ASSESSING THE BIODEGRADATION OF TOLUENE, ETHYLBENZENE AND RDX AND THE IDENTIFICATION OF THE MICROORGANISMS INVOLVED USING STABLE ISOTOPE PROBING AND HIGH THROUGHPUT AMPLICON SEQUENCING

By

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ABSTRACT

ASSESSING THE BIODEGRADATION OF TOLUENE, ETHYLBENZENE AND RDX AND THE IDENTIFICATION OF THE MICROORGANISMS INVOLVED USING STABLE ISOTOPE PROBING AND HIGH THROUGHPUT AMPLICON SEQUENCING

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Contamination of groundwater by organic pollutants is a worldwide environmental problem. Bioremediation is a viable option for cleaning and reclaiming sites contaminated with pollutants amenable to microbial transformation. However, our understanding of microorganisms playing key roles in bioremediation is still developing. In this study, specific aspects of biodegradation of three organic pollutants namely toluene, ethylbenzene and the nitramine explosive hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) were investigated. The overall objectives of this research were to 1) assess the biodegradation potential of toluene, ethylbenzene and RDX using soils from various sources, 2) identify the major groups of microorganisms responsible for the degradation of ethylbenzene and RDX in these samples and 3) assess the effect of a potential co-contaminant, isobutanol, on toluene biodegradation.

The first study examined the effect of isobutanol on the biodegradation of toluene under sulfate amended, nitrate amended or methanogenic conditions. The results indicated that toluene biodegradation was not greatly affected by isobutanol in five of the six experimental set-ups. However, toluene biodegradation was completely inhibited in one set of microcosms amended with sulfate and inocula from wastewater treatment plant activated sludge. This suggests that if co-contamination occurs, in some cases toluene degradation may be inhibited. In the second study, stable isotope probing (SIP) and high throughput sequencing were used with ethylbenzene degrading consortia to identify microorganisms benefiting from ^{13}C label uptake from

ethylbenzene (or metabolites). Several phylotypes were relatively more abundant in the heavy fractions from the labeled ethylbenzene amended soil microcosms compared to the controls indicating ^{13}C label uptake. This included phylotypes within the families *Oxalobacteraceae, Rhodospirillaceae, Xanthomonadaceae* and *Rhodocyclaceae* (*Proteobacteria*) as well as the genus *Gemmatimonas*. This work indicates microorganisms not previously linked to ethylbenzene degradation could have significant roles in the carbon uptake from this pollutant.

The third and fourth studies involved applying SIP and high throughput sequencing to investigate RDX degrading microbial communities. In the third study, microbial communities obtained from four soils previously unexposed to explosives were investigated. Sequences from the total DNA extracts of all soils illustrated an increase in abundance of *Brevundimonas* and/or unclassified *Bacillaceae 1* compared to the microbial communities in the initial soil or no RDX treatments. The fourth study investigated a RDX degrading community obtained from a Navy Base previously contaminated with explosives with and without glucose. The microbial communities in the total DNA samples indicated phylotypes classing as *Pseudomonadaceae* and *Acinetobacter* was more abundant in the presence and absence of glucose respectively. The SIP study also found that unclassified *Pseudomonadaceae* were primarily responsible for label uptake in both treatments. When glucose was present, *Comamonas* also increased in abundance following RDX degradation and was enriched in the heavy fractions, suggesting, for the first time, that this phylotype is also important for RDX removal. Overall, these data suggest both novel RDX degraders and previously reported RDX degraders were associated with RDX removal in these soils.

Dedicated to all the great people in my life

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KEY TO ABBREVIATIONS

BTEX - benzene, toluene, ethylbenzene and o-, p-, and m-xylenes

CFR – Code of Federal Regulations

DGGE – Denaturing gradient gel electrophoresis

DNA - deoxyribonucleic acid

LUST – leaking underground storage tanks

OTU - operational taxonomic unit

PCR - polymerase chain reaction

RDX – royal demolition explosive (hexahydro-1,3,5-trintro-1,3,5-triazine)

rRNA - ribosomal RNA

SIP – stable isotope probing

TRFLP - terminal restriction fragment length polymorphism

CHAPTER 1 INTRODUCTION

GROUNDWATER POLLUTION BY BTEX AND RDX

The chemicals benzene, toluene, ethylbenzene and the xylenes, collectively known as BTEX compounds, are a major source of groundwater contamination from leaking gasoline tanks. According the USEPA, as of March 2014, over half of a million releases have been confirmed in the United States from underground storage tanks (UST) containing gasoline (1). Groundwater contamination near military sites with the nitroamine explosive hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) is widespread due to the explosives manufacturing and handling particularly after World War II (2). Groundwater contamination by such organic pollutants threatens drinking water sources due to their toxic health effects. Bioremediation is a viable remedy option for these contaminants as they are amenable to bacterial degradation. In situ biodegradation of organic pollutants can depend on numerous factors including the microbial community, nutrient availability, physical and chemical factors and co-contaminant effects. It is also more appropriate to consider degradation of such pollutants under anaerobic conditions (as groundwater is often depleted in oxygen). The research presented in this thesis relates to the microorganisms involved in the biodegradation of the organic contaminants toluene, ethylbenzene and RDX.

BIODEGRADATION OF TOLUENE, ETHYLBENZENE AND RDX

Microorganisms that are able to metabolize or mineralize toluene under nitrate reducing (3-6), iron reducing (7, 8) and sulfate reducing (9) conditions have been isolated thus far. However, very little is known about the effects of a potential co-contaminant (isobutanol) on toluene degradation. To date, only three studies have investigated the effect of isobutanol on BTEX, two under aerobic conditions (10, 11) and one under anaerobic conditions(12). In this study, we extend on knowledge by investigating the effect of isobutanol on toluene (a model BTEX compound) in six different toluene degrading communities.

In contrast to toluene, only five anaerobic ethylbenzene degrading isolates exist. Among these, four can degrade ethylbenzene under nitrate reducing conditions (13). These include two *Azoarcus sp*. strains (*Aromatoleum aromaticum* EbN1 and EB1) (14-16) , *Dechloromonas aromatica RCB* (6, 17), *Georgfuchsia toluolica* (18) and *Desulfobacteraceae* Strain EbS7 (19). Interestingly, all except Strain EbS7 (*Deltaproteobacteria*) belong to the family *Rhodocyclaceae* (*Betaproteobacteria*).

Compared to the BTEX chemicals, many microorganisms have been associated with RDX degradation. Many anaerobic RDX degraders have been isolated from the phyla *Proteobacteria* (20-32) and *Firmicutes* (21, 29, 33-38) with one in the phylum *Fusobacteria* (28). Although contaminant degradation has been confirmed with pure cultures, it is still uncertain if these isolates can degrade ethylbenzene or RDX when present in a mixed culture. To address this unknown, stable isotope probing (SIP) was used in this research to link function to identity in mixed communities.

STABLE ISOTOPE PROBING AND HIGH THROUGHPUT SEQUENCING

SIP involves amending a stable isotope labeled (e.g. ^{13}C, ^{15}N) compound to a mixed microbial community, such as a soil or water sample (39). Isopycnic centrifugation of the extracted DNA in cesium chloride gradients allows the DNA to band based on it's buoyant density. Thus, this technique allows the separation of DNA that has incorporated the label (heavy DNA) from the background community DNA (unlabeled or light DNA). SIP studies have been used to identify microorganisms involved in biogeochemical cycles (40, 41) and environmental pollutant remediation (42-45). These studies employed finger printing techniques such as denaturing gradient gel electrophoresis (DGGE), terminal restriction fragment length polymorphism (TRFLP) and cloning and sequencing methods. Recently, researchers have combined SIP with high throughput sequencing to obtain a greater depth of information than that achievable with fingerprinting techniques or Sanger sequencing. (46-48). In this study, we combined SIP with high throughput amplicon sequencing (MiSeq Illumina) to identify the microorganisms in mixed communities involved in ethylbenzene/RDX biodegradation.

OBJECTIVES

The overall objectives were,

i) To assess toluene, ethylbenzene and RDX biodegradation potential in soils from various sources.

ii) To identify the major groups of microorganisms responsible for degradation of ethylbenzene and RDX.

iii) To assess the effect of a potential co-contaminant, isobutanol, on toluene biodegradation.

Specific objectives addressed in each chapter are as follows:

1. To assess the effect of a potential co-contaminant (isobutanol) on a model gasoline compound (toluene) under various electron acceptor conditions in laboratory microcosms (Chapter 2).
2. To identify the microorganisms responsible for carbon uptake from ethylbenzene under oxygen depleted conditions (Chapter 3).
3. To determine and compare the dominant RDX degraders in four uncontaminated soils and in a soil from a contaminated site (Chapters 4 and 5).

The research work presented under Chapters 2 and 3 were funded by an NSF grant awarded to Dr. Cupples entitled “Stable isotope probing to assess bioremediation of LUST contaminants: addressing existing MTBE/BTEX and probably future ethanol/BTEX contamination”. Chapter 2 addresses specific objective 1 and involved the investigation of isobutanol on toluene degradation in six toluene degrading communities. The results indicated that isobutanol had varying effects on toluene biodegradation. A modified version of the work in Chapter 2 was published (Jayamani, I and A. M. Cupples. 2013. Effect of isobutanol on toluene biodegradation in nitrate amended, sulfate amended and methanogenic enrichment microcosms. Biodegradation, 24: 657-663).

The study on ethylbenzene degradation under oxygen depleted conditions in agricultural soils is presented in Chapter 3. This study involved the investigation of ethylbenzene degradation in ~30 different experimental set-ups (different soils and redox conditions). From these, ethylbenzene degradation was noted only once. For this condition (nitrate reducing conditions in an agricultural soil), SIP and amplicon sequencing were used to identify the dominant ethylbenzene degrading microorganisms. . Bacteria belonging to the families *Oxalobacteraceae, Rhodospirillaceae, Xanthomonadaceae* and *Rhodocyclaceae* and the genus *Gemmatimonas* were linked to the assimilation of the ring labeled carbon from ethylbenzene. The sequencing data from the total DNA extracts from the ethylbenzene degrading microcosms showed that phylotypes belonging to the genus *Rhodanobacter* were the most dominant microorganisms. The work presented under Chapter 3 has been submitted for publication to a peer reviewed journal.

The research work presented in Chapters 4 and 5 were funded by a Strategic Environmental Research and Development Program (SERDP) grant entitled "Development of biomarkers for assessing in situ RDX biodegradation potential" awarded to Dr. Cupples. In Chapter 4, a comparative study of RDX degrading microbial communities obtained from four different uncontaminated soil sources is presented. Illumina sequencing revealed that phylotypes classifying either within the family *Bacillaceae 1* (contains RDX isolates) or the genus *Brevundimonas* (no known RDX degrading isolates) were the most abundant phylotypes in all four soils. In addition, a SIP study was conducted with samples for two of the soils (soil 9 and soil 10). In soil 9, sequences classifying as *Bacillaceae, Clostridiales* and *Tisserella* were the most abundant in the heavy labeled fractions compared to the control unlabeled fractions. In soil 10, phylotypes belonging to *Pusillimonas, Rhodococcus, Sedimentibacter* were enriched in the heavy labeled fractions in comparison to the fractions obtained from the unlabeled samples. The work presented under this chapter is being prepared for publication.

Chapter 5 involves the use of SIP and high throughput sequencing to identify the microorganisms responsible for RDX degradation in soils obtained from an area used to test denotations (a Navy Base in Virginia, US). This work also explores the use of glucose as an amendment to promote RDX degradation. Members of the *Pseudomonadaceae*, *Comamonas* (with glucose samples) and *Acinetobacter* (no glucose samples) were observed as the most abundant phylotypes in the total DNA extracts. Members of the *Pseudomonadaceae* family were also linked to label uptake from RDX, both in the presences and absence of glucose. The work in Chapter 5 has been submitted for publication to a peer reviewed journal.

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CHAPTER 2 EFFECT OF ISOBUTANOL ON TOLUENE BIODEGRADATION IN NITRATE AMENDED, SULFATE AMENDED AND METHONOGENIC ENRICHMENT MICROCOSMS

ABSTRACT

Isobutanol is an alternate fuel additive that is being considered because of economic and lower emission benefits. However, future gasoline spills could result in co-contamination of isobutanol with gasoline components such as benzene, toluene, ethyl-benzene and xylene (BTEX). Hence, isobutanol could affect the degradability of gasoline components thereby having an effect on contaminant plume length and half-life. In this study, the effect of isobutanol on the biodegradation of a model gasoline component (toluene) was examined in laboratory microcosms. For this, toluene and isobutanol were added to six different toluene degrading laboratory microcosms under sulfate amended, nitrate amended or methanogenic conditions. While toluene biodegradation was not greatly affected in the presence of isobutanol in five out of the six different experimental sets, toluene degradation was completely inhibited in one set of microcosms. This inhibition occurred in sulfate amended microcosms constructed with inocula from wastewater treatment plant activated sludge. Our data suggest that toluene degrading consortia are affected differently by isobutanol addition. These results indicate that, if co-contamination occurs, in some cases the *in situ* half-life of toluene could be significantly extended.

INTRODUCTION

Various additives have been added to gasoline in an effort to minimize the emissions and to reduce non-renewable fuel dependency. Approximately 77% of all gasoline in the US contained alcohol up to 10% v/vol as of the year 2009 (1). Currently, biologically produced isobutanol is being considered as an alternative gasoline additive as it convenient to use with existing car engines and pipelines, can be used pure or at any blend ratio and also can be mixed offsite (2). A recent success in obtaining microbially produced isobutanol from cellulosic material such as corn stove waste by genetically engineered bacterium (3) adds to the benefits of switching to isobutanol as a gasoline additive. However, assuming that future spills are inevitable, it would be wise to have an understanding of the fate of isobutanol in the environment as well as any effect it might have on the biodegradation of other gasoline components.

Previous research has revealed ethanol affects the fate of gasoline components in the environment. For example, due to its high hygroscopic nature, ethanol was found to produce a co-solvency effect resulting in increased BTEX aqueous concentrations (4, 5) and plume lengths (6). Ethanol is also preferentially degraded, causing depletion of oxygen, resulting in the inhibition of benzene degradation (7, 8). Further, ethanol biodegradation can consume subsurface nutrients and electron acceptors that would otherwise have been available for BTEX degradation (9). This in turn alters the composition of the local microbial population which could further result in reduced BTEX degradation (10). The concentration of ethanol also appears to affect BTEX degradation. At low ethanol concentrations, it was found to enhance BTEX biodegradation, in particular both aerobic and anaerobic benzene biodegradation (11, 12). In contrast, at higher concentrations, ethanol decreased the degradation rates BTEX compounds (9, 11, 13).

As ethanol exerts a high biochemical oxygen demand on the system, it is more relevant to discuss the effect of ethanol on BTEX biodegradation under anaerobic conditions (7). Corseiul et al.(1998) reported ethanol's detrimental effect on toluene degradation under iron and methanogenic conditions, but enhanced toluene degradation under sulfate reducing conditions (14). Others have reported only slight toluene degradation under denitrifying conditions (15). In contrast, Da Silva et al. (2005) reported that though ethanol was inhibitory under methanogenic conditions, it enhanced BTEX biodegradation with the addition of electron acceptors (12). At higher ethanol concentrations most studies report slow or no BTEX biodegradation (9, 11, 12, 15-17). Similar contradicting results were obtained in biodegradation studies with *n*-butanol, an isomer of isobutanol. N-butanol while enhancing biodegradation of gasoline (18), was also found to be toxic to cell growth even at a concentration of 1% v/v (19).

To date, only three studies (three aerobic and one anaerobic) have investigated the impact of isobutanol on BTEX biodegradation (20-22). Two of the studies used soil and groundwater samples collected from the same contaminated site in CA (20, 21) and found isobutanol had varying effects on BTEX degradation. The third study (22) used several agricultural soils degrading one or more of the BTEX compounds under aerobic conditions and concluded that isobutanol does not affect aerobic BTEX degradation. Here, we expand on this research by investigating the effect of isobutanol in six microbial communities derived from four different sources. The objective was to determine if the effect of isobutanol varied between samples from different sources (i.e. different microbial communities). More specifically, the effect of isobutanol on toluene degradation was investigated under nitrate amended, sulfate amended and methanogenic conditions, using samples from different sources. Toluene was selected as the representative BTEX compound because previous work in our laboratory identified the toluene degrading species in a number of these communities using stable isotope probing (SIP) (23). Thus, this enabled us to link the effect of isobutanol of specific toluene degrading species.

METHODS

The effect of isobutanol on toluene degradation was investigated in seven different experiments prepared using inocula from enrichment cultures previously developed in our laboratory. Of these, toluene was degraded under either methanogenic (one experiment), sulfate amended (three experiments), or nitrate amended (three experiments) conditions (Table 2.1). Each experiment consisted of 12 sample (live) microcosms and 2 control (killed) microcosms.

The microcosms for the methanogenic experiment were prepared using wastewater treatment plant digester sludge. The microcosms for the three nitrate amended experiments were prepared using either one of the three agricultural soils (corn crop) obtained from three different sources, two with low organic matter (agricultural soil 1 and 2) and one with high organic matter (agricultural soil 3). The microcosms for two of the sulfate amended experiments were prepared using wastewater treatment plant digester sludge and the microcosm for the third sulfate amended experiment was prepared using agricultural soil 1. A low concentration of isobutanol (5 mg/L) was chosen for six out of the seven experiments, following previous research (21). For the seventh set (sulfate amended experiment prepared with agricultural soil 1) the isobutanol concentration was still lower (~2.5 mg/L).

Microcosm preparation

All microcosms were prepared under strictly anaerobic conditions in an anaerobic chamber (Coy Laboratory Products, Grass Lake, MI). The microcosms were prepared in 60 mL clear wheaton serum bottles (Fisher scientific, Pittsburgh, PA) by adding 0.5 to 1 mL of enrichment culture to 40 mL of basal minimal media (24). When appropriate, sterile potassium nitrate or magnesium sulfate solution were added (final concentration of 1 g/L) as electron acceptors. The microcosms were sealed tight with a butyl rubber stopper with PTFE coating and an aluminum seal to maintain anaerobic conditions. Seven experiments were performed to investigate the effect of isobutanol on toluene degradation (one methanogenic, three sulfate amended and three nitrate amended). For each experiment, 14 microcosms were prepared, including 2 abiotic controls (heat sterilized), 6 no isobutanol replicate microcosms and 6 replicate microcosms with isobutanol. The concentration of isobutanol in the isobutanol containing replicate microcosms, was ~5 mg/L in all experiments except one sulfate amended experiment prepared with wastewater sludge enrichment, which was prepared with lower isobutanol concentration(~ 2.5 mg/L). The rationale for this extra experiment is discussed later. The microcosms were stored at room temperature without shaking.

Analytical methods

Toluene and isobutanol concentrations were investigated over time in all microcosms. The timing of sample removal varied depending on preliminary data collected on toluene degradation. Headspace toluene concentrations were measured using a Perkin Elmer gas chromatography equipped with a flame ionization detector (GC-FID). 200 µL headspace samples were sampled using a gas-tight syringe and injected through a DB-624 (Agilent) capillary column (length 30m, internal diameter 0.53mm and a film thickness of 0.5 um). Helium was used as the carrier and at 14 psi head pressure the elution time for ethylbenzene was 1.8 minutes. The aqueous phase toluene concentrations were calculated by applying Henry's law. Aqueous isobutanol concentrations were estimated using headspace isobutanol concentrations and mass balance, using the same GC protocol.

RESULTS AND DISCUSSION

The effect of isobutanol on toluene degradation was examined in seven experiments involving microbial communities from four different sources. As expected, toluene was degraded in all live samples but was not significantly depleted in the controls, indicating a biological removal mechanism. The time for toluene depletion varied, primarily depending on the electron accepting process. While the varying levels of organic matter in the different soils could affect sorption of toluene and thereby its bioavailability and biodegradation rates, previous studies show that it also desorbs rapidly when biodegradation occurs (25). In microcosms amended with nitrate and constructed with the agricultural soil 1 enrichment, toluene degraded in just over 20 days in all 12 live samples (Figure 2.1a). There was no significant difference in the time for toluene degradation between the samples amended and those unamended with isobutanol. Toluene was degraded more rapidly (under 5 days) in the microcosms derived from agricultural soil 2 and soil 3 enrichments (Figures 2.1b and 2.1c). Again, no effect of isobutanol on toluene degradation was noted for these samples.

Toluene degraded at a much slower rate in sulfate amended experiments compared to the nitrate amended experiments. In sulfate amended microcosms constructed from agricultural soil 1 enrichments, (Figure 2.2a), toluene degradation was completed in all live samples after approximately 90 days. In these samples, toluene degradation rates varied between replicates and between the treatments, with no clear trend being apparent. In contrast, in the sulfate amended microcosms constructed from the digester sludge enrichments (Figure 2.2b and 2.2c), toluene degradation was completely inhibited in the presence of isobutanol. Also, among the two different sulfate amended experiments constructed from digester sludge enrichments, the time for toluene degradation was slower in those amended with a lower concentration of isobutanol (Figure 2.2b), which was likely because these were prepared at a later date and the enrichment culture was less active. Toluene degradation was also slow in the methanogenic microcosms constructed from wastewater digester sludge enrichments. In these microcosms, toluene degradation occurred between day 60 and 137. As no measurements were taken between these timepoints, it is difficult to conclude if isobutanol had an effect on toluene degradation during this time (Figure 2.3). Also in these microcosms, isobutanol was in fact completely degraded by day 34 (see below).

Isobutanol degradation varied between the different experiments. Among the nitrate amended samples, it was slightly depleted in two of the three experiments to ~2-3 ppm (Figure 2.4a and 2.4c). In the third set of nitrate amended samples, the isobutanol concentration did not significantly change (Figure 2.4b). In the sulfate amended samples, isobutanol degradation was varied, taking between 21 and 118 days to degrade to ~1 ppm in the samples constructed with agricultural soil 1 enrichments (Figure 2.5a). In contrast, in the two experiments with the activated sludge samples, isobutanol degradation was rapid at both the lower (Figure 2.5b) and higher (Figure 2.5c) isobutanol concentrations. In these samples, isobutanol was degraded by approximately day 15. In the methanogenic toluene degrading microcosms, isobutanol degraded rapidly and was completely depleted by day 34 (Figure 2.6). Our results show that isobutanol is completely biodegradable in both the sulfate and nitrate amended microcosms and in the methanogenic microcosms. Isobutanol degraded much faster and along with toluene in the nitrate amended microcosms compared to the sulfate amended microcosms.

The presence of isobutanol had varying effects on toluene degradation in the six different microbial communities. In all three nitrate amended toluene degrading microbial consortia, the presence of isobutanol did not significantly alter the rate of toluene degradation. Schaefer et al (2010b) (21) reported that isobutanol actually increased the rate of BTEX biodegradation. They reported that isobutanol enhanced TEX biodegradation under nitrate reducing conditions and both ethanol and isobutanol enhanced benzene biodegradation under sulfate reducing conditions. Whereas, in the current study, in the sulfate amended samples derived from wastewater digester sludge, isobutanol actually inhibited toluene degradation. As previously mentioned, such mixed effects have been noted before for ethanol, where ethanol inhibited toluene degradation under methanogenic, iron reducing and nitrate reducing conditions, yet enhanced toluene degradation under sulfate reducing conditions (14). Schaefer et al (2010b) (21) also found that isobutanol could have a mixed effect on toluene degradation particularly under the presence of excess sulfate. Other studies have reported ethanol slowed TEX biodegradation rate in the absence of any electron acceptors (12, 21). These results show that the effect of isobutanol on BTEX biodegradation is likely not electron acceptor specific.

The current study found that the effect of isobutanol on toluene degradation varied between different toluene degrading communities. The major toluene degraders in three of the six inocula tested have been previously identified (23). The key degraders in agricultural soil 1 were identified as members of the family *Comamonadaceae*. In the microcosms derived from agricultural soil 1 enrichments, where isobutanol did not affect toluene biodegradation in sulfate amended microcosms, the major toluene degrader was identified as a member of the genus *Desulfosporosinus.* In the sulfate amended microcosms derived from digester sludge enrichments in which toluene degradation was inhibited in the presence of isobutanol the major toluene degrading organism was identified as a member of the family *Syntrophobacteraceae.* Site specific active microbial communities are often not considered as a input parameter in numerical models used to predict BTEX plume length (7). However, different microbial communities might be affected differently by isobutanol as seen here, and such numerical models may provide only partial information. Thus like ethanol the effect of isobutanol could be specific to the system in which contamination occurs (26). To our knowledge, this is the first study that has studied the effect of isobutanol on more than one toluene degrading consortia under different electron acceptor conditions.

CONCLUSIONS

Isobutanol illustrated a varying effect on toluene biodegradation in the different toluene degrading microbial communities. While the presence of isobutanol did not affect toluene degradation in five of the six experiments under nitrate reducing, sulfate reducing or methanogenic conditions, toluene degradation was inhibited in the digester sludge amended sulfate reducing microcosms. Also, in the experiments in which isobutanol did not affect toluene degradation, there was no apparent difference in the rate of degradation, suggesting it neither enhanced nor inhibited toluene degradation in these microcosms. Our results suggest that the effect of isobutanol on BTEX biodegradation could differ based on the site specific conditions, particularly the microbial consortia and their assimilative capacity. Thus, this study supports the need for further site specific studies on the effect of isobutanol on BTEX contaminants to further understand the overall impact of switching to isobutanol as a gasoline additive.

APPENDIX

Table 2.1. Experimental setup for nitrate amended, sulfate amended and methanogenic toluene degrading samples.

	Electron acceptor	**Enrichment culture source**	**No. of microcosms**			**Isobutanol concentration (mg/L)**
			Abiotic	**Live (no isobutanol)**	**Live (with isobutanol)**	
1	Methanogenic	Wastewater treatment plant activated sludge	2	6	6	5
2	Nitrate amended	Agricultural soil 1	2	6	6	5
3		Agricultural soil 2	2	6	6	5
4		Agricultural soil 3	2	6	6	5
5	Sulfate amended	Agricultural soil 1	2	6	6	5
6		Wastewater treatment plant activated sludge	2	6	6	5
7		Wastewater treatment plant activated sludge	2	6	6	2.5

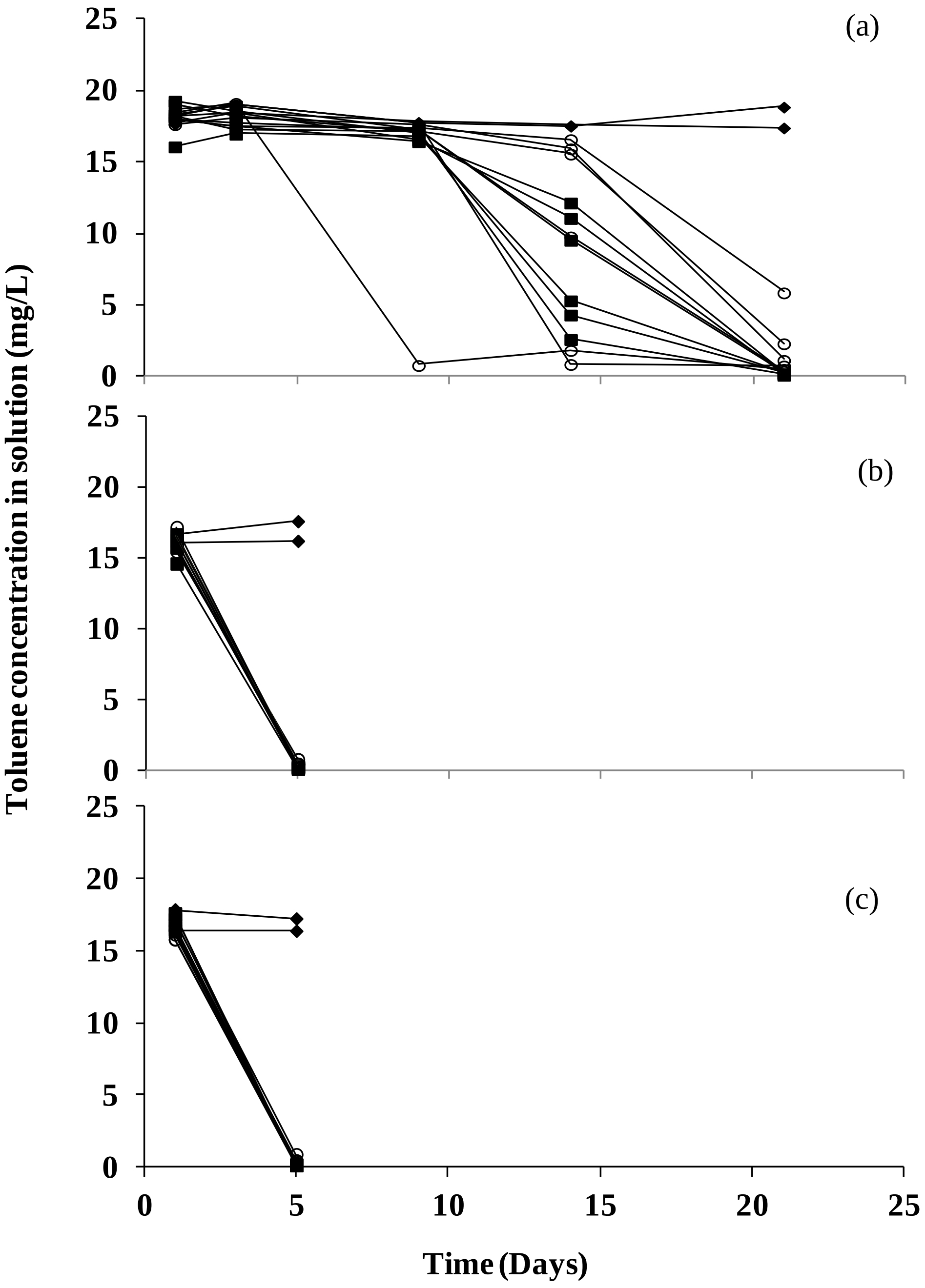

Figure 2.1. Toluene concentrations in nitrate amended microcosms derived from enrichments constructed from agricultural soil 1(a), agricultural soil 2 (b) and agricultural soil 3 (c).

The symbols represent abiotic control [♦], toluene only [○] and toluene and isobutanol [■] amended microcosms.

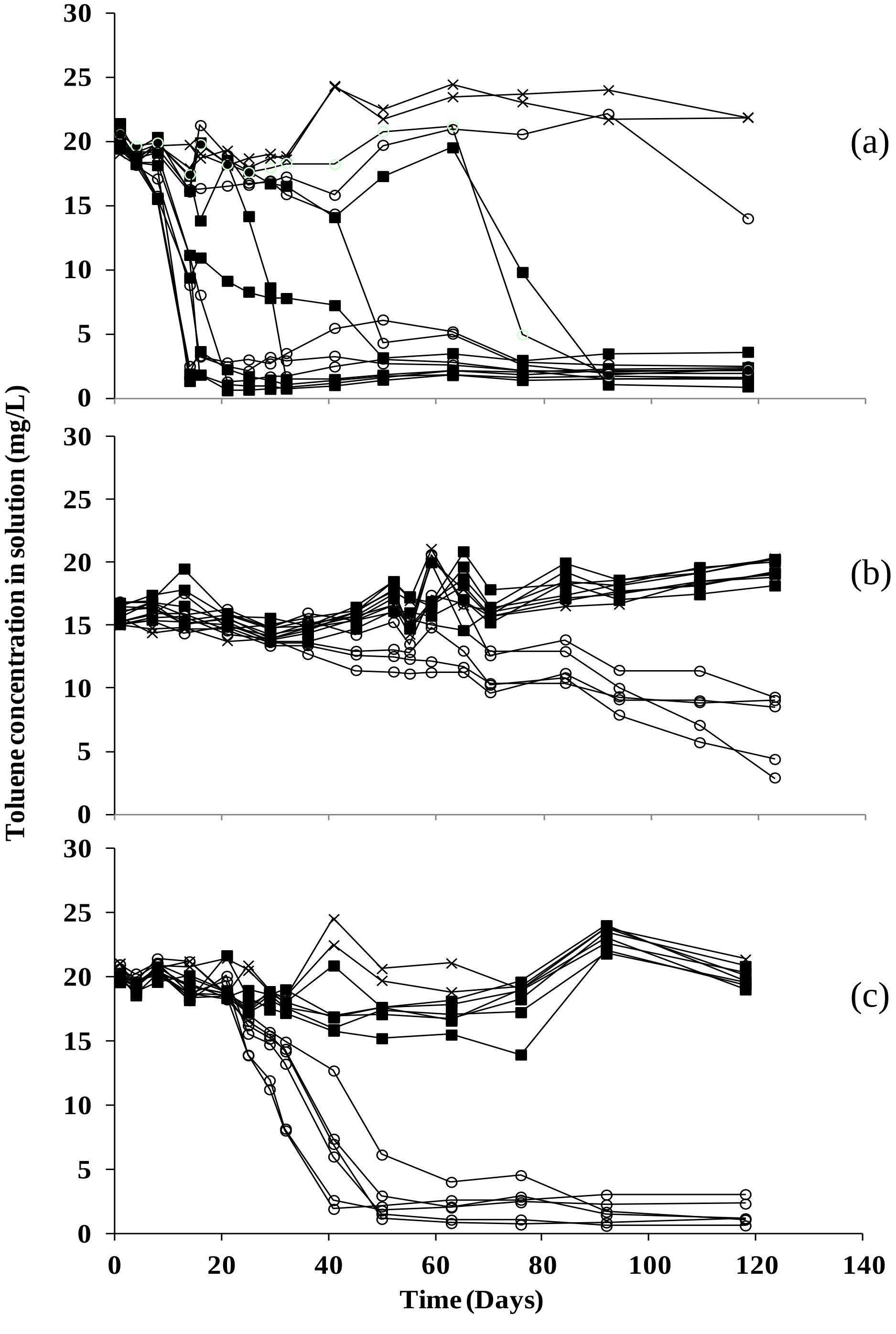

Figure 2.2. Toluene concentrations in sulfate amended microcosms derived from enrichments constructed from agricultural soil 1(a) wastewater treatment plant digester sludge with 2.5 ppm isobutanol (b), or with 5 ppm isobutanol (c).

The symbols represent abiotic control [╳], toluene only [○] and toluene and isobutanol [■] amended microcosms.

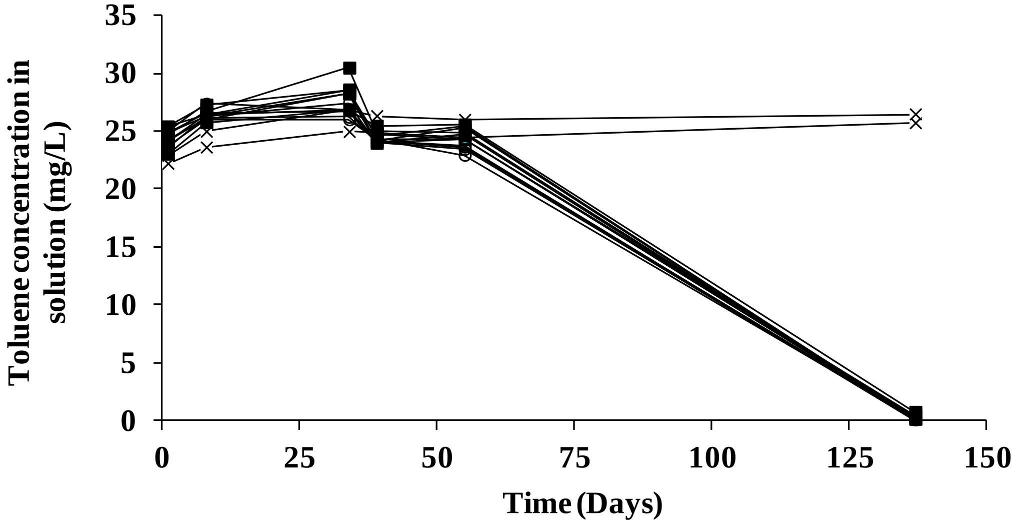

Figure 2.3. Toluene concentrations in methanogenic microcosms derived from enrichments constructed from wastewater treatment plant digester sludge.

The symbols represent abiotic control [╳], toluene only [○] and toluene and isobutanol [■] amended microcosms.

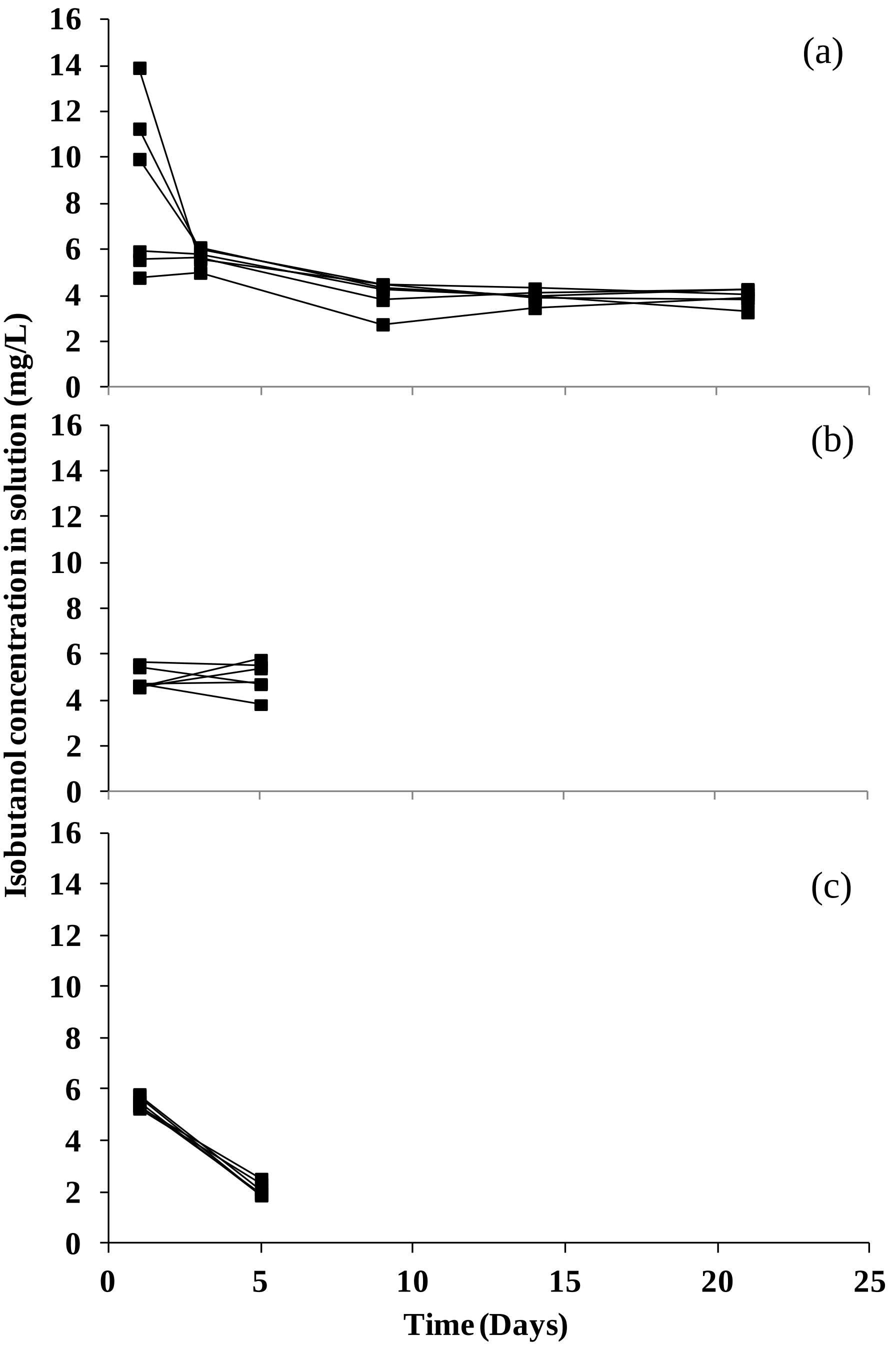

Figure 2.4. Isobutanol concentrations in nitrate amended toluene degrading microcosms derived from enrichments constructed from agricultural soil 1 (a), agricultural soil 2 (b) and agricultural soil 3 (c).

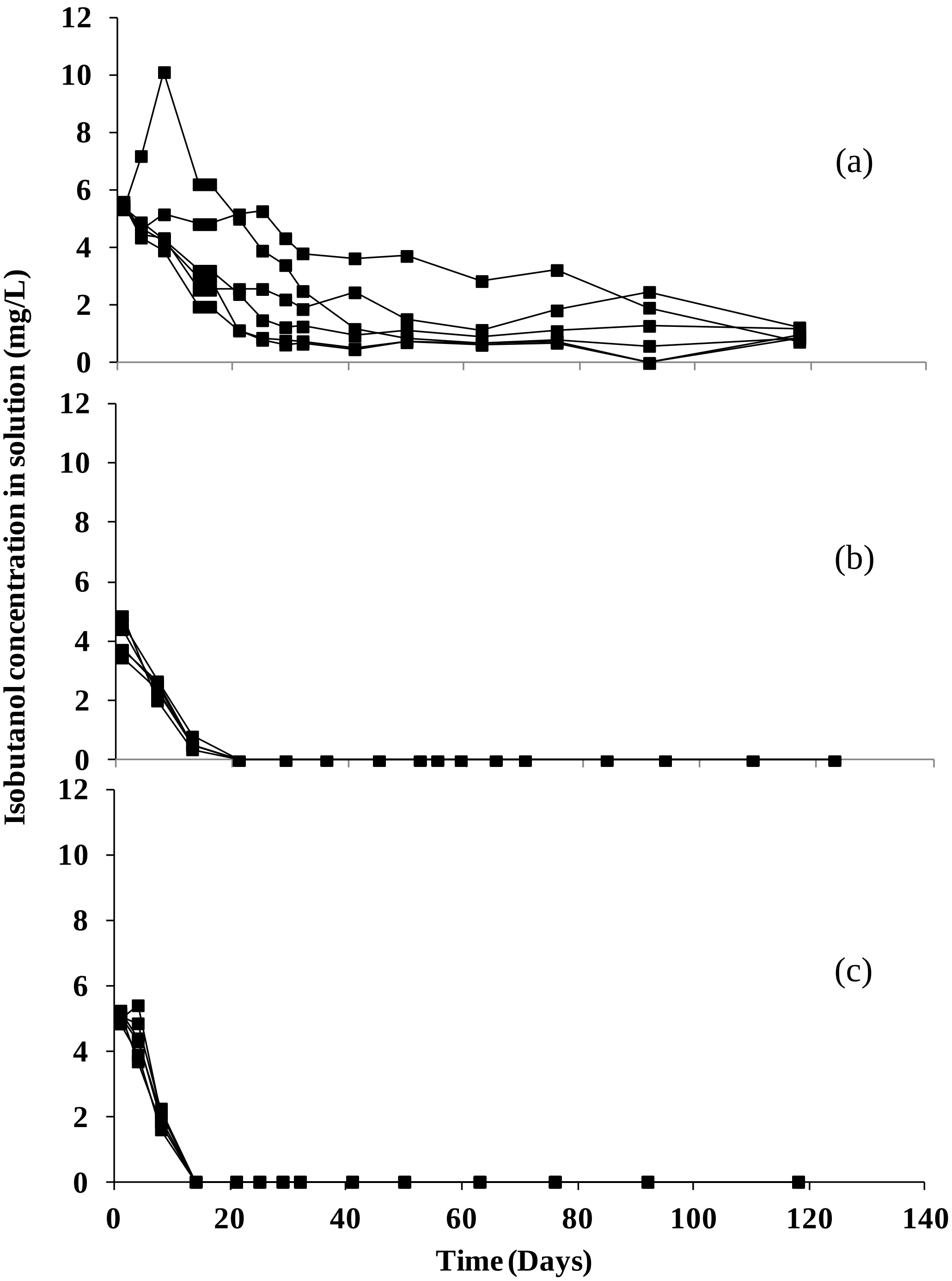

Figure 2.5. Isobutanol concentrations in sulfate amended toluene degrading microcosms derived from enrichments constructed with agricultural soil 1 (a), and wastewater treatment plant digester sludge 2.5 ppm isobutanol (b) or 5 ppm isobutanol (c).

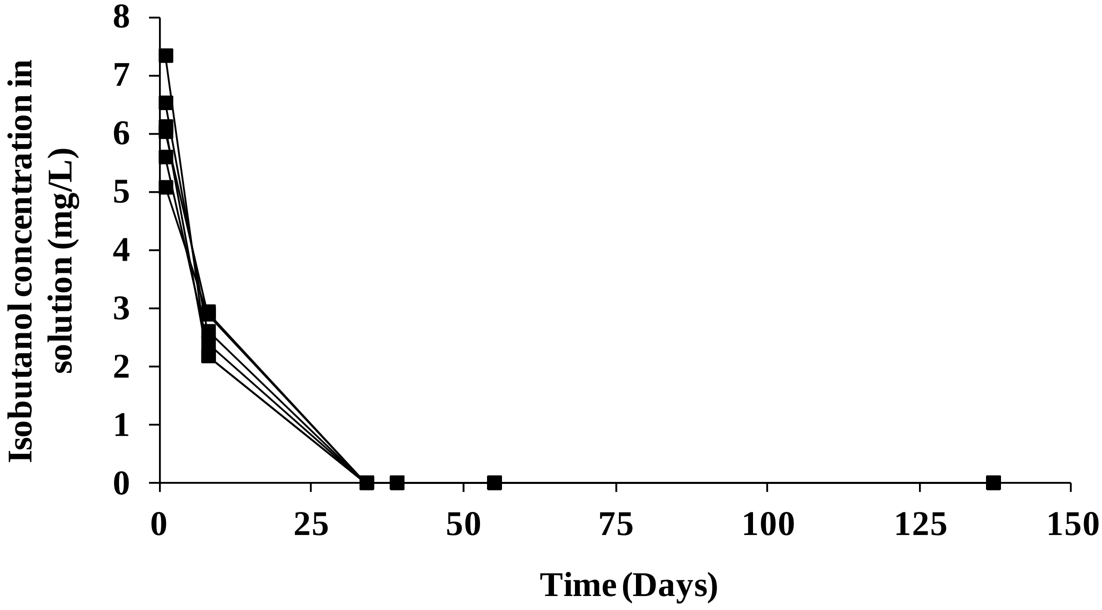

Figure 2.6. Isobutanol concentrations in methanogenic toluene degrading microcosms derived from enrichments constructed with wastewater treatment plant digester sludge.

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CHAPTER 3 STABLE ISOTOPE PROBING AND HIGH THROUGHPUT SEQUENCING IMPLICATES *OXALOBACTERACEAE, RHODOSPIRILLACEAE, XANTHOMONADACEAE* AND *RHODOCYCLACEAE* IN ETHYLBENZENE DEGRADATION

ABSTRACT

The overall aim was to identify the microorganisms responsible for carbon uptake from ethylbenzene (or ethylbenzene breakdown products) in soil microcosms using culture independent methods. Ethylbenzene degradation was observed in only one (nitrate amended agricultural soil) of the thirty-one different experimental setups (different amendments and inocula sources) tested. To determine which microorganisms were responsible for ^{13}C uptake, stable isotope probing (SIP) was combined with high throughput sequencing Illumina amplicon sequencing. For this, total genomic DNA and heavy fraction samples (following ultracentrifugation to separate light from heavy DNA) from the live control microcosms (amended with $^{12}C_6$ ethylbenzene) and sample microcosms (amended with $^{13}C_6$ ethylbenzene) were subject to Illumina amplicon sequencing. Several phylotypes were relatively more abundant in the heavy fractions from the $^{13}C_6$-labeled ethylbenzene amended soil microcosms compared to the heavy fractions from the live controls (from unlabeled ethylbenzene amended microcosms). This included unclassified phylotypes within the families *Oxalobacteraceae, Rhodospirillaceae, Xanthomonadaceae* and *Rhodocyclaceae* (*Proteobacteria*) as well as the genus *Gemmatimonas* (phylum *Gemmatimonadetes*). In addition, the total microbial community was characterized in six ethylbenzene degrading microcosms and was dominated by the genus *Rhodanobacter*

(relative abundance 11.7 – 15.6%) (family *Xanthomonadaceae*), although this phylotype did not illustrate ^{13}C uptake. *Azoarcus* (family *Rhodocyclaceae*), a known ethylbenzene degrader, was dominant in only two of the six microcosms (relative abundance 14.6 and 24.1%) and it also did not illustrate label uptake. The work indicates microorganisms not previously linked to ethylbenzene degradation have significant roles in the carbon uptake from this contaminant.

INTRODUCTION

Hydrocarbons such as benzene, toluene, ethylbenzene and xylene (BTEX) contained in gasoline have been released into the environment by accidental spillage or from leaking underground storage tanks. These compounds cause groundwater and sediment pollution posing an environmental and health threat due to their toxicity and mobility. BTEX remediation using microorganisms that can utilize these compounds as a carbon or an energy source is a feasible solution. However, under anaerobic conditions, BTEX biological removal is typically slow.

From the BTEX chemicals, toluene is the most studied and perhaps the most readily amenable to anaerobic biodegradation (1). Several microorganisms have been isolated that are able to metabolize or mineralize toluene under nitrate reducing (2, 3), iron reducing (4) and sulfate reducing (5) conditions (the majority belong to the phylum *Proteobacteria* and a few to *Firmicutes*). Isolates have also been shown to degrade *m*- or *o*-xylene under nitrate reducing (2), sulfate reducing (6) and iron reducing (7) conditions. In contrast, there have been reports of only three nitrate reducing, benzene degrading isolates (8, 9). After benzene, ethylbenzene is perhaps the next most difficult to degrade among the BTEX compounds. To date, anaerobic ethylbenzene degradation has been shown in several nitrate reducing or sulfate reducing bacteria (1). These include two *Azoarcus sp*. strains (*Aromatoleum aromaticum* EbN1 and EB1) (10-12) , *Dechloromonas aromatica RCB* (8, 13), *Georgfuchsia toluolica* (14) and *Desulfobacteraceae* Strain EbS7 (15). Interestingly, all except Strain EbS7 (*Deltaproteobacteria*) belong to the family *Rhodocyclaceae* (*Betaproteobacteria*). More information concerning the diversity of microorganisms involved in ethylbenzene degradation would contribute to our understanding of the biodegradation potential of this chemical at contaminated sites.

The current limited information on ethylbenzene degraders can, in part, be attributed to the limitations associated with traditional microbiological methods (only a minor % of existing bacteria can be cultured in the laboratory). Culture independent methods, such as stable isotope probing (SIP), can often circumvent these limitations. For example, SIP studies have identified novel microorganisms involved in geochemical cycles (16, 17) and in remediation of environmental pollutants (18-21) that were not previously identified using culture based techniques. SIP involves the addition of a labeled substrate (e.g. ^{13}C labeled toluene) to a mixed community, ultracentrifugation of the extracted DNA, then the analysis of the heavy fractions of the SIP gradient to identify the phylotypes responsible for label uptake. Previously, we have used terminal restriction fragment length polymorphism (TRFLP) and clone libraries (with traditional Sanger sequencing) to identify the organisms enriched in the labeled treatments during SIP experiments (22-27). However, extensive 16S rRNA gene clone libraries are needed to identify the enriched TRFLP fragments. In fact, researchers have previously reported they were unable to identify 25% (two out of eight) TRFLP fragments thought to be responsible for label uptake (28). Replacing TRFLP and clone libraries with next generation sequencing has the potential to provide a greater depth of information on the occurrence of specific phylotypes in SIP gradients.

For example, in 2011, researchers have used pyrosequencing on total DNA extracts in an SIP study to investigate the microorganisms involved in toluene degradation at a tar-oil-contaminated aquifer (29). Others have integrated pyrosequencing libraries with SIP identified sequences to determine the abundance of microorganisms in samples from the Deepwater Horizon soil spill (30) or to investigate ammonia oxidizers (31). Further, researchers have also used pyrosequencing on the SIP fractions (rather than on total DNA extracts) to, for example, investigate nitrogen-incorporating bacteria in petroleum contaminated artic soils (32), to study organic carbon uptake by bacterioplankton (33), to investigate ammonia and nitrite oxidation (31, 34-36), to study nonylphenol degraders in activated sludge (37), to study the gut microbiota of cotton leafworm (*Spodoptera littoralis*) (38) to investigate toluene degradation (39) and to study biological soil crusts (40).

In the current study, the overall aim was to identify microorganisms able to uptake ^{13}C from ethylbenzene (or ethylbenzene degradation products) in soil based microcosms, using culture independent methods (SIP and high throughput sequencing). For this, the relative abundance of phylotypes present in the heavy fractions of two labeled and two unlabeled ethylbenzene amended microcosms from one soil were compared. The phylotypes with a high relative abundance in the heavy fractions of the labeled ethylbenzene amended microcosm (compared to similar fractions in the unlabeled amended microcosms) were deemed responsible for incorporating the labeled carbon from ethylbenzene (or ethylbenzene degradation products). An underlying hypothesis of this work was that these techniques would produce a more diverse group of ethylbenzene degraders than those previously reported.

METHODS

Chemicals

Unlabeled and labeled ethylbenzene (Phenyl -$^{13}C_{6}$, 99%) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Other reagents were purchased from one or more of the following vendors: Fisher Bioreagent (Thermo Fisher Scientific, NJ, USA), Invitrogen (Life Technologies, Grand Island, NY, USA) and Sigma-Aldrich (St.Louis, MO, USA), unless stated otherwise.

Development of Ethylbenzene Degrading Microcosms

A wide variety of inocula and electron acceptor conditions were tested for ethylbenzene degradation using microcosms prepared under strict anaerobic conditions in an anaerobic chamber (Coy Laboratory Products, Grass Lake, MI). The inocula sources consisted of 12 different agricultural soil samples (collected from Michigan), activated sludge samples (from two wastewater treatment plants) and digested manure samples (from an anaerobic digester at Michigan State University). These sources were selected because previous research indicated BTEX degradation potential (23-27). The inocula sources were tested under nitrate amended, sulfate amended, iron amended and/or methanogenic conditions in the presence of an anaerobic minimal basal media (41). For this, triplicate microcosms were prepared with 6 to 10 g (wet weight) of the inocula source, 30 mL of the anaerobic basal media and 20 mg L^{-1} of unlabeled ethylbenzene in a 160 mL serum bottle. Electron acceptors were amended (1 g L^{-1} of SO_4^{2-} or NO_3^- or Fe^{3+}) as required. Microcosms were sealed using rubber stoppers and aluminum seals in the anaerobic chamber. All incubations were monitored for ethylbenzene removal for up to 8 weeks (data not shown). Among the 31 different incubations, only the triplicate microcosms prepared with an agricultural soil inocula amended with nitrate showed ethylbenzene degradation. This soil was used to inoculate the microcosms for the SIP study (prepared as above). For the SIP study, triplicate killed controls and four microcosms were prepared with 20 mg L^{-1} of unlabeled ethylbenzene (hereafter, called Unlabel EB 1, Unlabel EB 2, SIP Unlabel EB 1 and SIP Unlabel EB 2) and two live sample microcosms were prepared with 20 mg L^{-1} of labeled ethylbenzene (phenyl -$^{13}C_6$, 99%) (hereafter, called SIP Label EB 1 and SIP Label EB 2). From these, DNA extracted from SIP Unlabel EB 1, SIP Unlabel EB 2, SIP Label EB 1 and SIP Label EB 2 was used for SIP analysis (ultracentrifugation and fractionation) and total microbial community analysis. Whereas, DNA extracted Unlabel EB 1 and Unlabel EB 2 was only used for microbial community analysis. This allowed the comparison of the microbial community across six microcosms. All microcosms were incubated at room temperature (~20 °C), without shaking.

Analytical Techniques

Ethylbenzene biodegradation was monitored over time by measuring headspace ethylbenzene concentrations in the microcosms. Sampling days for the SIP microcosms were determined based on the results of the initial screening microcosms. For this, 200 µL of headspace samples were injected into a gas chromatograph (Perkin Elmer) attached to a flame ionization detector and an AT-624 capillary column (J & W Scientific; 30 m, 0.53 mm ID, 3.0 µm) with helium as carrier gas. The oven temperature was set to 150 °C while the injector port and detector temperature were set to 200 °C with an ethylbenzene elution time of 2.3 minutes. Nitrite and nitrate measurements were made only on day 1 and 22 using ion chromatography.

DNA Extraction and Isopycnic Centrifugation

Total genomic DNA was extracted from the six microcosms (two labeled and four unlabeled ethylbenzene amended) using the Power Soil DNA extraction kit following the manufacturer's instruction (MO BIO Laboratories, Inc. Carlsbad, CA) following the degradation of ethylbenzene. The extracted DNA from SIP Unlabel EB 1, SIP Unlabel EB 2, SIP Label EB 1 and SIP Label EB 2 was ultracentrifuged in cesium chloride gradients separately to obtain density-resolved gradients and fractions. Approximately 10 µg of total genomic DNA was mixed with a Tris-EDTA (pH 8.0) buffer and CsCl solution (no glycogen was added to the gradient). This mixture was added to a 5.1 mL Quick-Seal polyallomer tubes (1.3 x 5.1 cm, Beckman Coulter) the buoyant density (BD) of this mixture was adjusted to ~1.72 g mL^{-1} using a model AR200 digital refractometer (Leica Microsystems Inc.) and then sealed using a tube topper (Cordless quick-seal tube topper, Beckman). The tubes were then centrifuged at 178,000 x *g* for 46 hours at 20 °C in a Wx Sorvall Ultra 80 ultracentrifuge fitted with a Stepsaver 70 V6 Vertical Titanium Rotor (Thermo Scientific).

The samples were separated into 20 fractions (each 250 µL) by displacing the samples with molecular grade water. A syringe pump attached to a needle (BD, 23G and 1 inch) was used to displace samples from the top of the tube, as fractions were collected from the bottom by gravity, from heavy to light fractions. The BD of each fraction was calculated from the refractive index obtained using a refractometer. DNA from each of the fraction was recovered using a glycogen and ethanol precipitation. Precipitated DNA was then re-suspended in 30 µL PCR grade water and stored at -20 °C for further analysis. The DNA concentration in was quantified in these fractions using replicate measurements with the Qubit assay.

High Throughput Amplicon Sequencing (Illumina MiSeq)

Samples were subject to amplicon sequencing, following the protocol described elsewhere (42, 43) (Research Technology Support Facility or RTSF at Michigan State University). The fractions were analyzed to determine which microorganisms were enriched in the heavy fractions and were therefore responsible for carbon uptake from ethylbenzene (or ethylbenzene degradation products). Also, the total DNA samples were analyzed with high throughput sequencing to characterize the total microbial community present. The analysis included four heavy fractions from SIP Unlabel EB 1, SIP Unlabel EB 2, SIP Label EB 1 and SIP Label EB 2. To enable a comparison between fractions, the BD of the selected fractions from the labeled and unlabeled amended microcosms were similar (maximum difference ±0.01 g mL^{-1}) (Table 3.1). These fractions were selected because their BD values were similar to those previously reported by our group for SIP label enrichment (22-27). In addition, the fractions were selected based on the concentration of DNA in each fraction. Illumina specific fusion primers were used to amplify the V4 region of the 16S rRNA (42) and to add unique barcodes to samples in each well to enable pooling and sequencing. After the amplicons were checked on 1% agarose gel, equimolar amounts of the sample were pooled to normalize results, purified and then sequenced on the Illumina MiSeq™ Personal Sequencing System.

The data obtained from amplicon sequencing in the fastq file format was analyzed using Mothur (version 1.32.1) developed by Schloss (44) and the MiSeq standard operating procedure (SOP) developed by the same laboratory(45, 46). Briefly, barcodes were removed from the sequence data and contigs were made using the forward and reverse reads. Samples from one labeled and one unlabeled replica each containing 4 fractions and one total genomic DNA sample (thus a total of 10 samples) were analyzed together. The paired-end reads were used to make contigs, were analyzed for errors and then classified. Sequences that did not assemble well were removed from further analysis. Reads were checked for ambiguous bases and homopolymer length greater than 8 (following the Mothur SOP) to remove sequencing errors. These sequences were then aligned with the SILVA bacteria database (47) for the V4 region. Chimeras, mitochondrial and chloroplast lineage sequences were removed and then the sequences were classified into OTU's. The OTUs were then grouped into taxonomical levels with corresponding confidence levels. Rarefaction curves were generated using Mothur.

Cloning and Sanger Sequencing

A 16S rRNA gene clone library was constructed by amplifying, cloning and sequencing the total genomic DNA. The 16S rRNA gene was amplified using the universal primers: 27F 5'-AGAGTTTGATCMTGGCTCAG) and 1492R (5'-GGTTACCTTGTTACGACTT) (Operon Biotechnologies) using the following cycle program: initial denaturation step (94 °C for 5 min), 30 cycles of amplification (94 °C for 30 sec, 55 °C for 30 sec, 72 °C for 1.5 min) and final extension step (72 °C for 5 min). The amplicons were run on a 1% agarose gel and were then cut and purified using a gel purification kit (Qiagen). The purified amplicons were then cloned using the TOPO TA kit as per manufacturer's instruction. The cloned *Escherichia coli* cells were then plated on agar plates with 50 µg mL^{-1} ampicillin and grown over night. The colonies were screened for inserts using blue-white screening and PCR (M13F 5'-TGTAAAACGACGGCCAGT-3' and M13R 5'-AACAGCTATGACCATG-3'). The positive cells were submitted to RTSF for Sanger sequencing. The sequences obtained were then classified using the Ribosomal Database Project (RDP) "Classifier" (48) .

RESULTS

Ethylbenzene Biodegradation

Ethylbenzene degradation was observed in only one of the 31 different incubations over an 8 week period. The lack of ethylbenzene degradation may be a result of the rare occurrence of ethylbenzene degrading microorganisms (however, no molecular analysis of those samples not illustrating ethylbenzene degradation was performed). In the microcosms amended with agricultural soil and nitrate, >90% of the added ethylbenzene was degraded in 29 days in the sample microcosms, with no significant degradation in the killed abiotic controls (data not shown). There was an initial decrease of approximately 5 mg L^{-1} in all of the microcosms (live and killed) between day 1 and day 7. This was possibly due to sorption as no further decrease was noted in the killed control microcosms. Similar degradation rates were observed in the SIP microcosms prepared with this inoculum (Figure 3.1). However, replicate microcosms exhibited slightly different rates of ethylbenzene degradation.

No significant reductions in ethylbenzene concentrations were observed in the abiotic killed controls (after the initial sorption decrease in all microcosms). An initial lag in degradation was observed in all microcosms and following this, most degradation occurred in 7 to 10 days. There was also an 80% decrease in the nitrate concentration between day 1 and day 22 in the labeled and unlabeled samples with no significant decrease in the abiotic controls (data not shown).

High Throughput Amplicon Sequencing Analysis

Samples from fractions (Table 3.1) as well as total DNA were submitted for high throughput amplicon sequencing. The fractions selected were at the heavy end of the ultracentrifugation gradient (Figure 3.2). Approximately 90,000 final sequences were obtained per sample. Sequencing data were obtained for both the fractions as well as total DNA. The number of sequences obtained per run and the final number of sequences used for the OTU and phylotypes classification are listed (Table 3.2). Sequences that did not assemble well (>275 bp) were dropped from further analysis. Further, an average of approximately 3.3 % of the remaining sequences were also removed as they were either marked as chimeric by the Uchime algorithm (49) or as belonging to mitochondria or chloroplast lineage by the Bayesian classifier. The remaining sequences were used for analysis. These sequences were classified into OTU’s and taxons by splitting them into bins and clustering them at the order level within each bin at 97% similarity cutoff level. Sequences of clones from this study were submitted to Genbank under accession number KM362736-KM362822. The high throughput sequence data was submitted to SRA under Bioproject number PRJNA257333.

Microbial Community in Ethylbenzene Degrading Microcosms

Rarefaction curves indicated an adequate level of sequence coverage for the total community analysis of the six microcosms (Figure 3.3). As expected, no differences were noted between the coverage in the labeled compared to the unlabeled ethylbenzene amended microcosms. The OTU classification data of the total DNA samples into phylotypes was used to determine the community composition in each of the six microcosms. Figure 3.4 shows the phyla level classification of the sequences in the total genomic DNA samples from the six microcosms. A small percentage (4.3-8%) of the sequences were *unclassified Bacteria*. *Proteobacteria* was the most abundant phyla in all six microcosms (relative abundance of 58.7-79.1%). The remaining dominant phyla included *Firmicutes* (6.3-13.17%), *Actinobacteria* (2.9-8.4 %), *Acidobacteria*

(2.0-13.3%), and *Bacteroidetes* (0.8-4.6%). Microorganisms from both phyla *Gemmatimonadetes* and *Verrucomicrobia* were present in all six microcosms at levels between 0.9 and 1.9 %. Microorganisms in the phyla *TM7, Planctomycetes, Chloroflexi, Deinococcus-Thermus, Armatimonadetes, Chlamydiae* and *Nitrospira* were present on all six microcosms, but only at lower levels (0.007-0.3%). Whereas, microorganisms classifying within *BRC1, OD1, WS3, Chlorobi, Lentisphaerae* were present in some microcosms, but not in others (when present, <0.006%).

The relative abundance of all phylotypes with a relative abundance of > 1 % were compared across all six microcosms (Figure 3.5). This resulted in a comparison of fifteen phylotypes. In four of the six microcosms, *Rhodanobacter* (*Gammaproteobacteria*) was the most abundant phylotype (14.1-15.6%) and in the other two microcosms it was the second (14.2%) or third (11.7%) most abundant phylotype. In two microcosms, *Azoarcus* was the most abundant phylotype (24.1 and 14.6%), however, in the other four microcosms it was present at very low levels (<0.006 %). In four microcosms, unclassified *Rhodospirillaceae* was the second most dominant phylotype (10.7-14.1%) a family within the *Alphaproteobacteria*. In the other two microcosms, it was present at lower levels (2.7 and 5.1%). In all six microcosms, unclassified *Oxalobacteraceae* (*Betaproteobacteria*) was present at high levels (5.5-14.1%). Unclassified *Xanthomonadaceae* (the family containing *Rhodanobacter*) was also present in all six microcosms, but at lower levels (1.9-6.3%). Other unclassified families occurring in all six microcosms at lower levels include *Bacillaceae 1* (*Firmicutes*), *Chitinophagaceae* (*Bacteroidetes*) and *Comamonadaceae* (*Betaproteobacteria*). Three unclassified orders (*Bacillales, Rhodospirillales* and *Rhizobiales*), unclassified *Bacteria* and unclassified

Betaproteobacteria were also present in all six microcosms. In addition, two phylotypes classifying as *Thiobacillus* (*Betaproteobacteria*) and *Gemmatimonas* (phylum *Gemmatimonadetes*) were found in all six microcosms.

16S rRNA Gene Clone Library

A clone library was generated so that a comparison could be performed between traditional Sanger sequencing and high throughput sequencing data. The clone library consisted of a 120 clones (Table 3.3) belonging to eight different phyla: *Proteobacteria* (73.1%), *Firmicutes* (10.9%), *Bacteriodetes* (8.4%), *Acidobacteria* (5.0%), *Armatimonadetes* (0.8%), *Gemmatimonadetes* (0.8%), *TM7* (0.8%), and *Verrucomicrobia* (0.8%). Among the *Proteobacteria,* the majority belonged to *Betaproteobacteria* (45.8% of the total). The major phyla present in the clone library were relatively consistent with the high throughput sequencing results. That is, the Illumina results indicated the communities in the six microcosms were also dominated by *Proteobacteria* (58.7-79.1%), followed by *Firmicutes* (6.3-13.17%), *Acidobacteria* (2.0-13.3%), and *Bacteroidetes* (0.8-4.6%). Notably, no clones belonging to *Actinobacteria* were present in the clone library, however, this phylum exhibited a relative abundance of between 2.9 and 8.4 % in high throughput sequencing results. Further, the clone library did not contain representatives from 11 additional phyla that were found in the high throughput sequencing data. Consistent with the high throughput sequencing data, the clone library did contain a high number of *Rhodanobacter* (relative abundance of 10.0%) and unclassified *Oxalobacteraceae* sequences (25.0%). In contrast, the number of sequences of unclassified *Rhodospirillaceae* was much lower in the library (3.3%) compared to the Illumina data. *Azoarcus* was not present in the clone library, however, *Gemmatimonas* was present (0.83%). As expected, the Illumina results provided a more in depth representation of the microorganisms present in these ethylbenzene degrading communities.

Phylotype Relative Abundance Across SIP Fractions

The relative abundance of phylotypes in heavy fractions were compared between the labelled and unlabeled ethylbenzene amended microcosms to determine which phylotypes could be linked to label uptake. For this, the analysis focused on the most abundant phylotypes in fractions from the labeled ethylbenzene amended microcosms. The relative abundance of these phylotypes was then compared to their relative abundance in fractions from the unlabeled ethylbenzene amended microcosms. Overall, seven phylotypes were dominant in the heavy fractions from both of the labeled ethylbenzene amended microcosms (Figure 3.6). *Rhodanobacter* was the most abundant in these fractions, however, the relative abundance was similar in fractions from the labeled and unlabeled ethylbenzene amended samples, indicating no label uptake. Following this, unclassified *Oxalobacteraceae* and *Rhodospirillaceae* were also dominant. A comparison of the relative abundance of these phylotypes indicates label uptake likely occurred in one labeled ethylbenzene amended microcosm (SIP Label EB 2), but not the other (SIP Label EB 1). Unclassified *Xanthomonadaceae* and *Rhodocyclaceae* both illustrated a higher relative abundance in the fractions from the labeled ethylbenzene amended microcosms compared to the unlabeled amended microcosms. No difference in relative abundance was noted for unclassified *Bacillales* between treatments. Finally, the phylotype *Gemmatimonas* was more abundant in fractions from one labeled ethylbenzene amended microcosm (SIP Label EB 1). *Azoarcus* was not dominant in the heavy fractions of either labeled ethylbenzene amended microcosm.

DISCUSSION

Biodegradation studies using SIP aim to identify active organisms involved in chemical transformations that are typically not identified using culture based techniques (21, 50, 51). Combining SIP with high throughput sequencing has the added advantage of a greater depth of analysis (compared to Sanger sequencing). Although Illumina amplicon sequencing produces shorter reads even with paired reads (250 bp) compared to Sanger sequencing (up to ~1000 bp) (52, 53), previous studies have shown the method can still resolve the differences between microbial communities (42). The data generated in the current study indicated that one clone library (120 clones) with Sanger sequencing provided a weaker representation of the microbial community compared to the Illumina data. For example, *Actinobacteria*, a major phylum in the Illumina data, was absent from the clone library, as were 11 other phyla (that were present in the Illumina data). The contrasting results from these two sequencing methods are consistent with previous studies that used both techniques. The large amount of sequencing information from amplicon sequencing is an improved predictor of phylotype relative abundance (29, 32, 35) and thus has great potential for SIP studies.

Although *Rhodanobacter* was the most abundant phylotype in the majority of the six microcosms, it's relative abundance was similar in the fractions obtained from the labelled and unlabeled ethylbenzene amended microcosms, indicating it was not responsible for label uptake. Phylotypes from the families *Oxalobacteraceae* and *Rhodospirillaceae* appeared to be involved in carbon uptake from ethylbenzene in one microcosm (SIP Label EB 2). According to the Ribosomal Database Project (http://rdp.cme.msu.edu/index.jsp), *Oxalobacteraceae* (order *Burkholderiales*, class *Betaproteobacteria*) contains 11 genera and *Rhodospirillaceae (*order

Rhodospirillales, class *Alphaproteobacteria*) contains 27 genera. Any of these genera could be responsible for label uptake in this microcosm. The phylotype *Gemmatimonas* was more abundant in the fractions from one labeled ethylbenzene amended microcosm (SIP Label EB 1). This microorganism belongs to the phylum *Gemmatimonadetes*, which only contains one genus (with 15 known isolates), with the first isolate being obtained only relatively recently (2003) (54). The family *Xanthomonadaceae* (order *Xanthomonadales,* class *Gammaproteobacteria*) contains 21 genera and phylotypes within this family appeared to responsible for label uptake in both label amended microcosms. Again, any of these genera could be responsible for label uptake.

Phylotypes within the family *Rhodocyclaceae* were also more abundant in the fractions from both the labeled ethylbenzene amended microcosms compared to similar fractions from the unlabeled ethylbenzene amended microcosms. This pattern indicates phylotypes classifying as *Rhodocyclaceae* were involved in carbon uptake from ethylbenzene (or ethylbenzene degradation products). These results are consistent with pure culture studies, as the majority of anaerobic ethylbenzene degrading isolates belong to this family. Previous research has indicated phylotypes classifying as *Rhodocyclaceae* (*Azoarcus, Dechloromonas, Georgfuchsia* spp.) are capable of ethylbenzene degradation (8, 10, 11, 14). Also, previous studies have linked organisms from this family to transformation of several organic and inorganic substances such as phenantharene (55-57), naphthalene (58), other petroleum products (32), arsenic (59), uranium (60), chromium (61) and alkanes (62). Members of this family are also shown to degrade almost all of the BTEX compounds, particularly under nitrate reducing conditions (8-11, 15, 29, 63, 64). These findings indicate these microorganisms play an important role in monoaromatic hydrocarbon degradation in isolation as well as in mixed communities. Also, two ethylbenzene degrading strains, *Aromatoleum aromaticum* EbN1 and *Dechloromonas aromatica* RCB, were isolated from uncontaminated fresh water sediments. The other sulfate reducing ethylbenzene degrading bacterium was isolated from uncontaminated marine sediment, emphasizing the importance of ethylbenzene degrading bacteria in communities not previously exposed to monoaromatic or polyaromatic hydrocarbons (as in the current study).

In summary, combining the culture independent technique SIP with high throughput amplicon sequencing can be a useful approach for investigating the biodegradation of xenobiotics by minority organisms or by a community of organisms. The current work illustrates the utility of these methods for examining carbon pathways in soil communities. Although it was not possible to identify enriched phylotypes at the genus level, important families involved in carbon assimilation were identified. Interestingly, all classified within the *Proteobacteria*.

APPENDIX

Table 3.1. Buoyant density of heavy fractions from the labeled and unlabeled samples submitted for amplicon sequencing.

Microcosm name	**Buoyant Density of Fractions (g mL^{-1})**			
SIP Label EB 1	1.780	1.755	1.745	1.742
SIP Label EB 2	1.778	1.768	1.756	1.748
SIP Unlabel EB 1	1.778	1.759	1.747	1.740
SIP Unlabel EB 2	1.780	1.765	1.755	1.744

Table 3.2. Summary MiSEQ Illumina data generated from the fractions as well as total DNA extracted from labeled and unlabeled ethylbenzene amended microcosms.

	# of Sequences Following Make Contigs command	Final # of Unique Sequences	Final # of Sequences	% Chimeric	OTUs Per Fraction or Sample (Average ± Std dev)
SIP Label EB 1 and SIP unlabeled EB 1 with 4 fractions from each and total DNA samples from both	1758568	68926	869977	3.09	2622 ± 719
SIP Label EB 2 and SIP unlabeled EB 2 with 4 fractions from each and total DNA samples from both	2181584	81557	1075361	3.20	2758 ± 743
Unlabel EB 1 and Unlabel EB 2 total DNA	829470	26696	409141	3.13	1495±254

Table 3.3. Classification of 16S rRNA gene clones in the clone library.

Phyla	**Class**	**Order**	**Family**	**Full name**	**No. of Clones**
Acidobacteria	*Acidobacteria GP1*			*Gp1*	3
Acidobacteria	*Acidobacteria GP3*			*Gp3*	1
Acidobacteria	*Acidobacteria GP4*			*Gp4*	1
Acidobacteria				*Gp6*	1
Armatimonadetes				*Armatimonadetes_Gp4*	1
Bacteriodetes	*Flavobacteria*	*Flavobacteriales*	*Flavobacteriaceae*	*Flavobacterium*	3
Bacteriodetes	*Sphingobacteria*	*Sphingobacteriales*	*Chitinophagaceae*	*Ferruginibacter*	6
Bacteriodetes				*Unclassified Bacteriodetes*	1
Firmicutes	*Bacilli*	*Bacilllales*	*Alicyclobacillaceae*	*Tumebacilus*	1
Firmicutes	*Bacilli*	*Bacilllales*	*Bacillaceae*	*Bacillus*	5
Firmicutes	*Bacilli*	*Bacilllales*	*Paenibacillaceae 1*	*Unclassified Bacillales*	1
Firmicutes	*Bacilli*	*Bacilllales*		*Unclassified Bacillales*	6
Gemmatimonadetes	*Gemmatimonadetes*	*Gemmatimonadales*	*Gemmatimonadaceae*	*Gemmatimonas*	1
Proteobacteria	*Alphaproteobacteria*	*Rhizobiales*	*Brucellaceae*	*Unclassified Brucellaceae*	1
Proteobacteria	*Alphaproteobacteria*	*Rhizobiales*	*Methylobacteriaceae*	*Microvirga*	1
Proteobacteria	*Alphaproteobacteria*	*Rhizobiales*	*Phyllobacteriaceae*	*Aminobacter*	1
Proteobacteria	*Alphaproteobacteria*	*Rhizobiales*		*Unclassified Rhizobiales*	2
Proteobacteria	*Alphaproteobacteria*	*Rhizobiales*	*Xanthobateraceae*	*Pseudolabrys*	1
Proteobacteria	*Alphaproteobacteria*	*Rhodospirillales*	*Rhodospirillaceae*	*Unclassified Rhodospirillaceae*	4
Proteobacteria	*Betaproteobacteria*	*Burkholderiales*	*Alcaligenaceae*	*Pusilimonas*	1
Proteobacteria	*Betaproteobacteria*	*Burkholderiales*	*Comamonadaceae*	*Ramlibacter*	1
Proteobacteria	*Betaproteobacteria*	*Burkholderiales*	*Oxalobacteraceae*	*Herbaspirillum*	4
Proteobacteria	*Betaproteobacteria*	*Burkholderiales*	*Oxalobacteraceae*	*Massilia*	1
Proteobacteria	*Betaproteobacteria*	*Burkholderiales*	*Oxalobacteraceae*	*Unclassified Oxalobacteraceae*	30
Proteobacteria	*Betaproteobacteria*	*Burkholderiales*		*Unclassified Burkholderiales*	10
Proteobacteria	*Betaproteobacteria*	*Rhododcyclales*	*Rhodocyclaceae*	*Unclassified Rhodocyclaceae*	4
Proteobacteria	*Betaproteobacteria*			*Unclassified Betaproteobacteria*	4
Proteobacteria	*Gammaproteobacteria*	*Xanthomonadales*	*Xanthomonadaceae*	*Rhodanobacter*	12
Proteobacteria	*Gammaproteobacteria*	*Xanthomonadales*	*Xanthomonadaceae*	*Unclassified Xanthomonadaceae*	1
Proteobacteria	*Gammaproteobacteria*	*Xanthomonadales*	*Xanthomonadaceae*	*Luteimonas*	1
Proteobacteria				*Unclassified Proteobacteria*	8
TM7			*Unclassified TM7*	*TM7 genera incertae sedis*	1
Verrucomicrobia	*Spartobacteria*		*Unclassified Spartobacteria*	*Spartobacteria genera incertae*	1

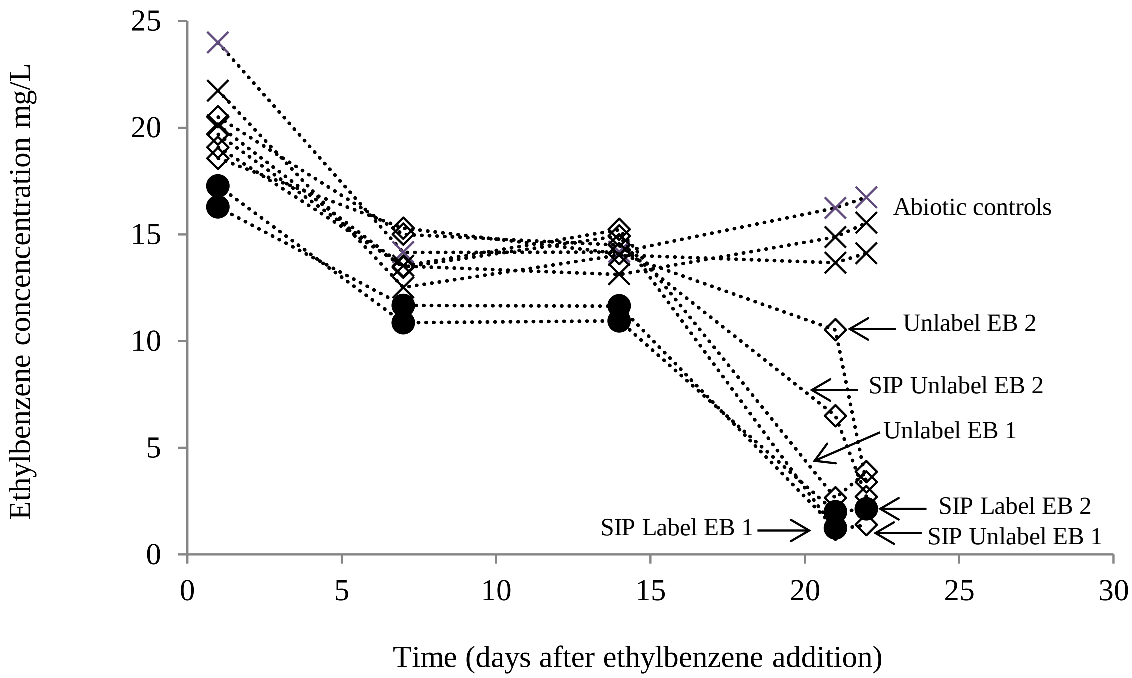

Figure 3.1. Concentration of ethylbenzene in microcosms over time (× - abiotic controls, open triangles - amended with $^{12}C_6$ ethylbenzene, closed circles - amended with $^{13}C_6$ ethylbenzene).

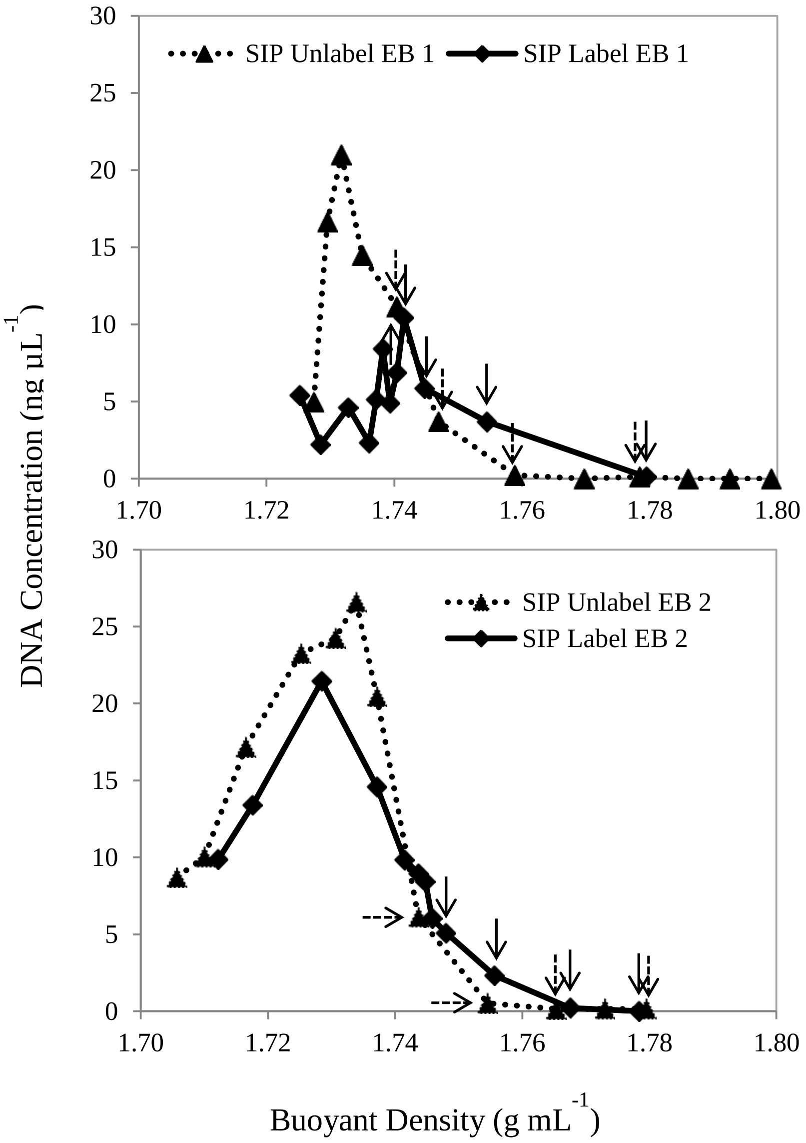

Figure 3.2. DNA concentration over the ultracentrifugation gradient in fractions from replicate microcosms amended with labeled or unlabeled ethylbenzene. The dashed arrows indicate the fractions that were sequenced from the unlabeled EB amended samples and solid arrows indicate the fractions that were sequenced from the labeled EB amended samples.

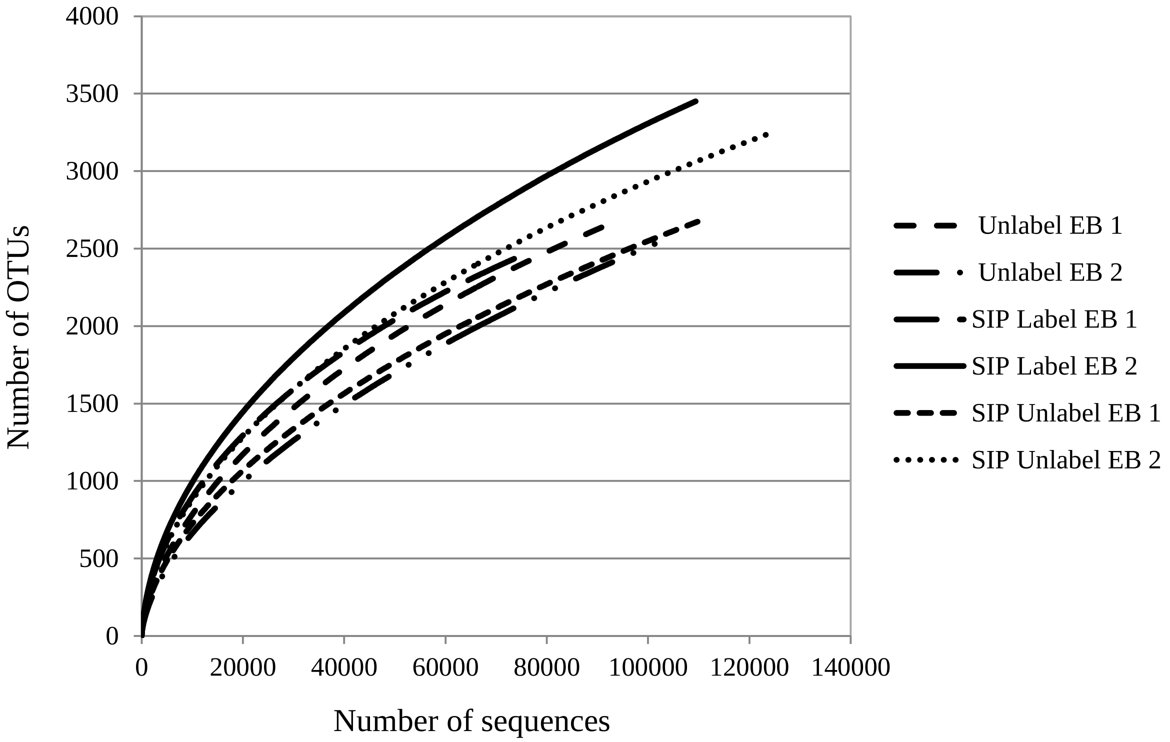

Figure 3.3. Rarefaction curves generated from sequencing data from total DNA extracts for all six microcosms.

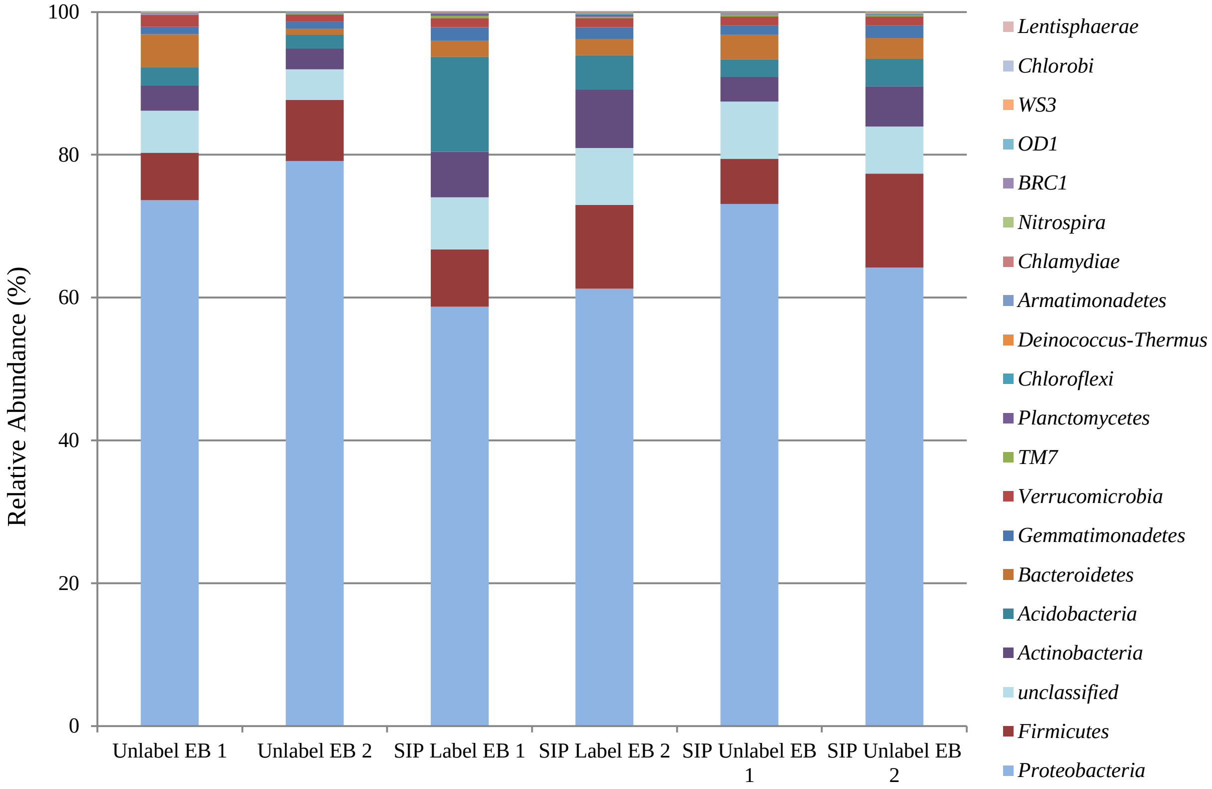

Figure 3.4. Comparison of relative abundance of sequences in total genomic DNA extracted from six microcosms amended with $^{13}C_6$ labeled or unlabeled ethylbenzene at phylum level classification.

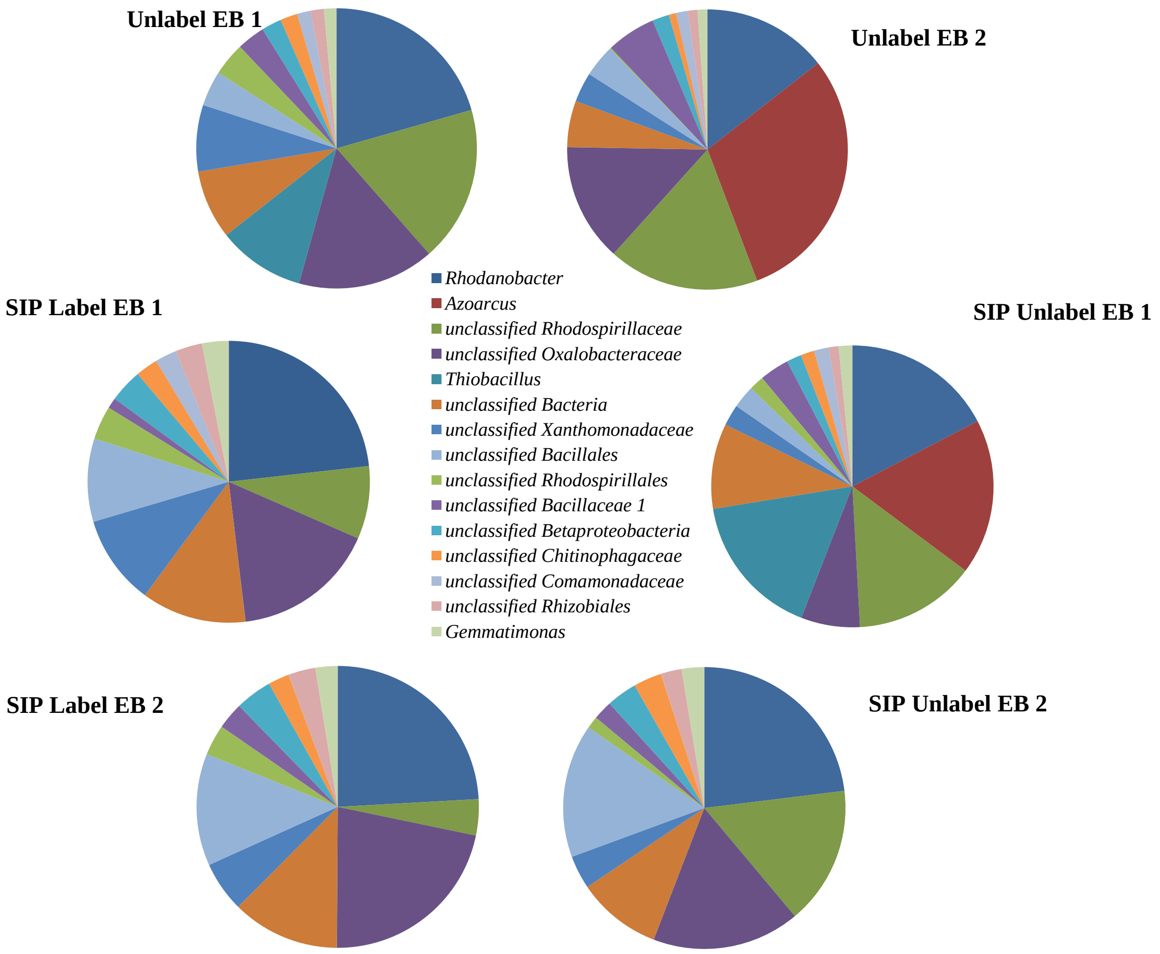

Figure 3.5. Relative abundance (%) of the most common phylotypes (>1%, at the lowest classification level) in all six microcosms (from total DNA extracts). The phylotypes are at the genus level, unless unclassified.

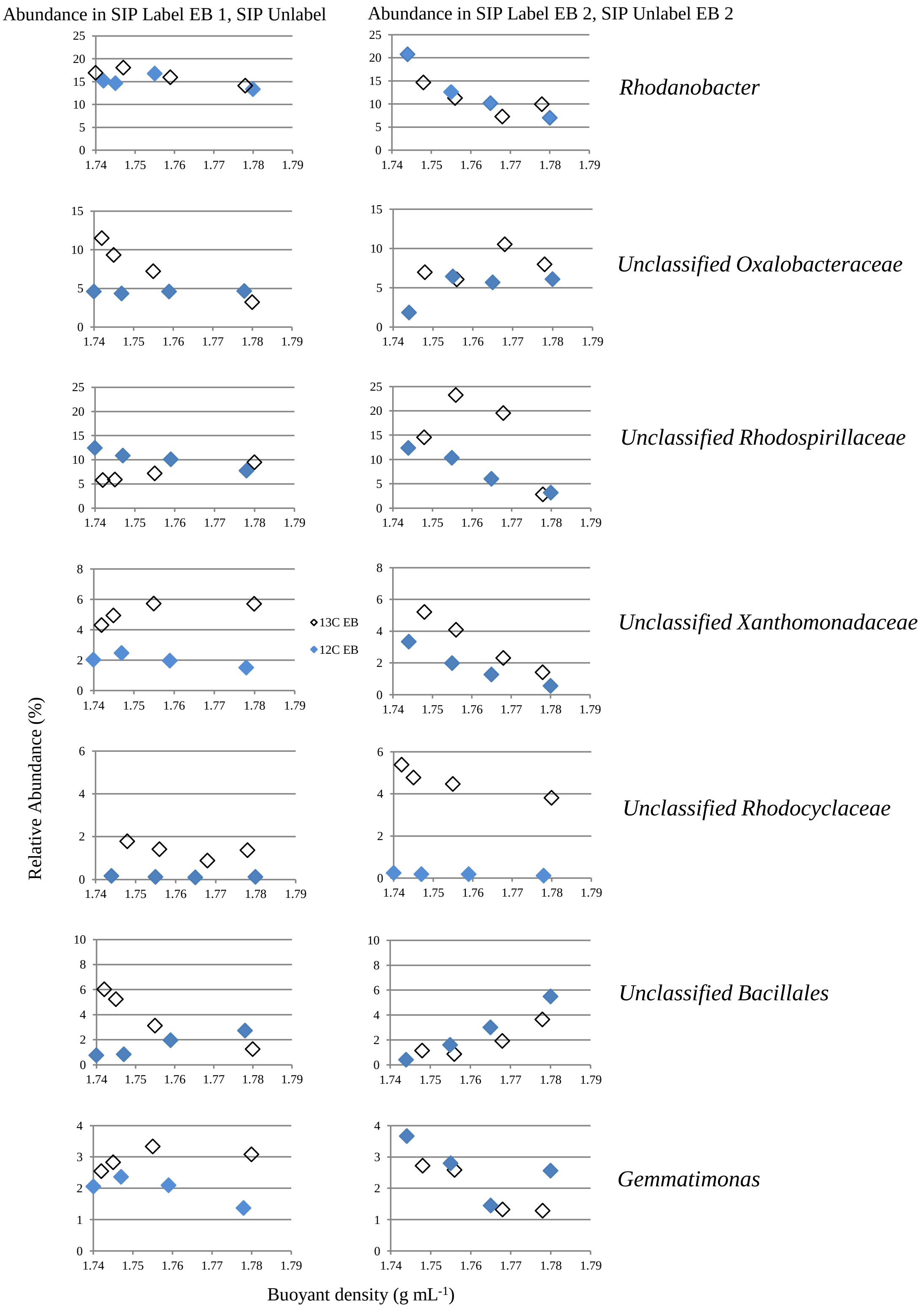

Figure 3.6. Relative abundance of the most common phylotypes in heavy fractions from ^{13}C ethylbenzene amended microcosms compared to those from ^{12}C ethylbenzene amended microcosms (SIP Label EB1 and SIP Unlabel EB1 on left, SIP Label EB2 and SIP Unlabel EB2 on right).

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CHAPTER 4 COMPARATIVE STUDY OF RDX DEGRADING MICROBIAL COMMUNITIES IN FOUR SOILS

ABSTRACT

The explosive hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) has contaminated many military sites. Recently, attempts to remediate these sites have focused on biostimulation to promote *in situ* RDX biodegradation. Although many RDX degrading isolates have been obtained in the laboratory, little is known about the potential of microorganisms to degrade this chemical in mixed microbial communities. The current study compared four soils to elucidate the microorganisms linked to RDX degradation. These soils were selected as they had no previous exposure to RDX, therefore their microbial communities offered an excellent baseline to determine changes following exposure to RDX. The work involved the collection of two sets of molecular data. First, high throughput sequencing was used to determine which phylotypes experienced an increase in relative abundance following RDX degradation. For this, DNA was sequenced from 1) the initial soil, 2) microcosms following RDX degradation and 3) control microcosms without RDX. Second, to ascertain which phylotypes were responsible for label (ring ^{15}N or ^{13}C) uptake from RDX, stable isotope probing (SIP) was performed on two soils.

The sequencing data provided valuable information on which phylotypes increased in abundance in RDX degrading microcosms compared to control microcosms. The most notable trend was the increase in abundance of *Brevundimonas* and/or unclassified *Bacillaceae 1* in the four soils

studied. These data indicate these two phylotypes are benefiting from RDX degradation under these conditions. Although members of the family *Bacillaceae 1* have previously been linked to RDX degradation, the genus *Brevundimonas* has not been previously associated with RDX degradation. SIP data indicated that phylotypes classifying within the *Firmicutes* and *Actinobacteria* (*Rhodococcus*) were involved in label uptake from RDX. Overall, these data suggest both novel RDX degraders and previously reported RDX degraders were associated with RDX removal in these soils.

INTRODUCTION

The manufacturing, transport and use of the nitramine explosive hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) has resulted in groundwater and sediment contamination at many military sites. RDX has moderate solubility, low sorption and low vapor pressure resulting in significant mobility in groundwater and sediments. Due to neurotoxic effects, the potential carcinogenic nature of RDX and widespread RDX contamination, the US EPA has issued a life-time drinking water health advisory level of 2 µg/L RDX. Although RDX was initially thought to be recalcitrant, the chemical has been shown amenable to biodegradation under both aerobic and anaerobic conditions. Thus, bioremediation has been considered a viable option to treat RDX contaminated sites. As many of these sites are oxygen depleted, it is more suitable to consider RDX biodegradation under anaerobic conditions.

Several bacteria have been isolated with the ability to degrade RDX under anaerobic conditions. These microorganisms classify within the phyla *Firmicutes*, *Fusobacteria* and *Proteobacteria*. The first anaerobic RDX degrading isolate obtained, *Clostridia bifermentans*, classified with the

Firmicutes (1). Other *Clostridia* isolates have also been reported with RDX degrading abilities, including, for example, *Clostridia* sp. EDB2 and *Clostridia acetobutylicum* (2-5). Other RDX degrading bacteria belonging to the *Firmicutes* include two *Acetobacterium* spp. (6, 7), two *Bacillus* spp. (8) and a *Desulfitobacterium* strain (9). *Fusobacteria* sp. HAQ-EB21 (10, 11) is the only anaerobic RDX degrading isolate from the phylum *Fusobacteria*.

The *Proteobacteria*, particularly the classes *Gamma* and *Deltaproteobacteria*, contain diverse anaerobic RDX degrading isolates. In the *Gammaproteobacteria*, RDX degrading bacteria have been isolated from the families *Enterobacteriaceae* (12-16), *Pseudomonadaceae* (8, 17), *Shewanellaceae* (10, 18, 19) and *Xanthomonadaceae* (20). Isolates belonging to *Cystobacteraceae* (9), *Geobacteraceae* (21) or *Desulfovibrionaceae* (11, 22, 23) within the *Deltaproteobacteria* are also able to degrade RDX.

Although much is known about pure cultures able to degrade RDX in the laboratory, very little is known about the microorganisms responsible for RDX degradation in mixed communities or at contaminated sites. Researchers have reported the microorganisms detected in field samples actively degrading RDX were generally not closely related to previously reported RDX degrading isolates (24). It is now widely recognized that only a small fraction of microorganisms can be isolated and cultivated in the laboratory (25), therefore, it is likely that many RDX degraders have yet to be identified. This information is important because if several key species were consistently linked to effective RDX degradation in mixed communities, their presence could provide strong evidence of RDX natural attenuation.

Recently, a small number of studies have used stable isotope probing (SIP) to determine which microorganisms were involved in carbon and/or nitrogen uptake from RDX (26-29). One report revealed the important of *Sphingobacteriales* (*Bacteroidetes*) in RDX degradation (28). A SIP (^{15}N labeled RDX) study by another group using samples from the Picatinny Arsenal Site, identified five phylotypes similar to known RDX degraders and ten phylotypes not previously linked to RDX degradation (29). A ^{13}C labeled RDX SIP study again using samples from one site, also revealed that novel phylotypes and phylotypes similar to known RDX degraders were responsible for label uptake (27). A recent SIP investigation (^{15}N labeled RDX) using material from the Eglin Air Force Base bombing range, reported that RDX degradation was spatially heterogeneous and dependent on carbon source addition (26). These researchers reported that *Rhodococcus* (a known RDX degrader) as well as several *Proteobacteria* were highly labeled with ^{15}N. These studies have led to the conclusion that RDX degrading microorganisms are phylogenetically more diverse than was previously thought.

In the past few years, SIP has been combined with high throughput sequencing. For example, researchers have examined the biodegradation of toluene (30), the thermophilic anaerobic conversion of microcrystalline cellulose (31), nitrogen incorporation from monoammonium phosphate in arctic soils (32) and carbon uptake in microbial fuel cells (33) using this technique. In the current study, SIP was combined with high throughput sequencing to compare the RDX degrading communities in four soils without prior exposure to RDX. This is the first investigation of RDX degrading communities in uncontaminated soils using the combined approaches. Further, is it is first study to use high throughput sequencing to compare the microbial communities between RDX degrading soils.

METHODS

Chemicals

Unlabeled and ring-labeled RDX ($^{13}C_3$, 99%; $^{15}N_3$, 98%) dissolved in acetonitrile were purchased from Cambridge Isotope Laboratories, Inc. (Tewksbury, MA). HPLC grade acetonitrile (≥99.8 % purity) was purchased from EMD Chemicals Inc. (New Jersey, USA). Other reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA), Fisher BioReagent (New Jersey, USA), or Invitrogen (Carlsbad, CA, USA) unless otherwise stated.

Experimental Design

Four agricultural soils were utilized in these experiments and, to our knowledge, these soils have not previously been exposed to RDX (Table 1). The soil microcosms for screening RDX degradation were established as previously described, with only slight modifications (28, 34). Briefly, for each soil, 60 mL serum bottles contained 1 g soil (wet weight), 20 mg L^{-1} unlabeled RDX dissolved in acetonitrile (as the sole nitrogen source), 201.6 mg L^{-1} glucose, and 4 mL of a minimal salts media (MSM) (28, 34).

For the high throughput sequencing and SIP studies, the microcosms were prepared with 1 g soil (wet weight), 1 mL of inocula from the corresponding RDX screening microcosm, 20 mg L^{-1} unlabeled or ring-labeled RDX dissolved in acetonitrile (as the sole nitrogen source), 201.6 mg L^{-1} glucose, and 4 mL of a minimal salts media (MSM). Triplicate live sample microcosms were amended with ring-labeled RDX ($^{13}C_3$, $^{15}N_3$), triplicate live and killed abiotic controls (autoclaved) microcosms were amended with unlabeled RDX ($^{12}C_3$, $^{14}N_3$). Two successive amendments of 20 mg L^{-1} of RDX were added to the live labeled and unlabeled RDX microcosms. The triplicate abiotic killed controls were prepared in a similar manner to the live microcosms except that they received only one amendment of unlabeled RDX. All microcosms were sealed with rubber stoppers and aluminum seals and were covered with aluminum foil to prevent photodegradation. The microcosms were incubated between 3 to 7 weeks in the dark at room temperature (~20 °C) without shaking while being monitored for RDX degradation.

Due to time constraints, only DNA extracted from soils 9 and 10 were ultracentrifuged for fraction analysis. However, total DNA extracts from all four soils, extracted from both replicates of the labeled RDX (hereafter called Labeled 1, Time t and Labeled 2, Time t) and unlabeled RDX microcosms (hereafter called Unlabeled 1, Time t and Unlabeled 2, Time t) were sequenced.

Additional live control microcosms, without RDX addition, were also prepared with all four soils and these were maintained under the same conditions as the live sample microcosms. Nucleic acids were extracted from these control microcosms (hereafter called No RDX, Time t) at the same time as those extracted from the RDX amended microcosms. Further, DNA was extracted from each soil prior to any manipulation (hereafter called No RDX, Time 0). These two sets of DNA extracts were sequenced to determine how the communities changed followed RDX degradation compared to the initial soil and the no RDX controls.

RDX Extraction and Analysis

RDX concentrations were determined using high performance liquid chromatography (HPLC) as previously described (34) with modifications. For this, 0.5 mL (extracted using a 1 mL BD syringe with a 21 gauge needle) was placed in a 1.7 mL sterile microcentrifuge tube. An equal volume of acetonitrile was added and the microcentrifuge tubes were shaken for 2 hours at room temperature to extract RDX. The samples were then centrifuged for 5 minutes at 10,000 x g and the supernatant was filtered using acetonitrile wetted filters (PVDF, 0.22 µm, Whatman) into HPLC amber vials (Sigma-Aldrich, St. Louis, MO, USA). External standards for the calibration curve were prepared with a dilution factor of 2 to account for the sample dilution at the liquid-liquid extraction step. HPLC analysis involved a Perkin Elmer (PE) series 200 autosampler; PE binary LC Pump 250; PE diode array detector 235C, at wavelength 255nm; Supelco C18 (25 cm X 4.6 mm, 5 µm) column; and isocratic conditions (40% acetonitrile and 60% 0.1% H_3PO_4 acidified deionized water) at a flow rate of 1 mL min^{-1}.

DNA Extraction and Isopycnic Centrifugation

As stated above, total genomic DNA from all four soils was extracted to establish the baseline microbial community (No RDX, Time 0). Total genomic DNA was also extracted from all sixteen microcosms (four soils, Labeled 1 & 2, Unlabeled 1 & 2, Time t) after the complete degradation of the second amendment of RDX. In addition, DNA was extracted from the corresponding live control microcosms at the same time (No RDX, Time t). All DNA extractions used the Power Soil DNA extraction kit (MO BIO Laboratories, Inc. Carlsbad, CA).

Isopycnic centrifugation was conducted for soils 9 and 10, following previously described procedures (35-40). DNA extracted from one labeled and one unlabeled microcosm for these soils was ultracentrifuged separately in Tris EDTA - Cesium Chloride (CsCl) gradients to obtain density gradient fractions. Approximately 3 to 5 µg of the total genomic DNA was added to a CsCl and TrisEDTA solution with an initial buoyant density (BD) of ~1.72 g mL^{-1} in a 5.1 mL

Beckmann Coulter Quick-Seal polyallomer tubes. Tubes were then sealed (Cordless quick-seal tube topper, Beckman) and centrifuged at 178,000 x g for 46 hours at 20 ^{o}C in a Wx Sorvall Ultra 80 ultracentrifuge fitted with a Stepsaver 70 V6 Vertical Titanium Rotor (Thermo Scientific). Each sample was then divided into 20 fractions (each 250 µL) by displacement with molecular grade water using a syringe pump system. The BD of all samples was measured using a model AR200 digital refractometer (Leica Microsystems Inc.) and the DNA from each fraction was precipitated using ethanol and glycogen. Precipitated DNA was then re-suspended in 30 µL PCR grade water and stored at -20 °C for further analysis.

Amplicon Sequencing and Data Analysis

Total genomic DNA extracted from four microcosms (Labeled 1, Labeled 2, Unlabeled 1, Unlabeled 2, Time t) for all four soils (soil 9, 10 , 11 and 12) and from the microcosms without RDX (No RDX, Time t) for all four soils were submitted for amplicon sequencing at Michigan State University's Research Technology and Support Facility (RTSF). Total genomic DNA extracted from all four of the initial soils (no RDX, Time 0) were also amplicon sequenced. Additionally, the first ultracentrifugation eight fractions with high BD values from soils 9 and 10 were also submitted for amplicon sequencing, in triplicate. Amplification of the V4 region and paired-end high throughput amplicon sequencing on the Illumina MiSeq platform at RTSF was conducted using a procedure previously described (41, 42).

Sequencing data obtained from the MiSeq platform Laboratory Information Management System (LIMS) was analyzed using Mothur v.1.33.2 (43) using the MiSeq standard operating procedure (44). The sequences data in the fastq format were processed using Mothur to remove the barcodes and these were then aligned to form contiguous sequences. The data was checked for sequencing errors and read length. The sequences were then aligned and checked for chimeras using UCHIME in Mothur (45). Following which, the sequences were classified into OTU's and phylotypes using the Ribosomal Database Project dataset within Mothur. The sequence data was then imported into Microsoft Excel for further analysis. High throughput sequences were also processed using the default pipeline option on MG-RAST (46) to create the MLTreeMap and the Heatmap with the following parameters, annotation source: RDP; maximum e-value cutoff :$1e^{-5}$; minimum % identity cutoff: 60%; minimum alignment length cutoff: 15. Illumina sequencing data were deposited to the NCBI Sequence Read Archive under Bioproject Number PRJNA263419.

RESULTS AND DISCUSSION

RDX Degradation

All four agricultural soils illustrated RDX degradation (Figure 4.1). The first amendment of RDX was degraded in 15 to 31 days and the second amendment was degraded in 7 to 12 days. No significant degradation of RDX was observed in the killed control microcosms. Among the four soils, RDX degradation was the fastest in the microcosms with soil 10 and was the slowest in the microcosms with soil 9.

Comparison at Phyla Level

Total genomic DNA from all treatments and live controls (all four soils) as well as DNA from the ultracentrifugation fractions (soils 9 and 10) were submitted for amplicon sequencing (116 samples in total). An average of >120,000 reads was obtained per sample (Table 2). On average, 27% of the sequences did not align properly into contiguous sequences resulting in read lengths >275 bp and these were dropped from the analysis. Another 2.3% (average) of the sequences were identified as chimeric or as non-bacterial (mitochondrial/chloroplast) by the Bayesian classifier within Mothur.

Phylotype classification and sequence abundance data were used to assess the microbial communities in each of the four soils (Figure 4.2). The relative abundance of each phyla for all four initial soils (No RDX, Time 0) were remarkably similar. That is, the phylotypes classified into *Firmicutes, Proteobacteria*, *Actinobacteria* (the three most dominant), *Bacteroidetes, Acidobacteria*, *Verrucomicrobia*, *Chloroflexi* and *Gemmatimonadetes.* In contrast, for all four soils, in both the RDX amended microcosms (Labeled, Unlabeled 1 & 2, Time t) and in the microcosms not amended with RDX (No RDX, Time t), the communities were primarily composed of *Proteobacteria* (particularly soils 10 and 11) and *Firmicutes* (particularly soils 9 and 12). This increase in *Firmicutes* is likely a result of oxygen depletion in these samples (the microcosms were sealed for more than 20 days). For the RDX amended samples, these results are somewhat expected, as the majority of anaerobic RDX degrading isolates belong to the *Firmicutes* and *Proteobacteria* (Table 1).

At the phyla level, for soils 9 and 12, the classifications were similar between the microcosms amended with RDX and those not amended with RDX. For soils 10 and 11, the abundance of *Proteobacteria* increased when RDX was amended. Although phyla level classifications provide a useful baseline to compare these communities, comparisons at the phylotype level are more informative and will be discussed below.

Microbial Community Analysis

The microbial communities were also compared using MLTreeMap (Figure 4.3a). Again, the phyla with the greatest representation and the most diversity included the *Proteobacteria* and *Firmicutes*. However, no clear trends between the treatments could be ascertained from this analysis. A heatmap was also generated with the sequencing data for all four soils and for the microcosms with and without RDX (Figure 4.3b). The image illustrates distinct trends between the four initial soils and the other treatments. As expected, the microbial communities from the four initial soils clustered together. Clear differences can be seen between the initial soils and the other treatments for the *Clostridia, Cloroflexi, Gammatimonadetes, Spirochaetia* and unclassified *Acidobacteria*. The heatmap did not provide any strong trends for the differences in community structure between the RDX amended microcosms and those not amended with RDX.

Rarefaction curves were also generated for the initial soils (No RDX, Time 0), for the microcosms amended with RDX (Labeled, Unlabeled 1 & 2, Time t) and for the microcosms without RDX (No RDX, Time t) (Figure 4.4a). The curves are all similar except for three (soils 10, 11 and 12) of the four initial soils, which illustrated a much steeper gradient, indicating incomplete sequence coverage for these samples. The curves for the other samples started to plateau, suggesting more complete sequence coverage.

Principal component analysis was also performed on the sequencing data (Figure 4.4b). Clustering occurred for each soil in the four samples amended with RDX (Labeled 1 & 2, Unlabeled 1 & 2). The clusters for soils 10 and 11 were close together, as were the clusters for soils 9 and 12. Separate clusters were also formed for each of the four initial soils (No RDX, Time 0) and for the four soil microcosms without RDX addition (No RDX, Time t).

To more precisely compare the relative abundance of phylotypes across treatments, the most abundant phylotypes (>1% relative abundance) for each microbial community were determined. The most abundant phylotypes were similar across all four initial soils (No RDX, Time 0) (Figure 4.5a). The majority of the abundant phylotypes classified within the *Firmicutes, Proteobacteria, Actinobacteria* and *Acidobacteria*, with only one in each of the *Chloroflexi, Verrucomicrobia*, *Gemmatimonadetes* and *Bacteroidetes*. Each of the four soils contained a large relative abundance of unclassified *Bacteria*. In general, the most abundant phylotypes in all four soils, included unclassified *Bacillales*, unclassified *Sphingomonadaceae, Arthrobacter*, unclassified *Actinomycetales*, unclassified *Actinobacteria, Acidobacteria Gp1* and *Gp 3* and unclassified *Chitinophagaceae*. The most abundant phylotypes in the microcosms not amended with RDX (No RDX, Time t) were different (Figure 4.5b). These phylotypes only classified within the *Firmicutes, Proteobacteria, Actinobacteria* and *Bacteroidetes*. The most abundant phylotypes were primarily *Firmicutes*, including *Clostridium XIVa, Tissierella, Sedimentibacter* and unclassified *Clostridiaceae 1*. In addition, the phylotypes *Rhodococcus* and unclassified *Chitinophagaceae* were abundant in three of the four soils. Not surprisingly, these data clearly indicate that the incubation conditions (sealed bottles with glucose and a minimal salts media) completely changed the microbial community structure. Interestingly, the microbial community that developed following incubation was similar between soils.

The most abundant phylotypes were also determined for the microcosms amended with RDX (Figure 4.6). Similar to the data discussed above (No RDX, Time t), the abundant phylotypes classified only within the phyla *Firmicutes*, *Proteobacteria*, *Actinobacteria* and *Bacteroidetes* (only for soils 10 and 11). Again, the majority of abundant phylotypes classified within the *Firmicutes*. Similar phylotypes were enriched across all four soils. For the *Firmicutes* this included *Sedimentibacter*, *Tissierella*, unclassified *Clostridiaceae 1*, unclassified *Bacillaceae 1*. Other common phylotypes included *Brevundimonas* (soils 10, 11 and 12), *Rhodococcus* and unclassified *Bacteria* (all four soils). In general, the relative abundance values were similar between replicates and between the labeled and unlabeled RDX amended samples.

Phylotypes Increasing in Abundance Following RDX Degradation

The communities were further examined to ascertain which microorganisms increased in abundance following RDX degradation compared to the initial soils and the microcosms without RDX. These data indicate which microorganisms are gaining a benefit from RDX degradation (perhaps as an energy, carbon or nitrogen source). For each soil, this involved the selection of the eight most abundant phylotypes in the microbial communities following RDX degradation. Following this, for each soil, the relative abundance of these eight phylotypes was determined in the initial soils and in the microcosms not amended with RDX. The comparison of these data for each soil has been summarized (Figure 4.7).

The data analysis produced two interesting common trends between the four soils. The phylotypes *Brevundimonas* (*Proteobacteria*, *Alphaproteobacateria*, *Caulobacterales, Caulobateraceae*) and *unclassified Bacillaceae* 1 (*Firmicutes, Bacilli, Bacillales*) were both dominant following RDX degradation compared to the controls (initial soil and no RDX microcosms) in at least three of the four soils. For example, *Brevundimonas* illustrated a clear increase in relative abundance following RDX degradation in all four replicates for soils 10, 11 and 12, compared to the controls. The same trend can be seen for unclassified *Bacillaceae 1* for soils 9, 11 and 12.

The family *Bacillaceae 1* contains 36 genera, any of which could have been involved in RDX degradation in the current study. *Bacillus* is the only genus within the family previously linked to RDX degradation (8). Members of this family were also dominant among the RDX degrading microbial community from a saturated contaminated surface soil (47) and have previously been reported to be involved in PCB (48) and PAH (49) degradation.

The phylotype *Brevundimonas* classifies within the *Alphaproteobacteria*. Although many known anaerobic RDX degraders belong to the phylum *Proteobacteria*, only one RDX isolate (*Methylobacterium* sp.) belongs to the *Alphaproteobacteria* (50). Therefore, the current study has provided new insights into the importance of this phylotype for RDX degradation. In other research, *Brevundimonas* was involved in degradation of an organophosphorus insecticide (51) and cellulose (52). They have also been associated with direct petroleum hydrocarbons degradation (53, 54) and with the root-associated bacterial communities of plants involved in phytoremediation (55).

The phylotypes *Rhodococcus*, *Tisserella* (soil 9), unclassified *Erysipelotrichaceae* (soil 10), unclassified *Alcaligenaceae* (soil 11) and *Flavobacterium* (soil 12) were also more abundant in the RDX treated samples in comparison to the no RDX controls, however, their relative abundance values were lower than *Brevundimonas* or unclassified *Bacillaceae* 1 (Figure 4.7).

Phylotypes Indicated In Label Uptake

Eight heavy fractions generated from the labeled and unlabeled RDX amended microcosms were sequenced for soils 9 and 10. The most abundant phylotypes in the heavy fractions from the labeled RDX amended samples were the focus of additional analysis, as these have previously been linked to label uptake (35-38). The relative abundance of these phylotypes were compared to their relative abundance values in the unlabeled fractions (Figure 4.8).

In soil 9, no clear enrichment of phylotypes was observed in the fractions from the labeled RDX amended microcosm compared to the unlabeled RDX amended microcosm (Figure 4.8a). Although the average relative abundance values were higher for unclassified *Bacillaceae 1*, unclassified *Clostridiales* and *Tissierella* from the labeled RDX amended microcosm, the standard deviations for each point were large. This trend could be attributed to any of three factors. One, the data was not normalized to DNA concentration or the number of 16S rRNA genes in each fraction (based on current studies in our laboratory, it is likely that the DNA concentration was considerably less in the unlabeled RDX amended fractions). Unfortunately, not enough fraction samples remained for this analysis. The second factor could be a dilution of the label between several phylotypes, resulting in no clear trend for any particular phylotype. Third, only the ring carbon and nitrogen was labeled, therefore uptake of any other nitrogen would not have been noted with this experimental design. As discussed above, for soil 9, unclassified *Bacilliaceae 1* was enriched following RDX degradation. Unfortunately, the SIP results (Figure 4.8a) are not strong enough to indicate this phylotypes was also responsible for ring label uptake.

The standard deviations were smaller for the fractions generated from soil 10 (Figure 4.8b). In this soil, phylotypes belonging to *Pusillimonas*, *Rhodococcus* and *Sedimentibacter* were more abundant in the fractions from the labeled amended samples compared to the fractions from the unlabeled RDX amended samples. These data indicate these phylotypes were likely involved in carbon or nitrogen uptake from RDX. All three phylotypes were among the most abundant microorganisms in the RDX degrading microbial communities. Unexpectedly, *Brevundimonas* was not enriched in the heavy fractions of soil 10 and this may be attributed by the inability of this microorganism to uptake the ring nitrogen or carbon.

Consistent with the fracion data for soil 10, many RDX degrading *Rhodococcus* isolates have been identified, including *Rhodococcus rhodochrous* 11Y (56), *Rhodococcus* sp. strain DN22 (57-60), *Rhodococcus* strain YH1 (61), and others (62-64). A recent SIP study also found *Rhodococcus* sp. to be associated with RDX degradation in explosive contaminated soils (26). Although previous research has reported RDX degradation by *Rhodococcus* under aerobic conditions, one report indicated RDX degradation under oxygen depleted or anaerobic conditions (63).

The other genera associated with label uptake in soil 10, *Sedimentibacter* and *Pusillimonas,* have not previously been associated with RDX biodegradation. Members of the genus *Sedimentibacter* were found to play a role in DDT transformation under iron reducing conditions

(65) and were one of the dominant microorganisms in a hydrocarbon contaminated aquifer (66). Members of the genus *Pusillimonas*, (*Proteobacteria*, *Betaproteobacteria*, *Burkholderiales*, *Alcaligenaceae*) were dominant members of microbial community capable of concurrently degrading both phenanthrene and arsenite (67). They were also associated to degradation of synthetic and natural estrogen (68) and high molecular weight PAHs (69).

In summary, the high throughput sequencing data provided valuable information on which phylotypes increased in abundance following RDX degradation compared to the initial soil and microcosms not amended with RDX. The most notable trend was the common increase in abundance of *Brevundimonas* and/or unclassified *Bacillaceae 1* in the four soils studied. The data indicate these two phylotypes are benefiting from RDX degradation under these conditions. Future research is needed to determine the important of these phylotypes at RDX contaminated sites.

APPENDIX

Table 4.1. The properties of the four agricultural soils used in this study.

Soil	Crop	Organic Matter %	Sand %	Silt %	Clay %	Textural Classification	Soil pH	Calcium mg/kg	Magnesium mg/kg	Potassium mg/kg	Phosphorus mg/kg	CEC meq/100g	OM Level
9	Corn	1.2	70	24	6	Sandy Loam	4.9	489	68	151	46	5	High
10	Corn	1	76	22	2	Loamy Sand	5.5	787	42	108	79	5	Low
11	Alfalfa	1.5	80	20	0	Loamy Sand	6.4	811	74	173	106	4.6	Low
12	Corn	2.2	86	14	0	Sand	6.1	1312	242	139	268	8.6	Low

Table 4.2. A summary of MiSEQ Illumina data generated from all genomic total DNA samples and fractions for Soil 9 and Soil 10.

	# of Sequences Following Make Contigs Command	Final # of Unique Sequences	Final # of Sequences	% Chimeric
Total DNA samples for four soils (No RDX Time 0 and t, Labeled 1 & 2, Unlabeled 1 &2) (24 samples)	2,845,956	75,964	1,570,985	10.00
Triplicates, 8 fractions, Soil 9, Labeled 1 (23 samples)	2,304,194	27,609	1,640,354	1.20
Triplicates, 8 fractions, Soil 9, Unlabeled 1 (23 samples)	2,275,419	27,118	1,646,062	1.20
Triplicates, 8 fractions, Soil 10, Labeled 1 (23 samples)	4,098,173	35,706	3,123,322	0.63
Triplicates, 8 fractions, Soil 10, Unlabeled 1 (23 samples)	3,286,585	25,614	2,516,050	0.33

Table 4.3. The classification of anaerobic RDX degrading isolates from previous studies.

Class	**Order**	**Family**	**Full name**	**Reference(s)**
Phylum *Firmicutes*				
Clostridia	*Clostridiales*	*Clostridiaceae*	*Clostridium bifermentans*	(1)
Clostridia	*Clostridiales*	*Clostridiaceae*	*Clostridium bifermentans (HAW-1, HAW-G3, HAW-G4, HAW- E3 and, HAW-EC1)*	(4, 5)
			Clostrdium sp. HAW-EB17	(11)
Clostridia	*Clostridiales*	*Clostridiaceae*	*Clostridium sp. EDB2*	(2)
Clostridia	*Clostridiales*	*Clostridiaceae*	*Clostridium acetobutylicum*	(3)
Clostridia	*Clostridiales*	*Clostridiaceae*	*Acetobacterium malicum sp. HAAP-1*	(3, 7)
Clostridia	*Clostridiales*	*Clostridiaceae*	*Acetobacterium paludosum*	(6)
Clostridia	*Clostridiales*	*Peptococcaceae*	*Desulfitobacterium sp.*	(9)
Bacilli	*Bacillales*	*Bacillaceae*	*Bacillus sp. HPB2 and HPB3*	(8)
Phylum *Fusobacteria*				
Fusobacteria	*Fusobacteriales*	*Fusobacteriaceae*	*Fusobacteria HAW-21*	(11)
Phylum *Proteobacteria*				
Gammaproteobacteria	*Enteriobacteriales*	*Enteriobacteriaceae*	*Serratia sp.*	(15)
Gammaproteobacteria	*Enteriobacteriales*	*Enteriobacteriaceae*	*Enterobacter sp.*	(12, 14)
Gammaproteobacteria	*Enteriobacteriales*	*Enteriobacteriaceae*	*Citrobacter sp.*	(13)
Gammaproteobacteria	*Enteriobacteriales*	*Enteriobacteriaceae*	*Morganella sp.*	(13, 14)
Gammaproteobacteria	*Enteriobacteriales*	*Enteriobacteriaceae*	*Klebsiella sp.*	(16)
Gammaproteobacteria	*Enteriobacteriales*	*Enteriobacteriaceae*	*Providencia sp.*	(13)
Gammaproteobacteria	*Pseudomonadales*	*Pseudomonadaceae*	*Pseudomonas sp.*	(8)
Gammaproteobacteria	*Alteromonadales*	*Shewanellaaceae*	*Shewenella sp.*	(11, 18, 19)
Gammaproteobacteria	*Xanthomonadales*	*Xanthomonadaceae*	*Strenophomonas sp.*	(20)
Deltaproteobacteria	*Desulfovibrionales*	*Desulfovibrionaceae*	*Desulfovibrio sp.*	(11, 22, 23)
Deltaproteobacteria	*Desulfuromonadales*	*Geobacteraceae*	*Geobacter sp.*	(21)
Deltaproteobacteria	*Myxococcales*	*Cystobacteraceae*	*Anaeromxyobacter sp.*	(9)

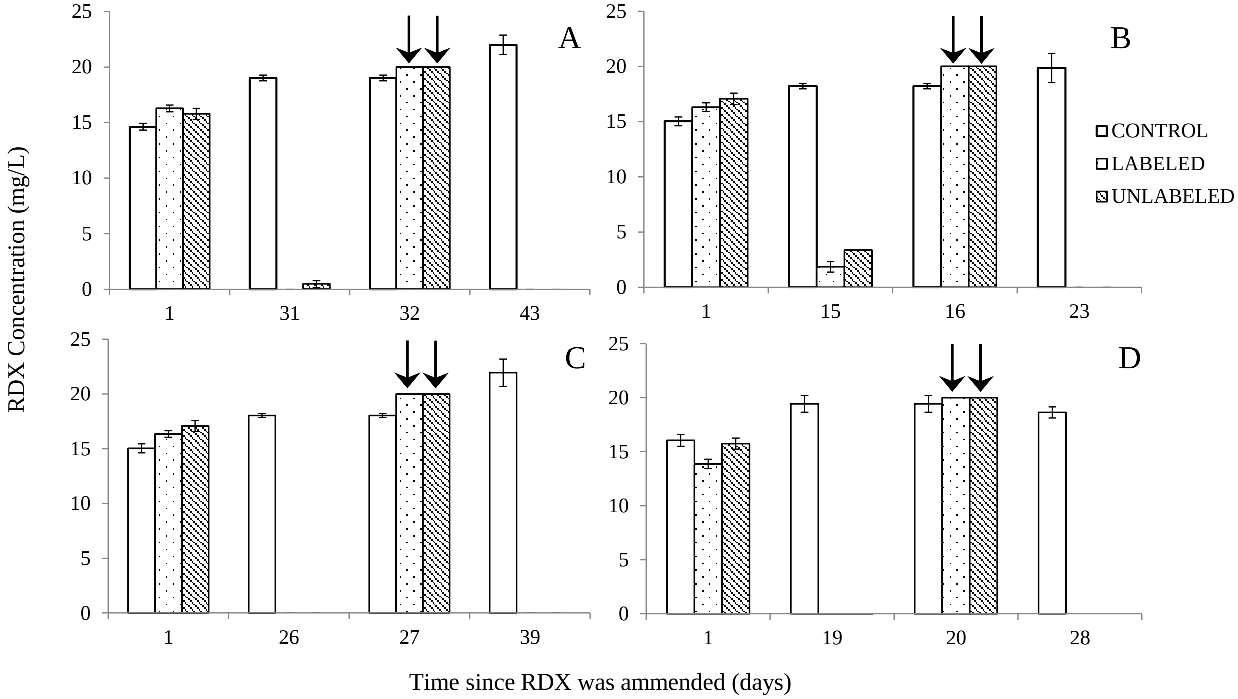


Figure 4.1. RDX in microcosms amended with labeled RDX ($^{13}C_3$, $^{15}N_3$), unlabeled RDX ($^{12}C_3$, $^{14}N_3$), and killed abiotic controls for Soil 9 (A), Soil 10 (B), Soil 11 (C), Soil 12 (D). Error bars denote standard deviation from triplicate micrososms. Arrows indicate hypothetical RDX concentrations on the day of respike.

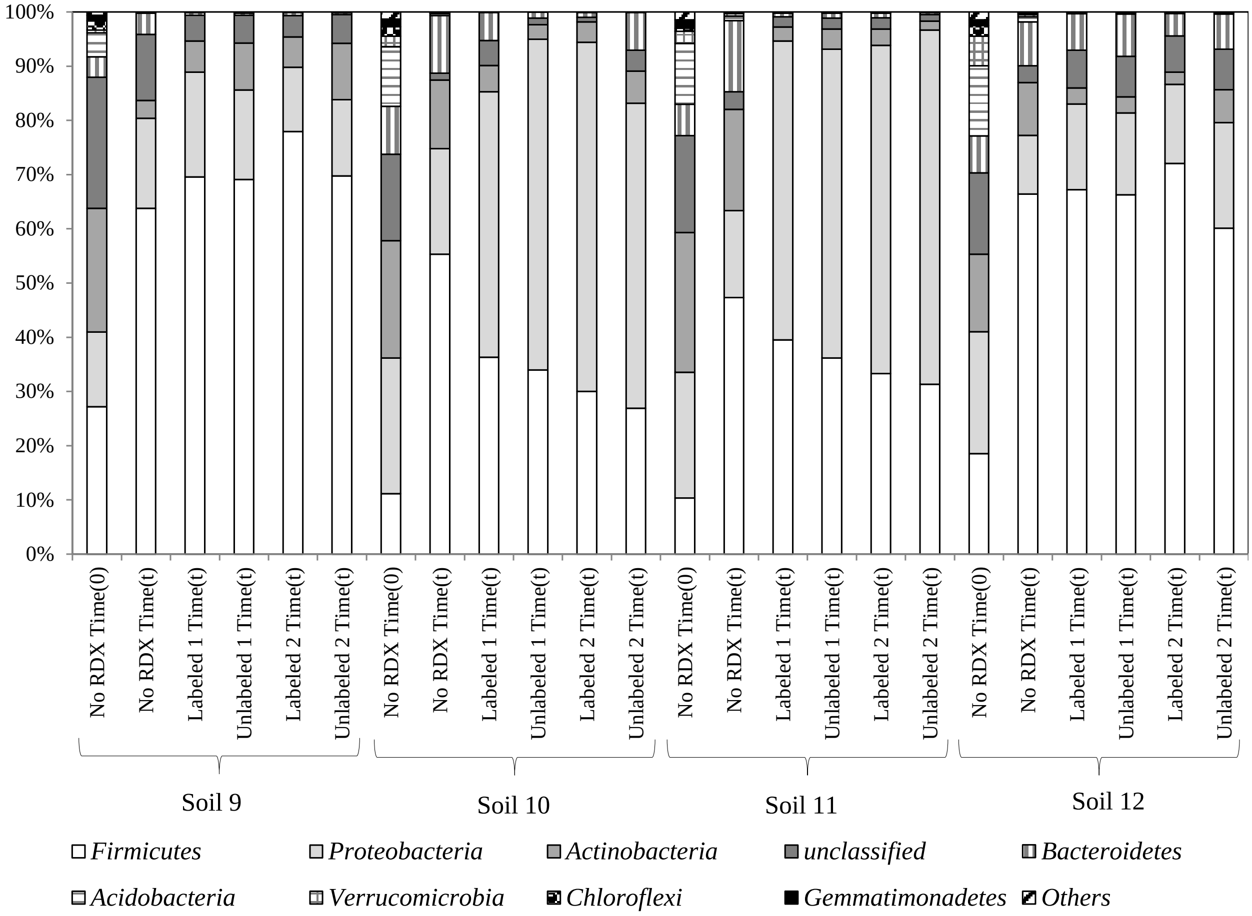


Figure 4.2. Phyla level comparison between the four soils of the communities in 1) the initial soils (No RDX, Time 0), 2) replicates of the labeled and unlabeled RDX amended microcosms (Labeled 1 & 2, Unlabeled 1 & 2, Time t) and 3) microcosms without RDX addition (No RDX, Time t).

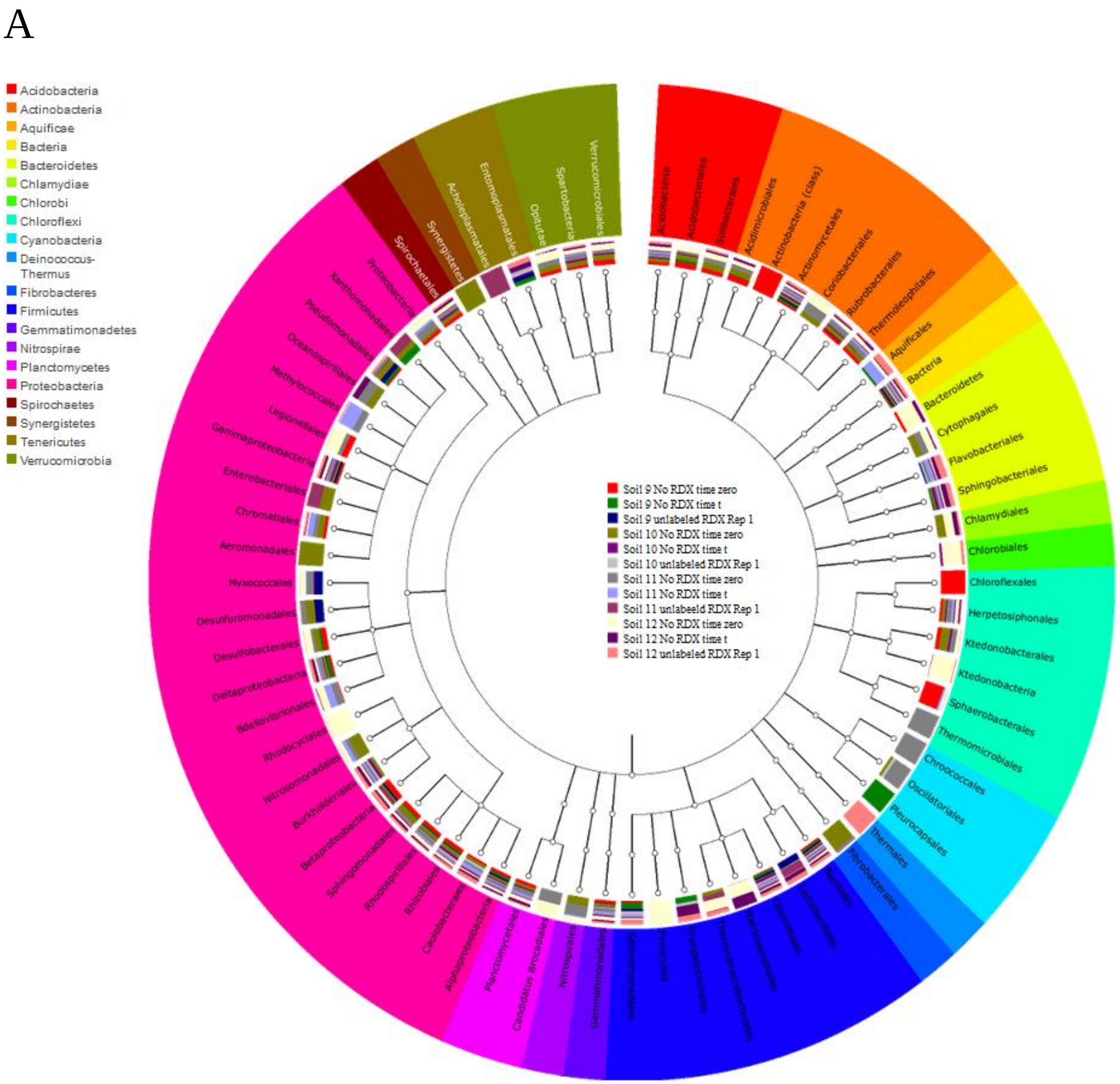
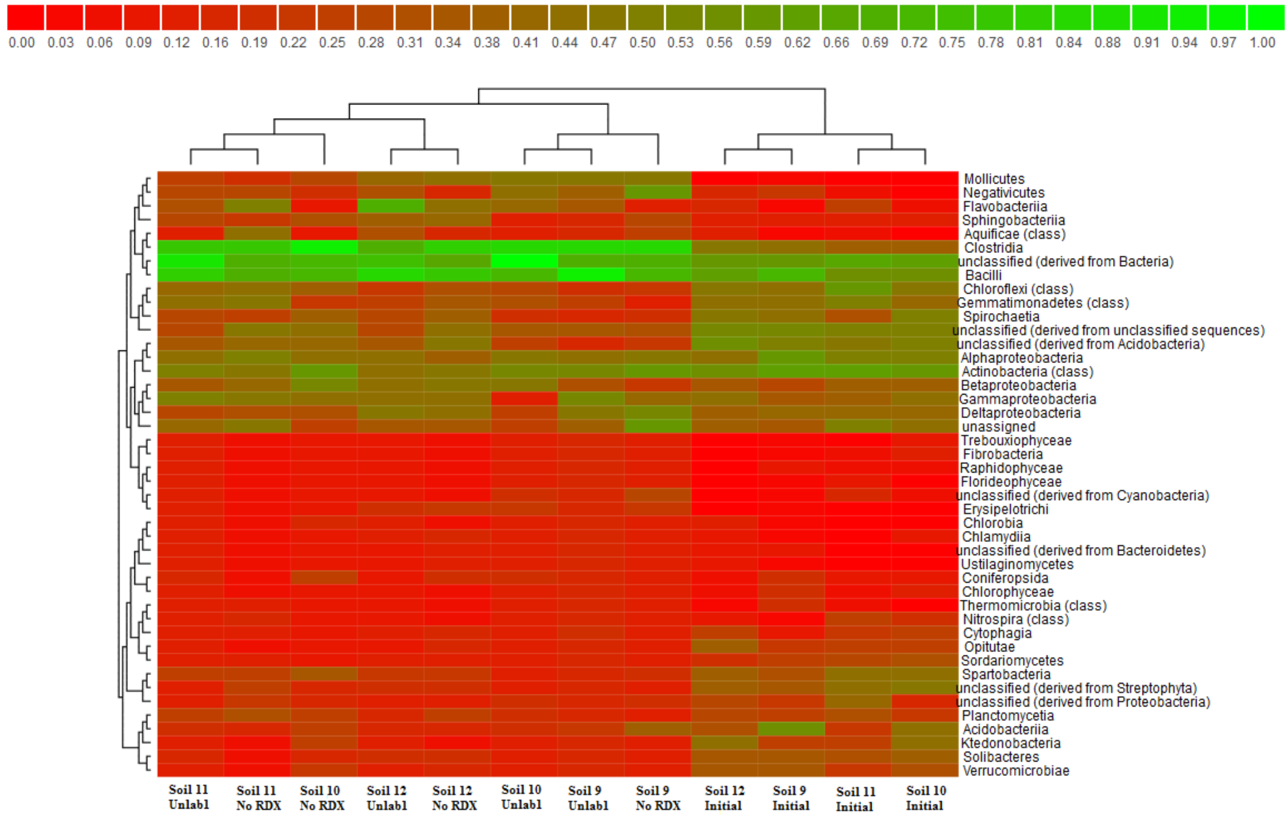


Figure 4.3. MLTree Map (A) and heatmap analysis (B) of the phylotypes within the initial soils and in samples with and without RDX. To simplify the presentation, only one replicate was used for the RDX amended samples.

Figure 4.3.(cont’d)

B



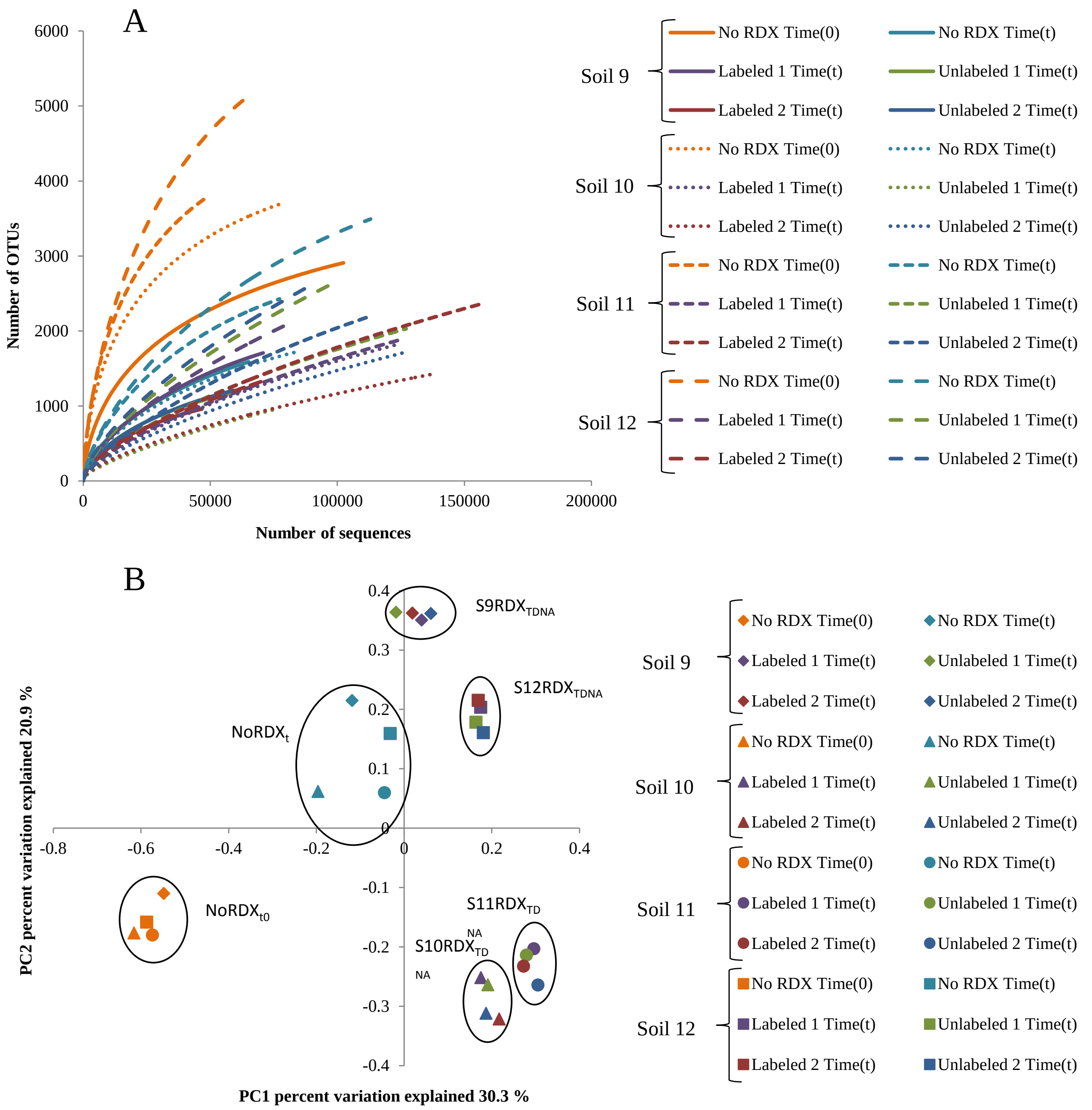


Figure 4.4. Rarefaction curves (A) and Principal Component Analysis (B) of sequencing data generated from all four soils, including total DNA extracts of the labeled RDX (Labeled 1 & 2, Time t), unlabeled RDX (Unlabeled 1 & 2, Time t), no RDX (No RDX, Time t) microcosms and the initial soils (No RDX, Time 0).

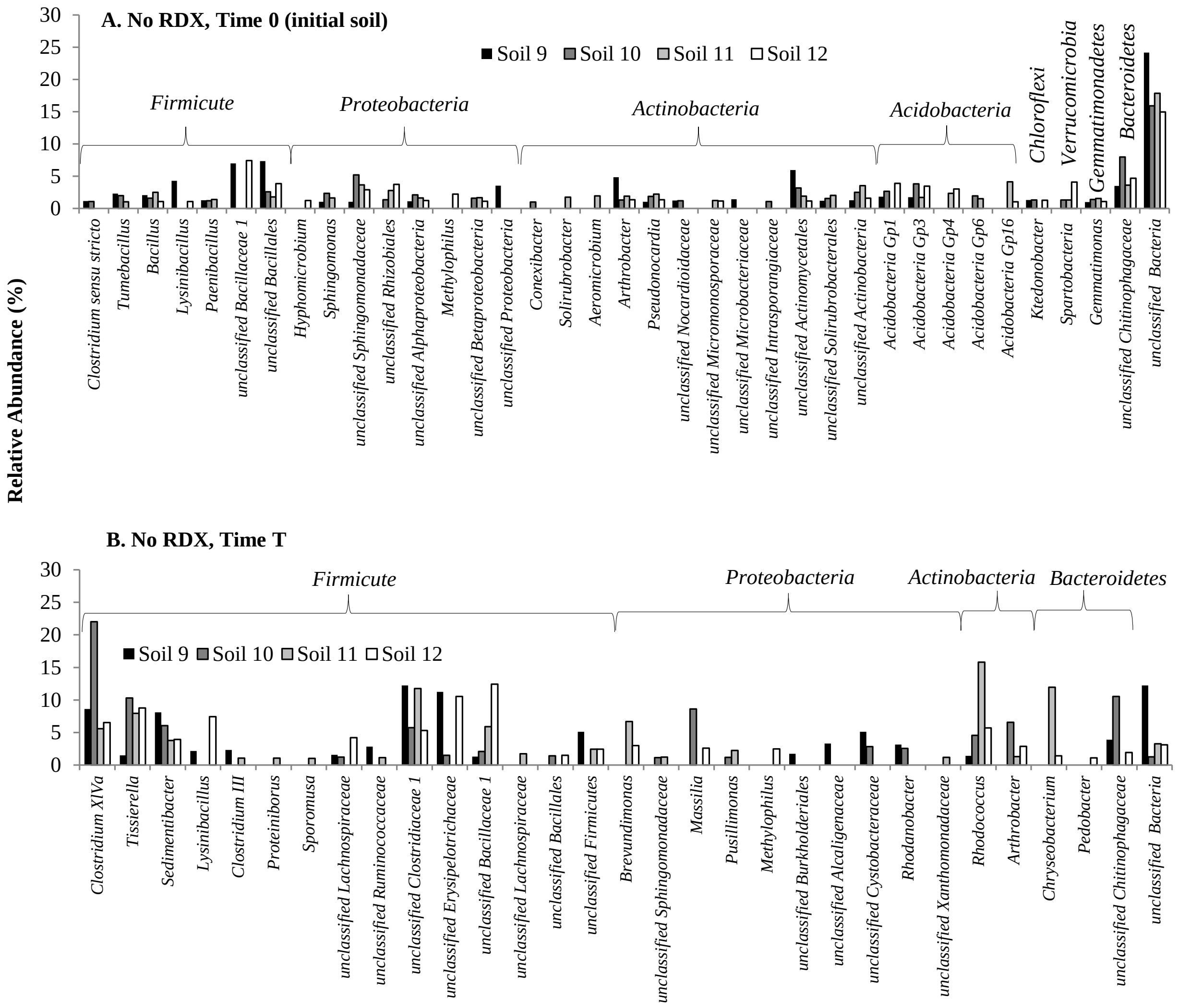


Figure 4 5. A comparison of the relative abundance (%) of the most common phylotypes (>1% , at the lowest classification level) in all four soils in the no RDX samples, including the initial soils (No RDX, Time 0) (A) and the microcosms without RDX (No RDX, Time t) (B).

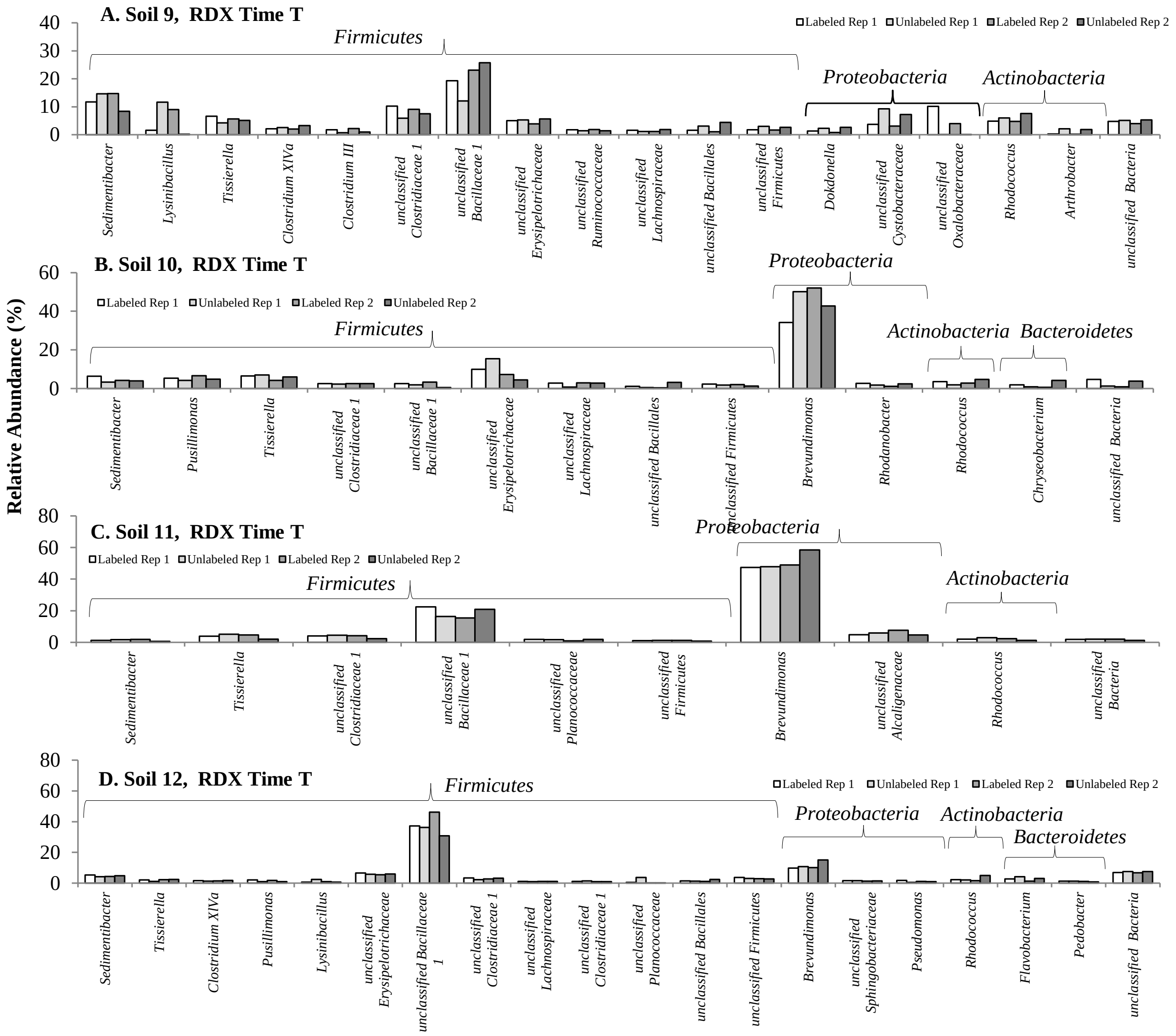


Figure 4.6. A comparison of the relative abundance (%) of the most common phylotypes (>1% , at the lowest classification level) in all four soils (A-D) following RDX degradation.

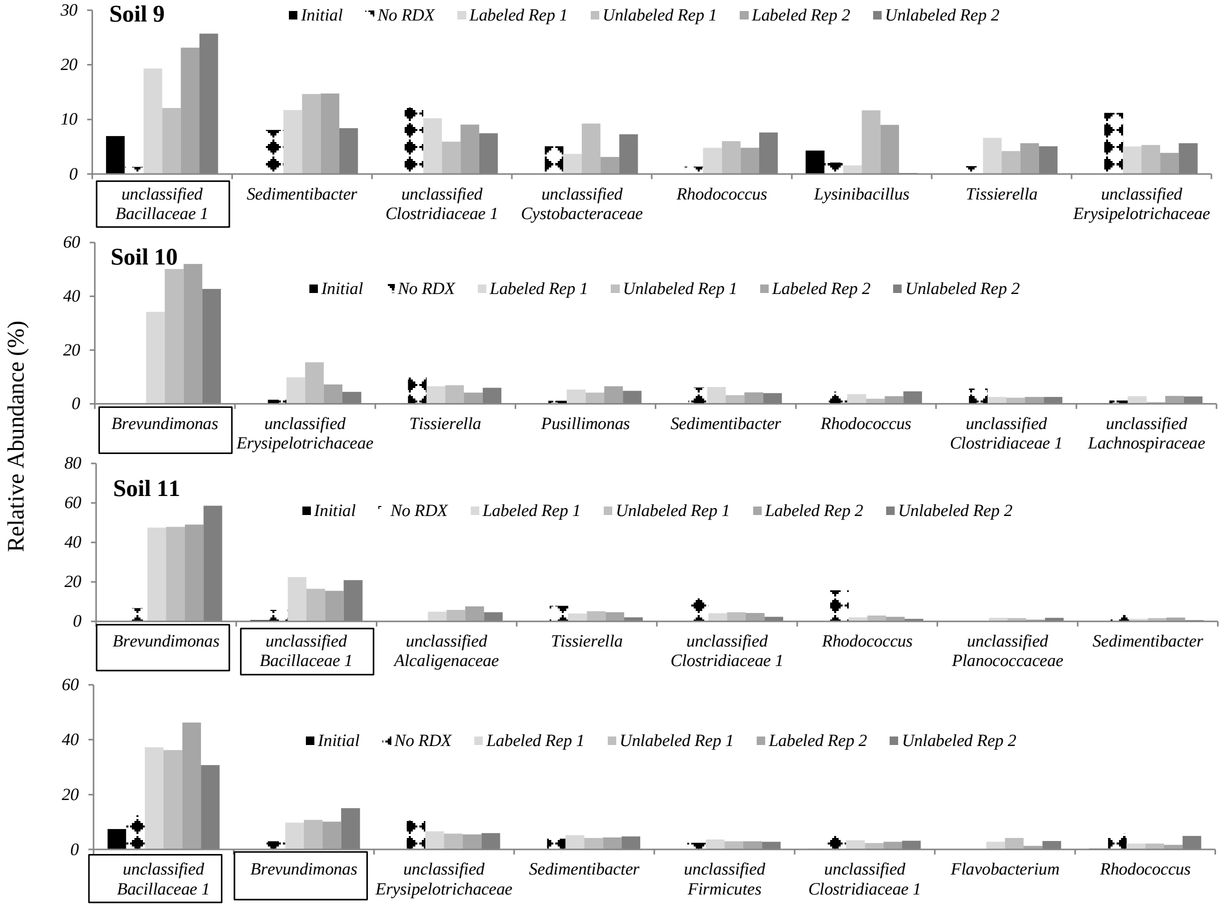


Figure 4.7. The relative abundance (%) of the eight most abundant phylotypes in the four RDX amended agricultural soils (Labeled 1 &2, Unlabeled 1 &2, Time t) compared to the RDX controls (initial soil and no RDX microcosms). Boxed phylotypes illustrate a greater abundance in RDX amended samples compared to the RDX controls for at least three soils. The phylotypes are at genus level, unless unclassified.

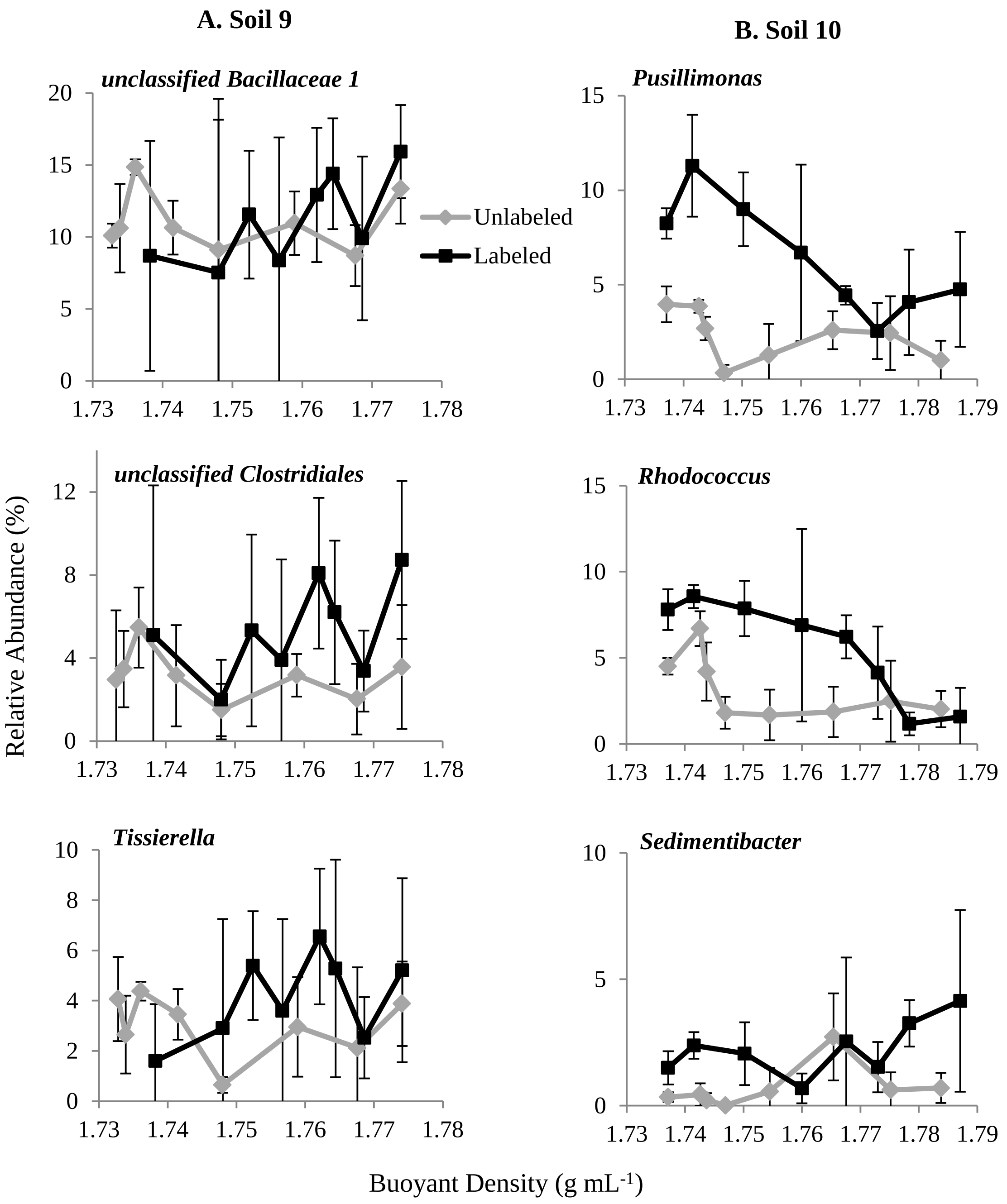


Figure 4.8. Relative abundance of the most common phylotypes in heavy fractions from the labeled RDX amended microcosms compared to those from the unlabeled RDX amended microcosms in Soil 9 (A) and Soil 10 (B). Bars illustrate standard deviations from triplicate sequencing data.

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CHAPTER 5 STABLE ISOTOPE PROBING REVEALS THE IMPORTANCE OF COMAMONAS AND PSEUDOMONADACEAE IN RDX DEGRADATION IN SAMPLES FROM A NAVY DETONATION SITE

ABSTRACT

This study investigated the microorganisms involved in RDX degradation from a detonation area at a Navy Base. Using Illumina sequencing, microbial communities were compared between the initial sample, samples following RDX degradation and controls not amended with RDX to determine which phylotypes increased in abundance following RDX degradation. The effect of glucose on these communities was also examined. In addition, stable isotope probing (SIP) using labeled ($^{13}C_3$, $^{15}N_3$) RDX was performed.

Illumina sequencing revealed several phylotypes were more abundant following RDX degradation compared to the initial soil and the no RDX controls. For the glucose amended samples, this trend was strong for an unclassified *Pseudomonadeae* phylotype and for *Comamonas*. Without glucose, *Acinetobacter* illustrated the greatest increase following RDX degradation compared to the initial soil and no RDX controls. *Rhodococcus*, a known RDX degrader, also increased in abundance following RDX degradation.

For the SIP study, unclassified *Pseudomonadaceae* was the most abundant phylotype in the heavy fractions in both the presence and absence of glucose. In the glucose amended heavy

fractions, *Comamonas* and *Anaeromxyobacter* were also present. Without glucose, the heavy fractions also contained *Azohydromonas* and *Rhodococcus*. However, all four phylotypes were present at a much lower level compared to unclassified *Pseudomonadaceae*. Overall, these data indicate unclassified *Pseudomonadaceae* were primarily responsible for label uptake in both treatments. When glucose was present, *Comamonas* increased in abundance following RDX degradation and was enriched in the heavy fractions, suggesting, for the first time, that this phylotype is also important for RDX removal.

INTRODUCTION

RDX (hexahydro-1,3,5-trinitro-1,3,5-triazine) is an explosive that has caused widespread soil and water contamination at many military sites in the US and worldwide. Such contamination has been associated with manufacturing and load-assemble-package processes performed during or after World War II and the Korean Conflict. Remediation of these sites has been initiated since the early 1980s, however many still have groundwater contaminated with nitroaromatics. The U.S. EPA has established a health advisory level in drinking water for RDX (2 μg/L), indicating the potential threat to humans. RDX is susceptible to aerobic or anaerobic biodegradation (1-10), therefore bioremediation offers a plausible approach for site clean-up. Anaerobic RDX degradation can occur via hexahydro-1-nitroso-3,5-dinitro-1,3,5-triazine (MNX), hexahydro-1,3-dinitroso-5-nitro-1,3,5-triazine (DNX), and hexahydro-1,3,5-trinitroso-1,3,5-triazine (TNX) (11), forming methanol and formaldehyde. Denitration has also been reported under anaerobic conditions, involving ring cleavage and methylene denitramine (MEDINA) formation (5, 9). Under aerobic conditions, denitration appears to be a common pathway (12). RDX denitration has been coupled with ring cleavage generating 4-nitro-2,4-

diazabutanal (NDAB) as a ring cleavage product (13). MEDINA has also been observed as a ring cleavage product (14, 15). In all, known RDX degrading aerobic or anaerobic isolates classify within the phyla *Firmicutes, Actinobacteria, Proteobacteria* (*Alpha*-, *Gamma*- and *Deltaproteobacteria*) and *Fusobacteria* (12). However, researchers have reported the microorganisms detected in field samples actively degrading RDX were generally not closely related to previously reported RDX degrading bacterial strains (16). The identification of RDX degraders at these sites is important for 1) determining if natural attenuation is a feasible approach across sites and 2) monitoring the populations of the microorganisms involved.

Although much is known about the pure cultures able to degrade RDX in the laboratory, very little is known about the microorganisms responsible for RDX degradation at contaminated sites. One group concluded that previously reported RDX degrading bacteria did not capture the microbial diversity associated with RDX bioremediation in groundwater, especially under typical biostimulation approaches (17). Indeed, it is now widely recognized that only a small percentage of microorganisms can be isolated and cultivated in the laboratory (18), therefore, it is likely that in situ RDX degraders have yet to be identified. However, recent advances in molecular biology have enabled a wealth of information concerning a range of microbial processes. A number of molecular approaches have been used to investigate RDX degraders in mixed communities, such as terminal restriction fragment length polymorphism (TRFLP), Sanger sequencing (17, 19-21) and stable isotope probing (SIP) (22-25). SIP is a culture independent method that targets only active organisms and involves sample exposure to a labeled substrate, incubation, nucleic acid extraction, ultracentrifugation to separate the labeled nucleic acid from the unlabeled background nucleic acid, and finally molecular analysis to identify the organism(s) responsible for label uptake. The method is advantageous because there is no requirement to work with pure cultures and so SIP can identify the microorganisms involved in label uptake in mixed cultures, e.g. soil samples. The method has been particularly valuable for detecting microorganisms involved in contaminant degradation (26-30).

To date, for RDX degradation studies, SIP has only been combined with TRFLP and/or Sanger sequencing. These approaches have their limitations because it is not always possible to sequence enough clones to identify the phylotypes involved in label uptake. In this study, we advance this methodology by combining SIP with high throughput sequencing to investigate the microorganisms responsible for label uptake from RDX. This is the first report of using both methods to examine RDX degraders.

Here, the objective was to identify the microorganisms involved in RDX degradation in soils from a detonation area at a Navy Base. The research also explores the effect of glucose on RDX degradation and the microorganisms involved. RDX biodegradation and bioremediation often involves the addition of a carbon source(31). Glucose was added in this study, based on previous research indicating enhanced RDX degradation in other soil samples when this substrate was added (24). The microbial communities were investigated both in the presence and in the absence of glucose. The research combined SIP and high throughput sequencing to provide two layers of data. Firstly, phylotypes illustrating an increase in relative abundance following RDX degradation compared to controls (initial soil and samples with no RDX added) were determined with Illumina sequencing. Secondly, DNA based SIP (^{15}N and ^{13}C RDX) was used to identify which microorganisms were involved in label uptake from RDX. The wealth of information provided by coupling high throughput sequencing with SIP has enabled the identification of novel RDX degraders and has provided data that can be used to design primers for the detection of these microorganisms at other RDX contaminated sites.

METHODS

Chemicals

Unlabeled and ring- labeled RDX ($^{13}C_3$, 99%; $^{15}N_3$, 98%) in acetonitrile (1,000 mg/L) were purchased from Cambridge Isotope Laboratories (Andover, MA). Other reagents were purchased from Sigma Aldrich® Corp. (St. Louis, MO) or Thermo Fisher Scientific Inc. (Waltham, MA), unless otherwise stated. HPLC grade acetonitrile was purchased from EMD Millipore (Chemicals), a division of Merck KGaA (Darmstadt, Germany).

Experimental Setup and DNA Extraction

The soil was collected from a Navy Installation in Virginia. Soil samples were first screened for their ability to degrade RDX. For this, duplicate killed controls (autoclaved) and live microcosms were prepared using 4 mL of a mineral salts media (MSM) (Thompson et al 2005), 1 mL of glucose (1 g L^{-1}), 1.5 g of soil (dry weight) and 10 mg L^{-1} of unlabeled RDX in amber serum bottles. Microcosms were sealed with rubber stoppers and aluminum seals and stored in the dark (~20 °C) without shaking. For the SIP study, microcosms were prepared with or without glucose. The serum bottles (30 mL) included 2 g of soil with either 4 mL of mineral media and 1 mL of glucose (1 g L^{-1} stock solution) or 5 mL of MSM. Then, 10 mg L^{-1} of labeled ($^{13}C_3$, $^{15}N_3$-RDX) or unlabeled RDX was added. A total of 2 killed controls (sterilized by autoclaving), 4 unlabeled controls (10 mg L^{-1} unlabeled RDX) and 2 labeled sample microcosms (10 mg L^{-1} of labeled

$^{13}C_3$, $^{15}N_3$-RDX) were prepared for each of the two treatments (with or without glucose). Two additional controls (no RDX controls) were also included, both were not amended with RDX and only one was amended with glucose. These controls were incubated under the same conditions as the above samples.

Following the degradation of the first addition of RDX (30 days), eight microcosms (replicate microcosms with labeled RDX and glucose, unlabeled RDX and glucose, labeled RDX and no glucose and unlabeled RDX and no glucose) and the two no RDX controls (with and without glucose) were sacrificed and the total genomic DNA was extracted. DNA was extracted using the Power Soil DNA extraction kit (MO BIO Laboratories, Inc. Carlsbad, CA) as per manufacturer's instructions. In the remaining replicate microcosms initially amended with unlabeled RDX (with and without glucose), a second amendment of 10 mg L^{-1} of RDX (unlabeled) was added. This was degraded under 20 days and the total genomic DNA was extracted from these samples. DNA was also extracted from the initial soil. In all, fifteen total genomic DNA extracts were submitted for Illumina sequencing (a list is provided below).

Analytical Methods

RDX extraction and analysis methods were modified from those previously described (Thompson et al 2005). Briefly, 200 µL from each microcosm was removed into 1.7 mL microcentrifuge tubes. RDX was extracted by adding equal volumes of acetonitrile and shaking the tubes at room temperature for 30 minutes. Following this, the tubes were centrifuged at 10,000 rpm for 5 minutes. The supernatant (350 µL) was filtered using 0.22 µm sterile filters into HPLC amber vials (Sigma). The HPLC parameters were as follows: injector volume, 20 µL;

isocratic conditions (40% acetonitrile and 60% 0.01% H_3PO_4-acidified water, 1 mL/min); Perkin Elmers (PE) Series 200 autosampler; PE binary LC Pump 250; Waters UV detector; wavelength 255 nm (detection limit was 500 µg/L).

Isopycnic Centrifugation

Total genomic DNA extracted from four microcosms with one amendment of RDX was subject to ultracentrifugation and fractioning. This involved two microcosms amended with glucose (labeled and unlabeled RDX amended) and two not amended with glucose (labeled and unlabeled RDX amended). The extracted DNA was ultracentrifuged with cesium chloride and tris-EDTA buffer solution. Specifically, 3 to 5 µg of DNA was added to a Tris-EDTA (pH 8.0) buffer and cesium chloride solution. The initial buoyant density (BD) of this mixture was adjusted to ~1.72 g mL^{-1} using a model AR200 digital refractometer (Leica Microsystems Inc.). This mixture was then loaded to a 5.1 mL Quick-Seal polyallomer tubes (1.3 x 5.1 cm, Beckman Coulter) and sealed using a tube topper (Cordless quick-seal tube topper, Beckman). The tubes were balanced in a Stepsaver 70 V6 Vertical Titanium Rotor (Thermo Scientific) and centrifuged at 178,000 x g for 46 hours at 20 °C in a Wx Sorvall Ultracentrifuge to obtain density gradients. The density gradients were separated into 20 fractions (each 250 µL) using gravity by displacing the samples by molecular grade water pumped by a syringe pump. Each fraction was mixed and sampled to measure their refractive index to calculate the buoyant density. The fractions were cleaned using a glycogen and ethanol precipitation to remove the cesium chloride and precipitate the DNA. They were then re-suspended in 30 µL PCR grade molecular water and stored at -20 °C until further analysis. The concentration of DNA in each fraction was quantified with the Quant-iT™ dsDNA High-Sensitivity Assay Kit using the Qubit® 2.0 Fluorometer.

Illumina MiSeq™ High Throughput Amplicon Sequencing

Both total genomic DNA extracts and selected fractions were submitted for high throughput amplicon sequencing following the protocol described elsewhere (32, 33) at the Research Technology Support Facility (RTSF) at Michigan State University. Fractions were selected based on the comparison of DNA concentrations over the buoyant density range in the fractions obtained from the unlabeled and labeled RDX amended microcosms. Those fractions from the labeled RDX amended microcosms illustrating a higher DNA concentration at the higher buoyant density values were selected for sequencing. This resulted in six fractions being sequenced for each of the four samples ultracentrifuged. Further, each fraction was sequenced with three replicates. In addition, fifteen total DNA samples were submitted for sequencing, including DNA extracted from 1) the soil prior to any incubation (called initial soil), 2) the two no RDX controls (one with glucose and the other without glucose), 3) replicate microcosms amended with labeled RDX and no glucose, 4) replicate microcosms amended with unlabeled RDX and no glucose, 5) replicate microcosms amended with labeled RDX and glucose, 6) replicate microcosms amended with unlabeled RDX and glucose, 7) replicate microcosms amended with two additions of unlabeled RDX and no glucose and 8) replicate microcosms amended with two additions of unlabeled RDX and one addition of glucose.

Illumina specific fusion primers were used to amplify the V4 region of the 16S rRNA (32) and to add unique barcodes to samples in each well to enable pooling and sequencing. After the amplicons were checked on 1% agarose gel, equimolar amounts of the sample were pooled to normalize results, purified and then sequenced on the Illumina MiSeq™ Personal Sequencing System. The amplicon sequencing data in the fastq file format was analyzed on Mothur version

1.33.0 from Patrick D. Schloss Laboratory (34) using the MiSeq standard operating procedure (35, 36). Barcode information was removed from the sequence data and contiguous sequences were created using the forward and reverse reads, were analyzed for errors and then classified. Samples were checked for the proper read length (<275 bp), ambiguous bases and homopolymer length greater than 8 to eliminate such sequences. These sequences were then aligned with the SILVA bacteria database (37) for the V4 region. Chimeras, mitochondrial and chloroplast lineage sequences were removed and then the sequences were classified into OTU's. The OTUs were then grouped into taxonomical levels with corresponding confidence levels. Rarefaction curves, Cho1 and Shannon values were determined for all DNA extracts using Mothur. Illumina sequencing data was deposited in the NCBI Sequence Read Archive under BioProject Number PRJNA264536.

Data Analysis

The most abundant phylotypes (>1% relative abundance) in all fifteen DNA extracts were determined and compared to ascertain differences and similarities between the microbial communities. The relative abundance of phylotypes was compared between the no RDX controls (initial soil and no RDX amended microcosms) to determine which phylotypes experienced an increase in abundance following exposure to RDX. In addition, the relative abundance (%) of the most abundant phylotypes in the fractions of the labeled RDX amended samples were determined. These abundance values were then compared to values from the fractions obtained from the unlabeled RDX amended samples. The purpose of this comparison was to determine which phylotypes had incorporated the ^{15}N or ^{13}C label from RDX and would thus be found in the heavier fractions.

RESULTS

RDX degradation occurred in all live microcosms within 30 days, whereas no removal of RDX was noted in the abiotic controls (data not shown). In the live microcosms, additional HPLC peaks appeared and when these were compared to analytical standards, they were identified as the mono-, di- and tri-nitroso derivatives of RDX. By day 30, all additional peaks had disappeared and DNA was extracted at this time. An extra amendment of RDX was added to four microcosms and this was removed (as were the metabolites) within 20 days. Again, DNA was extracted at this time.

High throughput sequencing was conducted on fifteen total genomic DNA extracts to investigate the microbial communities present following each treatment. For this, the most abundant phylotypes (>1% relative abundance) were compared between the microbial communities of each sample (Figure 5.1). The most abundant phylotypes in the initial soil microbial community was clearly different from the most abundant phylotypes in the other samples. For example, GP4 was the most abundant phylotype in the initial soil (12.8%), however in the other communities, it was present only at a low level (<2.1%). In addition, the three most common phylotypes in the other samples (unclassified *Pseudomonadaceae, Acinetobacter* and *Gracilibacter*) were present only at low levels in the initial soil community (<0.07%). As expected, the microbial communities were similar between the microcosms amended with labeled and unlabeled RDX. The addition of glucose also did not greatly change the most abundant phylotypes present. The four most abundant phylotypes in the microcosms amended with RDX in the absence of glucose were *unclassified Pseudomonadaceae, Acinetobacter, Gracilibacter* and *unclassified Bacteroidetes.* Whereas, in the presence of glucose and RDX, the four most abundant phylotypes were *Pseudomonadaceae, Acinetobacter, Gracilibacter* and *Comamonas.* Further, the communities that received two amendments of RDX were similar to those that received only one amendment.

Rarefaction curves (Figure 5.2) indicated that the majority of the populations were represented as these started to plateau for all of the samples. In contrast, the curve for the initial soil microbial community was still increasing, suggesting additional data is needed to represent the complete diversity of this sample. The total number of OTUs or species richness was estimated using the Chao1 estimator (Table 5.1). In general, the RDX and glucose amended microcosms appeared to have greater species richness compared to the RDX amended microcosms that did not receive glucose. The Chao1 value for the initial soil was notably higher than all of the other samples. The Shannon diversity value for the initial soil was also higher than all other Shannon values (Table 5.1). Again, the glucose amended samples illustrated higher Shannon values compared to the samples that were not amended with glucose.

To determine which microorganisms were enriched following RDX degradation, the relative abundance of each in RDX amended microcosms was compared to the initial soil and the no RDX control microcosms. Phylotypes with greater relative abundance in the RDX amended microcosms compared to the no RDX controls are shown (Figure 5.3). For the glucose amended samples, five bacteria illustrated an increase over the controls (Figure 5.3a). From these, three illustrated a larger increase in abundance compared to the controls, including unclassified *Pseudomonadeae*, *Pseudomonas* and *Comamonas*. For the microbial communities in the absence of glucose, *Acinetobacter* illustrated the greatest increase compared to the initial soil and no

RDX controls (Figure 5.3b). In the absence of glucose, *Rhodococcus* (a genus containing many known RDX degrading isolates) also increased in abundance, although it was at a much lower level than *Acinetobacter*. Interestingly, both treatments resulted in a greater relative abundance of *Comamonas* and *Sedimentibacter* in the RDX amended samples compared to the initial soil and no RDX controls.

Total DNA samples from the labeled and unlabeled RDX amended microcosms for both the glucose and no glucose samples were subject to ultracentrifugation and fractioning. The DNA concentration in each fraction was then determined (Figure 5.4). The DNA concentration was higher in the heavier fractions for the labeled RDX amended samples compared to the unlabeled RDX amended samples, indicating an enrichment of nucleic acids with ^{13}C and/or ^{15}N. This pattern occurred in both the glucose (Figure 5.4a) and the no glucose samples (Figure 5.4b). The heavy fractions were submitted for Illumina sequencing (three replicates for each) to determine which phylotypes were responsible for label uptake.

The three most abundant phylotypes in the heavy fractions from the labeled RDX amended samples were determined for both treatments (Figure 5.5). The sequencing data from the three replicates were similar, resulting in small error bars. From this analysis, only one phylotype (unclassified *Pseudomonadaceae*) was similar between treatments and, in both cases, it was the most dominant phylotype in the heavy fractions. In the glucose amended heavy fractions *Comamonas* and *Anaeromyxobacter* were also present, but at a much lower level compared to unclassified *Pseudomonadaceae* (Figure 5.5a). In the heavy fractions from the no glucose samples, *Azohydromonas* and *Rhodococcus* were present and again this occurred at a much lower level compared to the unclassified *Pseudomonadaceae* phylotype (Figure 5.5b). These data indicate unclassified *Pseudomonadaceae* were primarily responsible for label uptake in the soil microcosms, both in the absence and presence of glucose.

DISCUSSION

The potential for any site to biodegrade RDX is particularly difficult to predict because of the large number of RDX degrading isolates and the lack of correlation between these isolates and the species found at contaminated sites. To address this, the current study combined SIP and high throughput sequencing to provide an in depth analysis of the microorganisms linked to RDX degradation in samples from a Navy Base. The study utilized two layers of information to link RDX degradation with microorganism identity. Firstly, phylotypes illustrating an increase in relative abundance following RDX degradation, compared to controls (initial soil and samples with no RDX added) were determined. This approach is similar to using quantitative PCR to document growth on a substrate for specific phylotypes within mixed communities. It cannot be definitely stated that these microorganisms were growing using RDX as a carbon, nitrogen or energy source. However, because their abundance increased following RDX degradation, they were clearly benefiting from this process. Further, in replicate samples not exposed to RDX, this increase did not occur. Secondly, SIP was used to identify which microorganisms were involved in label uptake from RDX. Combining the results from these two approaches has provided an enhanced data set for documenting the microorganisms involved in RDX degradation.

To date, a range of molecular methods have been used to examine microbial communities at RDX contaminated sites, including terminal restriction fragment length polymorphism (TRFLP), lipid biomarker analysis (38), denaturing gradient gel electrophoresis (17, 19, 39), amplified 16S rDNA restriction analysis (ARDRA)(21), cloning and Sanger sequencing (17, 19-21). Only recently (2013), has high throughput sequencing (pyrosequencing, not in combination with SIP) been applied to examine RDX degrading microbial communities (40). Four studies have applied SIP to investigate RDX degradation (22-25). SIP has the advantage over these methods in that the labeled carbon or nitrogen derived from RDX can be linked to microorganism identity, providing more robust information on the microorganisms involved in RDX degradation.

The current study is the first to combine SIP with high throughput sequencing to investigate the microorganisms responsible for RDX degradation. Using high throughput sequencing, rather than Sanger sequencing, with SIP provides a much greater depth of information on the microorganisms involved. To our knowledge, SIP has been used in combination with traditional Sanger sequencing in four studies to examine RDX degradation (22-25). One study using ^{15}N and ^{13}C labeled RDX revealed the important of *Sphingobacteriales* in label uptake (24). Another SIP study (^{15}N labeled RDX) using samples from the Picatinny Arsenal Site identified five phylotypes similar to known RDX degraders and ten phylotypes not previously linked to RDX degradation (25). The authors reported that several of these phylotypes classified within the genus *Pseudomonas*. The authors did not report any enrichment of phylotypes from the *Betaproteobacteria*, indicating they did not find enrichment of the genus *Comamonas*. A more recent SIP study (2013), using ^{13}C labeled RDX, also reported the importance of *Pseudomonas* phylotypes in label uptake (23). The 2013 study also found that *Rhodoferax*, a genus within the same family (*Comamonadaceae*) as *Comamonas*, was responsible for label uptake. However, the research also reported label uptake over a range of phyla, including *Proteobacteria, Spirochaetes, Bacteroidetes, Firmicutes,* and *Actinobacteria* (23). The fourth SIP study (^{15}N labeled RDX), using material from the Eglin Air Force Base bombing range, found that *Rhodococcus* illustrated the greatest amount of label uptake (22). From the other seven phylotypes that were associated with label uptake, one classified (*Variovorax*) within the same family as *Comamonas* and another classified within the same order (*Burkholderiales*). The results obtained in the current study agree with previous SIP studies, indicating the importance of phylotypes within the families *Pseudomonadaceae* and *Comamonadaceae* in label uptake from RDX. In addition, similar to previous studies, the current research also found that *Rhodococcus* was associated with label uptake. Taken together, these data indicate that primers designed towards these phylotypes would be advantageous for investigating the feasibility of bioremediation across RDX contaminated sites.

Known RDX degrading isolates classify within four phyla, the *Firmicutes, Actinobacteria, Proteobacteria* and *Fusobacteria*. The phylum *Firmicutes* contains many RDX degrading bacteria and these fall within the class *Clostridia* or *Bacilli* (9, 10, 41-47). In the current study, unclassified *Clostridiaceae 1* and *Sedimentibacter* were enriched following RDX degradation, but were not linked with label uptake from RDX. The phylum *Actinobacteria* also contains RDX degrading isolates including many within the genus *Rhodococcus* (14, 48-55). Other *Actinobacteria* isolates include *Williamsia* sp. KTR4 (56), *Gordonia* sp. KTR9 (56, 57) and *Gordonia* sp. YY1 (19). The data from the current study indicate *Rhodococcus* phylotypes increased in abundance following RDX degradation and the SIP data suggest these phylotypes are responsible for lower levels of label uptake.

RDX degrading isolates in the phylum *Proteobacteria* are found within the *Alpha*-, *Gamma*- and *Deltaproteobacteria* classes (12). In the current study, *Anaeromxyobacter* was the only *Deltaproteobacteria* implicated in label uptake and previous research has indicated this genus is capable of RDX degradation (46). In the *Betaproteobacteria,* two phylotypes were associated with label uptake (*Comamonas* was particularly enriched and *Azohydromonas* was enriched to a lower level). Interestingly, no pure cultures of *Betaproteobacteria* have been shown to be capable of RDX degradation. The most dominant phylotype responsible for label uptake was a *Gammaproteobacteria,* unclassified *Pseudomonadaceae.* Any of the eight genera within this family (*Azomonas, Azorhizophilus, Azotobacter , Cellvibrio, Pseudomonas. Rhizobacter, Rugamonas, Serpens*) could have been responsible for label uptake. These results collaborate previous research, as many *Gammaproteobacteria* isolates are able to degrade RDX (1, 4, 5, 58, 59). When the unclassified *Pseudomonadaceae* partial 16S rRNA gene sequence was compared to those in Genbank it was found to be 100% similar to twenty uncultured bacterium sequences, seven *Pseudomonas* sequences, and four *Azotobacter tropicalis* sequences. In the current study *Pseudomonas* was also enriched following RDX degradation and again *Pseudomonas* isolates have been shown to be able to metabolize RDX (47, 60).

In summary, SIP was combined with high throughput sequencing to investigate the microorganisms involved in RDX degradation. To our knowledge, this is the first application of this combined approach to study RDX biodegradation. The study generated two important data sets. Firstly, high throughput sequencing data indicated several phylotypes increased in abundance following RDX degradation compared to the initial soil and no RDX controls. These trends suggest a growth benefit caused by the degradation of RDX as the microorganisms were directly benefiting from RDX or from RDX metabolites. Secondly, using SIP, two phylotypes were found to be primarily responsible for label uptake, indicating incorporation of the label into their DNA. It is not possible to conclude if they were assimilating the label from RDX or from RDX metabolites. The two phylotypes associated with label uptake included *Comamonas* and an unclassified *Pseudomonadaceae*. The high throughput sequencing data indicated these two phylotypes also increased in abundance following RDX degradation compared to the controls. Taken together, the two data sets implicate these phylotypes in RDX degradation, as their populations both increased following RDX degradation and the label was incorporated into their DNA. Other SIP and pure culture studies have also reported the importance of phylotypes within the *Pseudomonadaceae* for RDX degradation. In contrast to pure culture and SIP studies, the current research provided evidence of the importance of *Comamonas* in RDX degradation. These data suggest the presence of these microorganisms at contaminated sites should enhance natural attenuation efforts.

APPENDIX

Table 5.1. Chao1 and Shannon and lower and upper confidence intervals (CI) values for all microbial communities

	Chao1	**Lower 95% CI**	**Upper 95% CI**	**Shannon**	**Lower 95% CI**	**Upper 95% CI**
Initial Soil	6444	6331	6574	6.6	6.5	6.6
Glucose, no RDX	3469	3379	3575	3.4	3.4	3.4
Glucose, lab 1	4019	3928	4125	4.3	4.2	4.3
Glucose, lab 2	4404	4277	4552	4.3	4.3	4.3
Glucose, unlab 1	4974	4839	5130	4.2	4.2	4.2
Glucose, unlab 2	3183	3086	3296	4.1	4.0	4.1
Glucose, unlab 1, time 2	4261	4168	4368	4.4	4.4	4.4
Glucose, unlab 2, time 2	4718	4612	4840	4.5	4.5	4.5
No glucose, no RDX	4531	4427	4653	4.3	4.3	4.3
No glucose, lab 1	3435	3347	3538	3.8	3.7	3.8
No glucose, lab 2	3502	3406	3614	3.3	3.3	3.3
No glucose, unlab 1	3628	3512	3764	3.3	3.3	3.3
No glucose, unlab 2	3238	3155	3337	3.6	3.6	3.6
No glucose, unlab 1, time 2	4029	3929	4145	4.3	4.3	4.3
No glucose, unlab 2, time 2	3725	3622	3845	4.1	4.1	4.2

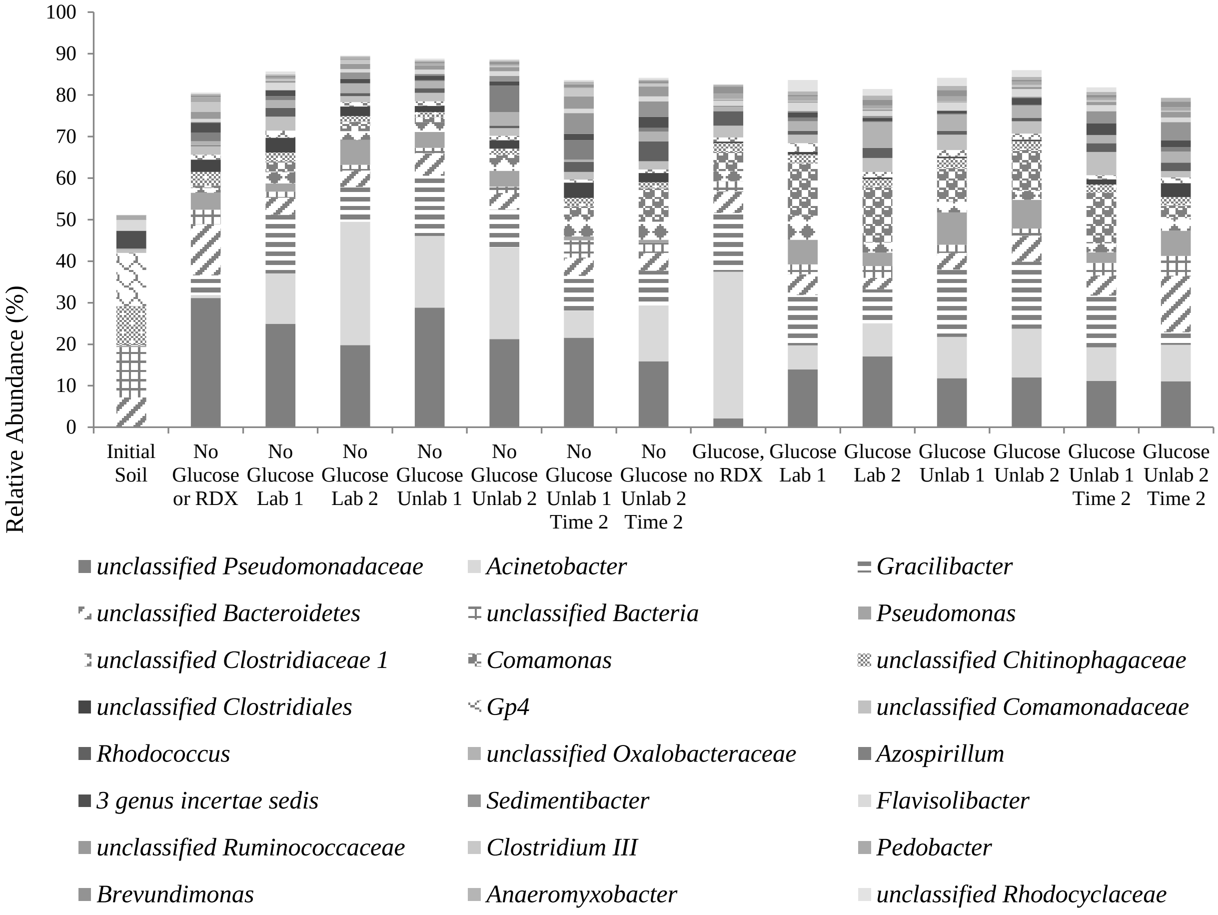

Figure 5.1. Relative abundance of the most common phylotypes in RDX amended microcosms (with and without glucose) compared to their abundance in the initial soil community and no RDX microcosms.

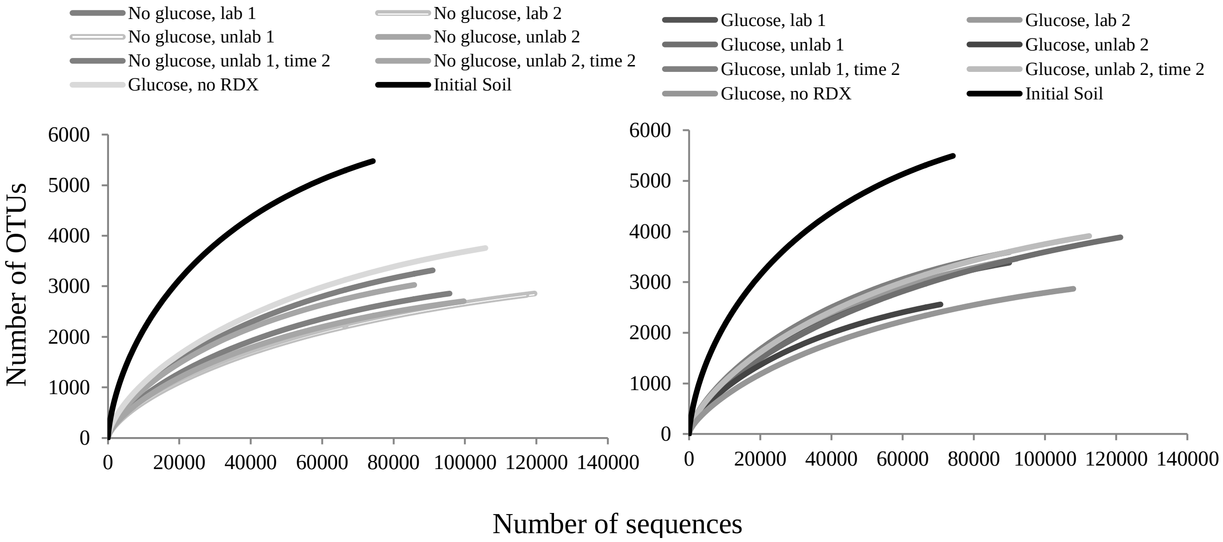

Figure 5.2. Rarefaction curves for the total microbial communities in the initial soil, RDX amended microcosms and the no RDX controls for both the no glucose amended (A) and glucose amended (B) microcosms.

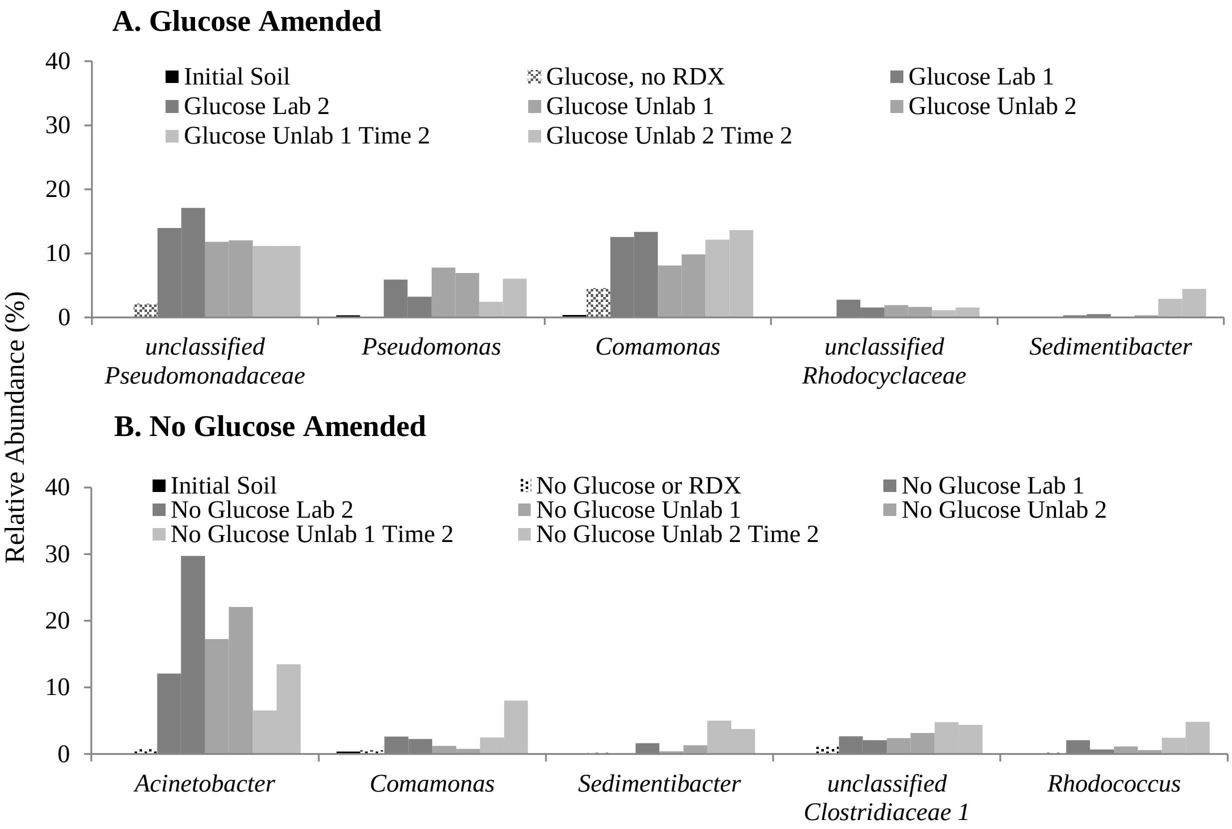

Figure 5.3. Relative abundance of phylotypes illustrating a difference in relative abundance between the RDX amended microcosms and the no RDX controls (initial soil and no RDX microcosm) in the glucose amended microcosms (A) and in the microcosms that were not amended with glucose (B).

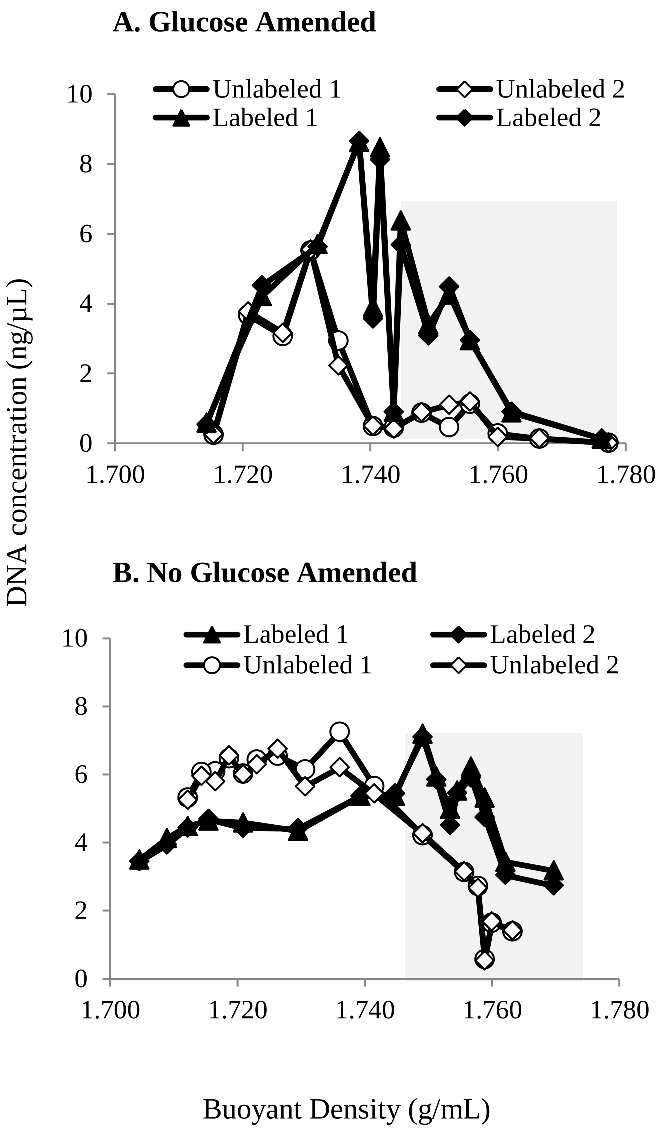

Figure 5.4. DNA concentration (ng/µL) in fractions across buoyant density gradients obtained from samples amended with labeled and unlabeled RDX, with (A) or without (B) the addition of glucose. Replicate lines represent duplicate DNA measurements. The shaded area represents the fractions analyzed with Illumina sequencing.

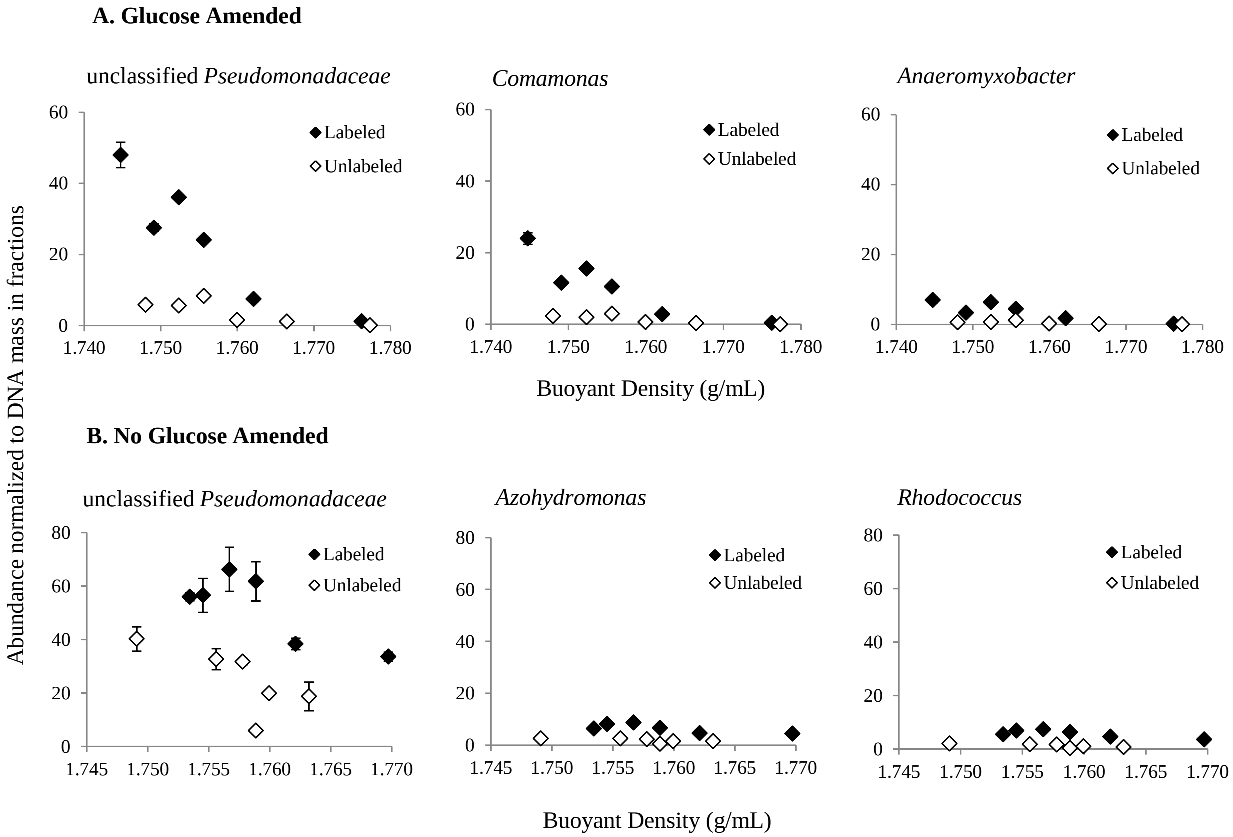

Figure 5.5. The three most abundant phylotypes in the heavy fractions from the labeled RDX amended microcosms compared to their abundance in fractions of similar buoyant density from the unlabeled RDX amended microcosms. Data are shown from those amended with glucose (A, top three graphs) and those not amended with glucose (B, bottom three graphs). The y-axis represents relative abundance in each fraction normalized by the DNA mass in each fraction (relative abundance times the DNA mass (ng)). Error bars represent standard deviations from three replicates and may be too small to be seen.

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