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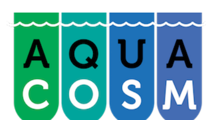

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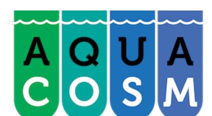

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Abstract	This deliverable is a Standard Operating Protocol (SOP) that describes the methods for sampling and analysis of phytoplankton from mesocosm experiments carried out in all aquatic environments (fresh and marine waters). It gathers best practice advice with a focus on sampling, counting and other analyses of phytoplankton as well as Quality Assurance/Quality Control (QA/QC) practices. Use of this SOP will ensure consistency and compliance in collecting and processing phytoplankton data from mesocosm experiments across the AQUACOSM community, in Europe and beyond.

Keywords	Phytoplankton Analysis, Sampling, Enumeration, Freshwater, Marine, Algae Utermöhl

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AQUA COSM

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1. Executive summary

This Standard Operating Procedure (SOP) describes methods for sampling, and analysis of phytoplankton from mesocosm experiments carried out in all aquatic environments (fresh and marine waters). It gathers best practice advice with a focus on sampling, counting and other analyses of phytoplankton as well as Quality Assurance/ Quality Control (QA/QC) practices. This SOP is based on EU Water Framework Directive and other related documents. It is designed to be compliant with this EU Directive (2000/06/EC) [1]. Use of this SOP will ensure consistency and compliance in collecting and processing phytoplankton data from mesocosm experiments across the AQUACOSM community, in Europe and beyond.

This SOP covers guidance on health, safety and environmental information, best practice advice on materials and methodology and QA/QC procedures to be followed during the sampling, analysis and counting of phytoplankton samples from mesocosm experiments. It applies only to the phytoplankton microscope investigation of the identification, composition, abundance, and biovolume estimation of phytoplankton samples.

2. Definitions and Terms

Biomass	• Total living organic material in a system or biological unit (e.g. taxon, functional group) [2] • Material composed of living organisms [3]
Biovolume	Total cell volume of a taxon or a functional group per volume (e.g. L^{-1} water sample) [2]
Cell volume	Total volume of a single phytoplankton cell [2]
Epilimnion	Upper water layer in stratified lakes that clearly differs from deep water layers in terms of water temperature. The depth of the epilimnion is defined as the warmed uppermost and mixed water layer, with a relatively homogeneous distribution of the water temperature during the summer stagnation period. It lies above the metalimnion (thermocline), which is the horizon of the largest vertical density change caused by differences in water temperature [2]
Euphotic zone	Zone within a water body where photosynthetic production is possible (gross primary production > respiration). It roughly corresponds to 2-2.5-times of the transparency (Secchi depth). [2]
Metalimnion	Transitional zone between the warm epilimnion and the cooler hypolimnion. It corresponds to the lake zone with the largest density change (thermocline; usually following the water-temperature gradient). According to the original definition of BIRGE (1897), the metalimnion corresponds to the zone where the change of water temperature within 1 m is at least 1 °C. [2]
Hypolimnion	Stratum of cool water below the thermocline in stratified lakes.
Pelagic zone	Open water zone of a lake, not close to the bottom or near the shore; in contrast to the benthic or bottom zone of a lake [2]

Phytoplankton	Community of free-floating, predominantly photosynthetic protists and cyanobacteria in aquatic systems. (in limnological analysis commonly excluding ciliates[1]). [2]
Secchi depth	Maximal depth at which a b/w segmented disk (after Angelo Secchi) is just visible from above the water surface. Measuring the Secchi depth (or transparency) allows determination of the turbidity of standing or running water after ON EN ISO 7027 [2]
Taxon	A phylogenetic unit (species, genus). Should be in accordance with current phylogenetic classification (algaebase.org as reference).
Thermocline	See Metalimnion [2]
Trophic state	The nutrient level of an aquatic system. Determines biomass and primary production of the community [2]
Utermöhl technique	Technique for the quantification of a phytoplankton through the sedimentation of a preserved phytoplankton sample; specifically, determination and counting of the organisms using an inverted microscope, calculation of the individual biovolumes, projection of the counted organisms to a volume unit and calculation of the total biovolume [2]

3. Cross References

The SOPs that will be provided by AQUACOSM will be listed here in the following versions when the different SOPs are completed.

The SOPs that will be provided by AQUACOSM by the end of 2017 will be for:

1. Phytoplankton (this SOP)
2. Zooplankton
3. Periphyton (Phytobenthos)
4. Water Chemistry (Physical and Chemical Elements of Water)
5. High-Frequency Data Collection

A general description for water sampling will be covered under the Water Chemistry SOP.

4. Materials and Reagents

Table 4-1 The materials and reagents used in analysis of phytoplankton

[1] Ciliates with endosymbiotic algae, such as *Mesodinium rubrum*, are often abundant in oligotrophic waters and belong functionally to phytoplankton

Name and concentration	Composition	Storage
Lugol’s Solution	Iodine based fixative most commonly used for analysis of phytoplankton using the Utermöhl method. See Annex I for detailed information about neutral, acidic and alkaline Lugol’s solution.	Fume Cabinet/Hood
Formaldehyde solution	Fixative to be used for phytoplankton analysis on epiflorescent microscope. See Annex I for detailed information about preparing formaldehyde solution.	Fume Cabinet/Hood

5. Health and Safety Indications

5.1 General Information

In this section, general guidance on the protection of health and safety while sampling, analysing and counting phytoplankton from mesocosm experiments will be provided to minimize the risk of health impacts, injuries and maximize safety. The users of this SOP are expected to be familiar with the Good Laboratory Practice (GLP) of World Health Organization (WHO) [4] and Principles on GLP of Organisation for Economic Co-operation and Development (OECD) [5]. Health and Safety Instructions of the mesocosm facility, if there are any, shall be followed properly to protect the people from hazardous substances and the harmful effects of them.

According to preventive employment protection measures to avoid accidents and occupational diseases (on-site or in the laboratory), the work should be practiced consistent with national and EU regulations (see the OSH Framework Directive 89/391/EEC, [6]). Other regulations and guidelines can be found in EU – OSHA website (European directives on safety and health at work [7]). All necessary safety and protective measures shall be taken by the users of this SOP and the scientist-in-charge shall ensure that those measures comply with the legal requirements.

The table below summarizes the hazards and risks and the measures for preventing them associated to the laboratory studies carried out on phytoplankton:

Table 5-2 Hazards and risks associated with laboratory work

Occupations at risk	Hazards/Risks	Preventive Measures
Laboratories	○ Risk of inhaling chemicals that are used for phytoplankton sample fixing	○ Use of fume cabinets/hood ○ Safe handling and transport of samples ○ Appropriate personal protection (protective gloves) and hygiene measures ○ Samples are stored into glass bottles to minimize diffusion of the fixative ○ Safe handling and transport of samples
	○ Accidental spills	○ Using of lidded cool boxes to deliver samples from field to laboratory ○ Using of lidded plastic boxes to store the sample bottles

5.2 Safety Instructions for Sampling

Please see the Water Chemistry SOP **(in preparation due by December 2017)** for safety instructions for sampling.

5.3 Working and Personal Protection (Safety) Equipment

Please see the Water Chemistry SOP **(in preparation due by December 2017)** working and personal protection equipment suggested for use in water sampling.

5.4 Use, Storage and Disposal of Reagents and Chemicals

The user of this SOP needs to visit the Safety Data Sheets (SDSs), which is provided by the manufacturer for any chemicals with necessary information for protection before using, storing and disposing the reagents as well as other chemicals. Only experienced personnel should be responsible for the use of reagents, preservatives and chemicals in a way compatible with the laboratory rules if available.

It is important to remember that use of formaldehyde as a fixative can result in “the generation of bis(chloromethyl)ether” - which is a potential human carcinogen [8]. In addition, formaldehyde, is an irritant and can enter the body via inhalation and damage the tissues seriously. For more information on formaldehyde, see the substance information ([9]) provided by European Chemicals Agency (ECHA).

5.5 Use, Storage and Disposal of the Equipment

If phytoplankton sampling from the waters is to be carried out with a single piece of equipment, this equipment needs to be cleaned in between two sampling events. [10]. The storage of equipment should be compatible with

the operating manual/instructions for use. Cleaning, disinfection and storage of equipment shall be taken care under the risk of parasites, diseases, foreign species, and pathogens.

6. Environment Indications

A plan for the disposal of mesocosm waste needs to be prepared prior to the experiments. The plan must be in competence with the EU Waste Legislation ([11]) and The List of Hazardous Wastes ([12]) provided by the European Commission. The Safety Data Sheets (SDSs) need to be revisited for the disposal of reagents and chemicals prior to waste disposal.

7. Methods

7.1 Prior to Sample Collection

7.1.1 Introduction

The AQUACOSM Standard Operating Procedure (SOP) on sampling, (qualitative and quantitative) analysis and counting of phytoplankton needs to be reviewed by the research team prior to initiating the sampling. The sampling method to be employed, sample type and volume, equipment and supplies needed for sample collection all shall be identified prior to the start of a sampling event.

7.1.2 The List of Required Equipment

The sampling equipment and supplies shall be provided by the AQUACOSM host facility. The following is a list of equipment and supplies that can be used for recordkeeping. The list of equipment needed during the sampling process is given in Section 7.2.1 and the list of equipment required for counting in Section 7.4.
The supplies for recordkeeping:

- ✓ Overview of mesocosm labels & treatments
- ✓ If mesocosms are stratified or contain macrophytes: Outline of sampling design (strata to be sampled)
- ✓ Field sheets (Metadata Sheets) (Field protocol; DQ from [2])
- ✓ Markers and pens/pencils
- ✓ Camera
- ✓ Other relevant paper work
- ✓ Notebook for contemporaneous notes if required

7.1.3 Calibration

The equipment should be checked. For sampler used in sampling and equipment for counting of phytoplankton, the following methodology, as provided by the EU FP7 226273 (WISER), documentation, shall be followed for calibration:

- Each **counting chamber** should be "marked with a unique mark or number and a note made of the counting chamber area. This is calculated by measuring the cover slip aperture (rather than the chamber itself) using either a Vernier gauge or the microscope stage Vernier if one is present. The mean of 5 diameters should be taken and the area of the chamber calculated using the formula πr^2" [13]. In addition, the chamber volumes should be measured accurately by weighing the chamber (counting chamber with cover slide + column of a determined volume + thick glass cover) and lid whilst empty, then fill with distilled water and re-weigh (e.g. 5 mL chambers can range from 4.7-5.2 mL). The weight in grams is equivalent to volume in mL. As a *best practice advice*, the calculation of volume should be repeated three times and the average of the three should be recorded. Both the diameters and the chamber volume should be recorded in a log book.

- The "**eyepiece/graticule** and objective combinations should be calibrated with a stage micrometer (e.g. 100µm x 10µm divisions) and the dimensions and areas of counting fields, transects and the whole chamber area should be calculated for each of the magnifications used" [13].

7.2 Water Sampling for Phytoplankton

7.2.1 Equipment for Sampling

(Some of the equipment below will be moved to the Water Chemistry SOP, when it is completed)

Sampling of phytoplankton is commonly carried out simultaneously with sampling for parameters of water chemistry and chlorophyll-a. For general water sampling, check the SOP for Water Chemistry. The items listed below are necessary for a water sample that is only used for phytoplankton analysis and does not consider any further parameters. Several alternatives for water samples are listed.

The following specific pieces of equipment are suggested for use in phytoplankton sampling [2]:

- ✓ Appropriate water sampler, depending on the type of sampling (stratified or integrated), and (e.g. Schröder/Schindler/Ruttner sampler for single strata; tube sampler for depth integrated samples)
- ✓ Clean & rinsed sampling containers in the field; sample bottles for subsequent preservation and storing of phytoplankton samples (Table 7-1 gives an overview of volumes)
- ✓ Supply of Lugol's iodine for preservation of samples
- ✓ A 10 or 20 µm plankton net can be used for concentrating phytoplankton. Net plankton samples can be used to supplement the species list from the quantitative sampling.

Sampling containers and sample bottles for preservation [14]:

- ✓ **Sampling containers:** Depending on the sampling procedure (especially whether water is collected for multiple parameters at one sampling event, or just for phytoplankton), water (the 'sample') from the experimental units is either filled directly into bottle for fixation ('sample bottle'), or a larger amount of water is collected in a container ('sampling container') and the sample bottle is later filled in the lab from the sampling container.
- ✓ **Bottles for storing phytoplankton samples – 'sample bottles':** The bottles should be selected according to the purpose of the study and the type of sample. For Lugol's fixation, transparent glass bottles (50-300 mL, Table 1) should be preferably used, as iodine will diffuse through standard plastic containers (e.g. PE, PP, PS). Transparent glass bottles should be preferred, since it is easier to see if the amount of Lugol's is enough through a transparent bottle. Sample bottles should be stored dark and cool. Keeping them in a fridge helps preserving the material (temperature ca. +4 - +8 °C). It is important that the user of this SOP ensure that sample bottles are stored and transported clean, ideally in their original packaging. Used bottles can be re-used, if cleaned and rinsed properly (otherwise cells sticking to the bottle walls might contaminate new samples).
- ✓ **Best practice advice:** "It is important that the bottle cap is securely tightened to avoid spillage of the sample and evaporation of the preservative. Utermöhl (1958) ([15]) recommended that the bottle is filled to 75-80% of its volume. This facilitates the homogenisation of the sample before dispensing into the sedimentation chamber" [16].

Labelling:

- ✓ Sampling containers and sample bottles needs to be labelled properly in the laboratory prior to sampling. The labels on the sampling bottles need to be standardized and provide information on name of the experiment, sampling date, mesocosm ID, and possibly depth with appropriate abbreviations of the

treatments of the experiments. Labels can be either printed or written using a permanent waterproof marker [14].

- ✓ As a best practice advice, a label template is provided in Annex II of this SOP.

7.2.2 Sampling

Representative sampling – samples need to be representative for the mesocosm unit of interest. In case other parameters (esp. nutrients, chlorophyll-a) are sampled on the same event, it is mandatory that all parameters are analysed from the same water sample (multiple sub-samples from one water sample).

A. Mixed mesocosms

In case mesocosms are mixed continuously, one water sample can be considered representative for the entire mesocosm. Here, samples should be taken near the center of the mesocosm as stated in both [13] and [17]. Care should be taken to avoid macrophytes, if present, while sampling. If it is unclear whether the whole water volume is efficiently mixed an initial series of samples can be taken in transects across the mesocosm and along the vertical axis (see point 7.2.2 B below).

B. Stratified mesocosms

If mesocosms waters are stratified or partially mixed, the sampling procedure must be determined after careful considerations on the water layer(s) to be sampled (e.g. sampling discrete water layers; sampling multiple depths with subsequent pooling to one combined representative sample; utilization of tube sampler). Absence of vertical temperature profiles indicates vertical mixing, but NOT necessarily homogenous distribution of motile organisms like (micro-) zooplankton. Heterogenous distribution of phytoplankton must also be assumed if mesocosms contain structuring elements (such as macrophytes), and has not been proven to be (practically) homogenous (see 7.2.2.A).

C. Composite and discrete sampling

According to WISER [13], representation of the whole phytoplankton community will be achieved, taking vertically integrated samples from the euphotic zone in thermally stratified mesocosm. Careful consideration of the sampling design is also required in case the mesocosms are stratified and include an aphotic zone (mesocosm depth > Z_{eu}[2]). In this case the integrated water sample would typically be taken from the euphotic zone (Z_{eu}).

For shallow mesocosms containing sediment and possibly macrophytes, a detailed sampling design for collecting samples at multiple horizontal and vertical positions might be needed [17]:

> As a best practice advice: "The entire water column, from the surface to approx. 5 cm above the sediment, is sampled from three positions in each enclosure: 10, 30 and 60 cm from the enclosure wall. Two samples are prepared: one to be used for chemical and phytoplankton analyses where water is sampled without touching the plants – and one to be used for zooplankton analyses, where water is sampled also close to the plants." "The best way to sample from the entire water column is by using tube sampler which sample from top to bottom. The diameter of the tube should not be too small to avoid zooplankton escaping during sampling (> 6 cm). If it is not possible to use a core sampler, samples can be taken with Ruttner water sampler from the surface (20 cm below the water surface), middle and the bottom (20 cm above the sediment). The sample in the middle should be adjusted according to the enclosure type and actual water depth" [16].

[2] Z_{eu} corresponds to 2-2.5 x Secchi depth

D. Recommended sample volume

Table 7-1 below gives a rough approximation about the required sample volume (total sample volume and volume for sedimentation): Note that the phytoplankton sample volume needs to be large enough to allow for preparation of multiple sedimentation samples (i.e. at least 3x the volume of the sedimentation chamber to be used).

Table 7-1 Recommended sample volumes and volumes for sedimentation according to trophic state of an aquatic system

Trophic state (TP µg L^{-1})	Sample bottle volume (ml)	Volume for sedimentation (ml)
Ultra-oligotroph (<<10 µg)	500	>=100 (concentrate)
Oligo-mesotroph (10-25 µg)	150-250	25-50
Meso-eutroph (15 - 45 µg)	50-150	5-25
Eutroph (>= 50 µg)	10-30	< 5 ml (dilute)

7.2.3 Sample Fixation

Phytoplankton samples are usually fixed by Lugol's solution (acidic or alkaline solution, see Annex 1). Lugol's iodine preserves the cells. In addition, iodine increases the specific density of cells and enhances sedimentation.

> "Use alkaline Lugol's solution (using sodium acetate buffer) or acidic Lugol's (which allows better sedimentation of buoyant cyanobacteria) as a preservative to reach a final concentration of about 0.5% in the sample, i.e. about 8 drops per 100 mL (or 2.5 mL for a 500 mL flask). The final concentration should give the sample a light brown/orange colour (whisky or cognac colour). Depending on the type of sample, reaching the colour can take a higher number of drops – in acid waters for instance" [13].

Lugol-preserved samples should be stored in dark and in temperature 4 to 8 degrees of Celcius until analysis. Samples should be analysed within few months. If longer-term storage is needed, intensity of coloration should be checked optically every 6 months to ensure sufficient concentration of the fixative (loss of coloration indicates loss of fixative by diffusion).

- ✓ As a best practice advice, it is important to **leave an-airspace of approximately 1-2 cm below the lid in the bottle**. If the bottle is filled to the top, mixing (by shaking) of the sample for preparation of the sediment chamber will be inefficient.

Fixation with formaldehyde (or glutaraldehyde) is used in combination with epifluorescence analysis (the sample is then filtered on a dark membrane filter prior to microscopic analysis and stained by a DNA dye). The advantage of this method is it allows reliable distinction of pigmented from non-pigmented cells. The proper procedure for preparing a formaldehyde solution is provided in the Annex 2 of this SOP.

- ✓ As a best practice advice; it is important that the Lugol's and especially formaldehyde solution are stored and used in the Fume Cabinets (Fume Hood) while using them for preserving samples. Material used in combination with form-/glutaraldehyde should be labelled for this purpose. It is recommended to clean this material using gloves. Country-specific regulations may apply esp. regarding the use of Formaldehyde and Glutaraldehyde.

7.3 Quantitative Analysis of Phytoplankton – general overview

The work flow of quantitative analysis of phytoplankton is summarized in Figure 7-1 below.

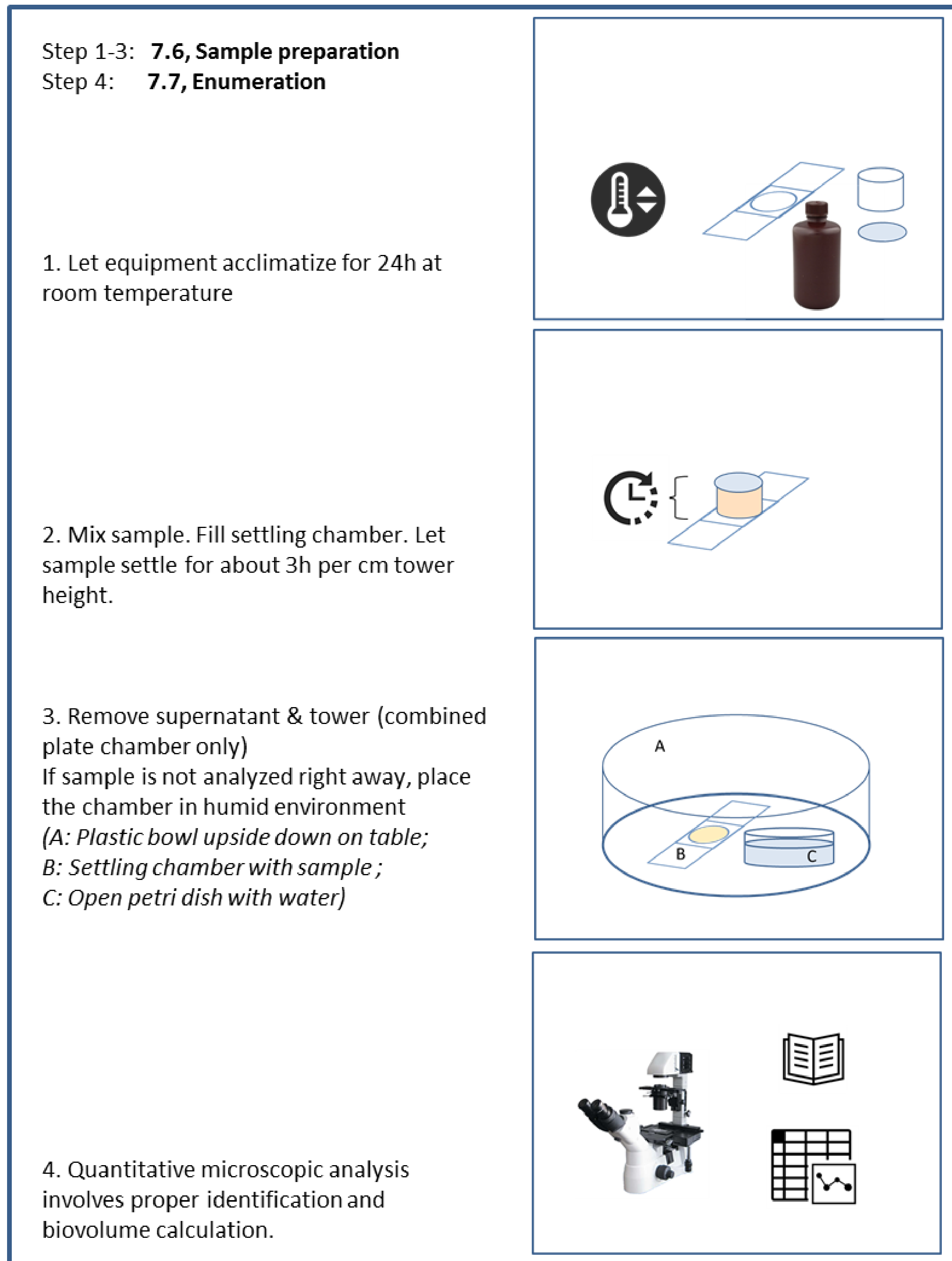

Figure 7-1 The Workflow of quantitative analysis of phytoplankton

7.4 The Equipment used in Counting Phytoplankton

The equipment that can be used in counting phytoplankton (adapted from WISER [16])

- "Sedimentation chambers of 5 to 50 ml capacity. Chambers should be approximately 25 mm in diameter. Sedimentation chambers of >= 100 mL should be avoided especially for small cells because of the high risk of uneven and non-quantitative sedimentation of small cells. Instead, dilute or concentrate samples if densities are very high or low (the concentration is too high if cells lay on top of each other; too low concentration is indicated if the required number of units counted per sample cannot be achieved, see below)". [16]
    - **Combined plate chambers**: A tower is placed on top of a base plate (Fig. 7-2.B-D). The sample is filled into the chamber. After sufficient time has passed (Table 7-2), the tower is removed.
    - **Tubular chamber**: The tower is permanently fixed on the settling chamber. Sample should be placed in humid chamber already for sedimentation to avoid formation of gas bubbles. (Fig. 7-2.A). Tubular chambers allow sedimentation of limited volume (5-10ml). Note that the height of chamber inc. tower must fit under the condenser unit of the microscope.

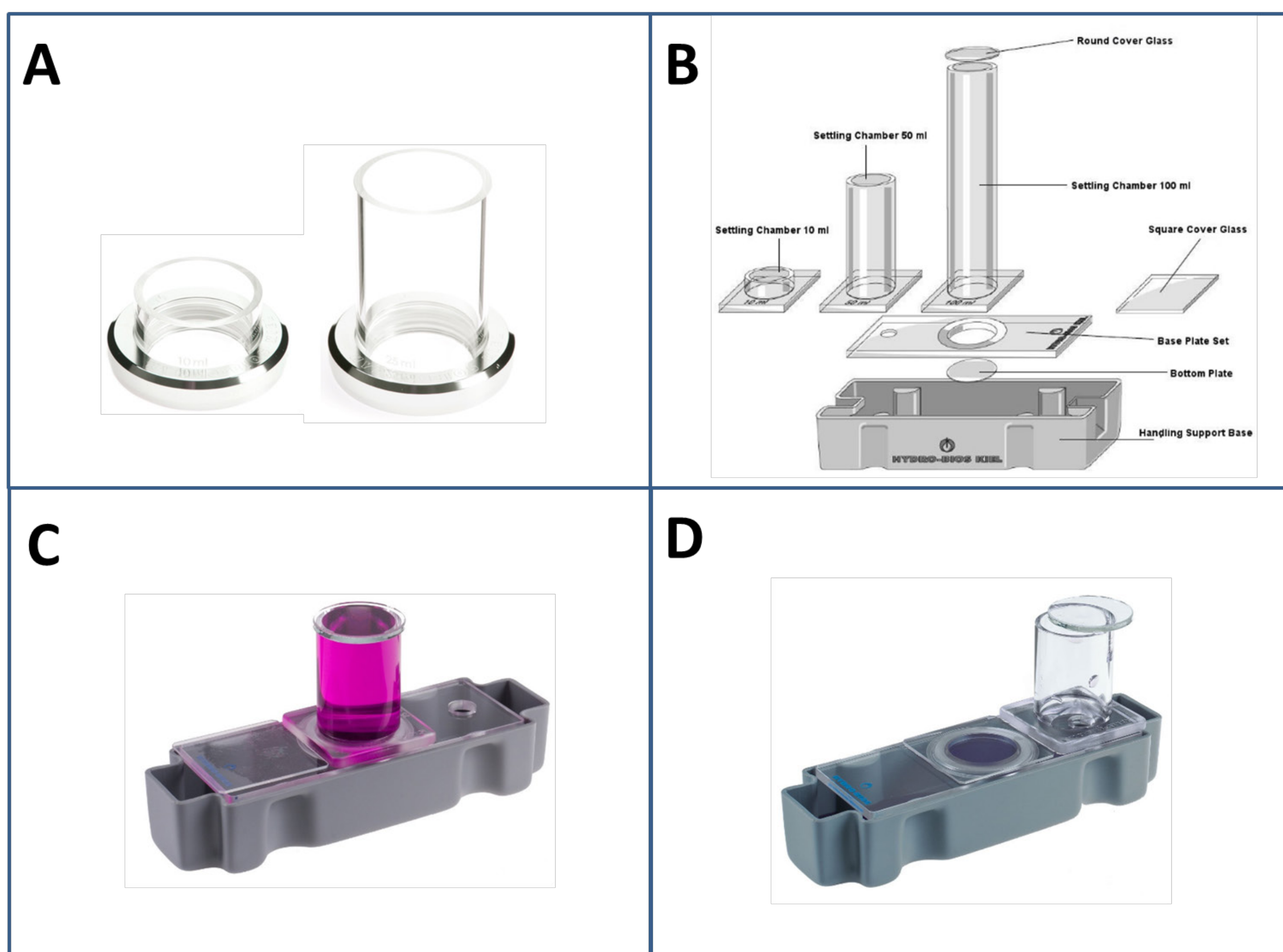

Fig. 7-2. Settling chambers. A: tybular chamber with fixed tower, different volumes. The sample volume stays in the chamber during analysis. B-D, combined plate chambers. B, accessoirs. C, chamber with sample. D, Chamber after removing the supernatant. The tower with the sample volume is gently pushed to the side by the cover glass plate. Pictures taken from from https://www.hydrobios.de/.

- ✓ **Best Practice Advice:** "The cleaning of sedimentation chambers is a critical part of the Utermöhl method. The chambers should be cleaned immediately after analysis to prevent salt precipitate formation. A soft brush and general purpose detergent should be used. To clean the chamber margin properly a tooth pick can be used. Usually it is sufficient to clean the chamber bottom without dissembling the bottom glass. Sometimes, however, it is necessary to separate the bottom glass from the chamber, either to clean it or to replace it. This is easily done by loosening the ring holding the bottom glass with the key. Care should be taken as the bottom glasses are very delicate. Counting chambers should be checked regularly to ensure that no organisms stick to the bottom glass. This can be achieved by filling the chambers with distilled water" [16].

- Inverted microscope with phase contrast (and/or Differential interference contrast) including:
  - Standard resources for microscopy (slides, cover slips, pipettes, cleaning paper, wash bottle etc.)
  - long working distance condenser with numerical aperture of >0.5
  - 10x or 12.5x binocular eyepieces; preferably one with a square grid, and another with a cross-hair graticule with scale bar (see Figure 7-3)
  - 10x, 20x and 40x phase &/or Differential Interference Contrast (DIC) objectives
  - ideally the microscope should be fitted with a digital camera coupled to image analysis system
  - a mechanical stage
- Supply of deionized/degassed or membrane filtered water is recommended for topping up, diluting and general cleaning" [13].

Figure 7-2 below demonstrates the suitable eyepiece graticules; Cross-hair graticule and Simple square.

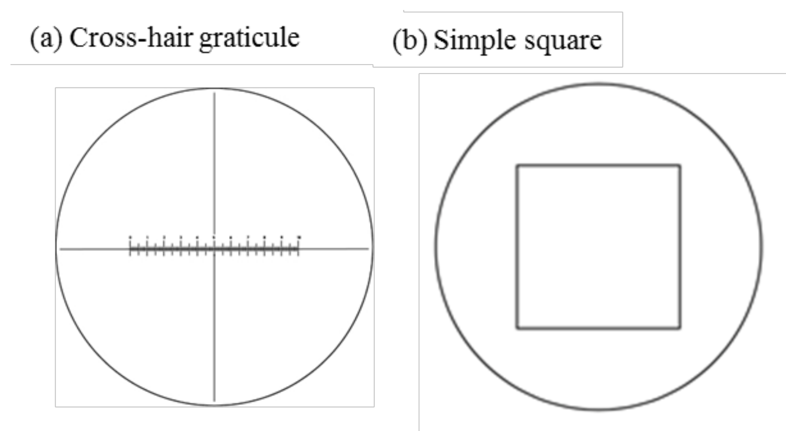

Figure 7-3 Examples of suitable eyepiece graticules (revised from [13])

7.5 Sample preparation

A. Acclimatization of sample and counting chambers

Samples and equipment involved in preparing Utermöhl samples should be acclimatized for 24 hours/overnight to room temperature. This is important in achieving a random sedimentation of algal cells on the bottom of the settling chamber [13].

B. Sample Mixing

Prior to sub-sampling, the sample should be mixed following the methodology provided by the WISER:

> "Just before taking a sub-sample to fill the sedimentation chamber, the sample is manually thoroughly mixed using a combination of alternating horizontal rolling and vertical tumbling (turning upside down) of the sample bottle for around 2 minutes. These actions should be gentle and not involve any vigorous shaking or vortex formation" [13].

C. Sub-Sample Volume

The exact volume of sample used to fill the chamber depends on the phytoplankton density **(see Table 7-1).**

A number of options are available for dealing with varying densities of phytoplankton (adopted from [13]):

- Use a sedimentation chamber of an appropriate size depending on how abundant the phytoplankton cells are (chlorophyll-a and/or nutrient concentration may serve as a guidance, see Table 7.1). If there is enough sample water to be used for several sedimentations, it is recommended to use a couple of different sedimentation chambers with different volumes to prepare a sample for microscopy. After correct sedimentation time, samples are checked with a microscope and a sample in which phytoplankton density is the most appropriate and evenly distributed is selected for the analysis.
- It is recommended to use settling chambers between 5 ml and 50 ml. Chambers with smaller or larger volumes likely result in uneven distribution of small cells.
- For very low densities, a pre-concentration step may be necessary. Let sample settle in a measuring cylinder - usually 250 mL is sufficient. Leave for 3 days and then draw off top water leaving 25 mL at bottom of cylinder (i.e. x10 concentration). If needed this can be repeated with up to 4 250 mL cylinders and the 4 lots of 25 mL then poured into a 100 mL measuring cylinder for a second pre-concentration to 10 mL (i.e. x100 concentration) [18].
- In ultraoligotrophic systems, large settling towers (>50 mL) can be employed for counting large phytoplankton ('microplankton', cells >=20µm; large cells sediment faster than nano- and picoplankton, hence the risk of uneven distribution is limited).
- For very dense samples, it is necessary to dilute the sample before adding it to the chamber. Add a known volume of sub-sample to a measuring cylinder and top-up to a measured volume with Lugol's preserved water, thereby ensuring proper mixing of the sample with the added water. The water used for dilution must be particle free. In freshwater analytics, deionized water can be used. In marine systems, seawater filtered by a 0.2 µm filter will be better as deionized water might cause cells to disintegrate.
- As alternative to dilution of samples, Palmer chambers can be used for enumeration of dominant taxa in very dense samples (volume of a Palmer chamber is typically 100µL). Palmer chambers do not necessarily warrant even distribution of cells. Ideally, the entire chamber is counted.

D. Proper Environment and Required Time for Sedimentation

The time required to ensure quantitative sedimentation of all particles including small phytoplankton depends on the height of the settling chamber. A good recommendation is to allow for 4h of sedimentation per centimeter of settling chamber height. Following the WISER guideline [13], one should allow "10 ml chambers to settle for at least 12 hours, 25 ml chambers for at least 24 hours, and 50 ml chambers for at least 48 hours. Note that too long a settling period (several days) increases the risk of disturbance and formation of air bubbles." (Table 7-2 below gives an overview about settling time).

Best practice advices:

- ✓ Even distribution of particles requires the settling chamber to be placed on an absolutely horizontal surface. Esp. for larger settling chamber (20 mL and more) it is advisable to check the horizontal orientation of the surface by a tubular level.
- ✓ The table used for preparing the settling chamber must not be vibrating or shaking. There should be no other equipment on the same table during sedimentation that might cause vibrations (like a vacuum pump). The table should not be placed on a hardwood floor which vibrates when people pass the room etc.
- ✓ Air bubbles inside the settling chamber disturb the vision and resolution when counting. To minimize air bubbles, ensure acclimation of material (see above) and store the settling chamber in a humid environment (Fig. 7-2). Such a space is easily created by placing the chamber next to a petri dish filled with water and covering both items under a plastic bowl turned upside down. When employing combined plate chambers, this procedure is relevant for the time after the settling tower has been removed.

In addition, HELCOM COMBINE [19]; a manual for monitoring the marine waters provided the minimum amount of settling times; which are dependent on the height of the chamber as well as the type of preservative used. The given settling time for specific chamber volumes and height when Lugol's is used as the preservative are tabularized below:

Table 7-2 Minimum amount of settling times for specific chamber volumes and height (adopted from [19]). The settling time depends mostly on height of the settling water column, with ca. 3 h per cm.

Volume of Chamber (cm^3)	Height of Chamber (cm)	Settling Time (h)
2-3	0.5 – 1	3
10	2	8
25	5	18
50	10	24

7.6 Taxonomy and Nomenclature

The nomenclature and taxonomy should follow best practice. At the moment, no standard taxonomic database is available to be used in the work of WFD partners. The former EU Funded projects with acrimony of WISER and REBECCA taxa list (found under freshwaterecology.info) is currently not updated and hence not a valid reference. IGB in Berlin is currently elaborating a taxa list [20] to be used for lakes. For Baltic Sea phytoplankton, the taxa list

of HELCOM Phytoplankton Expert group (PEG) [21] should preferably be used. The taxonomy and nomenclature of the HELCOM PEG list follows AlgaeBase. For general inquiries about phytoplankton taxonomy and nomenclature, AlgaeBase [22] is a good reference for both freshwater and marine taxa. The WORMS database (http://www.marinespecies.org/) is currentyl the best reference for marine taxa (and continuously synchronized with the AlgaeBase).

Recommended literature for identification of freshwater, brackish and marine taxa

- For freshwater phytoplankton, an extensive over view of available literature is given in [13].
- For marine phtoplankton, UNESCO/IOC [16] suggested the review of following literature for identification: Horner (2002) [23], Tomas (1997) [24] and Throndsen and others (2007) [25].
- For Baltic Sea phtoplankton, species lists are published on the webspace of the HELCOM Phytoplankton Expert Group [21].

✓ **Best practice advise**: It is highly recommended to speak to local experts about recommended literature and check with them about species lists of teh local habitats

Taxonomic resolution

The observed taxa are identified to the highest possible taxonomic level. It is very important to remember that **it is better to correctly identify algae to lower taxonomic level than misidentify to a higher level (e.g. report genus level if species cannot be identified unequivocally)**.

Notes on definition of phytoplankton

Phytoplankton is defined as the sum of suspended photosynthetic protists and cyanobacteria. However, the most commonly used method for analyzing phytoplankton (Utermöhl method in combination with Lugol's solution; this SOP) does NOT allow to distinguish pigmented (photosynthetic) from non-pigmented cells. Furthermore, the method is not suitable for quantitative analysis of ciliates in the sample (ciliates may be auto- mixo- or heterotrophic). The analyst should be aware about the following issues:

- **Auto- mixo- and heterotrophic protists**: The recent appraisal of widespread mixotrophy in phytoplankton [26] renders the traditional distinction of "autotrophic phytoplankton" and "heterotrophic protozoa" inoperative.
- There is **no general consensus whether heterotrophic protsists should be counted** (e.g. heterotrophic dinoflagellates such as *Gymnodinium helveticum*, *Protoperidinium sp.*). The identifier must be aware that Lugol fixed samples do not allow distinguishing pigmented from non-pigmented cells (fixation with formaldehyde in combination with epifluorescent microscopy is required, see 7.2, above). Heterotrophic taxa should be flagged in the datasheet when identified as such.
- Especially in freshwater studies, ciliates are typically not counted within phytoplankton analysis. Note, however, that **many ciliates are mixotrophic** (combine photosynthesis with phagotrophy).
- Many **ciliates will not be retained quantitatively in Lugol's fixed** samples If ciliates are to be counted, samples fixed with acidic Lugol's solution can be prepared for enumeration of microzooplankton, inc. ciliates [18]. If ciliates are a particular focus of a study, specific samples for ciliates should be prepared using appropriate fixatives (like Bouin's fixative [27]).
- In the **Baltic Sea** phytoplankton monitoring, the endosymbiotic ciliate *Mesodinium rubrum* is counted, since it contributes to primary production. Thus, *M. rubrum* should preferably be counted also within the

Baltic Sea mesocosm studies (in order to be able to compare the mesocosm results with the long-term monitoring results etc.).

7.7 Counting Procedure

An overview about the general principles for quantitative analysis of phytoplankton is provided by the WISER, as follows:

> "The quantitative analysis described here includes the identification, enumeration and calculation of biovolumes of Lugol's iodine preserved water samples. Analysis should be carried out using sedimentation chambers with an inverted microscope (Utermöhl technique).
>
> The preserved sample is thoroughly mixed by gently shaking the sample bottle and a sub-sample of known volume is placed in a sedimentation chamber. When the cells have settled to the bottom of the chamber, they are counted and identified using an inverted microscope.
>
> The statistical reliability of the analysis depends upon the distribution of phytoplankton units/cells within the sedimentation chamber and assumes that the cells are randomly distributed within the chamber.
>
> The counts for individual taxa are converted to phytoplankton biomass by using the cell/unit volume of the count units. The volumes are based on measurements made during counting or alternatively on available biovolume information for different taxa and size-classes." [13]

The following instruction on the counting procedure are mostly adopted from the corresponding WISER document [13].

It is useful to scan the sample at a variety of magnifications before the quantitative analysis is undertaken and to compile a list of the most frequent taxa before beginning to count.

Counting at different magnifications (steps A, B, C below)

In order to **obtain a reliable estimate of relevant size classes**, the counts should be carried out at 2-3 different size classes in the following manner (note that the effective magnification is the combined magnification of objective and ocular):

1) at low magnification (40x or 100x), a whole chamber count to pick up large taxa, followed by;

2) 2 transect counts at an intermediate magnification (200x or 250x), to enumerate "intermediate-sized" taxa (>20 µm) that are too small for the low-magnification count but too large to be reasonably counted using fields of view at high magnification, followed by;

3) a high magnification count (x400 or greater) using fields of view to pick up the small taxa. Aim to count 50-100 fields of view (i.e. at least 400 units assuming the recommended sample concentration).

More details are provided in the sections below.

A. Counting the whole chamber at low magnification for large taxa

Working at low magnification (x40 to x100) the whole chamber should be scanned in a series of horizontal or vertical transects (Fig. 7-3A) and the larger taxa (e.g. large dinoflagellates), large colonial or filamentous forms (e.g. *Microcystis, Fragilaria)* are counted. A cross-hair graticule eyepiece, or similar, (Figure 7-1) should be used if possible when counting the whole chamber. In horizontal transects, algae that lie between two horizontal lines are counted as they pass the horizontal line; algal objects that cross the top line are included whilst those crossing the bottom line are not and will be counted on the next transect (or vice versa; proceed accordingly for vertical transects).

B. Counting diameter transects

Algal cells larger than approximately 20 µm (e.g. *Cryptomonas*) are counted at 200x – 250x magnification in 2 randomly chosen diameter transects of the counting chamber (Figure 7-3B). The cross-hair eyepiece and method for counting algal objects described in the section above is used. The chamber is rotated between transect to randomly chosen positions.

C. Counting randomly selected fields

Small algae, <20 µm (e.g. *Rhodomonas*, small centric diatoms), should be counted in 50-100 randomly selected fields at x400 magnification (or greater) using a square, Whipple graticule, Miller Square or similar in the ocular eyepiece or the field of view to delineate the counting area. The number of fields counted should achieve a total count of approximately 400 phytoplankton units for the sample. Fields should be selected in a stratified-random way following the same pattern as the full chamber counts (Figure 7-3A). The counter must not look into the microscope when selecting a field – as this will result in non-random selection of fields.

A tally of the number of fields counted is required as well as the counts of individual identified algal units (cells, colonies or filaments).

When counting random fields, it is important to take a consistent approach to decide whether unicellular algal objects lying across the grid lines are counted in or out. A simple rule should be adopted as described in the CEN method (2006): unicellular algal cells crossing either the top or the left-hand side of the grid are not counted whilst those crossing the bottom or right-hand side of the grid are counted (Figure 7-3C).

For filaments and colonies, only the cells or filament length that is inside the field of view should be counted. Ideally, however, these larger taxa should usually be counted at lower magnification in transects or full chamber count.

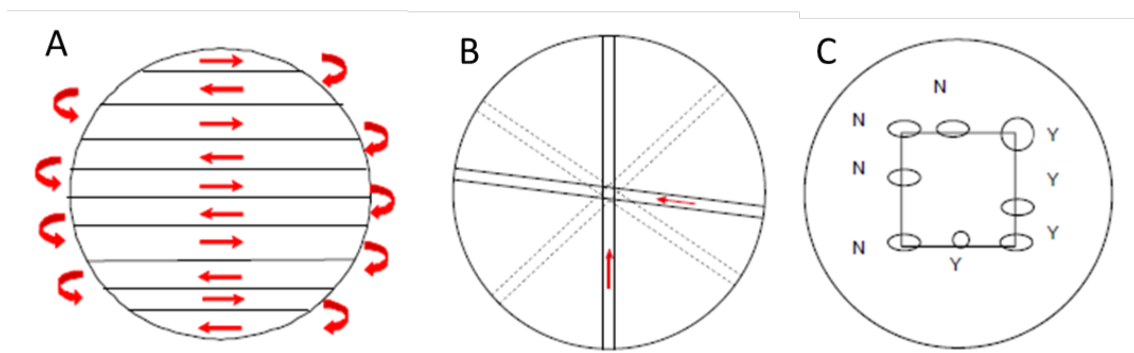

Figure 7-3 A:Counting method for whole chamber; horizontal transects, B:Counting method for diameter transects, C:Exemplified rule for counting cells at the edge of the field

Algal Objects and Counting Units

- Dead cells and empty loricas should not be counted (e.g. *Dinobryon*, *Cyclotella*)
- Littoral or benthic taxa should be counted and flagged as such (especially if mesocosms contain benthic compartment). In shallow systems, they can contribute a significant proportion of the sample.
- Heterocysts and akinetes of filamentous cyanobacteria should be counted (with separate measurements for biovolume estimates if present in large number).
- Unicellular picoplankton (<2 µm) should not be counted (unless using epifluorescence microscope).

Counting units are independent algal cells, chains, colonies or filaments/trichomes. One species or taxa may be present in the sample as different counting units and may be counted at different magnifications.
For example, *Microcystis* colonies are counted in the whole-chamber or transect but individual *Microcystis* cells (which may be present if colonies are disintegrating) are counted in random fields. Similarly, *Dinobryon* colonies may be counted in whole chamber or diameter transects, but single *Dinobryon* cells often need to be counted in random fields. Chain-forming algae (e.g. *Chaetoceros*) can be counted as single cells or colonies,

Counting units are assigned following practical considerations E.g. some filamentous forms do not allow distinction of individual cells. (e.g. counting *Planktothrix sp.* in units of 100µm filament length).

Species with a high variation of size can be counted in size-classes (e.g. Cryptomonadales <16 µm, 16-26 µm, >26 µm).

For analyses concerning Baltic Sea phytoplankton samples, counting units as given in the HELCOM PEG taxa and biovolume list [21] should preferably be used.

Identification and Coding

Species are coded as presented in the WISER_REBECCA taxa list [28] for phytoplankton available on the WISER intranet. The present updated taxa list is also included in the counter spreadsheet.

- **Calculation of phytoplankton biovolume**

Biovolumes must be measured for all taxa and it is done by assigning simple geometric shapes to each cell, filament or colony, measuring the appropriate dimensions and inputting these into formulae to calculate the cell volume. To obtain good biovolume estimates, species-specific biovolume should be estimated for all abundant taxa. For rare taxa, biovolumes from previous analyses (same system) or even from published resources can be used (e.g. [29],[30])

Hillebrand et al. [31] provides a comprehensive overview of formula for calculating biovolumes in phytoplankton. Most of the formula are available in the WISER excel spreadsheet for data entry (see → Data entry, below).

For analyses concerning Baltic Sea phytoplankton samples, taxon and size class -specific biovolumes given in the HELCOM PEG taxa and biovolume list [21] should preferably be used.

This SOP comes with an excel datasheet for entry of count data (adapted from [13]; see → Data entry, below). The sheet contains species lists and formula for the biovolume calculation for of each taxon. The cell dimensions that need to be measured depend on the underlying geometrical shape (see points listed below).
Measurements of the required cell dimensions (length, width, diameter) are made at an appropriate magnification using a calibrated ocular eyepiece, for instance, a Whipple Graticule. The eyepiece is rotated so that the scale is put over the required cell dimension and the measurement made by taking the ocular measurement

and multiplying by the calibration factor for that magnification and eyepiece combination. The measurements can be also made by image analysis. Per taxon, ca. 20 randomly chosen cells should be measured.

- **Biovolume estimation of unicellular taxa**

It is important to measure the linear dimensions of a number of individual units of all taxa observed in the sample. For taxa of more variable size (e.g. centric diatoms), and taxa that contribute significantly to the total biovolume (e.g. >5% of biovolume), at least 10 individuals should be measured.

For some species with external skeletons much larger than cell contents, e.g. *Dinobryon, Urosolenia*, the dimensions of the plasma/organic cell contents should be measured, not the external skeleton dimensions.

- **Biovolume estimation of filamentous taxa**

For filamentous taxa, the average biovolume can be approximated by estimating number of cells per filament multiplying by the mean biovolume of one cell.

Alternatively, it is possible to use the mean dimensions of filaments to calculate the biovolume of one filament multiplying by the number of filaments.

- **Biovolume estimation of colonial taxa**

For colonial taxa, count or estimate cell numbers and multiply by mean cell dimensions (often single measure of dimensions needed). Using colony/coenobium measurements – measure colony width and depth e.g. *Pediastrum* – with colony depth approximated as an individual cell diameter.

For dense colonies it may be difficult to estimate cell numbers. Some labs recommend estimating biovolume for e.g. Microcystis by applying a reduction factor on the biovolume of a colony (RF = reduction factor = 0.4; d = diameter of spherical colony; biovolume = RF x pi/6 x d^3) [32]. Similar to cellular biovolumes, density of colonies is not a constant factor but varies with age of colony etc. For a given sample, such a factor must be validated by careful microscopic inspection of a number of colonies.

- **Note on counting effort, taxon richness & diversity estimates**

If diversity parameters (taxon richness, evenness) are of interest, it is very important to maintain a constant counting effort among samples. A good standard is to count a minimum of 500 counting units per sample. Rarefaction analysis (as described in [33]) should be performed to check for sampling bias with regard to the parameter of interest. Constant counting effort also implies to balance detection limit relative to the biovolume of taxa (large taxa contribute over-proportionally to the total biovolume per sample). That is, the counting effort should be similar for different magnifications. As a general recommendation, the time spent on counting at different magnification should be in a similar range.

- **Data entry**

The counting can be carried out using different programs or spreadsheets. A template Excel spreadsheet that was prepared during the WISER project is recommended for freshwater taxa (https://www.aquacosm.eu/project-information/deliverables/). The spreadsheet contains the whole WISER REBECCA taxa list [28] and provides biovolume formulae for many of the common taxa (the overview of formula can also be used as a general reference for biovolume calculation). It also allows the raw data to be summarized. All required details must be input into the counting spreadsheet according to the accompanying instructions.

Data to be entered will include information on the sample 'site' (mesocosm ID etc) and date of collection, date of analysis, the person who carried out the count, information on the chamber and counting areas and the volume of sample used. For each taxon found, the number of units counted, the number of fields of view (or equivalent for whole chamber or diameter transects) in which it was counted and mean dimensions will be recorded for a given taxon. For taxa which are counted in different counting units (e.g. individual cells and filaments/colonies), it is important to fill in one row for cells counted and the other for filaments or colonies. For filaments and colonies, an estimate of the numbers of cells is also usually required to calculate biovolume/mL. Cells/mL and biovolume/mL for each taxon are automatically calculated once the count and mean dimensions are entered.

8. Quality Assurance and Quality Control (QA/QC)

According to [14], systematic or random errors can occur during the sampling operations. Systematic errors are generally due to the "poor sampling practices or equipment design failures" which are usually constant. On the other hand, random errors are generally unavoidable or unpredictable. One shall follow an effective Quality Assurance/Quality Control (QA/QC) strategy during the experiment to identify, quantify and control the errors. Standardization of sampling and analysis methods, taking replicate samples and analyses, or following a laboratory accreditation scheme are some examples for QA practices that can be followed during the experiment. Quality Assurance (QA) strategies are defined by the scientist-in-charge to ensure that the sample data meets Data Quality Objectives (DQO), which shall be defined prior to sampling. More information on DQOs can be found in the separate SOP on Data Quality Management (Reference will be provided when DQM SOP is completed). On the other hand, Quality Control (QC) is "the system of guidelines, procedures and practices designed to regulate and control the quality of products and services, ensuring they meet pre-established performance criteria and standards". The QC practices that can be followed are as follows: taking "sample blanks, replicates, splits", having and following "equipment calibration standards", determination of "sample container size, quality, use and preservative amount" prior to sampling.

The WISER project results highlighted the information below for QA of sampling and counting of phytoplankton:

> "1) Details of microscopes, chambers (individually identified and calibrated) and calibration of all ocular/objective combinations should be recorded in a note book and kept for reference. If fixed volume pipettes are used, these should be calibrated annually.
> 2) Checks for random distribution of sample should be done visually at low magnification for each sample. Some simple checks include:
> Comparing the number of observations in:
> (a) half a chamber with the other half
> (b) comparing counts in the 1st transect with the 2nd transect
> (c) comparing counts in the first 20 field of view with the next 20 fields.
> A more detailed check using simple Chi squared test should be done if a sample does not appear to be randomly sedimented or 1 sample every 3 months or so" [13].

In addition, the following were also suggested as a best practice advice by Brierley and colleagues (2007):

- If there is an aim for accreditation of phytoplankton analysis, ring-tests can be undertaken with a staged approach:
  1) "Determining mainly counting errors – group of analysts to count limited number of named taxa (1 to 3) or latex particles/pollen grains in set fields – can be done using photographs or videos
  2) Repeat transect or field counts by 2 or more analysts on real sample to check identification and counting errors.
  3) Full count comparisons
- Regular workshops should be held (3 - 4 times per annum) to carry out identification and ring tests, possibly combined with 1⁄2-1 day taught workshop on difficult groups"[34].

Moreover, UNESCO/IOC [16] determined a quality assurance procedure to follow when using Utermöhl technique. According to the document, the quality of the results are dependent on the skill of the analyst and, to ensure high quality results, one shall validate all steps of the method. The steps in the Utermöhl method to validate are determined in the document as follows:

i. "homogenization of sample
ii. sedimentation/sinking

iii. distribution on chamber bottom
iv. repeatability and reproducibility" [16].

Rott and others (2007) [35] suggested the use of both "intra-laboratory standardization and inter-(between) laboratory calibration (ring-test)" for quality assurance of quantitative analysis of phytoplankton. In addition, the use of the following results of his study as a pathway to assess counting precision:

- "Repeated subsample counts (pairs of transects, random fields etc.) evaluated separately can be simple and quick approaches for intra-laboratory scatter check, and can be helpful in identifying quality targets, which can be independent from the actual subsampling methods

- Minimum counting thresholds for unicellular taxa were found to be attained by counts of less than 50 in spite of fundamentally different approaches

- Although conventional size measurements and protocols (e.g**.** [36]**;** [37]**)** can be precise enough, traditional shape analysis can be improved. Microscopic observations combined with novel electronic methods **(**[38]**)** and flexible 3D electronic models **(**[39]**)** are recommended for future" [35].

Best practice advices:

✓ The mesocosm facilities can work in a network. 'Nordic Periphyton & Phytoplankton Group' (NPPG), is an example of such a network, and organizes annual meetings and practices intercalibration [40]. The group is made up of specialized algologists.
✓ In addition to NPPG, Nordic Microalgae [41] for the mesocosms in Nordic Region can be examined for information about microalgae and related organisms.
✓ For the Baltic Region, HELCOM Phytoplankton Expert Group (HELCOM PEG) project website [42] can be visited to receive information especially on Baltic Sea phytoplankton species and QA procedures followed.
✓ For sampling marine phytoplankton, PLANKTON*NET [43] data provider at the Alfred Wegener Institute for Polar and Marine Research (AWI) is suggested as an open access repository for plankton-related information.

For detailed QA methodology provided for sampling, analysis and counting of phytoplankton, please visit CEN - EN 15204 [44], Rott et al. [35] and WISER project [13].

According to the '*Common Implementation Strategy for the Water Framework Directive (2000/60/EC)*', the intercalibration exercises in between laboratories will provide a "continuous quality assurance system", by ensuring the results meet targeted levels [45]. In order to perform intercalibration in the AQUACOSM community, there shall be QA measures in each of the mesocosm facility. The common QA measures, that are determined based on this SOP and the valid sampling and analysis methods used during the experiments, that shall be taken by each mesocosm facility are listed as follows:

- "Field sampling and sample label
- Sample storage and preservation;
- Laboratory analysis" [45].

Further suggestions provided by [45] (not specific to phytoplankton analysis) are as follows:

- "Establishing routine internal quality control
- Participation in external QA schemes" [45].

Other QA/QC practices that must be carried out

- Visual check for clumping
- Consider the settling period (prior to initiation of the experiment (sampling) OR during counting and identification)
- Repeat the application of Iodine every 6 months
- Pay attention to the confidence level of microscopic enumeration of colonial or filamentous phytoplankton species
- Consider the resolution/degree of identification and take pictures of unidentified species or species which have unsure identification.

9. Dissemination activities related with the Deliverable

The SOP will be made available to all users of TA in AQUACOSM, and will also be publicly available for any user through the AQUACOSM webpage (https://www.aquacosm.eu/project-information/deliverables/)

10. Appendix

Appendix I

Standard recipe for Lugol's Solution (Adopted from [46]**)**

Equipment and Supplies

- ✓ Reagent water
- ✓ Glacial acetic acid
- ✓ Iodine
- ✓ Potassium iodide (KI)
- ✓ 1 L Volumetric flask
- ✓ Opaque 1 L container
- ✓ Glass funnel

Lugol's Solution preparation procedure

Step 1: In a fume hood, dissolve, 100 g of KI and 50 g of I2 in approximately 800 mL of reagent water in a 1 L volumetric flask.

Step 2: Mix until the chemicals are completely dissolved.

Step 3: Add 100 mL of glacial acetic acid and bring volume up to 1 L with reagent water. Add 1mL of Lugol's solution to each 100ml water sample within 2 hours of sample collection to obtain a final concentration of 1%. Store the solution in an opaque or brown glass container.

Important considerations

- Lugol's solution should be prepared maximum one week prior to the survey.
- Lugol's solution is stored in an opaque bottle labeled with the contents, date of preparation, and preparer's initials.

Recipes for alkaline, neutral and acidic Lugol's solution from [16]

Table 1. Recipes for Lugol's iodine solution (acidic, alkaline and neutral).
(from: Utermöhl 1958, Willén 1962, Andersen and Throndsen, 2004).

Acidic	Alkaline	Neutral
20 g potassium iodide (KI)	20 g potassium iodide (KI)	20 g potassium iodide (KI)
10 g iodine (I_2)	10 g iodine (I_2)	10 g iodine (I_2)
20 g conc. acetic acid	50 g sodium acetate	200 mL distilled water
200 mL distilled water	200 mL distilled water	

Formaldehyde Stock solution (Adopted from [46]**)**

Equipment and supplies

- ✓ Normal formaldehyde solution 40% v/v
- ✓ Deionized water
- ✓ 1 L Volumetric flask
- ✓ 1 L container
- ✓ Glass funnel

Formaldehyde Stock Solution preparation procedure

Step 1: Acquire the normal formaldehyde solution 40% v/v

Step 2: In a fume hood, measure 400 mL of the normal formaldehyde solution with a volumetric cylinder and add it in a 1 L volumetric flask using a glass funnel

Step 3: Fill the flask with water to obtain 1 L of Formaldehyde stock solution

Step 4: Transfer the contents of the flask in a Plastic container and cap it firmly. Store under fume hood.

Important considerations

- Formaldehyde is carcinogenic and should be handled with care. Proper safety equipment includes gloves and plastic goggles.
- The preparation of the formaldehyde Stock Solution should be done in a fume hood.

Appendix II

Label Standard (Revised from [46]**)**

Mesocosm Facility: .... Mesocosm Number: ....

Sample No:.. Sampler: ....

Secchi Depth: .... Max. Sampling Depth: ....

Sampling Depths: ....

Date and Time: .... Collector Initials: ....

Phytoplankton Sampling Field Datasheet (Revised from [46]**)**

Phytoplankton Sampling Field Sheet

Mesocosm Name: …………………………………………………………………………………………………….

Mesocosm Number…………………………………… Date and Time: ……………………………………………

SAMPLING POINT	GPS COORDINATES		Secchi Depth	Max. Sampling Depth (MSD)
	Longtitude (X)	Latitude (Y)		
1				
2				

No. Of Sample	Sampling Point	Sampler	Preservative
1			
2			
3			
4			
5			
6			

NOTES:

NAME & SIGNATURE:

11. References

[1] European Commission, “Directive 2000/60/EC of the European Parliament and of the Council of 23 October 2000 establishing a framework for Community action in the field of water policy,” 2000.

[2] G. WOLFRAM, K. DONABAUM, and M. T. DOKULIL, “Guidance on the Monitoring of the Biological Quality Elements Part B2 - Phytoplankton,” Vienna, 2015.

[3] ASTM International, “Standard Terminology Relating to Water,” 2013.

[4] WHO, *Handbook: Good Laboratory Practice (GLP): Quality practices for regulated non-clinical research and development*, 2nd ed. Switzerland: TDR/WHO, 2009.

[5] OECD, *OECD series on principles of good laboratory practice and compliance monitoring*. Paris, 1998.

[6] Council Directive, “89/391/EEC of 12 June 1989 on the introduction of measures to encourage improvements in the safety and health of workers at work,” *Off. J. Eur. Communities* , vol. 183, no. 29, p. 8, 1989.

[7] OSHA, “European directives on safety and health at work - Safety and health at work.” [Online]. Available: https://osha.europa.eu/en/safety-and-health-legislation/european-directives. [Accessed: 16-Jun-2017].

[8] D. on E. and L. S. Committee on Prudent Practices in the Laboratory, Board on Chemical Sciences and Technology, *Prudent practices in the laboratory : handling and management of chemical hazards*. National Academy of Sciences, 2011.

[9] ECHA, “Formaldehyde - Substance Information.” [Online]. Available: https://echa.europa.eu/substance-information/-/substanceinfo/100.000.002. [Accessed: 16-Jun-2017].

[10] Ministerium Für Ein Lebenswertes Österreich, “LEITFADEN ZUR ERHEBUNG DER BIOLOGISCHEN QUALITÄTSELEMENTE TEIL C - ARBEITSSICHERHEIT,” Wien, 2016.

[11] European Commission, “EU Waste Legislation.” [Online]. Available: http://ec.europa.eu/environment/waste/legislation/a.htm. [Accessed: 16-Jun-2017].

[12] European Commision, “COMMISSION DECISION of 3 May 2000 replacing Decision 94/3/EC establishing a list of wastes pursuant to Article 1(a) of Council Directive 75/442/EEC on waste and Council Decision 94/904/EC establishing a list of hazardous waste pursuant to Article 1(4) of Council Directive 91/689/EEC on hazardous waste (notified under document number C(2000) 1147) (Text with EEA relevance) (2000/532/EC),” *Off. J. Eur. Communities*, vol. 69, 2000.

[13] U. Mischke *et al.*, “EU FP7 226273, WISER deliverable D3.1-4: guidance document on sampling, analysis and counting standards for phytoplankton in lakes,” pp. 1–51, 2012.

[14] EPA (IRELAND), “Water Framework Directive: Sampling Manual for Rivers and Lakes,” 2016.

[15] H. Utermöhl, “Zur vervollkommnung der quantitativen phytoplankton methodik,” 1958.

[16] UNESCO/IOC, *MICROSCOPIC AND MOLECULAR METHODS FOR QUANTITATIVE PHYTOPLANKTON ANALYSIS*. Paris: O. (IOC Manuals and Guides, no. 55.) (IOC/2010/MG/55), 2010.

[17] Aarhus Universitet – National Environmental Research Institute (AU) and Middle East Technical University (METU), “EU FP7 244121, REFRESH Deliverable 3.4: Protocols for field experiments and monitor- ing along a climate gradient in task 2,” 2014.

[18] P. Pitta, A. Giannakourou, and U. Christaki, “Planktonic ciliates in the oligotrophic Mediterranean Sea: longitudinal trends of standing stocks, distributions and analysis of food vacuole contents,” *Aquat. Microb. Ecol.*, vol. 24, pp. 297–311, 2001.

[19] Baltic Marine Environment Protection Comission, “Monitoring of phytoplankton species composition,

abundance and biomass," 2017.

[20] IGB, "Taxaliste Phytoplankton O 2.17 | Drupal." [Online]. Available: http://www.igb-berlin.de/en/project/taxaliste-phytoplankton-o-217. [Accessed: 15-Jun-2017].

[21] Baltic Marine Environment Protection Comission, "HELCOM Phytoplankton Expert Group (HELCOM PEG)," *HELCOM*, 2016. [Online]. Available: http://www.helcom.fi/helcom-at-work/projects/phytoplankton. [Accessed: 05-Sep-2017].

[22] AlgaeBase, "AlgaeBase," 2017. [Online]. Available: http://www.algaebase.org/. [Accessed: 19-Jun-2017].

[23] R. A. Horner, *A Taxonomic Guide to some common marine phytoplankton*. Bristol, UK: Biopress Limited, 2002.

[24] C. R. Tomas, *Identifying marine phytoplankton*. San Diego: Academic press, 1997.

[25] J. Throndsen, G. R. Hasle, and K. Tangen, *Phytoplankton of Norwegian coastal waters*. Oslo: Almater Forlag AS, 2007.

[26] K. J. Flynn *et al.*, "Misuse of the phytoplankton-zooplankton dichotomy: The need to assign organisms as mixotrophs within plankton functional types," *J. Plankton Res.*, vol. 35, no. 1, pp. 3–11, 2013.

[27] C. A. JEROME, D. J. S. MONTAGNES, and F. J. R. TAYLOR, "The Effect of the Quantitative Protargol Stain and Lugol's and Bouin's Fixatives On Cell Size: A More Accurate Estimate of Ciliate Species Biomass," *J. Eukaryot. Microbiol.*, vol. 40, no. 3, pp. 254–259, May 1993.

[28] U. Mischke *et al.*, "Dataset: 'Phytoplankton' (according to the WISER project) www.freshwaterecology.info - the taxa and autecology database for freshwater organisms," *www.freshwaterecology.info*. [Online]. Available: http://linkinghub.elsevier.com/retrieve/pii/S1470160X15000758.

[29] C. T. Kremer, J. P. Gillette, L. G. Rudstam, P. Brettum, and R. Ptacnik, "A compendium of cell and natural unit biovolumes for >1200 freshwater phytoplankton species," *Ecology*, vol. 95, no. 10, p. 2984, Oct. 2014.

[30] I. Olenina *et al.*, "Biovolumes and size-classes of phytoplankton in the Baltic Sea," *HELCOM Balt.Sea Environ. Proc.*, vol. 106, no. 106, p. 144 pp, 2006.

[31] H. Hillebrand, C.-D. D?rselen, D. Kirschtel, U. Pollingher, and T. Zohary, "BIOVOLUME CALCULATION FOR PELAGIC AND BENTHIC MICROALGAE," *J. Phycol.*, vol. 35, no. 2, pp. 403–424, Apr. 1999.

[32] K. Olrik, P. Blomqvist, P. Brettum, G. Cronberg, and P. Eloranta, *Methods for quantitative assessment of phytoplankton in freshwaters. P. 1: Sampling, processing, and application in freshwater environmental monitoring programmes*. 1998.

[33] N. J. Gotelli and R. K. Colwell, "Quantifying biodiversity: procedures and pitfalls in the measurement and comparison of species richness," *Ecol. Lett.*, vol. 4, no. 4, pp. 379–391, 2001.

[34] B. Brierley, L. Carvalho, S. Davies, and J. Krokowski, "Guidance on the quantitative analysis of phytoplankton in Freshwater Samples," Edinburgh, 2007.

[35] E. Rott, N. Salmaso, and E. Hoehn, "Quality control of Uterm?hl-based phytoplankton counting and biovolume estimates?an easy task or a Gordian knot?," *Hydrobiologia*, vol. 578, no. 1, pp. 141–146, Mar. 2007.

[36] E. Hoehn, H. A. M. Ketelaars, and B. Ewig, *Erfassung und Bewertung von Planktonorganismen: Arbeitsgemeinschaft Trinkwassertalsperren eV Arbeitskreis Biologie*. Oldenbourg, 1998.

[37] J. Padisák and R. Adrian, "Biovolumen," in *Biologische Gewasseruntersuchung, Band 2.*, Gustav Fischer Verlag, 1999, pp. 334–368.

[38] D. Hepperle, "OPTICOUNT - Software for the enumeration of plankton," 2005.

[39] A. Lyakh, “The free form deformation of phyto- plankton models,” in *Lecture Notes in Computer Sciences 2331*, 2002, pp. 192–201.

[40] K. Vuorio, L. Lepistö, and A.-L. Holopainen, “Intercalibrations of freshwater phytoplankton analyses.,” *Boreal Environ. Res.*, vol. 12, no. 5, 2007.

[41] SMHI, “Nordic Microalgae.” [Online]. Available: http://nordicmicroalgae.org/. [Accessed: 19-Jun-2017].

[42] HELCOM, “HELCOM Phytoplankton Expert Group (HELCOM PEG).” [Online]. Available: http://helcom.fi/helcom-at-work/projects/phytoplankton/. [Accessed: 19-Jun-2017].

[43] Alfred Wegener Institute for Polar and Marine Research, “PLANKTON*NET.” [Online]. Available: http://planktonnet.awi.de/. [Accessed: 19-Jun-2017].

[44] CEN EN 15204, “Water quality–Guidance standard for the routine analysis of phytoplankton abundance and composition using inverted microscopy (Utermöhl technique),” 2006.

[45] European Commission, “Common Implementation Strategy for the Water Framework Directive (2000/60/EC) Monitoring under the Water Framework Directive,” Luxemburg, 2003.

[46] İ. Tuney and M. Maroulakis, “PHYTOPLANKTON SAMPLING METHODS,” 2014.