## Development of Monitoring Tools for Rotifers

Qualitative and quantitative methods are used in conjunction when screening for and tracking rotifers in algae cultures. The method for qualitative monitoring of rotifers uses small culture samples (~7ml) in test tubes that are left to settle under fluorescent lights (thus referred to as settling tubes). Rotifers present in the culture will tend to move towards the top of the culture, while the algae and other matter tends to settle to the bottom of the tube. Because of this separation, rotifers can be observed by eye in the transparent layer at the top of the culture. While this settling method allows for easy screening of many cultures at once, it has several disadvantages. The method is open to significant user bias and inexperienced users can easily record false negatives. Since settling tubes rely on the culture separation by gravity to be able to observe the rotifers, if the algae in the culture float to the top or do not settle, the method becomes difficult or even impossible. Large rotifers are easily seen while smaller rotifers are very difficult to see by eye. If quantification of the rotifers is desired, the sample can be counted manually in 1ml aliquots using a rotifer hemocytometer and dissecting scope. Both settling and counting are time consuming and have a threshold of detection of approximately >1 per 5ml. They are also limited in accuracy for quantification of rotifers that are present in low numbers. *Nannochloropsis* is primarily predated upon by smaller rotifers and does not significantly settle. It is also more sensitive to the presence of rotifers since much fewer rotifers are required to impact the culture than for larger algae. Thus, we sought to improve techniques for detecting and quantifying rotifers in algal cultures. Two approaches were taken for this. The first was to increase the volume being monitored in addition to a step to separate the algae from the rotifers by filtration rather than gravity. The second method involved qPCR, a method that has been successfully used to monitor microscopic algal pests in the past.

The filtering method involves filtering 10+ ml of culture through a 40µm filter, which is small enough to capture rotifers while allowing passage of algae and observing the sample via a dissecting microscope. Because the rotifers are transparent, the sample may be stained to aid visualization. This method works well for cultures that are not significantly flocked and that contain small algae. If the culture is not significantly flocked, larger sample volumes may also be used. The rotifers captured on the filter may also be quantified by manual counting through the microscope.

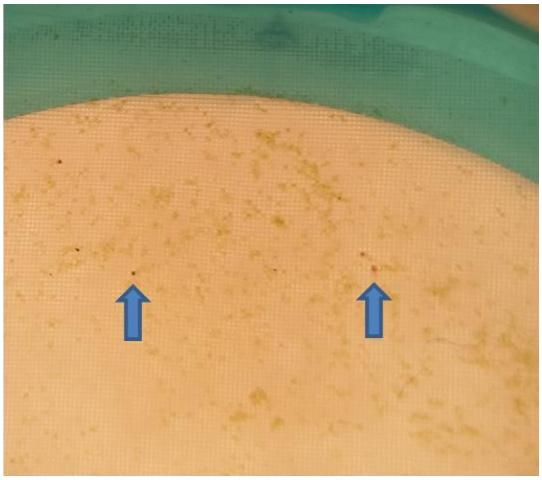

Figure 1. Visualization of rotifers. Left picture shows two different sized rotifers (large and small) in test tubes. Rotifers can be visualized by eye in transparent liquids, especially when present in high numbers, but if unsettled algae are present observation is difficult. Right hand picture shows visualization of rotifers on a filter after staining. Neutral red stain was added to culture containing rotifers prior to pouring through a ~40um filter and observation through a dissecting scope. Rotifers are observed to stain red (examples indicated by arrows).

Filtering and manually observing rotifers is quick, cheap, requires no specialized training or equipment and is thus useful for a production environment where composite samples are easily or already available and where all ponds contain the same culture and/or are routinely treated in the same way. Thus, this method was successfully deployed in 2015 production ponds. However, for the research environment where samples must be kept separate and reliable quantification data is required, this method is not suitable. qPCR is a powerful tool for quantification of DNA in a mixed sample, has been successfully used for algal pests, and may be applicable to rotifer monitoring. Rotifers isolated from Sapphire cultivation ponds were sequenced targeting the ITS1&2 regions All species identified fall within the genera *Brachionus* which share highly homologous ITS1&2 regions. Since all rotifers in this genus would be considered pests for *Nannochloropsis,* this represents an opportunity to design primers that detect multiple species of rotifer with one assay. The rotifer sequences were aligned, and primers designed to regions of similarity between multiple species. A primer set was successfully validated against multiple live rotifers and chosen for method validation.

The biggest obstacle to using a qPCR approach for rotifer monitoring is sample preparation since rotifers are present in relatively few numbers ($<10^3$/ml) as compared to algae and other pests ($\sim10^5$-$10^7$/ml) that the method has been used for in the past. However, the fact that they are multicellular should result in higher DNA per organism present in the sample. The sample prep method would be required to work with at least 10 ml of culture, be high throughput enough to handle up to 48+ samples and detect one rotifer per sample. Initially, six sample preparation methods were tested. Three methods showed promising data and were advanced for further testing. Since other methods were successfully being utilized for rotifer monitoring, developing the qPCR assay was deprioritized at this point. Further development and problem solving is planned.