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Bacterial and Archaeal direct counts: A faster method of enumeration, for enrichment cultures and aqueous environmental samples

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ABSTRACT

A new presence/absence method has been developed to count fluorochrome-stained bacterial and archaeal cells on membrane filters using epifluorescence microscopy. This approach was derived from the random distribution of cells on membranes that allowed the use of the Poisson distribution to estimate total cell densities. Comparison with the standard Acridine Orange Direct Count (AODC) technique shows no significant difference in the estimation of total cell populations, or any reduction in the precision of these estimations. The new method offers advantages over the standard AODC in considerably faster counting, as there is no need to discriminate between every potential cell visible on a field and fluorescent detritus, it is only necessary to confirm the presence of one cell. Additionally, the new method requires less skill, so has less reliance on expert counters, and that should reduce inter-counter variability. Although this work used the fluorochrome Acridine Orange, clearly the results are applicable to any fluorochrome used to count bacterial and archaeal cells. This method was developed using enrichment cultures for use with enrichment cultures and aqueous environmental samples where interfering detrital and mineral particles are minimal e.g., freshwater/seawater, therefore, it is not suitable for estimating total cells from sediment samples. This method has the potential for use in any situation where counts of randomly distributed items are made using a grid or quadrat system.

1. Introduction

Accurate estimation of the total number of cells in enrichment cultures or environmental water samples requires the use of a direct microscopic count method, and not a method that needs individual cells to demonstrate growth or colony formation as viable counts considerably underestimate total cell populations (Daley, 1979). Early versions of direct counting used either Coulter counters or light microscopy with counting chambers or, from 1933, membrane filters (Ehrlich, 1955; Francisco et al., 1973). Coulter counters are useful where samples are uniform and particle-free but where particulates exist miscounting can be a problem, and counting chambers are limited to cell concentrations greater than 10^6 – 10^7/mL, so generally requiring some sort of concentration technique for cells in natural waters (Collins, 1957). Early use of fluorescent dyes attempted to measure total fluorescence of a viewed microscope field and relates this to the cell abundance in that field. This proved problematic as each species of bacteria displayed a different fluorescence/cell count relationship, making the use of any such relationship with mixed environmental samples impossible (Ehrlich and Ehrmantraut, 1955). It also failed to differentiate stained cells from detritus and auto-fluorescence. The use of the fluorescent dye Acridine Orange (AO) with epifluorescence microscopy to just visualize cells was developed by Strugger (1948), and this was adapted for counting cells by Francisco et al. (1973). Daley and Hobbie (1975), further modified the method and Hobbie et al. (1977), published detailed methodology in what has become the prime cited reference for the Acridine Orange Direct Count (AODC) technique.

Since 1977 a number of articles have been published that seek to advance the technique by; investigating alternative fluorochromes, improving cell visualization on membrane filters, reducing the time taken to process samples and increasing the statistical precision of the counts. A variety of new fluorochromes have been tested against AO and, except for specific applications e.g., live/dead and active/inactive staining; only one, (DAPI) has, in the past, been adopted for general use (Maki and Remsen, 1981; Kepner and Pratt, 1994, and references therein). Until recently AO remained the fluorochrome of choice where samples contain particulates, and specifically for sediment samples, where the staining contrast between cells (blue/green) and background detritus (dull orange) is far superior to DAPI. Additionally, even with water samples, DAPI may give reduced counts when compared with AO (Suzuki et al., 1993). However, in recent years there has been increasing use of Sybr green (Shibata et al., 2006; Morono et al., 2009; Schippers et al., 2010), and this has now joined DAPI and AO as being in common usage for cell visualization and enumeration.

Methods to improve cell visualization on the membrane filters have included moving from self staining the polycarbonate membrane using Irgalan Black (Kirchman et al., 1982) or Sudan Black (Pedersen and Ekendahl, 1990) to the adoption of commercially produced black polycarbonate membranes (Lee and Deininger, 1999), ensuring the

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membrane is mounted damp and not dried out (von Münch and Pollard, 1997) and using slide mountants such as Citifluor AF2 to reduce the rate at which AO stained cells fade under UV light (Wynn-Williams, 1985).

Increasing the statistical precision of the data has generally conflicted with the aim of reducing counting time as increasing precision has usually meant increased counting. Hobbie et al. (1977) initially suggested that 10 fields containing, in total, at least 200 cells should be counted on a filter. However, the number of cells that are counted has tended to increase with von Münch and Pollard (1997) counting approximately 30 cells over 1–6 fields and then repeating this at least 12 times on the same filter membrane. Konda et al. (1994) prepared duplicate filters and counted at least 500 cells/filter and Pedersen and Ekendahl (1990) counted up to 600 cells/filter on duplicate filters. Given the time taken to count this number of cells tedium and operator fatigue are recognized problems (Kirchman et al., 1982; Roser et al., 1984: Martens-Habbena and Sass, 2006). Additionally, a degree of expertise is required to accurately identify large numbers of fluorescing particles as bacterial or archaeal cells rather than stained detritus or auto-fluorescing minerals.

Statistical analysis of the various components of cell counting by Kirchman et al. (1982), determined that increasing the number of fields counted beyond seven did very little to decrease sample variance and that increasing the number of filters that were counted was more useful, or if more than one subsample was available then counting only one filter from each of the subsamples was even better. However, these are based on 30 – 50 cells per field. They demonstrated that if the number of cells drops below 20 per field then the coefficient of variance rapidly increases, and here it makes sense to increase the number of fields counted (Montagna, 1982). Our approach to direct counting, based on that of Hobbie et al. (1977), consists of single samples where, from aqueous environmental samples, the number of cells per field is often considerably fewer than 20. Thus we count three filters each up to 200 cells, given a minimum viewing of 20 fields per filter, or 200 fields per filter, whichever occurs first.

This is nevertheless still a time-consuming and tedious procedure requiring expertise and taking experienced counters up to one hour to process a sample and inexperienced individuals substantially longer. The aim of this work is to develop a method to rapidly enumerate cells in more or less particle-free aqueous samples that requires reduced counting, less expertise in identifying bacterial and archaeal cells and saves time.

2. Theory

Many authors have noticed, and demonstrated, that the distribution of cells on a filter is random or approaching random and therefore follows, or approximates to, a Poisson distribution (Jones, 1974; Kirchman et al., 1982; Roser et al., 1984; Jones et al., 1989; Fry, 1990; Kepner and Pratt, 1994; Fischer and Velimirov, 2000). Naturally this is only true where cells are individual rather than clumped or filamentous, and this is one of the conditions of this approach to counting.

The Poisson distribution (Eq. (1)) is a special case of the binomial family of distributions, and occurs when the mean equals the variance. The mean usually

$$P = \frac{\lambda^n e^{\lambda}}{n!} \quad where\ \lambda = mean \tag{1}$$

has a low numerical value and this distribution is used when positive events are rare and random. Using an example from our laboratory, if 0.25 mL of a freshwater sample was prepared for counting, containing 0.5×10^6 cells/mL, then given our laboratory setup where there are a possible 36216 fields to view over the total filterable membrane area then the mean number of cells per field is only 3.45.

The Poisson equation expands into an infinite series of discrete probabilities for all possible numbers of events that can occur, and using the example above, and a standard count of 200 fields, then predicted values can be calculated for the different numbers of cells/field that can occur (Eq. (2)). If it is known that 200 fields were counted and also known how many fields contained 0 cells, 1 cell, 2 cells, etc. then it is possible to back-calculate to obtain the mean number of cells/field (λ) and thus make an estimation of the population density.

$$P = \frac{3.45^0 e^{-3.45}}{0!} + \frac{3.45^1 e^{-3.45}}{1!} + \frac{3.45^2 e^{-3.45}}{2!} + \frac{3.45^3 e^{-3.45}}{3!} + \frac{3.45^4 e^{-3.45}}{4!} +$$

Probability of;	0 cells/field	1 cell/field	2 cells/field	3 cells/field	4 cells/field
	= 0.0317	= 0.1095	= 0.1889	= 0.2173	= 0.1874
at 200 fields counted (probability x 200)					
Number of fields with;	0 cells	1 cell	2 cells	3 cells	4 cells
	= 6	= 22	= 38	= 43	= 37

(2)

Any single term in the Poisson expansion would serve this purpose, and this neatly separates the first term of the series (0 cells/field) from all of the others (1 or more cells/field) and allows a presence/absence approach to counting. Additionally, this first term further simplifies to $e^{-\lambda}$ as both λ^0 and 0! are equal to 1. An equation can then be derived that covers any size field area, any size filterable membrane area and any number of fields counted to produce a population estimate from presence/absence counts. Müller et al. (2011) have already demonstrated, with some complex mathematics, that it is theoretically possible to estimate abundance from such presence–absence maps.

3. Calculation

In the Cardiff laboratory setup for direct counting the total filterable area of the polycarbonate membrane is calculated to be 326 851 300 μm^2 and the sampling field is nominally an indexed grid of 10 × 10 squares measuring 95 μm × 95 μm overall on each side, equaling 9025 μm^2. Thus there are 36216 possible views per filter. Back-calculating from the Poisson term for numbers of occurrences of 0 cells/field the predicted number of cells/filter can be obtained from 36216 × (Ln 200 — Ln [number of 0 cells/field]) when 200 fields/filter are viewed. This can be simplified for general usage by converting natural logs to base 10 logs by multiplying by 2.3026 (Eq. (3)).

$$\text{Cells/filter} = 2.3026\left(\frac{A_T}{A_F}\right)\text{Log}_{10}\left(\frac{F}{Z}\right) \tag{3}$$

Where;

A_T	Total filterable area
A_F	Area of a field
F	Total number of fields viewed
Z	Number of fields containing 0 cells/field

After applying this equation the result is corrected for the volume of sample that was stained and any relevant dilution factor to produce an estimate of total cells/mL of the sample tested.

4. Method

4.1. Sample preparation

Two sets of experimental samples were used to test this procedure. In the first an enrichment pure culture of the Archaean *Methanococoides sp.* was used as the test cell. AODC of this culture gave the population size as (Log) 8.475 or 2.98×10^8 cells/mL. A dilution series was constructed of 9 × 1 mL volumes (M1 to M9) in plastic sterile (soaked in ethanol and dried in a laminar flow cabinet under UV light) Eppendorf vials (Fisher Scientific, Loughborough, UK). Each member of the dilution