

**South Dakota State University**  
**Open PRAIRIE: Open Public Research Access Institutional  
Repository and Information Exchange**

---

Civil and Environmental Engineering Faculty  
Publications

Department of Civil and Environmental  
Engineering

---

2-2015

## Performing a Nutrient Diffusion Substrata Test

Dillon J. Devitt

*South Dakota State University*, [dillon.j.devitt@sdstate.edu](mailto:dillon.j.devitt@sdstate.edu)

Kyungnan (Karen) Min

*South Dakota State University*, [kyungnan.min@sdstate.edu](mailto:kyungnan.min@sdstate.edu)

Suzette R. Burckhard

*South Dakota State University*, [Suzette.Burckhard@sdstate.edu](mailto:Suzette.Burckhard@sdstate.edu)

Joanita M. Kant

*South Dakota State University*, [Joanita.Kant@sdstate.edu](mailto:Joanita.Kant@sdstate.edu)

Follow this and additional works at: [https://openprairie.sdstate.edu/cvlee\\_pubs](https://openprairie.sdstate.edu/cvlee_pubs)

 Part of the [Civil and Environmental Engineering Commons](#)

---

### Recommended Citation

Devitt, Dillon J.; Min, Kyungnan (Karen); Burckhard, Suzette R.; and Kant, Joanita M., "Performing a Nutrient Diffusion Substrata Test" (2015). *Civil and Environmental Engineering Faculty Publications*. 3.  
[https://openprairie.sdstate.edu/cvlee\\_pubs/3](https://openprairie.sdstate.edu/cvlee_pubs/3)

This White paper is brought to you for free and open access by the Department of Civil and Environmental Engineering at Open PRAIRIE: Open Public Research Access Institutional Repository and Information Exchange. It has been accepted for inclusion in Civil and Environmental Engineering Faculty Publications by an authorized administrator of Open PRAIRIE: Open Public Research Access Institutional Repository and Information Exchange. For more information, please contact [michael.biondo@sdstate.edu](mailto:michael.biondo@sdstate.edu).

The background of the cover is a photograph of a grassy area with several trees. In the distance, a body of water and a building are visible. The text is overlaid on this image.

# **Performing a Nutrient Diffusion SubStrata Test**

**Dillon Devitt, Kyungnan (Karen) Min,  
and Suzette R. Burckhard**

*Edited by Joanita M. Kant*

Published by South Dakota State University (SDSU), Jerome J. Lohr College of Engineering, Civil and Environmental Engineering Department, and Water and Environmental Engineering Research Center, Brookings, SD 57007.

© 2015 by South Dakota State University, Brookings, South Dakota, USA.

All rights reserved, including the right to reproduce any part of this book in any form, except brief quotations, without written permission from the publisher.

This material is based upon work supported by the National Science Foundation under Grant No. (NSF 1037708). Any opinions, findings, and conclusions or recommendations expressed in this material are those of the author(s) and do not necessarily reflect the views of the National Science Foundation or of the colleges and universities with which the authors are affiliated.

**[www.sdstate.edu/weerc/](http://www.sdstate.edu/weerc/) (choose publications link)**

Cover photo: Site of former water works at Lake Kampeska, Watertown, South Dakota, 2007 (photo credit: Joanita M. Kant).

# Performing a Nutrient Diffusion Substrata Test

Dillon Devitt, Kyungnan (Karen) Min, and Suzette R. Burckhard

Edited by Joanita M. Kant

South Dakota State University

Jerome J. Lohr College of Engineering

Department of Civil and Environmental Engineering and

Water and Environmental Engineering Research Center

February 2015

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

## Table of Contents

INTRODUCTION .....	1
NDS PREPARATION .....	2
NDS FIELD INSTALLATION .....	4
CHLOROPHYLL MEASUREMENT .....	5
ANOVA ANALYSIS .....	7
ACKNOWLEDGEMENTS .....	8
REFERENCES .....	8

## 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 <sub>4</sub> Cl
3	K based Nitrate-N	0.5 M KNO <sub>3</sub>
4	Na based Nitrate-N	0.5 M NaNO <sub>3</sub>
5	Orthophosphate	0.5 M KH <sub>2</sub> PO <sub>4</sub>
6	Ammonium-N and orthophosphate	0.5 M NH <sub>4</sub> Cl and 0.5 M KH <sub>2</sub> PO <sub>4</sub>
7	K based Nitrate-N and orthophosphate	0.5 M KNO <sub>3</sub> and 0.5 M KH <sub>2</sub> PO <sub>4</sub>
8	Na based Nitrate-N and orthophosphate	0.5 M NaNO <sub>3</sub> and 0.5 M KH <sub>2</sub> PO <sub>4</sub>

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	☑	☑	☑
1 <sup>st</sup> N limited, 2 <sup>nd</sup> P limited	☑		☑
1 <sup>st</sup> P limited, 2 <sup>nd</sup> 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<sub>3</sub>
- Potassium phosphate, monobasic, KH<sub>2</sub>PO<sub>4</sub>
- Hot plate & stirrer
- Volumetric flasks
- Plastic L-bars
- PVC pipes and fittings
- 2.7cm glass crucible covers
- Ammonium chloride, NH<sub>4</sub>Cl
- Sodium nitrate, NaNO<sub>3</sub>
- 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 <sub>4</sub> Cl	6
3	K based Nitrate-N	20 g/L agar, 50.55 g/L KNO <sub>3</sub>	6
4	Na based Nitrate-N	20 g/L agar, 42.49 g/L NaNO <sub>3</sub>	6
5	Orthophosphate	20 g/L agar, 68.04 g/L KH <sub>2</sub> PO <sub>4</sub>	6
6	Ammonium-N and orthophosphate	20 g/L agar, 26.75 g/L NH <sub>4</sub> Cl, 68.04 g/L KH <sub>2</sub> PO <sub>4</sub>	6
7	K based Nitrate-N and orthophosphate	20 g/L agar, 50.55 g/L KNO <sub>3</sub> , 68.04 g/L KH <sub>2</sub> PO <sub>4</sub>	6
8	Na based Nitrate-N and orthophosphate	20 g/L agar, 42.49 g/L NaNO <sub>3</sub> , 68.04 g/L KH <sub>2</sub> PO <sub>4</sub>	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<sub>4</sub>Cl. Boil 200 mL of distilled water on a hot plate and stir. Add agar and NH<sub>4</sub>Cl 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, 22<sup>nd</sup> 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  $\mu\text{m}$  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.

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

Where,  $\text{ABS}_x$  = 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 ( $\text{cm}^2$ )

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						
<i>Groups</i>	<i>Count</i>	<i>Sum</i>	<i>Average</i>	<i>Variance</i>		
Control	-	-	-	-		
Nutrient Type	-	-	-	-		
ANOVA						
<i>Source of Variation</i>	<i>SS</i>	<i>df</i>	<i>MS</i>	<i>F</i>	<i>P-value</i>	<i>F<sub>crit</sub></i>
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)

## ACKNOWLEDGEMENTS

This project was funded in part by the National Science Foundation under Grant Number NSF 1037708. For technical assistance, we thank Beverly Klein, head chemist at the Water and Environmental Engineering Research Center (WEERC) at South Dakota State University.

## REFERENCES

1. Arar, E. J. (1997). *Method 446.0 - In Vitro Determination of Chlorophylls a, b, c<sub>1</sub> + c<sub>2</sub> and Pheopigments in Marine and Freshwater Algae by Visible Spectrophotometry*. National Exposure Research Laboratory, Office of Research and Development, U.S. Environmental Protection Agency, Cincinnati, Ohio.
2. Capps, K. A.; Booth, M. T.; Collins, S. M.; Davison, M. A.; Moslemi, J. M.; El-Sabaawi, R. W.; Simonis, J. L.; Flecker, A. S. (2011). Nutrient Diffusion Substrata: A Field Comparison of Commonly used Methods to Assess Nutrient Limitation. *Journal of the North American Benthological Society*, 30(2), 522-532.
3. Hauer, F. R.; Lamberti, G. A. (2007). *Methods in Stream Ecology 2<sup>nd</sup> Ed.*, Elsevier.

4. Rice, E. E.; Baird, R. B.; Eaton, A. D.; Clesceri, L. S. (2012), *Standard Methods for the Examination of Water and Wastewater (22<sup>nd</sup> Edition)*. American Public Health Association, American Water Works Association, and Water Environment Federation.
5. Rugenski, A. T.; Marcarelli, A. M.; Bechtold, H. A.; Inouye, R. S. (2008). Effects of Temperature and Concentration on Nutrient Release Rates from Nutrient Diffusing Substrates. *Journal of the North American Benthological Society*, 27(1), 52-57.