DIVERSITY AND MICROBIAL COMMUNITY STRUCTURE AT A FORMER MILITARY RANGES IN VIEQUES (PUERTO RICO)

By

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A thesis submitted in partial fulfillment of the requirement for the degree of

MASTER OF SCIENCE

in

BIOLOGY

UNIVERSITY OF PUERTO RICO

MAYAGÜEZ CAMPUS

December, 2008

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Abbreviations

(AFWTF)	Atlantic Fleet Weapons Training Facility
(ATSDR)	Agency for Toxic Substances and Disease Registry
(TNT)	Trinitrotoluene
(UXO)	Unexploded ordnances
(NSFS)	Naval gunfire support
(EMA)	Eastern Maneuver Area
(US EPA)	Environmental Protection Agency
(SIA)	Surface Impact Area
(LIA)	Live Impact Area
(TNT)	2,4,6- trinitrotoluene
(RDX)	Hexahidro-1,3,5-Trinitro-1,3,5-Triazina
(HMX)	Cyclotetramethylene tetranitramine
(DAT)	Diaminotoluene
(DNT)	Dinitrotoluene
(2,4-DNT)	2,4-dinitrotoluene
(2,6-DNT)	2,6-dinitrotoluene
(NO)	Nitric oxide
(MNX)	Hexahydro-1-nitroso-3,5-dinitro-1,4,5-triazine
(DNX)	Hexahydro-1,3-dinitroso-5-nitro-1,4,5-triazine
(TNX)	Hexahydro-1,3,5-trinitroso-1,3,5-triazine
(N_2O)	Nitrous oxide

(DGGE)	Denaturing Gradient Gel Electrophoresis
(T-RFLP)	Terminal Restriction Fragment Length Polymorphism
(TGGE)	Temperature Gradient Gel Electrophoresis
(RFLP)	Restriction Fragment Length Polymorphism
(OTU)	Operation taxonomic unit
(CA)	Correspondence analysis
(ARISA)	Automated ribosomal intergenic spacer analysis
(ANOSIM)	Analysis of similarities
(ANOVA)	Analysis of variance
(SIMPER)	Similarity Percentage
(GC)	Gas Chromatography
(HPLC)	High Pressured Liquid Chromatography
(dNTPs)	Dinucleotide triphosphates
(ddH_2O)	Double distilled water
(H_2O)	Water
(PAT)	Phylogenetic Assignment Tool
(LB/Amp/IPTG/X-gal)	Luria Bertani agar with 100 g/ml ampicillin, 0.5mM IPTG, and 80 g/ml X-Gal
(PCR)	Polymerase Chain Reaction
(RDP)	Ribosomal Data Project
(NJ)	Neighbor-joining
(BLK)	Bruhn, Lenke and Knackmuss medium
(SOIL)	Soil microcosm with out explosives addition

(SOIL-DNT)	Soil microcosm amended with 2,4-DNT
(SOIL-DNT/Ac)	Soil microcosm amended with 2,4-DNT and acetone as carbon source
(SOIL-RDX/Ac)	Soil microcosm amended with RDX
(SOIL-RDX)	Soil microcosm amended with RDX and acetone as carbon source
(SOIL-KILL-DNT)	Autoclaved soil microcosm amended with 2,4-DNT
(SOIL-KILL-RDX)	Autoclaved soil microcosm amended with RDX
(SED)	Marine sediment microcosm with out explosives addition
(SED-DNT)	Marine sediment microcosm amended with 2,4-DNT
(SED-DNT/Ac)	Marine sediment microcosm amended with 2,4-DNT and acetone as carbon source
(SED-KILL-DNT)	Autoclaved marine sediment microcosm amended with 2,4-DNT
(SED-KILL-RDX)	Autoclaved marine Sediment microcosm amended with RDX
(SED-RDX)	Marine sediment microcosm amended with RDX
(SED-RDX/Ac)	Marine sediment microcosm amended with RDX and acetone as carbon source

ABSTRACT

The eastern part of the island of Vieques (Puerto Rico) was subjected to live fire bombing by the US Navy and Marine Corps for approximately 60 years, leaving multiple unexploded ordnances in terrestrial and marine environments. The microbial community structure under different redox conditions were examined in soil and marine sediment samples exposed to 2,4-Dinitrotoluene (2,4-DNT) and Hexahidro-1,3,5-Trinitro-1,3,5-Triazina (RDX). Samples from the former training ranges were obtained and separately subjected to four treatments (no addition of explosives, 2,4-DNT or RDX, addition of 2,4-DNT or RDX and a carbon source, and a killed control with 2,4-DNT or RDX and a carbon source) under two redox conditions for four (4) weeks (oxic and sulfate-reducing). The influence of explosives on the bacterial community structure was analyzed by correspondence analysis (CA) of terminal restriction length polymorphism (T-RFLP) profiles and through the cloning and sequencing of 16S rRNA genes. Soil microbial communities exhibited the metabolic capability to mineralize 2,4-DNT under aerobic conditions as evidenced by the decreased of 2,4-DNT coupled with the stochiometric production of nitrite. Specific changes in microbial community during the active degradation process of 2,4-DNT (day 8 to 16) suggest an enrichment of possible degraders. Additionally, comparison of 2,4-DNT amended and un-amended clone libraries from day 8, showed an increment in the Comamonadaceae family (29 to 44%) in amended systems. The recovered Comamonadacea sequences were closely related to *Variovorax* genus. Finally, *in-silico* T-RF profiles of Comamonadaceae family are also dominant in T-RFLP profiles from 2,4-DNT amended microcosms. In contrast, soil concentration of RDX in aerobic microcosm did not decrease during the experiment but

significantly changed the microbial community T-RFLP profile, even more dramatic than 2,4-DNT treatments. In marine sediments, the persistence of RDX did not influence the microbial structure. Under sulfate reducing conditions, 2,4-DNT and RDX rapid decreases on soil and marine sediment microcosms even in abiotic controls, suggesting that under this conditions explosives are degraded or become less bio-available thus decreasing its impact on the ecosystem. It was observed as well that the addition of RDX to aerobic soil microcosms decreased the relative abundance of OTUs of the fragments related to the Commamonadaceae family. Therefore mix contaminations with RDX and 2,4-DNT in aerobic soil could affect the 2,4-DNT degrading community and thus interfering with its natural attenuation. Microbial community analysis provides key ecological criteria's to better understand the fate of pollutants and to identify indigenous microbial potential to assist environmental restoration.

RESUMEN

La isla de Vieques (Puerto Rico) fue utilizada por la Marina de los Estados Unidos como lugar de práctica militar siendo objeto de continuos bombardeos por un periodo aproximado de 60 años, dejando toneladas de municiones sin detonar en ambientes marinos y terrestres. El presente estudio pretende explorar, el destino y efecto del 2,4-DNT y RDX sobre las comunidades microbianas de Vieques. Las muestras se colectaron en suelo y sedimento marino de las antigua zonas de entrenamiento, estas fueron sujetas a cuatro distintitos tratamientos (sin adición de explosivo, con adición de 2,4-DNT o RDX, adición de 2,4-DNT o RDX y una fuente de carbono, y esterilización con adición de 2,4-DNT o RDX y una fuente de carbono) y dos condiciones de oxido-reducción (aeróbicas y sulfato reductoras) durante un periodo de 32 días. El efecto de los explosivos en la comunidad microbiana se determino por medio del análisis de correspondencia de los perfiles del T-RFLP y a través del la clonación y posterior secuenciación del gen 16s rRNA. Las comunidades microbianas del suelo poseen la capacidad metabólica para mineralizar 2,4-DNT bajo condiciones aeróbicas, como sugiere la evidencia de disminución de 2,4-DNT acoplada con la producción de estequiométrica de nitrito. Los cambios específicos en la comunidad microbiana durante el periodo activo de la degradación de 2,4-DNT (día 8 al 16) sugieren un enriquecimiento de los posibles degradadores. Adicionalmente, la comparación de las librerías de clones de los microcosmos de suelo del día 8, con y sin la adición de 2,4-DNT mostraron un enriquecimiento de la familia Commamonadacea (29 a 44%) en los microcosmos con adición de 2,4-DNT, la mayoría de estas secuencias se encuentran relacionadas con el genero *Variovorax* donde algunas especies como *V. paradoxus* han sido descritas como

degradadotas de 2,4-DNT in consorcios bacterianos. Finalmente, mediante la generación in-silico de fragmentos terminales de las secuencias de la familia Commamonadacea se demostró el enriquecimiento de estos fragmentos en el T-RFLP del microcosmo con adición de 2,4-DNT. Por el contrario, la adición de RDX mostró un efecto más fuerte en la comunidad del suelo donde se comporto como un contaminante persistente. En el caso de los sedimentos marinos, la persistencia del RDX no mostró un efecto aparente en la estructura microbiana lo cual sugiere que en el caso de liberación en sedimentos marinos RDX tiene un efecto más débil que la liberación de 2,4 DNT. Bajo condiciones de sulfato reducción, se observo una rápida disminución de la concentración 2,4-DNT y RDX los microcosmos del suelo y de sedimentos marinos, esta disminución de los explosivos se observo incluso en los controles abióticos, lo que sugiere que bajo condiciones de sulfato reducción los explosivos son rápidamente degradados o se disminuye su bio-disponibilidad y por lo tanto su impacto en el ecosistema. También se encontró que aquellos fragmentos terminales relacionados con la degradación de 2,4-DNT son inhibidos en el suelo por la adición de RDX, lo que sugiere que la liberación simultanea de RDX y 2,4-DNT en suelos bajo condiciones aeróbicas puede disminuir la degradación de 2,4-DNT y por lo tanto interferir con su atenuación natural en el ambiente. La identificación cambios en las comunidades microbianas y el uso de análisis ecológicos promueven el avance del entendimiento del destino y efecto de los contaminantes y permiten la identificación del potencial de los las comunidades microbianas nativas para determinar estrategias de restauración ambiental

INTRODUCTION

Effect of military activities on environmental and human health is an important concern in active or abandoned military training areas. During 60 years, the US Navy and Marine Corps used the eastern part of Vieques (an island-municipality of Puerto Rico) for live fire bombing and shelling practice (Vieques). This training facility was known as the Atlantic Fleet Weapons Training Facility (AFWTF). Although the AFWTF was officially closed on April 30, 2003, important focus of contamination persists in the area due to thousands of UXO's that persist on soils and marine sediments and could release explosives to the surrounding environment by core fragmentation. Residual explosive compounds, heavy metals and other chemicals associated to military activities are toxic to human and other living forms.

AFWTF military exercises had been associated to significant differences in the standardized cancer incidence rate for the civilian population in Vieques civil population (2, 69). Furthermore, the impact of military activities on Vieques marine and terrestrial plants has been studied. Studies have shown higher heavy metal concentrations (Pb, Cd, Ni and Cb) in *Syringodium filiforme, Panicum maximun, Calotropis procera, Sporobolus virginicus* collected at the bombing site compared to reference locations in Puerto Rico. The results suggest a significant contribution of military activities on heavy metal mobilization to marine and terrestrial vegetation. The transfer of pollutants to the base of the food web represents a major risk to human and other organism health (43).

Effects of military training activities in tropical soils and marine environments are scarcely studied. The understanding of microbial community structure in this disturbed area will support environmental restoration strategies and will help to predict effects of contamination disturbances on ground and marine environments. Additionally, military training areas are potential sites for finding explosive-degrading microorganisms due to the historical use of large amounts of munitions and the great number of unexploded ordnances (UXO) that persist on soils and marine sediments. In these habitats, the prolonged exposition to these compounds exerts a natural selection pressure that favors the evolution of explosive degradation pathways in organisms with a great metabolic flexibility such as microorganisms (32).

OBJECTIVES

- To evaluate the role of natural attenuation of 2,4-DNT and RDX in marine and terrestrial environments under different REDOX conditions at Vieques, Puerto Rico.

- To evaluate the influence of 2.4-DNT and RDX in marine and terrestrial microbial communities.

LITERATURE REVIEW

Vieques island and military activities

Vieques is a small island located about 7 miles across southeast of Puerto Rico mainland and 20 miles southwest of St. Thomas, U.S. Virgin Islands. It is 21 miles long by 4.5 miles width, with a surface of approx. 51 square miles. It is located between 18°10'N and 18°05'N latitude, and 65°35'W and 65°16'W longitude. Vieques has a population of about 9,106 habitants according to 2000 census, distributed mainly in two areas, Isabel Segunda and La Esperanza. Before the intervention of US Navy, the land was used mostly for sugar cane production and cattle grazing. Vieques has a tropical-marine climate with nearly constant temperature and an annual average of 79˚F. Precipitation patterns differ along the island. The western part of the island averages approx. 50 inches of rainfall per year, while the eastern part of the island is typically drier with an annual rainfall of about 25 inches per year (5).

Military activities started at Vieques in 1941, when the US Navy purchased 26,000 of the 33,000 acres that comprise the island. Between 1941 and 1950, Vieques was used as a live ordnance range of Navy and Marine Corps forces in support of World War II. In 1960, the Navy established naval gunfire support (NSFS) and air-to-ground (ATG) targets and subsequently began holding training exercises.

The Atlantic Fleet Weapons Training Area (AFWTA) of Vieques was conformed by four main areas: Eastern Maneuver Area (EMA), the former Surface Impact Area (SIA), the

Live Impact Area (LIA) and the Eastern Conservation Zone. The EMA was approx. a 10,673 acres area used as a maneuvering space. Several ranges were established at EMA for the training of marine amphibious units and battalion landing teams. Exercises consisted of amphibious landings, small arms fire, artillery and tank fire, shore fire control, and combat engineering tasks (3). In 1966, a series of small arms ranges and firing positions were established along the northern coast of the EMA and near the boundary with SIA (western end of the EMA), respectively. The SIA encompassed 11,070 acres, it was established in 1950s when several marine artillery targets were constructed, marine artillery was directed toward these targets from gun position within SIA and EMA. During mid 1970, bull eye targets were established in SIA for air-to-ground bombing with MK-76 and MK-106 practice bombs and inert rockets. The LIA was a 900 acres area where most of the training activities were performed, it was established in 1964 for naval training (5) exercises and in 1965 for aerial bombing. Since the middle 1970s, naval gunfire was practiced at the LIA. Between 1974 and 1998, a total weight of up to 27,000 tons of naval gunfire and air-to-ground artillery were fired to the LIA.

The predominant munitions used by the Navy in Vieques were 5”/54 caliber projectiles and Mk-82 aerial bombs. During the typical naval gunfire support training, 82% of the munitions used were non-explosive bombs and 18% were live bombs. Similarly air-to-ground ordnance delivery training used 85% of non-explosive bombs (3). The explosive charges were mainly mixes of different proportions of 2,4,6- trinitrotoluene (TNT), Hexahidro-1,3,5-Trinitro-1,3,5-Triazina (RDX), methyl-2,4,6-trinitrophenylnitramine

(tetryl), cyclotetramethylene tetranitramine (HMX), ammonium picrate (explosive D), and several combinations of these chemicals. Dinitrotoluene (DNT) was mostly used as munitions propellant. As a result of these practices, high amounts of unexploded ordnance (UXO) and remnants of exploded ordnance have been identified at the range areas and in the surrounding waters. After the analysis of possible hazardous chemicals on drinking water, two carcinogenic substances, RDX and TNT, were found in high concentrations in several fresh water sources at Vieques. In June 13th of 2003, due to the high risk for 'Vienqueses' and for terrestrial and marine ecosystems, Governor Sila M. Calderón petitioned the designation of the AFWTF to be included in the US EPA National Priority List (1).

Explosives biodegradation pathways

The abundance of natural compounds with nitro-functional groups is limited, but their presences in natural environments have been demonstrated. Some examples of naturally produced nitroaromatic compounds are: pyrrolnitrin, chloramphenicol, nitropolyuretane, oxypyrrolnitrin, and phidolopin (19). Explosives are xenobiotic nitro-substituted organic compounds and nitramines and their degradation pathway is a metabolic challenge for microorganisms due to the recent exposition and, the limited variety and quantity of nitro-substituted organic compounds (33).

Among explosives, 2,4-dinitrotoluene (2,4-DNT) is a nitroaromatic compound produced together with 2,6-dinitrotoluene (2,6-DNT) as sub-products of TNT synthesis. It is employed in polyurethane foams, ammunition, and dyes manufacturing (48, 59).

Degradation pathways for some nitro-aromatics have been observed in sites contaminated by ammunitions industries or by military activities. For example, *Burkholderia sp.* DNT and *Burkholderia cepacia* R34 are 2,4 DNT-degrading bacteria isolated from TNT manufacturing sites at the Radford Army Ammunition Plant (Radford, VA) and Waconda Bay near Volunteer Army Ammunition Plant (Chattanooga, TN), respectively (47, 59). Cloning and sequencing analysis of genes encoding for nitroarene degradation enzymes in *B. cepacia* R34 and *Burkholderia sp.* DNT revealed that 2,4 DNT degradation pathway evolved through gene recruiting from other aromatic compounds degradation pathways and amino acid metabolism (32). It is believed that 2,4 DNT degradation pathway has recently evolved through the assemblage by transposon insertions of oxygenase genes (supporting evidence includes remnants of genetic material of ancestral pathways, the primitive or inefficient regulation of enzymes synthesis, and the low catalytic specificity of enzymes) (33).

Dinitrotoluene can be used as carbon, nitrogen and energy sources in bacterial oxidative routes (Fig. 1). The 2,4 DNT aerobic degradation pathway starts with a hydroxylation reaction resulting in the formation of 4-methyl-5-nitrocatechol by a multicomponent dioxygenase similar to naphthalene dioxygenase (26). Methylnitrocatechol is then oxidized by methylnitrocathecol-monooxygenase to yield 2-hidroxy-5-methylquinone, which is reduced to produce 2,4,5-trihydroxytoluene. This compound is then oxidized by a trihydroxytoluene oxygenase that breaks the aromatic ring generating 2,4-dihydroxy-5-methyl-6-oxo-2,4-hexadienoic acid. Finally, this intermediate is transformed by known

enzymes involved in amino acid degradation to obtain piruvate and methyl-malonyl-CoA (33).

Lignin degrading fungi such as *Phanerochaete chrysosporium*, is able to transform 2,4-DNT to CO_2 and nitrous oxide (N_2O). In this case, the first nitro group is reduced to yield an amino dinitrotoluene, while a *o*-magnesium peroxidase catalyze the removal of the amino group (64). Alternatively, 2,4-DNT can be reduced to amino and azoxy compounds, however these products are not degradable and do not offer any selective advantage (60).

In contrast RDX is a cyclic nitramine widely used as a component of a wide number of explosives formulations. It can be degraded using composting systems, bioslurry reactors, soil flooded biopiles and bacterial pure cultures under aerobic and anaerobic conditions (29). Anaerobic degradation is catalyzed by nitroreductases of some Enterobacteriaceae (38). However, other microbial enzymes involved in RDX anaerobic degradation are still unknown. McCormick and coworkers (44) proposed a pathway in which nitroso compounds are transformed to hydroxylamine derivates before RDX ring cleavage. The degradation occurs in a sequential reduction of RDX to hexahydro-1-nitroso-3,5-dinitro-1,4,5-triazine (MNX), hexahydro-1,3-dinitroso-5-nitro-1,4,5-triazine (DNX) and hexahydro-1,3,5-trinitroso-1,3,5-triazine (TNX), generating hydrazines, formaldehyde and methanol. Hawari and coworkers (29) proposed a new pathway where novel metabolites, such as methylenedinitramine and bis(hydroxymethyl) nitramine, were formed by direct cleavage of RDX ring with the posterior production of nitrous oxide

(N_2O) as a major product, this initial enzymatic cleavage of the inner N-C-N bonds could be catalyzed by hydrolases (Fig. 2 path A).

Aerobically RDX can be degraded as well in pure culture of *Stenotrophomonas maltophilia*, *Rhodococcus* sp. strain A, *Rhodococcus* sp. strain D22 (5), *Williamsia* sp. KTR4, and *Gordonia* sp. KTR9 (63). Additionally, RDX is used as sole nitrogen source with three of its six nitrogen atoms incorporated into the biomass. Coleman and coworkers (17) found that a plasmid-encoded cytochrome P450 was responsible for RDX degradation in *Rhodococcus* strain D22. Cytochrome P450 2B4 from rabbit liver catalyzes the sequential transfer of two single electrons to RDX. The first electron causes denitrations to form compound I, and the second electron causes a second denitration to yield compound II (Fig. 3). The latter product is unstable in water and it is probably hydrolyzed by the incorporation of two hidroxyl groups from two H_2O molecules to produce a hypothetical compound III, and then occurs the spontaneous decomposition of compound III to produce 4-nitro-2,4-diazabutanal (9).

Explosive toxicity

Explosive toxicity has been documented in prokaryotic organisms as *Vibrio fisheri*, using bioluminescent as screening indicators for soil toxicity and mutagenicity (22). Trinitrotoluene (TNT) toxicity has also been studied in several eukaryotic model organisms, such as earthworms (52), white potworms (*Enchytraeus albidus*) and fishes (*Pimephales promelas*) (70). On earthworms, it was observed that sub-lethal concentrations of TNT affected its nervous system, caused blood disorders and weakened

its immunity (24). The US EPA has established that long-term exposition to TNT could result in urinary bladder tumors in rats and recognize TNT as a human carcinogen (1). Furthermore, exposure to high levels of DNT may affect blood and nervous systems in humans, and it may develop cancer in laboratory animals (4). Its toxicity has been studied in fish by gene based microarrays expression profiles. Results in *Pimephales promelas* showed adverse impacts of 2,4 DNT exposure in gene expression associated to lipid metabolism and oxygen transport (70).

A single organism toxicity screen, however, may not be a good assessment of the global health of a disturbed environment. Microbial community promise to be a more sensitive and comprehensive indicator of residual toxicity thus, serving as a good complement to the disappearance or sequestration of pollutants. Microbial response to a contaminant should quantitatively indicate the natural attenuation processes occurring, the nutritional status, and other features of the environment in concern; but its use as a risk assessment tool or risk prediction is still under study (66). For now, the understanding of microbial community structure in disturbed areas will support environmental restoration strategies and will help to predict effects of contamination disturbances on ground and water systems.

In the case of military training areas, shifts in microbial community structure could be an indicator of the biological effect of contaminants associated with military pollutants. Influence of explosives such as TNT, RDX and 2,4 DNT on microbial communities has been study in lakes (67), ground water (68), industrial soils (46), and military training

soils (50). Bhatt and coworkers (8) investigated microbial degradation of RDX in marine sediments from a coastal UXO field in the region of Oahu Island (Hawaii). In this study, microbial cultures from sediment samples mineralized up to 69% of RDX after 25 days of exposure. The methods used for accessing the bacterial groups within RDX-enrichment treatments were the isolation of degrading bacteria, Denaturing Gradient Gel Electrophoresis (DGGE), and 16S rRNA gene phylogenetic analyses. Their results showed that members of gammaproteobacteria (*Halomonas*) and sulfate-reducing deltaproteobacteria (*Desulfovibrio*) were the most abundant populations. *Marinobacter*, *Psudolaterobacter* and *Bacillus* were also identified.

Microbial community structure accessed by Terminal Restriction Fragment Length Polymorphism (T-RFLP) analysis

Cultivation-dependent methods give insufficient insight into the dynamics of microbial communities, due to the artificial effect of media and selection of fast growing organisms. Culture techniques generally homogenize environmental conditions, therefore they do not recreate the natural gradients of nutrients, either the physicochemical conditions and the structure of natural environments. Moreover, the assessment of microbial diversity and abundance can be misleading if only cultivable microorganisms are identified, since it has been estimated that nearly 99% of naturally occurring microbes cannot be cultured (31).

Culture-independent methods using molecular biology provide the opportunity to monitor non-cultivable microorganisms allowing a more realistic approach of exploring microbial diversity and abundance from natural environments (31). At a glance, metagenomics allows the identification of all microorganisms in complex environments (i.e. forest soil) through sequencing of complete environmental genomes. However, when the principal objective of the study is to compare and identify changes on microbial community structure, metagenomics becomes very time consuming and extensive laborious, therefore community fingerprinting methods have been developed to carry out comparative analyses such as DGGE, TGGE and T-RFLP. Each of these methods uses the properties of the gene chosen as a molecular marker to obtain a complete community fingerprinting, therefore diversity of specific taxonomic groups can be accessed by using group-specific primers (i.e. 16S rRNA universal primers for bacteria) (56).

T-RFLP is a variant of Restriction Fragment Length Polymorphism (RFLP) analysis. For T-RFLP fluorescent labeled universal primers (forward and/or reverse primers can be labeled) are used for PCR amplification of conserved regions, such as 16S rRNA genes. PCR products are then digested separately with several restriction enzymes and fragments are electrophoretically separated by size in an automated DNA analyzer. In RFLP all restriction products are detected, while in T-RFLP only the terminal fragment proximate to the labeled primer is detected by the sequencer and it is represented as a band. Complex samples will generate a profile with multiple bands, each band is graphed as a peak in an electropherogram, where the X-axis represents the sizes of the fragments and the Y-axis represents their fluorescence intensity. In a T–RFLP profile, each peak assumingly corresponds to one genetic variant or operation taxonomic unit (OTU), and

the intensity of the each band could be related to the relative abundance of each OTU in the sample, however caution has to be taken with this analysis since there are differences in rRNA gene copies among bacteria and the annealing selective effect of primers. The data obtained can be compared with data from *in silico* analyses of sequences in databases to infer the potential species composition of samples.

Some limitations of T-RFLP technique are: incomplete restriction digestion, the fact that several groups of organisms may share the same T-RF, and reported discrepancies between observed and predicted T-RF fragment size associated with the sequencing technology (37). Discrepancies, principally associated to capillary sequencer, are related to the fact that a different fluorophor is used as an internal size standard, and this could affect electrophoretic mobility (6).

Multivariate analyses in microbial ecology

Initially, microbial ecologists used to analyze T-RFLP data based on visual inspection of profiles. Similarity between samples was evaluated by identification of shared profiles. A shift on microbial communities was identified when relative abundance of peaks changes or when new peaks were observed. Even though, the visual inspection is still very helpful, several multivariate analyses have been used for T-RFLP analysis that allow the evaluation of significant differences between samples profiles and the correlation of patterns with measured environmental parameters. Statistical methods have been fully applied to summarize or describe the distribution and diversity patterns of plants and animals. Recently, microbial ecologists have applied multivariate analyses (i.e.

correspondence analysis, cluster analysis, principal component analysis) and statistical methods (i.e. ANOSIM) to test for significant differences between groups or clusters to explore large data sets and describe community diversity patterns.

Multivariate analysis is a branch of statistics that focuses in the examination of numerous variables simultaneously. Its purpose is to treat multivariate data as a whole, summarizing the data and revealing their structure. Ordination is a common multivariate analyses used in microbial ecology. Its purpose is to simplify the interpretation of data sets by organizing entities along gradients, which are defined by combinations of interrelated variables. Usually, it helps to reveal the relationship between the species composition at a site and the underlying environmental gradients. The premise is that a few major gradients can explain much of the variability on the data set (45).

Correspondence Analysis. Correspondence analysis (CA) is an ordination method that belongs to a set of procedures known as multidimensional scaling. Its procedure transforms similarities between samples or objects into distances and it represents them in a multidimensional space, maximizing the correspondence between samples and objects and determining the most influential parameters. It converts originally high-dimensional data set into dimensions small enough for visualization, resembling their original distances in the high-dimensional space. CA uses chi-square to standardize frequency values of cells in a contingency table and it displays the rows and the columns of a two-way contingence table as points in corresponding low-dimensional vector spaces (49).

Scatter plot interpretation of CA is based on distance, so proximity between points (samples or OTUs) is a measure of similarity (20).

CA has become a very popular method in microbial ecology allowing identification and understanding of microbial community composition and structure in different environments. It is generally applied to data obtained by fingerprinting techniques such as DGGE, ARISA and T-RFLP, to compare the correspondence between samples and OTUs and to determine whether patterns in microbial distribution could reflect differences in community composition (49). The proximity between sample and species points in the plot can be interpreted as the probability of occurrence of species in the sample or the probability of being more abundant than in other samples. Also, the distance of points around the centroid (axe X=0, Y= 0) and the edges of the plot can be used to evaluate the contribution of each species to the ordination (41).

CA has been applied in several studies to relate microbial community structure to specific environments, environmental processes, seasonal changes, specific substrates responses, and to evaluate long term effects of toxic carcinogenic substances. Ayala-del-Rio and coworkers (7) used T-RFLP and CA to demonstrate different community structures related to degradation rates of trichloroethene in fed-sequencing batch reactors, and they showed the selection of a stable community structure after long term exposition. Edlund and Jansson (18) used CA to link the identity and degradation activity of polyaromatic hydrocarbons by bacteria residing in Baltic Sea sediments.

ANOSIM and SIMPER. Analysis of similarities (ANOSIM) provides a way to tests statistically whether there is a significant difference between two or more groups. It is a procedure analogous to analysis of variance (ANOVA), which tests previously defined groups against random groups in an ordinate space (15). The output statistic, R_{ANOSIM}, goes from 0 to 1, where $R_{ANOSIM} = 0$ means no separation or difference among groups or no difference between composition of community structure; $R_{ANOSIM} < 0.25$ barely separated; $R_{ANOSIM} > 0.5$ separated but overlapping; $R_{ANOSIM} > 0.75$ well separated; while $R_{ANOSIM} = 1$ indicates a complete separation (16). ANOSIM can be applied to groups formed after component analysis to test for significance. Several applications of ANOSIM to microbial ecology encompass testing for spatial differences, temporal changes, and environmental impact (49). Through Similarity Percentage (SIMPER) analysis is also possible to statistically identify the taxa that are primarily responsible for differences between the groups (15), therefore coupling ANOSIM to evaluate groups and SIMPER to identify organism responsible for the differences, can help us to identify key OTUs responsible for separation of groups.

MATERIALS AND METHODS

Sampling

Two sets of samples of 500 g of soil sludge and marine sediment were collected from two locations near former practices ranges at the Eastern Maneuver Area at Vieques, Puerto Rico (Fig. 4, bottom). This location was selected over previous sites (see preliminary work in Appendix A) due to easier access for sampling. Samples were collected using wide mouth glass jars and stored at 4°C until further processing. At location 1 (18˚09'26.22" N, 65˚23'03.28" W), sludge soil was collected in area where rainwater from training range 3 and 4 converges to (Fig. 4, A). A second sample was collected at Puerto Negro Beach (18˚09'15.86" N, 65˚21'45" W) near range 6 (Fig. 4, B). This marine sediment sample was collected from surface to 10-15 cm of depth and stored as previously described.

Microcosms set-up

To evaluate the effect of RDX and 2,4-DNT on microbial community for both ground soil and marine sediment samples, four different treatments (non amended, amended with explosives, amended with explosives and acetone, and autoclaved amended control), (Table 1) were prepared under two different conditions: sulfate reducing and oxygenic conditions. Aerobic microcosm slurries were prepared by mixing 1:1 of sample with a nitrogen free BLK medium (13). Soil samples were mixed with regular BLK medium, while marine sediments were mixed with 3.5% NaCl BLK. Slurries were homogenized with a stirred bar during a 6 h period. Aerobic microcosms were prepared by pouring 6

ml of slurry and 34 ml of medium into 100 ml serum bottles. Non amended control microcosms (SOIL and SED) were set up without addition of explosives or acetone as carbon source. Microcosms amended with explosives as carbon and nitrogen source (SOIL-RDX, SOIL-DNT, SED-RDX and SED-DNT) were prepared by first air-drying 80 µl of 1000 ppm of 2,4-DNT or RDX in acetone into serum bottles, after which the slurry and the medium were added. Microcosms amended with explosives and acetones as carbon source (SOIL-RDX/Ac, SOIL-DNT/Ac, SED-RDX/Ac and SED-DNT/Ac) were prepared as explosive ammende microcosms but acetone was not evaporated. Abiotic control microcosms (SOIL-KILL-RDX, SOIL-KILL-DNT, SED-KILL-RDX AND SED-KILL-DNT) were prepared by triple autoclaving the serum bottles with slurry and media at 121°C for 20 min. After a last cooling to room temperature, 2,4-DNT or RDX diluted in acetone was added. Three replicates were set for each treatment and all microcosms were sealed with teflon-coated sterile rubber caps and aluminum seals.

The slurry and media under anaerobic sulfate reducing were prepared as described before, but BLK medium was amended with 30 mM lactate serving as electron donor and 45 mM sodium sulfate serving as electron acceptor (11). Microcosms were prepared by mixing 3 ml of slurry and 17 ml of BLK sulfate reducing medium into 40 ml serum bottles, additionally a sterile iron nail was introduced into the bottle to evidence sulfate reducing conditions. Smaller volumes and 15 vial replicates for each treatment were prepared in order to sacrifice a set of three replicates at each time point sampling; this to avoid change in electron acceptor conditions and/or vial pressure. Anaerobic microcosms were set as described for aerobic microcosms but a previous incubation time (2 to 3

weeks) was done before explosive addition. Once sulfate reducing conditions were observed (black sulphide deposit on the nail), 40 µl of 1000 ppm 2,4-DNT or RDX in acetone solution was added to the microcosms using a sterile syringe. Microcosms were sealed with blue butyl rubbers stoppers and aluminum seals and incubated statically at 30˚C.

Biodegradation of RDX and 2,4-DNT

For all microcosm treatments, triplicates of 800 µl sub-samples were collected at time intervals of 0, 2, 4, 8, 16 and 32 days. Two of the subsamples were processed immediately for chemical analysis while the other set was stored at -80ºC. For sampling anaerobic microcosms, vials of each treatment were sacrificed and processed, while for aerobic treatments, same vials were sampled several times. Explosive extraction was performed by mixing 1:1 sample and acetonitrile, followed by a 18 h shaking, then samples were centrifuged 5min at 14,000 *g* and the supernatant was collected in a 2 ml vials. RDX and 2,4-DNT were quantified by Gas Chromatography (GC) using 1 µl of sample injected into a Restek capillary column, RTX-5 (15 m x 0.25 mm ID x 0.25 µm df) and by High Pressured Liquid Chromatography (HPLC), using an Agilent 1100 series HPLC UV/VIS detector. Nitrite concentrations were determinate by colorimetric reaction using a small-scale nitrite assay (57).

Bacterial isolation

Aliquots of 100 µl from amended microcosms were platted on BLK solid media with XAD-7 resin (47) amended with 20 to 200 µM 2,4-DNT or RDX and incubated at 30°C for 7 days. Each morphotype was serially transferred three times to fresh solid BLK media. For selecting explosive degrading bacteria, the growth of each morphotype on BLK media with and without explosive was compared while 2,4-DNT mineralization was evaluated through nitrite production in axenic cultures.

DNA extraction and PCR amplification of 16S rRNA genes

DNA was extracted from 800 µl sub-samples using Q-biogene FastDNA® SPIN Kit for soil, and from pure bacterial using Q-biogene Fast DNA Kit. Polymerase Chain Reaction (PCR) reactions for 16S rDNA bacterial genes were amplified using a nested PCR. First PCR was performed with primer pair 8F (5'-AGAGTTTGATC CTGGCTCAG-3) and 1492R (5'-GGTTACCTTGTTACGACTT-3'), and then 1µl of a 1:50 dilution of PCR product was used as template for the second PCR using primer pair 519F (5'-CAGCMGCCGCGGTAATWC-3') and 1392R (5'-ACGGGCGGTGTGTRC-). For a PCR reaction approx. 100 ng of DNA template, 5µl of GoTaq polymerase 5X buffer, 2.5 µl of 25 mM $MgCl_2$, 0.5µl of dNTPs (10mM each), 1.0 µl of each primer (50 pM/µl), and 0.2 µl of GoTaq polymerase enzyme (Promega®) were used in a 25 µl reaction volume. The following steps were employed: 1 cycle at 95°C for 5min; 35 cycles, each consisting of 94°C for 1.5min, annealing at 52.6°C for 0.5min, and 72°C for 1min; and a final extension of 72°C for 5min. PCR reactions were performed in a MJ Mini^{tm} Personal

Thermal Cycler (BIO-RAD). The amplification products were visualized in 1% agarose-gel electrophoresis with ethidium bromide, which were run at 90 V for 30 min and observed using an ultraviolet transilluminator.

Terminal Restriction Fragment Length Polymorphism of 16S rRNA amplicons

For T-RFLP, primer 519F was labeled with Infra Red Dyes IR700 (LI-COR, Lincoln, NE). PCR products were separately digested with the following enzymes: *Msp*I, *Hae*III and *Rsa*I. Each digestion reaction consisted of 1.5 µl of 10X reaction buffer, 1 unit of each restriction enzyme, 12 µl of PCR product and 10 µl of double distilled water (ddH_2O) for a total reaction volume of 25 µl. The digestions were incubated at 37°C for 4h followed by 10min at 72°C to inactivate the enzymes and a 4°C final step.

An aliquot of 2 µl of restriction product was mixed 1:1 with IR2 stop solution (LI-COR, Lincoln, NE) and 1 µl was electrophoresed in a LI-COR Biosciences NEN®DNA Analyzer Model 4300 (LI-COR, Lincoln, NE). The acrylamide gel was prepared to a final concentration of 5.5 % by mixing acrylamide gel matrix (KB_{Plus}-LICOR) with BT buffer, adding 150 µl of 10 % ammonium per-sulfate and 20 ml of 15% of TEMED. The samples were denatured at 95 ºC for 5 min and kept at 4ºC until loading the gel. A gel pre-run consisted in a run of 20 min with 1X buffer (KB_{Plus}-LICOR) with the following parameters: 1,500 V voltage, 40mA current, and 40W of power . After pre-run, 1µl of each denatured sample was loaded and the gel was run for 3h using the same pre-run parameters. A KB_{Plus}-LICOR molecular sizing standard of 50-700 bp was used. T-RFLP

profiles were obtained and band analysis was performed using Gel-Pro Analyzer 4.5 (Media Cybernetics, Silver Spring, Maryland, USA).

Terminal Restriction Fragment Length Polymorphism (T-RFLP) analysis

The analysis of T-RFLP profiles was performed using two scripts written on CLisp (Haible et al., 2005): *PEAKS* for determining real peak bands and *BINNING* for possible aggregation of peaks as the same OTU (Appendix B). Final binned matrix was used to compare microbial communities from different samples. Matrix was exported to Paleontological statistics package ver. 1.79 (PAST) (28), which includes analyses such as CA, ANOSIM and SIMPER. Scatter plots generated by CA were used to identify grouping of T-RFLP microbial community profiles, while ANOSIM was used to evaluate these groups. In order to identify the relations between different treatments, ANOSIM was separately calculated for aerobic and sulfate-reducing microcosms. Similar size bands or peaks in T-RFLP profiles were used as OTUs and its relative abundance as a measure of OTU quantity. Morisita index previously used for T-RFLP comparison (54) was chosen as the similarity measured between groups.

SIMPER analysis was used only for those microbial communities distinguishably separated from non-amended control (based on ANOSIM) and OTUs primarily responsible for these differences between microbial communities were identified. Taxonomic inference of T-RFLP OTUs was done trough *in-silico* analysis of clone sequences.

Cloning of 16S rRNA gene and sequencing

Nested PCR product using 1392R/519F primer pair from SOIL (day 8) and SOIL-DNT (day 8) were used to construct two clobe libraries, A molar ratio of 3:1 PCR product to vector was cloned using pGEM (R)-T Vector System (Promega) as described by the manufacturer. Positive clones were identified by white/blue selection in Luria Bertani agar with 100 µg/ml ampicillin, 0.5 mM IPTG, and 80 µg/ml X-Gal (LB/Amp/IPTG/X-gal). White colonies were picked and transferred to 10 µl ddH_2O, an aliquot was streaked onto LB/Amp/IPTG/X-gal for its isolation and then the remnant was heated to 100°C for 10 min, centrifuged at 16,000 *g* for 5 min, and 1 µl was used as DNA template in colony-PCR using vector primer pair T7 and SP6. PCR reactions were performed as described and analyzed before, except for annealing temperature, which was 52°C. Positive clones were grown in 5ml of LB liquid media and plasmid extraction was performed using Wizard® Plus SV Minipreps DNA Purification System (Promega) as described by the manufacturer. The DNA concentration of plasmid isolation was measured spectrophotometrically at 260 nm and inserts were sequenced using SP6 vector primer using the services of the High-Throughput Genomics Unit (HTGU) at the University of Washington.

Sequence and phylogenetic analysis

Clone sequences were analized using Basic Local Alignment Search Tool (BLAST) from the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/BLAST). The phylogenetic relationships among clones from SOIL (day 8) and SOIL-DNT (day 8) libraries, were determined by constructing

phylogenetic trees. Sequence edition was done manually using BioEdit Sequence Alignment Editor (27), for this vector sequences were deleted and sequences that were inserted in a reverse orientation during pGEM cloning, their reverse-complement sequences were used. Reference sequences were obtained by submitting clone sequences in Sequence Match tool (rdp.cme.msu.edu/seqmatch/seqmatch_intro.jsp) at the Ribosomal Data Project (RDP), which found 495 nearest neighbors (Appendix E). ClustalW program (62) was used to perform two alignments, one with related organism and clone sequences containing the 519F primer sequence and the other with related organism and clone sequences containing the 1392R primer sequence. Even though all clone sequences overlapped, only 180 characters were shared between the ones containing the 519F and the 1392R primer sequences; for this reason sequences were split and analyzed in separate trees, which were constructed using Neighbor-joining (NJ) method included in the MEGA version 3.1 software (39) using 1,000 replicate trees. The first tree (519F) was composed of 27 sequences and the second tree (1392R) of 42 sequences. In order to further identify some clone sequences with ambiguous phylogenetic relations, additional reference sequences of related organisms were included to construct other five phylogenetic trees. Types of trees performed to identify each of clones are resumed in Table 9 and the reference sequences included in each phylogenetic tree are in appendix E (List 3-7).

For each clone library, clone sequences were subjected to unique genotype analysis using the PHYLIP package (21) and coverage analysis using ASLO program (http://www.aslo.org/lomethods/free/2004/0114a.html)(35) under the default parameters.

Each clone sequence library was aligned independently using ClustalW (62) and the aln files were used to construct a distance matrix using DNADIST (PHYLIP package) (http://evolution.genetics.washington.edu/phylip.html). Each DNADIST outfile was then used in DOTUR 1.53 software (53) applying the furthest-neighbor algorithm to assign sequences genotypes and calculate richness estimators and diversity indexes.

In-silico T-RFLP analysis of clones

Clone sequences were cut *in-silico* using BioEdit Restriction Map tool (27), to determine expected sizes of restriction fragments and to compare these fragments with the fragments generated by experimental T-RFLP, identification of sequences was determined looking for OTUs near the range (+/- 5pb) of the expected fragments (Table 12)

RESULTS

Biodegradation of RDX and 2,4-DNT

Microbial transformation of RDX and 2,4-DNT was evaluated in microcosms under aerobic and anaerobic conditions. Levels of RDX and 2,4-DNT were monitored for 32 days in all amended treatments. Chemical analysis of samples showed that the explosive extraction efficiency was affected characteristics of samples particles. The expected concentration of 2,4-DNT and RDX in microcosms was 11 and 9μM (Appendix D), respectively, however quantification of explosives right after addition ranged from 7.8 to 10.3μM for 2,4-DNT and 6.3 to 9.2μM for RDX. For marine sediments microcosms, 2,4-DNT concentrations ranged from 5.8 to 7.6μM, while RDX varied between and 5.2 to 6.9μM for RDX. This observation showed that the extraction of explosives on marine sediments is less effective that the observed for soil samples.

RDX and 2,4-DNT levels were evaluated in soil microcosms under aerobic conditions (Fig. 5, A and B). Concentration of RDX in autoclaved controls (Fig. 5. A, SOIL-KILL) slightly changed (4.6 % decrease) along incubation. Different results were observed for amended biological treatments, for SOIL-RDX treatment, RDX concentrations decreased 21 % in concentration (8.1 to 6.4 μM) after 32 days. In contrast, for SOIL-RDX/Ac treatments, RDX concentration decreased 100% (9.2 μM to non-detected levels) with a bigger decrement after day 4, and complete disappearance after 16 days. Production of nitrite was observed on SOIL-RDX treatment, however not evident decreased in concentration was observed. On the other hand in 2,4-DNT amended systems,

concentrations of 2,4-DNT on soil autoclaved microcosms (Fig. 5 B) decreased 17.5 % (10.3 to 8.5μM) a long the test. In contrast, 2,4-DNT concentration in SOIL-DNT treatments decreased in 94.4 % (7.2 to 0.4μM), followed by and increment of nitrite from none detected to 17μM at day 16 (Fig. 5, C). SOIL-DNT/Ac microcosms showed a similar decrease of 2,4-DNT, but without nitrite production.

In addition, RDX and 2,4-DNT concentration was also evaluated in marine sediment microcosms under aerobic conditions (Fig. 6A and 6B). Concentration of RDX in abiotic control SED-KILL-RDX showed a decreased of 17.7 % (7.9 to 6.5μM); similar results were observed for SED-RDX microcosms, where concentration decreased 13.3 % (from 6.5 to 5.7μM). In contrast, a 100% decreased of RDX was observed on SED-RDX/Ac microcosms (from 5.5μM to below detection levels by day 16). Therefore, the addition of acetone had a similar effect for RDX degradation in both soil and marine sediment microcosms. Moreover, Figure 5A and Figure 6A showed a very similar pattern, supporting the idea of a similar effect of the addition of an external carbon source. On the contrary, concentrations of 2,4-DNT on autoclaved marine sediments did not changed decreased along the experiment. Faster removal of 2,4-DNT was observed on SED-DNT and SED-DNT/Ac treatments when compared to its counterparts in soil microcosms (Fig. 5B and Fig. 6B).

In SED-DNT treatments, 2,4-DNT concentration decreased below detection limits within 48 hours, however no nitrite production was detected. Concentration of 2,4-DNT in SED-

DNT/Ac decreased to non-detectable levels, similarly to other acetone amended microcosms, not nitrite was produce (Fig. 6C).

Under anaerobic sulfate reducing conditions, RDX and 2,4-DNT concentrations rapidly decrease in both soil and marine sediments. Initial concentration of explosives in soil microcosms were 10.36μM for SOIL-KILL-DNT, 4.73μM for SOIL-DNT/Ac, 6.40μM for SOIL-KILL-RDX and 8.55μM for SOIL-RDX/Ac. In the case of marine sediments were 6.28μM for SED-KILL-DNT, non-detected in SED-DNT/Ac, 6.90μM for SED-KILL-RDX and non-detected in SED-RDX/Ac. Transformation processes of 2,4-DNT in SED-DNT/Ac and RDX in SED-RDX/Ac microcosms were extremely fast, and a complete transformation was observed as explosives were not detected at any time. In contrast, for other treatments from soil and marine sediments, the decrease of explosive concentration to non-detected levels took from 2 to 4 days. Abiotic transformation was also observed in both soil and marine sediments (SOIL-KILL -RDX, SOIL-KILL-DNT, SED-KILL-RDX and SED-KILL-DNT).

DNA extraction and PCR amplification of 16S rRNA genes

Total community DNA from microcosms samples was successfully extracted (Fig. 7). In order to determine the composition of microbial community by T-RFLP and cloning libraries, these DNA extractions were used as templates in PCR amplification of 16S rRNA genes using primer pair 8F and 1492R. In spite of evaluating several magnesium concentrations (2.5-5μM) and different annealing temperatures (50 to 55°C), no PCR amplification was obtained for any environmental sample, but positive amplifications

were always obtained when using genomic DNA from *Burkholderia* sp. In order to remove PCR inhibitors, several dilutions of the environmental DNA were tested (1:10, 1:15, 1:30, 1:50). A positive PCR amplification was achieved only with the template dilution 1:30, but the amplification product was weak and unspecific. In order to improve specificity and the PCR yield, a nested PCR strategy was implemented. First PCR amplification was performed using 1:30 template dilution and primer pair 8F and 1492R (using previously described PCR parameters except number of cycles were 25), then the PCR product was diluted to 1:50 and used as template for a second PCR using primer pair 519F and 1392R. This strategy yielded successful amplicons for all environmental DNA samples (Fig. 8). A similar approach was employed for 519F labeled primers (LICOR-IR800).

Terminal Restriction Fragment Length Polymorphism (T-RFLP) analysis

Correspondence analysis (CA) of T-RFLP. Microbial communities of aerobic microcosms from Puerto Negro (marine sediment) and Range (soil) were compared using CA (Fig. 10). Under static and aerobic conditions, the scatter plot showed a clear separation of distinct communities associated to the origin of the sample (Puerto Negro and Range sample) without overlapping communities, as shown by the 95% confidence ellipses. In contrast, microbial communities of these two sites, but incubated under static sulfate reducing conditions did show convergence (Fig. 11). It was observed that soil microbial communities had less variability than Puerto Negro marine sediment communities, as observed by the size of the 95% confidence ellipses.

Microbial community structure of soil microcosms was differentially affected by treatments (Fig. 12). Variability of data was explained by axis 1 and 2, accounting for 15.61 and 11.3% of variability, respectively. Microbial communities of treatment SOIL-RDX/Ac and SOIL-DNT/Ac were the most similar along the 32 days of incubation, their connecting lines intersected. SOIL-DNT was also very similar to SOIL-RDX/Ac at 0, 4 and 32 days, however microbial communities at days 8 and 16 were different from other days and type of treatment. Soil non-amended microbial community structure showed the largest variability, most of it during day 0 and 4, after which (day 8, 16 and 32) communities became more similar grouping near to other treatments. SOIL-RDX communities grouped separately from the other treatments.

For marine sediments microbial communities, different clustering groups were observed (Fig. 13). Variability of data was explained by axis 1 and 2, accounting for 8.56 and 14.67% of variability, respectively. SED-RDX/Ac, SED-DNT and SED-RDX communities showed a small variability along the 32 days of incubation, grouping closely in the scatter plot around point 0. Moreover, communities tended to overlap, as observed by the connecting lines. Non-amended treatment had higher variability than the observed for the other treatments, day 0 point overlapped with SED-RDX/Ac, SED-DNT and SED-RDX, however after day 8 non amended communities diverged from the other treatments. Finally, EB-DNT scatter points had a high variability within the treatment, day 0 was distant from the other day points while day 8 and 16 were the most similar. Even though SED-DNT/Ac communities were very variable, they consistently clustered apart from the other treatments

Soil and marine sediment microbial communities grouped closely under sulfate reducing conditions (Fig. 11), Scatter plots from different treatments overlapped within samples (Fig.14). Non-amended soil (SOIL) and marine sediment (SED), also grouped with amended samples, however at day 0 points were very distant from the main clustering group, suggesting a possible effect of incubation on microbial selection. Microbial communities from SED-DNT/Ac day 16 grouped apart from the rest.

ANOSIM. Analyses of similarities of soil aerobic microcosms (Table 2) showed that communities from different treatments overlapped but were clearly different (R_{ANOSIM} 0.5543). Additionally, a contrast between treatments allowed the identification of a community shift. SOIL-RDX microbial community showed the biggest difference compared to non-amended (SOIL) treatment (R_{ANOSIM} 0.50), both treatments sharing community composition but were clearly separated. SOIL-DNT/Ac and SOIL-DNT were different to non-amended, but overlapped more with this than SOIL-RDX (R_{ANOSIM} 0.47 and 0.40, respectively).

SOIL-RDX/Ac communities were the most similar to non-amended (SOIL) with a R_{ANOSIM} value of 0.23. In contrast, SOIL-RDX communities were completely separated from SOIL-DNT, SOIL-RDX/Ac and SOIL-DNT/Ac (R_{ANOSIM} 0.85, 0.99 and 1.00, respectively). SOIL-DNT communities overlapped with non-amended treatment and SOIL-RDX/Ac (R_{ANOSIM} 0.37), but were completely separated from SOIL-RDX and SOIL-DNT/Ac (R_{ANOSIM} 0.85 and 0.81, respectively). SOIL-RDX/Ac communities were very similar to non-amended, SOIL-DNT and SOIL-DNT/Ac but were completely different to SOIL-RDX communities (R_{ANOSIM} 0.99).

Analyses of similarities of marine aerobic microcosms (Table 3) showed that communities from different treatments overlapped, but were not completely similar (General R_{ANOSIM} 0.4514). Therefore treatments on marine sediments compared to soil microcosms had a smaller effect on the microbial community. Non-amended (SED) microbial communities were very similar to SED-RDX, SED-RDX/Ac (R_{ANOSIM} 0.06 and 0.23, respectively), but were clearly separated from SOIL-DNT and SOIL-DNT (R_{ANOSIM} 0.70 and 0.62, respectively). SED-DNT and SED-DNT/Ac microbial communities were well separated from each other (R_{ANOSIM} 0.66), therefore the addition of a carbon source did affect the composition of the community. SED-RDX/Ac communities were barely separated from non-amended (SED) and SED-RDX (R_{ASOSIM} 0.23 and 0.22, respectively). Additionally, SED-RDX/Ac communities were overlapped, but different from SED-DNT and SED-DNT/Ac (R_{ANOSIM} 0.72 and 0.66, respectively).

Analyses of similarities of soil and marine sediments under anaerobic microcosms (Table 4) showed that communities from different treatments overlapped, and they are scarcely separated (General R_{ANOSIM} 0.3036). Communities from the same sample did not show a clearly effect of treatments. Non amended SOIL communities were hardly separated from SOIL-RDX/Ac, SOIL-DNT/Ac and non amended SED (R_{ANOSIM} 0.24, 0.16 and 0.19, respectively). SOIL-RDX/Ac and SOIL-DNT/Ac communities were almost identical (R_{ANOSIM} 0.003). Non amended SED communities were also highly similar to ones from SED-RDX/Ac and SED-DNT/Ac (R_{ANOSIM} 0.20 and 0.08, respectively). In general, bigger differences were observed between amended treatments, (SOIL-RDX/Ac, SOIL-

DNT/Ac, SED-RDX/Ac and SED-DNT/Ac), compared to the high overlapping non-amended treatments (SOIL and SED). SOIL-RDX/Ac and SOIL-DNT/Ac communities were well separated to SED-RDX/Ac (R_{ANOSIM} 0.73 and 0.75, respectively), and overlapped but different to SED-DNT/Ac (R_{ANOSIM} 0.43 and 0.46, respectively).

SIMPER. After ANOSIM analysis, more dissimilar treatments to non-ameneded treatments were evaluated using SIMPER. The analysis was performed to determine the contribution of individual OTUs to dissimilarity, fragments were identify as follows R=*RsaI* , M=*MspI* and H=*HaeIII* . SIMPER analysis was not conducted for anaerobic microcosms since anaerobic microcosms tend to cluster together independently of the explosive addition. Treatments were contrasted using OTUs from T-RFLP profiles from day 4 to 32 in the following order: SOIL vs. SOIL-RDX, SED vs. SED-DNT/Ac, and SED vs SED-DNT/Ac. As a result several OTUs were identified as the responsible for dissimilarity among each comparison (Tables 2, 3 and 4).

The comparison of communities between non-amended soil (SOIL) and amended with RDX (SOIL-RDX) communities (Table 5) showed an enrichment of several OTUs 364-365-R, 202-205-M, 112-115-H, and 363-R OTUs in the RDX amended systems. In contrast, a decreased on 366-369-R, 221-224-H, 335-338-R, 374-377-H and 371-374-R OTUs was observed in the RDX amended systems. When marine sediment communities from non-amended treatments (SED) and 2,4-DNT amended treatment (SED-DNT) was contrasted (Table 6), enrichment of 641-645-M, 212-215-H, 366-369-R, 95-98-M, 229-232-R and 304-307-R OTUs was found in amended treatment. Less abundant OTUs in amended communities comprised 646-650-M, 359--362-R, 374-377-H, 137-140-R and

410-414-H. Finally, when non-amended marine sediment communities SED and SED-DNT/Ac were contrasted (Table 7), an enrichment of 366-369-R, 370-373-H, 384-387-R, 95-98-M, 355-358-R, 425-429-H and 229-232-R OTUs was observed in SED-DNT/Ac, while decrease of 374-377-H, 137-140-R, 359-362-R, 646-650-M OTUs was observed.

Cloning of 16S rRNA gene and sequencing

Microbial communities T-RFLP profiles coupled with clone library analysis were used to identify potential OTUs. A total of 42 sequences were obtained for non amended (SOIL day 8) library, while 37 sequences were obtained for SOIL-DNT day 8) library. Weblibshuff was used (http://libshuff.mib.uga.edu/) to quantify the coverage of libraries and determine significant difference between the compositions of their bacterial communities (Table 8). The results showed a coverage of 38% for SOIL-DNT (day 8) library and 31% for SOIL (day 8) library. webLIBSHUFF was also used to compare SOIL-DNT (day 8) and SOIL (day 8) libraries, a *p*-value of 0.290 was obtained for the first comparison and *p*-value of 0.713 was obtained for the reciprocal comparison.

Sequence and phylogenetic analysis

Microbial communities TRFLP profiles coupled with clone library analysis were used to identify potential OTUs. A total of 42 sequences were obtained for non amended (SOIL day 8) library, while 37 sequences were obtained for SOIL-DNT (day 8) library. Weblibshuff was used (http://libshuff.mib.uga.edu/) to quantify the coverage of libraries and determine significant difference between their bacterial composition (Table 8). The results showed a coverage of 38% for SOIL-DNT (day 8) library and 31% for SOIL (day 8) library. Weblibshuff was also used to determine if there was significant differences

between SOIL-DNT (day 8) and SOIL (day 8) libraries. Two p-values were found, the first *p*-value of 0.290 was obtained when SOIL-DNT was compared to SOIL, and the second *p*-value (0.713) was obtained when the reciprocal contrast SOIL and SOIL-DNT was performed. This result suggests lack of significant differences between the communities.

Sequence and phylogenetic analysis

Phylogenetic assignments of clone sequences were based on its inclusion or closeness to reference organism clades. Two NJ trees were built (Fig. 18 and 19) based on the insert sequencing direction, as described in Materials and Methods. Based on these trees, phylogenetic assignments were established for clones B5, B6, B8, B9, B10, B12, B19, B20, B21, B22, B25, B26, B27, B28, B30, B31, B33, B37, B40, B41 and B44 from SOIL (day 8) library and clones A2, A12, A14, A15, A16, A19, A20, A25, A27, A31, A34, A35, A36, A37, A42, A43, A47, A48 from SOIL-DNT. However, in order to unveil the phylogenetic relations of clones A23, B16, B24, B11, and A33 a new phylogenetic analysis was performed (Fig. 45 and 50) including more reference sequences from the phylum Firmicutes, one representative sequence from each genus for a total of 96 sequences (List 3). For clones A30, B18, B21, B23, B33, A17, A22, B15, B32, and B47, a new phylogenetic analysis was done including more reference sequences from the class beta-proteobacteria (List 4) to determine at which order they were more related to (Fig. 45, 46 and 47). A new analysis for clones A7, B42 and A27 was performed including more reference sequences from the class delta-proteobacteria (Fig. 30 and 31). In order to identify clones A28, A4, A8, B1 and B48 a sub set including the most related organisms

from Sphingobacteria class was used to identify the clones. Finally, in an attempt to relate clone B34 to known sequences, a new phylogenetic tree was performed including its BLAST hit sequences (List 7). Phylogenetic trees and zooms of branches that support the identification of clone sequences are shown in Figures 18 to 51.

Sequences were grouped by class, order and phylum, and their relative abundances were calculated (Fig. 17). In some cases, clone sequences could be assigned within a class or order (Table 11 and 10), however, clones A41, B31 and B34 could not be certainly assigned to one phylum and they were only identified as part of the Bacteria super-kingdom. Clones from SOIL-DNT and SOIL libraries are distributed consecutively as follows: phylum Firmicutes 7% and 8%, phylum Bacteriodetes 10% and 5%, phylum Proteobacteria 80% and 79%, and non-assigned phylum 3% and 5%. It was observed that Proteobacteria is the most abundant phylum in both clone libraries, and its relative abundance was very similar. However, the composition or each class within the Proteobacteria phylum showed differences. In SOIL-DNT and SOIL, beta-proteobacteria accounted for 71% and 67 %, alpha-proteobacteria 3% and 16%, and delta-proteobacteria 10% and 3%, respectively. Additionally, the beta-proteobacteria class showed big changes in the compositions of orders. Distribution of SOIL-DNT and SOIL clones into beta-proteobacteria orders was observed as follows: Burkholderiales 44% and 29%, Rhodocyclacles 0% and 8%, Neisseriales 3% and 8%, and non- order assigned 20% and 16%, respectively.

In-silico T-RFLP analysis of clones

Results showed that all of the generated fragments were also present in the T-RFLP (Fig. 16), thus validating T-RFLP profiles. Sequences from the Comamonadaceae family generated particular size fragments of 226 pb for *Hae*III, 96 pb for *Msp*I, and 373 and 140 pb for *Rsa*I. Together, these fragments can be related with the presence or absence of these organisms on the T-RFLP profiles. Interestingly, the relative abundance of these expected fragments was also higher in T-RFLP profiles from SOIL-DNT system at day 8 (Fig. 16), 221-222-223-224-H OTU for the 226 bp fragment, 95-87-98-M OTU for the 96 bp and 364-365-366-367-368-369-R OTU for the 373 fragment and 137-138-139-140-R OTU for the 140 pb fragment.

DISCUSSION

Biodegradation of RDX and 2,4-DNT

Microbial degradation of explosives was explored using nitrite production and substrate depletion as evidence of mineralization. For RDX degradation, lack of mineralization products suggests possible abiotic process responsible for lower levels of RDX. Instead, RDX addition to microcosms was evaluated in terms of its effect on microbial diversity and community structure.

In the case of 2,4-DNT, depletion of explosive levels could be linked to the stoichiometric production of nitrite as mineralization evidence. Degradation of 2,4-DNT was observed in SOIL-DNT aerobic microcosms (Fig. 5, B and C), where a concentration decreased in concentration was coupled to nitrite production. Production of nitrite (17 μM at day 16), nitrite production was nearly stoichiometric. Even though a smaller concentration of nitrite was expected (13.6 μM), this disparity on nitrite concentration could be explained by binding properties of soil, which could absorb or covalently bind 2,4-DNT to humic material. Therefore this abiotic process could diminish the initial extractable concentration but releasing it slowly later in the experiment.

Furthermore, nitrite production in SOIL-DNT microcosms must be related to 2,4-DNT addition because no production was observed in the biotic controls microcosms (SOIL), (Fig. 5, C). These results suggest that native microbial community from soil sample (Range 3 and 4) has the metabolic capability to degrade 2,4-DNT under aerobic

conditions, however further evidences are needed to confirm the origin of nitrite using quantitative techniques coupled with radiolabel nitrogen.

Decrease of 2,4-DNT to non-detected levels was also observed in SOIL-DNT/Ac, SED-DNT/Ac and SED-DNT aerobic microcosms, but nitrite was not produced (Fig. 5 and 6, B). Therefore, decrease in 2,4-DNT concentrations in this microcosms could be explained by transformation processes, such as the reduction of 2,4-DNT to forms like 2,4 DAT (diaminotoluene) and AMT (aminotoluene) (14).

Other possible reason that supports 2,4-DNT transformation is that high concentrations of carbon (as in the acetone amended microcosms) can also block DNT degradation pathways and favor co-metabolic transformations. The available carbon source stimulates growth of microorganisms, reducing oxygen concentration, inhibiting the function of oxygenases (involved in 2,4-DNT degradation pathways) and promoting 2,4-DNT unspecific transformation (47).

Finally, the decreased of 2,4-DNT can be due to polymerization of amino an nitroso forms of 2,4-DNT. Recent studies in marine sediments have shown that 2,4-DNT concentrations can be reduced with out mineralization by the formation of azo and hydrozo-polimers that irreversible bound sediment particles (71), so it is likely that this process is the responsible for 2,4-DNT decreased in SED-DNT/Ac and SED-DNT microcosms.

Decrease of RDX concentration was observed in aerobic static microcosms amended with acetone as a carbon source (SOIL-RDX/Ac and SED-RDX/AC). Degradation was more evident at day 4 for soil, and at day 8 for marine sediments (Fig. 5 and 6, A). Visual inspection of microcosm slurries evidenced the change of soil color to a darker gray color attributed to anaerobic conditions. Stoichiometric calculations of acetone as an electron donor, support total consumption of Oxygen in the serum bottle headspace (Appendix E). Decrease of oxygen levels favors anaerobic mechanisms of RDX degradation, such as chemical transformation and nitroreductases mediated .degradation.

In aerobic treatments SOIL-RDX and SED-RDX, no degradation of RDX was observed (Fig. 5 and 6, B). In contrast, when RDX was added to anaerobic microcosms, the explosive was rapidly degraded in both autoclaved (SOIL-KILL-RDX and SED-KILL-RDX) and biotic (SOIL-RDX and SED-RDX) microcosms. However, RDX concentration decreased faster on biotic than autoclaved systems, then biological activity stimulates faster decrease of RDX concentration in sulfate reducing conditions.

Terminal Restriction Fragment Length Polymorphism analysis

Correspondence Analysis of T-RFLP. In order to understand the effect and fate of explosives on military activity sites, laboratory microcosms should resemble microbial communities on the original samples. Static aerobic conditions for microcosms simulate best the natural environment, preserving the overall microbial structure. As observed in the correspondence analysis the microbial community structure did not change in a time-dependent pattern (Fig. 10), instead aerobic treatments showed clustering of same

treatment communities, evidencing the differential effect of treatments. These results contrast with the clustering patterns observed in the correspondence analysis of the preliminary study performed under shaking conditions (Appendix A). Preliminary analysis showed an overlap of microbial communities from different origin in a time-dependent pattern were the Axis 1 on the scatter plot was representative of temporal distribution of samples (Fig. A1). These results suggest that shaking conditions had a bigger effect homogenizing microbial communities than sample origin and treatment (Fig. A2). Shaking conditions affect spatial structure of microbial community, destroy natural gradients and decrease microbial diversity (34). Therefore, shaking conditions are not suitable to address the effect of explosives in natural microbial communities.

Correspondence analysis of all aerobic microcosms from soil and marine sediment showed a clear separation of communities by origin of sample. This relationship of clearly distinctive communities was observed by plotting confidence ellipses (95%) on the scatter plots (Fig. 10); in addition, the area of both confidence ellipses was similar in size, thus similar community variability was observed for Puerto Negro and Range sediments. These results suggest that static aerobic conditions affected equivalently soil and marine sediment microbial communities under aerobic conditions. In contrast, when correspondence analysis of anaerobic soil and marine sediment microcosms was performed (Fig. 11), the 95% confidence ellipses overlapped. This suggests that under sulfate reducing conditions, certain populations common in both samples are selected and/or inhibited favoring the convergence of both microbial communities. However, sulfate-reducing conditions differentially affected both samples. Soil microcosms

exhibited less variability (smaller confidence ellipse area) than the one observed for marine sediments. Differences on confidence ellipses of soil and marine sediment samples could be an indicator of a more cohesive and constrained microbial community on soil samples, and thus the bigger variability in marine sediments suggest a more diverse community response to sulfate reducing conditions in the marine environment.

Distances between treatment and non-amended control scatter points helps to elucidate the effect of treatments on microbial community. In addition, it is important to determine overall variability (confidence ellipses) within each treatment and how they compare to the non-amended systems. Correspondence analysis of all different treatments showed that non-amended communities have a bigger variability than the observed in amended systems. Bigger variability of microbial communities can be interpreted as a higher intrinsic diversity. In contrast, scatter plots from amended communities grouped more closely, which suggest that the addition of explosive tended to decrease the variability and diversity of microbial communities. Reduction in microbial diversity has been previously observed in pristine soils amended with TNT (23).

Correspondence Analysis shows that separation between scatter points of SOIL-DNT treatments indicate how microbial community change trough time as consequence of 2,4-DNT addition. It is observed that day 8 and 16 group separately from day 0, 4 and 32. Interestingly, these results coupled with the evidence of degradation at day 8 and 16, and showed a reestablishment of microbial community resembling the original community at day 0 (before 2,4-DNT addition).

ANOSIM. The analysis of similarities was performed to determine similarities of microbial community structure between amended and non-amended treatments. Similarities and thus differences were established using the R_{ANOSIM} Values. A bigger difference between the control and the amended treatment was interpreted as a stronger effect of treatment on the microbial community structure. Consequently, small differences between amended and control treatments reflect little changes on microbial community structure. Relations between treatments of aerobic and anaerobic microcosms (Table 2, 3 and 4) were graphically summarized for a better identification of microbial community changes (Fig. 15).

ANOSIM (Aerobic soil amended with RDX). Under aerobic conditions, non-amended soil microbial communities were the most different to SOIL-RDX communities, and therefore, addition of RDX without a carbon source strongly affected the initial community (R_{ANOSIM} = 0.50). RDX concentration on this microcosm was constant during the experiment, acting as a persistent contaminant. Additionally, total separation of SOIL-RDX community was observed when compared to other amended treatments (Fig. 15, A).

ANOSIM (aerobic soil amended with DNT). Addition of 2,4-DNT with acetone as carbon source (SOIL-DNT/AC) drove a shift in microbial community (R_{ANOSIM} =0.47) to a different but still overlapping one. Due to the lack of mineralization evidence in SOIL-DNT/Ac (no nitrite production) (Fig. 5), it is possible that 2,4-DNT had persisted in soil

as a reduced form (as expected under anaerobic conditions) partially affecting the composition of microbial community. However, this effect cannot be attributed to anaerobic conditions, because SOIL-RDX/Ac microbial community, that also seems to turn anaerobic, was very similar to non-amended SOIL community (R_{ANOSIM} = 0.23). SOIL-DNT treatment with nitrite production, showed that the addition of 2,4-DNT without a carbon source also impacted the microbial community (R_{ANOSIM} = 0.40); the change observed in this community can be related to the inhibition of populations, but also to the selection of degraders that could mineralize 2,4-DNT. SOIL-DNT community was more similar to SOIL (control) than previous treatments (SOIL-DNT/Ac and SOIL-RDX), this smaller effect is could be related to the non-persistence of explosive in the environment. Based on the results, a RDX release on Vieques soils under aerobic conditions will have a bigger impact on microbial community than a release of 2,4-DNT under these same conditions.

ANOSIM (aerobic marine sediments amended with RDX). A different effect of 2,4-DNT and RDX addition was observed in microbial community of the marine sediments (Fig. 15, B). Communities from RDX-amended systems were the most similar to non-amended systems. Persistence of RDX on marine sediments did not seem to affect the structure of the community, since SED-RDX microbial community (non-acetone added), which did not show any RDX degradation, was the most similar and almost identical to the non-amended system (R_{ANOSIM}= 0.06). In contrast, SED-RDX/Ac system, which did show RDX degradation, was less similar but still barely separated to non-amended system (R_{ANOSIM}=0.23). This effect was opposite to the one observed for soil microcosms, for

which the microcosms that presented degradation were more similar to the control, probably by the disappearance of the toxic substance. Interestingly, both RDX-amended communities (SED-RDX/AC and SED-RDX) were also very similar between them (R_{ANOSIM}=0.22). This could be explained by the type of soil particle that could has a strong capability to resist changes, possibly by binding some molecules, as RDX, and reducing their bioavailability. However, some organic substances, as acetone, can change explosive solubility (61), and it is suggested that acetone could had changed the solubility of RDX in marine sediment microcosms, so despite of RDX degradation in acetone-added microcosms, higher solubility of RDX could increased the bioavailability an thus had a bigger effect on microbial community that the treatment without acetone.

ANOSIM (aerobic marine sediments with DNT). Communities from 2,4-DNT systems were the most dissimilar to non-amended communities. SED-DNT/Ac and SED-DNT communities were separated but slightly overlapped with SED communities (R_{ANOSIM}=0.62 and R_{ANOSIM}=0.70, respectively). These results suggest that different explosives affect differently the marine samples, and it can be predicted that RDX will have a minor effect on the native microbial community than exposure to 2,4-DNT.

ANOSIM (anaerobic systems). Under sulfate reducing conditions, communities from amended and non-amended systems were almost totally overlapped (Fig. 15C). High overlapping communities were also evident by the ANOSIM R value for all anaerobic communities (R_{ANOSIM} = 0.3036). Under sulfate-reducing conditions 2,4-DNT and RDX underwent a rapid decreased in concentration, it is believed that 2,4-DNT is reduced to

amino forms while RDX becomes unstable a rapidly degrades. This decreased was also observed in abiotic systems, suggesting that the released of explosives on a sulfate reducing environment will not affect microbial community structure in the same extend that it was observed for aerobic systems. Sulfate reducing condition in marine sediments are likely to be found near shores as in Puerto Negro, were the continuous input of organic matter decreased the amount o dissolved oxygen, stimulating the used of sulfate as an electron acceptor (65).

Cloning of 16S rRNA gene and sequencing

Samples used in clone libraries were selected base on the evidence of possible explosive mineralization. Day 8 was chosen over other days due to the evidence of an active mineralization process based on the 2,4-DNT disappearances and the near stochiometric production of nitrite. No significant difference was obtained when the two libraries (SOIL and SOIL-DNT) were compared to each other, by webLIBSHUFF analysis. This result agrees with the one obtained by ANOSIM analysis, which showed SOIL-DNT community was very similar to SOIL (control) ($R_{ANOSIM} = 0.40$). However, small changes in microbial community structure non-evident in the library comparison could be explained by the inhibition of minor populations and/or the idea that degraders were already abundant in the non-amended treatments and therefore not significant changes were observed. SOIL-DNT library (37 sequences) and SOIL library (42 sequences) had coverage of only 38% and 31% of total bacterial community respectively (Table 8), therefore a larger sequencing effort may reveal additional differences between their community structures.

Phylogenetic and SIMPER analysis.

Phylogenetic assignment of clone sequences was carried out to explore SOIL and SOIL-DNT community composition changes at day 8. Both libraries showed a similar composition percentage of major phyla, Firmicutes, Bacteriodetes and Proteobacteria (Fig. 17). However, when analyzing the relative abundance of classes and orders of Proteobacteria, community changes were observed. One important difference between the libraries was the percentage of alpha-proteobacteria changing from 16% in non-amended to 3% in amended systems. This difference in alpha-proteobaceria abundance could be related to competitive displacement or as result of a detrimental effect of 2,4-DNT addition. BLAST best hits of the alpha-proteobacteria clones from SOIL library showed that B3 clone was more related to *Azospirillum lipoferum* (98% identity and 100% coverage), B19 and B25 were related to *Novosphingobium* (98% identity and 99% of coverage), and B27 sequence was very similar to *Methylobacterium* (99% identity and 100% coverage). Understanding the ecological role of these related organisms could reveal potential effects of 2,4-DNT at the former military ranges. For instance, *Methylobacterium* genus is known to produce phytohormones that can stimulate seed germination and plant development, *Azospirillum* genus is well known as nitrogen fixer essential in nitrogen cycle and *Novosphingobium* genus have been related to aromatic compound degradation. Then, release of 2,4-DNT could potentially inhibit microbial populations with key roles on the macro-biological ecosystem.

Class beta-proteobacteria encompassed for the majority of sequences in both libraries. Comparison of the different orders within beta-proteobacteria class showed an increase in

relative abundance of Burkholderiales order, 44% in amended libraries (SOIL-DNT) versus 29% in non-amended systems (SOIL) (Fig. 17). Phylogenetic analysis of clone sequences showed that most of the Burkhorderiales present in the amended libraries were related to the Comamonadaceae family (Table 10), These family encompasses metabolic diverse microorganisms with some genera previously associated to nitroaromaric degradation. Evidence of higher abundances of Comamonadaceae family was also observed in T-RFLP analysis from the same treatments as observed by *in-silico* restriction of clones. Interestingly, the results suggest that the fragments associated to Comamonadaceae family were also enriched in T-RFLP analysis, thus validating the use of T-RFLP supporting the idea of a positive selective effect of the Comamonadaceae family by the addition of 2,4-DNT to the soil microcosm.

BLAST analysis of related cultured sequences revealed that most of these Comamonadaceae sequences in amended systems had a high similarity percentage to *Variovorax* genus, which contains several isolates related to nitroaromatic degradation. For example, *Variovorax paradoxus* has been found to degrade 2,4-DNT in a consortium with other organisms (58), in which 2,4-DNT was degraded to 4-methyl-5 nitrocathecol (4M5NC) coupled to nitrite production. Therefore, the use of T-RFLP and clone libraries provides key evidence of 2,4-DNT degradation and their link to microbial populations with the metabolic capability of degrading nitroaromatic compounds.

Furthermore when comparing SOIL and SOIL-RDX communities by SIMPER, the OTUs previously identified as related to the degradation of 2,4-DNT, are absent in the SOIL-

RDX. Inhibition of populations associated to 2,4-DNT degradation by RDX could be a problem in cases were both are release to the environment as co-contaminants, therefore it could be hypothesize that the release of RDX and 2,4-DNT in soil aerobic environment could inhibit the 2,4-DNT degraders and thus interfere with natural attenuation.

CONCLUSIONS

Marine sediment particles strongly bind explosives making them less available, this characteristic of sediment particles could contribute to the natural attenuation of the explosives in marine sediments.

Input of organic matter into soil and marine sediments contributes to RDX degradation. High inputs of organic matter promotes anaerobic conditions were RDX is easily degradable. In the other hand, 2,4-DNT is reduced to amino compounds under anaerobic condition, these reduced forms are thought to polymerize and irreversible bind soil and marine sediment particles.

Under aerobic condition indigenous microorganisms of Vieques have the metabolic capability of degrading 2,4-DNT producing near stoichiometric amounts of nitrite. Phylogenetic analysis of clone libraries and T-RFLP analysis suggest that these microorganisms are related to know 2,4-DNT degraders of the Comamonadaceae family and that increased when degradation was active. Interestingly, this is the first report of presence of 2,4-DNT degraders in environments not related to explosives manufacturing.

TABLES

Table 1. General description of microcosms.

Microcosm	Treatment	Treatment description	Purpose of treatment
BIOTIC CONTROL	Biological control	None of the explosives added. (	To contrast microbial community with amended microcosms and to follow nitrite production by intrinsic nitrogen sources.
AMMENDED WITH EXPLOSIVE	Amended with 2,4-DNT or RDX	2,4-DNT or RDX as carbon and nitrogen source.	To evaluate microbial community changes due to addition of explosives, and to determine nitrite production as product of 2,4-DNT mineralization
AMMENDED WITH EXPLOSIVE AND ACETONE	2,4-DNT or RDX + Carbon source	2,4-DNT or RDX as nitrogen source and acetone as carbon source.	To evaluate microbial community changes due to addition of explosives, when acetone was added as carbon source. To determine nitrite production as product of 2,4-DNT mineralization
ABIOTIC CONTROL	Abiotic control with 2,4-DNT or RDX + Carbon source	Sterile serum bottles amended with 2,4-DNT or RDX and acetone.	To contrast with amended biological treatments to reveal biological degradation.

Table 2 Analysis of similarities (ANOSIM) among aerobic soil microcosms.

Treatment	CF (n/a)	CL (RDX)	CL (2,4DNT)	EB (RDX)	EB (2,4DNT)
CF (n/a)	-	0.50	0.40	0.23	0.47
CL (RDX)	0.50	-	0.85	0.99	1.00
CL(2,4DNT)	0.40	0.85	-	0.37	0.81
EB(RDX)	0.23	0.99	0.37	-	0.32
EB(2,4DNT)	0.47	1.00	0.81	0.32	-

Similarities between treatments are calculated using Morisita Index. Mean rank within 74.76, mean rank between 151.2, general R_{ANOSIM} : 0.5543.

Table 3 Analysis of Similarities (ANOSIM) between microbial communities of aerobic marine sediment microcosms.

Treatment	SED	SED-RDX	SED-DNT	SED-RDX/Ac	SED-DNT/Ac
SED	-	0.06	0.70	0.23	0.62
SED-RDX	0.06	-	0.40	0.22	0.55
SED-DNT	0.70	0.40	-	0.72	0.66
SED-RDX/Ac	0.23	0.22	0.72	-	0.60
SED-DNT/Ac	0.62	0.55	0.66	0.60	-

Similarities between treatments are calculated using Morisita Index. Mean rank within 94.08, mean rank 161. 8, general R ANOSIM : 0.4514.

Table 4 Analysis of Similarities (ANOSIM) between microbial communities of soil and marine sediment anaerobic microcosms.

Treatment	Soil			Marine Sediment		
	CF (n/a)	EB (RDX)	EB (2,4DNT)	CF (n/a)	EB (RDX)	EB (2,4-DNT)
SOIL	-	0.24	0.16	0.19	0.46	0.36
SOIL-RDX/Ac	0.24	-	0.03	0.51	0.73	0.43
SOIL-DNT/Ac	0.16	0.03	-	0.48	0.75	0.46
SED	0.19	0.51	0.46	-	0.20	0.08
SED-RDX/Ac	0.46	0.73	0.75	0.20	-	0.10
SED-DNT/Ac	0.36	0.43	0.46	0.08	0.10	-

Similarities between treatments are calculated using Morisita Index. Mean rank within 161.1, mean rank 227.2, general R_{ANOSIM} : 0.3036.

Table 5 SIMPER Analysis of soil aerobic communities of CF and CL-RDX treatments.

Taxon (OTUs)	Contribution	Cumulative %	Mean S-CF	Mean S-CL-R
364-365-R	6.016	14.05	0	36.1
366-369-R*	3.284	21.73	19.7	0
221-224-H*	1.93	26.23	22.3	15.8
202-205-M	1.84	30.53	10.9	20.6
335-338-R	1.52	34.08	10.2	1.04
112-115-H	1.509	37.61	3.92	12.5
374-377-H	1.472	41.05	12.3	10.3
371-374-R*	1.378	44.27	8.27	0
363-R	1.247	47.18	0	7.48
95-98-M*	1.19	49.96	25.8	25.3

OTUs label R were generated with *Rsa*I, H were generated with *Hae*III and M were generated with *Msp*I. Mean are calculated from relative abundance of T-RFLP profiles from day 4 to 32. Overall average dissimilarity 42.39

Table 6 SIMPER Analysis of Marine Sediment aerobic communities of CF and CL-D treatments

Taxon (OTUs)	Contribution	Cumulative %	Mean MS-CF	Mean MS-CL-D
646-650-M	3.585	9.411	21.5	0
641-645-M	2.084	14.88	0.639	13.1
212-215-H	1.946	19.99	12.5	22.7
359-362-R	1.922	25.04	11.5	0
366-369-R	1.875	29.96	26.2	28.5
95-98-M	1.833	34.77	30.2	33.4
229-232-R	1.452	38.58	7.58	15.1
374-377-H	1.321	42.05	21.6	13.8
137-140-R	1.219	45.25	23.3	18.6
410-414-H	1.095	48.13	23	18.2
304-307-R	0.9106	50.52	3.04	8.12

OTUs label R were generated with *Rsa*I, H were generated with *Hae*III and M were generated with *Msp*I. Mean are calculated from relative abundance of T-RFLP profiles from day 4 to 32. Overall average dissimilarity 38.09

Table 7 SIMPER analysis of marine sediment aerobic communities of SED and SED-DNT/AC treatments.

Taxon (OTUs)	Contribution	Cumulative %	Mean MS-CF	Mean MS-EB-D
366-369-R	3.29	6.764	26.2	34.3
374-377-H	3.088	13.11	21.6	3.09
370-373-H	2.758	18.79	0	16.5
384-387-R	2.426	23.77	0	14.6
137-140-R	2.424	28.76	23.3	8.96
95-98-m	2.163	33.21	30.2	42.9
359-362-R	1.922	37.16	11.5	0
355-358-R	1.834	40.93	6.86	17.8
646-650-m	1.704	44.43	21.5	11.3
425-429-H	1.633	47.79	0	9.8
229-232-R	1.477	50.83	7.58	12

OTUs label R were generated with *Rsa*I, H were generated with *Hae*III and M were generated with *Msp*I. Mean are calculated from relative abundance of T-RFLP profiles from day 4 to 32. Overall average dissimilarity 48.63

Table 8 Libshuff analysis of SOIL-DNT (day 8) and SOIL (day 8) clone libraries.

Library	Total No. of sequences	No. of unique OTUs	Coverage (Good's C)	Index of diversity	
				Shannon	1/Simpson
CL-S-D	37	28	38%	3.20796	47.57148
SOIL	42	35	31%	3.49416	107.625

Table 9 Phylogenetic tree types description for identification of clone sequences

Type	Tree description	RDP used sequences
I	The phylogentic tree was constructed using 27 sequences of the SOIL-DNT and SOIL libraries that contain the forward primer (519 F) and 495 sequences from "its nearest neighbors" obtain at the RDP using "Sequence Match"	Nearest neighbors
II	The phylogentic tree was constructed using 42 sequences of the SOIL-DNT and SOIL libraries that contain the reverse primer (1392 R) and 495 sequences from "its nearest neighbors" obtain at the RDP using "Sequence Match"	Nearest neighbors
III	The phylogentic tree was constructed using 27 sequences of of the SOIL-DNT and SOIL libraries that contain the forward primer (519 F) and 788 including all sequences browsed at the RDP.	Burkholderiales, Hydrogenophilales, Methylophilales, Rhodocyclales, Rhodospirillales, Rhizobiales, Sphingomonodales, genus:Streptococcus, genusAnaeromyxobacter, genus:Geobacter, genus:Syntrophobacter, Bacteroides, Chlorobi.
IV	The phylogentic tree was constructed using 42 sequences of the SOIL-DNT and SOIL libraries that contain the forward reverse (1392 R) and 788 including all sequences browsed at the RDP.	Burkholderiales, Hydrogenophilales, Methylophilales, Rhodocyclales, Rhodospirillales, Rhizobiales, Sphingomonodales, genus:Streptococcus, genus:Anaeromyxobacter, genus:Geobacter, genus:Syntrophobacter, Bacteroides, Chlorobi.
V	Subsets with in previous trees (zoom) for clones A23, B16, B24, B11, y A33	phylum Firmicutes.
VI	Subsets with in previous trees (zoom) for clones A30, B18, B21, B23, B33, A17, A22, B15, B32, B47	clase Beta-Proteobacteria
VII	Subsets with in previous trees (zoom) for clones A7 y B42	Delta-Proteobacteria.
VIII	Subsets with in previous trees (zoom) for clones A28, A4, A8, B1 y B48	Sphingobacteria

IX	Al clon B34 se le hizo de nuevo BLAST y se realizo una filogenia con todos los hits que no fueran solo bacteria,	Blast similar bacteria

A= SOIL-DNT (day 8), B=SOIL (day 8)

Table 10 Taxonomy of clones from SOIL-DNT (day8) library inferred from phylogenetic trees.

Clone	Most related taxonomic group	Taxonomic level	Other Taxonomic levels
A2	Sphingomonadales	***order***	class alpha-proteobacteria
A4	Sphingobacteriales	***order***	phylum Bacteroidetes
A7	Geobacter	***genus***	class Deltaproteobacteria order Desulfuromonadales family Geobacteraceae
A8	Chenotrichaceae	***family***	phylum Bacteroidetes order Sphingobacteriales
A12	Comamonadaceae	***family***	class Betaproteobacteria; order Burkholderiales
A14	Comamonadaceae	***family***	class Betaproteobacteria; order Burkholderiales
A15	Comamonadaceae	***family***	class Betaproteobacteria; order Burkholderiales
A16	Betaproteobacteria	***class***	
A17	Beta-Proteobacteria	***class***	phylum Proteobacteria
A19	Comamonadaceae	***family***	class Betaproteobacteria; order Burkholderiales
A20	Comamonadaceae	***family***	class Betaproteobacteria; order Burkholderiales
A22	Beta-Proteobacteria	***class***	phylum Proteobacteria
A23	Clostridiales	***order***	phylum Firmicutes class Clostridia
A24	Betaproteobacteria	***class***	
A25	Comamonadaceae	***family***	class Betaproteobacteria order Burkholderiales
A27	Syntrophobacter	***genus***	class Deltaproteobacteria order Syntrophobacterales; family Syntrophobacteraceae
A28	Sphingobacteriales	***order***	phylum Bacteroidetes
A30	Burkholderiales	***order***	class Betaproteobacteria;
A31	Neisseriaceaea	***family***	class Betaproteobacteria; order Neisseriales
A33	Streptococcaceae	***family***	phylum Firmicutes class Bacilli order Lactobacillales
A34	Burkholderiales	***order***	class Betaproteobacteria;
A35	Comamonadaceae	***family***	class Betaproteobacteria order Burkholderiales
A36	Anaeromyxobacter	***genus***	class Deltaproteobacteria order Myxococcales family Myxococcaceae
A37	Betaproteobacteria	***class***	
A41	Bacteria	***superkingdom***	
A42	Comamonadaceae	***family***	class Betaproteobacteria order Burkholderiales

A43	Comamonadaceae	***family***	class Betaproteobacteria order Burkholderiales
A44	Betaproteobacteria	***Class***	
A47	Comamonadaceae	***family***	class Betaproteobacteria; order Burkholderiales
A48	Burkholderiales	**order**	class Betaproteobacteria;

Table 11 Taxonomy of clones from SOIL (day8) library inferred from phylogenetic trees.

Clone	Most related taxonomic group	Taxonomic level	Other Taxonomic levels
B1	Chenotrichaceae	***family***	phylum Bacteroidetes order Sphingobacteriales
B2	Burkholderiales	***order***	class Betaproteobacteria;
B3	Rhodospirillaceae	***family***	class Alpha-proteobacteria order Rhodospirillales
B4	Comamonadaceae	***family***	class Betaproteobacteria order Burkholderiales
B5	Alpha-proteobacteria	***class***	
B6	Comamonadaceae	***family***	class Betaproteobacteria order Burkholderiales
B8	Neisseriaceaea	***family***	class Betaproteobacteria order Neisseriales
B9	Rhodocyclaceae	***family***	class Betaproteobacteria; order Rhodocyclales
B10	Sphingomonadaceae	***family***	class Alphaproteobacteria order Sphingomonadales
B11	Clostridiales	***order***	phylum Firmicutes class Clostridia
B12	Comamonadaceae	***family***	class Betaproteobacteria order Burkholderiales
B15	Beta-Proteobacteria	***class***	phylum Proteobacteria
B16	Clostridiales	***order***	phylum Firmicutes; class Clostridia
B18	Beta-Proteobacteria	***class***	phylum Proteobacteria
B19	Sphingomonadaceae	***family***	class Alphaproteobacteria order Sphingomonadales
B20	Azoarcus	***genus***	phylum Betaproteobacteria; order Rhodocyclales; family Rhodocyclaceae
B21	Beta-Proteobacteria	***class***	phylum Proteobacteria
B22	Comamonadaceae	***family***	class Betaproteobacteria; order Burkholderiales
B23	Burkholderiales	***order***	class Betaproteobacteria;
B24	Clostridiales	***order***	phylum Firmicutes; class Clostridia
B25	Sphingomonadaceae	***family***	class Alphaproteobacteria; order Sphingomonadales
B26	Burkholderiales	***order***	class Betaproteobacteria;
B27	Methylobacterium	***genus***	phylum Alphaproteobacteria; class Rhizobiales; family Methylobacteriaceae
B28	Comamonadaceae	***family***	class Betaproteobacteria order Burkholderiales
B29	Neisseriales	***order***	class Betaproteobacteria;
B30	beta-Proteobacteria	***class***	
B31	bacteria	***Super-kingdom***	
B32	Beta-Proteobacteria	***class***	phylum Proteobacteria
B33	Burkholderiales	***order***	class Betaproteobacteria;

B34	bacteria	***Super-kingdom***	
B37	Burkholderiales	***order***	class Betaproteobacteria;
B39	Proteobacteria	***phylum***	
B40	Neisseriaceaea	***family***	class Betaproteobacteria; order Neisseriales
B41	Rhodocyclaceae	***family***	class Betaproteobacteria; order Rhodocyclales
B42	Delta-proteobacteria	***class***	
B44	Comamonadaceae	***family***	class Betaproteobacteria order Burkholderiales
B47	Burkholderiales	***order***	class Betaproteobacteria;
B48	Chenotrichaceae	***family***	phylum Bacteroidetes order Sphingobacteriales

Table 12 Expected T-RF fragment lengths for 16S rRNA clone sequences using Hae*III*, Msp*I* and RsaI restriction enzymes

	In-silico-RFLP			Taxonomic levels			
Clones	*HaeIII*	*MspI*	*RsaI*	Phylum	Class	Order	Family
A 2	412	95	374	Proteobacteria	Alphaproteobacteria	Sphuingomonadales	-
A 16	208	96	21	Proteobacteria	Betaproteobacteria	-	-
A 21	226	96	140	Firmicutes	Betaproteobacteria	-	-
A 23	413	97	323	Firmicutes	Clostridia	Clostridiales	-
A 24	226	96	140	Proteobacteria	Betaproteobacteria	-	-
A 25	226	96	373	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae
A 33	412	201	112	Firmicutes	Bacilli	-	Streptococcaceae
A 34	226	96	373	Proteobacteria	Betaproteobacteria	-	-
A 36	71	142	140	Proteobacteria	Deltaproteobacteria	Myxococcales	Myxococcaceae
A 37	226	96	373	Proteobacteria	Betaproteobacteria	Burkholderiales	-
A 42	226	96	373	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae
A 44	226	96	184	Proteobacteria	Betaproteobacteria	-	-
A 47	226	96	140	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae
A 48	226	96	373	Proteobacteria	Betaproteobacteria	Burkholderiales	-
B 2	100	96	61	Proteobacteria	Betaproteobacteria	Burkholderiales	-
B 5	217	202	231	Proteobacteria	Alphaproteobacteria	-	-
B 8	377	96	373	Proteobacteria	Betaproteobacteria	Neisseriales	Neisseriaceaea
B 11	409	38	371	Firmicutes	Clostridia	Clostridiales	-
B 12	226	96	373	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae
B 16	413	129	323	Firmicutes	Clostridia	Clostridiales	-
B 24	71	106	375	Firmicutes	Clostridia	Clostridiales	-
B 26	226	96	373	Proteobacteria	Betaproteobacteria	Burkholderiales	-
B 30	>620	96	375	Proteobacteria	Betaproteobacteria	-	-

B 31	378	96	374	N/D	-	-	-
B 37	377	>618	373	Proteobacteria	Betaproteobacteria	Burkholderiales	-
B 39	100	97	373	Proteobacteria	-	-	-
B 40	377	96	373	Proteobacteria	Betaproteobacteria	Neisseriales	Neisseriaceaea
B 41	226	96	140	Proteobacteria	Betaproteobacteria	Rhodocyclales	Rhodocyclaceae

A=S-CL-D (day 8), B=S-CF(day 8)

FIGURES

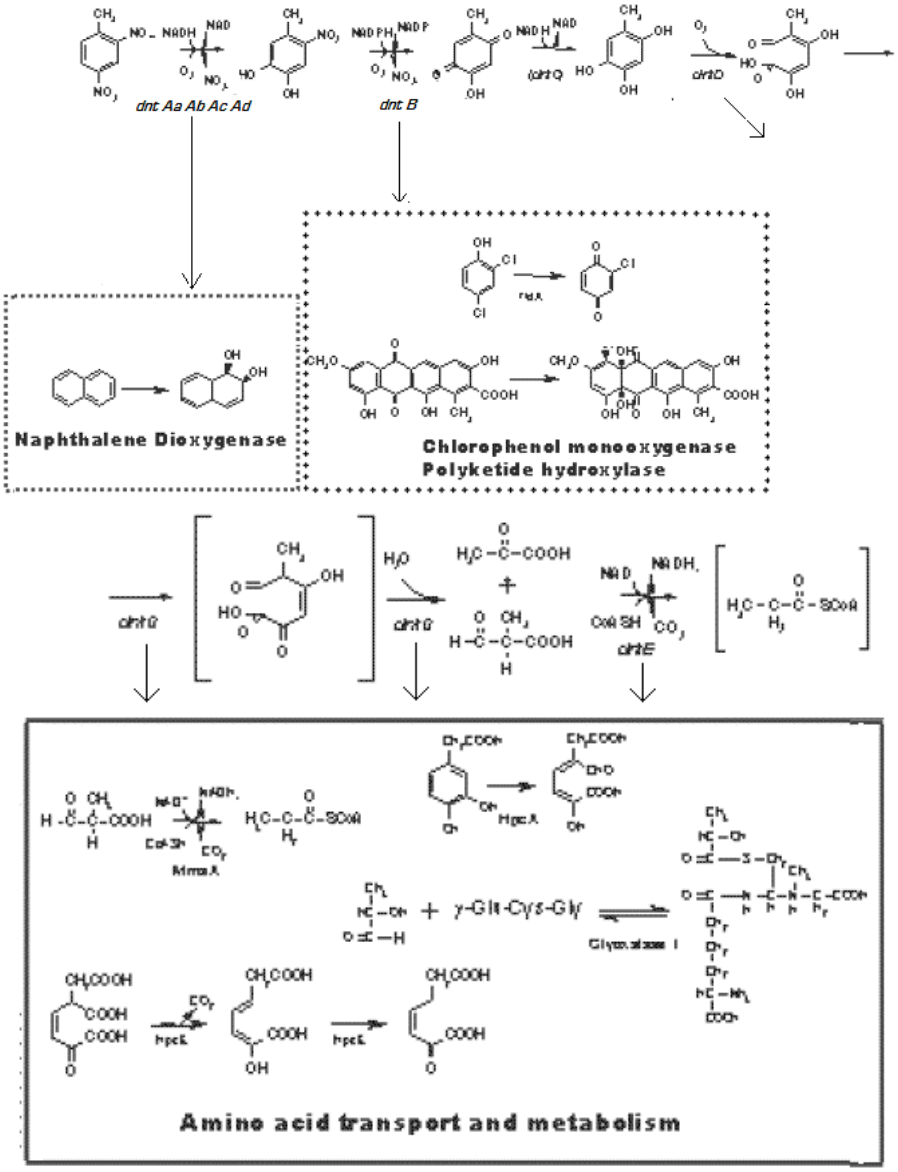


Figure 1 Degradation pathway of 2,4-DNT in *B. cepacia* R34, and its evolutionary relation with other enzymes. Figure modified from Johnson and Spain (2003).

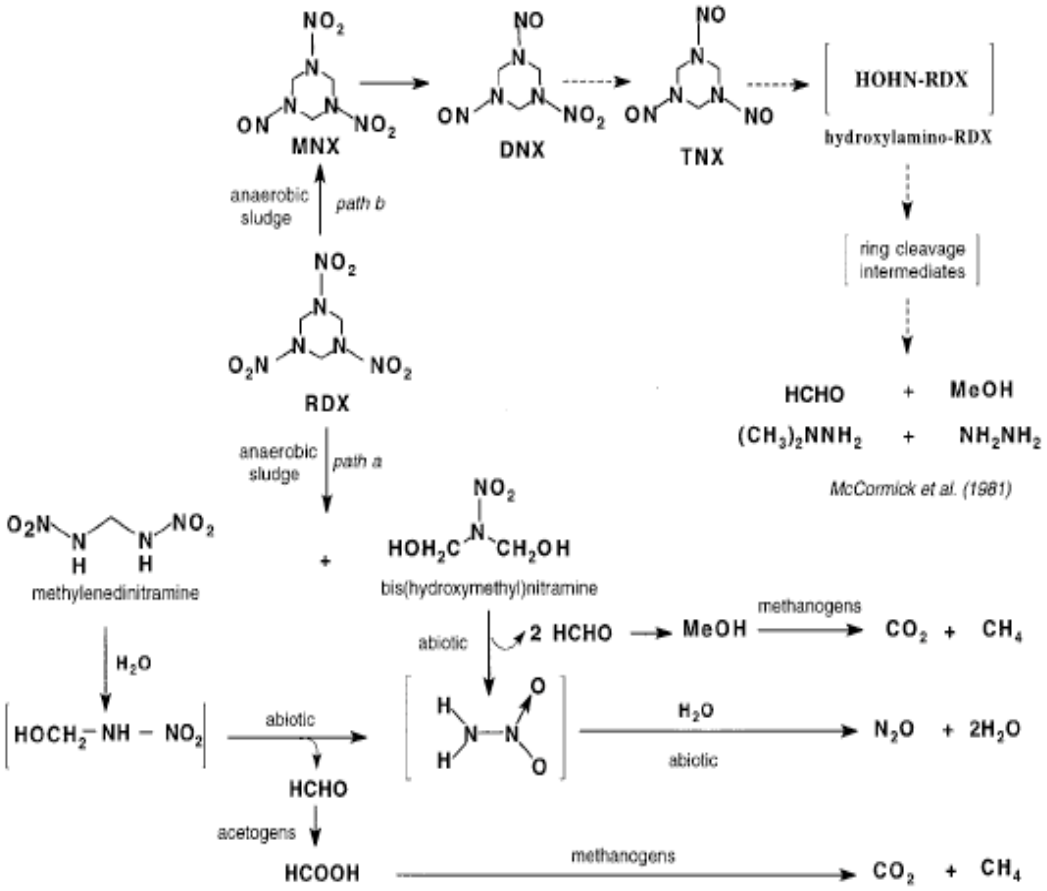


Figure 2 Degradation pathways of RDX under anaerobic sludge conditions. Pathway b was proposed by McCormick and coworkers (1981) while pathway "a" was proposed by Hawari and coworkers (2002). **Figure** was taken from last reference.

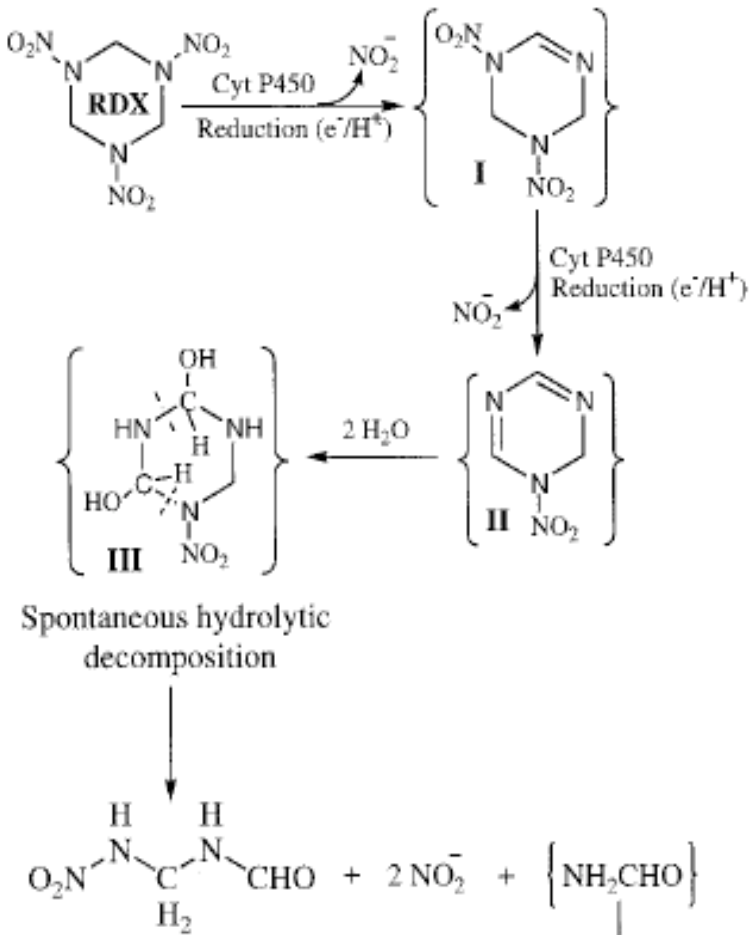


Figure 3 Pathway for RDX biotransformation catalyzed by rabbit liver cytochrome P450 2B4 (Cyt P450). Products in brackets were not detected. Image was taken from Brushan and coworkers (2003).

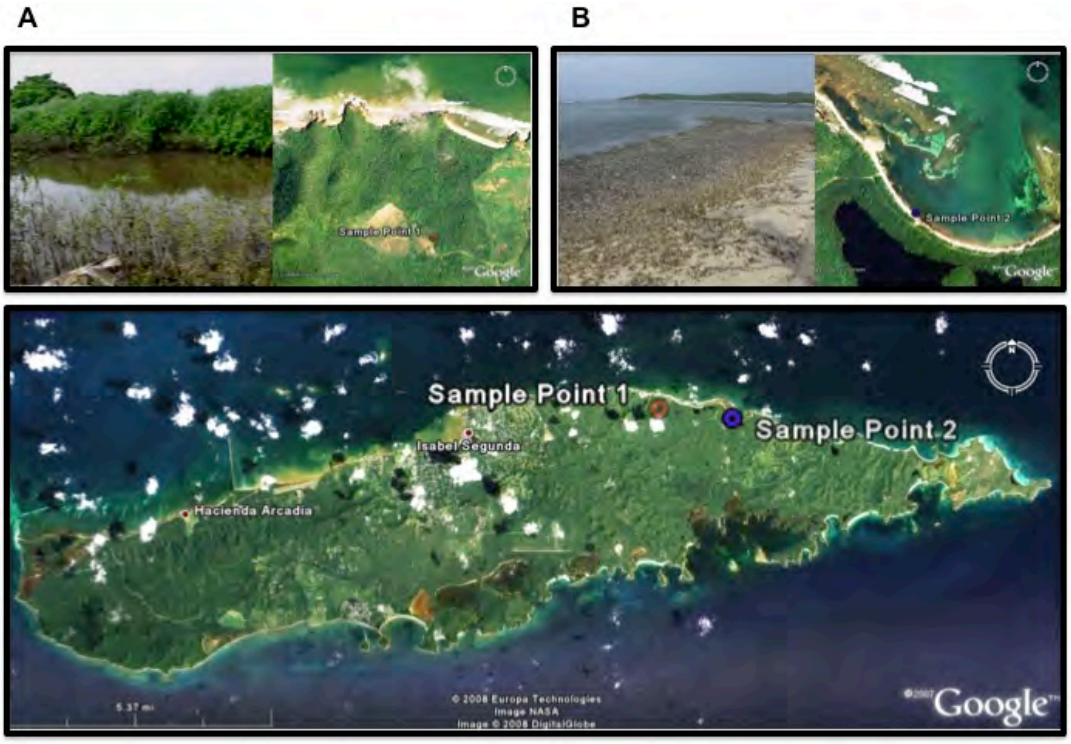


Figure 4 "Collection points at the AFWTF Vieques, Puerto Rico" Google Earth TM .

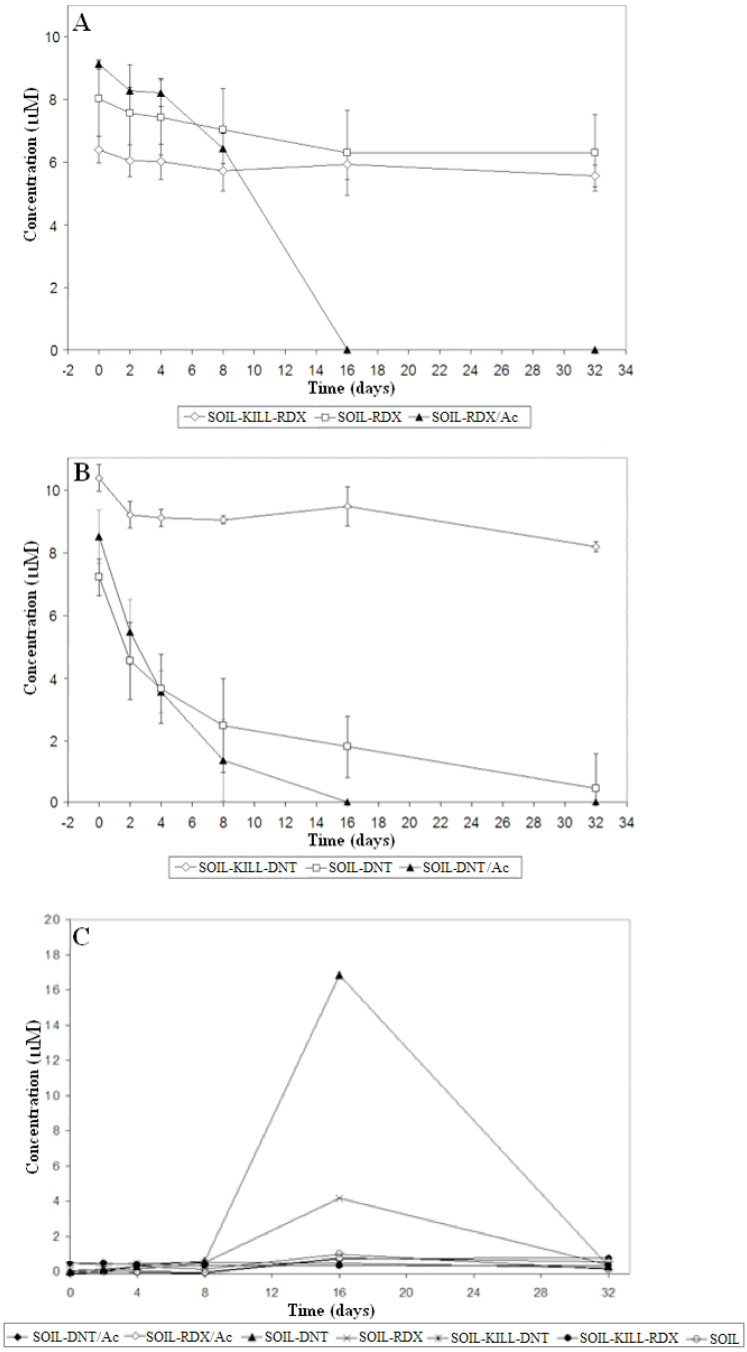


Figure 5 Quantification of RDX, 2,4-DNT and nitrite for soil aerobic microcosms under static conditions. (A) Microcosms amended with RDX. (B) Microcosms amended with 2,4-DNT. (C) Nitrite production of microcosms.

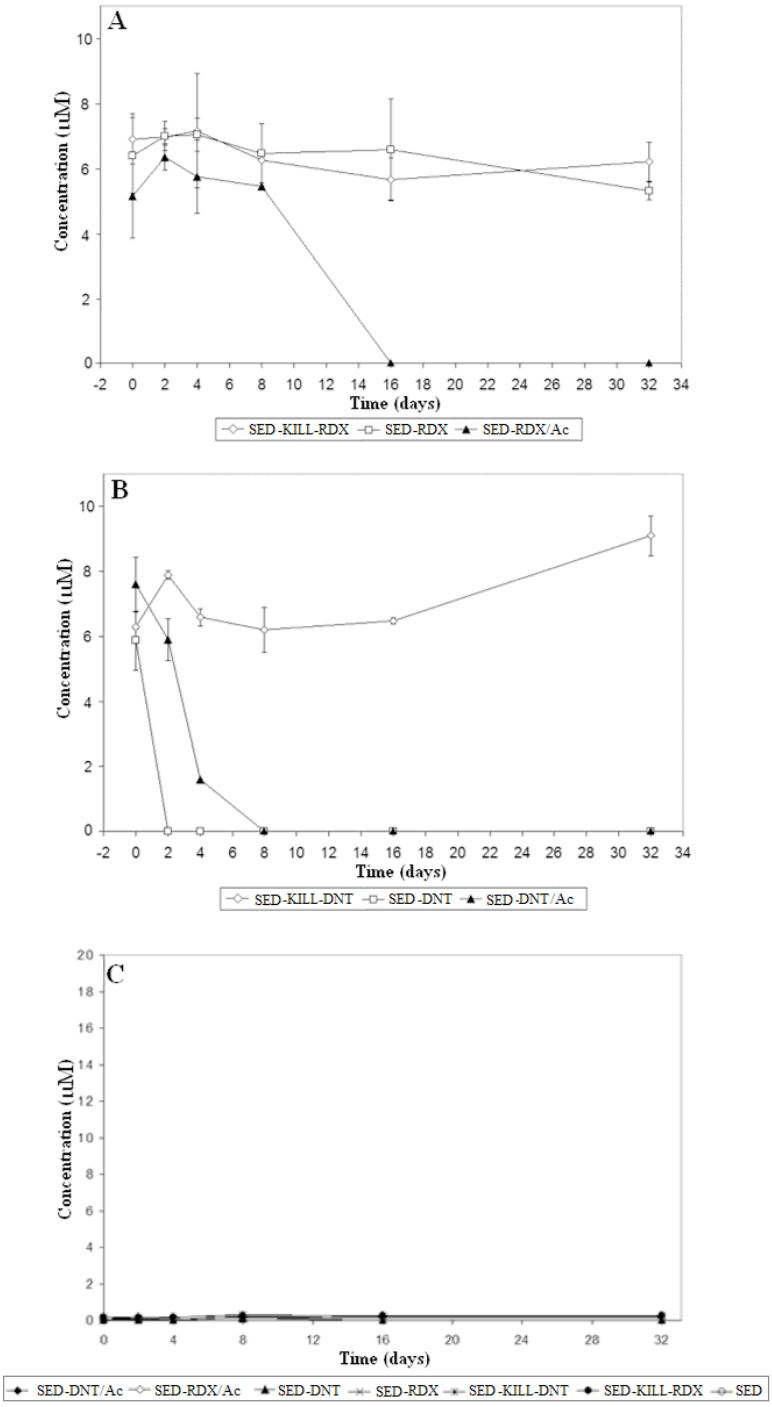


Figure 6 Quantification of RDX, 2,4-DNT and nitrite for marine sediments aerobic microcosms under static conditions. (A) Microcosms amended with RDX. (B) Microcosms amended with 2,4-DNT. (C). Nitrite production of different treatments.

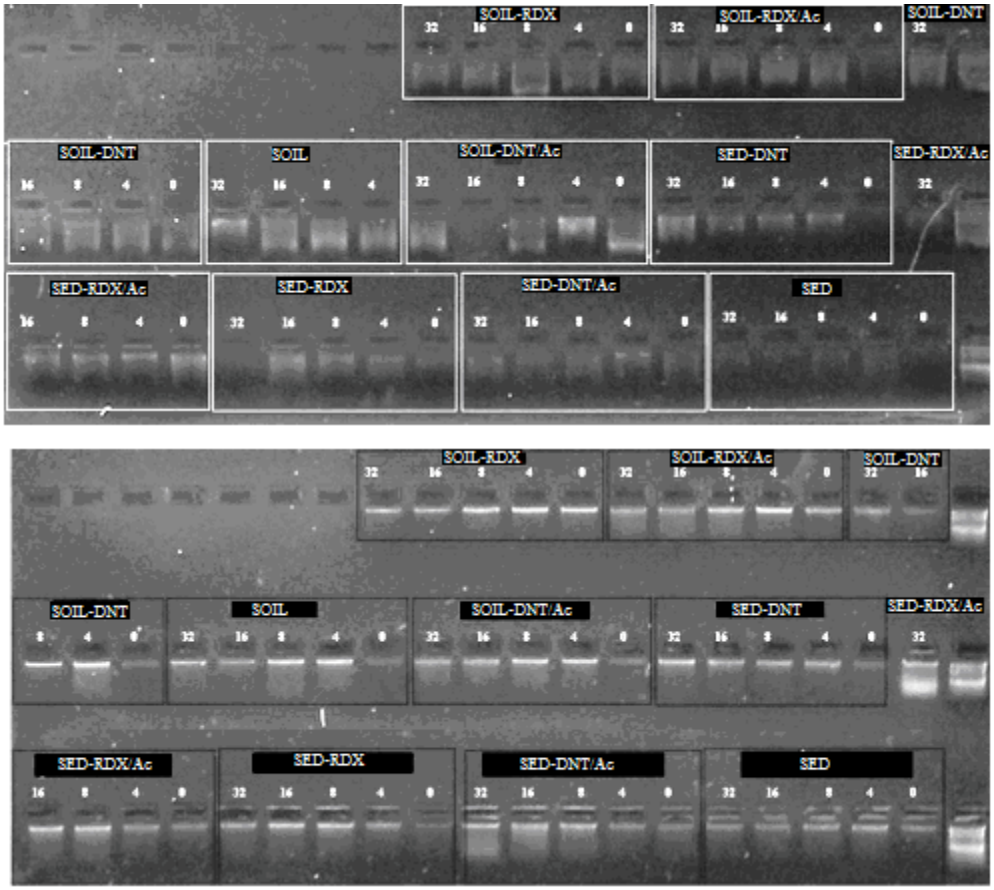


Figure 7 Gel electrophoresis of DNA extractions from soil and marine sediment microcosms. Five microlitres of each extraction reaction were loaded onto the agarose gel (1%). MW, 1Kb plus DNA ladder (Invitrogen).

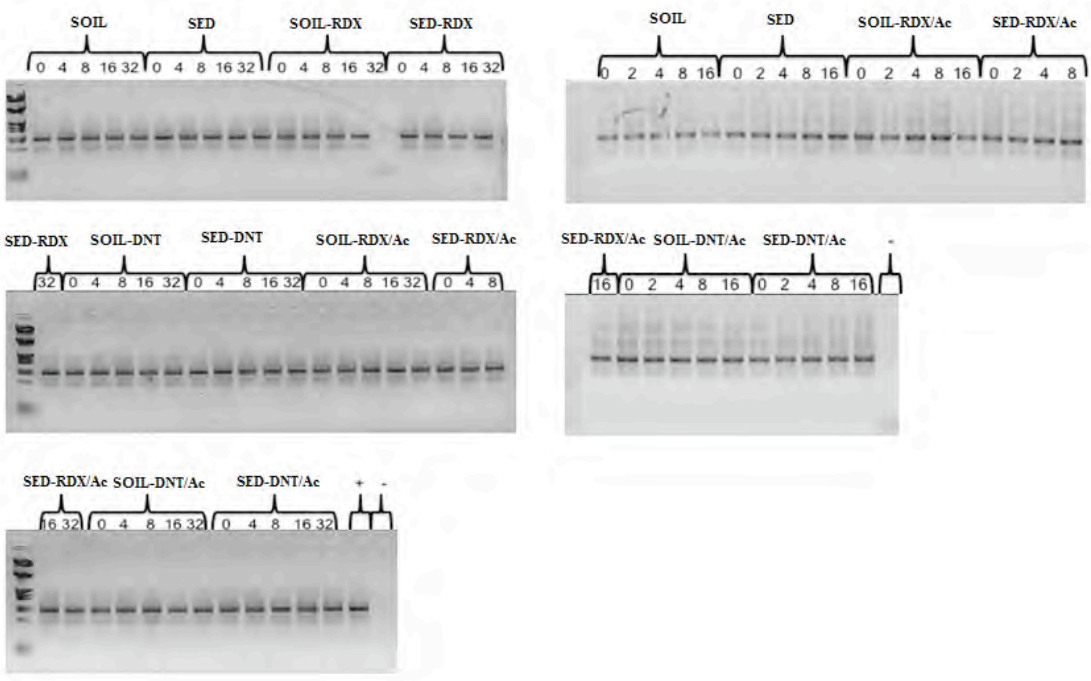


Figure 8 Gel electrophoresis of nested PCR amplifications of 16S rRNA genes using 519F and 1392R primer pair. (A) Aerobic soil and marine sediment microcosms. (B) Anaerobic soil and marine sediment microcosms. For each PCR product, 5 µl were loaded onto the agarose gel (1%). MW, 1Kb plus DNA ladder (Invitrogen)

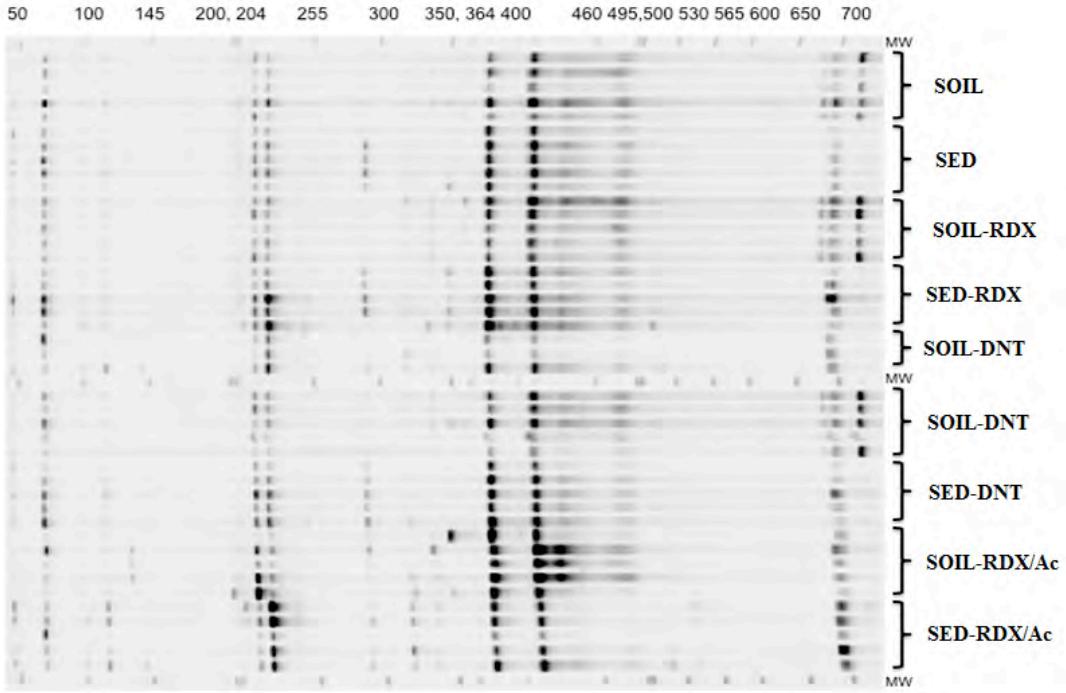


Figure 9 T-RFLP generated with *Hae*III from aerobic soil and marine sediments PCR amplifications.

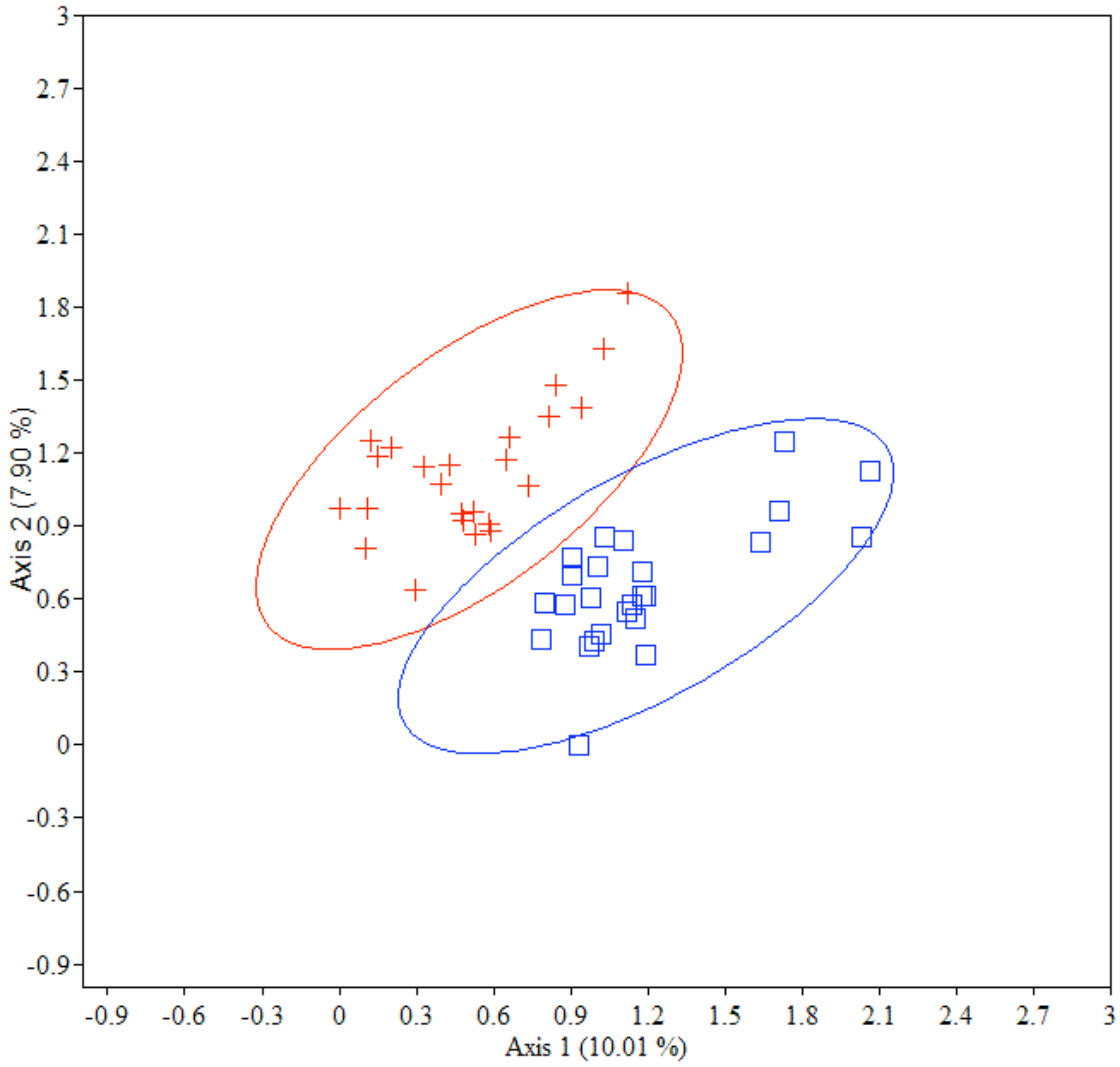


Figure 10 Correspondence analysis of T-RFLP profiles of 16S rRNA genes from aerobic microcosms under static conditions. Crosses represent soil community profiles, while blue squares represent marine sediment profiles. Ellipses represent 95% of confidence regions for the groups.

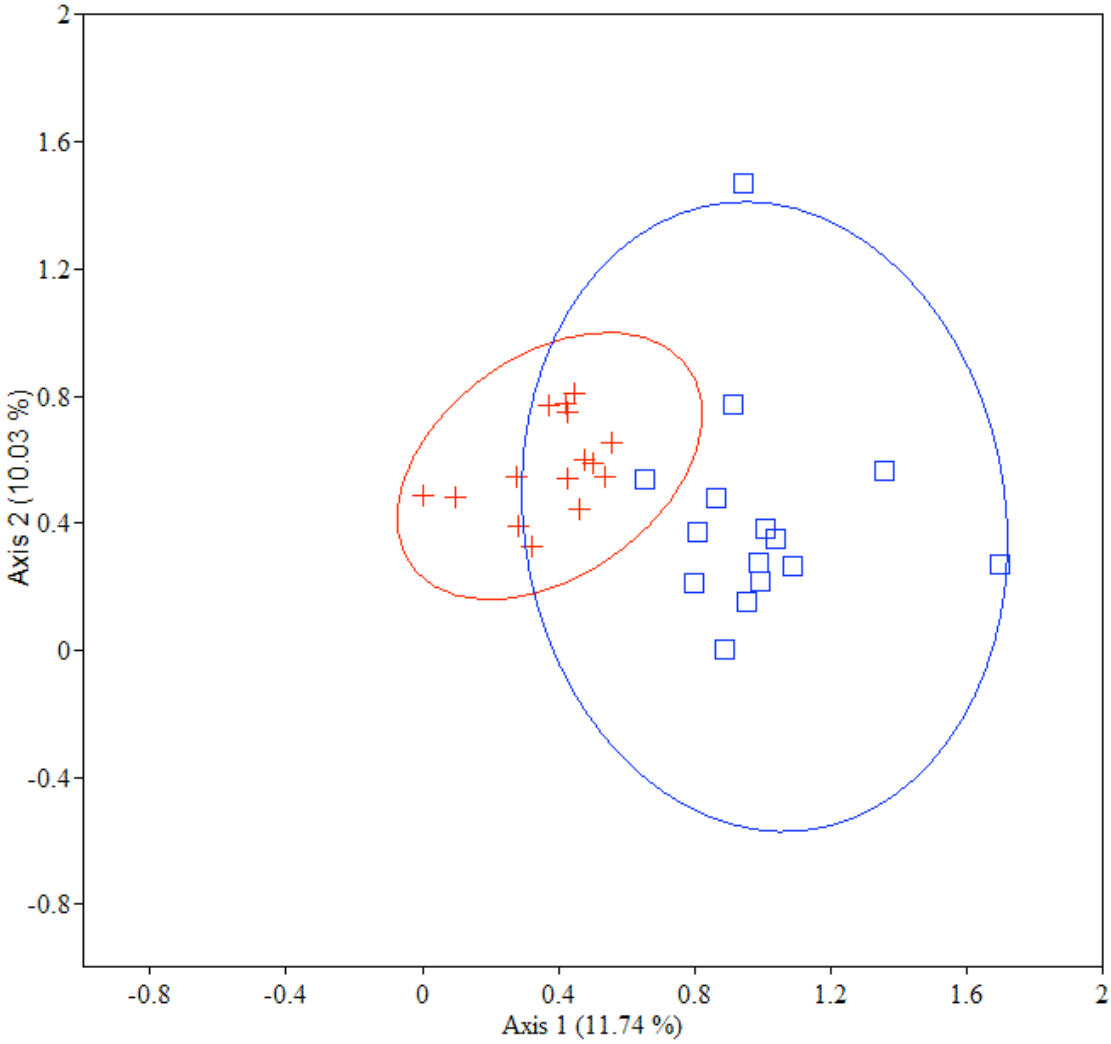


Figure 11 Correspondence analysis of T-RFLP profiles from 16S rRNA of anaerobic microcosms under static conditions. Red crosses represent soil communities, while blue squares represent marine sediment communities. Ellipses represent 95% of confidence regions for the groups**.**

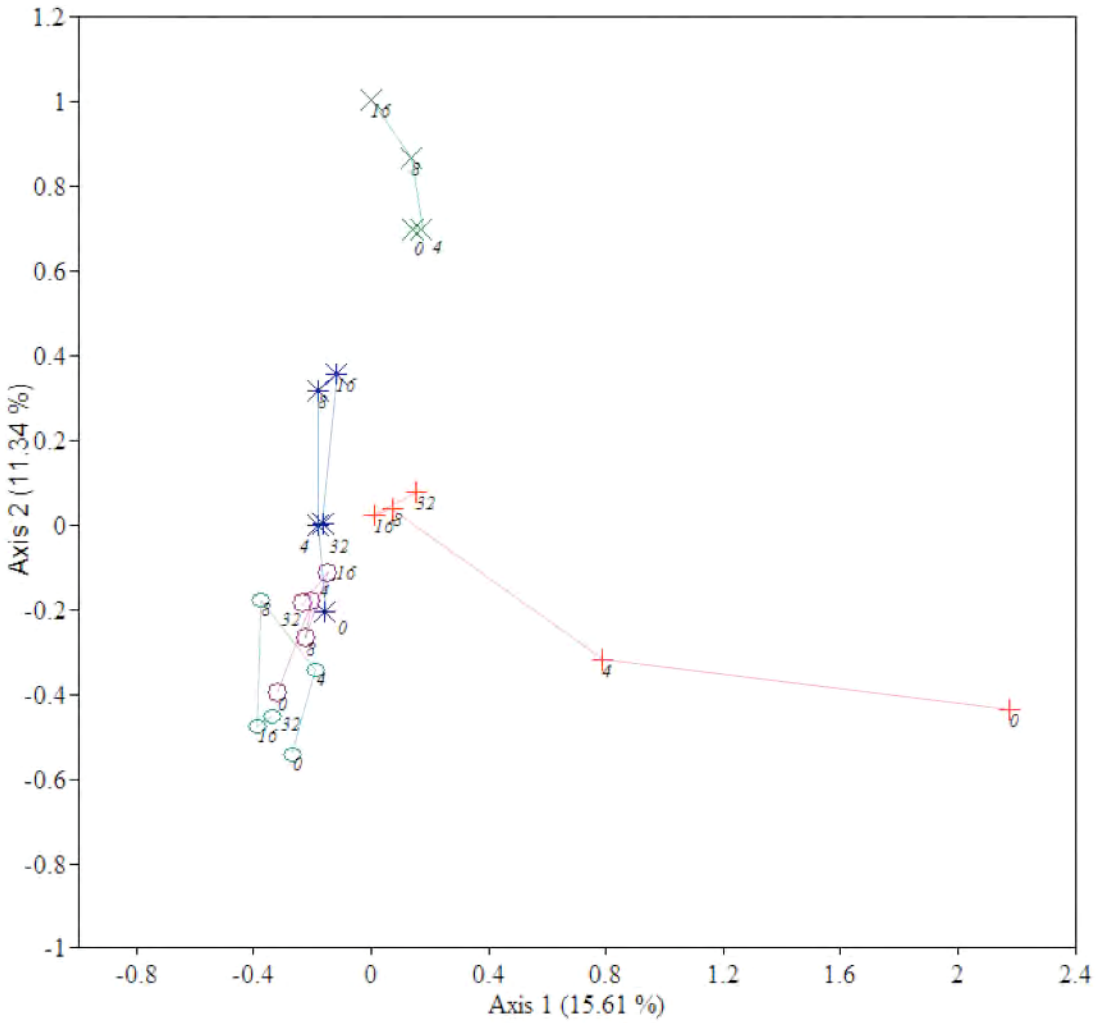


Figure 12 Correspondence analysis of microbial community structure from military impacted soil at Vieques Island. Aerobic microcosms treatments: Red Cruse SOIL, Green Cruse SOIL-RDX, Blue Stars SOIL-DNT, Blue oval SOIL-DNT/Ac, Purple circle SOIL-RDX/Ac.

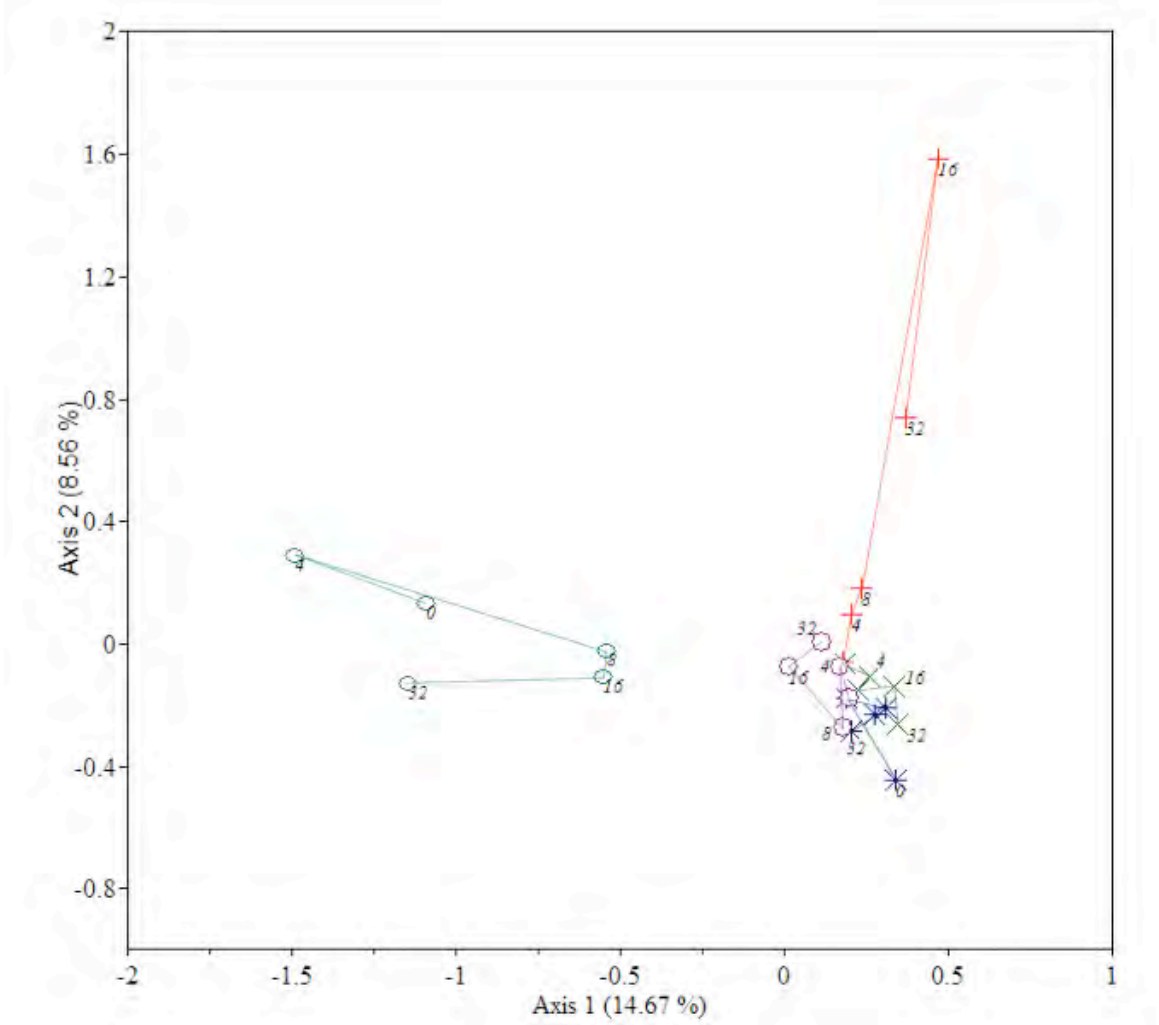


Figure 13 Correspondence analysis of T-RFLP profiles for marine sediment aerobic microcosms. Red Cruse SED, Green Cruse SED-RDX, Blue Stars SED-DNT, Blue oval SED-DNT/Ac, Purple circle SED-RDX/Ac.

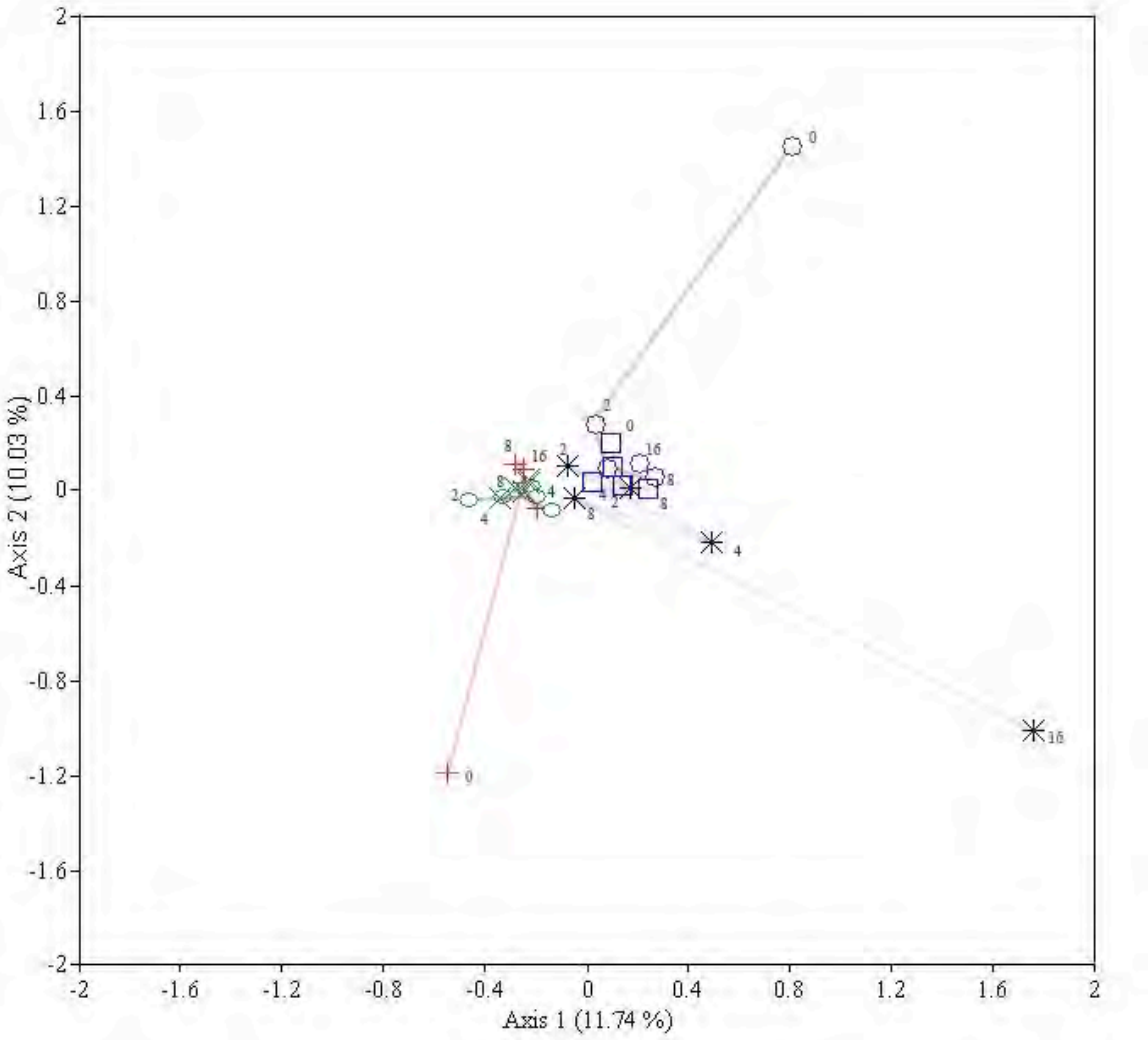


Figure 14 Correspondence analyses of T-RFLP profiles from soil and marine sediments microcosms of Vieques Island under anaerobic conditions. Red cross SOIL, blue oval SOIL-DNT/Ac, green crosses SOIL-RDX/Ac, purple circle SED, blue square SED-DNT/Ac, and blue start SED-RDX/Ac.

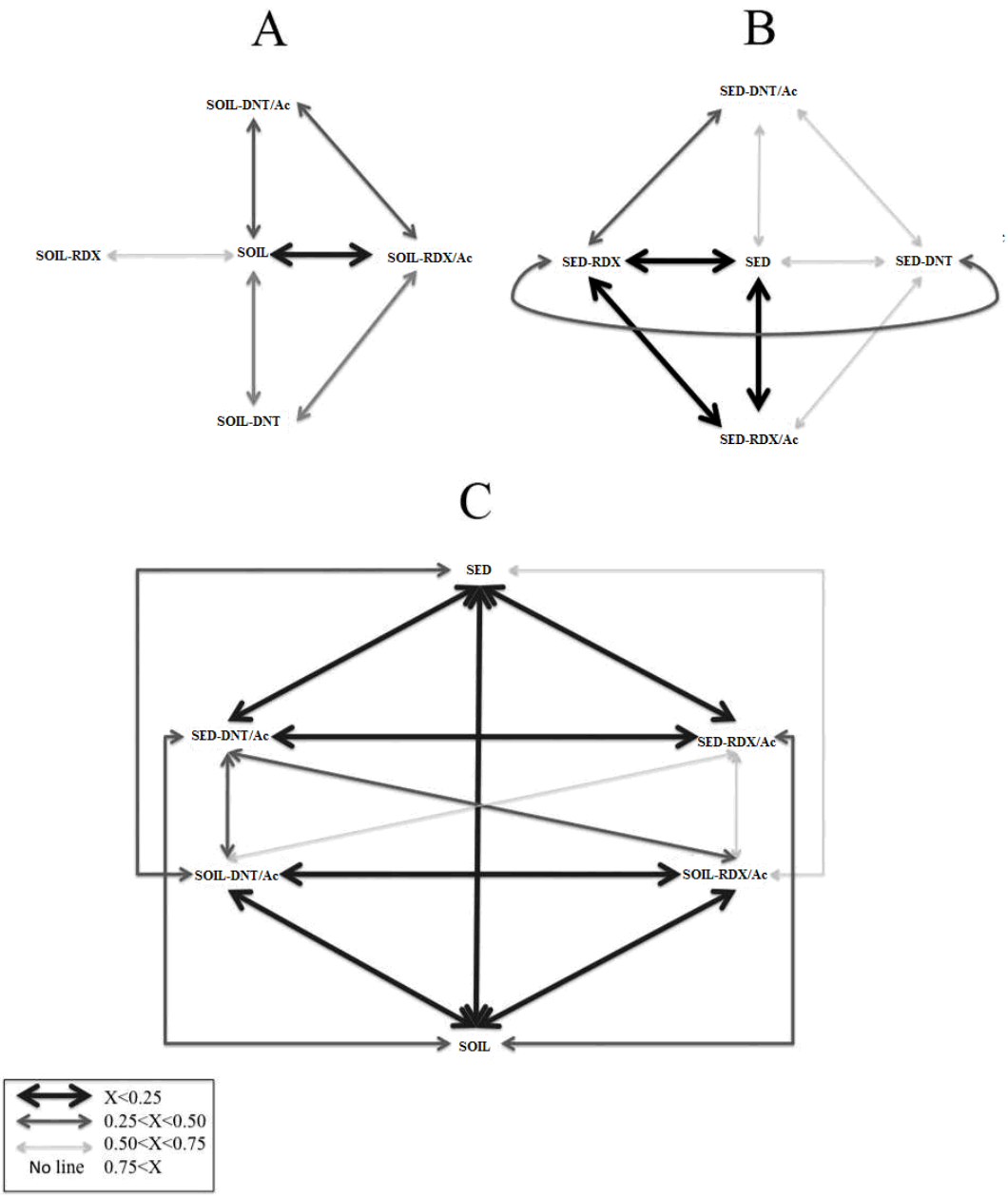


Figure 15 Relations between microbial communities from different treatments analyzed using ANOSIM. (A) Soil communities from aerobic microcosms. (B) Marine sediment communities from aerobic microcosms. (C) Communities from anaerobic soil and marine sediment microcosms.

Lines represent community relation base on ANOSIM R value**.** Absence on connecting represent well separated communities (R_{ANOSIM} > 0.75), thin lines represent well separated but overlapping communities

(0.75 > R_{ANOSIM} > 0.50), thick lines represent separating but overlapping communities (0.50> R_{ANOSIM} >0.25) and thicker lines correspond to more similar treatments (R_{ANOSIM} < 0.25).

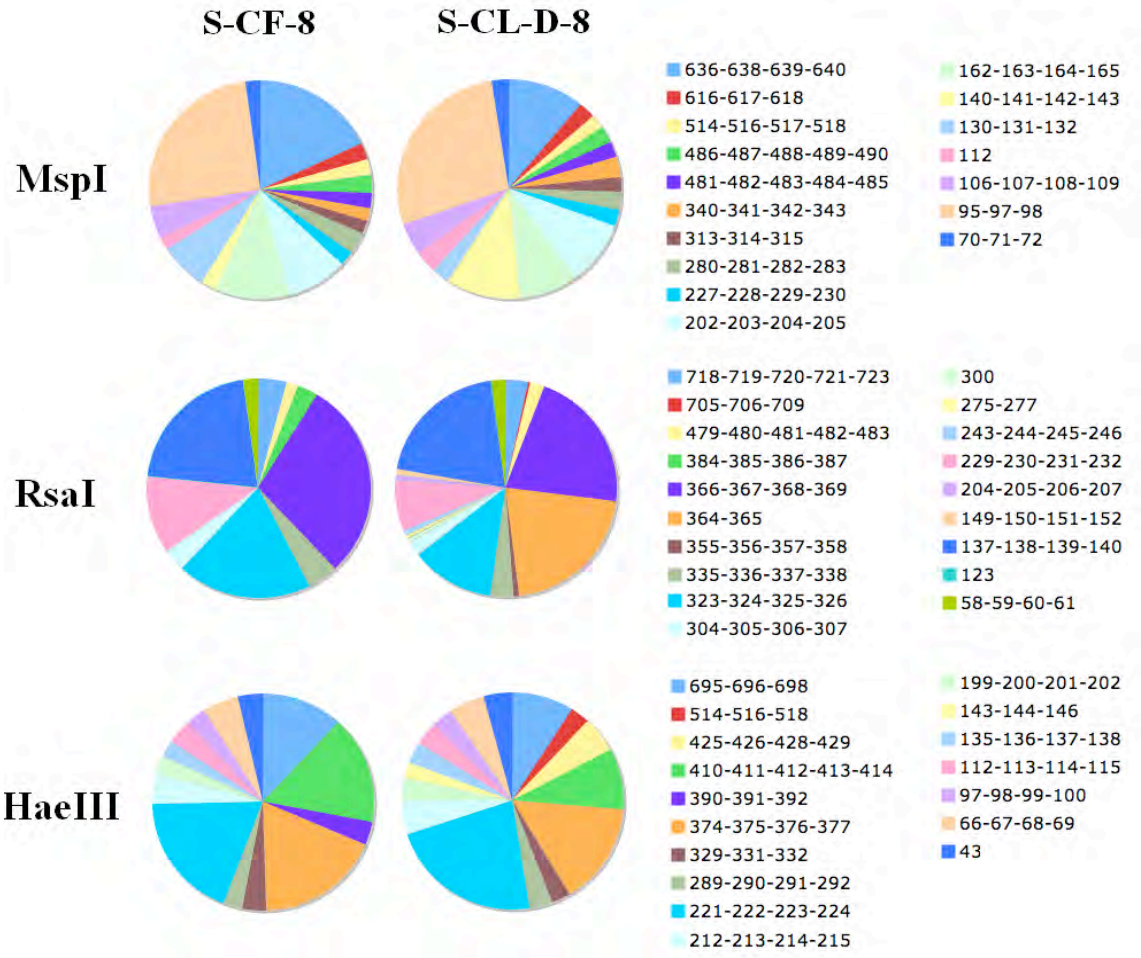


Figure 16 Relative abundance of T-RFLP OTUs from S-CF-8 and S-CL-D-8 samples, generating with *Msp*I, *Rsa*I and *Hae*III.

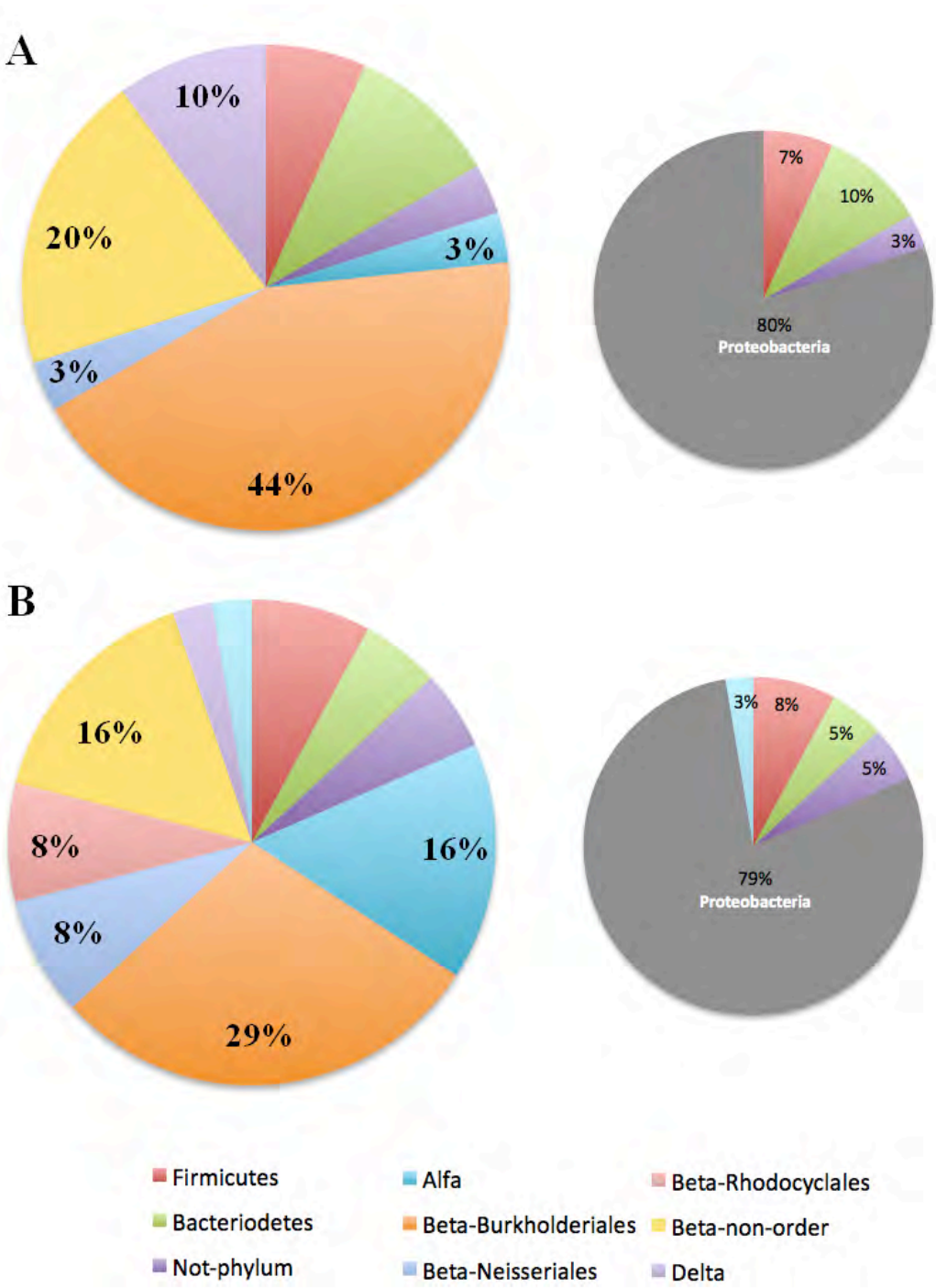


Figure 17 Percentage of different phylogenetic groups SOIL-DNT and SOIL at day 8 libraries.

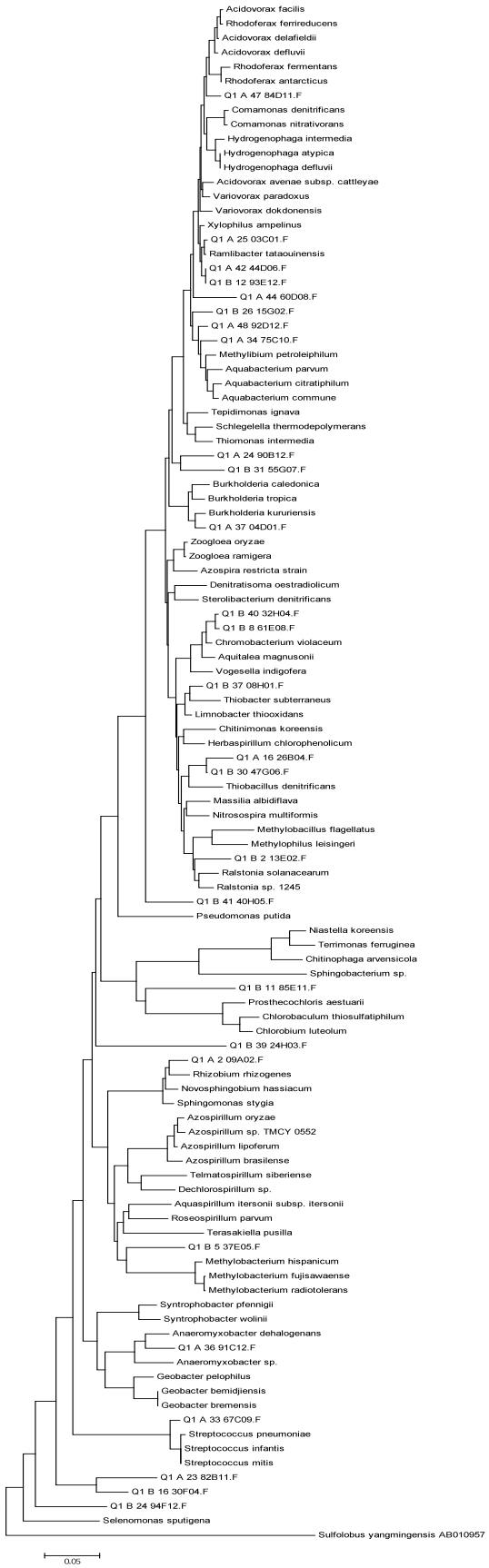

Acidovorax facilis
Rhodoferax ferrireducens
Acidovorax delafieldii
Acidovorax defluvii
Rhodoferax fermentans
Rhodoferax antarcticus
Q1 A 47 84D11.F
Comamonas denitrificans
Comamonas nitrativorans
Hydrogenophaga intermedia
Hydrogenophaga atypica
Hydrogenophaga defluvii
Acidovorax avenae subsp. cattleyae
Variovorax paradoxus
Variovorax dokdonensis
Xylophilus ampelinus
Q1 A 25 03C01.F
Ramlibacter tataouinensis
Q1 A 42 44D06.F
Q1 B 12 93E12.F
Q1 A 44 60D08.F
Q1 B 26 15G02.F
Q1 A 48 92D12.F
Q1 A 34 75C10.F
Methylibium petroleiphilum
Aquabacterium parvum
Aquabacterium citratiphilum
Aquabacterium commune
Tepidimonas ignava
Schlegelella thermodepolymerans
Thiomonas intermedia
Q1 A 24 90B12.F
Q1 B 31 55G07.F
Burkholderia caledonica
Burkholderia tropica
Burkholderia kururiensis
Q1 A 37 04D01.F
Zoogloea oryzae
Zoogloea ramigera
Azospira restricta strain
Denitratisoma oestradiolicum
Sterolibacterium denitrificans
Q1 B 40 32H04.F
Q1 B 8 61E08.F
Chromobacterium violaceum
Aquitalea magnusonii
Vogesella indigofera
Q1 B 37 08H01.F
Thiobacter subterraneus
Limnobacter thiooxidans
Chitinimonas koreensis
Herbaspirillum chlorophenolicum
Q1 A 16 26B04.F
Q1 B 30 47G06.F
Thiobacillus denitrificans
Massilia albidiflava
Nitrosospira multiformis
Methylobacillus flagellatus
Methylophilus leisingeri
Q1 B 2 13E02.F
Ralstonia solanacearum
Ralstonia sp. 1245
Q1 B 41 40H05.F
Pseudomonas putida
Niastella koreensis
Terrimonas ferruginea
Chitinophaga arvensicola
Sphingobacterium sp.
Q1 B 11 85E11.F
Prosthecochloris aestuarii
Chlorobaculum thiosulfatiphilum
Chlorobium luteolum
Q1 B 39 24H03.F
Q1 A 2 09A02.F
Rhizobium rhizogenes
Novosphingobium hassiacum
Sphingomonas stygia
Azospirillum oryzae
Azospirillum sp. TMCY 0552
Azospirillum lipoferum
Azospirillum brasilense
Telmatospirillum siberiense
Dechlorospirillum sp.
Aquaspirillum itersonii subsp. itersonii
Roseospirillum parvum
Terasakiella pusilla
Q1 B 5 37E05.F
Methylobacterium hispanicum
Methylobacterium fujisawaense
Methylobacterium radiotolerans
Syntrophobacter pfennigii
Syntrophobacter wolinii
Anaeromyxobacter dehalogenans
Q1 A 36 91C12.F
Anaeromyxobacter sp.
Geobacter pelophilus
Geobacter bemidjiensis
Geobacter bremensis
Q1 A 33 67C09.F
Streptococcus pneumoniae
Streptococcus infantis
Streptococcus mitis
Q1 A 23 82B11.F
Q1 B 16 30F04.F
Q1 B 24 94F12.F
Selenomonas sputigena
Sulfolobus yangmingensis AB010957
0.05

Figure 18 Phylogenetic Tree of clones from CL-S (day8) and SOIL (day 8) with the nearest neighbors using sequences with Forward primer (Type II)

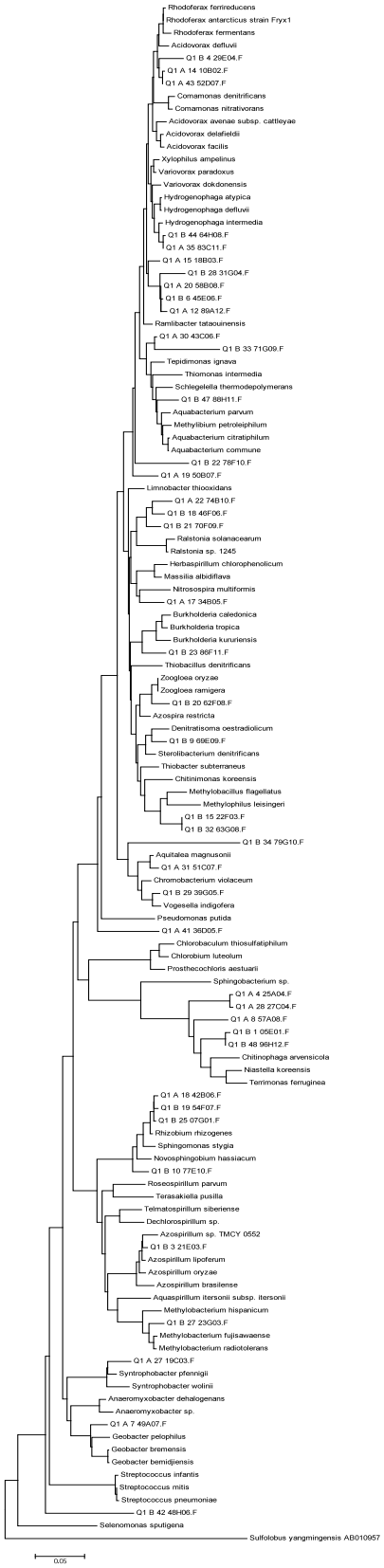

Rhodoferax ferrireducens
Rhodoferax antarcticus strain Fryx1
Rhodoferax fermentans
Acidovorax delfuvii
Q1 B 4 29E04.F
Q1 A 14 10B02.F
Q1 A 43 52D07.F
Comamonas denitrificans
Comamonas nitrativorans
Acidovorax avenae subsp. cattleyae
Acidovorax delafieldii
Acidovorax facilis
Xylophilus ampelinus
Variovorax paradoxus
Variovorax dokdonensis
Hydrogenophaga atypica
Hydrogenophaga defluvii
Hydrogenophaga intermedia
Q1 B 44 64H08.F
Q1 A 35 83C11.F
Q1 A 15 18B03.F
Q1 B 28 31G04.F
Q1 A 20 58B06.F
Q1 B 6 45E06.F
Q1 A 12 89A12.F
Ramlibacter tataouinensis
Q1 A 30 43C06.F
Q1 B 33 71G09.F
Tepidimonas ignava
Thiomonas intermedia
Schlegelella thermodepolymerans
Q1 B 47 88H11.F
Aquabacterium parvum
Methylibium petroleiphilum
Aquabacterium citratiphilum
Aquabacterium commune
Q1 B 22 78F10.F
Q1 A 19 50B07.F
Limnobacter thiooxidans
Q1 A 22 74B10.F
Q1 B 18 46F06.F
Q1 B 21 70F09.F
Ralstonia solanacearum
Ralstonia sp. 1245
Herbaspirillum chlorophenolicum
Massilia albidiflava
Nitrosospira multiformis
Q1 A 17 34B05.F
Burkholderia caledonica
Burkholderia tropica
Burkholderia kururiensis
Q1 B 23 86F11.F
Thiobacillus denitrificans
Zoogloea oryzae
Zoogloea ramigera
Q1 B 20 62F08.F
Azospira restricta
Denitratisoma oestradiolicum
Q1 B 9 69E09.F
Sterolibacterium denitrificans
Thiobacter subterraneus
Chitinimonas koreensis
Methylobacillus flagellatus
Methylophilus leisingeri
Q1 B 15 22F03.F
Q1 B 32 63G08.F
Q1 B 34 79G10.F
Aquitalea magnusonii
Q1 A 31 51C07.F
Chromobacterium violaceum
Q1 B 29 39G05.F
Vogesella indigofera
Pseudomonas putida
Q1 A 41 36D05.F
Chlorobaculum thiosulfatiphilum
Chlorobium luteolum
Prosthecochloris aestuarii
Sphingobacterium sp.
Q1 A 4 25A04.F
Q1 A 28 27C04.F
Q1 A 8 57A08.F
Q1 B 1 05E01.F
Q1 B 48 99H12.F
Chitinophaga arvensicola
Niastella koreensis
Terrimonas ferruginea
Q1 A 18 42B06.F
Q1 B 19 54F07.F
Q1 B 25 07G01.F
Rhizobium rhizogenes
Sphingomonas stygia
Novosphingobium hassiacum
Q1 B 10 77E10.F
Roseospirillum parvum
Terasakiella pusilla
Telmatospirillum siberiense
Dechlorospirillum sp.
Azospirillum sp. TMCY 0552
Q1 B 3 21E03.F
Azospirillum lipoferum
Azospirillum oryzae
Azospirillum brasilense
Aquaspirillum itersonii subsp. itersonii
Methylobacterium hispanicum
Q1 B 27 23G03.F
Methylobacterium fujisawaense
Methylobacterium radiotolerans
Q1 A 27 19C03.F
Syntrophobacter pfennigii
Syntrophobacter wolinii
Anaeromyxobacter dehalogenans
Anaeromyxobacter sp.
Q1 A 7 49A07.F
Geobacter pelophilus
Geobacter bremensis
Geobacter bemidjiensis
Streptococcus infantis
Streptococcus mitis
Streptococcus pneumoniae
Q1 B 42 48H06.F
Selenomonas sputigena
Sulfolobus yangmingensis AB010957
0.05

Figure 19 Phylogenetic Tree of clones from CL-S (day8) and SOIL (day 8) with the nearest neighbors using sequences with Reverse primer (Type II)

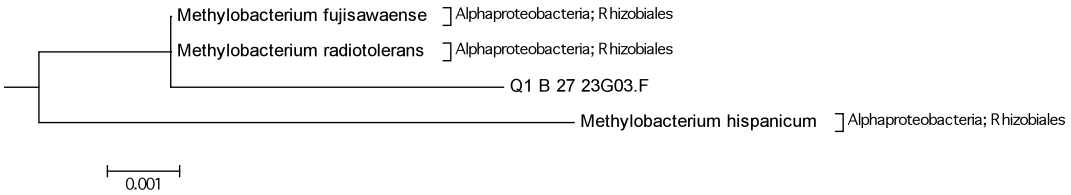


Figure 20 Zoom of branch from phylogenetic tree Type IV used to identify clone B27.

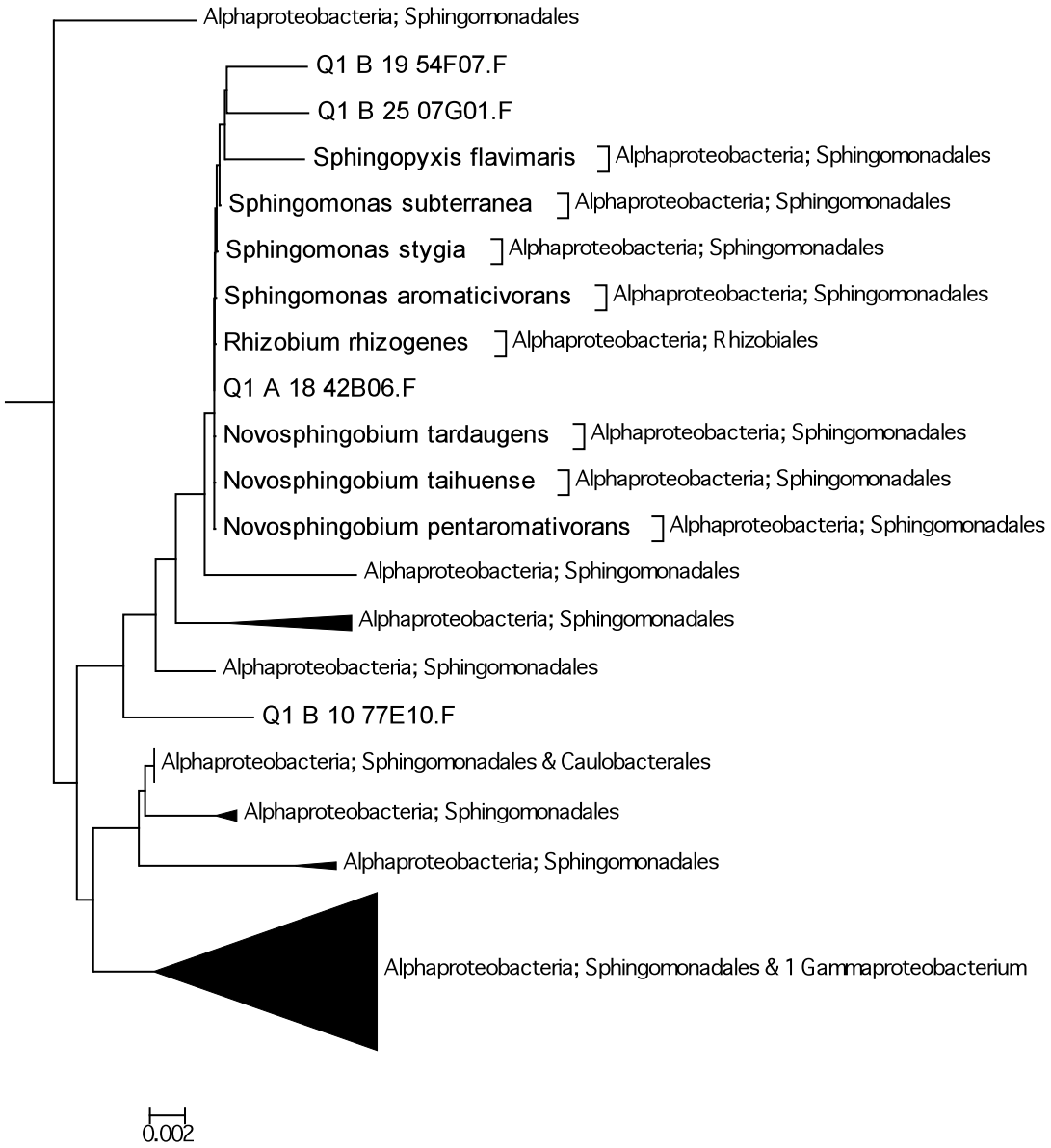


Figure 21 Zoom of branch from phylogenetic tree Type IV used to identify clones A18, B10, B19 and B25. *Sphingomonas* and *Novosphingobium* are genera from the family Sphingomonadaceae.

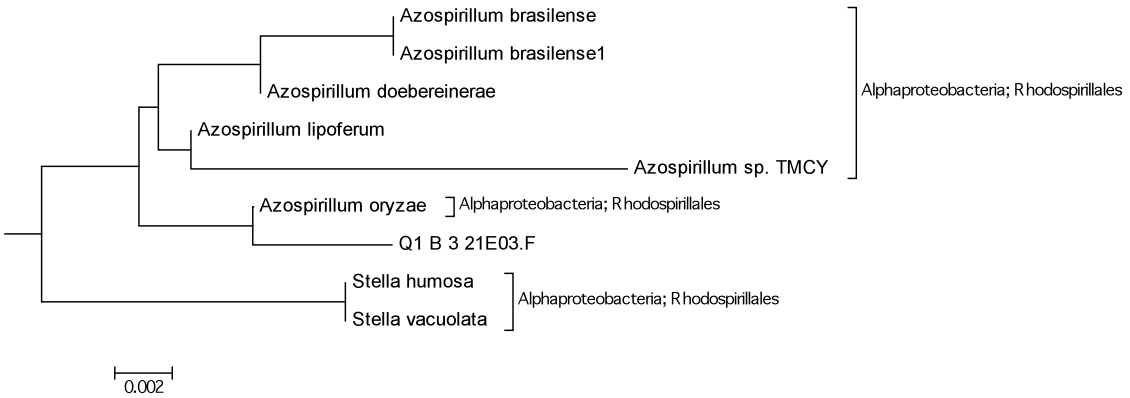


Figure 22 Zoom of branch from phylogenetic tree Type IV used to identify clone B3.

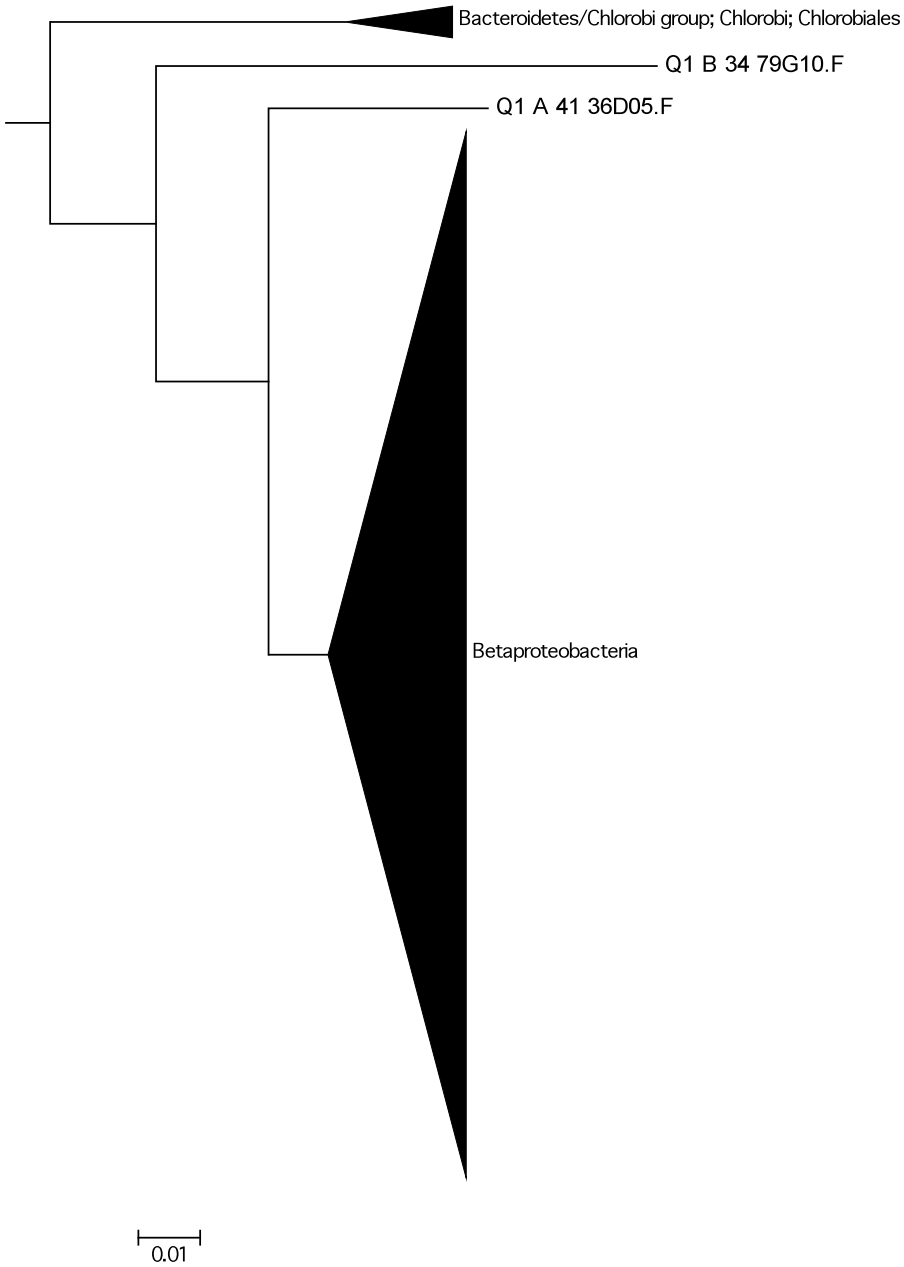


Figure 23 Zoom of branch from phylogenetic tree Type used used to identify clones A41 and B34.

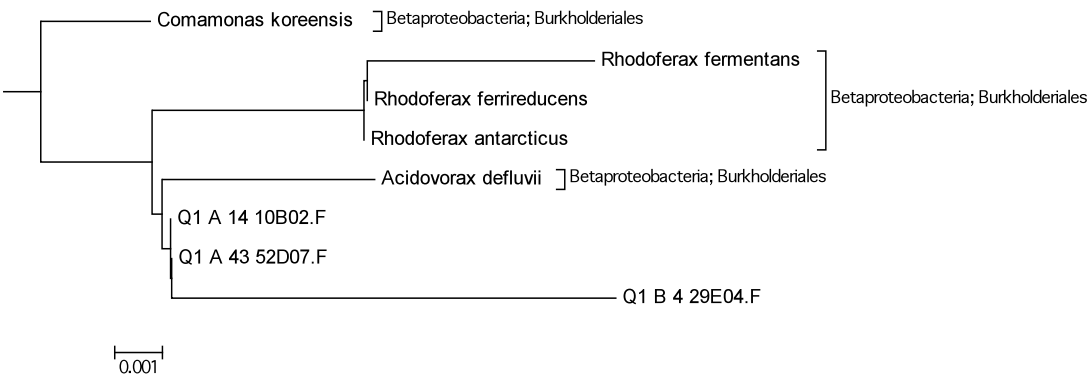


Figure 24 Zoom of branch from phylogenetic tree Type IV used to identify clones A14, A43 and B4. *Comamonas*, *Rhodoferax*, *Acidovorax* are genera from the family Comamonadaceae

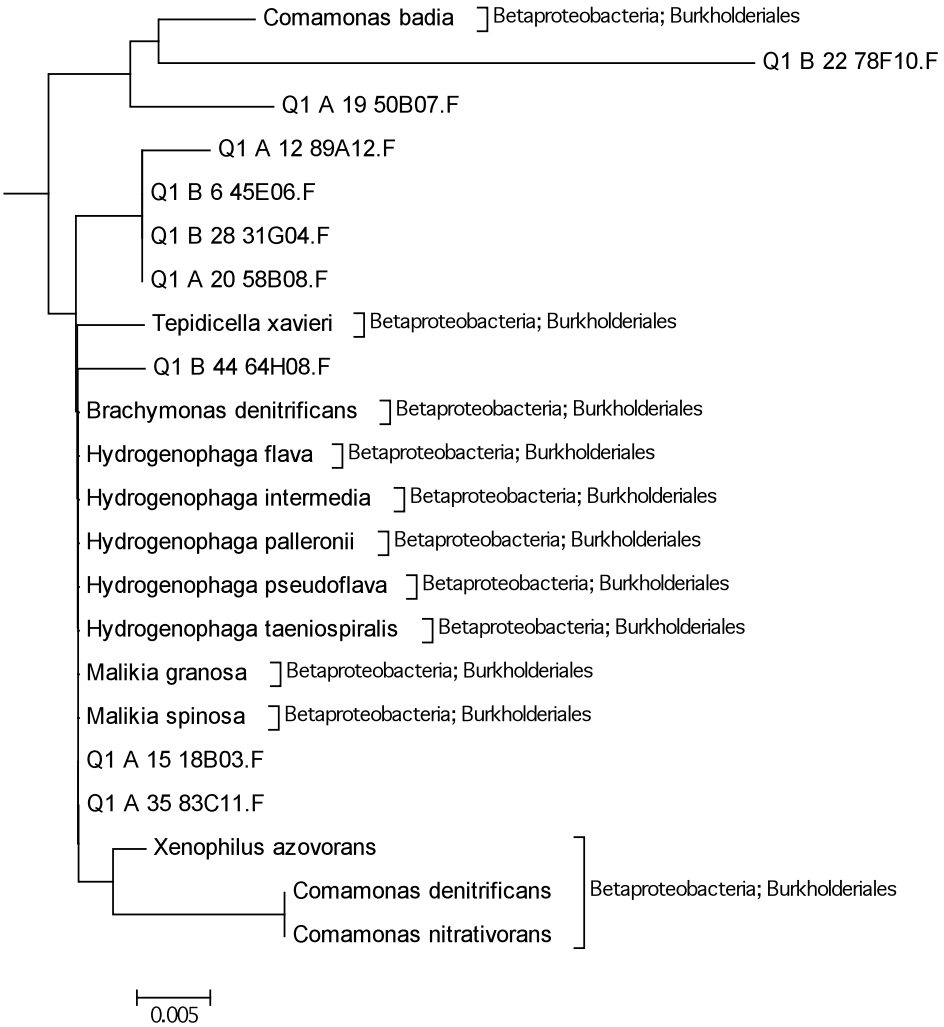


Figure 25 Zoom of branch from phylogenetic tree Type III used to identify clones A12, A15, A19,A20, A35, B6, B28, B44. *Comamonas, Hydrogenophaga, Malikia, Bachymonas, Xenophilu*s are genera from the family Comamonadaceae.

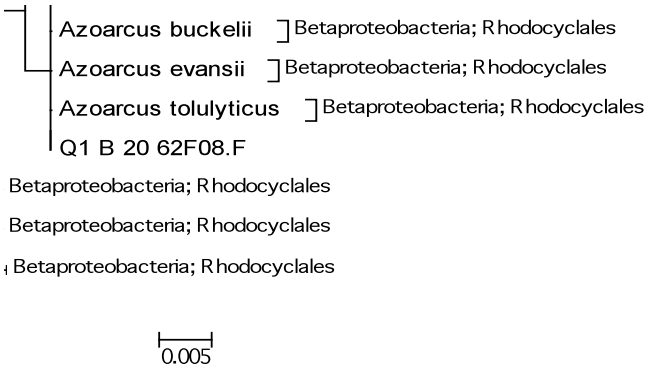


Figure 26 Zoom of branch from phylogenetic tree Type used to identify clone B20 .

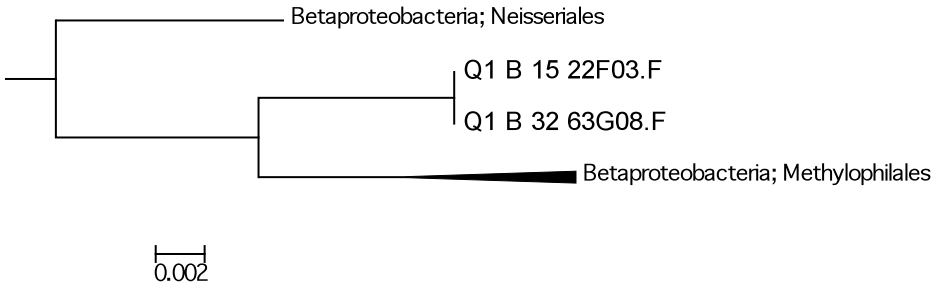


Figure 27 Zoom of branch from phylogenetic tree Type IV used to identify clones B15 and B32.

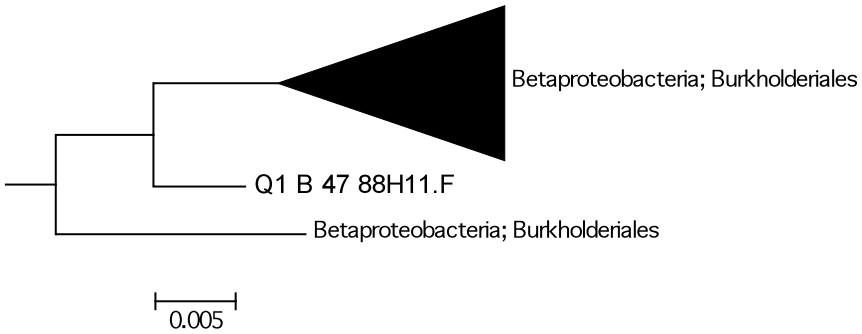


Figure 28 Zoom of branch from phylogenetic tree Type IV used to identify clone B47.

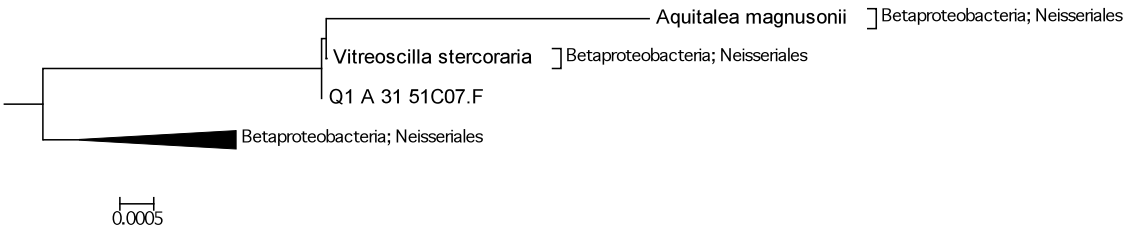


Figure 29 Zoom of branch from phylogenetic tree Type IV used to identify clone A31.

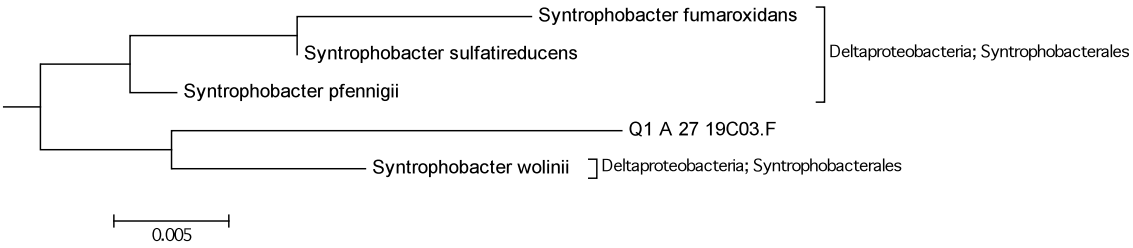


Figure 30 Zoom of branch from phylogenetic tree Type IV used to identify clone A27.

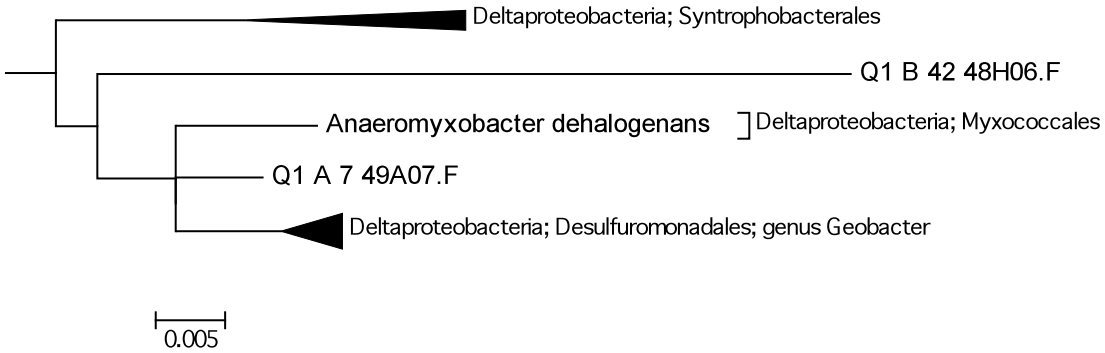


Figure 31 Zoom of branch from phylogenetic tree Type IV used to identify clones A7 and B42 .

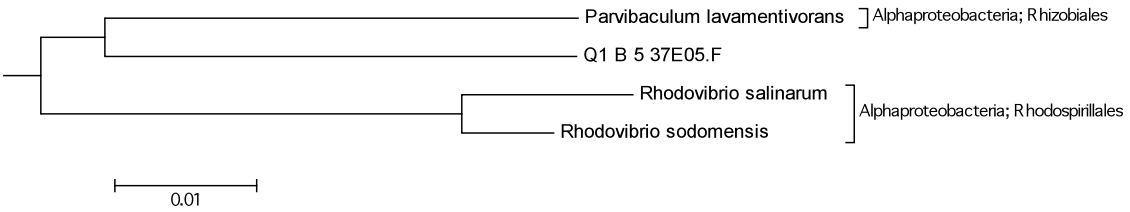


Figure 32 Zoom of branch from phylogenetic tree Type III used to identify clone B5.

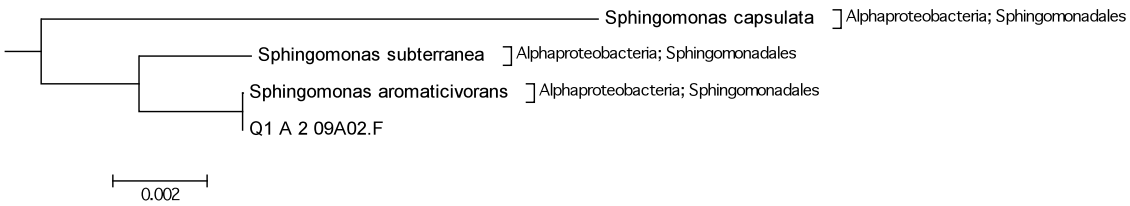


Figure 33 Zoom of branch from phylogenetic tree Type III used to identify clone A2.

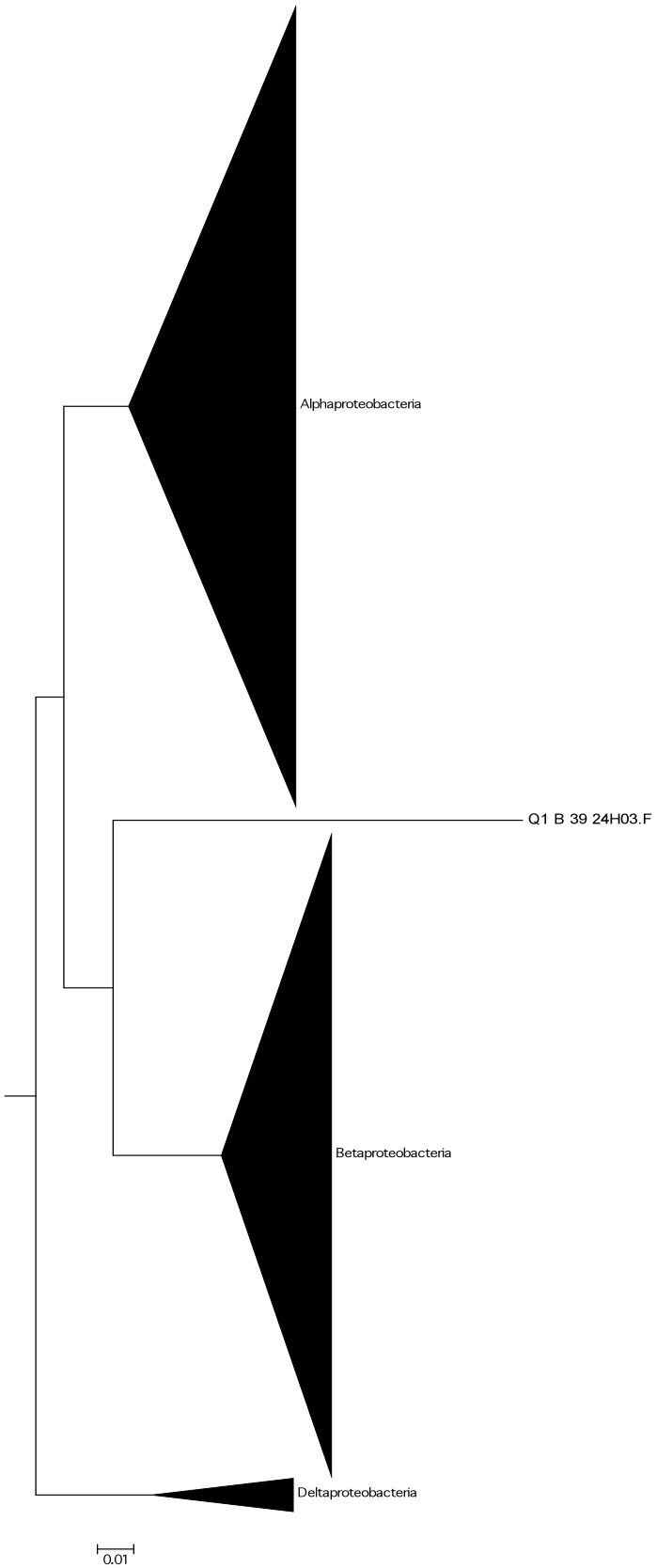


Figure 34 Zoom of branch from phylogenetic tree Type III used to identify Clone B39.

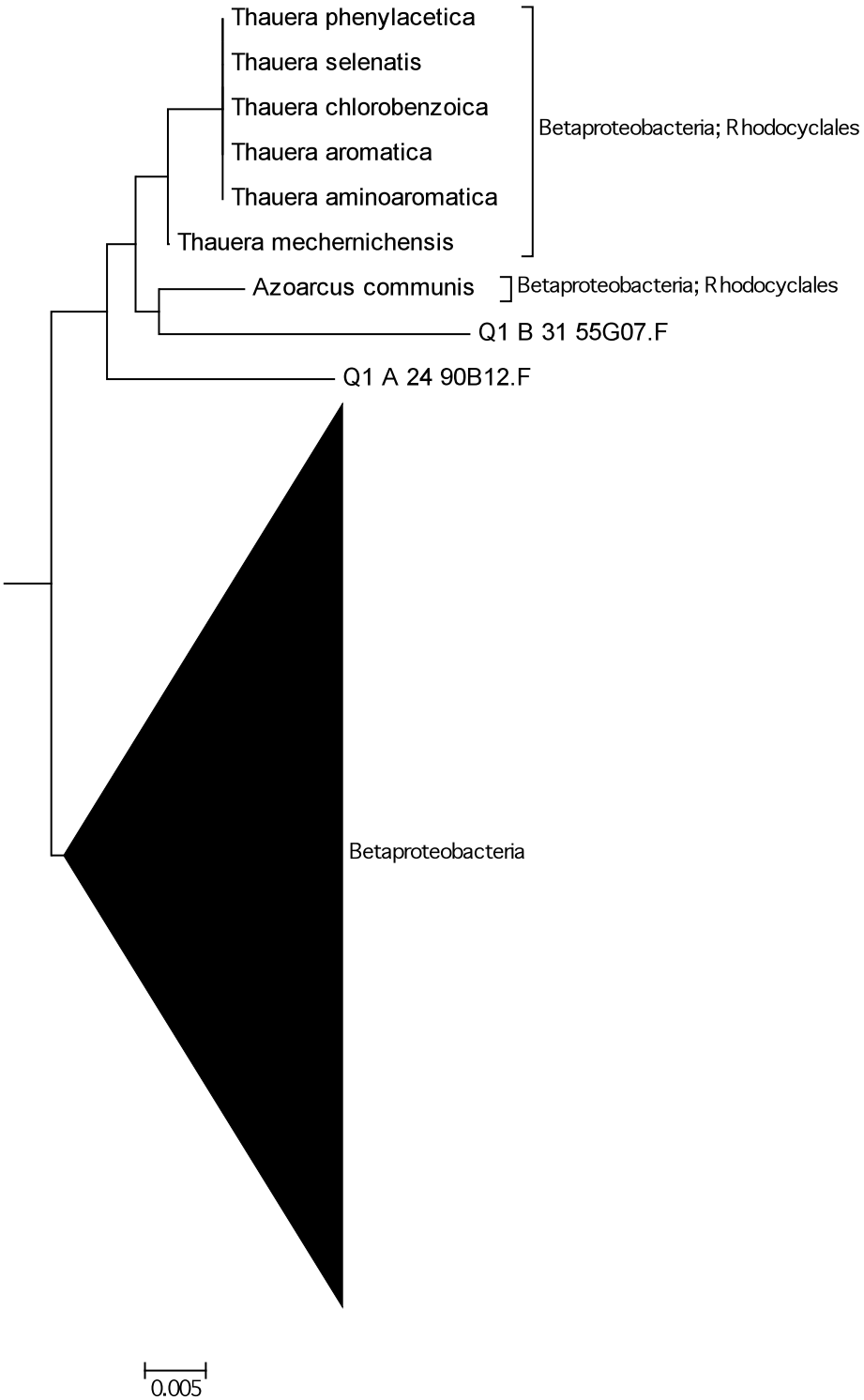


Figure 35 Zoom of branch from phylogenetic tree Type III used to identify clones A24 and B31.

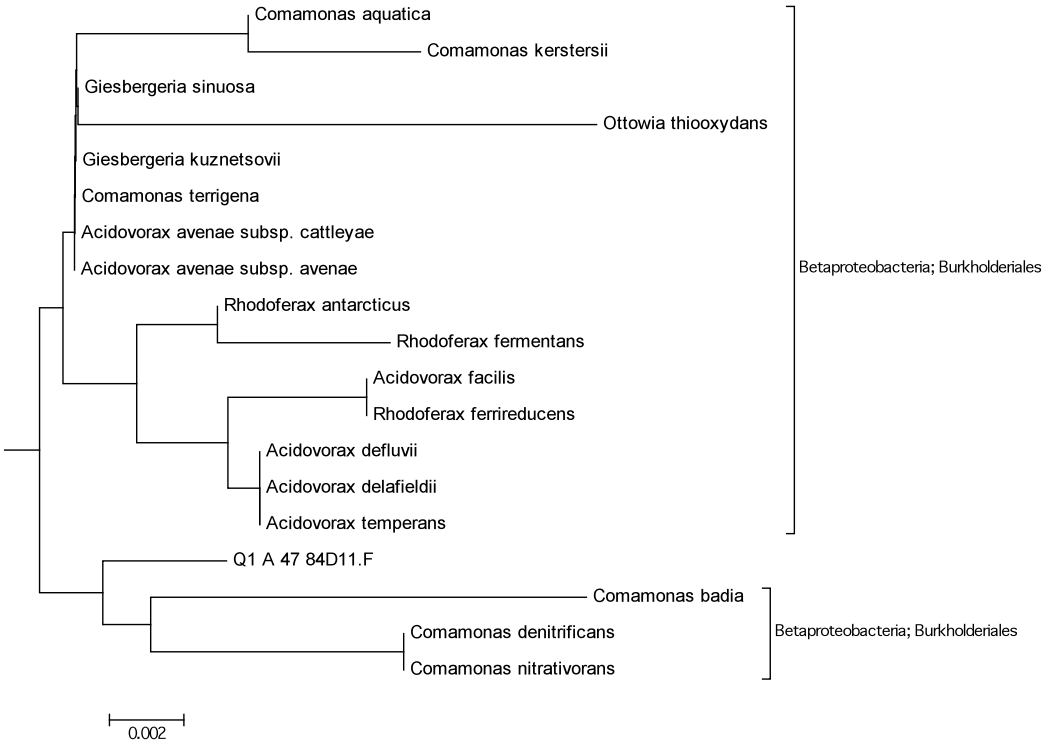


Figure 36 Zoom of branch from phylogenetic tree Type III used to identify clone A47. *Giesbergeria, Acidovorax, Rhodoferax, Comamonas* are genera from the family Comamonadaceae (Type III).

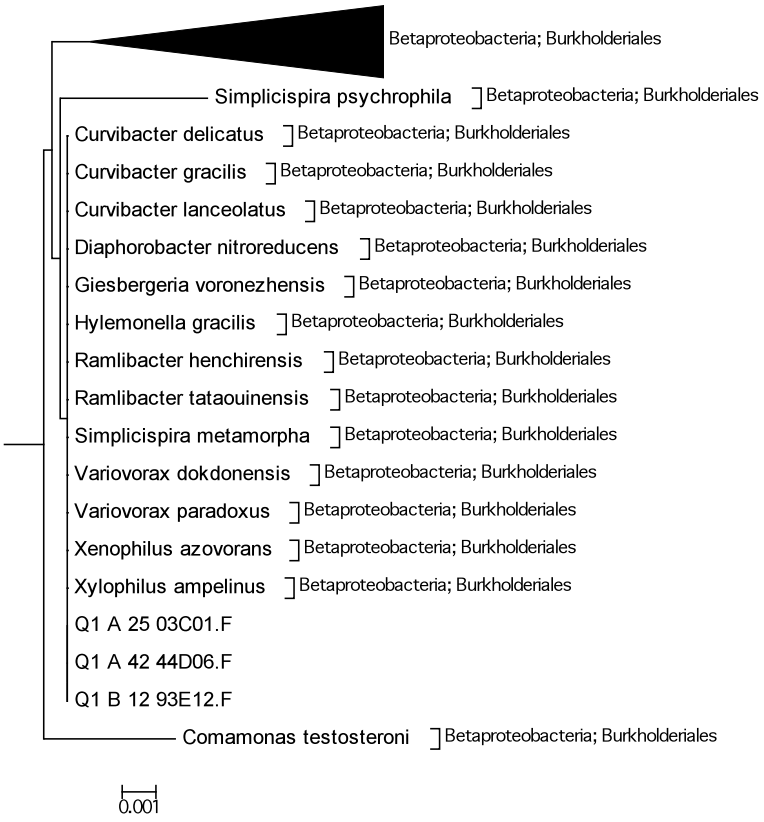


Figure 37 Zoom of branch from phylogenetic tree Type III used to identify clone A25, A42 and B12. *Curvibacter, Comamonas, Xenophilus, Simplicispira, Hylemonella, Diaphorobacter, Ramlibacter* are genera from the family Comamonadaceae (Type III).

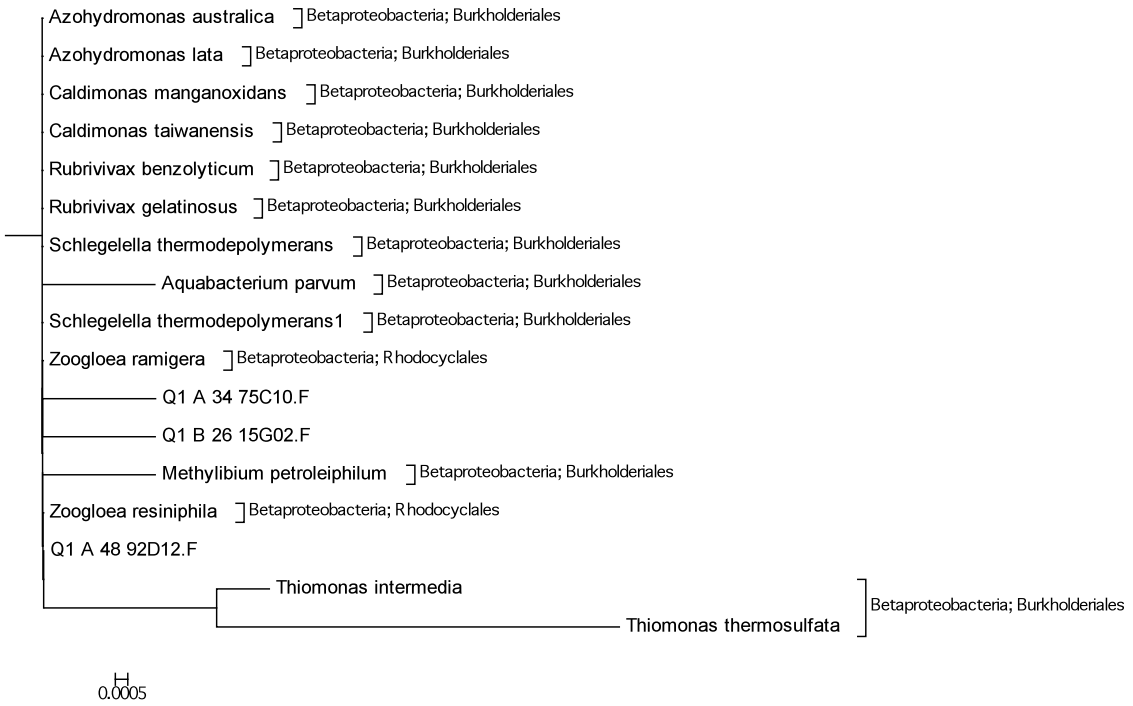


Figure 38 Zoom of branch from phylogenetic tree Type III used to identify clones A34, A48 and B26 (Type III).

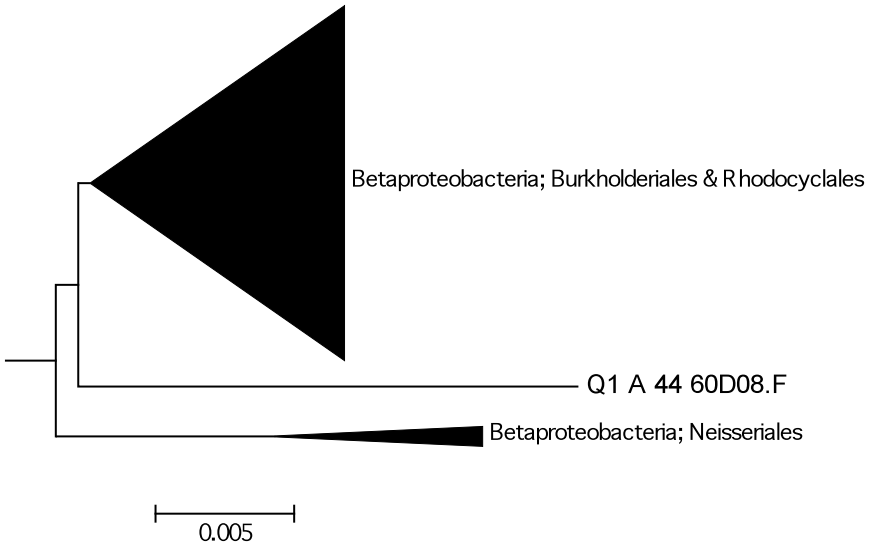


Figure 39 Zoom of branch from phylogenetic tree used to identify A44 clone (Type III).

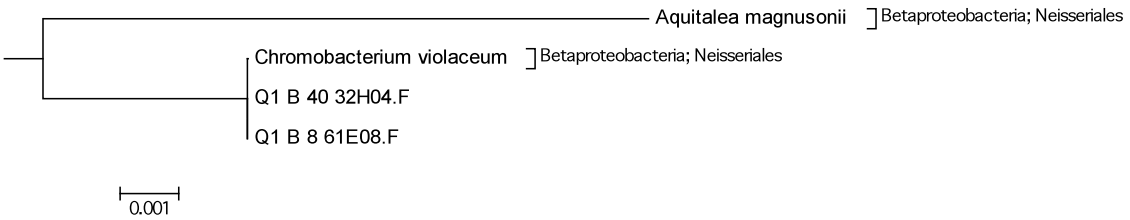


Figure 40 Zoom of branch from phylogenetic tree used to identify clone B8 and B40. (Type III). *Aquitalea and Chromobacterium* are genera from the family Neisseriaceae

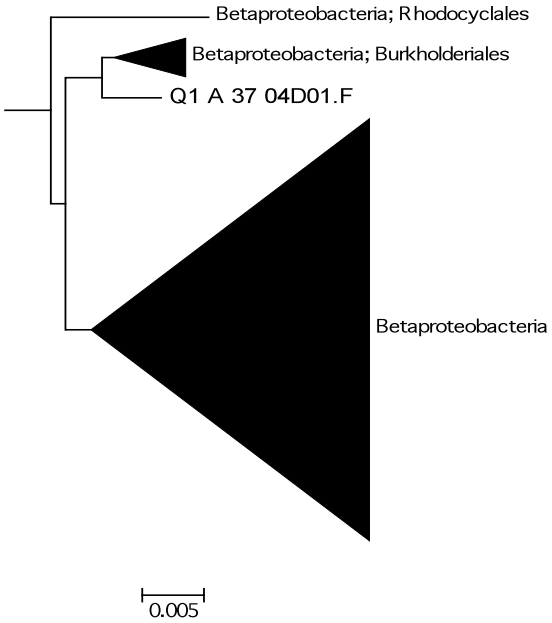


Figure 41 Zoom of Betaproteobacteria branch from phylogenetic tree Type III used to identify clone A37.

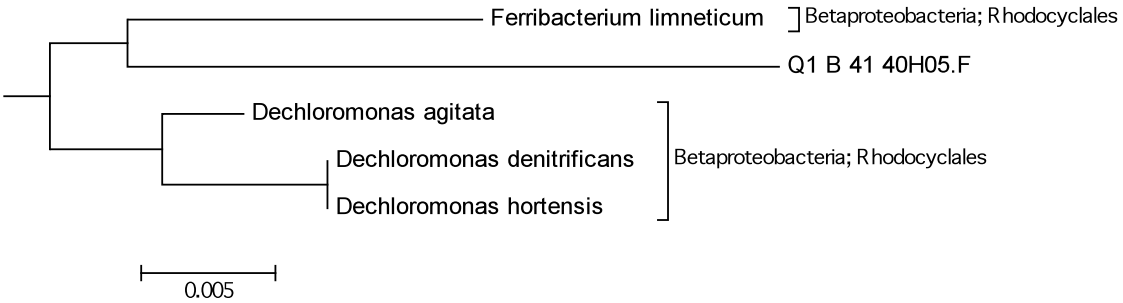


Figure 42 Zoom of branch from phylogenetic tree Type III to idenfied clone B41. Ferribacterium and Dechloromonas are genera from the family Rhodocyclaceae.

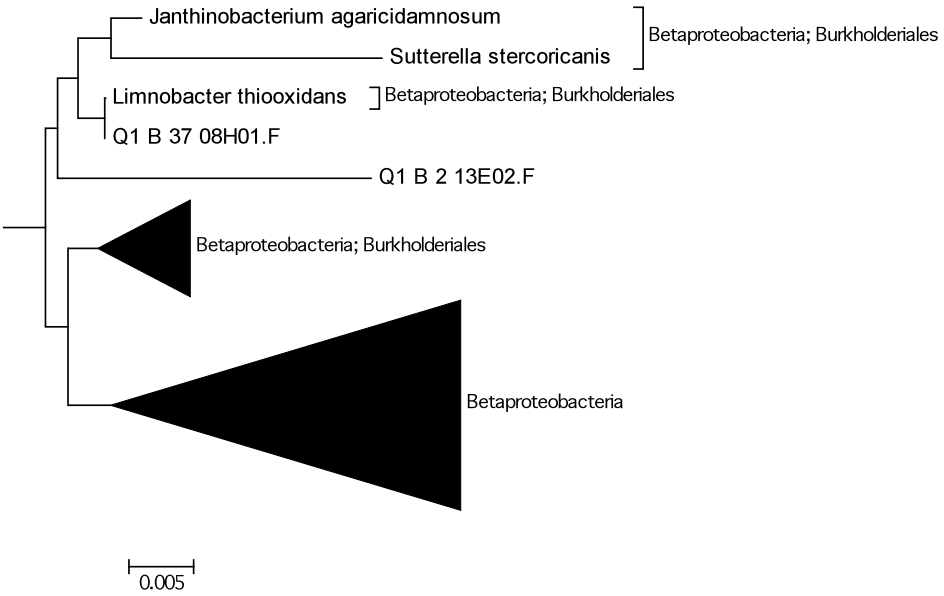


Figure 43 Zoom of branch from phylogenetic tree Type III used to identify clone B2 and B37.

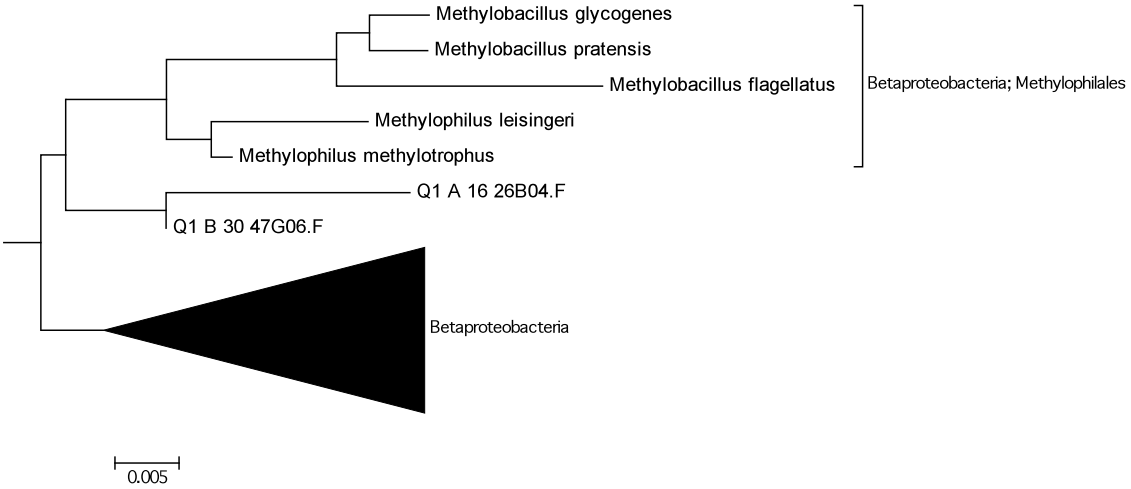


Figure 44 Zoom of branch from phylogenetic tree Type III used to identify clone A16 and B30.

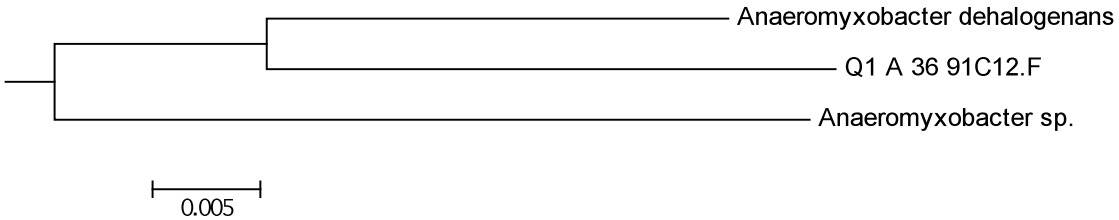


Figure 45 Zoom of branch from phylogenetic tree Type III used to identify clone A36.

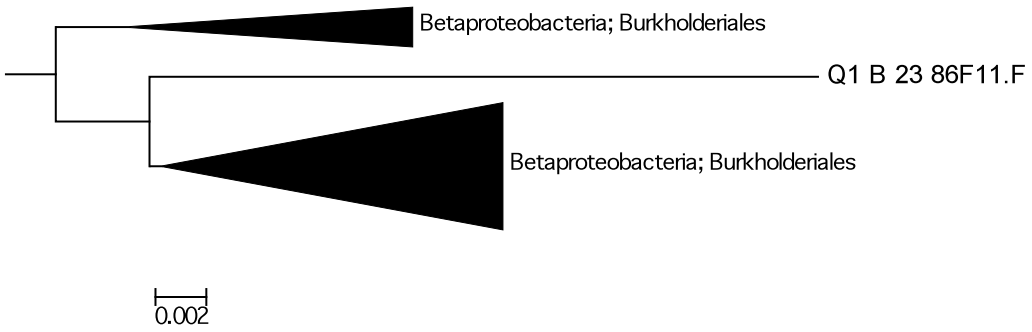


Figure 46 Zoom of branch from phylogenetic tree Type V used to identify clone B23.

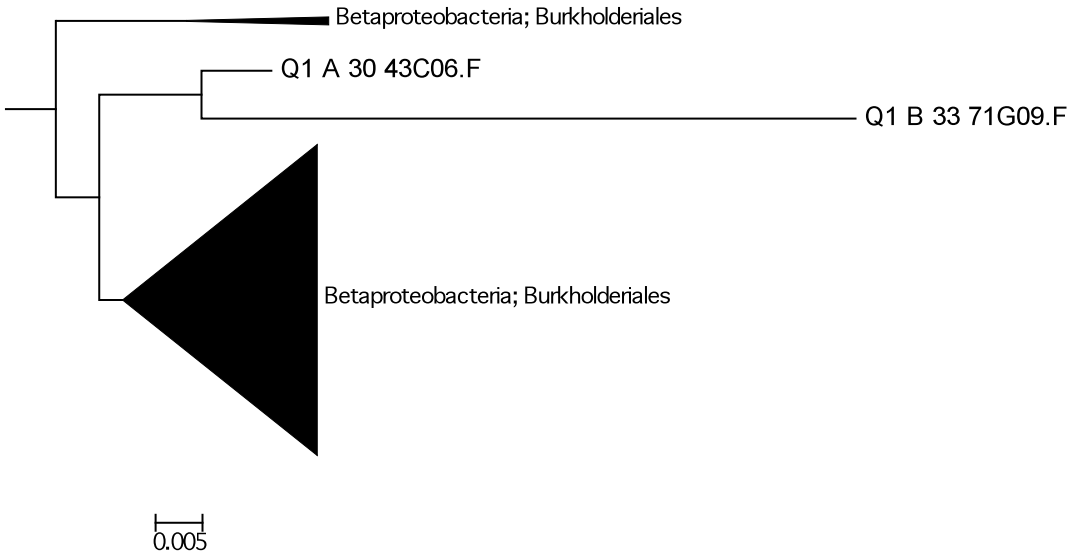


Figure 47 Zoom of branch from phylogenetic tree Type V used to identify clones A30 and B33 (Type V).

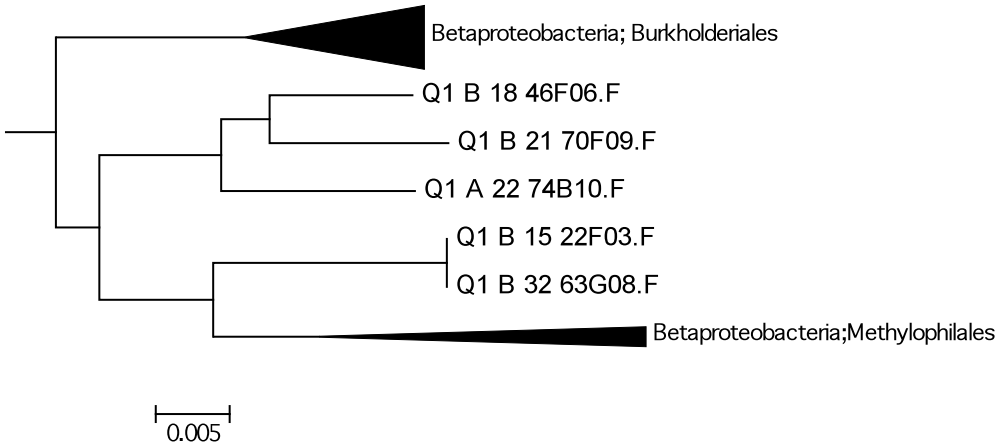


Figure 48 Zoom of branch from phylogenetic tree Type V used to identify clones A22, B15, B18, B21 and B32.

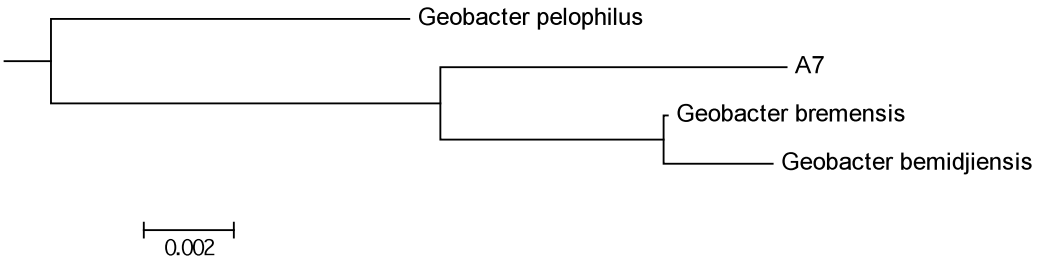


Figure 49 Zoom of branch from phylogenetic tree Type VI used to identify clone A7.

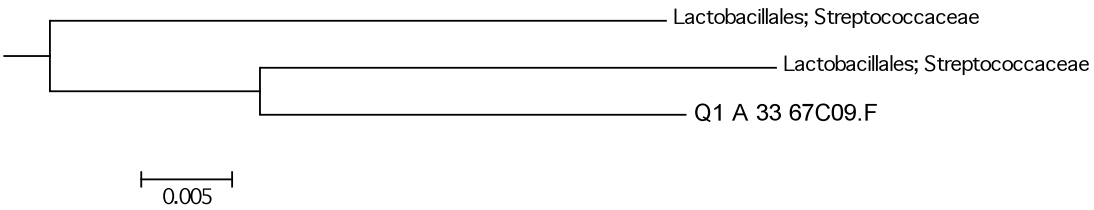


Figure 50 Zoom of branch from phylogenetic tree Type VII used to identify clone A33 (Type VII).

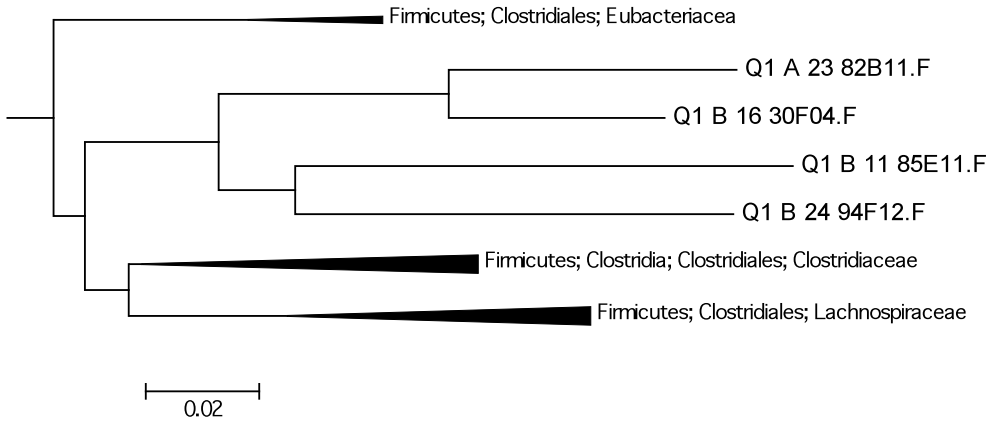


Figure 51 Zoom of branch from phylogenetic tree Type VII used used to identify clones A23, B11, B16 and B24.

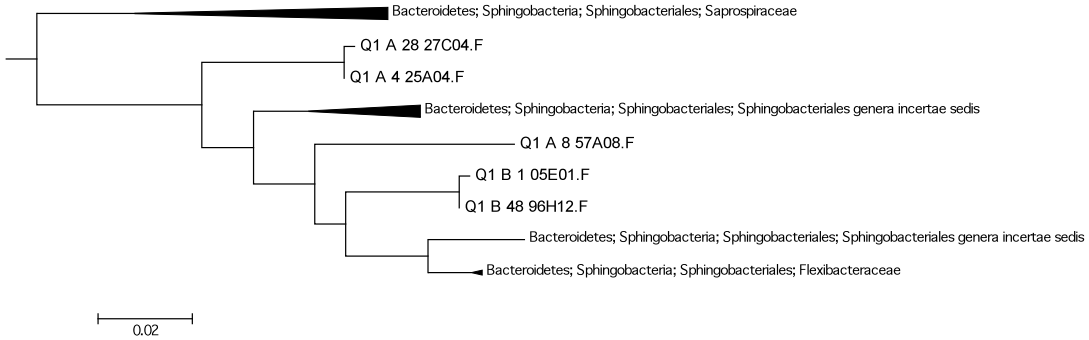


Figure 52 Zoom of branch from phylogenetic tree Type VIII used to identify clone A4, A8, A26, B1 and B48.

Appendix A. Preliminary study of 2,4-DNT effect and degradation

Materials and methods. Soil samples were collected from two different places at the AFWTF Laguna Anones (L) and Monte David (S) in the island of Vieques, Puerto Rico. Biotic microcosms (4 replicates) were prepared by mixing 3 ml slurry (of 1:1 water: soil) plus 17 ml of BLKN medium, spiked with 40 µl of 10mM explosive's solution (2,4-DNT). Abiotic controls (3 replicates) were prepared by autoclaving live microcosms, (explosive was added after autoclaving). Microcosms were incubated at room temperature at 260 rpm for about 9 days. Sampling and analysis Samples from the microcosms were collected from each of the vials at different times (0, 3 and 7 days). Explosive concentration was measured with HPLC and nitrate was quantified using the nitrate A, nitrate B Method (Colorimetric determination) as previously described.

Results and Discussion. Correspondence Analysis of microbial communities shows an initial separation (day 0) of distinct microbial communities from different locations, where laguna Anones (Vieques) and Guanica (Dry Forest, Puerto Rico) communities cluster nearest than Monte David (Vieques) (**Figure** 1A). After day 3 of incubation different location communities became more similar, clustering together independently of the treatment and origin. Evidence of normalization of communities is observed by overlapping of the confidence ellipses (95%).

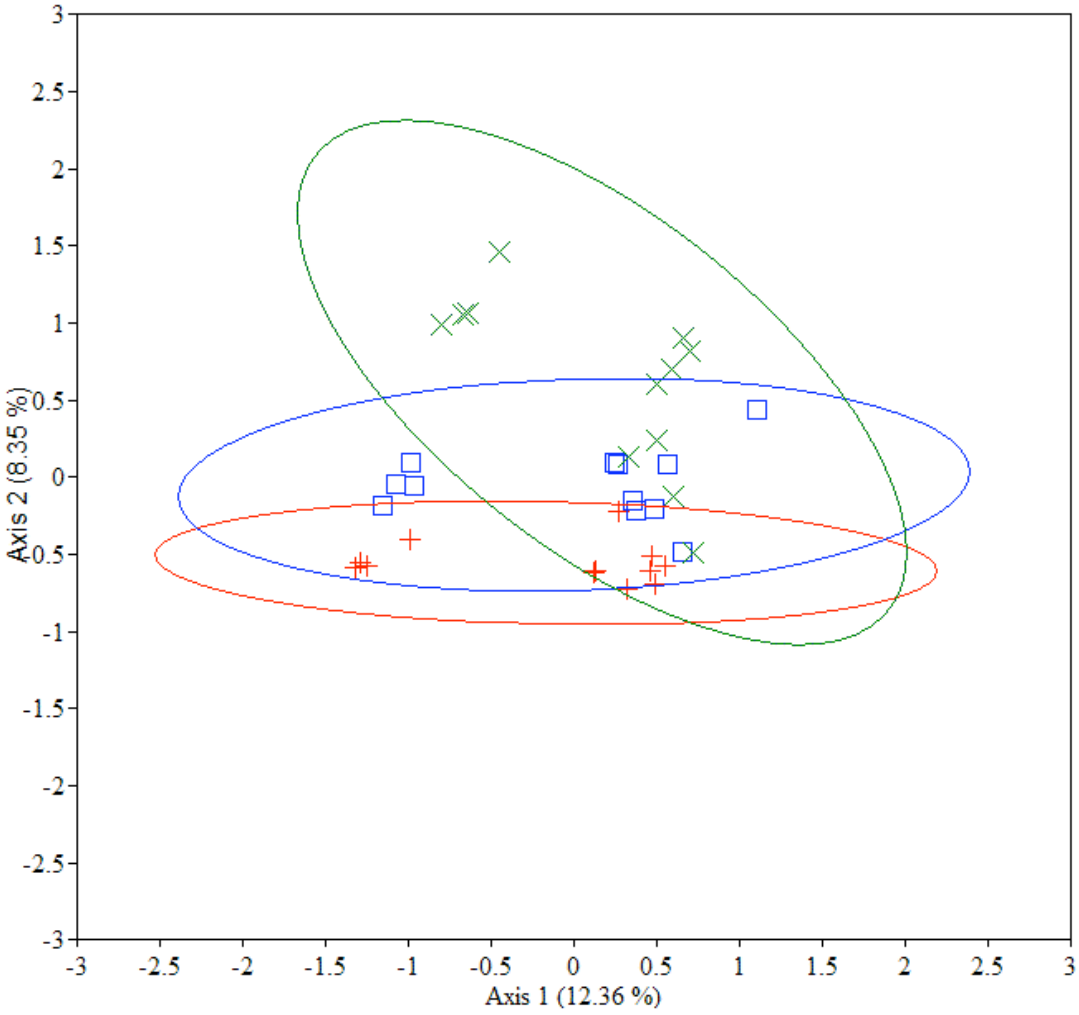


Figure A 1 Correspondence analysis of T-RFLP profiles of 16S rRNA of Vieques and Guanica Microcosms under shaking conditions. Green cruces represent Monte David Microcosms; Blue squares represent Guanica microcosms, Red cruces represent Laguna Anones Microcosms. Ellipses represent 95 % of confidence regions for the groups.

Effect of incubation over time could be explained by axis I (**Figure** 2), due to the clear clustering of communities by incubation days. Axis 1 of Correspondence Analysis explained 12.36 % of variability while Axis 2 explains 8.35%. Axis 2 separates scatter dots by sample location, grouping same location microcosms closer than different location. Guanica and Laguna Anones 2,4-DNT amended treatments are not clearly separated of non-amended microcosms, therefore 2,4-DNT effects on microbial

community structure is not evident with the concentrations used in this experiment. Monte David microcosms respond to addition of explosives, at day 0 non-amended and 2,4-DNT amended microcosms cluster together, however a differential effect of treatments on microbial communities is observed at day 3 and 7, separating non-amended and amended communities.

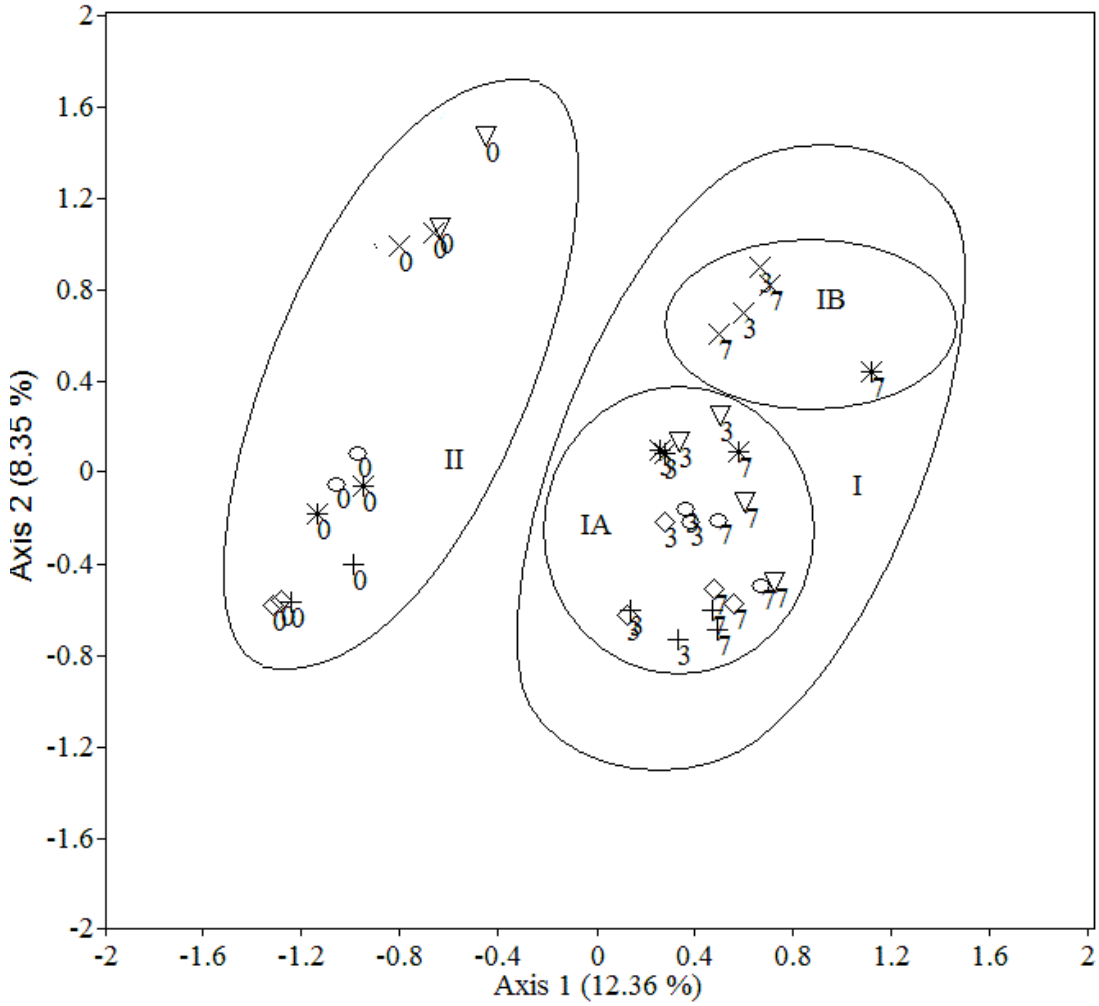


Figure A 2 Correspondence analysis of T-RFLP profiles of 16S rRNA of Vieques and Guanica Microcosms under shaking conditions. ▽ Monte David non-amended, ╳ Monte David with 2,4-DNT , ○ Guanica non-amended, ✳ Guanica with 2,4-DNT , ◇ Laguna Anones non-amended and ┼ Laguna Anones with 2,4-DNT . Grouping of samples was performed using cluster analysis of T-RFLP matrix, using Bray-Curtis to calculate similarity.

Correspondence analysis and Cluster Analysis clearly separates two groups with a similarity < 0.2 (bootstrap value 100), time 0 samples from all microcosms (II), and day 3 and 7 cluster (I),. In cluster II, same origin microcosms group apart from each other, Laguna Anones microcosms (bootstrap value 98), Guánica microcosms (bootstrap value 98) and Monte David microcosms (bootstrap value 98). Cluster I is subgroup into two cluster, the first cluster (IA) is formed by day 3 and 7 microbial communities of Guánica, Laguna Anones and none amended microcosms from Monte David, the second cluster (IB) is formed by day 3 and 7 microbial communities of 2,4-DNT amended microcosm from Monte David soil and one replicate from amended Guánica microcosms. Cluster IA is subdivided by day of sampling, day 3 and 7. As observed in the correspondence Analysis, inside the different cluster, Guánica communities are more similar to Laguna Anones than to Monte David.

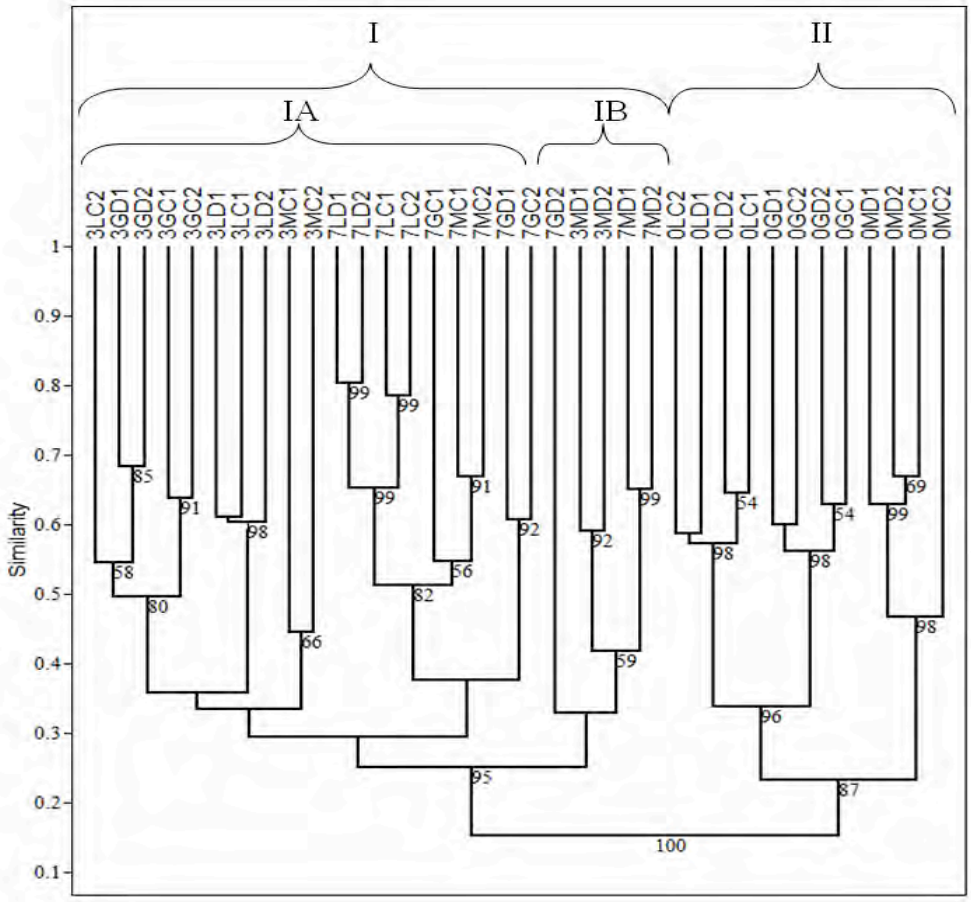


Figure A 3 Cluster analysis of 16S rRNA T-RFLP profiles, using Bray-Curtis index to calculate similarity measures, 1000 bootstraps were performed, only clusters with values superior to 50 are shown.

Mineralization of 2,4-DNT is coupled with nitrite (NO_2) production, so a decreased on 2,4-DNT with nitrite production could be used as an evidence of biological degradation. Concentration of 2,4-DNT on Guánica microcosms remains constant trough all sample days, no biological activity can be related to CL microcosms, additionally nitrite concentration decreased in all observed treatments (CA, CL and CF). Concentration of 2,4-DNT on Laguna Anones reduces from 3 mg/L to 1.5 mg/L however no difference is observed between amended (CL) and abiotic (CA) microcosms. Concentration of nitrite on Laguna Anones microcosms decreases on CL and CF treatments, an increase on abiotic CA microcosms is observed on day 3, at day 7 concentrations reduces out of the

quantification limit. Decrease on 2,4-DNT concentration of all treatments is observed on Monte David microcosms, 2,4-DNT decreases from 2.5 mg/l to 1.64 mg/L on CA and to 1.01 mg/L on CL microcosm. Nitrite production is observed also on CL microcosms to 49 mg/l.

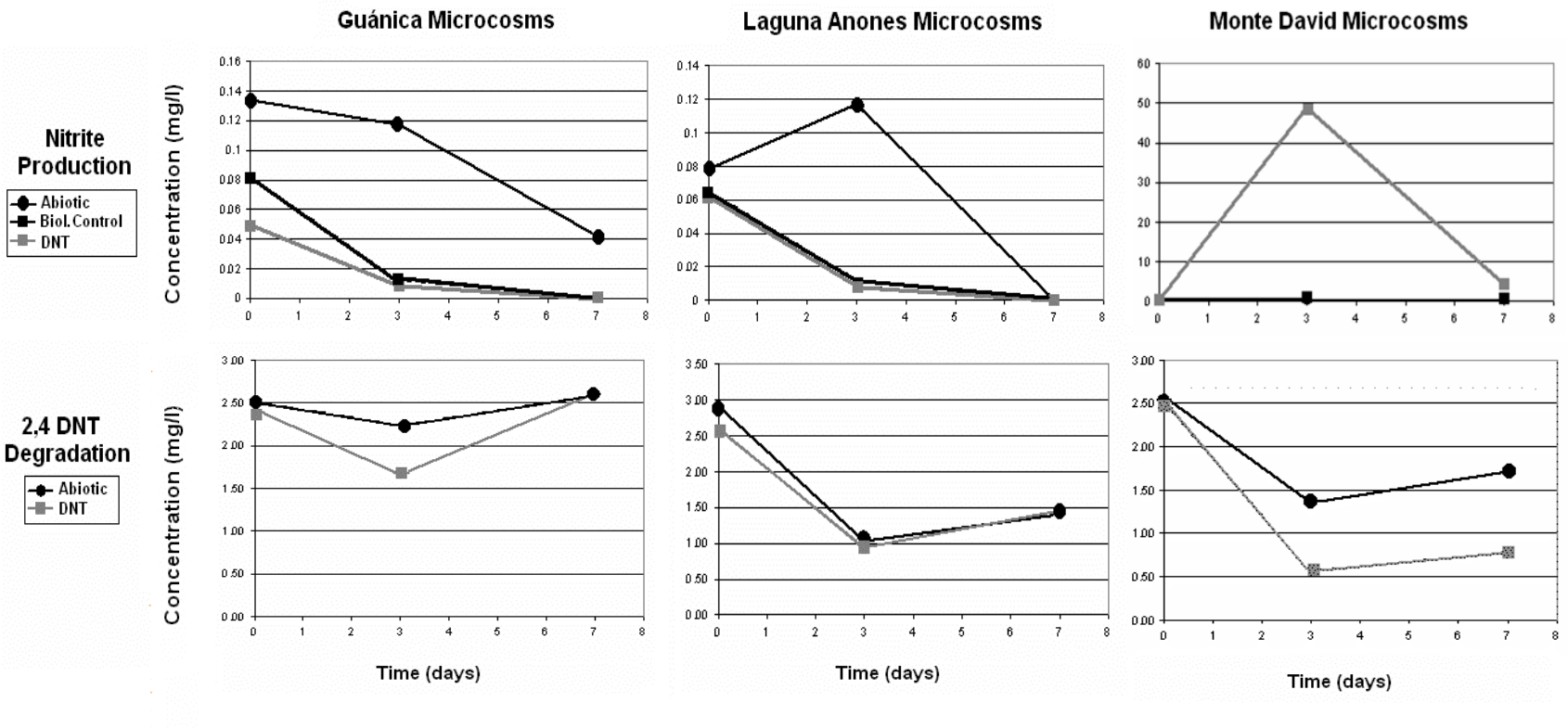


Figure A 4. 2,4-DNT and Nitrite concentration on Vieques and Guanica microcosms at time 0, 3 and 7 days.

Appendix B. Development of scripts for T-RFLP analysis software

Selection and binning of representative peaks in T-RFLP electropherogram is a fundamental step in the study of microbial community. Representative peaks are those peaks with a reproducible signal within different replicates, and which signal importantly contributes to establish a particular T-RFLP profile. The criteria used for selection of peaks will establish which peaks will be discarded for the analysis. Shared peaks from electropherograms generated with the same restriction enzyme do not always migrate equally in gel and capillary sequencers due to electrophoretic anomalies and thus calculation of exact fragment size must include a fragment size tolerance to group same size fragment as a shared genotype. Once all electropherograms peaks have been selected and binned, the results are generally stored in files containing the tabulated data and imported to a spreadsheet.

Detection of informative peaks in the electropherogram have been done using different criteria, such as deleting nonreproducible peaks between PCR replicates from the same DNA (40), selecting only peaks which height or area accounts for more than an specific percentage of total optical density or fluorescence (1%, (42) and 0.5%, (55)) or by using minimum height thresholds of fluorescent units (10). Also methods such as rarefaction have been use to determining which small peaks should be included in the analysis of T-RFLP (30). Recently, several software applications have been develop to aid peak selection for T-RFLP, some commercial programs include GeneMarker® software, GeneMapper ®software and torast (t-rflp operation results analysis software tool), also

non commercial applications have been develop such as T-RFLP Stats from IBEST (6) and dynamic programming algorithms (51). Most of the analysis software available has been designed to analyze ABI (Applied Biosystems) file formats, which difficult the use of these tools to address the same procedures with other sequencer files or from gel image analysis. Two T-RFLP scripts have been developed to address selection and binning of informative peaks from different sources using a standard text file.

T-RFLP Scripts. Common Lisp Implementation for ANSI (CLISP) version 2.38 (25) was selected as the language to develop these scripts CLISP is a general-purpose language that permits easy logical programming and definition of functions. Other important characteristic of CLISP is function iteration that is a powerful tool to find results with simple coding.

PEAKS script performed a recursive iteration to detect the minimal values that can separate fragment's peaks from noise baseline based in a modification of a statistical criteria previously proposed (6). Peak identification relies on the difference of signal intensity of background noise and labeled DNA fragments. Noise values cluster around the signal median of T-RFLP scan points (Figure B 1), while real peaks are distant from median values. Hence real peaks can be separated from background using a dispersal measure. Detection of real peaks is based on exclusion of values larger than the median plus three standard deviations ($\mu+3\sigma$), this calculation is done recursively until no larger values can be removed from data set and the script saves the last calculated value as the threshold to consider real peaks.

BINNING script is used to group similar length fragments (peaks) from different samples as the same OTU. The script explores all possible binning groups and organizes individual sets of data in a unified matrix, allowing easy modification and posterior analysis. BINNING used different criteria to detect possible binned groups, (1) fragment size, (2) congruence and (3) number of peaks within a possible OTU. The fragment size criteria established the maximum binning size of a OTU depending on fragment size as suggested for Automatic rRNA Intergenic Spacer Analysis (ARISA) by Brown and coworkers (12). Windows of 2, 3, 5, or 10 pb were used for fragments of 200-400, 400-700, 700-1000 and <1000 pb respectively. Congruence criteria discard binned groups that cluster two or more peaks of the same profile into the same OTU, script subdivide the grouping on 2 o more new OTUs. Finally the script counts the number of peaks that belong to each possible OTU and favor larger binned groups. Scripts output is a two space delimited text file, matrix merged file, and binning possible groups files. Final decision of binning groups is done using these two files in an Excel (Microsoft Office 2003) spread sheet were fragment lengths that are binned together are represented by their average size.

Results and Discussion. Several criteria for calculation of minimal real peak values were evaluated using electropherogram, histogram and percentile plots. PEAKS($\mu+3\sigma$) can effectively eliminate noise in the electropherogram separating peak values from background noise (A), real peak scan points in the evaluated electropherogram correspond to a 42% of total scan points (Figure B1 C, redline), this means that noise

background points are more frequent than real peak points (B). When using PEAKS (μ+20σ), a more strict selection of peaks is performed eliminating small peaks (A) and 60% of scan points (C). Finally, PEAKS μ+100σ criterion eliminates 92% of scan points (C) and several peaks in the electropherogram (A).

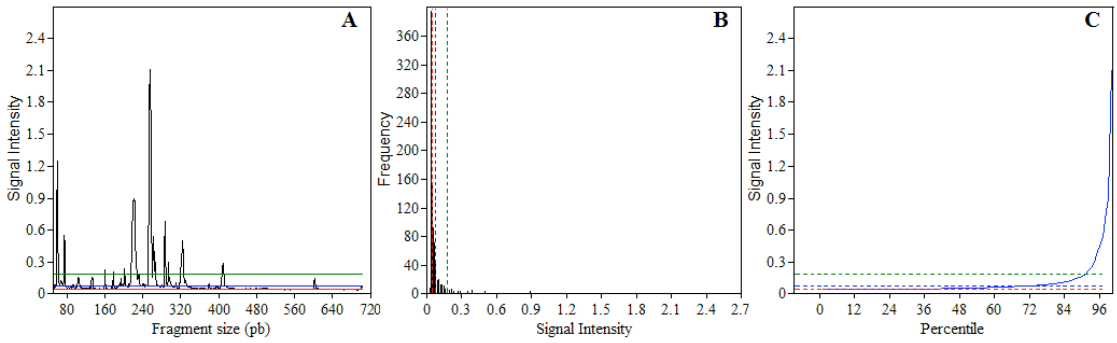


Figure B1. Electropherogram, histogram and percentile from Monte David microcosm digested with HaeIII. Red, blue and green lines represent the cutoff value calculated by PEAKS using μ+3σ (0.0439), μ+20σ (0.0674) and μ+100σ (0.1775) respectively.

Different samples were analyzed using PEAKS (μ+3σ) criteria (Fig. B1). Guanica microcosm T-RFLP profile has low signal intensity (A) compare with the other microcosms profiles due to low concentration of DNA digestion, nevertheless peaks signal is higher than the threshold line calculated by the script, this allow a successful elimination of noise independently from difference of digested product concentration (6). Cutoff values are calculated based in the intrinsic variability of data, so each sample has its own Peak detection threshold (Fig. B2).

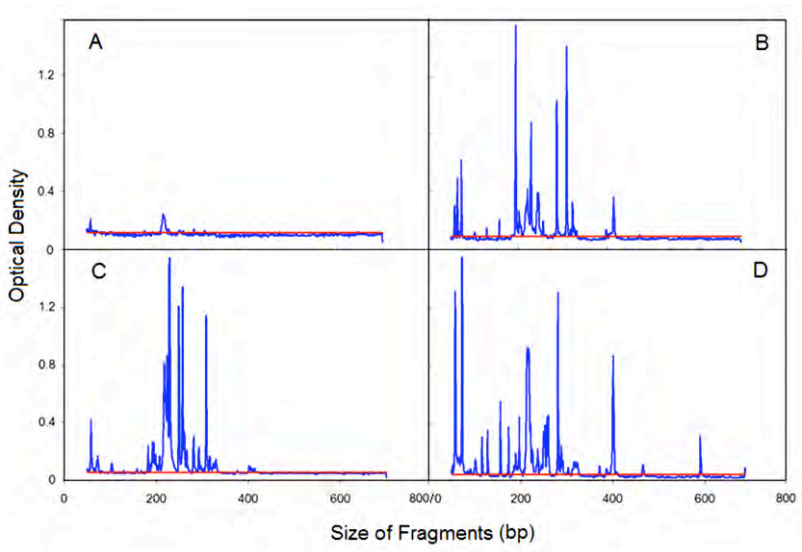


Figure B2. Electropherogram of 16S rDNA PCR amplifications from 2,4-DNT amended microcosms, digested with Hae III. A. Guanica (threshold= 0.112753294), B. Laguna Anones (threshold= 0.08060286), C. Monte David (threshold= 0.054272633) and Guanica-2 (threshold= 0.05083055). Red line represent calculated threshold using PEAKS(μ+3σ).

Appendix C. Steps for analysis of scan points from gel image analysis using.

In Order for the script to recognize, process and save the input file, several functions were constructed. Input files can be created exporting scan or defined peak data from the sequencer program to a spreadsheet (e.g., Microsoft Office).

1. Download CLisp-2.38 (scritps have been compiled in this version)

2. Download PEAKS and BINNING folders and save them inside Clisp-2.38 folder

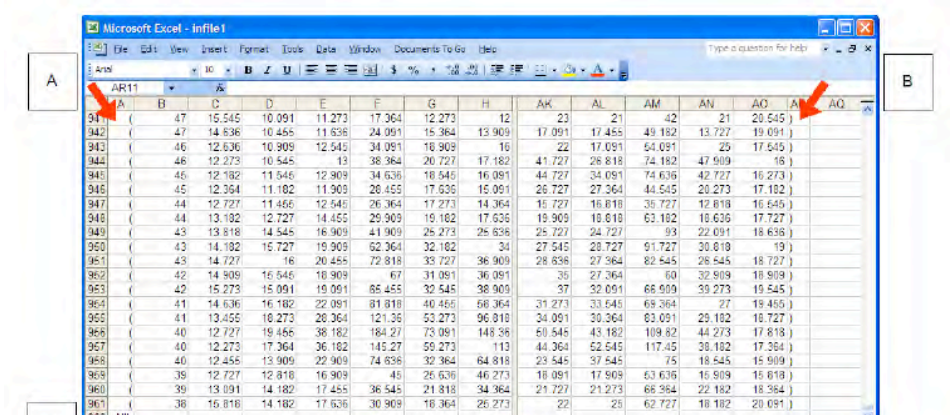
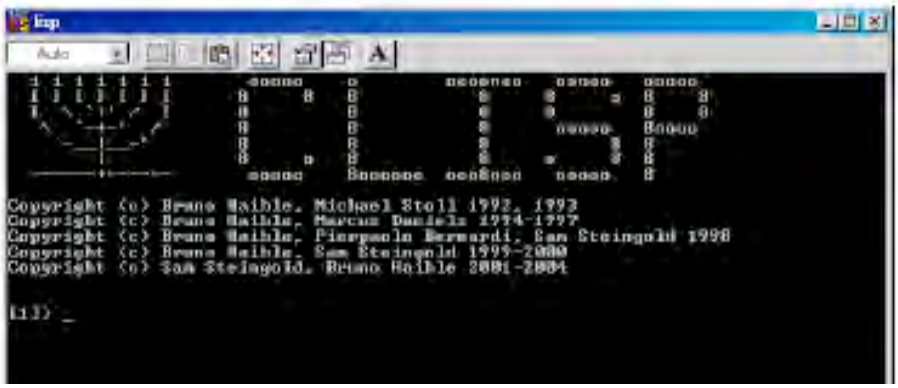


Figure C 1 File format of input file in Order to be recognized for the script

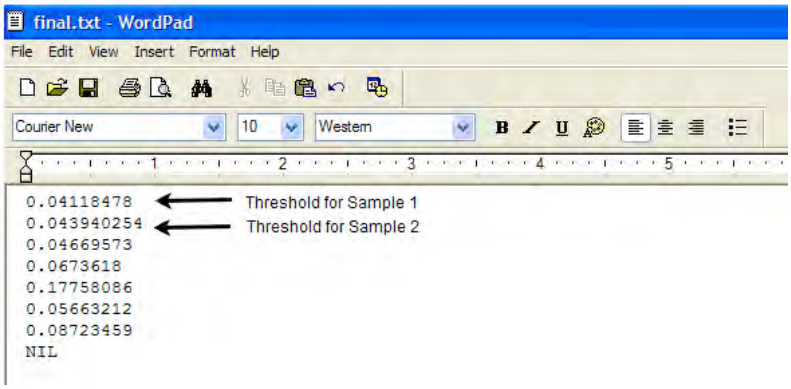
3. First column must have an open parenthesis (**Figure** # A),to group data in rows, and last row will end with NIL.
4. Second column will have the number belonging to the scan point.
5. Third and consecutive columns will have the optical density or fluorescent intensity of each scan point.
6. Last column must have a close parenthesis (**Figure**#, B)
7. Finally data must be saved as “infile.txt” a tab delimited text format and transfer the file to PEAKS folder.
8. Be sure that outfile.txt and final.txt files are empty otherwise open EasyErase folder copy both folders and paste them inside PEAKS folder.
9. Open trflp.txt file and modified functions “todos” and “final” changing X (number of columns in the file + 1), if your file has 35 lanes from gel image analysis, you should substitute X for 36.
10. Saved the modified file with the same name **trflp.txt**.

(load "trflp/Paso1.txt")
(load "trflp/Paso2.txt")
(todos X 0)
(load "trflp/Paso3.txt")
(load "trflp/Paso4.txt")
(load "trflp/picos.fas")
(final X 0)

11. Double click CLISP.exe and type at the prompt (load “PEAKS/Trflp.txt”), once the program finish running the script will show **>T**



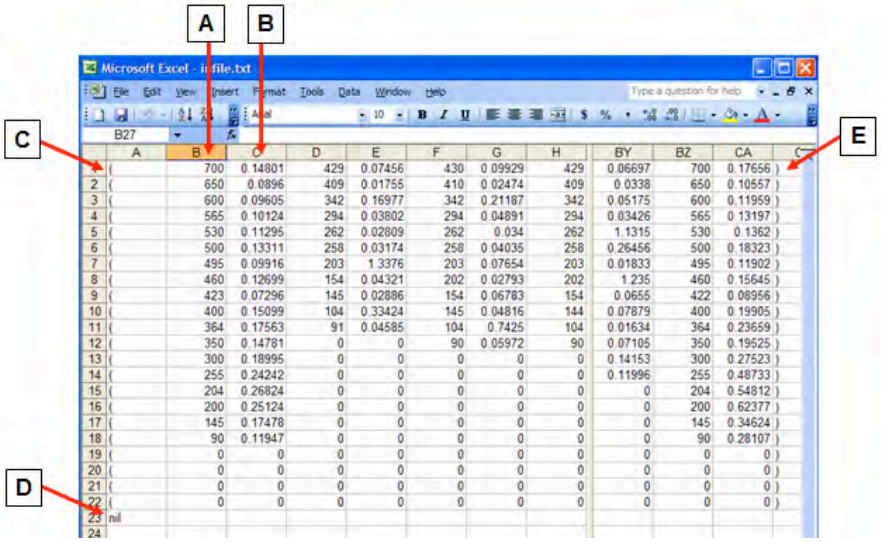
12. Open final.txt file, number will correspond to peak’s threshold for each of the lines at the original file.



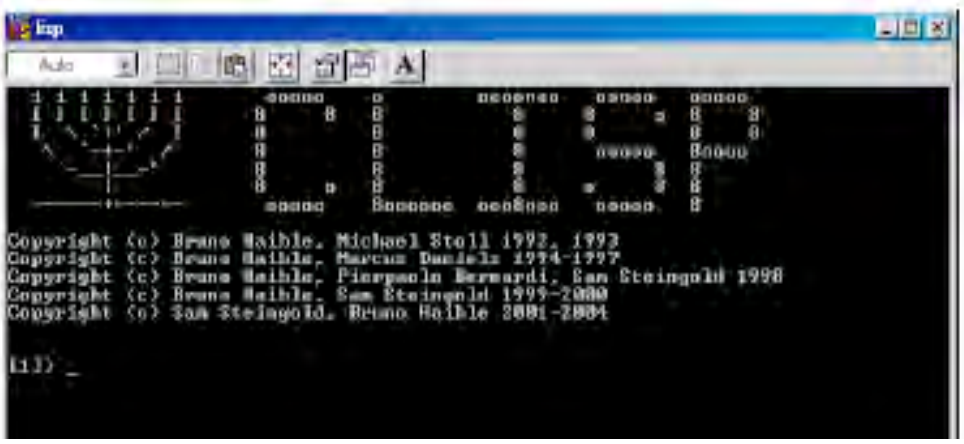
13. All scan points higher than threshold are real signal points, so every peak above this value must be considered for analysis.

B. Steps for binning of similar size fragments using BINNING

1. Once al real point have been identified, data must be put in the following format to be recognized by the script



2. Each sample set is composed by two columns first column (**Figure** # A) is the molecular weight of the fragment, second column is the signal intensity (**Figure** # B).
3. First column must have an open parenthesis to group data in rows (**Figure** # C), and last row will end with NIL (**Figure** # D).
4. Last column must have a close parenthesis (**Figure**#, E)
5. Finally data must be saved as "infile.txt" a tab delimited text format and transfer the file to BINNING folder.
6. Double click CLISP.exe and type at the prompt (load "binning/scripts/binning.txt"), once the program finish running the script will show **>T**



7. Several files are generated at <<clisp-2.38\Binning>> folder, **binned.txt** is a two lines file with all possible binned groups determined from de analysis and **matrix4.txt** is a general matrix formed from all T-RFLP profiles.
8. Choose of final binned groups must be done by the researcher using **binned.txt** file; final solution of groups must include binned groups that do not overlap, then this groups must be manually merged in the **matrix4.txt** file as one group

Further implementation of a scoring matrix is under development to determine the unique solutions.

APPENDIX D. Calculation of explosives concentration

Initial concentration of 2,4-DNT and RDX in microcosms

Information

BLK media +Slurry = 40 ml or 0.04 l
Explosives conc. = 1000 ppm (mg/l) or 1 g/l
2,4-DNT added vol. = 80 µl or 80 x10^{-6} l
RDX added vol. = 80 µl or 80 x10^{-6} l
2,4-DNT molar mass = 182.14 g/mol
RDX molar mass = 222.12 6/mol

Calculations

$$concentration \times volume = mass$$

$$(1\ g/l) \times (80 \times 10^{-6}\ l) = 80 \times 10^{-6}\ g$$

$$2{,}4DNT$$

$$\frac{80 \times 10^{-6}\ g \times 1 mol}{182 g} = 440\ nmoles$$

$$\frac{440 nmoles}{0.04 L} = 11 \mu M$$

$$RDX$$

$$\frac{80 \times 10^{-6}\ g \times 1 mol}{222.12 g} = 360\ nmoles$$

$$\frac{360\ nmoles}{0.04 L} = 9 \mu M$$

Approximately 440 nmoles of 2,4-DNT were added to the microcosms, so in case of total mineralization 880 nmoles of Nitrite (NO_2) are expected on the microcosm. Therefore in a final volume of 40 ml the expected concentration of nitrite must be around 22µM.

Concentration of Oxygen in the headspace of aerobic microcosms

Information

Serum vials volume = 100 cm_3
BLK media +Slurry = 40 cm_3
Headspace = 60 cm_3
Oxygen % on air = 21%

Calculations

$$number\ of\ moles = \frac{vol\,(cm^3)}{24000\ cm^3}$$

$$525 \times 10^{-6}\ moles = \frac{60\ cm^3 \times 0.21}{24000\ cm^3}$$

Results

$525 \mu moles$ of Oxygen atoms or $262.5 \mu moles$ of Oxygen molecules (O_2) expected on the EB aerobic microcosm.

Concentration of Acetone on aerobic microcosms (EB)

Information

BLK media +Slurry = 40 ml
Acetone added vol = 80 µl
Acetone density = 0.79 g/cm³
Acetone molar mass = 58.08 g/mol

Calculations

$$density = \frac{mass(g)}{volumen(cm^3_{\ or}\ ml)} \rightarrow density \times volumen = mass$$

$$(0.79g/ml) \times 0.08ml = 0.0632g$$

$$\frac{1mol \times 0.0632g}{58.08g} = 1088 \times 10^{-6}\ moles\ or\ 1088 \mu moles$$

Results. 1088 $\mu moles$ of Acetone are expected on the EB aerobic microcosm.
The following reaction shows the oxidation of acetone under oxygenic conditions.

$$4O_2 + C_3H_6O \rightarrow 3CO_2 + 3H_2O$$

Based on the following reaction four moles of Oxygen molecules are needed to oxidize one mole of Acetone, so the 265 µmoles of Oxygen molecules present on the microcosm headspace could oxidize 66.25 µmoles, because several other electron donor could be present in the slurry and media, oxygen must be rapidly consume leading to anoxic conditions.

APPENDIX E. List of Accession Numbers

List 1. Accession numbers from RDP using to construct the phylogenetic tree Type I and II.

S000010403, S000011652, S000116870, S000137057, S000137973, S000138443,
S000253432, S000413733, S000439318, S000002977, S000020300, S000436436,
S000543165, S000583256, S000434855, S000008159, S000011973, S000018037,
S000436442, S000431235, S000128561, S000130341, S000003861, S000021075,
S000010801, S000414108, S000011943, S000351835, S000351836, S000398099,
S000011476, S000112987, S000387422, S000089782, S000091005, S000004181,
S000395436, S000429252, S000441377, S000475150, S000021430, S000017844,
S000018317, S000130319, S000413471, S000437262, S000437264, S000458739,
S000539365, S000547982, S000843930, S000995202, S000394587, S000441842,
S000541574, S000128764, S000599485, S000103956, S000387423, S000392514,
S000413466, S000475022, S000016693, S000112393, S000112511, S000413473,
S000386315, S000462805, S000544176, S000364328, S000364342, S000413535,
S000468990, S000701177, S000018605, S000005350, S000007613, S000011492,
S000012284, S000016010, S000380772, S000428003, S000435922, S000437056,
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S000390729, S000390735, S000390738, S000428396, S000608915, S000544642,
S000401762, S000401763, S000434961, S000364327, S000435895, S000366462,
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S000544643, S000413736, S000019711, S000003872, S000008217, S000009277,
S000012680, S000014055, S000015039, S000017474, S000130044, S000130669,
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List 2. Accession numbers from RDP using to construct the phylogenetic tree Type III.

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List 3. Accession numbers from RDP using to construct the phylogenetic tree Type V.

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List 5. Accession numbers from RDP using to construct the phylogenetic tree Type VII.

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List 7. Accession numbers from RDP using to construct the phylogenetic tree Type III.

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EU548072, AY344056, AM295491, U62825 , EF019978, EF020153, EU693450, AY117557,
EU372837, AM398481, AY117554, AY117553, M22510, DQ417338, DQ417337, AY117572,
AY117567, EF626691, AM689950, AM396358, AY117569, AE016825, AB021385, AB017487,
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APPENDIX F. DNA Sequences from Clone Libraries

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72. Collection points at the AWTF Vieques, Puerto Rico. [map]. Scale undetermined. Generated by Alejandro Caro-Quintero; using “Google Earth” downloaded at http://earth.google.com/ (May 2008).