Sulfate Reducing	139, 163
	21	Methanogenic	139, 143
		Sulfate Reducing	143, 163
	28	Methanogenic	143
		Sulfate Reducing	163
Norfolk Harbor	14	Methanogenic	139, 325
		Sulfate Reducing	143
	21	Methanogenic	143
		Sulfate Reducing	143, 163, 182, 325
	28	Methanogenic	139, 163, 182, 325
		Sulfate Reducing	139, 163, 182, 325

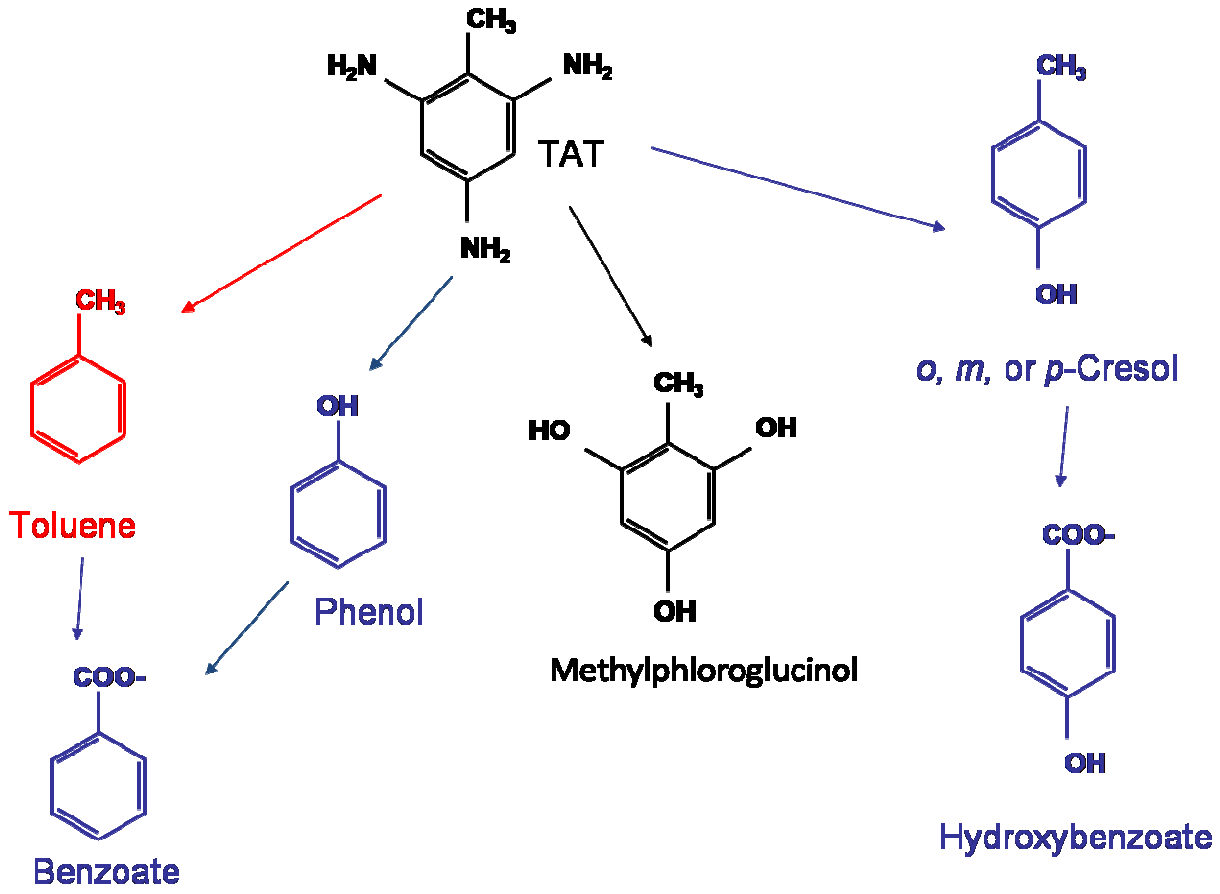


Figure 9. Metabolites of TNT degradation as determined by HPLC and/or LCMS analysis. Adapted from Esteve-Nunez et al., 2001 and Lovely and Lonergan, 1990.

Black- proposed TNT degradation pathway under anaerobic conditions and confirmed using LC/MS
Blue- novel TNT metabolites as seen by HLPC analysis
Red- confirmed novel TNT metabolite as seen by GC/FID analysis under both sulfidogenic and methanogenic conditions.

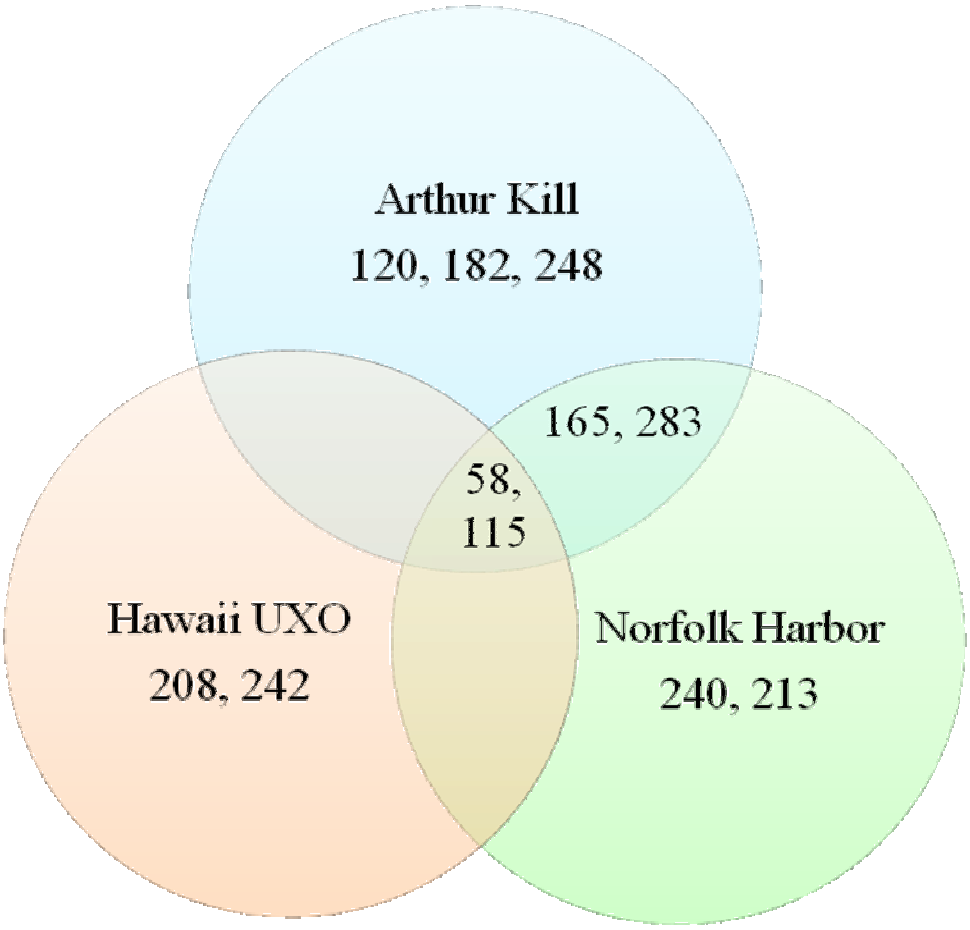


Figure. 10 The numbers inside of the Venn diagram represent the base pairs of TRFs seen with ^{13}C-label incorporation. This shows that there are 2 peaks that are universally represented at all sites (58 and 115 bps), shared peaks also exist between the populations present in Arthur Kill and Norfolk Harbor (165, and 283 bps). It also shows that there are peaks that are unique to the specific sites that were used as experimental innocula.

Table 5. Illustrates the appearance of metabolites in addition to TRFLP peaks detected at specific sites, reducing conditions and time points.

Experimental Conditions	*Metabolites Detected*	*TRFLP Peaks Detected*
Arthur Kill Methanogenic		
Day 21	TAT, Toluene	58, 115, 120, 283
Day 28	TAT	58, 120, 242, 283
Arthur Kill Sulfate Reducing		
Day 28	TAT, Benzoate, cresol	115, 242
Hawaii Sulfate Reducing		
Day 21	Methylphloroglucinol, TAT, cresol	58
Day 28	Benzoate	115, 216, 242
Norfolk Harbor Methanogenic		
Day 21	TAT, toluene	165, 242, 283
Day 28	Methylphloroglucinol	115, 216
Norfolk Harbor Sulfate Reducing		
Day 28	Methylphloroglucinol, cresol	115, 216

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CHAPTER 5
Conclusions and Future Works

5.1 CONCLUSIONS

This set of experiments has demonstrated that under anoxic conditions TNT can be used as a carbon and nitrogen source for bacteria and has allowed for the identification of the biological metabolites of this process.

In Chapter 2, it was determined that using stable-isotope probing (SIP) was possible in environmental samples using a labeled xenobiotic as a carbon source. Additionally, this work has shown that the time needed to see incorporation of a ^{13}C- or ^{15}N- label was reduced when using carrier DNA (*Halobacterium salinarium*) in the density separation gradients. This modification allowed for the optimization of SIP under laboratory conditions and facilitated the identification of the active population able to degrade TNT under anaerobic conditions.

Utilizing the modified SIP protocol, the study performed in Chapter 3 sought to detect the active members of the population capable of incorporating the ^{15}N-label into their DNA. TNT degrading cultures were incubated under sulfate reducing conditions with uniformly labeled ^{15}N-TNT or ^{13}C-TNT and the composition of the bacterial community was monitored. This experiment demonstrated that anaerobic bacteria were able to use TNT as both a nitrogen and carbon source for growth, confirming that stable isotope probing, with either ^{15}N or ^{13}C labeled nitroaromatic compounds, can identify the active members responsible for biodegradation from environmental samples. One example of these active degraders identified using TRFLP was a species most closely related to *Lysobacter taiwanensis* (93% identity over 400 bp). This species incorporated both the ^{13}C- and ^{15}N- label.

Finally, an attempt was made to identify the bacteria capable of incorporating the ^{13}C-label from TNT and to determine the biological metabolites produced from this TNT degradation. This work, outlined in Chapter 4, was conducted under both methanogenic and sulfate reducing conditions using sediments from 3 different marine or estuarine sites as innocula and ^{13}C-TNT. It was determined that there are bacteria present in all of these cultures that were capable of ^{13}C-label incorporation. These bacteria included a species most closely related to *Lysobacter taiwanensis* (93% identity over 400 bp), appearing at 58 bps, and *E. coli* sp. K12, matching at 95%, appearing at 115 bps. The different sites also contained peaks representative of bacteria that were unique to those sites. This suggests that there are a variety of bacteria capable of utilizing TNT as a carbon source present in a variety of geographic areas under both sulfate reducing and methanogenic conditions. The biological metabolites found from TNT degradation in this study were toluene, methylphloroglucinol, benzoate or hydroxybenzoate, and *o*-, *m*-, or *p*- cresol. The combination of this information provides insight into both the active TNT degraders in environmental samples and the biomarkers from TNT metabolism, which could prove to be useful in remediation strategies for impacted sites.

5.2 BIOLOGICAL DEGRADATION OF TNT

While is has been shown that TNT can be used as a terminal electron acceptor and as a sole nitrogen and carbon source under aerobic and anaerobic conditions, no organism has been isolated that is able to use TNT as a carbon source. Most likely if utilization is possible it will occur as part of a consortium of organisms. In the previous chapters, it has been shown that there are bacteria that can utilize TNT as a C and N source, additionally, that the metabolites of this degradation include toluene. This is significant

because toluene is not a dead-end metabolite and therefore it is possible for TNT to be completely mineralized under anaerobic conditions. This complete mineralization would most likely occur under anaerobic conditions because of the enhanced stability of the metabolic compounds when oxygen is not present. Finding the bacterium most likely responsible for the biological degradation of TNT will allow for more accurate genetic profiles to be developed. Having a better gene profile will aid in monitored natural attenuation by allowing for more accurate targets for PCR or other genetic probing mechanisms.

To better understand the process of TNT degradation the metabolic pathways that occur during this degradation need to be better understood. Detection methods for the determination of compounds present in the culture media should also be improved so that the metabolic pathway data is more definitive. Continued use of the HPLC and LCMS instruments will be able to give more accurate information to narrow down the possibility of the identity of the unknown metabolites. Known metabolites could then be used as biomarkers to determine if biodegradation of TNT is occurring at a specific site contaminated with TNT. The presence of biomarkers and bacteria able to degrade TNT could be used for the monitored natural attenuation (MNA) of a contaminated site. In addition to determining the actual pathway or pathways that organisms use to reduce TNT, it would be important to study the enzymes involved in the transformation and the degradation of TNT under both aerobic and anaerobic conditions. A complete picture of the degradation pathway could benefit remediation by adding terminal electron acceptors or readily used carbon substrates to speed the process along.

Lastly, microscopic and molecular techniques may be useful in determining if the bacteria present in a contaminated site are able to degrade the TNT, or use it as a carbon and/or nitrogen source. Fluorescent *in situ* hybridization (FISH) may be able to determine if bacteria are in proximity to TNT and if they have the genes needed to perform the degradation. Using 4'-6-Diamidino-2-phenylindole (DAPI) staining has shown that bacteria will closely associate with TNT most, likely in an effort to utilize the carbon or nitrogen present. DAPI is known to form fluorescent complexes with natural double-stranded DNA, showing a fluorescence specificity for AT, AU and IC clusters. See Figure 1. The TNT in these figures shows up yellow and the live bacteria shows up a lighter blue than the background. These show bacteria from the enrichment cultures closely associating with the TNT present, presumably using it for a growth substrate.

If there are genes present in the bacterial population identified in the stable-isotope probing (SIP) studies that are present in a high enough concentration to be tagged using FISH, it could be a viable technique in determining if there are bacteria present in a contaminated area that will degrade TNT. This type of an assay would be much faster and more efficient than utilizing enrichment cultures and other traditional monitored natural attenuation methods. Overall, knowing the genes involved with anaerobic TNT degradation and the metabolic pathway that is used, in addition to the possibility of being able to visualize the bacteria on the contaminant, it will aid in monitorned natural attenuation strategies and make them more efficient.

5.3 LIMITS OF DETECTION IN STABLE ISOTOPE PROBING

Stable-isotope probing (SIP) is still an emerging technique. While it is more accepted in the scientific community there are still questions as to its accuracy and how

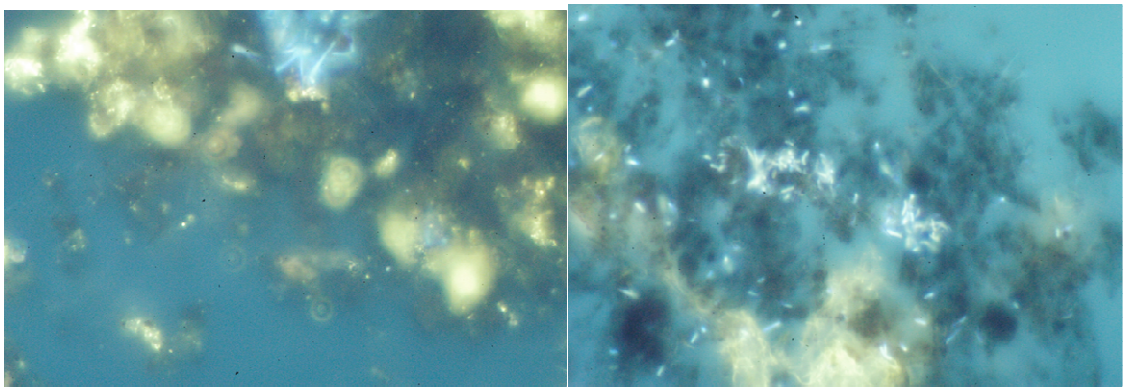
to best use the method in order to have optimal results. There have been issues with clean separation in the cesium chloride (CsCl) gradients in addition to different systems showing incorporation at different times (1, 2, 3, 4, 5). The use of "carrier DNA", typically archeal, can aid in the elimination of cross-contamination in the CsCl gradients (3).

However, there has not yet been a study to determine the limits of detection for SIP. In order to determine the limits of detection a known bacterium should be grown in liquid medium on a ^{13}C or ^{15}N substrate, and counted using an ocular grid. Adding a known quantity of this bacterium to soils and water that are of varied characteristics, i.e. clay, sand, or top soil, salt or fresh water, and perform a DNA extraction, followed by CsCl separation would allow for the labeled DNA to be separated from the unlabeled ambient DNA. Using primers that are specific to this bacterium, PCR could be performed to determine the limits of detection based on PCR amplification and DGGE or TRFLP if necessary. Determining the approximate limits of detection for different soil types, and water environments will allow for SIP experiments to be better designed and optimized to detect the users of the labeled substrate earlier in the time course of an experiment. This will lower the amount of cross-feeding and sloppy feeding that occurs and would illustrate a more accurate picture of the active population in the microcosm, especially those performing the metabolism of interest.

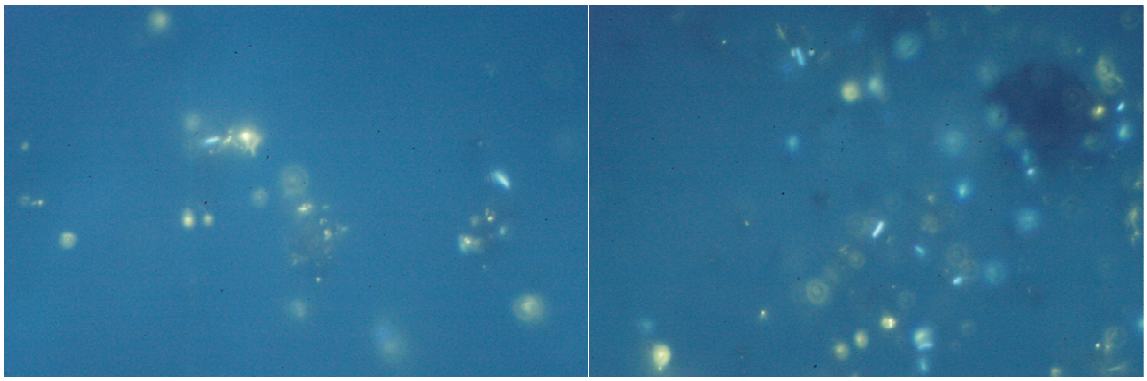
5.4 FIGURES

Figure 1. Shows both methanogenic (a and b) and sulfate reducing (c and d) cultures using the DAPI staining technique from the Hawaii UXO site enrichment cultures. TNT will auto-fluoresce yellow, the lighter blue rods and cocci are bacteria and the black is media components.

A. B.



C. D.



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