Indigenous soil bacteria and low moisture may limit but allow faecal bacteria to multiply and become a minor population in tropical soils

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Abstract The soil environment in Hawaii is generally characterised as sub-optimal but permissive to support the in situ growth of E. coli and enterococci. However, soil desiccation and competition for nutrients by major indigenous soil microflora have been identified as potential factors that could limit a rapid and continual growth of faecal indicator bacteria in this soil environment. Despite these limitations, the genetic capacities of E. coli and enterococci are robust enough to enable these bacteria to become established as minor populations of Hawaii’s soil microflora. Although the concentrations of E. coli and enterococci may have represented a fraction of the total soil microbiota, their presence in this habitat was very significant, for two important reasons: (a) soil was a major environmental source of E. coli and enterococci, and (b) the elevated counts of these bacteria in streams that routinely exceeded the EPA standards were due to run-off from soil. As a result, E. coli and enterococci were inadequate indicators to measure the degree of faecal contamination and potential presence of sewage-borne pathogens in Hawaiian streams.

Keywords Faecal indicator bacteria; microbial ecology; tropical soil; water quality

Introduction
Escherichia coli and enterococci are collectively called faecal indicator bacteria, because they are always found in the faeces of mammals and their concentrations in environmental waters are routinely used to measure the degree of faecal contamination as well as potential presence of human pathogens. To reduce the risk of infection from sewage-borne pathogens in recreational waters, EPA and WHO have developed water quality standards based on concentrations of faecal indicator bacteria that exceed ambient levels of these bacteria, and such standards are generally considered applicable to all geographic regions of the world. It should be noted that only data obtained from limited temperate regions of the world were used to establish ambient concentrations of faecal indicator bacteria in environmental waters, which led to the incorporation of the following two assumptions in the guidelines to interpret recreational water quality standards: (a) the only significant source of faecal indicator bacteria is faeces of human/warm-blooded animals or sewage, and (b) faecal indicator bacteria do not multiply to any significant degree under environmental conditions.

However, these faecal indicator bacteria have previously been reported to occur naturally in water, soil and on plants in tropical locations such as Hawaii (Fujioka et al., 1988; Hardina and Fujioka, 1991), Guam (Fujioka et al., 1999), Puerto Rico (Hazen, 1988; Rivera et al., 1988) and south Florida (Desmarais et al., 2002). These results indicated that the assumptions incorporated in the current guidelines to interpret water quality standards were not applicable to all regions, particularly tropical locations. In Hawaii (Hardina and Fujioka, 1991) and Guam (Fujioka et al., 1999), soil was identified as the primary source for the consistently elevated counts of faecal indicator bacteria in freshwater streams; rain/run-off was concluded to be the natural mechanism that transported soil-bound faecal
indicator bacteria into streams. Even though *E. coli* and enterococci can be readily and consistently recovered from most soils in Hawaii, one important aspect of their ecology, growth potential in natural soil environments, has essentially been unexplored. Based on preliminary studies (Byappanahalli and Fujioka, 1998; Fujioka and Byappanahalli, 2001), factors, such as soil moisture, indigenous microflora, and available nutrients, have been identified that can potentially restrict the growth of *E. coli* and enterococci in natural soil environments, although how these factors affected these bacteria were unclear. Therefore, the specific objective of this study was to characterise the effects of soil moisture and nutrient limitation (through competition by major indigenous soil microflora) on the ability of *E. coli* and enterococci to persist and multiply in the soil environment of Hawaii.

**Materials and methods**

**Soil samples and treatment**
All soil samples were obtained from various sources, such as a recreational beach, public parks, agricultural experimental farm (Waimanalo), and from grassy and shaded sites, within the campus of the University of Hawaii (located on the island of Oahu, Hawaii). The Waimanalo soil sample was sterilised by cobalt irradiation (1,750 krad, 33 h). Soil moisture content was determined for all samples collected; experimental soils used in the study were maintained at an optimum soil moisture level of 35%, which corresponded to 60% water-holding capacity (WHC). Bile salts #3 (Difco laboratories, Detroit, USA) were used as a selective inhibitor of non-faecal bacteria.

**Microbial assay**
Concentrations of indigenous faecal indicator bacteria (*E. coli*, enterococci) from soil samples were enumerated using the MPN method (APHA, 1992). Concentrations of culturable, heterotrophic bacteria in soil samples were enumerated by the spread plate technique, using soil extract agar (SEA) agar (SSSA, 1994) supplemented with cycloheximide (100 µg/mL). In certain experiments, pure cultures of *E. coli* (ATCC 25922) and *E. faecalis* (ATCC 29212) were used to inoculate the cobalt-irradiated soils; over time their concentrations in soil were determined by dilution plate technique using M-FC agar, *E. coli* (44.5°C, 24 h) and m-*Enterococcus* agar, *E. faecalis* (35°C, 48 h). Most experiments were conducted under laboratory conditions to simulate a predominant ambient temperature of 25 ± 2°C, and were protected from external contamination during the course of the experiment.

**Results**

**Effect of indigenous soil microflora on multiplication of *E. coli***
To obtain data that *E. coli* and enterococci were part of the soil microbiota, the relative culturable concentrations of faecal bacteria (*E. coli*, enterococci), and heterotrophic bacteria, which represent the most numerous group of soil microflora, were determined in six different soil samples collected on the campus of the University of Hawaii.

The results (Table 1) showed that the concentrations of heterotrophic bacteria were consistently high and ranged from $1 \times 10^8$ to $5 \times 10^8$ CFU/g dry soil. In the same soil samples, the concentrations of *E. coli* and enterococci were highly variable and relatively low, with counts ranging from $10^4$ to $10^5$ MPN/g dry soil. These results indicated that there were approximately 9,000–22,880,000 heterotrophic bacteria to every *E. coli* or enterococcus in these soil samples. However, since it was reasonable to assume that only 0.1% of total bacteria in soil could be cultured, there may be as many as $10^{10}$ indigenous soil bacteria to each *E. coli* or enterococcus.

The overwhelming ratio or number of indigenous soil bacteria relative to *E. coli* and enterococci provided direct evidence that these faecal bacteria constituted only a fraction
of the total soil microbiota. These results provided indirect evidence in support of our hypothesis that the major indigenous soil microflora efficiently extracted available nutrients from soil and thus restricted the growth of *E. coli* and enterococci *in situ*. To obtain more direct evidence in support of this hypothesis, an experiment was designed to measure growth of *E. coli* in natural soil supplemented with excess nutrients or small quantities of bile salts, an inhibitor of non-faecal bacteria. In this experiment, multiplication of natural populations of *E. coli* was monitored in soil samples that were supplemented with nutrients (glucose) or with bile salts and incubated at constant temperature (25°C) and adequate moisture content (35%) for 11 d. Glucose (1%) was used as a simple carbon source and 0.15% bile salts were chosen as the selective inhibitor of non-faecal bacteria, because when 0.15% of bile salts were added to soil extract agar, a reduction (94–99%) in the culturable heterotrophic bacteria was evident (Table 2).

The results (Figure 1) showed that in the unsupplemented soil (control sample), multiplication of *E. coli* was restricted to <1-log over the 11 d period. However, after 2 d incubation, *E. coli* numbers increased by 2-logs in soil supplemented with glucose, by 3-logs in soil supplemented with bile salts and by 5-logs in soil supplemented with glucose and bile salts. These results provided evidence that under nutrient-limited conditions (often the case in the soil environment) major indigenous microflora could significantly limit the growth potential of faecal indicator bacteria such as *E. coli*, most likely by more efficiently extracting available nutrients from soil. These results also indicated that *E. coli*, and most likely enterococci, have the capacity to multiply in soil but must wait for opportunities when nutrients become available.

### Table 1
Relative concentrations and ratios of culturable heterotrophic bacteria to *E. coli* and enterococci (Entero) in six soil samples from the University of Hawaii campus

<table>
<thead>
<tr>
<th>Soil moisture content (%)</th>
<th>28</th>
<th>44</th>
<th>29</th>
<th>32</th>
<th>35</th>
<th>44</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total heterotrophic bacteria (CFU/g dry soil)</td>
<td>$2.15 \times 10^8$</td>
<td>$1.01 \times 10^8$</td>
<td>$2.59 \times 10^8$</td>
<td>$1.99 \times 10^8$</td>
<td>$2.91 \times 10^8$</td>
<td>$5.63 \times 10^8$</td>
</tr>
<tr>
<td>Faecal indicators <em>E. coli</em> (MPN/g dry soil)</td>
<td>$2.94 \times 10^2$</td>
<td>$1.37 \times 10^2$</td>
<td>$2.20 \times 10^2$</td>
<td>$3.56 \times 10^1$</td>
<td>$1.48 \times 10^2$</td>
<td>$2.46 \times 10^1$</td>
</tr>
<tr>
<td>(MPN/g dry soil) Entero</td>
<td>$1.41 \times 10^3$</td>
<td>$1.14 \times 10^3$</td>
<td>$2.07 \times 10^4$</td>
<td>$2.11 \times 10^3$</td>
<td>$6.61 \times 10^2$</td>
<td>$6.64 \times 10^1$</td>
</tr>
<tr>
<td>Ratio of heterotrophic to faecal bacteria <em>E. coli</em></td>
<td>$7.31 \times 10^5$</td>
<td>$7.27 \times 10^5$</td>
<td>$1.18 \times 10^6$</td>
<td>$5.59 \times 10^5$</td>
<td>$1.96 \times 10^6$</td>
<td>$2.28 \times 10^7$</td>
</tr>
<tr>
<td>Enterococci Entero</td>
<td>$1.52 \times 10^5$</td>
<td>$8.90 \times 10^5$</td>
<td>$1.20 \times 10^4$</td>
<td>$9.00 \times 10^3$</td>
<td>$4.40 \times 10^5$</td>
<td>$8.48 \times 10^6$</td>
</tr>
</tbody>
</table>

**Figure 1** Growth responses of *E. coli* in natural Waimanalo soil when supplemented with (a) glucose, (b) bile salts and (c) bile salts + glucose at 25°C under constant moisture content (35%).
Effect of soil moisture on persistence and multiplication of *E. coli* and enterococci

To specifically assess the role of soil moisture on measurable counts of *E. coli* and enterococci, known concentrations of *E. coli* and *E. faecalis* were seeded into cobalt-sterilised soil: a treatment that eliminated the effects of indigenous microflora. One portion of this soil sample was allowed to naturally dehydrate at 25°C for 8 d while, under the same conditions, water was added as needed to the second portion to maintain the soil at approximately 35% moisture content. Concentrations of *E. coli* and *E. faecalis* were then determined in these two soil samples, using the plating method on selective media.

The results (Figure 2) showed that when soil moisture was maintained at 35%, concentrations of *E. coli* increased by approximately 1.5 logs over 2 d and maintained that concentration over 8 d. These results provided additional evidence that, when major indigenous soil microflora were not present, *E. coli* could utilise the available nutrients in soil to multiply. Under the same conditions, the concentrations of *E. faecalis* remained unchanged, indicating that *E. faecalis* required more complex nutrients for its multiplication.

The second set of soil samples was allowed to desiccate naturally, during which the moisture content was reduced at a fairly constant rate from 33% to 12% over 4 d at 25°C, and then stabilised to ~11% through 8 d (Figure 3). Under these conditions, the concentrations of *E. faecalis* remained fairly constant. In comparison, the concentrations of *E. coli* dropped by nearly 6-logs during the first 4 d. However, when moisture (35%) was added back to part of the soil sample, which had been allowed to dry for 4 d, concentrations of *E. coli* had increased by nearly 6-logs by d 6 (48 h after rehydration) and 6.5-logs by d 8 (96 h after rehydration) to levels similar to that before the soil was dehydrated. These results confirmed previous reports that Gram-negative bacteria were more susceptible to cellular injury and/or stress by unfavourable environmental conditions than Gram-positive bacteria.

### Table 2

<table>
<thead>
<tr>
<th>Location/sample site</th>
<th>Bacterial counts (CFU/g dry soil)</th>
<th>Reduction (%) in counts on SEA - BS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>On SEA</td>
<td>On SEA - BS</td>
</tr>
<tr>
<td><strong>Old Stadium Park</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Site 1</td>
<td>$6.27 \times 10^7$</td>
<td>$1.19 \times 10^6$</td>
</tr>
<tr>
<td>Site 2</td>
<td>$7.89 \times 10^7$</td>
<td>$9.47 \times 10^5$</td>
</tr>
<tr>
<td><strong>Ala Moana Beach Park</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Site 1</td>
<td>$6.96 \times 10^6$</td>
<td>$2.53 \times 10^5$</td>
</tr>
<tr>
<td>Site 2</td>
<td>$1.64 \times 10^7$</td>
<td>$9.85 \times 10^5$</td>
</tr>
</tbody>
</table>

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Figure 2 **Survival/multiplication of *E. coli* and *E. faecalis* introduced into cobalt-sterilised Waimanalo soil and incubated at 25°C under constant moisture content (35%)**
This showed that a drop in soil moisture to <20% could be expected to reduce recoverable concentrations of *E. coli*, but that this population was able rapidly to regain its numbers when environmental conditions became favourable.

**Discussion and conclusions**

The prevailing dogma is that the natural habitat of faecal indicator bacteria (*E. coli*, enterococci) is the gastrointestinal tract of mammals, and that these faecal bacteria will not multiply when discharged into the environment. However, the results of this, and previous (Hardina and Fujioka, 1991; Fujioka and Byappanahalli, 2001) studies showed that *E. coli* and enterococci had become established as minor populations in Hawaii’s soil environment. Further, it appeared that multiplication of these faecal bacteria in soil was slow and sporadic, for the following reasons: (a) the soil environment in Hawaii provided only sub-optimal conditions for their growth; (b) the major indigenous microorganisms, which were more numerous and better adapted to soil conditions, appeared to control the growth of *E. coli* and enterococci by out-competing these bacteria for a limited supply of available nutrients – in this scenario, growth of *E. coli* and enterococci was restricted and probably occurred only sporadically; (c) opportunities for multiplication of faecal indicator bacteria in soil were related to occasional conditions when excess nutrients become available (e.g. faecal droppings, decomposing organic matter and insects) and since *E. coli* requires simple nutrients and enterococci require complex nutrients, multiplication of these two bacteria in soil was believed to be sporadic and uncoordinated; and (d) a drop in soil moisture occurred regularly, and the culturability of *E. coli* was much more sensitive to a drop in soil moisture than were enterococci.

Our conclusion that faecal indicator bacteria could become established in two distinctly different habitats may be controversial, but it is supported by the principles of microbial ecology (Atlas and Bartha, 1993) which state that every environment will selectively support vigorous growth of those microbial populations whose optimum growth requirements coincide with those conditions provided by that environment. The predominant organisms in that habitat are called major indigenous microflora. However, this same environment can provide conditions (niches) for survival and limited multiplication of certain other microorganisms whose natural habitat may not be soil. These microorganisms are called minor indigenous microflora and are generally not recognised because they cannot be readily enumerated or their contributions cannot be determined.
However, the minor populations of faecal indicator bacteria in tropical soil environments can be readily cultured and enumerated. Moreover, their contributions are measurable because, in Hawaii and Guam, soil not only represents an environmental source of *E. coli* and enterococci but it also serves as the primary source for elevated levels of faecal indicator bacteria in stream waters on these islands (Hardina and Fujioka, 1991; Fujioka *et al.*, 1999). Since *E. coli* and enterococci were able to persist and multiply in soil, the concentrations of these faecal indicator bacteria in streams of Hawaii and Guam were inadequate indicators to measure the degree of faecal contamination and potential presence of sewage-borne pathogens.

**Acknowledgements**

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**References**


