

Assessment of Cell Counting Method Based on Image Processing for a Microalga Culture

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ABSTRACT

Routine monitoring of microalgal growth requires the use of one of several methods such as cell counting under the microscope and measuring optical density (OD) with a spectrophotometer. Each of these methods has their advantages and disadvantages. For example, counting cells under the microscope can be time consuming, but it provides the best estimate of cell growth. Measuring OD is much quicker, however it doesn't provide any information on cell numbers and debris in culture can interfere with OD measurements. Therefore, this study aimed to demonstrate the usefulness of an image processing approach for counting cells in a microalga culture. Results showed that highest correlations were observed between Utermöhl cell counts and OD measurements ($r=0.99$), ImageJ cell counts and OD measurements ($r=0.99$) and between Utermöhl and ImageJ cell counts ($r=0.99$). In the regression analysis, highest R^2 values were obtained for Utermöhl vs OD ($R^2=0.99$) and ImageJ vs OD ($R^2=0.99$). Counting algal cells with ImageJ allows the analyst to complete the procedure 4 times faster than with manual Utermöhl procedure.

KEY WORDS: Microalgae, Cell counting, Utermöhl method, Thoma counting chamber, ImageJ.

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1. Introduction

Microalgae are photosynthetic organisms acting as primary producers in all aquatic environments by fixing atmospheric CO₂ into inorganic molecules using solar energy (Guedes et al., 2011). In addition to their ecological importance, microalgae have various biotechnological uses. Their metabolites can be used in medicine as pharmaceutical raw material. In addition, they may produce pigment substances such as carotenoids and phycobiliproteins used in the food and feed industries (Chu, 2012). For example, the most common carotenoid produced by *Dunaliella* strains is β-carotene (Lee, 2008), which they produce to be protected against the ionization energy effects of sunlight (Schlipalius, 1991). *Dunaliella* species are commercially mass produced for the production of β-carotene (Borowitzka et al., 1990; Markovits et al., 1993).

Whether it is a mass culture or a small scale culture in a flask; growth of microalgae has to be followed by any of several methods. These methods range from counting cells under the microscope to measuring their pigments (Guillard and Sieracki, 2005) or measuring optical density (OD) with a spectrophotometer (Schultz, 1987). Each method has its advantages and disadvantages. For example, counting cells under the microscope can be time consuming, but it provides the best estimate of cell growth (Lund et al., 1958). Measuring OD is much quicker, but it doesn't provide any information on cell numbers and any debris in culture can interfere with OD measurements (Moheimani et al., 2013).

Aims of this research were (i) to compare different growth measurement methods for a microalgal culture and to establish a correlation between these different methods; (ii) to test a cell counting method based on image processing especially in comparison to manual cell counting under the microscope.

2. Materials and Methods

2.1. Culture and Growth Conditions

All growth measurements were performed using *Dunaliella* sp. AQUAMEB-21, obtained from AQUAMEB Culture Collection of Algae and Cyanobacteria (Aquatic Microbial Ecology and Biotechnology Laboratory, n.d.). This strain was isolated from Lake Tuz, Turkey. Length and width of

Dunaliella sp. AQUAMEB-21 changed between 7-17 μm and 7-19 μm, respectively. Therefore, these measurements were proper for easy observation of cells under the microscope. *Dunaliella* sp. AQUAMEB-21 strain was grown in Plymouth Erd-Schreiber (PES) medium (Tompkins et al., 1995) at 120 psu salinity. Medium was prepared with the Marmara seawater (27 psu) and salinity was adjusted with NaCl. The culture was grown in cabinets (Panasonic 352H, Osaka, Japan) at 23°C and at an irradiance of 120 μmol. photons. m⁻². s⁻¹ with dark-light cycles of 12 h:12 h. Light intensity and placement of the culture flask in the cabinet was adjusted by measuring the light intensity with a LI-COR quantum sensor (LI-COR, Lincoln, Nebraska, USA).

2.2. Sample Preparation, Optical Density Measurements and Manual Cell Counting

Exponential phase *Dunaliella* sp. AQUAMEB-21 culture was serially diluted to obtain 5 tubes in duplicates where the dilution factor was ½ in each step. Two hundred μl from each tube was used to obtain optical density values at 750 nm measured on a Biotek Epoch microplate reader (BioTek, Winooski, Vermont, USA). From each dilution, 3 ml sub-samples were fixed with 60 μl acidic Lugol's iodine solution (Edler and Elbrächter, 2010). Two mL of the fixed samples were transferred to Utermöhl settling chambers (Aquatic Research Instruments, ID, USA) and cells were allowed to settle for 24 hours (Ferrando et al., 2011). Cells in the settling chambers were counted with naked eye under an inverted Olympus CKX41 microscope (Olympus- Shinjuku, Tokyo, Japan) at 400 × magnification. At least 500 cells in random fields of a Whipple grid were counted. Number of cells per mL was calculated according to the formula below (Edler and Elbrächter, 2010);

$$\text{Field count ((No.)/mL)} = C \times A_t / A_f \times F \times V$$

where:

C= number of cells counted,

A_t= total area of settling chamber, mm²

A_f = area of a field, mm²

F = number of fields counted

V = volume of sample settled, mL.

Twenty μ l from each fixed sample was also used for cell counting on the Thoma cell counting chamber. There are two areas with 16 squares on the Thoma chamber. Ten μ l each was transferred onto the top and bottom of the chamber by a pipette and a coverslip was placed. Cells in each 16 large squares were counted and the mean of these two counts (i.e. [top count+ bottom count]/2) was multiplied by 10,000 to calculate the number of cells per mL of the culture.

2.3. Cell Counting on Image

Digital images for cell counting were obtained from the same Utermöhl samples used for manual cell counting using an inverted microscope at 400 \times magnification. In other words, an image was captured just after a Whipple grid was counted under the microscope. Therefore, the same number of fields was counted manually and by image processing. However, the area of a field (A_f) in the above formula was different in each method (i.e. Whipple grid vs image).

Image scales were added (20 μ m) and dimensions of the imaged area (A_f) were calculated using the camera's own software (Kameram, Turkey). JPG was selected as the image format. Afterwards, images obtained were imported into the ImageJ program. ImageJ version 1.50 was used for image processing. Latest versions can be downloaded from the ImageJ web site (ImageJ, n.d.).

The pathway of algal cell counting in ImageJ is described below and summarized in Figure 1.

Image analysis steps

1. Open image – ('File' > 'Open'),
2. (Optional): Calibrating ImageJ measurements using the scale bar on the original image. This is done by following the procedures described in the ImageJ user guide (ImageJ user guide, set scale, n.d.),
3. The image is converted to 8 bits to minimize color variation – ('Image' > 'Type' > '8-bit') (Figure 1A),

4. For better visualization of dark colored cells, subtract background – ('Process' > 'Subtract Background') adjust “Rolling ball radius” = “200.0 pixels (Pixel value can be adjusted according to the quality of the image)” – tick:

✓ “Light background (clarify the appearance of cells”),

✓ “Preview” – Click “OK” (Figure 1B),

5. Adjust Threshold – ('Image' > 'Adjust ' > 'Threshold') – move the bottom slider so that algal cells are highlighted and background particles are not visible. Also pay attention to separate adjacent algal cells – select "Default" and "Red" -click “Apply” (Figure 1C, D),

6. 'Process' > 'Binary' > 'Fill Holes' (Figure 1E),

7. (Optional) Set measurements for different analyses (These selections can be changed according to the desired analysis) – ('Analyze' > 'Set measurements') – tick (Figure 1F):

- ✓ “Area”
- ✓ “Mean gray value”
- ✓ “Standard deviation”
- ✓ “Min and max gray value”
- ✓ “Perimeter”
- ✓ “Integrated density”
- ✓ “Median” and “Area fraction”

8. Perform algal cell count– ('Analyze' > 'Analyze particles') – set “Size” = “40 (20-40 can be chosen for *Dunaliella* sp. Aquameb-21 culture) - Infinity”, “Circularity” = ”0.00–1.00” and “Show = Outlines (Shows the counted cells in a separate window)” – tick (Figure 1G):

- ✓ “Display results”
- ✓ “Include holes”
- ✓ “Summarize”– click “OK”

The size was determined by measuring cell lengths before image processing. It is advantageous to choose a larger size than the maximum cell size observed (i.e. 20 μ m) in the samples. This eliminates the probability of the software to count cell debris.

9. Shows the results – “Results” and “Summary” (Figure 1H).

10. The same formula given in section 2.2 for the Utermöhl settling method was also used to calculate the number of algal cells per mL using the ImageJ software.

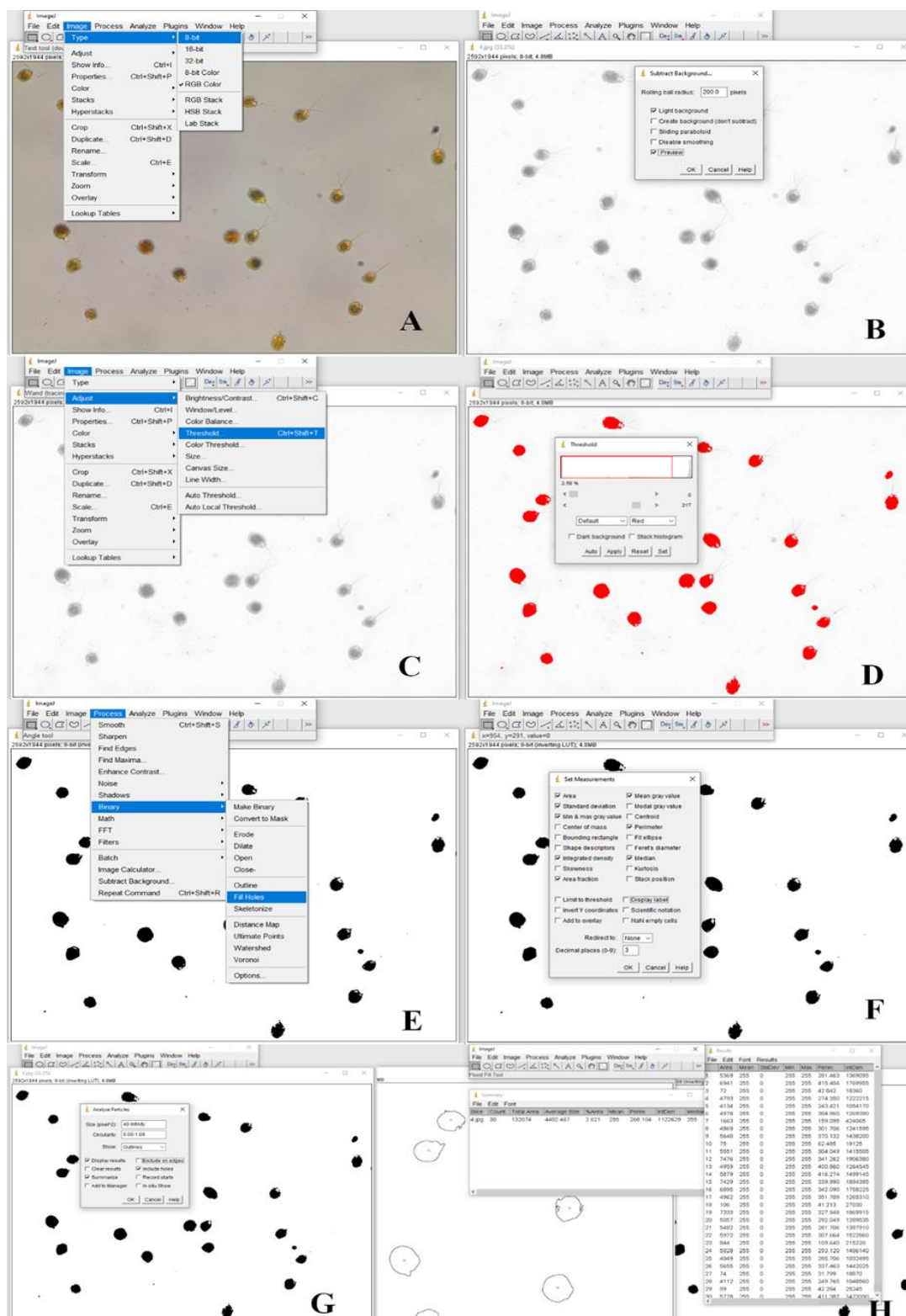


Figure 1. The pathway of algal cell counting method with ImageJ

2.4. Statistical Analysis

The relationships between OD, Thoma counting chamber, Utermöhl counting chamber and ImageJ cell counts were evaluated by regression analysis and

the Spearman correlation method with a linear model fit. R program was used for statistical analysis (Gentleman, 2008).

3. RESULTS

There was a high correlation between OD, counting chamber and Image J cell counts (Table 1). The highest correlation coefficients were observed between Utermöhl cell counts and OD measurements

($r=0.99$), ImageJ cell counts and OD measurements ($r=0.99$) and between Utermöhl and ImageJ cell counts ($r=0.99$). The lowest correlation coefficient was observed between Thoma and Utermöhl cell counts ($r=0.94$) (Table 1).

Table 1. Matrix for correlation analysis results between different cell counting methods.

	1	2	3	4
1-Thoma				
2-Utermöhl	0,94			
3- ImageJ	0,97	0,99		
4- OD	0,95	0,99	0,99	

Plots of cell counting methods as a function of optical density (Figure 2A, 2B, 2C) were significantly linear ($p < 0.04$). Highest R^2 values were obtained for Utermohl vs OD ($R^2=0.99$) and Image J

vs OD ($R^2=0.98$). On the other hand, the R^2 value for Thoma vs OD was 0.90.

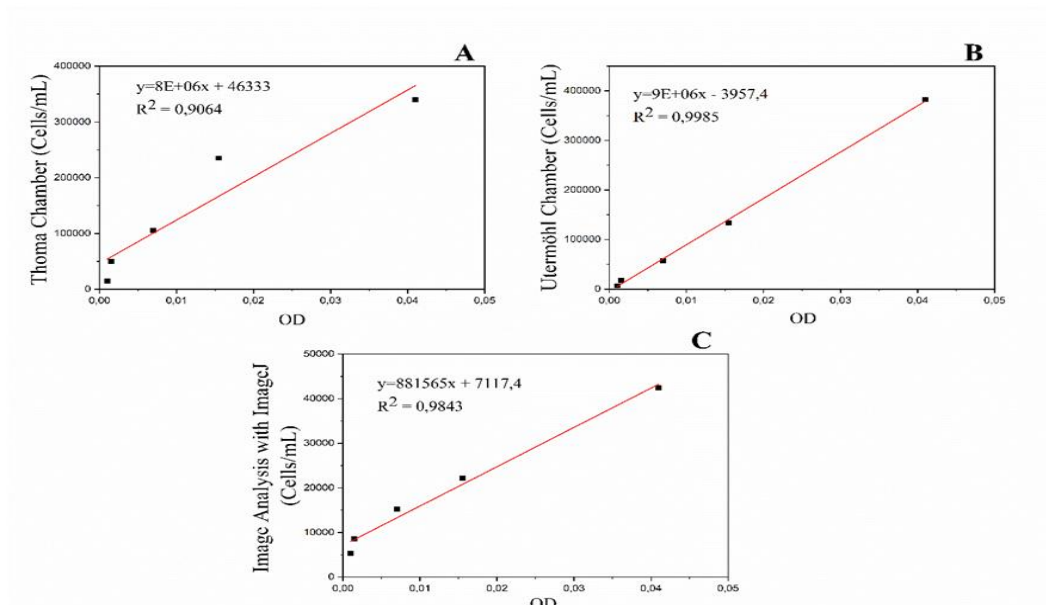


Figure 2. Results of regression analysis between optical density (OD) and cell counting methods (cells/mL). A: Thoma chamber vs OD, B: Utermöhl chamber vs OD, C: Image J counts vs OD.

The analyst in this study, on average, spent four minutes per counting field with the Utermöhl counting method. The time spent here varied according to the number of cells in the area. On the other hand, approximately one minute per field was spent for cell counting on ImageJ.

4. Discussion and Conclusion

This study examined the suitability of an image processing software for fast and reliable algal cell counts instead of manual counts under the microscope. Results suggested that routine monitoring of mono-algal cultures can be easily and correctly accomplished using the ImageJ software. Counting with image analysis reduces subjectivity and allows automation (Thomas and Paul, 1996). In addition, images can be stored so that they can be re-checked when necessary. Although not performed in this study, ImageJ also allows the analyst to properly measure the dimensions of cells to perform further biovolume calculations.

The use of optical density (OD) is an indirect method commonly used for monitoring microalgae biomass growth. Optical density (OD) is very practical; however, it doesn't give any information about the cell numbers and it might have interference from other particles (O'Brien et al., 2016). If the researcher chooses OD measurements over cell counts, then an initial regression analysis between OD and ImageJ cell counts as in Figure 2C will allow conversion of OD measurements to cell numbers. Results of this study suggest that the Thoma counting chamber may not be very suitable, at least for counting algal cells in this study. The Thoma cell counts had the lowest correlation coefficients against other measurement methods (Table 1) and the lowest R^2 value against OD measurement (Figure 2A). The dimensions of the Thoma counting chamber may not be suitable for entry and uniform distribution of large algal cells. However, the settling chamber used in the Utermöhl method allows all cells to be settled. For maximum accuracy, the entire chamber base can be counted, especially since cell distribution may be irregular (Hötzel and Croome, 1999). If only subsections of the Utermöhl chamber will be counted as in this study, then it is advised that a sample with

uniform cell distribution should be prepared (Guillard and Sieracki, 2005).

Grishagin (2015) demonstrated that it took 3.5 to 14 minutes to manually count the cells under the microscope depending on the dilution factor. In his automatic counts, 1.5 minutes were spent on 8 images. Similarly, in this study ImageJ cell counts were completed in a quarter of the time used for manual cell counting under the microscope.

ImageJ is a free and extensible program. ImageJ plugins or macros can be used to improve and automate results (Abràmoff et al., 2004). The development of automatic counting methods is expected to increase the work speed and reliability in routine monitoring of algal cultures.

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