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ORIGINAL PAPER

# Changes to the rumen bacterial population of sheep with the addition of 2,4,6-trinitrotoluene to their diet

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**Abstract** Previous work has shown that bacterial isolates from the sheep rumen are capable of detoxifying 2,4,6-trinitrotoluene (TNT) into polar constituents. In this study, the dietary effects of TNT on the sheep rumen microbial community were evaluated using molecular microbiology ecology tools. Rumen samples were collected from sheep fed with and without TNT added to their diet, genomic DNA was extracted, and the 16S rRNA-V3 gene marker was used to quantify changes in the microbial population in the rumen. Control and treatment samples yielded 533 sequences. Phylogenetic analyses were performed to determine the microbial changes between the two conditions. Results indicated the predominant bacterial populations present in the rumen were comprised of the phyla Firmicutes and Bacteroidetes, irrespective of presence/absence of TNT in the diet. Significant differences ($P < 0.001$) were found between the community structure of the bacteria under TNT (−) and TNT (+) diets. Examination of the TNT (+) diet showed an increase in the clones belonging to family Ruminococcaceae, which have previously been shown to degrade TNT in pure culture experiments.

**Keywords** 16S rRNA · Munitions · Rumen · Phylogenetics

**Abbreviations**

| | |
|---|---|
| TNT | 2,4,6-Trinitrotoluene |
| EPA | Environmental protection agency |
| DGGE | Denaturant gradient gel electrophoresis |
| LGCGPB | Low GC Gram-positive bacteria |
| OTUs | Operational taxonomic units |

## Introduction

Xenobiotic compounds such as nitroaromatic and nitrated polycyclic aromatic compounds have found widespread use in the manufacturing of explosives, pharmaceuticals, plastics, agrochemicals, and photocopy toners (Bryant and DeLuca 1991). Processes such as production of coal fly ash, incomplete combustion of fossil fuels, and processing of explosives have also produced significant amounts of nitroaromatic compounds (Spain 1995). One major

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nitroaromatic compound, 2,4,6-trinitrotoluene (TNT), was widely used in both World Wars rendering many sites contaminated with munitions and potential runoff hazards. According to the Environmental Protection Agency (EPA), TNT affects human, aquatic, and terrestrial life forms and lists it as a class C potential human carcinogen (Smets et al. 2007). TNT metabolites induce reproductive toxicity through oxidative DNA damage as shown by animal models and epidemiological studies (Homma-Takeda et al. 2002).

Attempts to develop biological system based technologies to degrade munitions such as TNT date back to as early as the 1970s (McCormick et al. 1976). Very few microorganisms have been reported to use TNT as their sole nitrogen (Esteve-Nunez and Ramos 1998) or carbon source (Montpas et al. 1997). Although, no efficient technology has been developed to remediate TNT, both bacteria (McCormick et al. 1976, Fuller and Manning 1997) and fungi (Stahl and Aust 1993) have been shown to be capable of degrading TNT to various degrees (Hawari et al. 2000).

The inability of most organisms to mineralize TNT is attributed to its structure, especially the symmetrical location of the nitro groups on the aromatic ring, the electro-negativity of the nitro groups, and subsequent misrouting of reactive intermediates (Hawari et al. 2000). Recent research has shown the necessity of anaerobic conditions for the complete mineralization of TNT (Nishino and Spain 2002). Two well characterized TNT biodegradation pathways are by the formation of a Meisenheimer complex (Williams et al. 2004; Ramos et al. 2005) and by the systematic reduction of the nitro group in the TNT molecule. In the Meisenheimer complex breakdown of TNT, nitrite is the final product formed. In the other method, TNT is reduced in a series of reactions targeting the nitro groups and is reduced to form 4-hydroxytolune (Esteve-Nunez et al. 2001). This molecule then enters the toluene degradation pathway. There are two enzymes involved in the degradation of TNT. One enzyme, nitroreductase, can be divided into two types based on the sensitivity to oxygen. The second enzyme belongs to the family of old yellow enzyme (OYE) and does not share any homology to the nitroreductase enzyme (Smets et al. 2007).

The rumen is known to be the most reductive ecosystem (Russell and Rychlik 2001, Bryant 1959) and provides a suitable ecosystem for the degradation of TNT. Previous research has found microorganisms from the sheep rumen capable of metabolizing munitions such as TNT to polar metabolites (Fleischmann et al. 2004; De Lorme and Craig 2009). The rumen consists of numerous microbes, consisting of protozoal, fungal, archaeal, and bacterial species (Krause and Russell 1996). The microorganisms present in the rumen convert cellulose-based carbohydrates to volatile fatty acids (VFAs), $NH_4$, $CO_2$, and $H_2$ under normal conditions. Most of these by-products are the result of fermentation and are taken up by the rumen wall. These fermentation by-products are utilized as a carbon source for energy for the animal while the hydrogen gas produced is utilized by methanogens as their energy source (Janssen and Kirs 2008).

The 16S rRNA gene has given microbiologists the necessary tool to identify and evaluate microbial communities by direct amplification (Woese et al. 1975, Woese et al. 1976). Most microbial ecologists, as well as microbial pathologists, have utilized the 16S rRNA gene marker to understand microbial community structure and function in ecosystems such as crenarcheal assemblages in soil (Sliwinski and Goodman 2004; Ochsenreiter et al. 2003), uncultured archaeal diversity (Nercessian et al. 2004), and in enumerating ocean bacteria (Rappe et al. 2002). The V3 region of the 16S rRNA gene marker (16S rRNA-V3) provides a suitable region for PCR amplification and classification of microorganisms (Muyzer et al. 1993). The 16S rRNA gene was used in this study to elucidate the changes to the sheep rumen bacteria and archaea with the addition of TNT to the sheep diet. The rumen samples were collected from a previous study that involved the assessment of TNT toxicity to sheep (Smith et al. 2008). The 16S rRNA-V3 gene marker was used to differentiate the two treatment conditions. Data were analyzed using the RDP II database and other phylogenetic tools.

## Materials and methods

### Animal diet, experimental setup, and whole rumen fluid collection

Rumen fluid was collected from four wethers ($n = 4$) used in a previous study (Smith et al. 2008). All sheep were fed a grass-hay and alfalfa mixture. The

(TNT −) control samples were collected from all four wethers prior to supplementing their diet with TNT. Three sheep ($n = 3$) were feed a diet supplemented with 33.1 mg of unlabeled TNT during a 21-day adaptation period. Rumen samples collected from these sheep constituted the TNT (+) treatment. All rumen samples were collected by rumenocentesis. The samples were transported overnight on dry ice and frozen at −80°C on arrival.

### Isolation of genomic DNA

The treatment samples were pooled and genomic DNA was extracted from both treatment and control groups using the Gentra puregene kit (Qiagen, Valencia, CA) combining both Gram-positive and Gram-negative extraction methods. DNA was quantified using a Nanodrop ND-1000 spectrophotometer (Thermo Fisher, Waltham, MA) and used as a template for setting up all polymerase chain reactions (PCRs).

### Primers and PCR conditions

The hypervariable region, V3, of the 16S rRNA gene was used as a marker for amplifying the rumen microbial population of the sheep. The primers and protocol for amplifying the 16S rRNA-V3 gene marker have previously been described (Muyzer et al. 1993). For amplifying the archaeal 16S-V3 gene products, a multiplex PCR approach was used. The first PCR reaction was carried out using the 27F forward primer (DeLong 1992) and the archaeal specific 934R reverse primer (Baker et al. 2003). The second round of PCR reactions was carried out using product from the first amplification as a template with specific V3 archaeal primers (Yu et al. 2008). All PCR reactions were carried out in triplicate.

PCR thermocycling was carried out using recombinant AmpliTaq® Gold polymerase (Applied Biosystems, Foster City, CA) in a PTC-200 thermocycler (MJ Research Inc., Watertown, MA). Each 50 µl PCR reaction contained: 75 ng of purified bacterial genomic DNA, 200 µmol of each dNTP, 5 µl of 10× PCR Buffer, 5 µl of 20 mM MgCl, 20 ng of bovine serum albumin (BSA), primers at 25 pmol each, 0.25 U polymerase, and the remaining volume made up with sterile water. PCR products were run on an agarose gel to visualize appropriate size bands and purified using the QIAquick PCR purification kit (Qiagen Inc., Valencia, CA) according to the manufacturer's recommendations.

### Denaturant gradient gel electrophoresis (DGGE) setup

The DCode system for DGGE (BioRad, Hercules, CA) was used to analyze PCR fragments. The 16S rRNA-V3 PCR products were separated on 8% polyacrylamide gel (37.5:1, acrylamide/bis-acrylamide) (BioRad) with a denaturing gradient of 30–65%. The 16S rRNA-V3 PCR products were mixed with 10 µl of 2× loading dye (BioRad) and brought to a final volume of 20 µl with TE (pH 8.0). A 100 ng per well of DNA was loaded into each DGGE lane. All gels were run for 16 h at 60 V in 1× Tris–acetate–EDTA (TAE) at 60°C. Gels were stained using a silver staining procedure (BioRad) according to the manufacturer's instructions and visualized on a fluorescent light box (Star X-ray, Amityville, NY). Gel images were captured using a Kodak DC120 camera.

### 16S rRNA-V3 library construction and plasmid extraction

PCR products were quantified (Nanodrop), cloned, and transformed into competent *Escherichia coli* (*E. coli*) cells using the TOPO® TA cloning kit for sequencing (Invitrogen Corporation, Carlsbad, CA) according to the manufacturer's recommendations. Transformants were spread onto petri dishes containing LB agar (EMD Chemicals Inc., Gibbstown, NJ) supplemented with 50 µg $ml^{-1}$ kanamycin sulphate (EMD). Plates were incubated overnight at 37°C. Clones were picked and grown overnight in 2× LB (Luria–Bertani) supplemented with 50 µg $ml^{-1}$ kanamycin. Sterile glycerol was added to the colonies to make a final concentration of 40% and stored at −20°C. The colonies were retransferred into TYGPN media (Elbing and Brent 2002) with 50 µg $ml^{-1}$ kanamycin for plasmid isolation as per published protocols (Engebrecht et al. 2000).

### Sequencing and phylogenetic analysis

Sequencing was performed using the BigDye® Terminator v. 3.1 Cycle Sequencing Kit (Applied

Biosystems, CA) using an ABI Prism® 3730 Genetic Analyzer at the Center for Genomic Research and Biocomputing (CGRB), Oregon State University. Single reads utilizing the T7 promoter were used to determine the nucleotide sequences. These were imported into the Geneious computer program (Drummond et al. 2007) and extracted. The sequences were checked for chimeras and the resulting FASTA file was used for further analysis. The RDP II Classifier (Wang et al. 2007) was used to sort sequences into their respective operational taxonomic units (OTUs). A confidence interval of 50% was used for the assignment of OTUs (Claesson et al. 2009). The LibCompare software of the RDPII database were used to compare whether the changes to the rumen microbial community was significantly different (at a confidence interval of 50%) when fed TNT. The Mothur software package (Schloss et al. 2009) was used for the analysis of data and the estimation of collectors and rarefaction curves. All sequence data were submitted to the GenBank database (Benson et al. 2010) under accession numbers GU304662–GU305194.

## Results

### DGGE analysis of bacterial and archaeal population

There were differences in the DGGE profiles between the TNT (−) and TNT (+) conditions when the 16S rRNA-V3 bacterial primers were used to amplify the DNA samples. By visual inspection, five bands were determined to be different between the two groups (Fig. 1). Although, there were differences in the band intensities between the TNT (−) and TNT (+) conditions for the same quantity of DNA loaded into the wells, the data was not quantified.

To estimate the changes to the archaeal population between the two treatment conditions, a second DGGE analysis was performed (Supplemental date, Fig. 2). Visual inspection of the gel indicated that there were no banding differences between the TNT (−) and TNT (+) conditions and, therefore, further downstream analysis such as cloning and sequencing were not performed to the archaeal samples.

A comparison of the differences between the two bacterial communities was carried out. PCR products were cloned, sequenced, and a phylogenetic analysis was performed.

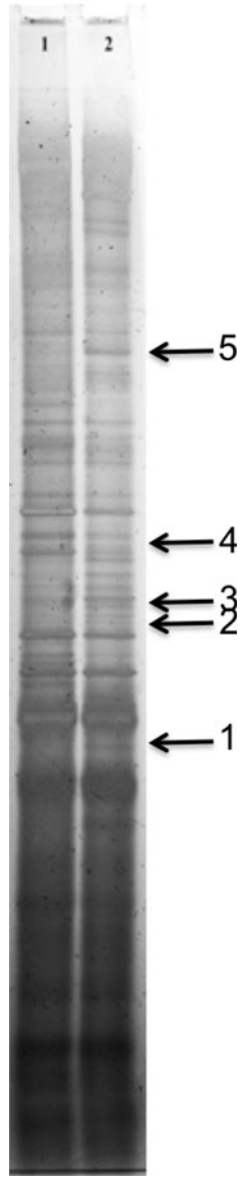


**Fig. 1** 16S rRNA V3-DGGE analysis of bacteria from the sheep rumen without TNT (−) and with TNT (+) added to their diet. A 30–65% denaturant gradient gel was cast, run for 16 h at 60 V, and stained with a silver staining kit. *Lane 1* represents the TNT (−) control and *lane 2* represents the TNT (+) treatment. The *arrows* indicate unique bands

### Phylogenetic classification of 16S rRNA under control and TNT diets

A total of 533 clones were used in the classification process of TNT (−) and TNT (+) treatments. There were 267 clones used in the classification of the TNT (−) and 266 clones for the TNT (+) treatments.

In both control and the treatment samples, the predominant microbial communities belonged to the

phyla Firmicutes and Bacteroidetes (Table 1). In the TNT (−) condition, out of 267 clones, the RDPII Classifier tool placed 121 clones into the phylum Firmicutes and 116 clones in the phylum Bacteroidetes. Of the 121 clones associated with the phylum Firmicutes, 48 sequences or 40% of the clones were classified only to the class/order level (data not shown). For the phylum Bacteroidetes, out of a total of 116 classified clones, 35 clones or 29.91% were classified to the class/order level (data not shown). The genus *Prevotella* (67 or 22.47% of the clones), associated with phylum Bacteriodetes, made up the single most abundant genus in the TNT (−) treatment. The second largest number of sequences associated with the genus *Fastidiosipila* (13 or 6.74% of the clones) that belongs to the phylum Firmicutes. Minor phyla in the TNT (−) treatment were members of TM7, Spirochaetes, Proteobacteria, Actinobacteria, and Tenericutes. Only ten clones or 3.75% of the total clones sequenced were unable to be classified under any previously known phyla.

Of the 266 clones in the TNT (+) treatment, the RDPII Classifier placed 154 clones in the phylum Firmicutes, 60 clones in the phylum Bacteroidetes, and 32 clones were unclassified bacteria (Table 1). Out of 154 clones in the phylum Firmicutes, 60 clones or 38.96% of the picked clones were classified only to the class/order level (data not shown). In the phylum Bacteroides, 24 clones or 40.00% of the total clones were classified to the class/order level (data not shown). The largest number of clones that associated with a single genus in the TNT (+) treatment belonged to *Prevotella* (22 or 8.27%), which belongs to phylum Bacteriodetes. The second largest number of sequences associated with the genus *Fastidiosipila* (13 or 6.74%) that belongs to the phylum Firmicutes (data not shown). Minor phyla were Spirochaetes, Actinobacteria, TM7, and Tenericutes. No sequences associated with the phylum Proteobacteria were found in the TNT (+) treatment.

## Phylogenetic analysis, Libshuff, LibCompare analysis of TNT (−) and TNT (+) samples

The total richness for both groups (TNT+ and TNT−) was estimated at 201 OTUs at 0.03% similarity (Table 2). The number of OTUs shared between the control and treatment groups was 42, which corresponds to 0.26% (Supplemental data, Fig. 2a). A ChaoI analysis estimate for the TNT (−) and TNT (+) conditions was 384.90 (Table 2). The results of this analysis assigned 199.50 OTUs to the TNT (−) control and 187.40 OTUs to the TNT (+) treatment. The number of OTUs shared between the two groups was 132.96, which corresponds to 0.52% (Supplemental data, Fig. 2b). The Shannon–Weaver Index

**Table 1** Tabulated classification of the 16S rRNA-V3 clones associated with TNT (−) and TNT (+) conditions at the phylum level using the Naïve Bayesian classifier of RDPII website at a confidence interval of 50%

| Phylum | Treatment | | | |
|---|---|---|---|---|
| | TNT* (−) | (%) Of clones | TNT* (+) | (%) Of clones |
| Actinobacteria | 2 | (0.75) | 6 | (2.26) |
| Bacteroidetes | 116 | (43.45) | 60 | (22.56) |
| Cyanobacteria | | | 1 | (0.38) |
| Firmicutes | 121 | (45.32) | 154 | (57.89) |
| Proteobacteria | 3 | (1.12) | | |
| Spirochaetes | 3 | (1.12) | 7 | (2.63) |
| Tenericutes | 1 | (0.37) | 1 | (0.38) |
| TM7 | 11 | (4.12) | 5 | (1.88) |
| Uncl bacteria | | (3.57) | 32 | (12.03) |

Trimmed and chimera checked DNA sequences from both the TNT (−) and TNT (+) treatments were submitted as a FASTA file to the Classifier tool of the RDPII database. The resulting bacterial hierarchy is represented as a table. The table also represents the percentage each phylum represents to the total sampled population

*Uncl bacteria* Unclassified bacteria

* Significant differences ($P < 0.001$) in the two conditions as analyzed by Libshuff analysis

**Table 2** Phylogenetic analysis of TNT (−) and TNT (+) clones based of the 16S rRNA-V3 analysis at the 1, 3 and 5% level

| Treatment | Number of sequences | Observed OTU | | | Estimated OTU | | | Shannon–Weaver | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | ND | 3% | 5% | ND | 3% | 5% | ND | 3% | 5% |
| TNT (−) | 267 | 156.00 | 102.00 | 83.00 | 527.00 | 199.50 | 159.00 | 4.61 | 3.93 | 3.70 |
| TNT (+) | 266 | 159.00 | 99.00 | 79.00 | 427.00 | 187.40 | 139.00 | 4.76 | 4.17 | 3.88 |

ND: No difference at (0.00 or 100% homology)

3%: OTU estimates at (0.03 or 97% homology)

5%: OTU estimates at (0.05 or 95% homology)

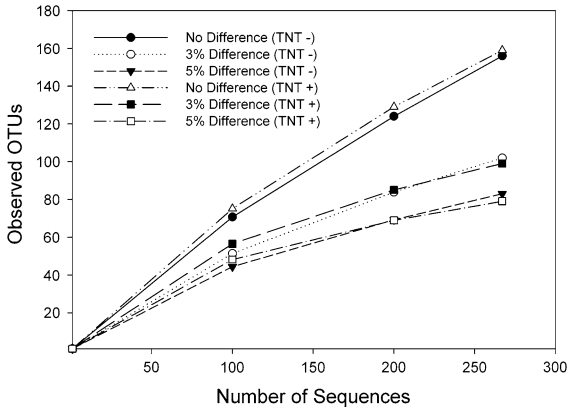


**Fig. 2** Rarefaction plot of TNT (−) and TNT (+) conditions with the16S rRNA-V3 primers as calculated by the mothur program. The estimates plotted represent the number of observed OTUs in relation to the number of sequences sampled. (*filled circle*) represents the observed OTUs at no difference in the TNT (−), (*open circle*) represents the 3% difference in the TNT (−), (*filled triangle*) represents the 5% difference in the TNT (−) condition, (*open triangle*) represents the observed OTUs at no difference in the TNT (+) treatment, (*filled square*) represents the 3% difference in the TNT (+) treatment, and (*open square*) represents the 5% difference in the TNT (+) treatment

(Table 2) suggests that the rumen is a complex ecosystem (Borneman and Triplett 1997). Rarefaction curves showed no trend toward reaching a plateau, which indicates the need for more sampling in order to observe all the diversity present in the rumen (Fig. 2).

A Libshuff analysis was performed to determine whether there was a significant difference to the microbial community structure when TNT was supplemented to sheep's diet. The statistical analysis indicated a significant difference ($P < 0.001$) between the two conditions. To establish the primary phyla/class/order contributing to the significant changes, a LibCompare analysis (Wang et al. 2007) was performed at a confidence interval of 50%. The analysis showed a significant difference in the clone libraries for the phyla Bacteroidetes ($P < 0.001$) and Firmicutes ($P < 0.001$) (Table 3). For the phylum Bacteroidates, there was a significant ($P < 0.001$) reduction in clone numbers in the TNT (−) control compared to the TNT (+) treatment. At the genus level, clones associated with the genus *Prevotella* were shown to be significantly different ($P < 0.001$) between the two conditions (Table 3). For the phylum Firmicutes, there was a significant ($P < 0.001$) increase in clone numbers in the control compared to the TNT (+) treatment. The class Erysipelotrichi showed a decrease in the number of clones in the control compared to the treatment group. The clones associated with the class Clostridia, identified specifically with the family Ruminococcaceae, showed a significant increase in the treatment compared to the control group ($P < 0.001$).

## Discussion

This study identified phylotypes (bacterial and archaeal) in the sheep's rumen capable of degrading munitions such as TNT in vitro. Bacteria have been shown to degrade TNT with the formation of polar metabolites (Spain 1995). There is little literature on rumen archaea involved in the degradation of TNT. In this study, there were visible changes to the banding pattern in the bacterial population between TNT (−) control and TNT (+) treatment conditions. Band five was the most prominently different band in the TNT (+) treatment (Fig. 1). The archaeal DGGE analysis, however, provided no evidence in community shifts in the rumen. Since no archaeal shifts were observed, all efforts were directed towards better understanding the changes in the bacterial population.

**Table 3** LibCompare analysis of TNT (−) and TNT (+) 16S rRNA-V3 clone library using the RDPII database at a confidence interval of 50%

| Rank | Name | No. of clones TNT (−) | No. of clones TNT (+) | Significance |
|---|---|---|---|---|
| Phylum | Bacteroidetes | 116 | 60 | $3.40E^{-7}$ |
| Class | Bacteroidetes | 102 | 55 | $1.08E^{-5}$ |
| Family | Prevotellaceae | 78 | 33 | $2.60E^{-6}$ |
| Genus | *Prevotella* | 68 | 22 | $1.16E^{-7}$ |
| Phylum | Firmicutes | 121 | 154 | $3.74E^{-3}$ |
| Class | Erysipelotrichi | 37 | 6 | $9.60E^{-7}$ |
| Family | Erysipelotrichaceae | 37 | 6 | $9.60E^{-7}$ |
| Class | Clostridia | 65 | 133 | $1.06E^{-9}$ |
| Family | Ruminococcaceae | 18 | 55 | $4.23E^{-6}$ |

Significance as measured by LibCompare analysis ($P < 0.001$)

There is considerable information about complete reduction of nitroaromatic compounds by microorganisms under both aerobic and anaerobic conditions (Esteve-Nunez et al. 2001; Spain 1995). For the complete mineralization of TNT, anaerobic conditions are necessary (Nishino and Spain 2002). Single bacterium can seldom completely mineralize TNT and a consortium is required for the mineralization process to occur in a short period of time (Nishino and Spain 2002). The rumen environment, due to its anaerobic conditions and microbial diversity, provides a very suitable ecosystem for the reduction of nitro groups into nitroso derivatives, hydroxylamines, and other intermediates.

The results of the present study are consistent with previous studies that have evaluated bacterial populations present in the intestinal ecosystems of other mammals. Based on a 16S rRNA analysis, the predominant populations in the sheep rumen have been shown to consist of microorganisms in the phyla Firmicutes and Bacteroidetes. Other minor phyla include Proteobacteria, Actinobacteria, Verrucomicrobia, Fusobacteria, Fibrobacteres, TM7, and Cyanobacteria (Ley et al. 2008; Dowd et al. 2008). Evaluation of ruminal microbial communities in cattle (Edwards et al. 2004; Whitford et al. 1998; Tajima et al. 2001), sheep (Koike et al. 2003), and some wild ruminants (Nelson et al. 2003; An et al. 2005) have shown them to be dominated by low GC Gram-positive bacteria (LGCGPB), particularly those related to the broad genus *Clostridium*. Members of the genus *Prevotella* are regarded as the most dominant bacterium in the rumen (Stevenson and Weimer 2007). The present analysis agrees with these observations in both the TNT (−) (68 clones) and TNT (+) (22 clones) conditions. The genus *Prevotella* was found to comprise the single largest clone population.

Results from the a feeding trial conducted by Smith et al. (Smith et al. 2008) to assess the toxicity of TNT to sheep indicated the radioactive carbon counts were predominately found in the feces (76.6%), urine (17.1%), and tissue (5.33%). Metabolites formed during the degradation of TNT were not identified as most of the radioactive counts of the parent $^{14}C$ labeled TNT were detected as polar metabolites in the urine in less than 6 h. These results show indirect evidence of the detoxification of TNT before adsorption of the products of digestion from the rumen to the animal. In the rumen, the enzymes responsible for most bacterial transformations of TNT are catalyzed by NADPH dependent nitroreducatase. Nitroreductases can be further divided into two groups based on their ability to metabolize nitro compounds in the presence of oxygen: Type I nitroreductase (oxygen insensitive) and Type II nitroreductase (oxygen sensitive) (McCalla et al. 1970, Peterson et al. 1979). *In silico* analysis of sequenced rumen fiber degraders provided evidence for the presence of nitroreductase enzymes (data not shown). The likelihood of rumen bacteria using TNT as a sole source of nitrogen or carbon has not yet been tested and it is currently accepted that the process of TNT degradation is an event of co-metabolism.

There are significant differences in bacterial communities in the rumen between the TNT (−) and TNT (+) conditions. The number of clones associated with phylum Firmicutes increased in the TNT (+) treatment compared to the number of clones associated with the phylum Bacteroidetes (Table 3).

Pure culture TNT incubation experiments showed that the bacteria associated with the Phylum Firmicutes (*Butyrivibrio fibrisolvens*, *Lactobacillus vitulinus*, *Selenomonas ruminantium*, and *Streptococcus caprinus*) completely detoxified 100 ppm TNT in less than 24 h with the appearance of metabolites (De Lorme and Craig 2009). Bacteria belonging to the phylum Bacteroidetes (*Prevotella bryantii* and *Prevotella ruminicola*) were also capable of degrading TNT at a slower rate with the appearance of metabolites (De Lorme and Craig 2009). The decrease in *Prevotella* clones in the TNT (+) treatment could be attributed to inter species competition and not the toxicity of TNT, as previous research has shown that *Prevotella* sp. are capable of degrading TNT in pure culture at 100 ppm levels (De Lorme and Craig 2009). Moreover, *in silico* analysis (data not shown) of sequenced *Prevotella* genomes indicated the presence of a nitroreductase enzyme system that is responsible for the breakdown of TNT. This finding leads to an assumption that either inter species competition or sampling is responsible for their decrease (Fig. 2).

Results from the present study clearly identify the family Ruminococcaceae is enriched under the TNT (+) treatment. This family includes rumen bacteria such as *Ruminococcus albus and Ruminococcus flavefaciens*, which are Gram-positive anaerobes belonging to cluster XIVa of the *Clostridium* subphylum (Rainey and Janssen 1995). *Ruminococcus* sp. has been studied largely due to its ability to efficiently degrade and use cellulose as a carbohydrate source. Previous research with the strains *Ruminococcus albus* and *Ruminococcus flavefaciens* has provided evidence for their ability to degrade TNT to 4-Amino-2,6-dinitrotoluene and 2-Amino-4,6-dinitrotoluene (De Lorme and Craig 2009). These rates of metabolite formation were found to be slower when compared to other rumen pure cultures such as *Butyrivibrio fibrisolvens* nxy, *Eubacterium ruminantium* GA195, and *Fibrobacter succinogenes* S85. In vitro results from the present study show that *Ruminococcus* sp. adapted and degraded TNT better in mixed communities.

This study provides evidence for *Ruminococcus* sp. to be involved in the breakdown of TNT. Further analysis using a 454 sequencing technique and quantitative PCR (qRT-PCR) will provide greater sequence data as well as real-time data on the changes to the *Ruminococcus* sp. in the rumen in the presence of TNT.

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