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# Water quality — Guidance on quantitative and qualitative investigations of marine phytoplankton

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# Water quality - Guidance on quantitative and qualitative investigations of marine phytoplankton

Qualité de l'eau - Guide pour l'étude quantitative et qualitative du phytoplancton marin

Wasserbeschaffenheit - Anleitung für die quantitative und qualitative Untersuchung von marinem Phytoplankton

This European Standard was approved by CEN on 29 July 2011.

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## Foreword

This document (EN 15972:2011) has been prepared by Technical Committee CEN/TC 230 “Water analysis”, the secretariat of which is held by DIN.

This European Standard shall be given the status of a national standard, either by publication of an identical text or by endorsement, at the latest by March 2012, and conflicting national standards shall be withdrawn at the latest by March 2012.

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# Introduction

Investigations of phytoplankton are an important part of marine environment monitoring. Phytoplankton responds rapidly to environmental changes (supply of nutrient salts, climate, light access etc.), and monitoring of occurrence, species composition and biomass may therefore in many cases be used to characterise the environment and the degree of impacts. The European Union Water Framework Directive therefore requires monitoring of marine phytoplankton as a part of assessments of ecological conditions. Investigations of phytoplankton are also included in monitoring programmes connected with other European directives (Urban Waste Water Treatment Directive, Habitats Directive), international conventions and national regulations. Monitoring of harmful/toxic phytoplankton is carried out both for the aquaculture industry and in connection with authorities' control of organisms for human consumption (shellfish). This requires uniform procedures for collection and quantification of phytoplankton.

Most principles for characterisation of environmental conditions based on phytoplankton require the use of quantitative methods, i.e. that the occurrence of species and quantities can be related to a known water mass. Data interpretation further requires information on the physical and chemical properties of the water body (supporting parameters). In order for environmental authorities to utilise the information, it is important that the investigations are comparable in space and time and that the data is of high scientific quality. This European Standard focuses on a limited selection of methods that can be documented precisely, reproduced and which have been in use for some time. This European Standard does not comprise chlorophyll determination. For chlorophyll determination see ISO 10260 [1].

**WARNING — Persons using this European Standard should be familiar with normal laboratory and fieldwork practice. This European Standard does not purport to address all of the safety problems, if any, associated with its use. It is the responsibility of the users to establish appropriate safety and health practices and to ensure compliance with any national regulatory conditions.**

## 1 Scope

This European Standard gives guidance for sampling, preservation, storage, quantification and qualitative analysis of phytoplankton from marine waters. Guidance for quantification is limited to the use of light microscopy with phase-contrast and epifluorescence.

This European Standard specifies:

— the development of the sampling programme;

— requirements for sampling equipment;

— procedures for sampling and treatment of samples in the field;

— methods for quantification;

— qualitative analysis.

This European Standard describes minimum requirements for environmental monitoring.

## 2 Normative references

The following referenced documents are necessary for the application of this document. For dated references, only the cited edition applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

EN 15204, *Water quality — Guidance standard on the enumeration of phytoplankton using inverted microscopy (Utermöhl technique)*

## 3 Terms and definitions

For the purposes of this document, the following terms and definitions apply.

**3.1**
**euphotic zone**
upper zone of a water body which is penetrated by light, and where the primary production over one or more days is equal to or greater than the respiration, i.e. a positive net production

NOTE Empirically, the euphotic zone is considered to extend down to the depth where 1 % of the surface light is present.

**3.2**
**phytoplankton**
community of free-living, suspended, mainly photosynthetic organisms in aquatic systems comprising Cyanobacteria and microscopic algae

[EN 15204:2006, 3.10]

**3.3**
**phytoplankton biomass**
mass of living matter comprising phytoplankton

NOTE The phytoplankton biomass can be expressed in terms of chlorophyll content, the carbon content of the algae (algal carbon) or the volume of the phytoplankton (biovolume).

**3.4**
**qualitative sample**
phytoplankton sample collected by plankton net, i.e. a concentrated sample where the size of the phytoplankton is mainly larger than the mesh diameter

NOTE Allows for recordings of taxon/taxa occurring in low concentrations.

**3.5**
**quantitative sample**
phytoplankton sample where a part of a body of water is enclosed so that the density of each taxon and their relative abundance per unit volume may be recorded

**3.6**
**receiving water body**
water body that receives an input of material, of either natural or anthropogenic origin

NOTE The term often appears in the context of anthropogenic input, for example, effluent from municipal waste water outlets or industrial processed water.

[EN ISO 16665:2005, 2.4]

**3.7**
**sampling station**
precise location where samples are collected

NOTE A sampling station is defined by its geographical position (OS National Grid Reference, latitude, longitude), its depth (relative to chart data and normalised to mean low water as given in tide tables) and any other invariant of physical conditions. The station is delineated using the given level of precision. In cases of doubt when revisiting sampling stations, emphasis should be placed on landmarks and water depth.

[EN ISO 16665:2005, 2.7]

**3.8**
**stratification**
two or more water bodies with different physical properties (temperature, salinity, density) lying upon each other

NOTE A thermocline separates the water bodies from each other.

**3.9**
**thermocline**
boundary layer between two horizontally arranged water bodies with different physical properties which change dramatically in the vertical

**3.10**
**water body**
body of water, standing or flowing; a lake, river, or other collection of water

**3.11**
**water transparency**
estimate of turbidity determined by recording the depth at which a white disc with black and white quarters lowered into the water is no longer visible from the surface

# 4 Aim and strategy for sampling phytoplankton

## 4.1 Sampling programme

Before a survey is carried out, the sampling programme shall be defined according to the individual aims of the survey and the required precision of results and their intended use. Sampling methodology, number of sampling stations, sampling frequency and methodology for sample processing will vary between different types of investigations. During development of the programme, consideration shall be given to hydrographic conditions in the survey area, information about local pollution sources, knowledge from previous surveys and any other conditions that may be of importance for the phytoplankton composition in the survey area.

For long-term baseline studies, it should be ensured that methodological changes do not have any influence on the results of time series.

## 4.2 Types of surveys

### 4.2.1 Pilot survey

Pilot surveys shall provide a general overview of the occurrence of phytoplankton and physical and chemical conditions in the water body of the survey area. In areas where larger scale environmental surveys or monitoring is required, there can be a need to carry out a pilot survey in advance. This pilot survey shall provide the necessary information for developing more specific sampling programmes. The requirements for equipment, methodology and repeatability are usually relatively simple. The phytoplankton samples shall be collected by plankton net and/or water samplers. Hydrographic data shall be collected with a continuous

recording probe or by use of a water collector and subsequent analysis of sampled water. The phytoplankton samples are processed in such a way that the most important taxon/taxa can be identified and quantified.

NOTE In this context, the most important taxon/taxa are those that are dominant in terms of numbers and/or biomass and taxon/taxa that have characteristic properties (indicators of eutrophication, producers of toxins etc.).

#### 4.2.2 Baseline survey and monitoring of biological diversity

Baseline surveys should be carried out for environmental monitoring and characterisation of biodiversity. Detailed analyses should be carried out on the relative species composition and abundance within the phytoplankton communities. The investigation shall provide the basis for characterising environmental conditions in the relevant areas in accordance with set criteria or by comparison with phytoplankton communities in other representative areas. The requirements for scientific documentation and ability to be replicated are usually relatively high.

If the baseline survey is carried out over time in one area, the investigation will provide a temporal description of natural variations of the phytoplankton communities and document any gradual changes (trend monitoring). The investigation shall be carried out at fixed stations and follow a carefully defined programme. At the start of investigations, in areas where the hydrographic conditions are insufficiently known, a preliminary investigation of the hydrographic conditions shall be carried out to identify the different water masses within the water column and to locate the thermocline depth before the formal sampling programme is established.

The investigations shall be carried out using quantitative methods. There should be specified requirements for station positioning, sampling depth, frequency and supporting parameters, such as chlorophyll-a concentration hydrographic measurements, nutrient analyses, *in situ* fluorescence and water transparency. Methods should be chosen so that the data can be used as a basis for comparison with baseline surveys in other areas. Additional qualitative samples (plankton net samples) can be taken to give a complete description of the species composition.

In areas where potential changes in environmental conditions are to be monitored, such as in impacted receiving waters or areas where there are plans for establishing activities that may cause environmental impacts, an initial baseline survey shall be carried out, and thereafter follow-up monitoring surveys.

#### 4.2.3 Specialised surveys - Monitoring of selected taxon/taxa

Specialised surveys are recommended for monitoring toxic and harmful algae (phytoplankton) for fish in aquaculture and shellfish production (such as blue mussels). Sampling shall be carried out at fixed locations and according to defined programmes where sampling depth, frequency and methodology are described. During sample processing, the occurrences of previously specified taxon/taxa shall be quantified. The investigations shall provide the basis for issuing warnings or to set in motion strategies for hazard reduction (mitigation).

#### 4.2.4 Verification survey

Verification surveys may be used to verify phytoplankton blooms detected by remote sensing techniques, for example in cases of discoloured water or on suspicion of mass occurrences of harmful algae (phytoplankton). The samples are collected by using a fine meshed plankton net and/or water samplers and further processed to identify the dominant taxon/taxa.

### 4.3 Sampling stations

Sampling stations shall be representative for the areas to be investigated or monitored. Back-eddies, more or less enclosed bays or other areas that are topographically or hydrographically anomalous shall be avoided unless the aim is specifically to investigate these.

If the area has been investigated previously or there is ongoing biological, physical and/or chemical sampling, the same stations shall be used, unless those stations will not be able to serve the current purposes.

Sampling should preferably be carried out from a boat. If sampling from quay or pier is necessary, there shall be sufficient water depth to avoid influence from the bottom sediment.

When using this means of sampling, the samples can become contaminated by micro-algae associated with hard substrates such as fouling on the quay/pier, macroalgae etc. The samples shall therefore be taken upstream and as far from the edge of the quay/pier as possible. For baseline surveys in receiving waters from industry, municipal discharges and aquaculture activities, the samples shall, as far as possible, be taken upstream of the plant(s). Sampling at a single station is usually sufficient. However, in particular or acute situations, sampling shall be carried out according to continual assessment of the most beneficial station positioning.

## 4.4 Sampling depth

In the case of a monitoring network operated in an international framework, sampling strategy shall be elaborated by the expert group of the member states. Samples for quantitative analysis may be collected either individually from fixed depths, as combined samples where samples from different depths are mixed together to form a single sample or as integrated samples, where sampling encompasses the entire water column.

Combined samples may be used in cases when an overview of the numerical dominating species is required and where it is not necessary to link the occurrence of phytoplankton to hydrographical conditions or chemical parameters.

In marine areas, where large fluctuations in the physical parameters within the water column occur, such as fjords or brackish waters, the samples should be taken both above and below the thermocline, because they can often form a boundary between different phytoplankton stocks. In marine areas where different water bodies meet, hydrographic measurements shall be carried out to identify stratified water layers of various origins down the water column, in order to ensure that the different water masses are adequately sampled.

For baseline surveys or trend monitoring, where the phytoplankton occurrences are related to chemical parameters such as nutrient levels, samples usually shall be taken from fixed depths. Standard sampling depths within the upper 100 m of open sea (recommended by ICES[1]) are 0 m, 5 m, 10 m, 20 m, 30 m, 50 m, 75 m and 100 m. This should be the case in areas where no vertical stratification is found. In fjord areas, additional sampling shall be carried out at 2 m. The number of sampling depths may be increased if there is information from, for example, hydrographic measurements on increased frequency of phytoplankton occurrence other than the recommended depth. If *in situ* fluorescence is measured, an additional sample may be collected from the depth at which the fluorescence maximum occurs. See A.2.2 for a description of fluorescence maximum.

The sampling depth from which samples are to be processed should be decided on an individual basis, based on the aim of the investigation. As a minimum, at least one water sample from every sampling date shall be processed. Samples from the same depths shall be processed throughout the entire duration of the programme, to ensure comparable data. Additional samples can be processed if necessary to get information about the phytoplankton composition at the thermocline or other interesting levels in the water column.

Integrated samples are often used in connection with specialised surveys and monitoring of particular taxon/taxa. For example, for monitoring of toxic or harmful phytoplankton in connection with fish or shellfish production, integrated samples shall be taken within the depth intervals at which the fish or shellfish are located if vertical stratification occur in the height of water concerned by the aquaculture livestock.

The net samples are used to complete the total species spectrum and for the determination of difficult taxa. The precise depth, or depth interval, at which the haul shall be carried out, shall be decided based on the aims of the sampling programme and/or the hydrographic conditions at the sampling location.

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[1] International Council for the Exploration of the Sea

### 4.5 Sampling frequency and duration

For baseline surveys monitoring, sampling shall be carried out at least twice per month. Sampling may be carried out more frequently during periods of the year where significant variations occur over short time-periods, for example during spring. The investigation shall cover the growth season of the phytoplankton over a period of one year. In many areas, the growth season actually covers the whole year.

For trend monitoring and studies of biodiversity, the sampling frequency shall be as for baseline surveys, but in addition, monthly sampling shall be carried out during the winter period. Investigations are recommended to be carried out over a period of at least 6 years, to document annual variations. However, this frequency may be determined by statistical analysis of the data and the needs for the survey.

In the case of specialised surveys for monitoring toxic phytoplankton in connection with health warnings about shellfish consumption, the sampling frequency shall comply with the current regulations set by the relevant authorities. For other specialised surveys, the sampling frequency and sampling period shall be adjusted according to the aims of the investigation.

### 4.6 Parallel water samples

In many cases, it may be appropriate to go back to previously collected samples to verify identifications and/or quantifications, split previously grouped taxa to lower taxonomic levels or to carry out other analyses of the collected material. Degradation of biological material will begin if sample containers are opened and air is introduced. Therefore, parallel samples preserved with buffered formaldehyde taken for future reference purposes shall be kept in separate containers. When planning the sampling programme it should be decided whether parallel water samples are to be collected.

## 5 Equipment

### 5.1 Equipment for quantitative sampling

Quantitative samples determining the density of the various taxon/taxa shall be collected using a water sampler, pipe or hose.

All water samplers shall be constructed so that they can be filled with water from a specified depth/depth interval. All water samplers should be made of non-toxic material. When using a hose, the lower end shall be sufficiently weighted to ensure that the hose is positioned vertically in the water column, and correctly takes an integrated sample from the relevant part of the water column. For an overview of various types of water samplers, see Annex B.

Additional equipment:

— weight for triggering closure of the water sampler;

— winch with counter or rope with metre marks;

— shackle to attach the wire/rope to the water sampler;

— mixing container, e.g. a plastic can or bucket with a lid for homogenising integrated samples.

### 5.2 Equipment for qualitative sampling

Qualitative samples for investigations of species occurrences should be taken using a plankton net, see Annex C.

In baseline surveys, where occurrences of small phytoplankton are important, a plankton net with mesh size from 5 µm to 10 µm shall be used to ensure that both nanoplankton and microplankton are sampled. For

specialised surveys, plankton nets with 20 µm mesh diameter may be used. However, if smaller species are to be monitored, the mesh size shall be adjusted according to the size of the species under investigation. If only the occurrences of larger-sized phytoplankton species are to be verified, the mesh size may be increased to > 20 µm.

NOTE 1 The mesh size given by the manufacturer should be considered as approximate because slight deviations may occur.

NOTE 2 Increasing the mesh size results in a loss of smaller phytoplankton cells. The most commonly used mesh sizes are 10 µm and 20 µm.

Additional equipment:

— if the plankton net is not equipped with a permanent collection cup and tap, a loose collection cup may be attached to the net;

— weight to increase sinking velocity, or to keep the net at the desired depth during vertical hauls;

— winch with counter or rope with metre marks;

— shackle for attaching the rope/wire to the plankton net;

— wash bottle to rinse down phytoplankton adhering to the inside of the plankton net.

## 5.3 Sample bottles

Sample bottles shall be made of material that does not affect the phytoplankton or the properties of the preservative before analysis. Samples that contain living phytoplankton shall be kept in the dark to prevent growth during storage. If the samples cannot be stored in the dark, the bottle used should not be pervious to daylight (for instance brown glass bottles). Samples fixed in iodine (Lugol) shall be stored in a dark place in transparent containers, such as glass bottles, in order to facilitate checking for bleaching of the iodine. The cork or screw cap shall be airtight to prevent evaporation. The usual volume of sample bottles is between 100 ml and 500 ml. If the phytoplankton concentration is suspected to be low (oligotrophic areas), larger bottle volumes shall be used. The sample containers should not be completely filled, so as to facilitate mixing of the sample before a sub-sample is extracted.

Samples for long-term storage shall be kept in bottles that are impermeable and non-reactive to the fixative. The cork or screw cap shall be watertight and as close to airtight as possible to prevent evaporation and introduction of oxygen that can lead to oxidation of the fixative during storage.

Plastic bottles or plastic containers may be used if the samples are not to be stored for extended periods, since fixatives such as Lugol's solution will become absorbed into or evaporate through plastic over time and the storage time shall not exceed 6 months. For monitoring of potentially toxin producing phytoplankton for the aquaculture industry, 25 ml plastic sample containers may be used.

Sample containers shall have a field for writing on, or be labelled with appropriate label stickers or tape. A waterproof marker pen or pencil should be used for marking samples.

## 5.4 Fixatives and preservatives

Substances used for fixation and preservation should as far as possible prevent the phytoplankton cells from changes in shape or other alterations in identifying characteristics, such that they can be identified by regular light microscopy throughout the entire storage period.

NOTE Lugol's solution and formaldehyde in water are the most commonly used. The solutions can be alkaline, neutral or acidic, each of which has slightly different properties and uses.

For fixation and preservation of quantitative phytoplankton samples (water samples) neutral Lugol's solution shall be used. Acid Lugol's solution should be used where it is not important to maintain calcareous structures. For fixation of qualitative samples (plankton net haul), a neutralised formaldehyde solution in water shall be used.

Annex D gives additional information on the use of various fixatives and preservatives, as well as how to prepare the solutions.

# 6 Survey procedures

## 6.1 Description of procedures

Before the start of field work, descriptions of methodology, safety instructions, personnel plan, overview of equipment and instruments, recording form, instructions for recording, procedures for securing records and samples as well as information on quality assurance requirements should be compiled.

## 6.2 Preparation

The following shall be carried out during equipment preparation:

— check that the plankton net is clean and in good condition, without holes or tears;

— check that the attachment of the rope/wire to the plankton net is sufficiently secure;

— rope shall be marked with metre marks in advance. The rope used shall not stretch when wet;

— check that the water sampler's closing mechanism is functioning well and that any seals are in order.

## 6.3 Defining the position of sampling stations

The position of sampling stations shall be defined unambiguously, such that they can easily be relocated by other investigators. Positions shall be defined using geographic co-ordinates and in accordance with a reference system (e.g. European Datum: ED-50, World Geodetic System: WGS-84), and also in relation to the UTM-system if appropriate. Positions shall be defined according to relevant guidelines. When sampling around an aquaculture installation, the concession number shall be provided.

When sampling from a boat the sampling strategy should specify if drift during sampling has to be prevented or not.

## 6.4 Operating the sampling device

### 6.4.1 Water samplers and devices for collecting integrated samples

When deploying a water sampler, the closing mechanism shall be checked to ensure it is properly open to allow an unhindered water throughflow. The water sampler shall be lowered vertically to the required depth at an even, moderate speed and then closed.

Hose samplers and other samplers for integrated sampling shall be lowered slowly to the required depth such that the water column from which the sample shall be taken is disturbed as little as possible by the sampling device.

### 6.4.2 Plankton net hauls

The plankton net shall be lowered to the required depth at which the sampling should either be carried out at or start from, and hauled vertically, at an angle or towed horizontally. For vertical hauls and those at an angle,

the depth interval across which the net is hauled shall be noted. For horizontal tows, the sampling depth shall be recorded.

The towing speed for plankton nets shall be kept slow, not exceeding 0,3 m/s for fine meshed nets (≤ 20 µm) and not exceeding 1 m/s for coarser meshed nets (> 20 µm).

When the plankton net reaches the surface, it shall be rinsed with seawater, so that all the material is gathered into the collection cup at the end of the net.

If there is insufficient material collected, multiple hauls shall be carried out. The plankton net shall be rinsed between each haul so that the sample material is gathered in the collecting cup. The mesh size of the plankton net shall be noted on the sample container.

Rinsing can be done by raising and lowering the net several times in the surface water whilst keeping the upper ring of the net above the surface, by lifting the net up and hosing with sea water from the outside or by holding the net at both ends and tipping the net up and down. Water remaining in the net shall be filtered through the mesh before transferring the sample to a sample container, such that the sample is concentrated as much as possible.

Prior to sampling at a new station, the plankton net shall be rinsed well, preferably with fresh water. If fresh water is not available, the equipment shall be rinsed with water from the new sampling location before it is deployed. The collecting cup shall be removed before rinsing or alternatively, if the net has a fixed collecting cup with a tap, this shall be opened to ensure a good throughflow of water.

## 6.5 Sample fixation

Fixing of phytoplankton shall be carried out immediately after the sample is transferred to the sample bottle in order to avoid loss of phytoplankton cells.

For quantitative analysis, 0,2 ml to 1,0 ml of Lugol's solution is added per 100 ml of water sample [13]. During phytoplankton blooming time, during which there is a very high concentration of phytoplankton, the quantity of fixative may be increased up to 2 ml per 100 ml sample. The sample should be golden brown in colour after fixation.

For qualitative analysis, plankton net samples should be fixed using a 20 % formaldehyde solution buffered with hexamethylentetramin ($C_6H_{12}N_4$) in a 3:1 sample to formaldehyde solution proportion. The quantity of fixative can be reduced considerably if there is only sparse material in the sample.

## 6.6 Samples for analysis of living material

If living phytoplankton shall be identified and quantified, the samples shall be kept dark and at a constant temperature equivalent to that at the sampling depth. If the water temperature at the sampling location is higher than 10 °C, the sample shall gradually be cooled down to 10 °C.

NOTE Warming of the sample, for example by exposure to sunlight, can result in destruction of phytoplankton cells.

Samples for analysis of living phytoplankton shall not be stored longer than 36 h. If both living and preserved material is to be examined, a double set of samples shall be collected; one for analysis of living material and one sample for preserved material.

## 6.7 Sample labelling and recording of additional information

Sample bottles shall be labelled. The label should contain the following information:

— name of site or sampling locality;

— UTM coordinates (or coordinates given in another specified geographic reference system if appropriate);

— date and time of sampling;

— sample type (water sample, plankton net haul);

— mesh size (for net hauls);

— sampling depth (single depth or integrated sample, e.g. 0 m to 10 m);

— fixative used;

— initials of personnel who collected the sample.

If the bottles are only numbered, the above information shall be entered into the field log/journal, together with any other additional information such as water transparency, current direction, weather conditions etc.

### 6.8 Sample storage

Phytoplankton samples to be processed within one week after collection shall be stored in the dark at room temperature to prevent photo oxidation. The maximum storage time for iodine (Lugol) fixed samples which have been stored in a dark and cool place (below 20 °C, but protected from frost) is 12 months. During this period, the samples shall be checked regularly and topped up with Lugol's solution if necessary (the sample shall be golden brown in colour). For long-term storage of iodine fixed samples, a buffered formaldehyde solution shall be added in accordance with EN 15204.

Samples of fixed phytoplankton that have been opened shall not be stored for a long time, as the phytoplankton will decompose when air is introduced to the sample. If needed, reference material may be collected and stored in suitable separate sample bottles.

### 6.9 Equipment maintenance

Equipment such as water samplers, hoses and mixing containers shall be rinsed in fresh water and dried before storage for future use. Drying shall be rapid to prevent unwanted phytoplankton growth within the hoses and tubes.

Plankton nets shall be rinsed thoroughly with fresh water to remove all plankton cells that may be attached to the net. At the same time, the nets shall be checked to ensure there are no tears or holes. Plankton nets shall be hung up to dry in an area protected from direct sunlight and sharp or pointed objects. Plankton nets shall be stored in a dark place.

Plankton nets shall be washed regularly in soapy water at least once a year. They shall be soaked for one day and then rinsed in abundant fresh water. After rinsing, they shall be placed in fresh water for one day and then dried and stored.

### 6.10 Requirements for personnel

Fieldwork shall be carried out by personnel with the required scientific background/ training.

## 7 Species identification and sample processing

### 7.1 Species identification

#### 7.1.1 Baseline survey and trend monitoring

As a general rule, all phytoplankton taxa shall be identified as precisely as possible. Dominant forms shall, if possible, be identified at species level. In those cases where it is not possible to identify dominant forms to

species level, these shall be separated out and identified to genus or class level (e.g. *Chrysochromulina* sp.1 or unidentified *Prymnesiophyceae* sp.1) as well as grouped into size fractions. Algae that cannot be identified to species or genus by using a regular microscope shall be grouped into size ranges within each class (e.g. size grouping of athecate dinoflagellates). Flagellates and other algae that cannot be identified shall be separated and grouped into size classes.

#### 7.1.2 Specialised surveys - monitoring of selected taxa

For monitoring of harmful and/or toxic phytoplankton, selected taxa shall be identified according to a pre-defined list. The taxa listed will depend on the situation and aim of the monitoring investigation. For issuing guidelines on shellfish consumption, the emphasis shall be placed on species toxic to humans, as highlighted by the relevant authorities. For monitoring around fish farms, the emphasis shall be placed on potentially ichthyotoxic species.

### 7.2 Methods for quantification

#### 7.2.1 Methods and detection limits

Various light microscopy methods exist for quantification of plankton algae. Phase contrast is needed to quantify many almost transparent cells. For surveys requiring a description of species composition, the sample material should usually be concentrated because many species naturally occur in low concentrations. The sample material may be concentrated by use of different types of filtration methods or by sedimentation in a sedimentation chamber, see EN 15204 for more detailed descriptions of quantification methods.

Taxon/taxa that occur in relatively high concentrations may be quantified directly from the sample, without prior concentration.

NOTE Such methods have higher detection limits because very small sample volumes are analysed and are therefore suitable for quantification of taxon/taxa that occur in high concentrations.

Before taking sub-samples, the samples shall be adapted to room temperature. The samples shall be thoroughly mixed by gently inverting and righting the bottle for 1 min to 3 min. Table 1 gives a selection of methods with their detection limits.

**Table 1 — Selected quantification methods for phytoplankton; sample volume refers to that of the subsample**

| Method | Principle | Sample volume ml | Detection limit cells/l |
|---|---|---|---|
| Sedimentation technique (Utermöhls method) | Concentration | 10 | 100 |
| | | 25 | 40 |
| | | 100 | 10 |
| Filtering | Concentration | 25 | 40 |
| | | 100 | 10 |
| Sedgewick-Rafter | No pre-treatment | 1 | 1 000 |
| Palmer-Maloney counting chamber | No pre-treatment | 0,1 | 10 000 |
| Drops on microscope slide | No pre-treatment | approximately 0,1 | approximately 10 000 |
| Haemacytometer (Fuchs-Rosenthal, depth 0,2 mm, 2 chambers counted) | No pre-treatment | 0,006 4 | 156 250 |

### 7.2.2 Sedimentation technique – Utermöhl method

Sedimentation techniques with subsequent analysis under inverse microscopy shall be used for baseline surveys and trend monitoring. The method is also recommended for pilot surveys.

Unless otherwise specified, subsamples of 10 ml, 25 ml or 50 ml shall be analysed. See Annex F for recommended sedimentation times. As a general rule, at least 500 cells shall be counted in total. For dominant, biomass relevant taxa, at least 50 cells of each taxon should be counted.

Sedimentation techniques shall be used for quantification of small, large and colony-forming phytoplankton. Based on these analyses, species lists shall be developed and the number of individuals (cells) of each species calculated per unit volume.

NOTE Sedimentation techniques provide the best precision and reproducibility using light microscopy [5] and give a near-complete overview of the species present that can be preserved. These techniques provide satisfactory quantification of small, large and colonial-forming phytoplankton, and are therefore recommended as a general method for quantification of phytoplankton [7], [14], [15].

The Utermöhls sedimentation technique for routine analysis of phytoplankton composition and concentration is fully described in EN 15204. Annex F gives a short description of the method, including estimation of counting uncertainty and statistical precision as a normative guideline for the amount of cells that need to be counted.

### 7.2.3 Filtration

Filtration techniques may be used in specialised surveys for selected species, particularly for quantification of larger species and those that retain their form after filtration. For quantification of diatoms, and flagellates, the filtration technique shall be combined with Utermöhl method or counting chambers (Palmer-Maloney or equivalent).

When using filtering techniques, sub-samples of 25 ml generally are analysed, but the filtration volume may be increased/reduced according to the concentration of the phytoplankton species being quantified. Quantification of large phytoplankton species with stable forms caught by the filter can be carried out immediately after filtration. Requirements as to the number of cells that shall be counted and statistical accuracy are as for the sedimentation techniques.

For analysis of picocyanobacteria filtered samples and epifluorescense, microscopy shall be used because picocyanobacteria are not detected with the Utermöhl method.

### 7.2.4 Quantification without concentration of the sample

Direct quantification in the sample without prior concentration shall be used where the phytoplankton occur in high densities, for example for verification investigations during intensive phytoplankton blooms. Small sized species undergoing blooms should be quantified using counting chambers, see Annex E. For larger sized blooming species, other quantification techniques such as sedimentation or filtration shall be used.

Drops placed on a microscope slide may be appropriate because this allows single cells to be manipulated, thereby ensuring correct species identification. To use this method for quantification, the volume on the microscope slide shall be known. The method shall not be used for quantification if the blooming species are large sized or have a low number of cells per unit volume.

Requirements for the number of cells to be counted and statistical accuracy are as for sedimentation and filtration techniques.

## 7.3 Methods for qualitative analysis

Qualitative analyses shall be carried out on plankton net samples to gain an overview of the phytoplankton composition. The samples shall be analysed under a regular light microscope. The sample container shall

stand undisturbed until all the material is sedimented (minimum 1 h). A Pasteur pipette or equivalent shall be used to take a subsample of the sedimented material, and a drop be placed on the microscope slide for examination.

The individual phytoplankton cells may be moved and turned, making identification easier. Thecate dinoflagellates may be coloured by Calcofluor [3] to make visible the cellulose plate and the seams between them.

If both water samples (quantitative) and plankton net samples (qualitative) are taken from a station, the qualitative sample should be analysed first. The species list compiled during the qualitative analyses should be used further during the quantitative analyses.

## 7.4 Determination of biomass

Calculation of phytoplankton biomass shall be carried out during baseline surveys and trend monitoring. The phytoplankton biomass can be expressed in terms of chlorophyll content, the carbon content of the algae (algal carbon) or the volume of the phytoplankton (biovolume). The total cell volume is calculated by measurements of the linear dimensions (length, width, height and/or diameter) of the phytoplankton taxa, as wet weight, or as cellular carbon content according to [10]. These results are then used in specific formulas for the various species/genera/group [4], [9]. The result of the measurement is then multiplied by the cell numbers per unit volume to give the total phytoplankton biomass per unit volume.

Calculation of phytoplankton biomass shall also be carried out in ecological surveys where the energy flux from one trophic level to another is calculated. For investigations where the biomass ratio between different phytoplankton taxa/groups or the total biomass shall be calculated, cellular carbon should be used as a measure of biomass.

## 7.5 Qualification requirements for personnel responsible for identification

Identifiers shall be able to document competence in identification of marine phytoplankton, preferably with reference to training courses in marine botany (phytoplankton flora) at university level or through practical experience of phytoplankton identification. Moreover, documentation is required to show that expertise is maintained and updated through participation in courses and practical identification work. Identifiers should participate in national/international interlaboratory comparisons for quantification and/or species determination of marine phytoplankton, when routines for this are available.

## 7.6 Literature

For species identification, the most recent identification literature appropriate to the investigation area, with synonym lists, shall be used. An updated list of literature used should be made available.

## 7.7 Reporting of result

For analyses of the total phytoplankton assemblages, a table shall be compiled for each analysed water sample, comprising a taxa list recorded to the lowest certain taxonomic level and concentration per unit volume of the various taxa or taxon groups. The following information shall be recorded for each analysed water sample:

— project name or cruise name;

— sampling date;

— sampling location (station name or position);

— sampling depth;

— person who counted the sample;

— method details (sedimentation volume, microscope type etc.);

— list of taxa and size classes with corresponding abundance and biomass.

For monitoring of potential toxin producing phytoplankton, the results shall be reported in accordance with the specifications issued by the regulatory authorities or other institute or client, as appropriate.

### 7.8 Data storage

All data shall be stored electronically in a database, so that it can be retrieved, compiled and presented in an appropriate manner. Extracts from the database shall be in a suitable form for further statistical analyses, such as diversity measures, biomass calculations and multivariate analyses. In addition, the data shall be linked to the measured environmental parameters such as salinity, temperature, stability, nutrients, light conditions etc.

# Annex A
(informative)

# Sampling and sample depth

## A.1 General

Samples for phytoplankton analysis are collected from various depths of the euphotic zone, because phytoplankton is usually unevenly distributed vertically. This unequal distribution is caused by different mechanisms. Phytoplankton blooms usually begin close to the surface and sink down as nutrients are used up. At the bottom of the euphotic zone, there is often an increase in nutrient concentrations. This can lead to a halt in the sinking process and result in an accumulation of phytoplankton biomass. Sometimes algae may accumulate in narrow layers as a result of gradients in density, nutrients etc. Aggregations in narrow layers can also occur among motile phytoplankton (dinoflagellates and flagellates). As these algae are able to swim, changes in their vertical distribution can occur during the course of a few hours. In addition, algae that can regulate their buoyancy (e.g. cyanobacteria) may be found in defined layers.

In many marine areas, there are fronts where different water bodies meet. In order to obtain an overview of the plankton flora in such areas, it is important to take samples from both sides of the front.

## A.2 Sampling depth

### A.2.1 Fixed depths in metres

Sampling at fixed depths is carried out by using a suitable water sampler, and the same sampling depth shall be used throughout the entire sampling period. Sampling depths shall be selected either on the basis of preliminary surveys or existing knowledge of the area. Fixed depths are often used for baseline surveys, trend monitoring and monitoring of biodiversity. It is also important that the sampling depths for chemical parameters and phytoplankton are concurrent.

### A.2.2 Fluorescence maximum

Information on the vertical distribution of photosynthic phytoplankton can be achieved by using *in situ* fluorescence sensor. If high fluorescence is measured at other levels than the fixed depths, it is recommended to take additional samples for phytoplankton analysis from these depths.

However, algal fluorescence is not a constant parameter, but one which is environmentally dependent. For example, both light availability and nutrient concentrations affect fluorescence. In addition, the portion of phytoplankton that is heterotrophic (lacking chlorophyll) is not detected in this way. For monitoring biodiversity it is important to detect as many species as possible.

## A.3 Integrated samples

Integrated water samples are collected by using a hose or tube, or by repeated deployment of a water sampler, such that samples are collected from the entire water column within a specified depth interval. An integrated sample from, for example, 0 m to 6 m can be taken by using a 6 m hose or pipe or by repeated use of a water sampler within, for example, the intervals 0 m to 2 m, 2 m to 4 m and 4 m to 6 m, and then combining the samples from the various depth intervals into a single mixed sample. Integrated water samples are often of such a large volume that they have to be emptied into a mixing vessel for homogenisation before a subsample is taken out.

The advantage of this type of sampling is that a single sample represents a large part of the water column. However, integrated water samples mask the vertical differences.

The relative concentration of phytoplankton that tends to occur in narrow layers, such as *Dinophysis*, will in an integrated sample be lower than the maximum concentration. The greater the depth interval over which the integrated sample is taken, the larger the dilution effect will be for the concentration of the various species, because the phytoplankton biomass generally declines down the water column. Nutrient concentrations, salinity and temperature vary with depth in stratified water. Therefore, integrated depth intervals should be restricted to layers of equal hydrographical and chemical conditions if they are to be related to phytoplankton occurrences.

Integrated samples (for example 0 m to 3 m, 0 m to 5 m, 0 m to 10 m, 0 m to 20 m) are often used for specialised surveys and monitoring. The sampling intervals shall however be adapted to the local conditions and the aims of the survey/ monitoring. For monitoring, for example, toxic or harmful phytoplankton for the shellfish and fish aqua culture industry, integrated samples shall be taken from the depth interval in which the shells are produced. The samples are collected with hose or water samplers to sample the whole of the relevant water layer.

## A.4 Combined samples

A combined sample is a sample where water from several depths is mixed together to form a single sample. The individual samples from each depth shall have the same volume. They may either be filled directly into sample bottles or poured into a mixing vessel from which a subsample is collected.

# Annex B
(informative)

# Water samplers

The Niskin water sampler takes samples from selected single depths and is available in several sizes. It is often used on research vessels to take samples from different depths. Several samplers, which can be individually triggered, are attached to a ring (rosette), which is lowered by a winch. This type of water collector often also has salinity and temperature sensors attached that record the water salinity and temperature at the various sampling depths.

Limnos and Ruttner water samplers also take samples from different depths, and are available in a range of sizes. These types of water samplers are easy to operate from small boats.

The Ramberg water sampler is two metres long and is used to take integrated water samples from a 2 m water layer, whereas KC-Denmark's integrated water samplers can take integrated samples from 1 m, 2 m and 3 m vertical profiles. The device can take integrated water samples from different water layers down through the water column.

Hose water samplers consist of a hose, the diameter of which can be varied as required. Hose samplers are used to take integrated samples. Rigorous cleaning procedures are necessary when using this particular type of sampling equipment. To avoid algal growth within the hose, which will cause contamination of the samples, it is important to clean the hose thoroughly with freshwater after use and to dry it well before its next use.

The integral water sampler (IWS) is equipped with a small motor unit on top which is controlled via the built-in pressure sensor and an electronic control unit. This will guarantee a complete sampling of the whole water column up to the desired depth. The sampling result will be documented via a protocol stored inside the sampler.

# Annex C
(informative)

# Plankton net samples

The principle of plankton net sampling is that the water is sieved through the pores in the mesh as the net is pulled through the water and those organisms larger than the pore diameter are retained within the net. With this kind of sampling an enrichment of phytoplankton is achieved. The net samples are used to get the total species composition, as also phytoplankton occurring in low concentration is collected.

In addition, net samples are advantageous for the determination of difficult taxa like diatoms or armoured (thecate) dinoflagellates. During microscopy (drops on a microscope slide) the phytoplankton cells can be turned and manipulated to observe their three-dimensional shape and specialised structures. For diatoms, further treatment may be necessary to remove the organic content of the cells to highlight the skeletal structures that form the basis for species identification.

Further, plankton net samples are suitable for staining of armoured dinoflagellates to reveal the structure of the plates, which is the basis for species identification.

Plankton net samples are usually collected as a supplement to quantitative samples.

# Annex D
(informative)

# Preservatives

Different fixation and preservation solutions are available, each with different properties and areas of application.

Lugol's solution has proved to be a suitable preservative for phytoplankton samples. The solution preserves the shape of the phytoplankton cells in flagellate species, the flagellae remain attached to the cells and the iodine staining of the phytoplankton cells gives a good contrast between the organisms and the background when using phase-contrast microscopy. For seawater samples where coccolithophorids are present, neutral or alkaline Lugol solution is appropriate. In other areas, acidic Lugol's solution is a good choice. If considered useful, a parallel sample fixed with hexamethyltetramin buffered formaldehyde may be taken as the acid dissolves coccoliths. In samples fixed with basic Lugol's solution, fungal infections may occur under certain circumstances, and for long-term storage, such samples is post fixed by adding a few drops of concentrated formaldehyde solution.

The disadvantage of Lugol's solution is that the brown colouring of iodine causes some discolouration of the organelles in the cells and the cellulose armouring of armoured dinoflagellates, making it difficult to use these features for identification. To reduce this effect, the sample may be bleached using thiosulphate ($S_2O_3^{2-}$) prior to analysis. Alternatively, a plankton net sample fixed in formaldehyde can be used for reference during the identification process.

Diatoms, calcareous flagellates and armoured dinoflagellates fix well in a formaldehyde solution. However, formaldehyde is unsuitable for fixation of naked phytoplankton cells because it increases the osmotic pressure within the cells, which can cause distortion of the cell shape and many flagellates lose their flagella. In addition, some naked phytoplankton forms disappear when the samples are fixed with formaldehyde (37 % formaldehyde), which causes underestimation of the phytoplankton biomass.

If the samples are to be stained with Calcofluor to enhance the pattern of plates on armoured dinoflagellates, it is an advantage to use neutral or basic Lugol's solution or a buffered formaldehyde solution, because acid prevents staining of the cellulose plates. It is also possible to increase pH (use NaOH) of a sample preserved with acidic Lugol's solution in order to get Calcofluor to stain the cellulose plates.

Different fixation and preservation agents are used depending on the objective of monitoring programs and on the investigated phytoplankton taxa which occur.

Each preservation agent has properties that affect both positively and negatively the phytoplankton cells that are to be preserved (Table D.1). It can therefore be necessary to take parallel samples.

**Table D.1 — Advantages and disadvantages of the preservatives**

| Agent | Pro | Contra |
|---|---|---|
| Acidic Lugol's solution | Good durability and fixation, especially for small flagellates and their flagella | Organelles may bleach, the use of thiosulfate might be necessary, the use of calcofluor is not possible |
| Neutral Lugol's solution or alkaline (basic) Lugol's solution | Durable fixation of Coccolithophorideae | Organelles may bleach; Fungal infections are possible, by the dropwise addition a long-term storage is possible |
| Acidic formaldehyd | Good durability and fixation, in particular organelles visible | The use of calcofluar is not possible; causes osmotic pressure changes within the cells, therefore not so suitable for preservation of small flagellates |
| Neutral, weakly basic formaldehyd solution | Chlorophyll fluorescence exists, durable fixation of Coccolithophorideae, usage of calcofluor to determine the plate texture from dinoflagellates possible | Causes osmotic pressure changes within the cells, therefore not so suitable for preservation of small flagellates |

*Acidic Lugol's solution*

| | |
|---|---|
| Potassium iodide (KI) | 100 g |
| Distilled water | 1 000 ml |
| Iodine ($I_2$) | 50 g |
| Acetic acid glacial (100 % $CH_3COOH$) | 100 g |

*Neutral Lugol's solution*

| | |
|---|---|
| Potassium iodide (KI) | 100 g |
| Distilled water | 1 000 ml |
| Iodine ($I_2$) | 50 g |

*Alkaline (basic) Lugol's solution*

| | |
|---|---|
| Potassium iodide (KI) | 100 g |
| Distilled water | 1 000 ml |
| Iodine ($I_2$) | 50 g |
| Sodium acetate ($CH_3COONa$) | 100 g |

*Neutral, weakly basic formaldehyde solution*

Analytical quality formaldehyde is diluted to 20 % (1 part concentrated solution to 1 part distilled water). 100 g hexamethylenetetramine (hexamine) $C_6H_{12}N_4$ is added to 1 l of the 20 % formaldehyde solution. Filter after one week to remove any precipitates.

*Acidic formaldehyde*

Equal amounts of analytical quality formaldehyde (37 %) and concentrated acetic acid are mixed.

**WARNING — Formaldehyde is allergenic and/or carcinogenic. Because of the health risks, use of formaldehyde shall be kept to a minimum. Processing of samples preserved with this compound shall be done either under a fume hood or using point fume extraction.**

# Annex E
(informative)

# Counting chambers for quantification without concentration of the phytoplankton cells

Note The Utermöhl method should be the standard method for quantification of phytoplankton. The methods described in this annex are applied only in very specific issues.

## E.1 Sedgwick – Rafter

The Sedgwick – Rafter chamber is a rectangular chamber (50 mm × 20 mm × 1 mm) with a volume of 1 ml, giving a theoretical detection limit of 1 000 cells per litre. The chamber is best suited to count large sized species. Due to the depth of the chamber (1 mm) it is difficult to use under light microscopy at high magnification (for example 40 ×). Algal cells smaller than 10 µm to 15 µm are therefore difficult to identify.

## E.2 Palmer – Maloney

The Palmer – Maloney chamber is circular (d = 17,9 mm, depth = 0,4 mm) with two channels on each side where the water sample can be added by use of, for example, a Pasteur pipette. The volume of the chamber is 0,1 ml when completely filled. Objectives up to 40 × magnification may be used if these have a long working distance. The chamber may be used for routine examination, but the chamber's detection limit of 10 000 cells per litre makes it unsuitable for counting species that occur in low concentrations.

## E.3 Haemacytometre

The Haemacytometre is a chamber that is available with two different depths, 0,1 mm and 0,2 mm, and various counting areas. For counting phytoplankton, the chamber type with greatest depth is usually used. This chamber consists of 2 rectangular counting fields separated into 16 quadrates, each of which measures. 1 mm × 1 mm. This gives a total volume of 0,006 4 ml and a theoretical detection limit of 156 250 cells per litre when counting is carried out in both counting fields. Plankton species larger than approximately 75 µm are seldom distributed evenly within the chamber and chain-forming phytoplankton often become attached to the edge of the counting field and can be underestimated. The chamber is therefore best suited for quantification of small, non chain-forming species that exist in high concentration (preferably more than 1 million cells per litre).

# Annex F
(informative)

# Utermöhl method

The Utermöhl method concentrates the sample material by sedimentation before identification and quantification. After homogenising of the sample, a sub-sample (usually 10 ml or 25 ml) is extracted into a tube placed over a horizontally orientated chamber with a transparent bottom plate. The phytoplankton cells are sedimented onto the bottom plate and are counted by use of inverse microscopy with phase contrast.

For Lugol fixed samples, the following sedimentation times are recommended [8] as shown in Table F.1.

**Table F.1 — Sedimentation times (chambers diameter = 26 mm)**

| Sedimentation volume<br>ml | Chamber height<br>cm | Sedimentation time<br>h |
|---|---|---|
| 2 | 1 | 3 |
| 10 | 2 | 8 |
| 25 | 4,5 | 12 |
| 50 | 10 | 24 |
| 100 | 20 | 48 |

Before starting counting, it should be checked whether the phytoplankton cells are evenly spread at the bottom of the chamber. If this is not the case, a new chamber has to be prepared.

Samples that have been sedimented for more than 4 d shall not be used for quantification.

For quantification of dominant, biomass relevant taxa, at least 50 cells of each species shall be counted. Quantification uncertainty is calculated using the following Equation (F.1):

$$95\ \%\ \text{confidence interval} = \pm\ 2 \times 100/\sqrt{n}\ \% \qquad \text{(F.1)}$$

where $n$ is the number of counted cells. If 100 cells of a given species are counted, the counting uncertainty will be ± 20 %. Large-sized species often occur in low numbers per unit volume, such that to achieve a quantification uncertainty of ± 50 %, 16 cells should be counted.

As a general rule, at least 500 cells shall be counted from the sample as a whole. Quantification of picoplankton (organisms < 2 µm) requires specialised techniques (epifluorescence) and therefore usually are not included in the analyses.

Usually, the number of cells present of each species is counted, whether the forms are chain-forming, colonial or solitary (single cells).

The area of the base of the counting chamber to be quantified depends on the density of cells in the sample. In cases of high cell density, quantification is only required from parts of a transect across the diameter of the counting chamber, providing that the cells are evenly distributed. If a whole transect is processed, phytoplankton cells that touch the right (or left) side of the line marker for the transect shall be quantified, whereas cells touching the other side of the line shall be excluded. If only individual quadrates are quantified, only cells touching the upper and right (or lower and left) line marks shall be included. Cells touching the other two sides shall be excluded. The same strategy shall be used for all countings. For a complete description of Utermöhls sedimentation technique and relevant statistical techniques for routine analysis of phytoplankton concentration and composition by use of inverse microscopy, see EN 15204.

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