

Determination of Bacterial Abundance using DAPI DNA binding stain and Epifluorescence microscopy.

This procedure describes the DAPI (4,6-Diamidino-2-phenylindole) assay for measuring bacterial abundance in seawater.

Definition: Bacterial abundance is given in terms of number of bacterial cells mL⁻¹ seawater.

Principal of analyses: Bacterial cells are preserved, stained with DAPI, concentrated onto a polycarbonate membrane filter and enumerated using UV excitation with epifluorescence microscopy. The individual cells are counted in fields of view of known area and the concentration of bacteria in the original sample is calculated.

Sampling

15 \pm 50 ml samples were collected from Niskin bottles into sterile Falcon tubes and fixed at a final concentration of 1% formalin, allowed to sit 10 minutes, then stored at 4C in the dark. Slides were prepared within 72 hours.

Reagent preparation

* DAPI: Stock solution is 1mg DAPI in 100ml Q water (makes 10ug/ml soln.) Store in freezer. Working solution is a dilution of the stock solution by approximately one half (5ug/ml). Sterile filter (0.2um) and keep working stock in fridge (4C), in dark (cover with foil).

* Irgalan Black: 200mg Irgalan Black, 2ml Acetic Acid (conc.), 98ml 0.2um filtered 2% formaldehyde. Store in fridge.

Slide Preparation

* Stain filters: Pour Irgalan Black solution into sterile dish and load 25mm/0.2um nuclepore filters into the stain, letting sit a minimum of 15 minutes. Filters cannot overstain. Filters are then rinsed in sterile (0.2um filtered) nanopure.

* Remove sample from fridge and invert several times to mix.

* Mount a 0.8um mixed cellulose ester (MCE) backing filter onto 25mm glass filtration tower base, followed by pre-blackened filter. Secure tower onto base using clamp.

* Add sample volume to tower using calibrated pipette.

* Turn on vacuum pump and allow water to filter.

* While water filters, prepare slide. Label with sample ID, mL filtered, and filter tower diameter. Prepare for mounting filter by placing a drop of immersion oil onto slide and smearing with coverslip. Also place a few small drops onto coverslip.

* Stop vacuum when only about 0.5mL of water remains.

* Add 0.5mL DAPI solution to each sample and let stain 3 minutes in the dark.

* Turn on vacuum pump and filter remaining volume. As soon as last liquid runs through, leaving vacuum on, take the blackened filter off the base (leave the backing filter in place, can be used several times) and mount filter onto prepared slide. Coverslip.

* Store slide boxes in Ziploc bag in freezer, with desiccant packs.

Direct counts

All slides were counted using an Olympus AX70 Epifluorescence Microscope housed at BIOS, using 100x oil immersion objective and ultraviolet filter set for DAPI. Bacteria are distinguished by distinct morphologies which brightly fluoresce blue under UV excitation. An eyepiece grid of known area was used during enumeration. A minimum of 10 fields of view are counted for each filter.

Calculations

Bacterial Abundance (cells L⁻¹) = (Cf * R)/Fs

Where:

Cf = mean number of cells per field

R = (active area of filter) / (area of field counted)²

Fs = volume of water filtered (liters)

Quality Control

Accurate measurements of sample filtered and preservative added are critical for accurate estimates. Use of calibrated pipettes is important.

Accurate, repeatable enumeration of cells by eye requires experience and a good microscope. New enumerators are cross trained with experienced microscopists by counting the same samples until reliable and consistent results are obtained.

There is no absolute standard for bacterial counts. Precision declines if too few or too many cells are concentrated on the filter. The amount of water filtered is a function of expected cell number. Immediately following slide preparation samples should be examined to ensure proper number of cells per field and even distribution. For our samples, approximately 30-60 cells per field on a 10x10um grid is ideal for counting.

References

BATS Methods Manual. Chapter 17. Determination of Bacterial Abundance. Updated by K.Orcutt 4/1997, pp. 111-114.version 4.

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