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Escherichia coli as a microbial indicator of fecal contamination in soils and surface waters: roles of environmental biotic and abiotic factors in modulating its occurrence and numbers.

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ABSTRACT

Recovery of bacterial indicators, such as *Escherichia coli*, from surface water is generally considered as the litmus test for its relatively recent contamination from fecal sources. Their presence, and numbers above a threshold defined by regulatory agency(ies), constitutes the basis of risk evaluation, communication, and management for human exposure to fecal pollution in recreational waters. It is therefore important to understand how their presence and numbers can be affected by environmental variables other than weather-related or other temporal and spatial variations in fecally-contaminated inputs.

Although rainfall is often correlated with higher numbers of bacteria in surface waters, to ascribe an observed increase in contamination levels from non-point sources solely to hydrological transport of microorganisms in increased land run-off is simplistic. Precipitation is only one environmental variable whereas the detectable numbers of bacterial indicators in surface waters are likely to be controlled by a plethora of physical, chemical and biological variables that are confounded in modulating the nutritional and physiological status of the bacteria and their ability to grow on conventional laboratory media.

Most routinely used detection methods for indicator bacteria are based on their isolation and quantitation in culture, often using growth media that contain enzyme substrates to give coloured or fluorescent colonies of the target organism(s). There is increasing evidence that many bacteria, including *E. coli,* may enter a form of dormancy or 'survival mode' when under stress of varying kinds. In this viable-but-not-culturable (VBNC) state the bacteria have an altered physiological status that could affect our ability to detect an undefined but potentially significant proportion of those present through routine culture assays. Under more favourable soil and water conditions such bacteria may 'recover' or regrow and resume the ability to be readily cultured. However, it is also possible to detect and even quantitate indicator bacteria by molecular methods without knowing their physiological status. In pure culture, or when suitably experimentally-labeled, one can also enumerate them using microscopy, flow cytometry or related techniques. Each method has limitations and advantages in the context of studying fate or behaviour of indicator bacteria in surface waters.

This chapter explores the survival and persistence of cultivable *E. coli* in surface waters, the interplay between *E. coli* as a member of the bacterioplankton and biofilms and its immediate neighbours, predators and phages. It also examines the extrinsic physicochemical environmental variables as they may influence numbers of indicator bacteria present, the intrinsic bacterial responses or adaptations that may permit their survival, and effects on the predator-prey relationship. The methods used to detect indicator bacteria are addressed together with the limitations of these methods and the consequences for the quality of the data obtained. An attempt has been made to separate the discussion into logical sections but there is inevitably some overlap that may require the reader's attention, and is necessary to avoid duplication. Furthermore, there are topics that are mentioned but cannot be addressed in detail even though they are relevant: an example is the cell-cell communication that occurs among members of a bacterial community and its interruption. This is considered outside the general scope because too little is known about its operation in situ in natural microbial assemblages. This chapter does not review the sources of *E. coli* to the soil or aquatic environments, though each of these may have somewhat different effects, nor does it address microbial transport; both of these issues are critical to risk analysis and risk management.

INTRODUCTION

Soils and waters are considered secondary habitats for enteric bacteria such as *E. coli* (Walk et al., 2007). Failure to recognize that *E. coli* can frequently survive and occasionally grow in such environments under appropriate conditions has lead to the common assumption that this indicator bacterium only decays following its introduction to soils and receiving waters. Often it is assumed that the *E. coli* decay rate is a constant, and consistent among environments. This is clearly untrue for different locations, and significant temporal differences in decay rates as well as non-linearity can be observed in environments that are subject to variation of conditions and inputs such as rivers (Springthorpe et al., 1993). Detection by culture of contaminating *E. coli* can occur for as little as 24-48 hours (Springthorpe, unpublished in situ observations in a storm water pond) or as long as 3-6 months or more (LeJeune et al., 2001; Davies et al., 1995; Cook and Bolster, 2007; Springthorpe et al., unpublished). While the numbers of detectable *E. coli* cells almost invariably decline in temperate climates following discharge, this decline is modulated by a host of extrinsic (environmental) factors and intrinsic bacterial responses and adaptations that may lead to persistence of a small fraction of the contaminating organisms. If such survivors can adapt to their new milieu, they also have the opportunity to reproduce if the right nutrients and conditions arise (Ferguson et al., 1996; Unc et al., 2006). One of the key difficulties in understanding this phenomenon is that *de novo* growth and recovery from a quiescent non-active state are confounded.

Survival of allochthonous bacteria like *E. coli* in the environment is affected by many biotic and abiotic factors. Biotic influences include grazing by protozoa, predation by *Bdellovibrio* and like organisms, virus-induced cell lysis and autolysis, interactions with other biota and intrinsic stress responses triggered by nutrient depletion or other stimuli. Abiotic factors in natural environments that play important roles include sunlight intensity, ambient temperature, pH and redox levels, lack of essential micronutrients and the presence of toxic/inhibitory chemicals as well as particulates. Enteric bacteria like *E. coli* are best adapted to grow in anaerobic or microaerophilic conditions with high nutrient concentration, relatively high temperature and a neutral or slightly alkaline environment. In drinking water systems, a more alkaline pH range, of about 8, was found to enhance survival (Grandjean et al., 2005).

There are some indications that the diversity of *E. coli* in a water source as measured by metabolic (Ahmed et al., 2006b), antibiotic resistance (Whitlock et al., 2002) or genotypic fingerprinting (Johnson et al., 2004; Lu et al., 2004) may be associated with the origin of contamination. Extent of survival in soils may also be associated with the origin of contamination (Unc and Goss, 2006).

Although the presence of *E. coli* in environmental samples in temperate climates is still considered to indicate relatively recent fecal contamination, there is a growing body of evidence that suggests some persistence and replication of *E. coli* in the secondary environment (Springthorpe et al., 1993; Unc et al., 2006; Beversdorf et al., 2007; Williams et al., 2007). The ubiquity and persistence of cultivable *E. coli* in tropical climates is now well recognized (Byappanahalli and Fujioka, 2004).

There are many different biotypes of *E. coli* and there is no reason to assume that they will all behave identically when faced with the same set of conditions although certain properties and responses appear to be highly conserved (Ihssen et al., 2007). Indeed data on extensive libraries of isolates suggest that the range of those found in rivers and beaches might differ significantly from those found in the contamination sources (McLellan, 2004). There are clear indications that some strains persist longer than others (Anderson et al., 2005). Are the strains that persist and replicate in the environment a specialized subset, or do individual strains possess specific survival skills independent of their subgroup? Which genes promote survival? How do different strains adapt/evolve in the secondary environment? Since survival is a prerequisite for transmission/acquisition, one could perhaps argue that those strains that are commensals rather than outright pathogens are likely to be more numerous and therefore may be those that survive the best in the secondary environment. There is not a great deal of evidence to support this but a recent study (Walk et al., 2007), that characterized *E. coli* strains from freshwater beaches of the great lakes basin, based on a PCR phylogrouping (Clermont et al., 2000), showed that many of the persistent strains belonged to the ECOR phylogroup B1. However, some isolates came from each of the phylogroups. In general, ECOR phylogroups A and B1 are more commonly considered to be commensals

whereas the pathogenic strains are more likely to be associated with phylogroups B2 and D (Clermont et al., 2000).
In water environments, there is a significant variability in the environmental survival capability of different strains of *E. coli* (Gordon et al., 2002), and therefore the instability of genetic fingerprints of fecal sources (Lu et al., 2005), or even for the same strain over time (Lu et al., 2004). Clearly more studies are needed to determine if these observations can be generalized and to determine which genotypes confer an adaptive advantage to the naturalized *E. coli* and allow them to persist and perhaps replicate. An alternate view could suggest that certain pathogenic strains may also be capable of survival, and this may be particularly true for O157:H7 isolates in some cases (LeJeune et al., 2001; Ogden et al., 2002; Williams et al., 2007).
While many of the major factors that contribute to the survival or decline of indicator bacteria in surface waters and soils are relatively well understood, there are likely to be additional influences that are less well understood and that are highly localized and significant to individual matrices and locales. Moreover, there is a tendency to consider each influence separately when the real predictor of *E. coli* presence and numbers is a continuously changing composite of many variables. It is obviously impossible to predict all these factors, and so what follows is a general discussion based on our current state of knowledge with a caveat that for any individual locale and time there is a degree of unpredictability that is a complex function of constituents of that environment.

REDUCTION OF *E. COLI* NUMBERS DUE TO EXTRINSIC BIOTIC PROCESSES

In natural soils, sediments and surface waters, where there are large populations of indigenous microorganisms and micro-invertebrates, and the extremes of unfavourable abiotic conditions do not tend to be present, biotic processes tend to be dominant among the influences decreasing *E. coli* numbers. However, the effects of physicochemical parameters are superimposed, and most likely to be important in surface layers or at the waters edge (UV, temperature), or in specific subsurface microenvironments and sediments (pH, redox). On the other hand, negative physicochemical influences are often exploited in engineered environments in attempts to reduce numbers of pathogens and indicator bacteria. Such actions reach beyond the intended targets and, while the negative stressors are maintained, may result not only in the dominance of the applied physicochemical factors but in the impairment or elimination of the potential biological controls.

Reduction of numbers by grazing/predation

The main biotic process by which autochthonous and allochthonous bacteria are eliminated is by predator grazing, most notably by protozoa (McCambridge and McMeekin, 1980; Barcina et al., 1997), with experimental clearance rates often in excess of 90% per day. Although some species or size specificity (Gonzalez et al., 1993; Pernthaler et al., 1996; Gonzalez, 1999) may exist, it is usual to consider predation of bacteria as non-specific (Sherr et al., 1989); there is no evidence of discrimination for or against bacterial indicators (Gonzalez, 1999). The main predators are free living protozoan ciliates (Epstein et al., 1992) and nanoflagellates (Novitsky, 1990; Barcina et al., 1992; Starink et al., 1996a), and the metazoan copepods, rotifers and cladocerans (Roff et al., 1995; Turner et al., 1998). Free living amoebae are also important grazers of bacterial biofilms (Huws et al., 2005). Predation by *Bdellovibrio* and *Bdellovibrio*-like organisms (Fratamico and Whiting, 1995; Rice et al., 1998, Davidov and Jurchevitch, 2004) can also be an important factor, especially on surficial biofilms (Williams et al., 1995a; Williams et al., 1995b), although their contribution to bacterivory is believed to be less significant than for protozoa (McCambridge and McMeekin, 1980). Interestingly, bdellovibrios have been reported to be able to acquire lipopolysaccharides apparently intact and unmodified from *E. coli* (Stein et al., 1992); this would reduce the energy requirements for synthesizing these macromolecules during growth of these bacterial predators. The importance of predation to *E. coli* decline is attested to by the many studies that have

inoculated *E. coli* into filtered water and found greatly extended persistence compared to unfiltered controls.

Estimates of the percentages of the protists that are active bacterivores ranged from 7-100% in aquatic systems with very different characteristics (Gonzalez, 1999). Particular types of predators may dominate in certain settings. For example, Sherr et al., (1989) found that ciliates were the major bacterial predator in tidal creek water whereas colourless flagellates were responsible in open estuary water. Wieltschnig et al. (1999) found that nanoflagellates were not the major bacterial predator in a eutrophic backwater environment and Starink et al. (1996a) indicate a more important role for heterotrophic nanoflagellates in the water column than in the sediment. It is clear that in some sediments and soils with fine grain size and low amounts of interstitial space there can be space limitations for protozoa that constrain the activities of certain types (Starink et al., 1996b; Wang et al., 2005). Furthermore, some sediment types may have fluctuating chemistries that may not be tolerated by certain protozoans (Starink et al., 1996b).

Seasonal and daily changes in bacterivory may be observed (Geller and Pinto, 1992; Marrase et al., 1992; Starink et al., 1996b; Jeppesen et al., 1997) with daily fluctuations being at least an order of magnitude smaller than seasonal fluctuations. Wikner and Hagstrom (1991) observed that although growth of bacteria was balanced by bacterivory on an annual basis, predation lagged bacterial growth in the spring, allowing bacteria to accumulate, whereas grazing exceeded bacterial growth in the winter. Interestingly, although water temperature clearly affected bacterivory, light cycles appeared to have no effect (Marrase et al., 1992), although it may be important in some cases (Berk et al., 1991). Therefore, the increased decline in bacterial numbers often observed during daylight hours may be more likely due to other factors such as exposure to UV and visible light as well as the potential for in situ generation of hydrogen peroxide and other oxidants.

In spite of the success of predation, predators and prey frequently co-exist and some of the population dynamics that allow this to happen have been discussed (Alexander, 1981). In particular, the ratios of bacteria to predators, the presence of predators of the bacteriovores or other means of protist decline may have significant effects and may change the microbial community composition (Starink et al., 1996b; Jürgens et al., 1999; Šimek et al., 2001; Šimek et al., 2002; Beardsley et al., 2003). In addition, autochthonous prey bacteria have developed a variety of strategies for escaping predation that have been reviewed recently (Matz and Kjelleberg, 2005; Pernthaler, 2005); fairly obvious approaches include extra-small or extra-large sizes (Pernthaler, 2005) swimming speed (Matz and Jürgens, 2005), 'clumping', colony formation and attaching to surfaces and particulates (Brettar and Hofle, 1992; Jin et al., 2004). The type of predation could alter the morphological appearance of bacterial biomass and 'protozoan resistant' morphotypes can be seen in ungrazed biomass (Jürgens et al., 1994; Jürgens et al., 1997). Allochthonous bacteria, freshly grown in manure or sewage and newly added in run off, including naïve *E. coli,* might be easily preyed on and thus their numbers may decline relatively fast (Chrzanowski and Šimek, 1990). Based on experimental introduction of labeled *E coli*, Arana et al. (2003) believe that differential effects of predation might reduce the importance of long-term persistence of *E. coli* cells added into aquatic systems. However, not all *E. coli* cells that enter water bodies are necessarily freshly grown or equivalent, and the effects of intrinsic bacterial stress responses may come into play (see below).

Less obvious strategies for bacteria to escape predation can include resistance to digestion and or toxin release. The former can lead to intracellular bacterial growth within the predator, and although this may turn the tables on the predator and eventually kill it as an intracellular pathogen, occasionally this evolves into an endosymbiotic relationship (Molmeret et al., 2005). The later may also deter or kill the predator depending on the location, type and severity of toxin release.

Interestingly, the importance of predation to the levels of indicator organisms is demonstrated by the more rapid loss of *E. coli* from drinking water systems using granular activated carbon (GAC) than those using nanofiltration. This was presumed to be due to the actively functioning ecosystem present in GAC systems (Sibille et al., 1998). While *Aeromonas hydrophila* and *Vibrio cholerae* (serogroup Non-O1) were able to colonize copepods in summer, other vibrios, *E. coli* and *Pseudomonas* could not (Dumontet et al., 1996). However, coliforms, including some *E. coli* can survive inside protozoa (King et al., 1988;

Barker et al., 1999). This suggests different ecological relationships and pairings between predator types and bacterial prey.
A further complication in this fascinating food web puzzle is the impact of predators on the organic nutrient pool in waters. Copepods, fed on bacteria can have a positive impact on bacterial production by increasing the soluble carbohydrates and free amino acid pools (Poulet et al., 1991; Peduzzi and Herndl, 1992; Pomeroy and Wiebe, 1993). Interestingly, this growth response to the eukaryotic predators was most pronounced in bacterial assemblages from oligotrophic waters rather than in the bacterioplankton of eutrophic systems.

Reduction of numbers by viral lysis and comparison with protozoan predation

Bacteria that survive predation might be eliminated from the environment by bacterial viruses, also known as phages (Brettar and Hofle, 1992). Phages of enteric bacteria are also introduced into the environment through input of fecal material; phages of coliforms such as *E. coli* are often termed coliphages. Although much is known about coliphages from laboratory studies, their impact on microbial ecology in natural waters and soils is much less well understood. In particular, the relative dominance or interplay of protozoa and phages in controlling bacterioplankton is of much interest but no real consensus. Some studies assess the impact of viral lysis as much less than that of protozoan predation (Pedrós-Alió et al., 2000; Choi et al., 2003) in the same setting, while others consider that both have significant impacts on the bacterial community (Jacquet et al., 2005). Both synergistic and antagonistic effects can be observed (Weinbauer et al., 2007). Observed results may depend on how and where experiments are performed, the feeding rates, relative densities, species present, etc. Predation is relatively non-specific whereas viral specificity is well recognized. Therefore, for a new microbial input into soils or receiving waters it is likely that the non-specific control mechanism can start immediately whereas one might anticipate that there might be a lag before the appropriate virus-bacterial contact is made and the virus concentration increases to a level where significant control can be effected. One laboratory-based study that compared these suggested that both sources of mortality were important with bacterivory predominant at days 2 and 4 and viral lysis predominant at days 7 and 9.
Although the evidence is sparse, bacterial mortality caused by viruses may be important under non steady-state conditions and when populations of the predatory eukaryotes crash. While both predation and viral lysis are to some extent density dependent, the rapid spread of viral infectivity is dependent on a sufficient 'pool' of susceptible bacteria in close proximity, such as may arise in spots where rapid growth of bacteria occurs on a locally available substrate. Thus, both sources of bacterial mortality may influence the ecology of bacterial indicator populations but perhaps in different fashions. Viral lysis of bacterial cells also enriches the pools of metabolizable compounds available near the lysed cells, but if these substrates are used to promote the growth of the susceptible cells then rapid lysis might ensue, and so the ultimate benefits of viral lysis may be for non-susceptible bacteria. Due to their large numbers in environmental samples, many viruses may become attached to bacterial cells whereas relatively few of them are infectious. Another factor to consider when examining viral lysis is the very large size discrepancy between viruses and their bacterial hosts. Given the potential effects of hydrodynamics on predation (see below), there are likely to be significant effects on the attachment of viruses to their bacterial hosts and also on viral dispersal.
Bacterial viruses are also subject to environmental degradation; they are inactivated faster at elevated temperatures that may be less deleterious to fecal bacteria but generally they survive better than enteric bacteria in natural waters (Duran et al., 2002; Allwood et al., 2003). They frequently exhibit different survival patterns than their bacterial hosts (John and Rose, 2004), but also different from each other under the same conditions (Brion et al., 2002). While it is inevitable that viruses might sometimes be digested intra- or extra-cellularly by other biota, the survival and infective capacity of these phages is also affected by abiotic environmental parameters.

Abiotic influences on predation and lysis

Given population and community adjustments to the normal variations in prevailing conditions, many systems may be in balance with regard to bacterial production and decay from all sources including predators. An important question that arises is whether all parts of this balanced system respond to perturbation from natural or anthropogenic disturbances in the same manner? There is some evidence to suggest that they do not. This is particularly important to understand in the case of *E. coli* because of its use in regulation as an indicator of fecal pollution inputs.

Salcher et al. (2007) studied the effects of phosphorus availability on predator-prey dynamics. They showed that while beta-proteobacteria were vulnerable to grazing, they were also able to be good competitors for phosphate, allowing for opportunistic growth strategies under limited grazer control and good nutrient availability, and perhaps compensating for their vulnerability to grazing. While *E. coli* was not specifically studied in this regard, it would be interesting to know if it responded competitively to phosphorous availability in this way. Cultivable *E. coli* has been shown to survive longer in the presence of phosphate in drinking water (Juhna et al., 2007). Perhaps a similarly efficient response to nutrients, in this case calcium ammonium nitrate, is responsible for the increased survival of cultivable *E. coli* in soils amended with the chemical fertilizer then in unamended soils (Estrada et al., 2004), though effects of the chemical in reducing predator numbers or predation cannot be ruled out.

Breitburg et al. (1999) have shown a variable response to trace element introduction; toxic effects were dependent on the timing of addition and the nutrient status (Breitburg et al., 1999) and might be affected by the presence of particulates (Boenigk et al., 2005). Lara et al. (2007) compared the flagellates and amoebae in soil polluted with polyaromatic hydrocarbons with an unpolluted control. They found higher numbers of cultivable protozoa in the polluted soil but reduced species diversity; certain types, including Acanthamoeba dominated. Depending on the species, the environmental conditions and the types and magnitudes of stressors, the presence of multiple stressors may increase or dampen the temporal and spatial variability seen in aquatic systems. In addition to anthropogenically introduced toxins, localized effects can be expected from a variety of toxic metabolites, including antibiotics. Do any such effects alter the survival of *E. coli*, either directly or through effects on predation or lysis? Probably they do, but to declare them as significant contributors to *E. coli* survival or decline in soils and waters is not supported by significant in situ evidence.

Physical perturbations through ‘small-scale’ turbulence appear to produce increased grazing rates on bacteria (Peters and Gross, 1994); changes in physiology and possibly behaviour of the protozoans were also implicated. The authors suggest that turbulence could be an important factor in control of aquatic bacteria by protozoans. However, they do not evaluate higher degrees of turbulence, perhaps from weather events, that might have detrimental effects on predator-prey contacts nor do they consider the potential increases in bacterial production that could arise from increased concentrations of nutrients brought about by turbulence and turbidity. Introduction of clays to surface waters, which may be most apparent under severe weather conditions, can be also associated with increased turbulence. Such introduction of clays to a freshwater planktonic community was shown to suppress profoundly many but not all of the planktonic predators without necessarily affecting bacterial densities (Jack et al., 1993; Boenigk and Novarino, 2004; Pfandl and Boenigk, 2006). Given the tendency for association of bacteria with particulates (De Souza, 2005) one might expect some increased adsorption of bacteria not already particle-associated. This suggests different planktonic dynamics depending on the level of clay present and that erosion of clays into receiving waters can potentially alter the bacterivory and potential for bacteria control. Resuspension of sediments (Jamieson et al., 2005) can also result in temporary reductions in predation (Fiordelmondo and Pusceddu, 2004). These phenomena may be particularly important in view of the observation of correlations between high *E. coli* levels and turbidity, often manifested in larger water bodies such as the Great Lakes by wave height (Ontario Ministry of Environment, 1984; Ontario Ministry of Health, 1989 and 1990; Francy and Darner, 1998). They may be important in modulating the observed levels of indicator bacteria where beaches are under the influence of erosion and non-point source pollution, and there is a failure to perform normal bacterial clearance

after a rainstorm. Thus, the observed levels of bacterial indicators, including *E. coli,* in water may have contributions from a) land run-off, b) resuspended sediments that can act as sinks for *E. coli*, c) increased cultivability (growth or recovery) because of more available nutrients, and d) reduced predation and or lysis. Each of these components can obviously vary according to the prevailing environments and weather conditions and may in part account for the high fluctuations in microbial numbers seen during routine sampling as well as the higher indicator numbers often associated with storm events.

Freshwater inflow into a marine environment also stimulated grazing on bacteria (Montagna, 1991). Tracing of ^{13}C added to a natural environment suggested labeling of 'biofilm scrapers', chironomids and copepods (Hall, 1995) but the study was done only in a headwater spring. It is not known if different predators would have been labeled in water of different quality/composition. (Carlsson et al., 1995) examined the effects of humic substances on different elements of the planktonic community. Although bacteria, ciliates and nanoflagellates all appeared to respond positively to the presence of humic substances, copepods did not. This may suggest distinct differences in prey species in the presence of different levels of humic material that can occur seasonally as well as at different locations.

AN ECOLOGICAL CONTEXT TO THE ENVIRONMENTAL SURVIVAL OF *E. COLI*

Allochthonous bacteria entering natural water bodies are immediately placed into direct competition with their autochthonous counterparts for the, often meagre, available resources. Enteric bacteria such as *E. coli*, delivered to an oligotrophic environment, will have great difficulty competing with the many indigenous bacteria that thrive under such conditions. They begin a period of starvation that may be rarely punctuated by small amounts of nutrients. Because of its rapid response to additional nutrients, *E. coli* may be able to exploit nutrient plugs that appear after upstream rainfall and recover cultivability or even grow (Springthorpe et al., 1993). Springthorpe et al. (1997) showed this in flow-through laboratory studies where, in response to short (10 minute) nutrient plugs non-cultivable *E. coli* became cultivable after short lags; however, the period of renewed cultivability under these circumstances was very short. For environments that are more degraded and eutrophic, where the diversity of indigenous microbes declines (Munawar et al., 1994; Torsvik et al., 1998), the *E. coli* may have a slightly better chance at survival, and perhaps occasional growth (Lim and Flint, 1989). In such environments, similar changes have been observed in species composition and relative abundance of planktonic species that are potential bacterial predators (Abdellatif et al., 1993).

When the combined effects of predation and viral lysis are considered, it is obvious that in most terrestrial and aquatic environments only a small percentage of the introduced *E. coli* will have the potential to survive these biotic influences. Many of those in receiving waters that do survive will tend to become attached to suspended solids, and some at least will settle into the sediments (Jeng et al., 2005) or on other submerged surfaces, or even beach sand for example (Beversdorf et al., 2007). Here the *E. coli* will encounter biofilms that are ubiquitous in natural environments and greater concentrations of nutrients than in the bulk water. Where favourable conditions exist, *E. coli* may even become a part of the biofilm community (Camper et al., 1996) as might have happened on beach sand (Beversdorf et al., 2007). Whether or not the *E. coli* is in the biofilm, it will certainly be in close proximity with other organisms and have much greater opportunities for interaction than can be found among bacterioplankton. Thus, it can more readily be affected by the activities of the other microorganisms. Such activities may be inhibitory/toxic or nutritive and so it will tend to be found in the more favourable locations where there are sources of appropriate nutrients (e.g. amino acids, vitamins, etc.) or where it can cross-feed on the wastes of other sediment residents. Furthermore, because of the proximity of other microorganisms, there are increased opportunities for exchange of genetic material, by recognized mechanisms, that may facilitate adaptation to new conditions and stressors or to new nutrient sources. In biofilms, there is also significant potential for chemical signaling (quorum sensing) to promote united fronts for action among the resident bacteria as well as means to counter these actions. As far as the authors know, no studies have been done to examine the potential for signals from bacteria such as *E. coli* to be interpreted by the

potential predator community. However, precedents exist for predators to know when the density of their prey makes it worth their approach (Danso and Alexander, 1975).

Phytoplankton tends to be mostly controlled by mineral nutrient availability, and this explains algal blooms associated with high nutrient content waters. Direct availability of mineral nutrients is considered to control bacterial growth (Grover, 2003) and mineral fertilization can extend *E. coli* survival in soils (Estrada et al., 2004). Bacteria, as a bulk compartment rather than individual species, are however less sensitive to the nutrient concentration (Chrzanowski and Grover, 2001) and may be more affected by significant changes in physical parameters of the environment. Ecological theory predicts that bacterial growth is likely to be enhanced by algal excretions of extracellular organic carbon (EOC), and that this also accentuates the competition between algae and bacteria for mineral nutrients (Bratbak and Thingstad, 1985). At low organic carbon concentrations algae are able to keep up with bacteria in their quest for mineral nutrients. Under steady state conditions where bacterial population increase is not controlled by another element of the system, algae would be outcompeted in their quest for mineral nutrients and basically participate in their own demise (Grover, 2000). However, protozoan grazing keeps bacteria under control and establishes a steady state equilibrium governed by nutrient availability and the metabolic activity of the ecosystem members, which in turn is also governed, as expected, by physical and chemical parameters of the environment. Another potential result of the presence of algal EOC is the localized reduction of competitive pressures among bacteria, which is to the advantage of the less competitive species and strains, as fecal bacteria are thought to be in natural environments.

Both *E. coli* and *Enterococci* were found to be ubiquitous in *Cladophora* algal mats collected at 10 locations all around the shores of Lake Michigan. The log mean densities were of 5.3 for *E. coli* and 4.8 for *Enterococci*. Both bacterial species survived in dry mats of *Cladophora* for over 6 months and they were revived easily after rehydrating the mats (Whitman et al., 2003). A direct comparison of the role of *Cladophora* mats on the survival of *E. coli* confirmed that *E. coli* within the mats survived longer than *E. coli* suspended in the same water but in the absence of *Cladophora* (Olapade et al., 2006). Cladophora might also harbor other human pathogens (Ishii et al., 2006b). A significant number of other bacterial groups, most notably sulphur reducers were also identified within the *Cladophora* mats. While the authors do not comment on this, this is an indication that *E. coli* are able to survive in multispecies environments supported on an EOC substrate; it would be of interest to identify if *E. coli* are favored by the low redox potential created around the hotspots of sulphur reduction.

Subsequent molecular fingerprinting of the *Cladophora* associated *E. coli* populations has shown them to be extremely diverse and different from *E. coli* populations collected from several known fecal sources (Byappanahalli et al., 2007). Moreover, *Cladophora* associated *E. coli* varied with the sampling location and temporally from one year to the next, for the same locations. In the same lake however, *E. coli* of fecal sources were identified in the epilithic periphyton, or rock-attached biofilms (Ksoll et al., 2007). Waterfowl was found to be the main source of *E. coli*, with the sewage sludge and periphyton-associated strains providing 8 to 28% of those identified. The presence of periphyton only *E. coli* strains also points to the possibility of naturalized *E. coli* strains in these rock-attached biofilms.

It is interesting to note here that some unicellular algae may be antagonists of a large number of fecal bacteria including *E. coli*. Fresh water algae *Hydrodictyon reticulatum* and the cyanobacteria *Aphanothece nidulans*, both associated with eutrophic waters, were found to reduce the number of recoverable enteric bacteria, while *Chlorella vulgaris* did not have any obvious impact on bacterial growth (Graf and Baier, 1981). It is unclear if the reduction in cell recovery were due to cellular death or due to inhibition of cultivability. Bacterial populations at diverse sites along the river Danube have been shown to depend mainly on intrinsic environmental parameters and be less correlated to the potential human impact along its shores; bacterial diversity was inversely correlated to the concentration of chlorophyll a (Williams et al., 2007), which is a universal indicator of phototrophic activity. This may corroborate the findings of Graf et al. (1981), which suggests that plant-associated algae may promote bacterial decay. Algal biomass entering water treatment facilities and exposed to water treatment chemicals can release biodegradable organic matter that can contribute to *E. coli* survival; this is especially true when ozone is used during primary disinfection (Bouteleux et al., 2005).

Signoretto et al., (2004) have found that putative VBNC *Enterococci* could be detected in both lake and marine waters. VBNC *Enterococcus faecalis* were typically attached to zooplankton (copepods) when available. There is no indication that attachment to copepods favors bacterial survival, but this does clearly indicate that zooplankton may constitute a significant sink of fecal bacteria in surface waters (Signoretto et al., 2005). The potential for *E. coli* to survive within protozoa has already been mentioned above and constitutes a special case of bacterial survival involving ingestion without digestion by protozoa. *Acanthamoeba polyphaga*, a soil and water biofilm protozoan, was found to support the growth of *E. coli* 0157:H7 through the release of organic carbon compounds, and also to protect ingested *E. coli* cells from external stresses. It has been shown that internalization without digestion of enteric organisms, including *E. coli*, by laboratory strains of two protozoa, *Acanthamoeba castellanii* and *Tetrahymena pyriformis*, is possible (King et al., 1988). It seems that the protozoan does not digest some of the ingested bacteria and it is speculated that the activation of the stationary phase rpoS-encoded sigma factor σS, may be responsible for triggering stress response changes in the *E. coli* cell, which allows it to avoid digestion (Barker et al., 1999). Protozoan predators that harbour *E. coli* may release it, or they may themselves be prey to organisms in higher trophic levels. The extent to which *E. coli* is further protected and allowed to survive inside other biota of varying size, and potentially be passed up the food chain by predation, is unknown, though there is some evidence that other bacteria such as legionellae may be passed on in this manner.

ABIOTIC STRESSORS AND INTRINSIC STRESS RESPONSES IN *E. COLI*

E. coli have long evolved in association with particular environments and their associated variables. Such stressors, often in combination, act on individual bacteria to modulate gene expression and allow an organism response that is most likely to favour species survival, if not always that of an individual organism. However, *E. coli* has evolved to be an organism of ubiquitous occurrence and is unlikely to be eliminated from natural environments by the levels of abiotic stressors alone. Nevertheless, their influence has clearly been shown in many studies.

As expected, *E. coli* is sensitive to temperatures above its growth range (and also to freezing), to UV radiation and to oxidative stresses (Barcina et al., 1997). Changes in pH can also have an impact (Cuthbert et al., 1950) but in most cases the soil, water or groundwater pH is not sufficiently extreme to be the sole factor in significant die-off. Desiccation in non-saturated soils is obviously a danger to all microorganisms (Rokitko et al., 2003) and, in conjunction with solar UV exposure, is considered to be the most effective abiotic eradicator of *E. coli* added to land (Williams et al., 2005). In surface waters, UV exposure may be accompanied by the generation in situ of hydrogen peroxide and hydroxyl radicals (Arana et al., 1992; Alam and Ohgaki, 2002).

Most water environments are oligotrophic and this low nutrient level adds another, and perhaps the largest, obstacle to the survival of the mostly copiotrophic *E. coli*. While *E. coli* are facultative organisms they prefer reduced environments and most pristine freshwater and marine environments tend to offer oxidative conditions. Hence, it is correct to assume that fecal microorganisms surviving initial predation, lysis and immediate elimination due to physical conditions in soil or water environments are continuously exposed to a combination of stresses that range from unavailability of nutrients, diurnal temperature shifts, light exposure, oxidative environments, pH and osmotic variations. How then is it possible that *E. coli* can be recovered, sometimes in significant numbers, from environmental samples?

Survival studies

There are numerous published studies of *E. coli* survival over more than four decades. Many use simple, contained and inoculated samples that lack the necessary realism to be useful as predictors of field effects. Others have been conducted in situ with diffusion chambers (McFeters and Stuart, 1972) or hollow-fibre devices (Springthorpe et al., 1993; Springthorpe et al., 1997). Although these have advantages of retaining known numbers of test organisms, and having real exposures to chemical components they

cannot give the total realism achieved by using tagged/identifiable organisms released in situ (Craig et al., 2004) into microcosms, or in the field. Unfortunately, however, the difficulties of knowing that the released organisms have been recovered in a quantitative fashion make 'realistic release' studies only useful to answer certain questions. In almost all cases, the measure of viability is taken as the ability to culture the cells on laboratory media. All studies where sediments and water samples are included have shown increased persistence in the sediments compared to the water.
Unfortunately, whichever type of study is conducted it is difficult or impossible to truly distinguish which cells are alive and those which are dead based on culture alone. Even live-dead stains have their limitations because cells that are apparently intact and viable might not be capable of growth.

Intrinsic properties of indicator bacteria that contribute to their survival

Escherichia coli is a facultative anaerobe that is able to obtain energy through aerobic respiration, anaerobic respiration, or fermentation. *E. coli* is very adaptable and is able to grow solely on glucose having the capacity to transform glucose into all the compounds it requires for growth. Under anaerobic growth conditions *E. coli* uses nitrates or fumarates as final electron acceptors (Reyes-Ramirez and Sawers, 2006). Under aerobic conditions growth results in the production of reactive oxygen species, which may inflict damage similar to senescence in higher organisms (Fredriksson and Nystrom, 2006) and may even possibly damage the capability of *E. coli* to grow anaerobically (Sutton et al., 2004). However *E. coli* can sense oxidative stress (Green and Paget, 2004) and produce protective enzymes such as superoxide dismutases and catalases capable of reducing reactive oxygen compounds (Iuchi and Weiner, 1996).
Many *E. coli* strains are motile and exhibit chemotactic behavior. In natural environmental matrices it is possible for microenvironments to exist that are not representative of the matrix as a whole. In such cases, it may be possible for a motile *E. coli* strains to avoid stress or acquire nutrients simply by negative-, or positive chemotaxis, respectively. Therefore *E. coli* has the capacity to respond to changes in cellular energy levels associated with changes in environmental oxygen, alternative electron acceptors, light and redox carriers that interact with metabolized carbon compounds (Taylor and Zhulin, 1998). However, such options may not be available to *E. coli*, and it has numerous mechanisms to react to and respond to stressors encountered in its secondary environment. Perhaps most important among these is that amino acid, carbon and phosphorus starvation lead to the so-called stringent response, which basically inhibits energy-consuming activities within prokaryotic cells (Ochi, 2007). The stringent response is capable of modulating the expression of a large number of genes and it manifests through the diversion of resources from growth and division towards amino-acid synthesis and survival (Caldeira de Araujo and Favre, 1985; Freedman et al., 1985; Grossman et al., 1985; Barcina et al., 1997; Shiba et al., 1997; De Wulf et al., 1999; van Delden et al., 2001; Knutsson Jenvert and Holmberg Schiavone, 2005; Ochi, 2007). Stringent response to amino-acid depletion and phosphate-phosphorus depletion (Rao et al., 1998) leads to intracellular accumulation of guanosine 3",5"-bis(diphosphate) (ppGpp) and guanosine 3"-diphosphate 5"-triphosphate (pppGpp), from GTP(GDP) using ATP as a phosphate donor (Irr, 1972; Ogawa and Sy, 1977). The two guanosine phosphate, ppGpp and pppGpp, directly or indirectly modulate a large number of genes relevant for the preservation of the microbial cell against environmental stresses and are now known to be global regulators in *E.coli* (Magnusson et al., 2005) with much broader roles for a wide range of prokaryotic and eukaryotic cells (Braeken et al., 2006).
This is accompanied by a significant reduction in the guanosine triphosphate (GTP) pool, as ppGpp inhibits inosine monophosphate (IMP) dehydrogenase, which is the key enzyme responsible for catalyzing the first step in the formation of guanine ribonucleotides from inosine monophosphate. ppGpp also binds to the RNA polymerase and thus represses rRNA synthesis. Depletion of GTP leads to morphological changes in the cell while the accumulation of polyphosphates affects physiological differentiation through inhibition of replication and transcription (Ochi, 2007), thus impairing the synthesis of DNA, RNA, tRNA, proteins, and nucleotide while stimulating synthesis of amino acids (Hou et al., 1999). Therefore growth is being traded off for persistence.

An up-shift in temperature, from 30 ºC to 44 ºC, was found to trigger a stringent response from *E. coli* similarly to the lack of amino-acids (Patterson and Gillespie, 1971) and it has been shown to result in an increase in the concentration of heat-shock proteins (Grossman et al., 1985). Cells lacking the capability of producing polyphosphate chains, due to the absence of the specific polyphosphate kinase enzyme, have reduced resistance to heat and oxidation stresses (Crooke et al., 1994).

Polyphosphate accumulation in cells may also indirectly trigger transcription of the RpoS gene, which leads to accumulation of σ^S factors. σ^S protein is a subunit of the ribosome that it is actively depleted during growth and accumulates as growth is slowed down. Over 70 dependent genes are linked to the σ^S factor (Loewen et al., 1998) and it has been shown to confer resistance against a large number of stresses (Hengge-Aronis, 2002). Accumulation of polyphosphates and/or the upregulation of RpoS in *E. coli* is considered to enhance cell resistance to UV-irradiation (Kornberg et al., 1999), osmotic and oxidative stress (Crooke et al., 1994; Shiba et al., 1997), temperature shifts (Shiba et al., 2000), and possibly metal toxicity directly by chelating available metals (Kornberg et al., 1999) or indirectly through induction of DNA repair enzymes (Al-Maghrebi and Benov, 2001).

Near-ultraviolet light (λ=366nm) has been shown to partially inactivate tRNA, therefore slowing down protein synthesis and the cell growth rate, with only a minor stringent effect. However this has not affected the cell division rates, leading only to reduction in cell size (Caldeira de Araujo and Favre, 1985). *E. coli* cultures exposed to visible light had diminished glucose uptake capability attained without cellular lysis, as it was determined by acridine-orange direct microscopy. One of the effects of exposure to visible light was the progressive loss of their capacity to multiply in bacteriological media (Barcina et al., 1989).

In soils, there may be significant dessication and associated osmotic shock as water evaporates. Such a state of water deficit is characterised by the accumulation of hydrophilins (Garay-Arroyo et al., 2000). These are proteins with a high glycine content and high hydrophilicity that are commonly synthesised by prokaryotes and eukaryotes as responses to dessication or osmotic shock.

The stress response in *E. coli* is extremely complex and involves large numbers of genes and proteins the activities of which are interwoven in an almost incomprehensibly coordinated manner. Purely biochemical changes can be accompanied by physical changes associated with certain cellular structures. One example is a response of bacterial porins that may diminish the capacity of *E.coli* to be influenced by its external environment (Iyer and Delcour, 1997; Liu and Ferenci, 1998; Ozkanca and Flint, 2002). Porins clearly influence the survival of *E. coli* in seawater (Gauthier et al., 1992; Darcan et al., 2003), as do intracellular levels of potassium and glutamate (Gauthier et al., 1991; Gauthier et al., 1993). *E. coli* survival is also enhanced by the accumulation of glycine betaine from its environment (Gauthier and Le Rudulier, 1990). While the reduced sensitivity to stressors may assist the survival process, reduced nutrient uptake will likely be an inevitable consequence that slows growth and response to growth inducing nutrients. Again, the importance of this inverse relationship between growth and survival is emphasized for environmental persistence of *E. coli*. Another consequence of reduced sensitivity to the environment suggests that stressed *E. coli* are likely to be less sensitive to environmental chemicals such as toxic metals and antibiotics (McMahon et al., 2007).

A recently reported independent phenomenon associated with stress responses is the increased vesicle release from stressed compared to non-stressed cells (McBroom and Kuehn, 2007), which appears to be related to the level of protein accumulation in the cell envelope. Such vesicle release improves bacterial survival under stress. It offers an opportunity to package and export stress products, and perhaps to communicate between cells? One could also speculate that it offers an opportunity to initiate the downsizing that is one of the recognized survival strategies for bacteria (Roszak and Colwell, 1987). Such vesicles might play a role in the recognized influence of prior culture conditions (Garcia-Lara et al., 1993; Gawande and Griffiths, 2005), and even in the ability of the 'dead' to communicate with the 'living' and influence their behaviour (Rowbury, 2003). In addition, the contents of such vesicles and the vesicles themselves may become part of the needed metabolite pool available to nutritionally-stressed bacteria.

Because much of the data on stress responses has been generated with densely populated bacterial cultures, the question arises as to whether similar phenomena are likely to be observed under more

realistic environmental densities; (Bodini et al., 2007) have begun to investigate this question and find marked similarities regardless of density.

When stress becomes a significant inhibitor: the viable but non-cultivable (VBNC) state.

The concept that stressor exposures can result in bacterial cells that are unable to be cultivated on conventional growth media has been mentioned above and is recognized as a widespread phenomenon in microbiology. However, what is not entirely resolved is whether this represents an adaptation for survival of the species under adverse conditions or is a debilitating sublethal injury to the cell (McDougald et al., 1998), or both, from which the exposed cell might occasionally be able to recover. This problem is not resolved by the discovery of toxin- genes and their products (e.g. MazF) that might induce dormancy (Inouye, 2006) and the overlapping phenomena of injury from, for example, chemicals such as chlorine. This also raises many questions regarding the interspecies and interstrain differences, the types(s) of stressors, the intensity of exposure to them and the duration of the exposure. It even spans the question of when the stress becomes too much and how the bacteria die?

In general under starvation bacteria enter a stationary phase and gradually lose cultivability. Frequently such non-cultivable cells can be revived early during starvation simply by providing the appropriate nutrients or other methods (McDougald et al., 1998). However, upon extended starvation they become moribund, and might lose their capacity to divide and be cultured even with the appropriate nutrients present (Aertsen and Michiels, 2004); frequently, direct microscopy indicates that these cells are still viable but non-dividing. This physiological state has been named as viable but non-culturable (VBNC). Shifts in environmental parameters, including during laboratory recovery efforts, may allow some of these moribund cells to regain their capacity to divide and thus be counted again as viable and culturable. However, it is very difficult to establish whether these moribund cells truly regain their capacity to divide, or whether a very small number of cells has remained viable and then have the capacity to grow if nutrition is provided. For example, reported recovery of chlorine-exposed *E. coli* in estuarine water (Bolster et al., 2005) could have resulted from recovery of injured cells, as suggested, or from growth of a few undamaged cells that remained. It is also currently disputable if VBNC just precedes cell death or is a physiological state that specifically allows cells to persist during periods of nutritional or chemical stress. Other environmental stresses that affect bacterial metabolic rates also lead indirectly to starvation with long-term exposure.

Since the concept of VBNC was first introduced by (Roszak and Colwell, 1987) many researchers have found it to be of significance for a wide variety of bacteria in various systems. VBNC may be induced by a variety of factors. The most common way to induce VBNC is by nutrient starvation. This way it has been shown that *E. coli* incubated in non-disinfected drinking water may enter VBNC and thus escape water quality testing (Bjergbaek and Roslev, 2005). *E. coli* incubated in oligotrophic groundwater can also enter the VBNC state. Stationary phase *E. coli* show a greater survival capability and this is directly related to the presence of the RpoS gene (Boaretti et al., 2003). We know that upon entering the stationary growth phase bacteria suffer from starvation, which likely triggers a stringent response and accumulation of the σ^S stress protein starting a cascade of molecular events that may lead to increase stress resistance. Gram-positive *Enterococci* were also found to enter the VBNC state in natural lake waters (Lleo et al., 1998). For *Aeromonas hydrophila* pre-starvation has been shown to delay the VBNC state (Mary et al., 2002). Cultures of *Ralstonia eutropha* added to activated sludge survived significantly longer when they were pre-starved (Watanabe et al., 2000).

Differences between the protein expression patterns of exponentially growing, starved, and viable but nonculturable (VBNC) *Enterococcus faecalis*, have been used to indicate that starvation and VBNC triggered by other stresses may actually reflect distinct physiological phases in the life of a cell (Heim et al., 2002). VBNC cells tend to reduce their size and change the structure of peptidoglycans in their cell walls (Signoretto et al., 2002). Survival of VBNC *E. coli* under nutrient and light stress is also associated with release of proteins, dissolved free amino acids and dissolved monomeric carbohydrates into the environment. It is obvious that such release of organic molecules might play a role in the transition to the VBNC state. *E. coli* incubated in supernatants previously colonized by cells in the VBNC state, survive

longer and there is a delay in the loss of their culturability (Arana et al., 2004). Therefore VBNC transitional states may be associated with a continuum of altruistic behaviors where both groups, the VBNC and the non-VBNC, may be favored.
Protein repair by L-isoaspartyl protein carboxyl methyltransferase (PCM), a hydrophobic protein (Boivin et al., 1995) found on the cell walls of vertebrate and some fecal organisms (Li and Clarke, 1992), plays a significant role in the long-term stress survival of *E. coli*, but only at alkaline pH, and it may function primarily to repair damage in cells that are recovering from nutrient limitation and in those cells that are able to divide during long-term stationary phase (Hicks et al., 2005).

VBNC can be triggered by a number of stress factors besides starvation. Wastewater treatment stresses applied during waste digestion are also considered to induce VBNC in *E. coli*; this leads to low counts of viable *E. coli* at the gate of the wastewater treatment facility, while allowing for fast regrowth once the treated cake is stored or land applied (Higgins et al., 2007). Exposure to toxic levels of copper sulfate induced reversible VBNC state in *E. coli* (Grey and Steck, 2001).

VBNC state does not affect antibiotic resistance (Lleo et al., 2003), virulence (Rahman et al., 1996) or infectivity (Pruzzo et al., 2002) of fecal bacteria and thus it is a mechanism through which organisms of significance to human health may survive environmental stresses undetected and re-infect.

Persister cells

A fraction of genetically homogeneous *E. coli* has been shown to survive exposure to stresses such as antibiotic treatment; these are termed persister cells. Unlike resistant mutants, cells regrown from persistent cells retain a similar spectrum of sensitivity to the original stress factor. It is considered that persistence is linked to pre-existent heterogeneity in bacterial population and that persistent phenotypes are generated both during active growth and during stationary growth phases with numbers of persister cells increasing as late exponential and stationary phases are achieved (Wiuff et al., 2005), and disappearing entirely if cells maintained in continuous culture in exponential growth. Persistence is believed to be achieved either through delayed growth or through continuous growth but at a rate slower than normal cells (Balaban et al., 2004). These two strategies allow for staggered growth within a bacterial population thus increasing the chances for at least some of the member cells to survive until more favorable growth conditions may occur.

It has been proposed that persister cells occur when inhibition of translation due to the overproduction of RelE toxins, shuts down cellular functions preventing antibiotics from corrupting their targets (Keren et al., 2004a; Keren et al., 2004b; Lewis, 2005). HipA toxin may also be involved (Korch and Hill, 2006). Shah et al. (2006) hypothesized that persister cells are dormant cells with a low level of translation but one of the obstacles to understanding persister cells is the difficulty of isolating them from the remainder of the population. They used a strain expressing a degradable GFP from a ribosomal promoter active only under rapid growth conditions to sort ‘dim’ persister cells from the bulk (bright) population. The ability to obtain a relatively pure population of persisters permitted an examination of their gene expression profile; approx. 5% of genes showed differential expression compared to the bulk population, but surprisingly an even greater difference from stationary phase cells. Persisters were apparently more like exponenential cells but with some downregulated genes for energy production and non-essential functions. The persistor transcriptome also pointed to toxin-antitoxin modules that may contribute to dormancy (Shah et al., 2006).
Although the persister cell phenomenon was initially observed in antibiotic resistance assays, it is logical to assume that it may offer ecological advantages against other stressors as well (Harrison et al., 2005).

Mutation under stress

Under sublethal stress there is a selective pressure operating that might cause new genotypes and phenotypes to arise and eventually predominate in a population. However, there remains a major controversy as to whether such events are a result of mutation occurring as a result of the stressor or simply of competitive growth variants (i.e. pre-existing mutants) that are the fittest for the ambient conditions (Roth et al., 2006). Interestingly, *E. coli* has been the subject of a long-running microbial evolution experiment where it has been propagated for thousands of generations in the same environment. Considerable parallel changes have been demonstrated: two interconnected networks governing DNA superhelicity and the stringent response have at the heart of beneficial adaptations of these experimental populations (Philippe et al., 2007).

Nevertheless, there are some arguments to be made that adaptation under stress involves mutations that occur as a direct result of the imposed stress. In particular there is reason, in modelling *E. coli* evolution in natural systems, to work with a starved batch culture model (Zinser and Kolter, 2004). Such cultures grown to stationary phase are then incubated without further introduction of nutrients or cells. The majority of cells lose viability fairly rapidly but this loss of viability rapidly declines and viable counts can remain after at least 5 years of incubation. Cells with a growth advantage in stationary phase (GASP) phenotype arise which have increased fitness and might coexist with or replace the originally dominant genotype(s) (Zinser and Kolter, 2004). Such GASP cells have been shown to be due to stable mutations that can arise relatively rapidly in starved populations (Zinser and Kolter, 2004). These authors provide a framework for discussion and future investigations of this issue.

Finkel and Kolter (1999) have studied evolution of microbial diversity among *E. coli* populations subjected to prolonged starvation; surviving cells were shown to be highly dynamic after meny months. As cultures aged, the fitter mutants did not exclusively dominate the population resulting in the co-existence of multiple forms. Vulić and Kolter (2001) have described evolutionary cheating as one means to increase proportions of a low occurrence mutant in a stationary phase population.

Bjedov et al. (2003) studied the potential evolutionary significance of stress-induced mutagenesis in aging colonies (MAC) of naturally-occurring *E. coli*. A large fraction of the isolates exhibited strain specific MAC, and the variability reflected the diversity of selective pressures in particular niches from which the strains were isolated. The authors conclude that irrespective of the causes, stress-induced mutations participate in adaptive evolution, and even concede the advantage that temporary mutator phenotypes can have for populations (Roth et al., 2006). Computer simulations suggest that stress-induced mutagenesis might be second order but the authors (Bjedov et al., 2003) state that it could also be a side-effect of some stress resistance mechanisms emerging from first order selection. In particular, they cite the possible involvement of transient mismatch repair in aging colonies. Some of the same authors have reviewed the causes and consequences of modulating DNA repair activity during stationary phase in *E. coli* (Saint-Ruf and Matic, 2006). They provide an excellent description of *E. coli* stationary phase characteristics and discuss the downwards modulation of DNA repair that occurs during stationary phase and its ecological and evolutionary consequences. Saint-Ruf and Matic (2006) have separately reviewed the effects of the environment on mutation rates.

Hersh et al. (2004) discussed adaptive mutation in the context of the *E. coli* Lac system and suggested two distinct mechanisms – double stranded DNA breaks and their potential error-prone repair, and adaptive amplification. A more recent study describes the dispersal and regulation of an adaptive mutagenesis cassette with mutagenic translation synthesis activity in the bacteria domain that has been shown to be under LexA regulation in several species (Erill et al., 2006). While the controversy of mutation versus selection is not solved, there is no reason too assume that both mechanisms cannot be operating simultaneously and that in part the experimental data is a function of the experimental model used.

UBIQUITY OF *E. COLI*

Evidence pointing at the capability of *E. coli* to adapt and become part of the native microbial population in the environment is mounting. Research carried in tropical soils in Hawaii, has shown soils to be sub-optimal but permissive to the support and growth in-situ of *E. coli* and *Enterococci*. Therefore it was concluded that in tropical soils the presence of *E. coli* in surface water following recharge events may not indicate fresh fecal contamination and it is only a reflection of the general presence of these organisms in the environment (Byappanahalli and Fujioka, 2004). Moreover, these organisms became established as a minor population in Hawaiian soils. Moisture was found to be the vital element favoring survival in a tidal zone soil in Florida (Solo-Gabriele et al., 2000). Marine sediments along warm beaches are significant sinks for *E. coli* and they also allow growth of *E. coli* in direct correlation with the available organic carbon (Lee et al., 2006). *E. coli* does survive and actively divides in tropical rivers showing insignificant variation in numbers (Jimenez et al., 1989). Testing of leaf surface and water samples collected from epiphytic flora in a tropical rain forest have shown *E. coli* to be always present suggesting that *E. coli* may be part of the phyllosphere microflora and not simply a transient bacterium of this habitat (Rivera et al., 1988).
Other experiments have evaluated the survival capability of *E. coli* in tropical marine waters. Marine waters are considered to be toxic to enteric bacteria through their salinity, light exposure and high-dissolved oxygen content. In general *E. coli* is considered to be a weak survivor in marine waters and thus *Enterococci* are usually considered to better indicate fecal contamination. Lopez-Torres et al. (1988) have evaluated side-by-side the survival capacity of *E. coli* and *Klebsiella pneumoniae*, the former being considered a nonsurvivor with the latter long considered a survivor in marine waters. The researchers have observed morphological changes in the *E. coli* subjected to nutrient stress (shortening and condensation) that were not noted in *K. pneumoniae*. However the respiration activity exhibited by *E. coli* was greater than *K. pneumoniae* even at high organic loads. Thus, based on physiological activity it was concluded that *E. coli* is as much a survivor in tropical marine waters as *K. pneumoniae*. The main contributor to *E. coli* persistence was the high input of organic waste nutrients from a rum distillery. *E. coli* numbers and activity responded to nutrient input even before measurable differences in oxygen concentration, pH and salinity could be observed.

Power et al. (2005) have reported the presence of three free-living encapsulated *E. coli* strains in two Australian lakes. The same three *E. coli* strains were responsible for blooms in the two distinct lakes, although they are 200 km apart. The phenotypic and genotypic profile of the strains could not be identified in the fingerprint database of 435 *E. coli* strains isolated from vertebrate sources. This suggests that some *E. coli* strains have evolved a lifestyle independent from vertebrate hosts and that their numbers in lake water is only a function of environmental parameters such as nutrient availability and temperature. Hence all evidence points out that tropical environments offer nutrients, warmth and humidity year-round and thus that explains the presence of established populations of putative enteric organisms.
Knowledge of the possibility for *E. coli* to actively degrade organic matter in temperate waters is not novel (Rao and Dutka, 1974). While eventually the notion of *E. coli* survival in warm tropical areas is becoming established de facto, *E. coli* and in general enteric organisms are still considered to be easily eliminated from soils and waters in colder climates. Recent investigations carried on soils around Michigan Lake have brought up the existence of soilborne *E. coli* strains that had fingerprints unique to specific soils and locations (Ishii et al., 2006a). The authors interpreted this to represent strains naturalized and autochthonous to the soil microbial community. Unc et al. (2006) were able to recover *E. coli* from clay soils, up to a depth of 70cm, after treating the soil with sterile municipal biosolids.
Clay, presence of plant roots and freezing does enhance survival of *E. coli* O57:H7 in soils. While generally root presence enhanced survival over survival in fallow soils, the type of plant may also alter the survival capacity. It was reported that roots of rye (*Secale cereale* L.) and alfalfa (*Medicago sativa* ROTH) led to longer survival in soil of *E. coli* O157:H7 than hairy vetch (*Vicia sativa* L.) or crimson clover (*Trifolium incarnatum* L.). Manure did not have any significant effect (Gagliardi and Karns, 2002).

Nevertheless, the combination of soil and type of added manure may have an impact on both the numbers and the survival capability of *E. coli* (Unc and Goss, 2006).

LABORATORY RECOVERY

Estimation of the level of fecal contamination of a specific environment is correlated to the number of *E. coli* recoverable on standard growth substrates. As we know, bacteria may be damaged by a wide variety of stress conditions, including nutrient starvation, oxygen radicals, heat, freezing temperatures, changes in pH, near-UV radiation, and osmotic as well as hydrostatic pressure. Adaptive molecular networks have evolved within bacteria to overcome the challenges of rapidly changing environments and to permit survival under conditions of stress. There is sufficient evidence to allow us to draw the conclusion that *E. coli*, or at least selected strains, do adapt to environmental conditions and do become part of the native population. The important practical question is how to monitor highly stressed cells in environmental samples and the food chain and what is the significance of their recovery.

The simplest techniques aimed at the recovery of injured or stressed fecal coliforms are aimed at removing any potential stress factor. Therefore the organisms are usually pre-incubated in a low nutrient, often non-selective medium, at temperatures around 35 °C (Stuart et al., 1977; Ozkanca et al., 2007). The low nutrient concentration is aimed at allowing the bacteria to recover while minimizing metabolic activity that may result in excessive free radicals, which may create an oxidative stress. Most common *E. coli* recovery substrates contain hydrolyzed organic extracts that provide a mixture of proteins and amino-acids (soy, yeast, beef or blood extracts), with carbohydrates (glucose or starch), added occasionally. Phosphorus is added either in organic form (casein) or often as mineral phosphates.

Given the preference of *E. coli* for anoxic environments, antioxidants are added to create reduced environments that have been shown to increase the potential resuscitation of stressed fecal bacterial populations (Alonso et al., 1999; Reissbrodt et al., 2002). Antioxidant amended substrates can also be used singly without a recovery step for samples ranging from food to water, groundwater (Shirey and Bissonnette, 1997; Jiang et al., 1998; Mizunoe et al., 1999). Recovery of *E. coli* from soils can benefit from employing similar recovery methods (Hepburn et al., 2002). Pre-treatment of soils with nutrient rich organic waste may help recovery of soil *E. coli* (Unc et al., 2006).

Human source *E. coli* suspended in distilled river water react very fast to a transient spike of tryptose phosphate broth. This indicates their capability to take rapid advantage of an organic source of nutrients (Springthorpe et al., 1997). Tests carried out in a salty marine and marsh waters have shown that, in general, *Enterococci* survive better than *E. coli* except for a nutrient rich salty marsh environment where there is large concentration of nutrients (Lessard and Sieburth, 1983).

Addition of iron, to phenol-treated *E. coli* has been shown to improve recovery (Harris and Richards, 1968). The authors suggested that iron might precipitate toxic compounds in the growth substrates. Supplementation of the recovery substrate with iron sources has been shown to improve recovery of Salmonella (Reissbrodt et al., 2000). Presence of iron compounds in the environment enhances survival of *E. coli*. (Grandjean et al., 2006) found iron to be essential for the survival of nutrient starved *E. coli* in drinking water.

Lyte et al. (1996a) have tested the growth of *E. coli* O157:H7 in “conditioned” media. This conditioned media was obtained by growing *E. coli* O157:H7 in the presence of norepinephrine, a mammalian neuro-endocrine hormone. They have observed that supplementation of fresh cultures with as little as 0.024% (v/v) norepinephrine conditioned medium resulted in increased growth as compared to controls, thereby indicating the presence of an autoinducer of growth. Norepinephrine and epinephrine are also known under the other name of adrenaline and are part of a larger group of monoamines - catecholamines - ubiquitous in human and animal intestinal system. They tend to be eliminated through urine and end up in wastewater. Seemingly they degrade easily in alkaline environments but are preserved in acidifieds media (Willemsen et al., 2007). Reissbrodt et al. (2002) have shown that the heat stressed EHEC *E. coli* and *Salmonella ssp.* were resuscitated in the presence of a heat stable autoinducer of growth, which is secreted by a number of enterobacterial species in the presence of norepinephrine (Freestone et al., 1999).

Therefore Reissbrodt et al. (2002) proposed the use of such an autoinducer for the recovery of Salmonella and *E. coli* from environmental samples. Moreover the presence of epinephrine or norepinephrine has the capability to restore virulence to enterohemorrhagic *E. coli* (Walters and Sperandio, 2006). Catecholamines thus do enhance growth of a number of human commensal and environmental strains of *E. coli* (Lyte and Ernst, 1992). Presence of catecholamines in the growth substrate was associated with iron uptake from iron-binding proteins, ^{55}Fe-complexed lactoferrin and ^{55}Fe-complexed serum transferrin (Freestone et al., 2002; O'Donnell et al., 2006). Enhanced production of shiga-like toxins it is also likely enhanced in the presence of norepinephrine (Lyte et al., 1996b). While tempting, it is currently difficult to estimate the resilience of catecholamines or any form of autoinducer molecule in the environment, or in organic wastes, or to extrapolate to their potential role in the environmental regrowth of fecal organisms.
It is nevertheless highly likely that the numbers of *E. coli* that we routinely detect in environmental sample is quite a large underestimate of those that may be present and viable at any one time. Moreover, as discussed earlier, their numbers can be modulated continuously and in combination by inputs, nutrients, weather and turbidity. Thus, one has to question whether they form the ideal indicator of fecal pollution in environmental samples. Nevertheless, there is no universally accepted and easy to measure alternative and their continued use for this purpose is likely. A further note that comes from the discussion in this chapter is the differences in survival that are clear among different isolates of *E. coli* and also depending on their niches within soils, sediments and the water environment. This has potential implications for the accuracy of microbial source tracking, quantitative microbial risk assessments, modelling of microbial populations and the efficacy of regulatory standards.

CONCLUDING DISCUSSION

Given the importance of *E. coli* as an indicator we have a relatively poor understanding of its microbial ecology and it would desirable to improve this if it is to continue as our fecal pollution indicator of choice. Introduction of fecal material into waters occurs either through direct discharge of organic wastes, or through diffuse input associated with surface runoff and tile drainage. These inputs are usually associated with a significant increase in the numbers of fecal organisms in water. Other increases can be triggered by waves and turbidity resuspending *E. coli* from sediment sinks.
Diversity of *E. coli* in surface water tends to be at its highest during rainy seasons, when many genotypes found in water samples may be associated with diffuse source of contamination such as wildlife or pastures. Diversity is diminished significantly during drier seasons clearly suggesting that only a limited number of strains have the capacity to survive extensively Extensive die-off of *E. coli* is expected following their release in the environment. The combination of these factors does raise questions about the reliability of detailed fingerprinting methods for the potential identification of *E. coli* sources.
Most of these environmentally established *E. coli* are non-pathogenic. However other evidence suggests that enterohemorrhagic *E. coli* have the potential to persist and grow in water environments. Esterase activity based measurements in river water samples from an agricultural watershed and an industrial area has shown that *E. coli* O157:H7 existed in both viable and inactive forms. This points at their survival capability and potential to retain or re-gain metabolic activity and regrowth (Tanaka et al., 2000).
Newly introduced *E. coli* tend to die-off rapidly and survivors attach to suspended particles and eventually settle at the water's bottom (Brettar and Hofle, 1992). Lake sediments offer short and long term sinks for freshly fecal and naturalized *E. coli* extending their survival (Ishii et al., 2007), while temperature increases lead to *E. coli* regrowth (He et al., 2007). Production and participation in multi-species biofilms may enhance survival of *E. coli* in aquatic environments (Ksoll et al., 2007). Biofilm formation in aquatic environments seems to be enhanced by the availability of low-molecular organic matter and mineral nutrients in the absence of competitors. Seasonal effects have been observed on the species composition of biofilms (Olapade and Leff, 2005). It is of interest to note the results of (Colon-Gonzalez et al., 2004), which suggest that under anoxic conditions *E. coli* losses its capability to form biofilms.
It is safe to assume that stress resistance of *E. coli* strains reaching soil or water environments can be

roughly classified along a continuum ranging from surviving to non-surviving strains. It is likely that the position of any given strain along this continuum is a function of the interaction between environmental stresses and the capacity of each individual strain to counteract stress impacts. The stress resistance pattern varies widely among *E. coli* strains. Homeostasis is important to bacterial cells and therefore each strain achieves a specific equilibrium between stress resistance and growth, in accordance to the common stress patterns in their environment. Excessive cost associated with the synthesis of stress protectants may reduce the capacity of vegetative *E. coli* to compete in oligotrophic environments (Ferenci and Spira, 2007).

Bacteria growth is directly related to the availability of mineral nutrients. Under steady state situations the amount of available mineral nutrients is dependent on the rate at which they are made available through mineralization of organic matter and the rate at which predators recycle the ingested nutrients (Grover, 2003). If predators recycle little of the ingested material and mineralization is hindered by environmental conditions then the bacteria must compete for a shrinking pool of mineral nutrients and thus they need to employ energy saving mechanisms, which may lead them to reduce their metabolism and shift from energy consuming to energy saving mechanisms. This may result in the apparition of VBNC cells or, if stresses persist, die-off. The behavior of each individual strain of *E. coli* is expected to be dependent on their stress related enzymatic arsenal, and their capabilities to timely deploy it. Thus, the more extensive the stress the lower the number of strains that can adapt. This may explain the differences in the survival of *E. coli* in tropical versus temperate climates, or in water versus soils. Presence of algal EOC, mitigates *E. coli* survival and also allows for a greater *E. coli* diversity. Following the same reasoning one would expect that changes in the nutritional status of the environment, such as brought by addition of organic nutrients (Unc and Goss, 2006; Unc et al., 2006), mineral nutrients (Estrada et al., 2004), or possibly increased mineralization rates as shown along plant roots (Gagliardi and Karns, 2002), would favor general bacterial growth and thus growth of *E. coli* that did survive through longer or shorter stress episodes.

Isolation and identification of *E. coli* from most non-agricultural soils is difficult at best, and in most situations their recoverable number is low. Nevertheless, changes in the nutritional status of some of such soils may allow VBNC or just very low numbers of *E. coli* to become active and increase their numbers to levels where they become easily detectable. Land application of biosolids may prove to be one of such nutrient augmentation event that can leads to re-activation of dormant and low levels of *E. coli* in soils (Unc et al., 2006). Given the lack of information on the presence of potential molecular signals and growth enhancers in biosolids we cannot at this point speculate on their potential impact on environmental recovery and re-growth.

E. coli have the genetic equipment to sense and react to various stresses (Hersh et al., 2004) from nutritional deficiencies to temperature, reactive oxygen and metal compounds. It has even been shown that under certain protective environments they can even survive desiccation. Hence its continuous presence in soil and water environments should be assumed even if standard recovery efforts may fail to indicate its presence. *E. coli* have also the capacity to satisfy their nutritional requirements in a wide range of environmental conditions, and also seems to have the potential to take promptly advantage of newly available nutrient sources, made available through mineralization of naturally occurring root exudates, algal excreted organic carbon compounds or from other dead bacteria. Addition of organic and mineral nutrients through contamination events has also a positive effect on *E. coli* growth. Such complex nutrient sources may possibly even revive cells found in various stages of metabolic decline, and possibly not only due to nutrient stimulation. Certain *E. coli* strains have been shown to adapt and become part of the natural environmental populations and therefore their numbers at any given time is more often likely independent of contaminant events. Seasonal climatic factors and their impact on physical, chemical and biological parameters of the environment are therefore more likely to govern the numbers of recoverable *E. coli* throughout seasons.

Hence we propose that further research be carried towards the understanding of the ecological role of new and naturalized *E. coli*, the role of contaminant sources in the enhanced recovery of *E. coli* in soil and

water as well as the role of each contaminant source in defining the composition of resistant *E. coli* population in environment.

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