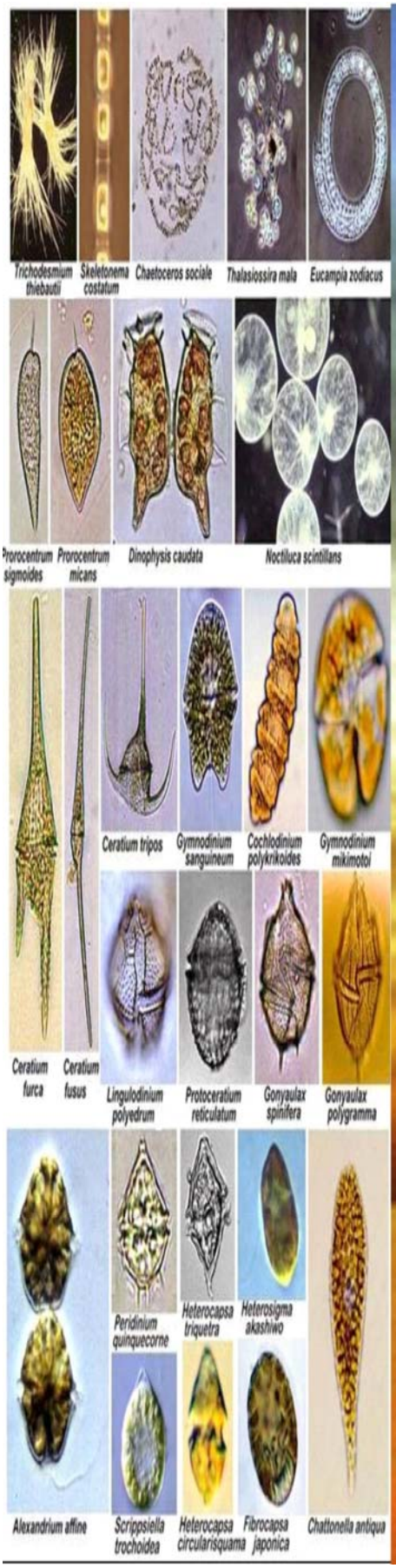

PHYTOPLANKTON MANUAL

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Manual for Phytoplankton Sampling and Analysis in the Black Sea

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Table of Contents

This document is intended for use as a guide to harmonize the procedures among the different labs in the Black Sea countries for phytoplankton monitoring. The methods for phytoplankton microscope analysis refer to inverted and light microscope. Methods for analyzing autotrophic picoplankton are not included in this manual.

The first edition has been finalized during the Plankton Workshop, held in Odessa between 15-19 August, 2005 under the GEF/UNDP Black Sea Ecosystem Recovery Project (BSERP)-RER/01/G33/A/1G/31, attended by representatives of all Black Sea countries.

The developers of the checklist software are Oleksandra Sergeyeva, Kseniia Skuratova IBSS, Sebastopol, Ukraine

This updated edition has been discussed and contributed by the participants of the Phytoplankton Workshop, held in Istanbul between 21-23 June 2010, organized by the Black Sea Commission for the protection of the Black Sea against pollution, under the UP-GRADE Black Sea Scene Ptoject, FP7 , No 226592, List of participants is presented in *Annex D-1*.

1. INTRODUCTION

1.1 Basic documents used

This manual is based on the contents of other phytoplankton manuals and documents, as well as experience from within the Black Sea Region itself. The following reports and documents have been of particular help in compiling this manual:

- HELCOM Manual (http://www.helcom.fi/Monas/CombineManual2/PartC/CFrame.htm).

- Intergovernmental Oceanographic Commission of ©UNESCO. 2010. Karlson, B., Cusack, C. and Bresnan, E. (editors). Microscopic and molecular methods for quantitative phytoplankton analysis. Paris, UNESCO. (IOC Manuals and Guides, no. 55.) (IOC/2010/MG/55), 110 p.

- Standard Operating Procedures for Algae and Aquatic Plant Sampling and Analysis North Carolina Department of Environment and Natural Resources Division of Water Quality January 2003 (JAN-03) Version Protocol for Monitoring Phytoplankton.

- A report by the marine biodiversity monitoring committee (Atlantic maritime ecological science cooperative, Huntsman marine science center) to the ecological monitoring and assessment network of environment Canada by Jennifer L. Martin. The EMAN Ecosystem Monitoring Protocols and the Community-Based Monitoring Protocols for Marine and estuarine ecosystems, Canada.

- Phytoplankton guild sampling, preservation and laboratory analysis (prepared by Marina Cabrini, Laboratorio di Biologia Marina, via A. Piccard 54, 34010 Trieste, for a team of European phytoplankton experts working on the project TWReference-net)..

- Guide to UK Coastal Planktonic Ciliates © 2001 DJS Montagnes, University of Liverpool http://www.liv.ac.uk/ciliate/.

- REPORT OF THE ICES/HELCOM Steering Group on Quality Assurance of Biological Measurements (SGQAB) in the Baltic Sea ICES Headquarters, Copenhagen 25–28 February 2003 Conseil International pour l'Exploration de la Mer, Palægade 2–4 DK–1261 Copenhagen K Denmark..

- ICES SGQAB REPORT 2005ACME:06 Ref. C, E, HICES/HELCOM Steering Group on Quality Assurance of Biological Measurements in the Baltic SeaICES Headquarters22 – 25 February 2005.

- ISO/IEC 17025. *General requirements for the competence of testing and calibration laboratories* and prEN 15204 (under Approval: 2005-11): Water quality - Guidance standard for the routine analysis of phytoplankton abundance and composition using inverted microscopy (Utermöhl technique).

1.2 Phytoplankton – definition and rationale

The largest plant community in the oceans are the free-drifting, microscopic phytoplankton. These single celled organisms use solar energy by photosynthesis to survive. Though normally existing in solitary form, they may form large chains or spherical shaped colonies, some large enough to see with the naked eye. Despite their small individual size, Phytoplankton are of enormous importance in the aquatic food chain creating a food source for a variety of organisms, fish and shellfish which in turn provide a food source for larger animals. In addition, the phytoplankton provide most of the oxygen we breathe, and fossilised phytoplankton are associated with deposits of oil and benthic deposits (sapropell in the Black Sea).

Microalgal cells can range in size from less than 1 mm to greater than 1 000 µm, and in some cases can multiply to more than one million cells per liter during a high peak bloom. The phytoplankton evolved into a diverse group of photosynthetic organisms that are divided into categories according to size:

- Microplankton - 20-200 µm.
- Nanoplankton - 10-20 µm.
- Ultraplankton - 2-10 µm.
- Picoplankton less than 2 µm.

Picoplankton and ultraplankton tend to be more common in the open ocean than the coastal waters, whereas the larger nanoplankton and microplankton, such as diatoms and dinoflagellates, tend to dominate coastal waters.

Phytoplankton is responsive to the physical and chemical conditions in the aquatic environment. Sometimes their rapid reproduction causes nuisance growths or blooms. Algal blooms have dramatic effects on water chemistry, most notably pH and dissolved oxygen (DO). When algae remove carbon dioxide during photosynthesis they raise the pH by increasing the level of hydroxide. The opposite reaction occurs during respiration when carbon dioxide is produced lowering hydroxide and lowering the pH.

Microscopic algae are a concern for the water quality and ecosystem health. They can cause taste and odor problems, water discoloration, or form large mats that can interfere with boating, swimming, and fishing.

Reasons for the importance of phytoplankton monitoring when assessing the environmental status and biodiversity of marine ecosystems include:

- Much of our information on photosynthesis, nutritional requirements, roles of vitamins, and studies of protoplasm comes from studies of algal physiology.

- Algae play an important role in the production of oxygen and other organic materials.
- They serve in the food chain as a food source for species such as protozoa, rotifers, copepods, cladocerans, euphausiids, clams, mussels, various fish (such as herring) and even some whales.
- Algae serve as the first step in the system of energy transfer through aquatic food webs. All aquatic animals in both fresh and salt water require these small plants to maintain their existence.
- There is a growing speculation that the occurrences and blooms of phytoplankton in coastal waters are increasing world wide, especially those that have negative effects.
- Algae are more and more becoming a great biotic indicator of environmental changes, and/or human induced alterations.

Marine environmental policy has increased in importance and a number of directives has been developed to monitor water quality. The Water Framework Directive (WFD) uses phytoplankton as one of the ecosystem components required to monitor the quality status of marine and freshwater bodies. Phytoplankton is also a required biological component of the EU Marine Strategy Framework Directive, devised to protect and conserve the marine environment. The seInternational Maritime Organization (IMO) adopted the Ballast Water Convention in 2004 although it has not yet been ratified. This convention includes a ballast water discharge standard whereby ships will be required to treat or manage ballast water to ensure that no more than 10 organisms per mL in the size category >10 μm - < 50 μm and no more than 10 organisms per m3 >50 μm are discharged.

1.3 The main objectives of phytoplankton community analysis

Analysis of phytoplankton species composition abundance and biomass is carried out for the following purposes:

- To describe temporal trends in phytoplankton species composition, their abundance, biomass and abundance of blooms.
- To describe spatial distribution of phytoplankton species, their abundance, biomass and blooms.
- To identify key phytoplankton species (e.g. dominating, harmful and indicator species).

1.4 Phytoplankton communities in the Black Sea

Phytoplankton research in the Black Sea date back to the end of XIX beginning of XX century. The extensive investigations on phytoplankton biodiversity, community structure and time-spatial variability (see national biodiversity reports- Konsulov ,1998, Öztürk , 1998, Petranu,1998, Zaitsev & Alexandrov, 1998, Komakhidze & Mazmanidi, 1998) and the references there in) conducted at basin-wide scale reveal that the specific features of the Black Sea, its geographical location in the temperate zone, hydrology and nutrient regime determine to a great extent the natural pattern of phytoplankton dynamics. Originally a highly productive sea with an intensive winter-spring maximum and less intensive autumn peak of phytoplankton proliferation attributed to the natural variability of the ecosystem (Bologa et al. 1984, Mikaelyan, 1997). In summer due to nutrients depletion related to the water stratification the phytoplankton

biomass is maintained low. The succession of phytoplankton communities follows normally the sequence small diatoms- large diatoms-dinoflagellates-diatoms, with less representatives of silicoflagelates, coccolithophores and xanthophytes (Bodeanu, 1989).
During the early sixty the usual diatoms/dinoflagellates biomass ratio in spring was10:1 (Bodeanu et al., 1997) in contrast to 1970-1990 when frequent late-spring and summer phytoplankton blooms have been recorded, dominated preferentially by fast growing small microalgae, that altered the main taxonomic groups ratio to 1:7. An increase of the share of opportunistic species was well documented and a number of "exotic" species proliferating in the Black Sea basin (Gomoiu et al., 2001, Mihnea, 1997, Nesterova, 2001, Zaitsev & Öztürk, 2001 and the references there in, Moncheva, Kamburska, 2002.)

There is a general agreement that the Black Sea ecosystem evolution in relation to anthropogenic pressure could be relatively subdivided into reference (pristine) period (1954-1973) and a period of progressive anthropogenic pressure (1974-1993) featured by alteration of phytoplankton communities structure and function (Vinogradov et al., 1992, Finenko et al., 2001, Churilova et al, 2002, Stelmach et al., 2002, Yunev et al., 2002) and related ecosystem deterioration effects (anoxia, decreased water transparency, mass mortalities etc.- Mee, Mihnea, 2002, Zaitzev, Mamaev, 1997).
The reported changes during the late 90ies and the recent period (such as reduced amplitude of seasonal oscillations, a decrease of phytoplankton monospecific blooms and their critical levels attained, especially in summer, the increased dominance of diatoms, the shift of the seasonal succession to the Black Sea ecosystem natural cycle) manifest some signatures of recovery related to the reduction of the land-based nutrient load into the basin. The instability of this trend however questioned the role of anthropogenic eutrophication as a single driving force, in favour of "top-down" and climatic control (Yunev et al, 2002, Oguz et al., 2002, Moncheva et al., 2001). A comprehensive overview of the recent trends in phytoplankton biodiversity and growth patterns at basin-wide scale is included in. State of the Environment of the Black Sea (2001-2006/7), the BSC, 2008.

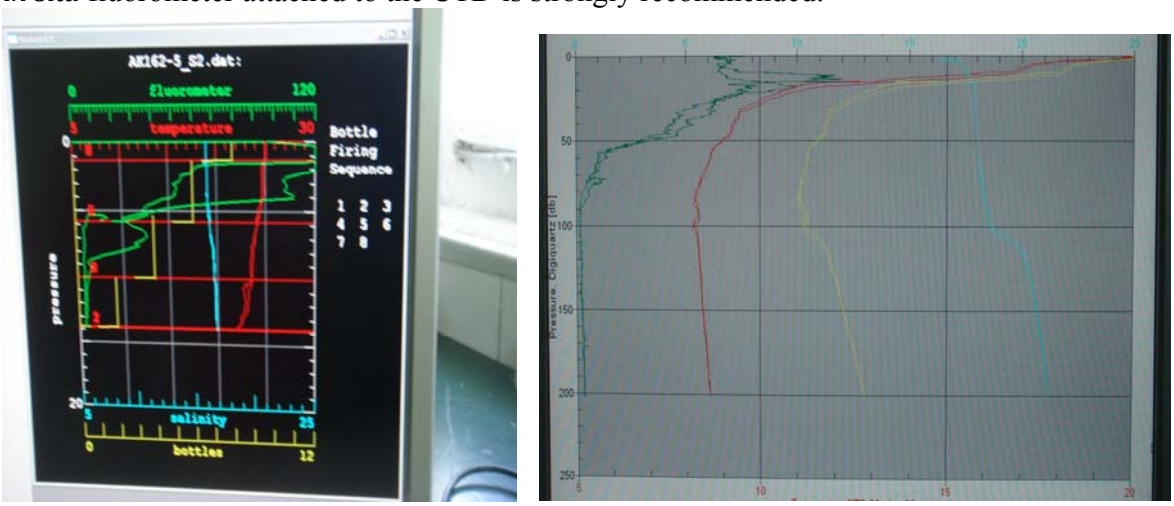
2. SAMPLING

2.1 Site selection

The sampling design originally depends on the purpose of the monitoring program and the available resources.
Initial sampling sites are dependent on water exchange, depth, water column stability, proximity to land-based sources of pollution. There should also be a sampling location near fresh water exchange, or river run-off ensuring all types of predefined water bodies (*sensu* WFD) e.g. coastal, transitional and offshore sites are included. In order to reflect the Black Sea specific physical oceanography an on-offshore transect of stations within the 12 miles zone is recommended. Sites such as bridges, wharfs, and intertidal areas are not suitable for sampling. The availability of long-term data (monitoring stations) is an advantage.

2.2 Depth

The sampling depths are determined depending on the vertical profile of the water column and in addition to the standard sampling levels (surface-1m under the surface, 3-5m, 10m, 25m, 50m, 75m and bottom sample – 1m above the bottom) sampling at the vertical gradients is extremely important (thermocline, pycnocline, nutricline and the chl.a subsurface maximum). Therefore an *in situ* fluorometer attached to the CTD is strongly recommended.



Vertical profile of water column as inferred from a CTD cast equipped with a n in situ fluorimeter

2.3 Frequency and seasonality

As phytoplankton shows a substantial seasonal variation, sampling needs to cover the entire growth season, which in the Black Sea extends over the entire year. For monitoring purposes it is recommended that samples should be taken at least twice per month but weekly sampling is desirable during bloom periods since species concentration and composition can vary significantly in 8-10 days. Sampling at this frequency also aids in determining seasonal patterns

and trends. For practical reasons Black Sea monitoring program for phytoplankton should include sampling on monthly bases at least at one "impacted" and one "reference" monitoring station.

2.4 Algal Blooms

Most algal problems occur when environmental conditions are favorable to a particular taxon, or group of taxa, and permit the formation of a "bloom". In the Black Sea algal blooms are mostly excessive growths (cell density or biomass) in the surface 5-10m, (Bodeanu et al, 1998) or in particular sections of the water column, sometimes forming dense filamentous mats (Mickaelyan et al.1997).
Phytoplankton bloom data are used (*sensu* WFD) in concert with chlorophyll–*a*, nutrients, salinity and dissolved O_2, Secchi depth measurements for evaluations of water and ecological quality.

2.4.1 Phytoplankton bloom detection in the field

Phytoplankton blooms are visually detected by discolored water or surface films. Fortunately in the Black Sea, toxic effects have not been detected, irrespective of the presence of species, cited as toxic in other areas of the world ocean (Moncheva et al., 1996) with the exception of Prorocentrum lima (Leighfield et al., 2002,Vershinin et al., 2006, Mortona et al., 2009). The relationship between algal blooms, DO and pH can be used to detect non-visual phytoplankton blooms. Phytoplankton samples should be collected for evaluation when: DO > 110% saturation or DO > 9.0 mg/ l and pH > 8.0 and of course during cases of, foam appearance, fish kills etc.

2.4.2 Phytoplankton bloom measures

Three measures are considered in bloom determination: a) chlorophyll-a; b) total unit density; and c) biovolume. Additional important features are bloom frequency and duration, that count for the most of the cases of hypoxia and associated zoobenthos and fish mortalities in the Black Sea. The levels that phytoplankton growth/density must exceed to be considered a bloom may be set to standardize the communication of ecological quality evaluations.

Chlorophyll-a
Chlorophyll-*a* measurements are estimates of algal biomass and are often used as the primary measure in phytoplankton bloom determination. Typically, annual maximum chlorophyll-a values are about three time annual mean chlorophyll-a values, but such ratios are clearly dependent on sampling seasonality and frequency, so should be used with caution.

Unit density and Biovolume
The number of algal units, expressed as units/l, is an estimate of phytoplankton abundance and the biovolume, expressed as mm^3/m^3, is an estimate of phytoplankton biomass [mg/m^3] within the water column. Although there are a lot of uncertainties of what constitutes a bloom (Smayda,

1977) in the Black sea concentrations exceeding >1x10^6 [cells/l] for a single species or > 5x10^6 cells/l for 2-3 species or biomass >10000 mg/m^3 are normaly considered "bloom" – Fig.1.

Fig.1.Discoloration of water during a phytoplankton bloom, chlorophyll a> 30 µg/l, phytoplankton biomass > 13 000 mg/m3 (photo: S. Moncheva)

2.4.3 Evaluation forms

Should include:

- Waterbody
- Collector's name
- Location (latitude and longitude)
- Visual description (water colour, foam etc.)
- Date collected
- DO (mg/l or % saturation)
- Salinity (ppt) and/or conductivity [if measured] (µMhos/cm^3)
- pH [if measured [on site]
- Secchi depth [if measured] (m)
- Water temperature (°C)
- Nutrient concentrations [if sampled]
- Chlorophyll-a [if sampled] (µg/l)

Chlorophyll-*a* samples should be taken at phytoplankton blooms, fish mortality events and taste and odour evaluations.

2.5 Equipment

The equipment for field sampling include:
CTD rosette
5-10 L Niskin, Go Flow plastic bottles
Plankton net (10 µ or 20 µ mesh size)
In situ fluorimeter
Secchi disk

PVC bucket
Sampling bottles (1-3 l)
10 ml pippetes
Reagent for fixation

A sampling bottle should meet the following requirements, some of which depend on the duration of the storage of the sample:

- The bottle must be clean and easily be cleaned.
- The bottle must not be permeable to the preservative used.
- The combination of bottle and screw cap should ensure a closure that is watertight (to facilitate homogenization) and almost gastight (to minimize evaporation) to allow long periods of storage.
- The bottle should be transparent unless the sample cannot be stored in the dark. In that case is should not be pervious to daylight (for instance, brown glass medicine bottles). If kept in transparent bottles the sample can easily be examined with respect to the state of preservation and the presence of aggregates.
- The neck of the bottle must be wide enough for filling the counting chamber.
- Bottles should not be filled completely with sampling water to facilitate homogenization (preferably fill to around 80 %).

2.6 Methodology

There are various methods for collecting samples for phytoplankton analysis, depending on whether a quantitative or qualitative analysis is desired. Irrespective of methods, samples should be preserved soon after collection and where possible, live samples should also be examined.

2.6.1 Qualitative sampling

Plankton net sampling - involves lowering standard plankton net to just off bottom and towing it gently in a vertical direction to obtain an integrated sample throughout the water column. Plankton nets have the advantage of simultaneous collection of larger quantities of plankton, giving larger concentrations of the rarer cells and aiding in their identification. The best size for mesh spacing is 20 μm (sometimes 10 μm). The ratio of net length to net mouth diameter should be between 3:1 and 5:1. In order to account for horizontal patchiness of algal species the plankton net may be towed in a horizontal direction at depth 0.5-1m bellow the surface.

This method does not allow for accurate counting of algal species. In addition, some phytoplankton species pass through the net, or burst or disintegrate when stressed or coming in contact with the net.

2.6.2 Quantitative sampling

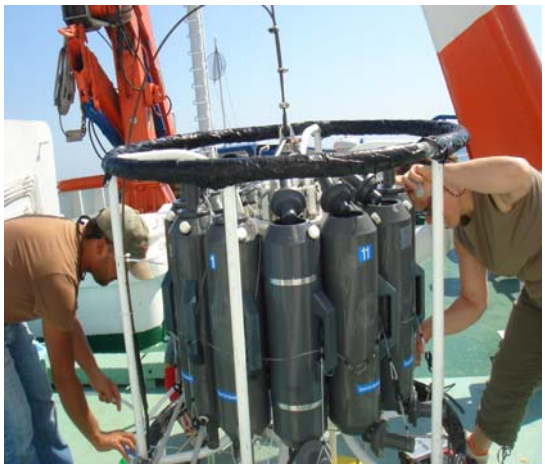
For quantitative phytoplankton sampling the most conventional procedure is sampling at discrete depths *(see section 2.2)* by sampling water bottles (5-10 L Niskin, Go Flow plastic bottles), normally attached to CTD-rosette system – Fig.2. In addition, a plankton net sample is desirable

to help species identification in the case there are problematic species in the quantitative sample as well as for detection of rare species and biodiversity analysis.

For ship-of-opportunity, a single sample from the mixed surface layer can be taken by a pump.

It is recommended to take a net sample from the 0-20 m water column in order to obtain a concentrated plankton sample (Martin and Wildish, 1992). This sample serves as a support for species identification

Very helpful to identify difficult species is to observe living material. Plankton net with a 10 µm mesh-size is recommended. In case of higher concentration it is advisable to use a net with 25 mesh-size.



CTD with attached sampling bottles

phytoplankton net (20 µm mesh size)

Tube and pipe for integrated water sampling

3. SAMPLE PRESERVATION AND STORAGE

Net and bottles samples should be preserved immediately. Sub-samples to be studied alive can be kept fresh for a few hours in an open container in a refrigerator. They can be kept unpreserved for up to 24 h in 0.5-1.0 dm^3 polypropylene or polyethylene bottles at in <u>situ</u> temperature if it is below +10°C, or 1 month in a deep freezer.

3.1 Fixatives/preservatives

Preservatives are toxic by definition. Delicate organisms without strong cell walls may collapse before fixation is complete. To minimize this effect it is important that the fixative agent enters the cell quickly, which may be achieved by the addition of dimethyl sulphoxide (Trondsen, 1978). Some preservatives, *e.g.,* acid solutions of Lugol, may eventually lead to the loss of some groups of organisms.

Preservatives have to meet the following requirements:

- The effect of the agent on the loss of organisms by chemical shock or otherwise must be known beforehand.
- The preservative must effectively prevent the microbial degradation of organic matter at least during the storage period of the samples.
- The preservative must guarantee a good recognition of taxa at least during the storage period of the samples.

The most frequently used preservatives in phytoplankton research are Lugol's solution (and adaptations of it) and formaldehyde-based solutions. For specific organism groups or advanced research methods use may be made of several other preservatives, for example, glutaraldehyde (Taylor, 1993) osmium tetroxide or, for delicate flagellates, a mixture of glutaraldehyde, acroleïc acid and tannic acid (Van der Veer 1982). Nevertheless, the more common fixatives currently employed are:

- **Formaldehyde neutralized with hexamethylentetramine.** The solution is prepared by diluting 1 litre of pro analysis grade formalin (=40 per cent formaldehyde) with 1 litre of distilled water. 100 g hexamethylenetetramine is added to 1 litre of the 20% formaldehyde solution. The fixing agent have to be fresh-prepared some days before the samples collection, kept at 5-6 °C and put in the dark glass bottles. Water samples are fixed with 40 ml/l of 20 % neutralized formaldehyde. Water samples should be kept in the dark for long term storage, formaldehyde should be added to give a concentration of 4 %.
  Formaldehyde action as a fixative is based on the principle of protein-cross-linking (by the formation of hydrogen bridges between protein molecules). As a result organic tissues and cellular structures are preserved in a form that can be stored for long periods of time. Formaldehyde, however, slowly transforms into formic acid and methanol (Cannizzaro reaction), which negatively affects fixation and preservation. For this reason formaldehyde should not be kept in stock too long and preferably should be applied with

a buffer added, for instance hexamethylene tetramine or disodium tetraborate (Borax) – $Na_2B_4O_3x10H_2O$ (check to receive a final pH=8). The transformation into formic acid and methanol can be counteracted if the solution is prepared from paraformaldehyde, a polymer of formaldehyde. If the concentration of formaldehyde exceeds 20 %, there is a risk of precipitation (Smayda, 2002).

- **Formaldehyde acidified with acetic acid**. Formalin-acetic acid (FAA) is inexpensive and seems to be the best preservative for preservation over long periods of time. It is prepared by combining equal volumes of formaldehyde (37%) and glacial acetic acid. Five ml FAA are added to a sample of 250ml. Most species except the "naked" flagellates preserve well in FAA.

- **Lugol's solution** of iodine and acetic acid (Sournia, 1978). Lugol's iodine fixative (0.25-0.5 ml acid Lugol's solution per 100 cm^3) is also widely used, but has the same limitations as FAA. It may be purchased commercially or made by mixing 200 g potassium iodide, 100 g crystalline iodide, 2000 ml distilled water, and 190 ml glacial acetic acid . Add 10 drops Lugol's iodine to each 200 ml sample. Samples preserved in Lugol's fixative are sensitive to light and do not have a long shelf life. If coccolithophorids need to be preserved with the coccoliths intact, a parallel sub-sample should be fixed with 0.5-1.0 cm^3 alkaline Lugol's solution. Clear, colorless 200 cm^3 iodine-proof bottles with tightly fitting screw caps should be used for iodine-preserved material. With such bottles it is easy to see when the iodine becomes depleted and more preservative needs to be added. Glass containers should be used for sample storage because the silica from the glass helps preserve the diatom cell wall. The samples should be counted as soon as possible. They should be stored dark and cool. Samples stored for more than one year are of little use.

- **Acid Lugol's solution** (Willen ,1962). Consists of: 200 cm^3 distilled or deionized water, 20 g potassium iodide (KI), 10 g resublimated iodine (I_2) and 20 cm^3 glacial acetic acid (cone. CI^COOH). Mix in the order listed. Make sure the previous ingredient has dissolved completely before adding the next. Store in a tightly stoppered glass bottle in cool. Add 0.5-1.0 cm^3 per 200 cm^3 sample.

- **Alkaline Lugol's solution** (modified after Utermohl ,1958). Replace the acetic acid of the acid solution by 50 g sodium acetate (CH_3COONa). Use a small part of the water to dissolve the acetate. Lugol's solution can be stored in a dark bottle at room temperature for at least 1 year**.**

- **Buffered glutarahaldehyde.** For further identification with an electron microscope, material can be preserved in buffered glutarahaldehyde fixative, (2% GTA with sodium cacodylate of barate/borax, a combined buffered glutaraldehyde/osmium tetraoxide cold fixative (GTA/OsO_4) has been strongly recommended.

Neutralized formaldehyde gives incomparable results to Lugol's solution and should not be used, except at a few coastal stations where long time series are already established using formaldehyde.

Many unarmoured dinoflagellates and naked flagellates are damaged or severely deformed by formalin and Lugol's solutions, so an inspection of live material is desirable. The living cells must also not be subjected to the heat from a microscope lamp for more that a few minutes due to their extreme sensitivity.

3.2 Advantages and disadvantages of formaldehyde

3.2.1 Advantages

- Good fixing and preserving agent for algae with a more rigid cell wall.
- Cell wall structures and other characteristics like eye spots remain visible.
- When stored properly in appropriate bottles samples will stay in good condition for many years without attention.
- Auto-fluorescence of chlorophyll *a*, though decaying, remains intact for at least several days if the samples are stored in the dark continuously.

3.2.2 Disadvantages

- Formaldehyde is irritating already at very low concentrations in the air and may lead to allergic reactions.
- Some algal species can be distorted or cannot be recovered in the sample at all.
- Organisms may shrink resulting in lower cell volumes and calculated biomasses.

3.3 Advantages and disadvantages of Lugol's

3.3.1 Advantages

- Better for accurately quantifying than many aldehyde-based fixatives (Throndsen 1978).
- Lugol's solution is relatively harmless compared to aldehyde-based or other more toxic fixatives. Iodine enhances the sinking of cells in settling chambers.
- Lugol's stains cells a dark brown colour, makingcounting easier.
- However, Lugol's fixed material can be processed in several ways: SEM (Montagnes & Taylor 1994), DAPI, protargol staining (Montagnes and Lynn, 1993)

3.3.2 Disadvantages

- Lugol's masks chlorophyll fluorescence, which may be needed to recognize mixotrophic species (Gifford & Caron, 2000).
- Lugol's dissolves hard structures such as coccoliths and diatom frustules and, therefore, is not ideal for long-term storage of many plankton taxa
- Lugol's stains cells a dark brown colour, which obscures some of the characteristic features of ciliates (e.g. macronucleus). However, darkly stained specimens can be cleared with sodium thiosulphate.
- Lugol's does not necessarily preserve the cell shape and size of live specimens. Comparison of live and Lugol's-fixed material is not always possible.

3.4 Sample Storage

3.4.1 Living samples

Living samples of surface waters for preliminary analysis should be kept in the dark at a temperature of between 4 and 10 °C. Samples taken from ambient water at a higher temperature may need to be gradually cooled to avoid damage to phytoplankton cells.

NOTE. In samples with a very high density of organisms, blooms or surface scums, depletion of oxygen should be prevented by diluting the sample, by leaving a large amount of space for air in the bottle or by exposing it to a small amount of light. The last option requires practical experience in determining the right exposure without inducing growth.

3.4.2 Preserved samples

Samples preserved with Lugol's solution (or aldehydes) should always be stored in the dark and cooled to t 5 ^{o}C (not higher than 10°C), unless they are analysed within a week. In that case they can be stored in the dark at room temperature. The only reason for cooled storage is to slow down the rate of physical and chemical processes that lead to a reduction in the quality of the sample. Storage in the dark is always necessary to prevent photo-oxidation and, in formaldehyde samples, preserve auto-fluorescence of pigments. If oxidation is prevented the maximum storage time of Lugol preserved samples in the dark and below 5° C is 6 months. Preservation and storage for periods of years is only possible after addition of formaldehyde.

QUALITY CONTROL Samples that are preserved in the field after sampling should be checked after a couple of days for oxidation of the Lugol. The sample must have a 'cognac'-like colour. If not, Lugol's solution should be added until the sample has regained this colour.

4. PHYTOPLANKTON ENUMERATION AND DENSITIES

4.1 Equipment

microscope (iverted or light compound)*
25-50 ml PVC bottles with screw top caps for archive of phytoplankton concentrated samples
system for sub-sample preparation-decantation of surface water
settling chambers (Utermoll)
measuring cylinders (Class A)
counting chambers (Palmer-Maloney, Sedgwick-Rafter)
glass microscope cover slips rectangular, 22 x 50mm, #1 thickness
micrometer or video-interactive image analysis system

Essential parts of sample analysis include identification and counting. The precision of these analysis depend equally on the quality of the microscopes, the counting chambers and the associated procedures and the professional qualification/skills of the counter

*In the development of phytoplankton manuals, there has been (and is) a trend to reject phytoplankton enumeration undertaken under conventional microscopes and to focus exclusively on enumeration analyses under inverted microscopes only. It is likely that this accounts for one of the key differences in phytoplankton enumeration results obtained by different laboratories and it is strongly recommended that inverted microscopes (and ancillary equipment) are purchased for future use by all Black Sea laboratories. However, inverted microscopes are more expensive than conventional compound light microscopes, and the aim of this manual is to improve quality assurance of phytoplankton analysis in all laboratories in the region. Thus, the cell enumeration section is divided into two major sub-sections (*sections 4.3 and 4.4*), dealing with sample preparation and analysis using inverted and conventional microscopes.

Optical properties. The optical properties of the microscope determine the discriminating potential and hence the identification possibilities. The microscope should be equipped with a condensor with a NA of at least 0.5 and plan objectives with a NA of 0.9 or more. In particular cases phase-contrast or Nomarski interference-contrast illumination can be advantageous for the examination of details. If an inverted microscope is not available to use, a conventional compound microscope with several magnifications (for example - 10X, 40X and 100X oil immersion objectives) can be used instead..

Illumination A powerful light source is needed, particularly for phase contrast and photomicrography and may be powered by a regulating transformer. A field diaphragm is necessary to set up Koehler illumination. In most cases bright-field illumination is the best choice for counting phytoplankton. The use of phase-contrast or Nomarski interference-contrast illumination is advantageous for examination of details in some organisms or species, which may be hardly visible under bright-field illumination, for instance details of weakly silicified diatoms and small flagellates. In special cases, for instance the identification of thecate dinoflagellates or situations where it is necessary to discriminate between cyanobacteria and other bacteria, fluorescence microscopy may be useful.

Objective and ocular lenses. A 10X and/or 20X (phase) objective(s) should be available for counting of the larger organisms, which are easy to distinguish and to identify. A 20X objective of at least NA (numerical aperture) 0.5 (for instance a plan-apo objective which has a resolution of at least 1 micrometer) may be very useful for critical identifications. A 40X phase and a 60X or 100X plan-apo oil immersion objective (NA > 0.9 with a resolution of about 0.3 micrometer) should be present for more critical examination of pico- or nanoplankton. A binocular tube should be used equipped for counting by wide-field x10 (or more) eyepieces. One eyepiece should be equipped with a calibrated ocular micrometer. The other eyepiece may be equipped for counting by two crossing threads or counting square.
Sharpness (resolution) The resolution of the image is determined by a set of factors of which the numeric aperture describes the performance of the condenser or the objective.

The NA of the objective lens. With a NA of 0.9 or more, immersion oil must be used. The NA has a large influence on the resolution of the objective and is directly coupled to the quality of the objective. The best lenses which also correct for such phenomenon like peripheral blurring are the so-called plan-lenses: plan-achromatic, plan-apochromatic and planfluorite, of which the plan-apochromatic one produces the clearest view. Normally these lenses have, at the appropriate magnification of 40–60 X, a NA of 0.90 or more.

The NA of the condenser. In general the condenser must be adjusted to the NA of the objective used in such a way that the NA of the condenser diaphragm is 0.7–1 times the NA of the objective. A smaller diaphragm results in a loss of resolution and an increased contrast. For inverted microscopes a NA of the condenser diaphragm of about 0.5 is standard. Condensers with a high NA are not applicable with sedimentation chambers with a height of 4 mm or more.

The thickness of the glass bottom of the counting chamber. The thickness should preferably not exceed 0.2 mm. With some objectives there is the possibility to correct for the (higher) thickness of the chamber's bottom, but the resolution of these combinations is far below the critical minimum performance for counting phytoplankton.

Other aspects of image resolution. Only organisms that have settled completely can be studied under optimal conditions. When free floating or resting on for instance setae, the observation of organisms suffers under poor resolution and the limited ability to focus properly. Although the resolution of the objective is the most important factor determining the image quality, the difference in contrast of the details against the background is also important. Higher scattering of light (e.g., by denaturised proteins of protoplasm) will result in a darker image. Absorption may result in colour, which gives the object contrast.

Sedimentation chamber. The most important characteristic is the thickness of the base plate as it directly affects the image quality. The thickness should preferably not exceed 0.2 mm. The generally used standard type (Utermoll chambers) has a separate column which can be shifted aside when the algae are settled on the bottom.

4.2 Sub-sample preparation

4.2.1 Sample homogenisation

The first critical step in preparing a sample for microscopic analysis is homogenisation of the sample. During sample storage seston settles out and (small) algae become indiscernible by incorporation in detritus aggregates or by adhesion to other large algae firmly, but gently, in irregular jerks to homogenise the contents. cells. Resuspension and separation of particles can be achieved by shaking the sample. Manual shaking should be standardized precisely- a combination of alternating horizontally rolling and vertical turning upside down of the sample bottle for 1 – 3 min. is a better alternative than traditional shaking.

NOTE 1 Shaking vigorously may lead to the disintegration of fragile colonies. However, this is only a problem if colony size has to be determined. When a lot of small bubbles are produced (which will adversely affect sedimentation), the vigorous shaking should be done not later than one hour before starting the sedimentation followed by gentle re-shaking just before taking the subsample.
For some diatoms, it may be necessary to remove some of the cell contents and the organic part of the cell wall, in order to determine the valve structures and involves removing the preservative by centrifuging several times and replacing the liquid with distilled water, acid cleaning, and embedding in a mounting medium.

4.3 Cell counts using an inverted microscope

The pre-concentration of phytoplankton samples is time consuming and contributes to the overall measuring error. If possible this step should be avoided but might be unavoidable for samples with an extremely low phytoplankton abundance.

Water samples (1-3l depending of the sampling depth) that have been collected from sampling bottles can be analyzed quantitatively by preparing a sub-sample by one of the the following methods:

- Sedimentation and surface water removal
- Filtration
- Use of settling chambers

4.3.1 Surface water removal

Although often reserved for phytoplankton enumeration using a conventional microscope, surface water removal may be used to increase the density of phytoplankton in a sample prior to the use of settling chambers.
Sedimentation is most frequently used and can proceed in the original sample bottle if it is made of transparent material. Alternatively measuring cylinders (1 or 2 l) should be used that can be stoppered and have measuring bars with a known precision (Class A). Some organisms may not settle at all but keep floating or adhere to the cylinder wall. Upon sedimentation, the cylinders should be placed in the dark, at a constant ambient temperature and in the absence of vibrations,

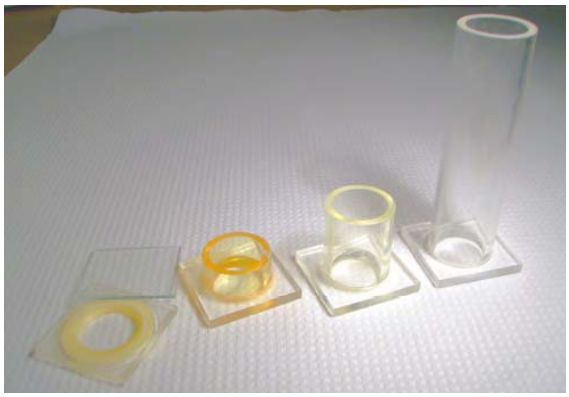
preferably in the immediate vicinity of the place where the decantation is carried out for a period of not less than 20 days. To avoid loosing organisms adhered to the wall one may once a week quickly rotate the cylinders a quarter turn along their axis while leaving them standing on the table.
At the end of the settling period, carefully siphon off the top of the water column without disturbing the settled materials. Decant the remaining sample from the graduated cylinder into a pre-labeled 25-50 ml bottle. Rinse the cylinder two times with about 2 mL of distilled water and empty the rinse water into the bottle. This is the archived sample.
For decantation a low pressure can be applied using a water pump. The mouth tip of the tube can be a Pasteur pipette.
Generally, about 25-50 mL of the sample should remain in the cylinder (the sampling bottle).

4.3.2 Filtration

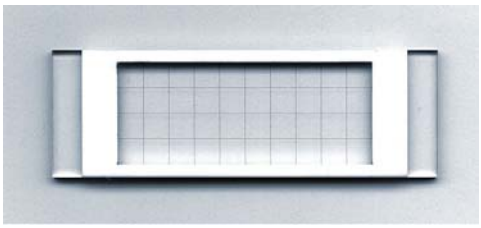
Filtration using plankton gauzes with mesh sizes of 10 µm or 25 µm is not appropriate for a quantitative concentration of phytoplankton in general, but can be used to concentrate organisms with sizes more than 10 µm or 25 µm (length-breadth). It is important to moisten the filter before filtration and to prevent it from drying out during the filtration process. When samples have to be concentrated validation must be carried out to assess the recovery of the different algal taxa in concordance with the objectives of the analysis.

4.3.3 Settling chambers

The settling chamber technique is the more widely used and was originally described by Utermohl (1958) and later Hasle (1978). It requires the use of settling chambers that can be purchased as a unit and also requires the use of an inverted microscope for enumeration. They are available for various volumes, such as 5, 10, 25 and 50 ml, depending on the desired size of subsample – Fig.3.



Utermol sedimentation chambers:
bottom plate with cover glass,
10 mL, 25 mL and 50 mL chambers



Sedgwick-Rafter chmber with Grid

Generally 50 mls are settled. When no phytoplankton bloom is present, 50 ml chambers may be considered suitable for microscope determination. For phytoplankton determination, phase contrast inverted-microscope with 400x magnification should be used. Cell counting should be made on the whole counting chamber for the less abundant species and on diameter transects or random fields for the dominant species. Cell abundance is determined taking into account the ratio of the observed surface with the total surface of the counting chamber. The minimum sample size (number of cells to count) is 400 cells.
It is a general phenomenon that after settling, the distribution of particles on the bottom of a chamber is very variable. After filling the chamber and closing it with a glass cover, the fluid flow, induced by pipette action or final mixing, will rapidly come to rest . Normally, this process will take less than a minute and will not cause any irregular distribution. Constant temperature is essential to avoid convection flows within the chamber.
Chambers with sedimentation depths of > 5 mm give convection flows inducing non-random distribution.
The sedimentation should take place at room temperature and out of direct sunlight. In order to minimise evaporation the sedimentation chamber may be covered with a plastic box and a Petri dish containing water should be placed beside the chamber.

Filling the counting chamber and the settling of particles

After homogenisation of the sample one or more millilitres should be poured into the counting chamber. The exact volume depends on the phytoplankton density and the surface to volume ratio of the counting chamber. Apart from the accuracy of the pipette used, an error will be introduced by stochasticity of this subsampling. This error can be estimated by Poisson statistics, in which the variance is equal to the mean number of objects. For example, a 1 ml subsample with a mean of 10,000 objects ml^{-1} or more in the sample will have a coefficient of variation of less than 1 %. However, for separate species with a very low abundance this subsample error might become significant.

NOTE 1 The filling of the counting chamber is most crucial, as it will affect the final distribution of settled particles. Random distribution allows for simple and uniform counting strategies and statistical procedures to assess measurement uncertainty (*Annex C*).

NOTE 2 For small subsamples, 1–5 ml pipettes with removable tips are sometimes used. To minimise selective sucking (especially of large taxa like *Ceratium*) the end of the tip is often cut off to widen the opening (to ± 2–3 mm).

The following points should be noted for optimal filling of sedimentation chambers:

- Ensure that all equipment (including filling tips etc.) is allowed to equilibrate in the ambient temperature of the room where the analyses are to be performed.
- Place the chamber on a horizontally placed small plateau with a low specific heat (for instance a thin acrylic plate).
- Wait until all materials have reached an equal temperature.

- When filling the chamber, try not to touch it with your fingers as body heat is quickly transferred to the chamber wall.
- Take enough sample and dilute if necessary, to fill the chamber in one instance. Do not add (Lugol) water to the chamber to fill it up when the subsample is small.
- Dilute the subsample if necessary, to ensure that the concentration of particles is sufficiently low to prevent clogging of particles by adhesion.
- When it becomes obvious that many algal cells are obscured by adhesion to detritus, the subsample can be improved by prolonged and/or more intensive shaking of the sample and appropriate dilution.
- Close the chamber with a cover glass. Avoid air bubbles!
- The sedimentation should take place in the dark at a constant ambient temperature that is similar to the temperature of the subsample. Avoid vibrations!.

QUALITY CONTROL When incomplete sedimentation has been observed or when there are specific sampling or historical indications that buoyant algae may be present, the fluid in the upper column of the chamber should be centrifuged at an appropriate speed to estimate whether the amount of buoyant algae is significant. Furthermore it should be realized that in certain samples some small blue-green cells remain in suspension just above the bottom glass. This can be checked by focusing above the bottom prior to counting. If necessary gas vesicles of cyanobacteria can be collapsed, after which a new sample can be prepared. Collapsing of gas vesicles can be easily achieved by putting a sample in a large syringe from which the needle has been removed, leaving an opening of 1 or 2 mm in diameter. By smashing the needle end firmly against a wall, while holding the piston with your hand, the sudden increase of pressure inside the syringe will collapse the gas vesicles.
Settling time is dependent on the height of the chamber and the preservative (e.g. Hasle, 1978 and Rott, 1981). The times given below are recommended as minimum. If vibration caused e.g. by traffic is a problem, the minimum time should not be significantly exceeded, otherwise it is suggested that counting be performed within four days. Sedimented samples not counted within a week should be discarded. Separated bottom chambers not counted immediately should be kept in an atmosphere saturated with humidity.

For seawater samples preserved with formaldehyde a settling time of at least 16 h per cm is recommended (Hasle 1978). For Lugol's-preserved seawater samples the minimum settling times in the table below can be used. A sedimentation time of 16 h per cm is often applied, even though the real settling time will be closer to 4 h/cm. Too long a sedimentation time (more than 24 to 36 hours) can cause bleaching of the sedimented algae making the counting more difficult.

Chamber volume [ml]	**Chamber hight [cm]**	**Settling time [h] Lugol's solution**
2	1	3
10	2	8
25	5	16
50	10	24
100	20	48

One hundred ml chambers should be used with caution since convection currents are reported to interfere with the settling of plankton in chambers taller than five times their diameter. Such chambers can be used only when phytoplankton is very sparse, as in late autumn and winter. For such samples it is recommended to scan the whole chamber bottom.
If the cells are too strongly stained by iodine for comfortable identification, surplus iodine can be chemically reduced to iodide by dissolving a small amount of sodium thiosulphate ($Na_2S_2O_3.5\ H_2O$) in the aliquot to be sedimented.
After sedimentation the chimney of the sedimentation chamber is gently slid off from the bottom plate and replaced by a cover glass. Care should be taken not to introduce airbubbles at this stage. The transfer of the bottom plate to the microscope will not affect the distribution of the settled phytoplankton cells if there are no air bubbles present. The bottom plate is placed on the inverted microscope and the phytoplankton cells are identified and counted.

4.3.4. Counting procedure

The quantitative analysis should start with a scan of the entire chamber bottom at a low magnification. In order to save time and to achieve a reasonable accuracy in counting, the sedimented sample should first be examined for general distribution of cells on the chamber bottom, and the abundance and size distribution of the organisms.. During this scan it is also convenient to make a preliminary species list, which may help to select the counting strategy. If the distribution is visually uneven the settled sample should be discarded. If this occurs consistently, measures should be taken to eliminate the sources of disturbance. How much of the chamber area should be counted and the magnification to be used is dependent on the size of the organisms and their abundance, and on the kind of counting units used (see the details bellow).

The following **natural counting units** are recommended:

CELL: All non-colonial unicellular species:
Detonula
Leptocylindrus

COLONY: *Chaetoceros*
Skeletonema (and other chain-forming diatoms)
Planktonema (and other filamentous green algae)
Microcystis (incl. *Aphanocapsa*)
Phaeocystis etc.

COENOBIUM: with a ± fixed number of cells (n):
Coelastrum (8, 16)
Crucigenia (4)
Pediastrum (4, 8, 16, 32, etc.)
Scenedesmus (2, 4, 8) etc.

SOME COLONIAL ALGAE are most conveniently counted as groups of four cells, e.g.:
Chroococcus
Merismopedia

UNIFORM COLONIES:
Count the number of cells in e.g. 1/4 of the colony. Multiply with a factor of 4. Report number of cells per l.

NON-UNIFORM COLONIES:
Divide into smaller areas. The number of cells in one area is counted. Multiply with the number of "small areas". Report number of cells per l.

FILAMENTS to be counted in lengths of 100 um. Report number of 100 pm pieces per dm^3.

Anabaena
Aphanizomenon
Oscillatoria
(other filamentous blue-green algae)

The following combinations of objectives and oculars are recommended for quantitative microscopy with the inverted microscope:

	Microplankton > 20 µm	Nanoplankton 2- 20 µm	Picoplankton<2µm
Objective	10x or 16x or 25x	40x or 63x	100x
Oculars	12.5-16x10-12.5x	12.5-16x10-12.5x	12.5-16x10-12.5x

Small microplankton species can preferably be counted together with the nanoplankton when they occur in abundance, or they can be counted using an objective with intermediate magnification, 20-25x. A grid of 5x5 squares in one of the oculars is very helpful when counting dense fields of small cells.

At least 50 counting units of each dominating taxon should be counted, and the total count should exceed 400. The approximate 95 % confidence limits of a selected number of counted units are given below. They have been calculated according to the formula

$$95\%CL = n \pm 2 \times \frac{100}{\sqrt{n}}$$

where n is the number of units counted. (Actually the error is not symmetrical, but increasingly asymmetrical with lower counts. Thus, for four units counted the theoretical limits are -73 to +156 %.) (Kozova and Melnik, 1978).

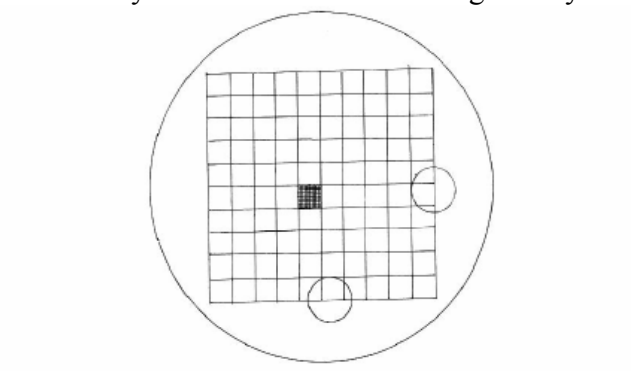
Number of Counted units	95 % CL (%)
4	100
5	89
7	76
10	63
15	52
20	45
25	40
40	32

50	28
75	23
100	20
200	14
400	10
500	8.9
700	7.6
1000	6.3
2000	4.5
5000	2.8
10000	2.0

It should be recognized that these are not maximum errors as the statistics assume perfectly random-distribution of cells on the bottom of the sedimentation chamber which is probably never realized.

With species for which the counting unit is smaller than the individual, e.g. some colonial forms, chain forming diatoms, and filamentous species with average filament length in excess of 100 µm, the distribution of the counting units will be aggregated even in perfectly sedimented samples. The number of counted units should be increased in the ratio average size of individual/size of counting unit.
Reliable quantitative counting of the picoplankton fraction could not be expected as already mentioned it requires (epi)fluorescence microscopy and specific (DAPI staining) technique.

To facilitate counting the use of **Whipple disc** is also recommended. It is inscribed with an accurately ruled grid that is subdivided into 100 squares (Figure below).. It is the responsibility of the analyst to know the area of the grid they are using.



An example of a Whipple disc grid
To identify and enumerate the complete algal assemblage in a sample:

1. Place a prepared Utermöhl chamber on the microscope stage.
2. Record sample data on a phytoplankton bench sheet (see
3. Randomly select a disc to be analyzed.
4. Identify (to species when possible) all taxa and the number of their cells and units within the entire whipple disc area or touching the right and bottom grid lines.

5. Continue to analyze randomly selected disc until 100 units of the most abundant taxon is reached *.
6. Record the number of discs analyzed, the algae found and the number of their cells and units on the bench sheet.
7. Tabulate the total number of units and cells for each taxon identified and record the totals into the space provided on the bench sheet.
8. Tabulate the total number of cells and units for the entire analysis and write it in the space provided on the bench sheet.
9. Enter bench sheet data into the database.

*Exceptions to this rule are:
Sparse samples that cannot be concentrated, analyze at least 0.5% of the Utermöhl chamber (i.e. 40 grids at 300X magnification).
Diverse samples with no true dominant taxa; analyze until a total of 400 units is reached.

Number of algae per field or grid

For optimum identification and counting procedure the final number of algae per microscopic field or grid should be considered. The ideal density is (subjective) dependent on the relative size of the algae (visibility) and the number of non-algae particles (detritus etc) in combination with the skills of the analyst. Evaluation can be carried out by counting a relevant sample at different densities.

Counting procedure

The right counting procedure is dependent on the information needed. As microscopic phytoplankton analyses are time consuming and therefore costly, it is important to optimize this strategy.

Preliminary study

Generally, a preliminary study of living samples should be made as preservation by Lugol's iodine or formaldehyde often obscures diagnostic identification characteristics. Living samples permit the use of auto-fluorescence of chlorophyll *a* for the distinction between small cyanobacteria and eubacteria. They are also valuable to distinguish between Xanthophyceae and Chlorophyceae and to identify delicate flagellates of the Chrysophyceae. Living flagellates may be concentrated by their positive phototaxis. If the sample bottle is wrapped in aluminium foil leaving the bottleneck exposed to the ambient light, the flagellates can be sampled from the bottleneck after approximately one hour. Many species can be more easily identified in the final preserved sample when they have been observed alive previously.

QUALITY CONTROL A preliminary taxon check-list should be available for consultation during the final analysis of the preserved sample. Any observed difference should be further investigated. Any taxa that are uncommon in the waterbody/region etc. should be highlighted and expert confirmation sought.

How many grids or algal objects to count

When validation demonstrates that the Poisson distribution is a suitable model the number of grids and or objects that should be counted to achieve a specific preset precision can be estimated. The easiest way is to set precision (D) by which one wishes to estimate the number of objects in a chamber according to

$$D = \frac{\text{standard error}}{\text{arithmetic mean}} = \frac{1}{\bar{x}}\sqrt{\frac{s^2}{n}} = \frac{1}{\bar{x}}\sqrt{\frac{\bar{x}}{n}} = \frac{1}{\sqrt{n\bar{x}}}$$

in which n is the number of grids counted and the mean number of objects per grid. If a precision of 5 % in the estimation of the mean number of objects per grid is required, it can be calculated that $n\bar{x} = \left(\frac{1}{0.05}\right)^2 = 400$ objects should be counted. Note that it makes no difference whether 10 grids are counted with 40 objects per grid or 80 grids with just 5 objects. If we consider the precision in terms of percentage confidence limits the above formula must be multiplied by t^2 (Student's t) which results in: $\Sigma x = \frac{t^2}{D^2}$

In this case, a count of 400 objects will be sufficient to reach 95 % confidence .

Detection limit

The detection limit is an important performance characteristic in phytoplankton surveys. It can be defined as the minimum concentration of a specific taxon or group at which it will be detected with 99 % certainty. Below this limit, detection is a matter of probability. The limit of detection from an identification point of view, corresponds with the laboratory species list. The probability with which an object is detected can be determined by Poisson statistics according to:

$P(x>0) = 1-e^{-\mu nv}$ for P = 0.99 this gives: $n = \ln(0.01)/(-\mu v)$

in which n - is the number of microscopic grids counted, μ - is the mean number per grid and v - is the volume of sample in the chamber.

NOTE Very often in microscopic analysis protocols the number of objects to be counted is fixed. As a consequence of sample difference a variation in detection limit within the same sample series will occur which renders comparison difficult.

Counting cells, colonies and filaments

In routine analyses algal objects situated at the edge of the counting grid should be counted 50/50 rule (counting the cells on the edge of only half of the microscope field (grid). This rule holds for single cells only. For coenobia this rule determines whether the coenobium is counted or not. For colonies and filaments the best approach is to count only cells inside the grid.
When the number of cells per colony or filament is of importance a special protocol should be applied. Generally this requires a lower magnification (200X). Care should be taken to ensure that sampling, transport and homogenisation do not alter unduly the colony or filament size

Quality assurance To ensure high quality results all steps of the method must be validated. Ideally this is performed on natural samples, but in some instances it may be helpful to spike the sample with cultured algae. Steps in the Utermöhl method to validate are
• homogenisation of sample

• sedimentation/sinking
• distribution on chamber bottom
• repeatability and reproducibility

Ultimatley the quality of the result from this method is dependent on the skill of the analyst. The variation of parallel samples counted by the same analyst and the variation in parallel samples counted by different analysts are two of the most important considerations in quality assurance (Willén 1976). When possible laboratories should take part in interlaboratory comparisons.

4.4 Cell counts using a conventional light compound microscope

- Sedimentation and surface water removal
- Filter-transfer-freezing (FTF) of the sample.
- Centrifugation.

4.4.1 Surface water removal *(see section 4.3.1)*

4.4.2 Filter-transfer-freeze methodology

It requires the following equipment:
distilled water, 25 mm diameter polycarbonate filters (1 μm pore size), forceps, 25 mm filter tower assembly, -20^{o}C freezer, a vacuum pump and flask, volumetric pipette, methanol, coverslips, microscope slides, acrylic coating and nail polish.

A filter is placed on the base of the filter tower assembly and 2-3 ml distilled water is drawn through the filter. A 10 ml sub-sample of a well mixed sample is drawn through the filter assembly and the tower assembly is rinsed with distilled water. The filter with the phytoplankton is then air-dried before removing it from the tower assembly. Meanwhile, a ring is drawn with a wax pencil on the microscope slide and a drop of distilled water is placed in the center. The filter is then placed onto the water on the slide with the sample side down. The slide with the filter is then transferred to a -20^{o}C freezer for 5 minutes, where the sample contents become part of the ice formed on the slide. The filter can then be removed and discarded, and the slide allowed to dry. A coverslip is then placed on the slide with a drop of preservative. Nail polish is applied around the edges of the coverslip to seal it to the slide. The slide is then ready for enumeration with a light microscope. All organisms on the slide are counted.

4.4.3 Centrifugation

10-15 ml centrifuge skrew tubes should be used. The samples should be centrifuged at 1000 g for 20 min. Centrifugation is inappropriate for fragile taxa and colonial species, due to disintegration and cell destruction.

Cell counts

For phytoplankton cell counting under conventional compound microscope the settling chambers are not appropriate. Instead counting chambers are used such as Palmer-Maloney, Sedwick-Rafter etc. Due to the low height of the chambers, the time for phytoplankton settling in the chambers will not exceed ½ hour. All the procedures described in *Section 4.2.3*. should be followed.

4.5 Recording the number of algal cells

Algal cell counts are required for quantitative analysis of phytoplankton density and to calculate biovolume, an estimate of algal biomass, in a sample.

Choose to count random fields or along transects. Both methods (inverted microscope and compound microscope) involve counting phytoplankton cells in a chamber, by counting either random fields or along transects.

Choose one of the following.

Determine random fields: Using a high dry microscope objective (40-45x objective, 400-450x total system magnification), identify and enumerate algae in selected, random fields. From each Palmer-Maloney counting cell or Utermöhl chamber, enumerate between 8 and 50 fields; use a second counting cell or sedimentation chamber, if necessary.
Choose a random starting place in the upper left-hand quadrant of the counting cell and approximate the number of fields that must be analyzed (400 natural units need to be counted with a minimum of 8 and maximum of 100 random fields). Develop a pattern that allows for equal probability of landing in any area of the cell or chamber with the exception of the edges and the center. A maximum pattern with 50 fields is made by having a grid of 8 x 8, subtracting 3 or 4 fields in either direction of the center.

Determine transects: Using a high dry microscope objective (40-45x objective, 400- 450x total system magnification) with a calibrated stage, identify and enumerate algae along transects, either horizontally or vertically across the chamber of the counting chamber or Utermöhl plate chamber. Without looking into the microscope, choose a location near the left edge in the upper third of the chamber (if vertical transects are analyzed, choose a location near the top edge in the left third of the chamber). Make a transect by moving only the horizontal stage control (or vertical control for vertical transects) a measured distance. Develop a pattern for the transects that will avoid the center and edges of the chamber. A second counting chamber or Utermöhl chamber can be used, if necessary . Enumerate 400 natural units

- Identify and enumerate all algal forms in the field of view:
- Enumerate algal forms using natural counting units: colony, filament, or unicell.
- Identify algal forms to the lowest possible taxonomic level (differentiate diatoms to the lowest practical taxonomic level).
- Count the number of algal cells comprising each multicellular counting unit.
- Tabulate the data on a bench sheet
- Count only “living” units (not empty cells) as part of the required 400 natural algal units.
- Add and record the tallies of each taxon on the bench sheet.

- Record the number of cells for multicellular counting units in parenthesis beside the tally of natural counting units.
- Record the number of fields or total length of the transect for the area that was observed as well as the Volume of the counting chamber scanned.

However, counting all the algal cells of all the algal units found during an analysis is impractical. Therefore, an average number of cells in a taxon's unit can be determined and then applied to the total number of units counted when the analysis is completed. This procedure can be performed before, during, or after the analysis using any style of microscope with the appropriate magnification.

To obtain an average number of cells in a taxon's unit:

- 1. Enumerate the number of cells in at least 10 units (n>10) of the selected taxon.
- 2. Record the number of cells of each unit on the bench sheet
- 3. Make a note on the bench sheet stating the count is of cells not units.
- 4. Place parentheses around these numbers to indicate that these are the number of cells in the unit that will be used to determine their average
- 5. When the analysis is completed determine the average number of cells in a unit by:
- $$\frac{Total\ Cells}{Total\ Units} = Average\ number\ of\ cells\ per\ unit$$
- Where: Total Cells = the total number of the particular taxon's cells that were counted to be averaged; and
- Total Units = the total number of the particular taxon's units in which cells were counted to be averaged.
- 6. Multiply the average number of cells per unit by the total number of units counted.
- 7. Record this number in the appropriate box on the bench sheet.

4.6 Density calculations

Density should be expressed as unit density [units/l] or cell density [cells/l].
Unit densities are calculated for each individual taxon in the assemblage and for the entire assemblage. The unit density of each individual taxon is calculated first and then the density of all the taxa are summed together to provide total density. The same procedure and equations are used to calculate individual and total cell density.

5. SEMI-QUANTITATIVE ANALYSIS OF PHYTOPIANKTON SAMPLES

Microscopic determination is the only method to get information on the species composition of phytopiankton samples. This information is needed in order to reveal changes in the phytopiankton communities in time and space The counting of cell number is time consuming, and when, mainly information on phytoplankton species composition is needed (ship-of-opportunity transects, additional vertical samples), a semi-quantitative counting method can be used instead of the quantitative one. In this method, all the taxa will be identified and listed, but their abundance is estimated using a semi-quantitative ranking (cf. Leppanen *et al*., 1995).

5.1 Inverted microscope

For the analysis, the inverted microscope technique is used. At least half of the chamber bottom should be analysed using small magnification (10X objective) and two bottom transects with larger magnification (40X objective). The semi-quantitative ranking should be done using a scale from one to five:

1. very sparse, one or a few (less than five of the > 20 μm fraction) cells or units in the analyzed area = in the sedimentated sample
2. sparse, slightly more cells or units in the analyzed area
3. scattered, irrespective of the magnification several cells or units in many fields of view
4. abundant, irrespective of the magnification several cells or units in most the fields of view
5. dominant, irrespective of the magnification many cells or units in every field of view

The ranking is sample specific, and several species can also get the same ranking, even the highest one. When the accurate abundance of a species (e.g. a potentially toxic one) should be counted, at least 20 fields (with 40X objective), or one transect (with 10X objective) should be analyzed.

5.2 Conventional light compound microscope

The same procedures (*see 5.1.)* are required for species identification and counting on light microscope. The counting chambers used are also of various volumes, depending on the abundance of the sample (Palmer-Maloney, Sedgwick, etc).

6. TAXONOMIC IDENTIFICATION

6.1 Introduction

Generally, there are just two methods of identifying organisms: by matching and by elimination. Matching depends on the availability of a comprehensive set of specimens or illustration and keen observation. Elimination depends on good knowledge of species-specific diagnostic characteristics. Identification based on illustrations alone should be avoided. If the accompanying text is properly written, it will include more information on variation and salient and diagnostic morphological features of a given species.

NOTE The availability of validated historical data of a specific sampling location and if available the results of a preliminary study of the living sample will expedite the analysis and prevent misidentification.

QUALITY CONTROL Proper identification should be controlled by setting up one or more taxon lists depicting all taxa identified by the analysts of the laboratory. Identification characteristics, literature etc. of the taxa should be added to this list (reference system). A (digital) image collection should be added whenever necessary. As taxonomy is dynamic in nature, splitting and lumping of taxa names will change and species will arise or disappear. Introductions of exotic species also would change the species list. It is important to keep track of these changes. Also the identification skills of analysts will normally improve in time by training and experience. So regularly the species list will be extended with species, which were not recognized before. Especially for long-term monitoring series this kind of information is inevitable. On a regular basis intra-laboratory comparison tests should be performed to avoid/minimize identification differences between analysts. It is also advisable, in addition to nationally or internationally organised inter-laboratory studies, to organize these kinds of control with related laboratories (dealing with comparable phytoplankton communities). A representative natural sample should be selected and analysed.
In principle two methods can be followed during the comparative examination. The easiest way is to analyse grid by grid by switching the microscope from one analyst to the other or a complete transect at once. After a grid or transect has been analysed by all participants the individual results should be directly compared and evaluated. A more advanced method is by using a computer controlled XY-stage which can reproduce identical series of random grids. In this way different analysts can carry out a standard survey after each other. After the last analyst has finished the analysis of each counted grid can be reviewed together.

Accurate identification is essential especially when establishing a data base for future reference. As previously indicated, where possible, live samples should be examined to determine species identification and preserved samples could be used for enumeration purposes.

The identification of species can be limited by the microscope used. For many diatoms and dinoflagellates it is possible to identify cells to genus and species but for some taxa, submicroscopic structures that are only shown by electron microscopy, are becoming more important to identification (Vershinin, Velikova, 2008). Where it is not possible to make an

identification of a particular organism, it should be identified to a taxonomic level at which the identifier is certain.

Due to the many different species of algae, references for identification are extensive and identifications are not condensed in a simple key. The existing identification books (IB) for the Black Sea phytoplankton species are too old and incomplete, so different IB composed for species from other basins are normally used (see the *section 6.2*.). The unification although crucial is difficult due to the usage of advanced identification techniques that come up with new findings that totally change the traditional position of a given species or a taxonomic group (*See Section 6.3.*) Thus for example recently many species have changed between *Gymnodinium* and *Gyrodinium* so it should be decided which names to use. The IB of Tomas Carmelo (1997) should be used as a principle IB and in order to avoid misinformation, on the bench sheet the IB used for the identification of species missing in Tomas should be mentioned – here a list of recommended IB

It is a prerequisite to follow the systematic nomenclature developed by WoRMs (http://www.marinespecies.org) for unification of taxonomic lists. Examples of web site providing additional information for phytoplankton analysis is given bellow

Examples of web sites that provide useful information for phytoplankton analysts	
Species information	**URL**
AlgaeBase	www.algaebase.org
World Register of Marine Species, WoRMS	www.marinespecies.org
IOC-UNESCO Taxonomic Reference List of Harmful Micro Algae	www.marinespecies.org/hab/index.php
European Register of Marine Species, ERMS	www.marbef.org
Integrated Taxonomic Information System, ITIS	www.itis.gov
Micro*scope	starcentral.mbl.edu/microscope/
Plankton*net	www.planktonnet.eu
Encyclopedia of Life	www.eol.org
Black Sea phytoplankton checklist	http://phyto.bss.ibss.org.ua

6.2 Identification books in use within Black sea countries

The following list of taxonomic references are currently used within the Black Sea Region:

1. Carmelo, T.R. [editor] (1997) Identifying Marine Phytoplankton, Academic Press USA. 858 pp.
2. Chretıennot-Dınet, M.J. (1990) Atlas du phytoplancton marin, Vol. III – Chlorarachinio-phyceees, Chlorophycees, Chrysophycees, Cryptophycees, Euglenophycees, Eustigma-

tophycees, Prasinophycees, Prymnesiophycees, Rhodophycees, Tribophycees, Paris.. 261 pp.
3. Cupp, E.E. (1943) Marine plankton diatoms of the West coast of North America // Bull. Scripps Inst. Oceanogr., Vol. 5.
4. Delgado, M. & Fortuna, J.M. (1991) Atlas de Fitoplancton del Mar Mediterraneo.Sci. Mar.,55, spl. 1: 1-133. Barcelona, Spain.
5. Dinet, C. (1990): Atlas Du Phytoplankton Marin. Vol. 3. Editions du CNRS. Paris, 1990.
6. Dodge, J.D. (1985) Atlas of Dinoflagellates. A Scanning Electron Microscope Survey. Botany Department, Royal Holloway & Bedford Colleges, (University of London) Egham, Surrey. Farrand Press, London.
7. Faust, M.A., Larsen, J. & Moestrup Ø. (1999) Potentially toxic phytoplankton 3. Genus Prorocentrum (Dinophyceae). In: ICES identification leaflets for plankton. Ed. Lindley J. A. DK-1261 Copenhagen K, Denmark. P. 1-24.
8. Fensome, R.A., Taylor, F.J.R., Norris, G. *et al*. (1993) A classification of living and fossil dinoflagellates. Hanover, Pennsylvania..351 pp.
9. Fukuyo, Y. (2000) Atlas of Dinoflagellates prepared for Harmful Algal Bloom Program of IOC and HAB project of WESTPAC/IOC, distributed by the Grant-in-Aid for Scientific Research (B) No.09556043 from the Ministry of Education, Science, Sports, and Culture of Japan. (CD-ROM ver. 1.5/Nov. 1, 2000).
10. Gemeınhardt, K. & Shıller, J. (1930) Silicoflagellatae, Coccolithineae, Leipzig, 273 pp.
11. Gerhard, D. (1974) Marines phytoplankton eine Auswahl der Helgolander planktonalgen (Diatomeen, Peridineen). Georg Thieme Verlag Stuttgart.
12. Gollerbah, M.M., Kosınskaıa, E.K. & Polıanskıı, V.I. (1953) Sinezelenie vodorosli (Blue-green algae), Moskow. 652 pp.(in Russian)
13. Hallegraeff, G.M. (2002) Aquaculturists guide to harmful Australian microalgae, School of Plant Science, University of Tasmania, Hobart, Tasmania, Australia, 136 pp.
14. Hallegraeff, G.M., Anderson D.M. & Cembella A.D. (2003) Manual on harmful marine microalgae. Paris, UNESCO. 793 pp.
15. Hallegraeff, G.M. (1993) A review of harmful algal blooms and their apparent global increase // Phycol., 32, 79-99.
16. Hendey, N.I. (1964) An Introductory Account of the Smaller Algae of British Coastal Water Part V: Bacillariophyceae (Diatoms). London.
17. Horner, R.A.A. (2002) Taxonomic guide to some common marine phytoplankton. 195 pp.
18. Hustedt, F. (1930). Die Kieselalgen Deutschlands, Osterreichs und der Schweiz. Kryptogramen-Flora / G.L. Rabenhorst, Vol. 7 (1). (In German.)
19. Ivanov, A.I. (1965) Characteristics of the qualitative composition of Black Sea phytoplankton [Kharakteristika kachestvennogo sostava fitoplanktona Chernogo morya], [in:] Investigations of the plankton of the Black Sea and Sea of Azov [Issledovaniya planktona Chernogo i Azovskogo morey], Naukova dumka, Kiev, 17-35. (In Russian.)
20. Jahnke, J. (1992) Dominant species in phytoplankton blooms. Emiliania huxleyi (Lohmann) Hay and Mochler. In: ICES identification leaflets for plankton. Ed. Lindley J. A. DK-1261 Copenhagen K, Denmark. P. 1-4.
21. Kiselev, I.A. (1950) Armoured flagellates [Pancirnye zhgutikonoscy], Izdatelstvo AN SSSR, Moscow-Leningrad, 279 pp. (In Russian.)
22. Komakhidze, A. & Mazmanidi, N. [eds] (1998). Black Sea Biological Diversity. Vol. 8. Georgia. Black Sea Environmental Series, New York.167 pp.

23. Komarek, J. & Anagnostidis, K. (1989). Modern approach to the classification system of cyanophytes. 4. Nostocales, Arch. Hydrobiol. Suppl., 82/3, Algolog. Stud., 96, pp. 247-345.
24. Komarek, J., Anagnostidis, K. (1999) Cyanoprokaryota, 1 Teil. Chroococcales. Süsswassers, von Mitteleuropa, 19/1, Gustav Fischer Verlag, Stuttgart, 548 pp.
25. Komarek, J. & Fott, B. (1983) Chlorophyceae (Grünalgen), O. Chlorococcales Das Phytoplankton des Süsswassers. Systematik und Biologie. Band 16, 7. Teil. 1. Hälfe. Stuttgart, E. Schweizerbart'sche Verlagsbuchhandlung (Nägele u. Obermiller), 1044 pp.
26. Konovalova, G.V. (1998) Dinoflagellatae (Dynophyta) of The Far Eastern Seas of Russia and Adjacent Waters of The Pacific Ocean, Vladivostok Dalnauka, 298pp (In Russian.)
27. Konovalova, G.V., Orlova, T.U. & Pautova, L.A. (1989) Atlas of Phytoplankton of The Japan Sea, Vladivostok Dalnauka. (In Russian.)
28. Konsulov, A. [ed]) (1998) Black Sea Biological Diversity. Vol. 5. Bulgaria. Black Sea Environmental Series, New York. 131 pp.
29. Krammer K. & Lange-Bertalot H. (1986) Band 2/1: Bacillariophyceae, 1. Teil.Naviculaceae [In]: Ettl Y., Gerloff J., Heynig H., Mollenhauer D. [Eds], Susswasserflora von Mitteleuropa, 2/1, VEB Gustav Fischer Verlag, Jena. 876 pp.
30. Krammer, K. & Lange-Bertalot, H. (1988) Band 2/2: Bacillariophyceae, 2. Teil.Epithemiceae, Surirellaceae [In]: Ettl Y., Gerloff J., Heynig H., Mollenhauer D. [Eds], Susswasserflora von Mitteleuropa, 2/2, VEB Gustav Fischer Verlag, Stuttgart-Jena. 596 pp.
31. Krammer, K. & Lange-Bertalot, H. (1991a) Band 2/3: Bacillariophyceae, 3. Teil.Centrales, Fragilariaceae, Eunotiaceae [In]: Ettl Y., Gerloff J., Heynig H., Mollenhauer D. [Eds], Susswasserflora von Mitteleuropa, VEB Gustav Fischer Verlag, Stuttgart-Jena. 576 pp.
32. Krammer, K. & Lange-Bertalot, H. (1991b) Band 2/4: Bacillariophyceae, 4. Teil.Achnanthaceae [In]: Ettl Y., Gartner G., Gerloff J., Heynig H., Mollenhauer D. (Eds), Susswasserflora von Mitteleuropa, VEB Gustav Fischer Verlag, Stuttgart-Jena. 436 pp.
33. Larsen, J. & Moestrup, Ø. (1992) Potentially toxic phytoplankton 2. Genus Dinophysis (Dinophyceae). In: ICES identification leaflets for plankton. Ed. Lindley, J.A. DK-1261 Copenhagen K, Denmark. pp. 1-12.
34. Moestrup, Ø. & Larsen, J. (1992). Potentially toxic phytoplankton 1. Haptophyceae (Prymnesiophyceae). In: ICES identification leaflets for plankton. Ed. Lindley, J.A. DK-1261 Copenhagen K, Denmark. pp. 1-11.
35. Nagy-Toth, F. & Barna, A. (1998) Unicellular green algae (Chlorococcales), Cluj. 200 pp. (In Romanian.)
36. Öztürk, B. [compiler] (1998) Black Sea Biological Diversity. Vol. 9. Turkey. Black Sea Environmental Series, New York, 144 pp.
37. Petranu, A [compiler] (1998) Black Sea Biological Diversity. Vol. 4. Romania. Black Sea Environmental Series, New York. 314 pp.
38. Popova, T.G. (1955) Evglenovie vodorosli, [Euglenophyta], vol VII., Moskva. 283 pp.
39. Priddle, J. & Fryxell, G. (1985) Handbook of common plankton diatoms of the Southern Ocean: Centrales except the genus Thalassiosira . Cambridge University Press,.
40. Proshkina-Lavrenko. (1955) Plankton Diatoms of The Black Sea, Academy of Sciences USSR. (In Russian.)

41. Proskina-Lavrenko, A.I. (editor) (1950) [Diatoms]Diatomovii analiz, Vol. III - Opredeliteli iskopaemih i sovremenih ditomovih vodoroslei, Poriadoc Pennales. 698 pp.
42. Proskina-Lavrenko, A.I. (editor) (1951) Diatomovie vodorosli, Moskow. 619 pp.(In Russian)
43. Proskına-Lavrenko, A.I. (editor) (1955) [Plankton Diatoms from the Black Sea] Diatomovie vodorosli planktona Cernovo Moria, Moskow. 220 pp.(in Russian)
44. Rhicard, M. (1987) - Atlas du phytoplancton marin, vol. II - Diatomophycees, Paris. 294 pp.
45. Schiller, J. (1933) Dinoflagellatae (Peridineae), Leipzig, 2 vol.. (In German.)
46. Skov, J., Lundholm, N., Moestrup & Ø. Larsen, J. (1999) Potentially toxic phytoplankton 4. The diatom genus Pseudo-nitzschia (Diatomophyceae/Bacillariophyceae). In: ICES identification leaflets for plankton. Ed. Lindley J. A. DK-1261 Copenhagen K, Denmark. pp 1-23.
47. Sournia, A. (1986) Atlas Du Phytoplankton Marin. Vol. 1. Editions du centre national de la recherche scientifique. Paris,
48. Wood, E.J.F. (1963). Dinoflagellates in the Australian region // Austral. J. Mar. Freshwater Res. Vol. 5, № 2, 172-351.
49. Zaitsev, Yu. & Alexandrov, B. (compilers) (1998) Black Sea Biological Diversity. Vol. 7. Ukraine. Black Sea Environmental Series, New York. 351 pp.
50. Zaitsev, Yu. & Öztürk, B. (2001) Exotic species in the Aegean, Marmara, Black, Azov and Caspian Seas. Publ. by Turkish Marine Research Foundation, Istanbul, Turkey. 267 pp.

6.3 Additional references of recent taxonomic developments

The following taxonomic references, through not routinely or widely used in the Black Sea Region, are valuable sources of information, since they provide detail of the evolution of marine phytoplankton taxonomy in addition to those references cited above:

51. Daugbjerg, N. G Hansen, J. Larsen and. Moestrup. (2000). Phylogeny of some of the major genera of dinoflagellates based on ultrastructure and panial LSU rDNA sequence data, including the erection of three new genera of unarmoured dinoflagellates. Phycologia 39: 302-317.
52. Meier K.J., D. Janofske, and H. Willems (2002). New Calcareous Dinoflagellates (Calciodinelloideae) from the Mediterranean Sea. Universitat Bremen, Fachbereich 5 - Geowissenschaften, Postfach 330440, D-28334 Bremen, Germanv J. Phycol. 38, 602-615
53. Dodge J. D. and S. Toriumi. (1993) A Taxonomic Revision of the Diplopsalis Group (Dinophyceae).1993. Botanica Marina Vol. 36, pp. 137-147,
54. De Solas. M. F, Ch.. S. Bolch., L. Bates., G. Nash, S. W. Wright., G. M. Hallegraeff. (2003). Takayama Gen. Nov. (Gymnodiniales, Dinophyceae), A New Genus of Unarmored Dinoflagellates with Sigmoid Apical Grooves, including the Description of Two New Species'. 2003. J. Phycd. 39,1233-1246
55. Haywood A., K. A. Steidinger, E. W. Tmby., P. Bergquist, P. L. Bergquist., J Adamson and L. MacKenzie (2004). Comparative Morphology And Molecular Phylogenetic Analysis of Three New Species of the Genus Karenia (Dinophyceae) from New Zealand. J. Phycol. 40, 165-179 © 2004 Phycological Society of America.

56. Moestrup H. G.,., K. R. Roberts (2000) Light and electron microscopical observations on the type species of Gymnodinium, G. fuscum (Dinophyceae).. Phycologia, Volume 39 (5), 365-376
57. Vershinin A.O. and Velikova. V.V. (2008) New Species to the Black Sea Phytoplankton List and Some Commonly Occurring Light Microscopy Misidentifications. Botanica Marina. In press.
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60. Gómez, F., , D. Moreirab and P. López-Garcíab, 2010. Neoceratium gen. nov., a New Genus for All Marine Species Currently Assigned to Ceratium (Dinophyceae). Protist, Volume 161, Issue 1, January 2010, Pages 35-54

7. BIOMASS DETERMINATION

The biomass is determined based on morpho-metric measurement of phytoplankton units. A list of all species is necessary and a concent agreement for the definition of cell relative geometric forms in order to calculate cell volume with the same geometric transformation for each taxon as described in detail in (Edier, 1979). It is assumed normally that the plasma volume is equal to cell volume (PV = CV unit: μm^3) except for diatoms, which need additional calculations (Edler, 1979).
For the Black sea efforts have been made to simplify the measurements and biovolume calculation by estimating a correction coefficient (k), reflecting the cell fullness (Bryantzeva et al., 2005). The approach releal statisticaly reliable similarity for a small fraction of species, out of the 250 species included in the document.

A new automated system, phytoplankton data-base and Black Sea check-list are under current development (*see Annex A – Phytoplankton check list*).

7.1 Diatoms

Different methods for calculation of the plasma volume have been suggested by Smayda (1965) and Strathmann (1967). The method recommended here is a modification of that given by Strathmann to include pennate diatoms. Plasma volume (PV) equals cell volume (CV) minus vacuole volume (VV).

The vacuole volume is calculated by subtracting plasma thickness c from the measurements of length, breadth etc. If a measurement extends from cell wall to cell wall, 2 c should be subtracted from the measured value. When using a measurement such as radius that is not bounded by the cell wall and thus contains only one layer of plasma, only c should be subtracted. Values of c have been given by Smayda, 1965 and are in the range of 1 µm.
Due to the high content of organic matter in the vacuole, only 90 % of the vacuole volume should be subtracted from the cell volume to give the plasma volume.

PV = CV-(0.9*VV) unit:μm^3

7.2 Calculation of a cell's reference biovolume

All cells should be measured (µm) with an ocular reticule (micrometer) at 400X magnification or greater. To determine a cell's reference biovolume the following steps are necessary:

- Select the equation or set of equations that best fits the morphology of the algal cell from the biovolume check-list (annex A)
- Prepare a wet mount of the sample that contains the alga being evaluated.
- Locate a specimen on the microscope slide.

- Take the necessary measurements (in microns) using the ocular reticule or micrometer , or the image analysis system
- Positions of the specimen can be changed by lightly tapping the coverslip with the tip of a pencil or pen. Practice is required to reposition the specimen correctly.
- Record the measurements on the reference biovolume calculation worksheet.
- Measure the cells of at least 15 specimens (preferably from more than one sample and during different seasons since this calculation will ultimately represent an "ideal" or reference biovolume).
- Determine the average size of a cell by adding all related measurements together then dividing by the number of measurements taken.
- Use the average of these measurements in the selected equation to determine the reference biovolume of the cell.

7.2 Carbon biomass

Carbon biomass of planktonic organisms is a fundamental parameter in ecosystem models and biogeochemical carbon budgets. Temporal and spatial variability in total and export primary production can be quantified and predicted only if the carbon content of the major plankton organisms is known. Carbon is the principal structural component of both heterotrophic and phototrophic organisms and is the basis for community-wide as well as group-specific comparisons of biomass and bioenergetics. Estimates of carbon biomass of plankton organisms are usually made by converting microscopic size measurements to cell volumes, which are then expressed in carbon biomass using empirically or theoretically derived carbon to volume ratios. This assures comparability of data when regional data bases are composed and further used in various regional studies. For carbon content calculations there are several different equations reported in the literature (Montagnes DJS, Franklin DJ (2001), Stratman, 1967, Menden-Deuer & Lessard 2000). It is recommended to apply the the equations recommended by (Menden-Deuer & Lessard 2000) as follows:

$\log C = -0.541 + 0.811 (\log V)$ (1) – diatoms<3000 μm^3

$\log C = -0.933 + 0.881 (\log V)$ (2) - diatoms >3000 μm^3

$\log C = -0.353 + 0.864 (\log V)$ (3) - dinoflagellates

$\log C = -0.642 + 0.899 (\log V)$ (4) - coccolithophores

$\log C = -1.026 + 1.088 (\log V)$ (5) - chlorophytes

$\log C = -0.583 + 0.860 (\log V)$ (6) - small flagellates, cryptophytes and cyanophytes

8. RECORDING DATA

All data of the analysis must be recorded on an approved bench sheet.

8.1 Bench Sheets

The bench sheets on quantitative or qualitative analysis are kept indefinitely. Data on the bench sheets must be written legibly so that other individuals may read and understand notes, identifications and counts. The total number of cells and units must be calculated and recorded in the appropriate box on the bench sheet when a quantitative analysis is completed. These totals are used later after the data is entered to verify that the numbers of units and cells entered is equal to the number of units and cells on the bench sheet.
Bench sheet data must include:

Bench-sheet for phytoplankton reporting

DATASET-NAME:
*PROJECT:
*PLATFORM/SHIP :
*STATION COORDINATES:
*GEOGRAPHIC-COVERAGE:
*DATE & HOUR :
*BOTTOM DEPTH :
*SAMPLING DEPTH
*OBSERVED-PARAMETERS: (wind, current, chemistry)
*MEASURED/DETERMINED-PARAMETERS:
*COMPUTED/CONVERTED-PARAMETERS:

SAMPLING INSTRUMENTS AND METHODS:

Sampling instrument/equipment:
Sample volume :
Sample preservation and handling:
Responsible for data collection:

DETAILED TAXONOMIC-IDENTIFICATION and ANALYSIS:

Method of sample concentration:
Volume of the sub-sample analyzed:
Type of microscope used :
Magnification used:
Type of the counting chamber:
Area of the chamber analyzed:
Number of cells/ units counts:
Biovolume determination method:
Identification system/books:
Image library:
Storage media:

***QUALITY CHECK Procedure (available basic documents):**

Data entry (double entry):

***TAXONOMISTS IN CHARGE OF THE DETERMINATION:**

(take part in sampling and sample-handling Workshops, Taxonomic training Workshops, Intercalibrations , in ring-tests)

The metadata format presented here has been also adopted by UP-GRADE BS SCENE Working expert group on phytoplankton data QC/QA

9. PHYTOPLANKTON-RELATED ENVIRONMENTAL STATUS INDICATORS

9.1 Introduction

The WFD imperative target "achieving good ecological quality of the water bodies by 2015", ultimately suggests the urgency of development of reliable indicators of ecosystem health. The metaphor of the "health" of an ecosystem as an analog for human health has been used for over a half century, at least since Aldo Leopold (1941) wrote about "land sickness." However, there are significant limitations, both theoretical and practical, in the application of this concept in the way we understand and measure human health (Carpenter et al., 2001).

Implicit in the concept of health is how humans value the performance of an ecosystem in terms of the goods and services it provides. Nonetheless, there have been significant recent advances in the conceptualization and measurement of ecosystem health (Costanza et al., 1997;) that permit the measurement of coastal ecosystem health more rigorously.

Any useful notion of ecosystem health should involve both the structure (the species and populations involved) and function (the flow of energy and materials) of the ecosystem.

Ecosystem health should include the three components of vigor, organization, and resilience (V-O-R) (Costanza ,1992), in which**:**

- **Vigor** embodies the throughput or productivity of the ecosystem.

- **Organization** represents not only species diversity, but also the degree of connectedness of the constituent species (complexity of trophic and other interactions). This suggests that systems issues concerning resilience and recovery need to devise system indicators of the strength and number of interactions (Peterson et al., 1998, Reynolds and Smayda, 1998).

- **Resilience** refers to an ecosystem's ability to maintain structure and patterns of behavior in the face of stress. Resilience is the ability of a social-ecological system to undergo, absorb and respond to change and disturbance, while maintaining its functions and controls (Carpenter *et al*., 2001).

A healthy ecosystem, then, is one that is actively producing (V), maintains its biological organization over time (O), and is resilient to stress (R). It is a normative concept, involving interpretation based on human values of the overall ecosystem. Consequently, the way we try to monitor the health of a coastal ecosystem is to measure selected indicators, or small pieces of the ecosystem, to represent the whole.

From scientific viewpoint we have to reflect the complexity of the ecosystem understanding the mechanisms and functional pathways of its operation, while from policy view point (WFD) we have to explain it by simple, easy to measure, robust and easy to interpret descriptors. Below are

listed potential phytoplankton structural and functional indicators that need further testing and refinement.

Phytoplankton structural indicators

- Total biomass [mg/m3] /chlorophyll a [μg/l]
- Total abundance [cells/l]
- Taxonomic structure (taxonomic ratios).
- Species richness/mo of species and related indices
- Species diversity (indices)
- Size structure
- Dominant (key, "engineer" species)
- Phytoplankton blooms (species, abundance/biomass)
- Harmful species (hypoxia/anoxia).
- Exotic species/Ratio native/exotic.
- Sphearicity index (Bryantseva, 2005)
- Morphometrical diversity (Bryantseva, 2010)

Phytoplankton functional indicators

- Primary productivity.
- Functional groups- r/K strategy C-S-R functional groups (Reynolds and Smayda ,1998; Smayda and Reynolds, 2001)
- Morpho-functional indices (Phytoplankton surface index- Minicheva, 2003)
- Autotrophs/heterotrophs.
- Life cycle (vegetative cells/cysts)
- Succession pattern.

Unfortunately indices such as Shannon- Weaner Biodiversity index, Pielou evenness index, species richness, exotic index etc. are applicable to long-term data series while for monitoring purposes they are less informative. Composite indices such as the Trophic state Index (TRIX) developed by Vollenweider *et al*. (1998), phytoplankton efficiency index have a number of constraints that limit their usefulness as a monitoring tool (Moncheva *et al*, 2002).

9.2 Black Sea-specific phytoplankton-related environmental status indicators

At a workshop on the development of indicators of eutrophication of the Black Sea (Istanbu,l 25-30 September 2000, EU TACIS Project: ENVRUS9602: Phase 2) the following list of indicators were agreed upon:

- Concentration of chlorophyll-a, μg/l
- Biomass of each phytoplankton species, mg/m^3
- Density of each phytoplankton species, cells/l
- Phytoplankton species composition, % composition of key groups (number and biomass); for example, dinoflagelates: diatoms
- Annual maximum density of each blooming species, cells/l

- Specific production of dominant species, mg C. d^{-1}
- Ratio of the total biomass of phyto-: zooplankton

10. QUALITY CONTROL AND QUALITY ASSURANCE (QC/QA)

10.1 Introduction

To help meet the needs of the EU Water Framework Directive and to satisfy the aim of the Black Sea laboratories to improve the quality of analytical results, a widely accepted standard for phytoplankton sampling and sample treatment/analysis is required. Such a standard cannot give a single best counting protocol as the questions underlying monitoring programmes are too diverse in character. This guidance is intended, therefore, to provide a uniform framework to optimize and validate 'house' methods and protocols in a comparable and standardized way.. In order to reduce the high variance in the results of phytoplankton analysis, standardization of as many steps as possible of the analytical procedures is necessary. Comparability of data is achieved through a standard statistical method for the estimation of measurement uncertainty.
The normative document which constitute provisions of the European Standard is ISO/IEC 17025. "*General requirements for the competence of testing and calibration laboratories"* . For more details see "Guidelines for QC/QA of biological data-phytoplankton" (Moncheva, 2010).

10.2 Equipment

Everybody should use the same equipment (*See Section2.5)*

10.3 Sampling protocol

Everybody should use the same methodology and sample from the same depths (standard depths, and depths in relation to thermeocline/halocline/nutricline). Ensure representative transitional, coastal and offshore water sites are included in the sampling programme. Every time a phytoplankton sample is collected, a sample should also be collected and analysed for chlorophyll-a content. Parallel sampling of hydrophysical and chemical parameters is strongly recommended.

10.4 Sample preservation

Everybody should use the same fixative: Due to the large number of samples and the long-time required for processing in order to avoid storage problems borax buffered formaldehyde will be used for fixation. In specific cases (coccolothophorid blooms etc.) a parallel sample fixed with other preservative is desirable (*see section 3.1)*

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10.5 Sub-sampling – validation of homogenization

Validation of homogenisation can be performed at best with the use of a Sedgwick-Rafter or similar counting chamber as these chambers give the smallest counting errors.
As input for the test two perpendicular transects or diagonals of microscopic grids are counted.

The following three-step procedure is recommended for the validation test:

1. Variance to mean ratio

A first test should reveal if the objects can be considered to be randomly distributed among the counted grids. The easiest way is to determine the variance to mean ratio or index of dispersion (I), which gives a good approximation to χ^2 for n−1 degrees of freedom. It is calculated according to:

$$I = \chi^2 = \frac{s^2(n-1)}{\overline{x}} \qquad v = n - 1$$

In which n is the number of grids, $\overline{x}$ is the mean number of objects, s^2 is the variance of the number of objects and *v* is the degrees of freedom.

For $v < 30$ the critical values of $\chi 2$ can be found in standard chi-squared tables as found in most standard textbooks on statistics. Agreement with a Poisson series is accepted at the 95 % probability level if $\chi 2$ value lies between the critical values of 0.975 and 0.025.

If no reproducible and acceptable random distribution in agreement with a Poisson series can be obtained, no reliable counting data can be produced. However when dealing with slightly contagious ($\sigma 2 > \mu$) distributions a Poisson approximation is still applicable when μ is small (< 5) and 1/k (index of dispersion: $k = \frac{\overline{x}^2}{s^2 - \overline{x}}$) is fairly small (< 0.2) (Table below).

Table Maximum allowable variance for Poisson approximation (μ = mean, σ2 = variance).

μ	σ^2
0,5	0.55
1	1.20
2	2.80
3	4.80
4	7.20
5	10.00

2. Serial randomness

A second step in the validation procedure is testing whether the separate taxa (species) are randomly distributed among themselves. This so-called serial randomness can be tested with a Run-test. In this case a run is a sequence of identical algae observed in a small diagonal band bounded on either side by another algae or no algae. The same sample as used for overall randomness can be used. The Run test can be applied for two or more taxa. When one taxon has an abundance of at least 30 objects in the scan, the expected mean amount of runs is calculated according to:

$$\mu_u = \frac{N(N+1) - \sum n_i^2}{N}$$

in which N = total number of objects and ni is the number of objects of taxon i.

NOTE It is not unlikely that the distribution of algal objects is random and that the run test reveals no clustering (objects are independent) whereas a gradient in number of algae is still observed. It might be possible that towards the chamber wall irrespective of the taxa more or less algae have sedimented. This pattern can best be evaluated by clustering objects among several adjacent grids and presenting the results graphically and testing for randomness again. If it still passes the test the only concern will be to count random grids inside the chamber. In the case of random grids the probability of counting a specific region is proportional to the surface area of that region relative to the total surface area of the counting chamber. If this is not taken care of a systematic error may result.

3. Comparison of taxon composition

A final check can be carried out to test whether the taxon composition in different subsamples is identical. To test whether N samples are homogeneous with respect to the true proportion of the taxa, $\chi 2$ should be calculated. The critical value of $\chi 2$ can easily be found in most statistical textbooks. When 'rare' taxa are included in the counts they should be grouped according to the rule that a minimum count of 1 is allowable if less than 20 % has an abundance of less than 5.

10.6 Sample analysis

All analysts should use the same technique and same approach. Although recent developments recommend inverted microscope technique for phytoplankton monitoring, both inverted microscope and conventional light compound microscope (used by majority of the Black Sea labs) should result in similar counts when used correctly.

10.6.1 Biovolume/biomass estimation

All analysts should use the same methodology (*see Section 7*).

To avoid the difference in calculating the biomass among the Black Sea labs a taxonomically up-dated Black Sea phytoplankton species check list with agreed geometric formulas has been developed and is attached to this manual. An automated system for biovolume calculation and phytoplankton data-base is in progress of development. For more details see Annex A

10.6.2 Phytoplankton enumeration

All users should use same methodology (*see Section.4*.). For unification of counting procedure – Palmer-Maloney cells will be used for counting high density (blooms) samples and Noggot's or Sedgewick-Rafter's for less abundant samples. Counting of at least 400 units per sample is compulsory.

10.6.3 Taxonomy

To avoid taxonomic complications Tomas Carmelo (1997) will be used as a basic identification book, and all the other available IB and sources (*see Section 6.2*) for species that are not found in Tomas. A regional network of experts has been established that will be consulted on all identification issues/uncertainties. Where equipment/sample integrity allows, identification uncertainties should be addressesed using fresh samples, fixed samples or photographs. When necessary, confirmation should be sought from specialists outside of the Region.

10.6.4 Re-analysis

Ten percent of all phytoplankton samples should be re-analysed by another analyst, and the results compared for QA purposes (*see Annex B.)*

10.6.5 Training and inter-laboratory comparability testing

All analysts to participate in annual quality assurance inter-comparability tests. Based on the results of these, more frequent comparisons may be necessary. The exercises should be organized and samples collected on a rota basis by BSIMAP phytoplankton laboratories, and should include samples from transitional, coastal and offshore water sites. Because of problems with sample integrity, it is recommended that only fixed/preserved samples be used for such exercises.

10.6.6 Repeatability and reproducibility

The determination of the performance characteristics repeatability and reproducibility should be carried out with natural samples from a range of relevant conditions. For proper interpretation it should be stated whether the sample was rich or poor in algae, detritus etc.. Quantitative results should be assessed on a couple of most numerous species or, less preferably, on the level of green algae, diatoms, cyanobacteria and others. The measured error should be compared with the expected stochastic error based on Poisson statistics to get insight into the performance of the procedure and analyst.

10.6.7 Uncertainty

A measurement cannot be properly interpreted without knowledge of its uncertainty. Uncertainty of a final result encompasses the uncertainties of the whole measurement process (sampling, stability, subsampling, homogeneity, identification, quantification etc.). The general procedure is to specify, identify the sources, quantify, and calculate the combined (expanded) uncertainty. It can be expressed in the form of a standard deviation. In biological methods it should be considered that uncertainties are sometimes qualitative in nature (misidentification) and difficult to combine with other uncertainties into a combined one. In these cases specific solutions should be selected, based on common sense. Often the absolute statistical limits are difficult to assess, particularly when no standards or other reference methods exist. In these cases the mean value obtained in inter-laboratory studies organized among proficient laboratories can be adopted as practical limits.
For further details *see Annex C.*

10.7 Reporting/data storage

A standardized data storage/reporting developed for all BSIMAP phytoplankton monitoring labs. The reporting format should automatically calculate the agreed list of phytoplankton quality indicator metrics. Because some of these metrics also rely on zooplankton data, there is a need to coordinate development of phytoplankton and zooplankton reporting formats and data software, and to ensure that these formats are compatible with the Black Sea Information System software currently being developed for the Black Sea Commission Permanent Secretariat.

Annex A.

CHECK LIST OF BLACK SEA PHYTOPLANKTON, REFERENCE BIOVOLUMES and DATA BASE

Species checklist with reference list of biovolumes is one of the most important components of the Quality Control procedure for the phytoplankton data from Black Sea region. It will help to provide comparable and homogeneous data sets of phytoplankton for the Black Sea basin. The list of phytoplankton species was developed as dynamic web-site, available on-line: http://phyto.bss.ibss.org.ua

Software (*Oleksandra Sergeyeva, Kseniia Skuratova IBSS, Sebastopol, Ukraine*)

The special software for creation of online marine species checklists was developed. This software is based on wiki engine and has special developed functions which make it easy to add, delete, move species and add any type of structured information in the form of patterns, which can be easily added by the checklist administrator on request of users.
Each species has the corresponding page, where all information is placed either in form of predefined patterns or in the form of text, images, tables etc.

Taxonomy
The name of each species is checked towards WoRMS nomenclature. Also all available synonyms are attached to the species name. For each species the APHIAID with direct link to WoRMS is placed. If species cannot be found in WoRMS – Algaebase is used. For those species that could not be found in WoRMS and Algaebase special mark is placed on the page.

Information sources

The number of information sources analyzed is increasing continuously. The actual information can be accessed on-line: http://phyto.bss.ibss.org.ua

By November 2010, 27 information sources have been analyzed and species from them were included to the list:

Boicenco L. Updated list from Bodeanu N., 1987-88. Structure et dynamique de l'algoflore unicellulaire dans les eaux du littoral Roumain de la Mer Noire. Research Marines, nr.20/21, 19-250

Bryantseva Yu., 2000.Changes in structural characteristic of phytoplankton in the Black Sea. Sevastopol, 2000.

Bryantseva Yu., 2010. Sevastopol Bay phytoplankton monitoring database.

Dereziuk, N., 2008. List of Phytoplankton Species Observed in the Water Area near the Zmiinyi Island in 2004-2007. In: Zmiinyi Island. Ecosystem of Coastal Waters: monograph / V.A.Smyntyna, V.I. Medinets, I.O. Suchkov [et al.]; Odesa National I.I.Mechnikov University. - Odesa : Astroprint, 2008. - XII, p.208-218.

Gomez F., Boicenko L.,2004. An annotated checklist of dinoflagellates in the Black Sea. Hidrobiologia. 2004. V. 517. P. 43–59.

Gvarishvili Ts., 2010. Phytoplankton species composition along the Georgian Black Sea coast.

Ivanov A.I., 1965 Characteristics of the qualitative composition of Black Sea phytoplankton. In: The study of plankton of the Black and Azov seas. Kiev: Naukovs. Dumka, 17-35.

Kiselev I.A., 1950. Thecate Flagellates (Dinoflagellata) of the Seas and Freshwater Reservoirs of the USSR, USSR Acad. Sci. Publ. House, Moscow, Leningrad (1950).

Komakhidze, A. & N. Mazmanidi, 1998. Black Sea Biological Diversity, Georgia. Black Sea Environmental Series, Vol. 8. United Nations Publications, New York, 167 pp.

Krakhmalnyy A.F., Panina Z.A., Krakhmalnyy M.A. Dinophyta. Algae of Ukraine: Diversity, Nomenclature, Taxonomy, Ecology and Geography/ Edited by P.M.Tsarenko, S.P.Wasser, E.Nevo. Vol. 1. Cyanoprocaryota, Euglenophyta, Chrysophyta, Xanthophyta, Raphidophyta, Phaeophyta, Dinophyta, Cryptophyta, Glaucocystophyta, Rhodophyta. Ruggell. A.R.A. Gantner Verlag K.G./Liechtenstein. 2006.- P. 470- 532.

M.Turkoglu, T.Koray, 2002. Phytoplankton species succession and nutrients in the Southern Black Sea (Bay of Sinop), Turkish Journal of Botany 26 (2002), pp. 235–252.

Mikaelyan A.S., Senichkina L.G., Pautova L.A., Georgieva L.V., Dyakonov V., 2008 Cell volumes of phytoplankton of the Black Sea

Moncheva S. (IO-BAS), 2010. Species lists based on sampling identifications and literature. Bulgarian waters

Morozova-Vodyanitskaya N.V., 1948. Phytoplankton of the Black Sea: Part 1 Tr. Sevast. Biol. Stantsii 6, 39–72.(1948)

Morozova-Vodyanitskaya N.V., 1954. Phytoplankton of the Black Sea: Part 2 Tr. Sevast. Biol. Stantsii 8, 11-99. (1954)

Nesterova D.A., Terenko L.M., Terenko G. V., 2006 Phytoplankton species list in: Northen-West part of the Black Sea: biology and ecology // Kiev, Naukova dumka, 2006, pp. 557 – 576

O.Yasakova, 2010. Phytoplankton of the northern-eastern part of Black Sea 1995 -2009

Ozturk, B., 1998. Black Sea Biological diversity, Turkey. Black Sea Environmental Series. United Nations Publications, New York, 144 pp.

Pereyaslavtseva, S. M. 1886, Protoza of the Black Sea. Notes of. Novorossiysk naturalists society, V.10, N2: 79-144. , (in. Russian)
Petranu A., 1997. Black Sea Biological Diversity, Romania. Black Sea Environmental Series, Vol. 4, United Nations Publication, New York, 314 pp.
Petrova-Karadjova, S. Moncheva , 1998. Biological devrsity of the pelagic ecosystem in the Bulgarian Black Sea- Phytoplankton. In: Konsulov, A.[ed], Black Sea Biological diversity, Bulgaria. Black Sea Environmental Series, Vol. 5. United Nations Publications, New York, 131 pp.
Polikarpov I.G., Saburova M.A., Manzhos L.A., Pavlovskaya T.V., Gavrilova N.A., 2003 Microplankton biological diversity in the Black Sea coastal zone near Sevastopol (2001-2003). In: Modern condition of biological diversity in near-shore zone of Crimea (the Black sea sector)
Proshkina-Lavrenko A.I., 1955. Diatomovye vodorosli planktona Chernogo morya. M. –L.: AN SSSR, 1955. 222 c.
Reinhard, L.V. 1909. The Black Sea phytoplankton from the Kertch Bay, the Bosporus and Marmara Sea. Trud. Obstch. Ispit. Prir. Pr. Harkovsk. Univ. 13: 3-31.
Senicheva M.I., 2008. Species diversity, seasonal and annual variability of plankton microalgae near Crimea coast.// The Black Sea microalgae: problems of biodiversity preservation and biotechnological usage. NAS of Ukraine, Institute of Biology of the Southern Seas. – Sevastopol. 2008. – Ch. I p. 5-18
Senichkina L. G., Altukhov D. A., Kuzmenko L. V., Georgieva L. V., Kovaleva T. M., Senicheva M. I. (2001) Species diversity of Black Sea phytoplankton in the southeastern coast of Crimea. Karadag: History, biology, archaeology. Collection of papers dedicated to 85th anniversary of Karadag Scientific Station, Simferopol, Sonat, 119 - 125.
Zaitsev, Y. P. & B. G. Alexandrov, 1998. Black Sea Biological Diversity,Ukraine. Black Sea Environmental Series, Vol. 7. United Nations Publications, New York, 351 pp.

The number of information sources to include in the species list is unlimited and will be regularly updated upon the demand of the phytoplankton specialists.

Information sources types:
The information sources were divided into 5 types:

- List based on literature analysis
- List extracted from database
- List extracted from published paper, which is based on monitoring(scientific) research of scientist(s) and includes analysis of real collected data
- Personal list, based on monitoring(scientific research) of scientist (not published as article)
- Identification book

Important: for the moment the species list is composed by technically combining of different lists. Further check should be applied and set of flags should be used to mark doubtful for the Black Sea species.

Additional information attached to the species:

Phytogeography (genesis), ecological group, trophic status, harmful effect etc.

First record

The year of the first record and the name of the person who identified the species in the Black Sea with reference are included to the species page where available.

Occurrence

For each species the occurrence in national countries waters is added with corresponding references

Min, max, average volume

Min, average, max cell volumes (in cubic microns) were added to the species from databases and publications:

Cell volumes of phytoplankton of the Black Sea A.S.Mikaelyan, L.G.Senichkina, L.A.Pautova, L.V.Georgieva, V.Dyakonov

Bryantseva Yu., 2000. Changes in structural characteristic of phytoplankton in the Black Sea.

Dereziuk, N., 2008. List of Phytoplankton Species Observed in the Water Area near the Zmiinyi Island in 2004-2007

Bryantseva Yu., 2010. Sevastopol Bay phytoplankton monitoring database 2008 -2009

Biovolume shapes (*Bryantsteva Y., IBSS, Sebastopol, Ukraine*)

The most commonly used traditional biomass calculation method for microalgae is cell biovolume, which is calculated from measured linear dimensions under microsope. Automated or semiautomated methods are not yet fully developed, and conventional light-microscope-based biovolume estimates are still in use. Few attempts have been made to standardize the calculation of algal biovolume. As a result, different sets of equations were used by different researchers

The efforts have been made to create one reference list of biovolumes for Black Sea microalgae. Thus for each species in the checklist the appropriate suggested figure to calculate biovolume was attached. For detailed research of morphometric characteristics of the community the more precise figure is also suggested where possible.

For cross check of suggested volumes with other publications the shapes from the following publications are attached to each species:

Olenina, I., Hajdu, S., Edler, L., Andersson, A., Wasmund, N., Busch, S., Göbel, J., Gromisz, S., Huseby, S., Huttunen, M.,Jaanus, A., Kokkonen, P., Ledaine, I. and Niemkiewicz, E. 2006 Biovolumes and size-classes of phytoplankton in the Baltic Sea HELCOM Balt.Sea Environ. Proc. No. 106, 144pp.

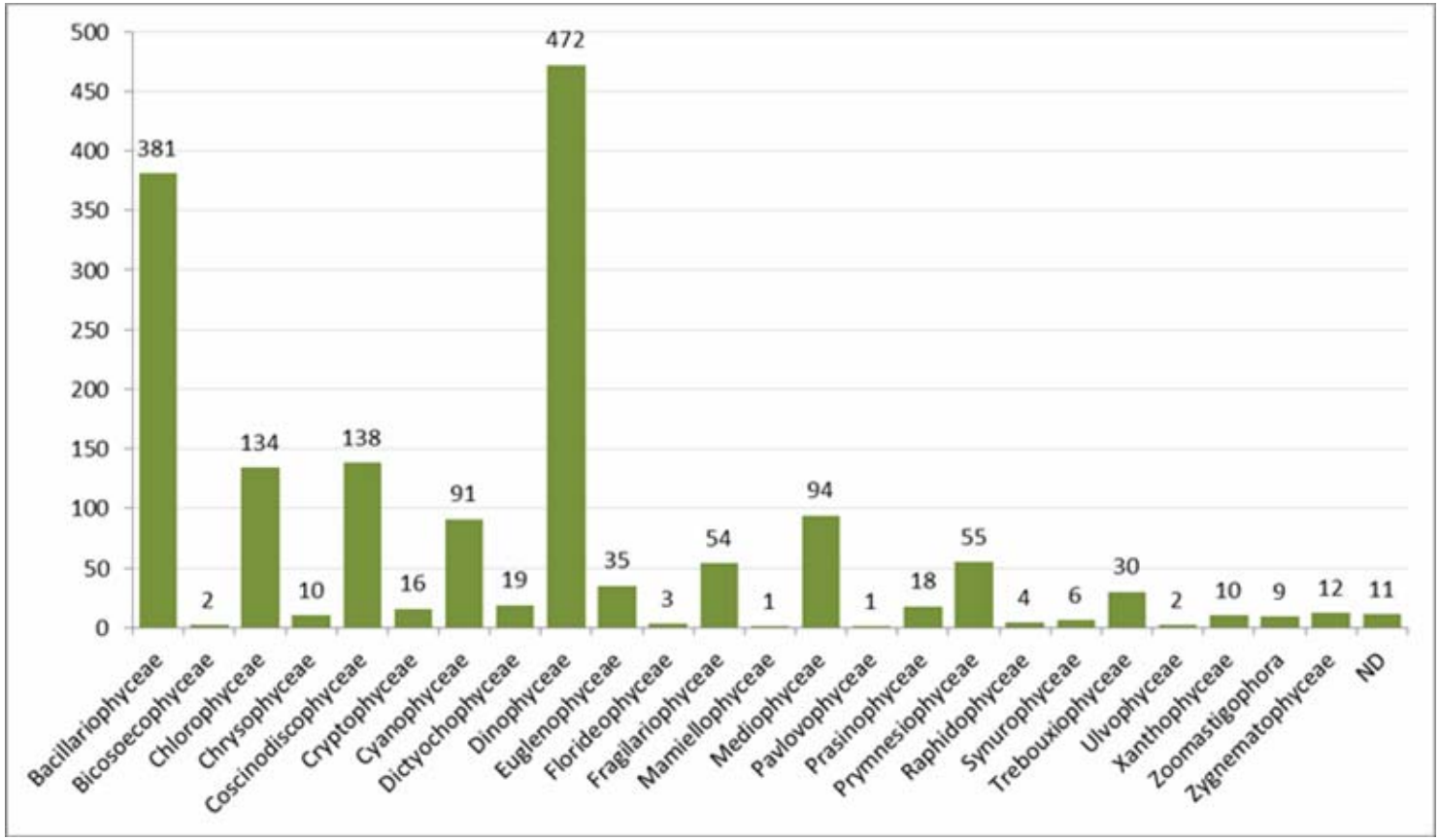
Vadrucci M.R., Cabrini M., Basset A. Biovolume determination of phytoplankton guilds in transitional water ecosystems of Mediterranean Ecoregion // Transitional Waters Bulletin. 2007 ; 2 : P. 83 — 102

Hillebrand, H., Dürselen, C. D., Kirschtel, D., Pollingher, D. and Zohary, T. (1999) Biovolume calculation for pelagic and benthic microalgae. J. Phycol., 35, 403–424

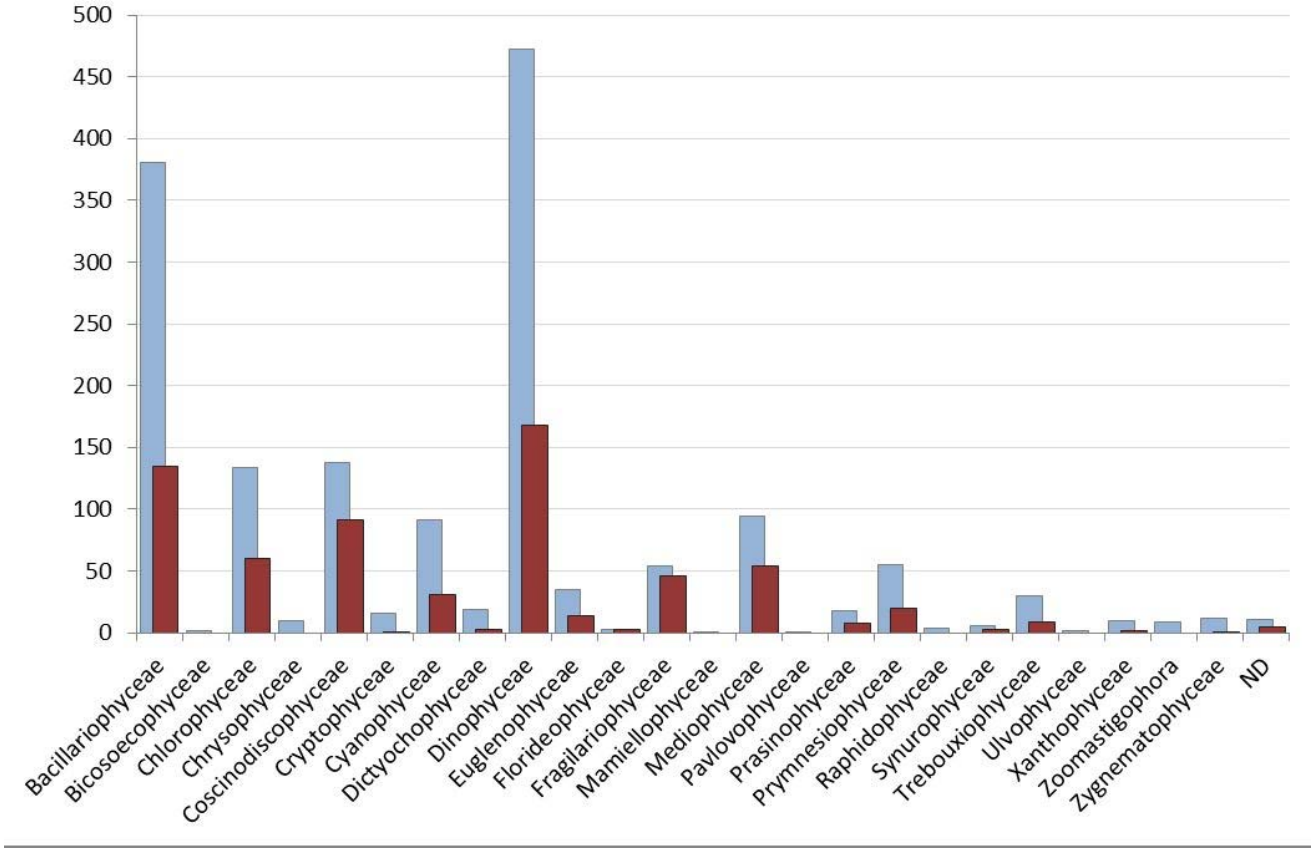
In the publications Vadrucci M.R. et al., 2007 and Hillebrand H., 1999 shapes are suggested at the genus level, not at species level, except those genera where species are highly varied in shapes.

So far in the Black Sea check list 1608 species from 24 classes are listed (Table, Figs bellow)

Class	N of species	%
Bacillariophyceae	381	23.68
Bicosoecophyceae	2	0.12
Chlorophyceae	134	8.33
Chrysophyceae	10	0.62
Coscinodiscophyceae	138	8.58
Cryptophyceae	16	0.99
Cyanophyceae	91	5.66
Dictyochophyceae	19	1.18
Dinophyceae	472	29.33
Euglenophyceae	35	2.18
Florideophyceae	3	0.19
Fragilariophyceae	54	3.36
Mamiellophyceae	1	0.06
Mediophyceae	94	5.84
Pavlovophyceae	1	0.06
Prasinophyceae	18	1.12
Prymnesiophyceae	55	3.42
Raphidophyceae	4	0.25
Synurophyceae	6	0.37
Trebouxiophyceae	30	1.86
Ulvophyceae	2	0.12
Xanthophyceae	10	0.62
Zoomastigophora	9	0.56
Zygnematophyceae	12	0.75



Number of species by classes in Black Sea phytoplankton checklist



Comparison of number of species by classes in Black Sea phytoplankton checklist (blue) and from Ivanov, 1965(red)

The species check-list is attached as a stand alone pdf file

Annex B. CONTROL CHARTS FOR BIOLOGICAL MEASUREMENTS

For quality control while measuring biological variables, the Shewhart charts (in this case: R-charts for duplicate samples), where the criteria for evaluation of testing results is based on statistically calculated values, are used. The main advantage of Shewhart charts is the possibility to monitor the testing process; nevertheless, there is still the disadvantage that an accepted statistical deviation may be greater than the maximum deviation set by the method. The **control chart** for duplicate samples can be constructed as follows: run one duplicate sample within every batch of samples. For phytoplankton, run every tenth sample or at least one sample per batch as duplicate, counting two subsamples from the same sample (approximately 10% of all samples). Calculate standard deviation SR from the following equation

$$S_R = \pm \sqrt{\frac{\Sigma(|\Delta x_i|/2)^2}{n-1}}, \text{ where}$$

n is the number of pairs of duplicate samples.

(SR can be calculated on the basis of testing results of duplicate samples taking into account at least ten analytical series.)

Plot the difference between testing results [Δxi]/2 *versus* time.

Plot the standard deviation SR *vs*. time. The construction of control charts can be done using any statistical software. The warning limit of the analysis precision is two standard deviations. It is recommended then to check the analytical procedure for avoiding further errors. The results of analysis are acceptable if the limit of three standard deviations is not exceeded.

The quality control chart is intended to identify changes in random or systematic error. The following criteria for out-of-control situations are recommended for use with Shewhart charts:

- 1 control value being outside the action limits (upper) UA and [lower] LA; or
- 2 consecutive values outside warning limits UW and LW; or
- 7 consecutive control values with rising tendency; or
- 7 consecutive control values with falling tendency; or
- 10 out of 11 consecutive control values being on one side of the central line.

Stochastic errors

The result of a measurement or assessment deviates from the true value because of the existence of a number of systematic and random errors. Particularly in biology important sources of random error are those introduced by the sampling and sub-sampling of biological items. These errors are stochastic by nature and should be considered separately. As one cannot do better than theoretical probability distributions predict, the calculation of these errors is an important tool in the design of biological and ecological studies.

Elimination of systematic errors

To check for systematic errors, several different trueness control samples are analyzed. To detect errors depending on the reagents or the method, control samples should be used whose concentrations cover the entire measuring range. As a minimum, a trueness control sample in the lower and one in the upper part of the working range should be used. In the event of a systematic error with results predominantly being higher or lower than the actual values, a step by step examination should by performed to find the reason for this bias. Exchanging experimental parameters, such as reagents, apparatus or staff, might help to identify quickly this type.

Improving precision
The precision can also be improved by a step-by-step approach to find the causes of random error. The total precision of an analytical method can be improved by examining its individual procedural steps to find the one which contributes most to the total error.

Plausibility control.
There could be errors which may not be detected by a statistical approach to quality control. In most cases, this concerns errors influencing individual analyses in a batch, but not ones before or after. This type of error can only be revealed by means of plausibility controls – checks on the observed value in relation to expectations based on previous knowledge. Such knowledge may be based on chemical consideration, for example checks on the equivalence of anions and cations in a sample, or on prior expectation, for example that COD will be greater than BOD. A successful approach to plausibility control requires that appropriate background information is available.

Data entry accuracy
Data are entered into the database. These data are checked for accuracy by a comparison of original records to entered data. Every entry must be reviewed to check that the required data has been recorded correctly. The total number of cells and units must be tabulated and written on the bench sheet. These numbers must be compared to entered data and a check mark placed in the designated line to verify that the totals have been checked. Any discrepancies between original and entered data must be rectified.

Interpretation
Reports should make a clear distinction between the objective reporting of data collected during the study, interpretation of these results, and the author's opinions. The basis of interpretation and opinions should be stated, and relevant authorities cited. Factors that may influence interpretation (e.g., unusual weather conditions) should also be stated. Ecological data are often processed into relatively simple classification schemes. In situations where such schemes are used, it is important that the risks of misclassification (based on errors and uncertainties in the method and data) are stated. This is particularly important where results lie close to class boundaries.

All reported data should be in standard digital formats.

Annex C. MEASUREMENT UNCERTAINTY

An abundance or composition estimate cannot be properly interpreted without knowledge of its uncertainty. Uncertainty of the final result of a microscopic analysis encompasses the uncertainties of the whole measurement process (sampling, stability, subsampling, homogeneity, identification, and quantification). A clear distinction should be made between quantitative and qualitative uncertainty. Qualitative uncertainty refers to mis- and non-identification of taxa. This part of uncertainty can only be dealt with in a general statement based on inter- (if available) and intra-laboratory comparisons. As yet there is no way to combine qualitative- and quantitative uncertainty estimates in one meaningful overall uncertainty. However, when a final assessment in the form of an index value is carried out it is possible to combine both qualitative and quantitative uncertainty on the level of the index. This should be part of an index sensitivity analysis.

Quantitative uncertainty

When it has been demonstrated by validation that the procedure of homogenization, subsampling and sedimentation has been brought into a state of statistical control, a meaningful uncertainty statement can be developed. Measurement uncertainty can be thought of as the sum of the intralaboratory reproducibility and the trueness. Trueness is difficult to assess as the true value is actually always unknown. When available, trueness may be derived from the deviation of the mean score in interlaboratory studies. Another approach can be obtained by using some other method as for instance SR-chamber or electronic particle counters as a reference method. Interlaboratory reproducibility can be assessed by recounting a representative set of samples under different relevant conditions.

Quantitative uncertainty is dependent on the abundance of the specific taxon in the sample. This means that in the same sample the uncertainty of the abundance estimate of one taxon might be smaller or greater than that from another taxon. In general the uncertainty will increase with a decrease in abundance.

$$X^2 = \frac{(x_{11} - n_1\hat{p}_1)^2}{n_1\hat{p}_1} + \frac{(x_{21} - n_1\hat{p}_2)^2}{n_1\hat{p}_2} + \ldots\ldots + \frac{(x_{k1} - n_1\hat{p}_k)^2}{n_1\hat{p}_k}$$
$$+ \frac{(x_{12} - n_2\hat{p}_1)^2}{n_2\hat{p}_1} + \frac{(x_{22} - n_2\hat{p}_2)^2}{n_2\hat{p}_2} + \ldots\ldots + \frac{(x_{k2} - n_2\hat{p}_k)^2}{n_2\hat{p}_k} \qquad (df = (N - 1)(k - 1))$$
$$+ \frac{(x_{1N} - n_N\hat{p}_1)^2}{n_N\hat{p}_1} + \frac{(x_{2N} - n_N\hat{p}_2)^2}{n_N\hat{p}_2} + \ldots\ldots + \frac{(x_{kN} - n_N\hat{p}_k)^2}{n_N\hat{p}_{k1}}$$

in which $\hat{p}_i = x_i' / n'$ and

$$\sum x_1^2 / n_1 = (x_{11}^2 / n_1) + (x_{12}^2 / n_2) + \ldots\ldots + (x_{1N}^2 / n_N)$$

As a general rule it holds that if random samples of size n are drawn from a non-normal population the distribution of the mean will tend towards normal as n increases in size. This so-called central limit theorem can be applied to Poisson series when n > 30 in which n is the number of grids and the mean number of a taxon or group per grid. *x x.* In this case estimation of confidence limits for the estimate of the mean is straightforward according to:

$$\bar{x} \pm t_{0,05(2),\nu} \sqrt{\frac{\bar{x}}{n}}.$$

In which n is number of grids/fields counted per chamber, $\bar{x}$ the mean number of a particular alga or group per grid/field and ν = n−1.

The confidence limits represent only the uncertainty in the estimation of the mean for a particular chamber. The reproducibility error should be added by counting different chambers etc. and then calculating the overall variance. Then the confidence limits can be calculated according to

$$\bar{x} \pm t_{0,05(2),\nu} \sqrt{\frac{s_p^2}{n+m}}.$$

in which ν = (n−1)+(m−1) n is number of grids/fields counted per chamber, m is the number of chambers and s_p^2 the overall variance which is the sum of $\bar{x}$ and the reproducibility variance. s_R^2.

This variance should encompass, if relevant, different analysts, days, chambers etc. When nm < 30 the simplest way is to cluster those taxa which do not fulfil the constraint of nm > 30 until nm > 30 and then calculate the measurement uncertainty for this clustered group as a whole. Otherwise a direct method for the calculations of the confidence limits for the poisson parameter can be applied. The best approach to calculate confidence limits for the total count irrespective of the number of grids or objects counted is:

Lower 1−α confidence limit $L_1 = \frac{\chi^2_{(1-\alpha/2),\nu}}{2}$

in which ν = 2X

Upper 1−α confidence limit $L_2 = \frac{\chi^2_{(\alpha/2),\nu}}{2}$

in which ν = 2(X+1)

and X = number of objects counted.

This approach for calculating the confidence limits for the Poisson parameter results in an asymmetrical confidence interval. When a significant part of a chamber has been screened (for instance for large diatoms or desmids) the Poisson series is still applicable. The recommended procedure to carry out is to calculate the confidence limits according to the above mentioned

method for calculating confidence limits for the Poisson parameter. To estimate the total number for the whole chamber the count and its confidence limits are multiplied by the ratio between sample and subsample size.

NOTE When dealing with colonies, it should be realized that the measurement uncertainty as calculated above, refers to the number of colonies and not to the number of cells. For the mean number of cells per colony for which the distribution pattern is unknown the central limit theorem should be applied. The number of cells in at least 30 replicate colonies should then be counted in order to estimate the mean.

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