

# Manual for Marine Monitoring in the

# COMBINE

## Programme of HELCOM

### Part C

## Programme for monitoring of **eutrophication** and its effects

Annex C-12

Guidelines concerning  
bacterioplankton abundance determination



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# ANNEX C-12: GUIDELINES CONCERNING BACTERIOPLANKTON ABUNDANCE DETERMINATION

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## 1. INTRODUCTION

The total biomass of bacterioplankton constitutes an indicator of nutrient status in aquatic environments and thereby an indicator of eutrophication. The variable is estimated by direct microscopy and image analysis following methodology published in refereed international scientific journals.

Bacterioplankton biomass analysed by manual direct microscopy is relatively simple and economic. It may therefore be conducted by most laboratories. Image analysis provides a better estimate of biovolume, save counting effort and is operator independent. As a state variable changes in bacterial biomass is subjected to more complicated interpretation than bacterial growth rate, as both growth, mortality and e.g. competition may constitute reasons to observed variations. Ideally therefore both variables are included in a monitoring programme, but may be evaluated also individually.

### 1.1 BACKGROUND

Bacterioplankton constitutes about half of the living mass of secondary producers in many aquatic environments. Bacteria also account for half of the secondary production in many aquatic environments, and are comparable to the productivity of phytoplankton in some environments <sup>1</sup>.

Bacterioplankton are osmotrophs feeding on dissolved organic carbon and dissolved mineral nutrients. They often live like solitary cells free floating, but may also grow attached to particle surfaces. Bacterioplankton typically divide by binary fission, are rod shaped, spherical or c-shaped with an average dimension of 0.6  $\mu\text{m}$ . Small heterotrophic flagellates are their main predators.

Both the biomass and productivity of bacterioplankton is shown to increase with increasing nutrient status in aquatic environments <sup>2,3</sup>. Time series of bacterioplankton biomass also show proper power to detect trends (J. Wikner unpubl. data). Monitoring of bacterioplankton is therefore motivated to follow the nutrient status of marine environments.

### 1.2 PRINCIPLE

Bacterioplankton in aquatic environments are rather small ( $\approx 0.5 \mu\text{m}$ ) and at low abundance ( $10^9 \text{ dm}^{-3}$ ) as compared to bacteria in culture. To create sufficient contrast in the microscope samples are stained with a fluorescent dye like acridine orange (AO)<sup>4</sup>. This is a planar aromatic molecule that binds unspecifically to nucleic acid, but also other cellular components. Staining of the whole cell allow estimation of cell size. AO fluoresce in visible light (red/orange) at illumination with wave lengths 450 – 490 nm.

The sample is filtered on to a blackened filter and stained in the filter funnel. All bacteria will thereby be placed in one focal plane, concentrated and become easier to focus. The filter is mounted on a glass slide and a drop of paraffin oil added, before the cover slip is applied to achieve minimal light diffraction.

A statistically determined number of microscopic fields are counted by aid of an ocular grid or image digital camera. The size of each field is determined by a micrometer scale. The filtered area is determined by the inner diameter of the filter funnel. The average number of bacteria per microscopic field is calculated, and

the number of bacteria on the filtered area is determined by multiplying with the ratio between filtered area and microscopic field area.

By image analysis cell volume and morphology type may also be estimated<sup>5</sup>. The bacterial volume may also be estimated manually by comparison with an ocular scale. The volume is calculated by geometric functions for cylinders and spheres. The bacterial volume may then be used to calculate the cell biomass from known carbon-to-volume relationships.

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### 1.3 EXTENT

Samples from oceanic, brackish and fresh waters may be analysed. Filtration of a sample volume providing about 30 cells per microscopic field mean that cell concentrations from  $1 \times 10^7$  cells  $\text{dm}^{-3}$  may be detected.

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### 1.4 DISTURBANCES

The sample should be preserved prior to filtration and filtration the same day as sampling should be aimed at.

Use well rinsed sample containers. Sample bottles and solutions added to the samples must be free from contaminating particles that may be stained. Make sure that the filtering proceeds at an expected rate. Too rapid filtration may indicate a broken filter, erroneously applied filter or too high vacuum.

The background should typically be near black with a good contrast to the bacterial cells. Try to find a remedy to high background before counting the sample.

Two different qualities of filters have occurred at the market. The darker is required for proper microscopy.

Check the settings of the microscope at each counting session. Changes in type of objective, ocular or other lenses may change the magnification factor. The settings should match the directives under item 8.7 or your customized settings.

If many organic aggregates occur in the sample, a significant occurrence of particle bound cells may be found. The total concentration of bacteria may then be under estimated. In this case pre-treat the sample with detergent and sonication according to item 4.3

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### 1.5 CONTAMINATION RISK

See to that the sampling towers are well rinsed with particle free water after each usage to reduce remaining bacteria. Background preparations from Milli-Q purified deionized water, as described under item 4.3 C, should be made at each filtration occasion. By experience contamination from filter funnels, air or other sources is negligible if this standard operating procedure is followed.

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### 1.6 SAFETY

Acridine orange and formaldehyde are harmful substances. Handling of these chemicals shall be done in a ventilated hood and with gloves. Preferably formaldehyde should be used on a ventilated bench as the gas

phase is heavier than air. Mouth protection should be used when working with acridine orange powder as it may cause cancer.

## 2. PREPARATION

### 2.1 CLEANING AND PURIFICATION

#### SAMPLE BOTTLES

Sample bottles are washed with detergent and warm water, followed by rinsing with Milli-Q water. The bottles are air dried and stored with cap or stopper to avoid contamination.

#### FILTER EQUIPMENT

The filter equipment should be washed after each filtering occasion. The filter manifold is rinsed with warm water followed by Milli-Q water. The same cleaning procedure is done with the filter funnel. Pay specific attention to the filter supports. Strive for lack of remaining colour from the stain.

#### GLASS SLIDE AND COVER SLIP

Glass slides and cover slips directly from the packages may be used without further cleaning. See to that no larger particles remains that may interfere with planar application of the cover slip.

#### MICROSCOPE

The ocular and objective should be cleaned annually or as required by competent staff. Use lens paper and chemical pure gasoline. For more comprehensive cleaning consider to use microscope technician from the supplier.

### 2.2 SAMPLE IDENTIFICATION

All sample bottles should be labelled with cruise identity, sampling station, variable, sample depth and year. Use water resistant tape and marker pen. The glass slide is labelled with water resistant marker pen with cruise identity, sampling station, sample depth, filtered volume and filtration date.

### 2.3 REAGENTS

Formaldehyde crystals may form with time in its concentrated form (37%). Therefore, concentrated formaldehyde is filtered through 25mm Ø Acrodisc® filter, 0,2 µm pore size at earliest 1 week before sampling.

Mix 30 mg acridine orange in 10 cm<sup>3</sup> of Milli-Q water. Use mouth protection and gloves.

### 2.4 PRIOR TO SAMPLING

Pure sample bottles (e.g. 50 cm<sup>3</sup> glass bottles) are added 2.0 cm<sup>3</sup>, 0.2 µm filtrated, 37% formaldehyde. Close with a rubber stopper and plastic wrapping (Parafilm™) or other closure. Provide an aluminium seal for bottles lacking a screw cap, to be applied after sampling.

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## 2.5 PROTOCOL

A protocol for logistic data (Station, coordinates, date, time, etc.) should be used during the sampling

## 3. SAMPLING

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### 3.1 SAMPLING

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#### 3.1.1 SAMPLING STRATEGY

It is recommended to take at least 2 samples at different representative depths of the monitored layers of the water column. Surface layer and deepest layer are made priority to. Layers are defined by hydrographic profiles. Required power of the data and natural variability set the required number of samples.

A sampling frequency of 8 samples per year is required to get confident annual estimates (J. Wikner, unpubl. results). Samples should be distributed in the seasonal curve to provide a good coverage of different levels (more samples during the productive season).

An economic alternative is to allocate at least 2 samples to a representative month with limited inter-annual variation. This strategy, however, result in a lower power to detect trends and less ability to cover changes in seasonality. August is recommended based on current experience. Low frequency stations should preferably be evaluated together with high frequency stations located in the same sea area.

It is advocated that at least one high frequency station of 18 samples per year is monitored in each country. This allows an analysis of intra-annual variation and follow changes in seasonal dynamics.

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#### 3.1.2 SAMPLING METHOD

Sampling may be performed with a rosette sampler or Niskin bottles attached to a wire.

Rinse a Vogel-pipette with sample water and discard. Add new sample liquid and pour 50 cm<sup>3</sup> to a labelled sample bottle with formaldehyde added. Mix the sample by turning the bottle up-side-down 5 times.

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### 3.2 PRESERVATION/PROCESSING

Preservation is provided by the filtered formaldehyde (1.4 % final conc.) in the prepared sample bottle.

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### 3.3 STORAGE

Store the preserved samples at 4 °C. A preserved sample may be stored in the refrigerator (4 °C) for 7 days before making the microscope slide. Microscope slides with filter mounted may be stored for at least 70

days in the freezer (- 20 °C) without loss of cells<sup>6</sup>. Store the glass slides horizontally to avoid that the immersion oil pours away.

Dry filters may be stored for at least 70 days in the freezer (- 20 °C).

## 4. METHOD DESCRIPTION

### 4.1 REAGENTS

Prepare acridine orange, immersions oil, and Milli-Q water according to item 9.

### 4.2 CALIBRATION SOLUTION

Calibration standard with fluorescent beads are primarily used for image analysis systems, but may be applied at manual counting to control settings and magnification. Prepare fluorescent micro-spheres according to item 9.

### 4.3 SAMPLE PROCESSING

Collect fresh Milli-Q water in two sterile plastic tubes (e.g. Falcon 50 cm<sup>3</sup>). One tube (A) is used for rinsing the pipette between the transfer of samples to the filter funnels. The other tube (B) is used to provide Milli-Q water when rinsing filters after staining.

If the samples have a large amount of aggregates and particle bound bacteria they need to be sonicated before analysis. This may be the case for coastal stations during some seasons<sup>7</sup>. A preserved sample is added Triton X-100 (0.001% final conc.) and natriumpyrophosphate (10 mM final conc.). Mix the sample by a vortex for 5 s. Sonicate the sample in ice bath for 30 s at 75 Watt power.

1. Clean the filter support by a paper towel, close the valve and place a few drops of Milli-Q water on the support
2. Mount a GF/C filter and wet it with Milli-Q water. Apply a black 0.2 µm polycarbonate filter (e.g. Poretics<sup>®</sup>). Apply vacuum so that the filter lies flat on the GF/C filter. Close the vacuum source, let the pressure disappear and close the valve
3. Make a background sample where a filter without sample addition is stained and destained with Milli-Q water in the same way as the other samples
4. Shake the sample flask violently for 10 s to homogenise the sample. Open the lid and transfer the appropriate volume of sample (typically 3-7 cm<sup>3</sup>), aiming at least 30 cells per microscopic field (same tip as under item C may be used). Use a sample volume table for different stations and seasons developed by experience. See an example for the Gulf of Bothnia in item 11.1. Rinse the pipette tip with Milli-Q water (Falcon Tube A)
5. Filter all samples at a vacuum of -13 kPa (-100 mm Hg, 0.87 bar, 14 psi ) to the filter surface appear dry. Close the vacuum let the pressure disappear and close the valve

6. Fill a syringe with acridine orange. Mount a 0.2 µm Acrodisc® filter. Add 15 drops (about 0.75 cm<sup>3</sup>) of acridine orange to each sample. The filter should be completely covered by the stain. Incubate for 5 min.  
In the mean time label the glass slides according to item 2.2. Apply immersion oil according to item I. See to that the background-filter is on the same glass slide as a sample to aid focusing
7. Filter the stain through the filters. Close the vacuum, let the pressure disappear and close the valve. Wash the filter by adding 1 cm<sup>3</sup> of Milli-Q water from Falcon tube B with a clean tip. Filter the liquid through the filter until it appear dry and leave the vacuum on. Remove the filter funnels
8. Pick up the filter with forceps. Air-dry the filter 45 s by slowly moving it in the air until dry. The filter may be labelled and stored in this condition
9. Apply a drop of immersion oil where filters are supposed to be mounted. Two 25 mm filters may be mounted on each glass slide. Spread the immersion oil drop on an area larger than the filter, by using the filter itself. Place the filter on the oil film. Add a drop of oil on top of the filter. Mount the cover slip and let the oil spread under the whole cover slip by capillary forces. Occasionally a slight pressure on the cover slip by the forceps may aid the spreading of the oil. If two filters are mounted large cover slips covering both may be used. Store the glass slides in slide holders at -20 °C until microscopic analysis
10. Wash the filter funnels after the filtration according to item 2.1. See item 3.3 for storage of samples
11. Preserved liquid samples and glass slides are stored until the annual quality assurance is done

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#### 4.4 CALIBRATION

Check that the settings of the microscope (lenses and filter sets) match with that expected (cf. item 8.6 and 11.4). Use a standard preparation of fluorescent beads with known size to calibrate the microscope and image analysis system if applied. Run standard beads in the beginning of each session and compare the results with a control diagram.

The control diagram should include bead abundance and bead size as a function of analysis date.

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#### 4.5 ANALYSIS

Analysis of microscope slides with bacterioplankton on filters may be done manually or by image analysis.

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##### 4.5.1 IMAGE ANALYSIS

Microscopic fields may be photographed by a digital video camera, images stored on digital media and the particles analysed by image analysis software. This is a preferred analysis as it is operator independent, possible to calibrate, reduce manual counting effort and is economic.

Specific procedure and settings should be done according to specification for camera and software used. An example of an image analysis system routinely applied for monitoring analysis for 5 years is available in item 11.4

#### 4.5.2 MANUAL ANALYSIS

1. Manual analysis may be used if image analysis can not be accomplished. This will introduce operator dependence and less accurate biovolume estimates. Check that the correct equipment is installed and settings used (item 8.6). Check that the run time of the mercury lamp is not exceeded (max. 200 hours). Record the run-time in a log journal
2. Count the number of cells in 20 microscopic fields, distributed in a representative way over the filter surface. Avoid looking in the microscope while moving position. If areas with markedly heterogeneous distribution occur, a new slide should be prepared
3. Count cells that are rod-shaped, spherical and c-shaped particles, cells appearing solitary or in colonies. Irregular particles are likely not bacteria and should be excluded. Extremely tiny particles may be viruses and should be neglected
4. Use the large square in the ocular grid. Choose a sample volume resulting in an average of 30 cells per field (use item 11.2). Count 20 cells per sample or more than 300 cells in total. Standard error of the average of microscopic fields should not exceed 10 %. Count more fields in that case

## 5. CALCULATIONS

### 5.1 CALCULATION FUNCTIONS

#### 5.1.1 TRANSFORMATION OF CELL NUMBERS

The average number of cells per microscopic field ( $BA_f$ ) and particles on the corresponding background filter ( $BA_{bkg}$ ) is used to calculate the concentration of bacteria ( $N_b$ ) in the sample according to:

$$N_b = F_f \times \frac{(BA_f - BA_{bkg})}{V \times d} \quad (1)$$

where  $V$  is the filtered volume,  $F_f$  the magnification factor (5.1.2), and  $d$  the dilution factor due to formaldehyde (50/52=0.9615).

#### 5.1.2 MAGNIFICATION FACTOR

The magnification factor  $F_f$  is the filtered area (i.e. inner diameter of the filter funnel) ( $A_f$ ) divided by the area of the camera image or the ocular grid ( $a$ ) according to:

$$F_f = \frac{A_f}{a} \quad (2)$$

This factor depends on the ocular, lenses and objective on the microscope and camera according to item 8.6. Use a micrometer scale to measure the area of the captured image from the CCD camera or ocular grid square (item 8.5).

### 5.1.3 VOLUME CALCULATION

The volume estimate is made on each sample with image analyses. When doing manual microscopy an estimate should be made at 4 occasions during the year.

The bacterial ( $V_b$ ) is calculated from estimated cell length and cell radius assuming a cylinder with two half spheres according to:

$$V_b = \frac{4\pi r^3}{3} + \pi r^2(l - 2r) \quad (3)$$

where  $l$  is the cell length and  $r$  the cells radius. For cocci the length is <1.5 times the width. The radius is calculated by an estimate of the cell area in the image analysis software according to Blackburn et al. 1998<sup>5</sup>.

### 5.1.4 CARBON DENSITY

The carbon density per cell is determined from biovolume by a volume dependant function<sup>8,9</sup> according to:

$$m_b = 0.12 \times v_b^{0.7} \quad (4)$$

where  $m_b$  is the carbon content of the bacterium in  $\text{pg cell}^{-1}$  and  $v_b$  the bacterial volume in  $\mu\text{m}^3 \text{cell}^{-1}$ . The constant 0.12 is a conversion factor with the unit  $\text{pg carbon } \mu\text{m}^{-3}$ . The carbon biomass may be converted to  $\text{mol dm}^{-3}$  by dividing with the molar weight for carbon ( $12 \text{ g mol}^{-1}$ ). Bacterial volume may be interpolated between samplings if missing. Literature values (e.g.  $0.07 \mu\text{m}^3$ ,<sup>5</sup>) may provide a rough estimate of the biovolume as within year variation has been shown to be low ( $\pm\text{SD } 10\%$ )<sup>5</sup>.

### 5.1.5 BACTERIAL BIOMASS

The bacterial biomass ( $B_b$ ) is determined as

$$B_b = N_b \times m_b \quad (5)$$

## 5.2 CALCULATIONS

Primary data from the image analysis or manual count is readily calculated to appropriate parameters in a calculation software like Microsoft Excel (item 11.1). Calculated data can be aggregated using a pivot table procedure. Compiled data are entered into a database according to item 7.

## 5.3 PRECISION AND ACCURACY

The detection limit of the method is estimated to  $5.0 \times 10^6 \text{ cells dm}^{-3}$ . The calculation is based on 1 cell per 5 fields on average multiplied by the magnification factor.

The standard error for microscopic fields should be less than 17 % (action limit of  $3 \times \text{SD}$ ) when performing image analysis and 10 % at manual count (due to fewer but larger fields in the former technique).

Otherwise count more fields. At high heterogeneity consider making a new slide or sonicate the sample

according to item 4.3. The standard deviation between 2 homogenous samples has been estimated to  $\pm 2$  %.

The accuracy of the method is based on the difference in morphology between, other plankton cells bacteria and abiotic particles. For manual count specific care should be taken to distinguish coccoid cyanobacteria (1-2 cells) during juli-august, when they may approach 10% of the heterotrophic bacterial community. Inter-calibration with other laboratories is desirable as objective standards are missing.

In image analysis coccoid cyanobacteria are discarded based on size criteria. The accuracy is not operator sensitive, but depend on the ability of the neural network to distinguish bacterial cells from similarly sized and shaped particles.

The measurement uncertainty has been determined according to the standard of measurement uncertainty in chemical analysis of the European Union <sup>10</sup>.

The standard uncertainty corresponds to standard deviation and is estimated from several identified variance components of the method. The bacterial biomass shows a low uncertainty of  $\pm 23$  %, approximately corresponding to a 95 % confidence interval (Table 1). The estimate of average number of cells per microscopic field contributes with the greatest uncertainty (i.e. variation between microscopic fields). The uncertainty is therefore similar in the unit cells  $\text{dm}^{-3}$ .

**Table 1:** Measurement uncertainty for bacterial biomass. The expanded uncertainty (*U*) is presented based on the expansion factor 2.

Parameter	Unit	Value	U (%)
<b>Bacterial biomass</b>	$\mu\text{g carbon dm}^{-3}$	28	23

## 6. QUALITY ASSURANCE AND EVALUATION

When performing manual counting the standard error for the average number of cells per microscopic field should be below 10% in manual analysis. Increase the number of counted field otherwise.

### 6.1 CONTROL CHARTS

Run a standard of fluorescent beads at each session when performing image analysis. Bead abundance and volume is plotted against date.

The Milli Q background should show values of  $4 \times 10^5$  cells  $[\text{filtered area}]^{-1}$  ( $\pm 6.4 \times 10^5$ ) based on image analysis estimates. The background value should be divided by the sample volume prior to subtraction from the corresponding sample. When the image analysis system finds no particles an error message may be returned. These background values should be set to zero manually.

Standard deviation (i.e. variance between microscopic fields) for samples and Milli-Q backgrounds are plotted versus date.

Duplicate samples should be analysed regularly amounting to about 10% of the total number of samples. Plot the standard deviation of duplicates against date.

## 6.2 EVALUATION

After a full year of data set has been collected, values are scrutinized by plotting bacterial concentration for each station versus date and depth, respectively.

Evaluate estimated bacterial concentration versus empirically derived seasonal and depth abundance. Coupling to substrate and local values should show an expected seasonal variation with highest values during late summer. Bacterial abundance should typically be higher in the trophic layer than in the deep water.

For all control charts it's advised that samples differing more than  $2 \times SD$  from average values are checked, and actions should be taken for samples deviating more than  $3 \times SD$ . If causes of errors can be identified they are attended to. If no source of error can be identified the sample maybe left as an extreme value or is labelled by quality code "questionable value". Be careful not to delete values without proper reason.

## 6.3 HARDWARE CALIBRATION

The camera image size has been determined with a microscopic scale with the 63x objective (item 11.4). The image area for the camera was determined with aid of the micrometer scale. Height and width was determined to  $107.4 \mu\text{m}$  and  $132.8 \mu\text{m}$ , respectively ( $14258 \mu\text{m}^2$ ), using a scale to measure dimensions on the computer screen image.

The ocular counting square size is determined in a similar way when performing manual counting. A typical size for a large counting square in the ocular is  $70 \times 70 \mu\text{m}$ , and  $21 \times 21 \mu\text{m}$  for a small square.

Filtered area was determined from the diameter of the filter funnel ( $21\,000 \mu\text{m}$ ). The filter area ( $\pi r^2$ ,  $3.464 \times 10^8 \mu\text{m}^2$ ) and image area was used to determine the magnification factor according to equation under item 5.1.2 to 24292.

Number of pixels per  $\mu\text{m}$  for the CCD camera was determined with the "Analysis: Line" option in the image capture software *Wasabi*. The microscopic scale was projected with a regular light source and captured in *Wasabi* as an image. A line measure in *Wasabi* determined the length in pixels between two scale lines. The distance between the scale lines was divided by the length in pixels giving  $0.0986 \mu\text{m pixel}^{-1}$  for the system. This value should be entered in the "Calibration" cell in LabMicrobe (item 11.4.4). LabMicrobe round this value to 0.1 but use the precise value.

## 7. REPORTING FORMAT

Make required calculation in a calculation software like Microsoft Excel and aggregate data from different images using pivot tables. Variable specific parameters are given in table 2. A similar table may be set up for Milli-Q and bead results. Also add logistic and quality code information according to standards of the International Council for the Exploration of the Sea (ICES) and HELCOM.

For internal laboratory data handling it is recommended to record preparation date and time of the samples (link Milli-Q background with samples), and analysis date and time which link bead standards with samples.

**Table 2:** Reporting format of bacterial biomass shown with units and typical values.

Parameter	Unit	Digits	Function	Categories	Acronym	Value ex.
<b>Bacterial abundance</b>	cells dm <sup>-3</sup>	3	5.1.1, 5.1.2	Depth, Standard	BACTABU	1.30E+09
<b>±SD Bact. conc.</b>	cells dm <sup>-3</sup>	2	Statistics	Depth, Standard	BASDCON	1.10E+08
<b>Number of fields</b>	number	1	-	Depth, Standard	BANPIC	5
<b>Counted cells</b>	cells	3	-	Depth, Standard	BACOUCEL	500
<b>Bacterial biovolume</b>	µm <sup>3</sup> cell <sup>-1</sup>	3	5.1.3	Depth, Standard	BACVOL	0.07
<b>±SD Biovolume</b>	µm <sup>3</sup> cell <sup>-1</sup>	2	Statistics	Depth, Standard	BASDVOL	0.006
<b>Preparation date-time</b>		10	-	Depth, Standard	BAPDAT	05.09.2026 13:50
<b>Analysis date-time</b>		10	-	Depth, Standard	BAADAT	05.10.2001 14:30

**Table 3:** Calculated parameters.

Parameter	Unit	Digits	Function	Categories	Acronym	Value ex.
<b>Carbon density</b>	fg cell <sup>-1</sup>	3	5.1.4	Depth	BACTDENS	19
<b>Bacterial biomass</b>	µg carbon dm <sup>-3</sup>	3	5.1.5	Depth	BACTBIOM	24

“Statistics” mean that established statistical functions are used.

Valuable variables of explanation encompass bacterial growth rate (whole community, cells  $\text{dm}^{-3} \text{day}^{-1}$ ), temperature ( $^{\circ}\text{C}$ ), total phosphorus ( $\mu\text{mol dm}^{-3}$ ), total nitrogen ( $\mu\text{mol dm}^{-3}$ ) bacterivorous flagellates (cells  $\text{dm}^{-3}$ ) and oxygen concentration ( $\text{mol O}_2 \text{dm}^{-3}$ ).

Data should be reported to national and international databases. If primary data is not archived by these databases the operator should store primary data locally.

Images and primary data may be archived on digital media for time determined by quality assurance directives.

## 8. EQUIPMENT

### 8.1 FILTER

Blackened polycarbonate filters with pore size  $0.22 \mu\text{m}$ , diameter 25 mm are used to capture bacteria. Glassfiber filter (e.g. Whatman GF/C), 25 mm diam. are used as filter supports.

Two qualities of blackened filter have occurred on the market. The blacker type is required for epifluorescence microscopy.

### 8.2 MULTI-FILTERING UNIT

A multifilter-unit with 12 filter sets for 25 mm diameter filters is recommended. Filter funnels may be made of stainless steel or glass.

### 8.3 SUPPLIES

Plastic syringe:  $5 \text{ cm}^3$ , Plastipak, Becon and Dickonson. Pincett, Millipore, Glass slides, Menzel-Gläser,  $76 \times 26 \text{ mm}$ . Cover slip, Menzel-Gläser,  $24 \times 60 \text{ mm}$ , #0, Sample bottles: Glass,  $50 \text{ cm}^3$ . Gloves: Powder free vinyl gloves.

### 8.4 STERILE FILTER

$0.2 \mu\text{m}$  glassfiber filter, sterile, non-pyrogenic (e.g. Acrodisc<sup>®</sup>, GelmanScience).

### 8.5 MICROSCOPIC SCALE

A microscopic scale constitutes the basis for abundance estimates and size determination of bacteria in the microscope and image analysis system. One example is S8-Stage Mic., (Graticules Pyser-SIG LTD., Great Britain). A calibration certificate should be provided with the microscope scale.

### 8.6 MICROSCOPE

An example of a microscope system is given below. Other systems with similar capacity may be applied.

Zeiss Axiovert 100:

Ocular: 10 x/20, adapted for glasses, prod. nr. 44 40 32  
Oil-objective: Planapo 63 x/1.4, oel, 160, prod. nr. 44 04 81  
Colour filter set: Acridine orange: FS 09: 450-490, FT 510, LP 520  
FS15: BP546, FT580, LP590  
DAPI: FS 02; G 365, FT 395, LP 420  
Ocular holder: ∞/1x

This setting gives a magnification factor of 24292 for the Camera ORCA-ER (Hamamatsu®). Zeiss counting grid (PI10x/18 nr. 44 41 32) gives a factor for the small square 318054. The large square has the magnification factor 29695.

For manual counting and other camera systems a 100x objective (e.g. Zeiss Neofluar 100 x/1.30, oel, 160/-, Ph 3, prod. nr. 46 18 21-9903) may also be used. Determine the magnification factor for each system.

## 8.7 SONICATOR

A sonicator disrupts aggregates based on high frequency sound. A typical tip size is 5 mm diameter. The sonicator should be able to generate the given power.

## 9. CHEMICALS AND SOLUTIONS

### ACRIDINE ORANGE

30 mg acridin orange (Merck®, best. nr. 1.14281.0010) is dissolved in 10 cm<sup>3</sup> Milli-Q water. The solution is filtered through a 0.2 µm sterile filter (Acrodisc®) directly into the filter funnel. Working solution is stored at 4 °C in the dark for at most 8 weeks.

Always use gloves when handling acridine orange, as it's a carcinogen. When working with its powder form use a mouth protection. Working solution of acridine orange should be disposed of according to local directives.

### FORMALDEHYDE

37 % formaldehyde is filtered through a 0.2 µm sterile filter (Acrodisc®). Use gloves and eye protection. A ventilated hood preferably with evacuation downwards should be used, when formaldehyde vapour is heavy.

### IMMERSIONS OIL

Cargille non-drying Immersion oil for microscopy., Type A, formula code 1248. Cat. No. 16482 (R.P. Cargille laboratories, Inc. Cedar Grove, N.J. 007009, USA). The oil may be stored at 4 – 40 °C. It does not contain solvents or polychlorinated biphenyl (PCB) compounds.

### TRITON® X-100

The final concentration in samples to be sonicated is 0.001 % (v/v).

#### NATRIUMPYROPHOSPHATE

Tetra- $\text{Na}_2\text{P}_2\text{O}_7 \cdot 10 \text{H}_2\text{O}$ , is diluted in Milli-Q water. The final concentration in a sample to be sonicated is 10 mM.

#### SUSPENSION OF FLUORESCENT BEADS

Fluorescent latex beads (Duke Scientific, Polymere Microspheres™ Green Fluorescing, 1% solids,  $1,4 \cdot 10^{11}$  beads  $\text{cm}^{-3}$ , diameter 0.519  $\mu\text{m}$ , CV < 5 %, Cat. No. G500) are used to calibrate the image analysis system. The average volume of the bead is 0.073  $\mu\text{m}^3$  (0.063-0.085  $\mu\text{m}^3$ ). The small bacterial cells cover only 5 pixels, and larger deviation may occur in reality.

Bead standards are prepared by filtering beads at an expected concentration of  $1 \cdot 10^9$  beads  $\text{dm}^{-3}$ . Make two standards that are given unique codes. Store in standards the refrigerator at 4 °C. Standards prepared from the same solution should have  $\pm$ CV of 21 % (n=4) for cell numbers and 15 % (n=4) for cell volume. The same standard has been applied for many years without clear changes in abundance or size of beads.

## 10. REFERENCES

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## 11. APPENDICES

### 11.1 CALCULATION OF PARAMETERS

Primary data from the image analysis or manual microscopy may be transformed to desired parameters by calculation in LabDatabase (calculation module to LabMicrobe, Bioras<sup>®</sup>) or Microsoft Excel. Calculation of cell concentration, carbon density per cell and carbon biomass is in the latter case done in same sheet as primary data. In a second sheet data for each image is aggregated to give average values per samples by a pivot table. Processed data are stored together with logistic data in a local data base. Report to national and international databases are done according to directives from ICES.

### 11.2 SAMPLE VOLUME

The tables below give volumes of sample in millilitre that by experience gives desired cell density per microscopic field on the filters as a function of month. Upper row show depth in meters

Bothnian Bay													Öre estuary							
Month/ m	0	1	4	8	1	1	2	4	6	8	9	10	12	Month/ m	0	1	4	8	1	2
					4	5	0	0	0	0	0	0	0						4	0
Jan	5	5	5	5	5	5	5	5	7	7	7	7	7	Jan	5	5	5	5	5	5
Feb	5	5	5	5	5	5	5	5	7	7	7	7	7	Feb	5	5	5	5	5	5
Mar	5	5	5	5	5	5	5	5	7	7	7	7	7	Mar	5	5	5	5	5	5
Apr	5	5	5	5	5	5	5	5	5	5	5	5	5	Apr	5	5	5	5	5	5
May	5	5	5	5	5	5	5	5	5	5	5	5	5	May	4	4	4	4	4	4
Jun	5	5	5	5	5	5	5	5	5	5	5	5	5	Jun	4	4	4	4	4	4
Jul	4	4	4	4	4	4	4	4	4	4	4	4	4	Jul	3	3	3	3	3	3
Aug	4	4	4	4	4	4	4	4	4	4	4	4	4	Aug	4	4	4	4	4	4
Sep	4	4	4	4	4	4	4	5	5	5	5	5	5	Sep	4	4	4	4	4	4

<b>Oct</b>	4	4	4	4	4	4	4	6	6	6	6	6	6	<b>Oct</b>	4	4	4	4	4	4
<b>Nov</b>	5	5	5	5	5	5	5	6	6	6	6	6	6	<b>Nov</b>	4	4	4	4	4	4
<b>Dec</b>	5	5	5	5	5	5	5	6	6	6	6	6	6	<b>Dec</b>	5	5	5	5	5	5

Botnian Sea													
Month/m	0	1	4	8	14	15	20	40	60	80	90	100	200
<b>Jan</b>	5	5	5	5	5	5	5	5	7	7	7	7	7
<b>Feb</b>	5	5	5	5	5	5	5	5	7	7	7	7	7
<b>May</b>	5	5	5	5	5	5	5	5	7	7	7	7	7
<b>Apr</b>	5	5	5	5	5	5	5	5	5	5	5	5	5
<b>May</b>	5	5	5	5	5	5	5	5	5	5	5	5	5
<b>Jun</b>	5	5	5	5	5	5	5	5	5	5	5	5	5
<b>Jul</b>	4	4	4	4	4	4	4	4	4	4	4	4	4
<b>Aug</b>	4	4	4	4	4	4	4	4	4	4	4	4	4
<b>Sep</b>	4	4	4	4	4	4	4	5	5	5	5	5	5
<b>Oct</b>	4	4	4	4	4	4	4	6	6	6	6	6	6
<b>Nov</b>	5	5	5	5	5	5	5	6	6	6	6	6	6
<b>Dec</b>	5	5	5	5	5	5	5	6	6	6	6	6	6

### 11.3 Import format

The table shows the structure for bacterioplankton concentration data to be imported to the local database.

Station	CruiseCode	SampDateTime	Depth	BactCon	BactConq	BaSDCon	BaNPic	BaCounCel	BaVol	BaVolq	BaSDVol	PrepDateTime
<b>Sampling station</b>	Cruise code (YYYYnn)	Date and time for sampling (yyyy-mm-dd_hh:mm)	Sampling depth (meter)	Bacteria conc. in (celler dm <sup>-3</sup> )	Quality code	Standard deviation for bacterial conc. (celler dm <sup>-3</sup> )	Number of analysed images per filter	Total number of cells counted	Bacterial volume in µm <sup>3</sup>	Quality code for volume	Standard deviation for volume (µm <sup>3</sup> )	Time and date of filtration (yyyy-mm-dd_hh:mm)

Corresponding data are archived for Milli-Q backgrounds and beads. A column for Analysis date-time and type of particle should then be added. Preparation date-time connect samples with the corresponding sample. Analysis date-time connect samples and Milli-Q backgrounds with a standard estimate.

#### 11.4 EXAMPLE OF IMAGE ANALYSIS

In the following section an example of a procedure and settings for image analysis of microscope slides with aquatic bacteria is presented. The protocol has been applied for routine monitoring of bacterioplankton at Umeå Marine Sciences Centre for 5 years.

The system employs a digital charged couple device (CCD) camera and specifically developed software for image analysis of bacterioplankton. The software is developed in a LabView® environment and applies a neural network technology to recognize bacterial cells among other particles in the sample (LabMicrobe™, Manufacturer: DiMedia®). This presentation may aid tuning of also other image analysis systems.

##### 11.4.1 CAPTURE OF IMAGES

**A** Check that the correct equipment is installed and settings applied on the microscope, as it may affect the magnification. Record the burning-time for the mercury lamp via the counter - max. 200 hours). The computer screen should be set at “highest 32 bits” and a resolution of 1280x1024, 60Hz.

**B** Start the main current on the camera control unit. Start the software *Wasabi*. The window “ORCA Control” should appear. Check that the settings match item 8.6 and 8.7.

**C** Run a standard slide with fluorescent microspheres of known abundance and size according to item 9.4. Compare analysis results and check that they stay within 3 standard deviations of the average. Typical values for a standard are  $1.6 \times 10^9$  cells  $\text{dm}^{-3}$  (SD  $\pm 0.13 \times 10^9$ ) and  $0.093 \mu\text{m}^3$  (SD  $\pm 0.0096$ ).

**D** In the “ORCA control” menu typical start values are 200 ms for fluorescent beads and 350 ms for sea water samples. “Gain” and “Offset” should be 0.

Image analysis is done on all samples and a background slide. Add one drop of immersion oil on the cover slip. Use first filter set 09 (Zeiss) according to item 8.5. Focus the bacteria with the oculars. Close the light passage to the oculars and open it to the camera. Filter set 15 may be used if better contrast and lower background is achieved.

**E** Choose “Live Image” in the “Image” menu. A window with the live video image of the sample should appear. Fine tune the focus based on the live image.

**G** Open the “LUT” function measuring the level of exposure of the image. The exposure meter should be yellow and cover more than 50% of the full range. Red bar mean over exposure. Adjust the exposure if required to meet the criterion above.

A check of particle intensity can be made by the procedure “Analysis: Line”. The intensity of the strongest particle should not exceed 2500 units. The background should be even and about 300 units. Other settings of the LUT function need not to be changed.

**H** When you are satisfied with the image click "Image:Acquire". Label the file with station<space>cruise number (NN)<space>depth with fullstop and one decimal according to the format "A13 04 6.0m". Use space between all categories, but not between 6.0 and m. In the LabMicrobe module "Analyze" station, cruise and depth will be distributed into columns. Image number is added by *Wasabi* automatically. Morphology type is added by "Analyze". Do not use a hyphen. Extra information of 61512 bytes is always stored with the image (always 1344 × 1024 pixel, 16 bits signed).

**I** estimates of sea water sample require 5 images to be taken, distributed over a representative area of the filter. Exit *Wasabi* when images have been stored.

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#### 11.4.2 ANALYSIS OF IMAGES

**A** Open the program "LabMicrobe" and window "BatchMicrobe". Choose the appropriate log file. No file extension should be added.

Press the right arrow in the menu bar. A new navigation window should appear. Select the desired folder by entering it and press "Select current directory". Make the corresponding choice for the result file. Binary images (less memory requirement) do not need to be saved, and loose information of dividing cells.

Check that the settings of the log file for BatchMicrobe are correct when required (item 8.7). This is done in LabMicrobe by opening the log in question by "Operate:Datalogging:Retrieve". Choose log number and press "OK".

**B** The image analysis is starting automatically when the target folder is chosen. All files in the defined source folder will be processed (0.1 s per image). The image analysis system chooses and characterizes particles according to the settings in the log file. The selection done by the neural network is based on training sets of natural brackish water bacteria used for the development of the software 1995. See Blackburn et al. 1998 for further specification.

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#### 11.4.3 PROCESSING OF PRIMARY DATA

**A** Images analyzed by BatchMicrobe requires further processing. Start the program "Biovolume". Start extraction of biovolume data by clicking the arrow button in the upper left corner. Select folder where the result files from the BatchMicrobe analysis are stored. One file per image should be present. Select name for the result file and its folder. The biovolume extraction will start immediately. If required the result files can be open in a calculation program like Excel and file names adjusted.

**B** Bacterial abundance and biovolume of the whole community is aggregated (median value) from primary data with the module "Analyze". Use values for field data according to item 11.4. Choose settings for largest and smallest cell volume by the cells provided. Start the analysis with the arrow button in the upper left corner. Give name to the source file and result file.

All files created under item 11.4.3B can should then be compiled in an calculation software like Excel to give average values per volume for all morphology types in the community. Calculations according to item 5 should be used. Results are reported according to item 5.2. Also the Milli-Q background is analyzed in the

same way and subtracted from the sample values. Note that Milli-Q values should be divided by the same volume as the corresponding sample to be correct.

#### 11.4.4 IMAGE ANALYSIS INSTRUMENTATION AND SETTINGS

**CCD camera:** C4742-80-12AG (synonymous with ORCA ER, Hamamatsu<sup>®</sup>) with control unit may be used. A C-screw thread connects the camera with the microscope. The camera can be cooled to -30°C.

**Computer:** Personal computer, Pentium processor 2.66 GHz , 1 Gbite RAM memory, TEAC.

##### *Control unit for the CCD camera*

Control of the CCD camera is done by the software Wasabi in the window "ORCA control". The following settings had worked well for routine use of acridine orange stained water samples:

##### **Buttons**

**Focus mode:** Off, **Light mode:** Off, **Auto exposure:** Off

##### **Camera**

The exposure time is chosen so that the coloured bar in the LUT-window is light green to yellow, and cover at least 70% of the range. The exposure is then optimal relative the dynamic range of the camera. The exposure time is not expected to vary extensively between samples. Auto exposure may be used to make a first adjustment of the exposure time. Typical values achieved are:

**Exposure time:** 350 ms for sea water samples, 200 ms for fluorescent beads

**Gain:** 0

**Offset:** 0

##### **Auto exposure (AE)**

**Sensitivity:** 0.0% Set minimal frequency for light strength used for calculation of exposure time.

**Hysteresis:** 5.0% Lock light strength according to *Sensitivity* in relationship to maximum possible value.

##### **Format**

Set the size of the shown image. Large image size should be used.

**Superpixel:** 1x1

**Subarray:** Large

##### **Trigger**

**Camera timing:** Intern

##### **Temperature**

Control the working temperature of the camera.

“Temperature” show the actual temperature of the camera.

**Cooling:** ON **Auto:** *Unclear*

**Target temperature:** 20°C

*Software*

**Image capture:** WASABI 1.4.0.4 control software for the camera unit.

**Image analysis:** LabView 3.1, Image analysis library. Concept Vi (LabView macro) with copy protection, LabMicrobe 1.0 och BatchMicrobe 1.0 by Nicholas Blackburn, DiMedia®.

*Settings for WASABI*

Images are saved with “Aquire” in WASABI. Correct settings are done with “Aquire” with preview.

**Aquisition area:** Top: 0, Left: 0, Width: 1344, Height: 1024.

**Resolution:** Full

**Bits per pixel:** 16BPP

**Average:** 1

**Clip negative pixel to zero:** unmarked, **Aquire to new window:** Unmarked, **Keep live image:** Marked.

A captured image is stored as a .TIFF-fil (6.0) with 16 bits per pixel with no compressin. **Strip size:** 10. Typical size is 2 755 532 bytes for an image file (see under “Properties” for the file).

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#### 11.4.5 SETTINGS FOR LABMICROBE

Standard setting for LabMicrobe is given below. They are entered in LabMicrobe and saved as a log-file. Call the correct log-file when performing an analysis.

Only **Pixel size (calibration)**, **Neural network** parameters, **Classifier tolerance**, **Threshold values** and **File options** in LabMicrobe’s log files are used by BatchMicrobe. “Threshold parameter’s” is not used when the flip for “Parameter threshold” is off.

At delivery of LabMicrobe the neural network is trained to select bacterial morphology types (Short rod (=cocci), Long rod and C-shaped). The standard cell sets are those used during the development of the software. See Blackburn et al. 1998 for details.

*The LabMicrobe window*

The following settings should be used for the camera and microscope systems presented above:

**Status window:** Gives current activity

**Save binary image:** Save an image with less information and memory requirement. Not routinely used when counting bacteria.

**Threshold data:** Min. 2 Max. 10000

**Palette:** Binary

**Data params:** Area (calibrated)

**Calibration:** 0,0986 (rounded to 0,10 by the software. Entered value is used)

**Processing:** Show the image file under processing. End at final image file when the image set is done.

**Particle:** 1

**Classifier tolerance:** High.

**All particles, Plot species, Select species and Identified particles** change depending on image analysed.

**Parameter threshold:** Set to desired interval during manual analysis. Determine what particles that are shown with a large symbol in the diagram. Does not affect the analysis with done with BatchMicobe. The handle should typically be down (off).

*"Other options->" (extend the window to the right).*

Read raw file 1 **Read Raw file**

16 bits signed 5 **File data type**

**Offset to data**=3020. The offset value may be required to be adapted to a specific image type. TIFF format is complex and save also other information with the image. The off set value is determined as the total file size in bytes subtracted with the pixel area times 2.

Offset = 2 755 532 -(1344x1024x2)=3020

Don't use min max 0 **Use Min max**

0,00 **Optional min. value**

0,00 **Optional max. value**

Little Endian (Intel) 1 **Byte Order**

**Width**=1344, **Height**=1024.

**Mark local maxima:** Down

**Network:** 0; 0,0; 5,13

**Size independency:** Down

**Labels:** 0,?

*Reconstruct and collect window*

**Number of frequencies:** 4

**Other:** 4

**Plot particles:** 1

**Continue:** Green

*Parameters window*

**CVI Parameters:** Area (calibrated) and Number of holes labelled black.

**User Parameters:** Particle# and Hemisphrod labelled

*Morphology window*

**Minimum particle size:** 1

See also the manual of the image analysis software provided by DiMedia®.

*Batch Microbe window*

**Save binary Image:** Off (=down).

**LabMicrobe log number:** 0

**Mark local maxima:** Down

**Data file extension:** (blank)

*Analyze window*

Min and max set the limits for the biovolume range that is included in the analysis.

**Min. size:** 0.01

**Max. size:** 0.40

**# bins (mode):** 10.