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Performing a Nutrient Diffusion Substrata Test

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Performing a Nutrient Diffusion Substrata Test

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A White Paper presented to Oglala Lakota College for use in pre-engineering class exercises.

Table of Contents

INTRODUCTION

Available nutrients, primarily nitrogen (N) and phosphorus (P), control the growth of periphyton in lakes and streams. The nutrient diffusing substrata (NDS) is an economic and replicable way to determine whether an aquatic environment is limited by a particular nutrient (Capps et al., 2011). The NDS commonly uses clay pots, plastic cups, or periphytometers (Rugenski et al., 2008). The NDS in this paper uses plastic cups based on *Methods in Stream Ecology* (Hauer and Lamberti, 2007). The agar solution is mixed with 8 different types of nutrients in Table 1. Each nutrient type has 6 replicates. The control is an agar solution with no nutrient addition.

Table 1. Nutrient Composition for the NDS Test

No.	Types of Nutrient	Nutrient Concentration
1	Control	None
2	Ammonium-N	0.5 M NH_4Cl
3	K based Nitrate-N	0.5 M KNO_3
4	Na based Nitrate-N	0.5 M $NaNO_3$
5	Orthophosphate	0.5 M KH_2PO_4
6	Ammonium-N and orthophosphate	0.5 M NH_4Cl and 0.5 M KH_2PO_4
7	K based Nitrate-N and orthophosphate	0.5 M KNO_3 and 0.5 M KH_2PO_4
8	Na based Nitrate-N and orthophosphate	0.5 M $NaNO_3$ and 0.5 M KH_2PO_4

The mixture is poured into polyethylene containers with a glass crucible cover so that the nutrient enriched agar solution diffuses through a permeable glass crucible cover, and algae grow on the surface of it. After the NDS containers are placed in the stream for 2 to 3 weeks, algae growth is measured as *chlorophyll a*. Chlorophyll is bound within most living plant cells as a key biochemical component for photosynthesis. Algae and other phytoplankton are the most common photosynthetic organisms in surface water. Chlorophyll exists in living cells in four forms: a, b, c, and d, with *chlorophyll a* being the most dominant. Limiting nutrient is determined based on the impact of the nutrient on the relative *chlorophyll a* production using a one-way analysis of variance (ANOVA) tool as shown in Table 2.

Table 2. Determination of Limiting Nutrient in the NDS Test

Interpretation	N impact	P impact	Interaction N x P
N limited	☑		
P limited		☑	
N and P colimited			☑
N and P colimited	☑	☑	
N and P colimited	☑	☑	☑
1st N limited, 2nd P limited	☑		☑
1st P limited, 2nd N limited		☑	☑
Not limited by N or P			

NDS PREPARATION

1. The following apparatus and materials are needed.

- 1 oz polyethylene hinged-lid containers
- BioReagents™ granulated agar
- Potassium nitrate, KNO_3
- Potassium phosphate, monobasic, KH_2PO_4
- Hot plate & stirrer
- Volumetric flasks
- Plastic L-bars
- PVC pipes and fittings

- 2.7cm glass crucible covers
- Ammonium chloride, NH_4Cl
- Sodium nitrate, $NaNO_3$
- Balance
- Distilled water
- Beakers
- Cable ties
- Aluminum foil

2. Cut out the container lid to fit a 2.7cm glass crucible cover as shown in Figure 1.

Figure 1.   Preparation of NDS Containers

3. Table 3 summarizes 8 different types of nutrient mixtures used in NDS. Each nutrient type has 6 replicates. Label each container accordingly.

Table 3. NDS Nutrient Solution Preparation

No.	Types of Nutrient	Chemical Composition	Replicates
1	Control	20 g/L agar	6
2	Ammonium-N	20 g/L agar, 26.75 g/L NH_4Cl	6
3	K based Nitrate-N	20 g/L agar, 50.55 g/L KNO_3	6
4	Na based Nitrate-N	20 g/L agar, 42.49 g/L $NaNO_3$	6
5	Orthophosphate	20 g/L agar, 68.04 g/L KH_2PO_4	6
6	Ammonium-N and orthophosphate	20 g/L agar, 26.75 g/L NH_4Cl, 68.04 g/L KH_2PO_4	6
7	K based Nitrate-N and orthophosphate	20 g/L agar, 50.55 g/L KNO_3, 68.04 g/L KH_2PO_4	6
8	Na based Nitrate-N and orthophosphate	20 g/L agar, 42.49 g/L $NaNO_3$, 68.04 g/L KH_2PO_4	6

4. Preparation of "Control": Weigh 4 g of agar. Boil 200 mL of distilled water on a hot plate and stir. Add 4g of agar to distilled water while stirring. Mix solution until it turns from a cloudy to a transparent amber color. Remove the agar solution from heat and fill 6 containers. Let them cool. Close lids and keep the containers in the refrigerator.

5. Preparation of "Ammonium-N": Weigh 4g of agar and 5.35 g of NH_4Cl. Boil 200 mL of distilled water on a hot plate and stir. Add agar and NH_4Cl to distilled water while stirring. Mix solution until it turns from a cloudy to a transparent amber color. Remove the agar solution from heat and fill 6 containers. Let them cool. Close lids and keep the containers in the refrigerator.

6. Repeat Step 4 for each nutrient type.

7. Once all the containers have been filled and cooled to room temperature, securely fasten them to L-bars using cable ties and weighted PVC frame as shown in Figure 2. CAUTION: If a surface of the glass crucible cover is exposed to air for more than an hour, the agar and nutrient solution will dry. If drying occurs, discard the nutrient solution and make a new one.

Figure 2. NDS Containers in a PVC Frame

8. Wrap the NDS containers in aluminum foil and keep them in the refrigerator.

NDS FIELD INSTALLATION

Set up the NDS in the stream with the following consideration:

- All the containers should be completely submerged for the entirety of the test.
- The glass crucible covers should be close enough to the surface so that sunlight can penetrate water and promote algae growth.
- Both the containers and PVC frame should be secured in the stream during the test.
- The NDS should be installed in a place to minimize sediment build-up on top of the glass crucible covers.

The NDS should remain in the water for a minimum of 14 days until algae growth is visible. When the test is complete, take out the NDS from the stream, wrap it in aluminum foil, and keep in the refrigerator. CAUTION: If algae on the glass crucible cover dry or are exposed to direct sunlight, the *chlorophyll a* will degrade. If this happens, the test shall be discarded and repeated.

CHLOROPHYLL MEASUREMENT

The chlorophyll can be measured by a spectrophotometer, fluorometer, and high-performance liquid chromatography. The *chlorophyll a* concentration can be determined by either Jeffrey and Humphrey's trichromatic equation or Lorenzen's pheopigment-corrected *chlorophyll a* equation. This paper presents the spectrophotometer method using trichromatic equation based on *Standard Methods for the Examination of Water and Wastewater, 22nd Edition* (Rice et al., 2012) and *EPA Method 446.0* (Arar, 1997). The chlorophyll degrades under direct sunlight. Avoid exposure to light during the test. The following apparatus and materials are needed.

- Mortar and pestle
- Spectrophotometer with less than 2 nm band width
- Centrifuge
- Polyethylene centrifuge tubes

- Acetone
- 1 cm cuvettes
- Aluminum Foil
- Pipettes

1. Take out the permeable glass crucible cover using tweezers as shown in Figure 3. Avoid touching algae and do not wash out sediments. If the chlorophyll analysis is delayed, wrap the sample in aluminum foil and keep in dark freezer for up to 3 weeks.

Figure 3. Glass Crucible Cover after a Field NDS Test

2. Place the glass crucible cover in the mortar. Crush it with the pestle until it is a sandy consistency.

3. Move the crushed mixture into a clean polyethylene centrifuge tube. Transfer all the remains in the mortar to the centrifuge tube by washing with acetone. The total volume of acetone should be 10 mL. Close the centrifuge tube and wrap it in aluminum foil. Leave it in a dark refrigerator for more than 4 hours and less than 24 hours to extract chlorophyll to acetone.

4. Place the tube into the centrifuge and spin it for 20 minutes at 10,000 rpm. The centrifuge must have a lid so that the samples are not exposed to light. Carefully take out the tube from the centrifuge.

5. Transfer 3 mL of the supernatant from centrifugal tube to a 1 cm cuvette.

6. Set the wavelength to 750 nm. Zero the absorbance reading with acetone as a blank. Record the absorbance of a sample. If the absorbance at 750 nm is greater than 0.005, filter the mixture using 0.45 μm membrane filter and polyethylene syringe filter holder.

7. Set the wavelength to 664 nm. Zero the absorbance reading with acetone as a blank. Read the absorbance of a sample. If the absorbance reading is less than 0.1, use greater width of cuvette for absorbance reading or extract chlorophyll with less volume of acetone than 10 mL in Step 3. The minimum acetone volume is 5 mL.

8. Set the wavelength to 647 nm. Zero the absorbance reading with acetone as a blank. Read the absorbance of a sample.

9. Set the wavelength to 630 nm. Zero the absorbance reading with acetone as a blank. Read the absorbance of a sample.

10. Calculate the *chlorophyll a* concentration using the following equation.

$$Chloropyll\ a\ (\mu g/cm^2) = \frac{(11.85 \times ABS_{664} - 1.54 \times ABS_{647} - 0.08 \times ABS_{630}) \times V}{A \times L}$$

Where, ABS_x = Absorbance at wavelength x nm − Absorbance at 750 nm
V = Volume of acetone used for extraction (mL)
A = Surface area of a glass crucible cover exposed for algae growth (cm^2)
L = Cuvette light path width (cm)

ANOVA ANALYSIS

The ANOVA is performed to determine whether the specific nutrient has significant impact on algae growth. The chlorophyll values for each nutrient type are compared with those of the control. The ANOVA is available on statistical software as well as Microsoft Excel. This paper presents the Excel ANOVA analysis.

1. Arrange the *chlorophyll a* values on Excel worksheet as shown in Table 4.

   Table 4. Example of the ANOVA Analysis Set-up on Microsoft Excel

Control	Nutrient Type to be compared
Replicate 1	Replicate 1
Replicate 2	Replicate 2
Replicate 3	Replicate 3
Replicate 4	Replicate 4
Replicate 5	Replicate 5

2. Make sure that the analysis tool pack Excel add-on is active. Click the Data Ribbon. Select Data Analysis and then select "Anova: Single Factor" from the Data Analysis pop-up window.

3. For Input Range, select the values in Table 4. Determine the Output Options; a table similar to Table 5 will be produced.

   Table 5. Example of the Output of the ANOVA Excel Analysis.

   Anova: Single Factor

   SUMMARY

Groups	Count	Sum	Average	Variance		
Control	-	-	-	-		
Nutrient Type	-	-	-	-		

   ANOVA

Source of Variation	SS	df	MS	F	P-value	F_{crit}
Between Groups	-	-	-	-	-	-
Within Groups	-	-	-			
Total	-					

4. The ANOVA Excel analysis produces more information; however, record only the values for F and F_{crit}.

5. Summarize the ANOVA analysis result with the F and F_{crit} values similar to Table 6. The 95% confidence interval is the default and recommended. For the specific nutrient type compared to the control, greater F value than F_{crit} indicates that the nutrient type has a significant impact on algae growth.

Table 6. Example of the ANOVA Excel Analysis Result at 95% Confidence Interval

Nutrient Type	F value	F_{crit} value	Significant Difference?
Ammonium-N	-	-	Is F > F_{crit} (Y/N)
K based Nitrate-N	-	-	Is F > F_{crit} (Y/N)
Na based Nitrate-N	-	-	Is F > F_{crit} (Y/N)
Orthophosphate	-	-	Is F > F_{crit} (Y/N)
Ammonium-N and orthophosphate	-	-	Is F > F_{crit} (Y/N)
K based Nitrate-N and orthophosphate	-	-	Is F > F_{crit} (Y/N)
Na based Nitrate-N and orthophosphate	-	-	Is F > F_{crit} (Y/N)

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