A quantitative FlowCAM analysis of diatoms in Otsego Lake, New York, with an emphasis on method implications

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ABSTRACT

A study was performed from January to May 2015 that was intended to investigate environmental change as revealed in sediment cores over the last 90 centuries in Otsego Lake, NY. Various conditions of the lake and local environment can be characterized based on the species of diatoms that were present in the cores. To fulfill this purpose, three methods were investigated as to their ability to obtain absolute diatom abundances from different core depths. Diatoms were chosen as a paleoecological proxy for environmental changes because they are highly sensitive to environmental conditions, respond rapidly to changing conditions and preserve well in sediment (Smith and Flocks 2010). This paper documented the results from core analyses and highlighted limitations of the petrographic microscope, FlowCAM and ImageJ software.

Diatoms were separated into two orders of Pennales and Centrales (westerndiatoms.colorado.edu) by creating diluted slide smears to take photomicrographs. Due to the lack of anatomical detail that could be observed using a petrographic microscope, limitations arose that regarded species level identification. FlowCAM, a benchtop particle analyzer, photographed and counted particles from a sample through a fluid stream. Parameters that characterized particles were displayed such as length, circularity and transparency. An image library was created to isolate shapes and sizes that resembled certain diatoms into a folder, which could be useful to obtain absolute abundances. Before samples could be introduced into the FlowCAM, an ImageJ software program was utilized to measure diatom dimensions. ImageJ assisted with the classification of Pennales and Centrales further into more distinct groups based on their minor anatomical and shape differences. Ultimately, due to the steep learning curve of the FlowCAM, methods were ineffective in determining diatoms abundances. However, mitigations could be made to lead to a successful quantitative analysis.

BACKGROUND

Otsego Lake is located in northern Otsego County, New York with Cooperstown at the southern end. Otsego County was completely glaciated during the Pleistocene Epoch, which deposited a variety of dense, unsorted clay, sand, gravel and boulders over the land surface (Isachsen 1991). Due to the glacial erosion, Otsego Lake is found in a glacially carved, over-
deepened valley, at an elevation of approximately 364 meters. Otsego Lake serves as the headwaters of the Susquehanna River in Otsego County (Harman and Albright 1997).

Sediments contain the remains of diatoms, mollusk shells, algal material, woody debris, mineral particles, and charcoal. These components provide a mosaic of lake processes and changes in the local environment over the last 90 centuries. Siliceous diatom valves rarely decompose in sediment, making them a useful proxy for the ecological analysis of Otsego Lake. The distribution of diatoms can relate to the trophic status of a lake. Their various ecosystem niches are sensitive to changes in pH, salinity, temperature and nutrient concentrations. Generally, lakes with low productivity are considered oligotrophic. Nutrients at higher concentrations lead to mesotrophic and eventually eutrophic lakes (Arnold 2002). Different diatom species function as different environmental indicators. Studies have been done that examined the history of lake conditions as revealed by diatoms in sediment cores (Fritz 1993, Arnold 2002).

Diatoms (Bacillariophyta) are distinguishable in two orders, Pennales and Centrales. Diatoms can be seen in different orientations under a microscope. The ‘valve view’ refers to the view of the ‘face’ of a diatom frustule, which classifies their symmetry. Conversely, the profile view is referred to as the girdle view. Pennales, or pennate diatoms, are bilaterally symmetrical. Their general outline may be boat-shaped or rod-shaped. In the center of many pennate diatoms is an unsilicified groove, known as the rapine. The Centrales, or centric diatoms, are radially symmetrical and lack rapines. The outline of centric diatoms is typically circular, oval or elliptical. These outlining differences are not always true for the two morphological groups. Diatom identification must be based on the anatomical pattern of their internal vales (Round 1990).

Near the Biological Field Station in Cooperstown, NY, six sediment cores were probed in Rat Cove in Otsego Lake by a modified Livingstone-type drive rod corer of which two cores were analyzed for diatom abundances. Each core, or drive, was about 1 meter long. A number of drives were extracted from these locations. For the purpose of this study, samples taken from the cores were mentioned in relation to where the sediment/water interface meets.

At location A (OTS – 2014 – 02 - B), a core was extracted on 1 February 2014 in a deep part of the lake, close to a protruding delta (Figure 1). Total, there were ten drives taken that collected about 6 meters of sediment. Drive 2 began at 100 cm below the sediment/water interface (Figure 2). The core contained very dark sediment with little to no layers. Samples were extracted and analyses were made from 115, 155 and 195 cm below the sediment/water interface.
At location C (OTS – 2012 – 05 – 12C), a 1.6-meter long core was probed on 12 May 2012 within three drives. Each subsequent drive overlapped the last drive by 50 cm. This area is closer to the shore than location A (Figure 1). From drive 2, which began at 25 cm below the sediment/water interface, samples at 70 and 97 cm were extracted for analysis (Figure 3). This

Figure 1. Map of core locations in Otsego Lake just north and east of the Biological Field Station, Otsego County, NY, in Rat Cove.

Figure 2. Sediment core that was extracted from a deeper part of Otsego Lake at location A, drive 2. The core began at 100 cm below the sediment/water interface.
core contained a variety of organic material, few white gastropods in layers of brown-grey mud. Woody debris, leaf remains and peat were dispersed in darker, rich sediment in the middle section of the core.

In addition, at location C, samples at 77-137 cm below the sediment/water interface (sampling every 2 cm) from drive 3, were observed in this study. Drive 3 began at 75 cm below the sediment/water interface. This core contained organic material, shells and gastropods embedded in dark brown/black mud. There was an abrupt mud/clay mixture layered in the middle of the core. Previously, sediment slides were created and analyzed in this study from 77 and 79 cm below the sediment/water interface of drive 3.

**METHODS**

In this study, the use of an Olympus Petrographic Polarizing Microscope was used to manually identify Centrales or Pennales. Slide smears were created and analyzed of location A and location C at various depths. Samples were introduced into the FlowCAM through a fluid stream for a diatom abundance calculation. Dimensions of the diatoms were measured with ImageJ. Measurements assisted with diatom identification. In addition, measurements helped determine what objective lens and flow cell would be appropriate for the FlowCAM to maximize data collection of a wide range of diatom sizes.

To initially confirm that diatoms were present in the core, photomicrographs were analyzed of previously created slides of location C at 77 and 79 cm below sediment/water interface. Slide smears were created of location C at 97 cm below the sediment/water surface to examine physical characteristics of diatoms on a deeper scale. The core was sampled with a metal spatula in 2 cm increments. We avoided obtaining samples that were potentially disturbed...
by the coring barrel by digging through the soil surface for collection. Each 1x1x1 cm sample was placed in separate sample bottles, then hydrated with deionized water. A tiny amount of the samples was transferred onto individual glass microscope slides via pipette. The addition of detergent broke up surface tension to allow easier dispersion of particles across the slide. Samples were dried on a hot plate and weighed. Photomicrographs were taken at 40x magnification (Figure 4a and 4b).

Diatoms were visually characterized based on their shapes. Any diatoms that appeared elliptical or round with no raphie were assumed to be Centrales (Figure 4a). Oppositely, boat-shaped, rod-shaped or elongated diatoms with possible raphie (central area) were assumed to be Pennales (Figure 4b). However, diatoms cannot be characterized based on their shape outline. Diatom identification was loosely interpreted since anatomical features within their frustule were not always visible (Round 1990).

Inspection of photomicrographs revealed that HCl increased the visibility of diatoms that were on the slide smears. Location C premade slides at 77 and 79 cm below the sediment/water interface (HCl absent), displayed a large amount of organic material, which covered the diatoms partially. In contrast, location C at 97 below sediment water interface (+HCl) resulted in much less organic debris (Figure 4). HCl addition caused samples from location C to effervesce.

The Pennales and Centrales were broken down further into more specific groups based on their minor anatomical and shape differences (Table 2). A species name was not assigned to these diatoms. However, they were classified based on their distinct visual differences between each one. To do this, ImageJ scaled sample photomicrographs and measure diatom dimensions. An aspect ratio, or proportional relationship between an object's width and height, was calculated for each diatom observed of location C at 77 and 79 cm below the sediment/water surface. A value close to 1 indicated that the diatoms distance in length was similar to its distance in width.
Figure 4a. A centric diatom found at location C (Drive 2) + HCl at 97 cm below the sediment/water interface.

Figure 4b. A pennate, boat-shaped diatom found at location C, drive 2 (300 µm sieve + HCl) at 97 cm below the sediment/water interface.
A dynamic imaging particle analysis tool, known as the bench top FlowCAM, at the Biological Field Station was utilized to retrieve abundances of diatoms (Figures 5 and 6). This machine photographed each individual particle in a flow stream of water and lake sediment. Subsequently, the photographs of diatom shaped particles that the FlowCAM took were compared to previously acquired photomicrographs for species identification. Although a 4x objective allowed for a huge range of sizes to be photographed in a large field of view, any particle under 100 µm was extremely hard to identify.

The following analyses were performed with FlowCAM-stored data of the samples. Particles were sorted by shape and size to isolate certain species of diatoms into an image library as templates. Figure 7 displays Pennales that have been narrowed down based on their similar boat-shaped characteristic within a remote folder. Subsequently, FlowCAM automatically scanned and filtered the database for similar images that matched the criteria provided. The data were then instantly enumerated into concentrations of specific particle characteristics.

Figure 5. FlowCAM, the dynamic imaging particle analysis tool used for quantifying diatoms.
Figure 6. Sample addition to the FlowCAM for image processing.

Figure 7. FlowCAM image library of boat-shaped pennate diatoms isolated into a folder. Summary statistics of particles is displayed on the bottom left.
The more template images that are placed aside from the particles, the better chance the FlowCAM has in isolating exact shape matches. The diatom library required clear, individual images of the desired shape. This rose to the issue of the FlowCAM’s inability to perform this technique due to plant debris and fibers blocking the field of view of some diatom images (Figure 8).

Figure 8. Image of fibrous material from a run through FlowCAM (4x objective and 300 µm flow cell). Location A at 195 cm below the sediment/water interface (300 µm sieved).

Three samples of location C at 70 and 97 cm below the sediment/water surface were run through the FlowCAM. Due to the fact that creating a library in FlowCAM did not isolate Pennales and Centrales (Figure 6), each image page was roughly counted by the naked eye. The number of diatoms and particles from each total run were added to calculate an absolute abundance of Pennales and Centrales (Table 1).
The FlowCAM was equipped with a 4x magnification objective with a correlating 300 µm flow cell and 1mL syringe. These parameters were recommended by the company’s Configuration Optimization Guide in the FlowCAM Manual (2011). The maximum detectable size was 300 µm. The machine was focused by running 50 µm specialized pre-diluted focus microspheres through the field of view. In addition, to balance between grey-scale and color measurement, the intensity mean value was set to be approximately 150. Before and after each use, the system was flushed with ultra pure water to ensure particles did not melt into the flow cell.

Prior to presenting sediment samples into the FlowCAM, samples were sieved through a 300-µm mesh screen to leave a broad range of diatoms (Location A at 115, 155 and 195 cm below sediment/water surface; Location C at 77-137 cm below sediment/water surface sampled every 2 cm). The sieve was folded into a funnel and the sediment was completely filtered into a beaker with deionized water. Approximately 1-2 drops of the remaining sediment sample was introduced from a funnel set above the FlowCAM and pumped at a rate of 20 fps (Figure 5). Drawing up prepared sediment of less clumped particles ensured that soil clumps would not get lodged in the flow cell. In addition, detergent was added to ensure smooth flow of sediment by reducing surface tension and adhesion.

The samples were pretreated with 5-15 drops of HCl of location A at 115, 155 and 195 cm below the sediment/water interface and of location C at 70 and 97 cm below the sediment/water interface. The addition of HCl was intended to reduce organic matter and carbonate composition. To observe if diatom visibility increased due to the partial dissolution caused by HCl, slide smears were created of HCl pretreated samples and compared to non-pretreated samples.

RESULTS AND INTERPRETATIONS

The original strategy to isolate certain diatom shapes into a FlowCAM library to retrieve abundances did not work (Figure 9). When the database was scanned for similar particles, the FlowCAM isolated other particles such as minerals, plant debris and other sediment. Therefore, numeric estimations by the naked eye was executed to count the number of diatoms at certain depths at each location. FlowCAM and photomicrograph data of location C at depths of 70, 97 cm (Figure 9), 77 and 79 cm (Table 2; Figure 10) below the sediment/water interface were compared to FlowCAM data of location A at the depths of 115, 155 and 195 cm (Figure 4 + HCl) below the sediment/water surface. Location C showed a Centrale (Figure 4a) and a Pennale (Figure 4b) at 97 cm below the sediment/water surface. Oppositely, location A showed very few shapes that could be classified as diatoms (Figure 9). All of the samples appeared to be fibrous, organic, tissue-like, and contained minerals such as calcite and quartz.
Figure 9. Location C at 97 cm below the sediment/water interface. There are 3 unknown, possibly pennate, rectangular-shaped diatoms within the cluster of images.

The core observations at location C, 77 and 79 cm below the sediment/water interface, showed that there were Pennales and Centrales present (Figure 10). There was an array of different diatom species as seen in column 1 of Table 2. However, without having a strong view of raphie, we were limited in terms of accuracy while identifying diatoms by species name.

In Figure 10a, photomicrographs showed approximately two Centrales and four Pennales. In Figure 10b, the collection of diatoms counted were four Centrales and five Pennales. There were seven Pennales and one Centrale in the field of view in Figure 10c. Figure 10d displayed one Pennale and Figure 10e displayed two Pennales. In Figure 10f, there was a possible broken diatom valve present along with an assortment of algal material covering many Centrales and Pennales.

In Table 1, location C at a depth of 97 cm below the sediment/water surface, revealed absolute abundances of 1.52E-3 for Pennales and 2.2E-4 for Centrales. At a depth of 45 cm, Pennales were 7.58E-4 and Centrales were 2.53E-4. This also indicated that there were a higher abundance of diatoms in the shallower water (location C) in comparison to deeper in the lake (location A). Figure 9 provides an example of a FlowCAM photosequence of these images.
In Table 2, diatoms at location C at 77 and 79 cm below sediment/water surface were characterized and separated into groups that highlighted their individual features. There were six different possible species found shown in column 1 of Table 2. The pennate, boat-shaped diatoms in row 1 displayed an aspect ratio between 3.06-4.86 µm. The longer diatoms with a narrower width in row 2 had slightly higher aspect ratios between 5.77 – 8.68 µm. Diatoms with the same, elongated shape except small size (row 4) had an aspect ratio ranging from 3 – 4 µm and two other outliers with 5.58 and 8.28 µm. It was a possibility that these diatoms belong in column 2, but it was uncertain because photomicrographs did not display enough detail. The Pennale was similar to row 2, except with a thicker width, and had an aspect ratio of 3.95 µm. This ratio, closer to a value of 1, showed that an increased width to be closer to the length size caused the aspect ratio to decrease.

<table>
<thead>
<tr>
<th>Depth (cm)</th>
<th>Pennales</th>
<th>Centrales</th>
<th>Total number of particles</th>
</tr>
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<tbody>
<tr>
<td>45</td>
<td>3 (7.58E-4)</td>
<td>1 (2.53E-4)</td>
<td>3956</td>
</tr>
<tr>
<td>72</td>
<td>26 (1.52E-3)</td>
<td>4 (2.2E-4)</td>
<td>17076</td>
</tr>
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Table 1. Location C, drive 2 + HCl, 300 µm sieve and 4x objective in FlowCAM. The absolute abundance of diatoms is in parenthesis beside the numeral output of diatoms.
<table>
<thead>
<tr>
<th>Diatom type</th>
<th>Figure in paper</th>
<th>Length, µm</th>
<th>Width, µm</th>
<th>Aspect ratio (length/width)</th>
<th>Depth below sediment/water interface, cm</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Pennate: Boat-shaped; raphe</td>
<td>-</td>
<td>104.5</td>
<td>21.5</td>
<td>4.86</td>
<td>77</td>
</tr>
<tr>
<td></td>
<td>10c</td>
<td>32.6</td>
<td>9.3</td>
<td>3.51</td>
<td>77</td>
</tr>
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<td></td>
<td></td>
<td>72</td>
<td>20</td>
<td>3.60</td>
<td>79</td>
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<tr>
<td></td>
<td>10a</td>
<td>38.8</td>
<td>12.7</td>
<td>3.06</td>
<td>77</td>
</tr>
<tr>
<td></td>
<td>10f</td>
<td>34.9</td>
<td>9.1</td>
<td>3.84</td>
<td>79</td>
</tr>
<tr>
<td></td>
<td>10e</td>
<td>25.9</td>
<td>8</td>
<td>3.2375</td>
<td>79</td>
</tr>
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<td>2. Pennate: Elongated; narrow</td>
<td>-</td>
<td>54.4</td>
<td>7.6</td>
<td>7.16</td>
<td>77</td>
</tr>
<tr>
<td></td>
<td>10b</td>
<td>-</td>
<td>10.9</td>
<td>-</td>
<td>77</td>
</tr>
<tr>
<td></td>
<td></td>
<td>-</td>
<td>72.9</td>
<td>7.59</td>
<td>79</td>
</tr>
<tr>
<td>3. Pennate: Smaller species of above diatom</td>
<td>10f</td>
<td>35.6</td>
<td>4.3</td>
<td>8.28</td>
<td>79</td>
</tr>
<tr>
<td></td>
<td></td>
<td>33.8</td>
<td>7</td>
<td>4.83</td>
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<tr>
<td></td>
<td>10c</td>
<td>25.3</td>
<td>5.4</td>
<td>4.69</td>
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<td>12.9</td>
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<td>10.9</td>
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<td>3.76</td>
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<td>5.4</td>
<td>4.54</td>
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<td>13.6</td>
<td>3.7</td>
<td>3.68</td>
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<tr>
<td></td>
<td>10b</td>
<td>25.5</td>
<td>5.9</td>
<td>4.32</td>
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<td></td>
<td></td>
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<td>5</td>
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<td></td>
<td></td>
<td>24.6</td>
<td>6.7</td>
<td>3.67</td>
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<tr>
<td>4. Pennate: Thicker width, elongated with small, rounded valve tips</td>
<td>8b</td>
<td>80.1</td>
<td>20.3</td>
<td>3.95</td>
<td>79</td>
</tr>
<tr>
<td>5. Pennate: elliptical</td>
<td>-</td>
<td>8.1</td>
<td>6</td>
<td>1.35</td>
<td>77</td>
</tr>
<tr>
<td></td>
<td></td>
<td>19.7</td>
<td>14.6</td>
<td>1.35</td>
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<td>8.4</td>
<td>1.21</td>
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<td>21</td>
<td>13.2</td>
<td>1.59</td>
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<tr>
<td>6. Centric</td>
<td>10b</td>
<td>13.9</td>
<td>13.9</td>
<td>1.00</td>
<td>79</td>
</tr>
</tbody>
</table>

Table 2. Measured diatoms by ImageJ of location C at 77 and 79 cm below the sediment/water interface.
Figure 10a. A diverse assortment of diatoms found from premade slides of location C at 77 cm below the sediment/water interface.

Figure 10b. A diverse assortment of diatoms found from premade slides of location C at 79 cm below the sediment/water interface.
Figure 10c. A diverse assortment of diatoms found from premade slides of location C at 79 cm below the sediment/water interface.

Figure 10d. A diverse assortment of diatoms found from premade slides of location C at 77 cm below the sediment/water interface.
Figure 10e. A diverse assortment of diatoms found from premade slides of location C at 77 cm below the sediment/water interface.

Figure 10f. A diverse assortment of diatoms found from premade slides of location C at 79 cm below the sediment/water interface.
DISCUSSION

When diatom identification was attempted, photomicrographs were compared to FlowCAM images. In the photomicrograph image (Figure 10c), which was treated with HCl (Location C at 97 cm below the sediment/water interface), it was difficult to be certain if the rectangular-shaped diatom was a Pennale or Centrale. The same sample run through FlowCAM, for a particale of similar shape, lacked the detail which would allow species level identification. Therefore, the diatoms under 100 µm were very difficult to classify due to lack of anatomical detail and less than perfect focus.

For future work, two runs through the FlowCAM with two different sized objectives (4x and 10x) and correlating flow cells should be done to increase shape visibility. This would capture the full size ranges of diatoms. A 10x objective with a 100 µm flow cell would ultimately retrieve larger, more visible images of the smaller diatom sizes up to 100 µm (FlowCAM Manual 2011).

By observing the minor anatomical differences in the diatom valves within photomicrographs, Pennales and Centrales were split into different groups from location C (Table 2). Diatoms were measured in ImageJ to further separate by size. A few of the diatoms in row three (larger; elongated) could be grouped with row two (smaller elongated, possible raphie). The raphie could not be distinguished. When the petrographic microscope was focused to a different degree, diatoms raphie were either highlighted or hidden. This sensitivity and variation presents a huge limitation in diatom identification because there are so many species that resemble one another. An SEM microscope would show the internal anatomy of the diatom in detail, ultimately making the species groups in Table 2 more accurate.

To compute an absolute diatom abundance using the FlowCAM, a library was created to isolate certain species shapes into a folder (Figure 7). However, a limitation with this method was that the identification of species based on their common shape outline was not possible. When the database was scanned for similar particles to create the library, the FlowCAM isolated other particles such as plant debris, minerals and other sediment. When plant debris covered part of the diatoms, this caused the FlowCAM to inaccurately isolate material such as mineral fragments and plant fibers. In addition, a diverse library of similar shapes and sizes that matched diatom characteristics increased the probability of isolating particles that resembled diatoms. However, when there were many cluttered and broken diatom valves within the sediment samples, it made the construction of the library difficult because the images to choose from were limited.

Since the amount of photos that displayed a diatom shape was limited, the FlowCAM’s versatility was not utilized to its full potential. Therefore, this assay could not be utilized since diatom abundance results would be skewed, as some of the isolated debris was not diatom-
shaped. A way to mitigate this would be to pretreat the samples with hydrogen peroxide to dissolve excess sediment (Battarbee 1973). This would make FlowCAM and photomicrograph diatom images easily distinguished. Since the application of HCl caused increased visibility diatoms in this study, it was believed that an H$_2$O$_2$ technique would greatly increase visibility.

It was possible that the FlowCAM was not utilized to its full imaging potential, as there was a steep learning curve associated with the machine. It would take time to learn all of its features. FlowCAM’s particle parameter table should be utilized to assist with the isolation of diatoms when an image library was created (Figure 7). It was shown in Ide (2007) that the accuracy of plankton identification by image analysis in the FlowCAM was limited. However, Buskey (2006) concluded that the FlowCAM was less tedious and time-consuming method than microscopy. When target cells were added to natural plankton samples, the image recognition software correctly identified 80–90% of the target cells, but incorrectly identified 20–50% of non-target cells.

Petrographic microscope analyses were originally used to identify diatoms by creating slide smears in hopes of retrieving an absolute abundance. However, small samples extracted from large areas of the core created an analysis that was not highly representative to the whole core. Settling-tank assays could obtain a quantitative analysis of diatoms and had been studied since Scherer (1994). Warnock (2015) showed an improvement in this preparation method by using large settling chambers, small samples, and an absence of aliquot subsampling to improve statistical results. Uniform particle distributions were obtained on slide smears for a quantitative analysis, which created a more representative analysis of the core as a whole (Battarbee 1973).

Diatoms were separated from Pennales and Centrales groups based on their minor anatomical and shape differences. Based on whether diatoms were oriented in valve view or profile view onto a slide smear, their anatomical detail was difficult to distinguish, which served as a limitation. Their varying orientations created a difficulty with identifying them. In addition, focus variations of the microscope highlighted different anatomical structures within the diatoms.

**CONCLUSION**

Due to a steep learning curve, FlowCAM’s automated database scan did not isolate diatoms from other particles completely to compute an absolute abundance (Figure 7). As a result, diatoms were counted manually within the FlowCAM database, which concluded that the shallower core possibly contained a higher number of diatoms than the deeper core. Loose interpretations, through the petrographic microscope, of diatoms were made to classify Pennales and Centrales into further morphological groups based on their common shape outline, size and
internal structures (Table 2). The alternative technique of running the same sample through FlowCAM with both 4x and 10x objectives could produce successful quantitative results. This would ultimately photograph a larger range of diatom sizes relative to the field of view.

REFERENCES


