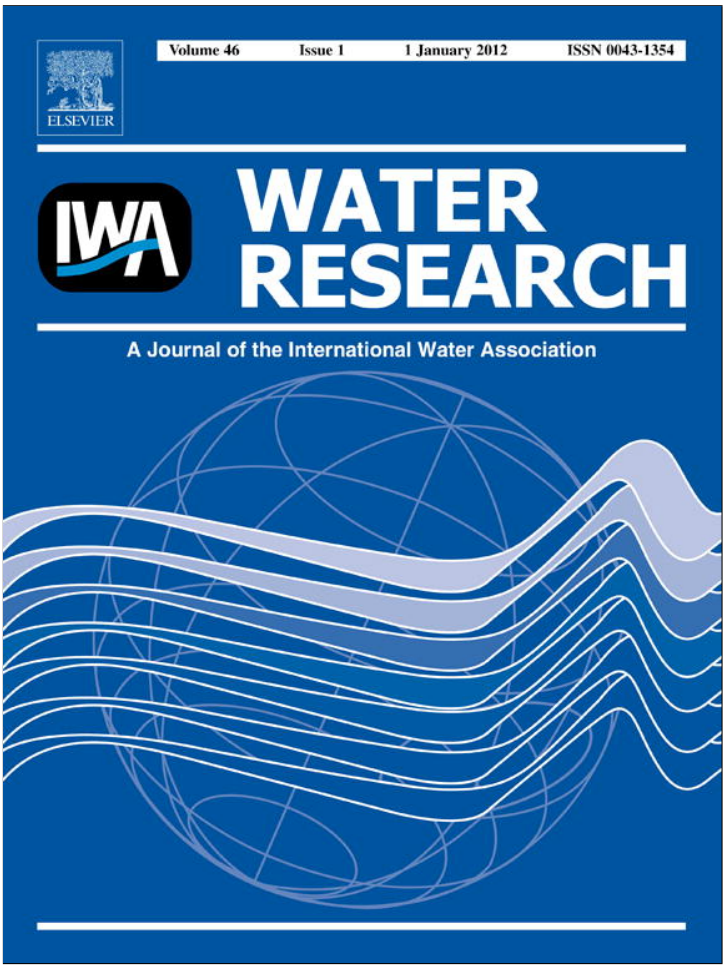

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High diversity and differential persistence of fecal *Bacteroidales* population spiked into freshwater microcosm

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ABSTRACT

Bacteroidales markers are promising indicators of fecal pollution and are now widely used in microbial source tracking (MST) studies. However, a thorough understanding of the persistence of *Bacteroidales* population after being released into environmental waters is lacking. We investigated the persistence of two host specific markers (HF183 and CF193) and temporal change of *Bacteroidales* population over 14 days in freshwater microcosms seeded with human or bovine feces. The concentrations of HF183/CF193 and *Escherichia coli* were determined using quantitative polymerase chain reaction (qPCR) and standard cultivation method, respectively. Shifts in the *Bacteroidales* population structure were fingerprinted using PCR-denaturing gradient gel electrophoresis (DGGE) and subsequent sequencing analysis targeting both 16S rDNA and rRNA-transcribed cDNA. Both HF183 and CF193 decayed significantly faster than *E. coli* but the decay curves fit poorly with first-order model. High diversity of *Bacteroidales* population was observed for both microcosms, and persistence of different species in the population varied. Sequence analysis indicated that most of the bovine *Bacteroidales* populations in our study are unexplored. DGGE and decay curve indicated that RNA decayed faster than DNA, further supporting the use of rRNA as indicator of metabolically active *Bacteroidales* population. Evaluations with more realistic scenarios are warranted prior to extending the results of this study to real field settings.

1. Introduction

Bacteroidales, primarily from the family *Prevotellaceae* and *Bacteroidaceae*, are strict anaerobes. Members of the *Bacteroidales* group have been suggested as an alternative indicator of recent fecal contamination (Kreader, 1995) because of i) their higher abundance in fecal populations than traditional fecal indicator bacteria (FIB) (Eckburg et al., 2005), ii) the low potential to grow outside of the host due to their anaerobic metabolism (Kreader, 1995, 1998), and iii) the correlation with the presence of fecal pathogens (Walters et al., 2007). In addition, certain fecal *Bacteroidales* lineages are host specific (Bernhard and Field, 2000), the presence of which enables identification of the source of fecal pollution.

Polymerase chain reaction (PCR) based microbial source tracking (MST) methods targeting host specific *bacteroides*

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gene markers have been developed and increasingly applied to evaluate the source of fecal pollution in environmental water in different geographical locations (Fremaux et al., 2009; Gawler et al., 2007; Gourmelon et al., 2007). Quantitative PCR methods have also been utilized to assess the abundance of host specific markers in natural waters (Kildare et al., 2007; Layton et al., 2006). Before qPCR data can be reliably used to quantify the contribution of fecal contamination from each specific source, two properties concerning *Bacteroidales* markers need to be considered (Schulz and Childers, 2011): namely the marker distribution and abundance within different host species, and the relative persistence/survival of markers and/or marker cells in the non-intestinal environments under the interaction of various environmental factors. Understanding the survival of *Bacteroidales* spp are also important in the development of proper MST models for health risk predication (Balleste and Blanch, 2010).

Previous studies indicated that certain fecal *Bacteroides* spp cells could survive from only a few hours to a few days in environmental water (Bae and Wuertz, 2009; Balleste and Blanch, 2010; Kreader, 1998), while the host specific *Bacteroides* DNA marker could persist up to weeks (Bae and Wuertz, 2009; Kreader, 1998; Walters and Field, 2006, 2009). Environmental factors, including temperature, the presence of predators, salinity, and sunlight, have been reported to affect the survival of *Bacteroidales* species and persistence of *Bacteroidales* DNA (Bell et al., 2009; Kreader, 1998; Okabe and Shimazu, 2007; Savichtcheva et al., 2005). *Bacteroidales* are a deeply divergent and diverse group of microorganisms based on 16S rRNA phylogeny. A high diversity of *Bacteroidales* from animal feces was also reported (Jeter et al., 2009). In a realistic scenario for MST study, the clades that the host specific *Bacteroidales* markers detect may include a variety of species, genera, and families from a wide range of sources (individuals), which may not necessarily share the same survival profiles in the environment (Schulz and Childers, 2011). In order to accurately attribute the fecal pollution to different sources, persistence of the whole *Bacteroidales* population should be considered. But the few studies investigating the persistence of *Bacteroidales* spp. were confined to only one to limited species (Balleste and Blanch, 2010; Kreader, 1998). Divergence in the response of different *Bacteroidales* species to environmental stresses has been detected (Balleste and Blanch, 2010; Wilkins et al., 1978); it is therefore improper to infer the environmental persistence of the *Bacteroidales* population in feces from the pattern of only a few species. Thus, there is a gap of information on the ecology and environmental behavior of fecal *Bacteroidales* populations. Using clone library analysis, Schulz and Childers (Schulz and Childers, 2011) indicated that diverse lineages of *Bacteroidales* survived similarly, but the use of DNA in their study failed to provide information on the survival of live cells under those conditions.

Our objective in this study was to determine the persistence pattern of a fecal *Bacteroidales* population once they are released into environmental waters. Freshwater microcosms inoculated with human or bovine feces were incubated for a period of 14 days; population compositions of *Bacteroidales* spp. in the microcosm were fingerprinted with both a DNA and RNA based DGGE approach targeting 16S rRNA sequence. The persistence of the fecal *Bacteroidales* population was compared with persistence of two host specific *Bacteroidales* markers. The persistence of the standard fecal indicator, *Escherichia coli*, was also monitored during the entire incubation, using standard culture methods, for comparison. This may be the first study to evaluate the persistence pattern of metabolically active fecal *Bacteroidales* population in the extra-intestinal environment.

2. Materials and methods

2.1. Microcosm setup and sampling

Fresh human fecal samples were collected within 24 h of use from 4 healthy adult volunteers in different families with a sterile utensil and stored in a sterile 50 ml tube. On the day of experimental setup fresh cattle fecal samples were collected immediately after excretion from 4 bovines in 2 cattle farms. Fecal samples were transported in the dark on ice to the laboratory immediately after the collection. Two types of microcosm were constructed: the human microcosms in which human fecal samples were spiked into 28 L water, and the bovine microcosm in which cattle fecal samples were spiked. For each host, 40 g (wet weight) of fecal sample (10 g from each individual) was homogenized by mixing and suspended into 300 ml sterile PBS solution. Large fecal particles were removed and the fecal slurry was stored on ice until use.

Fresh water was collected in autoclaved containers from a canal near the Indian River Research and Education Center in Fort Pierce, FL and stored overnight in the dark at room temperature before the start of the experiments. The initial turbidity of the fresh river water was 31 nephelometric turbidity units (NTU). To check for the presence of *Bacteroidales* spp. prior to the microcosm experiment, 500 ml of freshwater samples were concentrated by membrane filtration and subjected to direct DNA extraction in triplicates using PowerSoil DNA extraction kit as described in Section 2.3. The presence/absence of host specific *Bacteroidales* markers HF183 and CF193 in this source water was checked by qPCR as described in Section 2.5.

The microcosm was designed to simulate the environmental water conditions fecal microbiota may encounter after being released from the host, such as radiation, fluctuating ambient temperature, and the possible presence of bacterivorous predators. Microcosms were set up in a roofed greenhouse without sides. The polycarbonate roofing blocks most of the UV but allows 90% visible light transmission (http://www.usgr.com/greenhouse-coverings/polycarbonate.php). Sterile glass aquariums with dimensions of 25.4 by 50.8 by 25.4 cm (W/L/H) were used as containers with an air stone installed at the bottom of each aquarium for aeration and constant mixing of the water column. Human fecal *Bacteroidales* microcosms were established in triplicates by inoculating 100 ml of the human fecal slurry into the aquarium filled with 28 L of freshwater. The bovine microcosms were conducted using the same method. The final concentration of feces in the microcosm was 46 mg wet weight per 100 ml water. The outside of the side wall and bottom of the

aquarium was wrapped with opaque material to ensure light penetrated only from the top opening of the microcosm. FEP-Teflon film (1/20 mm thick) which allows 96% of natural light penetration was used to cover the opening (top) of the microcosms to reduce water evaporation during the incubation. Slight evaporation could still be observed on each sampling event. We calculated daily evaporation loss of water using the following equation: $v = [(L \times W \times \Delta H) - S]/14$, where v is the daily evaporation loss of water; L and W are the length and width of the microcosm, respectively; ΔH is the difference between the initial water level and the final water level, and S is the total volume of water sampled during the 14 days incubation. The density of targets in the microcosms was corrected accordingly to account for the loss of evaporation.

Sampling was conducted at day 0 (1 h after the inoculation), 2, 4, 6, 8, 10, 12 and 14 for marker concentration analysis, DGGE profiling of *Bacteroidales* population and *E. coli* enumeration. Sampling was conducted at approximately the same time on each sampling day. For each microcosm, around 150 ml of water was collected in a sterile container for nucleic acid extraction and subsequent DNA/RNA analysis. Water (10 to 100 ml) was collected for *E. coli* enumeration. Water samples were transferred back to the laboratory on ice and processed immediately.

2.2. E. coli *enumeration*

E. coli densities were determined, in triplicate, using standard membrane filtration methods (USEPA method 1623). A preliminary experiment was conducted to determine the initial concentration of *E. coli* in the microcosm. Dilutions ranging from one hundreds to one tenth were made for accurate enumeration. The concentration of *E. coli* was expressed as CFU per 100 ml sample.

2.3. *Nucleic acid extraction*

Canal fresh water: Five hundred ml of source water was concentrated by membrane filtration using nitrocellulose filters (0.45 μm pore size, 47 mm diameter). The filter was aseptically folded, cut and transferred into the PowerBead tube included in the PowerSoil™ DNA kits (MO BIO Laboratories, Inc., Carlsbad, CA). DNA from the microorganisms on the filters was extracted using PowerSoil™ DNA kits following the manufacturer's instruction with some modification as described previously (Liang et al., 2008). Eluted DNA was stored at −80 °C.

Microcosm sample: A total of 120 ml water sample was centrifuged at 15000 rpm for 20 min at 4 °C using an Eppendorf 5430R microcentrifuge in 50 ml Nalgene™ Oak Ridge polypropylene centrifuge tubes (Fisher Scientific). DNA/RNA from each sample was immediately extracted from the pellet using AllPrep™ RNA/DNA Mini kit (Qiagen, Valencia, CA) following the manufacturer's instruction. Extraction control using double stilled water was included each day the extraction was conducted. DNA eluted in 50 μl buffer EB was stored at −80 °C. RNA eluted in 30 μl RNase free water were immediately subjected to DNase treatment using TURBO DNA-free™ Kits (Ambion, Foster City, CA) following the manufacturer's suggestions to remove co-eluted trace DNA. RNA supernatants were transferred to RNase free 0.2 ml vial and subjected to reverse transcription.

2.4. *Reverse transcription*

Reverse transcription was conducted with an Eppendorf Mastercycler (Eppendorf) using High Capacity RNA-to-cDNA master mix (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. Two μl of RNA was mixed with 1 × RNA-to-cDNA master mix in a 20 μl reaction volume. The reactions were performed by incubating the sample at 25 °C for 5 min, 42 °C for 30 min, and 85 °C for 5 min. Contamination of genomic DNA was tested for using master mix without reverse transcriptase (−)RT included in the kit. cDNAs transcribed were stored at −80 °C. cDNAs were subjected to qPCR and PCR-DGGE analysis.

2.5. *Quantification of host specific* Bacteroidales *marker DNA and cDNA*

Human specific primer HF183 and bovine specific primer CF193 (Bernhard and Field, 2000) were paired with 265R (Seurinck et al., 2005) for human and bovine specific marker quantification, respectively. Quantitative PCR was performed with a CFX96™ Real-Time PCR Detection System (Bio-rad, Hercules, CA) in a 20 μl reaction volume containing 10 μl iQ™ SYBR® Green Supermix (Bio-rad, Hercules, CA), 0.2 μM of each forward and reverse primers and 2 μl template DNA/cDNA. A universal thermal cycling protocol (initial denaturation step of 94 °C for 2 min, followed by 40 cycles of 94 °C for 15 s and 60 °C for 60 s) was used for the quantification of both markers. A final melt curve analysis was performed to determine the presence or absence of nonspecific amplification products. All samples were run in triplicate and a negative control was included for each analysis. Standard curves were generated using a serial dilution of purified plasmid DNA containing each host specific sequence.

2.6. *PCR-DGGE and sequence analysis*

DNA extracted and cDNA transcribed were amplified by PCR using primers Bfr-F/Bfr-R-GC (Liu et al., 2003) and then subjected to DGGE analysis. This primer pair was originally designed for the identification of the group *Bacteroidales fragilis, which included the species Bacteroides caccae, Bacteroides distasonis, Bacteroidales eggerthii, Bacteroidales fragilis, Bacteroidales merdae, Bacteroidales ovatus, Bacteroidales stercoris, Bacteroides thetaiotaomicron, Bacteroides uniformis, and Bacteroidales vulgates* (Liu et al., 2003). Since then it has been widely used for the detection of *Bacteroidales*. spp from human and animal fecal microbiota (Li et al., 2007, 2009; Pang et al., 2005; Yuan et al., 2011). A 40 bp GC-clamp was attached to the 5′ end of Bfr-R to facilitate band separation on DGGE. All PCR reactions were conducted in an Eppendorf Mastercycler (Eppendorf). Two μL of DNA extract or 5 μl cDNA was used as template in a reaction volume of 25 μL containing 1.5U Taq DNA polymerase and 1× PCR buffer (20 mM Tris–HCl, 50 mM KCl, pH 8.4) with 0.3 μg μl^{-1} BSA. The PCR protocol consisted of denaturation at 95 °C for 2 min followed by 35 cycles of 95 °C for 30 s, amplification at 54 °C for 30 s and elongation at 72 °C for 30 s and a final elongation step at 72 °C for 30 min. Amplification products were checked for size (270 bp) and

yield by standard 2% (w/v) agarose-0.5× Tris-borate-EDTA (TBE) gel electrophoresis with GelStar staining.

Twenty μL of PCR products were subjected to DGGE in a SE 600 Ruby Standard Dual Cooled Vertical Unit (GE healthcare, Piscataway, NJ). Denaturant gradient was pre-tested to sufficiently separate bands on the gel. The 0.75 mm thick gel containing 8% (w/v) polyacrylamide (37.5:1 acrylamide/bis-acrylamide) and a 30–60% denaturant gradient (100% denaturant is defined as 7 M urea and 40% (v/v) formamide) were electrophoresed for 16 h 30 min at 80 V and 60 °C in 1 × TAE buffer. Gels were stained with GelStar nucleic acid stain and gel images were captured using the Gel Doc XR imaging system (Bio-rad, Hercules, CA).

Bands of interest were excised from the DGGE gel and stored overnight in 100 μL of water at 4 °C to allow the DNA to passively diffuse out of the gel strips before re-amplification. Ten microliter of eluted DNA was then used as template for PCR amplification with primer pair BfrF/Bfr-R-GC as described above, and the products were subjected to DGGE to check for their migration. Confirmed DNA fragments were re-amplified using primer pair BfrF/Bfr-R and purified from a 2% (w/v) agarose gel using Qiaquick Gel extraction Kit (Qiagen, Valencia, CA) and cloned into pCR2.1-TOPO vector (Invitrogen, Carlsbad, CA). The ligation products were chemically transformed into *E. coli* (strain TOP10) using the TOPO TA Cloning Kit (Invitrogen, Carlsbad, CA) following manufacturer instructions. Recombinant colonies were identified on LB medium containing ampicillin (50 mg ml^{-1}) and X-Gal (5-bromo-4-chloro-3-indolyl-b-D-galgacto-pyranoside: 0.1 mM). One insert with the right size confirmed with PCR from each clone library was purified using the Wizard Plus Minipreps DNA purification system (Promega, Madison, WI) and sequenced with the Big-Dye Terminator Cycle Sequencing Kit v 3.1 on the AB 3730xl DNA analyzer (Applied Biosystems, Foster City, CA). Sequences were edited by Chromas (version 2.33), checked for chimera by the RDP CHECK-CHERIMA program, and compared with the NCBI database using BLASTN to obtain estimates on closest phylogenetic affiliates. Sequences obtained in this study were deposited in the European Molecular Biology Laboratory (EMBL) database under accession numbers JF831366-JF831420.

2.7. Data analysis

To normalize the data, the concentrations of markers and *E. coli* were transformed to natural logarithm (Ln). Decay rate of each host specific *Bacteroidales* marker and *E. coli* were calculated using the following standard exponential growth/decline equation: $k = -[\ln(N_t) - \ln(N_0)]/t$, where k = the decay rate with a unit of d^{-1}, N_t = the geometric mean concentration of Bacteroides marker or *E. coli* at time t, N_0 = the geometric mean concentration of *Bacteroides* marker or *E. coli* at time zero, t = time (days), Time t was determined by the days when the last sampling event occurs (14 day) or when the markers could not be detected by qPCR. Only data points above the assay limit of detection were used in the regression model. Time required for 99% of the initial population to decay (t_{99} value) was calculated as: $t_{99} = (-2/k)$. Analysis of variance (ANOVA) and a Turkey's post-hoc test were performed using SAS software (SAS Institute Inc, 2009) to evaluate the difference among the mean decay values.

DGGE gel images were analyzed using a GelCompar II package (Applied Maths, St-Martens-Latem, Belgium). We defined a band as "dominant" if the intensity of this band comprised of 5% or more of the total intensity in a sample lane. A threshold value of 5% relative to the maximum value in a lane was set when doing band search, band with value below the threshold value was defined "invisible". The Shannon-weaver index was calculated according to the following equation: $H = -\sum[P_i \times \ln(P_i)]$ where P_i is the relative probability of the band in a gel lane as calculated as $P_i = ni/N$, in which ni is the band intensity of a particular band and N is the sum of the intensities of all the bands in a lane as judged by peak height in the densitometric curves. The richness (R) is a count of the number of bands in a community. Evenness was calculated as: $E = H/\ln R$.

3. Results

Human specific (HF183) and ruminant specific *Bacteroidales* markers (CF193) were not detected by qPCR in the freshwater collected from a canal. The average salinity in the microcosms throughout the study was 0.10‰ and 0.12‰ for human feces treatment and bovine feces treatment, respectively. Average pH was 7.3 and average dissolved oxygen (DO) concentration was 6.3 mg l^{-1} for both treatments. Daily temperature in this area of Florida, as obtained from Florida climate center, fluctuates between 4 and 26 °C with an average of 15 °C during the experiment period (Feb, 2010). The average solar irradiation in this area is 4.12 kWh/m^2day (http://www.solarpanelsplus.com/solar-calculator), with an average irradiation level of 3.7 kWh/m^2day inside the greenhouse.

3.1. *Decay dynamic of host specific* Bacteroidales *markers DNA/RNA and* E. coli *in the microcosms*

As shown in Fig. 1A, after remaining relatively unchanged in the first two days of inoculation, the natural log transformed concentration of CF193 DNA decreased linearly from day 2 to day 8 before declining sharply from day 8 to day 10 and become undetected thereafter. The persistence of CF193 RNA largely resembled that of the DNA markers: its log transformed concentration declined only slightly in the first two days, subsequently linearly decreased to day 8, and then was lower than the limit of detection. Persistence profile of the human marker HF183 differed greatly from CF193 (Fig. 1B). Changes in the slope of curve indicated that HF183 decayed in a biphasic mode. From day 0 to day 6 HF183 DNA declined gradually, and the decay tended to become slower from day 6 to day 12. HF183 was still detectable at day 14. Concentration of HF183 RNA declined substantially in the first two days. It then decayed at a lower rate until it dropped below the detection limit after day 10.

During the entire sampling period the concentration of *E. coli* ranged from 16,300 to 500 CFU/100 ml for the human microcosms and from 1080 to 133 CFU/100 ml for the cattle microcosms. *E. coli* in both microcosms were above the water quality standard through the study. In the human microcosm natural Log transformed concentration of *E. coli* increased slightly at the first two days before it declined steadily until

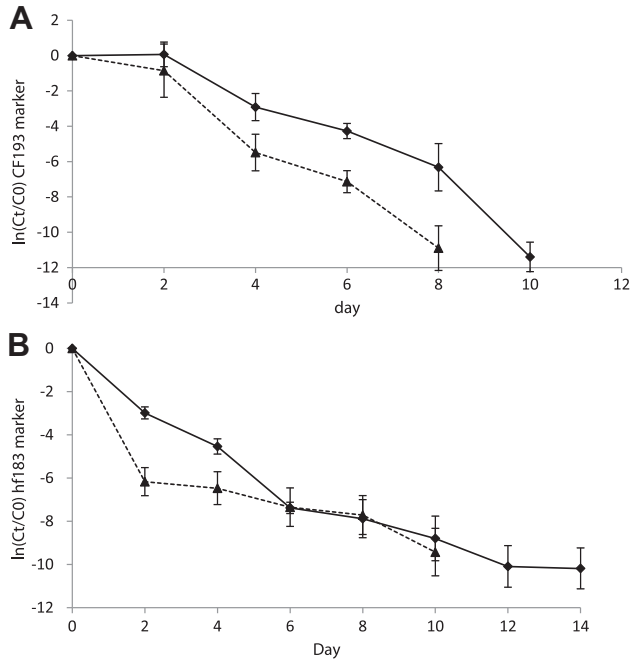

Fig. 1 – Persistence of (A) bovine specific *Bacteroidales* marker CF193 and (B) human specific *Bacteroidales* marker HF183 DNA and RNA in microcosms. Solid line: DNA marker, dotted line: RNA marker.

the end of the experiment. In contrast no post-inoculation growth phase was observed for *E. coli* in the bovine microcosm: it declined exponentially at a much lower rate of $-0.24\ d^{-1}$ throughout the incubation. Concentration of *E. coli* dropped by 3 Lns in the bovine microcosms by the end of the experiment (Fig. 2).

To make direct comparison between the persistence of host specific markers DNA/RNA and that of *E. coli*, in spite of the poor fitting, a first-order decay model was used to describe the decay process of all targets. The mean decay rates over the entire incubation period were calculated (Table 1), which in general were consistent with previous studies (Schulz and Childers, 2011). *E. coli* persistence tended to be greater than *Bacteroidales* marker DNA and RNA in the freshwater microcosms, as the decay rates of *E. coli* were significantly smaller than those of the *Bacteroidales* markers ($p = 0.023$). Average

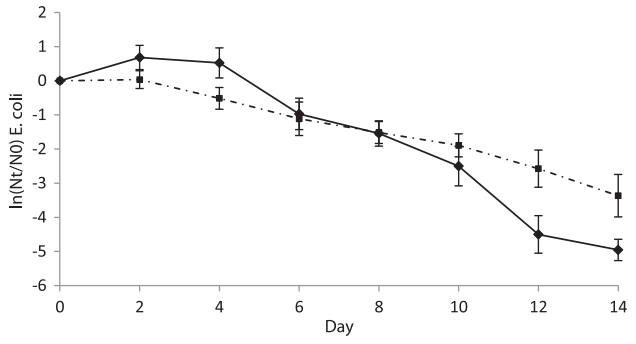

Fig. 2 – Survival of standard indicator *E. coli* in microcosms inoculated with bovine (dot line) and human feces (solid line).

Table 1 – Decay rates and t_{99} values from regression lines of inactivation models for each individual genetic markers and fecal indicator bacteria (*E. coli*) in the freshwater microcosms.

Markers	K (d^{-1})	t_{99} in day
CF193 DNA	−0.73	2.75
CF193 RNA	−0.94	2.12
Bovine *E. coli*	−0.24	8.32
HF183 DNA	−0.73	2.72
HF183 RNA	−0.96	2.08
Human *E. coli*	−0.35	5.65

t_{99} values for *E. coli* were 8.32 day in the bovine microcosms and 5.65 day in the human microcosms. The t_{99} value for both marker DNAs were surprisingly similar: 2.75 day for CF193 and 2.72 day for HF183. As expected, RNAs were less persistent than DNA with a t_{99} value of 2.12 day for CF193 and 2.08 day for HF183, respectively. However, no significant difference occurred between the k value of the DNA marker and RNA marker ($p = 0.13$).

3.2. *DGGE analysis of the* Bacteroidales *DNA and RNA in the microcosms*

Because three replicates sampled at the same time point produced nearly identical DGGE profile, only one replicate was used for analysis. A total of 30 bands of distinct mobility were observed on DGGE profile from bovine microcosms (marked from 1 to 30 in Fig. 3), reflecting a high diversity of *Bacteroidales* community structure. For the DGGE fingerprint generated from DNA, predominant band profiles did not change significantly between day 0 and day 8 with similar Shannon's diversity (H) (Fig. 5): only 2 faint bands lost at day 4 (band No 11 and 26), 3 faint bands lost at day 6 (band No 2, 3 and 24) and 3 bands lost at day 8 (band no 21, 25 and 28). H value declined abruptly from day 8 to day 10: the number of bands decreased from 21 at day 8 to 8 at day 10; the relative intensities of the 8 bands were greatly reduced although they remained detectable till the end of the experiment (day 14). RNA DGGE fingerprints at day 0 to day 4 were very similar to those of DNA with H value slightly lower than that of DNA at the same sampling point. At day 6, 16 bands were lost on RNA DGGE profile, as compared to the DNA DGGE pattern, resulting in a significantly lower H value. Band number and intensity declined significantly from day 8 and no band is detectable on day 12. BLAST search in the NCBI database showed that the majority of the sequences obtained from bovine microcosms had closest matches to sequences from uncultured *Bacteroidales* lineages that were derived from various environments such as bovine manure, human intestine, human skin, gull feces, and chicken feces (26 out of 30 in Table 2), indicating the largely unexplored nature of the bovine *Bacteroidales* population. The only 4 sequences related to cultured *Bacteroidales* species were band 13 (98% similarity to *Bacteroides dorei*), band 16 (95% similarity to B. *fragilis*), band 20 (96% similarity to B. *fragilis*) and Band 27 (97% similarity to B. *uniformis*).

Similar to the bovine microcosms, a high diversity of *Bacteroidales* was detected from the human microcosms, with a total of 25 discrete band profiles (marked from 1 to 25 in

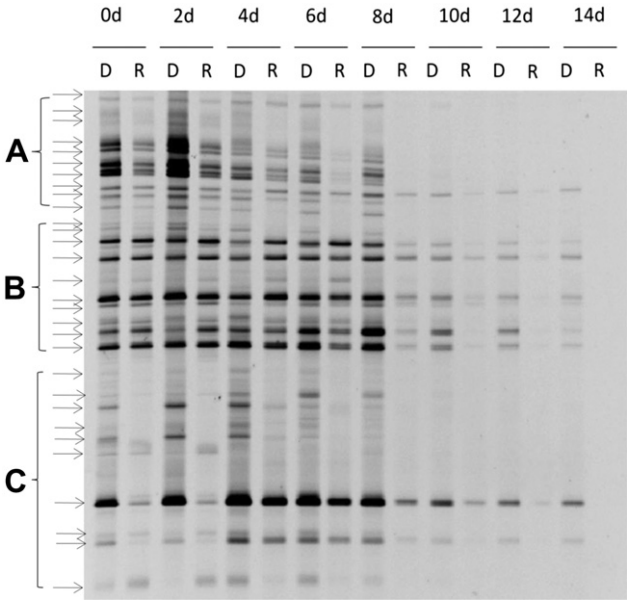

Fig. 3 – DGGE profile of *Bacteroidale* 16S rDNA fragments from DNA (D) and rRNA (R) based community in microcosms inoculated with bovine fecal material. Bands were numbered from top to bottom: 1-30. A indicates band from number 1 to number 10, B denotes bands from number 11 to number 20, C denotes bands number 21–30.

Fig. 4). Clear time dependency in DGGE pattern was observed. The DNA DGGE band profile was relatively unchanged from day 0 to day 8, with only minor reduction in H value at day 8 (Fig. 5). Band number declined from 17 at day 10 to 6 at day 14, accompanied by the lowering of band intensity, indicating fast decay of DNA during this period. The RNA DGGE profile resembled that of DNA in the first two days with a slightly

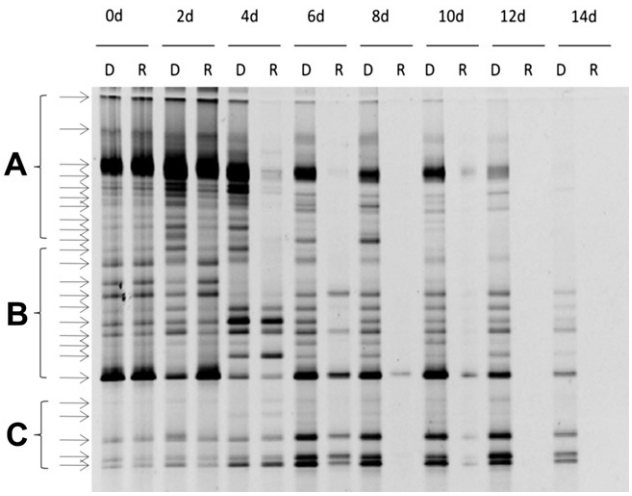

Fig. 4 – DGGE profile of *Bacteroidale* 16S rDNA fragments DNA (D) and RNA (R) based community in microcosms inoculated with human fecal material. Bands were number from top to bottom 1–25. A indicates band from number 1 to number 10, B denotes bands from number 11 to number 20, C denotes bands number 21–25.

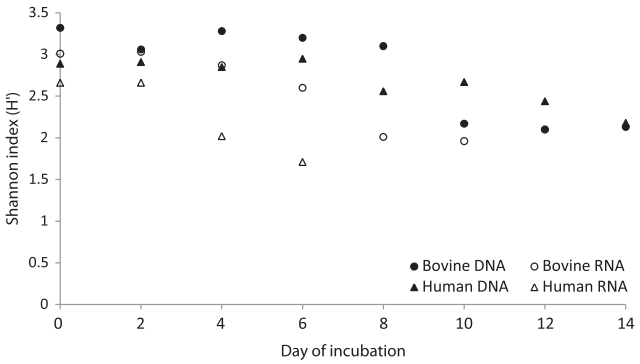

Fig. 5 – Change in Shannon's diversity (H) with time as calculated from DGGE profiles of *Bacteroidales* 16S rDNA and rRNA.

lower H value, after which the H value experienced a marked reduction. Number of bands dropped from 9 at day 4 to 4 at day 10 and became completely invisible at day 12. Unlike bovine microcosm, BLAST similarity search in the database revealed that a much larger percentage of the sequences (15 out of a total of 25) obtained from human microcosms was affiliated to cultured *Bacteroidales* genus, including *B. thetaiotaomicron*, *B. fragilis*, *B. vulgates*, *B. xylanisolvens*, *B. caccae*, *B. uniformis* and *B. dorei*, with similarity levels ranging from 96% to 100%. Two fifth of the sequences were closely related to uncultured *Bacteroidales* species from different environments, including human, turkey and chicken feces (Table 3).

4. Discussion

Our primary objective was to investigate the persistence of phylogenetically different *Bacteroidales* populations with time in a setting that incorporates various environmental stresses that may influence their survival, including the fluctuating ambient temperature, sunlight and the possible presence of bacterivorous predators. This information is not only necessary to proportionate the contribution of fecal pollution from different sources; it is also helpful to bridge the gap in the ecology and environmental behavior of fecal *Bacteroidales* population. In our study we targeted both rDNA and rRNA to detect *Bacteroidales* markers and to determine temporal change of *Bacteroidales* population. The diversities of *Bacteroidales* communities from human and bovine microcosm declined as time elapsed. Decay of host specific markers DNA was much slower than the decay of marker cells that were metabolically active as represented by rRNA. Both targets decayed faster than the standard fecal indicator bacteria, *E. coli*, suggesting that *Bacteroidales* was a less conservative indicator of human health risk as compared with *E. coli*.

4.1. DNA vs RNA

Minimal information on the persistence and survival of host specific *Bacteroidales* marker cell in the environment is available (Bae and Wuertz, 2009; Balleste and Blanch, 2010; Walters and Field, 2009). Molecular methods relying on the detection

Table 2 – Sequence affiliation of bands obtained from *Bacteroidales* 16S rDNA/rRNA DGGE profile in microcosms seeded with bovine feces and the last day of band detection during the 14 days incubation.

Band position	Bovine *Bacteroidales* DGGE band identification	Similarity %	Last day of detection	
	Closest matches in NCBI database		DNA	RNA
1	GU939540.1 Uncultured Bacteroidaceae bacterium clone R_247.13-1	97	8	6
2	HQ993031.1 Uncultured bacterium clone HuMC-B44	97	4	N/A
3	JF160912.1 Uncultured bacterium clone ncd1853f02c1	98	4	N/A
4	AY978897.1 Uncultured bacterium clone NF59	95	8	6
5	GU939318.1 Uncultured Bacteroidaceae bacterium clone HC_811.32-1	98	8	4
6	GU107994.1 Uncultured bacterium clone HFV09_555	97	8	4
7	EU533564.1 Uncultured bacterium clone Ch6b_1633	99	8	4
8	EU913596.1 Uncultured *Bacteroidales* bacterium clone Fhc77	97	14	8
9	GU939593.1 Uncultured Bacteroidaceae bacterium clone R_347.41-1	97	8	4
10	GU939318.1 Uncultured Bacteroidaceae bacterium clone HC_811.32-1	98	8	2
11	FJ220013.1 Uncultured Bacteroides sp. clone RacWA-23	98	2	0
12	AY978418.1 Uncultured bacterium clone KO17	99	8	2
13	JF298878.1 Bacteroides dorei strain EBA14-8	98	14	8
14	GU939494.1 Uncultured Bacteroidaceae bacterium clone PA_525.16-2	95	14	10
15	GU362577.1 Uncultured bacterium clone Bac.I-11m.h435	100	2	6
16	HM007585.1 Bacteroides fragilis strain DSM 9671	95	14	8
17	HQ992999.1 Uncultured bacterium clone HuMC-A3	97	8	6
18	HQ992999.1 Uncultured bacterium clone HuMC-A3	97	8	6
19	DQ905531.1| Uncultured bacterium clone 014-g8	98	14	8
20	JF298885.1 Bacteroides fragilis strain EBA21-17	96	14	8
21	DQ826965.1 Unidentified bacterium clone H2-20	98	6	0
22	HQ992999.1 Uncultured bacterium clone HuMC-A3	98	8	0
23	GU939318.1 Uncultured Bacteroidaceae bacterium clone HC_811.32-1	96	8	4
24	GQ047288.1 Uncultured bacterium clone nbw1011a03c1	98	4	0
25	HQ992999.1 Uncultured bacterium clone HuMC-A3	99	6	0
26	HQ896759.1 Uncultured Bacteroidetes bacterium clone 31	99	2	2
27	JF298891.1 Bacteroides uniformis strain EBA25-2	97	14	12
28	GU939540.1 Uncultured Bacteroidaceae bacterium clone R_247.13–1	96	6	2
29	JF160912.1 Uncultured bacterium clone ncd1853f02c1	98	14	8
30	HQ896738.1 Uncultured Bacteroidetes bacterium clone 2	98	8	2

of DNA directly from environmental samples lack the ability to differentiate live and dead cells because DNA from various origins, including live/dead cells, cell in viable but non-culturable status, and extracellular sources, will be detected. Research on the use of propidium monoazide (PMA) indicated promise to distinguish DNA from live cells (Bae and Wuertz, 2009), but the high turbidity in some environmental waters may seriously affect the penetration of PMA into live cells and thus the reliability of these methods. Analysis targeting rRNA, on the other hand, offers an alternative for the detection of metabolically active cells because the number of ribosomes per cell is a good indicator of the overall activity of the cell (Schaechter et al., 1958) and rRNA usually degraded quickly when the cell becomes less active. In addition, studies targeting both rDNA and rRNA have the potential to elucidate not only the presence of the markers but also the activity of the species to which the markers are related (Eichler et al., 2006; Walters and Field, 2009). In this study both the decay rate estimation and the decay curve analysis indicated that rRNA decayed faster than rDNA. This tendency was also clearly evidenced by DGGE profile analysis, further supporting the use of rRNA as indicator of metabolically active *Bacteroidales* population. As compared to other studies that indicated *Bacteroidales* spp. die off fast in oxygenated waters (Balleste and Blanch, 2010; Kreader, 1998), the prolonged persistence of *Bacteroidales* RNA observed in this study, however, was in contrast to the assumption that RNA degraded fast upon the death of cell. This discrepancy may be caused by a number of contributing factors. It is possible that some *Bacteroidales* can still grow or remain alive for an extended time in low oxygen micro-niches or a portion of the *Bacteroidales* population enters into viable but none cultivable (VBNC) status (Bell et al., 2009) in which certain level of rRNA was still maintained. Alternately, the complex secondary structure of the RNA may prevent the fast decay after cell death (Rodgers, 1970); or similar to the behavior of extracellular DNA (Lorenz and Wackernagel, 1994) some rRNA molecules may bind on suspended solid particles and thus protect it from enzymatic degradation in the microcosms.

We centrifuged the water samples to collect sufficient *Bacteroidales* biomass for DNA/RNA extraction. Although often used to concentrate microbial biomass for environmental water sample (Dick et al., 2010; Okabe and Shimazu, 2007; Walters and Field, 2009), this gravity based step may result in the loss of considerable amounts of the extracellular DNA/RNA because only those in intact cells or associated with solid particles will be captured and subsequently extracted. Considering the extended persistence of extracellular DNA in the environment compared to intact cells, the loss of the free DNA in this form may result in overestimation of decay rates. When alternative procedures, such as membrane filtration, are used to collect biomass, it is possible that different decay

Table 3 – Sequence affiliation of bands obtained from *Bacteroidales* 16S rDNA/rRNA DGGE profile in microcosms seeded with human feces and the last day of band detection during the 14 days incubation.

Band position	Human *Bacteroidales* DGGE band identification	Similarity %	Last day of detection	
	Closest matches in NCBI database		DNA	RNA
1	EF709128.1 Uncultured *Bacteroidales* bacterium clone MS194A1_F09	98	10	2
2	HQ896759.1 Uncultured Bacteroidetes bacterium clone 31	98	10	2
3	JF298890.1 Bacteroides thetaiotaomicron strain EBA25-1	100	12	4
4	DQ456039.1 Uncultured bacterium clone CFT112F7	98	4	2
5	GU939215.1 Uncultured Bacteroidaceae bacterium clone FS_133.66-1	100	12	2
6	DQ827205.1 Unidentified bacterium clone H5-59	99	10	2
7	FJ210103.1 Uncultured Bacteroides sp. clone BAC_B2_53.T7	98	10	2
8	JF298885.1 Bacteroides fragilis strain EBA21-17	98	4	2
9	JF298890.1 Bacteroides thetaiotaomicron strain EBA25-1	99	4	2
10	AB510697.1 Bacteroides caccae gen	99	8	2
11	JF298873.1 Bacteroides vulgatus strain EBA12-12	98	4	2
12	JF298887.1 Bacteroides xylanisolvens strain EBA22-11	98	10	2
13	JF298877.1 Bacteroides vulgatus strain EBA7-36	99	2	2
14	JF298887.1 Bacteroides xylanisolvens strain EBA22-11	99	14	6
15	JF298878.1 Bacteroides dorei strain EBA14-8	96	12	4
16	FJ509956.1 Uncultured bacterium clone 16slp74-02b01.p1k	97	12	4
17	JF298890.1 Bacteroides thetaiotaomicron strain EBA25-1	99	14	6
18	JF298882.1 Bacteroides caccae strain EBA18-15	97	10	2
19	JF298878.1 Bacteroides dorei strain EBA14-8	96	12	4
20	JF298885.1 Bacteroides fragilis strain EBA21-17	99	14	10
21	EU913596.1 Uncultured *Bacteroidales* bacterium clone Fhc77	98	12	0
22	HQ992999.1 Uncultured bacterium clone HuMC-A3	99	4	4
23	JF298891.1 Bacteroides uniformis strain EBA25-2	99	14	10
24	JF298882.1 Bacteroides caccae strain EBA18-15	97	14	6
25	HM443005.1 Uncultured Bacteroidetes bacterium clone SS506	97	14	10

dynamic may be obtained. We considered this in the data interpretation.

4.2. *PCR-DGGE analysis of* Bacteroidales *populations*

The high diversity of *Bacteroidales* observed in this study is generally in agreement with previous studies (Jeter et al., 2009). A total of 30 band types were observed from bovine microcosms and 25 band types from human microcosms. This may be an underestimation of the *Bacteroidales* because it is possible that some bands are common to different individuals (Pang et al., 2005). Most sequences from the bovine microcosms were closely affiliated with uncultured *Bacteroidales* from various sources, suggesting that bovine *Bacteroidales* is a very deep order and its diversity is largely under-explored (Dick et al., 2005; Lamendella et al., 2007). The detection of *B. thetaiotaomicron* affiliated sequences from human microcosm was in agreement with previous studies that this species dominates in human *Bacteroidales* mirobiota. *B. fragilis*, *B. uniformis* and *B. dorei* were detected from both microcosms, indicating they are shared by both hosts. Noticeably, bands affiliated with *B. fragilis* from human (band 20) and bovine (band 16 and band 20) DGGE profiles persisted until the end of the experiment. This is consistent with the finding that this species has a relatively high oxygen tolerance and can survive in aerobic conditions for extended period of time (Baughn and Malamy, 2004; Rocha et al., 2003).

As indicated by DGGE analysis, complicated population trends of persistence were observed, possibly caused by the heterogeneity of *Bacteroidales* populations present in the microcosms. It is reasonable to claim that *Bacteroidales* population in the feces used as inoculum contained different subpopulations at various physiological status, and different strains respond differently to a range of environmental conditions (Balleste and Blanch, 2010). Although *Bacteroidales* population from human and bovine microcosm exhibited differential diversity and band patterns, some common phenomena were still evident: 1) diversity of both microcosms decreased with time, which is expected because of the decline in richness attributed to the limited potential of most *Bacteroidales* to grow in aerobic conditions; 2) most samples collected from both microcosms in the first 8 days had a richness of more than 10, implying a uniform persistence pattern for most genotypes of *Bacteroidales*, which is consistent with the finding of Blanch (Balleste and Blanch, 2010) that DNA from all three species of *Bacteroidales* decays similarly in freshwater microcosm; and 3) several bands were present on the DGGE profile throughout the experiment, revealing the ability of some members of the *Bacteroidales* population to persist over a long period of time, possibly in a VBNC status or attached to suspended solid to prevent the fast degradation of DNA. Our results were generally in agreement with the findings of a recent study investigating the persistence of *Bacteroidales* populations in microcosms spiked with sewage and manure influent (Schulz and Childers, 2011).

4.3. *Decay curve analysis*

A close examination of the decay curve indicated that neither host specific *Bacteroidales* markers nor *E. coli* decayed linearly

and the patterns fit poorly with a first-order model. The difference in the shape of the decay curve was possibly caused by heterogeneity of marker cells present in the microcosms. Each indicator may have its specific persistence pattern as determined by its intrinsic properties including cell wall composition, sensitivity to oxygen and ability to grow extra-intestinally. Previous studies have also shown the insufficiency of using single-parameter model in the description of bacterial decay (Bae and Wuertz, 2009; Crane and Moore, 1986; Gonzalez, 1995). The deviation of decay curves from linearity observed in this study suggested that comparing decay rate from different studies without reference to the raw data should be conducted with caution because large variation may be introduced if inappropriate methods are used. This is clearly illustrated by the persistence profile of HF183 in this study: decay rate estimated over the course of the experiment was −0.7 for DNA and −0.9 for RNA, which were much different from the rates derived from the exponential stage: −1.2 for DNA and −3.1 for RNA (data not shown). One specific need for future studies, as advocated by Schulz and Childers (2011), is to have raw data available for reliable comparison between studies and the development of models.

The decay rates of HF183 (−0.7) and CF193 (−0.7) DNA markers during the entire incubation period were lower than those observed in freshwater in similar studies (HF813 at rate of −1.7 and CF193 at rate of −1.0) (Walters and Field, 2009). This discrepancy may be attributed to the difference of environmental parameters during the incubation such as temperature, sunlight exposure, and the level of predators: strong correlations between temperature and the inactivation of *Bacteroidales* spp. have been reported (Balleste and Blanch, 2010; Bell et al., 2009; Kreader, 1998; Okabe and Shimazu, 2007). Microcosms in our study experienced a fluctuation of temperature ranging from 4 °C to 18 °C with an average of 11 °C during most of the incubation time. The highest temperature (26 °C) was observed in only one day at the late stage of the test. It is possible that temperature variation over such a wide range may be conducive to the persistence of marker DNA. The effects of sunlight on the die-off of microorganisms are variable (Bae and Wuertz, 2009; Dick et al., 2010; Walters et al., 2009). We established the microcosms in a polycarbonate roofed greenhouse, which prevented the direct exposure to sunlight and reduced the UV radiation penetrated into the microcosms, thus delaying the decomposition of marker DNA. Moreover, the presence of predators in the water significantly influences the persistence of *Bacteroidales* spp. (Bell et al., 2009; Kreader, 1998; Okabe and Shimazu, 2007). The water collected from the canal during the winter time (Feb 2010) in this study might contain lower levels of bacterivorous predators. The strain specific differences as discovered above may also contribute to the long persistence of marker DNA. Other factors, such as the type of inocula, the starting concentration of markers, and the time of the year when the experiment was carried out may also dramatically affected the decay of *Bacteroidales*, making direct comparison between the two studies difficult.

In this study, although qPCR monitoring of the decay of host specific markers and PCR-DGGE profiling the persistence of *Bacteroidales* population targeted different region of 16S rRNA, some common patterns could still be perceived. For instance, PCR-DGGE profiles of DNA indicated an abrupt decline of bovine *Bacteroidales* populations after day 8, and the DGGE profile of RNA showed a sharp decrease after day 6. Decay curves of the bovine marker revealed the same trend. This similarity possibly indicates the ubiquity of bovine markers in different lineages of *Bacteroidales* populations. Although this conclusion is in conflict with the finding of Silkie and Nelson (2009) that bovine specific markers comprised only 4% of the total *Bacteroidales*, other studies revealed the high variability of human specific *Bacteroidales* marker HF183 in different individual host, with concentrations ranging from non-detectable to $>10^9$ markers g^{-1} wet feces (Seurinck et al., 2005). It is highly probable that the same level of viability may occur in the case of bovine markers.

4.4. *Implication for future research*

Although we designed the microcosms to simulate environmental conditions, because environmental parameters that *Bacteroidales* may encounter are too complex and unpredictable to replicate in the laboratory, further evaluations are warranted before extending the findings of this study to real field settings. For instance, a dialysis tube that provides a continuous flow-through system may be positioned *in situ* in natural scenarios to monitor the decay of *Bacteroidales* (Bae and Wuertz, 2009; Balleste and Blanch, 2010). Mixed source samples such as sewage and wastewater that are more representative of the natural pollution scenarios (Schulz and Childers, 2011) should be used in future studies. In addition, sediment resuspension contributed a significant portion of bacteria pollution to water columns (Fries et al., 2008) and, compared to water, the survival of indicator bacteria was enhanced in sediments (Dick et al., 2010). So the presence of sediment may also significantly affect the survival pattern of *Bacteroidales* populations. Further, because high levels of fecal indicators were detected in runoff water from agricultural land (Howell et al., 1995), it is essential to determine the influence of agricultural practices, such as the application of chemical fertilizers and pesticides, that may lead to the poor water quality on the persistence of *Bacteroidales* population in agricultural runoff.

5. Conclusion

- Using DGGE analysis of 16S rRNA genes, we observed a high diversity of *Bacteroidales* populations from both human and bovine microcosms, indicating the complexity of *Bacteroidales* spp. Most of the sequences from bovine microcosm are affiliated with uncultured B species, suggesting the largely unexplored nature of *Bacteroidales*.
- Diversity of *Bacteroidales* decreased with time for both DNA and RNA based analysis because of the decline of *Bacteroidales* richness due to its anaerobic metabolism.
- Persistence patterns of the dominant *Bacteroidale* species are uniform, some members of the *Bacteroidales* population persisted over the course of the 14 day incubation.
- As indicated by previous studies, a first-order model is not sufficient to describe the decay pattern of both markers and *E. coli* because of the presence of shoulder and tail time.

- Difference in the decay rates in this study from previous work may be caused by the difference in environmental parameters.
- Further investigations are needed to determine the persistence pattern of *Bacteroidales* populations in more natural scenarios.

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