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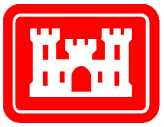

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Characterization of Microbial Communities in an Anaerobic Fluidized Bioreactor Treating TNT Using Molecular Techniques

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Foreword

This study was conducted for Directorate of Military Programs, Headquarters, U.S. Army Corps of Engineers (HQUSACE) under 622720D048, "Industrial Operations Pollution Control (6.2 Exploratory Development)," Work Unit U60, "Biological Treatment of Munitions Production Waste." The technical monitor was Dr. Stephen W. Maloney, Environmental Processes Branch (CN-E).

The work outlined in this report was performed by the Department of Civil and Environmental Engineering at the University of Illinois at Urbana-Champaign (UIUC) for CN-E, Installations Division (CN), Construction Engineering Research Laboratory (CERL) located in Champaign, IL. Dr. Robert Sanford is an Assistant Professor, Dr. Lutgarde Raskin is a Professor, and Soon Hwan Oh is a graduate student, all from the Department of Civil and Environmental Engineering at UIUC. Dr. Neal R. Adrian is the CERL (CN-E) principal investigator. The technical editor was Linda L. Wheatley, Information Technology Laboratory. Dr. Ilker Adiguzel is Chief, CN-E; Dr. John T. Bandy is Chief, CN; and Gary W. Schanche (CVT) is the associated Technical Director. The Acting Director of CERL is William D. Goran.

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1 Introduction

Background

Pinkwater is a wastewater contaminated with residual explosive chemicals such as 2,4,6-trinitrotoluene (TNT) and cyclotrimethylene-trinitramine (RDX). Untreated pinkwater can pose a serious problem to water quality because the residual explosive chemicals are mutagenic and carcinogenic (Kaplan and Kaplan 1982). For example, TNT is suspected to have toxicological effects on a number of organisms, including humans, fish, and algae (Won and Disalvo 1976; Harvey et al. 1990). Exposure to TNT causes pancytopenia, a disorder of the blood-forming tissues characterized by a severe decrease in the number of leukocytes and erythrocytes in mammals, including humans (Harris and Kellermeyer 1970).

The current approach for treating pinkwater is adsorption by granular activated carbon (GAC), which is expensive (Concurrent Technologies Corporation 1995). In contrast, the biotransformation of explosive residuals by microorganisms has attracted much interest as a more economical remediation approach. One method of treatment uses an anaerobic fluidized-bed granular activated carbon (AFB-GAC) bioreactor, a system that combines the advantages of physical adsorption by GAC with biodegradation mediated by anaerobic microorganisms. Little is known, however, about the microbial community in such anaerobic bioreactor systems used to treat pinkwater. A better understanding of this community, therefore, particularly the characterization of the explosive-degrading populations, is important for developing optimal treatment systems for pinkwater. Knowledge about the active explosive-degrading population will help develop strategies for enhancing this group of bacteria, rather than simply attempting to enhance the target activity by increasing the activity of the whole community. Identifying the active degrading population may also lead to process indicators that are more effective in assessing the treatment process and may be useful as a potential indicator for early detection of impending reactor upsets.

The characterization of microbial communities by conventional culture-based techniques results in biases, since only a small fraction of microorganisms can be cultured. For example, it has been estimated that only 1 to 5 percent of the total microbial community in soil can be cultured (Bakken 1985). In the case of anaerobes, this problem is exacerbated because of low growth rates and fastidious

anaerobic culture conditions. Nucleic acid hybridization techniques that use oligonucleotide probes to target regions of the 16S and 23S ribosomal ribonucleic acid (rRNA) can be used to identify microbial populations in the environment (Stahl et al. 1988). One of the biggest advantages of this technology is the ability to detect and identify microbial populations without having to culture them (Holben and Tiedje 1988). The hybridization assay used in this study involves extraction of RNA from bioreactor samples, denaturation of the RNA, and immobilization of RNA on nylon membranes. Subsequently, the membranes are hybridized with radiolabeled oligonucleotide probes specific for certain target populations. The amount of probe that hybridizes is proportional to the amount of RNA from the target population that was originally present in the bioreactor sample.

Community structure has also been evaluated using polymerase chain reaction (PCR)-based techniques that amplify portions of the 16S ribosomal deoxyribonucleic acid (rDNA). Denaturing-gradient gel electrophoresis (DGGE) has been used to quantify and identify to at least the genus level the predominant microbial populations in a community (Muyzer, DeWaal, and Uitterlinden 1993). Microbial Insights, Inc. (Rockford, TN) has conducted some DGGE analyses with samples from the AFB bioreactors and found that several different types of microbes are present. Amplification of 16S rDNA using PCR can be used to generate a ribosomal clone library. Each 16S rDNA clone sequence can be determined, which also permits the identification of predominant microbial populations in environmental samples. Other PCR-based methods such as terminal restriction fragment length polymorphisms (t-RFLP) may also provide useful information that can be used to characterize the microbial community (Liu et al. 1997). Future integration of these methods into the analysis of the microbial communities in the AFB reactors will continue.

Objectives

The objectives were to study and characterize the microbial communities in two anaerobic bioreactors and to evaluate the impact of TNT on the microbial community in the same reactors. A better understanding of the microbial community will enable researchers to develop strategies to assess the performance of anaerobic bioreactors treating pinkwater as well as to optimize the biological treatment process.

Approach

To study the microbial ecology of explosive chemical biotransformation, AFB bioreactors were set up by the Construction Engineering Research Laboratory (CERL) to treat TNT in simulated wastewater. Sand was used as a support medium (instead of GAC) to facilitate the analysis of the wastewater and the microbial community. This medium will enable researchers to better distinguish between the AFB bioreactor microbial community in reactors with and without the addition of TNT.

CERL took samples weekly and provided them to Soon Hwan Oh, a graduate student of Dr. Sanford. RNA was extracted from the samples and analyzed as outlined in Chapter 2 to aid in characterizing the microbial community of the reactors.

Mode of Technology Transfer

Results of this research will direct further laboratory studies that will eventually lead to operational guidance for full-scale bioreactors used for the treatment of pinkwater.

2 Materials and Methods

Reactor Description

Two anaerobic bioreactors have been operated by CERL since August 1998. One reactor, designated "+TNT," received a synthetic feed containing ethanol and TNT (440 µM), while the other reactor, designated "Control," was fed only ethanol. The TNT mass loading was gradually increased from 646 mg day^{-1} on 22 March 1999 (after 9 weeks of sampling) until it was 1260 mg TNT day^{-1} on 6 April, almost double the initial loading rate. The reactors were sampled for 12 weeks at the 2X loading rate (except for a 2-week period in May) until the first week of August 1999, when RDX was substituted for TNT. Sand was used for the AFB support medium in both reactors. According to the phospholipid fatty acid (PLFA) analysis (data from Microbial Insights), over 95 percent of the biomass in the reactors was attached to the sand as a biofilm.

Reactor Sampling

Mixed liquor samples (100 mL) were taken weekly and transferred to 15-mL conical tubes. Samples were further dispensed into 2-mL screw-cap tubes, centrifuged at 7200 X g for 15 min, and the supernatant was decanted. Sand samples were also taken weekly and directly transferred to the 2-mL screw-cap tubes after carefully removing the supernatant. Cell pellets and sand-associated biofilm were frozen immediately in a dry ice/ethanol bath for 10 min and stored at -80 °C until RNA extraction.

Extraction of RNA

RNA from the reactor samples was extracted by a low-pH hot-phenol extraction method (Stahl et al. 1988). After the final chloroform extraction step, RNA was precipitated by adding 0.5 volumes of 7.5 M ammonium acetate and 0.9 volumes of isopropanol and overnight storage at -20 °C. RNA was collected by centrifugation at 16,400 X g for 30 min. The precipitated RNA was washed with 80 percent ethanol. Air-dried pellets were resuspended with 50 to 100 µl of filtered distilled water and stored at -80 °C until needed for membrane hybridization analysis.

Quality and Quantification of RNA

The extracted RNA was evaluated by polyacrylamide gel electrophoresis (PAGE) (3.3 to 10 percent discontinuous gel) (Alm and Stahl 2000). RNA concentrations were estimated by measuring the absorbance at a wavelength of 260 nm and assuming that each optical density (OD) unit corresponds to 0.5 mg RNA. RNA samples were diluted to 100 ng/µL and used as a working stock solution for membrane hybridization analysis.

Oligonucleotide Probes and Reference RNAs

Probes were chosen based in part on what one would expect to observe in an anaerobic bioreactor operating under methanogenic conditions and on earlier studies that involved the preliminary characterization of the microbial community in the bioreactors. Membrane hybridizations were performed using oligonucleotide probes specific for the domain *Bacteria* — the alpha-, beta-, and gamma-Proteobacteria subclasses, and the *Cytophaga/Flavobacteria* phyla. To quantify archaeal populations, oligonucleotide probes were used that were specific for the domain *Archaea* — the *Methanococceae,* the *Methanobacteriaceae,* the *Methanomicrobiales,* and the *Methanosarcinales*. In addition, to be able to normalize the hybridization response obtained with specific probes, a universal probe was used that hybridizes to the 16S rRNA of almost all organisms. To quantify the hybridization signals obtained with each probe, appropriate reference rRNAs were also applied to the hybridization membranes. Table 1 lists the oligonucleotide probes used in this experiment, their sequences, target organisms, and reference organisms.

Table 1. Oligonucleotide probes used in hybridizations.

Probe name (OPD[1])	Sequence (5' – 3')	Target group	Reference Organism	Td (°C)
S-*-Univ-1390-a-A-18	GACGGGCGGTGTGTACAA	virtually all organisms		44
S-D-Bact-0338-a-A-18	GCTGCCTCCCGTAGGAGT	virtually all *Bacteria*		55
S-Sc-aProt-0019-a-A-17	CGTTCG(C/T)TCTGAGCCAG[2]	α Proteobacteria subclass	*Azospirillum brasiliense*	53
L-Sc-bProt-1027-a-A-17	GCCTTCCCACTTCGTTT	β Proteobacteria subclass	*Alcaligenes faecalis*	58
L-Sc-gProt-1027-a-A-17	GCCTTCCCACATCGTTT	γ Proteobacteria subclass	*Acinetobacter lwoffii*	58
S-P-CytFlav-319-a-A-18	TGGTCCGTGTCTCAGTAC	*Cytophaga/ Flavobacterium* phylum	*Flavobacterium ulignosum*	55
S-D-Arch-0915-a-A-20	GTGCTCCCCCGCCAATTCCT	virtually all *Archaea*		56
S-F-Mcoc-1109-a-A-20	GCAACATAGGGCACGGGTCT	*Methanococcaceae*	*Methanococcus thermolithotrophus*	55
S-F-Mbac-0310-a-A-22	CTTGTCTCAGGTTCCATCTCCG	*Methanobacteriaceae*	*Methanobacterium formicicum*	57
S-O-Mmic-1200-a-A-21	CGGATAATTCGGGGCATGCTG	*Methanomicrobiales*	*Methanogenium organophilum*	53
S-O-Msar-0860-a-A-21	GGCTCGCTTCACGGCTTCCCT	*Methanosarcinales*	*Methanosarcina barkeri*	60

[1] Oligonucleotide Probe Database.

[2] This probe is a mixture of two probes.

Membrane Hybridization

Membrane hybridizations were performed as described in detail by Raskin et al. (1994). Oligonucleotide probes were 5'-end labeled with ^{32}P using T_4 bacteriophage polynucleotide kinase and ^{32}P-ATP as a substrate. RNA extracted from the reactor samples was diluted to 0.5 ng/µl and denatured with 2 percent of glutaraldehyde for 10 min at room temperature. Using a slot-blotter, 100 µl of RNA was applied to membranes (Magna Charge, Micron Separation Inc, Westbora, MA) in triplicate. After air drying, the RNA was immobilized by baking for 2 h at 80 °C and the membranes were prehybridized for at least 2 h. The membranes were hybridized overnight at 40 °C, then washed twice with 100 ml of wash buffer (1 percent of sodium dodecyl sulfate [SDS] and 1X sodium chloride sodium citrate [SSC] [0.15 M NaCl and 0.015 M sodium citrate] – pH 7.0) at 40 °C for 1 h. The membranes were then subjected to a final wash for 30 min at the temperature of dissociation (T_d) of each specific oligonucleotide probe. The hybridization results were quantified using an Instant Imager (Packard Instrument Co., Meriden, CT).

3 Results

RNA Extraction

Samples were taken from the +TNT and control bioreactors once a week for 40 weeks beginning in January 1999 according to the schedule summarized in Table 2. This table also indicates when the TNT loading was gradually increased from 685 mg/day to 1,370 mg/day starting at week 9. Extractions have been completed for the first 20 weeks. Because more than 95 percent of the biomass was associated with the biofilm support media (sand) (data from Microbial Insights), most RNA extractions were performed for the sand samples. The extracted RNA showed both the 16S and 23S bands of the RNA as determined by PAGE, indicating that the extracted RNA was intact (Figure 1). Membrane hybridization analyses were completed on reactor samples taken between 1/27/99 and 6/16/99 (Table 3). Only one mixed liquor sample for each reactor was included in the hybridization experiment (3/10/99 sample).

Reactor samples were also taken weekly between weeks 20 and 40 as shown in Table 2. RNA extraction has been completed for the samples designated in Table 2. The reactor feeding was changed during this latter period (weeks 20 to 40) with TNT replaced by RDX in the +TNT reactor.

Membrane Hybridization Results

The bacterial and archaeal rRNA levels in both reactors (% bacterial rRNA and % archaeal rRNA) during the first 20-week period are shown in Figure 2a and b. In both reactors, approximately 20 to 40 percent and 60 to 80 percent of the rRNA consisted of archaeal and bacterial rRNA, respectively. During the first 9 weeks, the levels of bacterial and archaeal rRNA were similar for both reactors. After 9 weeks, however, when the TNT loading was increased, the proportion of *Archaea* decreased in the +TNT reactor while the *Bacteria* increased.

The largest differences in archaeal population levels between the two reactors were observed for the *Methanosarcinales* and *Methanomicrobiales* (Figures 3 and 4). The *Methanosarcinales*, which contain all acetate-utilizing methanogens, were predominant in the control reactor, accounting for about 80 percent of the

Archaea. In the +TNT reactor, the *Methanosarcinales* decreased from about 50 percent to 20 percent of the *Archaea* after the TNT loading was increased (Figure 3). In contrast, the H_2-utilizing *Methanomicrobiales* were the predominant methanogens in the +TNT reactor after the TNT loading was increased, accounting for about 80 percent of *Archaea* (Figure 4). In the control reactor, however, the *Methanomicrobiales* were barely detectable. During weeks 16 and 17, when the +TNT reactor did not receive TNT, the *Methanomicrobiales* decreased and the *Methanosarcinales* populations increased. This trend was reversed when the TNT feed was reestablished by week 18 (Figures 3 and 4). No significant changes occurred with either the *Methanobacteriales* or the *Methanococcaceae* populations in both reactors. The *Methanobacteriales* were fairly stable in both reactors accounting for 20 to 30 percent of the *Archaea* during the whole 20-week period analyzed (Figure 5a). Although some fluctuations occurred in the +TNT reactor, these changes did not appear to correspond directly to the presence of TNT. The activity of the *Methanococcaceae* accounted for less than 10 percent of *Archaea* in both reactors (Figure 5b).

Table 2. Reactor sampling schedule for nucleic acid extraction and for fluorescent *in situ* hybridization (FISH).

week	Nucleic acid extraction sample					FISH sample		week	Nucleic acid extraction sample					FISH sample	
	Date	control sand	+TNT sand	control ML[a]	+TNT ML[a]	control sand	+TNT sand		Date	control sand	+TNT sand	control ML[a]	+TNT ML[a]	control sand	+TNT sand
0	1/21/99	2[g](1)[h]	2(1)	1	1	0	0	20	6/16/99	4(1)	4(1)	2	2	1	1
1	1/27/99	2	2	2	2	1	1	21	6/30/99	4	4	2	2	1	1
2	2/3/99	2(1)	2(1)	2	2	1	1	22	7/7/99	4(1)	4(1)	2	2	1	1
3	2/10/99	2	2	2	2	1	1	23	7/14/99	4	4	2	2	1	1
4	2/17/99	4(1)	4(1)	2	2	1	1	24	7/21/99	4(1)	4(1)	2	2	1	1
5	2/24/99	4(1)	4(1)	2(1)	2(1)	1	1	25	7/28/99	4	4	2	2	1	1
6	3/3/99	4(1)	4(1)	2	2	1	1	26[e]	8/4/99	4(1)	4(1)	2	2	1	1
7	3/10/99	4(2)	4(2)	4(1)	2(1)	1	1	27	8/18/99	4(1)	4(1)	2	2	1	1
8	3/17/99	4(1)	4(1)	2(1)	2(1)	1	1	28	9/1/99	4(1)	4(1)	2	2	1	1
9[b]	3/24/99	4(1)	4(1)	2	2	1	1	29	9/8/99	4	4	2	2	1	1
10	3/31/99	4(1)	4(1)	2	2	1	1	30	9/15/99	4(1)	4(1)	2	2	1	1
11[c]	4/7/99	4(1)	4(2)	2	2	1	1	31	9/21/99	4	4	2	2	1	1
12	4/14/99	4(1)	4(1)	2	2	1	1	32	10/5/99	4(1)	4(1)	2	2	1	1
13	4/21/99	4(1)	4(2)	2	2	1	1	33	10/19/99	4(1)	4(1)	2	2	0	0
14	4/28/99	4	4	2	2	1	1	34	10/26/99	4	4	2	2	1	1
15	5/5/99	4(2)	4(2)	2	2	1	1	35	11/2/99	4	4	2	2	1	1
16[d]	5/12/99	4	4	2	2	1	1	36	11/9/99	4	4	2	2	1	1
17[d]	5/19/99	4(2)	4(1)	2	2	1	1	37	11/16/99	5	5	2	2	1	1
18	5/28/99	4(1)	4(1)	2	2	1	1	38[f]	11/23/99	4	4	2	2	1	1
19	6/7/99	4	4	2	2	1	1	39	11/30/99	12	12	2	2	1	1

[a] Mixed liquor.
[b] Initiation of TNT-loading increase (3/22).
[c] TNT loading at double initial rate in +TNT reactor.
[d] TNT not present in the influent in +TNT reactor.
[e] TNT replaced by RDX in +TNT reactor.
[f] RDX loading was decreased to half the original loading.
[g] Number of tubes prepared for nucleic acid extractions
[h] Number ( ) of tubes used for nucleic acid extractions

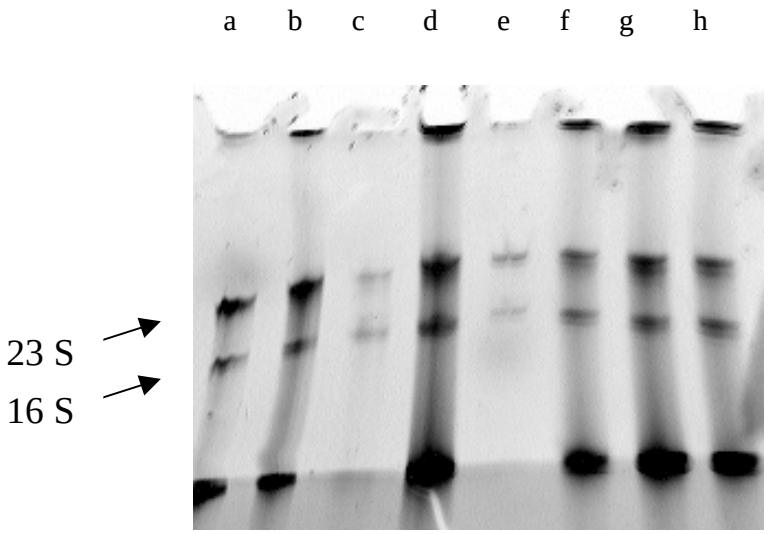


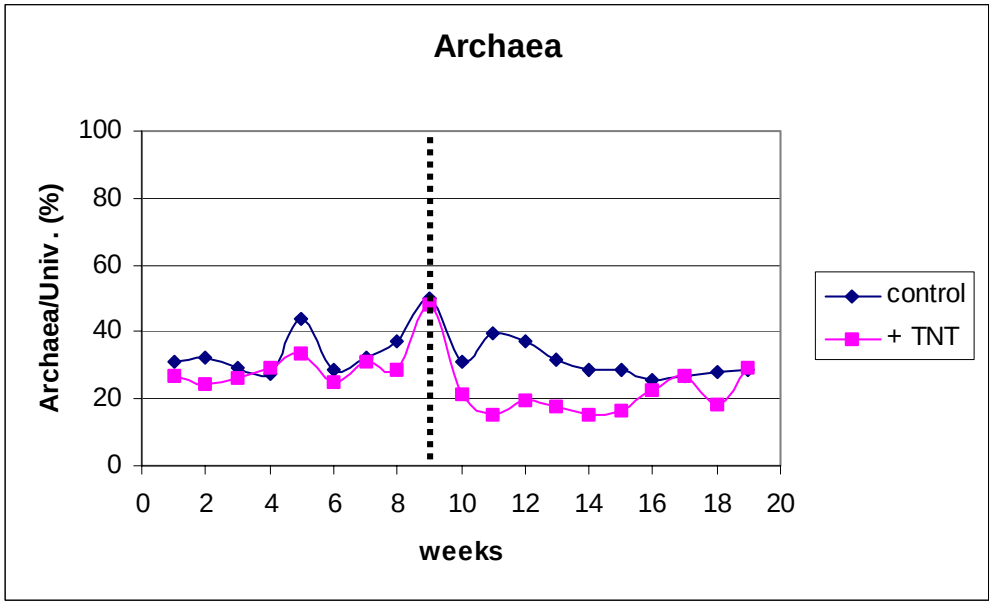
Figure 1. 3.3% PAGE gel of RNA extracted from reactor samples. Lane a is 3/10 reactor +TNT-mixed liquor, b is 3/10 reactor +TNT-sane, c is 4/21 reactor +TNT-sane, d is 4/28 reactor control-sane, e is RNA standard 62.5 ng, f is 4/28 reactor +TNT-sand, g is 5/12 reactor control-sand, and h is 5/12 reactor +TNT-sand.

Table 3. RNA samples used in membrane hybridizations.

Week[a]	Date	Sample	ng[b]		Sample	ng[b]
1	1/27/99	control-sand	24.3		+TNT-sand	19.3
2	2/3/99	control-sand	45.5		+TNT-sand	23.3
3	2/10/99	control-sand	23.8		+TNT-sand	27.5
4	2/17/99	control-sand	42.1		+TNT-sand	36.0
5	2/24/99	control-sand	54.4		+TNT-sand	31.5
6	3/3/99	control-sand	32.3		+TNT-sand	36.7
7	3/10/99	control-sand	63.2		+TNT-sand	33.3
8	3/17/99	control-sand	32.1		+TNT-sand	24.6
9	3/24/99	control-sand	45.2		+TNT-sand	32.0
10	3/31/99	control-sand	20.4		+TNT-sand	15.5
11	4/7/99	control-sand	50.7		+TNT-sand	36.8
12	4/14/99	control-sand	38.9		+TNT-sand	44.4
13	4/21/99	control-sand	26.1		+TNT-sand	39.1
14	4/28/99	control-sand	28.5		+TNT-sand	37.4
15	5/5/99	control-sand	18.1		+TNT-sand	25.2
16	5/12/99	control-sand	20.8		+TNT-sand	55.6
17	5/19/99	control-sand	17.7		+TNT-sand	21.8
18	5/28/99	control-sand	33.2		+TNT-sand	25.0
19	6/16/99	control-sand	16.6		+TNT-sand	3.5
	3/10/99	control-ML[c]	51.4		+TNT-ML[c]	25.7

[a] Designated week of sample taken (see Table 2).
[b] Amount of RNA (ng) applied to the membrane, as determined by universal probe.
[c] Mixed liquor.

a



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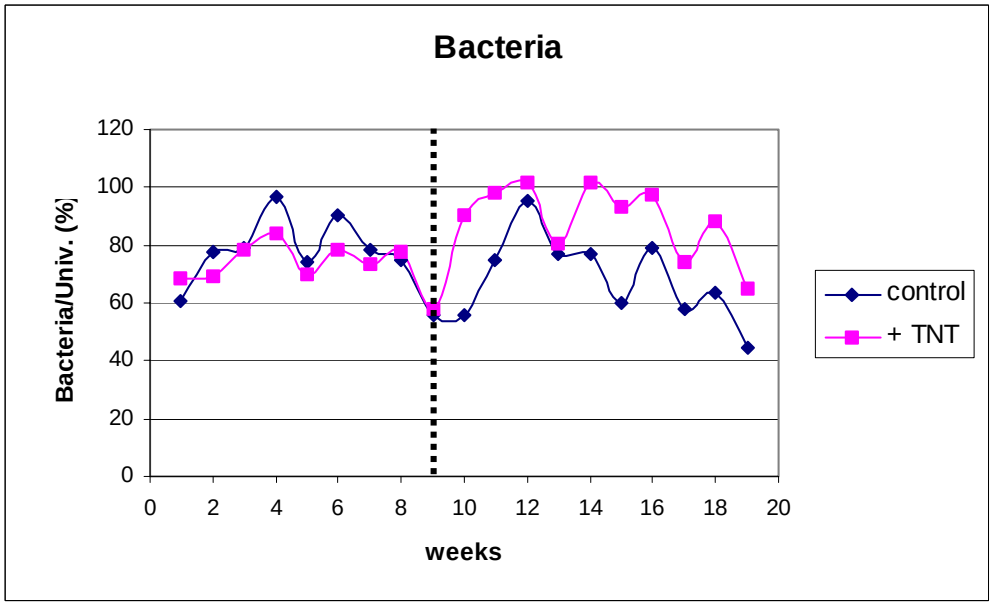


Figure 2. Proportion of the *Bacteria* and *Archaea* 16S rRNA in the control and +TNT reactors. The hybridization signals obtained with the archaeal specific probe (S-D-Arch-0915-a-A-20) and the bacterial specific probe (S-D-Bact0338-a-A-18) were divided by the signals obtained with the universal probe (S-*-Univ-1390-a-A-18) and expressed as a percentage. Dashed line indicates when TNT loading increased.

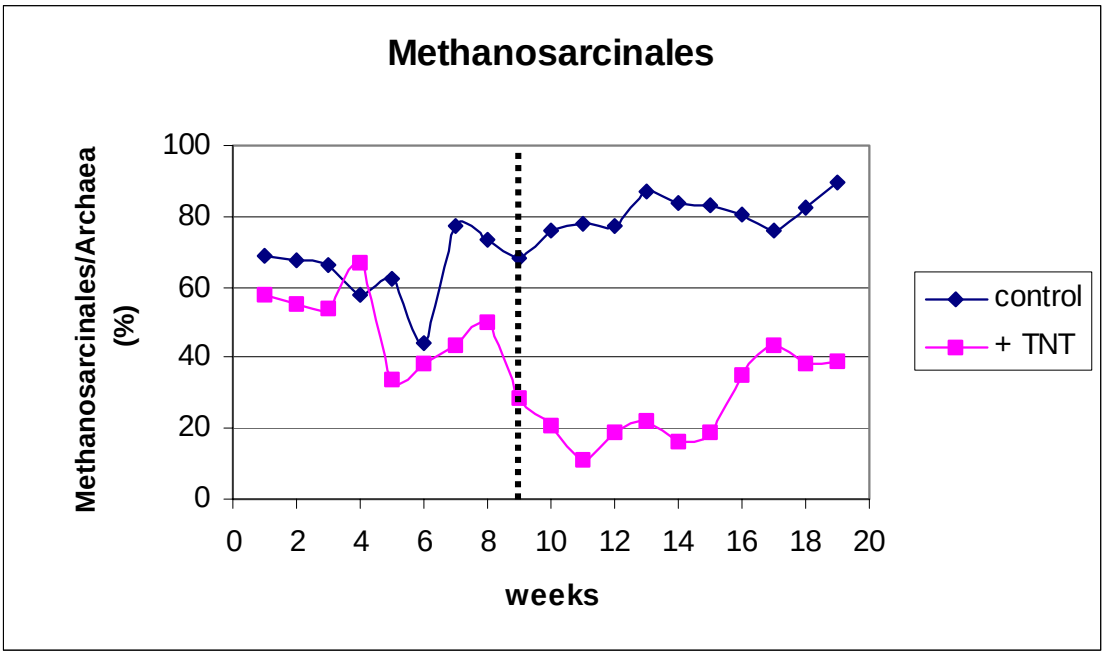


Figure 3. Proportion of the *Methanosarcinales* 16S rRNA in the control and +TNT reactors. The percentage of *Methanosarcinales* was obtained by calculating the ratio of the hybridization signal with the *Methanosarsinales* specific probe (S-O-Msar-0860-a-A-21) to the signal with S-D-Arch-0915-a-A-20. Dashed line indicates when TNT loading increased.

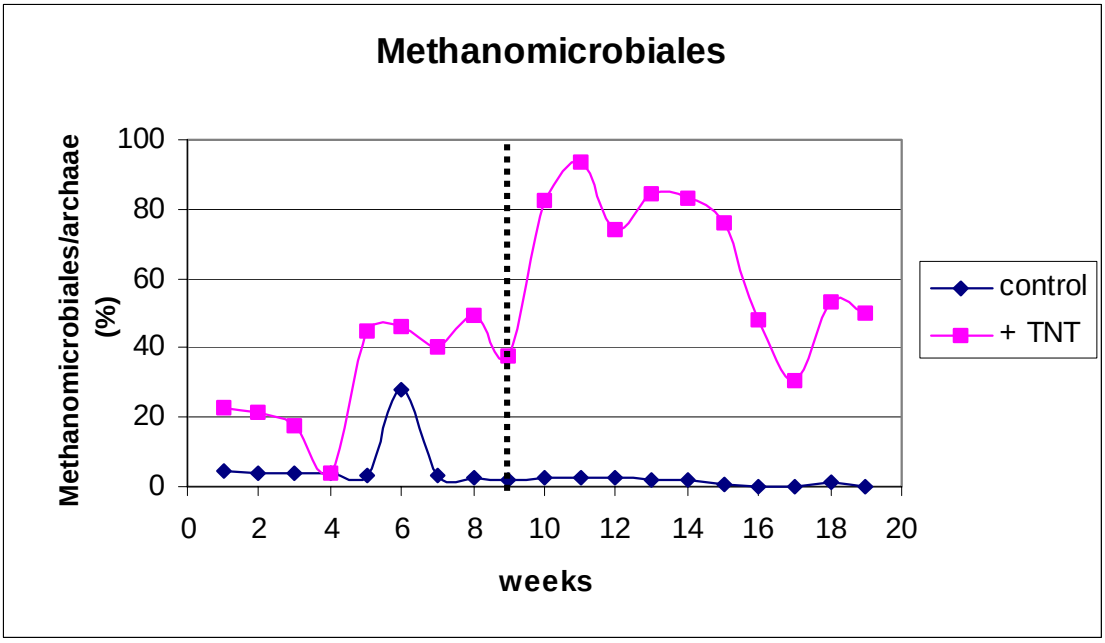
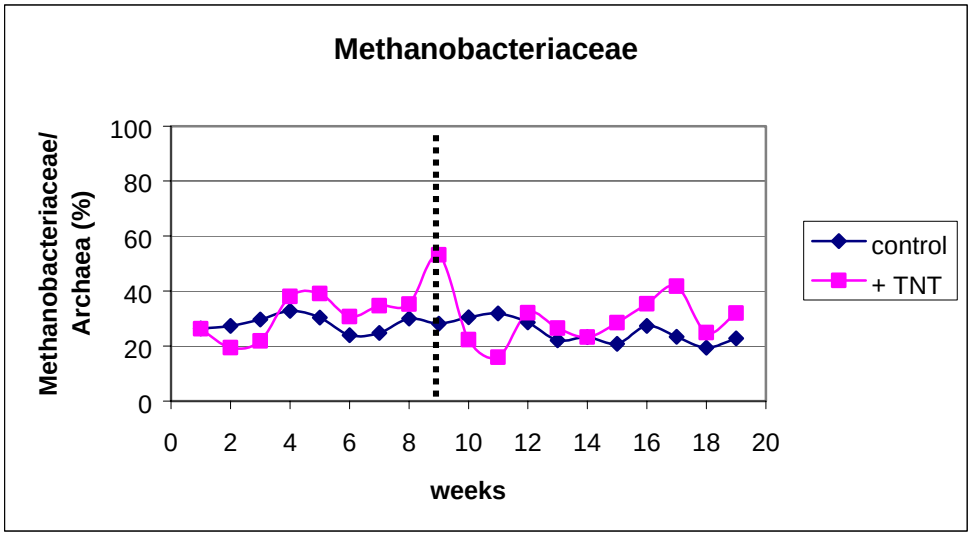


Figure 4. Proportion of the *Methanomicrobiales* 16S rRNA in the control and +TNT reactors. The percentage of *Methanomicrobiales* was obtained by calculating the ratio of the hybridization signal with the *Methanomicrobiales* specific probe (S-O-Mmic-1200-a-A-21) to the signal with S-D-Arch-0915-a-A-20. Dashed line indicates when TNT loading increased.

a



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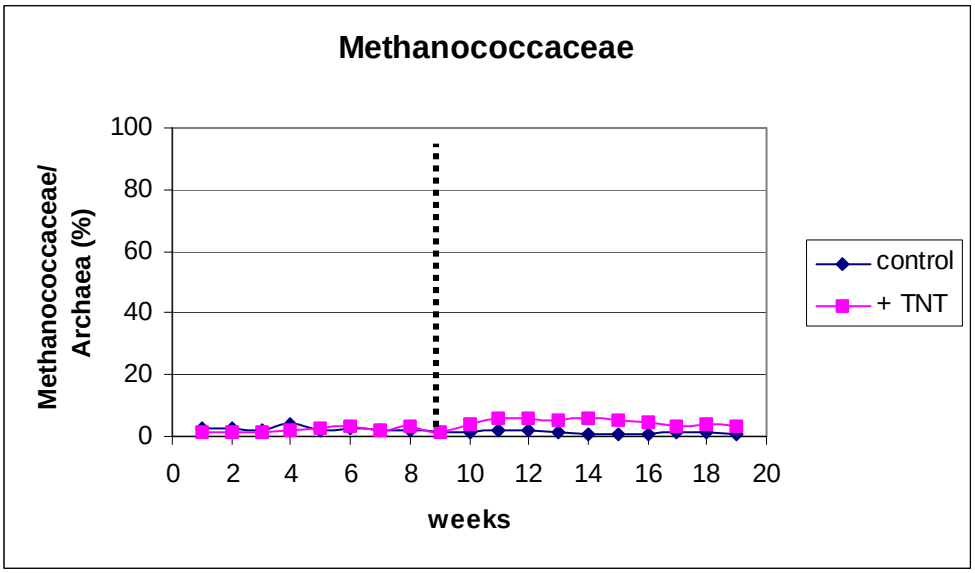


Figure 5. Proportion of the *Methanobacteriales* and *Methanococcaceae* 16S rRNA in the control and +TNT reactors. The percentage of *Methanobacteriales* (a) was obtained from the ratio of the hybridization signal with the *Methanobacteriales* specific probe (S-F-Mbac-0310-a-A-22) to the signal with S-D-Arch-0915-a-A-20. The percentage of *Methanococcaceae* (b) was obtained from the ratio of the hybridization signal with the *Methanococcaceae* specific probe (S-F-Mcoc-1109-a-A-20) to the signal with S-D-Arch-0915-a-A-20. Dashed line indicates when TNT loading increased.

The hybridization results using bacterial-specific probes also showed a significant effect of TNT, with the rRNA hybridizing to the alpha-Proteobacteria probe increasing when the TNT loading was increased (Figure 6). The hybridization signals obtained by the alpha-Proteobacteria probe increased almost twice to 60 percent of *Bacteria* after 9 weeks in the +TNT reactor whereas, in the control reactor, the signal was about 35 percent of *Bacteria* continuously (Figure 6). The probe (S-Sc-aProt-0019-a-A-17) used in this experiment, however, is not exclusively specific to alpha-Proteobacteria and is known to hybridize to several

members of the delta-Proteobacteria. It is possible that the increased hybridization signal in the +TNT reactor can be attributed to microorganisms outside the alpha-Proteobacteria. Hybridization results indicated that the proportion of the *Cytophaga/Flavobacteria* group in the sand biofilm accounted for approximately 10 percent of the 16S rRNA in both reactors (Figure 7). Gamma- and beta-Proteobacteria accounted for a very small proportion of *Bacteria* — less than 10 percent and less than 1 percent of bacterial 16S rRNA respectively in both reactors (Figure 8a and b).

With the exception of the *Cytophaga/Flavobacteria* group, the mixed liquor sample from both reactors showed similar levels of rRNA from all other methanogen and bacterial groups. The *Cytophaga/Flavobacteria* group accounted for a major proportion of the rRNA (45 percent of *Bacteria*) in the mixed liquor from the +TNT reactor (data not shown). Less than 10 percent of the bacterial 16S rRNA was attributed to this group in the control reactor. The lower percentage indicates the possibility of different bacterial community structures between the sand and mixed liquor in the +TNT reactor.

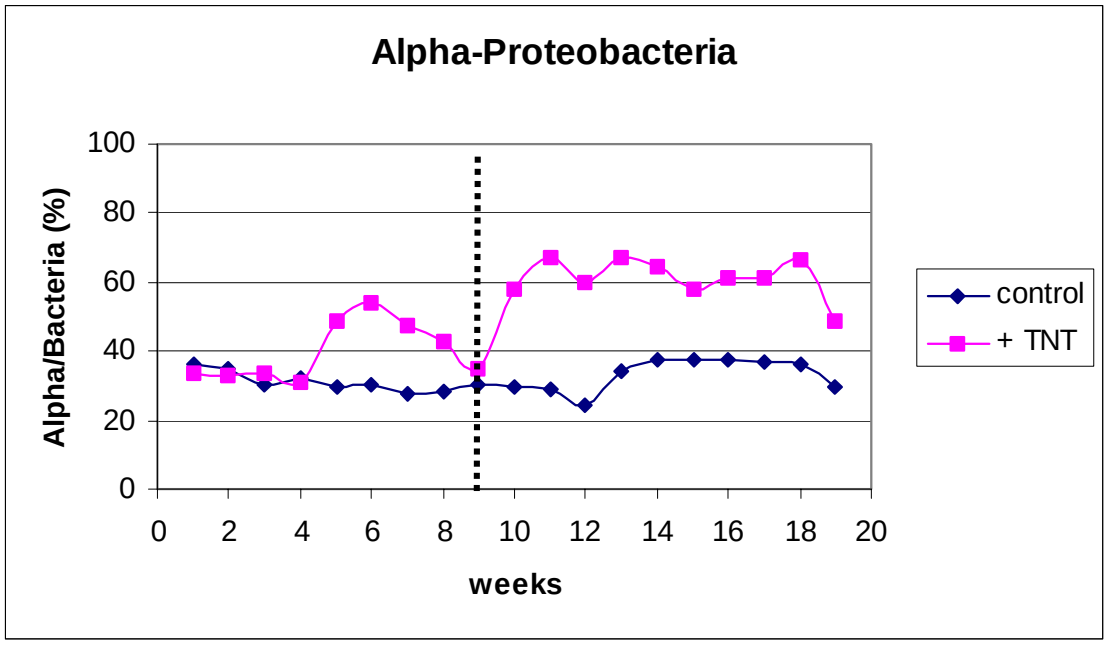


Figure 6. Microbial 16S rRNA hybridizing to the alpha-Proteobacteria probe in the control and +TNT reactors. The percentage of rRNA hybridizing to alpha-Proteobacteria probe was obtained from the ratio of the hybridization signal with the probe (S-Sc-aProt-0019-a-A-17) to the signal with the bacterial specific probe (S-D-Bact0338-a-A-18). Dashed line indicates when TNT loading increased.

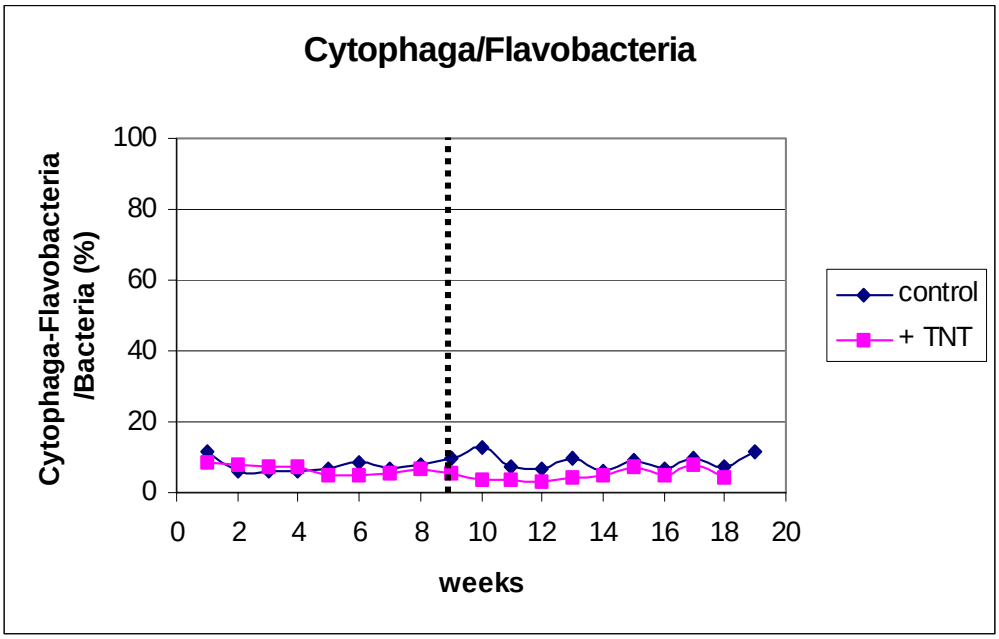
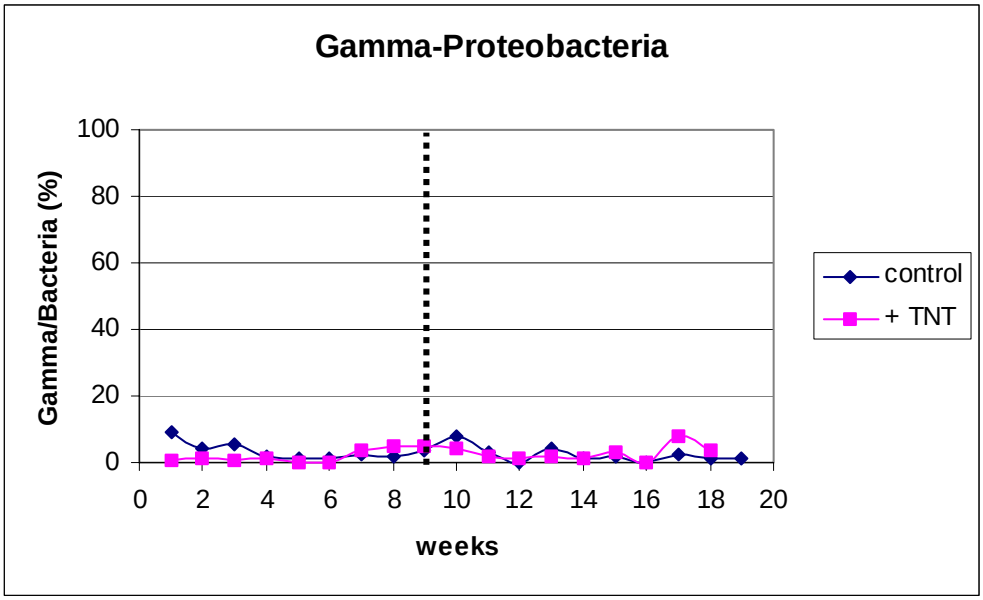


Figure 7. Proportion of *Cytophaga/Flavobacteria* 16S rRNA in the control and +TNT reactors. The percentage of *Cytophaga/Flavobacteria* was obtained from the ratio of the hybridization signal with the *Cytophaga/Flavobacteria* specific probe (S-P-Cyt-Flav-319-a-A-18) over the signal with S-D-Bact0338-a-A-18. Dashed line indicates when TNT loading increased.

a



b

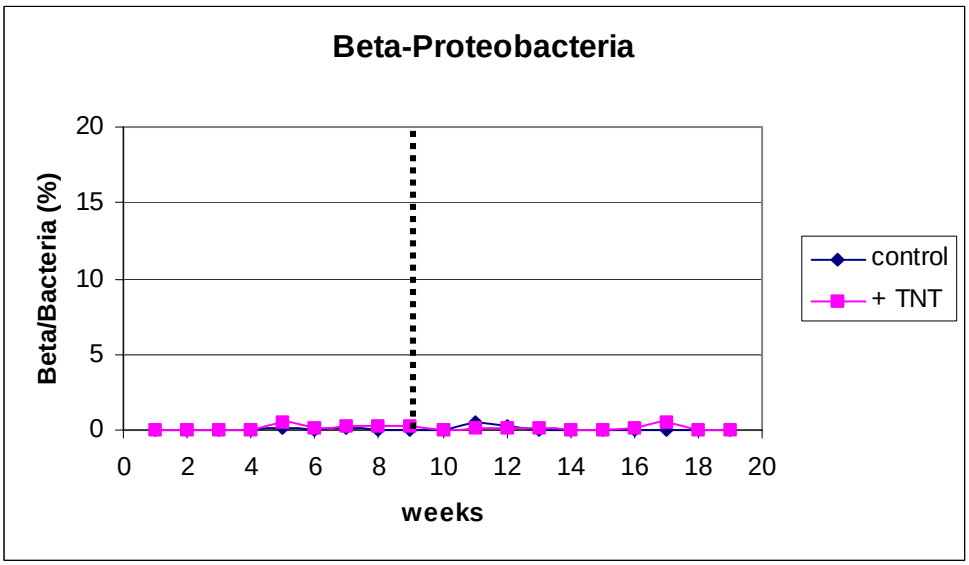


Figure 8. Proportion of gamma- and beta-Proteobacteria rRNA in the control and +TNT reactors. The percentage of gamma-Proteobacteria (a) was obtained from the ratio of the hybridization signal with the gamma-Proteobacteria specific probe (L-Sc-gProt-1027-a-A-17) to the signal with S-D-Bact0338-a-A-18. The percentage of beta-Proteobacteria (b) was obtained from the ratio of the hybridization signal with the beta-Proteobacteria specific probe (L-Sc-bProt-1027-a-A-17) to the signal with S-D-Bact0338-a-A-18. Dashed line indicates when TNT loading increased.

4 Discussion and Conclusions

TNT was used as a model substrate to investigate the effect of pinkwater on microbial community structure in an AFB bioreactor. RNA was extracted from reactor samples, and membrane hybridization analysis was performed using different oligonucleotide probes. In the control reactor, the *Methanosarcinales* were the predominant methanogens accounting for 80 percent of *Archaea*, while in the +TNT reactor, they accounted for only 20 percent of *Archaea* after the TNT loading was doubled. This result indicates that TNT has a significant impact on the acetate-utilizing methanogens. This conclusion is corroborated to some extent by the persistence of acetate in the +TNT reactor (Jae Kim personal communication). The *Methanomicrobiales* are the predominant methanogens (80 percent of *Archaea*) in the +TNT reactor, while they are mostly undetectable in the control reactor. This result is unusual as the difference is particularly large in the *Methanomicrobiales* 16S rRNA between the two reactors. Perhaps TNT inhibits only certain methanogen populations, such as the *Methanosarcinales*, which gives a selective advantage to the *Methanomicrobiales* when TNT is present. Alternatively, it is possible that the change in the *Archaea* that occurs with TNT contributes to the discrepancy between these two reactors with the *Methanomicrobiales*, however, this drop in *Archaea* is not enough to account for the relative increase observed in the +TNT reactor. Further work should be done to evaluate the impact of the changing methanogen community structure on the stability of the treatment system.

The RNA that hybridized to the alpha-Proteobacteria probe accounted for 60 percent of the bacterial rRNA after TNT loading was doubled in the +TNT reactor. An analysis of this alpha-Proteobacteria probe sequence indicated that it would also hybridize to rRNA from *Bacteria* in the delta-Proteobacteria. Because of the ambiguous specificity of the alpha-Proteobacteria probe, further studies are necessary. Previous data from Microbial Insights, for example, indicate that the delta-Proteobacteria genera *Geobacter* and *Pelobacter* are present in both reactors. The alpha-Proteobacteria probe hybridizes to strains in both of these genera, indicating that it is reasonably possible that the increase observed in the +TNT reactor is due to the presence of one of these microorganisms. The *Cytophaga/Flavobacteria* group accounted for about 10 percent of *Bacteria* while the sum of the gamma- and beta-Proteobacteria accounted for less than 10 percent of the bacterial 16S rRNA. The presence of higher levels of the *Cytophaga/*

Flavobacteria group in one sample of the mixed liquor compared to the sand biofilm in the +TNT reactor indicates the possibility of a different community structure in these two environments. Additional molecular analyses of selected mixed liquor samples will be completed to validate this hypothesis.

In summary, the hybridization results indicate that the presence of TNT significantly impacts both the archaeal and bacterial community structures. The reduction in acetate-utilizing methanogens could be problematic to reactor operation in the long term; however, no effects were obviously apparent on the performance of the reactor. Hybridization results do suggest that microorganisms with 16S rRNA detected by the alpha-Proteobacteria probe may be involved in TNT reduction to tri-amino toluene (TAT), since this group increased when the TNT loading was doubled. Future efforts will focus on characterizing the populations targeted by this probe.

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14. ABSTRACT

Pinkwater, a hazardous wastewater containing 2,4,6-trinitrotoluene (TNT), is produced during munition production or demilitarization operations. Anaerobic bioreactors are under investigation as an alternative to activated carbon for treating pinkwater. This research studied the microbial community structure of an anaerobic bioreactor treating a synthetic feed containing ethanol and TNT. Samples from the reactor were taken weekly, ribonucleic acid (RNA) was extracted, and membrane hybridizations were performed using ologonucleotide probes specific for the domains *Bacteria* and *Archaea*. The bacterial and archaeal ribosomal RNA (rRNA) were similar between the two reactors, but, after doubling the TNT loading, the archaeal rRNA decreased and the bacterial rRNA increased. In response to the increased TNT loading, the α-Proteobacteria increased to 60 percent of the bacterial rRNA, but remained unchanged in the control reactor. In the control reactor, the *Methanosarcinales* accounted for about 80 percent of the archaeal rRNA, while, in the +TNT reactor, they decreased from about 50 percent to 20 percent after increasing the TNT loading. The *Methanomicrobiales* accounted for about 80 percent of the *Archaea*, while they were barely detectable in the control reactor. The findings indicate that TNT significantly affects the bacterial and archaeal community structure. Several strategies are suggested for assessing and optimizing anaerobic bioreactors treating pinkwater.

15. SUBJECT TERMS
TNT, munitions waste, anaerobic fluidized bioreactors, pinkwater, hazardous waste management

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