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NOTE

Treatment and splitting of samples for bacteria and meiofauna biomass determinations by means of a semi-automatic image analysis system

Laurenz Thomsen

Christian Albrechts Universität Kiel, SFB 313, Olshausenstr. 40, W-2300 Kiel 1, Germany

ABSTRACT: Treatment and splitting of samples for bacteria and meiofauna biomass determinations by means of a low cost semi-automatic image analysis system is described. The technique allows enumeration, size measurement and biomass calculation of stained bacteria and meiofauna from the same sample. An example of size spectra and biomass of bacteria and meiofauna around a macrofauna organism tube inhabited by *Echiurus echiurus* from the German Bight is presented. These results demonstrate enhanced biomass and a shift of size-class distribution of these microorganisms inside the tube as compared to the ambient sediment.

The determination of abundance, size-classes and organic carbon content of marine microorganisms is of interest to a wide range of marine scientists. The discovery and analysis of biomass spectra characteristics of benthic communities have led to considerable new insight into dynamics of these communities which are characterized by a gradient of biomass according to logarithmic size-classes (Schwinghammer 1983). Marine microbiologists expend much effort in determining numbers and biomass of marine bacteria (Meyer-Reil 1983, Bratbak 1985). Meiofauna ecologists are concerned with sizes, total counts and biomass of natural samples (Gerlach 1978, Jensen 1984).

Foraminiferal population dynamicists, for example, are concerned with foraminiferal sizes in relation to physiological and physical conditions and growth rates (Lutze 1965, Altenbach 1985, Linke 1986). Following the equation of allometric growth (Bertalanffy 1960), a double logarithmic graph of test length compared to organic carbon content shows an adequate correlation, so the relation between test length and biomass can be computed (Altenbach 1987). Despite the importance of this information there is no reliable method for its rapid estimation. In the case of nematodes and foraminifera, accurate data can be obtained using an eyepiece micrometer but these measurements are time consuming. In the case of smaller bacteria, accurate estimation of size, biovolume and biomass is hampered by their small size (Meyer-Reil 1983, Bratbak 1985). Automated image analysis has been used since the 1950's for counting and sizing of a wide variety of objects (Pettipher & Rodriques 1982, Caldwell & Germida 1985, Siereacki et al. 1985, Bjørnsen 1986, Estep et al. 1986). However, the accurate estimation of biovolume and biomass of extremely small objects like sediment bacteria is prevented by limitations in both hardware and software. The smallest bacteria are represented by only a few screen picture elements (pixels), making their volume estimation inprecise. Systems running fully automatically are unable to differ between fluorescent stained detritus and bacteria. Image analysis systems developed by Bjørnsen (1986) and Estep et al. (1986) included several important hardware and software improvements, like additional magnification lenses, useful for the analysis of bacterioplankton. These systems are composed of a powerful array-processor (Ibas, Artec) with an additional host computer, a moonlight camera and an epifluorescence microscope.

In this article a method for the treatment and splitting of samples is presented which enables the rapid size-determination of bacteria and meiofauna from the same sediment sample with a new low-cost image-analysis system. The cost of the entire system is below that for a single component of the systems mentioned above.

Treatment and splitting of samples. For bacteria and meiofauna biomass determinations, 1 cm^3 of sample was preserved on board ship in 10 cm^3 of buffered 2 % formalin (filter-sterilized using 0.2 µm cellulose-nitrate membranes). A flow diagram for processing the samples is given in Fig. 1.

Bacteria. In the laboratory a 1 cm^3 subsample was

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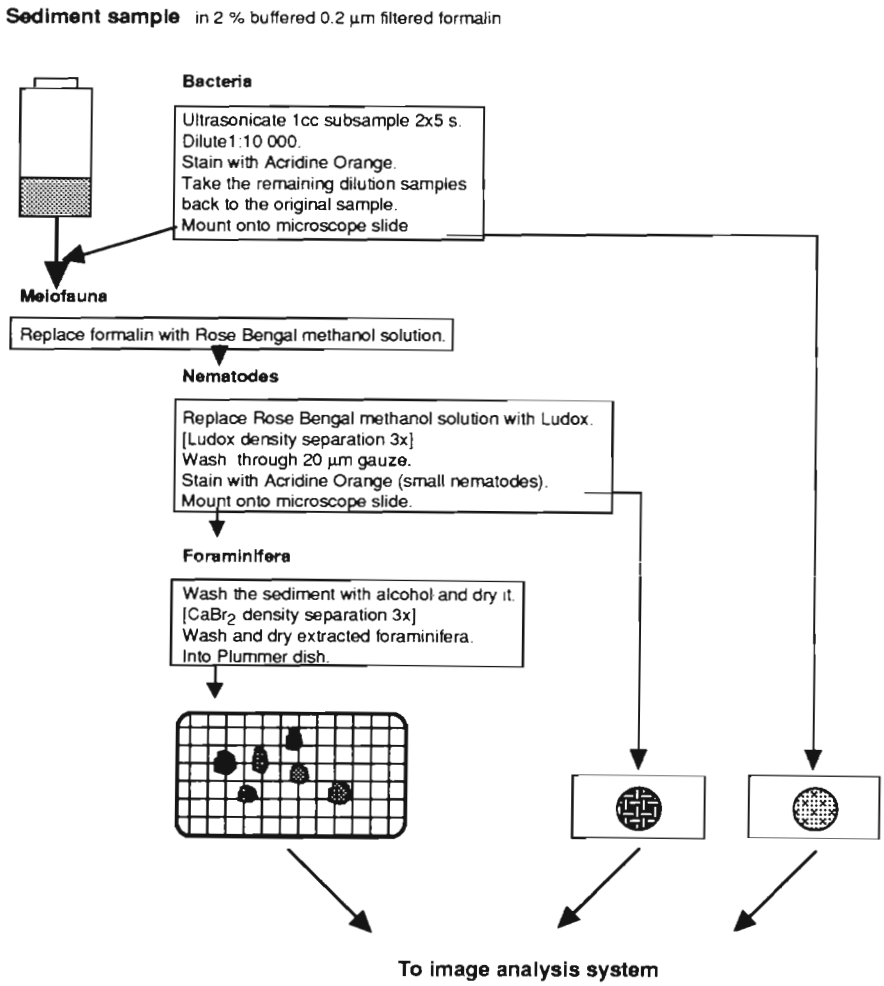

Fig. 1. Flow diagram of treatment and splitting of samples

sonicated (2 × 5 s, 40 kHz Branson sonifier 250) and diluted to final concentration of 1:10 000. The samples were filtered onto Nuclepore filters (0.2 µm pore size; prestained in Sudan black), stained with Acridine Orange, and counted by epifluorescence microscopy (Zeiss 'Standard' fluorescence microscope). Bacteria in each sample were photographed for biovolume estimations (Kodak Ektachrome film ASA 400, colour slides). These pictures were projected onto the computer screen of the image analyser with a film-video processor (TAMRON-fotovix, 4× magnification) and the sizes of 100 to 200 bacteria from each sample were measured.

Soft meiofauna. The remaining subsamples of the dilution series were returned to the original sample. The samples were stained with Rose Bengal and the soft meiofauna was extracted from the sediment by diluted Ludox-TM (δ 1.21) density separation (described by de Jonge & Bouwmann 1977). The extracted meiofauna samples were washed on a 40 µm mesh sieve, sorted and taken to the image analyser for biomass estimations.

Foraminifera. The remaining sediment sample was carefully washed with alcohol and dried. Another density separation with calcium bromide (δ 1.65) (described by Thomsen 1989) was carried out to separate the foraminifera from the sediment by flotation, decantation and sieving. The foraminifera samples were washed on a 20 µm mesh sieve, dried, sorted and taken to the image analyser for biomass estimations.

The image analysis system. The system consists of an Atari 1040ST computer (68 000 CPU, 1MB RAM, 760kB floppy-disk), an Atari Genlock system and a television set (Sony Trinitron, 14") used as computer monitor. For bacteria biomass determinations the system is connected to a TAMRON film-video processor (colour slides of the bacteria). For meiofauna biomass determinations the system is connected to a dissecting microscope (Wild M8) with a Panasonic video camera. Video-pictures of the images from the microscope or the film-video processor are transferred to the computer via the Genlock system by creating a high resolution camera-like video picture of the sample on the computer monitor (Fig. 2). The software developed for

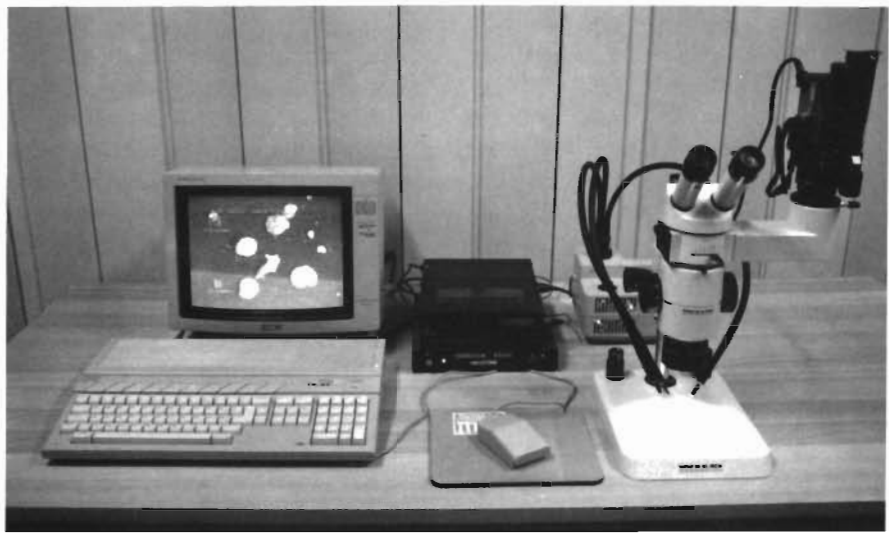

Fig. 2. The image analysis system for meiofauna biomass determinations showing dissecting microscope with video camera (right), Genlock system (center) and Atari computer with monitor (left). For bacteria biomass determinations a film-video processor is used instead of the dissecting microscope and video camera. Images are displayed on the computer monitor via the Genlock system. The Atari computer processes the measurement, analyzes the data, stores the data on a disk and presents the statistical graphics

the biomass estimations is handled by the pulldown menus and mouse commands typical of an Atari application. The basic functions of analysing the objects on the computer screen are accomplished using 'push buttons' on the monitor with the mouse. The program is written in 'C' language and can be purchased commercially ('Biomass', Softwares finest, Fleethörn 64, W-2300 Kiel 1, Germany).

Measuring. After the image analysis system is switched on, the computer program starts with a manually entered calibration routine that allows the calculation of a given distance in nanometers, micrometers or millimeters, a value which is used by the program to convert measured scales to absolute distances. The Genlock system transfers the video picture of the sample onto the computer screen, where the measurements are made by drawing the lines of length and width on the image by hand, using the mouse. Based on the data, the computer calculates length, width, volume and length-to-width ratio of the measured objects. Therefore, no additional array processor is needed. The analysis of the images is done by the Atari computer. Based on the estimation of the volume from body length, both wet weight and biomass of the measured object are calculated by the computer. A problem arose that the smallest bacteria were found to occupy too few pixels to allow proper measurement. This was solved through magnification by the film-video processor. Thus a sphere of 0.5 µm diameter occupied ca 30 pixels in length on the computer screen after magnification.

Table 1. Summary of conversion factors for volume and biomass estimations

Formula	Source
Bacteria	
A: length/width >2 B: length/width <2	
A: Volume $(\pi/4) \times$ width[µm]$^2 \times$ (length[µm] − width[µm]/3)	
B: Volume $(\pi/6) \times$ length[µm] $\times$ width[µm]^2	
C(g) = $1.1 \times$ volume $\times 10^{-13}$	Meyer-Reil (1983)
Nematoda	
Volume[nl] = length[µm] $\times$ width[µm]$^2/16\times10^5$	Andrassy (1956)
Wet weight[µg] = volume[µl] $\times$ 1.1	Wieser (1960)
C[ng] = wet weight[µg] $\times 10^3/8$	Jensen (1984)
Foraminifera	
Volume[µm]$^3 = \pi/6 \times$ length[µm] $\times$ width[µm]^2	Murray (1967)
Volume[µm]$^3 = \pi/6 \times$ width[µm] $\times$ length[µm]^2	
Volume[µm]$^3 = \pi/12 \times$ length[µm] $\times$ width[µm]^2	
C[ng] = (volume $\times$ 0.06) $\times$ 1000	Altenbach (1985)
Allometric relation between test length and organic carbon content:	
C[µg] = $0.5456 \times 10^{-5} \times$ maximum test length[µm]$^{1.77}$ (*Elphidium excavatum*, Rotaliina, Foraminifera)	Altenbach (1985)

Table 2. Accuracy of the image analysis system on bacterial sized objects. Comparison of manufacturer's stated sizes of 4 fluroescent microsphere size-classes to their sizes as determined using the computer system. Values are given as a percentage of the 'true' values (supplied by the manufacturer)

Manufacturer's size		Image analyser	
Diameter ([µm])	Vol (μm^3)	Diameter (%)	Vol. (%)
0.21	0.005	109.5	128.6
0.57	0.097	100.9	103.6
0.73	0.204	101.6	105.4
1.74	2.758	98.6	96.6

The introduced background noise and blooming of the objects was suppressed by using 'false colours'. For those foraminiferal species for which the allometric relation between test length and biomass had already been calculated (Altenbach 1985) only the maximum test length was measured. The entire data file was transferred to a statistical analysis and graphic presentation program. Table 1 shows the conversion factors that were used to calculate volume and biomass data. The accuracy of the system was tested by comparing the manufacturer's stated sizes of 4 fluorescent microsphere size-classes to their sizes as determined using the computer system. Some 150 fluorescent spheres of each size were analysed with the image analysis system. Results are presented in Table 2. For spheres with diameters of 1.73, 0.73 and 0.57 µm, the image analysis system estimated diameters and volumes within 6 % of the mean values as stated by the manufacturers. Spheres of 0.21 µm diameter were overestimated in diameter by 10 %, and in volume by 29 %. A deviation of 0.01 µm easily occurs in measuring these smallest bacteria owing to the limited optical resolution of the light microscope. However the crucial problem to be solved is still the differentiation between stained bacteria and particles.

Case study. During RV 'Poseidon' Cruise 146-4 to the German Bight, sediment samples around a macrofauna zoobenthos tube inhabited by *Echiurus echiurus* were collected with small cut-off syringes radially outward from the tube at 1 cm depth intervals down to a depth of 8 cm where the burrow of *Echiurus echiurus* was located. Fig. 3 depicts the difference between the logarithmic size spectra of bacterial biomass inside the tube and that of those in the ambient sediment at a depth of 5 cm. In comparison to the surrounding sediment the distribution of bacterial biomass inside the tube shifts to higher size classes. Inside the tube peak biomass occured in the 3.2 to 5.6 $\times 10^{-5}$ ng ind.$^{-1}$ size class. Bacteria of the 1.8 to 3.2 $\times 10^{-5}$ ng ind.$^{-1}$ size class dominated in the ambient sediment. Inside the tube bacterial carbon was ca 70 to 80 % of the total bacterial and meiofauna biomass with lower values in the ambient sediment where the total bacterial biomass decreased rapidly (Fig. 4). Foraminifera dominated the meiofauna biomass inside the tube (10 to 15 % living foraminifera) and in the ambient sediment (5 to 10 % living foraminifera). Contribution of nematodes to the total living carbon was about 1 to 5 %.

Using the system described, the measurement and statistical evaluation of e.g. 100 bacteria, 100 foraminifera and 100 nematodes was possible in about 4 h. The precision represents a considerable improvement over manual procedures.

The treatment and splitting of samples together with image analysis is a promising tool for enumeration and size measurement of stained bacteria and meiofauna when variations in cell number, size-classes and biomass are studied.

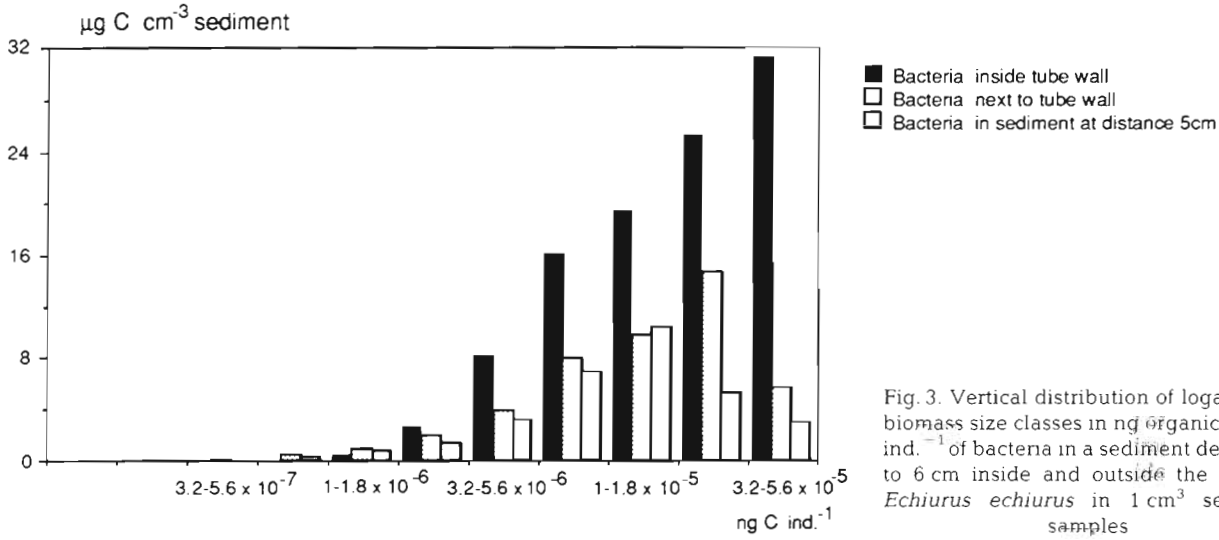

Fig. 3. Vertical distribution of logarithmic biomass size classes in ng organic carbon ind.$^{-1}$ of bacteria in a sediment depth of 5 to 6 cm inside and outside the tube of *Echiurus echiurus* in 1 cm^3 sediment samples

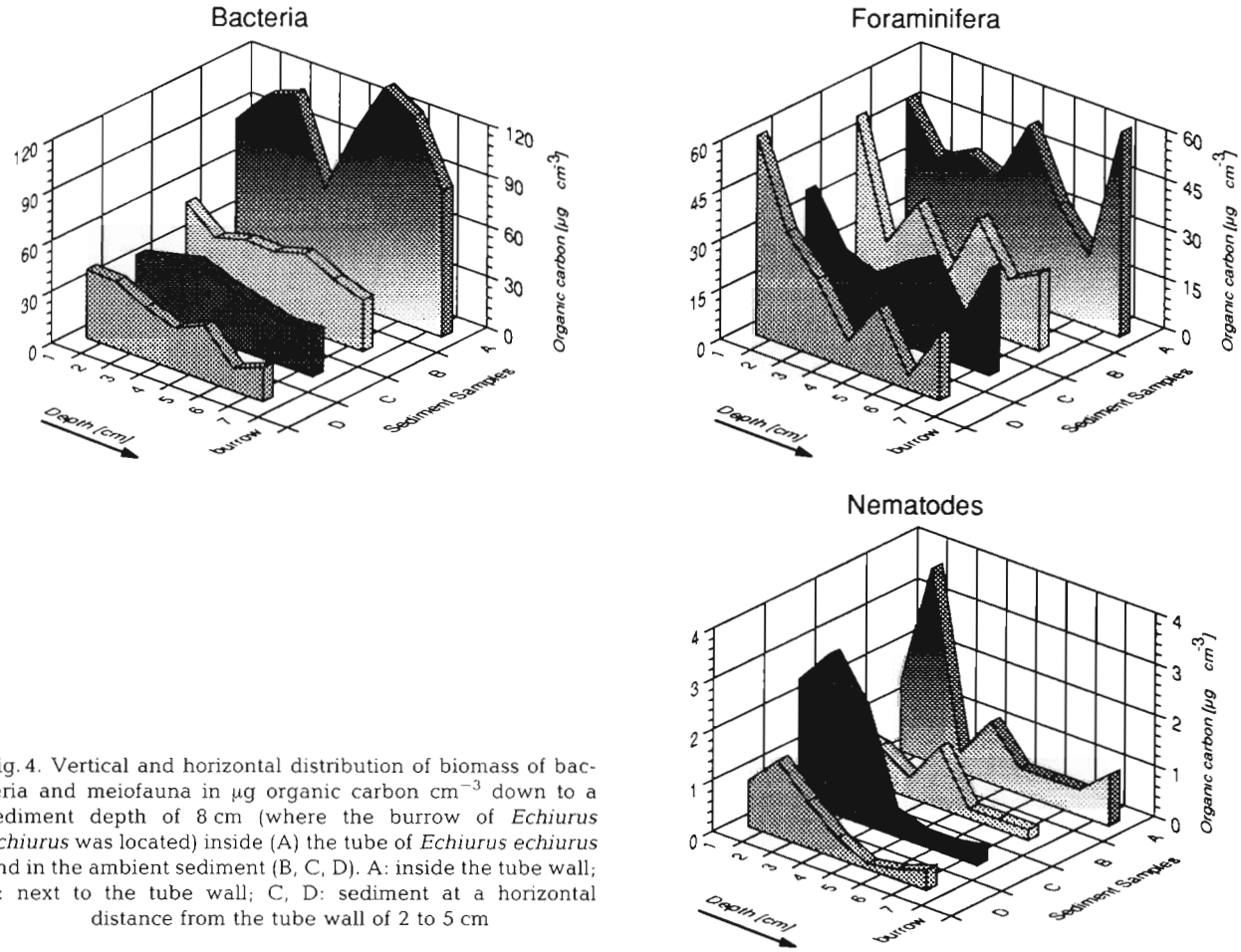

Fig. 4. Vertical and horizontal distribution of biomass of bacteria and meiofauna in µg organic carbon cm^{-3} down to a sediment depth of 8 cm (where the burrow of *Echiurus echiurus* was located) inside (A) the tube of *Echiurus echiurus* and in the ambient sediment (B, C, D). A: inside the tube wall; B: next to the tube wall; C, D: sediment at a horizontal distance from the tube wall of 2 to 5 cm

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