1. Procedure summary

This procedure details the steps required to count the number of rotifers present in a sample.

Related Procedures

Pond Sampling and Data Collection	LC-01-003-001

Procedure impacts and concerns

Safety	Proper PPE for this procedure: safety glasses, safety toe shoes and gloves. Nitrile gloves should be worn when handling pond samples. The MSDS/SDS for chemicals used in this SOP should be reviewed.
Quality	NA
Delivery	Samples should be counted the day of collection in order to be an accurate representation of the rotifer number in that sample.
Environmental	Pond samples are properly disposed of in the evaporation basins.
Cost	NA
Compliance	Compliance with OSHA's Hazardous Waste Operations and Response, and Hazardous Communication Standard in addition to the Sapphire Energy, Inc. Chemical Hygiene Plan is required (see 29 CFR 1910.120 and 1200).

Responsibilities and owners

Document Owner	Manage content and distribution	Kalli Lambeth
Process Owner	Responsible for content and process validation	Alina Corcoran
Site Manager	Responsible for implementation and conformance	Becky Ryan

2. Process

2.1 Process description

Rotifers can be considered a pest or a biological crop protection tool. In either situation, it is important to be able to quantify the population of rotifers in a given pond. This procedure describes how to prepare subsamples of a sample, stain rotifers and count rotifers to quantify rotifer abundance.

This protocol can be used to count rotifers in lab cultures and in pond samples.

2.2 Process diagram: Work Instruction

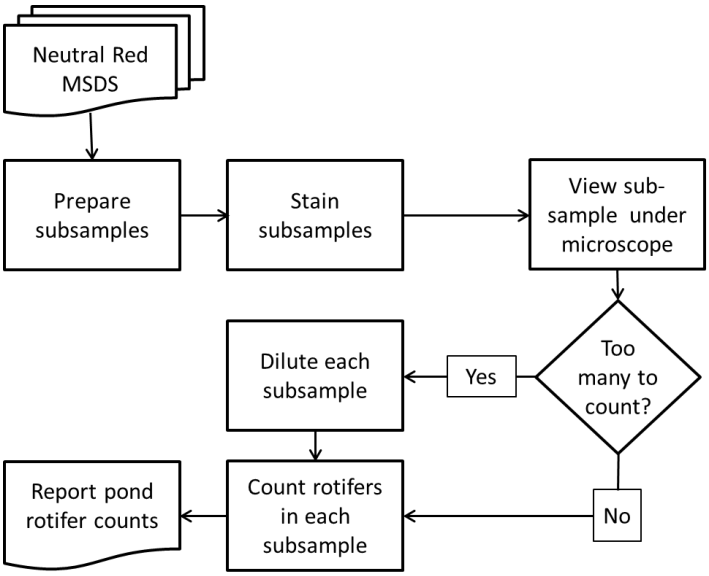

Figure 1: Counting Grid (developed at Sapphire)

2.3 Process steps

1. Obtain three 1.5mL snap cap tubes (or equivalent) per sample for analysis, put them in a holding rack and label appropriately with sample name and subsample #1-3.
2. Put 20mL of 1000ppm Neutral Red (see note) in the bottom of each tube. Vortex the neutral red before use.
3. Obtain a 1000µl pipette and tips. Set the pipette to 1000µl.
4. Carefully swirl or invert the rotifer sample to mix it thoroughly.
5. Immediately after mixing, take 1mL from the middle of the sample. See note.
6. Dispense the 1mL into the tube labeled subsample #1. The sample will self-mix with the neutral red as you dispense it into the tube.
7. Repeat steps 4-6 for the other two subsamples.
8. Incubate the samples at room temperature for 15-20 minutes.
9. While the samples stain, obtain the following:
    a. Low magnification compound microscope,
    b. Counting grid (see Fig. 1),

Step 2 Note: To mix 1000ppm stock of Neutral Red dilute 10mg of neutral red powder into 10mL of DI water.

Step 5 Note: It is important to minimize time between step 4 and 5 since rotifers will tend to gather in certain parts of the sample.

Step 13 Note:
The magnification level is

c. Pipette set to 100 µl and appropriate pipette tips.

10. When staining is complete, pipette mix subsample #1 and dispense 100µl of the subsample onto a square of the counting grid. See "Variations" in 2.5.
11. Repeat step 10 until the entire subsample has been aliquoted onto the counting grid. This should require 10 squares.
12. Place the counting grid on the microscope stage and turn the light on.
13. Center a square with a 100 µl sample and bring the rotifers into focus. It is recommended that the magnification be between 1X-4X. See Note.
14. Count the number of rotifers in the 100 µl sample. See Note. See "Troubleshooting" in 2.4.
15. Repeat step 14 for the other nine squares containing 100 µl.
16. Add the number of rotifers in each of the ten squares to obtain the #Rotifers/mL for subsample 1.
17. If you diluted the subsample in order to count (troubleshooting: step 1 and 2) You will need to multiply your count by the dilution factor to obtain the #Rotifers/mL.
18. Repeat steps 10-17 for the other two subsamples. Obtain an average and standard deviation of the three subsamples. The average is your #Rotifers/mL for the initial sample.

subjective to the user; however it is advised to use a magnification at which the entire sample can be viewed through the objective. This allows you to see all the rotifers in a sample at once, greatly reducing the odds of double-counting a rotifer.

Step 14 Note:
Rotifers will be stained a pink to red color from the neutral red stain.

2.4 Troubleshooting:

1. **Too many rotifers to count:**

Dilute sample with water/media until a countable number is achieved. Ideally the diluted sample should yield a rotifer concentration of 50-100/mL. Note dilution factor.

2. **It's difficult to see the rotifers because the culture is dense:**

For samples already on the grid: Use a pipetter to move 50µl of each 100 µl sample to a new square. If necessary, then add 50 µl of water/media to each square to dilute the rotifers and make it easier to count them.
For samples still in an epi tube: Dilute 500 µl of each stained subsample into 500 µl of water/media and then count. A greater dilution may be required. Note dilution factor.

3. **I can't tell if it's a rotifer:**

Center the object of question and zoom in for a better look.

4. **The rotifers are moving too fast to count**

Put the samples in the fridge or freezer for 5-10 minutes. The rotifers warm up fast so chill the counting plate too.The counting plate with sample on it can be put in the freezer but be careful not to spill it.

2.5 Variations:

1. **Using fewer aliquots:**

It may be possible, depending on rotifer density and counter skill, to aliquot the subsample into less than ten squares.
Eight squares of ~125uL or five squares of 200uL is commonly used instead of ten squares of 100uL.

2. **Using smaller subsamples**

In some situations, it may be acceptable to use 500uL subsamples instead of 1mL subsamples. This greatly reduces counting time but may increase standard deviations. 1mL samples are suggested for monitoring in experiments.

500uL samples are suggested for daily pond monitoring.

3. Required documents

Input documents
NA
Output documents
NA

4. Document control

Revision history

R0 – Kalli Lambeth	3/20/2012
R1 – Kalli Lambeth	10/7/2014
R2 – Kalli Lambeth	5/6/2016

Document approval

Becky Ryan	2/13/2015

Document reviewers

Phil Lee	11/13/2014
Raechel Roberts (safety committee)	11/25/2014