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3 Early detection and quantification of zooplankton grazers in algal cultures by
4 FlowCAM

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51 **Abstract:**

52 Algal cultivation and biomass production for biofuel and other products have
53 many advantages over terrestrial crops. However, sustainable algal cultivation,
54 especially in open ponds, is difficult due to opportunistic zooplankton grazers. There
55 is an urgent need for the development of methods and procedures for early detection
56 and quantification of zooplankton in algal cultures. In this study, the FlowCAM was
57 able to detect and quantify *Brachionus calyciflorus*, a model organism, in algal
58 cultures with a detection limit of < 1 individual/mL for *B. calyciflorus* in an algal
59 (*Chlorella* sp.) cell density of 10^7 cells/mL. The methodology also allowed successful
60 monitoring of rotifer population growth at low density (<1 individual/mL of *B.*
61 *calyciflorus*) in dense algal cultures. Furthermore, the methodology was also effective
62 in detecting and quantifying other zooplankton grazers including ciliates,
63 *Poteroochromonas* sp., and *Euplotes* sp. in outdoor open raceway cultures.

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65 **Keywords:** Algae, Contamination, Culture Crash

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1. Introduction

Microalgal-based biofuels are considered a promising renewable energy source and have the potential to be a significant feedstock to meet transportation fuel demands [1-4]. Microalgal-derived biofuels have several advantages. First, the high carbon dioxide uptake rate of microalgae can help reduce greenhouse gas emissions from the combustion of fossil fuels [5, 6]. Second, microalgae have high growth rates and lipid content with the ability to grow continuously throughout the year. Therefore, the potential for oil production is estimated to be more than 10 times greater than conventional biofuel crops such as rapeseed and canola [7-9]. Moreover, microalgae can be cultivated in brackish water and wastewater on non-arable land, which provides less reliance on potable water resources and less competition for arable lands [10-12]. Sustainable and large-scale microalgae cultivation must be achieved in order to produce algae biomass for biofuels and other products.

However, microalgae also represent a food source for other aquatic organisms including amoebas, ciliates, rotifers and other zooplankton. Some rotifer species, under optimal conditions, can ingest 200 algal cells per minute per individual, which means these organisms could consume all the microalgae in the cultivation system within a relatively short period of time leading to a culture crash [13]. Moreno-Garrido and Cañavate [14], for example, showed that the high grazing capacity of ciliates led to a complete elimination of the algal culture within two days. Therefore, grazing by these zooplankton predators must be controlled and prevented. Recently, several studies have been published on the control of zooplankton grazers in algal cultures by different chemical compounds [14-18], but few reports have focused on early detection of grazers to prevent their adverse effects on algal cultures [19].

Early detection is essential if potential growers seek to control and prevent the negative effects of zooplankton grazers, including reduced biomass productivity and culture crashes. An effective detection technique should be able to recognize and/or detect potential grazers at low concentration (i.e., <10 individuals/mL) in dense algal cultures (as high as 10^8 cells/mL) in a reasonable period. Microscopic enumeration is a commonly applied monitoring protocol for assessing culture health and the presence of zooplankton grazers, but is tedious and time consuming [20]. The FlowCAM (Fluid Imaging Technologies LLC, Maine, USA) is a combination of a flow-cytometer and a camera that employs continuous digital imaging to characterize particles in a fluid sample [21]. Recently the application of FlowCAM was described in studies on phytoplankton and zooplankton [22-26]. In this study, we selected a freshwater rotifer, *Brachionus calyciflorus*, as a model organism and a novel FlowCAM based methodology was developed to assess its detection limit and quantification of *B. calyciflorus* in cultures of *Chlorella vulgaris*. Applicability of this methodology was then assessed and verified in outdoor raceway cultures.

2. Materials and Methods

2.1 Algae cultivation

The freshwater algae *Scenedesmus acutus* LRB 0401 and *Chlorella vulgaris* LRB 1201 were cultivated in 800 mL conical glass tubes in the laboratory. BG-11 Medium (see <http://www.ccap.ac.uk/media/documents/BG11.pdf>) was used as the algae growth medium. Cultures were maintained at 25°C, provided continuous illumination ($220 \mu\text{mol}/\text{m}^2 \cdot \text{s}$), and were bubbled with compressed air at a flow rate of 400 mL/min (with 2% CO_2).

Outdoor open raceway algae cultures were cultivated in 100L raceway ponds with BG-11 Medium. Log-phase algal suspensions from the above laboratory cultures were used as inoculums for those raceway ponds. All the raceway ponds were washed with bleach solution and rinsed by distilled water before use and between experiments. Algal cultures were mixed by paddle wheels driven by an electric motor at 30 rpm and aerated with CO₂ at 2 L/min. The depth of algal culture in the pond was 15 cm.

2.2 Rotifer cultivation

The freshwater rotifer, *Brachionus calyciflorus*, was started from lyophilized cysts purchased from Florida Aqua Farms Inc. (Dade City, Florida, USA). The rotifer cysts were hatched in Petri dishes containing 20 mL sterilized water for approximately 24 hours and then the rotifers were transferred to 500mL flasks containing 100 mL of BG-11 medium and maintained at 20°C under constant light. The rotifers were provided with fresh *Chlorella vulgaris* UTEX395 daily for maintenance.

2.3 Experiment procedure

2.3.1 Rotifer detection in algae culture by FlowCAM

Four different densities (0.1, 1.0, 10, and 100 individuals/mL) of *B. calyciflorus* cultures were mixed with three different culture densities (10⁵, 10⁶, and 10⁷ cells/mL) of the unicellular green microalgae, *C. vulgaris* LRB1201, respectively. Those samples were then analyzed by the FlowCAM under AutoImage Mode with a

4X objective and FC300 flow cell. During previous open raceway cultivation, the density of *C. vulgaris* LRB1201 had the potential to reach 10^8 cells/mL but often crashed by the zooplankton prior to reaching this density, while the density of *S. acutus* LRB0401 outdoor cultures could reach as high as 10^7 cells/mL (1 g/L). Therefore, we defined 10^7 cells/mL as high density algal cultures in our experiments.

2.3.2 Monitor rotifer growth in laboratory algal cultures.

A “real world scenario” was designed to evaluate the capability of FlowCAM to detect rotifers at low density and monitor their growth in algal cultures. 300 *B. calyciflorus* neonates were suspended in 1 L *C. vulgaris* LRB1201 culture with an algal density of 5.0×10^6 cells/mL. Once *B. calyciflorus* was added, the algal cultures were cultivated in triplicate under laboratory conditions. The population counts of *B. calyciflorus* were monitored daily by the FlowCAM and the haemocytometer.

2.3.3 Monitor contaminant organisms in open raceway algal cultures

S. acutus LRB0401 cultures were cultivated in 100L open raceways. Samples from those cultures were collected and analyzed by the FlowCAM and microscopy (haemocytometer) every day to monitor the population of different contaminant organisms during a 14-day cultivation period.

2.4 Sample preparation

The densities of algal cells and rotifers were determined by microscopic enumeration. Algal cell counting was performed on an Olympus BH-2 microscope (Olympus, Japan) by counting replicate samples using a haemocytometer (Hausser Scientific, Horsham, PA, USA), while rotifer and other zooplankton enumeration were performed using either a 50- μ L Nageotte bright line haemocytometer (Hausser Scientific, Horsham, PA, USA) or a 1-mL Sedgwick-Rafter chamber. Each counting continued until at least 100 individuals of an organism were reached. All haemocytometer measurements were performed in triplicate [27, 28].

Preliminary comparisons between microscopy and the FlowCAM were performed by employing the 50 μ m standard calibration beads (Thermoscientific Inc, USA) to assess the accuracy of FlowCAM. The standard beads samples were diluted from the product to prepare different concentrations (from 10 beads/mL to 3000 /mL) and were examined using both the FlowCAM (under AutoImage mode) and the haemocytometer.

2.5 FlowCAM operational parameters

A Bench Top B3 series FlowCAM (Fluid Imaging Technologies, Inc. Maine, USA) was employed in this study. The 50 μ m standard beads and an interactive calibration procedure in the software VisualSpreadsheet (Version 3.4) were used as calibration for size measurement and quantification using the FlowCAM.

Before each count, the FlowCAM was primed manually with the sample to be counted. The focus of the camera view was adjusted to ensure clear images of the

desired objects and the flow cell was cleared of bubbles. The parameters including size range and frame speed were set at the beginning of each run.

For samples containing larger protozoa (i.e., rotifers and *Euplotes* sp.), samples were pumped at a flow rate of 1.0 mL/min through a FC300 flow cell and observed under a 4X magnification lens equipped on the FlowCAM. For samples containing smaller contaminant organisms (i.e., ciliates and *Poteroochromonas* sp.), samples were pumped at a flow rate of 0.1 mL/min through a FC100 flow cell and observed under a 10X magnification lens. Images were taken in the AutoImage Mode and at a specific frame speed to ensure maximum imaged sample volume.

2.6 Data analysis

All the FlowCAM analyses were carried out in triplicates. Data from the FlowCAM were exported to Microsoft Excel files. Statistically significant differences between groups (the FlowCAM counting results at different algae densities) were determined by one-way analysis of variance (ANOVA, $p < 0.05$ was considered significant). Data was pre-examined for their normality and homoscedasticity. Coefficient of determination (R^2) was calculated to evaluate the linear regression fit between the results from FlowCAM and haemocytometer.

3. Results and discussion

3.1 Calibration of FlowCAM for sizing and counting

Table 1 provides a comparison of the size of the standard beads determined from the FlowCAM and the certified value labeled on the product. The results suggest that size estimation by the FlowCAM ($49.9 \pm 1.7 \mu\text{m}$) was confirmed using 50 μm standard beads ($50.2 \pm 1.0 \mu\text{m}$).

The standard beads were also accurately counted by the FlowCAM in AutoImage mode, in which beads were imaged and counted at regular intervals as they passed through the flow cell. Figure 1 shows the bead counts by the FlowCAM compared to haemocytometer counts. Bead concentrations ranged from 10 to 3000 beads/mL. The results show strong agreement between the two methods ($R^2 = 0.9984$) and that there was a linear relationship over the entire density range.

3.2 Rotifer quantification in algae culture by FlowCAM

Although several articles have successfully employed the FlowCAM to detect and quantify phytoplankton and zooplankton in different environments [20, 22, 23, 26, 29-32], initial tests in our study were performed to determine the quantification capability and the detection limit of a FlowCAM on *B. calyciflorus*, a common freshwater rotifer, under varying algae densities. According to the results (Figure 2), the FlowCAM was able to detect and enumerate rotifers as low as 1 individual/mL, at all algal density levels. No statistical difference was detected between the results obtained by the FlowCAM under different algae densities ($p = 0.42, 0.20$, and 0.42 for results of 100, 10, and 1 individuals/mL at the three algae densities, respectively). At higher algal density (10^7 cells/mL) and very low rotifer density (0.1 individuals/mL), the performance of FlowCAM (0.04 ± 0.03 individual/mL) was significantly different (ANOVA between groups, $p = 0.008$, $df = 2$, $F = 9.18$) from the performance at lower

algae densities (0.14 ± 0.04 individuals/mL at 10^5 cells/mL and 0.16 ± 0.01 individuals/mL at 10^6 cells/mL, respectively). It was technically not possible for the FlowCAM to detect rotifers in those cultures with densities higher than 10^7 cells/mL, under the configurations employed (FC300 flow cell and 4X magnification objective) in our initial tests, since the high algae cell density may be beyond the desired image capturing and processing capacity of the FlowCAM (SI Figure 1). The FlowCAM could detect and quantify rotifers at a density as low as 0.1 individuals/mL, demonstrating that the FlowCAM was capable of providing early warning and detection of large size zooplankton grazers, such as rotifers, in algal culture, which would provide additional time to treat the algal culture prior to a culture crash.

When the rotifer density decreased, the sample volume pumped and imaged by the FlowCAM increased, in order to increase the opportunity for FlowCAM to capture a rotifer. At a rotifer density of 0.1 individuals/mL, the sample volume required for analysis was between 10 to 20 mL. However, the FlowCAM could still complete the counts for ultra-low densities of rotifers within 10 minutes, compared to the more time consuming manual microscopic counts. For samples with dense algae cultures, sequential dilution may be needed to assist the FlowCAM in identifying and quantifying rotifers in those samples. Other options that may be considered to improve the application of FlowCAM under high algae density is using 10X or higher magnification objectives with or without dilution of the samples. However, large rotifers have the potential to block the flow cell when using the higher magnification objectives and therefore needs to be taken into account using sample pretreatment methods to separate rotifers and other large zooplankton from algae cultures. The FlowCAM also has the ability to detect and measure the specific fluorescence intensity (i.e. fluorescence from chlorophyll) from the target particles triggered by the

laser beam of the FlowCAM under Trigger Mode. This property could offer additional options to differentiate the rotifers in high density algal cultures by measuring the fluorescence intensities of rotifers and algal cells and only capture the rotifers which have relative low fluorescence intensity. More studies are required for the application of FlowCAM on the early detection of zooplankton grazers for algae production.

3.3 Application of FlowCAM methodology for zooplankton detection and monitoring

3.3.1 Rotifer growth monitoring in laboratory algal cultures

The comparison of the data is shown in Figure 3. The FlowCAM could detect and quantify the rotifer density as low as 0.3 individuals/mL in the algal cultures (0.29 ± 0.15 individuals/mL). According to the results, FlowCAM could detect the existence of the rotifer *B. calyciflorus* 3-4 days earlier than with the microscopic haemocytometer method. Therefore, the capability of FlowCAM to detect rotifer grazers at the early stage when its population is relatively low suggests that it could be a powerful technique for early detection of zooplankton grazers in algal cultivation systems.

3.3.2 Application of FlowCAM for monitoring contaminant organisms in open raceway algae cultures

When monitoring the contaminant zooplankton in open raceway cultures, the FlowCAM was capable of detecting many different zooplankton species in the open

pond cultures, including ciliates, *Poterioochromonas* sp., *Euplotes* sp., and *Vorticella* sp. (SI Figure 2). Ciliates, *Poterioochromonas* sp., and *Euplotes* sp. were used to assess the accuracy of enumeration by the FlowCAM, compared to microscopy (Figure 4). The population data acquired by both FlowCAM and haemocytometer suggest that the results from the two different counting technologies were comparable when the densities of the contaminating organisms were higher than 10 individuals/mL. When the density was lower than 10 individuals/mL, the FlowCAM could still detect and quantify those protozoa species in the algae culture, while the haemocytometer method typically could not, let alone quantify the contaminant. This indicates that the FlowCAM could provide a 3-5 day earlier warning system for the producers of algae biomass to take effective actions to control the contaminating grazers and to prevent culture crash.

4. Conclusions

The FlowCAM demonstrated the ability to accurately size and image particles compared to a standard microscopic method. The FlowCAM demonstrated the capability to reliably detect and quantify a freshwater rotifer, *B. calyciflorus*, at a density as low as 0.1 individuals/mL in algal cultures over a variety of algal cell densities. Similar results were exhibited when outdoor open pond cultures were contaminated with a diverse set of zooplankton grazers. The FlowCAM-based detection methodology could be employed in algal mass culture (open pond or other cultivation systems) monitoring for early detection in order to detect, mitigate and control diverse zooplankton grazers and prevent algal culture crashes.

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Table 1. Size measurement of 50 µm standard beads by FlowCAM

Certified Value (Mean ± SD)	50.2 ± 1.0 µm
Measured Value (Mean ± SD)	49.9 ± 1.7 µm

Figure 1. A comparison of average and standard deviation of bead counting results between FlowCAM and hemacytometer.

Figure 2. Average rotifer (*B. calyciflorus*) quantification at different algal cell densities by FlowCAM in AutoImage mode. Error bars represent standard deviation.

Figure 3. Comparison of average rotifer (*B. calyciflorus*) density using FlowCAM (triangle dot) and haemocytometer (square dot). Error bars represent standard deviation.

Figure 4. Comparison of enumeration of three zooplankton species using FlowCAM and hemacytometer in *Scenedesmus* sp. open pond cultures. a) ciliates, b) *Poterioochromonas* sp., and c) *Euplotes* sp.. Error bars represent standard deviation.

Figure 1.

Figure 2.

Figure 3.

Figure 4.