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**Survival and persistence of host-associated *Bacteroidales* cells and DNA in
comparison with *E. coli* and *Enterococcus* in freshwater sediments as
quantified by PMA-qPCR and qPCR**

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propidium monoazide, quantitative PCR

ABSTRACT

Decay of the fecal source identifier *Bacteroidales* in sediments has not been studied until now. Two types of microcosms inoculated with human, cow and dog feces were constructed to investigate the survival and persistence of host-associated *Bacteroidales* cells and their DNA, respectively, in freshwater sediments: (i) a completely anaerobic microcosm where feces were entirely mixed with sediments for estimating decay of *Bacteroidales* in oxygen-free sediments at two temperatures (6°C and 20°C) and (ii) a core microcosm where feces in the overlying water column settled on top of undisturbed core sediments. Quantitative PCR (qPCR) along with propidium monoazide (PMA) was used to differentiate between genetic markers present in intact cells and total intracellular as well as extracellular marker DNA. Regulated fecal indicator bacteria were measured by cultivation (*E. coli* and *Enterococcus*) and qPCR (*Enterococcus*) in relation to *Bacteroidales* associated host markers. In anaerobic microcosms, the survival and persistence of *Bacteroidales* cells and DNA in sediments were considerably extended, especially at the lower temperature of 6°C, with two-log reduction times (T_{99}) >56 d (cells) and >169 d (DNA). *Bacteroidales* DNA persisted up to five times longer than cells in anaerobic microcosms at 6°C, whereas decay rates of cells and DNA were not significantly different at 20°C in anaerobic microcosms. In core microcosms, the levels of *Bacteroidales* cells and DNA decreased approximately six times more slowly in sediments than in overlying water; T_{99} values of *Bacteroidales* cells and DNA were 6-9 d (water) and 29-82 d (sediment). The survival of universal, human-, ruminant- and dog-associated *Bacteroidales* cells in sediments was similar in both microcosms under each given condition, as was the persistence of DNA. Decay rate constants of *Bacteroidales* cells and DNA were comparable with those of cultivable *Enterococcus* and *E. coli* cells in core sediments while *Enterococcus* DNA levels fluctuated

without noticeable decay. The prolonged persistence of host-associated *Bacteroidales* suggests that sediments should be considered in practical applications of microbial source tracking, because they can act as non-point sources of fecal markers.

1. INTRODUCTION

Impaired water quality caused by microbial contamination has been continuously reported in the United States. Among 3,762 coastal recreation beaches monitored in 2012, 40% had one or more advisory or closure notices due to an excess of applicable bacteria levels, which indicates the possible presence of disease-causing pathogens from sewage or fecal pollution in the water (USEPA 2013). Since the 1980s, cultivable *Enterococcus* and *Escherichia coli* (*E. coli*) have been used as regulated fecal indicator bacteria (FIB) to monitor fecal contamination in recreational waters because they are abundant in gastrointestinal tracts of humans and animals and, therefore, can indicate the presence of pathogenic microbes (USEPA 1986). The recently released USEPA guideline for recreational water quality criteria provides revised recommendations for cultivable FIB levels based on latest health studies and also includes a molecular testing method for the rapid detection of enterococci (USEPA 2012b).

Along with regulated FIB monitoring in recreational waters, microbial source tracking (MST) techniques using quantitative polymerase chain reaction (qPCR) are being increasingly applied in water quality monitoring programs to identify the sources of fecal pollution by detecting host-associated gene sequences (Wuertz et al. 2011). Members of the order *Bacteroidales* are the most widely used fecal identifiers of humans, livestock and domestic animals in qPCR-based MST studies, and the performance of a variety of host-associated *Bacteroidales* assays was tested in an inter-laboratory comparison study (e.g., Boehm et al. 2013; Ebentier et al. 2013; Layton et al.

2013; Raith et al. 2013; Schriewer et al. 2013). In order to be effectively applied as fecal identifiers in MST applications, especially when fecal age of contamination is of interest, the decay kinetics of host-associated *Bacteroidales* must be known, because divergent marker decay may complicate the comparison of detected levels of host markers in environmental matrices. The decay of fecal *Bacteroidales* in natural waters as a function of different environmental stressors, including temperature, predation, salinity and light, has been studied extensively (for a recent analysis, see Bae and Wuertz 2015). By comparison, little is known about the fate and transport of host-associated *Bacteroidales* cells and DNA in sediments that largely contain oxygen-limited zones. While *Bacteroidales* cells do not survive in water due to their obligate anaerobic physiology (Bae and Wuertz 2015), they may persist longer in sediments or even grow. The presence of host-associated *Bacteroidales* in sediments can lead to erroneous conclusions about the incidence of recent fecal contamination since sediment resuspension may contribute microbial concentrations to the overlying water column. Therefore, it is important to understand the fate and transport of *Bacteroidales* in sediments to improve our ability to identify contributing host sources in MST applications. In general, qPCR-based MST methods detect genetic markers from both live and dead cells since nucleic acids can persist after cell death for days to weeks. To exclude the detection of compromised cells, propidium monoazide (PMA)-qPCR can be used to differentiate between genetic markers in intact cells and total (including intracellular and extracellular) genetic markers (Bae and Wuertz 2009a). We recently validated a cell detachment method comprised of hand-shaking and Tween 80, and applied PMA-qPCR to *Bacteroidales* in sediments (Kim et al. 2014).

The purpose of this study was to investigate the survival and persistence of host-associated *Bacteroidales* cells and DNA in freshwater sediments. Two types of microcosm experiments

were designed to estimate i) the decay kinetics of host-associated *Bacteroidales* cells and DNA in completely anaerobic sediments mixed with feces and ii) the fate and transport of *Bacteroidales* in non-mixed core sediments when introduced from the overlying water column. The inactivation of regulated FIB, *Enterococcus* and *E. coli*, was also assessed.

2. MATERIALS AND METHODS

2.1. Sediment and water collection

Freshwater sediments were collected from a freshwater beach in South Lake Tahoe, NV (38°59'22"N, 119°57'11"W). For the anaerobic sediment microcosm experiment, a total of 7 kg of surface sediments at a depth of approximately 0 – 2 cm was obtained using sterilized scoops and placed in a 7-l sterile container. The container was filled to the top with freshwater in the field to minimize oxygen penetration into the sediment sample. An additional water sample was also collected by submerging a 7-l sterile container at knee depth in freshwater. For the core sediment microcosm study, undisturbed sediment cores were obtained using 20-cm long acrylic columns (inner diameter of 5-cm) with a sharpened edge at the bottom. Thirty-three columns were slowly inserted to a depth of 10 cm below the sediments surface and tightly capped inside the water column using sterilized rubber stoppers. Sediment and water samples were placed on ice and transported to the laboratory immediately after collection. After arrival, sediments and water samples were stored at 4°C in the dark until used within 48 h. Initial water parameters including pH, conductivity and salinity were measured with a handheld instrument (YSI 63, YSI Inc., Ohio, USA) during sample collection (Table S1). Dry weights of sediment samples were determined by measuring weight differences before and after drying sediments at 105°C for 24 h. Sediment particle size distribution was analyzed using six sets of sieves ranged from No. 10 (2-

mm) to No. 230 (63- μ m) after dehydration of sediments under 105°C for 24 h (Sheldrick and Wang 1993) (Table S1). Total organic carbon (TOC) contents of sediments were determined using the acid fumigation with combustion method (Harris et al. 2001) (Table S1). Nitrogen and phosphorus in sediment samples were quantified using the Total Kjeldahl Nitrogen method and the Olsen-P method, respectively (Olsen and Sommers 1982; Horneck and Miller 1998) (Table S1).

2.2. Fecal inocula preparation

Fresh human, cow, and dog feces were collected in Davis, CA using sterile disposable spoons and placed on ice during transportation. For the anaerobic microcosm experiment, a total of 15 g of feces from five individuals of each host were added into a one-liter freshwater sample and vigorously mixed for 5 min. For the core microcosm experiment, a total of 10 g of human feces from two adults, 10 g of cow feces from five cows, and 8 g of dog feces from five dogs were mixed together with 100 ml sterile PBS and vortexed for 5 min. The fecal mixture in PBS was pre-filtered using 100- μ m pore sized sterile Nylon Net filters (Millipore Co., Billerica, MA, USA) to eliminate large debris. A few drops of resazurin sodium salt (Sigma-Aldrich Co., Saint Louis, MO, USA) were added into both types of fecal inocula as an anaerobic indicator. Aliquots of fecal inocula were analyzed using qPCR to ascertain the presence of host-associated *Bacteroidales* markers. Once confirmed, fecal inocula were used for microcosm construction within 24 h of initial collection of fecal samples.

2.3. Microcosm design

Two types of laboratory microcosm experiments were conducted: i) an anaerobic microcosm and ii) a core microcosm study.

2.3.1. Anaerobic microcosm

A Coy anaerobic chamber Type B (Coy lab products Inc., Grass Lake, MI, USA) was used for the construction of anaerobic microcosms. For all microcosms, a total of 3 kg of sediment was placed in a sterile 4-l container followed by hand-mixing with one liter of freshwater spiked with fecal inocula for 10 min using a sterile wooden stick. After homogeneous distribution of the fecal mixture in sediment samples, approximately 25 g of wet weight of sediments was filled into each 30-ml crimp top serum bottle (outer diameter of 36.6 mm, height of 62.8 mm) (Sigma-Aldrich Co., Saint Louis, MO, USA) using sterile pipettes, where the narrow pipette ends had been removed. Excess water on top of sediments was removed, then three milliliter of deoxygenized sterile freshwater was gently introduced above the sediments to submerge them during the experiment. After addition of sediment and water, microcosms were tightly capped with butyl septa and aluminum crimp seals (Sigma-Aldrich Co., Saint Louis, MO, USA) using a vial crimper. After construction, a total of 72 microcosms were removed from the anaerobic chamber and immediately transferred to temperature-controlled rooms. Thirty-six microcosms were placed at 6°C and 20°C simulating winter and summer temperatures in the field, respectively. Microcosms were incubated in the dark for up to 42 d, and triplicate microcosms were sacrificed at each sampling point for microbial analysis. Samples were collected from the anaerobic microcosms 0, 2, 3, 5, 7, 14, 21, 28, 35, and 42 d after inoculation. Anaerobic conditions inside the microcosms were confirmed before sampling with the colorless overlying water in the microcosms in the presence of resazurin. On the day of sampling, each microcosm was opened under ambient conditions, and 3 g of sediment was collected using a sterile 10-ml pipette without a front end and placed in a sterile 50-ml Falcon tube, after gently decanting the overlying water without sediment disturbance in the microcosm. For detachment of *Bacteroidales* and FIB from

sediment subsamples, 30 ml of 1% Tween 80/NaOH pH 7.0 solution was added to the Falcon tube and the tubes were vigorously shaken by hand for 2 min (Kim et al. 2014). After 10 min, the supernatant from each Falcon tube was collected and used for microbial analysis.

2.3.2. Core microcosm

For the core microcosm study, sediment core columns half-filled with sediments and water without headspace were opened under ambient conditions by detaching the rubber stoppers on the top. The overlying water was gently removed using a 50-ml sterile pipette until 100 ml remained above sediments. Next, two milliliters of fecal inocula comprising of human, cow, and dog feces in PBS was gently added into the overlying water and mixed without disturbing the sediment surface. A rubber stopper was loosely placed back on top of core columns to reduce dirt settling and water evaporation during incubation. After construction, core sediment microcosms were incubated at 20°C in the dark for up to 28 d. Microcosms were sacrificed 0, 1, 2, 3, 4, 7, 10, 14, 21 and 28 d after incubation. Due to leakage in several core microcosms during setup, triplicate sediment samples were obtained by sampling one microcosm three times at each sampling date. On sampling days, water samples were carefully collected from the upper 50 ml of overlying water using a sterile 50-ml pipette and used for microbial analysis. The remaining water over the sediment core was carefully discarded without resuspension of sediments. Sediment core samples were collected by extruding the sediment core slowly from the bottom and separating sediments by depth with sterile spatulas. The top 2 cm of sediment surface (hereafter referred as top layer) and sediments between 4 and 6 cm deep (hereafter referred as middle layer) were used for microbial analysis after homogenizing each sediment layer using a sterile wooden stick for 1 min. Triplicate subsamples of 3 g sediment from each layer (top and middle) were treated in the same manner as applied in the anaerobic microcosms experiment for

microbial analysis. Redox potentials in the top and middle layers of sediments were measured by placing built-in platinum pin type electrodes into a sediment core using an Oakton pH/Con 510 Benchtop Meter (Oakton Instruments, Vernon Hills, IL, USA). A calomel reference electrode (Fisher Scientific Inc., Santa Clara, CA, USA) in conjunction with the platinum electrodes was placed in the overlying water during the measurement.

2.4. PMA treatment and nucleic acid extraction

One milligram of PMATM dye (Biotium Inc., Hayward, CA, USA) was dissolved in 1 ml of 20% dimethyl sulfoxide. Fifty microliters of PMA solution was added to 950 µl of sediment eluant or water subsamples to prepare 100 µM of final concentration of PMA in the mixture (Bae and Wuertz 2009a). After 5 min of incubation in the dark at room temperature, samples were exposed to strong light for 10 min using a 650-watt halogen light lamp placed 20 cm apart from the samples to cross-link PMA to DNA of impaired *Bacteroidales* cells or free DNA in samples (Nocker et al. 2006). After PMA treatment, samples were stored at -20°C prior to nucleic acid extraction. Genomic DNA (gDNA) in samples treated with or without PMA was extracted using the PureLink® Viral RNA/DNA Mini Kit (InvitrogenTM, Carlsbad, CA, USA) according to the manufacturer's manual. Background concentrations of DNA in raw sediment and water samples before spiking of feces were determined after extraction with the UltraClean Water DNA Kit (Mo Bio Laboratories, Inc., Carlsbad, CA, USA) according to the manufacturer's instructions. This method had a lower detection limit, which was necessary for these unspiked samples.

2.5. *Bacteroidales* and *Enterococcus* qPCR

TaqMan qPCR assays for *Bacteroidales* targeting a region of the 16S rRNA gene (Kildare et al. 2007) and *Enterococcus* large subunit ribosomal RNA (23S rRNA) (Haugland et al. 2005) were

performed using the StepOne™ Real-Time PCR System (Applied Biosystems®, Foster City, CA, USA). Universal (BacUni) as well as human- (BacHum), ruminant- (BacCow), and dog-associated (BacCan) *Bacteroidales* qPCR assays were used to quantify host-associated *Bacteroidales* markers. For *Bacteroidales* analysis, each 25-μl qPCR reaction volume contained 10 μl of nucleic acid extract, diluted from 3-to 81-fold in nuclease-free water (Ambion®, Austin, TX, USA), and final concentrations of 1x TaqMan® Environmental Master Mix 2.0 (Applied Biosystems®, Foster City, CA, USA), 400 nM (each) of forward and reverse primers, and 80 nM of probe. DNA plasmids containing target host-associated *Bacteroidales* sequences were extracted using a QIAprep Spin MiniPrep Kit (Qiagen, Valencia, CA, USA) and quantified by a NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). 10-fold serial dilutions (10^8 to 10^1 gene copies per reaction) were used in six-replicates for generating the standard curves for each assay. The *Enterococcus* qPCR assay was also prepared in a total volume of 25 μl containing 10 μl of the diluted DNA extracts and a final concentration of 1x TaqMan® Environmental Master Mix 2.0, 1 μM (each) of forward and reverse primers, and 80 nM of probe. The *Enterococcus* qPCR standard curve was generated from six 10-fold dilutions (10^6 to 10^1 gene copies per reaction) of *Enterococcus faecalis* (ATCC 29212) genomic DNA in six replicates (USEPA 2012a). Briefly, *E. faecalis* genomic DNA was extracted from *E. faecalis* grown at 37°C in brain heart infusion broth for 24 h. The total DNA concentration in the extract was determined at OD₂₆₀ with a spectrophotometer. The *E. faecalis* concentration in gene copies of target sequence per microliter in the extract was calculated by dividing the total DNA concentration by 3.6 (fg per genome) and multiplied by 4 (large subunit ribosomal RNA gene copies per genome) (USEPA 2012a). The lower limits of quantification for each qPCR assay were determined using the standard curves. For environmental sample analysis, four serial

dilutions (3- to 81-fold) were employed to assess inhibition in qPCR analysis. Triplicate positive and negative controls were run along with analytical duplicate of triplicate samples on every 96-well plate. The thermal cycling conditions applied were 2 min at 50°C and 10 min at 95°C, followed by 40 cycles at 94°C for 15 s and 60°C for 60 s. Genetic marker concentrations of *Bacteroidales* and *Enterococcus* were reported as gene copies (gc) per ml of water or g of dry weight of sediments. Background concentrations of universal *Bacteroidales* and *Enterococcus* target markers in raw water samples were less than 10 gc ml⁻¹ of freshwater. In sediments, background concentrations of universal *Bacteroidales* were about 1,000 gc g⁻¹ of dry weight of sediments. Human-, ruminant-, and dog-associated *Bacteroidales* were less than 150 gc g⁻¹ of dry weight of sediments. *Enterococcus* target marker in initial sediment was less than 500 gc g⁻¹ of dry weight of sediments. For spiked sediments and water samples, sample limits of detection (S_{LODs}) were determined as before (Schriewer et al. 2010). The S_{LOD} for sediment was calculated with a small modification of the S_{LOD} in water by exchanging water concentration factor with sediment elution factor. For samples collected from microcosms, the S_{LODs} of *Bacteroidales* and *Enterococcus* in water were less than 200 and 400 gc ml⁻¹, respectively. The S_{LODs} of *Bacteroidales* and *Enterococcus* in sediment were less than 2,500 and 5,000 gc g⁻¹ of dry weight of sediments, respectively.

2.6. Cultivable *E. coli* and *Enterococcus* enumeration

In an anaerobic microcosm experiment, cultivable *E. coli* and *Enterococcus* cells were enumerated using a membrane filtration method according to EPA methods 1604 (USEPA 2002b) and 1600 (USEPA 2002a), respectively. Eluants from sediment samples were diluted with sterile phosphate buffer saline and processed for FIB enumeration. In the core microcosm study, cultivable FIB in water and sediment eluants were estimated by the Colilert-18 and Enterolert

(IDEXX, Westbrook, MN, USA) methods for *E. coli* and *Enterococcus*, respectively, according to the manufacturer's instructions. The concentration of FIB was reported as colony forming units (CFU) or most probable number (MPN) ml⁻¹ of water or g⁻¹ of dry weight of sediments. Autochthonous concentrations of cultivable *E. coli* and *Enterococcus* cells in raw freshwater and sediments samples were analyzed using Colilert-18 and Enterolert, respectively, before microcosm construction. The numbers of *E. coli* and *Enterococcus* cell in raw freshwater and sediments were less than 1 MPN 100 ml⁻¹ of water and 10 MPN g⁻¹ of dry weight of sediments, respectively.

2.7. Decay rate calculation and statistical analysis

Decay rates and the time required for a two-log reduction of initial concentration, T₉₉, were calculated by a first-order exponential decay model containing a lag period before the beginning of logarithmic decay: $N = N_0 \times e^{-k(t-t_0)}$, where t_0 is time at the end of lag period, t is time at any point after lag period, N is the number of gene copies or cultivable FIB ml⁻¹ or g⁻¹ at time t , N_0 is the initial concentration, and k is the first-order decay rate constant. An exponential-plus-linear model ($N = N_0 \times e^{-kt} + y_0$) was used to fit the data for cultivable *E. coli* and *Enterococcus* in 6°C anaerobic microcosms, where y_0 is a constant and the other terms are as previously noted, since growth or inactivation of FIB was observed in the first few days followed by no further decay for the rest of the experimental period of 42 d. Nonlinear regression model fitting was performed using SigmaPlot™ 12 (Systat Software, Inc., San Jose, CA, USA), and the regression coefficients including slope, standard error of estimate, and R² were reported. Decay rate constants (k) were compared using a paired t-test or one-way analysis of variance (ANOVA) using SigmaPlot™ 12. Differences were considered significant when the p -value was less than 0.05.

3. RESULTS

3.1. *Bacteroidales* in anaerobic microcosms

Anaerobic microcosms were initially completely mixed and contained *Bacteroidales* and FIB throughout. Host-associated *Bacteroidales* cells and DNA were enumerated using PMA-qPCR and qPCR, respectively. Interestingly, we observed rapid increases in *Bacteroidales* cell concentrations in the first 2 d of incubation at both temperatures of 6°C and 20°C. Therefore, the decay rates of *Bacteroidales* cells were analyzed after the post inoculation lag phase (t_0) of 2 d (Table 1). There were considerable differences in decay rates of host-associated *Bacteroidales* in 6°C and 20°C anaerobic microcosms (Fig. 1). In 6°C anaerobic sediments, *Bacteroidales* cells and DNA gradually decayed with average T_{99} values of 100 d and 323 d, respectively. The decay rates of *Bacteroidales* from different host groups were not statistically different in 6°C anaerobic sediments ($p\text{-value}_{(\text{cell})} = 0.220$, $p\text{-value}_{(\text{DNA})} = 0.054$; Table 3). In 20°C anaerobic sediments, *Bacteroidales* cells and DNA decayed approximately 1.7 times (cell) and 5 times (DNA) faster than those in 6°C microcosms, and the differences were statistically significant ($p\text{-value} < 0.05$ for both cells and DNA). Similar to 6°C microcosms, there was no difference in the decay rates among different host markers in 20°C sediments ($p\text{-value}_{(\text{cell})} = 0.051$, $p\text{-value}_{(\text{DNA})} = 0.608$; Table 3). *Bacteroidales* cells decayed faster than their DNA in 6°C sediments ($p\text{-value} = 0.001$), while there was no difference in 20°C sediments ($p\text{-value} = 0.085$) (Table 3).

3.2. *Bacteroidales* in core microcosms

In the core microcosm experiment, the physicochemical parameters including water pH, turbidity, salinity and sediment redox potential at two different depths (top layer, 0 - 2 cm; middle layer, 4 - 6 cm) were monitored before microcosms were sacrificed. Overlying water pH ranged from 6.0

to 7.3 during the 28-d experiment (Fig. S1(A)). The pH dropped at the beginning of the experiment after the fecal spike was added but gradually increased with time. Turbidity rapidly decreased in the water column during the first two days of incubation; turbidity in the water column was around 50 NTU at the beginning of experiment and then decreased to less than 20 NTU after 2 d. Salinity stayed constant with values below 0.2 ppt throughout the experiment. The range of redox potential was between 102 and 187 mV for the sediment top layer and between 45 and 177 mV for the sediment middle layer (Fig. S1(B)).

Within the first five days, the concentrations of *Bacteroidales* cells and DNA in the water column significantly decreased by more than one order of magnitude and could no longer be detected after 14 d (Fig. 2). Unlike the rapid decrease of *Bacteroidales* levels in the water column, the concentrations of *Bacteroidales* cells and DNA in the sediment core decreased relatively slowly with decay rate constants of approximately 0.1 d^{-1} (Table 2). In top and middle sediment layers, the survival of *Bacteroidales* cells and persistence of their DNA were comparable ($p\text{-value}_{(\text{top})} = 0.984$, $p\text{-value}_{(\text{middle})} = 0.468$; Table 3). In addition, there was no significant difference in inactivation of host-associated *Bacteroidales* among different target markers in the top layer ($p\text{-value}_{(\text{cell})} = 0.955$, $p\text{-value}_{(\text{DNA})} = 0.952$; Table 3) and middle layer ($p\text{-value}_{(\text{cell})} = 0.149$, $p\text{-value}_{(\text{DNA})} = 0.713$; Table 3). Although the decay rate constants of *Bacteroidales* cells and DNA in the sediment middle layer usually appeared lower compared to those in the top layer, there was no statistically significant difference ($p\text{-value}_{(\text{cell})} = 0.724$, $p\text{-value}_{(\text{DNA})} = 0.946$).

3.3. FIB in anaerobic and core microcosms

3.3.1. Anaerobic microcosms

In 6°C anaerobic microcosms, cultivable *Enterococcus* cells (cENT) increased about 10-fold in the first 7 d with a growth rate of 0.191 d^{-1} , and the numbers remained constant for the remaining experimental period (Fig. 3A; Table 1). Cultivable *E. coli* cells (EC) decreased approximately six-fold during the first 5 d with a decay rate constant of 0.500 d^{-1} , but no further reduction in cell counts was observed. The concentrations of *Enterococcus* 23S rRNA genetic marker (tENT) including DNA of intact and impaired cells as well as extracellular DNA were highly variable ranging from 2×10^5 to $2 \times 10^7\text{ gc g}^{-1}$ of dry weight of sediments throughout the experimental period. In 20°C anaerobic microcosms, cENT and EC counts increased rapidly within the first 2-3 d by about one and two orders of magnitude, respectively, followed by a gradual decline (Fig. 3B). The decay rate constants revealed that cENT decayed faster than EC in the 20°C anaerobic microcosms ($k_{\text{(cENT)}} = 0.125\text{ d}^{-1}$, $k_{\text{(EC)}} = 0.051\text{ d}^{-1}$, $p\text{-value} = 0.007$). The levels of tENT were highly variable over the experimental period and did not decrease at either temperature.

There was a major difference in the persistence of tENT and host-associated *Bacteroidales* cells and DNA in the 20°C microcosms, although the decay rate of tENT could not be analyzed by the first-order decay model (there was no decay). For example, host-associated *Bacteroidales* cells and DNA gradually decreased more than one order of magnitude, whereas tENT continuously fluctuated over two orders of magnitude during the experimental period. Post-inoculation growth was observed for intact *Bacteroidales* cells and cENT at both temperatures. While intact *Bacteroidales* cells gradually decayed at both temperatures after the initial growth, the cENT concentration decreased only at 20°C and stayed constant at 6°C.

3.3.2. Core microcosms

In core microcosms, the levels of cENT, EC and tENT were measured at two sediment depths as well as in the overlying water column (Fig. 4). In the overlying water, cENT decreased rapidly after one day with a decay rate constant of 0.620 d^{-1} (Table 2). In the sediment core, cENT concentrations increased approximately two orders of magnitude within the first 3 d in the top and middle layers followed by a gradual decrease. The decay rate constant, k , of cENT was approximately 0.2 d^{-1} for both top and middle layer. The levels of EC increased in the overlying water and in the sediments in the first 2 d of incubation. As observed for cENT, EC decreased rapidly in the overlying water ($k = 1.021\text{ d}^{-1}$) and relatively slowly in the sediment core ($k_{(\text{top})} = 0.172\text{ d}^{-1}$, $k_{(\text{middle})} = 0.126\text{ d}^{-1}$). tENT was detected throughout the experimental period without noticeable decay in the overlying water as well as in the sediment core (Fig. 4C). There was no marked difference in decay rates between cultivable FIB and host-associated *Bacteroidales* cells and DNA in the core sediments ($p\text{-value} > 0.05$; Table S2).

4. DISCUSSION

This is the first study on the decay of the fecal source identifier *Bacteroidales* in freshwater sediments as detected by PMA-qPCR for cells and qPCR for DNA. In anaerobic microcosms, post-inoculation growth of *Bacteroidales* cells was observed, that is, host-associated *Bacteroidales* cell numbers increased by one to two orders of magnitude during the first two days of incubation before the onset of exponential decay. In addition, *Bacteroidales* concentrations in the sediments of core microcosms exhibited lag phases in the first few days. Thus, a modified first-order decay model including an initial lag period ($N = N_0 \times e^{-k(t-t_0)}$) was applied to describe the microbial decay. The post-inoculation lag-phase before the exponential inactivation of microbial populations has been reported previously for *Bacteroidales* decay in fresh and seawater microcosm experiments without sediments (Walters and Field 2009;

Ballesté and Blanch 2010; Green et al. 2011). As observed in our study, under completely anaerobic conditions simulating a worst-case scenario in environmental sediments, obligate anaerobic *Bacteroidales* could even proliferate before inactivation triggered by predation or physicochemical stressors. Recently, a biphasic decay model consisting of initial rapid inactivation followed by slower die-off has been proposed to describe the different resistances of *Bacteroidales* subpopulations in natural waters (Bae and Wuertz 2009b, 2012, 2015; Dick et al. 2010; Green et al. 2011; Marti et al. 2011; Solecki et al. 2011). In the present study, however, the decay of *Bacteroidales* markers from intact cells and total DNA was best described with a single first order decay model. The absence of a rapid initial decrease is likely due to the extended survival and persistence of *Bacteroidales* cells and DNA in sediments.

4.1. Decay of *Bacteroidales* in anaerobic microcosms

Completely anaerobic microcosms simulated the worst-case scenario of fecally derived cells existing under suitable redox conditions in the environments. The survival and persistence of cells and DNA of *Bacteroidales* markers (BacUni, BacHum, BacCow, and BacCan) was considerably extended in sediments kept at the lower temperature of 6°C (average $T_{99(\text{cell})} = 100$ d, $T_{99(\text{DNA})} = 323$ d). Given that the microcosms were incubated under oxygen-free conditions in our experiment, it is plausible that reduced activity of predators and decreased degradation rates of *Bacteroidales* rather than a decreased dissolved oxygen level at low temperature contributed to the prolonged survival and persistence of host-associated *Bacteroidales* cells and DNA in sediments. Our results showed that sediments can act as a non-point source of fecal pollution, because obligate anaerobic *Bacteroidales* that have shown rapid decay in water can persist considerably longer in sediments. Even the T_{99} values of *Bacteroidales* cells and DNA in microcosms kept at 20°C were > 43 d, indicating that anaerobic *Bacteroidales* may persist for an

extended period also in summer once they become associated with sediments under low-oxygen conditions.

There was no significant difference in inactivation rates between cells and DNA in 20°C anaerobic microcosms (average $k_{(\text{cell})} = 0.082 \text{ d}^{-1}$, $k_{(\text{DNA})} = 0.073 \text{ d}^{-1}$; $p\text{-value} = 0.085$), while *Bacteroidales* intact cells decayed faster than DNA at 6°C (average $k_{(\text{cells})} = 0.051 \text{ d}^{-1}$, $k_{(\text{DNA})} = 0.016 \text{ d}^{-1}$; $p\text{-value} = 0.001$). Our results suggest that seasonal variation in persistence of host-associated *Bacteroidales* cells and DNA in sediments must be considered in interpreting quantitative MST data. For example, use of PMA in sediments may not be necessary when monitoring in the summer; however, *Bacteroidales* intact cell levels may be overestimated in the winter if MST monitoring for sediments is solely based on *Bacteroidales* DNA.

Universal and human-, ruminant-, and dog-associated *Bacteroidales* markers showed similar decay for each given temperature, and this makes a direct comparison of detected marker concentrations in sediments possible in MST monitoring.

4.2. Fate of *Bacteroidales* in core microcosms

4.2.1 Water column

In core microcosms, the concentrations of host-associated *Bacteroidales* cells and DNA decreased rapidly in the overlying water column with approximate T_{99} values of 8 d, compared to the top and middle sediment layers where the cells and DNA decreased approximately six times more slowly. The disappearance of host-associated *Bacteroidales* in the water column can be attributed to the combined effect of *Bacteroidales* die-off in water and settling velocity. Assuming that *Bacteroidales* cell size and density ranged from 1 to 2.5 μm and 1.09 to 1.13 g cm^{-3} , respectively (Liu et al. 2006), and that the overlying water density was 1.0 g cm^{-3} , the

average settling velocity for *Bacteroidales* cells was about 1 cm d⁻¹ according to Stokes' law (Thomann and Mueller 1987). It would have taken up to 5 d for cells in the overlying water to settle onto surficial sediments. Removal of *Bacteroidales* DNA from the water column could have been accelerated by attachment to particles in the water column. However, *Bacteroidales* DNA concentrations did not decrease considerably in the overlying water column during the first 2 d when turbidity was greatly reduced (Fig. S1(A)). Therefore, we suggest that inactivation of *Bacteroidales* DNA rather than sedimentation contributed to the decrease in DNA in the overlying water column. Compared to other studies on *Bacteroidales* decay in water (see Bae and Wuertz 2015), our core microcosm results showed longer survival and persistence of *Bacteroidales* cells and DNA in the overlying water column of approximate T₉₉ values of 8 d. For example, values for T₉₉ of human-associated *Bacteroidales* genetic markers (qHF183 and BacHum) in a freshwater batch experiment incubated at 15°C were ≤ 3 d (Dick et al. 2010). The same host-associated *Bacteroidales* markers (BacUni, BacHum, BacCow, and BacCan) for cells and DNA as used in our study were followed in dialysis bags in a freshwater microcosm at an average temperature of 22°C without light exposure, yielding T₉₉ values of approximately 2 (intact cells) and 4 d (DNA) (Bae and Wuertz 2012). In the present study, the extended persistence of *Bacteroidales* cells and DNA in the overlying water column of core microcosms was likely influenced by relatively low oxygen concentrations caused by the lack of water circulation compared to the continuous flow study of Bae and Wuertz (2012).

4.2.2 Sediments

In sediment cores, the inactivation rates of *Bacteroidales* were not significantly different in the top and middle layers ($p\text{-value}_{(\text{cell})} = 0.724$, $p\text{-value}_{(\text{DNA})} = 0.946$). The average redox potentials were approximately 150 mV and 110 mV for the top and middle layers, respectively (Fig. S1(B)).

These values are below the range of aerobic respiration for microorganisms of 300 to 500 mV (DeLaune and Reddy 2005). The redox potentials at each depth are within the ranges of denitrification, manganese reduction, and iron reduction at pH 6-7. Given that the top and middle layers had similar redox potential ranges as well as other sediment characteristics such as particle size distribution and nutrient condition (Table S1), it is not surprising that *Bacteroidales* cells at the two sediment depths had comparable decay profiles. Similar to results obtained from anaerobic microcosms, *Bacteroidales* cells and DNA had comparable decay rates in core sediments incubated at 20°C ($p\text{-value}_{\text{(top)}} = 0.984$, $p\text{-value}_{\text{(middle)}} = 0.468$), suggesting that qPCR-based MST methods without PMA application can give information about the presence of cells in freshwater sediment in practical monitoring. Further, the universal and different host-associated markers (human, ruminant, and dog) showed similar decay ($p\text{-value}_{\text{(top)}} = 0.955$, $p\text{-value}_{\text{(middle)}} = 0.149$ for intact cells; $p\text{-value}_{\text{(top)}} = 0.952$, $p\text{-value}_{\text{(middle)}} = 0.713$ for DNA). Similar decay profiles of different host-associated markers in both microcosm types in our experiment suggest that differential resistance to oxygen between host-associated *Bacteroidales* strains may not be significant in their decay when they were associated with freshwater sediments where oxygen is limited. In qPCR-based MST applications, it is essential to understand the environmental persistence of target host markers, especially when the contribution of diverse fecal hosts is to be compared. Our results showed that the relative contribution of fecal source groups in sediments can be determined by direct comparison of measured marker concentrations in summer season.

4.3. Comparison of decay of FIB and *Bacteroidales*

In anaerobic sediment microcosms, we found an initial increase in concentrations of *Bacteroidales* cells during the first two days of incubation at both 6°C and 20°C. Similarly, a

post-inoculation increase was also detected for cultivable enterococci (kept at 6°C and 20°C) and *E. coli* (kept at 20°C). The initial growth of bacteria in the present study may be attributed to the combined effect of presence of moisture, enhanced availability of nutrients in sediments and a large surface area for biofilm formation, providing microorganisms a favorable niche to proliferate. The anaerobic conditions could have enhanced the growth of *Bacteroidales* cells with obligate anaerobic physiology. The gradual decay of *Bacteroidales* following the post-inoculation increase may have been the consequence of nutritional competition between microorganisms, predator activity, and physical and chemical constraints in a closed system. In anaerobic sediments kept at 20°C, universal and host-associated *Bacteroidales* cells and DNA mostly showed comparable or lower decay rates ($k_{\text{(cell)}} = 0.056 - 0.107 \text{ d}^{-1}$, $k_{\text{(DNA)}} = 0.063 - 0.083 \text{ d}^{-1}$) than cultivable enterococci ($k = 0.125$). Cultivable *E. coli* cells had a relatively low decay rate at 20°C ($k = 0.051 \text{ d}^{-1}$), probably due to enhanced survival or possible proliferation of a subpopulation of *E. coli* from fecal sources under the given conditions.

In core sediments, the die-off rates of cultivable FIB cells ($k = 0.126 - 0.206 \text{ d}^{-1}$) and universal and host-associated *Bacteroidales* cells ($k = 0.056 - 0.160 \text{ d}^{-1}$) and DNA ($k = 0.076 - 0.152 \text{ d}^{-1}$) were similar. Our results revealed that obligately anaerobic *Bacteroidales*, which have been considered to decay rapidly in water, can survive as long as or even longer than facultative enterococci when the host markers become associated with sediments in environments. Importantly, the *Enterococcus* 23S rRNA genetic marker did not decay neither in the overlying water column nor in sediment cores. This result could be due to the presence of viable but non-culturable (VBNC) *Enterococcus* cells or the extended persistence of *Enterococcus* DNA after cell death. The long-term persistence of *Enterococcus* DNA in sediments calls into question the

reliability of water quality criteria based on the detection of *Enterococcus* DNA as a predictor of health risks in surface water.

4.4. Implication and limitations

Overall, the prolonged persistence of host-associated *Bacteroidales* cells and DNA in freshwater sediments showed that sediments can act as a non-point source of fecal identifiers in aquatic environments. Host-associated *Bacteroidales* have been considered an effective tool for detecting recent or ongoing fecal contamination due to their rapid decay in water (Bae and Wuertz 2009b; Walter and Field 2009; Ballesté and Blanch 2010; Tambalo et al. 2012). However, our study strongly suggests that sediments should be considered in MST applications in aquatic environments because reintroduction of *Bacteroidales* from sediments can contribute to the microbial population observed in the water column. Further research is necessary to determine the relationship of the survival and persistence of the *Bacteroidales* markers in sediments with those of pathogenic bacteria. Also the quantitative estimation of microbial release from sediments into the overlying water column will provide a better foundation to assess health risks associated with sediment resuspension.

5. CONCLUSIONS

- The survival and persistence of host-associated *Bacteroidales* cells and DNA was prolonged in completely anaerobic sediments, especially at the lower temperature of 6°C. In core sediment microcosms, the levels of *Bacteroidales* cells and DNA rapidly decreased in the overlying water but persisted longer in sediments. Results of this study suggest that sediments can act as a non-point source of fecal markers and that seasonal differences should be considered in MST field monitoring studies.

- *Bacteroidales* DNA persisted longer than cells at 6°C but had comparable decay rate constants to cells at 20°C. The similar decay kinetics of cells and DNA in sediments at higher temperatures representing summer conditions suggests that the application of PMA-qPCR for sediments may not be necessary in practical MST monitoring during the summer season.
- The universal as well as human-, ruminant- and dog-associated *Bacteroidales* genetic markers in sediments showed similar decay kinetics under each condition tested, which indicates that the quantification of target markers in sediments can be directly used to analyze relative contributions of fecal source groups without further adjustments for differential decay.
- *Bacteroidales* cells and DNA had similar inactivation rates compared with cultivable FIB cells in core sediments, showing that anaerobic *Bacteroidales* can survive as long as facultative FIB when the host markers become associated with sediments in aquatic environments. The use of qPCR assays for *Enterococcus* in freshwater environments in the context of recreational water quality is called into question because *Enterococcus* DNA did not show any noticeable decay in either anaerobic or core sediments.
- The present study is the first to investigate the survival of *Bacteroidales* cells and the persistence of their DNA in freshwater sediments using PMA-qPCR and qPCR, respectively. Understanding the fate of host-associated *Bacteroidales* cells and DNA in sediments will advance our ability to determine the significance of sediments as potential sources of fecal markers in MST applications.

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Table 1. Decay rate constant (k) and T_{99} of host-associated *Bacteroidales*, *Enterococcus*, and *E. coli* in sediments of anaerobic microcosms incubated at 6°C and 20°C^a

Temp	Target ^b	Cells				DNA			
		k (d ⁻¹) [standard error]	t_0 (d)	R^2	T_{99} ^c (d)	k (d ⁻¹) [standard error]	t_0 (d)	R^2	T_{99} (d)
6°C	BacUni	0.034 [0.015]	2	0.52	134	0.012 [0.005]		0.50	389
	BacHum	0.049 [0.007]	2	0.90	94	0.011 [0.006]		0.39	429
	BacCow	0.040 [0.022]	2	0.42	116	0.027 [0.004]		0.91	169
	BacCan	0.082 [0.020]	2	0.76	56	0.015 [0.003]		0.84	307
	ENT ^d	-0.191*[-0.109]		0.71	>>42 ^e				>>42
	EC	0.500[0.209]		0.89		NA ^f			
20°C	BacUni	0.071 [0.010]	2	0.98	64	0.063 [0.004]		0.98	73
	BacHum	0.093 [0.016]	2	0.82	50	0.075 [0.017]		0.82	61
	BacCow	0.056 [0.010]	2	0.96	82	0.071 [0.007]		0.96	65
	BacCan	0.107 [0.015]	2	0.93	43	0.083 [0.010]		0.93	55
	ENT	0.125 [0.021]	2	0.96	37				>>42
	EC	0.051 [0.006]	3	0.96	91	NA			

^a The decay model is as follows: $N = N_0 \times e^{-k(t-t_0)}$, where t_0 is the time at the end of lag period, t is the time at any point after the lag period, N is the number of gene copies or number of cultivable FIB ml⁻¹ or g⁻¹ at time t , N_0 is the initial concentration of decay curve, and k is the exponential decay rate constant.

For cultivable FIB at 6°C microcosms, the exponential-plus-linear model ($N = N_0 \times e^{-kt} + y_0$) was used, where y_0 is a constant because growth or inactivation was observed for the first a few days followed by stable concentrations. Decay rate constant marked by an asterisk indicates growth rate in the microcosm.

^b BacUni, universal *Bacteroidales*; BacHum, human-associated *Bacteroidales*, BacCow, ruminant-associated *Bacteroidales*; BacCan, dog-associated *Bacteroidales*; ENT, *Enterococcus*; EC, *E. coli*.

^c Time for log-2 reduction calculated based on the first-order decay model.

^d *Enterococcus* gene copies increased over time and hence there was no decay.

^e Decay of *Enterococcus* cells and DNA was not observed during the 42-d experimental period.

^f NA, not analyzed.

Table 2. Kinetic parameters of host-associated *Bacteroidales*, *Enterococcus*, and *E. coli* in different matrices of core microcosms^a

Matrix	Target ^b	Cells				DNA			
		k [standard error] (d ⁻¹)	t_0 (d)	R^2	T_{99} ^c (d)	k [standard error] (d ⁻¹)	t_0 (d)	R^2	T_{99} (d)
Overlying water	BacUni	0.639 [0.097]		0.80	7	0.597 [0.095]		0.82	8
	BacHum	0.487 [0.121]		0.84	9	0.554 [0.125]		0.83	8
	BacCow	0.613 [0.194]		0.78	8	0.601 [0.057]		0.96	8
	BacCan	0.648 [0.131]		0.84	7	0.753 [0.168]		0.78	6
	ENT	0.620 [0.234]	1	0.62	7				>>28 ^d
	EC	1.021 [0.089]	2	0.98	5				NA ^e
Top layer (0-2 cm)	BacUni	0.110 [0.037]		0.71	42	0.120 [0.040]		0.65	38
	BacHum	0.092 [0.027]		0.72	50	0.125 [0.044]		0.58	37
	BacCow	0.121 [0.047]		0.60	38	0.120 [0.030]		0.78	38
	BacCan	0.096 [0.044]		0.57	48	0.152 [0.061]		0.60	30
	ENT	0.206 [0.102]	3	0.60	22				>>28
	EC	0.172 [0.867]	2	0.87	27				NA
Middle layer (4-6 cm)	BacUni	0.070 [0.015]	3	0.87	66	0.086 [0.032]	3	0.67	53
	BacHum	0.056 [0.033]	3	0.48	82	0.076 [0.035]	3	0.56	60
	BacCow	0.160 [0.044]	3	0.79	29	0.133 [0.044]	3	0.46	35
	BacCan	0.091 [0.035]	4	0.73	51	0.102 [0.036]	3	0.69	45

	ENT	0.152 [0.041]	3	0.84	30	>>28
	EC	0.126 [0.051]	3	0.70	37	NA
651	^a The decay model is as follows: $N = N_0 \times e^{-k(t-t_0)}$, where t_0 is the time at the end of lag period,					
652	t is the time at any point after the lag period, N is the number of gene copies or number of					
653	cultivable FIB ml ⁻¹ or g ⁻¹ at time t , N_0 is the initial concentration of decay curve, and k is the					
654	exponential decay rate constant.					
655	^b BacUni, universal <i>Bacteroidales</i> ; BacHum, human-associated <i>Bacteroidales</i> , BacCow,					
656	ruminant-associated <i>Bacteroidales</i> ; BacCan, dog-associated <i>Bacteroidales</i> ; ENT, <i>Enterococcus</i> ;					
657	EC, <i>E. coli</i> .					
658	^c Time for log-2 reduction calculated based on the first-order decay model.					
659	^d Decay of <i>Enterococcus</i> DNA was not observed during the 28-d of experimental period.					
660	^e NA, Not analyzed.					

Table 3. Comparison of decay rates of *Bacteroidales* cells and DNA for different host markers in sediments for each microcosm type

Microcosm		<i>p</i> -value ^a		
		Compare host markers ^b		Intact cells vs DNA
		Intact cells	DNA	
Anaerobic microcosm	6°C	0.220	0.054	0.001
	20°C	0.051	0.608	0.085
Core microcosm	Top layer	0.955	0.952	0.984
	Middle	0.149	0.713	0.468
	layer			

^a One-way ANOVA was used for comparison of decay rates. A *p*-value of less than 0.05 was considered significant. Bold indicates significantly different value.

^b Host markers: BacUni, BacHum, BacCow, and BacCan.

Figure legends

Figure 1. Decay of host-associated *Bacteroidales* cells measured by PMA-qPCR and DNA measured by qPCR in anaerobic microcosms at 6°C (A, C, E, G) and 20°C (B, D, F, H); concentrations of universal *Bacteroidales* (A-B), human-associated (C-D), ruminant-associated (E-F), and dog-associated *Bacteroidales* (G-H). Error bars represent the standard error of triplicates. Open and closed circles represent target concentrations of *Bacteroidales* cells and DNA, respectively. The dashed lines were plotted using the first-order decay model.

Figure 2. Decay curves of host-associated *Bacteroidales* cells (A, C, E, G) and DNA (B, D, F, H) in the overlying water and sediments at two depths (top layer, 0-2 cm depth; middle layer, 4-6 cm depth) in core microcosms; concentrations of universal *Bacteroidales* (A-B), human-associated (C-D), ruminant-associated (E-F), and dog-associated *Bacteroidales* (G-H). The data are shown as mean $\pm$ standard error of triplicates. Gray squares represent target concentrations in the overlying water. Closed and open circles denote target concentrations in the sediment top layer and middle layer, respectively. The dotted and dashed lines were plotted using the first-order decay model.

Figure 3. Survival of cultivable fecal indicator bacteria (FIB), *Enterococcus* and *E. coli*, cells and persistence of *Enterococcus* 23S rRNA genetic marker in anaerobic microcosms at 6°C (A) and 20°C (B). Error bars represent the standard error of triplicates. Closed and open circles represent target concentrations of cultivable *Enterococcus* (cENT) and *E. coli* (EC), respectively. The

triangle denotes the number of *Enterococcus* (tENT) DNA measured by qPCR. The dashed lines were plotted using the first-order decay model.

Figure 4. Survival of cultivable *Enterococcus* (A) and *E. coli* cells (B) and persistence of *Enterococcus* 23S rRNA genetic marker (C) in the overlying water and the sediments at two depths (top layer, 0 – 2 cm depth; middle layer, 4 – 6 cm depth) in core microcosms. Error bar represents standard error of triplicates. Gray squares represent target concentrations in overlying water. Closed and open circles denote target concentrations in the sediment top and middle layer, respectively. The dotted and dashed lines were plotted using the first-order decay model.