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Methodology

Overview:
Algae are a heterogeneous group of microorganisms that include prokaryotes and eukaryotes with one common characteristic: namely, they all contain chloroplasts, which allow them to photosynthesize. In the environment, algae can cause problems for swimming pool owners by growing in the water. Algae can also cause problems in surface waters, such as lakes and reservoirs, due to algal blooms that release toxins. More recently, algae are being evaluated as novel sources of energy via algal biofuels. Blue green algae are actually bacteria classified as cyanobacteria. Cyanobacteria not only photosynthesize, but also have the ability to fix nitrogen gas from the atmosphere. Other algae are eukaryotic, ranging from single-celled organisms to complex multicellular organisms, like seaweeds. These include the green algae, the euglenoids, the dinoflagellates, the golden brown algae, diatoms, the brown algae, and the red algae. In soils, algal populations are frequently 10^6 per gram. These numbers are lower than corresponding numbers for bacteria, actinomycetes, and fungi, mostly because the sunlight required for photosynthesis cannot penetrate far beneath the soil surface.

Because algae are phototrophic, obtaining energy from photosynthesis and carbon for biomass from carbon dioxide, they can be grown in growth media consisting entirely of inorganic nutrients and without an organic carbon substrate. The lack of organic substrate precludes the growth of heterotrophic bacteria. Using an inorganic growth medium, algae originally present in soil or water can be quantitated by the most probable number (MPN) method. The MPN method relies on successively diluting a sample, such that the algae themselves are diluted to extinction. The presence of algae in any dilution is determined by a positive sign of growth in the medium, which is typically a green slime of algae that results from photosynthesis. Use of replicate tubes at each dilution and a statistical evaluation of the number of tubes positive for growth at any given dilution allows for the number of algae present in the original sample to be calculated. MPN tables have been developed and published specific to a particular MPN design, including the number of replicates used at each dilution.

Procedure:
1. Weigh out a 10 g sample of soil that has been kept moist for 2-3 days.

2. Prepare a 10-fold dilution series by adding the 10 g of soil into 95 ml of Modified Bristol's Solution (**Figure 1**).

    2.1. To create Modified Bristol's Solution, dissolve the following in 1,000 ml of tap water: 0.25g $NaNO_3$, 0.025g $CaCl_2$, 0.075g $MgSO_4^- \cdot 7H_2O$, 0.075g K_2HPO_4, 0.018g KH_2PO_4, 0.025g NaCl, and 0.5 mg $FeCl_3$.

3. Continue the dilution series by the addition of 1 ml of Suspension A to 9 ml of Bristol's

Solution and additional sequential dilutions.

4. Inoculate 5 replicate tubes of Bristol's Solution with 1 ml each, for the dilutions 10^{-2} to 10^{-6} (**Table 1**).

5. Incubate the capped tubes for up to 4 weeks in an area exposed to sunlight.

6. Observe the tubes for algal growth once every 7 days. Tubes with algal growth appear green.

Representative Results:

Figure 2 is an example of representative results.

p_1 is chosen to be the number of replicate tubes of the highest dilution (least concentrated in soil) that has the highest number of positive tubes. Here, the replicates from Tube B do not count, because those of Tube C are from a higher dilution. In contrast, the number of tubes from Tube D that show a positive sign of growth is less than those from Tube C. So, p_1 = 5.

p_2 and p_3 are chosen to be the number of tubes in the next two higher dilutions that show a positive sign of growth. Thus, p_2 = 3 and p_3 = 1.

The value for p_1 can be found by looking down the first column in **Table 2**. The same is done in the p_2 column. Then, the value of p_3 (across the top) intersects the two defined by the values of p_1 and p_2. In this example, the value is 1.1 organisms ml^{-1}.

Dividing the value for p_3 by the concentration of soil in the dilution leads to p_2. In this example, this is Tube D.

$$\frac{1.1 \text{ organisms}}{ml} \text{ x } \frac{1 \text{ ml}}{10^{-4} \text{ g soil}} = 1.1 \text{ x } 10^4 \text{ organisms per g soil}$$

Thus, in this example, there were 1.1 x 10^4 algae cells per g of soil.

Applications:

The MPN methodology is useful, because it allows estimation of a functional population based on a process-related attribution. In the example, the functional process was photosynthesis undertaken by algae, which allowed for growth in the absence of organic carbon. This allowed for total algal populations in soil to be enumerated.

MPN is also used to estimate the number of a particular type of microbial pathogens in water, such as *Salmonella*, utilizing the resistance of *Salmonella* to malachite green.

A further application is the estimation of mycorrhizal fungi by inoculating soil dilutions onto a plant host and looking for root colonization by the fungi.
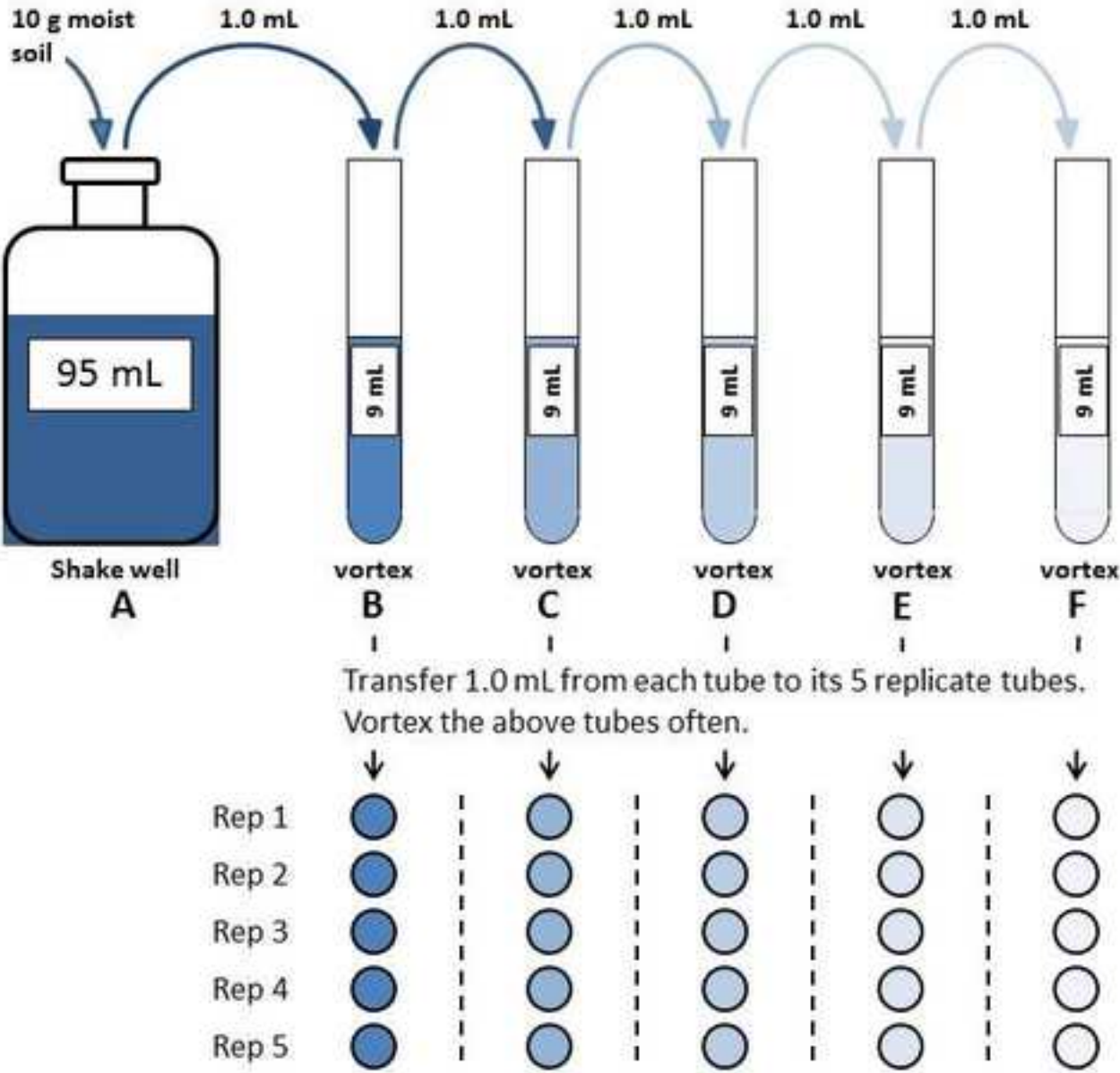
Legend:
Figure 1: How to make a 10-fold dilution series.

Figure 2: Hypothetical outcome of an algae enumeration experiment. Shaded tubes indicate the presence of algae. Unshaded tubes represent the absence of algae.

Table 1: Tubes and dilutions.

Table 2: Most probable numbers for use with the experimental design in this exercise.

Make a 10-fold dilution series

	10^{-2} B	10^{-3} C	10^{-4} D	10^{-5} E	10^{-6} F
Rep 1	●	●	○	○	○
Rep 2	●	●	●	○	○
Rep 3	●	●	○	●	○
Rep 4	●	●	●	○	○
Rep 5	●	●	●	○	○
		$P_1 = 5$	$P_2 = 3$	$P_3 = 1$	

TUBE	DILUTION
B	10^{-2}
C	10^{-3}
D	10^{-4}
E	10^{-5}
F	10^{-6}

$p1$	$p2$	Most probable number for indicated values of p_3					
		0	1	2	3	4	5
0	0	--	0.018	0.036	0.054	0.072	0.090
0	1	0.018	0.036	0.055	0.073	0.091	0.11
0	2	0.037	0.055	0.074	0.092	0.11	0.13
0	3	0.056	0.074	0.093	0.11	0.13	0.15
0	4	0.075	0.094	0.11	0.13	0.15	0.17
0	5	0.094	0.11	0.13	0.15	0.17	0.19
1	0	0.020	0.040	0.060	0.080	0.10	0.12
1	1	0.040	0.061	0.081	0.10	0.12	0.14
1	2	0.061	0.082	0.10	0.12	0.15	0.17
1	3	0.083	0.1	0.13	0.15	0.17	0.19
1	4	0.11	0.13	0.15	0.17	0.19	0.22
1	5	0.13	0.16	0.17	0.19	0.22	0.24
2	0	0.045	0.068	0.091	0.12	0.14	0.16
2	1	0.068	0.092	0.12	0.14	0.17	0.19
2	2	0.093	0.12	0.14	0.17	0.19	0.22
2	3	0.12	0.14	0.17	0.20	0.22	0.25
2	4	0.15	0.17	0.20	0.23	0.25	0.28
2	5	0.17	0.20	0.23	0.26	0.29	0.32
3	0	0.078	0.11	0.13	0.16	0.20	0.23
3	1	0.11	0.14	0.17	0.20	0.23	0.27
3	2	0.14	0.17	0.20	0.24	0.27	0.31
3	3	0.17	0.21	0.24	0.28	0.31	0.35
3	4	0.21	0.24	0.28	0.32	0.36	0.40
3	5	0.25	0.29	0.32	0.37	0.41	0.45
4	0	0.13	0.17	0.21	0.25	0.30	0.36
4	1	0.17	0.21	0.26	0.31	0.36	0.42
4	2	0.22	0.26	0.32	0.38	0.44	0.5
4	3		0.33	0.39	0.45	0.52	0.59
4	4	0.34	0.40	0.47	0.54	0.62	0.69
4	5	0.41	0.48	0.56	0.64	0.72	0.81
5	0	0.23	0.31	0.43	0.58	0.76	0.95
5	1	0.33	0.46	0.64	0.84	1.1	1.3
5	2	0.49	0.7	0.95	1.2	1.5	1.8
5	3	0.79	1.1	1.4	1.8	2.1	2.5
5	4	1.3	1.7	2.2	2.8	3.5	4.3
5	5	2.4	3.5	5.4	9.2	16	--