Biphasic Decay Kinetics of Fecal Bacteria in Surface Water
Not a Density Effect

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Abstract: The decay of fecal bacteria in surface water often follows a biphasic pattern with the apparent first-order rate constant relatively high during a first phase and lower in a second one. This could be the result of population heterogeneity in resistance due to various mechanisms (different strains, genetically or nongenetically differentiated cells, different growth or cell cycle stage, clumping, hardening), or the specific decay rate could be directly or indirectly affected by the cell density (e.g., quorum sensing). All these mechanisms can theoretically produce a biphasic decay pattern and are consistent with the literature. However, they are fundamentally different and lead to different behavior of mechanistic total maximum daily load models, so identifying the correct mechanism is important. This technical note presents the results of a study aimed at determining if a density effect is involved. Laboratory decay experiments on the pure strain *Escherichia coli* cells in phosphate buffer were conducted over a range of initial densities. The results show that the rate constant changes after a certain time, rather than at a certain density, which is inconsistent with a density effect. As the experiments were performed with a pure strain, the resistant fraction cannot be attributed to a different strain. Further research is needed to identify the mechanism responsible for the population heterogeneity.

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Introduction

Pathogen pollution of surface waters is one of the most important environmental problems faced by society. The U.S. EPA (2004) estimates that about 13% of streams and 17% of estuaries are impaired by pathogens. In 2006, 32% of beaches had at least one advisory due to the presence of pathogen indicators (U.S. EPA 2007). Pathogens and their indicators enter surface waters from a number of sources, including sanitary, storm and combined sewer discharges, direct runoff, and others (e.g., birds, boats). After discharge, the density generally decreases due to a number of processes (Thomann and Mueller 1987; Chapra 1997; Boehm et al. 2005; Liu et al. 2006), including dilution, dispersion, settling, predation, and decay (also called die-off). However, densities can also increase due to growth in soil or sediments (e.g., Solo-Gabriele et al. 2000). Decay varies depending on the type of pathogen or indicator and numerous environmental factors, including temperature, salinity, solar irradiance, and various water chemistry parameters. Quantitatively estimating the density decrease is important, because it controls the assimilative capacity of the receiving water, a critical component of a total maximum daily load analysis.

This technical note is concerned primarily with the decay process, which has first to be defined. Fecal bacteria are traditionally enumerated by culturing, which strictly speaking does not measure “alive” but “culturable” cells. There is a difference, because many bacteria, including *E. coli*, can exist in a “viable but not culturable” (VBNC) or dormant state (Smith et al. 1994; Winfield and Groisman 2003). So quantifying density as culturable can be misleading, especially in the context of “die-off” or “decay.” Also, because VBNC cells can be infective (Colwell et al. 1996), this may not be the most relevant measure from a public health risk perspective. However, it is consistent with past decay studies and current monitoring practices. Therefore, alive is defined here as culturable and decay is defined as loss of culturability. The decay process is typically modeled using a first-order kinetic model, originally proposed by Chick (1908, 1910), with the rate constant a function of environmental variables (Thomann and Mueller 1987; Chapra 1997). However, numerous studies, as reviewed in the following paragraph, have found a biphasic pattern, consisting of a first period with relatively high apparent first-order rate constant and a second period with a lower constant.

Frost and Streeter (1924) analyzed field data from the Ohio River during summer and winter conditions and concluded that decay of fecal bacteria, including *E. coli*, was biphasic (Phelps 1944; Velz 1970). Orlob (1956) reviewed data from 13 experiments on the decay of *E. coli*, coliforms, and *E. typhosa* under various temperatures and experimental conditions in sea-
water, about half of which exhibited a biphasic pattern. Geldreich and Kenner (1969) performed laboratory decay experiments in filtered storm water at two temperatures. The bacteria studied included *Streptococcus faecalis*, *Streptococcus faecalis* var. *lignifaciens*, *Streptococcus bovis*, *Aerobacter aerogenes*, fecal coliform (FC), and *Salmonella typhimurium*. All of the bacteria showed a biphasic pattern in at least one of the two experiments. Dutka and Kwan (1980) studied decay of *E. coli*, *Streptococcus faecalis* and *Salmonella thompson* using in situ membrane diffusion chambers. All experiments exhibited a clear biphasic pattern. Fujioka et al. (1981) studied the decay of FC and fecal streptococci (FS) in river water, seawater, and phosphate buffer in dark and sunlight conditions. Decay of FC and FS in dark seawater and FC in sunlight was biphasic, but the other experiments did not exhibit this pattern. Smith et al. (1994) studied decay of *E. coli*, *Enterococcus faecalis*, *Salmonella typhimurium*, and *Yersinia enterocolitica* in diffusion chambers exposed to seawater. Culturable cells were quantified using plate counts on two different media. *E. coli* did not show a biphasic pattern, but data from at least one of the two enumeration methods showed a biphasic pattern for the other bacteria. Munro et al. (1995) studied the decay of *E. coli* and *Salmonella typhimurium* in filtered sterilized seawater. Experiments were conducted with wild type and mutants (related to stress resistance) from cultures at various growth conditions (exponential, stationary). Several of their experiments exhibited a biphasic pattern. Bogosian et al. (1996) studied the decay of *E. coli* in sterile and nonsterile river water and sterile seawater at different temperatures. Culturable cells were quantified using plate counts and most-probable number methods. All experiments showed a biphasic pattern. Medema et al. (1997) studied decay of *E. coli*, fecal enterococci, and *Clostridium perfringens* in sterilized and natural river water at two different temperatures. Two experiments, *E. coli* and *C. perfringens* at 15°C in natural river water, exhibited a biphasic pattern. Easton et al. (2005) followed the densities of total coliforms, *E. coli*, and enterococci from raw sanitary sewage and pure strain *E. coli* O157:H7 in laboratory diffusion chambers immersed in river water at two temperatures. All experiments showed a biphasic pattern. These studies demonstrate that biphasic decay is a common phenomenon. However, it is not always observed. Several of the above-referenced studies did not show an obvious biphasic pattern in all experiments. There are also numerous decay studies where a biphasic pattern is not obvious in any of the experiments (e.g., Xu et al. 1982; Rhodes and Kator 1988).

Several mechanisms may be responsible for the biphasic decay pattern. There could be two subpopulations with different resistances to decay (Frost and Streeter 1924). This could simply be due to the presence of different strains. Also, as mutant populations of *E. coli* can emerge in a matter of days in stationary phase (Zambrano et al. 1993; Finkel 2006), the resistant fraction could be due to recent mutation. Another possibility is nongenetic cell differentiation. For death by antibiotics, the presence of a small fraction of “persistor” cells leads to a biphasic decay pattern (Balaban et al. 2004; Lewis 2007). The growth condition (e.g., exponential versus stationary phase) can have a significant effect on the decay rate (Gauthier et al. 1988; Munro et al. 1995), and population heterogeneity in this factor at the time of inoculation could cause heterogeneity in the decay rate. Intrapopulation variability in other factors, like cell cycle stage, or clumping may also play a role. The resistant fraction could also develop after inoculation. For inactivation of viruses by chemical agents, a “hardening” was postulated as leading to a reduction in the decay rate over the course of the experiment (Gard 1960). Another possibility is a density effect that causes a change in resistance once the cells reach a certain threshold density. Although this “density effect” hypothesis has existed for many years (e.g., Phelps 1944), it has received renewed attention (e.g., Easton et al. 2005) with the recent realization of the ubiquity of quorum sensing in bacteria, including *E. coli* and *S. typhimurium* (Surette and Bassler 1998; Miller and Bassler 2001). Also, in a related phenomenon, the deliberate inactivation of bacteria using disinfectants, a biphasic pattern has been observed (Chick 1908, 1910; Haas and Morrison 1981) and a density effect has recently been demonstrated (Kaymak 2003; Li 2004). Theoretically, all of these mechanisms can generate a biphasic decay pattern, and they can generate a monophasic pattern, when the resistant fraction is negligible or the density is above or below the threshold value. Therefore, these mechanisms are consistent with the above reviewed literature. However, the underlying models are fundamentally different and produce different results when incorporated into mechanistic water quality models. Consider the simplified case of a time-variable discharge into a lake, and biphasic decay with a very high rate constant for the first phase and a negligible one for the second one. The resistant fraction model would predict that the receiving water density will always be proportional to the load, because the resistant fraction (which is all that remains after rapid decay of the labile one) is proportional to the discharge. The density effect model would predict a constant density, because decay stops once a threshold value is reached (which happens rapidly due to the high rate constant for the first phase), regardless of the external load. Understanding which mechanism is responsible for the biphasic decay pattern is therefore of fundamental importance from an environmental engineering perspective.

Here the writers are specifically concerned with the role of a density effect. As all mechanisms can theoretically generate a biphasic decay pattern, the previous studies cannot be used to identify the responsible mechanism. A number of laboratory decay experiments with pure strain *E. coli* in phosphate buffer were performed. The key to isolating the density effect is to conduct experiments over a range of initial densities, as illustrated in Fig. 1. If the apparent first-order decay rate constant (the slope) changes at the same time (and different densities) for all experiments, the biphasic pattern is consistent with population heterogeneity [Fig. 1(a)]. If the rate constant changes at the same density (and different time) for all experiments, the biphasic pattern is consistent with a density effect [Fig. 1(b)].

**Materials and Methods**

Pure strain *E. coli* (ampicillin-resistant, ATCC 31698) were cultured in Luria-Bertani media with 50 μg/mL ampicillin at 37°C. Cultures were grown in static falcon tubes for 20 h to stationary phase (OD_{595}=1.0) (OD=optical density) with an approximate density of 10^{10} colony-forming units (cfu) per 100 mL. Cultures were vortexed to reduce clumping and several treatments were prepared with various target initial *E. coli* densities ([C1]₀) in sterile phosphate buffer solution (PBS) (EPA method 1603, U.S. EPA 2002). Cells were not washed. The experiments were done with an ampicillin-resistant laboratory strain and PBS, rather than a recent isolate and natural water. This was done to simplify the protocol, control the experiment, and eliminate other factors (e.g., contamination, changes in water quality). The literature indicates that the biphasic decay pattern is common across
strains and experimental conditions, which suggests it is a fundamental property of fecal bacteria that can be studied with any strain and water type. Duplicate treatments with \( C_T(0) \) of approximately \( 10^6, 10^5, \) and \( 10^4 \) cfu per 100 mL were used. Experiments were done in series with new cultures for each duplicate treatment. The treatments consisted of 1 L, kept in 2 L culture flasks with cotton-gauze stoppers on stir plates at 500 rpm, 20°C, and continuous ambient fluorescent laboratory light. Density measurements were made on a daily basis for 5–7 days, using membrane filtration (0.45 \( \mu \)m sterile filter paper) using the patented Coliscan membrane filter (MF) defined substrate medium (Micrology Laboratories, Goshen, Ind.). The Coliscan MF medium was selected, because it enumerates \( E. coli \) and other bacteria (different colony colors) and thus checks for contamination, which was not detected. For each treatment and time point measurement, three volumes were filtered covering a range of densities. The densities from the acceptable filters (20–80 cfu per filter) were averaged or the one closest to the acceptable range was used. Ninety five percent of the measurements had at least one acceptable filter.

### Results and Discussion

All experiments show a clear biphasic pattern (Fig. 2). Densities decrease rapidly for about 2 days and then increase slightly for the remainder of the experiments (discussed further in the following). The important feature is that the apparent first-order rate constant (the slope in Fig. 2) decreases at the same time for all experiments. This is clearly consistent with population heterogeneity in resistance. If the rate constant would change at the same density, the results would be consistent with a density effect. In other words, the results resemble the idealized pattern in panel (a) of Fig. 1, rather than panel (b). Our experiments were limited to a single strain of \( E. coli \) and experimental conditions (temperature, light, water chemistry). However, it is hypothesized that the biphasic decay pattern observed for other fecal bacteria and conditions is also due to population heterogeneity and not a density effect.

A simple resistant fraction model (see the Appendix) can reproduce the general pattern of the experiments. The parameters (Table 1), the initial resistant fraction \( [f_R(0)] \), and the first-order decay rate constants for the labile \( (k_L, \text{day}^{-1}) \) and resistant \( (k_R, \text{day}^{-1}) \) fractions, were estimated by minimizing the sum of the error squares (in log space) using the Microsoft Excel Solver optimization plug-in. The estimated values are within the range of the literature (Table 1). This resistant fraction biphasic decay model is readily implemented in operational water quality models.

More experimental work is needed to identify the mechanism responsible for the population heterogeneity underlying the biphasic decay pattern. However, the writers’ results shed some light on the question. First, because experiments were conducted with a pure strain, the resistant fraction is not a different strain. Second, the experiments suggest that the resistant fraction is not just resistant to decay, but also is multiplying (the negative value for \( k_R \) is significant, see Table 1). Some cell division may occur in the absence of growth by production of smaller or “dwarf” cells (e.g., Roszak and Colwell 1987). Another possibility is that they are mutants growing on nutrients released by dead cells, like stationary phase mutants in spent media (Zambrano et al. 1993; Finkel 2006).

### Appendix. Resistant Fraction Biphasic Decay Model

Biphasic decay can be modeled by splitting the total \( (T) \) bacteria population into labile \( (L) \) and resistant \( (R) \) fractions

\[
C_T(t) = C_L(t) + C_R(t)
\]

where \( C \) (cfu/100 mL) is the bacteria density with subscripts \( T, L, \) and \( R \) indicating total, labile, and resistant. Decay for both frac-
tions is assumed to follow first-order decay kinetics. Without other kinetic (e.g., predation) and transport (advection, dispersion, settling) processes or external sources, the mass balance equation for the labile fraction is

\[ \frac{dC_L(t)}{dt} = -k_L C_L(t) \]  

(2)

where \( k_L \) (day\(^{-1}\)) is the first-order decay rate constant for the labile fraction. The equation for the resistant fraction has the same form. Eq. (2) and the corresponding equation for the resistant fraction can be solved analytically and combined

\[ C_L(t) = C_L(0)[(1 - f_R(0))\exp(-k_L t) + f_R(0)\exp(-k_R t)] \]  

(3)

where \( f_R(0) \) is the initial resistant fraction. Eq. (3) constitutes the model used for the laboratory decay experiments. Biphasic decay can also be simulated using existing operational water quality models, like WASP (Wool et al. 2001), RCA (HydroQual 2004), or QUAL2K (Chapra and Pelletier 2003), by using different state variables corresponding to the different fractions or separate model runs for each fraction (if the model only supports one state variable).

References


