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thesis entitled

IDENTIFICATION OF ACTIVE SOIL RDX BIODEGRADING MICROORGANISMS USING ^{15}N STABLE ISOTOPE PROBING

presented by

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has been accepted towards fulfillment
of the requirements for the

M.S. degree in Environmental Engineering

Alison Cupples
Major Professor's Signature

12.14.09
Date

IDENTIFICATION OF ACTIVE SOIL RDX BIODEGRADING MICROORGANISMS USING ^{15}N STABLE ISOTOPE PROBING

By

Indumathy Jayamani

A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

MASTER OF SCIENCE

Environmental Engineering

2009

ABSTRACT

IDENTIFICATION OF ACTIVE SOIL RDX BIODEGRADING MICROORGANISMS USING ^{15}N STABLE ISOTOPE PROBING

By

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^{15}N DNA stable isotope probing was used to identify microorganisms responsible for degradation of hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) from soil microcosms. An agricultural soil was amended with either unlabeled or labeled ($^{13}C_3{}^{15}N_3$) RDX along with added mineral salts medium and glucose. Following RDX degradation monitored through HPLC analysis, DNA was extracted from both sets of microcosms. The DNA samples were then subject to isopycnic density gradient ultracentrifugation, fractionation, followed by terminal restriction fragment length polymorphism on 'heavy' fractions. One fragment was dominant in heavy fractions of ^{13}C ^{15}N-RDX amended samples, but not in the unlabeled controls indicating label uptake by this organism from RDX. Sequencing of the total DNA indicated the organisms involved in RDX transformation belonged to the class of *Sphingobacteria* and *Acidobacteria*. These organisms have not been associated with RDX degradation so far, but have been noted for their ability to degrade many other xenobiotic compounds such as MTBE, BTEX, tetracyclines and PCBs. This study indicates the potential for identifying more non-indigenous RDX degrading microorganism from uncontaminated soils.

ACKNOWLEDGEMENTS

I am thankful to my research advisor Dr. Cupples for giving me the opportunity to work on this project. She also needs to be thanked for her continuous support and guidance without which this thesis would not be possible. I also would like to thank Dr. Marsh, for his guidance and for generously allowing me to use the GeneScan Software at his laboratory. I am also thankful to Dr. Hashsham for being on my committee and as well a mentor.

Many thanks to Strategic Environmental Research and Developmental Program (SERDP) for funding this work through the 2008 SEED grant (ER1606).

Thanks to Michael Manzella a doctoral student from Microbiology and Molecular Genetics, for his contribution to this project. My extended thanks to Melissa Knapp and Weimin Sun for their valuable advice, guidance and troubleshooting techniques whenever I needed them. Thanks to Yanlyang Pan, Daniel Williams and Dr. Cha for their help with the analytical instruments. My special thanks to faculty and staff at the Department of Civil and Environmental for their continuous support.

The success of this study is due to the support of the faculty, staff and students at Michigan State University and I am thankful to all who contributed either directly or indirectly.

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ABBREVIATIONS

bp	base pair(s)
CL20	2, 4, 6, 8, 10, 12-hexonitrohexazaisowurtzitane
CsCl	cesium chloride
CsTFA	cesium trifluoroacetate
DGGE	denaturing gradient gel electrophoresis
DNX	hexahydro-1,3-dinitroso-5-nitro-1,3,5-triazine
DNA	deoxyribose nucleic acid
dNTP	dinucleotide triphosphate
EDTA	ethylenedinitraminetetraacetic acid
EPA	Environmental Protection agency
GTN	glycerol trinitrate, nitroglycerine
HMX	high melting explosive, octahydro-1,3,5,7-tetranitro-1,3,5.7-tetrazocine
HPLC	high performance liquid chromatography
kb	kilobase pair(s)
LB	Luria Bertani broth
MeBr	methyl bromide
MeCl	methyl chloride
MNX	hexahydro-1-nitroso-3,5-dinitro-1,3,5-triazine
PAH	polyaromatic hydrocarbon
PCB	polychlorinated biphenyls
PCR	polymerase chain reaction
PETN	pentaerithritol tetranitrate

rDNA	ribosomal DNA
rRNA	ribosomal RNA
SIP	stable isotope probing
RDX	royal demolition explosive, hexahydro-1,3,5-trinitro-1,3,5-triazine
RNA	ribose nucleic acid
T.DNA	total DNA
TNT	2,4,6-trinitrotoluene
TNX	hexahydro-1,3,5-trinitroso -1,3,5-triazine
TRFLP	terminal restriction fragment length polymorphism
X-gal	5-bromo-4-chloro-3-indolyl-®-D-galactopyranoside

1.0 INTRODUCTION

This thesis is divided into four chapters and three appendices. Chapter one provides an overview and introduces RDX as a problem contaminant. Chapter two provides a review of the biological degradation pathways and strains isolated for transformation of RDX. This is followed by a review of the molecular technique stable isotope probing (SIP) and its applications in bioremediation and microbial ecology. Chapter four presents the experimental methods, materials, key results, discussion and conclusions from the present study.

1.1 General

Xenobiotic compounds are chemicals that are human made many of which are present in the environment at a relatively high concentration. Examples are pesticides, herbicides, pharmaceuticals, explosives, benzene, toluene, ethylbenzene & xylenes (collectively known as BTEX), methyl tert-butyl ether (MTBE), polycyclic aromatic compounds (PAH's) and polychlorinated biphenyls (PCB's). Environmental contamination by organic xenobiotic compounds is a growing global problem (1). These contaminants persist and accumulate in the environment depending upon their fate and transport mechanisms. One family of xenobiotics that has been of concern since the early 1900's is the explosives.

Explosives are materials which when suitably initiated, undergoes very rapid self-propagating decomposition resulting in the release of energy. Detonation of explosives produces more stable products, release of heat and a sudden pressure effect by the action

of heat. Explosive chemicals are in general high in nitrogen and oxygen content. Widely used explosives include nitrate esters (Example: glycerol trinitrate, nitroglycerine (GTN) and pentaerithritol tetranitrate (PETN)), nitroaromatics (Example: picric acid and 2,4,6-trinitrotoluene (TNT)) and nitramines (Example: hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX), octahydro-1,3,5,7-tetranitro-1,3,5.7-tetrazocine (HMX), 2, 4, 6, 8, 10, 12-hexonitrohexazaisowurtzitane (CL-20)).

1.2 RDX

The nitramine explosive RDX is the most widely used explosive after TNT and is the most important military high explosive in the United States (82). RDX was also the first nitramine explosive to be developed. It was discovered in the 1890's by Hans Hemmings, when he introduced it as a medicine. It was patented and produced in 1920's by direct nitration of hexamine. The U.S. Bachmann Process for continuous generation of RDX was developed in the 1940's (23). It is aspowerful as the nitrate esters but less sensitive and hence preferred (7). It is primarily used in combination with other plastic explosives and detonators and is rarely used alone.

1.3 Toxicity of RDX

The USEPA has classified RDX as a class C human carcinogen (90) and has recommended a life time health advisory of 2μg RDX/L. RDX exerts its primary toxic effects on the central nervous system in addition to affecting the gastrointestinal and renal tracts (35) in humans and laboratory animals. However, experiments with rats (25), zebra fish (71) and earthworms (79) have revealed that RDX also affects the reproductive system or produces smaller offspring in exposed animals. RDX has also been shown to

bio-accumulate in plants and animals (45, 81) denoting a potential danger through food chain transfer to higher level organisms. RDX was once used as a rat poison and has been found to cause weight loss and affect offspring size in rats (58). RDX was also shown to be toxic to humans. RDX processing plant employees who were possibly exposed to powdered RDX through inhalation, suffered convulsions and unconsciousness (51). A 3 year old child who had ingested RDX pellets become epileptic while all her other body functions were found normal (95). All of these observed effects on humans and other animals were short term and were reversible. However, the toxic and carcinogenic nature of RDX is still questionable and the dangers associated with it should not be overlooked.

1.4 RDX as an environmental contaminant

RDX was extensively used both during and after World War II. Although it is not commercially manufactured in the United States, it is still produced, handled, packaged and deployed at various army ammunition sites in United States (82). These activities have caused contamination of land, water and air. In addition, past practices of improper disposal of wastewater from RDX production plants has also caused land and water pollution (87).

RDX contamination is a significant problem in several countries, including United States, United Kingdom, Canada, Germany and Australia (82). In the United States alone, explosives have been found in 115 sites at 25 installations amounting to 45,000 tonnes of contaminated soil (82). Maximum levels of RDX contamination (e.g. 27 g RDX/kg soil)

at some sites are significantly above the U.S. Environmental Protection Agency's recommended clean up level of 5.8 mg RDX per kg soil (47, 82).

Contamination of ground water due to leaching of explosives from soil is also a problem. Alhough RDX has low water solubility (maximum 38 mg/L at 20°C, (82)), its low sorption coefficient (K_d =0.8 L/kg,(82)) makes it a potential ground water pollutant. Thus RDX's potential to migrate quickly in soil and pollute potential water sources makes it a contaminant of concern. Also, the natural attenuation of RDX in water is shown to produce nitrate, a known contaminant (19).

1.5 Study objectives

1. To screen environmental samples for RDX biodegradation.
2. To identify the microorganisms responsible for RDX biodegradation in these complex communities using ^{15}N stable isotope labeling.

2.0 BIOREMEDIATION OF RDX – A REVIEW

A summary of RDX biodegradation pathways and the degradation products are presented in this chapter. This chapter also provides a list of known RDX degraders and their corresponding proposed biodegradation pathways. The information is presented by categorizing these pathways and degraders as aerobic, anaerobic or fungal degraders. A brief note on the microorganisms that degrades RDX metabolites is provided at the end of this chapter.

2.1 General

RDX has been considered recalcitrant but a review of the literature reveals the potential for biodegradation. Microbial mediated degradation of RDX was first illustrated in 1981 by McCormick et. al. (66). Since then, many bacterial strains and fungi have shown to be capable of degrading RDX under various conditions. RDX biodegradation takes place under aerobic, anaerobic and microaerobic (46), nitrate reducing (39), sulfate reducing (18), methanogenic (3), manganese reducing (22), iron-reducing (54, 75) and acetogenic conditions (4). RDX biotransformation has also been reported to occur in different types of environments: surface, subsurface, vadose zone, marine (12, 99), aquifer (10), fresh water and sewage sludges (9).

In many cases, RDX degradation is achieved by adding a carbon source and.or another nitrogen source resulting in a diauxic growth (17). In contrast to this, RDX degradation by other organisms is inhibited by the presence of other organic or inorganic nitrogenous compounds (72).

2.2 Biodegradation pathways and products

Degradation of RDX occurs through different pathways depending upon the conditions and the microorganism(s). The molecular structures of RDX and a number of the reported degradation products are illustrated in Figure 2.1. As summarized by Crocker et. al. (2006) (32) three mechanisms have been proposed for RDX degradation: two-electron reduction, denitration and direct enzymatic cleavage. The two electron reduction mechanism involves the addition of redox equivalents ($2e^-/2H^+$) to RDX, to form the reduced nitroso-derivatives and the hypothesized hydroxylamino-derivatives and triamino-derivatives. Denitration of RDX occurs by the addition of a single electron forming the anion radical RDX^- followed by ring cleavage. Enzymatic cleavage of RDX refers to the breaking of C-H bonds or C-N bonds or N-N bonds in the RDX molecule by direct enzymatic attack.

There are seven proposed RDX degradation pathways based on these three basic degradation mechanisms(32) as summarized by Crocker et. al. (2006) (32) (Figure 2.2). They include (a) the reduction of RDX to nitroso derivatives before ring cleavage first observed and proposed by McCormick et. al. (1981) (66); (b) the reduction of RDX to 1,3,5-triamino-1,3,5-triazine first observed and proposed by Zhang and Hughes (2003) (98); (c) the reduction of RDX via *Aspergillus niger* nitrate oxidoreductase enzyme first observed and proposed by Bhushan et. al. (2000) (14); (d) the direct enzymatic cleavage of RDX first observed and proposed by Hawari et. al. (2000) (46); (e) the anaerobic denitration of RDX first observed and proposed by Zhao et. al. (2002) (100); (f) the intial reduction to MNX followed by denitration of RDX first observed and proposed by Zhao

et. al. (2003) (103); and (g) aerobic denitration of RDX first observed and proposed by Fournier et. al. (2002) (37).

The reported degradation products (intermediate and end products) of RDX are hexahydro-1-nitroso-3,5-dinitro-1,3,5-triazine (MNX), hexahydro-1,3-dinitroso-5-nitro-1,3,5-triazine (DNX), hexahydro-1,3,5-trinitroso-1,3,5-triazine (TNX), 4-nitro-2,4-diazabutanal (NDAB), methylenedinitramine (MDNA), carbon dioxide, methanol, formaldehyde, nitrite, nitrate, nitrous oxide and triamino-RDX. In addition, several hydroxylamino-derivatives are postulated to form as intermediates during RDX biodegradation. The enzymatic ring cleavage products are still unknown. The degradation products also differ based upon the electron acceptor (O_2) conditions. The ring nitroso degradation products (reduced forms of RDX) are reported to form only during anaerobic transformation of RDX and have not been observed during aerobic degradation of RDX. Formaldehyde, when formed as a result of RDX degradation, is believed to be transformed to methanol and formic acid and further to carbon dioxide and methane when acetogenic and methonogenic bacteria are present (32). To date, the majority of studies have used traditional laboratory culture techniques to isolate RDX degrading bacteria. A summary of known RDX degrading bacteria has been provided (Tables 2.1., 2.2., 2.3).

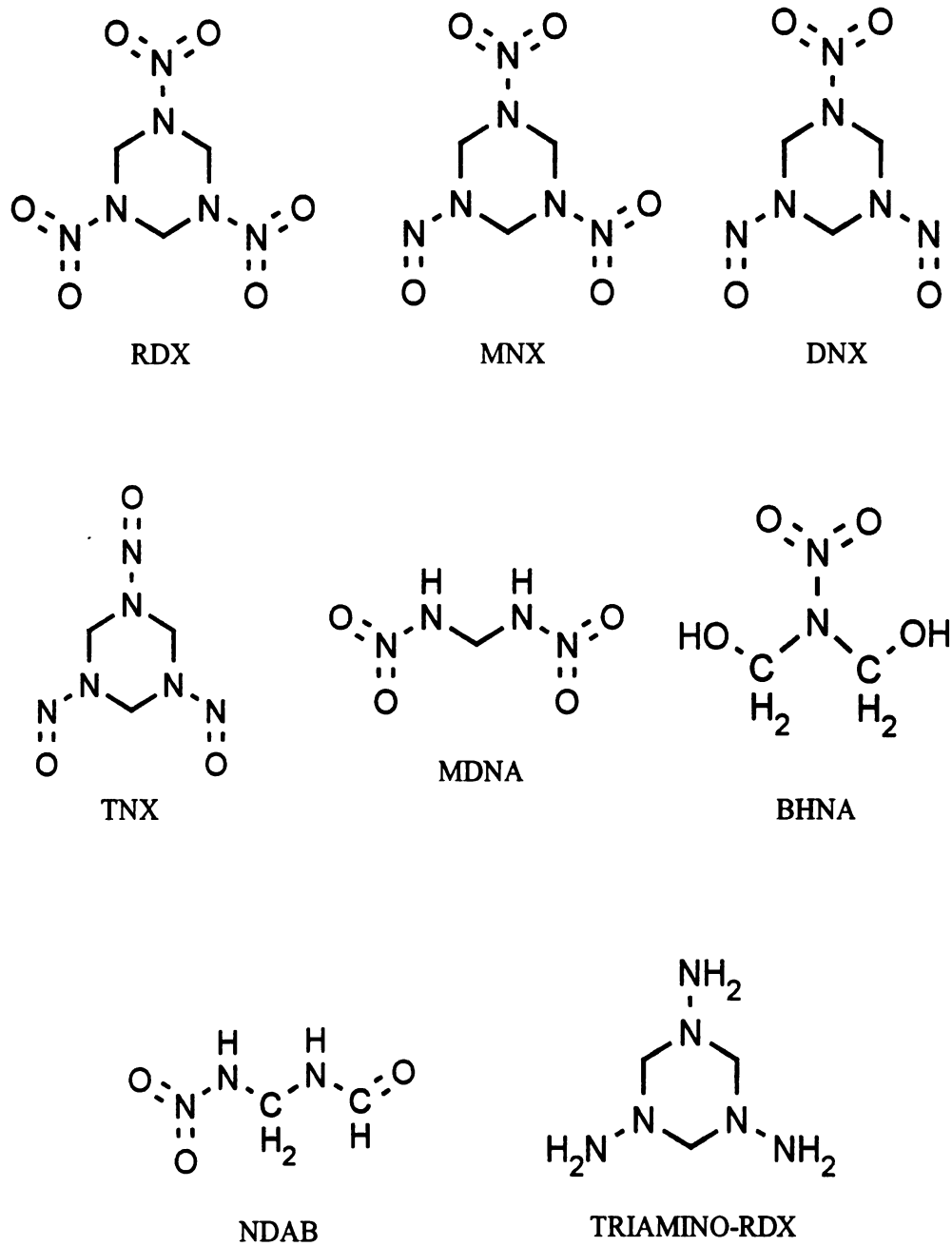


Figure 2.1 - Molecular structures of hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX), -1,3-dinitroso-5-nitro-1,3,5-triazine (DNX), hexahydro-1,3,5-trinitroso-1,3,5-triazine (TNX), methylenedinitramine (MDNA), bis-(hydroxymethyl)nitramine (BHNA), 4-nitro-2,4-diazabutanal (NDAB) and triamino-RDX

Figure 2.2 - Proposed biodegradation pathways for RDX (reproduced from Crocker et. al. (2006) (32)). *Path a* Reduction of RDX to nitroso derivatives followed by ring cleavage. *Path b* Reduction of RDX to triamino-RDX. *Path c* Reduction of RDX via *Aspergillus niger* nitrate oxidoreductase enzyme. *Path d* Direct enzymatic cleavage of RDX. *Path e* Anaerobic denitration of RDX. *Path f* Denitration of RDX via the reductive intermediate MNX. *Path g* Aerobic denitration of RDX

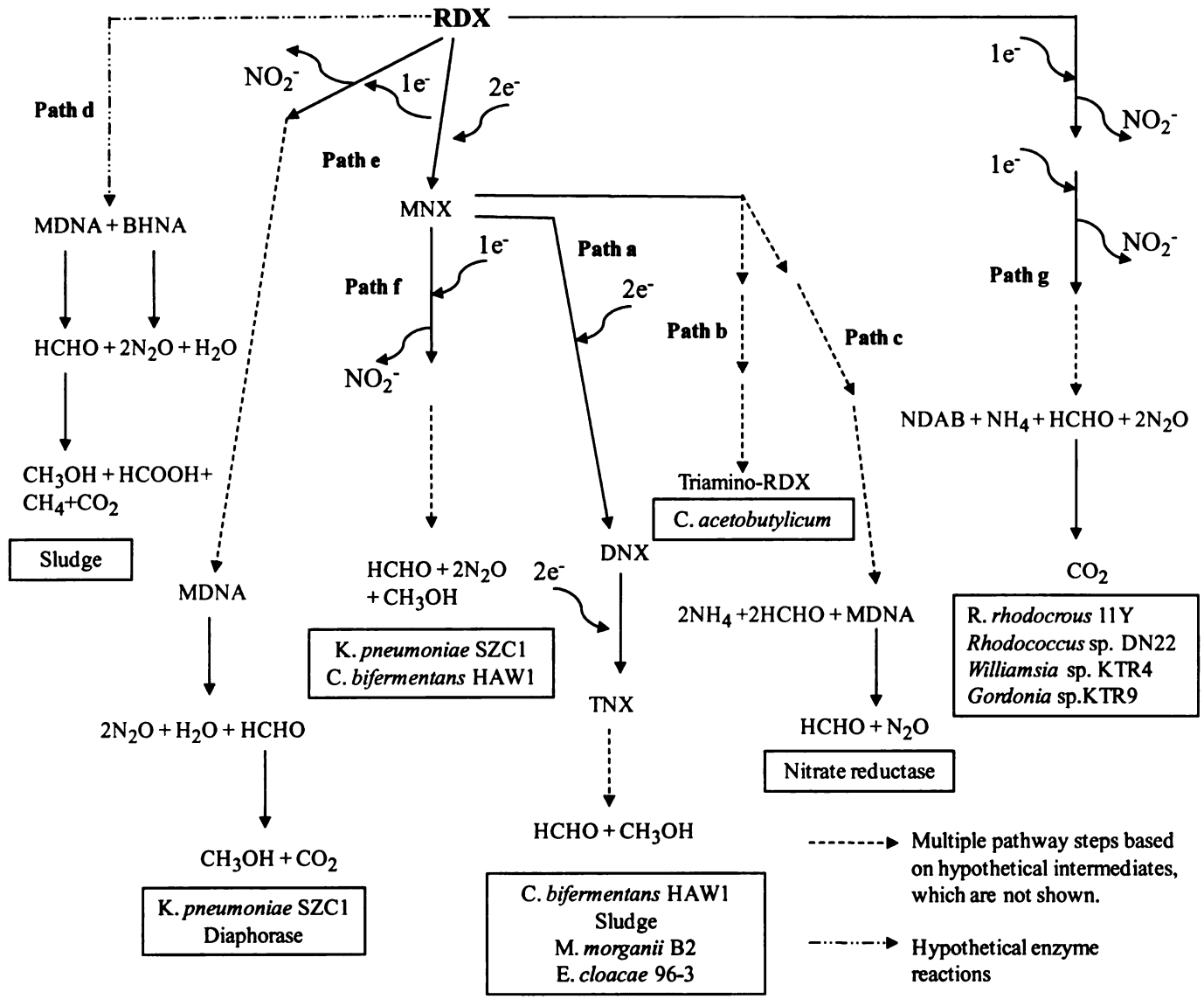

RDX
Path d
Path e
Path f
Path a
Path b
Path c
Path g
NO_2^-
1e-
2e-
MDNA + BHNA
HCHO + $2N_2O$ + H_2O
CH_3OH + HCOOH+ CH_4+CO_2
Sludge
MNX
HCHO + $2N_2O$ + CH_3OH
K. pneumoniae SZC1
C. bifermentans HAW1
Triamino-RDX
C. acetobutylicum
NDAB + NH_4 + HCHO + $2N_2O$
CO_2
R. rhodocrous 11Y
Rhodococcus sp. DN22
Williamsia sp. KTR4
Gordonia sp.KTR9
MDNA
$2N_2O$ + H_2O + HCHO
CH_3OH + CO_2
K. pneumoniae SZC1
Diaphorase
DNX
TNX
HCHO + CH_3OH
C. bifermentans HAW1
Sludge
M. morganii B2
E. cloacae 96-3
$2NH_4$ +2HCHO + MDNA
HCHO + N_2O
Nitrate reductase
Multiple pathway steps based on hypothetical intermediates, which are not shown.
Hypothetical enzyme reactions

Table 2.1 - RDX degrading strains and possible biodegradation pathways

Strain	Proposed pathway†	Reference
Morganella morganii B2	A‡	(52, 53)
Providencia rettgeri B1	A	(52)
Citrobacter freundii NS2	A	(52)
Stenotrophomonas maltophila PB1	ND	(17)
Serratia marcescens	A	(97)
Rhodococcus Sp. Strain DN22	G	(31, 37)
Enterobacter cloacae strain 96-3	A	(53)
Rhodococcus rhodochrous sp. 11Y	G	(83)
Klebsiella pneumoniae Strain SCZ-1	A, E, F	(100)
Clostridium bifermentans HAW-1	A, F	(103, 104)
Clostridium bifermentans HAW-G3.	A, F	(104)
Clostridium acetobutylicum	C	(98)
Desulfovibrio desulfuricans HAW-ES2	A, F	(104)
Acetobacterium malicum HAAP-1	E, F	(3)
Shewanella halifaxensis sp. HAW-EB4	A, F	(102, 105)
Shewanella sp. HAW-EB1	A, F	(105)
Shewanella sp. HAW-EB2	A, F	(105)
Shewanella sp. HAW-EB5	A, F	(105)
Clostridium bifermentans HAW-G4	A, F	(104)
Clostridium bifermentans HAW-E3	A, F	(104)
Clostridium bifermentans HAW-HC1	A, F	(104)
Desulfovibrio sp. HAW-EB18	A, F	(105)
Clostridium sp. HAW-EB17	A, F	(105)
Fusobacteria isolate HAW-EB21	A, F	(105)
Shewanella sediminis sp. HAW-EB3	A, F	(101, 105)
Methylobacterium sp. strain BJ001	A	(92)
Methylobacterium organophilum	A	(92)
Methylobacterium extorquens	A	(92)
Methylobacterium rhodesianum	A	(92)
Clostridium sp. EDB2	F	(13, 15)
Williamsia sp. KTR4	G‡	(88)
Gordonia sp. KTR9	G	(88)
Acetobacterium paludosum	ND	(84)
Halomonas sp. HAW-OC4	C	(12)
Marinobacter sp. HAW-OC1	C	(12)
Pseudoalteromonas sp. HAW-OC2	C	(12)

† Figure 2.2 explains the 7 different proposed pathways using the same alphabet codes.
‡ ND Not enough data to determine the pathway

Table 2.1 – RDX degrading strains...continued.

Strain	Proposed pathway†	Reference
*Pseudoalteromonas sp.*HAW-OC5	C‡	(12)
Bacillus sp. HAW-OC6	C	(12)
Rhizobium rhizogenes BL	ND	(55)
Burkholderia sp.	ND	(55)
Pseudomonas putida	ND	(29)
Fifteen strains belonging to phyla *Actinobacteria, -Proteobacteria and γ-Proteobacteria*	ND	(80)

† Figure 2.2 explains the 7 different proposed pathways using the same alphabet codes.
‡ ND Not enough data to determine the pathway

Table 2.2 - RDX degrading fungi and possible biodegradation pathways

Fungi	Proposed pathway†	Reference
Phanerochaete chrysosporium	ND‡	(8, 36, 85)
Aspergillus niger	C	(14)
Cladosporium resinae	ND	(8)
Cunninghamella echinulata varelegans	ND	(8)
Cyathus pallidus	ND	(8)
Rhodotorula HAW-OCF1	ND	(11)
Bullera HAW-OCF2	ND	(11)
Acremonium HAW-OCF3	C, E	(11)
Penicillium HAW-OCF5	ND	(11)
Cladosporium cladosporioides	ND	(55)

† Figure 2.2 explains the 7 different proposed pathways using the same alphabet codes.
‡ ND Not enough data to determine the pathway

2.3 Anaerobic biodegradation

RDX biodegradation was first studied under anaerobic condition in mixed cultures obtained from contaminated soil or industrial sludge (46, 66). McCormick et. al. (1981)

(66) reported the first confirmed anaerobic microbial degradation of RDX. They reported the formation of formaldehyde and methanol indicating ring cleavage. The authors also reported the detection of hydrazines, and postulated a two-electron pathway for RDX degradation (Figure. 2.2., Path a). This pathway also was shown to be followed by many bacterial strains that were later isolated including *Klebsiella pneumoniae* strain SCZ-1, *Clostridium bifermentans* strain HAW-1, *Shewanella halifaxensis* strain HAW-EB4, and *Shewanella sp.*, *Methylobacterium sp.*, Enterobacteria, *Shewanella sp.* HAW-EB2, as a major or minor pathway (38, 52, 92, 100, 102, 103, 105). A type I nitroreductase that was cloned and sequenced from *Enterobacter cloacae* strain 96-3 degraded RDX through nitroreductase activity (53) and also oxidized NADPH in the presence of RDX.

An alternate pathway that begins with two electron reduction was observed in a study with the cell-free extracts of *Clostridium acetobutylium* (98) (Figure 2.2., path b). This strain utilized H_2 as electron donor and transformed RDX to mono-,di, tri-nitroso derivatives and mono-, di-, tri-amino derivatives. These compounds did not disappear during the study and hence RDX was not completely mineralized in this pathway. A nitrate oxidoreductase was extracted from the fungi *Aspergillus niger* (14) and was shown to transform RDX in the presence of NADPH as electron donor. Though MNX and MDNA were observed as intermediate degradation products, they were further transformed to nitrous oxide, formaldehyde and ammonium ion (Figure 2.2., Path c).

Another anaerobic pathway significantly different from that postulated by McCormick et al was postulated by Hawari et. al. (2000) (46) (Figure 2.2., path d). In this pathway, the RDX ring was degraded by enzymatic attack on the C-N bonds, leading to the generation

of the hydroxylamine intermediates, MDNA and BHNA. These intermediates further decomposed in water to nitrous oxide, methanol, formic acid and formaldehyde.

Denitration of RDX appears to be a major route of RDX biotransformation (32). Anaerobic denitration of RDX could occur directly or after a reduction step in which RDX is transformed to MNX first and then denitrified (Figure 2.2., path e, f). Anaerobic denitration involves a single electron transfer to form the anion RDX^- and subsequent loss of a nitro group. This destabilizes the molecule leading to ring cleavage and formation of MDNA. Alternatively, RDX could be first reduced to MNX followed by denitration. These routes can either be a major or minor pathway in different organisms (100, 103).

2.4 Aerobic degradation

The first report on aerobic biodegradation of RDX identified three pure strains of *Corynebacterium* capable of utilizing RDX as a sole source of nitrogen (96). Later several strains including *Stenotrophomonas maltophia* PB1, *Rhodococcus sp.* strain DN22 and 11Y, *Williamsia sp.* KTR4 and *Gordonia sp.* KTR9 were isolated with the capability to degrade RDX aerobically (17, 31, 83, 88). Although aerobic and anaerobic denitration involves the loss of nitro group, they are both slightly different mechanisms. Aerobic denitration of RDX involves two one-electron transfers leading to the loss of two nitro groups resulting in ring cleavage (37). As a result NDAB, nitrous oxide, ammonium, formaldehyde and carbon dioxide was formed (Figure 2.2., path g). The aerobic bacteria *Williamsia sp.* and *Gordonia sp.* isolated by Thompson et. al. (2005)

(88), degrade RDX using this pathway and utilize it as a carbon, nitrogen and energy source suggesting that they are able to link the catabolic pathway for RDX with the anabolic pathway for carbon and nitrogen assimilation (32).

Although RDX biodegradation has been studied from the early 1980's, only recently has a gene corresponding to RDX degradation been identified. The *xplA* gene encoding a constitutively expressed, fused flavodoxin-cytochrome P450 enzyme was successfully identified from *Rhodococcus rhodochrous sp.* 11Y (83). Bhushan et .al. (2003) (16) later provided evidence that RDX degradation catalyzed by a rabbit liver cytochrome P450 enzyme and the strain *Rhodococcus* DN22 produced the same degradation products (NDAB). They also found that RDX biotransformation by the *xplA* protein and the rabbit liver cytochrome P450 enzyme was three times faster under anaerobic conditions when compared to aerobic conditions (16). Roh et. al. (2009) (80) recently used this gene to design specific primers and identify RDX degrading strains by combining it with a powerful molecular probing technique called stable isotope probing (SIP, chapter 3).

2.5 Fungal biodegradation

A white rot fungi *Phanerochaete chrysosporium was* found to transform both RDX and TNT to carbondioxide and nitrous oxide (36, 85). However the studies conducted with *P. chrysosporium* did not observe any other biodegradation products at detectable concentrations, therefore pathways has not been completely elucidated. Further studies have shown several other fungi are capable of degrading RDX (8, 11, 55).

2.6 Degradation of metabolites

Although many bacteria mineralize RDX to harmless gaseous product, some metabolites such as NDAB, MNX are recalcitrant in certain biodegradation systems (37, 88). Thus there is a concern over producing more toxic intermediates during RDX bioremediation. However, growing evidence indicates a number of degradation products can be degraded by other organisms (Table 2.3) therefore biodegradation of RDX continues to be a valuable cleanup technique.

Table 2.3 - Microorganisms that degrade RDX metabolites

Strain/Fungi	**RDX metabolite**	**Reference**
Methylobacterium sp. JS178	NDAB	(38)
Phanerochaete chrysosporium	NDAB	(37)
Klebsiella pneumoniae Strain SCZ-1	MNX	(100)

3.0 STABLE ISOTOPE PROBING – A REVIEW

This chapter provides a brief summary of stable isotope probing methodology. It also outlines the different markers that are used in SIP studies and their advantages and disadvantages. Following this a review of SIP studies, involving microbial ecology is presented. Subsequently, a review of a select number of studies utilizing DNA-SIP for bioremediation of environmental contaminants is provided. Finally, a brief note on the various isotopes used in SIP experiments along with their methodological consideration is presented.

3.1 General

In addition to an understanding of the contaminants physico-chemical properties, having an accurate insight of microbial community and function is necessary to assess microbial degradation of xenobiotic contaminants. Because less than 1% of the total microbial population is cultivable (89), culture independent techniques that use molecular biomarkers facilitate a more holistic study of microbial community . Although 16S rRNA genes are useful to determine the phylogeny of organisms present in a sample, such information does not always provide insight into function *in situ*. Metagenomics, an approach to develop gene libraries of the environmental genome, has offered a new way to assess the microbial community and functions of even uncultivable microorganisms.

Metagenomics, may fail to identify low-abundance species, and may not completely represent the diversity. The introduction of a method called stable isotope probing (SIP) has changed this limitation. Stable isotope probing involves the use of an isotopically

labeled compound and aims at linking function to identity while attempting to maintain experimental conditions closer to *in situ*. The method is based on the uptake of a label by microorganisms in mixed cultures and analysis of the biomarkers obtained from the labeled microorganisms to reveal identity of organisms that were involved. Figure 3.1 provides an overview of the method. In the first step, the environmental sample is incubated with labeled or unlabeled substrate (killed and live) in microcosms. After the DNA has been extracted from the labeled sample and live control, it is subjected to ultracentrifugation and fractionation, followed by terminal restriction fragment length polymorphism (TRFLP) to identify the phylotypes that are quantitatively dominant in the 'heavy' fractions of samples but not controls.

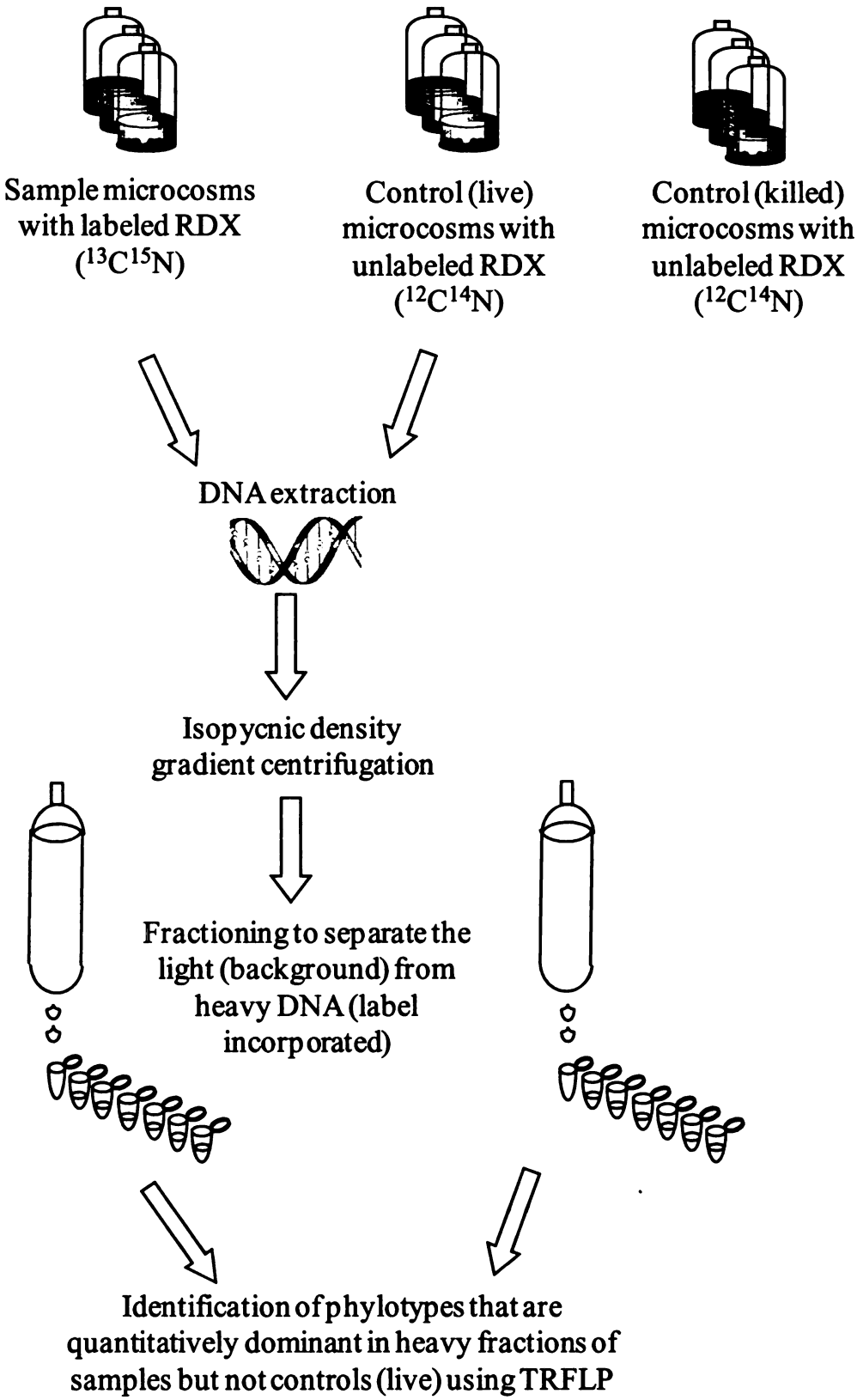


Figure 3.1 - Overview of DNA-SIP using fractionation and TRFLP

3.2 Markers used in SIP

Phospholipid fatty acids (PLFA) and nucleic acids have been extensively used as biomarkers for SIP. Phospholipid fatty acids were the first biomarkers used, in a study by Boschker et. al. (1998) (21) . This study was focused on organisms noted for their biogeochemical processes, the sulfate-reducing bacteria that utilized acetate as carbon source and methane reducing bacteria in aquatic sediments, using ^{13}C labeled acetate and methane respectively. Their findings showed that a gram-positive *Desulfotomaculum acetoxidans* were dominant in ^{13}C-labeled acetate uptake and not the most widely studied *Desulfobacter spp.* A type I methanotrophic bacteria possibly belonging to the genera *Methylobacter* or *Methylomicrobium* was identified as the dominant methane oxidizing organism in this study.

Although attempts to use stable isotopes to study phylogeny and functionality were used during the 20^{th} century, the term 'stable isotope probing' came into existence after its first use by Radejewski et. al. (2000) (78). These researchers grew microorganisms on ^{13}C labeled CH_3OH and separated the ^{13}C (heavy) DNA from ^{12}C (light) DNA by isopycnic density gradient centrifugation in a CsCl/EtBr gradient. The heavy DNA was used to construct 16S rRNA clone libraries. The dominant methylotrophs were identified as bacteria from the α-*Proteobacteria* and *Acidobacterium*.

The third type of biomarker that followed PLFA and DNA was RNA. RNA stable isotope probing was first performed and proved practical by Manefield et. al. (2002) (65). These

researchers used the approach to study phenol degrading communities in an industrial bioreactor. Total community DNA and RNA were extracted at various time points and the researchers showed the level of label enrichment in RNA was much higher than in DNA during the same time period. The ^{13}C labeled RNA was separated from unlabeled RNA by equilibrium (isopycnic) density gradient centrifugation in CsTFA, fractionation and was then analyzed using reverse transcriptase-PCR (RT-PCR) and denaturing gradient gel electrophoresis (DGGE). They identified the organism that dominated carbon acquisition from phenol belonging to the genus *Thauera*.

All three biomarkers (PLFA, DNA, RNA) have been widely used under a variety of experimental conditions, substrates, time of exposure, label incorporation etc and each has its own advantages and disadvantages. As summarized by Neufeld JD et. al. (2007) (74), PLFA-SIP is the most sensitive of these biomarkers and DNA-SIP is the least sensitive as the label incorporation depends upon DNA replication, which is slow. However, the highly sensitive PLFA is not useful in studying uncultured bacteria as the unique PLFA patterns of all microorganisms are not known (34). The need for longer incubation time or increased substrate concentration (that does not mirror the in situ conditions) to result in better separation of the heavy and light background nucleic acid material in DNA/RNA-based SIP, can be overcome by fractionation in CsCl or CsTFA (73). The shortcomings of nucleic acid SIP include cross feeding, if the study uses longer incubation periods and requirement of higher than typical or *in situ* substrate conditions. Nonetheless, nucleic acid SIP, particularly DNA-SIP is still considered a unique approach to link function to identity for microorganism in complex samples. In addition,

functional genes can be targeted (41). Though DNA-SIP is ideal to identify organisms that use a particular compound as their sole source of carbon or nitrogen, the spectra of its use in recent times has widened to study organisms that use other environmentally important compounds as described below.

3.3 DNA based SIP

3.3.1 General methodology

Stable isotope probing requires the use of a labeled substrate (preferably highly labeled) supplied to a mixed community sample. Such experiments have been conducted in microcosms or directly *in situ*. Following label uptake, the total DNA is extracted and subject to equilibrium (isopycnic) density gradient centrifugation to separate labeled and unlabeled DNA. The gradient is then either fractioned or the labeled (heavy) DNA is extracted using a needle and syringe for downstream analysis. The DNA in the heavy fractions (or "heavy DNA") is PCR amplified and can be fingerprinted using tools such as TRFLP, DGGE, cloning and sequencing. Alternatively, real-time PCR with specific primers can be used to quantify the targeted organisms at various times points to identify dominant species. Besides using the SSU rRNA genes for phylogenetic analysis, functional marker genes that encode enzymes for specific activity can be used to target microorganisms of geochemical importance.

3.3.2 DNA SIP in microbial ecological studies

DNA SIP has been extensively used to study microbial communities of environmental importance. In particular, it has been utilized in studies of various C_1 compounds, denitrifiers and rhizosphere-microbial interactions. The pioneering DNA-SIP study (78) illustrated the dominant methylotrophs in an oak forest soil belonged to *Acidobacterium* and α-*Proteobacteria*, which were previously not associated with methanol assimilation. They utilized primers specific to the *mxaF* gene that codes α-subunit of the methanol dehydrogenase to target the dominant methylotrophs in the 'heavy' DNA. Many studies that followed also utilized DNA SIP with both 16S rRNA and functional genes to identify active methylotrophs in various natural environments(67).

Methanotrophs have been widely studied using DNA-SIP. Morris SA et. al. 2002 (70) characterized the active methanotrophic population in a peat soil using 16S rRNA genes and three other functional genes encoding CH_4 oxidation pathway. They identified a novel methanotrophic organism closely related to *Methylocella palustris* along with organisms from α subclass of *Proteobacteria* as active organisms in methane assimilation. Another conducted DNA-SIP using $^{13}CH_4$ in microcosms that closely relate to environmental conditions, in microcosms amended with water and microcosms amended with mineral salts medium (27). While treatments best reflecting the environmental conditions had cross feeding and slow label uptake, the samples amended with mineral salts medium labeled a less diverse population of methanotrophs. They

observed the diversity of the treatments amended with moisture, with 80% type I methanotrophs and 20% type II methanotrophs best reflected the actual in situ diversity.

In a study focused on ammonia oxidizing bacteria, although the *Nitrosomonas sp.* dominated in fresh water and a *Nitrosospira sp.* dominated in brackish water, further investigation with ^{13}C DNA SIP revealed that *Nitrosomonas sp.* were still the dominant active ammonium oxidizers in marine environment (40). Similarly in a recent study, Jia et. al. 2009 (50) found that Archaea are dominant by population, even though bacteria dominate ammonia oxidation in agricultural soils. DNA-SIP was also applied to study acetate or methanol assimilating bacteria under nitrate reducing conditions in sludge (43, 76) using ^{13}C acetate. Both studies identified the dominant degraders as closely related to *Comamonadaceae* and *Rhodocyclaceae* in the subclass of β-*Proteobacteria.*

Miller et. al. (2004) (69) identified methyl chloride and methyl bromide degrading organisms in soils where these gases are naturally released. While the organisms from *Burkholderia* dominated MeBr degradation, organisms related to *Rhodobacter*, *Lysobacter* and *Nocardioides* were found to degrade MeCl. In a later study using DNA-SIP to investigate MeCl utilizing bacteria, it was reported that DNA-SIP can identify a more diverse group of organisms involved in biodegradation compared to enrichment and isolation techniques (20).

3.3.3 DNA SIP in bioremediation studies

Bioremediation of persistent xenobiotics has proved to be effective and ecologically friendly (91). A wide variety of microorganisms capable of utilizing many pollutants have been isolated under laboratory settings. However, those isolated and characterized in a laboratory may not be able to degrade the pollutant under typical environmental conditions. Thus identifying active degraders (whether dominant or not) using DNA-SIP is important for the ultimate application of bioremediation technologies.

There have been many pollutants studied using DNA. Most are carbon rich organic compounds studied using ^{13}C isotope labeling. A field based study conducted by DeRito et. al. (2005) (33) utilized DNA-SIP under three different ^{12}C and ^{13}C phenol doses and enrichment conditions to identify both the primary active phenol degraders and the organisms that assimilate the $^{13}CO_2$ respired from phenol degradation. While a diverse group of phenol degraders (α-,β-,γ-*Proteobacteria*) were found in the single dosed unenriched setup, the primary phenol degraders in the enriched setup were found to be members of the genera *Kocuria* and *Staphylococcus*. They also identified a *Pseudomonas* as the dominant cross-feeders of the ^{13}C from phenol degradation.

Many organisms have been isolated with the ability to utilize a variety of the PAH compounds as a carbon source. Singleton et. al. (2005) (86) studied organisms that degrade naphthalene, phenanthrene and salicylate in a bioreactor. Combining ^{13}C DNA with DGGE enabled these researchers to determine that salicylate and naphthalene were

transformed by *Pseudomonas* and *Ralstonia* species, while phenantharene was primarily utilized by a bacterium related to the genus *Acidovorax*.

In addition PCB degradation has been studied using SIP. While cultivation studies indicated the *Rhodococcus sp.* dominated in biphenyl degradation, DNA-SIP combined with T-RFLP analysis identified that the *Pseudonocardia sp.* dominated in biphenyl utilization (56, 57). BTEX compounds have also been a focus of study (2, 5). Luo et. al. (2009) (62) identified a novel TM7 strain capable of degrading toluene using ^{13}C DNA-SIP.

3.3.4 Isotopes and methodological considerations

Ideally an isotope of any element present in the DNA (C, H, N or O) could be used in SIP. However, the higher the proportion of the element in the biomarker the greater the signal will be. This is because SIP depends on the increase in buoyant density of the DNA and the higher the label in the DNA the higher the increase in DNA buoyant density. Thus the ^{13}C label is extensively used as it provides results in high label incorporation and therefore a clear separation of the labeled and unlabeled DNA. However, in recent times (24, 80) there have been attempts to use ^{15}N label to study several nitrogen rich compounds. Interestingly, ^{15}N isotope labeling was used as early as 1958 by Meselson and Stahl (68) to study DNA replication.

Laboratory culture based techniques have isolated many strains degrading nitrogen rich compounds. However there is great interest in identifying organisms that are responsible for degradation *in situ*, or under experimental conditions representing in situ conditions. The low percentage of nitrogen in DNA (average C/N ratio in DNA, 2.1:1) (26) and the risk of toxicity of these compounds if present in higher concentration restrict the amount of label incorporation and are important limitations to this approach. The required 40 -50 atom% ^{15}N-DNA (variable based on the G+C content) enrichment for a clear separation of labeled and unlabeled DNA is high compared to the required 20% atom ^{13}C-DNA enrichment or the 10% atom ^{13}C-RNA enrichment (26).

Thus nitrogen-labeling SIP might not yield a strong signal, and hence methodological changes have been proposed (26). These include, centrifuging the DNA-CsCl mixture at a low speed for longer duration (140000 × *g* for 69 hours) and employing 100% labeled and unlabeled DNA from pure culture to use as boundaries. In addition to these considerations, the amount of ^{15}N labeled DNA obtained and used for downstream analysis is also vital in a successful SIP study.

Thus with the application of these methodological changes and understanding its limitations ^{15}N stable isotope labeling can be applied to study biological uptake of environmentally important compounds.

4.0 IDENTIFICATION OF HEXAHYDRO-1,3,5-TRINITRO-1,3,5-TRIAZINE DEGRADING MICROORGANISMS USING 15N STABLE ISOTOPE PROBING

In the current study, ^{15}N DNA stable isotope probing was used to identify microorganisms responsible for degradation of hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) from soil microcosms. Following label uptake, the extracted DNA was subject to ultracentrifugation and fractionation. Terminal restriction fragment length polymorphism (TRFLP) was utilized to identify phylotypes that were quantitatively dominant in heavy fractions in samples but not in the controls. The results indicate the organisms involved in RDX transformation belonged to the class of *Sphingobacteria* and *Acidobacteria*. This chapter details the methodology used including both analytical and molecular methods. This chapter also provides the TRFLP, cloning and sequencing results, followed by the discussion and conclusions. Finally, a brief note on future studies is provided.

4.1 Introduction

Hexahydro-1,3,5-trinitro-1,3,5-trazine (RDX) is a nitramine explosive that has been widely used since World War II and has caused significant contamination of land and water in and around military ranges at United States. In particular, the physical-chemical properties of RDX, including low water solubility, low sorption to soil and nonvolatile nature, have resulted in significant groundwater contamination. For example, RDX has been found at concentrations as high as 36 mg/L in groundwater in the Iowa Army Ammunition Plant (87). RDX has shown to be toxic to humans, terrestrial animals and

aquatic system. The U.S. EPA has classified RDX as a type C carcinogen and estimated a life time exposure health advisory of 2 μg of RDX/L.

Two common methods for contaminated site cleanup are incineration and composting (82). While incineration does not remove RDX completely (48) it also potentially creates more toxic products. In contrast, composting (94) reduces the toxicity and mutagenicity of the contaminants potentially resulting in successful engineered bioremediation. Numerous microorganisms have been isolated with the potential to transform RDX to less harmful end products under either anaerobic or aerobic conditions (Table 2.1, 2.2). However, successful *in situ* bioremediation requires knowledge on the microorganisms able to transform the contaminant under conditions typical of the contaminated site, i.e. mixed culture, complex samples. This represents a knowledge gap for effective bioremediation of RDX, because the majority of information on RDX degrading microorganisms has originated from pure culture or enrichments experiments.

To address this knowledge gap, one study has recently (2009) attempted to identify the microorganisms responsible for RDX degradation in mixed culture, complex samples using stable isotope probing (SIP) (80). These researchers used ^{15}N - ring labeled RDX to enrich the DNA of RDX degrading microorganisms from explosive contaminated groundwater. The extracted heavy DNA was PCR amplified using both 16S rRNA gene and the *xplA* functional gene (83) specific to RDX degradation and the active RDX degraders were identified through 16Sr RNA gene sequencing as belonging to *Actinobacteria*, α-*Proteobacteria* and γ-*Proteobacteria*.

Here we present a study utilizing ^{15}N DNA stable isotope probing to identify potential RDX degraders from a soil previously unexposed to the contaminant but likely to contain a diverse microbial community.

4. 2 Materials and methods

4.2.1 Chemicals

Unlabeled RDX and ring labeled RDX ($^{15}N_3$, $^{13}C_3$; 50% N Labeled) (>99%) dissolved in acetonitrile were purchased from Cambridge Isotope Laboratories (Andover, MA, USA). Reagents were either purchased form Sigma-Aldrich (St.Louis, MO, USA), Fisher BioReagent (New Jersey, USA), Invitrogen (Carlsbad, CA, USA) unless otherwise stated. Acetonitrile (HPLC grade; ≥99.8% purity) was purchased from EMD Chemicals Inc (New Jersey, USA).

4.2.2 Soil incubations

Soil samples used were either collected from agricultural sites (previously unexposed to RDX) or BTEX contaminated sites in Michigan. The agricultural sites had been previously amended with biosolids from a wastewater treatment plant, with the last application being within 1 to 4 years before sample collection. Soils were manually sorted, homogenized, air dried and sieved through a 4 mm screen after collection and stored at 4 °C until use (<1.5 years). In total, ten different soils were tested for RDX degradation under O_2 rich or depleted conditions (Appendix A). Test microcosms (triplicate killed controls and live samples) were constructed with unlabeled RDX to determine RDX degradation potential. Stable isotope probing was conducted only on one soil (referred to as Soil 3).

Microcosms were constructed as previously described (88). Briefly, microcosms were assembled with soil (2 g; wet weight), a mineral salts medium (MSM), glucose (5.6 mM)

and RDX (45 μM or 90 μM) and were incubated in the dark on a shaker. The MSM was prepared as previously described (88). Final masses (per liter) in each microcosm were as follows: KH_2PO_4, 0.218 g; K_2HPO_4, 0.278 g; $MgSO_4.7H_2O$, 0.16 mg; $FeSO_4.7H_2O$, 1.6 mg; $CaCl_2.2H_2O$, 0.024 mg; $MnCl_2.4H_2O$, 0.4 mg; H_3BO_3, 0.04 mg; $ZnCl_2$, 0.04 mg; $CuCl_2$, 0.024 mg; $Na_2MoO_4.2H_2O$, 0.008 mg; $CoCl_2.6H_2O$, 0.4 mg; $NiCl_2.6H_2O$ 0.04 mg; $Na_2MO_4.2H_2O$, 0.008 mg; $CoCl_2.6H_2O$, 0.4 mg; $NiCl_2.6H_2O$, 0.04 mg; and Na_2SeO_3, 0.4 mg.

The SIP study involved microcosm samples amended with labeled or unlabeled RDX as well as autoclaved controls. Although all microcosms (50 mL or 160 mL) were closed with a rubber seal and aluminum crimp, a select number were also aerated between sampling days. All microcosms were briefly exposed to air during sampling, performed on day one and when RDX was expected to be removed completely (based on preliminary studies). Microcosms were prepared in duplicates or triplicates, covered in heavy-duty aluminum foil (to prevent RDX photo degradation) and were shaken at room temperature (~20 °C).

Microcosms for testing the extraction efficiency of RDX were constructed as described above (only live unlabeled samples). The SIP study involved two different RDX concentrations (45 μM and 90 μM) dissolved in acetonitrile.

4.2.3 RDX extraction and HPLC analysis

RDX extraction and analysis were as previously described (88). Briefly, sampling for RDX involved mixing and removal of 1 mL using a wide tip sterile serological pipette into a Nalgene Oak Ridge High-Speed FEP Centrifuge Tubes with Tefzel ETFE screw caps. RDX was extracted by adding equal volumes of acetonitrile and sonicating for 18 hours at 15 °C. The ultrasonic bath (Fischer Scienctific) was coil cooled by circulating cooled deionized water. At the end of 18 hours, the tubes were centrifuged, at 2900 rpm for 20 minutes. The supernatant (600 µL) was filtered using acetonitrile wetted filters (PVDF, 0.22 µm, Whattman). All samples were analyzed on the same day as extraction to minimize potential for RDX degradation.

HPLC analysis involved the following conditions and instrumentation: injector volume: 20 µL for samples and 10 µL for standard; isocratic 40% acetonitrile and 60% 0.1% H_3PO_4 acidified deionized water; mobile phase flow rate: 1 mL/min; Perkin Elmer series 200 autosampler; PE binary LC Pump 250; PE diode array detector 235C, wavelength 255 nm; column: Supelco Reverse Phase PAH C18 (25 cm X 4.6 mm, 5 µm).

4.2.4 DNA extraction and ultracentrifugation

Following the complete removal of RDX, genomic soil DNA from the live labeled and unlabeled microcosms were extracted using the PowerSoil DNA extraction kit (MO BIO Laboratories, Inc., Carlsbad, CA) as per manufacturer's instructions. Ultracentrifugation was performed in Quick –Seal Polyallomer tubes (Beckman Coulter) in a Thermo Sorvall WX ultra series centrifuge equipped with a step saver rotor system (70V6) for 46 hours at

178127 × *g* and 20 °C. All extracted DNA from a single microcosm (approximately 100 ng or more) was added to a Beckman Centrifuge tube along with a TE/CsCl solution. Buoyant densities (BD) were calculated by measuring the refractive index with a model AR200 digital hand-held refractometer (Leica Microsystems Inc.) before the tubes were sealed (Quick-Seal tube topper, Beckman Coulter). The initial buoyant density of the TE/CsCl solution was adjusted to 1.7828 g mL^{-1}, and that of the DNA and TE/CsCl solution to 1.7276 to 1.7285 g mL^{-1}.

Following isopycnic gradient centrifugation, the DNA was divided into fractions (20-26 fractions) using a fractioning system (Beckman Coulter) and a syringe pump (Kd scientific). Deionized water was pumped into the top of the ultracentrifugation tubes and DNA-TE/CsCl mixture was collected from the bottom (heaviest DNA collected first) in volumes of 150 µL. The BD of each fraction was determined by measuring the refractive index with a model AR200 digital refractometer (Leica Microsystems, Inc.). The DNA was separated from the CsCl in each of the fractions by overnight glycogen-ethanol precipitation. The purified DNA was stored at -20 °C until further analysis.

4.2.5 TRFLP and sequencing

Heavy fractions (first 10 -12 fractions that had detectable DNA on 1% agarose gel) were analyzed by 16S rDNA terminal restriction fragment length polymorphism (TRFLP) using standard procedures (60). Universal primers 27F-FAM (5'-AGAGTTTGATCMTGGCTCAG, 5' end-labeled with carboxyfluorescein) and 1492R

(5'-GGTTACCTTGTTACGACTT) (Operon Biotechnologies) were utilized for PCR of all fractions. The PCR reaction mix included the following: 10 μL of template (varying weight based on fraction DNA concentration); 10 μL of 10x PCR buffer; 0.2mM of dNTP mix; 50 pmols of 27F-FAM; 50 pmols of 1492R; 2.5 units of Taq; and molecular biology grade water to a final volume of 100μL. The PCR program was: 94 °C (5 min); 94 °C (30 secs), 55 C (30 secs), 72 °C (1.5 min) (30 cycles); 72 °C (5 min). 15 μL of the PCR products were run on a 1% agarose gel and the first 10 to 12 heavy fractions that had a band on the gel were chosen for further analysis.

The PCR products were purified using a Qiagen PCR Purification kit following the manufacturer's instruction and concentrated in a 30 μL volume of elution buffer. 13 μL of the purified product (200 to 800 ng) was digested in a 15 μL digestion volume using 15 units of Hae III restriction enzyme (restriction site: CCGG). The digested DNA samples were analyzed in duplicates using Capillary Electrophoresis (ABi 3730 Genetic Analyzer, Research Technology Support Facility, Michigan State University). The percent abundance of fragments was analyzed using Genescan software.

Total DNA was PCR amplified (as described above with a 30 minutes extension step) and cloned into *Escherichia coli* TOPO 10 cells using TOPO TA cloning kit (Invitrogen Corporation). The *E. coli* cells were grown on LB broth (25 g L^{-1}) solidified with 15 g agar L^{-1} in the presence of 50 μg ampicillin mL^{-1} for 16 hours at 37°C. The combined DNA from first four heavy fractions of one of the triplicates was also used to construct

clone libraries. Individual colonies were isolated and grown in LB broth with ampicillin (50 $\mu g\ mL^{-1}$) for upto 16 hours and checked for growth. The clones with inserts were verified by PCR using M13 forward (5'-TGTAAAACGACGGCCAGT-3') and M13 reverse (5'-AACAGCTATGACCATG-3') primers and the plasmids were extracted using QIAPrep miniprep system (Qiagen, Inc.) and sequenced (using M13 forward and M13 reverse primers) at the Research Technology Support Facility at Michigan State University The Ribosomal Database Project's (Center for Microbial Ecology, Michigan State University) analysis tool called "Classifier" was used to assign taxonomic identity. The clustalW2 web tool (European Bioinformatics Institute, European Molecular Biology Laboratory, United Kingdom) was utilized to align sequences.

4.3 Results

4.3.1 RDX biodegradation

The extraction efficiency of RDX was 121.90 (± 6.38) % (non autoclaved samples only) tested with soil 6, (Appendix A). The RDX concentrations, measured at various time points for all soils, under various conditions, are summarized in a tabular form Appendix A and are illustrated in bar charts in Appendix B. Among the study soils, RDX was degraded over a span of 2 to 3 weeks only in microcosms setup with soils 3, 4 (in the presence of acetonitrile) and 7, 8, 9 and 10 (in the absence of acetonitrile), whereas little or no degradation was observed in the corresponding autoclaved controls. However all degradations occurred only when the microcosms were unopened (no oxygen diffusion) between day 1 and the last sampling day (varied between 11-49 days).

In soils 3 and 4 (with acetonitrile), 45 μM RDX was degraded in ~11-14 days and 90 μM RDX was degraded in ≤16 days. Soil 5 showed only slight degradation (significantly different from the controls as per ANOVA test). Soils 1, 2 and 6 showed no degradation under conditions tested. Soils 7, 8, 9 and 10 degraded ~45 μM RDX (no acetonitrile) in 16 days.

Following these preliminary RDX degradation experiments, microcosms were constructed for SIP, using soil 3 with both 45 μM and 90 μM ring labeled RDX ($3N^{15}3C^{13}$) dissolved in acetonitrile. After 11 and 16 days respectively, the RDX concentration was below detection level (<500 ppb) in all labeled samples and unlabeled

live control microcosms, while no or little degradation was observed in the killed control samples (Figure 4.1 and Figure 4.2).

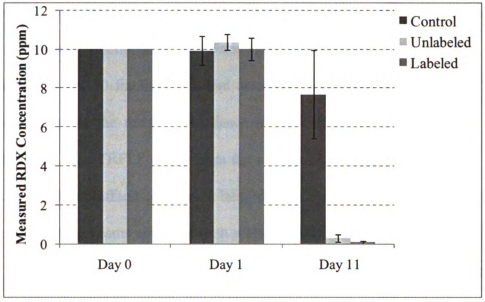


Figure 4.1 - RDX concentration in SIP microcosms (triplicate live controls, killed controls and labeled samples) set up with soil 3 (Intial RDX concentration – 45 μM) and with acetonitrile

All microcosms were allowed to go to O_2-depleted conditions.

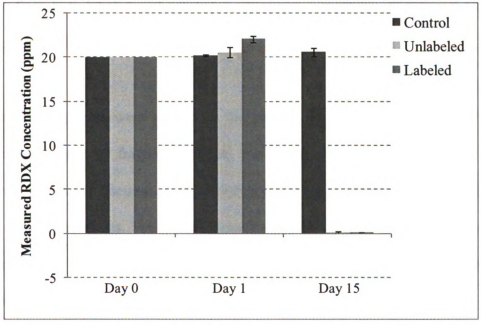


Figure 4.2 - RDX concentration in microcosms (triplicate live controls, killed controls and labeled samples) set up with soil 3 (Intial RDX concentration – 45 μM) and with acetonitrile

All microcosms were allowed to go to O_2-depleted conditions.

4.3.2 TRFLP results of SIP

DNA extracts from the labeled and unlabeled RDX amended soil samples were subject to ultracentrifugation, fractionation of ultracentrifuged samples, followed by TRFLP analysis on the first 10 fractions that had detectable amplified DNA. The TRFLP data were used to assess the relative abundance of each fragment in fractions of varying buoyant density. The TRFLP trends from the microcosms amended with 45 μM did not show any significant difference between labeled and unlabeled fractions. However, in the fractions from microcosms amended with a higher concentration of RDX (90 μM), one TRFLP fragment (260 bp) showed a trend of label uptake in two of the three triplicates. In other words, this fragment was of higher relative abundance in the heavier fractions from labeled samples when compared to the heavier fractions (of comparable BD) from the unlabeled samples (triplicates) (Figure 4.3). TRFLP profiles of the first few heavy fractions from both labeled and unlabeled samples are presented in figure 4.4 and 4.5, showing the dominance of 260-bp fragment.

While many fragments were present in the heavier fractions of both the labeled and unlabeled samples, only fragments of size 260 bp showed a trend of increased relative abundance, in heavier fractions from labeled samples when compared to heavier fractions from unlabeled treatments. The relative abundance of the other peaks were similar in both treatments. Some terminal fragments (data not shown) had variable trends in each of the triplicates and were not considered, as the trend was not consistent.

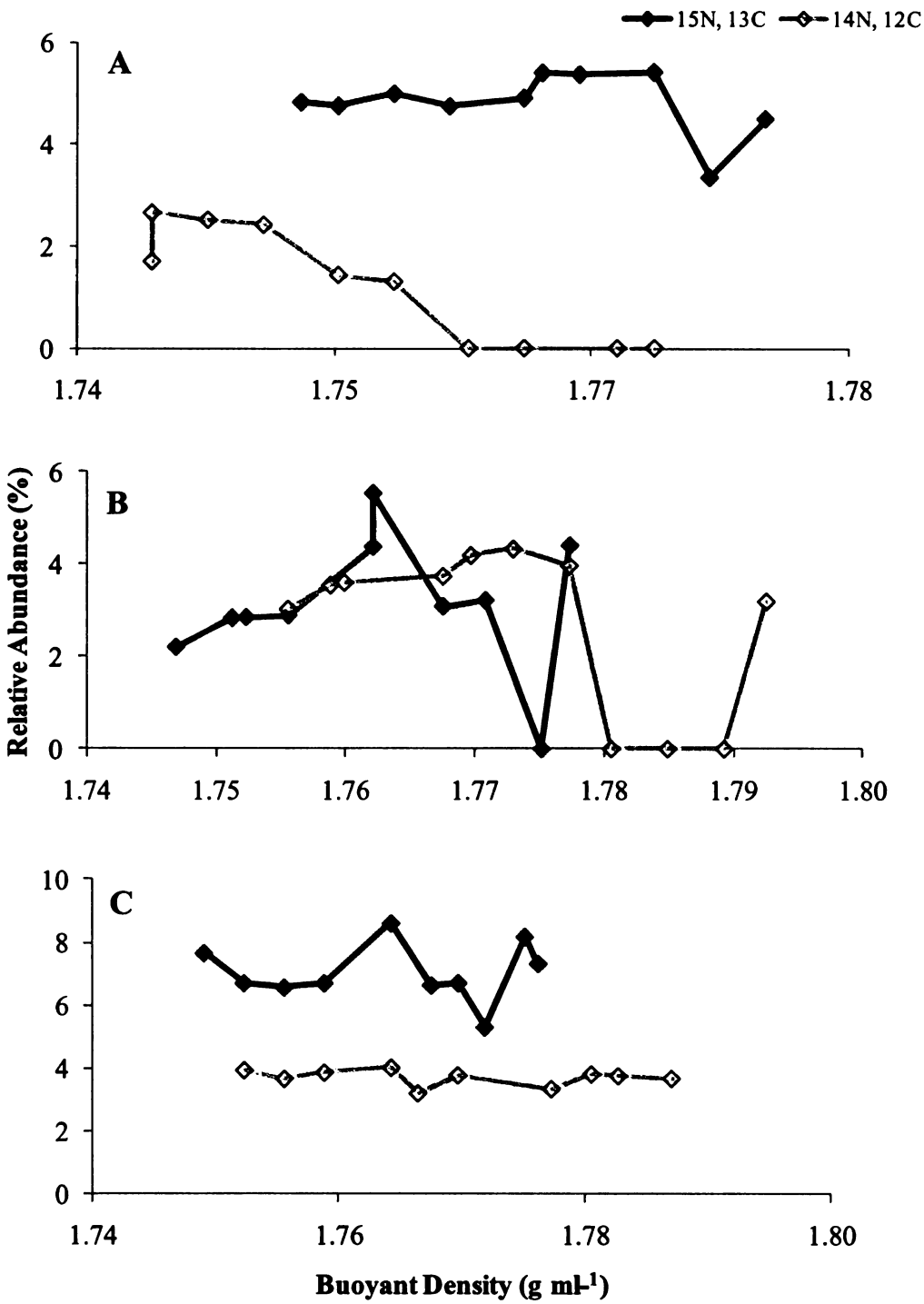


Figure 4.3 - The relative abundance of the fragment of length 260 bp over a range of buoyant density from DNA extracted from triplicate microcosms (A, B, C) on day 16 from samples amended with 90μM of labeled and unlabeled

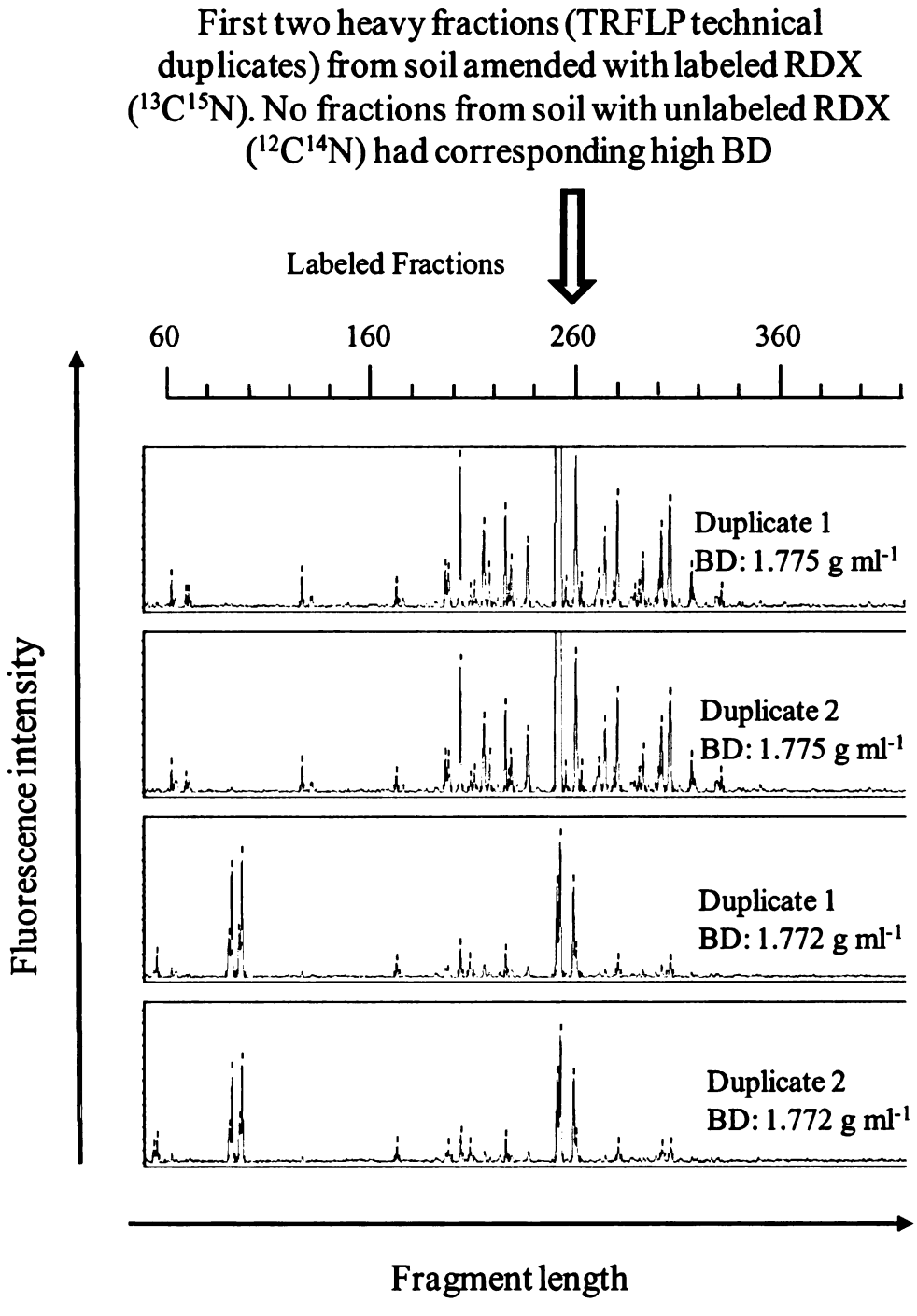


Figure 4.4 - TRFLP profiles of first two heavy fractions from soil amended with labeled RDX ($^{13}C^{15}N$) showing 260-bp fragment and its abundance

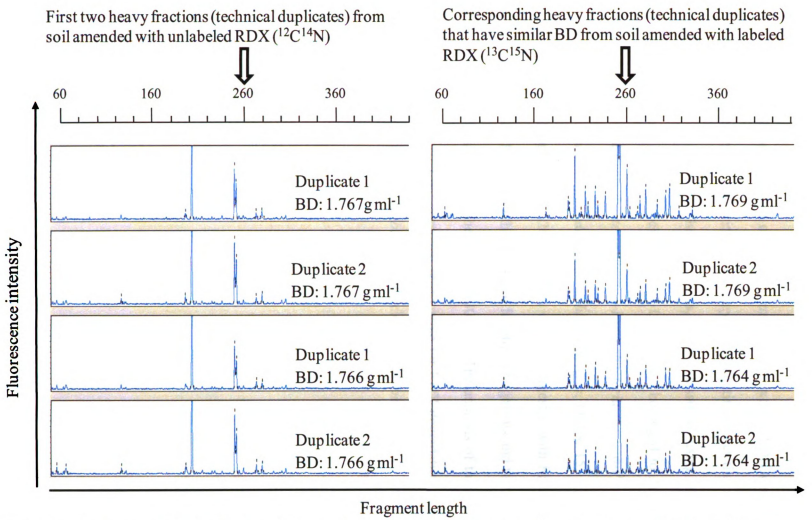


Figure 4.5 - Dominance of 260-bp fragment in heavy fractions from labeled samples in comparison to abundance in heavy fractions (of corresponding BD) from unlabeled samples

4.3.3 Sequencing

Partial 16S rRNA gene sequences obtained from total soil extracted DNA were virtually digested (restrictionmapper.org) with HaeIII enzyme, to identify clones that corresponded to the fragments of interest. The sequences, when classified using the Classifier (Ribosomal Database Project, Michigan State University), belonged to *Actinobacteria*, *Acidobacteria*, *α-Proteobacteria*, *γ-Proteobacteria*, *δ-Proterobacteria*, *Verrucomicrobiae*, *Gemmatimonadetes* and *Sphingobacteria*. Of the 155 clones, 19 had terminal fragment lengths of 258-264 bp when virtually digested with HaeIII restriction enzyme. This slight difference in the measured length of fragments and that predicted by sequence data has observed in other studies (30, 60). The analysis of these partial 16S rRNA sequences indicated the organism responsible for RDX degradation might belong to either of the following: *Sphingobacteria* (18 clones), *Acidobacteria* (1 clone).

4.4 Discussion

The extraction efficiency of RDX has been found to be high in previous studies and correlates well with the value observed here when employing an acetonitrile and sonication extraction procedure (6). Since the microcosms were setup and allowed to go to O_2 depleted conditions the time when RDX degradation begins in not known. Notably, no degradation was observed in microcosms that were aerated. The initial amount of O_2 varied based on the volume of the bottle used. The limited amount of data generated did not allow the calculation of RDX half lives, however others have reported values between 94 to 154 days (49).

The trend of increased relative abundance of fragment 260 bp, in heavier fractions from labeled samples when compared to heavier fractions from unlabeled treatments, indicates label uptake by the organism represented by this fragment. Unfortunately, there was no opportunity to control for label cross feeding in these experiments.

Although many strains have been isolated with RDX biodegradation potential there has ben only one previous study that has employed SIP to identify RDX biodegraders active in situ (80). In that study, RDX degradation was examined in microcosms constructed with material from RDX contaminated aquifer and groundwater. The microcosms were constructed with unlabeled or ring-^{15}N-labeled RDX. Following RDX uptake, the DNA from the labeled RDX amended microcosms were extracted and ultracentrifuged in CsCl-EtBr solution. The ^{15}N labeled heavy DNA was extracted using a needle and syringe, as

opposed to fractioning as employed in the current study. The 'heavy DNA' was amplified PCR amplification employing specific primers targeting the *xplA* gene. This gene has been associated with RDX biodegradation. They derived 5 xplA-like genes and showed that the 16S rRNA gene sequences of the clones from the 'heavy DNA' containing RDX biodegraders belonged to *Actinobacteria*, α-*Proteobacteria* and γ–*Proteobacteria*.

In the current study, the SIP data indicate an organism belonging to the phylum *Bacteriodetes* and class *Sphingobacteria*. These are Gram negative bacteria found in grow in aerobic or anaerobic conditions (44). *Sphingobacteria* have been isolated from a variety of environments and enrichments including, oil-contaminated sediments, consortium of polycyclic aromatic hydrocarbon (PAH) degraders, community of trichloroethylene (TCE) degraders and community of dentrifiers (28, 61, 64, 77, 93). Though *Sphingobacteria* have not been previously linked with RDX biodegradation they have been noted for their ability to biotransform a number of xenobiotic compounds such as methyl tert-butyl ether (MTBE) (59), tetracycline (42) and polychlorinated biphenyls (PCBs) (63). Many strains of *Acidobacteria* have been enriched in the past with ability to degrade MTBE (59) and BTEX. Previous studies have primarily isolated RDX degrading microorganisms from explosive contaminated soil or water. In this study RDX degraders were studied in an agricultural soil. Since organisms from neither of these classes of bacteria have been associated with RDX degradation before, our results suggest that RDX biodegradation might be possible by phylogenetically diverse microbial populations.

4.5 Conclusion and future studies

Bioremediation has the potential to be an effective tool for cleanup of sites contaminated with RDX. However, the approach requires knowledge of microorganisms that are capable of metabolizing the compound, the necessary site conditions for successful remediation, the biodegradation pathway, and the end products produced. In addition, insight into the enzymes responsible for RDX biodegradation provides the ability to probe for other organisms that possess similar functionality. This study aimed at identifying microorganisms capable of utilizing RDX by employing SIP. To our knowledge this is the first study to use ^{15}N DNA-SIP with fractioning and TRFLP to study RDX biodegradation. The partial 16S rRNA sequences of the RDX degrading bacteria were classified as *Sphingobacteria* or *Acidobacteria*. As suggested by Binks et. al. (1995) (17) using non-indigenous microorganism and linking their survival with contaminant provides for a reliable bioremediation strategy and requires identification of non-indigenous strains capable of degrading the pollutant. All RDX degrading strains isolated so far have been isolated from explosive contaminated soil or water and this is the first study that identified RDX degrading microorganisms from an agricultural soil amended with biosolids.

However it is not known whether the organism was indigenous to the agricultural soil or to the biosolids. Further studies to identify the biodegradation products of RDX biodegradation would clearly be useful. As mentioned earlier (Chapter 2) confirming that RDX mineralization occurs is crucial to ensure no toxic byproducts accumulate in the system (66). Employing mass spectrometry to identify intermediates and biodegradation

end products will also facilitate the identification of the biodegradation pathway. Specific functional genes to RDX degradation (*xplA*) could be used for PCR amplification to compliment the current study. Real-time PCR using specific functional genes helps in studying the relative abundance of these genes in fractions (labeled and unlabeled) of varying buoyant density profile. Further conducting SIP over time will help in identifying and avoiding any cross feeding issues if any.

APPENDIX A

Table A.1 - List of soils tested and experimental details

Soil No.	Soil Type	Crop Grown	Type of Setup	RDX degradation
1	Agricultural	Alfalfa	With 45 μM RDX, with 100 μL acetonitrile and was not aerated	No
2	Agricultural	Corn	With 45 μM RDX, with 100 μL acetonitrile and was not aerated	No
3	Agricultural	Soybean	1. With 45 μM RDX, with 100 μL acetonitrile and was not aerated	Yes
			2. With 45 μM RDX, with 100 μL acetonitrile and was aerated	No
			3. With 90 μM RDX, (acetonitrile evaporated) and was not aerated	No
			4. With 90 μM RDX, with 200 μL acetonitrile and was not aerated for stable isotope probing	Yes
4	BTEX Contaminated	NA	1. With 45 μM RDX, with 100 μL acetonitrile and was not aerated	Yes
			2. With 45 μM RDX, with 100 μL acetonitrile and was aerated	No
5	BTEX Contaminated	NA	With 45 μM RDX, with 100 μL acetonitrile and was aerated	No
6	Agricultural	Corn	With 45 μM RDX, with 100 μL acetonitrile and was aerated	No
7	Agricultural	Soybean	With 45 μM RDX, (acetonitrile evaporated) and was not aerated	Yes
8	Agricultural	Light red kidney	With 45 μM RDX, (acetonitrile evaporated) and was not aerated	Yes
9	Agricultural	Corn	With 45 μM RDX, (acetonitrile evaporated) and was not aerated	Yes
10	Agricultural	Corn	With 45 μM RDX, (acetonitrile evaporated) and was not aerated	Yes

APPENDIX B

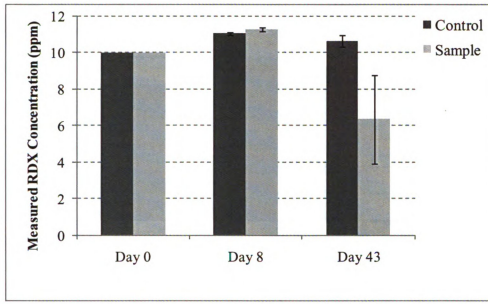


Figure B.1 - RDX concentration in microcosms (triplicate live and killed controls) set up with soil 1 and was allowed to go to O_2-depleted conditions

- No significant degradation on both day 8 and day 43 as verified by ANOVA test.

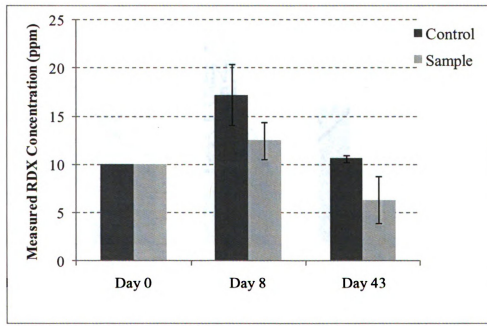
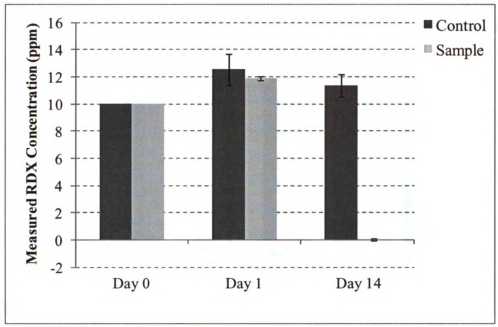


Figure B.2 - RDX concentration in microcosms (triplicate live and killed controls) set up with soil 2

- No significant degradation on both day 8 and day 43 as given by ANOVA test.

(A)



(B)

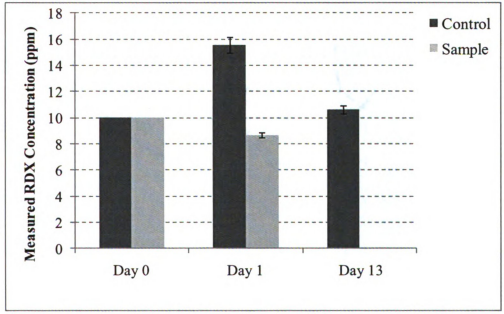
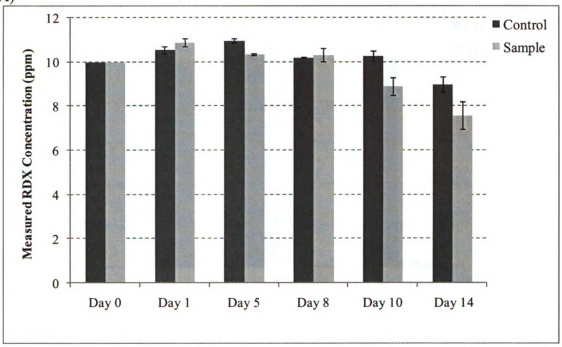


Figure B.3 - RDX concentration in microcosms (triplicate live and killed controls) **set up with** soil 3 and was allowed to go to O_2-depleted conditions (160 mL bottles)

- Figure (A) and (B) are results of similar but repeated experiments setup. 45**μM** RDX was degraded in 13 to 14 days.

(A)



(B)

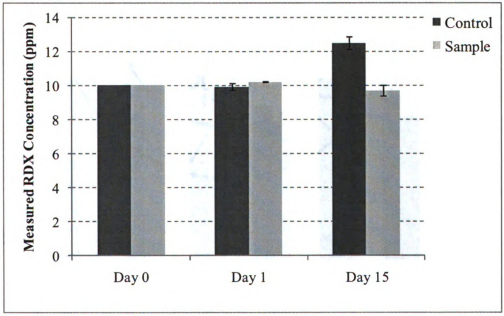


Figure B.4 - RDX concentration in microcosms (triplicate live and killed controls) set up with soil 3 and was aerated daily (160 mL bottles)

- Figure (A) and (B) are results of similar but repeated experiments, (A) continuously sampled (B) sampled on day 1 and day 15 only. No significant degradation was observed as verified by ANOVA test.

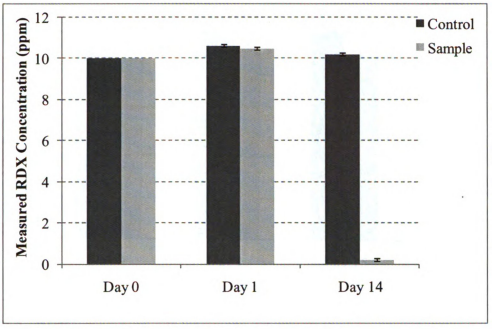


Figure B.5 - RDX concentration in microcosms (triplicate live and killed controls) set up with soil 4 and was allowed to go to O_2 depleted conditions (160 mL bottles).

- 45μM RDX was degraded in 14 days.

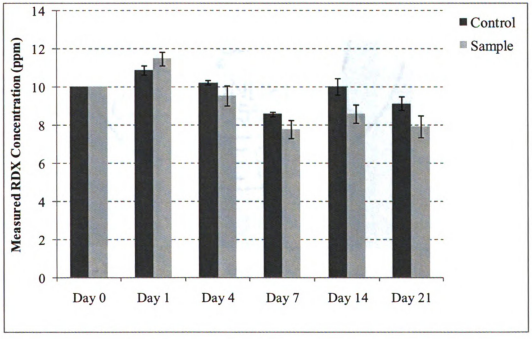


Figure B.6 - RDX concentration in microcosms (triplicate live and killed controls) set up with soil 4 and was aerated daily (160 mL bottles)

- No significant degradation until day 14 as verified by ANOVA test.

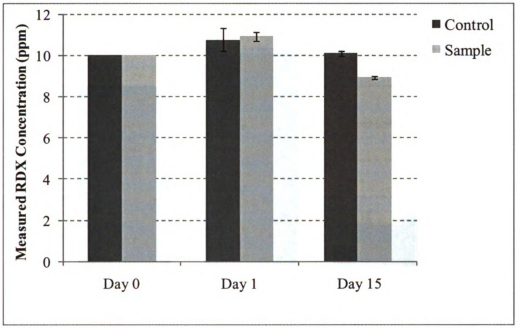


Figure B.7 - RDX concentration in microcosms (triplicate live and killed controls) set up with soil 5 and was aerated daily (160 mL bottles)

- No significant degradation until day 15 as verified by ANOVA test.

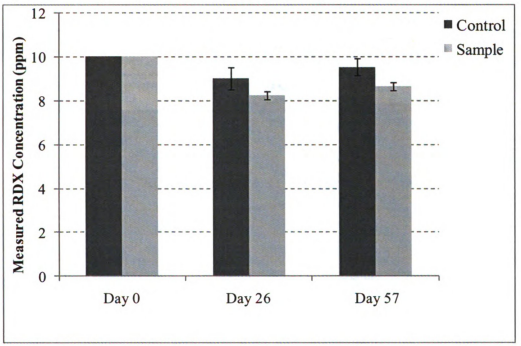


Figure B.8 - RDX concentration in microcosms (triplicate live and killed controls) set up with soil 6 and was allowed to go to O_2-depleted conditions

- No significant degradation until day 57 as verified by ANOVA test.

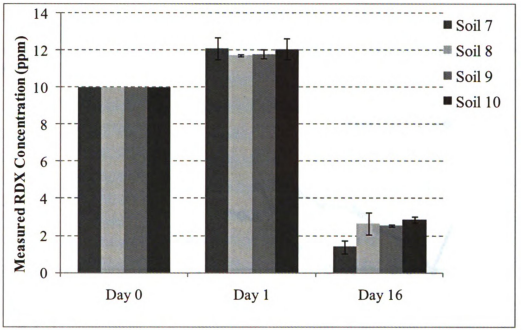


Figure B.9 - RDX concentration in microcosms (duplicate live) set up with soil 7, 8, 9, 10 and no acetonitrile (O_2-depleted conditions)

- 45μM RDX was degraded in 16 days.

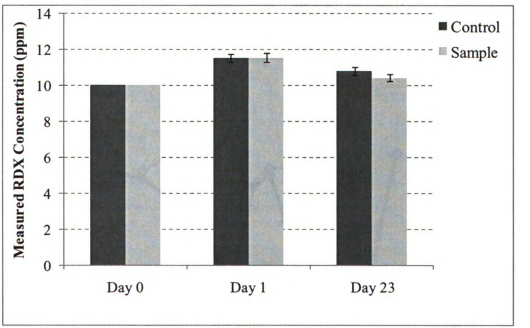


Figure B.10 - RDX concentration in microcosms (triplicate live and killed controls) set up with soil 8, no acetonitrile and was aerated daily

- No significant degradation until day 23 as verified by ANOVA test.

APPENDIX C

This section presents the relative abundance plots of T-RF's from SIP study setup with 90 μM RDX.

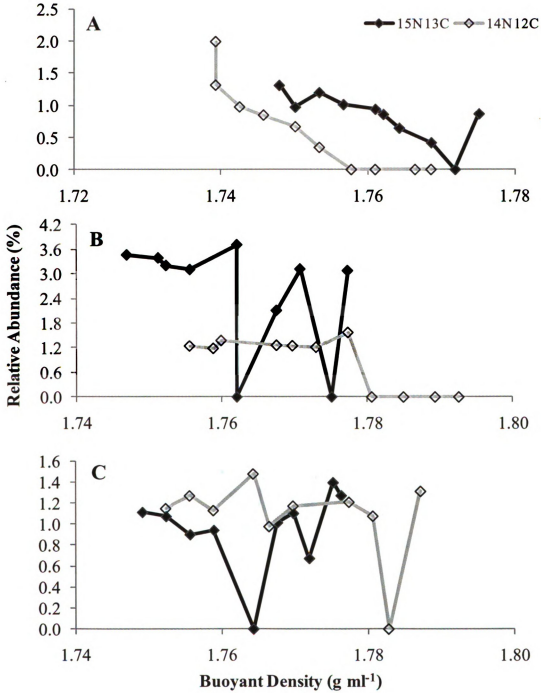


Figure C.1 - The relative abundance of the fragment of length 63 bp over a range of buoyant density from DNA extracted from triplicate microcosms (A, B, C) on day 16 from samples amended with 90 μM of labeled and unlabeled RDX

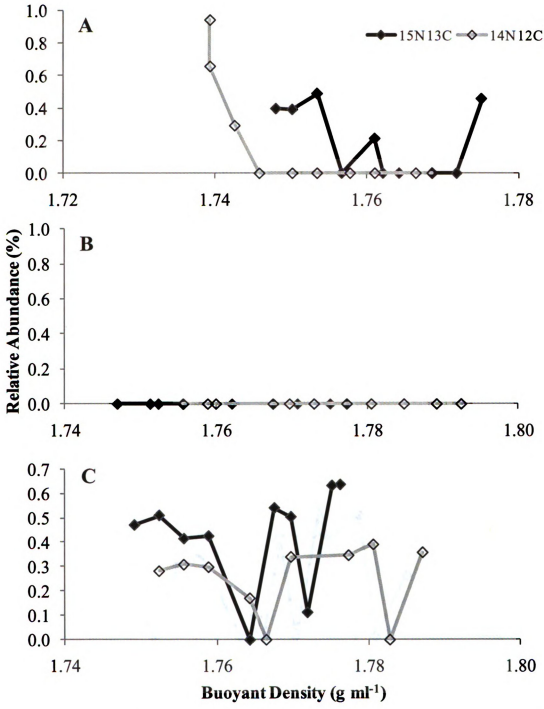


Figure C.2 - The relative abundance of the fragment of length 70.5 bp over a range of buoyant density from DNA extracted from triplicate microcosms (A, B, C) on day 16 from samples amended with 90 μM of labeled and unlabeled RDX

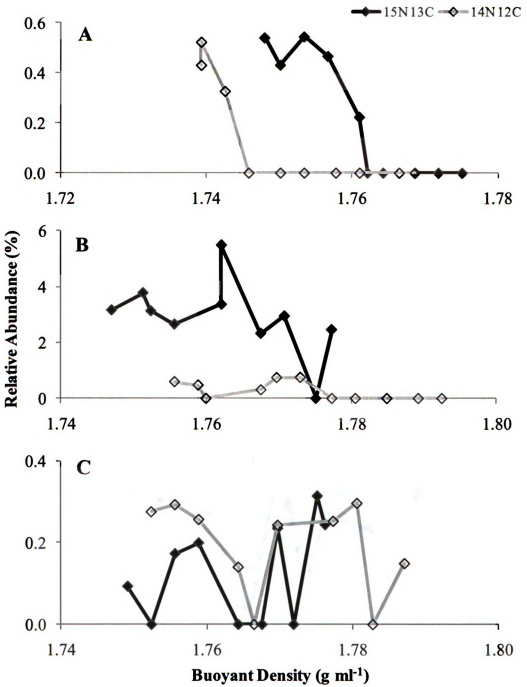


Figure C.3 - The relative abundance of the fragment of length 72 bp over a range of buoyant density from DNA extracted from triplicate microcosms (A, B, C) on day 16 from samples amended with 90 μM of labeled and unlabeled RDX

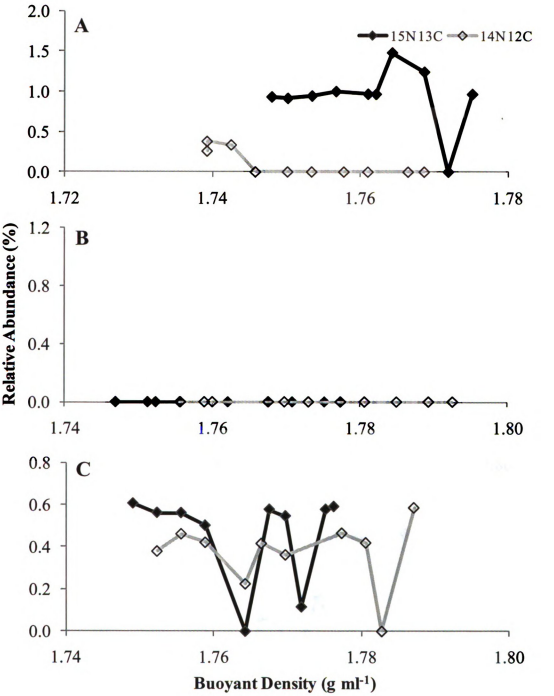


Figure C.4 - The relative abundance of the fragment of length 126 bp over a range of buoyant density from DNA extracted from triplicate microcosms (A, B, C) on day 16 from samples amended with 90 μM of labeled and unlabeled RDX

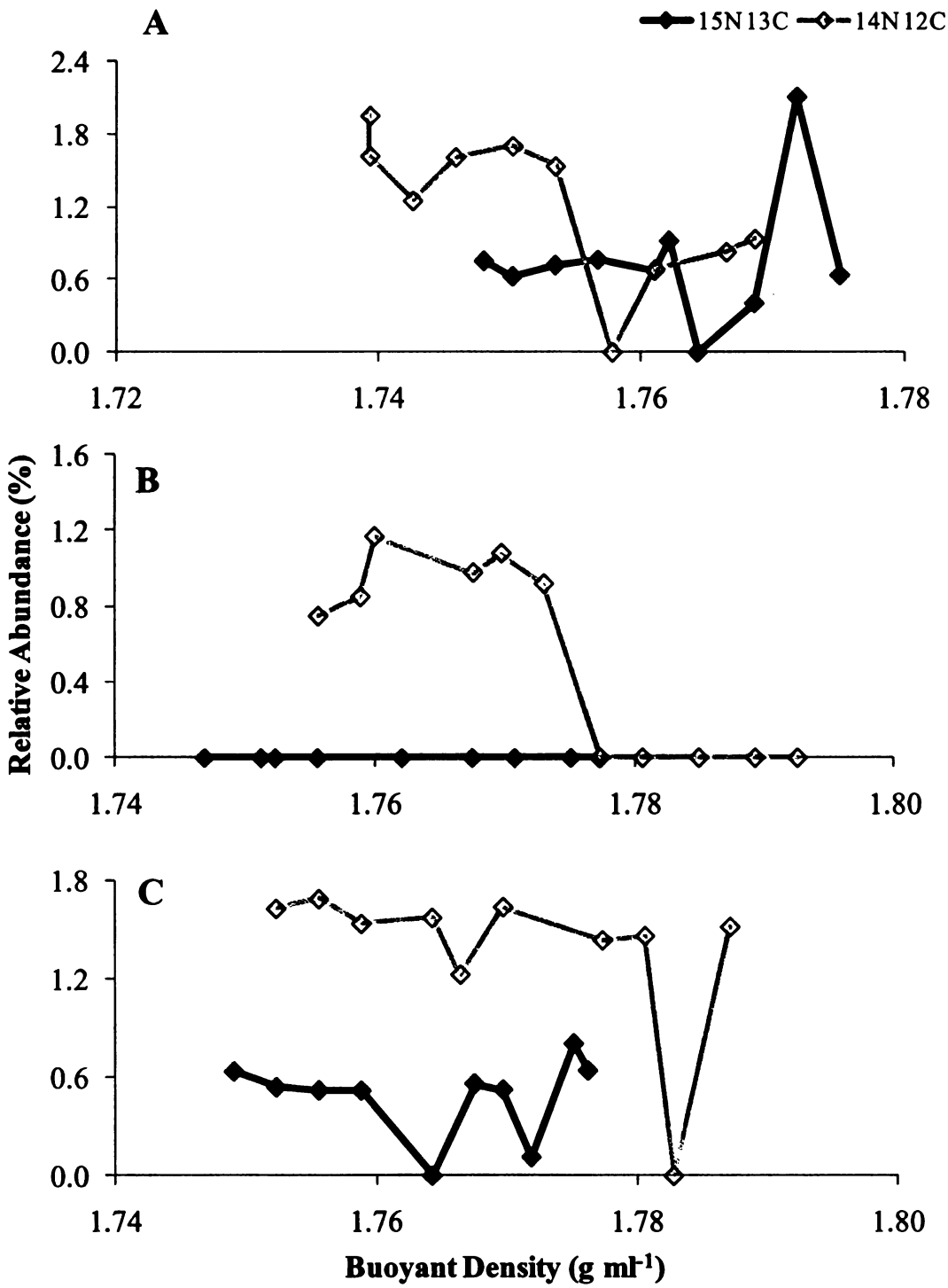


Figure C.5 - The relative abundance of the fragment of length 172 bp over a range of buoyant density from DNA extracted from triplicate microcosms (A, B, C) on day 16 from samples amended with 90 μM of labeled and unlabeled RDX

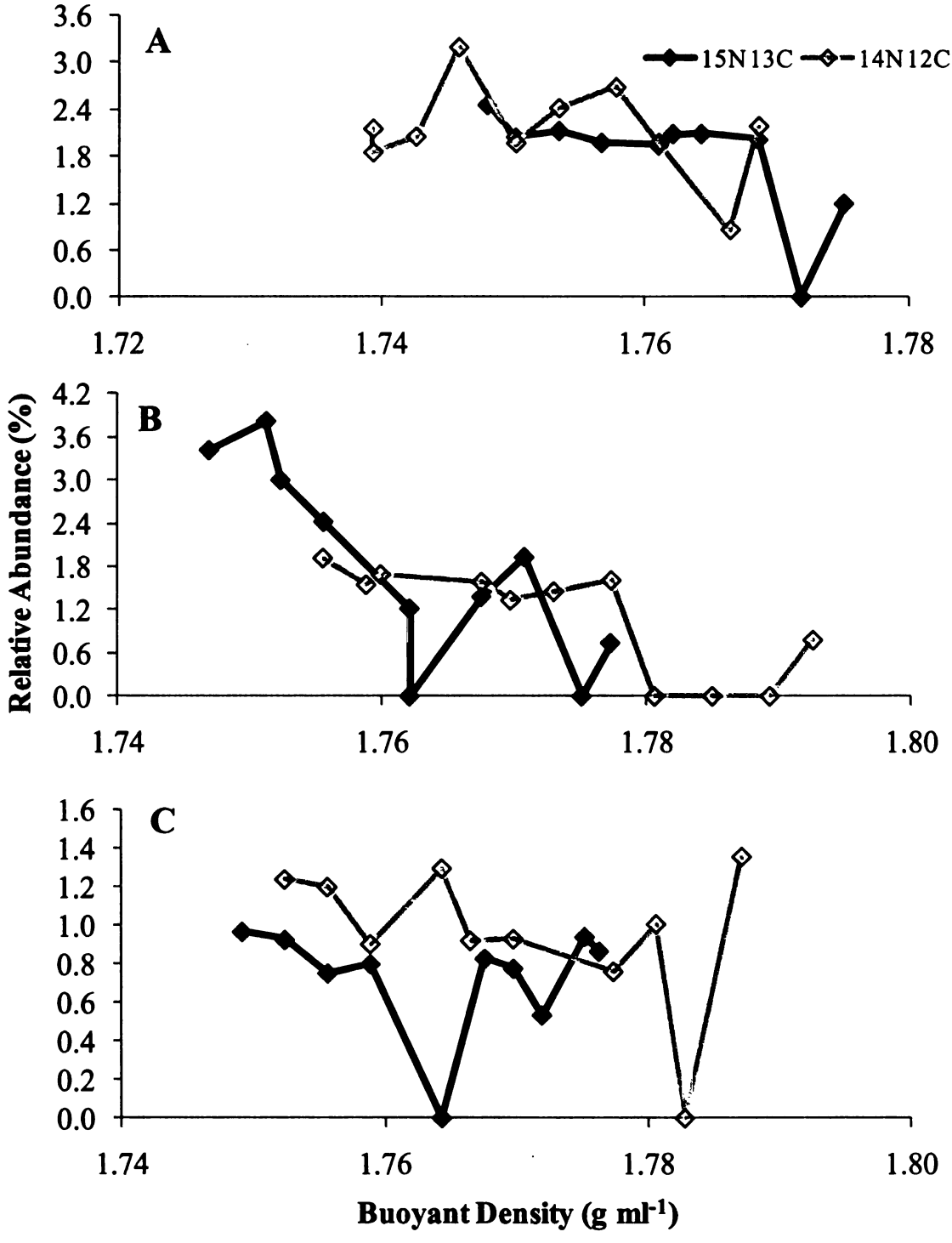


Figure C.6 - The relative abundance of the fragment of length 196 bp over a range of buoyant density from DNA extracted from triplicate microcosms (A, B, C) on day 16 from samples amended with 90 μM of labeled and unlabeled RDX

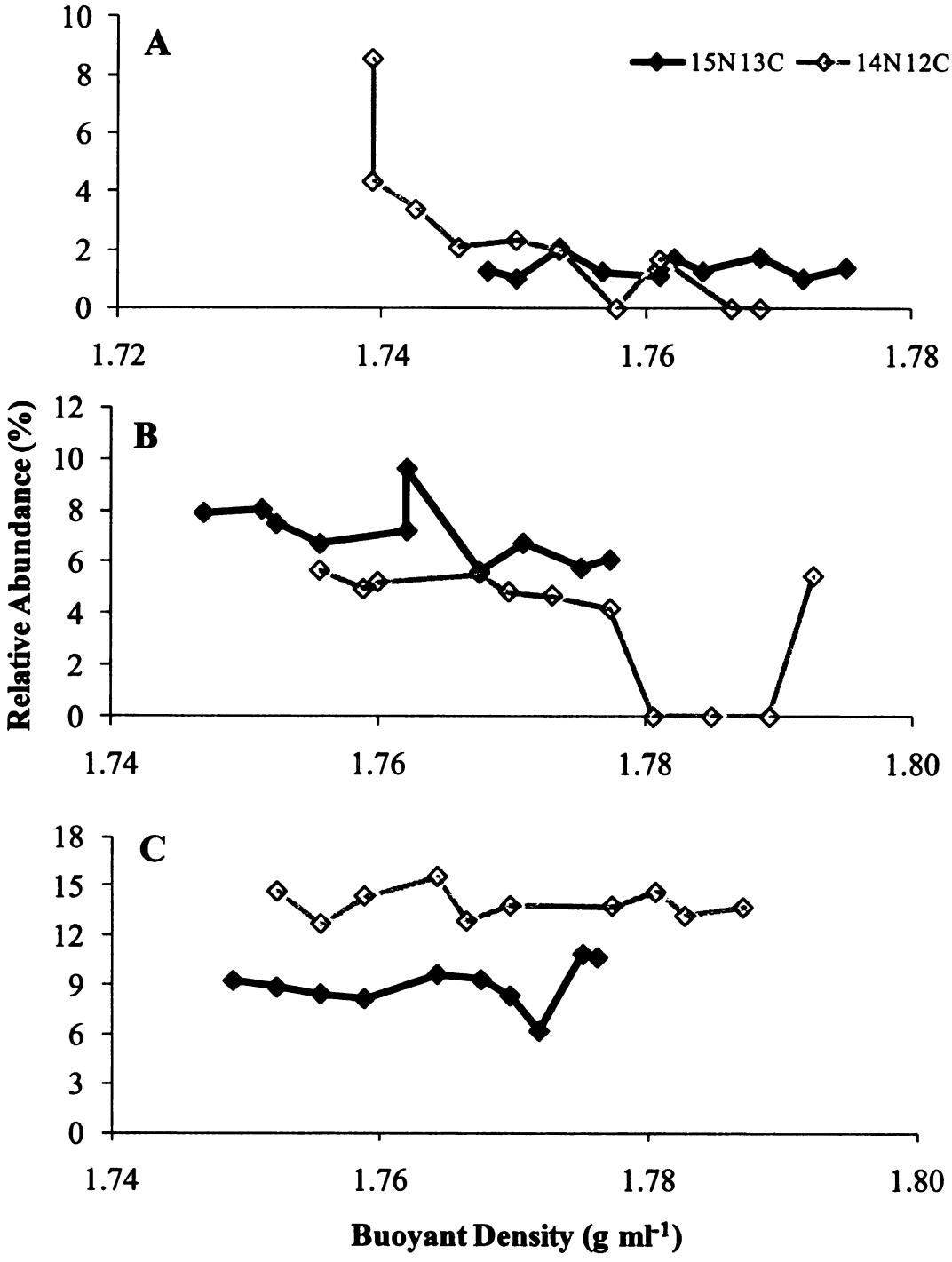


Figure C.7 - The relative abundance of the fragment of length 198 bp over a range of buoyant density from DNA extracted from triplicate microcosms (A, B, C) on day 16 from samples amended with 90 μM of labeled and unlabeled RDX

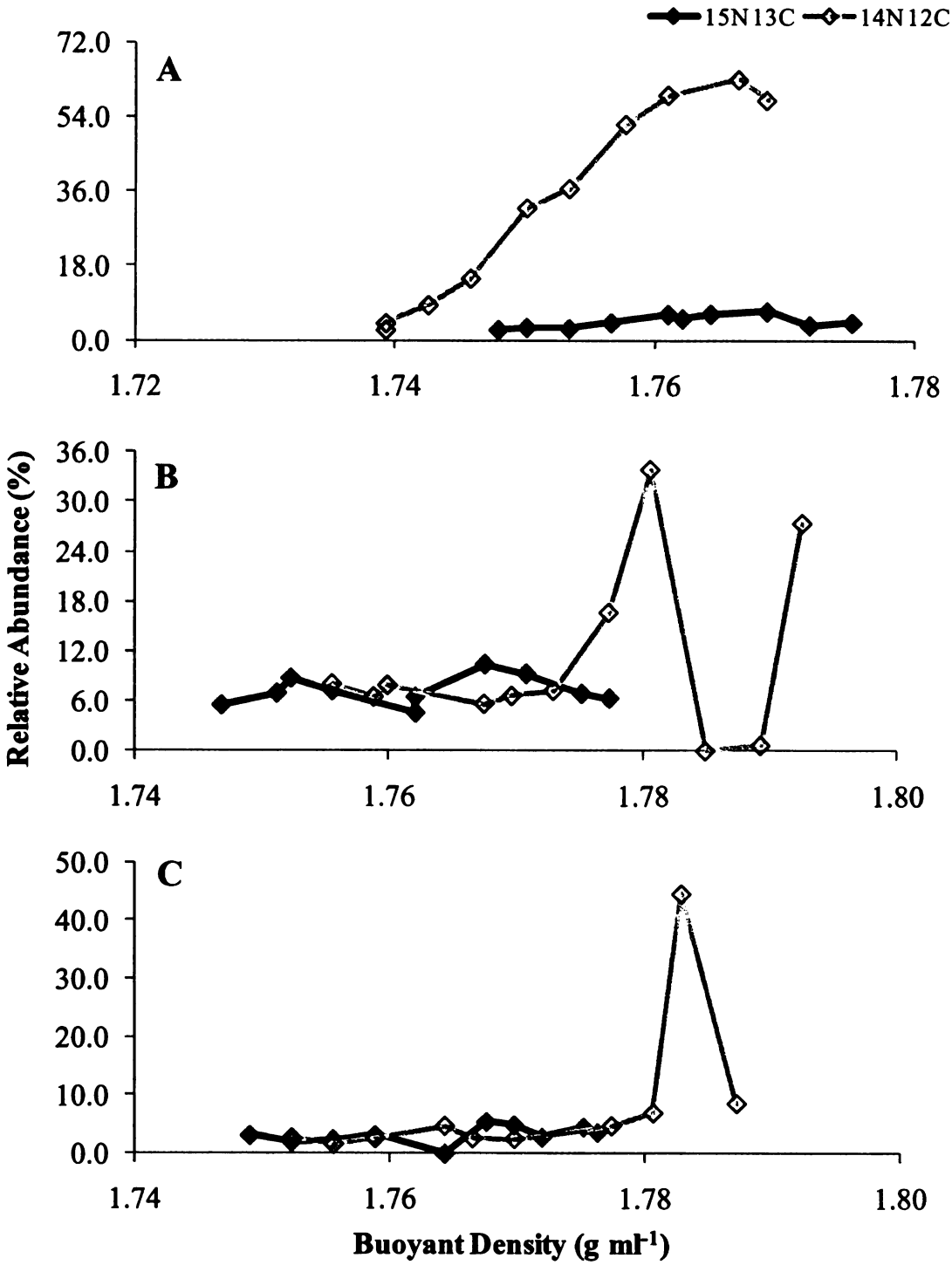


Figure C.8 - The relative abundance of the fragment of length 204 bp over a range of buoyant density from DNA extracted from triplicate microcosms (A, B, C) on day 16 from samples amended with 90 μM of labeled and unlabeled RDX

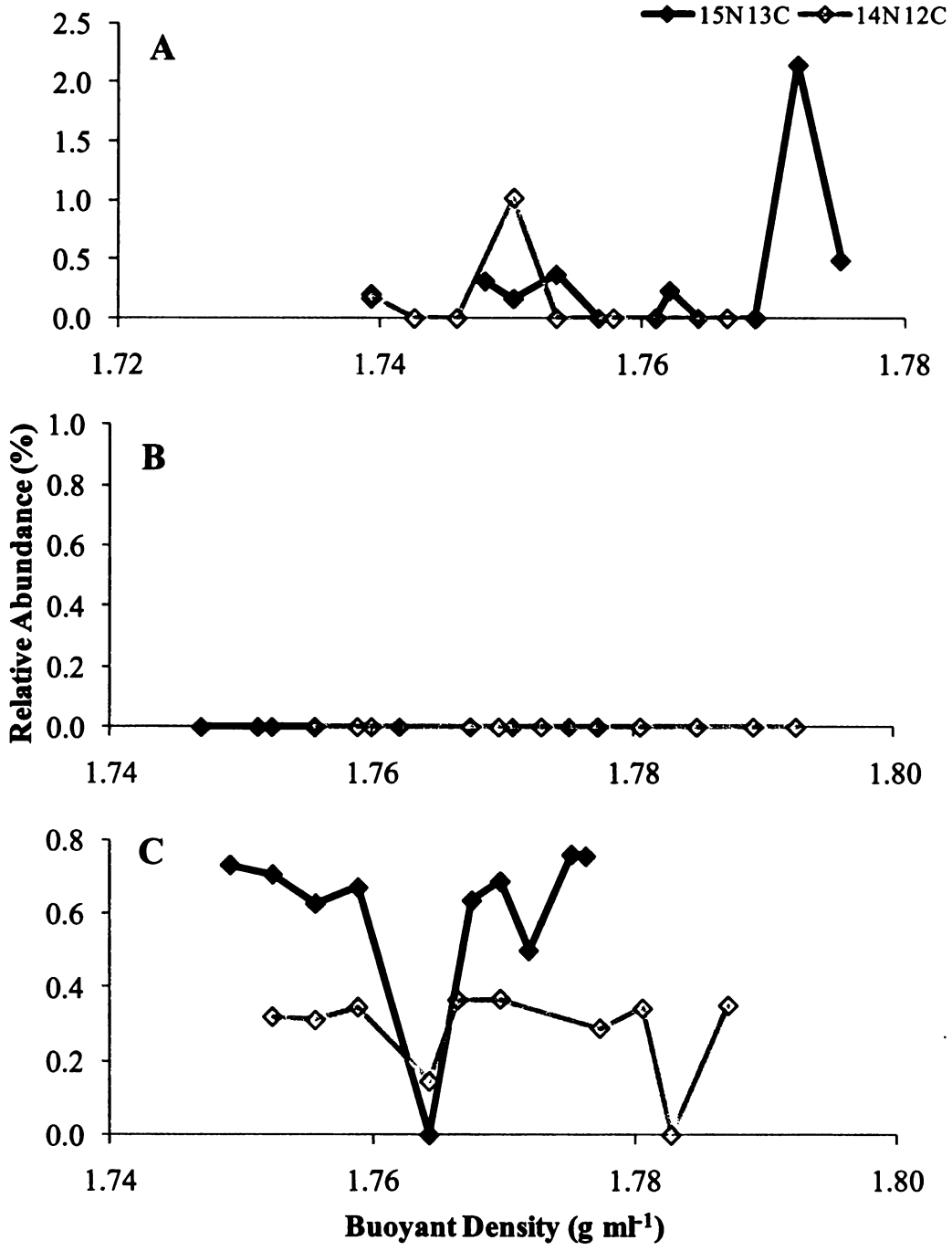


Figure C.9 - The relative abundance of the fragment of length 208 bp over a range of buoyant density from DNA extracted from triplicate microcosms (A, B, C) on day 16 from samples amended with 90 μM of labeled and unlabeled RDX

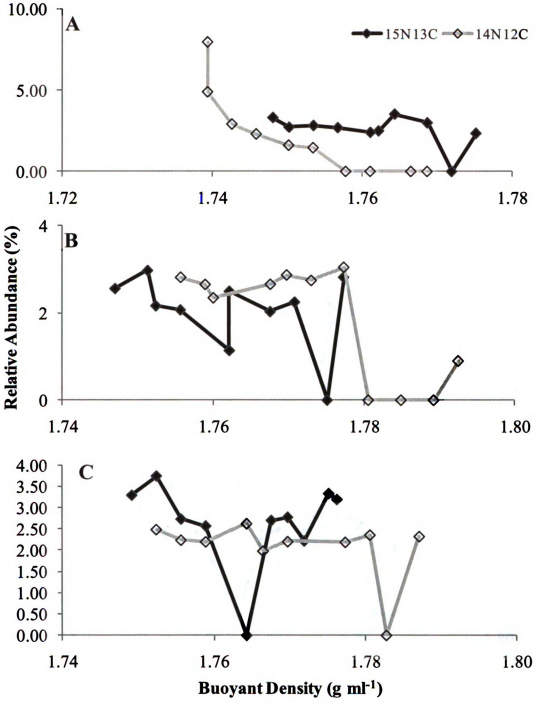


Figure C.10 - The relative abundance of the fragment of length 215 bp over a range of buoyant density from DNA extracted from triplicate microcosms (A, B, C) on day 16 from samples amended with 90 µM of labeled and unlabeled RDX

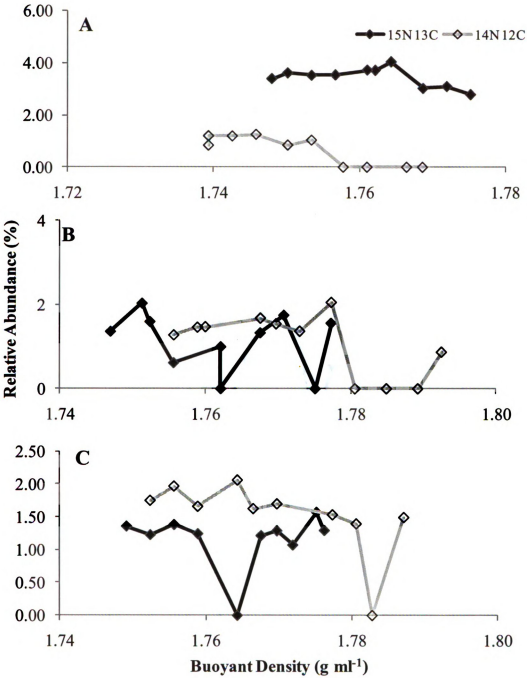


Figure C.11 - The relative abundance of the fragment of length 226 bp over a range of buoyant density from DNA extracted from triplicate microcosms (A, B, C) on day 16 from samples amended with 90 µM of labeled and unlabeled RDX

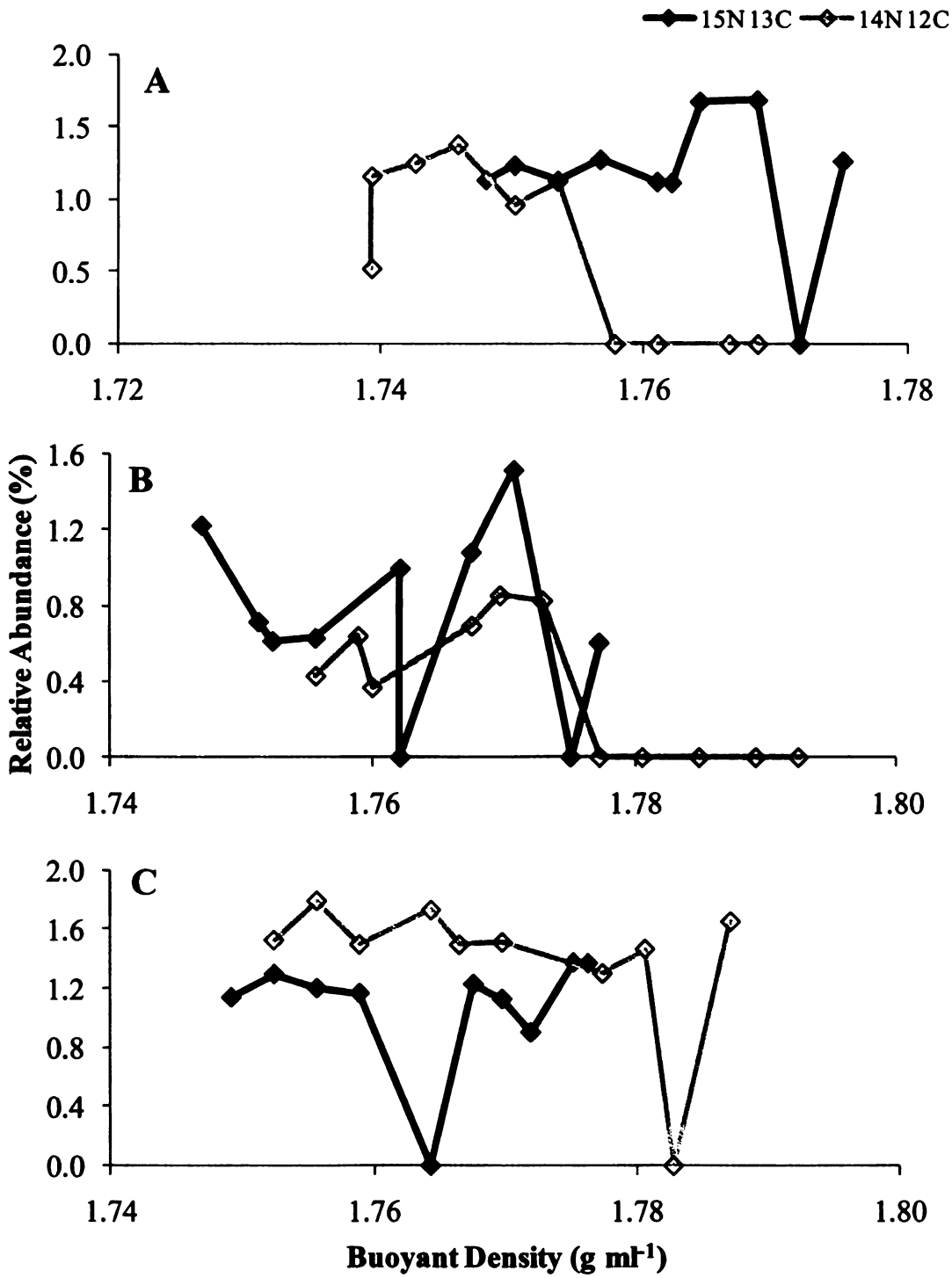


Figure C.12 - The relative abundance of the fragment of length 228 bp over a range of buoyant density from DNA extracted from triplicate microcosms (A, B, C) on day 16 from samples amended with 90 μM of labeled and unlabeled RDX

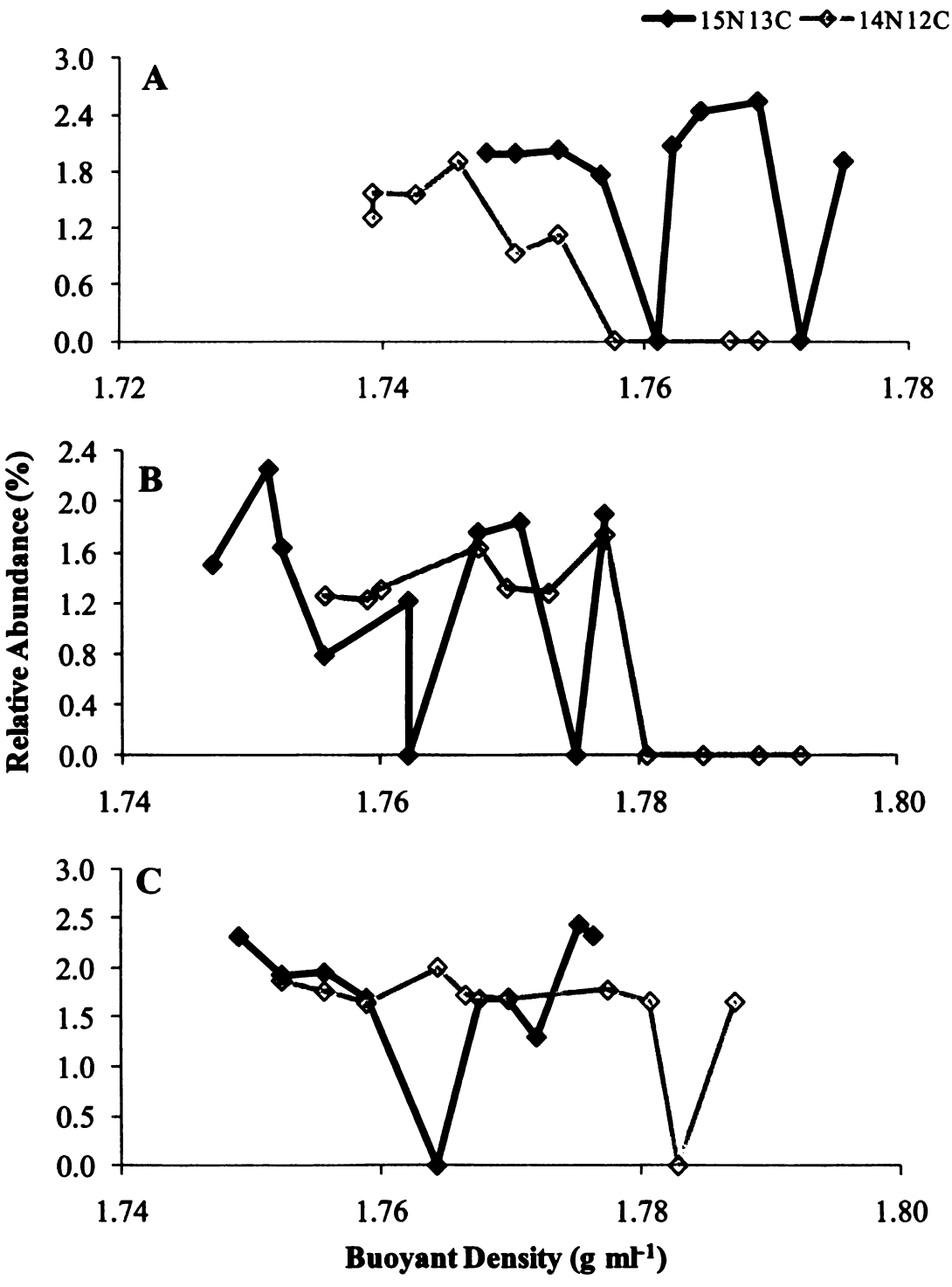


Figure C.13 - The relative abundance of the fragment of length 236 bp over a range of buoyant density from DNA extracted from triplicate microcosms (A, B, C) on day 16 from samples amended with 90 μM of labeled and unlabeled RDX

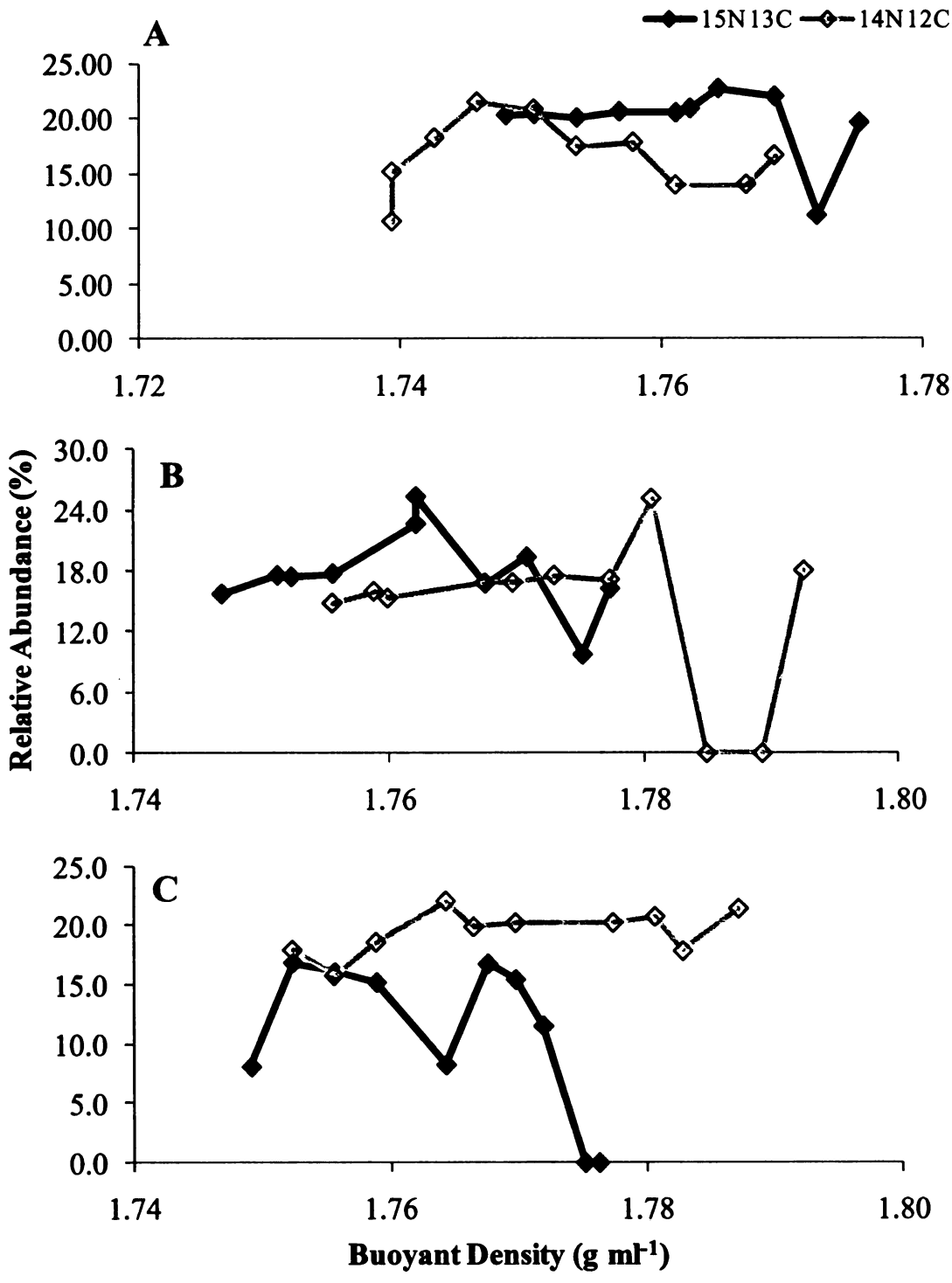


Figure C.14 - The relative abundance of the fragment of length 251 bp over a range of buoyant density from DNA extracted from triplicate microcosms (A, B, C) on day 16 from samples amended with 90 μM of labeled and unlabeled RDX

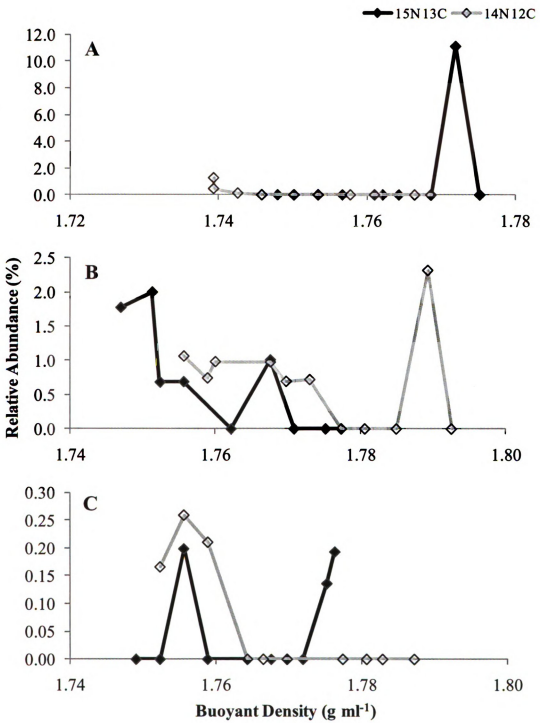


Figure C.15 - The relative abundance of the fragment of length 258 bp over a range of buoyant density from DNA extracted from triplicate microcosms (A, B, C) on day 16 from samples amended with 90 μM of labeled and unlabeled RDX

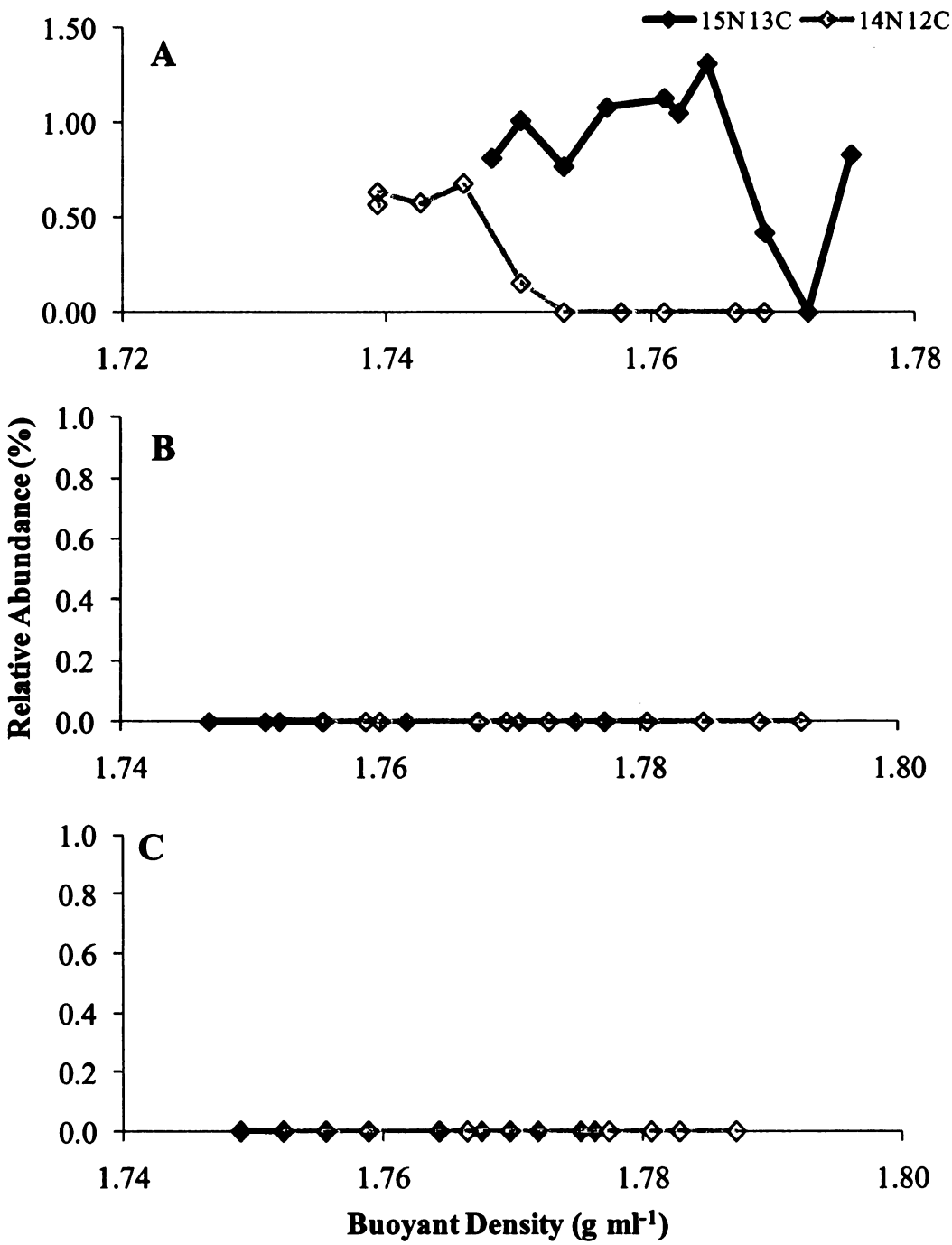


Figure C.16 - The relative abundance of the fragment of length 263 bp over a range of buoyant density from DNA extracted from triplicate microcosms (A, B, C) on day 16 from samples amended with 90 μM of labeled and unlabeled RDX

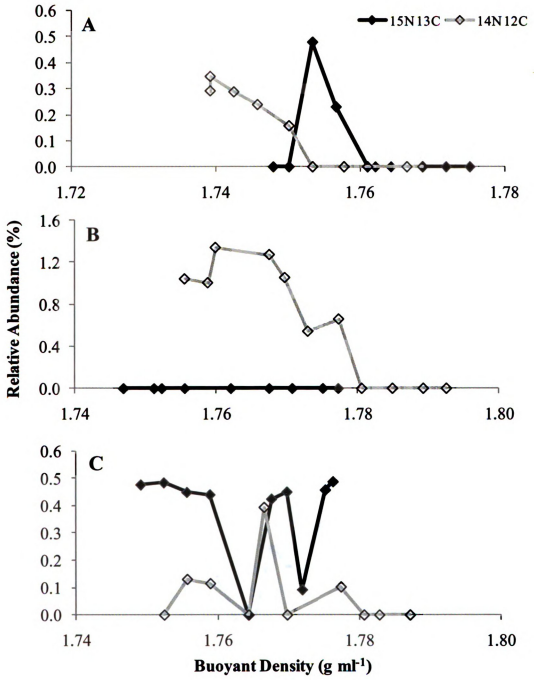


Figure C.17 - The relative abundance of the fragment of length 271 bp over a range of buoyant density from DNA extracted from triplicate microcosms (A, B, C) on day 16 from samples amended with 90 μM of labeled and unlabeled RDX

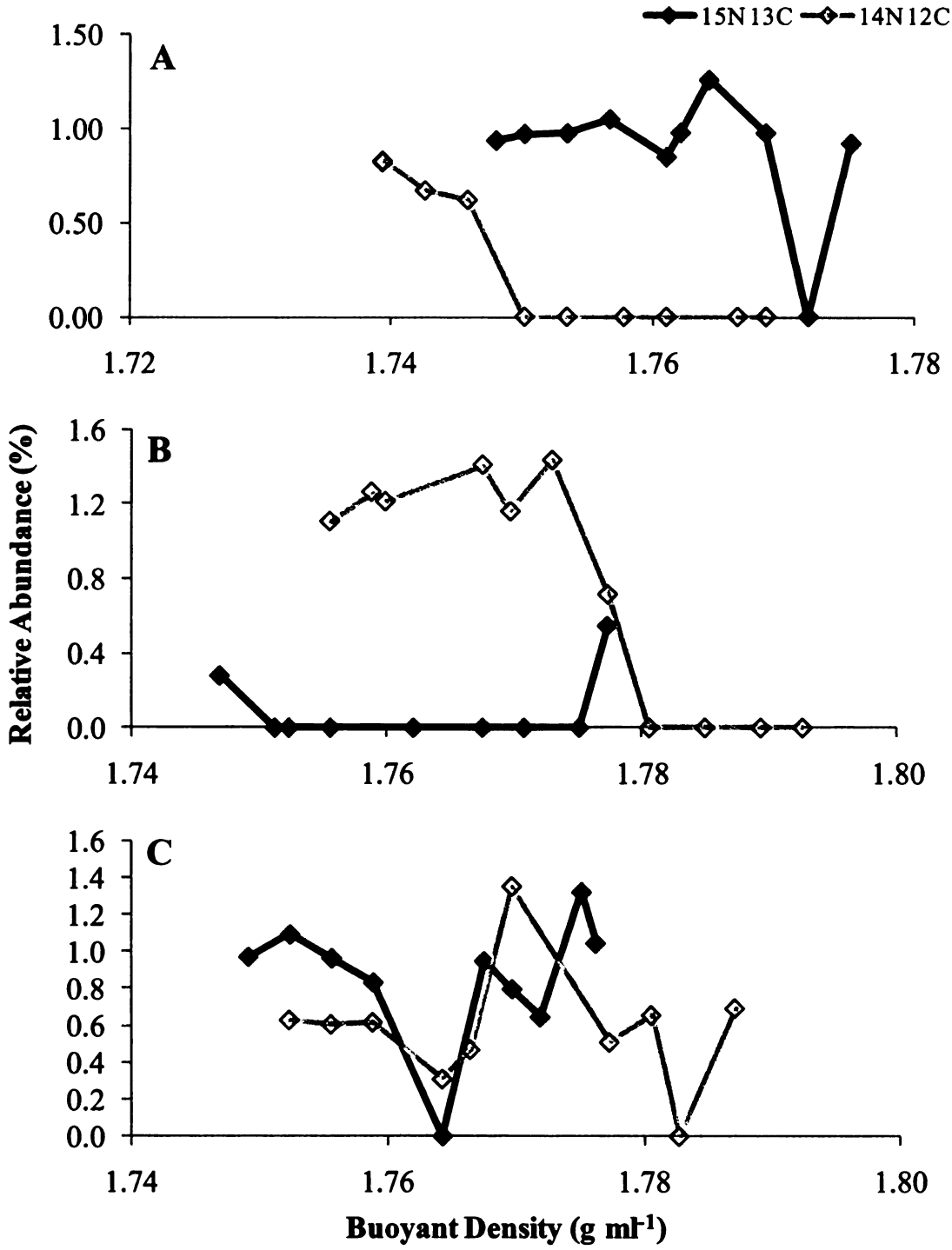


Figure C.18 - The relative abundance of the fragment of length 272 bp over a range of buoyant density from DNA extracted from triplicate microcosms (A, B, C) on day 16 from samples amended with 90 μM of labeled and unlabeled RDX

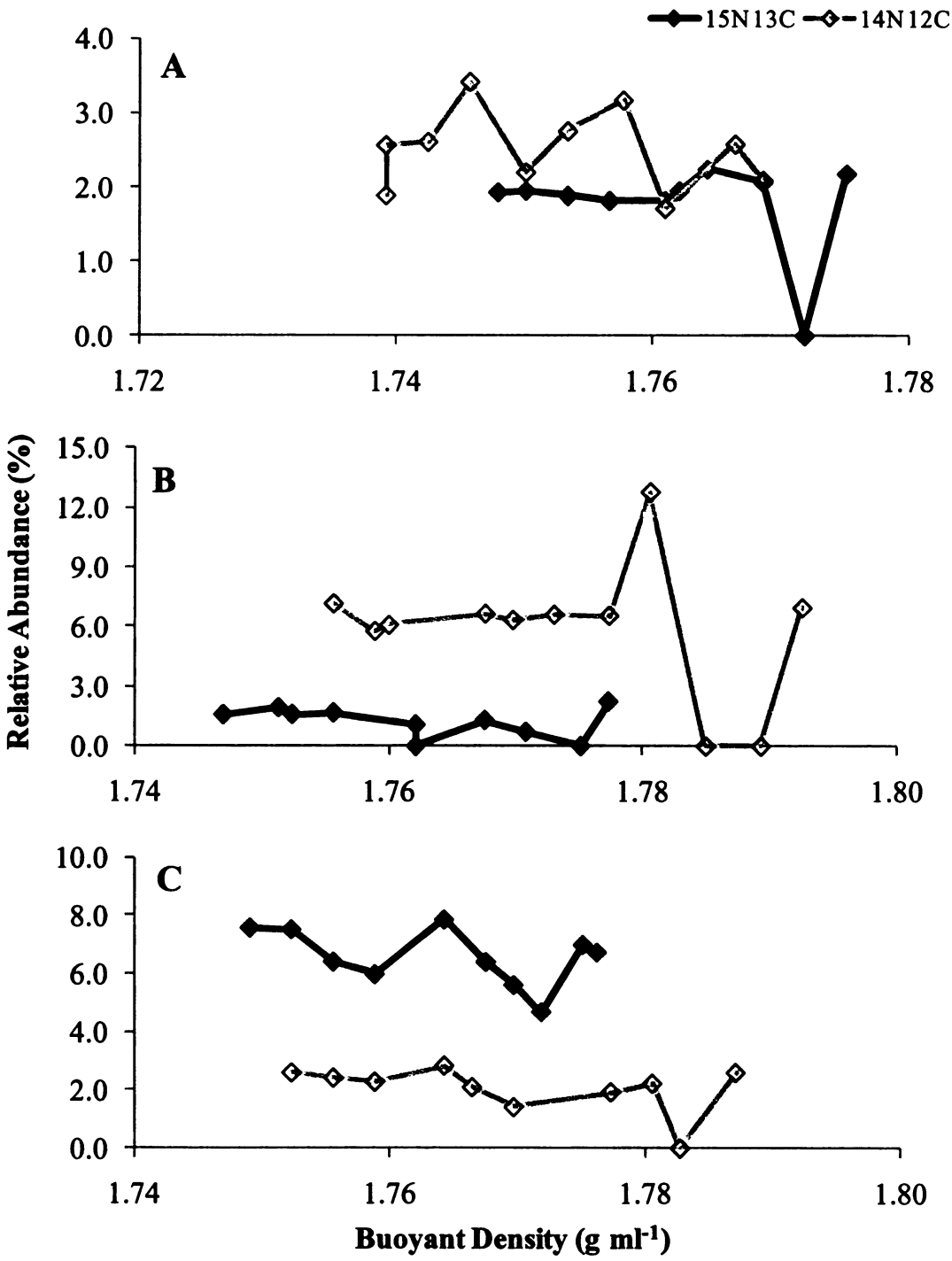


Figure C.19 - The relative abundance of the fragment of length 274 bp over a range of buoyant density from DNA extracted from triplicate microcosms (A, B, C) on day 16 from samples amended with 90 μM of labeled and unlabeled RDX

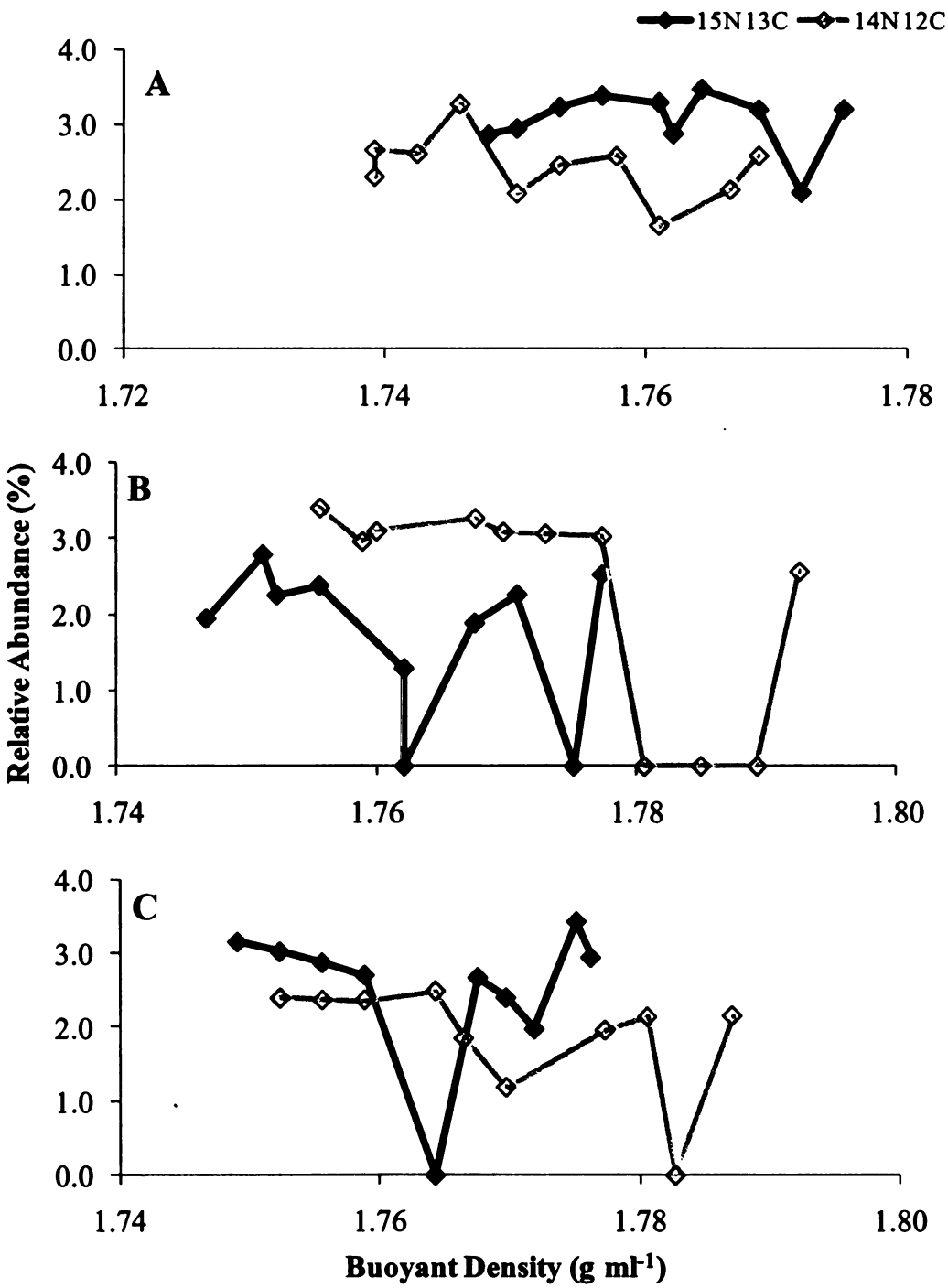


Figure C.20 - The relative abundance of the fragment of length 281 bp over a range of buoyant density from DNA extracted from triplicate microcosms (A, B, C) on day 16 from samples amended with 90 μM of labeled and unlabeled RDX

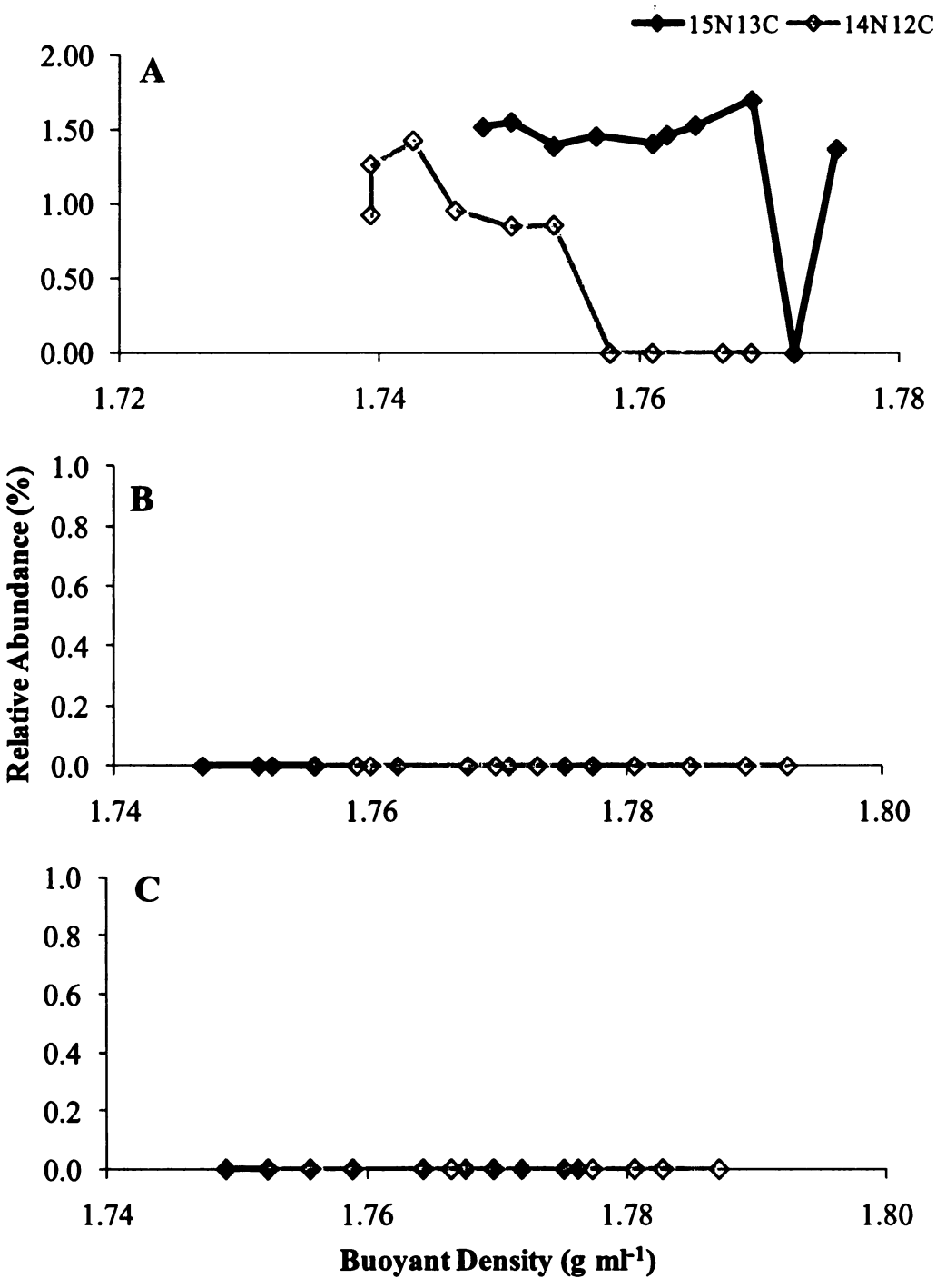


Figure C.21 - The relative abundance of the fragment of length 292 bp over a range of buoyant density from DNA extracted from triplicate microcosms (A, B, C) on day 16 from samples amended with 90 μM of labeled and unlabeled RDX

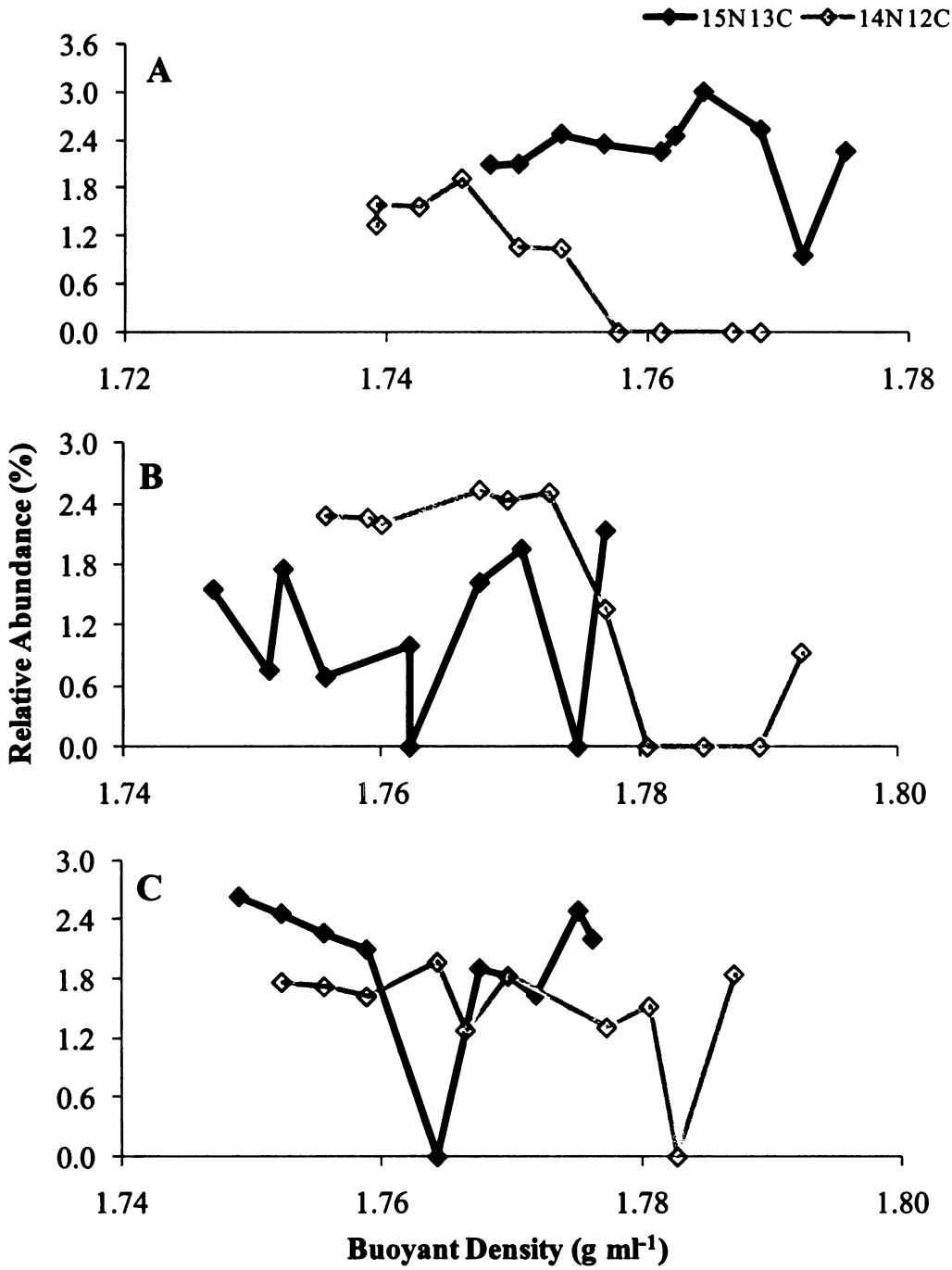


Figure C.22 - The relative abundance of the fragment of length 302 bp over a range of buoyant density from DNA extracted from triplicate microcosms (A, B, C) on day 16 from samples amended with 90 μM of labeled and unlabeled RDX

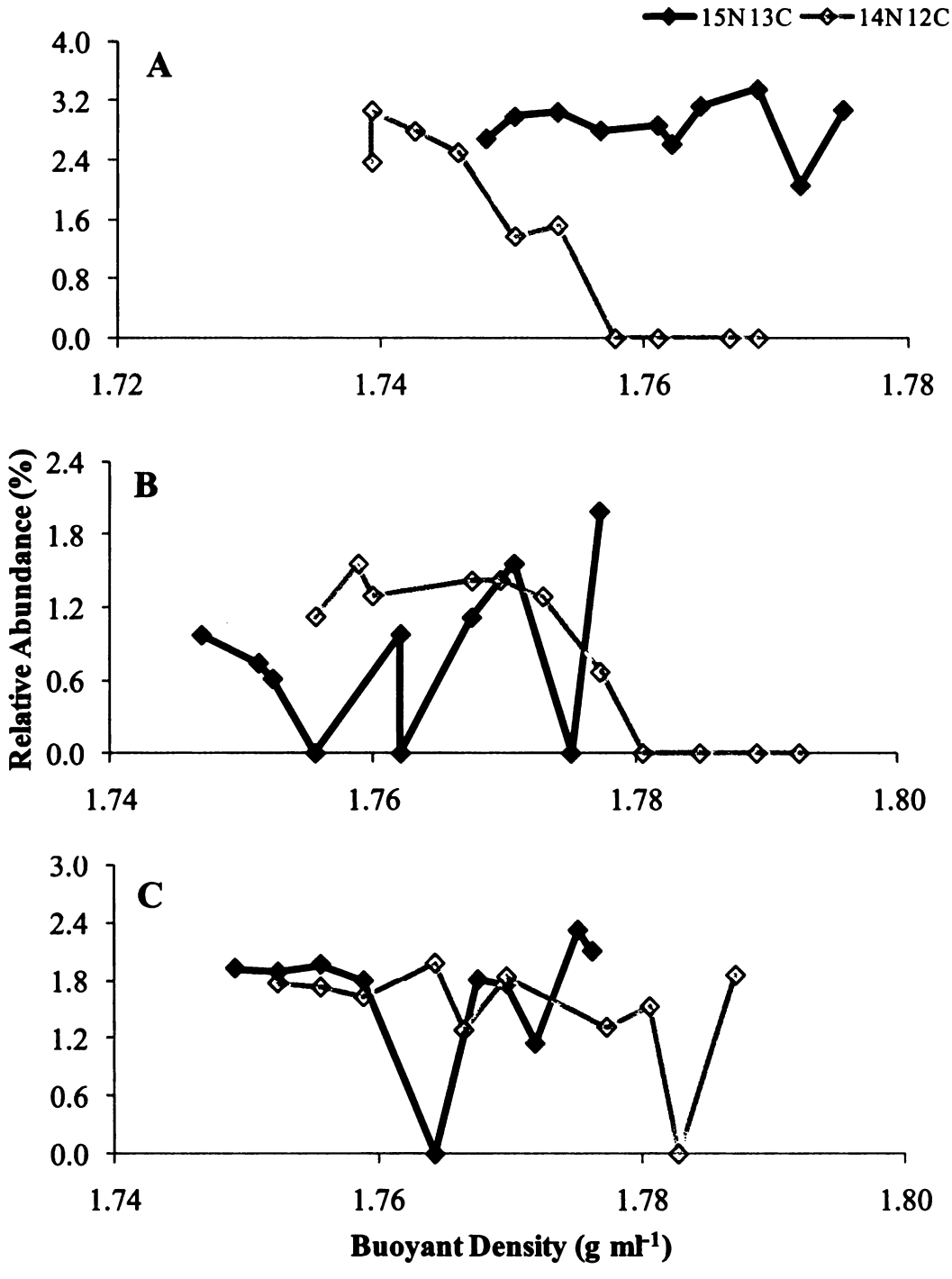


Figure C.23 - The relative abundance of the fragment of length 306 bp over a range of buoyant density from DNA extracted from triplicate microcosms (A, B, C) on day 16 from samples amended with 90 μM of labeled and unlabeled RDX

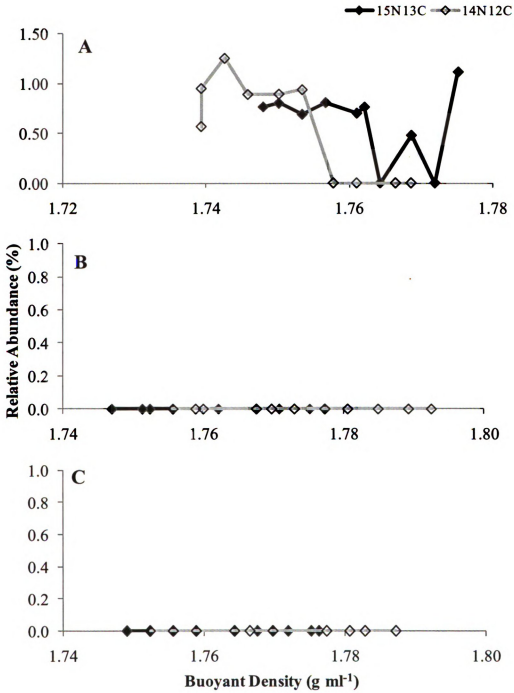


Figure C.24 - The relative abundance of the fragment of length 316 bp over a range of buoyant density from DNA extracted from triplicate microcosms (A, B, C) on day 16 from samples amended with 90 µM of labeled and unlabeled RDX

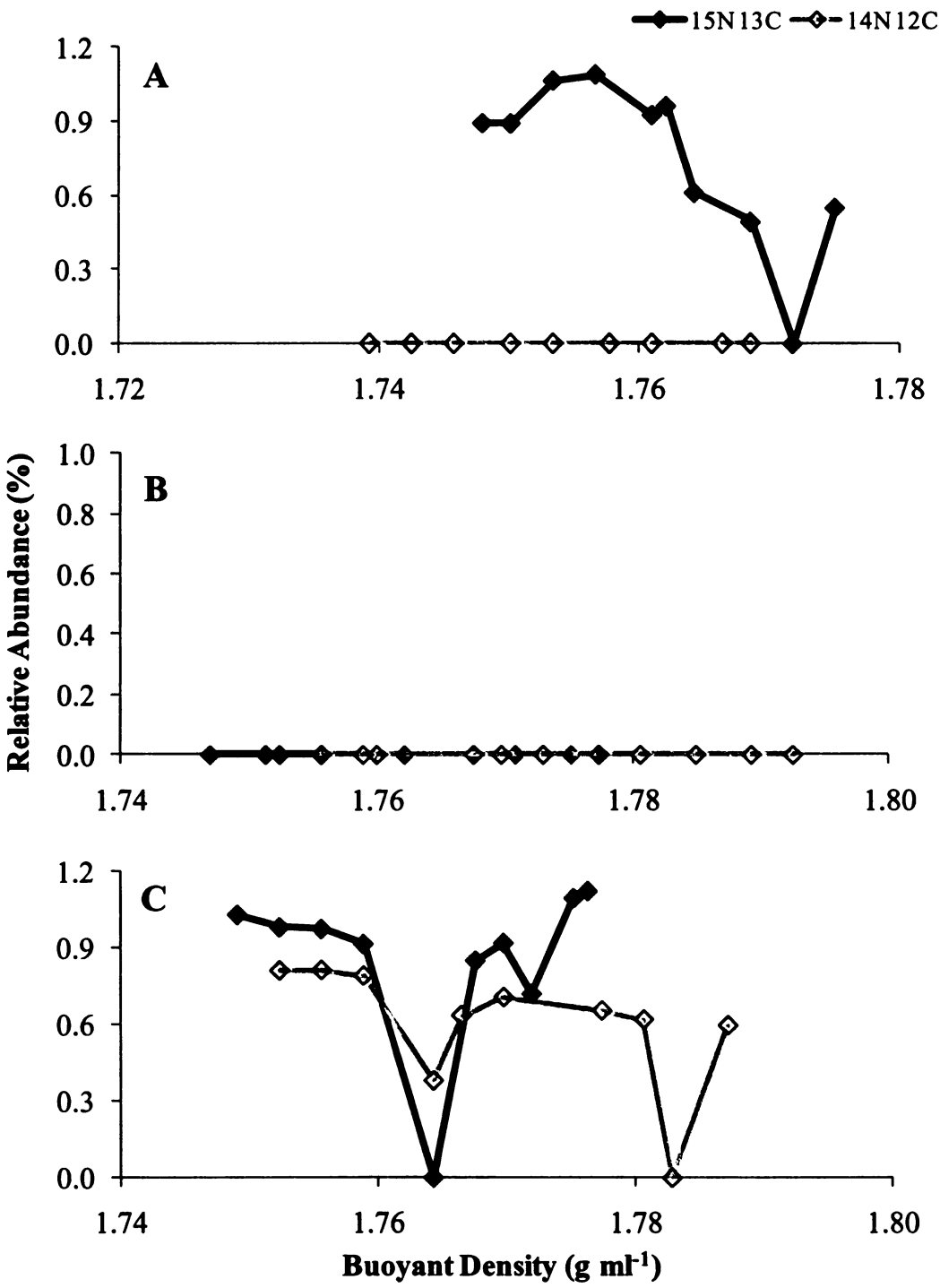


Figure C.25 - The relative abundance of the fragment of length 328 bp over a range of buoyant density from DNA extracted from triplicate microcosms (A, B, C) on day 16 from samples amended with 90 μM of labeled and unlabeled RDX

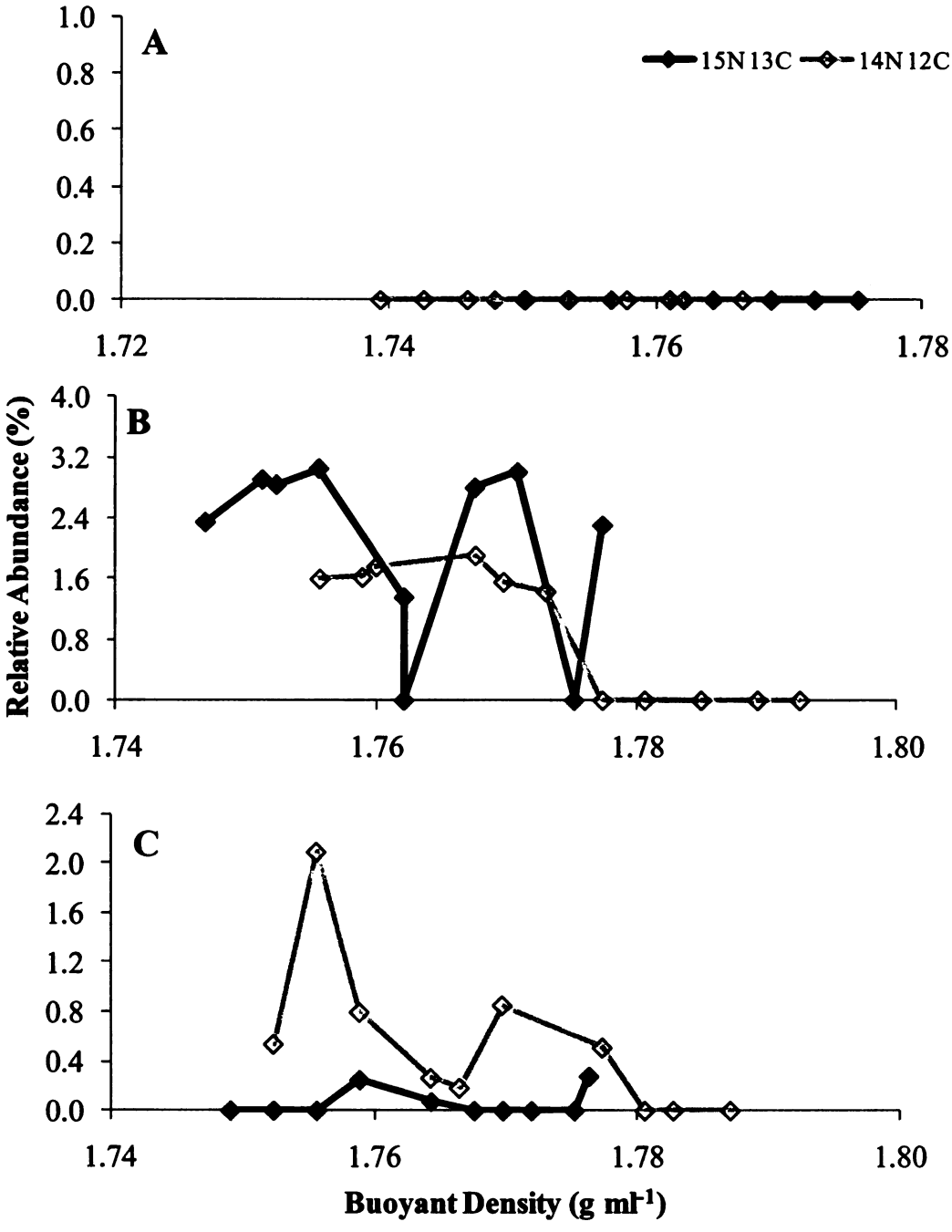


Figure C.26 - The relative abundance of the fragment of length 402 bp over a range of buoyant density from DNA extracted from triplicate microcosms (A, B, C) on day 16 from samples amended with 90 μM of labeled and unlabeled RDX

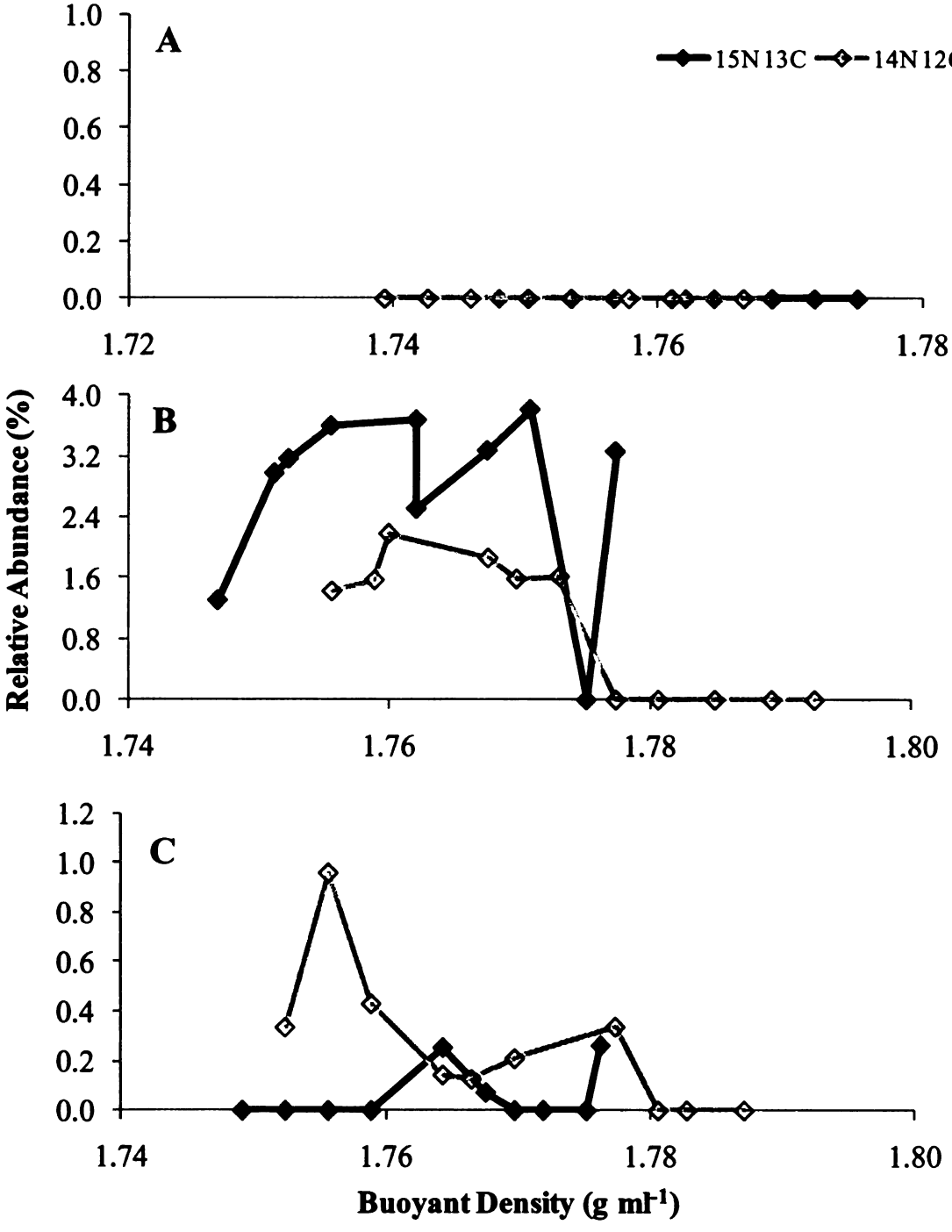


Figure C.27 - The relative abundance of the fragment of length 404 bp over a range of buoyant density from DNA extracted from triplicate microcosms (A, B, C) on day 16 from samples amended with 90 μM of labeled and unlabeled RDX

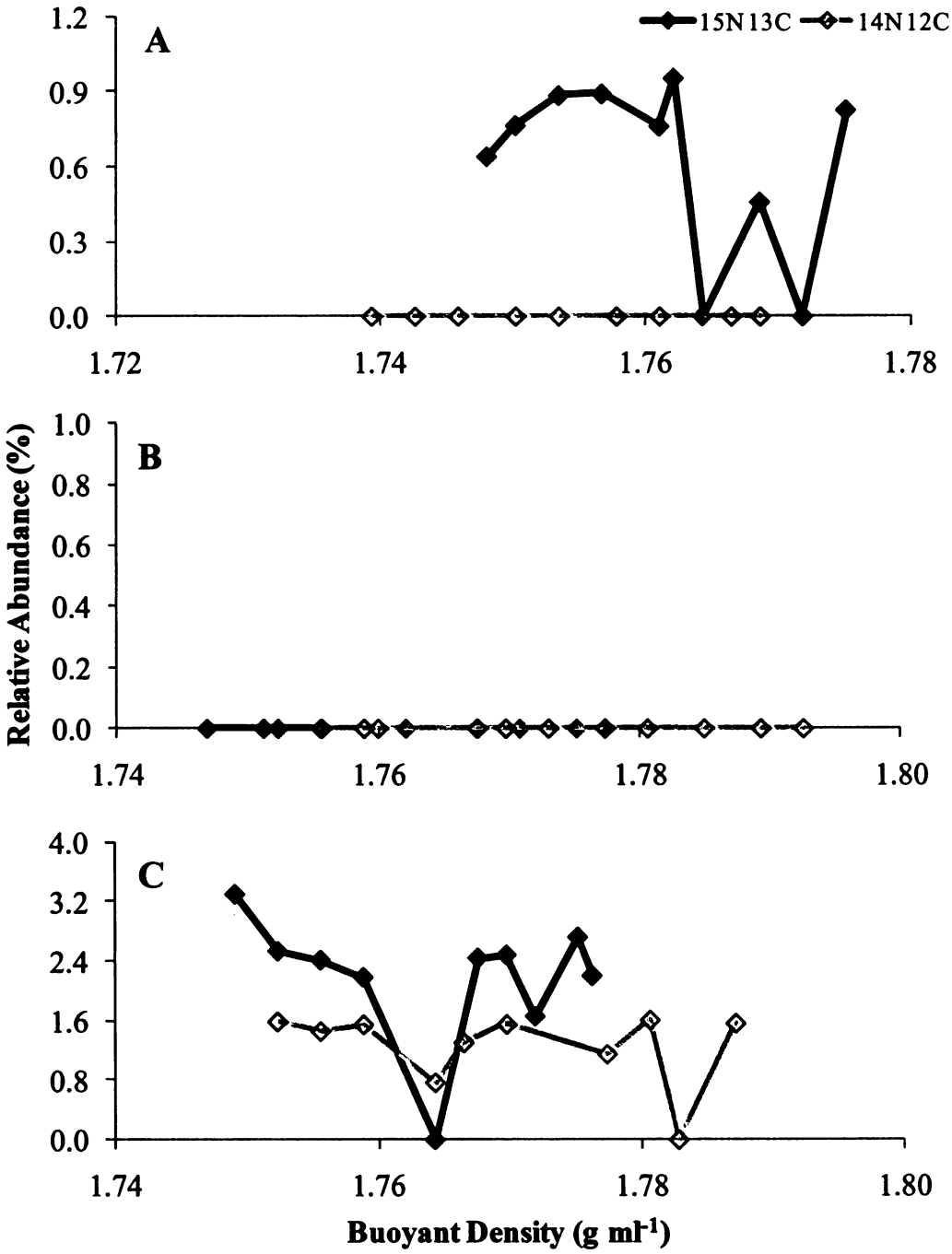


Figure C.28 - The relative abundance of the fragment of length 448 bp over a range of buoyant density from DNA extracted from triplicate microcosms (A, B, C) on day 16 from samples amended with 90 μM of labeled and unlabeled RDX

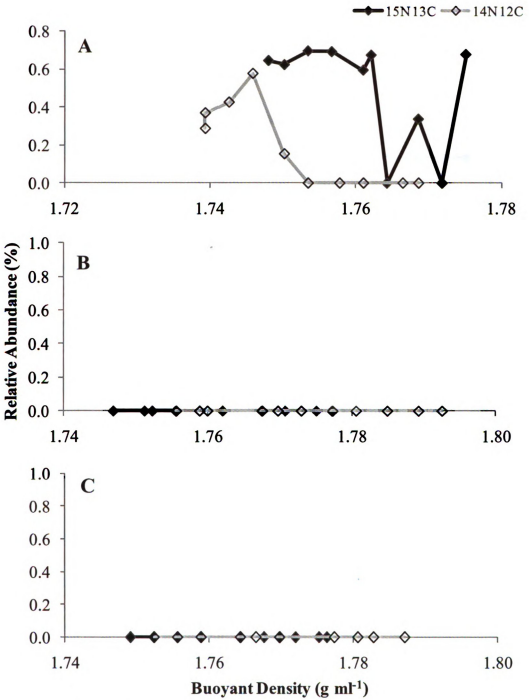


Figure C.29 - The relative abundance of the fragment of length 462 bp over a range of buoyant density from DNA extracted from triplicate microcosms (A, B, C) on day 16 from samples amended with 90 μM of labeled and unlabeled RDX

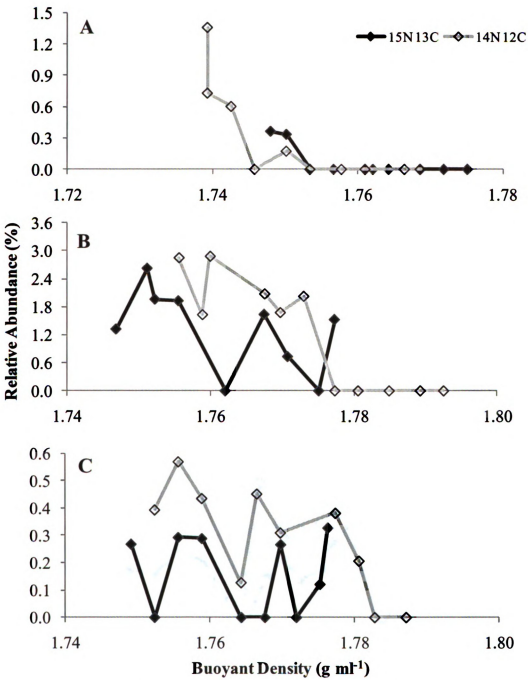


Figure C.30 - The relative abundance of the fragment of length 885 bp over a range of buoyant density from DNA extracted from triplicate microcosms (A, B, C) on day 16 from samples amended with 90 μM of labeled and unlabeled RDX

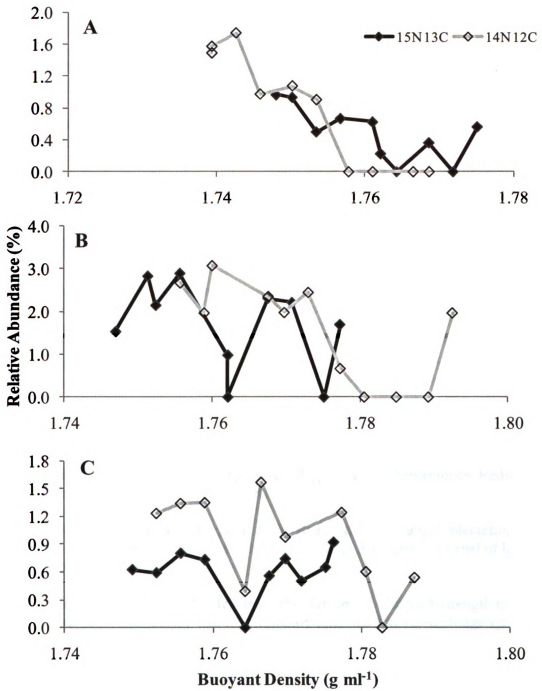


Figure C.31 - The relative abundance of the fragment of length 920 bp over a range of buoyant density from DNA extracted from triplicate microcosms (A, B, C) on day 16 from samples amended with 90 μM of labeled and unlabeled RDX

REFERENCES

1. **Abhilash, P. C., S. Jamil, and N. Singh.** 2009. Transgenic plants for enhanced biodegradation and phytoremediation of organic xenobiotics. Biotechnology Advances **27:**474-488.

2. **Aburto, A., and A. S. Ball.** 2009. Bacterial population dynamics and separation of active degraders by stable isotope probing during benzene degradation in a BTEX-impacted aquifer. Revista Internacional de Contaminacion Ambiental **25:**147-156.

3. **Adrian, N. R., and C. M. Arnett.** 2004. Anaerobic biodegradation of hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) by Acetobacterium malicum strain HAAP-1 isolated from a methanogenic mixed culture. Current Microbiology **48:**332-340.

4. **Adrian, N. R., C. M. Arnett, and R. F. Hickey.** 2003. Stimulating the anaerobic biodegradation of explosives by the addition of hydrogen or electron donors that produce hydrogen. Water Research **37:**3499-3507.

5. **Andreoni, V., and L. Gianfreda.** 2007. Bioremediation and monitoring of aromatic-polluted habitats. Applied Microbiology and Biotechnology **76:**287-308.

6. **ATSDR.** 1995. Toxicological profile for RDX. Agency for toxic substances and disease registry, U.S Department of health and human services.

7. **Bailey, A. M., S. G.** 2000. Explosives, Propellants & Pyrotechnics. Redwood Books, Trowbridge, Wiltshire, UK.

8. **Bayman, P., S. D. Ritchey, and J. W. Bennett.** 1995. Fungal interactions with the explosive RDX (hexahydro-1,3,5-trinitro-1,3,3-triazine). Journal of Industrial Microbiology **15:**418-423.

9. **Bell, B. A., and G. J. Hardcastle.** 1984. Treatment of a high-strength industrial-waste in a continuously fed, intermittently operated, activated-sludge system. Journal Water Pollution Control Federation **56:**1160-1164.

10. **Beller, H. R.** 2002. Anaerobic biotransformation of RDX (hexahydro-1,3,5-trinitro-1,3,5-triazine) by aquifer bacteria using hydrogen as the sole electron donor. Water Research **36:**2533-2540.

11. **Bhatt, M., J. S. Zhao, A. Halasz, and J. Hawari.** 2006. Biodegradation of hexahydro-1,3,5-trinitro-1,3,5-triazine by novel fungi isolated from unexploded ordnance contaminated marine sediment. Journal of Industrial Microbiology & Biotechnology **33:**850-858.

12. **Bhatt, M., J. S. Zhao, F. Monteil-Rivera, and M. Hawari.** 2005. Biodegradation of cyclic nitramines by tropical marine sediment bacteria. Journal of Industrial Microbiology & Biotechnology **32:**261-267.

13. **Bhushan, B., A. Halasz, and J. Hawari.** 2006. Effect of iron(III), humic acids and anthraquinone-2,6-disulfonate on biodegradation of cyclic nitramines by Clostridium sp EDB2. Journal of Applied Microbiology **100:**555-563.

14. **Bhushan, B., A. Halasz, J. Spain, S. Thiboutot, G. Ampleman, and J. Hawari.** 2002. Biotransformation of hexahydro-1,3,5-trinitro-1,3,5-triazine catalyzed by a NAD(P)H: Nitrate oxidoreductase from Aspergillus niger. Environmental Science & Technology **36:**3104-3108.

15. **Bhushan, B., A. Halasz, S. Thiboutot, G. Ampleman, and J. Hawari.** 2004. Chemotaxis-mediated biodegradation of cyclic nitramine explosives RDX9 HMX, and CL-20 by Clostridium sp EDB2. Biochemical and Biophysical Research Communications **316:**816-821.

16. **Bhushan, B., S. Trott, J. C. Spain, A. Halasz, L. Paquet, and M. Hawari.** 2003. Biotransformation of hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) by a rabbit liver cytochrome p450: insight into the mechanism of RDX biodegradation by Rhodococcus sp strain DN22. Applied and Environmental Microbiology **69:**1347-1351.

17. **Binks, P. R., S. Nicklin, and N. C. Bruce.** 1995. Degradation of hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) BY *Stenotrophomonas-maltophilia* PB1. Applied and Environmental Microbiology **61:**1318-1322.

18. **Boopathy, R., M. Gurgas, J. Ullian, and J. F. Manning.** 1998. Metabolism of explosive compounds by sulfate-reducing bacteria. Current Microbiology **37:**127-131.

19. **Bordeleau, G., M. M. Savard, R. Martel, G. Ampleman, and S. Thiboutot.** 2008. Determination of the origin of groundwater nitrate at an air weapons range using the dual isotope approach. Journal of Contaminant Hydrology **98:**97-105.

20. **Borodina, E., M. J. Cox, I. R. McDonald, and J. C. Murrell.** 2005. Use of DNA-stable isotope probing and functional gene probes to investigate the diversity of methyl chloride-utilizing bacteria in soil. Environmental Microbiology **7:**1318-1328.

21. **Boschker, H. T. S., S. C. Nold, P. Wellsbury, D. Bos, W. de Graaf, R. Pel, R. J. Parkes, and T. E. Cappenberg.** 1998. Direct linking of microbial populations to specific biogeochemical processes by C-13-labelling of biomarkers. Nature **392:**801-805.

22. **Bradley, P. M., and R. S. Dinicola.** 2005. RDX (hexahydro-1,3,5-trinitro-1,3,5-triazine) biodegradation in aquifer sediments under manganese-reducing conditions. Bioremediation Journal **9:**1-8.

23. **Brown, G. I.** 1998. The big bang - A history of explosives. Sutton Publishing, Bridgend.

24. **Buckley, D. H., V. Huangyutitham, S. F. Hsu, and T. A. Nelson.** 2008. N-15(2)-DNA-stable isotope probing of diazotrophic methanotrophs in soil. Soil Biology & Biochemistry **40:**1272-1283.

25. **Burdette, L. J., L. L. Cook, and R. S. Dyer.** 1988. Convulsant properties of cyclotrimethylenetrinitramine (RDX) - spontaneous, audiogenic and amygdaloid kindled seizure activity. Toxicology and Applied Pharmacology **92:**436-444.

26. **Cadisch, G., M. Espana, R. Causey, M. Richter, E. Shaw, J. A. W. Morgan, C. Rahn, and G. D. Bending.** 2005. Technical considerations for the use of N-15-DNA stable-isotope probing for functional microbial activity in soils. Rapid Communications in Mass Spectrometry **19:**1424-1428.

27. **Cebron, A., L. Bodrossy, N. Stralis-Pavese, A. C. Singer, I. P. Thompson, J. I. Prosser, and J. C. Murrell.** 2007. Nutrient amendments in soil DNA stable isotope probing experiments reduce the observed methanotroph diversity. Applied and Environmental Microbiology **73:**798-807.

28. **Chen, J., Z. Y. Wang, Y. F. Jiang, H. F. Qian, W. Zhang, and J. M. Chen.** 2009. Assessment of the Bacterial Community for Denitrifying Removal of Nitric Oxide in a Rotating Drum Biofilter by Denaturing Gradient Gel Electrophoresis. Environmental Engineering Science **26:**1189-1196.

29. **Cho, Y. S., B. U. Lee, and K. H. Oh.** 2008. Simultaneous degradation of nitroaromatic compounds TNT, RDX, atrazine, and simazine by Pseudomonas putida HK-6 in bench-scale bioreactors. Journal of Chemical Technology and Biotechnology **83:**1211-1217.

30. **Clement, B. G., L. E. Kehl, K. L. DeBord, and C. L. Kitts.** 1998. Terminal restriction fragment patterns (TRFPs), a rapid, PCR-based method for the comparison of complex bacterial communities. Journal of Microbiological Methods **31:**135-142.

31. **Coleman, N. V., D. R. Nelson, and T. Duxbury.** 1998. Aerobic biodegradation of hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) as a nitrogen source by a Rhodococcus sp., strain DN22. Soil Biology & Biochemistry **30:**1159-1167.

32. **Crocker, F. H., K. J. Indest, and H. L. Fredrickson.** 2006. Biodegradation of the cyclic nitramine explosives RDX, HMX, and CL-20. Applied Microbiology and Biotechnology **73:**274-290.

33. **DeRito, C. M., and E. L. Madsen.** 2009. Stable isotope probing reveals Trichosporon yeast to be active in situ in soil phenol metabolism. Isme Journal **3:**477-485.

34. **Dumont, M. G., and J. C. Murrell.** 2005. Stable isotope probing - linking microbial identity to function. Nature Reviews Microbiology **3:**499-504.

35. **Faust, R. A.** 1994. Toxicity summary for hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX). Oak Ridge National Laboratory.

36. **Fernando, T., and S. D. Aust.** 1991. Biodegradation of munition waste, TNT (2,4,6-trinitrotoluene) and RDX (hexahydro-1,3,5-trinitro-1,3,5-triazine) by Phanerochaete chrysosporium. Acs Symposium Series **468:**214-232.

37. **Fournier, D., A. Halasz, J. Spain, P. Fiurasek, and J. Hawari.** 2002. Determination of key metabolites during biodegradation of hexahydro-1,3,5-trinitro-1,3,5-triazine with Rhodococcus sp strain DN22. Applied and Environmental Microbiology **68:**166-172.

38. **Fournier, D., S. Trott, J. Hawari, and J. Spain.** 2005. Metabolism of the aliphatic nitramine 4-nitro-2,4-diazabutanal by Methylobacterium sp strain JS178. Applied and Environmental Microbiology **71:**4199-4202.

39. **Freedman, D. L., and K. W. Sutherland.** 1998. Biodegradation of hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) under nitrate-reducing conditions. Water Science and Technology **38:**33-40.

40. **Freitag, T. E., L. Chang, and J. I. Prosser.** 2006. Changes in the community structure and activity of betaproteobacterial ammonia-oxidizing sediment bacteria along a freshwater-marine gradient. Environmental Microbiology **8:**684-696.

41. **Friedrich, M. W.** 2006. Stable-isotope probing of DNA: insights into the function of uncultivated microorganisms from isotopically labeled metagenomes. Current Opinion in Biotechnology **17:**59-66.

42. **Ghosh, S., M. J. Sadowsky, M. C. Roberts, J. A. Gralnick, and T. M. LaPara.** 2009. Sphingobacterium sp strain PM2-P1-29 harbours a functional tet(X) gene encoding for the degradation of tetracycline. Journal of Applied Microbiology **106:**1336-1342.

43. **Ginige, M. P., J. Keller, and L. L. Blackall.** 2005. Investigation of an acetate-fed denitrifying microbial community by stable isotope probing, full-cycle rRNA

analysis, and fluorescent in situ hybridization-microautoradiography. Applied and Environmental Microbiology **71:**8683-8691.

44. **Hamana, K., T. Itoh, Y. Benno, and H. Hayashi.** 2008. Polyamine distribution profiles of new members of the phylum Bacteroidetes. Journal of General and Applied Microbiology **54:**229-236.

45. **Harvey, S. D., R. J. Fellows, D. A. Cataldo, and R. M. Bean.** 1991. Fate of the explosive hexahydro-1,3,5-triazine (RDX) in soil and bioaccumulation in bush bean hydroponic plants. Environmental Toxicology and Chemistry **10:**845-855.

46. **Hawari, J., A. Halasz, T. Sheremata, S. Beaudet, C. Groom, L. Paquet, C. Rhofir, G. Ampleman, and S. Thiboutot.** 2000. Characterization of metabolites during biodegradation of hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) with municipal anaerobic sludge. Applied and Environmental Microbiology **66:**2652-2657.

47. **Hundal, L. S., J. Singh, E. L. Bier, P. J. Shea, S. D. Comfort, and W. L. Powers.** 1997. Removal of TNT and RDX from water and soil using iron metal. Environmental Pollution **97:**55-64.

48. **Isbister, J. D., G. L. Anspach, J. F. Kitchens, and R. C. Doyle.** 1984. Composting for decontamination of soils containing explosives. Microbiologica **7:**47-73.

49. **Jenkins, T. F. B., Claudia ; Ranney, Thomas A.** 2003. Stability of CL-20, TNAZ, HMX, RDX, NG, and PETN in Moist, Unsaturated Soil.

50. **Jia, Z. J., and R. Conrad.** 2009. Bacteria rather than Archaea dominate microbial ammonia oxidation in an agricultural soil. Environmental Microbiology **11:**1658-1671.

51. **Kaplan, A. S., C. F. Berghout, and A. Peczenik.** 1965. Human intoxication from RDX. Archives of Environmental Health **10:**877-883.

52. **Kitts, C. L., D. P. Cunningham, and P. J. Unkefer.** 1994. Isolation of 3 hexahydro-1,3,5-triazine-degrading species of the family enterobacteriaceae from nitramine explosive-contaminated soil. Applied and Environmental Microbiology **60:**4608-4611.

53. **Kitts, C. L., C. E. Green, R. A. Otley, M. A. Alvarez, and P. J. Unkefer.** 2000. Type I nitroreductases in soil enterobacteria reduce TNT (2,4,6-trinitrotoluene) and RDX (hexahydro-1,3,5-trinitro-1,3,5-triazine). Canadian Journal of Microbiology **46:**278-282.

54. **Kwon, M. J., and K. T. Finneran.** 2008. Hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) and octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX) biodegradation kinetics amongst several Fe(III)-reducing genera. Soil & Sediment Contamination **17:**189-203.

55. **Lee, S. Y., and B. W. Brodman.** 2004. Biodegradation of 1,3,5-trinitro-1,3,5-triazine (RDX). Journal of Environmental Science and Health Part a-Toxic/Hazardous Substances & Environmental Engineering **39:**61-75.

56. **Leigh, M. B., V. H. Pellizari, O. Uhlik, R. Sutka, J. Rodrigues, N. E. Ostrom, J. H. Zhou, and J. M. Tiedje.** 2007. Biphenyl-utilizing bacteria and their functional genes in a pine root zone contaminated with polychlorinated biphenyls (PCBs). Isme Journal **1:**134-148.

57. **Leigh, M. B., P. Prouzova, M. Mackova, T. Macek, D. P. Nagle, and J. S. Fletcher.** 2006. Polychlorinated biphenyl (PCB)-degrading bacteria associated with trees in a PCB-contaminated site. Applied and Environmental Microbiology **72:**2331-2342.

58. **Levine, B. S., E. M. Furedi, D. E. Gordon, J. M. Burns, and P. M. Lish.** 1981. 13 week toxicity study of hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) in fisher 344 rats. Toxicology Letters **8:**241-245.

59. **Liu, H., J. Yan, Q. Wang, U. G. Karlson, G. Zou, and Z. Yuan.** 2009. Biodegradation of methyl tert-butyl ether by enriched bacterial culture. Current Microbiology **59:**30-34.

60. **Liu, W. T., T. L. Marsh, H. Cheng, and L. J. Forney.** 1997. Characterization of microbial diversity by determining terminal restriction fragment length polymorphisms of genes encoding 16S rRNA. Applied and Environmental Microbiology **63:**4516-4522.

61. **Llado, S., N. Jimenez, M. Vinas, and A. M. Solanas.** 2009. Microbial populations related to PAH biodegradation in an aged biostimulated creosote-contaminated soil. Biodegradation **20:**593-601.

62. **Luo, C. L., S. G. Xie, W. M. Sun, X. D. Li, and A. M. Cupples.** 2009. Identification of a Novel Toluene-Degrading Bacterium from the Candidate Phylum TM7, as Determined by DNA Stable Isotope Probing. Applied and Environmental Microbiology **75:**4644-4647.

63. **Luo, W., E. M. D'Angelo, and M. S. Coyne.** 2008. Organic carbon effects on aerobic polychlorinated biphenyl removal and bacterial community composition in soils and sediments. Chemosphere **70:**364-373.

64. **Macbeth, T. W., D. E. Cummings, S. Spring, L. M. Petzke, and K. S. Sorenson.** 2004. Molecular characterization of a dechlorinating community resulting from in situ biostimulation in a trichloroethene-contaminated deep, fractured basalt aquifer and comparison to a derivative laboratory culture. Applied and Environmental Microbiology **70:**7329-7341.

65. **Manefield, M., A. S. Whiteley, R. I. Griffiths, and M. J. Bailey.** 2002. RNA stable isotope probing, a novel means of linking microbial community function to Phylogeny. Applied and Environmental Microbiology **68:**5367-5373.

66. **McCormick, N. G., J. H. Cornell, and A. M. Kaplan.** 1981. Biodegradation of hexahydro-1,3,5-trinitro-1,3,5-triazine. Applied and Environmental Microbiology **42:**817-823.

67. **McDonald, I. R., S. Radajewski, and J. C. Murrell.** 2005. Stable isotope probing of nucleic acids in methanotrophs and methylotrophs: A review. Organic Geochemistry **36:**779-787.

68. **Meselson, M., and F. W. Stahl.** 1958. The replication of DNA in *Escherichia coli*. Proceedings of the National Academy of Sciences of the United States of America **44:**671-682.

69. **Miller, L. G., K. L. Warner, S. M. Baesman, R. S. Oremland, I. R. McDonald, S. Radajewski, and J. C. Murrell.** 2004. Degradation of methyl bromide and methyl chloride in soil microcosms: Use of stable C isotope fractionation and stable isotope probing to identify reactions and the responsible microorganisms. Geochimica Et Cosmochimica Acta **68:**3271-3283.

70. **Morris, S. A., S. Radajewski, T. W. Willison, and J. C. Murrell.** 2002. Identification of the functionally active methanotroph population in a peat soil microcosm by stable-isotope probing. Applied and Environmental Microbiology **68:**1446-1453.

71. **Mukhi, S., and R. Patino.** 2008. Effects of hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) in zebrafish: General and reproductive toxicity. Chemosphere **72:**726-732.

72. **Nejidat, A., L. Kafka, Y. Tekoah, and Z. Ronen.** 2008. Effect of organic and inorganic nitrogenous compounds on RDX degradation and cytochrome P-450 expression in Rhodococcus strain YH1. Biodegradation **19:**313-320.

73. **Neufeld, J. D., M. G. Dumont, J. Vohra, and J. C. Murrell.** 2007. Methodological considerations for the use of stable isotope probing in microbial ecology. Microbial Ecology **53:**435-442.

74. **Neufeld, J. D., M. Wagner, and J. C. Murrell.** 2007. Who eats what, where and when? Isotope-labelling experiments are coming of age. Isme Journal **1**:103-110.

75. **Oh, B. T., and P. J. J. Alvarez.** 2002. Hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) degradation in biologically-active iron columns. Water Air and Soil Pollution **141**:325-335.

76. **Osaka, T., S. Yoshie, S. Tsuneda, A. Hirata, N. Iwami, and Y. Inamori.** 2006. Identification of acetate- or methanol-assimilating bacteria under nitrate-reducing conditions by stable-isotope probing. Microbial Ecology **52**:253-266.

77. **Owsianiak, M., L. Chrzanowski, A. Szulc, J. Staniewski, A. Olszanowski, A. K. Olejnik-Schmidt, and H. J. Heipieper.** 2009. Biodegradation of diesel/biodiesel blends by a consortium of hydrocarbon degraders: Effect of the type of blend and the addition of biosurfactants. Bioresource Technology **100**:1497-1500.

78. **Radajewski, S., P. Ineson, N. R. Parekh, and J. C. Murrell.** 2000. Stable-isotope probing as a tool in microbial ecology. Nature **403**:646-649.

79. **Robidoux, P. Y., C. Svendsen, J. Caumartin, J. Hawari, G. Ampleman, S. Thiboutot, J. M. Weeks, and G. I. Sunahara.** 2000. Chronic toxicity of energetic compounds in soil determined using the earthworm (Eisenia andrei) reproduction test. Environmental Toxicology and Chemistry **19**:1764-1773.

80. **Roh, H., C. P. Yu, M. E. Fuller, and K. H. Chu.** 2009. Identification of Hexahydro-1,3,5-trinitro-1,3,5-triazine-Degrading Microorganisms via N-15-Stable Isotope Probing. Environmental Science & Technology **43**:2505-2511.

81. **Sarrazin, M., S. G. Dodard, K. Savard, B. Lachance, P. Y. Robidoux, R. G. Kuperman, J. Hawari, G. Ampleman, S. Thiboutot, and G. I. Sunahara.** 2009. Accumulation of hexahydro-1,3,5-trinitro-1,3,5-triazine by the earthworm Eisenia andrei in a sandy loam soil. Environmental Toxicology and Chemistry **28**:2125-2133.

82. **Seth-Smith, H. M. B.** 2002. Microbial degradation of RDX. University of Cambridge.

83. **Seth-Smith, H. M. B., S. J. Rosser, A. Basran, E. R. Travis, E. R. Dabbs, S. Nicklin, and N. C. Bruce.** 2002. Cloning, sequencing, and characterization of the hexahydro-1,3,5-trinitro-1,3,5-triazine degradation gene cluster from Rhodococcus rhodochrous. Applied and Environmental Microbiology **68**:4764-4771.

84. **Sherburne, L. A., J. D. Shrout, and P. J. J. Alvarez.** 2005. Hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) degradation by Acetobacterium paludosum. Biodegradation **16:**539-547.

85. **Sheremata, T. W., and J. Hawari.** 2000. Mineralization of RDX by the white rot fungus Phanerochaete chrysosporium to carbon dioxide and nitrous oxide. Environmental Science & Technology **34:**3384-3388.

86. **Singleton, D. R., S. N. Powell, R. Sangaiah, A. Gold, L. M. Ball, and M. D. Aitken.** 2005. Stable-isotope probing of bacteria capable of degrading salicylate, naphthalene, or phenanthrene in a Bioreactor treating contaminated soil. Applied and Environmental Microbiology **71:**1202-1209.

87. **Talmage, S. S., D. M. Opresko, C. J. Maxwell, C. J. E. Welsh, F. M. Cretella, P. H. Reno, and F. B. Daniel.** 1999. Nitroaromatic munition compounds: environmental effects and screening values. Reviews of Environmental Contamination and Toxicology **161:**1-156.

88. **Thompson, K. T., F. H. Crocker, and H. L. Fredrickson.** 2005. Mineralization of the cyclic nitramine explosive hexahydro-1,3,5-trinitro-1,3,5-triazine by Gordonia and Williamsia spp. Applied and Environmental Microbiology **71:**8265-8272.

89. **Torsvik, V., and L. Ovreas.** 2002. Microbial diversity and function in soil: from genes to ecosystems. Current Opinion in Microbiology **5:**240-245.

90. **U.S.EPA.** 1988. Drinking water health adivisory for hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX). Office of Drinking Water, Washington, DC.

91. **Uhlik, O., K. Jecna, M. B. Leigh, M. Mackova, and T. Macek.** 2009. DNA-based stable isotope probing: a link between community structure and function. Science of the Total Environment **407:**3611-3619.

92. **Van Aken, B., J. M. Yoon, and J. L. Schnoor.** 2004. Biodegradation of nitro-substituted explosives 2,4,6-trinitrotoluene, hexahydro-1,3,5-trinitro-1,3,5-triazine, an octahydro-1,3,5,7-tetranitro-1,3,5-tetrazocine by a phytosymbiotic Methylobacterium sp associated with poplar tissues (Populus deltoides x nigra DN34). Applied and Environmental Microbiology **70:**508-517.

93. **Van Beilen, J. B., and E. G. Funhoff.** 2007. Alkane hydroxylases involved in microbial alkane degradation. Applied Microbiology and Biotechnology **74:**13-21.

94. **Williams, R. T., P. S. Ziegenfuss, and W. E. Sisk.** 1992. Composting of explosives and propellant contaminated soils under thermophilic and mesophilic conditions. Journal of Industrial Microbiology **9:**137-144.

95. **Woody, R. C., G. L. Kearns, M. A. Brewster, C. P. Turley, G. B. Sharp, and R. S. Lake.** 1986. THE NEUROTOXICITY OF CYCLOTRIMETHYLENETRINITRAMINE (RDX) IN A CHILD - A CLINICAL AND PHARMACOKINETIC EVALUATION. Journal of Toxicology-Clinical Toxicology **24:**305-319.

96. **Yang, Y., X. Wang, P. Yin, W. Li, and P. Zhou.** 1983. 3 strains of Cornebacterium degrading cyclo tri methylene tri nitro amine. Weishengwu Xuebao **23:**251-256.

97. **Young, D. M., P. J. Unkefer, and K. L. Ogden.** 1997. Biotransformation of hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) by a prospective consortium and its most effective isolate Serratia marcescens. Biotechnology and Bioengineering **53:**515-522.

98. **Zhang, C. L., and J. B. Hughes.** 2003. Biodegradation pathways of hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) by Clostridium acetobutylicum cell-free extract. Chemosphere **50:**665-671.

99. **Zhao, J. S., C. W. Greer, S. Thiboutot, G. Ampleman, and J. Hawari.** 2004. Biodegradation of the nitramine explosives hexahydro-1,3,5-trinitro-1,3,5-triazine and octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine in cold marine sediment under anaerobic and oligotrophic conditions. Canadian Journal of Microbiology **50:**91-96.

100. **Zhao, J. S., A. Halasz, L. Paquet, C. Beaulieu, and J. Hawari.** 2002. Biodegradation of hexahydro-1,3,5-trinitro-1,3,5-triazine and its mononitroso derivative hexahydro-1-nitroso-3,5-dinitro-1,3,5-triazine by Klebsiella pneumoniae strain SCZ-1 isolated from an anaerobic sludge. Applied and Environmental Microbiology **68:**5336-5341.

101. **Zhao, J. S., D. Manno, C. Beaulieu, L. Paquet, and J. Hawari.** 2005. Shewanella sediminis sp nov., a novel Na+-requiring and hexahydro-1,3,5-trinitro-1,3,5-trinitro-degrading bacterium from marine sediment. International Journal of Systematic and Evolutionary Microbiology **55:**1511-1520.

102. **Zhao, J. S., D. Manno, C. Leggiadro, D. O'Neill, and J. Hawari.** 2006. Shewanella halifaxensis sp nov., a novel obligately respiratory and denitrifying psychrophile. International Journal of Systematic and Evolutionary Microbiology **56:**205-212.

103. **Zhao, J. S., L. Paquet, A. Halasz, and J. Hawari.** 2003. Metabolism of hexahydro-1,3,5-trinitro-1,3,5-triazine through initial reduction to hexahydro-1-nitroso-3,5-dinitro-1,3,5-triazine followed by denitration in Clostridium bifermentans HAW-1. Applied Microbiology and Biotechnology **63:**187-193.

104. **Zhao, J. S., J. Spain, and M. Hawari.** 2003. Phylogenetic and metabolic diversity of hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX)-transforming bacteria in strictly anaerobic mixed cultures enriched on RDX as nitrogen source. Fems Microbiology Ecology **46:**189-196.

105. **Zhao, J. S., J. Spain, S. Thiboutot, G. Ampleman, C. Greer, and J. Hawari.** 2004. Phylogeny of cyclic nitramine-degrading psychrophilic bacteria in marine sediment and their potential role in the natural attenuation of explosives. Fems Microbiology Ecology **49:**349-357.