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ORIGINAL ARTICLE

Evaluation of detachment methods for the enumeration of *Bacteroides fragilis* in sediments via propidium monoazide quantitative PCR, in comparison with *Enterococcus faecalis* and *Escherichia coli*

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Bacteroides, cell detachment, *Enterococcus*, *Escherichia coli*, microbial source tracking, propidium monoazide (PMA), sediments.

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Abstract

Aims: The aim was to develop an optimized detachment method for separating *Bacteroidales* from sediments to allow enumeration via PMA-qPCR. The effectiveness of four different detachment treatments in removing *Bacteroides fragilis* was compared as a function of time as well as in relation to *Enterococcus faecalis* and *Escherichia coli* as detected by cultivation and qPCR.
Methods and Results: Cells were inoculated into four sediments from sea water (SW) and freshwater (FW) beaches. Sediment samples were taken on days 1 and 7 and subjected to four different treatments for separation of micro-organisms. On day 1, the detachment treatments performed equally well in removing intact *Bact. fragilis* cells. In contrast, 7 days later the detachment treatment with Tween 80 and handshaking (TH) resulted in up to eightfold higher 16S rRNA gene concentrations of intact and total *Bact. fragilis* cells compared to other detachment treatments. Total *Ent. faecalis* cells based on the 23S rRNA gene were also preferentially recovered by treatment TH. Cultivable *Ent. faecalis* or *E. coli* numbers detached from sediments were similar for all methods in most sediments tested.
Conclusions: Handshaking and 1% Tween 80/NaOH (pH 7·0) eluant was the most efficient technique to recover intact as well as total *Bact. fragilis* cells in sediment samples with different salinities and after prolonged sediment cell contact time.
Significance and Impact of the Study: The optimized detachment method enables the application of PMA-qPCR to sediment samples to detect the presence of *Bacteroidales* cells and their DNA in future microbial source tracking studies.

Introduction

Library- and cultivation-independent microbial source tracking (MST) methods based on quantitative polymerase chain reaction (qPCR) enable quantitative monitoring of target micro-organisms in water by detecting their gene sequences of interest in a relatively short time compared to conventional cultivation methods (Wuertz *et al.* 2011). Members of the order *Bacteroidales* have been increasingly used as alternative faecal indicator bacteria (FIB) as well as source identifiers in MST due to host specificity and their abundance in the gastrointestinal tract (Savichtcheva and Okabe 2006; Kildare *et al.* 2007; Schriewer *et al.* 2010). In addition, *Bacteroidales* are obligate anaerobes and hence exhibit limited potential for regrowth in surface water after release into the environment. This reduces the likelihood of counting bacteria that are not indicative of recent faecal contamination, as has been observed for conventional FIB (Walters and Field 2006; Bae and Wuertz 2009b). However,

qPCR cannot discriminate between DNA from intact and impaired cells because DNA can persist after cell death (Walters and Field 2009). Potential health risks may thus be overestimated. Recently, the application of propidium monoazide (PMA) prior to qPCR has been shown to be a useful technique for the discrimination of DNA from intact and impaired cells by inhibiting DNA amplification from damaged cells during qPCR (Nocker *et al.* 2007; Bae and Wuertz 2009a). PMA can penetrate the membrane of impaired cells and bind to their DNA upon light exposure thus reducing the likelihood of DNA amplification. By combining qPCR with PMA (PMA-qPCR), it is possible to detect intact *Bacteroidales* cells in wastewater and storm water containing solids concentrations up to 1000 mg l^{-1} (Bae and Wuertz 2009a).

PMA-qPCR has not been applied to detect intact *Bacteroidales* cells in sediments as sediment particles can hinder the photolysis of PMA by blocking light exposure of impaired cells. While survival of obligately anaerobic *Bacteroidales* is limited in surface waters (Walters and Field 2006; Okabe and Shimazu 2007; Bae and Wuertz 2009b, 2012), it may be possible for cells to grow or persist longer in sediments with anaerobic conditions. In addition, sediments can provide favourable environments for certain micro-organisms in terms of nutrient availability (Davies *et al.* 1995; Craig *et al.* 2004), protection from sunlight inactivation (Lee *et al.* 2006), predation (Hartz *et al.* 2008; Feng *et al.* 2010) and biofilm formation (Balzer *et al.* 2010). Release of those cells in sediments into the overlying water can occur during times of turbulence (Le Fevre and Lewis 2003; Craig *et al.* 2004; Ferguson *et al.* 2005). To effectively evaluate these effects, better methods for detaching microbes from sediment particles are needed.

The objectives of this study were (i) to compare the effectiveness of four detachment treatments in removing intact and total *Bacteroides fragilis* cells from four types of sediments with different characteristics, (ii) to test whether the performance of detachment methods changes with cell-sediment contact time, and (iii) to determine the most appropriate detachment treatment for separating *Bacteroidales* cells from sediments for the application of PMA-qPCR. The detachment of the faecal indicator bacteria *Enterococcus faecalis* and *Escherichia coli* was also compared with that of *Bact. fragilis*.

Materials and methods

Preparation of bacterial strains

Bacteroides fragilis ATCC 25285 purchased from ATCC was grown in thioglycolate broth (Anaerobe system, Morgan Hill, CA) under anaerobic conditions using GasPak anaerobic jars (Becton Dickinson Microbiology system, Cockeyscille, MD) at 37°C for 3 days. An *E. coli* strain was isolated from the UC Davis Arboretum waterway (Davis, CA) using membrane cultivation technique on mTEC agar (USEPA 2002), followed by growth in Luria-Bertani (LB) broth at 37°C for 24 h. The identity of isolated *E. coli* strains was evaluated using the API 20 E (bioMérieux, Durham, NC) and verified as *E. coli* with 99·5% accuracy. *Enterococcus faecalis* ATCC 29212 was grown in brain heart infusion broth at 37°C for 24 h. Cell densities of *E. coli* and *Ent. faecalis* were measured before they were spiked into experimental bottles using membrane cultivation techniques with mTEC agar and mEI agar, respectively (USEPA 2002, 2006). The concentrations of genetic marker BacUni targeting the 16S rRNA gene of intact and total *Bacteroidales* cells (Kildare *et al.* 2007) were estimated using PMA-qPCR (Bae and Wuertz 2009a).

Collection of sediment and water samples

Sediments as well as overlying water samples were collected from two sea water beaches and one freshwater creek of Half Moon Bay in El Granada, California, and one freshwater beach in South Lake Tahoe, Nevada (Table 1). The stations had varying salinities and distinct differences in sediment grain sizes ranging from very fine to very coarse sand. Surface sediments at a depth of approx. 0–2 cm were collected from each site at knee depth of water using sterilized plastic scoops that had been soaked in a 10% bleach solution and rinsed with DI water, and placed in sterile Nalgene polypropylene bottles. Bottles were filled to the top with field water to minimize direct air contact with sediment samples. Additional water samples were collected by directly submerging sample bottles at a depth of 30 cm below the water surface. The sediment and water samples were placed on ice in the dark and immediately transported to the laboratory, where they were stored in the dark at 4°C until being processed. The particle size distribution of sediments was analysed using different pore sizes of sieves after dehydration of sediments. Dry weights of sediments were determined after drying at 105°C for 24 h. Organic carbon contents of sediments were calculated based on a 58% weight loss during 2 h at 360°C in a muffle oven after initial drying at 105°C (Nelson and Sommers 1996). The initial concentrations of *Bacteroidales* and *Enterococcus* target gene markers as well as cultivable FIB of sediment and water samples were also analysed.

Attachment and detachment procedure

Attachment of three bacterial isolates onto sediment particles was performed with four sediment samples from different stations (Table 1). Sediment samples were individually homogenized by hand mixing with sterilized

Table 1 Location, salinity and pH of water column, and median diameter, water content, and organic carbon content of sediments

				Water property		Sediment property		
Location	Latitude N	Longitude W	Station	Salinity (‰)	pH	Median grain diameter (mm)	Water content (%)	Organic carbon content (%)
Half Moon Bay, Beach	37°30′09″	122°29″27″	1	30·1	7·3	0·12	33·8	0·94
Half Moon Bay, Beach	37°30′00″	122°28′14″	2	29·6	7·9	0·21	24·1	0·52
Half Moon Bay, Creek	37°30′14″	122°29′13″	3	0·1	7·9	0·20	25·8	0·43
Lake Tahoe, Beach	38°59′22″	119°57′11″	4	0·0	7·4	1·47	21·3	0·18

spatulas for 5 min before use. Forty grams (wet weight) of each sediment sample was placed in four sterile 500-ml wide mouth bottles followed by adding 40 ml of water sample collected from the individual sampling station. Fifty microlitre each of the *E. coli*, *Ent. faecalis*, and *Bact. fragilis* strains was spiked into the overlying water of each sediment bottle. After that the bottles were loosely capped and placed in a GasPak anaerobic jar to prevent the decay of anaerobic *Bact. fragilis* during incubation. The cell concentration in each inoculum was approx. 4×10^{10} gene copies ml^{-1} of intact *Bact. fragilis* cells, 3×10^{11} gene copies ml^{-1} of total (intact and impaired) *Bact. fragilis* cells, 8×10^{8} CFU ml^{-1} of cultivable *Ent. faecalis* cells, 3×10^{12} gene copies ml^{-1} of total (intact and impaired) *Ent. faecalis* cells and 2×10^{9} CFU ml^{-1} of cultivable *E. coli* cells. The GasPak anaerobic jar was placed in the dark at 4°C and shaken at 120 rev min^{-1} for 18 h (C25KC incubator shaker; New Brunswick Scientific, Edison, NJ) to reach equilibrium conditions for sorption/desorption of micro-organisms to sediment particles. To collect sediment subsamples on day 1, the overlying water in the bottle was temporarily transferred to a sterile Falcon tube without disturbing the sediment surface using a sterile pipette. After that, sediments in the bottle were homogenized for 10 s by gently mixing with a sterile wooden stick. Then, 2 g of sediments was collected in triplicates from the bottle with a sterile 10-ml pipette from which the tip had been aseptically removed and placed in three new sterile Falcon tubes. The previously removed overlying water was replaced in the bottle. The bottle containing sediments and water was placed back in the GasPak anaerobic jar, followed by incubation in the dark at room temperature for 7 days to estimate the effect of extended cell-sediment contact time. At the end of the incubation, additional sediment samples were collected.

For detachment of micro-organisms associated with sediment particles, two grams of collected sediment subsample was combined with 20 ml of eluant in a 50-ml Falcon tube followed by one of two treatments (Fig. 1). One per cent (v/v) of the nonionic surfactant, polysorbate 80, also known by the brand name Tween 80 (Fisher Scientific, Fair Lawn, NJ), and 1× of Ultrapure MB grade 10× phosphate-buffered saline (PBS) (USB Corporation, Cleveland, OH) were selected as eluants (Yoon and Rosson 1990; Boehm *et al.* 2009). One drop of Antifoam Y-30 Emulsion (Sigma-Aldrich, St. Louis, MO) was added into the Tween 80

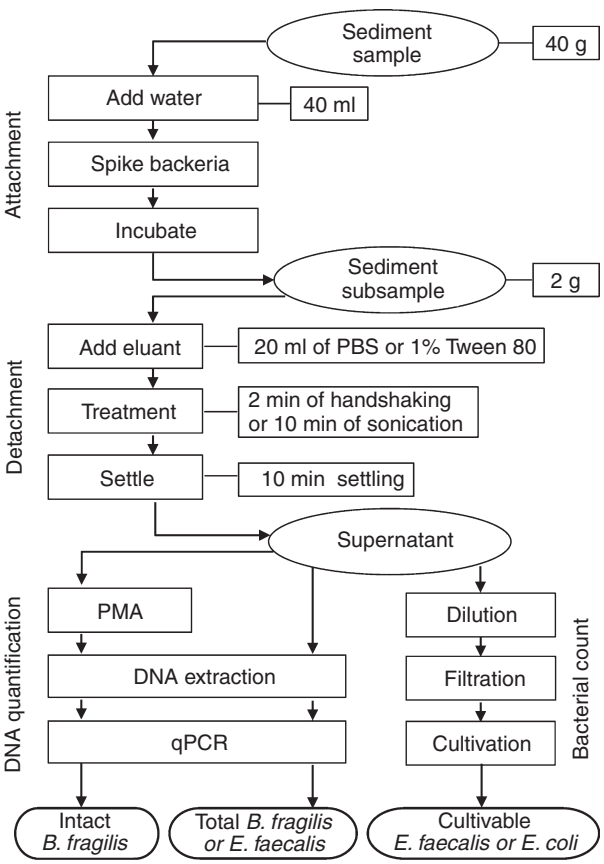

Figure 1 Flow diagram of the attachment/detachment of target micro-organisms and their enumeration procedure. The concentrations of micro-organisms in each inoculum were approx. 4×10^{10} gene copies ml^{-1} of intact *Bacteroides fragilis* cells, 3×10^{11} gene copies ml^{-1} of total (intact and impaired) *Bact. fragilis* cells, 8×10^{8} CFU ml^{-1} of cultivable *Enterococcus faecalis* cells, 3×10^{12} gene copies ml^{-1} of total (intact and impaired) *Ent. faecalis* cells and 2×10^{9} CFU ml^{-1} of cultivable *Escherichia coli* cells. Fifty microlitre of each inoculum was spiked into individual sediment samples.

solution followed by adjusting the pH to approx. 7·0 with NaOH solution. In addition, two different treatments (2-min handshaking and 10-min sonication) were chosen from previous studies (Ellery and Schleyer 1984; Epstein *et al.* 1997; Craig *et al.* 2002; Ferguson *et al.* 2005; Boehm *et al.* 2009). Mechanical shaking such as blending was reported to yield low separation efficiency due to an increased chance for cell injury during the treatment (Ellery and Schleyer 1984; Epstein and Rossel 1995; Boehm *et al.* 2009). For this reason, blending or vortexing was not used in this study. Handshaking was performed by one individual, and the effect of analysts in handshaking treatment is known not to be significant (Boehm *et al.* 2009; Cao *et al.* 2011). Handshaking was performed by vigorously shaking approximately twice per second, using a hand over an arc of about 10 cm (Boehm *et al.* 2009), and sonication was carried out using a Bransonic® Ultrasonic Cleaner (250W, 44 kHz, Model 8510E-MT; Branson, Danbury, CT). Hence, four combinations of detachment treatments, PBS with handshaking (PH), PBS with sonication (PS), Tween 80 and handshaking (TH), and Tween 80 and sonication (TS), were utilized for detachment of *Bact. fragilis*, *Ent. faecalis* and *E. coli.*

After the application of a detachment treatment, particles were allowed to settle for 10 min. Supernatants from each Falcon tube were collected and used for enumeration of *E. coli, Ent. faecalis* and *Bact. fragilis.* Total suspended solids (TSS) concentrations in supernatants after 10 min of settling were found to be <1000 mg l^{-1}, which is within the suitable range for PMA-qPCR analysis (Bae and Wuertz 2009a).

Enumeration of faecal indicator bacteria

Cultivable *E. coli* and *Ent. faecalis* cells were enumerated using a membrane filtration method according to EPA methods 1103·1 (USEPA 2002) and 1600 (USEPA 2006), respectively. Supernatants from separation treatments were diluted with sterile PBS and filtered using a 0·45-μm pore size sterile membrane (Millipore Corp., Bedford, MA). For *E. coli*, the membrane filters were transferred to mTEC agar and incubated at 35°C for 2 h and then at 44·5°C for 22 h. After incubation, filters were placed on sterile pads saturated with urea broth for 20 min for confirmation. For *Ent. faecalis*, the membrane filters were placed on mEI agar and incubated for 24 h to enumerate cultivable *Enterococcus* in supernatant samples. The results were expressed as CFU g^{-1} of dry weight of sediments.

PMA treatment and nucleic acid extraction

PMA (Biotium Inc., Hayward, CA) was prepared and stored as described before (Nocker *et al.* 2007; Bae and Wuertz 2009b). The previously optimized PMA concentration and light exposure period for *Bacteroidales* in water (Bae and Wuertz 2009a) was applied to an aliquot of supernatant to estimate the 16S rRNA gene concentration from intact *vs* total *Bact. fragilis* cells. Nucleic acids of PMA-treated or untreated supernatant samples were extracted using the Invitrogen PureLink Viral RNA/DNA extraction Kit (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions.

Quantitative PCR of *Bacteroidales* and *Enterococcus*

TaqMan qPCR assays targeting the 23S rRNA gene of *Enterococcus* (Haugland *et al.* 2005) and the 16S rRNA gene of *Bacteroidales* (Kildare *et al.* 2007) were performed in a StepOne Real-Time PCR System (Applied Biosystems, Foster City, CA). Each 25-μl qPCR reaction volume contained 12·5 μl of TaqMan Environmental Master Mix 2·0 (Applied Biosystems), 10 μl of nucleic acid extract, and appropriate concentrations of forward and reverse primers and probe according to the original publications. Thermal cycling conditions were 2 min at 50°C and 10 min at 95°C, followed by 40 cycles of 15 s at 95°C and 1 min at 60°C. A serial dilution approach was employed for each sample to mitigate potential inhibitors during qPCR (Schriewer *et al.* 2011). The sample limits of detection (S_{LOD}) of *Bacteroidales* and *Enterococcus* genetic markers were calculated as previously described (Schriewer *et al.* 2010).

Statistical analysis

Statistical analysis was performed using IBM SPSS Statistics 20.0 (Chicago, IL). Results of day 1 were evaluated by two-way analysis of variance (ANOVA) with salinity as a blocking factor in testing for statistical differences among the detachment treatments and sediments. Friedman's two-way ANOVA by ranks was used to test for statistical differences of the genetic marker concentrations of total *Bacteroidales* cells in sediments obtained from different detachment treatments on day 1, because inequality of variances was present in sample data. The effect of detachment treatments on intact and total bacterial cells on day 7 was analysed separately for individual sediments by one-way ANOVA, followed by *post hoc* pairwise comparisons (Tukey or Tamhane's T2 test). Differences were considered significant when the *P*-value was <0·05.

Results

Ambient concentrations of *E. coli* and *Enterococcus* were <1 colony forming unit (CFU) per ml of water or g of dry weight of sediments. The genetic marker BacUni

concentrations of total *Bacteroidales* cells, including both intact and impaired cells as well as extracellular DNA, in initial water and sediment samples were $<10^5$ gene copies ml^{-1} of water or g^{-1} of dry weight of sediments.

Comparison of bacterial detachment treatments after 1 day

Cell numbers removed from various types of sediments after 1 day of incubation were compared using two-way ANOVA with salinity as a blocking factor (Table 2). Recoveries of target micro-organisms were calculated by dividing the amount of separated bacteria from sediments by the amount of bacteria attached to the sediments. The attached amount was the difference between the total amount of bacteria inoculated and that detected in the overlying water column. Assuming no decay under the given condition, the recovery of total *Bact. fragilis* from each sediment was 4–38% on day 1 (Table S1). The four sediments were classified as sea water or freshwater sediments based on the salinity of the overlying water. Target concentrations achieved with the different treatments varied considerably with sediment type ($P < 0{\cdot}05$), regardless of whether viable counts or qPCR was used to enumerate. In contrast, counts of intact or cultivable cells separated from the same sediment type with different methods were not significantly different on day 1. The effect of detachment treatment on separation of intact *Bact. fragilis* cells, as determined by PMA-qPCR, was not significant ($P > 0{\cdot}05$). In addition, separated cultivable cell counts of *Ent. faecalis* and *E. coli* from sediments showed no significant differences among detachment treatments ($P > 0{\cdot}05$). The results indicate that on day 1, the different detachment treatments used separated intact and cultivable cells in the sediments equally well. All methods yielded comparable amounts of the genetic marker of total *Bact. fragilis* cells from associated sediment particles ($P > 0{\cdot}05$). In contrast, the *Ent. faecalis* genetic

Table 2 Enumeration of *Bacteroides fragilis*, *Enterococcus faecalis* and *Escherichia coli* separated from sea water and freshwater sediments using various detachment treatments after 1 day of incubation*

		Detachment treatment§				*P*-value¶	
Target micro-organism†	Sediment‡	PH	PS	TH	TS	(S)	(T)
Intact	S1	31 ± 4·4	18 ± 1·5	9·1 ± 0·8	31 ± 0·4		
Bact. fragilis	S2	17 ± 3·9	12 ± 3·2	10 ± 0·9	6·1 ± 0·9		
(10^5 gc g^{-1})	S3	67 ± 19	93 ± 23	48 ± 27	46 ± 13	<0·001	0·085
	S4	81 ± 12	88 ± 6·7	69 ± 7·0	63 ± 8·9		
Cultivable	S1	4·4 ± 3·5	0·9 ± 0·2	0·5 ± 0·4	0·0 ± 0·0		
Ent. faecalis	S2	4·5 ± 3·0	1·0 ± 0·2	0·1 ± 0·0	0·0 ± 0·0		
(10^4 CFU g^{-1})	S3	25 ± 7·4	24 ± 10	5·8 ± 4·7	0·4 ± 0·3	0·003	0·140
	S4	8·0 ± 0·8	10 ± 1·8	NA**	NA		
Cultivable	S1	76 ± 26	120 ± 53	96 ± 59	120 ± 45		
E. coli	S2	35 ± 11	40 ± 7·6	59 ± 16	49 ± 15		
(10^4 CFU g^{-1})	S3	180 ± 55	110 ± 11	120 ± 21	45 ± 14	0·010	0·527
	S4	200 ± 17	100 ± 17	150 ± 46	120 ± 84		
Total	S1	140 ± 24	140 ± 4·2	180 ± 11	63 ± 7·5		
Bact. fragilis††	S2	32 ± 4·2	29 ± 1·2	120 ± 1·4	40 ± 6·7		
(10^6 gc g^{-1})	S3	18 ± 2·9	14 ± 2·4	85 ± 2·1	61 ± 6·0	<0·001‡‡	0·075‡‡
	S4	21 ± 7·4	14 ± 0·7	14 ± 1·3	17 ± 1·8		
Total	S1	3·8 ± 0·4	3·2 ± 0·3	4·5 ± 0·2	3·2 ± 0·2		
Ent. faecalis††	S2	3·7 ± 0·2	3·5 ± 0·1	4·9 ± 0·1	3·8 ± 0·2		
(10^8 gc g^{-1})	S3	2·6 ± 0·2	3·7 ± 0·5	4·4 ± 0·2	2·8 ± 0·4	<0·001	<0·001
	S4	1·8 ± 0·4	1·8 ± 0·0	3·2 ± 0·1	3·0 ± 0·1		

*Results shown are means ± standard errors.

†Unit is gene copies or colony forming units g^{-1} of dry weight of sediments.

‡S1 and S2 are sea water sediments from the station 1 and 2, while S3 and S4 are freshwater sediments from the station 3 and 4. Detailed description of sediments is given in Table 1.

§PH, PBS with handshaking; PS, PBS with sonication; TH, Tween 80 with handshaking; TS, Tween 80 with sonication.

¶Statistical differences (*P*-values) among sediments (S) and detachment treatments (T) were analysed using 2-way ANOVA for a block design with salinity as a blocking factor. *P*-values <0·05 were considered significant differences among separation treatments.

**NA, not analysed due to technical difficulties.

††Total *Bact. fragilis* or *Ent. faecalis* includes DNA from both intact and impaired cells as well as extracellular DNA.

‡‡Friedman's two-way ANOVA by ranks was used due to the presence of inequality of variances.

marker concentration in total cells after detachment treatment TH was significantly higher than after treatments with PH, PS or TS ($P < 0{\cdot}05$).

Comparison of detachment treatments after 7 days

The effect of different detachment treatments on removing *Bact. fragilis*, *Ent. faecalis* and *E. coli* after 7 days of incubation was examined in individual sediments by one-way ANOVA and hence without using salinity as blocking factor (Table 3). The reason for not using two-way ANOVA was that the *Bact. fragilis* cell numbers in sea water sediments had decreased by a factor of at least 20 compared to the levels in freshwater sediments, indicating more rapid decay in marine environments, which resulted in unequal distribution of cell counts. The magnitude in the difference varied between sediment types and was generally greater for freshwater sediments with more cells. For sediments from station 1, the effect of detachment treatments on separation of intact *Bact. fragilis* cells, as measured by PMA-qPCR, could not be evaluated because intact cell numbers decreased below the S_{LOD} (about 3×10^4 gene copies g^{-1}) during the 7 days. The concentrations of cultivable *Ent. faecalis* cells separated from station 1 sediments were not significantly different among the four detachment treatments ($P > 0{\cdot}05$) (Tables 3 and 4). Likewise, no significant difference was observed in the amount of cultivable *E. coli* released by each treatment in the sediments ($P > 0{\cdot}05$). In the sediment sample from station 2, each detachment treatment yielded similar numbers for the genetic marker of intact *Bact. fragilis* cells ($P > 0{\cdot}05$), while more cultivable *Ent. faecalis* cells were detected with detachment treatment PS compared to treatments TH and TS ($P < 0{\cdot}05$). The concentrations of cultivable *E. coli* yielded by different treatments were not significantly different ($P > 0{\cdot}05$). At stations 3 and 4, copy numbers of the marker gene in intact *Bact. fragilis* cells varied significantly with detachment treatment ($P < 0{\cdot}05$); however, the *post hoc* pairwise comparison could not confirm the significance. The same was found

Table 3 Enumeration of *Bacteroides fragilis*, *Enterococcus faecalis* and *Escherichia coli* separated from different sediments using various detachment treatments after 7 days of inoculation*

		Detachment treatment§				
Target micro-organism†	Sediment‡	PH	PS	TH	TS	*P*-value¶ (Treatment)
Intact	S1	n.d.**	n.d.	n.d.	n.d.	
Bact. fragilis	S2	0·5 ± 0·2	0·6 ± 0·2	0·7 ± 0·2	0·3 ± 0·2	0·56
(10^5 gc g^{-1})	S3	4·9 ± 1·3	25 ± 3·3	9·6 ± 2·1	8·4 ± 6·5	0·03††
	S4	11 ± 0·3	34 ± 7·2	24 ± 2·7	8·6 ± 0·4	0·01††
Cultivable	S1	1·2 ± 0·8	0·3 ± 0·0	2·6 ± 1·2	0·2 ± 0·2	0·15
Ent. faecalis	S2	2·5 ± 0·9	4·3 ± 0·9	0·4 ± 0·1	0·4 ± 0·3	0·01
(10^4 CFU g^{-1})	S3	170 ± 82	400 ± 60	170 ± 140	12 ± 5·8	0·03††
	S4	2·8 ± 0·9	8·4 ± 5·2	6·5 ± 5·2	5·9 ± 5·1	0·85
Cultivable	S1	0·3 ± 0·1	0·2 ± 0·1	0·1 ± 0·0	0·4 ± 0·3	0·33††
E. coli	S2	0·5 ± 0·1	0·4 ± 0·2	0·2 ± 0·0	0·1 ± 0·0	0·10
(10^4 CFU g^{-1})	S3	990 ± 320	3200 ± 1200	2600 ± 150	1800 ± 1100	0·34
	S4	210 ± 24	340 ± 70	120 ± 33	290 ± 50	0·05
Total	S1	0·6 ± 0·1	0·2 ± 0·0	1·4 ± 0·1	0·5 ± 0·1	<0·001
Bact. fragilis‡‡	S2	31 ± 5·2	29 ± 1·2	120 ± 1·7	41 ± 7·9	<0·001
(10^6 gc g^{-1})	S3	9·8 ± 3·2	16 ± 4·8	12 ± 8·5	24 ± 13	0·64
	S4	8·7 ± 2·1	45 ± 9·1	70 ± 6·8	63 ± 4·0	<0·001
Total	S1	2·8 ± 0·3	2·1 ± 0·1	5·5 ± 0·3	3·2 ± 0·4	<0·001
Ent. faecalis‡‡	S2	5·2 ± 0·2	5·6 ± 0·1	35 ± 1·2	23 ± 6·8	<0·001
(10^8 gc g^{-1})	S3	2·6 ± 0·3	2·4 ± 0·1	4·3 ± 1·2	2·7 ± 0·3	0·27
	S4	7·2 ± 1·1	4·6 ± 0·5	11 ± 0·8	6·5 ± 0·1	0·001

*Results shown are means ± standard errors.

†Unit is gene copies or colony forming units g^{-1} of dry weight of sediments.

‡Details of sediments are given in Table 1.

§PH, PBS with handshaking; PS, PBS with sonication; TH, Tween80 with handshaking; TS, Tween 80 with sonication.

¶Statistical differences (*P*-values) among detachment treatments in each sediment were analysed using 1-way ANOVA. *P*-values <0·05 were considered significant differences among separation treatments.

**n.d., not detected.

††Statistic of Welch's test replaced ANOVA as unequal variance existed in data. Significances <0·05 were considered significant differences between groups.

‡‡Total *Bact. fragilis* or *Ent. faecalis* includes DNA from both intact and impaired cells as well as extracellular DNA.

Table 4 Summary of statistical analyses. Effects of detachment treatments on bacterial separation were tested using 2-way ANOVA on day 1 and 1-way ANOVA for each sediment on day 7. *Post hoc* pairwise comparison results are shown in parentheses in italics and significant differences in bold

		P-value*				
			Day 7			
Cell type	Target micro-organism	Day 1	S1	S2	S3	S4
Intact/cultivable cells	*Bacteroides fragilis*	0·09	NA†	0·56	**0·03**‡	**0·01**‡
	Enterococcus faecalis	0·14	0·15	**0·01** (*TH,TS<PS*)	**0·03**‡	0·85
	Escherichia coli	0·53	0·33	0·10	0·34	0·05
Total cells§	*Bact. fragilis*	0·08	**<0·001** (*PH,PS,TS<TH*)	**<0·001** (*PH,PS,TS<TH*)	0·64	**<0·001** (*PH<PS,TS,TH*)
	Ent. faecalis	**<0·001** (*PH,PS,TS<TH*)	**<0·001** (*PH,PS,TS<TH*)	**<0·001** (*PH,PS<TS,TH*)	0·27	**0·001** (*PH,PS,TS<TH*)

**P*-values <0·05 were considered significant.

†NA, not analysed.

‡Robust tests of equality of means (Welch's test) suggested a significant difference among detachment treatments; however, *post hoc* pairwise comparison (Tamhane's T2 test) could not confirm the significance.

§Total cells include intact and impaired cells.

for cultivable *Ent. faecalis* cell counts in sediments from station 3. Cultivable *Ent. faecalis* cell counts released from station 4 sediments were comparable for the four treatments ($P > 0{\cdot}05$). In addition, for sediments from stations 3 and 4, no great difference was observed in the amount of cultivable *E. coli* cells separated by each of the detachment treatments in the individual sediments. Overall, with regard to intact and cultivable bacterial separation, the detachment treatments PS and TH separated comparable or higher amounts of intact *Bact. fragilis* cells from individual sediments. The treatment TH also produced numbers of cultivable *Ent. faecalis* cells that were similar to other treatments, except for sediments from station 2. For cultivable *E. coli*, the numbers of cells separated did not vary by treatment within one sediment type.

The marker gene copies of total *Bact. fragilis* and *Ent. faecalis* cells from both intact and impaired cells were also compared for individual sediments using one-way ANOVA after 7 days (Table 3). When the genetic marker concentration of total *Bact. fragilis* cells was analysed, different patterns appeared compared to the gene concentrations of only intact *Bact. fragilis* cells. The detachment treatment TH resulted in an eightfold increase in the detached BacUni genetic marker of total cells compared to the other three treatments in sediments from stations 1, 2 and 4. Results were similar for the *Ent. faecalis* marker gene. However, for sediments from station 3, the effect of detachment treatment TH was not significantly different compared to the other three treatments ($P > 0{\cdot}05$) for both *Bact. fragilis* and *Ent. faecalis* genetic marker of total cells. Thus, in all but one sediment samples, detachment treatment TH resulted in increased yields of the 16S rRNA and 23S rRNA genetic markers in total bacterial cells with any of the sediments tested.

Discussion

On day one, more intact *Bact. fragilis* cells as well as cultivable *Ent. faecalis* and *E. coli* cells were detached from freshwater sediments than from sea water sediments. Given the fact that micro-organisms more easily attach to particles in solutions of higher ionic strength and of smaller grain sizes (Derjaguin and Landau 1941; Verwey and Overbeek 1948; Dong *et al.* 2002), it is unlikely that more bacterial cells were attached to the freshwater sediments, which had a lower ionic strength and larger grain sizes compared to the sea water sediments. Instead, target micro-organisms may have been loosely attached to the freshwater sediments and hence were removed more easily.

In comparing the four detachment treatments for separation of intact *Bact. fragilis* and cultivable FIB including *Ent. faecalis* and *E. coli*, all detachment treatments resulted in similar yields for each bacterial strain and sediment type after 1 day of incubation (Table 4). After 7 days, however, the intact *Bact. fragilis* or cultivable *Ent. faecalis* cell numbers released from individual sediments varied among different detachment treatments. Previous studies have shown that natural biofilms were formed within 7 days on submerged surfaces by suspended micro-organisms in surface water (Jackson *et al.* 2001; Augspurger *et al.* 2010). Hence, the extended cell-sediment contact time may have allowed bacteria more time to form biofilms on surfaces by enhancing the formation of extracellular polymeric substances (EPS). Harsher detachment methods were required in other studies to separate bacteria forming biofilms (Buesing and Gessner 2002; Ferguson *et al.* 2005; Amalfitano and Fazi 2008; Boehm *et al.* 2009), which supports the notion of contact time affecting the choice of treatment method.

The differences in detached intact *Bact. fragilis* cell numbers among treatments in sediments 3 and 4 on day 7 were due to higher yields obtained with detachment treatments PS (PBS with sonication) and TH (Tween 80 with handshaking). For the two physical techniques, handshaking and sonication, the magnitude of shear stress is higher in the case of sonication. Regarding chemical treatment used in the present study, PBS was used as an eluant to detach micro-organisms from sediments by minimizing cell lysis during treatment. Therefore, using PBS during sonication may have reduced the risk of microbial destruction during the strong physical treatment. Surfactants such as Tween 80 have been applied for the release of micro-organisms in sediments due to their ability to lower the surface tension between sediments and associated micro-organisms, thereby facilitating particle dispersion (Yoon and Rosson 1990; Epstein and Rossel 1995). Moreover, Tween 80 is a nonionic surfactant, which can result in greater repulsion between sediments and micro-organisms because the thickness of the electrical double layer increases with decreasing ionic strength of the surfactant (Bolster *et al.* 2001). The use of Tween 80 combined with handshaking successfully separated tightly associated *Bact. fragilis* cells from sediments on day 7.

On the other hand, the detachment treatments TS and PH yielded fewer intact *Bact. fragilis* cells in the same sediments. An explanation could be that the relatively harsh detachment method TS resulted in fewer intact *Bact. fragilis* cells due to an increased chance of cell injury; the less aggressive detachment treatment PH presumably led to lower cell counts, because of inactive disruption of sediment particles. Although severe shear forces can be destructive to micro-organisms, sonication has been efficiently applied to remove micro-organisms from various sediments (Ellery and Schleyer 1984; McDaniel and Capone 1985; Craig *et al.* 2002). The discrepancy with our study results may be caused by the different detachment techniques and sediment characteristics between studies. Therefore, the techniques for removing various micro-organisms from different types of sediments should be carefully chosen to achieve satisfactory detachment with minimum damage to attached cells. Micro-organisms associated with smaller particles, such as silt or clay, may need more energy to be separated because fine-grained silt and clay have a relatively large surface area per unit volume compared to sand, which may have elevated sites for attached micro-organisms.

Cultivable *Ent. faecalis* cell counts separated by TH in sediment 2 were not as high as for PS after 7 days, and it is possible that salt ions released from the sediments may restrain the desorption ability of Tween 80. This result, however, was observed only for cultivable *Ent. faecalis* in sediments of station 2. Regarding cultivable *E. coli*, detachment treatments appear to be equally effective for detachment of cultivable cells from sediments. These results agree with a previous study where the approximately twenty treatments tested did not result in significantly different FIB concentrations in sand samples (Boehm *et al.* 2009). Indeed, in all but one case, cultivable FIB numbers released by the four detachment treatments tested in the present study were similar in each of the four sediments.

With regard to the genetic marker of total cells for either *Bact. fragilis* or *Ent. faecalis*, treatment of samples with TH produced higher or comparable numbers from each of four sediment samples (Table 4). The difference in detached DNA concentrations of total *Bact. fragilis* cells among detachment treatments increased up to eightfold on day 7. The detachment treatment PS, which performed well for separation of intact *Bact. fragilis* as well as cultivable *Ent. faecalis* cells, did not yield significantly greater genetic marker concentrations compared to TH in all but sediments from station 3. We propose that DNA from either impaired *Bact. fragilis* cells or extracellular DNA was more effectively detached from sediments by detachment treatment TH compared to other treatments. Impaired cells ought to be more easily removed from sediment particles because exopolysaccharides causing adhesion of micro-organisms to sediment particles are degraded after cell death (Riis *et al.* 1998). Obtaining measurements of genetic markers from both intact and total cells is important because the ratio of intact cell marker DNA and total marker DNA can help estimate the age of recent faecal contamination in environments (Bae and Wuertz 2009b). In the present study, the detachment treatment TH involving 2 min of handshaking and 1% Tween 80 yielded greater DNA from sea water and freshwater sediments with minimized cell lysis; thus, this method is appropriate for PMA-qPCR analysis of *Bacteroidales*.

One limitation of this study was that *Bact. fragilis* spiked as an inoculum did not represent the diversity of indigenous *Bacteroidales* in each sediment sample. However, the controlled conditions allowed for a direct comparison of detached numbers of *Bact. fragilis* in different sediments. The TSS concentrations in supernatants after 10 min of settling were low enough to apply PMA to all four sediments tested. However, PMA application may be inhibited in samples with higher amounts (>1000 mg TSS l^{-1}) of silt and clay due to limited photolysis through the particles remaining in supernatant after 10 min. Thus, the selection of the appropriate detachment methods should take sediment type into consideration. The background concentrations of BacUni marker in the sediments prior to inoculation were close to detection limits and were not expected to increase much during the incubation; hence, experimental controls without inoculation were not included in the experimental design.

Using sediment samples spiked with known concentrations of cells, we estimated the recovery of target organisms from sediments on day 1. We established detachment methods that worked for sediments on days 1 and 7 because 1 week was a reasonable length of time to represent recent faecal pollution introduced into a watershed. The optimized detachment method has been successfully applied to separate *Bacteroidales* from sediments in a faecal pollution monitoring project conducted in our lab. In a long-term microcosm experiment using fresh and sea water sediments contaminated with human faecal waste, the detachment method TH yielded human-associated *Bacteroidales* cells and DNA from sediments at concentrations ranging from $7{\cdot}2\times10^{5}$ gc g^{-1} to $9{\cdot}5\times10^{3}$ gc g^{-1}, and the standard deviations were fairly low during the 30 days of experimental period (M. Kim and S. Wuertz, unpublished data).

In conclusion, the detachment method comprised of handshaking and Tween 80 was the most efficient technique to recover *Bact. fragilis* intact cells and total DNA in the sediments tested. The validated detachment treatment in this study will help expand our understanding of the survival of host-associated *Bacteroidales* cells and the persistence of their DNA in sea water and freshwater sediments by enabling the application of PMA-qPCR to micro-organisms present in sediment samples. In this way, the contribution of environmental *Bacteroidales* in sediments can be estimated in future microbial source tracking studies.

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Conflict of Interest

No conflict of interest declared.

References

Amalfitano, S. and Fazi, S. (2008) Recovery and quantification of bacterial cells associated with streambed sediments. *J Microbiol Methods* **75**, 237–243.

Augspurger, C., Karwautz, C., Mußmann, M., Daims, H. and Battin, T.J. (2010) Drivers of bacterial colonization patterns in stream biofilms. *FEMS Microbiol Ecol* **72**, 47–57.

Bae, S. and Wuertz, S. (2009a) Discrimination of viable and dead fecal *Bacteroidales* bacteria by quantitative PCR with propidium monoazide. *Appl Environ Microbiol* **75**, 2940–2944.

Bae, S. and Wuertz, S. (2009b) Rapid decay of host-specific fecal *Bacteroidales* cells in seawater as measured by quantitative PCR with propidium monoazide. *Water Res* **43**, 4850–4859.

Bae, S. and Wuertz, S. (2012) Survival of host-associated *Bacteroidales* cells and their relationship with *Enterococcus* spp., *Campylobacter jejuni*, *Salmonella enterica* Serovar Typhimurium, and Adenovirus in freshwater microcosms as measured by propidium monoazide-quantitative PCR. *Appl Environ Microbiol* **78**, 922–932.

Balzer, M., Witt, N., Flemming, H.C. and Wingender, J. (2010) Faecal indicator bacteria in river biofilms. *Water Sci Technol* **61**, 1105–1111.

Boehm, A.B., Griffith, J., McGee, C., Edge, T.A., Solo-Gabriele, H.M., Whitman, R., Tsao, Y., Getrich, M. *et al.* (2009) Faecal indicator bacteria enumeration in beach sand: a comparison study of extraction methods in medium to coarse sands. *J Appl Microbiol* **107**, 1740–1750.

Bolster, C.H., Mills, A.L., Hornberger, G.M. and Herman, J.S. (2001) Effect of surface coatings, grain size, and ionic strength on the maximum attainable coverage of bacteria on sand surfaces. *J Contam Hydrol* **50**, 287–305.

Buesing, N. and Gessner, M.O. (2002) Comparison of detachment procedures for direct counts of bacteria associated with sediment particles, plant litter and epiphytic biofilms. *Aquat Microb Ecol* **27**, 29–36.

Cao, Y., McGee, C.D., Griffith, J.F. and Weisberg, S.B. (2011) Method repeatability for measuring *Enterococcus* in southern California beach sands. *Lett Appl Microbiol* **53**, 656–659.

Craig, D.L., Fallowfield, H.J. and Cromar, N.J. (2002) Enumeration of faecal coliforms from recreational coastal sites: evaluation of techniques for the separation of bacteria from sediments. *J Appl Microbiol* **93**, 557–565.

Craig, D.L., Fallowfield, H.J. and Cromar, N.J. (2004) Use of macrocosms to determine persistence of *Escherichia coli* in recreational coastal water and sediment and validation with in situ measurements. *J Appl Microbiol* **96**, 922–930.

Davies, C.M., Long, J.A.H., Donald, M. and Ashbolt, N.J. (1995) Survival of fecal microorganisms in marine and fresh-water sediments. *Appl Environ Microbiol* **61**, 1888–1896.

Derjaguin, B.V. and Landau, L.D. (1941) Theory of the stability of strongly charged lyophobic sols and of the adhesion of strongly charged particles in solutions of electrolytes. *Acta Physicochim URS* **14**, 633–662.

Dong, H.L., Onstott, T.C., Ko, C.H., Hollingsworth, A.D., Brown, D.G. and Mailloux, B.J. (2002) Theoretical prediction of collision efficiency between adhesion-deficient bacteria and sediment grain surface. *Colloids Surf B Biointerfaces* **24**, 229–245.

Ellery, W.N. and Schleyer, M.H. (1984) Comparison of homogenization and ultrasonication as techniques in extracting attached sedimentary bacteria. *Mar Ecol-Prog Ser* **15**, 247–250.

Epstein, S.S. and Rossel, J. (1995) Enumeration of sandy sediment bacteria: search for optimal protocol. *Mar Ecol Prog Ser* **117**, 289–298.

Epstein, S.S., Alexander, D., Cosman, K., Dompe, A., Gallagher, S., Jarsobski, J., Laning, E., Martinez, R. *et al.* (1997) Enumeration of sandy sediment bacteria: are the counts quantitative or relative? *Mar Ecol-Prog Ser* **151**, 11–16.

Feng, F., Goto, D. and Yan, T. (2010) Effects of autochthonous microbial community on the die-off of fecal indicators in tropical beach sand. *FEMS Microbiol Ecol* **74**, 214–225.

Ferguson, D.M., Moore, D.F., Getrich, M.A. and Zhowandai, M.H. (2005) Enumeration and speciation of enterococci found in marine and intertidal sediments and coastal water in southern California. *J Appl Microbiol* **99**, 598–608.

Hartz, A., Cuvelier, M., Nowosielski, K., Bonilla, T.D., Green, M., Esiobu, N., McCorquodale, D.S. and Rogerson, A. (2008) Survival potential of *Escherichia coli* and enterococci in subtropical beach sand: implications for water quality managers. *J Environ Qual* **37**, 898–905.

Haugland, R.A., Siefring, S.C., Wymer, L.J., Brenner, K.P. and Dufour, A.P. (2005) Comparison of *Enterococcus* measurements in freshwater at two recreational beaches by quantitative polymerase chain reaction and membrane filter culture analysis. *Water Res* **39**, 559–568.

Jackson, C.R., Churchill, P.F. and Roden, E.E. (2001) Successional changes in bacterial assemblage structure during epilithic biofilm development. *Ecology* **82**, 555–566.

Kildare, B.J., Leutenegger, C.M., McSwain, B.S., Bambic, D.G., Rajal, V.B. and Wuertz, S. (2007) 16S rRNA-based assays for quantitative detection of universal, human-, cow-, and dog-specific fecal *Bacteroidales*: a Bayesian approach. *Water Res* **41**, 3701–3715.

Le Fevre, N.M. and Lewis, G.D. (2003) The role of resuspension in enterococci distribution in water at an urban beach. *Water Sci Technol* **47**, 205–210.

Lee, C.M., Lin, T.Y., Lin, C.C., Kohbodi, G.A., Bhattl, A., Lee, R. and Jay, J.A. (2006) Persistence of fecal indicator bacteria in Santa Monica Bay beach sediments. *Water Res* **40**, 2593–2602.

McDaniel, J.A. and Capone, D.G. (1985) A Comparison of procedures for the separation of aquatic bacteria from sediments for subsequent direct enumeration. *J Microbiol Methods* **3**, 291–302.

Nelson, D.W. and Sommers, L.E. (1996) Total Carbon, Organic Carbon, and Organic Matter. In *Method of Soil Analysis Part 3: Chemical Methods* ed. Sparks, D.L., Page, A.L., Helmke, P.A., Loeppert, R.H., Soltanpour, P.N., Tabatabai, M.A., Johnston, C.T. and Sumner, M.E. pp. 961–1010. Madison, WI: Soil Science Society of America and American Society of Agronomy.

Nocker, A., Sossa-Fernandez, P., Burr, M.D. and Camper, A.K. (2007) Use of propidium monoazide for live/dead distinction in microbial ecology. *Appl Environ Microbiol* **73**, 5111–5117.

Okabe, S. and Shimazu, Y. (2007) Persistence of host-specific *Bacteroides-Prevotella* 16S rRNA genetic markers in environmental waters: effects of temperature and salinity. *Appl Microbiol Biotechnol* **76**, 935–944.

Riis, V., Lorbeer, H. and Babel, W. (1998) Extraction of microorganisms from soil: evaluation of the efficiency by counting methods and activity measurements. *Soil Biol Biochem* **30**, 1573–1581.

Savichtcheva, O. and Okabe, S. (2006) Alternative indicators of fecal pollution: relations with pathogens and conventional indicators, current methodologies for direct pathogen monitoring and future application perspectives. *Water Res* **40**, 2463–2476.

Schriewer, A., Miller, W.A., Byrne, B.A., Miller, M.A., Oates, S., Conrad, P.A., Hardin, D., Yang, H.H. *et al.* (2010) Presence of *Bacteroidales* as a predictor of pathogens in surface waters of the central California coast. *Appl Environ Microbiol* **76**, 5802–5814.

Schriewer, A., Wehlmann, A. and Wuertz, S. (2011) Improving qPCR efficiency in environmental samples by selective removal of humic acids with DAX-8. *J Microbiol Methods* **85**, 16–21.

USEPA (2002) Methods 1103.1: *Escherichia coli* (*E. coli*) in water by membrane filtration using membrane-thermotolerant Escherichia coli agar (mTEC), EPA 821-R-02-020.

USEPA (2006) Method 1600: enterococci in water by membrane filtration using membrane-Enterococcus indoxyl-ß-D-glucoside agar (mEI), EPA 821-R-06-009.

Verwey, E.J.W. and Overbeek, J.T.G. (1948) *Theory of the Stability of Lyophobic Colloids*. New York: Elsevier.

Walters, S.P. and Field, K.G. (2006) Persistence and growth of fecal *Bacteroidales* assessed by bromodeoxyuridine immunocapture. *Appl Environ Microbiol* **72**, 4532–4539.

Walters, S.P. and Field, K.G. (2009) Survival and persistence of human and ruminant-specific faecal *Bacteroidales* in freshwater microcosms. *Environ Microbiol* **11**, 1410–1421.

Wuertz, S., Wang, D., Reischer, G.H. and Farnleitner, A.H. (2011) Library-independent bacterial source tracking methods. In *Microbial Source Tracking: Methods, Applications, and Case Studies* eds. Hagedorn, C., Blanch, A.R. and Harwood, V.J. pp. 75–126. New York, Dordrecht, Heidelberg, London: Springer.

Yoon, W.B. and Rosson, R.A. (1990) Improved method of enumeration of attached bacteria for study of fluctuation in the abundance of attached and free-living bacteria in response to diel variation in seawater turbidity. *Appl Environ Microbiol* **56**, 595–600.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Percent recovery of cells and DNA±SEM.