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On the accuracy of assessing copepod size and biovolume using FlowCAM and traditional microscopy

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In this paper, based on the biovolume estimation of different genera of copepods, we present the fact that only the Area-based Diameter (ABD) algorithm of FlowCAM has the efficiency to measure the bio-volume of copepods better than traditional microscopy. Also, we have demonstrated that the efficiency of the Equivalent Spherical Diameter (ESD) algorithm of FlowCAM over traditional microscopy is lesser while assessing the copepod biomass, and it depends on the morphological characteristics of various copepod genera. The ESD algorithm overestimates (8-140 times) while traditional microscopy method underestimates (2-8 times) the copepod biovolume, chiefly because of the inclusion of the entire image field for volume estimation in the former case and avoidance of extended body parts such as appendages in the latter case. These observations have special implications in aquatic environmental monitoring as many of the modern researchers prefer FlowCAM as a better tool to accurately quantify plankton biomass.

[**Keywords**: Plankton, Zooplankton, Copepod, Biovolume, Microscopy, FlowCAM, Arabian Sea]

Introduction

Zooplankton, particularly mesozooplankton, occupies the second trophic level in the aquatic food web, and they consume phytoplankton and get consumed by fishes. Monitoring zooplankton is a well-accepted practice in aquatic research to understand the trophic status and ecological response of an aquatic system[1,2]. Among different taxonomic groups of zooplankton, copepods form the most abundant one and play a vital role in linking primary producers with higher level consumers[3]. The plankton biovolume, a size and shape-dependant factor, mostly determines the biomass[4]. As a substitute to traditional microscopy, many recent researchers use the equipment, FlowCAM, to quantify plankton communities and estimate their biovolume[5-8].

Flow CAM estimates the bio-volume of plankton from their two-dimensional images[9]. Studies have suggested that FlowCAM could rapidly count and size micro - and nano-plankton[5-8,10]. The application of FlowCAM for zooplankton quantification has been presented recently[11] and the primary objective of this paper is to provide conclusive information on the limitations and advantages associated with the estimation of the zooplankton biovolume, especially of copepods, using a FlowCAM and traditional microscopy and discuss the accuracy and errors associated with each method.

Materials and Methods

Mesozooplankton samples were collected from the western Bay of Bengal and the eastern Arabian Sea using standard zooplankton net tows during May - June 2014. Formalin-preserved mesozooplankton samples were first sorted into different taxonomic groups and then copepods were taxonomically classified to the order level. The specimens were washed with saline water before analysis to remove excess formalin. Copepods belonging to different taxonomic orders were selected for the present analysis using traditional microscopy and FlowCAM, the details of which are presented in Table 1. Firstly, the body dimensions (length and width) of each specimen were measured using an Olympus BX53 microscope (Fig. 1a).

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Table 1 - The Dominant forms of Copepods analysed for their Biovolume through Microscopy and FlowCam Methods

Sl. No.	Order (No. of specimens)	Genus/Species
1	Calanoida (25)	*Acartia danae* *Acartia erythraea* *Acartia* sp. *Calanopia elliptica* *Calanopia* sp. *Centropages furcatus* *Centropages* sp. *Euchaeta indica* *Nannocalanus* sp. *Subeucalanus* sp. *Temora turbinata* *Undeuchaeta plumosa*
2	Poecilostomatoida (5)	*Copilia mirabilis* *Corycaeus anglicus* *Corycaeus speciosus* *Oncaea venusta* *Sapphirina stellata*
3	Cyclopoida (5)	*Oithona nana* *Oithona plumifera* *Oithona similis* *Oithona* sp.
4	Harpacticoida (5)	*Macrosetella gracilis*
5	Monstrilloida (5)	Species unknown

The prosome was considered as elliptical and urosome as cylindrical to estimate the biovolume of copepods[12]. Then the specimens were analysed through FlowCAM with image processing software (Visual Spreadsheet IV). In FlowCAM, 1mm field of view (FOV) flow chamber was fixed with the combination of a 2X objective lens and specimen images were captured in autoimage mode; also, the ABD and ESD algorithm based biovolume data were generated (Fig. 1b-c). In ABD algorithm of the FlowCAM, the diameter measured by the number of grey scale pixels of the binary image of copepods is automatically converted to a circle with same number of pixels. Subsequently, from the pixel volumes of the images, total biovolume of the individual is generated. In the case of ESD algorithm, the mean of 36 diameter values measured at every 5° angle of the specimen image is considered to estimate biovolume.

Results and Discussion

For a scientific evaluation of the length and biovolume estimation of copepods using traditional microscope and FlowCAM, the length, width and volume of copepods measured by both methods were compared. The results showed a significant variations ($p<0.005$) in the dimensions of the copepods measured using the FlowCAM and traditional microscope, especially for individuals belonging to Calanoida, Harpacticoida, and Cyclopoida. However, such noticeable variation between the two methods was insignificant in the case of individuals belonging to the orders Monstrilloida and Poecilostomatoida ($p>0.05$).

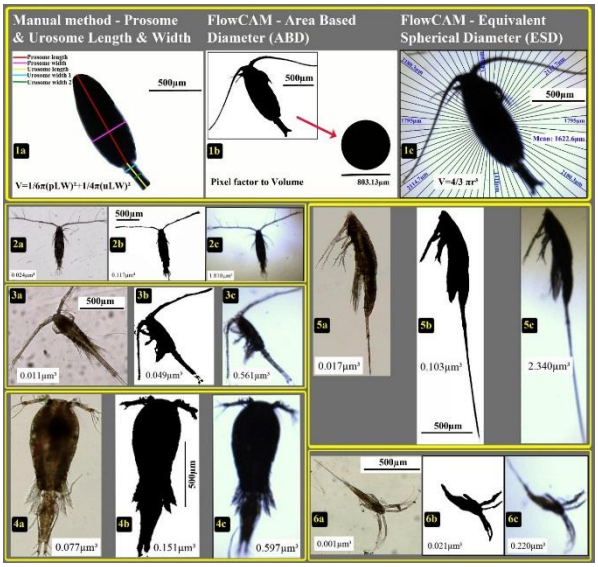

Fig. 1 - The biovolume estimation of copepod by (1a) traditional microscopy (1b) FlowCam ABD algorithm and (1c) FlowCam ESD algorithm. Biovolume estimations of dominant (2a-c) Calanoids, (3a-c) Cyclopoids, (4a-c) Poecilostomatoids, (5a-c) Harpacticoids and (6a-c) Monstrilloids presented. In all panels (a) represents traditional microscopy, (b) represents ABD algorithm and (c) represents ESD algorithm based measurements. The estimated biovolume (µm³) by each method is mentioned at the bottom of each panel.

In most cases, the biovolume of copepods measured using ABD and ESD algorithms of the FlowCAM varied significantly from the value obtained through traditional microscopy. The ABD volume was 6 times and ESD volume 141 times higher than traditional microscopy in the case of copepod order Harpacticoida. Similarly, ABD diameter was 5 times and ESD diameter was 43 times higher than the results provided by traditional microscopy for Cyclopoid copepods (Fig. 2). Microscopy is considered to be the most accurate method accepted worldwide to measure the size and biovolume of individual copepods. In traditional microscopy, however, the protruded body parts of copepods such as appendages cannot be accounted easily in biovolume estimations due to many practical troubles involved in doing so[13,14]. In FlowCAM analysis

methods, the ESD algorithm is recommended for measuring spherical and elliptical three-dimensional particles and, therefore, this algorithm is believed to be more suitable for copepods[15]. However, the present study shows that ESD algorithm is likely to overestimate the dimensions of copepods with long appendages as in this method the entire field of the image of the specimens is considered for biovolume estimations (Fig. 1c).

The ABD biovolume of copepods was found to be higher than the microscopy volume, due to the contribution of the extended body parts such as antenna and other cephalic and thoracic appendages (Fig. 1b). Volume calculated as per ESD algorithm was as many as 8-140 times higher than the ABD and traditional microscopy measured volumes. This can be attributed to the extended body parts such as appendages, which increase the image size and equivalent spherical diameter of the specimen to a large extent (Fig. 1c). In ESD algorithm, the overestimation of biovolume is very high for copepods of the orders of Harpacticoida, Monstrilloida and Calanoida. The extended caudal setae of Harpacticoida and Monstrilloida and the long antennae of the calanoid copepods tend to cause large overestimation in ESD biovolume (Fig. 2).

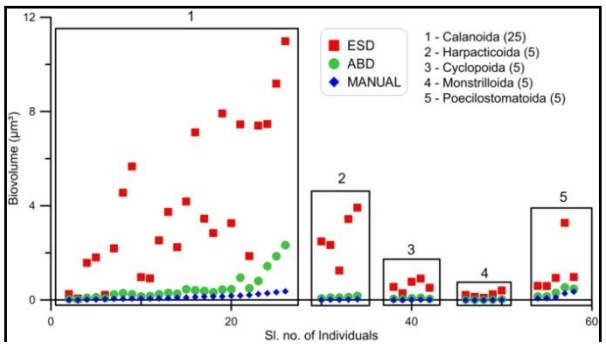

Fig. 2 - Bio-volume estimated in Manual and FlowCam based on ABD and ESD algorithms for five dominant copepod orders. In all cases, ABD algorithm and Traditional (manual) microscopy methods show comparable results. Significant over estimation in ESD algorithm, particularly in the case of Calanoids is evident.

ESD algorithm gets accuracy only when the specimens have a spherical or elliptical shape with short or no extended appendages. Conversely, in all the cases, ABD algorithm selects only the darkened region of the image to generate the biovolume and is hence, more accurate. In essence, it was evident in the present study that the ESD algorithm overestimates (8-140 times) and traditional microscopy underestimates about 2-8 times of the copepods biovolume because of the inclusion of entire image field for volume estimation in the former case and the avoidance of extended body parts in the latter.

Conclusion

Having accepted the fact that the time required for imaging estimation of bio-volume of plankton is remarkably lower in FlowCAM analysis (~3000 specimen/5min) as compared to the manual microscopy method (1 specimen/5min),the present study showed that FlowCAM ESD algorithm overestimates (8-140 times) copepod biovolume, whereas traditional microscopy underestimates (2-8 times) it. This was due to considering the entire image field for volume estimation in ESD algorithm and the avoidance of extended body parts of copepods in traditional microscopy. The ABD algorithm of FlowCAM provides a better estimation of copepod biovolume compared to traditional microscopy as this method also considers the appendages and other extended portions of the copepod for biomass estimation.

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