Microbial Productivity from Aquatic Samples

Author: MW Howard, Ph.D

Objective:

The purpose of this protocol is to enumerate microbial cells in water from various domains of NEON within the United States using propidium iodide (PI) staining, black filter filtration, and epifluorescence microscopy. Data from this protocol will be used to determine microbial productivity from sites across the United States.

This protocol is an on-slide staining technique adapted from Boulos et al.[1]; modifications include change of fluorophore and concentration, mounting media and volume of sample filtered.

Materials and Equipment

Record all materials and reagents used in support of testing below:

Required Reagents:

- Propidium Iodide (30 µM working stock)
- MilliQ water (particle-free or 0.22 µm filtered water)

Required Equipment:

- Fume Cabinet
- Oven (to dry glassware)
- 47-mm and/or 25-mm glass vacuum filter holders (including fritted glass funnel and base, aluminum clamp and stopper)
    - All glassware must be particle free.
    - Wash glassware using Alconox, rinse in copious amounts of MilliQ water and dry in oven prior to use
- Black polycarbonate membranes (47-mm or 25-mm as appropriate)
- Vacuum manifold and pump
- Pipettes
- ProLong Diamond (or similar) aqueous mounting media
- Coverglass and slides
- Immersion oil
- Epifluorescence microscope

Procedure:

1) **Record** all sample names, volume filtered and other information as needed**.**

[1] Boulos et al. J. Microbial Methods 37(1):77-86 (1999)

2) **Staining:**

a. Thaw working staining solution (30 µm PI). **This stain is light sensitive**; thaw aliquot in the dark (or covered completely with foil).

b. Soak black filters in MilliQ water for 1-5 minutes prior to use. Take note of which orientation the filters are in when placed for soaking. The shiny vs non-shiny side of the filter will not be able to be identified after soaking.

c. Place filters shiny side up on the filter manifold.

d. *Critical Step:* Filter sample
   i. Add appropriate volume of each sample (or dilution). This should be recorded and is needed to calculate microbial productivity.
   ii. Turn on vacuum and slowly filter sample at low (<10 psi) vacuum until all liquid has been pulled through the filter. **Filter to near-dryness, but do not dry the filter – this will result in poor images.**

e. Stain Sample
   i. This step must occur in the dark. Make sure to work in a dark fume hood or dark area on the bench.
   ii. Gently remove filter from apparatus with forceps and lay the filter face up on the slide.
   iii. Place slide in dark humid box (sealable box lined with damp paper towels/kimwipes and covered with foil).
   iv. Stain for **10 minutes +/- 1 minutes** in the dark.
   v. Stop staining by folding a dry kimwipe over itself a few times. Put the slide on the kimwipe and hold it at a slight angle, just enough for the solution to soak gently into the kimwipe.
   vi. Immediately place 20 µL of ProLong Diamond Antifade Mountant onto the center of the filter and place a coverslip on top. Wrap the slide and coverslip in a kimwipe, then gently but firmly press any excess mountant out the edges and soak it up in the kimwipe. Try not to slide the coverslip relative to the slide at this point.
   vii. Allow ProLong to cure for 24 hr at room temperature (RT) in the dark. Slides should be stored at -20 ^{o}C in slide box for long term storage after 24 hours.

3) Capture Images from stained slides.

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a. Observe slides within 5-7 days.
b. Take images on microscope using a 40X or 100X (preferred) objective lens and save in appropriate data file folder. Image fields should be randomly selected from across the filter. Name images such that they can be tracked back to the specific slide they are acquired from.

Note:

- Counting will be from recorded images, not through the oculars. Analysis of samples is based on the images, so it is imperative to take numerous *in focus* images.
- When taking images, use the fine focus adjustment to maintain focus – but make certain that all images are taken from the same focal plane.
- Images should be of organisms on the filter and not those in between the filter and the coverslip.

- Avoid collecting fields near the edge of the filter and around any air bubbles. If a filtered sample has many blank areas or air bubbles, it may indicate a filtration problem and should be noted.

4) Assisted Automated Image Analysis using ImageJ

Note*:* Bacteria densities will range, but generally should be between 5-250 cells per field.

a. Open images using ImageJ software
b. Examine the focus of each image per set
    - Select images that are totally in focus and can be easily adjusted

c. In ImageJ, adjust the parameters of each image for consistent background by selecting "Image" → "Adjust" → "Brightness and Contrast"

d. Select a contrast range and annotate it on the analysis paperwork

    i. Have multiple images from the same set opened simultaneously
    ii. Check the setting and ensure that the background staining is eliminated uniformly from each image to be counted
    iii. *Note: the contrast adjustment will vary between different samples and magnifications, but MUST be the same on all images taken for each slide and magnification.* Be sure to adjust each set before beginning analysis

e. In the toolbar, select "Image" → "Adjust" → "Color Threshold"

f. Adjust the selections in the manager and annotate the values for Saturation and Brightness of each image analyzed

g. Observe each image and adjust each set consistently. *Do not adjust the Hue setting. This will only change the intensity of the color and not the threshold.*

h. Once all appropriate adjustments are complete, select "Analyze" → "Analyze Particles"

    i. Type in the range 20-2000 as the particle size range to be counted. This is a blanket approach to limit the number of objects identified as microbial cells to those between 20 and 2000 pixels^2 with no relationship to magnification.

    ii. Click on "Show Overlay Masks," "Summary," and "Display Results"

    iii. The number of counted particles will appear in the "Summary" tab and individual areas are tabulated in "Display Results"

    Required if an image has a scale bar: When looking at the image overlay, be sure check the scale bar and omit it from the analysis. This is necessary as the image scalebar is counted as an object when performing the analysis.

i. Record all particles that the software counts, and count any particles that the software has missed.

i. For example, if the software counts 10 particles, put this number in the 'Counted' column
ii. If there are 3 additional particles that should be included, count them manually and put them in the 'Additional' column.
iii. Calculate the total number of particles for each image by adding the 'counted' and 'Additional' columns.

j. Repeat procedures c through k for all images for each slide across all domains analyzed

5) Determine Microbial Productivity.

Calculate Microbial Cell counts using data captured in step 4 and the following formula:

$$Cf = \frac{(Average\ number\ of\ cells/field)\times R}{Fs}$$

Where:

Cf = average number cells/mL
R = active area of filter/area of field counted
Fs = volume of initial sample filtered
- For example, if 1 mL of sample is filtered, Fs=1, if 200 μL of sample is filtered, Fs = 0.2
- Fs must consider any dilution made during sample processing for an accurate number to be calculated.

Filter Size	**Active Filter Area** (cm^2)	**Area of Image Counted (**cm^2)	**Objective Used**	**Conversion factor (R)*** **
47 mm	9.6	4.86 E-05*	100X	197,530.86
	9.6	8.6 E-05**	40X	111,636.60
25 mm	2.1	4.86E-05*	100X	43,209.88
	2.1	8.60E-05**	40X	24,420.51

* Images are 80.56 µm x 60.36 µm using the 100X objective
** Images are 107.13 µm x 80.27 µm using the 40X objective
*** R factor calculated for a specific Zeiss Axiovision A2 Epifluorescence Microscope and color AxioCam MRc5 camera and calibrated Axiovision software. R factor must be calculated for each microscope and camera used for image capture and analysis.