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Complete List of Authors:	Liu, Rulong; Shanghai Ocean University, Shanghai Engineering Research Center of Hadal Science and Technology, College of Marine Sciences; The Hong Kong University of Science and Technology, Division of Life Science Yeung, Leo T. C.; The Hong Kong University of Science and Technology, Division of Life Science Ho, Pui-Hei ; The Hong Kong University of Science and Technology, Division of Life Science Lau, Stanley C.K.; The Hong Kong University of Science and Technology, Division of Life Science; The Hong Kong University of Science and Technology, Division of Environment
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Tracking the relative concentration between *Bacteroidales* DNA markers and culturable *Escherichia coli* in fecally polluted subtropical seawater: potential use in differentiating fresh and aged pollution

Rulong Liu, Leo T. C. Yeung, Pui-Hei Ho, Stanley C. K. Lau

Rulong Liu. Shanghai Engineering Research Center of Hadal Science and Technology, College of Marine Sciences, Shanghai Ocean University, Shanghai 201306, China; Division of Life Science, The Hong Kong University of Science and Technology, Clear Water Bay, Hong Kong (Email: rlliu@shou.edu.cn)

Leo T. C. Yeung and Pui-Hei Ho. Division of Life Science, The Hong Kong University of Science and Technology, Clear Water Bay, Hong Kong (Emails: L.T.C. Yeung, leoyeung13@gmail.com; P.H. Ho, nicoleho.nic@gmail.com)

Stanley C. K. Lau. Division of Life Science and Division of Environment, The Hong Kong University of Science and Technology, Clear Water Bay, Hong Kong (Email: scklau@ust.hk)

***Corresponding author:**

Stanley C. K. Lau

Division of Life Science and Division of Environment, The Hong Kong University of Science and Technology, Clear Water Bay, Hong Kong

Email: scklau@ust.hk;

Phone: +852-2358-7329; Fax: +852-2358-1559

Abstract

Routine water quality monitoring practices based on the enumeration of culturable *Escherichia coli* provides no information about the source or age of fecal pollution. An emerging strategy is to use culturable *E. coli* and the DNA markers of *Bacteroidales* complementarily for microbial source tracking. In this study, we observed consistently in seawater microcosms of three different conditions that culturable *E. coli* decayed faster ($T_{99} = 1.14 - 4.29$ day) than *Bacteroidales* DNA markers did ($T_{99} = 1.81 - 200.23$ day). Concomitantly, the relative concentration between *Bacteroidales* DNA markers and culturable *E. coli* increased over time in all treatments. Particularly, the increase during the early stage of the experiments (before T_{99} of *E. coli* was reached) was faster than during the later stage (after T_{99} of *E. coli* was attained). We propose that the tracking of the relative concentration between *Bacteroidales* DNA markers and culturable *E. coli* provides an opportunity to differentiate a pollution that is relatively fresh from one that has aged. This method, upon further investigation and validation, could be useful in episodic pollution events where the surge of *E. coli* concentration causes non-compliance to the single sample maximum criterion that mandates high frequency follow up monitoring.

Keywords: *Bacteroidales*, *E. coli*, decay, fecal pollution, age

For decades, culturable *Escherichia coli* (cEC) served as an indicator of fecal pollution in freshwater (USEPA 2006) and seawater (HKEPD 2014). It is a common practice for routine water quality monitoring programs that the water of a given location is sampled and analyzed on a weekly or even monthly basis (HKEPD 2014; USEPA 2015). Very often non-compliance to water quality standard would not come to a regulatory agency's attention until the next scheduled monitoring. When monitoring is infrequent, it is often not known whether the non-compliance is due to a pollution incident that has occurred shortly prior to the monitoring or an incident that has occurred days or weeks before (Sokolova et al. 2012).

The differentiation of fresh and aged pollution can be useful for health risk assessment, since the persistence of fecal pathogens varies widely in the aquatic environment. For example, it took the intact cells of *Salmonella enterica* serovar Typhimurium 122 h to undergo 99 % reduction of the initial concentration in freshwater under diurnal sunlight, and it only took the DNA of adenovirus 72 h and the intact cells of *Campylobacter jejuni* 34 h to attain the same under identical conditions (Bae and Wuertz 2012). Contrarily, norovirus and the endospores of *Bacillus* have been reported to persist for weeks or longer (Boyle et al. 2008; Seitz et al. 2011). Ideally, the persistence of cEC as fecal indicator bacteria should be comparable to that of the most recalcitrant pathogens. Nevertheless, as the more labile pathogens deactivate more quickly, a pollution event that has occurred recently should carry pathogens of higher diversity and infectivity than an event that has aged. However, the concentrations of cEC obtained in routine water quality monitoring, as a function of input scale, population decay and dispersal, provide little information whether a pollution being detected is fresh or aged (Sokolova et al. 2012).

Another limitation of cEC counting-based routine water quality monitoring is the lack of information about the source(s) of fecal pollution (e.g. humans, farm animals and wildlife). This hinders not only the identification of the source of impairment (e.g. sewage discharge vs. farm runoff) but also the assessment of health risks, since different animal species often carry

different variety of fecal pathogens (Harwood et al. 2014). Although DNA fingerprinting of cEC can be used for microbial source tracking, the broad host range of *E. coli* can limit its use in microbial source tracking (Savichtcheva and Okabe, 2006). In recent years, the host-specific 16S rRNA gene markers (DNA markers hereafter) of *Bacteroidales* have emerged as promising alternatives for microbial source tracking (Liu et al. 2012). A tiered approach that couples the monitoring of cEC count as an index of pollution level followed by PCR detection of *Bacteroidales* DNA markers as source identifier has been proposed (Noble et al. 2006; Liu et al. 2015).

Building upon the tiered approach of microbial source tracking, here we demonstrate using the data of the current study and a previous study (Table 1) that the monitoring of the relative concentrations between the DNA markers of *Bacteroidales* and cEC is potentially useful for the differentiation of a pollution incident that is aged from one that is relatively fresh. The persistence of fecal microbes is dependent on a number of intrinsic and extrinsic factors, such as the physiology and genetic diversity of the microbes and the prevailing environment conditions. It is a common practice to use T_{99} (i.e. the amount time required to attain 99 % reduction of the initial concentration) as a metric for the persistence of fecal microbes in the aquatic environments (Bae and Wuertz 2012; Jenkins et al. 2012). Here we use T_{99} of cEC as a delimiter to differentiate fresh and aged pollution; we define pollution as relatively fresh when its associated cEC population is on decay towards 99 % reduction of the initial concentration (i.e. before T_{99} is reached), and as aged when the reduction is > 99 % (after T_{99} is reached).

In the current study, we conducted an outdoor experiment with two trials of a microcosm that was comprised of a 27 L glass aquarium filled with 19 L untreated seawater and 1 L raw sewage. Upon the adding of the raw sewage to the seawater at 9 am of the day that the experiment began (0 h), the microcosm was placed outdoor without obstruction to

sunlight, receiving on average 13.49 MJm^{-2} of global solar radiation each day with daily maximum intensity of UVA ranging from 11.3 to 61.0 Wm^{-2} during the course of the experiment (Hong Kong Observatory). The total water level was 20 cm deep, allowing sunlight to penetrate to the bottom of the microcosm. Water temperature in the microcosm was maintained at ca. 26°C (typical for summer days in Hong Kong) using a run-through water jacket pumping from the sea surface. The microcosm was continuously aerated using $0.22 \text{ }\mu\text{m}$ -filtered air to keep the seawater normoxic (dissolved oxygen concentration ca. $8 \text{ }\mu\text{g ml}^{-1}$), amidst the respiration of the fecal community. To reduce water evaporation, the microcosm was covered with a Dupont FEP-Teflon film ($50 \text{ }\mu\text{m}$ thick) that was reported to allow the transmission of $> 96 \%$ of solar radiation (Teflon 1996). Water level and salinity in the microcosm was observed regularly. Water loss due to evaporation was replenished using autoclaved double-distilled water so as to maintain water salinity at $30 \pm 1 \text{ ‰}$. Further description of the microcosm conditions are provided in Supporting Information.

The experiment lasted for 4 days until the initial concentration of cEC had reduced by $> 99.9 \%$ (i.e. 3 log) (Fig. 1 and 2). During this period of time, triplicate water samples (200 ml each) were taken from the microcosm on the schedule specified in Table 1. The water samples were $0.22 \text{ }\mu\text{m}$ membrane-filtered. Bacterial cells captured on the membranes were subjected to the enumeration of cEC using CHROMagarTM ECC, and the determination of the copy numbers of the general (AllBac) and human-specific (HF) *Bacteroidales* DNA markers using SYBR[®] Green dye-based qPCR (Liu et al. 2015). Further information of the procedure of DNA extraction, and the conditions and quality control of the qPCR assays can be found in Supporting Information.

At 0 h of the experiment, seawater in the two trial microcosms was observed carrying $3.40 \times 10^5 - 3.44 \times 10^6 \text{ CFU } 100 \text{ ml}^{-1}$ of cEC, $4.73 \times 10^9 - 1.08 \times 10^{10} \text{ copy } 100 \text{ ml}^{-1}$ of AllBac, and $5.07 \times 10^8 - 2.17 \times 10^9 \text{ copy } 100 \text{ ml}^{-1}$ of HF (Fig. 1 & 2). In trial one, the $\log_{10}$ transformed

ratio of the concentrations between AllBac and cEC (AllBac:cEC hereafter) was 4.19 whereas that between HF and cEC (HF:cEC hereafter) was 3.29 (Fig. 1). In trial two, the values for AllBac:cEC and HF:cEC were 3.29 and 2.53, respectively (Fig. 2). These values are in line with what had been reported for human feces and sewage that AllBac was 2 to 6 orders of magnitude more abundant than cEC, and that HF as a major *Bacteroidales* lineage was 1 to 2 orders of magnitude less abundant than AllBac (Gourmelon et al. 2010; Stoeckel et al. 2011).

The changes in the concentrations of cEC, AllBac and HF over the next 4 days were analyzed using the GInaFit software package for the fitting of the data to decay models, and the determination of decay rate constants and T_{99} values (Geeraerd et al. 2005). The results of decay model fitting indicated for both microcosm trials of the experiment that cEC went on population decay immediately after 0 h whereas AllBac and HF exhibited a lag time of > 1 day (i.e. a “shoulder” in the decay curves) before population decay (Fig. 1 & 2; Table 2). The decay of cEC, as observed using cultivation, was primarily associated with cell death and/or the loss of culturability in viable cells (Green et al., 2011). Contrarily, the decay of *Bacteroidales* DNA markers, as observed using qPCR of target genes, was independent of the state of cellular viability or culturability. Solar radiation, particularly the UV spectrum, is strongly bactericidal. It can directly and indirectly (through reactive oxygen intermediate) damage the nucleic acid, protein and lipids of bacterial cells, resulting in mutation, inactivation of metabolism, membrane damage, or death (Sinton et al. 2002). However, the effects of solar radiation on metabolic inactivation were quicker than on DNA or membrane damage, as shown for the bacterioplankton community in the Mediterranean coastal waters (Alonso-Saez et al. 2006). Therefore, we suggest that the differences between the time required for the onset of the decay of cEC (immediate) and *Bacteroidales* DNA markers (> 1 day) can be in part explained as due to solar radiation having more immediate effect on the deactivation of metabolism (hence the decay of cEC) than to the damaging of DNA and/or

cell membrane (hence the loss of *Bacteroidales* DNA markers) (Liu et al. 2008).

Nonetheless, the specific decay pattern of each bacterial indicator varied considerably between trials (Fig. 1 & 2; Table 2). For example, AllBac and HF had shorter “shoulder lengths” and more rapid decay in trial one than in trial two. The decay of cEC in trial one was log-linear for two days followed by a tail whereas the decay in trial two was biphasic with a relatively rapid but short 1st phase and a slower but longer 2nd phase. Biphasic decay is believed to be the effects of multiple environmental stresses (Bae and Wuertz 2009 and 2012). We suggest that such between-trial differences were due to the variations of one or more intrinsic or extrinsic factors associated with the experiment, including but not limited to weather conditions, and the genetic diversity and physiological status of the fecal microbes.

Despite the between-trial variations in decay pattern of individual indicator, the values of AllBac:cEC and HF:cEC exhibited a trend of increase over the four days of incubation in both trials. In trial one, the value of AllBac:cEC varied between 3.65 and 4.19 during the first 4 h. Then it increased to 5.26 by the end of day one and further, by a smaller magnitude, to 5.94 in the next three days (Fig. 1). In trial two, the value of AllBac:cEC increased from 3.28 to 5.07 during the first 12 h, and hovered between 4.34 and 4.87 towards the end of the experiment (Fig. 2). As a lineage within *Bacteroidales* (Seurinck et al. 2005), the changes in the concentration of HF and the value of HF:cEC mirrored those of AllBac and AllBac:cEC, respectively.

Generally, the values of AllBac:cEC and HF:cEC increased in relatively large magnitudes during the first day, as cEC decayed towards 99 % reduction of the initial concentrations ($T_{99} = 1.13$ day for trial one and 1.14 day for trial two) and the *Bacteroidales* DNA markers remained relatively recalcitrant (Fig. 1 & 2). After that, the magnitude of increase reduced when *Bacteroidales* DNA markers started to decay (trial one) or the decay of cEC started to enter the slower 2nd phase (trial two). The fluctuations of the AllBac:cEC and

HF:cEC values observed for the short intervals of sampling during the first few hours may be associated with the uneven distribution of the fecal microbes in the microcosms.

Many studies have reported that *Bacteroidales* DNA markers and cEC decayed at different rates under the same environmental condition. It is primarily attributed to the differences in the physiology of the two bacteria (e.g. *E. coli* being facultative anaerobic vs. *Bacteroidales* being strictly anaerobic) and the differences in the working principles of the detection methods (cultivation vs. PCR detection of DNA markers) (Green et al. 2011; Jeanneau et al. 2012). Based on the results of the microcosm experiment described above, we suggest that the differential decay between *Bacteroidales* DNA makers and cEC is potentially useful for estimating the status of a pollution event being detected. A fast increase in the relative concentration between *Bacteroidales* DNA markers and cEC may suggest a relatively fresh pollution (cEC associated with the pollution is decaying towards 99 % reduction of the initial concentration) whereas a slow increase may suggest a pollution that has aged for a while (cEC population has decayed by > 99 % of the initial). Nonetheless, the experiment was conducted under a single condition (i.e. outdoor for a few days, aerated) with a single source of *E. coli* and *Bacteroidales* (i.e. sewage). It was not known whether similar results would be obtained when experiments were conducted using other fecal sources and under different environmental conditions.

To corroborate our results, we re-analyzed the data of two seawater microcosms of our previous work that had fecal source and incubation setting different from the experiment discussed above (Table 1). The setup of the two microcosms is described in details in Liu et al. (2015). Briefly, the microcosms were kept under normoxic (dissolved oxygen ca. 8 $\mu\text{g ml}^{-1}$) or hypoxic (dissolved oxygen < 1 $\mu\text{g ml}^{-1}$) condition by purging the seawater with 0.22 μm -filtered air or N₂ gas (purity > 99.9 %). The seawater was 0.22 μm -filtered and autoclaved for the purpose of investigating the effects of normoxia and hypoxia on bacterial decay

without the confounding effects of bacterial competition, viral lysis and protozoan grazing. Slurry of feces collected freshly from feral cows was spiked to each microcosm to a final concentration of ca. 0.01 g (w.t.) ml⁻¹. The microcosms were maintained indoor under darkness at 26 °C for 11 days until cEC had decayed by > 99.9 % of the initial concentration. The concentrations of cEC, and AllBac and CF (the DNA marker for cow-associated *Bacteroidales*) were monitored over time (Table 1). Information about qPCR assay for the detection of CF can be found in Supporting Information.

AllBac decayed log-linearly under the hypoxic condition and in a biphasic manner under the normoxic condition with the 1st phase accounted for 86.46 % (trial one) and 99.03 % (trial two) reduction of the initial concentrations (Table 2; see Liu et al. 2015 for the decay curves). The decay of CF was primarily log-linear, except in trial one of the normoxic treatment where biphasic decay was observed (1st phase = 83.31 % reduction). cEC decayed in a biphasic manner in both treatments with the 1st phase accounted for > 99.80 % reduction of the initial concentration. Generally, cEC decayed at significantly higher rates than AllBac and CF did, as compared on the basis of the regression slopes ($p \leq 0.005$).

Concomitant with the differential decay was the relative concentrations between *Bacteroidales* DNA markers and cEC exhibiting a trend of increase over time (Fig. 3). We compared the magnitude of changes in the values of AllBac:cEC and CF:cEC before and after the cEC population in a microcosm had decayed by 99 % of the initial. The T₉₉ values of cEC ranged from 2.96 to 4.29 days in the two trials of the two microcosms (Table 2). To align these values with the monitoring schedule of the experiment (Table 1), we used day 0 - 4 as the period that cEC was on decay towards 99 % of the initial concentrations, and day 4 – 11 as the period after. For the hypoxic treatment, the values of AllBac:cEC increased by the magnitude of 1.85 (trial one) and 1.68 (trial two) between day 0 and day 4 while the values of CF:cEC increased by 2.09 (trial one) and 1.72 (trial two) during the same period of time (Fig.

3). Subsequently, between day 4 and 11, the increase was by smaller magnitudes of 0.48 (trial one) and 0.67 (trial two) for AllBac:cEC, and 0.58 (trial one) and 0.84 (trial two) for CF:cEC. Similar results were obtained for trial one of the normoxic treatment but not for trial two (Fig. 3). For trial one, the value of AllBac:cEC increased by the magnitude of 1.05 between day 0 and 4, followed by a smaller magnitude of 0.38 between day 4 and 11 whereas the values of CF:cEC increased by 1.11 and 0.40 during the two periods, respectively. However, for trial two, the magnitude of increase during the period between day 0 and 4 (AllBac:cEC = 0.57 and CF:cEC = 0.76) were smaller than during the period after (AllBac:cEC = 1.02 and CF:cEC = 0.81).

The microcosms reported in Liu et al. (2015) and the current study together represent an array of fecal sources (sewage vs. cow) and environmental conditions (light vs. dark; normoxia vs. hypoxia) that are relevant to the marine environment of the subtropical Hong Kong. We have consistently observed in all microcosms that the decay of *Bacteroidales* DNA makers was generally slower than the loss of culturability in *E. coli*, and that concomitant with the differential decay was the relative concentrations between the two bacterial indicators increasing over time. Using T_{99} of cEC in a microcosm to delimit fresh and aged pollution, we observed in five of the six microcosms (except for the normoxic treatment in trial two of indoor experiment, Fig. 3) that the magnitudes of the increase in the relative concentration between *Bacteroidales* DNA makers and cEC (1.69 – 2.09) were larger during the period prior to T_{99} than the period after (0.48 – 0.84). Nonetheless, the microcosm of trial two of the normoxic treatment would still have the relative concentration increased for a larger magnitude in the earlier stage than in the later stage of the experiment if the delimiter was shifted to the next sampling point (day 6) of the microcosm experiment.

Many regulatory agencies have established water quality criteria based on (i) maximum concentration of *E. coli* present in a single water sample, and (ii) geometric mean / 90th

percentile / statistical threshold value of *E. coli* concentrations in water samples collected over a period of time (USEPA 2006; HKEPD 2014). The single sample maximum criterion is important for waters that are monitored infrequently or prone to episodic pollution; in the event of non-compliance, follow-up monitoring with high sampling frequency would usually be in place. We propose that, upon successful validation, the tracking of the pattern of change in the relative concentration between *Bacteroidales* DNA makers and cEC during such follow-up monitoring (e.g. once per day) would provide an opportunity to estimate whether the pollution being detected is relatively fresh or aged - a fast increase in the relative concentration would suggest the former whereas a slow increase would suggest the latter. The method can be conveniently added to the tiered approach of pollution monitoring that others have proposed to implement microbial source tracking within the current regime of *E. coli*-based water quality monitoring (Noble et al. 2006; Reischer et al. 2008).

Whether T_{99} of cEC is a practical delimiter of fresh vs. aged pollution, and what magnitude of increase in the relative concentration is indicative of fresh or aged pollution need to be defined in further investigations under field conditions that include dilution and other environmental factors. Indeed, *Bacteroidales* was observed to decay faster in batch-type microcosms than in flow-through experiments in the field (Bae and Wurtz 2015 and references therein). The method that we propose relies on the monitoring of the change in the relative concentration between *Bacteroidales* DNA markers and cEC, and is thus independent of the initial ratio of the two during the onset of a pollution event. Nonetheless, we have tested only two fecal sources. It would be useful for further investigations to include fecal sources that differ widely in the relative population sizes between *Bacteroidales* and *E. coli* and to include the monitoring of pathogen diversity in relation to the changes in the relative concentrations.

Emerging evidence indicates that fecal bacteria, including *E. coli* and *Bacteroidales*, are

capable of persisting in environmental matrices such as sediment and submerged phyllosphere (Vierheilig et al. 2012; Weidhass et al. 2015). The cross reactivity of *Bacteroidales* DNA markers with non-target animal sources or even non-fecal source is another noteworthy problem (Lamendella et al. 2009; Vierheilig et al. 2012; Harwood et al. 2014). These issues concern all microbial source tracking studies, including the current one. In fact, the detection of CF reported in this study was performed using a newly designed primer to alleviate the effects of cross reactivity with non-target animal sources (Liu et al. 2015).

A previous study proposed the use of the ratio between the host-associated *Bacteroidales* DNA markers and culturable indicator bacteria (e.g. *E. coli*) for the apportionment of pollution to different animal sources (Wang et al. 2013). It was concluded that the apportionment would be distorted as the pollution aged, due to the differential decay between host-associated DNA markers and culturable indicator bacteria (Wang et al. 2013). The method that we propose in the study is indeed to capitalize on such differential decay. Therefore, further testing should also include the feces of multiple animal sources and evaluate whether it would be possible to determine the status of the pollution (fresh vs. age) contributed by each source by tracking the concentration of each host-associated *Bacteroidales* DNA marker relative to cEC.

Others have proposed using viable or metabolically active *Bacteroidales* cells as the detection target to identify recent pollution, since the strictly anaerobic *Bacteroidales* are unlikely to survive for long in the aquatic environment (Green et al. 2011; Bae and Wuertz 2015). While these methods are potentially useful, they require additional procedures of reverse transcription of 16S rRNA molecules (for metabolically active cells) or propidium monoazide treatment (for cells with intact membrane) prior to qPCR assays. In this study, we propose that qPCR detection of *Bacteroidales* DNA markers (inclusive of cells that were viable or non-viable, and cells that were metabolically active or inactive) in reference to the

decay of cEC can provide metrics to evaluate the status of a pollution event as relatively fresh or aged. This method is based upon our findings that cEC decayed at higher rates than *Bacteroidales* DNA makers did. Many studies have reported such differential decay, but there were also reports that *Bacteroidales* DNA markers decaying at higher rates than cEC or that the two bacterial indicators decaying at similar rates (Walters and Field 2009; Dick et al. 2010; Jeanneau et al. 2012; Ahmed et al. 2014). Such variations can potentially confound the monitoring method being proposed in this study. Nonetheless, the results in this study provide a foundation for further investigation and validation of method under different settings that involve other types of water (e.g. freshwater and estuarine) and fecal sources under a wider range of environmental conditions in other climate regions (e.g. temperate, tropical) in the lab and in the field. Ultimately, a modeling approach that incorporates hydrological parameters, prevailing environmental conditions, and the decay rates of *Bacteroidales* DNA markers and cEC would be required to implement the tracking of the relative concentrations of the two bacterial indicator for the differentiation between fresh and aged pollution.

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Supporting Information

Materials and methods. Supporting information for microcosm conditions, DNA extraction, qPCR conditions, performance characteristics of qPCR assays, alleviating the effects of PCR inhibitors present in DNA samples.

Table S1. Primers for qPCR enumeration of the 16S rRNA gene markers of total (AllBac), human-specific (HF) and cow-specific (CF) *Bacteroidales* as well as the cytochrome b gene of the barnacle *Balanus amphitrite* serving as DNA recovery control (DRC).

Table S2. Performance characteristics of qPCR assays.

Figure legends:

Figure 1. Trial 1 of the microcosm conducted in the current study under sunlight with raw sewage spiked to untreated seawater. Data shown in (a) and (b) are changes in mean concentrations ± 1 S. D. ($n = 3$) of the 16S rRNA gene markers of total (AllBac) and human-associated (HF) *Bacteroidales*, and culturable *E. coli* (cEC) during the course of the experiment. Curve fitting was determined using the GInaFit software. The dotted line in (b) indicates the reduction of cEC concentration by 99 % of the initial. The mean concentrations of AllBac and HF relative to that of cEC at each time point are $\log_{10}$ -transformed and shown as AllBac:cEC and HF:cEC in (c) with the vertical dotted line indicating the time required for cEC to attain 99 % reduction of the initial concentration.

Figure 2. Trial 2 of the microcosm conducted in the current study under sunlight with raw sewage spiked to untreated seawater. Data shown in (a) and (b) are changes in mean concentrations ± 1 S. D. ($n = 3$) of the 16S rRNA gene markers of total (AllBac) and human-associated (HF) *Bacteroidales*, and culturable *E. coli* (cEC) during the course of the experiment. Curve fitting was determined using the GInaFit software. The dotted line in (b) indicates the reduction of cEC concentration by 99 % of the initial. The mean concentrations of AllBac and HF relative to that of cEC at each time point are $\log_{10}$ -transformed and shown as AllBac:cEC and HF:cEC in (c) with the vertical dotted line indicating the time required for cEC to attain 99 % reduction of the initial concentration.

Figure 3. The re-analysis of the data of the microcosm experiment published in Liu et al.

(2015). The microcosms containing autoclaved seawater spiked with cow feces were incubated under darkness, and were purged with air and N₂ to maintain normoxic and hypoxic conditions, respectively. The experiments were conducted in two trials with different batches of seawater and cow feces. Data shown are the log₁₀-transformed mean concentrations (n = 3) of the 16S rRNA gene markers of total (AllBac) and cow-associated (CF) *Bacteroidales* relative to that of culturable *E. coli* (i.e. AllBac:cEC and CF:cEC) at each time point. The vertical dotted lines indicate the time required for cEC to attain 99 % reduction of the initial concentration in each microcosm.

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Table 1. Conditions of microcosm experiments. The results in this study are derived from the data of an experiment (one microcosm) that was conducted during the current study, and the re-analysis of the data of an experiment (two microcosms) that was reported in Liu et al. (2015). All microcosms were conducted in two trials.

Microcosm No.	Conditions					Monitoring schedule	Reference
	Fecal source	Seawater	Light	Oxygen	Water temp. (°C)		
1	Raw sewage	Untreated	Sunlight [*]	Normoxic [§]	26	0, 2, 4, 6, 12, and 24 h; 1.5, 2, 3, and 4 d	Current study
2	Cow	Filtered & autoclaved	Absent [†]	Normoxic [§]	26	0, 1, 2, 4, 6, 8 and 11 d	Liu et al. (2015)
3				Hypoxic [¶]			

^{*}Sunlight through a 50 µm-thick Dupont FEP-Teflon film

[†]Wrapped in black plastic lining

[§]Dissolved oxygen = ~ 8 µg mL⁻¹

[¶]Dissolved oxygen = ~ 1 µg mL⁻¹

Table 2. Decay rates of culturable *E. coli* (cEC), and the 16S rRNA gene markers of general (AllBac), human-associated (HF) and cow-associated (CF) *Bacteroidales* in seawater microcosms. Each microcosm was conducted in two trials. Data were fitted to decay models using the Glnafit software (B: biphasic; L: log-linear; S: shoulder; T: tail). Data shown for biphasic decay include decay rate constants for the first (k_1) and second (k_2) phase (day^{-1}). Log-linear decay involves only one decay constant (k_1). For decay curves having a “shoulder” preceding the decay, “shoulder length” (SL) is provided to indicate the length of the period (day). The time required for a bacterial indicator to attain 99 % reduction of the initial concentration is indicated by T_{99} (day).

Microcosm No.	Conditions	Trial	cEC					AllBac					HF/CF				
			Model	SL	k_1	k_2	T_{99}	Model	SL	k_1	k_2	T_{99}	Model	SL	k_1	k_2	T_{99}
1	Normoxic + Sunlight	1	L-T	-	4.08	-	1.14	S-L-T	1.50	47.76	-	1.62	S-L-T	1.52	16.8	-	1.81
		2	B	-	23.04	1.20	1.13	S-L	3.48	7.92	-	4.07	S-L	3.25	6.48	-	3.96
2*	Normoxic + Darkness	1	B	-	1.19	<0.01	2.96	B	-	0.73	<0.01	-	B	-	0.89	<0.01	28.62
		2	B	-	1.09	<0.01	3.68	B	-	0.59	<0.01	51.28	L	-	0.40	-	11.54
3*	Hypoxic + Darkness	1	B	-	1.59	0.14	4.07	L	-	0.19	-	24.26	L	-	0.16	-	200.23
		2	B	-	1.27	<0.01	4.29	L	-	0.19	-	13.42	L	-	0.04	-	113.08

*the values shown were re-calculated from published data in Liu et al. (2015), with the permission from Applied Microbiology and Biotechnology.

Figure 1. Trial 1 of the microcosm conducted in the current study under sunlight with raw sewage spiked to untreated seawater. Data shown in (a) and (b) are changes in mean concentrations $\pm$ 1 S. D. ($n = 3$) of the 16S rRNA gene markers of total (AllBac) and human-associated (HF) *Bacteroidales*, and culturable *E. coli* (cEC) during the course of the experiment. Curve fitting was determined using the GInafit software. The dotted line in (b) indicates the reduction of cEC concentration by 99 % of the initial. The mean concentrations of AllBac and HF relative to that of cEC at each time point are log₁₀-transformed and shown as AllBac:cEC and HF:cEC in (c) with the vertical dotted line indicating the time required for cEC to attain 99 % reduction of the initial concentration.

253x225mm (300 x 300 DPI)

Figure 2. Trial 2 of the microcosm conducted in the current study under sunlight with raw sewage spiked to untreated seawater. Data shown in (a) and (b) are changes in mean concentrations ± 1 S. D. ($n = 3$) of the 16S rRNA gene markers of total (AllBac) and human-associated (HF) *Bacteroidales*, and culturable *E. coli* (cEC) during the course of the experiment. Curve fitting was determined using the GInaFit software. The dotted line in (b) indicates the reduction of cEC concentration by 99 % of the initial. The mean concentrations of AllBac and HF relative to that of cEC at each time point are log₁₀-transformed and shown as AllBac:cEC and HF:cEC in (c) with the vertical dotted line indicating the time required for cEC to attain 99 % reduction of the initial concentration.

252x218mm (300 x 300 DPI)

Figure 3. The re-analysis of the data of the microcosm experiment published in Liu et al. (2015). The microcosms containing autoclaved seawater spiked with cow feces were incubated under darkness, and were purged with air and N₂ to maintain normoxic and hypoxic conditions, respectively. The experiments were conducted in two trials with different batches of seawater and cow feces. Data shown are the log₁₀-transformed mean concentrations (n = 3) of the 16S rRNA gene markers of total (AllBac) and cow-associated (CF) *Bacteroidales* relative to that of culturable *E. coli* (i.e. AllBac:cEC and CF:cEC) at each time point. The vertical dotted lines indicate the time required for cEC to attain 99 % reduction of the initial concentration in each microcosm.

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Supporting Information

Material and Methods

Microcosm conditions

In the current study, we conducted an outdoor experiment with two trials of a microcosm that was comprised of a 27 L glass aquarium filled with 19 L untreated seawater and 1 L raw sewage. The aquarium was cleaned (household bleach) and rinsed (autoclaved double-distilled water) prior to the filling of seawater freshly collected off the coast of Hong Kong (Table 1). The seawater had no detectable *Bacteroidales* DNA marker or cEC as determined using the methods described in the main text of the manuscript. Upon the adding of the raw sewage to the seawater at 9 am of the day that the experiment began (0 h), the microcosm was placed outdoor without obstruction to sunlight, receiving on average 13.49 MJm^{-2} of global solar radiation each day with daily maximum intensity of UVA ranging from 11.3 to 61.0 Wm^{-2} during the course of the experiment (Hong Kong Observatory). The total water level was 20 cm deep and had turbidity of 10.42 NTU in trial one of the experiment and 24.40 NTU in trial two after the addition of sewage, allowing sunlight to penetrate to the bottom of the microcosm. These turbidity values were within the range of the annual average of $7.5 - 29.5$ NTU reported for Hong Kong waters (HKEPD 2014).

Water temperature in the microcosm was maintained at ca. 26°C (typical for summer days in Hong Kong) using a run-through water jacket pumping from the sea surface. The microcosm was continuously aerated using $0.22 \mu\text{m}$ -filtered air to keep the seawater normoxic (dissolved oxygen concentration ca. $8 \mu\text{g ml}^{-1}$), amidst the respiration of the fecal community. The DO concentration fell within the range of $5 - 10 \mu\text{g ml}^{-1}$ typically observed for the surface waters in Hong Kong (HKEPD 2014). To reduce water evaporation, the microcosm was covered with a Dupont FEP-Teflon film ($50 \mu\text{m}$ thick) that was reported to allow the transmission of $> 96\%$ of

solar radiation (Teflon 1996). Water level and salinity in the microcosm was observed regularly. Water loss due to evaporation was replenished using autoclaved double-distilled water so as to maintain water salinity at 30 ± 1 ‰. Water pH in the microcosm varied between 7.7 and 8.0 during the experiment.

DNA extraction

Each water sample collected from the microcosms was divided into two equal volumes for the enumeration of cEC using CHROMagarTM ECC and *Bacteroidales* DNA gene markers in both viable and non-viable cells using SYBR[®] Green dye-based qPCR following the procedures described in Liu et al. (2015). The samples for qPCR were filtered through 0.22 µm polycarbonate membranes. Cells captured were lysed using lysozyme and achromopeptidase. Each cell lysate was spiked with a 345 bp PCR-amplified cytochrome b gene of the barnacle *Balanus amphitrite* (10^7 copies µl⁻¹; 10 µl) as DNA recovery control (DRC) (Gregoris et al. 2009). Total DNA was extracted using the phenol-chloroform method (Liu et al. 2010) and the final volume of the extract were 100 µl for each sample. The copy numbers of DRC and the general (AllBac) and human-specific (HF) *Bacteroidales* DNA markers in each DNA sample were quantified using previously reported qPCR assays (Table S1). All values of *Bacteroidales* DNA marker concentration reported in this manuscript were corrected using the recovery rate of DRC: Conc. of DNA marker detected / (Conc. of DRC detected / Conc. of DRC spiked).

qPCR conditions

All qPCR assays were performed in duplicate 20 µl reactions, each containing 4 µl of DNA and 200 nM of each primer in 1 X GoTaq[®] qPCR Master Mix (Promega). The thermal

cycles were: initial denaturation (95 °C) for 10 min, followed by 40 cycles of denaturation (95 °C) for 15 sec, and annealing and extension (60 °C for HF, CF, and DRC; 62 °C for AllBac) for 1 min. Standard curves were constructed using plasmids of each *Bacteroidales* DNA marker described below.

Performance characteristics of qPCR assays

Standards curves of Ct value vs. DNA marker copy number were constructed using serially diluted linearized plasmids of each marker. Eight and four standard curves were obtained for CF and HF, respectively, while twelve were obtained for AllBac and DRC. Three replicate reactions were used for each copy number of a DNA marker on a standard curve. As a measure of the variability of Ct values among replicates, the coefficient of variations (CV) was calculated using the mean and standard deviation of the Ct values of the three replicates ($CV = \text{standard deviation} / \text{mean} \times 100 \%$). The mean of all CV values obtained for the standard curves of each DNA marker was $< 0.81 \%$ (Table S1).

The dynamic range of the standard curves of each DNA marker was $2 \times 10^1 - 2 \times 10^6$ copy per reaction for AllBac and CF, $4 - 4 \times 10^5$ copy per reaction for HF, and $2 \times 10^2 - 2 \times 10^7$ copy per reaction for DRC. Within their dynamic range, the linearity ($R^2 = 0.988 - 1.000$) and amplification efficiency ($10^{(-1/\text{slope})} - 1 = 90.0 - 101.8 \%$) of the standard curves fell within the acceptable range of 0.985 - 1.000 and 90 - 110 %, respectively (Eurogentec) (Table S1).

PCR inhibitors present in DNA samples

All DNA samples were diluted so as to alleviate the effects of PCR inhibitors. The level of dilution required was determined on the basis of the difference in the Ct values between two successive 10-fold dilutions of a sample (ΔCt). Inhibition was considered significant when ΔCt

73 was < 3 (Dick et al. 2010). Ten-fold dilution (0.1x) was applied to all samples in study.

Draft

74 Table S1. Primers for qPCR enumeration of the 16S rRNA gene markers of general (AllBac), human-specific (HF) and cow-specific
 75 (CF) *Bacteroidales* as well as the cytochrome b gene of the barnacle *Balanus amphitrite* serving as DNA recovery control (DRC).
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Marker	Organism (gene)	Primer	Sequence (5' - 3')	Annealing temperature(°C)	Amplicon size (bp)	Reference
AllBac	All <i>Bacteroidales</i> (16S rRNA)	AllBac-296F AllBac-412R	GAGAGGAAGGTCCCCCAC CGCTACTTGGCTGGTTCAG	62	116	Layton et al. 2006
HF	Human-specific <i>Bacteroidales</i> (16S rRNA)	HF183F Bac265R	ATCATGAGTTCACATGTCCG TACCCCGCCTACTATCTAATG	60	82	Seurinck et al. 2005
CF	Cow-specific <i>Bacteroidales</i> (16S rRNA)	CF128F CF302R	CCAACYTTCCCGWTACTC TCAGAACCCCTAGACATCG	60	174	Bernhard and Field 2000 Liu et al. 2015
DRC	Barnacle <i>Balanus amphitrite</i> (cytochrome b)	CY-F CY-R	GGACACTGCATGCTAATGGA AGGCAGCAGCCATAGTCAAG	60	144	Gregoris et al. 2009

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79 Table S2. Performance characteristics of qPCR assays. Four standard curves were obtained for HF, eight were obtained for CF whereas
80 twelve were obtained for AllBac and DRC. All of the curves of the same DNA marker resulted in the same dynamic range. The values
81 of linearity (R^2), slope and amplification efficiency were determined for each curve using data obtained within the dynamic range; data
82 shown for each marker are the range and mean (in parenthesis) of all curves. The mean efficient of variation (CV) for each marker was
83 calculated using Ct values from all curves.
84

	DNA Marker			
	AllBac	CF	HF	DRC
No. of standard curves	12	8	4	12
Mean CV (%)	0.63	0.41	0.81	0.53
Dynamic range (copies reaction ⁻¹)	$2 \times 10^1 - 2 \times 10^6$		$4 - 4 \times 10^5$	$2 \times 10^2 - 2 \times 10^7$
R^2	0.987 – 0.998 (0.996)	0.999 – 1.000 (0.999)	0.994 – 0.999 (0.996)	0.994 – 0.999 (0.998)
Slope (-ve values)	3.30 – 3.59 (3.46)	3.20 – 3.59 (3.58)	3.34 – 3.51 (3.40)	3.32 – 3.58 (3.43)
Amplification efficiency (%)	90.0 – 101.8 (94.6)	90.0 – 90.3 (90.1)	92.9 – 99.1 (97.1)	90.2 – 100.0 (95.6)

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References for supporting materials

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