Bacteria Enumeration by Epifluorescence Microscopy using DAPI Stain
Florida Coastal Everglades LTER

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Equipment:

- Leica Epifluorescent microscope with a Hg bulb, blue filter (BG 38) and ocular quadricule divided into 100-grid square.
- 100 µl, 1000 µl and 5 ml adjustable pipettes.
- Filter towers.
- Vacuum pump.

Supplies:

- 0.2-µl pore-size, 25-mm diameter, polycarbonate membrane filters.
- Polypropylene bottles for sampling.
- Microscope slides.
- 25 x 25 mm cover glass.
- Non-fluorescence immersion oil.
- Glass 20 ml scintillation vials.

Solutions and Reagents:

- Phosphate buffered saline (PBS) solution:
  5 mM $NaH_2PO_4.H_2O$ (M.W.137.99)
  5 mM $Na_2HPO_4.7H_2O$ (M.W. 268.07)
  130 mM NaCl (M.W. 58.44)

  1. For 1L dissolve 0.69 g $NaH_2PO_4.H_2O$, 1.34 g $Na_2HPO_4.7H_2O$ and 7.6 g NaCl in 800 ml of distilled water.
  2. Adjust pH to 7.5 with 1N HCl or 1N NaOH (1N 40g NaOH - 1 L)
  3. Bring to 1 L with distilled water.
  4. Autoclave at 121°C for 15 minutes.
  5. Store PBS in the refrigerator between uses.

- 20% PBS buffered formaldehyde fixing agent for bacterial samples.

  1. For 1 L add 540.5 ml of 37% formaldehyde to 459.5 ml PBS buffer. Store this solution in a sterile 1000 ml container.
  2. Filter sterilized the 20% PBS formaldehyde solution through a 0.2 µm filter into a 50 ml centrifuge tubes.

- Preparation of DAPI (M. W. 350.25) formula $C_{16}H_{17}Cl_2N_5$

1. Wrap a new glass scintillation vial in aluminum foil.
2. Add 10 mg (whole container) of DAPI into the glass vial, and then add 20 ml of sterile, distilled water.
3. Keep vial at 4ºC.

CAUTION: Use caution when using DAPI; it can potentially bind with your own DNA if you spill it on yourself. A pair of latex gloves is adequate protection while using DAPI.

1. Sample collection

1. Water samples are collected in clean, sample-rinsed dark polypropylene bottles filled by hand.
2. Samples are kept on ice during the sampling day.
3. Samples are stored at 4ºC in the laboratory and processed the next day.

2. Sample preservation and staining

2.1 Sample Preservation

1. Use 1 ml PBS buffered formaldehyde per 10 ml sample (final concentration of formaldehyde in sample is 2%).
2. Mix each sample thoroughly.
3. Store samples at room temperature for no more than 2 weeks before making slides.

2.2 Sample staining.

1. Soak black polycarbonate membrane filters (0.2 μm pore size) in sterile, distilled water prior to use.
2. Place filters shine side up on filter manifold.
3. Add 5 ml of sterile, distilled water in filtration well.
4. Add 0.5 ml of sample to the filtration well.
5. Add 25 μl DAPI in the filtration well.
6. Cover the filter tower with dark polypropylene bottles (to block any light from hitting the filter) and allow standing for 20 minutes.
7. After 20 minutes, gently vacuum away the solution on top of the filter.
8. Rinse away any unincorporated stain with 5 ml of sterile, distilled water.
9. Place 1 drop of sterile, distilled water on a clean microscope slide and place the filter (sample side up) onto slide.
10. Place 1 drop of non-fluorescent immersion oil on top of the filter and cover with a cover slip.
11. Keep the slides in the dark until they can be counted on the epifluorescent microscope.

2.3 Bacterial Enumeration

1. Place a drop of immersion oil on top of the coverslip and place the slide onto the microscope stage.
2. Bacteria densities (with the proper dilution) should be at least 30 organisms per field.
3. Count at least 10 fields (to achieve a final count of 300 bacterial cells).
4. Calculate final bacterial densities using the following equation (from Wetzel and Likens, 1991).

Bacteria ml^{-1} = (membrane conversion factor * ND)
Membrane conversion factor = Filtration area/area of micrometer field
N = Total number of bacteria counted/number of micrometer fields counted
D = Dilution factor; volume of sample stained/total volume of sample available

Selected Citations:

Lebaron, P, Catala, P, and Parthuisot, N. 1998. Effectiveness of SYTOX green stain for bacterial viability assessment. Applied and Environmental Microbiology. 64:2697-2700.

Noble, RT, and Fuhrman, JA. 1998. Use of SYBR Green I for rapid epifluorescence counts of marine viruses and bacteria. Aquatic Microbial Ecology. 14: 113-118.

Weinbauer, MG, Beckman, C, and Höfle, MG. 1998. Utility of green fluorescent nucleic acid dyes and aluminum oxide membrane filters for rapid epifluorescence enumeration of soil and sediment bacteria. Applied and Environmental Microbiology. 64:5000-5003.

Wetzel, RG and Likens, GE. 1991. Limnological Analyses, Second Edition. Springer-Verlag. 391 pp.