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Report of the ICES/OSPAR/HELCOM Steering Group on Quality Assurance of Biological Measurements (STGQAB)

13–15 February 2007

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Conseil International pour l'Exploration de la Mer

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Contents

Executive summary

The STGQAB met between the 13th and 15th February 2007 at ICES headquarters in Copenhagen, Denmark. Eight members from 6 countries participated in the meeting. In addition Hanna Paulomäki, Finland, from the HELCOM Secretariat (13th to 14th of February) and Claus Hagebro, Denmark, from the ICES Secretariat, attended the meeting.

Please see ANNEX 4 for a summary list of the proposed recommendations.

On request from HELCOM the group reviewed the recently moved COMBINE guidelines for editorial changes. Hanna Paulomäki from the HELCOM Secretariat was present the first two meeting days to revise the documents on line.

The meeting also reviewed proposals of technical revision of phytoplankton and zooplankton guidelines respectively, and recommended actions by responsible expert groups.

The data validation guidelines were further developed and a common format for Standard Operating Procedures proposed. The meeting again requests that relevant working groups review the data validation guideline before being adopted to the COMBINE guidelines.

Due to the revision of the data validation guideline, a reorganization of the subchapters of part B, and subsequently also of part C and D, in the COMBINE guidelines was proposed.

It was noted that the recommendations for HELCOM MONAS had not been considered at its meeting 2006. Relevant recommendations from 2006 are therefore still provided also in this report (ANNEX 5).

The work done in various expert groups and workshops was reviewed and appreciated. It was suggested to establish connection with the new Planning Group for Phytoplankton and Microbial Ecology group. Under the BSRP-project the group working with phytobenthos was recommended to extend its members to all HELCOM countries before their proposal of revision of COMBINE guidelines is adopted.

An update of the JAMP guidelines is suggested in harmony with international standards and directives. Update is also recommended by linking JAMP guidelines with JAMP quality assurance documents. STGQAB also proposes to develop common guidelines for HELCOM and OSPAR in the future.

The results of a questionnaire regarding implementation of quality assurance guidelines were compiled. It was concluded that the implementation of the guidelines is not yet fully accomplished. Further work to make laboratories aware of the guidelines is recommended.

The members of STGQAB recommend that special quality assurance groups are maintained also in the future to secure the status of quality assurance and to promote confident environmental assessments from HELCOM and OSPAR.

1 Opening of the meeting

The meeting was opened by the chairman. The joint HELCOM/OSPAR/ICES group is chaired by Mr. Johan Wikner. Representatives from ICES, Finland, Germany, Norway, Russia, Sweden and The Netherlands were present at the meeting. For list of participants see ANNEX 1. Participants gave an introduction of their own area of expertise and experiences in biological QA work.

2 Adoption of the agenda

The chairman introduced the agenda and Terms of Reference (ToR) of the meeting. The agenda is prepared by the Chair based on the requests from HELCOM, OSPAR and ACME. The agenda was adopted with some minor changes (see ANNEX 2).

3 Appointment of a Rapporteur

Ms. Petra Schilling was appointed as the rapporteur.

4 Presentation of HELCOM Ecological Objectives

Ms. Hanna Paulomäki from the HELCOM Secretariat gave a short presentation on the HELCOM monitoring programmes and the Baltic Sea Action Plan (see ANNEX 6). The presentation introduced the concept and structure of the Action Plan as well as its linkages to the HELCOM monitoring activities. The presentation highlighted the importance of monitoring and quality of data in order to give scientifically sound policy advice for the decision makers.

5 Review and propose editorial changes on the moved and updated HELCOM COMBINE guidelines

Minor editorial changes were proposed and taken care of by the present representative from the HELCOM secretariat.

During the discussion on updating the HELCOM COMBINE guidelines it became clear that the structure of part C should be improved because the Annexes C-2 to C-10 are the most important descriptions of the analytical methods. Therefore a new structure of Chapter C.4 and Annexes C-2 to C-10 is recommended in ANNEX 7 of this report The Chapter C.4 should contain the following three subchapters instead of ANNEXES:

C.4.1	Hydrographic variables
C.4.2	Hydrochemical variables
C.4.3	Biological variables.

In the following this new structure is followed. In consequence a new numbering within the subchapters will be necessary. STGQAB recommends that this should take place via the HELCOM secretariat.

Generally all relevant documents of the HELCOM COMBINE manual should be made available as ".doc-file" (editable document) for experts and for storage in the archive. It is the responsibility of HELCOM secretariat to arrange it before the next revision will be undertaken.

5.1 Phytoplankton production

The meeting recommended a revision of the Annex C-5 Phytoplankton primary production (part C). It was proposed that this chapter should be included in part C as a subchapter C.4.3.3 Phytoplankton primary production (see ANNEX 7). A proposal for a revised guideline should be prepared for STGQAB for their next meeting in 2008. Sweden took on the responsibility as lead country.

5.2 Soft bottom macrozoobenthos

Minor editorial changes of the Annex C-8 Soft bottom macrozoobenthos (part C) were proposed and taken care of by the present representative from the HELCOM secretariat. It was proposed that this chapter should be included in part C as Subchapter C.4.3.7 Soft bottom macrozoobenthos (see ANNEX 7).

5.3 Phytobenthic plant and animal communities

A new title was suggested by STGQAB: Macrophytobenthos and associated macrofauna. It was proposed that this chapter should be included in Part C as a subchapter C.4.3.8 Macrophytobenthos and associated macrofauna (see ANNEX 7). Minor editorial changes were proposed and taken care of by the present representative from the HELCOM secretariat. STGQAB recommends changing the existing pdf-document into a word-document.

5.4 Coastal fish monitoring

A new title was suggested by STGQAB: Coastal fish. It was proposed that this chapter should be included in Part C as a subchapter C.4.3.9 Coastal fish (see ANNEX 7). STGQAB recommends changing the existing pdf-document into a word-document. It is recommended to check the coastal fish guideline by fish experts in the context of EU-WFD to take note of new developments and to update the existing guideline. ICES is asked to nominate the relevant ICES expert group to STGQAB.

5.5 Bacterioplankton growth

The updated bacterioplankton growth guideline is attached as ANNEX 8. As already recommended in STGQAB Report 2006 STGQAB endorsed the submission to HELCOM MONAS 10/2007 for adoption into the HELCOM COMBINE Guidelines Part C. It was proposed that this chapter should be included in Part C as a subchapter C.4.3.5 Bacterioplankton growth (see ANNEX 7).

5.6 Bacterioplankton abundance

The updated bacterioplankton abundance guideline is attached as ANNEX 9. As already recommended in STGQAB Report 2006 STGQAB endorsed the submission to HELCOM MONAS 10/2007 for adoption into the HELCOM COMBINE Guidelines Part C. It was proposed that this chapter should be included in Part C as a subchapter C.4.3.6 Bacterioplankton biomass (see ANNEX 7).

6 Review and propose on updated guidelines for Phytoplankton in the HELCOM COMBINE manual (ToR d)

After some years of searching for a reliable method of phytoplankton carbon estimation, the HELCOM Phytoplankton Expert Group (PEG) in 2005 agreed to apply the formulas of Menden-Deuer and Lessard (2000). PEG submitted a draft for a new carbon calculation chapter of the COMBINE Manual to the STGQAB meeting in 2006. A decision was postponed at that meeting. After a revision, this new method is qualified to replace the old method in the Manual.

A further revision became necessary with the adoption and publication of a new biovolume calculation file (Olenina *et al*., 2006), which has to replace the old strategy in the Manual. Consequently, PEG revised the complete section on "Phytoplankton species composition, abundance and biomass", including the biovolume and carbon calculation. It was proposed that this updated chapter should be included in Part C as a subchapter C.4.3.2 Phytoplankton species composition, abundance and biomass. The updated guideline is attached as ANNEX 10.

STGQAB reviewed and recommends revising the phytoplankton guideline according to the proposal by the HELCOM Phytoplankton Expert Group.

7 Review and propose on updated guidelines for Zooplankton in the HELCOM COMBINE manual

Germany presented the draft from Mr. Lutz Postel, Chair of HELCOM Zooplankton Expert Network (ZEN) of an updated Chapter 6.2 Biomass which is based on the development of carbon biomass conversion factors in respect to length/biomass determination of mesozooplankton (see ANNEX 11).

During the discussion on reviewing the guidelines for zooplankton the following recommendations were given:

1) Subchapter 6.1 Abundance
STGQAB recommends considering whether or not it is necessary to revise this subchapter by ZEN and WGZE and to provide a draft for the next meeting of STGQAB in 2008.

2) Subchapter 6.2. Biomass
The proposed method of carbon biomass determination (ANNEX 11) should be included in the zooplankton guideline. But ZEN should add an explanation of abbreviations of stages of development of table 3. STGQAB recommends to delete the example *Eurytemora longicornis* in Table 1, first line (see ANNEX 11) because this species is neither included in Table 2 nor in Table 3.
ZEN should also consider whether the previous text with the old methodology of measuring wet and dry weight of animals should nevertheless be kept for comparison purposes of historical data presented in ICES and/or other databases. If old data are required then it may be complemented with modern knowledge. Otherwise ZEN should give guidance for the use of the historical data based on the old methodology. A proposal should be provided before the next meeting of STGQAB in 2008.

3) Chapter 7 Data reporting
STGQAB recommends that ZEN should provide a table containing all necessary parameters to be reported to the ICES data centre. This table should be used as an example for the reporting protocol. STGQAB recommends that ZEN should cooperate with ICES data centre in this kind of matter. To evaluate the current database, extraction of zooplankton data from the ICES data base should be carried out and the quality of these data should be assessed.

4) Chapter 8 Quality Assurance
This chapter should be revised and clarified. The estimation of errors at the different stages of sampling and counting procedure has to be assessed as the range of maximum and minimum values. The sources of errors should be listed.

It was proposed that the updated zooplankton guideline should be included in Part C as a subchapter C.4.3.4 Mesozooplankton (see ANNEX 7). In this case a new structure and renumbering of the zooplankton guideline is necessary. STGQAB asked ZEN to provide a revised draft of zooplankton guideline for the next meeting of STGQAB in 2008.

8 Review and propose new data validation guidelines for the HELCOM COMBINE manual (ToR c)

For rapid finding of information by users it is desirable that guidelines are harmonized regarding quality and organization.

8.1 Reviewing proposed revision of Part B of the COMBINE manual

STGQAB was concerned that recommendations from STGQAB Report 2006 were not considered and commented by relevant ICES expert groups during 2006. It was stated that the recommendations from STGQAB Report 2006 were still valid and the group would appreciate comments from relevant ICES expert groups (STGQAC, MCWG, WGSAEM, WGMDM) on these matters before next HELCOM MONAS meeting in 2007. The ICES secretariat is asked to forward the recommendations to the relevant ICES expert groups for comments before the next HELCOM MONAS meeting so that STGQAB will be in a position to prepare a revised proposal before MONAS 10/2007.

STGQAB points out that there were different versions of HELCOM COMBINE manuals available on the Internet and it was not distinguishable which version was up to date. HELCOM secretary should ensure that the versions are dated with a clear referral to the current valid version.

A revised proposal for a new structure of Chapter B.4 is attached as ANNEX 12. ANNEX 15 contains the proposal of a revised Chapter B.4 based on the recommendations from STGQAB Report 2006. Because Annex B-8 up to Annex B-17 are technical notes on special parameters, they should be included in appropriate sections of Parts C and D (see ANNEX 13: Table of rearranging of Annexes of Part B). Because some Annexes of Part B should be relocated to Part D, also a revision of Part D is recommended. STGQAB has asked ICES to forward the proposals to the relevant ICES expert groups (STGQAC, MCWG, WGBEC).

8.2 Template for HELCOM guidelines

Mr. J. Wikner proposed a draft for a Standard Operating Procedure which can be useful for a description of chemical and biological analytical procedures (ANNEX 16).

STGQAB recommends adopting a standard format for the Standard Operating Procedures in the HELCOM COMBINE guidelines. The standard form should be placed in the HELCOM COMBINE guideline in Part B as a new Annex B-5 for Chapter B.2.4 “Documentation, Standard operating procedure”. A proposal of a revised Chapter B.2 is given in ANNEX 14. STGQAB asked STGQAC to notice and comment the draft for a Standard Operating Procedure and the revised Chapter B.2 before the next meeting of STGQAB in 2008.

9 Report from the last HELCOM MONAS meeting

9.1 Minutes of HELCOM MONAS 9/2006, Agenda Item 6

The meeting considered the minutes of the MONAS 9/2006 meeting and took note of the conclusions. Further STGQAB noticed that the MONAS 9/2006 did not consider or comment on the recommendations from STGQAB 2006 to HELCOM in the attachment to the MONAS 9/2006 document 6.3.2.

It was decided that some of the recommendations from 2006 are still valid. STGQAB requests that recommendations from 2006 are considered at the MONAS 10/2007 in the updated form of the report of 2007 STGQAB meeting (ANNEX 5).

10 Review the activities of the HELCOM Phytoplankton expert group (ToR a)

The Chair of the Phytoplankton Expert Group (PEG) Mr. N. Wasmund gave a report (see ANNEX 17) on the background and the aims of HELCOM PEG, the running HELCOM-PEG project and last years activities. The last workshop and training course on phytoplankton was held in Helsinki, 25–29 September 2006. It was attended by 32 participants (including guests). The training course concentrated on small dinoflagellates. The Terms of Reference of the workshop were determined by the project requirements, requests of MONAS and STGQAB to PEG and long-term tasks of the PEG.

The workshop focused on the following items:

- Update of the phytoplankton biovolume file,
- Update of the COMBINE manual
- Elaboration of an Indicator Fact Sheet
- Comparability of different sampling devices
- Regular update of the PhytoWin counting software

The latter point has urgently to be solved in the nearest future, as the PhytoWin counting software is not maintained by the software producer. The import of the updated biovolume file into the software cannot be done by the user himself, nor will it be done by the software producer unless properly paid by HELCOM.

STGQAB sees a possible solution in a public invitation to tender for buying or developing a new counting programme at a reasonable price.

11 Review the outcome of the ICES Phytoplankton workshop (ToR b)

Mr. Max Latuhihin gave a short report of an ICES workshop “Time Series Data relevant to Eutrophication Ecological Quality Objectives (WKEUT)” held in September 2006 in Tisvildeleje, Denmark. The aim of this workshop was to support the development of eutrophication-related Ecological Quality elements and EcoQO’s. For practical reasons the focus of this workshop was restricted to the analysis of phytoplankton time series. In doing so, the workshop addressed several questions on long-term trends in physical features, nutrients and phytoplankton behaviour. A wide variety of data was presented by 21 participants, coming from 12 different countries in Europe and North America. The length of the time series varied from 10 to 40 years.

Some highlights of the workshop outcome were:

- Effects of elevated nutrient levels and North Atlantic Oscillation index (NAO) patterns could be detected in many datasets but are not uniform, both gradual as well as sudden changes occur in a given dataset and differ between datasets
- In general there is no convincing evidence that harmful algal blooms, either in intensity or bloom species selection, are linked to eutrophication processes, to elevated nutrient concentrations or to altered nutrient ratios; Blooms of *Phaeocystis globosa* in the Belgian coastal waters and the Wadden Sea are possibly a unique exception to this finding
- Indicator species and communities having general application are not evident in the time series considered

- Although good correlations with NAO related changes were shown on a decadal scale, the datasets were found to be inadequate to evaluate long-term climate change.

A few recommendations with a relation to QA were:

- Continuation of collecting long-term time series is necessary in order to evaluate eutrophication and climate variations on phytoplankton processes in particular
- Statistics: close cooperation between biologists and statisticians is needed, analysis should be done using statistics appropriate to the intended use of the analysis
- Taxonomy: experienced taxonomists are required in order to provide good quality control of species identification
- Species coding needs to be standardised to facilitate migration of time series to a common data centre.

Full report of the workshop is available at http://www.ices.dk/iceswork/wgdetailace.asp?wg=WKEUT

Most of the analyses are to be published in the Journal of Sea Research, planned to be issued during the second half of 2007. Work will continue in a newly established ICES Planning Group for Phytoplankton and Microbial Ecology (PGPYME).

STGQAB recommends establishing a link to this group on the field of QA/AQC matters and asks the ICES secretariat to undertake the necessary steps.

12 Review the activities of the HELCOM MONAS Zooplankton Expert Network (ZEN)

STGQAB took note of the activities from the HELCOM Zooplankton Expert Network and recommends continuing the activities of this HELCOM group.

The Second HELCOM ZEN Workshop took place onboard RV "Aranda" from 14–19 August 2006 with 10 participants from Finland, Estonia, Latvia, Poland, Germany and Sweden. During the Workshop, under field conditions, small modifications of the sampling procedure were tested and their potential influences on the results were discussed. A procedure for the planned ring test checking the accuracy of the analysis procedures and the taxonomic skills of the participating laboratories was discussed.

There are some national activities in zooplankton assessments, e.g. German compilation of changes in the Baltic Sea zooplankton since the 1950s that could be used for the preparation of a disciplinary thematic assessment of zooplankton in the Baltic Sea and data access for this task will be investigated. The valuable long-time series of zooplankton was discussed and the importance of their continuing within the EU-WFD and EU-Marine Strategy strongly underlined. ZEN cooperates with the ICES WGZE in preparation of the Annual status report of the North Atlantic. ZEN supports the SCOR WG 125 on Global Comparisons of Zooplankton Time Series. ZEN and BMB WG 29 work together in preparation of the Mesozooplankton Atlas of the open Baltic Sea which will be published at the end of 2007. The ratios of length to carbon (carbon mass factors for mesozooplankton taxa of the Baltic Sea) were presented and first proposals for the revision of the biomass part of the zooplankton guideline were prepared by ZEN.

13 Report from the Annual Science Conference of ICES 2006

None of the participating members of the group had information from the Annual Science Conference 2006 to share.

14 Report from last years OSPAR meetings (SIME, ASMO)

The ICES representative Mr. Claus Hagebro presented the minutes of last years OSPAR meetings in SIME and ASMO. The meeting noted the comments from SIME regarding the lack of satisfactory answers to the requests in the OSPAR 2006 ICES work programme. In addition to the comments made by SIME, some guidance had been received from the OSPAR Secretariat before the STGQAB meeting.

15 Review and update the JAMP guidelines for benthos, phytoplankton and chlorophyll-a according to good current practice and/or international standards for acceptability of biological sampling and analytical practices required by monitoring programmes (ToR e)

There is a need for a general update of the guidelines since they do not meet current QA-standards. However, review and update of the JAMP guidelines is a major task and the suggestion from STGQAB is that this work should be done by experts in the light of ISO/CEN standards on biological surveys, some of them still drafts, and that it also should consider the final outcome of the ongoing work of the North Eastern Atlantic-Geographic Intercalibration Groups (NEA-GIGs) in the context of the EU-WFD. June 2007 is the deadline for the protocols from these groups. The exercise and its results are described in the Intercalibration Report – Coastal GIGs, September 2006 (Jowett, 2006). The Norwegian Quality Index, NQI (Index 1, Rygg, 2006) for benthic invertebrates was applied in the NEA-GIG intercalibration exercise.

Further documentation on the geographic intercalibration work can be downloaded from: http://forum.europa.eu.int/Public/irc/jrc/jrc_eewai/library?l=/milestone_reports/milestone_june_2006&vm=detailed&sb=Title and more additional informative documents are:

- Rygg, B. 2006. Developing indices for quality-status classification of marine soft-bottom fauna in Norway. Norwegian Institute for Water Research. NIVA-report OR-5208. 33pp.
- Jowett, D. 2006. Milestone 6 Report – Coastal GIGs. European Commission, Directorate General, Jrc - Joint Research Centre, Institute of Environment and Sustainability. 47pp.

STGQAB recommends updating the JAMP guidelines for benthos, phytoplankton and chlorophyll-a, by including or pointing to the critical quality assurance factors and priority QA actions that are tabulated in document 2002-15 (Appendix 1, tables 1 – 5 in: “JAMP guidelines on quality assurance for biological monitoring in the OSPAR area”).

Further, STGQAB recognizes that there is a common need for updated guidelines in HELCOM and OSPAR, and that this could be handled in cooperation between the two commissions.

STGQAB recommends that the secretariats of OSPAR and HELCOM together take necessary initiatives to start a process towards a coordinated update and harmonisation of guideline documents, taking into account the development combined with the EU WFD and the Marine Strategy. STGQAB will be the natural body entitled to comment on the draft guidelines.

16 Review and evaluate the status of implementation and the practical use of OSPAR/ICES quality assurance guidelines in marine monitoring and assessment programmes in the

OSPAR/ICES area and provide guidance for future assessment programmes (ToR f)

Evaluation of the status of implementation of quality assurance guidelines has now been finalised on the basis of the outcome of the Questionnaire that OSPAR circulated under the OSPAR contracting parties (Annex 18). A compilation of the questionnaire is presented in ANNEX 19.

The following observations were made when comparing the results:

- Response was achieved from eight countries: France, Germany, the Netherlands, Northern Ireland, Norway, Portugal and Spain
- A total of 10 laboratories filled in the questionnaire, for most of the countries just one lab each, except for Northern Ireland (4)
- All responding laboratories were governmental or non-profit, no commercial organisation answered the questionnaire
- It is unknown how many labs received a questionnaire

STGQAB observed that for – at least – some of the responding countries more than one lab received the questionnaire. In many countries several labs are involved in national monitoring programmes. STGQAB noticed that at least one lab of the responding countries did not receive the questionnaire.

Almost all of the responding labs (9 out of 10) contribute to chemical monitoring, 8 out of 10 also contribute to macrobenthos and phytoplankton measurements. There’s a small contribution of the respondents to ecotoxicology, coastal fish and zooplankton (4 out of 10) and to phytobenthos (2 out of 10). All responding labs take part in one or more types of QA/AQC activities, at least in between laboratory exercises, but also on an international level. JAMP guidelines are in use by many of the respondents (9 out of 10). The HELCOM COMBINE manual and ICES Times documents seem less used in the OSPAR area (1 out of 10 and 3 out of 10 labs respectively). Some laboratories are aware of all guidelines mentioned (concerning biological measurements), some of them just partially. Labs that contribute to a particular field should be aware of the relevant guidelines, which is not always the case.

STGQAB recognizes that, due to the limited response, this questionnaire just gives a partial view on the current status of implementation of the guidelines on a national and international level.

STGQAB concludes that implementation and practical use of OSPAR/ICES guidelines is not yet fully accomplished. Many countries do have a certain level of QA/AQC. But, in particular when it comes to biological measurements, laboratories are sometimes not aware of the relevant guidelines. Therefore it is necessary to take further steps to inform labs about the existence of available guidelines.

STGQAB noted that no further action respectively information is observed from the BEQUALM secretariat concerning the collection of information on QA schemes operating across Europe (recommendation 15 of STGQAB 2006).

17 Review the progress with, and offer further advice on the development of QA of biological measurements in relation to OSPAR JAMP products (ToR g)

With reference to the SGQAE report 2005 STGQAB discussed this item and confirmed that more guidance from OSPAR is needed to clarify the expected role of STGQAB. With respect to measurements of phytoplankton in general more attention is paid under Agenda Item 15.

18 Review the outcome of the Water Framework Directive intercalibration exercise with respect to biological quality elements and assess this as the basis for ongoing quality assurance of biological measurements within OSPAR (ToR h)

Only a draft of WFD intercalibration technical report, Part 3 – Coastal and Transitional Waters, was available at the meeting because the GIG meeting takes place in parallel. Therefore STGQAB decided to review the outcome of the intercalibration exercises with respect to biological quality elements during the next meeting in 2008.

19 Any other business

STGQAB noticed the absence of some OSPAR Contracting parties who have been attending this meeting for the past years and expressed their concern for this. STGQAB stated that it is important that HELCOM and OSPAR Contracting parties ensure participation of experienced experts from the contracting parties in the STGQAB meetings; otherwise the group is not able to respond adequately to all the requests allocated to the group. With respect to OSPAR matters, so far no Co-Chair from OSPAR has been nominated. This person could fulfil a facilitating role in that context..

19.1 Statement for the future existence of STGQAB

The STGQAB members recommend ICES to continue the Steering Groups for quality assurance issues.

Continuation of the quality assurance Steering Groups will secure that quality assurance issues are given sufficient attention in the ICES, HELCOM and OSPAR organizations. These groups also promote a similar level of quality being achieved for quality elements (indicators) across disciplines and sea areas.

If these issues were to be distributed to other working groups with broader objectives, quality issues might be set aside. This could undermine the confidence for environmental assessments from ICES, HELCOM and OSPAR in the future.

The groups may be viewed as important referees of the work by other working groups in the mentioned organizations. The STGQAB and STGQAC thereby foster a regular feature of the important quality assurance work.

For the future the STGQAB members foresee that quality routines regarding analytical precision (international standards, guidelines, accreditation of laboratories) will have developed to minimize uncertainty from this level of information. Instead more of the resources of these groups may be allocated to reduce uncertainties by proper design of monitoring programmes, routines and techniques for data evaluation, compilation of quality indices and overall assessments.

19.2 Revised guidelines for phytobenthos

Mr. Georg Martin, Estonia, provided a short report from the Baltic Sea Regional Project (BSRP) group for phytobenthos monitoring by E-Mail (see ANNEX 20).

The second meeting of the BSRP phytobenthos group was organised in May 2006 in Saaremaa Island, Estonia. At this meeting the scheme used in the previous meeting was used (combination of lectures and actual field exercise). The main focus was on application of under water video techniques for monitoring and mapping of phytobenthic communities.

Sixteen participants from BSRP recipient countries and Finland took part in this meeting. Different available under water video techniques were studied and tested in the field against actual coverage estimations made by scuba divers.

Main outcome of the meeting was:

- The current substrate coding system presented in the HELCOM Phytobenthos Guidelines should be revised and is not suitable for use with under water video methods. The system should be developed covering the full range of ecologically significant substrate types and applicable in different areas of the Baltic Sea
- Taxonomic knowledge of phytobenthos species should be increased in many laboratories. For this purpose international effort should be taken in form of taxonomic workshops (as an example of regular taxonomic workshops of HELCOM Phytoplankton expert group)
- Underwater video systems presented at the workshop are useable for the purpose of habitat mapping and could be used in the combination with scuba and other quantitative methods
- Further intercalibration of mapping techniques should be done actually in the field during common field work

The BSRP group for phytobenthos monitoring propose STGQAB to

- Establish the project group covering the development of methods and coordination of phytobenthos monitoring activities in the Baltic Sea area
- Ensure further development of HELCOM COMBINE guidelines in accordance with latest developments in phytobenthos monitoring techniques and methods
- Update the existing HELCOM COMBINE guidelines according to the proposal made by BSRP lead laboratory Phytobenthos Monitoring

STGQAB notes that a recommendation to establish a phytobenthos monitoring group was made to the responsible organizations already in 2006. The current BSRP group is acknowledged for its important work to improve the guidelines, which are in line with the Water Framework Directive. However, the group is asked to extend its members also to other HELCOM countries prior to being mandated to revise the current guidelines. Establishment of a formal expert group of phytobenthos monitoring will be the way to ensure development of HELCOM COMBINE guidelines.

STGQAB recommends submission of a proposal for revised guidelines for phytobenthos from an extended HELCOM phytobenthos expert group well before its next meeting in February 2008.

In the future STGQAB in general recommends that working groups for indicators are established as joint groups of both HELCOM and OSPAR to promote the development of common guidelines, based on international standards and the development following the Water Framework Directive and the EU Marine Strategy.

20 Date and Venue of the next meeting

STGQAB suggested the next meeting should take place at the ICES headquarters from February 5th -7th 2008.

21 Adoption of a draft report

The draft report was adopted on 22nd February 2007.

22 Closing of the meeting

The meeting was closed at 17:00 o'clock on 15th February 2007.

Annex 1: List of participants

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Annex 2: Agenda

Meeting of the ICES Steering Group of Quality Assurance of Biological Measurements.

ICES Main office, H.C. Andersen Blvd. 44-46, Copenhagen, Denmark

13-15th of February 2007. Meeting starts at 8:30 Tuesday 13th of January.

Chair: Johan Wikner Co-Chair: Pending

Agenda

1) Opening of the meeting

2) Adoption of the Agenda

3) Appointment of a Rapporteur

4) Presentation of HELCOM Ecological Objectives, Hanna Paulomäki

5) Review and propose editorial changes on the moved and updated HELCOM COMBINE guidelines
    5.1 Phytoplankton production
    5.1.1 Measuring protocol figure for primary production to Annex C-5.
    5.2 Soft bottom makrozoobenthos
    5.3 Phytobenthic plant and animal communities
    5.4 Coastal fish monitoring
    5.5 Bacterioplankton growth
    5.6 Bacterioplankton abundance

6) ToR d): Review and propose on updated guidelines for Phytoplankton in the HELCOM COMBINE manual
    6.1 Revised phytoplankton guideline

7) Review and propose on updated guidelines for Zooplankton in the HELCOM COMBINE manual
    7.1 Revised mesozooplankton guideline

8) ToR c): Review and propose new data validation guidelines for the HELCOM COMBINE manual
    8.1 Reviewing proposed revision of part B of the COMBINE manual
    8.2 Template for HELCOM biology guidelines

9) Report from the last HELCOM MONAS meeting
    9.1 HELCOM MONAS 9 2006: Minutes of HELCOM MONAS 9_2006, Agenda Item 6

10) ToR a): Review the activities of the HELCOM Phytoplankton expert group

11) ToR b): Review the outcome of the ICES Phytoplankton workshop.

12) Review the activities of the HELCOM MONAS Zooplankton Expert Network (ZEN)

13) Report from the Annual Science Conference of ICES 2006

14) Report from last years OSPAR meetings
    14.1 SIME
    14.2 ASMO

15) ToR e): Review and update the JAMP guidelines for benthos, phytoplankton and chlorophyll-a according to good current practice and/or international standards for acceptability of biological sampling and analytical practices required by monitoring programmes.

16) ToR f): Review and evaluate the status of implementation and the practical use of OSPAR/ICES quality assurance guidelines in marine monitoring and assessment

programmes in the OSPAR/ICES area and provide guidance for future assessment programmes.

17) ToR g):Review the progress with, and offer further advice on the development of QA of biological measurements in relation to OSPAR JAMP products

18) ToR h): Review the outcome of the Water Framework Directive intercalibration exercise with respect to biological quality elements and assess this as the basis for ongoing quality assurance of biological measurements within OSPAR.

19) Any other business.
    19.1 Statement for the future existence of STGQAB
    19.2 Revised guidelines for phytobenthos

20) Date and venue of the next Steering group meeting.

21) Adoption of a draft report

22) Closing of the meeting

Annex 3: STGQAB terms of reference for the next meeting

The **Steering Group on Quality Assurance of Biological Measurement** [STGQAB] (Chair: J. Wikner, Sweden) will meet in Copenhagen, Denmark from 5–7th February 2008 to:

a) review the activities of the HELCOM Phytoplankton expert group

b) review a proposal of revised HELCOM COMBINE guideline for Phytoplankton primary production

c) review the outcome of the HELCOM Zooplankton Expert Network

d) review and propose on updated guideline for Zooplankton biomass determination in the HELCOM COMBINE guideline

e) review the outcome from other ICES Expert groups to the proposal of data validation guidelines for the HELCOM COMBINE guideline

f) review a proposal for revision of the phytobenthos guideline from the BSRP Phytobenthos work group

g) review and propose on updated JAMP guidelines for benthos, phytoplankton and chlorophyll a for OSPAR

h) review international and national interlaboratory comparisons and propose measures as required

i) review the status of quality assurance in relation to Water Framework Monitoring Programmes and review the outcome of the intercalibration exercises with respect to biological quality elements

j) maintain a watching brief on developments of the European Marine Strategy in relation to Quality Assurance

k) review relevant studies in OSPAR/HELCOM participating countries in relation to Quality Assurance

STGQAB will report by DATE 2008 to the attention of the ACME and the Marine Habitat and Oceanography Committees.

Supporting Information

PRIORITY:	HIGH
Scientific justification and relation to action plan:	Terms of Reference a) - f), h) These items are of utmost importance for quality assurance and quality control in the HELCOM/OSPAR monitoring and assessment scheme, which is under harmonisation with EU WFD requirements and in future with EU Marine Stratgy. This will involve the review of received material and following acceptance in order to maintain and increase the level of QA/AQC in the COMBINE programme. Terms of Reference g) - h) These items focus on QA/AQC requirements in the OSPAR monitoring and asessment area. There is an urgent need to improve the implementation and the practical use of OSPAR/ICES quality assurance guidelines. Terms of Reference i) – j) These items focus to QA/AQC reqirements of European Directives (EU-WFD, EU Marine Strategy) to ensure the harmonisation of HELCOM and OSPAR monitoring schemes.
Resource requirements:	
Participants:	Scientist from ICES Member Countries and HELCOM/OSPAR Contracting Parties, involved in QA of monitoring of biological variables. The Group is normally attended by some 10–15 members and guests.
Secretariat facilities:	Meeting room and secretary assistance is required
Financial:	No financial implications.

Linkages to advisory committees:	ACME, ACE
Linkages to other committees or groups:	STGQAC, MCWG, WGZE, ZEN, PGPYME, PEG, BEWG, SGNSBP, WGECO, WGDIM, WGSAEM, MHC, OCC, Baltic Committee
Linkages to other organizations:	HELCOM, OSPAR, EU
Secretariat marginal cost share:	

Annex 4: Recommendations

Outstanding recommendations from STGQAB 2007.

RECOMMENDATION	ACTION
To HELCOM.	
1. STGQAB recommends a reorganisation and a new numbering of the sub-chapters of part C in the COMBINE guideline (Agenda Item 5, ANNEX 7).	HELCOM MONAS, HELCOM secretariat
2. STGQAB recommends revising the guidelines of phytoplankton primary production guideline in the HELCOM COMBINE guideline part C (Agenda Item 5.1). Sweden is asked to provide a proposal for the next meeting of STGQAB in 2008.	HELCOM MONAS, Chair of STGQAB
3. STGQAB recommends implementing the guidelines of Bacterioplankton growth and Bacterioplankton abundance in the HELCOM COMBINE guideline part C (Agenda Item 5.5 and 5.6, ANNEX 8 and 9).	HELCOM MONAS
4. STGQAB recommends revising the phytoplankton guideline in the HELCOM COMBINE guideline part C according to the proposal by the phytoplankton expert group (PEG)(Agenda Item 6, ANNEX 10).	HELCOM MONAS
5. STGQAB recommends revising the text and the content of the Zooplankton guideline by the Zooplankton Expert Network group in collaboration with WGZE, and provide a draft for the next meeting of STGQAB in 2008 (Agenda Item 7, ANNEX 11).	HELCOM MONAS and Chairs of the HELCOM Zooplankton Expert Network and of WGZE
6. STGQAB recommends that HELCOM secretary should ensure that the versions of HELCOM COMBINE manual are dated with a clear referral to the current valid version (Agenda Item 8.1).	HELCOM MONAS, HELCOM secretariat
7. STGQAB recommends a general revision of part B of the HELCOM COMBINE guideline, following a review by other expert groups (Agenda Item 8.1 and 8.2, ANNEX 12, 13, 14, and 15 and 16).	HELCOM MONAS, All members of STGQAB, STGQAC, MCWG, WGSAEM, WGDIM
8. STGQAB recommends a general revision of part D of the HELCOM COMBINE Manual (Agenda Item 8.1 and 8.2, ANNEX 13).	HELCOM MONAS, all members of STGQAB, STGQAC, MCWG, WGBEC, WGDIM
9. STGQAB recommends adopting a common content and format for Standard Operating Procedures (Agenda Item 8.2, ANNEX 16).	HELCOM MONAS and Chair of STGQAC
10. STGQAB recommends securing availability of phytoplankton counting software (Agenda Item 10).	HELCOM MONAS and Chair of the HELCOM Phytoplankton Expert Group
11. STGQAB recommends reviewing the outcome of the intercalibration exercises with respect to biological quality elements during the next	HELCOM MONAS

meeting in 2008 (Agenda Item 18).	
12. STGQAB recommends submission of a proposal for revised guidelines for phytobenthos from extended HELCOM expert group well before next STGQAB meeting in February 2008 (Agenda Item 19.2).	HELCOM MONAS and Chair of the group for phytobenthos monitoring BSRP
13. STGQAB recommends establishing a joint ICES/HELCOM/OSPAR expert group on phytobenthos (Agenda Item 19.2).	HELCOM MONAS, OSPAR, ICES and Chair of the group for phytobenthos monitoring BSRP
To OSPAR	
14. STGQAB recommends updating the JAMP guidelines for benthos, phytoplankton and chlorophyll-a by including or linking the critical quality assurance factors and priority QA actions to existing documents in JAMP (Agenda Item 15)	OSPAR, ICES and Chair of BEWG and new established PGPYME
15. STGQAB recommends that the secretariats of OSPAR and HELCOM together take necessary initiatives to start a process towards a coordinated update and harmonisation of guideline documents (Agenda Item 15).	OSPAR, HELCOM, ICES
16. STGQAB recommends informing laboratories of the available quality assurance guidelines (Agenda Item 16, ANNEX 18).	OSPAR
17. STGQAB recommends reviewing the outcome of the intercalibration exercises with respect to biological quality elements during the next meeting in 2008 (Agenda Item 18).	OSPAR
18. STGQAB recommends establishing a joint ICES/HELCOM/OSPAR expert group on phytobenthos (Agenda Item 19.2).	OSPAR, HELCOM, ICES and Chair of the group for phytobenthos monitoring BSRP
To ICES	
19. ICES is asked to nominate a relevant ICES expert group to revise the coastal fish guideline (Agenda Item 5.4).	ICES
20. STGQAB asks ICES to forward the proposals of reviewing of HELCOM COMBINE guidelines Parts B, C and D to HELCOM and the Chairs of relevant ICES expert groups before their next meetings and to provide STGQAB with comments from these expert groups in good time before MONAS 10/2007 to ensure providing MONAS with an updated proposal (Agenda Item 8.1).	ICES
21. STGQAB recommends establishing a link to the Planning Group for Phytoplankton and Microbial Ecology group (PGPYME) on the field of QA/AQC matters (Agenda Item 11).	ICES, Chair of new established PGPYME
22. The STGQAB members recommend ICES to continue the ICES/HELCOM/OSPAR Steering Groups for quality assurance issues (Agenda Item 19.1).	ICES

Annex 5: Outstanding recommendations from STGQAB report 2006

Outstanding recommendations from STGQAB 2006 that were not considered by HELCOM MONAS 9.

RECOMMENDATION	ACTION
To HELCOM	
1. STGQAB recommends HELCOM to include chapters on bacterioplankton(biomass and growth) in COMBINE manual part C.	HELCOM MONAS
2. STGQAB recommends HELCOM to consider the establishment phytobenthos expert/project group.	HELCOM MONAS
3. STGQAB recommends HELCOM to consider to change the status of zooplankton to mandatory in the MON-PRO scheme	HELCOM MONAS and MON-PRO
4. STGQAB recommends to consider to change the status of bacterioplankton biomass, bacterioplankton growth and primary production to optional parameters in MON-PRO scheme.	HELCOM MONAS and MON-PRO

Annex 6: Presentation of HELCOM Ecological Objectives by H. Paulomäki

HELCOM monitoring and the Baltic Sea Action Plan

Hanna Paulomäki
HELCOM

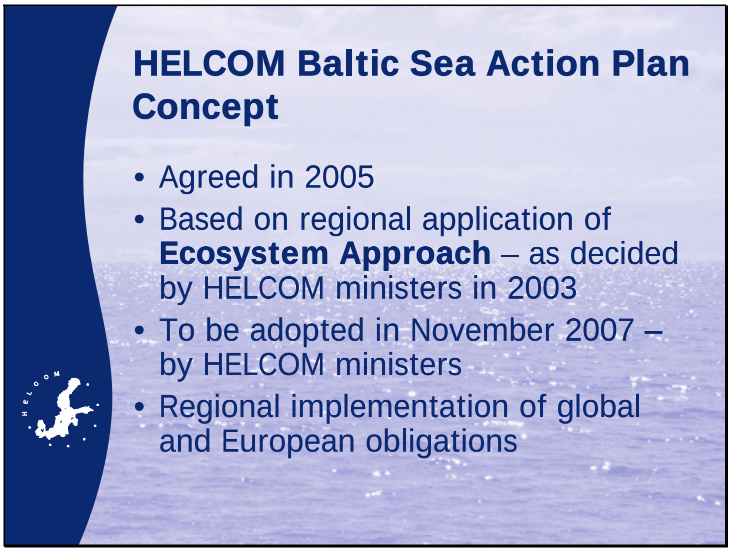
Contents

HELCOM Baltic Sea Action Plan

- Concept
- Ecological Objectives
- Progress

Relation to COMBINE

- Ongoing assessments
- Indicators



HELCOM Baltic Sea Action Plan Concept

- Agreed in 2005
- Based on regional application of **Ecosystem Approach** – as decided by HELCOM ministers in 2003
- To be adopted in November 2007 – by HELCOM ministers
- Regional implementation of global and European obligations

BSAP Concept

- Overall vision and strategic goals
- Ecological objectives
- Indicators and targets
- Concrete actions identified to achieve agreed objectives

Progress

First Stakeholder Conference

- March 2006 – adoption of ecological objectives

Four priority areas

- Lead Country outlines
- Expert Workshops; indicators and targets
- Ad hoc Task Force meetings; actions

Second Stakeholder Conference

March 2007 – first draft HELCOM Baltic Sea Action Plan

VISION: A healthy Baltic Sea environment, with diverse biological components functioning in balance, resulting in a good ecological status and supporting a wide range of sustainable human economic and social activities

- HELCOM Ecological Objectives
  - Describing key issues within each of our four environmental priority areas
    - Reflect central ecosystem functions
    - Highlighting topics of common concern

Goals and Ecological Objectives

Goals

- Baltic Sea unaffected by eutrophication
- Favourable status of Baltic Sea biodiversity
- Baltic Sea life undisturbed by hazardous substances
- Maritime activities carried out in an environmentally friendly way

Objectives

- Concentrations of nutrients close to natural levels
- Clear water
- Natural oxygen levels
- Natural level of algal blooms
- Natural distribution and occurrence of plants and animals

- Natural landscapes and seascapes
- Thriving and balances communities of plants and animals
- Viable populations of species

- All fish safe to eat
- Healthy wildlife
- Concentrations of hazardous substances close to natural levels
- Radioactivity at pre-Chernobyl level

- No illegal pollution
- Safe maritime traffic without accidental pollution
- Efficient response capacity
- No introduction of alien species
- Minimum air pollution
- Zero discharges from offshore platforms

Content of the BSAP

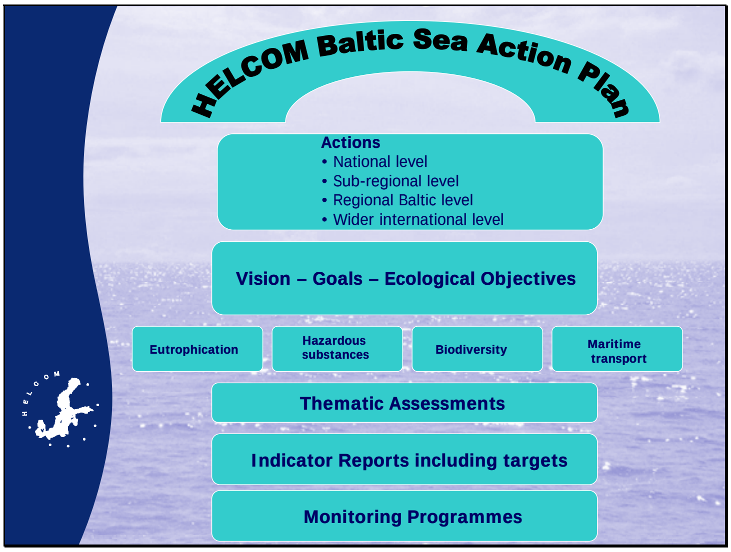
Actions, based on:

- Ecological state we want
- Cost-effectiveness

Actions, to be taken at:

- Sector/plant level
- international, regional, national level

Timetable and review mechanism



HELCOM Baltic Sea Action Plan

Actions
- National level
- Sub-regional level
- Regional Baltic level
- Wider international level

Vision – Goals – Ecological Objectives

Eutrophication | **Hazardous substances** | **Biodiversity** | **Maritime transport**

Thematic Assessments

Indicator Reports including targets

Monitoring Programmes

HELCOM monitoring and assessment strategy

- Adopted in 2005
- how the contracting States commit themselves to carry out their national monitoring programmes and work together to produce joint assessments

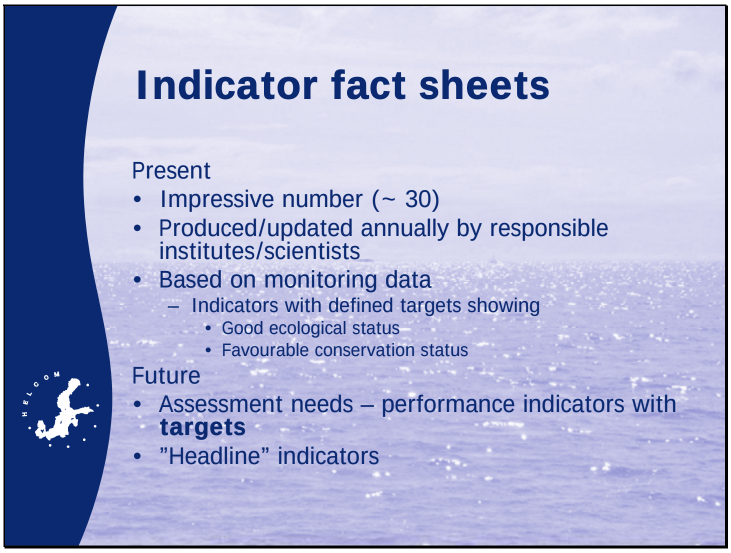
HELCOM monitoring programmes

- PLC-Air and PLC-Water
  - Airborne and waterborne sources and inputs;
- **COMBINE**
  - the state, impacts and changes in marine environment
  - water, biota including coastal fish, and sediment, physical forcing;
- MORS
  - sources and inputs of artificial radionuclides
  - state and changes in the marine environment;
- Surveillance of deliberate illegal oil discharges
- Inventory of marine accidents
- Transport statistics - AIS

Relation to BSAP

Ongoing holistic and integrated assessments

- Hazardous substances by 2007
- Eutrophication by 2008
- Biodiversity and nature protection 2008/09
- Maritime activities



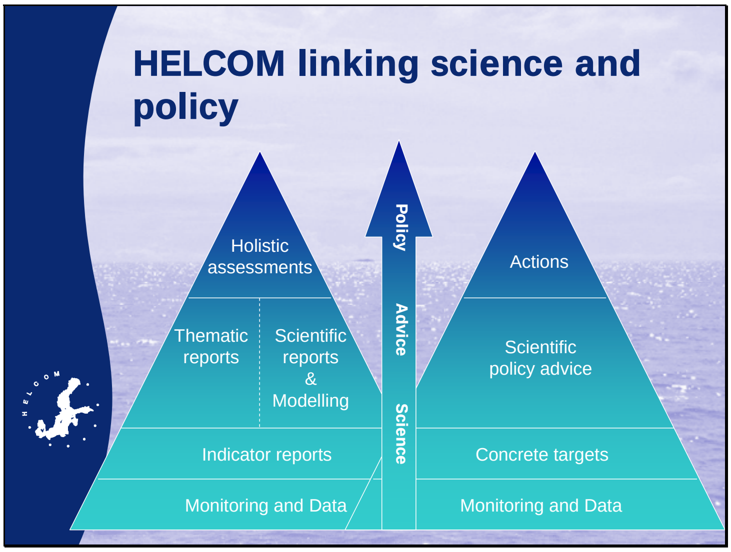
Indicator fact sheets

Present

- Impressive number (~ 30)
- Produced/updated annually by responsible institutes/scientists
- Based on monitoring data
  - Indicators with defined targets showing
    - Good ecological status
    - Favourable conservation status

Future

- Assessment needs – performance indicators with **targets**
- ”Headline” indicators



HELCOM linking science and policy

Holistic assessments

Thematic reports

Scientific reports & Modelling

Indicator reports

Monitoring and Data

Policy

Advice

Science

Actions

Scientific policy advice

Concrete targets

Monitoring and Data

Thank you!

For more information please contact:

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(HELCOM)
Katajanokanlaituri 6 B
FI-00160 Helsinki
Finland
www.helcom.fi

Annex 7: Proposal of a new structure of HELCOM COMBINE guidelines Part C

Part C. Programme for monitoring of eutrophication and its effects

C.1. Introduction

C.2. Sampling stations

C.3. Sampling programme as committed by the Contracting Parties

C.4. Directives for sampling and analysis of hydrographic, chemical and biological variables

C.4.1 Hydrographic variables

- C.4.1.1 Salinity
- C.4.1.2 Temperature
- C.4.1.3 Determination of dissolved oxygen
- C.4.1.4 Calculation of solubility and saturation of dissolved oxygen
- C.4.1.5 Hydrogen sulphide
- C.4.1.6 Light attenuation
- C.4.1.7 Current speed and direction

C.4.2 Hydrochemical variables

- C.4.2.1 Nutrients
- C.4.2.2 Particulate and dissolved matter
- C.4.2.3 Humic matter
- C.4.2.4 pH
- C.4.2.5 Alkalinity
- C.4.2.6 Anoxic waters
- C.4.2.7 Trace metals (Cd, Pb, Cu, Co, Zn, Ni, Fe) including mercury in sea water
- C.4.2.8 Persistent organic compounds in sea water
- C.4.2.9 Organic Carbon in Sea water

C.4.3 Biological variables

- C.4.3.1 Phytoplankton chlorophyll-a
- C.4.3.2 Phytoplankton species composition, abundance and biomass
- C.4.3.3 Phytoplankton primary production
- C.4.3.4 Mesozooplankton
- C.4.3.5 Bacterioplankton Growth

C.4.3.6 Bacterioplankton Biomass

C.4.3.7 Soft bottom macrozoobenthos

C.4.3.8 Macrophytobenthos and associated macrofauna

C.4.3.9 Coastal fish

Annex C-1 Tables listing sample stations

Annex 8: Proposal of a new guideline concerning Bacterioplankton growth determination(HELCOM COMBINE guidelines part C)

HELCOM **COMBINE MANUAL** ***PART C*** **CHAPTER C.4.3.5**	Bacterioplankton growth	**ISSUE NO.: 1** **REVISION N0: 0** **PAGE NO: 29 (19)** **ISSUE DATE: 07-02-13** **APPROVED BY:** **SIGNATURE:**

1 Introduction

Bacterioplankton growth rate is an indicator of the nutrient status in aquatic environments. It is an estimate of the consumption of organic carbon in the ecosystem and therefore closely related to the biochemical oxygen demand *in situ* (cf. BOD_7). Bacterioplankton growth rate thereby indicates the rate of oxygen consumption that may lead to oxygen deficiency in the water column when exceeding oxygen supply. The growth rate indicator may be used in all aquatic environments.

The original method is published in international scientific journals and has been used in many marine research studies since the beginning of the 1980´s. The method has been part of the Helsinki commission guidelines for a longer period of time (Baltic Sea Environment Proceedings No. 27D). The current protocol is an adaptation from Smith and Azam [1].

Bacterial growth rate is a relatively unambiguous indicator of the flux of organic matter through the pelagic ecosystem [2]. Even if the relationship between the factors specific growth rate and abundance may vary, their product representing growth rate reflects the substrate supply of organic matter to the bacterioplankton community. Density limitation (i.e. competition) or other limiting factors (i.e. inorganic nutrients, temperature) do not therefore directly appear to control the bacterioplankton community growth rate at typical environmental conditions. This agrees with empirical observations that bacterial growth rate over larger scales correlates with trophic status of a system [2, 3], and at smaller scales between water layers and seasons [4]. This is true for a growth rate range covering several orders of magnitude.

Bacterioplankton growth rate may be complemented by bacterial abundance and biovolume estimate providing better precision in the biomass production values. This also allows a deeper understanding of whether specific growth rate or bacterial abundance explains changes in community growth rate.

1.1 Background

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

The rate of bacterial biomass production was suggested as an indicator of the consumption of organic carbon in an ecosystem by Billen et al. [2]. A positive relationship between nutrient status and bacterial growth, as well as biomass, across different ecosystems has been demonstrated in independent studies [2, 3]. Increased organic production is detected by the variable, whether due to increased phytoplankton growth or import of organic matter from river and waste water discharge.

Bacterial biomass production is closely linked to biological oxygen demand in an environment, and bacterioplankton accounts for about 50% of the oxygen demand in aquatic environments [5]. This is due to oxygen constituting the major electron acceptors in aerobic environments and that bacteria channel a large part of the carbon flux in aquatic ecosystems. Bacterioplankton respiration is therefore an important cause of oxygen depletion when eutrophication prevails, as more than 80% of the marine secondary production occurs in the pelagic environment [6, 7].

1.2 Principle

Bacterial growth rate is estimated by uptake of the DNA base thymidine that is radioactively labelled. Thymidine has been shown to be almost exclusively taken up by heterotrophic bacteria in natural samples [1, 8]. Uptake by photosynthetic plankton does not seem to interfere significantly. The synthesis of DNA in a cell is coupled to cell division. Before a cell may divide, the DNA should have doubled to provide all genetic information required for the cell. Thereby twice as much thymidine should have been incorporated when the cell is ready for division.

Thymidine labelled with tritium (^{3}H) in the methyl group is used. The amount of thymidin taken up is thereby proportional to the amount of radioactivity taken up.

The amount of thymidine taken up is transformed to the number of cells produced by empirical knowledge of the amount of thymidine per cell on average. The theoretical conversion factor correlates relatively well with the empirically derived, but is typically slightly below the latter. The reason is that thymidine pools within the cell dilute the added radioactive thymidine, leading to some underestimation of the true thymidine incorporation by theoretical factors. The fact that some bacteria do not assimilate thymidine, and that some predation on bacteria occur during incubation, also leads to somewhat higher empirical conversion factors.

Bacterial cell growth may be transformed to biomass production by knowing the carbon content per cell. Further transformation to oxygen consumption can be used by using literature values of growth efficiency and respiration quotient (RQ).

1.3 Extent

Bacterial growth rate may be applied in freshwater as well as oceanic salinity (0-35). The requirement is that radioactive thymidin is added in sufficient excess to natural extra-cellular pools (e.g. 25 nmol dm^{-3} tritiated thymidine in brackish water). The applied conversion factor should also be valid for the studied environment.

1.4 Disturbances

Avoid exposing the sample to markedly different temperature or light irradiance compared to *in situ* conditions. Ice-cold (0°C) TCA solutions and tubes are essential for the precipitation step. Be careful to pre-cool solutions and tubes prior to use.

Careful removal of the supernatant after centrifugation is a critical step in the procedure. The sample tubes should be kept at room temperature during this procedure to avoid formation of mist on the tube walls. Use a glass pipette (Pasteur) where the tip has been narrowed by heating over a gas burner. Remove all liquid to the bottom, following the side opposite to where the precipitate is expected. Also remove any drops under the lid.

Do not use latex protection gloves as they may create fluorescence.

1.5 Contamination risk

Work antiseptically with sterile tips and tubes. Avoid especially contaminating the thymidine stock solution by carefully removing aliquots for each experiment to a sterile tube.

Keep the samples away from any biocide (e.g. formaldehyde, Lugol solution, Latex rubber, TCA etc.).

1.6 Safety

Isotope

Tritiated [metyl-^{3}H] thymidine with a specific activity of typically 80 000 Ci mol^{-1} and concentration of 12.5 µmol dm^{-3} (1mCi/ml) is used. The isotope is a ß-emitter and has a range of 10 mm in water. Protect face and eyes from concentrated stock solution. Use a laboratory coat and protective gloves. Diluted working solution should be handled according to laboratory procedures.

TCA

TCA is corrosive on eyes, skin and mucous membrane. Vapour and dust may cause irritation and harm lungs. Use mouth protection, protective gloves and laboratory coat when weighting the substance. Work in a ventilated hood.

2 Preparations

2.1 Cleaning and purification

Use tubes well rinsed with Milli-Q water for sub-samples the sampling bottles, or taken directly from the bag. These tubes may be re-used following rinsing with Milli-Q water. All bottles and tubes should be clean and not have been in contact with biocides like TCA or formaldehyde.

Tips for e.g. automatic pipettes may be re-used following rinsing with Milli-Q water. Only use tips exclusively for each solution.

Rinse the tip with Milli-Q-water between each depth when dispensing water samples.

2.2 Identification of sample

50 ml polypropylene tubes for sub-sampling should be labelled with variable, sample depth and replicate (if applied).

Micro-centrifuge tubes (1.5 ml) are labelled with cruise, station, depth and treatment on the lid with water resistant marker pen.

Place samples in proper order to simplify data treatment.

2.3 Reagents

Isotope

Tritiated [methyl-^{3}H] thymidine according to item p. 9 is used.

Trichloroacetic acid (TCA)

TCA contains a lot of crystal water. 100 % TCA is prepared by mixing 500 g TCA (e.g. Merck, ProAnalysi) with 227 l Milli-Q water. 5 % and 50 % TCA is prepared from the concentrated solution by dilution with Milli-Q water.

TCA is corrosive for eyes, skin and mucous membrane. Vapour and dust may be irritating and cause lung damage. Use mouth protection and laboratory coat. Work in a ventilated hood.

Scintillation liquid

Touluene- and Xylene free scintillation liquid is recommended (e.g. Optiphase HiSafe, Wallac OY). The scintillation liquid should be possible to mix with water.

Ice

Crushed ice may be used as cooling medium for TCA tubes.

2.4 Before cruise/sampling

Eppendorf tubes (1.5 ml) are placed in 5 ml scintillation vial without lid. The tubes are labelled with cruise, station, depth and treatment. Place the tubes in the order that results are wanted to appear in the scintillation file or print out.

At the beginning of the sampling day incubator for tubes and cold centrifuge are switched on for pre-cooling to the desired temperature. Label tubes for sub-sampling (i.e. 50 ml Falcon tubes) with station and depth, and place them in racks. Have two thermoses ready labelled “Above thermocline” and “Below thermocline”, respectively. Have a glass pipette (Pasteur-type) with thin tip attached to a vacuum source (e.g. water tap vacuum device). Bench surfaces used with radioactive samples should be covered with protective paper.

2.5 Protocol

A sampling protocol for logistic data according to ICES recommendation should be used.

3 Sampling

3.1 Sampling

3.1.1 Sampling strategy

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

A sampling frequency of 10 samples per year is required to get confident annual estimates (J. Wikner, unpubl. results). Samples should be distributed in the seasonal curve to provide a good coverage of different levels (more samples during the productive season).

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

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

3.1.2 Sampling method

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

Water samples are collected according to HELCOM guidelines.

A Milli-Q rinsed polypropylene tube is rinsed once with sample water before a sample of 50 ml is collected. Store the tube with a closed lid as close to *in situ* temperature as possible until start of the incubation according to item 4.4.1.

Fill the thermoses with water from the surface and deep water layer to be used for incubation of samples from depth with similar temperature.

3.2 Preservation/processing

Processing is done within 1 hour from sampling according to item 3.3 and 4. At rough weather processing may wait up to 8 hours. Note delays exceeding 1 hour in the protocol.

3.3 Storage

Tubes with sub-samples are stored as close to in *situ* temperature as possible. Refrigerator or other incubators may be used.

Samples in micro-centrifuge tubes with 50% TCA added may be stored at 4°C for up to 7 days before processing.

Micro-centrifuge tubes with TCA precipitated material in scintillation liquid may be stored at room temperature and in the dark until analysis in a scintillation counter. Counting should be done within 5 days.

4 Method description

4.1 Reagents

Make 50 % (w/v) TCA and 5 % (w/v) TCA in sufficient volume to last at least one cruise. Store working solutions of TCA in polypropylene tubes (e.g. Falcon®) submerged in ice slurry during the whole processing procedure.

Withdraw the volume of [methyl-^{3}H] thymidine that is required to run analysis at one station to a sterile (fresh) Micro-centrifuge tube.

4.2 Calibration solutions

None.

4.3 Processing

4.3.1 Preparations

Switch on the cold centrifuge to pre-cool the rotor to +4°C. Centrifuges without cooling may be put in a refrigerator. If an incubator like micro-centrifuge tube ThermoStat plus is used, it is set to +2°C. If a cold plexiglass block is used, it should be tempered at least one hour before use at -20°C.

4.3.2 Uptake of labelled thymidine

- For every sample depth two Micro-centrifuge tubes are filled with 1 ml of sample. Additional replication per depth may be applied as appropriate. Replicates should be measured on at least one station per cruise for analytical quality assurance. The same tip may be used for all depths provided that rinsing with two sample volumes Milli-Q water is done in between
- Add 100 µl 50 % TCA to the background treatment samples, mix 3 s with a blender and incubate for 5 min. TCA stop cell activity in the samples

- Withdraw the amount of [^{3}H-methyl] thymidine that is required for one station from the stock solution to a clean micro-centrifuge tube. Add 2 µl of thymidine to samples and then background samples. The same tip may be used for all tubes, by placing the drop of isotope on the wall above the water surface in the sample tube. Mix the tubes 3 s in a blender.
- Place the tubes in the thermos with closest temperature to the water depth of the sample. Note time of incubation start, specific activity and batch number in the protocol. Incubate for 1 hour. If a cooled plastic block is used, cool it at -20 °C in the mean time
- Stop the incubation by placing the micro-centrifuge tubes in the cooling device used at +2 °C for 5 min. Note the stop time in the protocol

4.3.3 Precipitation of bacterial biomass with TCA

- Add 100 µl 50 % TCA to the samples (*not* the background vials) and mix for 3 s. The TCA solutions should be ice-cold at this step. Incubate the samples at +2 °C or on ice for 5 min. If centrifugation can't be done directly, samples may be stored in this condition at +4 °C for up to 7 days
- Place the micro-centrifuge tubes in a cooled (+4 °C) centrifuge with the "necks" facing outwards and towards the rotor. Samples should not be frozen at this stage. Centrifuge the micro-centrifuge tubes at 16000 × g (13000 rpm, see item 8 Equipment) for 10 min. If not all tubes fit in the rotor, store the remaining tubes in the refrigerator
- Place the micro-centrifuge tubes in a tube rack at room temperature. Remove the supernatant with a Pasteur pipette with a thin tip using a vacuum source. Note that the supernatant is radioactive. Be very careful to remove all liquid. Also remove all mist and droplets on the tube wall and under the lid. The typically invisible pellet is located in the tube bottom facing outward from the rotor. Some precipitate may however stick to the tube wall on the same side. Don't touch the pellet
- Wash the pellet and tube with 5 % TCA. Make sure that no air bubbles are left in the bottom of the tube so that the pellet is washed properly. Close the lid, mix the sample 5 s and turn it up-side down to also wash the inside of the lid
- Centrifuge the micro-centrifuge tubes with the "necks" facing outward at 16000 × g (13000 rpm) for 10 min. Remove the supernatant as above
- Add 1 ml of scintillation liquid to each tube. Close the lid and mix 5 s on a blender. Hang thee micro-centrifuge tubes in the 5 ml scintillation vials. Store the samples as defined in item 3.3.

4.4 Calibration

The scintillation should be calibrated with sealed standards, typically provided by the manufacturer. Record calibration date and result. Change standards before the expiration date.

Quench correction curve installed by the manufacturer is typically used.

4.5 Analysis

Scintillation counting can be done in the 5 ml scintillation vials with micro-centrifuge tubes.

Run standards before the samples and follow the manual of the scintillation counter.

Record the results preferably in a computer file to minimize errors while entering values manually and save time. Data may also be printed.

The samples are counted in the [^{3}H]-window and settings generating disintegrations per minute (DPM) values from counts per minute (CPM) based on a quench curve installed by the manufacturer (see item 4.4).

The following settings have successfully been used in a Beckman LS6500 scintillation counter for counting tritium:

```
ID: 3H, 5MIN, DPM

USER            : 1              COMMENT:

PRESET TIME     : 5.00

DATA CALC       : SL DPM   H#:   : YES  SAMPLE REPEATS : 1   PRINTER::    STD
COUNT BLANK     : NO       IC#   : NO   REPLICATES     : 1   RS232   :    OFF
TWO PHASE       : NO       AQC   : NO   CYCLE REPEATS  :                    1
SCINTILLATOR    : LIQUID   LUMEX   : NO            LOW SAMPLE REJ    :      0
LOW LEVEL       : NO       HALFLIFE CORRECTION DATE:     none


ISOTPE 1:       3H    %ERROR: 2.00     FACTOR:1.000000     BKG. SUB:    0


BACKGROUND QUENCH CURVE: Off          COLOR QUENCH CORRECTION: On


QUENCH LIMITS     LOW: 2.672      HIGH: 316.80
```

5 Calculations

5.1 Calculation functions

5.1.1 Transformation of DPM to cell growth

The amount (mol) of incorporated ^{3}H-thymidine ml^{-1} h^{-1} (n_{ty}) is calculated as

$$\Delta n_{ty} = \frac{(dpm_s - dpm_b) \times 4.5 \times 10^{-13}}{v \times \Delta t \times SA} \quad (1)$$

where

dpm_s=disintegration per minute in the sample (average of replicates if present)

dpm_b= disintegration per minute in the background (average of replicates if present)

$4.5*10^{-13}$=conversion factor (dpm ==> Ci)

v= sample volume (cm^3)

Δt= incubation time (hours)

SA= specific activity for [^{3}H]-thymidine (Ci mol^{-1})

Bacterial growth in cells (P_c) is calculated as

$$P_c = \Delta n_{ty} \times TCF \times 24 \times 1000 \quad (2)$$

where *TCF* is the thymidine cell conversion factor. A conversion factor empirically determined for the Baltic Sea area of 1.4×10^{18} cells [mol thymidine]$^{-1}$ (n=73, ±SE=$0{,}1\times 10^{18}$) is recommended. This factor seems independent of growth rate and is close to the theoretical factor for coastal environments [4, 8-12].

The factors 24 and 1000 transform cells cm^{-3} h^{-1} to cells dm^{-3} day^{-1}.

5.1.2 Bacterial biomass production

Cell production is transformed to bacterial biomass production (P_b, mol carbon dm^{-3} day^{-1}) with the function

$$P_b = P_c \times m_b \quad (3)$$

The factor m_b is the carbon content of cells on average in the sample in µmol C cell^{-1}. See the standard operating procedure for bacterioplankton biomass for a definition.

5.1.3 Bacterial oxygen consumption

Bacteral oxygen consumption, $\Delta O_2^{\mathrm{bact}}$, may be calculated from P_b , bacterial growth efficiency, *BGE*, and the respiration quotient, *RQ*, according to:

$$\Delta O_2^{bact} = P_b \times \frac{1 - BGE}{BGE} \times RQ \quad (4)$$

Estimates of BGE are currently uncertain and vary with at least nutrient status. Recalculation of bacterial growth to bacterial oxygen consumption is therefore a crude estimate of the latter. The best estimate of BGE is probably obtained by the function reported by Del Giorgio and Cole, 1998 [13]

$$BGE = \frac{0.037 + 0.65 \times P_b}{1.8 + P_b} \quad (5)$$

where *Pb* is the bacterial growth rate in µg C dm^{-3} h^{-1}. The bacterial growth efficiency average 0.27 in the reported data set, which is close to constants used in the literature. The uncertainty of the function has not been reported.

Values of 0.9 has been used for the respiration quotient (RQ), based on a weighted average for respiration of carbohydrate (weight 0.5), protein (weight 0.33) and fatty acids (weight 0.17) [14]. This RQ is also in good accordance with results from experiments on a marine bacterium [15].

5.1.4 Standard deviation

The standard deviation (SD_{tot}) for replicates at one sample depth is calculates as the square sum of both sample and background treatments according to:

$$SD_{tot} = \sqrt{\left(SD_s^2 + SD_b^2\right)} \quad (6)$$

where SD_s och SD_b are the standard deviation for samples and backgrounds, respectively.

5.1.5 The variations coefficient

The variation coefficient (CV_{tot}) is calculated as:

$$CV_{tot} = \frac{SD_{tot}}{\overline{m}} \quad (7)$$

where m is the average netto dpm based on the difference between samples and background.

5.2 Calculations

Values for assimilated thymidin (dpm), background and other factors are entered in a database or calculation software according to table 2. Calculation functions according to item 5.1 are applied.

The calculation should return parameters and units according to Table 3 in item 7.

5.3 Measurement uncertainty

The measurement uncertainty has been determined according to the standard of measurement uncertainty in chemical analysis of the European Union [16].

The standard uncertainty corresponds to standard deviation and is estimated from several identified variance components of the method. The assimilation of thymidine shows a low expanded uncertainty of ±21%, approximately corresponding to a 95% confidence interval (Table 1). Conversion factors contribute with the greatest uncertainty.

Table 1 Measurement uncertainty for bacterial growth rate. U is the expanded uncertainty with a factor 2.

Parameter	Unit	Value	U (%)
Bacterial growth	µmol C dm^{-3} day^{-1}	0.29	±21

The coefficient of variation (±CV) for netto dpm should stay below 20% in productive waters. During the winter season values may be somewhat higher. Values above ±60 % should be scrutinized.

Background values should stay below 100 dpm and average 30 dpm.

The detection limit corresponds to 100 dpm netto uptake of thymidine (+2 x SD). This corresponds to 1×10^7 cells dm^{-3} day^{-1} or 0.02 µmol dm^{-3} day^{-1}, approximately the same in carbon or O_2. Typical growth rates in mesotrophic environments are 20 times higher.

6 Quality assurance and evaluation

6.1 Control charts

Duplicate samples should be run regularly corresponding to about 10% of the samples. Plot the standard deviation of duplicates against date in a control chart.

Background values are plotted in control chart.

6.2 Evaluation

For evaluation of all charts use alarm (2 × SD) and action limits (3 × SD). Values above the action limit should be evaluated for potential error sources. If errors are found, they are corrected with date, motivation and signature added. If no error can be identified, values are labelled as extreme values or questionable values.

It's recommended plotting a full year of data at the end of the year of measurement, to get a good view of the seasonality and depth variation. Sample dpm and growth rate parameters may be plotted against date and depth. Values should be plausible and not differ more than 3 × SD from the average values during a given season. Values should also show an expected variation with depth, where surface values typically are greater than those in deeper water.

Correct found errors and note date, motivate change and sign the change made. Label deviating values as extreme or questionable values if no errors can be identified. Avoid deleting values without proper reason.

7 Reporting

Enter data in a data base or calculation program as described in Table 2, together with logistic data to identify the sample according to recommendation by the International Council for Exploration of the Sea (ICES).

Table 2 Primary database variables and units.

PARAMETER	UNIT	DIGITS	FUNCTION	CATEGORY	ACRONYME	VALUE EX..
Sample radioactivity	dpm	3	-	Depth	BGSAMDPM	1000
Background radioactivity	dpm	3	-	Depth	BGBKGDPM	40
Specific activity	Ci mol^{-1}	3	-	Depth	BGSPACTY	82000
TCF†	cells mol^{-1}	3	-	Depth	BGTCF	$1.4\text{x}10^{18}$
Sample volume	cm^{-3}	3	-	Depth	BGSAMVOL	1
Start of incubation	tt.mm	4	-	Depth	BGINCST	10.18
End of incubation	tt.mm	4	-	Depth	BGINCEN	11.20
Respiration quotient	-	2	-	Depth	BGRQ	0.9
Bact. growth efficiency	%	2	5	Depth	BGGREFF	0.3
Date of calibration (scint.)	01-09-26	6	-	Depth, standard	BGICD	01-10-04

† Thymidin conversions factor transforming uptake of thymidine in mol to cells produced.

Table 3 Calculated parameters of bacterial growth rate.

THYMIDINE UPTAKE RATE	MOL CM^{-3} H^{-1}	3	5.1.1.	DEPTH	BGTHYUP	5.11×10^{-15}
Thym. uptake rate ±SD	mol cm^{-3} h^{-1}	2	5.1.1, 5.1.5	Depth	BGTHYCV	4.13×10^{-16}
Bacterial cell production	cells dm^{-3} day^{-1}	3	5.1.1	Depth	BGCELLP	$1.84\text{x}10^{8}$
Bact. Prod. ±SD	cells dm^{-3} day^{-1}	2	5.1.4, 5.1.5	Depth	BGCCELCV	$1.12\text{x}10^{7}$
Bact. Carbon production	µmol C dm^{-3} day^{-1}	3	5.1.2	Depth	BGCARPR	0.29
Bact. Carbon prod. ±SD	µmol C dm^{-3} day^{-1}	2	5.1.4, 5.1.5	Depth	BGCARCV	0.02
Bact. oxygen consump.	µmol dm^{-3} day^{-1}	3	5.1.3	Depth	BGCOXYCO	0.60
Bact. oxygen ±SD	µmol dm^{-3} day^{-1}	2	5.1.4, 5.1.5	Depth	BGCOXYCV	0.05

Use quality codes according to ICES directives.

Use ICES format when reporting logistic information with each value.

Valuable variables of explanation include bacterial biomass (whole community, µmol C dm^{-3}), bacterial volume (median, µm^{3} cell^{-1}), bactivorous flagellates (flagellates dm^{-3}), temperature (°C), total phosphorus (µmol dm^{-3}), total nitrogen (µmol dm^{-3}) and oxygen (µmol dm^{-3}). Substrate variables may also be used if available, where total DOC is a crude indicator of substrate availability.

8 Equipment

Plastic- and glass ware

- Polypropylene tubes (50 ml) with lid (e.g. Falcon®)

- 1.5 ml micro-centrifuge tubes of polypropylene (e.g. Eppendorf®)
- Scintillation vials (6 ml). (e.g. Beckman Mini Poly-Q-vial)
- Pipette tips 0.5-10 µl, 10-100 µl, and 100-1000 µl
- Pasteur pipettes of glass with a narrow tip. Narrow the tip by melting the pipette over a gas burner, gently pulling each end of the pipette apart. Break the pipette at the narrowest position.

Refrigerated centrifuge

A refrigerated centrifuge for micro-centrifuge tubes (1.5 ml) that can achieve the desired g-force and 4 °C is required. One example is a Beckman GS-15R with rotor F2402. Centrifuges that do not manage refrigeration may be run inside a refrigerator. The rotor should be chilled before applying the samples.

Water vacuum device

A vacuum pump with capacity of at least -400 mmHg is required. A Pasteur pipette with narrowed tip is connected to a water vacuum device attached to a regular tap to remove the supernatant. This also discards the radioactive liquid directly into the sink. Alternatively a water trap may be installed between the vacuum source and the Pasteur pipette.

Automatic pipettes

Calibrated pipettes covering volume ranges of 0.5-10 µl, 10-100 µl and 100-1000 µl is required. A motor driven pipette is recommended for dispensing liquid to many samples.

Refrigerated incubator

A refrigerated incubator with room for at least 14 micro-centrifuge tubes is recommended to use (e.g. Eppendorf ThermoStat plus, prod nr. 5352 000.010 + 5364 000.016). A temperature of +2°C has been found to give an optimal precipitation of cell material by TCA. The incubator should be pre-chilled for 30 min.

Cooling rack

A solid cooling rack (e.g. plastic) with holes for 1.5 ml micro-centrifuge tubes may be used to chill samples, as an alternative to a refrigerated incubator. The rack should be chilled at -20°C. The rack keeps sufficient cooling capacity for 15 min. at room temperature. Store the rack in the freezer when not in use.

Scintillation counter

A scintillation counter with internal quench correction is recommended. One example is Beckman Coulter™ LS 6500 Multi-Purpose Scintillation Counter (cat.nr. 510656). Beckman's™ software for digital collection of data to a computer file is used.

Tube blender

A laboratory blender for tubes is recommended. One example is_Vibrofix VF1, Jankel & Kunkel, IKA-Labortechnik.

9 Chemicals and solutions

Isotope

A fresh solution of tritiated thymidine less than 8 weeks old from activity date should be used. Make sure the isotope has the desired specific activity (about 80 000 Ci mol^{-1}) Note the date of arrival and volume used on the vial. Store the isotope in the refrigerator.

Tritiate [methyl-^{3}H] thymidine (e. g. Amersham order no. TRK 686) with specific activity of 80 000 Ci mol^{-1} and concentration of 12.5 µmol dm^{-3} (1mCi ml^{-1}) is used. Withdraw the volume required for one station to a clean micro-centrifuge tube to minimize the risk of contamination. Use sterile tips with the pipettes.

The [methyl-^{3}H] thymidine is a ß-emitter where electrons have a range of about 10 mm in water. Minimise handling of the concentrated stock solution. Use protective gloves and a laboratory coat. Discard the diluted isotope according to local regulations.

Trichloroacetic acid (TCA)

Trichloroacetic acid contains large amounts of crystal water. 100% (w/v) of TCA is made by mixing 500 g of TCA powder (e.g. Merck, ProAnalysi) with 227 cm^{3} of Milli-Q water. Diluted solution of 50 % and 5 % are made from the stock solution by dilution with Milli-Q water.

TCA is corrosive on eyes, skin and mucous membrane. Vapour and dust may cause irritation and damage on the lungs. Use mouth protection and protective gloves and a laboratory coat. Work in a ventilated hood with TCA powder.

Scintillation liquid

Toluene- and Xylen free scintillation liquid is recommended (e.g. Pharmacia OptiPhase HiSafe 3, Wallac OY, prod. no. 1200-437).The scintillation liquid should be possible to mix with water.

Milli-Q water

Milli-Q water is made from deionized water that is further purified through an ion exchange resin and 0.2 µm filter. Devices producing Milli-Q water is manufactured by e.g. the Millipore ® Company.

Ice

Crushed ice may be use as a cooling medium for tubes with TCA.

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Annex 9: Proposal of a new guideline concerning Bacterioplankton abundance determination (HELCOM COMBINE guidelines part C)

HELCOM **COMBINE manual** *Part C* Chapter C.4.3.6	***Bacterioplankton abundance***	Issue no.: 1 Revision n0: 0 Page no: 42 (19) Issue date: 07-02-13 Approved by: Signature:

1 Introduction

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

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

1.1 Background

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

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

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

1.2 Principle

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

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

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

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

1.3 Extent

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

1.4 Disturbances

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

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

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

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

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

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

1.5 Contamination risk

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

1.6 Safety

Acridine orange and formaldehyde are harmful substances. Handling of these chemicals shall be done in a ventilated hood and with gloves. Preferably formaldehyde should be used on a ventilated bench as the gas phase is heavier than air. Mouth protection should be used when working with acridine orange powder as it is may cause cancer.

2 Preparation

2.1 Cleaning and purification

Sample bottles

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

Filter equipment

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

Glass slide and cover slip

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

Microscope

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

2.2 Sample identification

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

2.3 Reagents

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

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

2.4 Prior to sampling

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

2.5 Protocol

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

3 Sampling

3.1 Sampling

3.1.1 Sampling strategy

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

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

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

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

3.1.2 Sampling method

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

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

3.2 Preservation/processing

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

3.3 Storage

Store the preserved samples at 4 °C. A preserved sample may be stored in the refrigerator (4 °C) for 7 days before making the microscope slide. Microscope slides with filter mounted may be stored for at least 70 days in the freezer (- 20 °C) without loss of cells [6]. Store the glass slides horizontally to avoid that the immersion oil pours away.

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

4 Method description

4.1 Reagents

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

4.2 Calibration solution

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

4.3 Sample processing

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

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

a) Clean the filter support by a paper towel, close the valve and place a few drops of Milli-Q water on the support b) Mount a GF/C filter and wet it with Milli-Q water. Apply a black 0.2 µm polycarbonate filter (e.g. Poretics©). Apply vacuum so that the filter lies flat on the GF/C filter. Close the vacuum source, let the pressure disappear and close the valve

c) Make a background sample where a filter without sample addition is stained and destained with Milli-Q water in the same way as the other samples

d) Shake the sample flask violently for 10 s to homogenise the sample. Open the lid and transfer the appropriate volume of sample (typically 3-7 cm^3), aiming at least 30 cells per microscopic field (same tip as under item **C** may be used). Use a sample volume table for different stations and seasons developed by experience. See an example for the Gulf of Bothnia in item 11.1. Rinse the pipette tip with Milli-Q water (Falcon Tube A)

e) Filter all samples at a vacuum of -13 kPa (-100 mm Hg, 0.87 bar, 14 psi) to the filter surface appear dry. Close the vacuum let the pressure disappear and close the valve

f) Fill a syringe with acridine orange. Mount a 0.2 µm Acrodisc® filter. Add 15 drops (about 0.75 cm^3) of acridine orange to each sample. The filter should be completely covered by the stain. Incubate for 5 min.
In the mean time label the glass slides according to item 2.2. Apply immersion oil according to item **I**. See to that the background-filter is on the same glass slide as a sample to aid focusing

g) Filter the stain through the filters. Close the vacuum, let the pressure disappear and close the valve. Wash the filter by adding 1 cm^3 of Milli-Q water from Falcon tube B with a clean tip. Filter the liquid through the filter until it appear dry and leave the vacuum on. Remove the filter funnels

h) Pick up the filter with forceps. Air-dry the filter 45 s by slowly moving it in the air until dry. The filter may be labelled and stored in this condition

i) Apply a drop of immersion oil where filters are supposed to be mounted. Two 25 mm filters may be mounted on each glass slide. Spread the immersion oil drop on an area larger than the filter, by using the filter itself. Place the filter on the oil film. Add a drop of oil on top of the filter. Mount the cover slip and let the oil spread under the whole cover slip by capillary forces. Occasionally a slight pressure on the cover slip by the forceps may aid the spreading of the oil. If two filters are mounted large cover slips covering both may be used. Store the glass slides in slide holders at -20 °C until microscopic analysis

j) Wash the filter funnels after the filtration according to item 2.1. See item 3.3 for storage of samples

k) Preserved liquid samples and glass slides are stored until the annual quality assurance is done

4.4 Calibration

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

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

4.5 Analysis

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

4.5.1 Image analysis

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

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

4.5.2 ManUal analysis

a) Manual analysis may be used if image analysis can not be accomplished. This will introduce operator dependence and less accurate biovolume estimates. Check that the correct equipment is installed and settings used (item 8.6). Check that the run time of the mercury lamp is not exceeded (max. 200 hours). Record the run-time in a log journal

b) Count the number of cells in 20 microscopic fields, distributed in a representative way over the filter surface. Avoid looking in the microscope while moving position. If areas with markedly heterogeneous distribution occur, a new slide should be prepared

c) Count cells that are rod-shaped, spherical and c-shaped particles, cells appearing solitary or in colonies. Irregular particles are likely not bacteria and should be excluded. Extremely tiny particles may be viruses and should be neglected

d) Use the large square in the ocular grid. Choose a sample volume resulting in an average of 30 cells per field (use item 11.2). Count 20 cells per sample or more than 300 cells in total. Standard error of the average of microscopic fields should not exceed 10 %. Count more fields in that case

5 Calculations

5.1 Calculation functions

5.1.1 Transformation of cell numbers

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

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

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

5.1.2 Magnification factor

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

$$F_f = \frac{A_t}{a}$$

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

5.1.3 Volume calculation

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

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

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

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

5.1.4 Carbon density

The carbon density per cell is determined from biovolume by a volume dependant function: [8, 9] according to:

$$m_b = 0.12 \times v_b^{0.7}$$

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

5.1.5 Bacterial biomass

The bacterial biomass (B_b) is determined as

$$B_b = N_b \times m_b$$

5.2 Calculations

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

5.3 Precision and accuracy

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

The standard error for microscopic fields should be less than 17 % (action limit of 3 × SD) when performing image analysis and 10 % at manual count (due to fewer but larger fields in the former technique). Otherwise count more fields. At high heterogeneity consider making a new slide or sonicate the sample according to item 4.3. The standard deviation between 2 homogenous samples has been estimated to ±2 %.

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

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

The measurement uncertainty has been determined according to the standard of measurement uncertainty in chemical analysis of the European Union [10].

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

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

PARAMETER	UNIT	VALUE	U (%)
Bacterial biomass	µmol carbon dm^{-3}	2,34	23

6 Quality assurance and evaluation

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

6.1 Control charts

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

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

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

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

6.2 Evaluation

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

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

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

6.3 Hardware calibration

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

The ocular counting square size is determined in a similar way when performing manual counting. A typical size for a large counting square in the ocular is 70 × 70 µm, and 21 × 21 µm for a small square.

Filtered area was determined from the diameter of the filter funnel (21 000 µm). The filter area (πr^2, 3.464×10^8 μm^2) and image area was used to determine the magnification factor according to equation under item 5.1.2 to 24292.

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

7 Reporting format

Make required calculation in a calculation software like Microsoft Excel and aggregate data from different images using pivot tables. Variable specific parameters are given in table 1. A similar table may be set up for Milli-Q and bead results. Also add logistic and quality code information according to standards of the International Council for the Exploration of the Sea (ICES) and HELCOM.

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

Table 1 Reporting format of bacterial biomass shown with units and typical values.

PARAMETER	UNIT	DIGITS	FUNCTION	CATEGORIES	ACRONYM	VALUE EX.
Bacterial concentration	cells dm^{-3}	3	5.1.1, 5.1.2	Depth, Standard	BACTCONC	1.30E+09
±SD Bact. conc.	cells dm^{-3}	2	Statistics	Depth, Standard	BASDCON	1.10E+08
Number of fields	number	1	-	Depth, Standard	BANPIC	5
Counted cells	cells	3	-	Depth, Standard	BACOUCEL	500
Bacterial biovolume	μm^3 $cell^{-1}$	3	5.1.3	Depth, Standard	BACVOL	0.07
±SD Biovolume	μm^3 $cell^{-1}$	2	Statistics	Depth, Standard	BASDVOL	0.006
Preparation date-time		10	-	Depth, Standard	BAPDAT	05-09-26 13:50
Analysis date-time		10	-	Depth, Standard	BAADAT	05-10-01 14:30

Table 2 Calculated parameters.

PARAMETER	UNIT	DIGITS	FUNCTION	CATEGORIES	ACRONYM	VALUE EX.
Carbon density	fmol $cell^{-1}$	3	5.1.4	Depth	BACTDENS	1.55
Bacterial biomass	µmol	3	5.1.5	Depth	BACTBIOM	2.02

	carbon dm^{-3}					

”Statistics” mean that established statistical functions are used.

Valuable variables of explanation encompass bacterial growth rate (whole community, cells dm^{-3} day^{-1}), temperature (°C), total phosphorus (µmol dm^{-3}), total nitrogen (µmol dm^{-3}) bactivorous flagellates (cells dm^{-3}) and oxygen concentration (mol O_2 dm^{-3}).

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

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

8 Equipment

8.1 Filter

Blackened polycarbonate filters with pore size 0.22 µm, diameter 25 mm are used to capture bacteria. Glassfiber filter (e.g. Whatman GF/C), 25 mm diam. are used as filter supports.

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

8.2 Multi-filtering unit

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

8.3 Supplies

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

8.4 Sterile filter

0.2 µm glassfiber filter, sterile, non-pyrogenic (e.g. Acrodisc®, GelmanScience).

8.5 Microscopic scale

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

8.6 Microscope

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

Zeiss Axiovert 100:

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

This setting gives a magnification factor of 24292 for the Camera ORCA-ER (Hamamatsu®). Zeiss counting grid (Pl10x/18 nr. 44 41 32) gives a factor for the small square 318054. The large square has the magnification factor 29695.

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

8.7 Sonicator

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

9 Chemicals and solutions

Acridine orange

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

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

Formaldehyde

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

Immersions oil

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

Triton® X-100

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

Natriumpyrophosphate

Tetra-$Na_2P_2O_7$*10 H_2O, is diluted in Milli-Q water. The final concentration in a sample to be sonicated is 10 mM.

Suspension of fluorescent beads

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

Bead standards are prepared by filtering beads at an expected concentration of $1x10^9$ beads dm^{-3}. Make two standards that are given unique codes. Store in standards the refrigerator at 4 °C. Standards prepared from the same solution should have ±CV of 21 % (n=4) for cell numbers and 15 % (n=4) for cell volume. The same standard has been applied for many years without clear changes in abundance or size of beads.

10 References

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Annex 10: Proposal for updating guidelines for Phytoplankton in the HELCOM COMBINE manual

Formerly Annex C-6 should refer now to Chapter C.4.3.2 Phytoplankton species composition, abundance and biomass (see ANNEX 7).

C.4.3.2 Phytoplankton species composition, abundance and biomass

Phytoplankton serves as an indicator of the effects of eutrophication. Nutrient enrichment may give rise to shifts in phytoplankton species composition and biomass. Furthermore, an increase in the frequency, magnitude and duration of harmful phytoplankton blooms may occur.

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

a) to describe temporal trends in phytoplankton species composition, their abundance, biomass and intensity of abundance of blooms

b) to describe spatial distribution of phytoplankton species, their abundance, biomass and blooms

c) to identify key phytoplankton species (e.g. dominating, harmful and indicator species)

C.4.3.2.1 Sampling

Phytoplankton species composition, their abundance and biomass in the euphotic zone form the basis for the determination of temporal trends of phytoplankton. High frequency sampling at a number of stations covering all basins in the Baltic Sea area is needed to reveal trends. As phytoplankton shows a substantial seasonal variation, sampling needs to cover the entire growth season, which in parts of the Baltic Sea extends over the entire year. In addition to the sampling at fixed sampling stations, ships-of-opportunity transects, satellite images and aerial surveillance help to identify variability in temporal and spatial extent of phytoplankton. Synoptic surveys are necessary for the study of the extension of phytoplankton blooms.

For the purpose of quantitative studies in the open sea, the minimum requirement is to take an integrated sample from 0-10 m depth using a hose (Lindahl, 1986). In Coastal Monitoring Programme (CMP), the sample from 0-1 m or an integrated sample (0-10 m) could be analysed.

The same integrated sample should be used for chlorophyll determination and, if desired, primary production.

An additional sample, 10 – 20 m, is recommended.

The 0 – 10 m integrated sample may be approximated by pooling equal amounts of water from the depths 0 – 1 m, 2.5 m, 5 m, 7.5 m and 10 m.

The integrated sample should be thoroughly mixed in a bucket. One subsample of 200 cm^3 is drawn from the well-mixed sample for quantitative phytoplankton counts.

For ship-of-opportunity and helicopter sampling a single sample from the mixed surface layer can be taken.

If there is a subsurface chlorophyll a maximum an additional sample may be taken at this depth, using a water sampler. 200 cm^3 of this sample is drawn for at least qualitative analysis of phytoplankton species composition. Another subsample is taken for chlorophyll a determination.

It is recommended to take a net sample from the 0 – 20 m water column in order to obtain a concentrated plankton sample. This sample serves as a support for species identification. Observing living material facilitates identification. A plankton net with a 10 µm mesh-size is recommended. In case of higher concentration it is advisable to use a net with 25 µm mesh-size.

C.4.3.2.2 Preservation and storage of samples

Preservatives

Acid Lugol's solution (Willén 1962):

200 cm^3	distilled or deionized water
20 g	potassium iodide (KI)
10 g	resublimated iodine (I_2)
20 cm^3	glacial acetic acid (conc. CH_3COOH)

Mix in the order listed. Make sure the previous ingredient has dissolved completely before adding the next. Store in a tightly locked glass bottle cooled.

Alkaline Lugol's solution (modified after Utermöhl 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.

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.

Preservation

Net samples to be studied alive can be kept fresh for a few hours in an open container in a refrigerator.

For preservation of net or water samples, 0.5 – 1.0 cm^3 acid Lugol's solution have to be added immediately to 200 cm^3 sample. If coccolithophorids need to be preserved with the coccoliths intact, a parallel subsample should be fixed with 0.5 – 1.0 cm^3 alkaline Lugol's solution. Clear, colourless 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.

They should be stored dark and cool and counted as soon as possible. Samples stored for more than one year are of little use.

C.4.3.2.3 Qualitative determinations

Net samples are recommended to be studied for the identification of sparsely occurring microplankton species with a standard research microscope. The advantages include potentially higher resolution, thinner preparations and the possibility to turn the cells around by tapping the cover glass. This is especially helpful when examining the plate structure of dinoflagellates. Dinoflagellate plates are also well studied using the epifluorescence method with Calcofluor (Andersen & Throndsen, 2003).

C.4.3.2.4 Quantitative determinations (phytoplankton counting)

The recommendation is based on the counting technique with an inverted microscope as described by Utermöhl (1958).

<u>Settling procedure</u>

Before sedimentation the sample should be adapted to room temperature to avoid excessive formation of gas bubbles in the sedimentation chambers. Gas bubbles will adversely affect sedimentation, the distribution of cells in the bottom-plate chamber, and microscopy.

Immediately before the sample is poured into the sedimentation chamber, the bottles should be shaken firmly but gently in irregular jerks to homogenize the contents. Too violent shaking will produce a lot of small bubbles, which may be difficult to eliminate. If the sample must be shaken vigorously in order to disperse tenacious clumps, this should not be done later than one hour before starting sedimentation.

The chambers should be placed on a horizontal surface and should not be exposed to temperature changes or direct sunlight. Covering the settling chamber(s) with an overturned plastic box will provide a fairly safe and uniform environment for sedimentation. If moistened tissue paper is included under the hood, problems caused by evaporation will be reduced considerably. It is essential that the supporting surface is vibration free, since vibrations will cause the cells to collect in ridges.

Settling time is dependent on the height of the chamber and the preservative (e.g. Hasle, 1978 and Rott, 1981). The times given in Table 1 are recommended as minimum. If vibration 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.

Table 1: Settling time in phytoplankton samples, preserved with Lugol solution, in dependence of the volume of the sedimentation chamber

VOLUME OF CHAMBER (CM^3)	HEIGHT OF CHAMBER (CM)	SETTLING TIME (H)
2	1	3
10	2	8
50	10	24
100	20	48

Sedimentation chambers of 100 cm^3 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 (Nauwerck, 1963 and Hasle, 1978). Such chambers can be used only when phytoplankton is very sparse, as in late autumn and winter. For such samples it is recommended scanning 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 ($Na_2S_20_3$. $5\ H_20$) in the aliquot to be sedimented.

<u>Counting procedure</u>

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. The settled sample should be discarded if the distribution is visually uneven, one-sided or in ridges, indicating convection or vibration, respectively. If this occurs consistently, measures should be taken to eliminate the sources of disturbance.

Counting begins at the lowest magnification, followed by analysis at successively higher magnification. For the sake of adequate comparison between samples, regions and seasons, it is important to always count the specific species at the same magnification. In special situations, such as bloom conditions, however, this may not be possible. Large species, easy to identify (e.g. *Ceratium* spp.), which are usually also relatively sparse are counted at the lowest magnification and over the entire chamber bottom. Smaller species are counted at higher magnification and possibly on only a part of the chamber bottom.

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 5 x 5 squares in one of the oculars is very helpful when counting dense fields of small cells.

The recommended magnifications for different phytoplankton sizes are listed in Table 2.

Table 2 Recommended magnification for counting of different size classes of phytoplankton (From Edler and Elbrächter, in prep.).

SIZE CLASS	MAGNIFICATION
0.2 – 2 µm (picoplankton)*	1000 x
2 – 20 µm (nanoplankton)	100 – 400 x
>20 µm (microplankton)	100 x

*** picoplankton cannot be properly analysed using the Utermöhl method**

Counting the whole chamber bottom is done by traversing back and forth across the chamber bottom. The parallel eyepiece threads delimit the transect, where the phytoplankton are counted (Fig. 1.). Phytoplankton cells crossing the upper thread are counted, but not those crossing the lower thread.

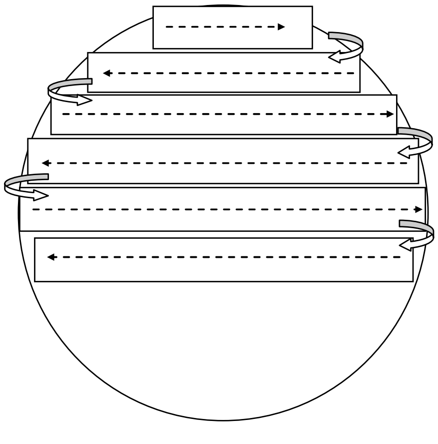

Figure 1 Traversing the whole chamber bottom with the parallel eyepiece threads to indicate the counted area. (From Edler and Elbrächter, in prep.)

Counting part of the chamber bottom can be done in different ways. If half the chamber bottom shall be analysed every second transect of the whole chamber counting method is counted. If a smaller part shall be analysed, one, two, three or more diameter transects are counted. After each transect is counted the chamber is rotated 30-45°

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. The common counting unit is the cell. This applies also to colonies with irregular numbers of cells. Estimation of cell numbers in small-celled and densely-packed colonies may be realised by visual dividing of the colony into smaller areas, counting cell numbers in one area and multiplying with the number of "small areas".

Colonial algae which occur regularly as groups of four cells or a multiple are most conveniently counted and reported as colonies, e.g.:

Choricystis
Crucigenia
Crucigeniella
Desmodesmus
Dictyosphaerium
Elekatothrix
Gonium
Merismopedia
Pandorina
Pediastrum
Scenedesmus
Tetrastrum
Willea

Filamentous cyanobacteria are to be counted in lengths of 100 µm. Numbers of 100 µm pieces per litre are reported.

While counting, the species/individuals have to be allocated to size classes according to the scheme of Olenina et al. (2006). This is important for a reliable biovolume calculation.

At least 50 counting units of each dominating taxon should be counted, and the total count should exceed 500. All cells should be counted and reported even if fewer counted units progressively will decrease the precision of the count and increase the statistical error of the population estimate. The approximate 95 % confidence limits of a selected number of counted units are given in Table 3. They have been calculated according to the formula:

$$95\% \text{ C.L.} = n \pm 2 \times (100/\sqrt{n})\%$$

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 % (Lund *et al.*, 1958, Kozova and Melnik, 1978).

Table 3 The approximate 95 % confidence limits of a selected number of counted units.

COUNT	95 % C.L. (%)
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. The statistics assume perfectly random-distribution of cells on the bottom of the sedimentation chamber, a condition which is probably never realized. The several subsampling steps involved also tend to increase the variance (cf. Venrick, 1978a; Venrick, 1978b).

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 variance will be higher, and the precision accordingly lower. If it is necessary to keep the error within the same limits as for "randomly" distributed units, the number of counted units should be increased in the ratio average size of individual/size of counting unit.

The number of counting units per dm^3 sea water is calculated by multiplying the number of units counted with the coefficient C, which is obtained from the following formulas:

$$C\,(dm^{-3}) = \frac{A \times 1000}{N \times a_1 \times V} \quad \text{or } C\,(dm^{-3}) = \frac{A \times 1000}{a_2 \times V}$$

where

A = cross-section area of the top cylinder of the combined sedimentation chamber
the usual inner diameter is 25.0 mm, giving A = 491 mm^2 (the inner diameter of the bottom-plate being irrelevant)
N = number of counted fields or transects
a_1 = area of single field or transect
a_2 = total counted area
V = volume (cm^3) of sedimented aliquot

Reliable quantitative counting of the picoplankton fraction requires fluorescence microscopy.

When counting phytoplankton in a sedimentation chamber, it is suitable to count protozooplankton (e.g. ciliates and colourless flagellates). This recommendation is also valid for these forms. However, it must be stressed that the protozooplankton are a separate group and must not be mixed with the phytoplankton. Thus, they must not be included in abundance or biomass values of phytoplankton.

Cleaning of the sedimentation chambers

After use no part of the combined sedimentation chamber should be allowed to dry out before it is carefully cleaned. Dried phytoplankton or formalin preservative may be quite difficult to remove. The separate parts are first rinsed under running tap water, and then soaked for a few minutes in Lukewarm water with some nonabrasive detergent added, thereafter cleaned with a soft brush or soft tissue paper, and rinsed with tap water. The sedimentation chamber may also be cleaned with 95% ethanol. Finally, they are given a rinse with deionised or distilled water, and are put away to dry. Special care should be taken not to scratch either end of the top cylinder and the entire upper surface of the bottom plate. The sedimentation chamber may also be cleaned with 95% ethanol.

C.4.3.2.5 Biomass determinations

Introduction

Biomass data are a much better descriptor of phytoplankton than abundance, especially because the latter is strongly influenced by the highly abundant picoplankton and small nanoplankton, which can be analysed only with limited certainty. Thus, biomass data are preferred for characterising spatial and temporal phytoplankton patterns and modelling. Depending on the purpose of the investigation, phytoplankton biomass can be expressed as cell volume (or weight) or carbon. The transformations to cell volume are based on measurements of the size of the species and the adaptation of the shapes to geometrical figures. The mandatory geometric formulas, size groups and the resulting biovolumes per counting unit are compiled in the paper of Olenina et al. (2006). In a further step, the carbon content should be calculated because organic carbon is the universal component of organisms and is the energy source transported along the food chain.

Biovolume calculation

As specified in Section C.4.3. 2.4, the species/individuals have to be allocated to size classes according to the scheme of Olenina et al. (2006) during the counting process. The individual biovolumes of the different counting units have to be multiplied with their abundance to get the biovolume per dm^3.

$$\text{Biovolume}_{\text{taxon}}\ [mm^3\ dm^{-3}] = \text{abundance}\ [dm^{-3}] \times \text{VCU} \times 10^{-9}$$

VCU = volume of counting unit (in μm^3)

From the bio volume data, the biomass (wet weight) can simply be derived by a rough assumption of a plasma density of 1 g cm^{-3}.

Carbon content calculation

In the previous guidelines (HELCOM 1988) it was recommended to calculate the carbon content from the plasma volume by a constant factor. Since the calculation of the plasma volume of diatoms bears a lot of uncertainties and, moreover, the conversion factor is not constant in reality, the calculation of carbon has been suspended for some years.

New formulas by Menden-Deuer and Lessard (2000) take the decrease in specific carbon content with cell size into account and calculate the carbon content of diatoms directly from the cellular biovolume without plasmavolume calculation.

The general formula for phytoplankton is:

$$\text{Carbon [pg C cell}^{-1}] = 0.216 \times \text{CV}^{0.939}$$

Diatoms require a particular formula because of their lower specific carbon content:

$$\text{Carbon [pg C cell}^{-1}] = 0.288 \times \text{CV}^{0.811}$$

If cell aggregates are the counting unit (CU), their carbon content has to be calculated via the cells carbon by the following formulas. It has to be differentiated between counting of cell packages (e.g. 100 cells of *Microcystis* as a CU) and filaments (e.g. 100 µm of *Nodularia* as a CU). In filaments, the cell length has to be known.

For multi-cell colonies:

$$\text{Carbon [pg C CU}^{-1}] = 0.216 \times \text{CPU} \times (\text{VCU/CPU})^{0.939}$$

For filaments:

$$\text{Carbon [pg C CU}^{-1}] = 0.216 \times \text{LCU/CL} \times (\text{VCU*CL/LCU})^{0.939}$$

CU = counting unit
VCU = volume of counting unit (in μm^3)
CPU = number of cells per counting unit
CL = cell length (in µm)
LCU = length of counting unit (mostly 100 µm)

The calculation of the carbon content is non-obligatory, but if executed it has to be done according to the given formulas.

C.4.3.2.6 Semi-quantitative analysis of phytoplankton samples

Introduction

Microscopic determination is the only method to get information on the species composition of phytoplankton samples. This information is needed in order to reveal changes in the phytoplankton communities in time and space and e.g. to estimate the potential toxicity of a bloom. 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 (Leppänen *et al.*, 1995).

Counting procedure

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). All the species found should be listed using the HELCOM counting software with the net option, so when recorded they get the smallest ranking 1 automatically. The semi quantitative ranking should be done using a scale from one to five. The ranking is sample specific, and several species can also get the same ranking, even the highest one.

1 ***very sparse***, one or a few (less than five of the >20 µm fraction) cells or units in the analysed area = in the sedimented sample

2 ***sparse***, slightly more cells or units in the analysed 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

When the accurate abundance of a species (e.g. a potentially toxic one) should be counted, at least 20 fields (with 40 x objective), or one transect (with 10 x objective) should be analysed.

The sedimentation chambers etc. should be cleaned as for the quantitative analysis.

C.4.3.2.7 References

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Annex 11: Proposal for updating guidelines for Zooplankton in the HELCOM COMBINE manual

Formerly Annex C-7, Chapter 6.2 including chapter 7 and 8 and References, should refer now to Chapter C.4.3.4 Mesozooplankton. A new structure and renumbering of this chapter is necessary.

6.2 Carbon biomass determination

Parallel to the identification and counting procedure (see 6.1) the carbon biomass should be estimated for each individual. This allows the calculation of the carbon biomass of different taxonomic zooplankton groups and by summarising the results of the single taxa of the total sample, respectively. If the carbon biomass length equation is available (see 6.2.2), the calculation of carbon biomass should be based on length measurements (see 6.2.1) otherwise the calculation should base on average carbon biomass conversion factors (see 6.2.3).

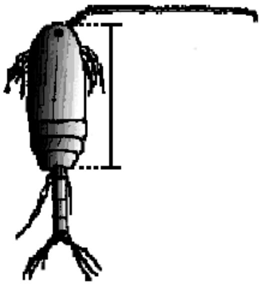
6.2.1 Length measurements of carbon biomass determination

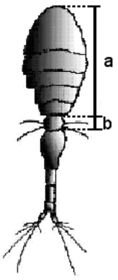
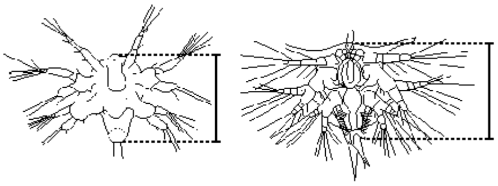
The required lengths should be measured by means of an eyepiece micrometer or with the aid of image analysis software. Prior to measurement, both systems have to be calibrated with a calibrated object micrometer for each microscope. This applies to the objectives and oculars too. All length measurements have to be standardised, because length to carbon ratios are based on a defined measuring mode (see Table 1).

At least, all individuals per dominant taxonomic group and sample have to be measured.

For length measurements of formalin fixed samples no shrinking factors need to be taken into account (Postel et al. 2007).

Table 1 Recommendations for length or body measurements

TAXA	MEASURING MODE	REMARKS
Calanoid copepods e.g.: *Pseudocalanus* sp., *Paracalanus* sp., *Acartia* sp., *Centropages hamatus,* *Temora longicornis,* *Eurytemora longicornis*		prosoma length

Cyclopoid copepods e.g.: *Oithona similis* *Cyclops* sp.		thoracal length (a)
Nauplia of copepods		
Cladocerans e.g.: *Evadne nordmanni* (a) *Podonidae* (b)	 (a) (b)	

6.2.2 Calculation of carbon biomass based on length measurements

If the carbon biomass length equation is available the calculation of carbon biomass [µg C / Ind.] should preferably base on length measurements L [µm / Ind] (see 6.2.1). The length to carbon biomass equations apply to all taxa regardless of their stage of development. All potential seasonal and / or regional environmental influences which could affect individual carbon biomass will also be taken into account by these equations.

The following regression equation has to be used:

$$\text{Carbon Biomass } [\mu g \text{ C / Ind.}] = a \times L^{b}$$

Table 2 contains the coefficients a and b for eleven selected taxa (Postel et al. 2007). This list will be completed by further taxa as soon as appropriate data are available.

For *Acartia bifilosa* and *Centropages hamatus* the length to carbon ratios are larger in May than in the rest of the year. Therefore, special equations are provided for both taxa.

Table 2 Coefficients a and b for calculation of carbon biomass based on length measurements. The estimated individual specific carbon biomass [µg C / Ind.], base on the individual specific length measurements [µm], varied within upper and lower confidence limits on the 95 % probability level (Postel *et al*, 2007).

Taxon		*A*	*B*
Acartia bifilosa		$3.8665*10^{-9}$	3.0253
	May only	$1.4388*10^{-9}$	3.1868
Acartia longiremis		$4.7756*10^{-8}$	2.6178

Acartia tonsa		$1.4390*10^{-8}$	2.8146
Centropages hamatus		$1.7298*10^{-8}$	2.8323
	May only	$6.2923*10^{-9}$	2.9903
Cyclops sp.		$5.5751*10^{-8}$	2.6510
Evadne nordmanni		$1.4663*10^{-9}$	3.0342
Oithona similis		$5.3930*10^{-10}$	3.3959
Paracalanus sp.		$1.6220*10^{-6}$	2.1934
Pseudocalanus sp.		$2.3223*10^{-6}$	2.1312
Podonidae		$1.8756*10^{-7}$	2.4600
Temora longicornis		$2.2744*10^{-9}$	3.2093

6.2.3 Calculation of carbon biomass based on average conversion factors

If length measurements or length to carbon biomass relations are not available, average conversion factors should be used for the calculation of individual specific carbon biomass. The abundance of a taxon has to be multiplied by the appropriate conversion factor and thus results in carbon biomass concentration of the taxon.

Most conversion factors originate from results of rapid-cryofixation (Köster 2003, Postel et al. 2007). In some cases, data have been used from literature sources based on cryofixation (Latja and Salonen, 1978; Kankaala, 1987; Kankaala and Johansson, 1986). To ensure comparability to data which base on rapid-cryofixation those factors were corrected by multiplying them by 0.8 (Postel *et al.*, 2007).

Some carbon biomass data are correlated to temperature and/or salinity. This indicates a seasonal and /or regional variability. This aspect needs further investigation and will be considered in the nearest future.

The carbon biomass conversion factors are presented in Table 4.

Table 3 Carbon biomass conversion factors [µg C / Taxon] ([1]Latja and Salonen, 1978 adapted after Postel, 2007; [2]Kankaala, 1987; Kankaala and Johansson, 1986 adapted after Postel, 2007)

TAXON	STAGES OF DEVELOPMENT	CARBON BIOMASS CONVERSION FACTOR
Acartia sp.	N	0.164
	C I_III	0.426
	C IV_V	1.243
Acartia bifilosa	F	2.439
	M	2.294
Acartia longiremis	F	1.521
	M	1.394
Acartia tonsa	F	2.093
	M	2.038
Alaurina composita	–	0.232
Balanus sp.	N	0.731
	Cypris L	2.199
Bivalvia	L	0.884

Bosmina sp.	–	1.405
Centropages hamatus	N	0.150
	C I_III	0.686
	C IV_V	1.940
	F	3.617
	M	2.864
Cyclops spp.	C I_III	0.389
	C IV_V	1.182
	F	3.080
	M	2.791
Eurytemora affinis[2]	N	0.140
	C I_III	0.540
Eurytemora affinis	C IV_V	0.952
	F	2.512
	M	1.720
Evadne nordmanni	–	1.382
Fritillaria borealis	–	1.312
Gastropoda	L	1.722
Gymnolaemata (syn.Bryozoa)	L	0.534
Keratella sp.[1]	---	0.019
Limnocalanus macrurus[2]	N	0.230
	C I_III	1.140
	C IV_V	3.750
	F	7.690
	M	7.020
Oikopleura dioica	---	2.160
Oithona similis	N	0.095
	C I_III	0.143
	C IV_V	0.271
	F	0.492
	M	0.552
Pseudocalanus / Paracalanus sp.	N	0.523
	C I_III	1.886
	C IV_V	3.257
Pseudocalanus sp.	F	4.340
	M	4.745
Paracalanus sp.	M	5.650
Podon sp.	---	2.197
Polychaeta	L	1.160
Synchaeta	---	0.198
Temora longicornis	N	0.285
	C I_III	0.706
	C IV_V	1.924
	F	3.590
	M	2.818

7. Data reporting

Data should be reported according to ICES data reporting formats.

8. Quality Assurance

[The quality assurance instructions should be in accordance with the recommendations given in the SGQAB Report of 1998, section 10, part B.

The following additional items should be included:

- determination of uncertainty of measurements
- participation in ring tests;
- intercalibration of equipment;
- participation in scientific workshops when e.g. recent taxonomical changes have been implemented.

9. References

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Annex 12: Proposal for a new structure of chapter B.4 in the HELCOM COMBINE manual

- **B.4 Validation guidelines**
  - **B.4.1 Sampling**
  - **B.4.2 Validation of analytical methods**
    - **B.4.2.1 Selectivity**
    - **B.4.2.2 Sensitivity**
    - **B.4.2.3 Limit of Detection (LOD), limit of quantification (LOQ), Lower limit of application (LLOA)**
      - **B.4.2.3.1 Calculation of LOD for methods with normally distributed blank values**
      - **B.4.2.3.2 Calculation of LOD for chromatographic methods**
      - **B.4.2.3.2 Calculation of LOQ**
    - **B.4.2.4 Range**
    - **B.4.2.5 Accuracy**
      - **B.4.2.5.1 Estimation of random errors**
      - **B.4.2.5.2 Estimation of systematic errors (biases)**
    - **B.4.2.6 Measurement uncertainty**
      - **B.4.2.6.1 The „bottom-up “procedure to estimate uncertainty**
      - **B.4.2.6.2 The „top-down“ model to estimate uncertainty using data of interlaboratory comparison exercises**
      - **B.4.2.6.3 The „top-down“-model to estimate uncertainty for individual laboratories**
    - **B.4.2.7 Robustness/interference**
  - **B.4.3 Validation of data**
    - **B.4.3.1 Data checks applied to individual data points and variables**
    - **B.4.3.2 Data checks applied by database managers**

ANNEX B-1 Validation of established analytical methods

ANNEX B-2 Protocol for evaluation of measurement uncertainty from in-house control measurements

ANNEX B-3 Checklist for implementing an internal audit programme

ANNEX B-4 Examples of reference materials for internal quality control

ANNEX B-5 Format for Standard Operation Procedure

Annex 13: Table of rearranging of ANNEXES of part B

ANNEX CODE	TITLE	PROPOSED ACTION	NEW LOCATION/ANNEX CODE	JUSTIFICATION
B-1	Principal components of a quality manual	Include into text	B.2.1 General	Very short annex, better presented in text
B-2	Validation of established analytical methods	Keep as it is	Annex B-1 Validation of established analytical methods	
B-3	Quality audit	Keep as it is, rename	Annex B-3 Checklist for implementing an internal audit program	
B-4	Technical note on Measurement Uncertainty	Include into text	B.4.2.6 Measurement uncertainty	
B-4 Appendix 1	Protocol for evaluation of measurement uncertainty from in-house control measurements	Convert to new annex	Annex B-2 Protocol for evaluation of measurement uncertainty from in-house control measurements	
B-5	General remarks on sampling	Include into text	B.4.1 Sampling	
B-6	Examples of reference materials for internal quality control	Table B-6.1. should divided in 3 separate tables: Currently available CRMs in Sediments (Table B-6.1) /Biota (Table B-6.2)/ Water (Table B-6.3) for marine monitoring programmes (January 2001).	Annex B-4 Examples of reference materials for internal quality control	Table 6.1. is currently too long and not well arranged
B-7	Units and conversions	Delete	Omitted	Duplication of B.1.4.1 Units
B-8	Technical note on the determination of hydrographic parameters	Include into text of part C	C.4.1 Hydrographic variables	Technical note on special parameter
B-8, Appendix 1	Technical note on the determination of salinity and temperature of seawater	Include into text of part C and subdivide in separate chapters	C.4.1 Hydrographic variables C.4.1.1 Salinity C.4.1.2 Temperatur	Technical note on special parameter
B-8, Appendix 2	Technical note on the determination of dissolved oxygen in seawater	Include into text of part C	C.4.1 Hydrographic variables C.4.1.3 Determination of dissolved oxygen	Technical note on special parameter
B-8, Appendix 3	Recommended equations for the calculation of solubility and saturation of dissolved oxygen in seawater	Include into text of part C	C.4.1 Hydrographic variables C.4.1.4 Calculation of solubility and saturation of dissolved oxygen	Technical note on special parameter
B-8, Appendix 4	Technical note on the determination of hydrogen sulphide in seawater	Include into text of part C	C.4.1 Hydrographic variables C.4.1.5 Hydrogen sulphide	Technical note on special parameter
B-9	Technical note on determination of nutrients	Include into text of part C	C.4.2 Hydrochemical variables	Technical note on special

			C.4.2.1 Nutrients	parameter
B-10	Chemical analysis of anoxic waters	Include into text of part C	C.4.2 Hydrochemical variables C.4.2.6 Anoxic waters	Technical note on special parameter
B-11	Technical note on the determination of heavy metals and persistent organic compounds in seawater	Include into text of part D	Part D	Technical note on special parameter
B-11, Appendix 1	Technical note on the determination of trace metals (Cd, Pb, Cu, Co, Zn, Ni, Fe) including mercury in seawater	Include into text of part D	Part D	Technical note on special parameter
B-11, Appendix 2	Technical note on the determination of persistent organic compounds in seawater	Include into text of part D	Part D	Technical note on special parameter
B-12	Technical note on the determination of heavy metals and persistent organic compounds in biota	Include into text of part D	Part D	Technical note on special parameter
B-12, Appendix 1	Technical note on biological material sampling and sample handling for the analysis of persistent organic pollutants (PAHs, PCBs and OCPs) and metallic trace elements	Include into text of part D	Part D	Technical note on special parameter
B-12, Appendix 2	Technical note on the determination of polycyclic aromatic hydrocarbons in biota	Include into text of part D	Part D	Technical note on special parameter
B-12, Appendix 3	Technical note on the determination of chloronated biphenyls and organochlorine pesticides in biota	Include into text of part D	Part D	Technical note on special parameter
B-12, Appendix 4	Technical note on the determination of trace metallic elements in biota	Include into text of part D	Part D	Technical note on special parameter
B-12, Appendix 5	Technical note on the determination of total mercury in marine biota	Include into text of part D	Part D	Technical note on special parameter
B-13	Technical note on the determination of heavy metals and persistent organic compounds in marine sediments	Include into text of part D	Part D	Technical note on special parameter
B-13, Appendix 1	Technical note on the determination of polycyclic aromatic hydrocarbons in marine sediment	Include into text of part D	Part D	Technical note on special parameter
B-13, Appendix 2	Technical note on the determination of chlorinated biphenyls in marine sediment	Include into text of part D	Part D	Technical note on special parameter
B-13, Appendix 3	Technical note on the determination of heavy metals in marine sediment	Include into text of part D	Part D	Technical note on special parameter

B-14	Technical note on the measurement of pH in seawater	Include into text of part C	C.4.2 Hydrochemical variables C.4.2.4 pH	Technical note on special parameter
B-15	Technical note on the measurement of total alkalinity in seawater	Include into text of part C	C.4.2 Hydrochemical variables C.4.2.5 Alkalinity	Technical note on special parameter
B-16	Technical note on co-factor analysis	Delete	Omitted	Duplication of B.3.6
B-17	Technical Note on the determination of organic carbon in seawater	Include into text of part C	C.4.2 Hydrochemical variables C.4.2.9 Organic carbon	Technical note on special parameter
New annex	Format for Standard Operating Procedure for methods presented in C and D	Addition	Annex B-5 Format for Standard Operating Procedure for methods presented in C and D	

Annex 14: Proposal of a revised chapter B.2 in part B of HELCOM COMBINE guidelines

B.2. The Quality System

B.2.1 General

'Quality system' is a term used to describe measures which ensure that a laboratory fulfills the requirements for its analytical tasks on a continuing basis. A laboratory should establish and operate a Quality System adequate for the range of activities, i.e., for the type and extent of investigations, for which it has been employed.

The basic principles for establishing a quality system are fixed in ISO 9000 series and EN ISO/IEC 17025 (2005).

The Quality Management System should be formalized in a Quality Management Manual which must be maintained and kept up-to-date.

The person responsible for authorization and compilation of the Quality Management Manual should be identified, and an identification of holders of controlled copies should be listed in the manual.

The Quality System must contain a statement of the intentions of the laboratory top management in relation to quality in all aspects of its work (statement on Quality Policy).

The quality management manual should contain, for example, the following items or their equivalent:

1. Scope
2. References
3. Definitions
4. Statement of quality policy
5. Organization and management
6. Quality system audit and review
7. Personnel
8. Accommodation and environment
9. Equipment and reference material
10. Measurement, traceability and calibration
11. Calibration and test methods
12. Handling of calibration and test items
13. Records
14. Certificates and reports
15. Sub-contracting of calibration or testing
16. Outside support services and supplies
17. Complaints

B.2.2 Scope

The laboratory's scope should be formulated in terms of:

- The range of products, materials or sample types tested or analysed;
- The types of tests or analyses carried out;
- The specification of method/equipment/technique used;
- The concentration range and accuracy of each test and analysis.

B.2.3 Organization, Management and Staff

B.2.3.1 Organization

The Quality System should provide general information on the identity and legal status of the laboratory and should include a statement of the technical role of the laboratory (e.g., employed in marine environmental monitoring).

The following information must be included in an organizational chart:

- Technical Manager, Quality Manager, and any deputies;
- General lines of responsibility within the laboratory (including the relationship between management, technical operations, quality control and support services);
- The lines of responsibility within individual sections of the laboratory;
- The relationship between the laboratory and any parent or sister organizations.
- The appropriate chart should show that, for matters related to quality, the Quality Manager has direct access to the highest level of management at which decisions are taken on laboratory policy and resources, and to the Technical Manager.

B.2.3.2 Management

Job descriptions, qualifications, training and experience are necessary for:

- Technical Manager;
- Quality Manager;
- Other key laboratory managerial and technical posts.

Job descriptions should include:

- title of job and brief summary of function,
- person or functions to whom jobholder reports,
- person or functions that report to jobholder,
- key tasks that jobholder performs in the laboratory,
- limits of authority and responsibility.

The **Technical Manager**:
The Quality System should include a statement that the post-holder has overall responsibility for the technical operation of the laboratory and for ensuring that the Quality System requirements are met.

The **Quality Manager:**
The Quality System should include a statement that the post-holder has responsibility for ensuring that the requirements for the Quality System are met continuously and that the post-holder has direct access to the highest level of management at which decisions are taken on laboratory policy or resources, and to the Technical Manager.

The Quality System should state explicitly the Quality Manager's duties in relation to control and maintenance of documentation, including the Quality Manual, and of specific procedures for the control, distribution, amendment, updating, retrieval, review and approval of all documentation relating to the calibration and testing work of the laboratory.

B.2.3.3 Staff

The laboratory management should define the minimum levels of qualification and experience necessary for engagement of staff and their assignment to respective duties.

Members of staff authorized to use equipment or perform specific calibrations and tests should be identified.

The laboratory should ensure that all staff receive training adequate to the competent performance of the tests/methods and operation of equipment. A record should be maintained which provides evidence that individual members of staff have been adequately trained and their competence to carry out specific tests/methods or techniques has been assessed. Laboratory managers should be aware that a change of staff might jeopardize the continuation of quality.

B.2.4 Documentation, Standard Operating Procedures

Necessary documentation includes:

- a clear description of sampling equipment;
- a clear description of all steps in the sampling procedure;
- a clear description of the analytical methods (Standard Operating Procedures: SOPs);
- a strict keeping of ship and laboratory journals;
- instrument journals;
- protocols for sample identification;
- clear labelling of samples, reference materials, chemicals, reagents, volumetric equipment, stating date, calibration status, concentration or content as appropriate and signature of the person responsible.

Standard Operating Procedures are detailed descriptions of processes or workflows by precisely work specification and defining responsibilities for each task.

The following types of SOPs can be differentiated:

1. Descriptive SOPs
2. SOPs of procedures
3. SOPs of methods
4. SOPs of equipment

A proposal for a Standard Operating Procedure is given in Annex B-5.

B.2.5 Laboratory Testing Environment

Samples, reagents and standards should be stored and labelled so as to ensure their integrity. The laboratory should guard against deterioration, contamination and loss of identity.

The laboratory should provide appropriate environmental conditions and special areas for particular investigations.

Staff should be aware of:

- the intended use of particular areas,
- the restrictions imposed on working within such areas,
- the reasons for imposing such restrictions.

B.2.6 Equipment

As part of its quality system, a laboratory is required to operate a programme for the necessary maintenance and calibration of equipment used in the field and in the laboratory to ensure against bias of results.

General service equipment should be maintained by appropriate cleaning and operational checks where necessary. Calibrations will be necessary where the equipment can significantly affect the analytical result.

The correct use of equipment is critical to analytical measurements and this equipment must be maintained, calibrated and used in a manner consistent with the accuracy required of data. For certain chemical analysis, one should consider that measurements can often be made by mass rather than by volume.

Particularly for trace analyses, contamination through desorption of impurities from, or uncontrolled determinand losses through sorption on, surfaces of volumetric flasks can be significant. Therefore, special attention should be paid to the selection of appropriate types of material (quartz, PTFE, etc.) used for volumetric equipment and its proper cleaning and conditioning prior to analysis.

Periodic performance checks should be carried out at specific intervals on measuring instruments (e.g., for response, stability and linearity of sources, sensors and detectors, the separating efficiency of chromatographic systems).

The frequency of such performance checks will be determined by experience and based on the need, type and previous performance of the equipment. Intervals between checks should be shorter than the time the equipment has been found to take to drift outside acceptable limits and should be given in the equipment list.

B.2.7 Quality Audit

The ISO 9000 and ISO 14000 series emphasize the importance of audits as a management tool for monitoring and verifying the effective implementation of an organizations quality and/or environmental policy. Audits are an essential part of conformity assessment activities such as accreditation.

In EN ISO/IEC 17025 (2005) it is stated that laboratories should periodically, and in accordance with a predetermined schedule and procedure conduct

- internal audits of its activities to verify that its operations continue comply with the requirements of the management system,
- reviews of the laboratory's management system and testing and/or calibration activities to ensure their continuing suitability and effectiveness, and to introduce necessary changes or improvements.

The audit is a systematic, independent and documented process for obtaining audit evidence and evaluating it objectively to determine the extent to which the audit criteria are fulfilled.

Guidelines for auditing are given in EN ISO 19011 (2002). Programs for internal audits may be based upon a check list developed by APLAC (2004), see Annex B-3.

Internal audits are conducted by the organization itself for management review and other internal purposes, and may form the basis for an organization's self-declaration of conformity. External audits are conducted by parties having an interest in the organization, such as customers or by external, independent auditing organizations, such as accreditation bodies.

Annex 15: Proposal of a revised chapter B.4 in part B of HELCOM COMBINE guidelines

B.4 Validation guidelines

Validation means the whole process by which the research community receive the necessary information to - assess the ability of a procedure to obtain reliable results

- determine the conditions under which such results can be obtained
- define the limitations of the procedure.

According to EN ISO /IEC 17025 (2005) Section 5.4.5.1: “Validation is the confirmation by examination and the provision of objective evidence that the particular requirements for a specific intended use are fulfilled.”

B.4.1 Sampling

The appropriate design of the sampling programmes, the selection of proper equipment and the assignment of well trained personnel are the basis for effective and well-founded sampling and have to dovetailed with the monitoring programmes described in Part C and D of the manual.

Sampling for the performance of analytical investigation has to be oriented towards the particular analytical task. Different aspects of sampling programmes are comprehensively dealt with in articles by Kratochvil and Taylor, 1981, the ACS Committee on Environmental Improvement (ACS, 1983), and Garfield, 1989.

Based on information provided by the above-mentioned authors, an acceptable sampling programme should include the following:

1) a predetermined sampling plan that takes into account the specific purpose of the investigations, including the number of replicates of samples, the contaminants to be determined, their expected concentration range, and the type of matrix to be analysed;
2) sample collection by personnel trained in the sampling techniques and procedures specified;
3) a written sampling protocol or a standard operating procedure (SOP)
4) maintenance of the sample integrity by
5) using sampling devices that have been found to be suitable for the particular purpose,
6) avoiding contamination of samples from the use of unclean equipment,
7) using transportation and storage procedures that ensure that the composition of the sample or the concentrations of the variables are not altered (checks of sample stability);
8) instructions for labelling the sample specifying its identity;
9) a record that demonstrates an unbroken control over the sample from collection to its final disposition.

Detailed guidelines on sampling for chemical analysis will be dealt with at a later time. Recommendations from other bodies or working groups will be taken into consideration when available.

Detailed information on sampling for biological measurements is found with each separate variable in part C.

B.4.2 Validation of analytical methods

On the basis of the specifications developed in the items under Section B.3, the method must now be examined to determine whether it actually can produce the degree of specificity and confidence required. Accordingly, the objective of the validation process is to identify the performance of the analytical method and to demonstrate that the analytical system is operating in a state of statistical control.

When analytical measurements are 'in a state of statistical control', it means that all causes of errors remain the same and have been characterized statistically.

According to EN ISO /IEC 17025 (2005), Section 5.4.5.2 "The laboratory shall validate non-standard methods, laboratory-designed/developed methods, standard methods used outside their intended scope, and amplifications and modifications of standard methods to confirm that the methods are fit for the intended use. The validation shall be as extensive as is necessary to meet the needs of the given application or field of application. The laboratory shall record the results obtained, the procedure used for the validation, and a statement as to whether the method is fit for the intended use."

The very purpose of a method validation is to conclude whether the method is fit for the intended use.

A full validation of analytical methods has to be applied:

- When a standardized analytical method is applied for the first time
- When a standardized or other accepted analytical method undergoes substantial change (e.g., change of important chemicals, automatisation, scaling down, etc.)
- When a published but non standardized analytical method shall be used
- When an analytical method is developed in-house for subsequent routine use
- When an analytical method is developed in-house as a solution to a unique analytical problem
- When a method is used outside its intended scope (e.g., other matrices or other concentration intervals).

A limited validation procedure can be used for:

- Methods already in use in the laboratory (previously validated), where minor modifications have been carried out.
- Standard methods (ISO, EN or national standards).
- Standard methods that have been subject to minor modification.
- Other generally accepted methods, where information on scope is given (e.g., methods taken directly from HELCOM or OSPAR guidelines or from Grasshoff et al., "Methods of Seawater Analysis").

The validation of analytical methods should include the following steps:

- Definition of the scope of the analytical method
- Identification of the analytical method performance characteristics that have to be validated (see below)
- Selection of suitable experiments to determine required performance characteristics
- Realisation of suitable experiments to determine required performance characteristics
- Documentation, revision and maintenance of analytical method validation results

The performance characteristics include:

- Selectivity,

- Sensitivity,
- Range,
- Limit of detection (LOD),
- Limit of quantification (LOQ)
- Accuracy (precision, trueness)
- Measurement uncertainty

The procedure for limited validation will generate information on:

- Limit of detection
- Linearity
- Range
- Accuracy (precision, trueness)

These performance characteristics should be clearly stated in the documented method description so that the suitability of the method for a particular application can be assessed. Special information will be found with each separate analytical method at a later time (*this has to be checked*).

In the following, a brief explanation and, where appropriate, guidance on the estimation of these parameters is given.

B.4.2.1 Selectivity

….

B.4.2.2 Sensitivity

….

B.4.2.3 Limit of Detection (LOD), limit of quantification (LOQ), Lower limit of application (LLOA)

….

B.4.2.3.1 Calculation of LOD for methods with normally distributed blank values

….

B.4.2.3.2 Calculation of LOD for chromatographic methods

….

B.4.2.3.2 Calculation of LOQ

….

B.4.2.4 Range

….

B.4.2.5 Accuracy

….

B.4.2.5.1 Estimation of random errors

....

B.4.2.5.2 Estimation of systematic errors (biases)

....

B.4.2.6 Measurement uncertainty

The principal aim of analytical work is to gain information on the material under investigation. This information always constitutes a probability distribution determined by a random error and a systematic error inherent in the analytical procedure used. A systematic error can act as an additive or as a multiplicative shift. Systematic errors are superimposed by the random error. Analytical practice shows that there is always some doubt about the correctness of a stated result, even when all the suspected sources of error have been taken into account and the appropriate corrections have been applied. This is due to the uncertainty regarding the correction factors and the uncertainty arising from random effects, which cannot be eliminated, although they can be reduced by increasing the number of observations. The result of an analysis after careful consideration of all error sources may by chance be very close to the true value. However, the uncertainty can still be very large, simply because the analyst is very unsure of how close that result is to the true value. Consequently, a measurement cannot be properly interpreted without the knowledge of the uncertainty associated with the result.

The concept of expressing or estimating the uncertainty of measurements was developed to inform the final users of the analytical data concerning how much allowance must be made for the possibility that repetition of the test will give a different value (Horwitz, 1998). This information is particularly necessary when analytical results are not used by the data originator, as is the regular case in the assessment of data from environmental monitoring programmes. This technical note provides information on how the uncertainty of measurement of the analytical methods used in the HELCOM COMBINE programme can be estimated, so that it would be possible to judge whether or not the accuracy (trueness and precision) of the method meets the requirements of this programme. It should be taken into account that the requirements on accuracy depend on the aims and the purpose of the monitoring programme.

In accordance with the current version of the International Vocabulary of Basic and General Standard Terms in Metrology (ISO, 1993), the ISO Guide (ISO, 1995) defines Measurement Uncertainty as a parameter, associated with the result of a measurement, that characterises the dispersion of the values that could reasonably be attributed to the measurand. The following definitions apply (EURACHEM/CITAC, 2000):

Standard uncertainty

$u(x_i)$ uncertainty of the result x_i of a measurement expressed as a standard deviation

Combined standard uncertainty

$u_C(y)$ standard uncertainty of the result y of a measurement when the result is obtained from the values of a number of other quantities, equal to the positive square root of a sum of terms, the terms being the variances or covariances of these other quantities weighed according to how the mesasurement results varies with these quantities

Expanded standard uncertainty

U quantity defining an interval about the result of a measurement that may be expected to encompass a large fraction of the distribution of values that could reasonably be attributed to the measurand, an expanded uncertainty is calculated from a combined standard uncertainty u_C and a coverage factor *k* using $U = k \, x \, u_C$

Coverage factor

k numerical factor used as a multiplier of the combined standard uncertainty in order to obtain an expanded uncertainty, the choice of the factor k is based on the level of confidence desired, k = 2 for an approximate level of confidence of 95 %

The expanded uncertainty (estimated combined standard uncertainty multiplied by the coverage factor *k*, usually *k* = 2 for a confidence limit of 95 %) should be reported for individual monitoring parameters in the form of a standard deviation or confidence interval together with information on how it was determined.

Generally, there are two main approaches to estimating the uncertainty of analytical measurements or an analytical procedure, respectively.

According to the original ISO approach (Horwitz, 1998), the uncertainty of analytical results is derived by listing all of the possible errors in the form of standard deviations. Using this error budget model, the combined standard uncertainty can then be calculated as the square root of the sum of squares of the individual error components. This „bottom-up“ approach of ISO assumes that an analytical method can be structured into small, simple steps, and that an individual standard uncertainty can be attributed to all of these steps, sometimes based on a best guess of experienced analysts.

A „top-down“ view on estimating the combined standard uncertainty through interlaboratory comparisons was developed by the Analytical Methods Committee of the Royal Society of Chemistry of the UK (1995). Both systematic and random errors of individual laboratories become random in an interlaboratory comparison or in a laboratory proficiency testing scheme, provided that identical and homogeneous samples are analysed. Following ISO 5725 (1994), the within- and between-laboratory variance can be calculated and combined in the reproducibility of the interlaboratory comparison. Only in case that the same analytical procedure is used, the calculated reproducibility of the interlaboratory comparison can be considered as the standard uncertainty of measurement.

A further „top-down“ approach is provided by the Nordic Committee on Food Analysis(1997) based on experimental data generated in the individual laboratory /6/. According to this proposal, the combined standard uncertainty is characterised as the internal reproducibility standard deviation calculated from method validation data or using information of internal quality assurance measures (analysis of certified reference materials, participation in proficiency testing schemes or use of reference methods). In the case that analytical results are compared to those of a reference method, a BCR information can be used to estimate the uncertainty (BCR, 1998).

A comprehensive description of all of these approaches can be found in a recently released EURACHEM/CITAC Guide (2000).

B.4.2.6.1 The „bottom-up“ procedure to estimate uncertainty

The „bottom-up“ approach to estimate the uncertainty of analytical results seems to be rather impractical (Horwitz, 1998), because it does not include the outcome from interlaboratory comparison or from laboratory proficiency testing schemes in marine monitoring available today. Therefore, if information on the uncertainty of analytical data generated in the COMBINE programme is needed, the „top-down“ approach should be preferred. The procedure by which the uncertainty of measurements is calculated depends on the requirements of the monitoring programme.

B.4.2.6.2 The „top-down“ model to estimate uncertainty using data of interlaboratory comparison exercises

As stated in the EURACHEM/CITAC Guide (2000), the reproducibility standard deviation of interlaboratory comparison exercises which are carried out according to ISO 5725 (1994) or Thompson and Wood (1993) can be used as the combined standard uncertainty for methods operating within their defined scope, provided that contributions of additional factors (i.e. sampling error, inhomogeneous distribution of the analyte) have been shown to be negligible. The sampling error and its inclusion in the uncertainty budget require special investigations. The same is true for the uncertainty of cofactor determinations carried out in the COMBINE programme to support monitoring results. Annex B-2 gives an example on the uncertainty estimation using data of an interlaboratory comparison.

B.4.2.6.3 The „top-down“-model to estimate uncertainty for individual laboratories

In case an individual laboratory is requested to provide information on uncertainty of measurement in connection with reported data, the approach proposed by the Nordic Committee on Food Analysis (1997) should be followed. The so called internal reproducibility standard deviation can be derived easily from internal quality control charts or from special investigations carried out to determine the internal reproducibility.

According to the EURACHEM/CITAC Guide (2000), an uncertainty estimation based on validation studies and routine quality control requires the best available estimate of the overall bias and the precision together with a quantification of any uncertainties associated with effects incompletely accounted for in the method performance studies (i.e matrix effects, different concentration levels, calibration model used). Information on the overall bias and precision should be available from routine quality control charts, which are to be established in the COMBINE monitoring laboratories. For example, the overall bias is best estimated by the analysis of an appropriate certified reference material (CRM) analysed several times over a period of time or regularly between routine monitoring samples. In that case, the combined standard uncertainty is simply the combination of the standard deviation associated with these determinations and the uncertainty in the CRM value (see ANNEX B-2 for an example). To study the influence of additional parameters on their contribution to the uncertainty budget, special investigations are needed. This is normally carried out in the form of robustness or ruggedness testing during method validation studies (Youden and Steiner, 1975). Robustness studies provide information on the variability of analytical results caused by small deviations from the experimental design prescribed by the analytical method.

B.4.2.7 Robustness/interference

….new chapter to be compiled by a qualified expert group

B.4.3 Validation of data

Data validation is the first step in assessing data quality and is a standardized process for judging the analytical quality and usefulness of discrete sets of environmental data. It is a decision making process during which established quality control criteria are applied to the data. Data which meet all defined validation criteria are accepted as qualified and can be used as needed. Data which do not meet one or more validation criteria have to be rejected and can not be used at all. These rejected data have to be flagged and depending on the intended use of data the applicability of these data is restricted after examining the reasons of rejection.

The knowledge of the “total uncertainty” consisting of sampling variability and measurement errors places the end users of data in a position to assess the usability of data sets in the context of previously defined data quality objectives. The specific data quality objectives have be defined before any data are collected. Data assessment is necessary to ensure that all activities connected with sampling, analysing and supplying environmental data meet the objectives of the HELCOM monitoring programme. This includes data verification, data validation and data quality assessment of all steps of data collection.

It should be known which factors the sampling and measurements influence: size, age and stage of development of organisms, sampling conditions, sample handling, season, external factors (stress, climate, hydrodynamic parameters, physical and chemical conditions), experience of scientist/expert.

The intention of data validation is

- To produce a consistent database with validated data of a known quality
- To evaluate the internal, spatial, temporal and physical consistency of the data
- To compare data to identify errors, bias or outliers

There are three levels of data evaluation:

- By the originator of the data and/or the data submitter
- By the data centre
- By the end-user.

B.4.3.1 Data checks applied to individual data points and variables

This means the verification of “raw” data (primary and secondary data) by the originator of the data before submitting data to the data centre by plausibility checks presupposing that appropriate quality assurance mechanisms are established:

- Check the compliance of the data with defined format and conformity with agreed taxonomic nomenclature;
- Check the expected interval range;
- Check the measurement units;
- Range checks;
- Check for outliers;
- Check for missing information/data;
- Check for deviations from programme, guidelines, methodologies used in previous years;
- Check for availability of essential metadata (e.g. method and QA information);
- Check for availability of important accompanying parameters (for example co-factors see B.3.5);
- Reference controls or use of functional dependences for controls;
- Data trend controls;
- Control samples;
- Reference collections.

These checks should be carried out by a qualified person or team. All data which are not plausible should be listed in a validation report. This report should contain:

- The doubtful values;
- Date and time of occurrence;
- The validation criteria which were failed and the reasons;
- A proposal for the further use of these suspect data (for example: delete or suitable flag or repeating analysis if possible).

B.4.3.2 Data checks applied by database managers

A central data management system should guarantee safe archiving (regular back-ups, computer virus checks, multiple storage, etc.) and access to the data.

The qualifications of the data managers and programmers are of importance for the effective management of the data. A scientific background of the data manager is highly recommended, as well as training of both data managers and programmers in order to meet up-to-date standards.

The verification of data by special routines of the data centre before submitting the data to reports and assessments should be achieved by answering the following questions:

- Are the correct data provided;
- Are the data complete (missing stations, missing intervals, completeness of accompanying parameters/information e.g. track plots, cruise summary, cruise reports, statistics on types and amount of data);
- Are there new data provided (new stations, new parameter);
- Check the compliance of the data with defined format and conformity with agreed taxonomic nomenclature, expected interval range, measurement units, range checks (data range tests);
- Check for deviations from programme, guidelines, methodologies (sampling/analysis procedures) used in previous years;
- Perform quick-look visualisation of the data/information (e.g. track plots or charts);
- Check for outliers;
- Data trend controls;
- Incorporation of data quality flags.

It is recognised that decisions regarding the overall acceptability of multivariate data arising from the analysis of biological communities can be difficult to arrive at, since elements of the submitted information may be unsuitable for some purposes, but nevertheless sufficient for others. For example, deficiencies in species identification may preclude the use of a submitted data set in “biodiversity” assessments, but the responsible laboratory may return biomass data of acceptable quality for the same samples, which may then be useful in assessments of ecosystem function. Criteria for determining the acceptability of data from surveys of biological communities to meet specified information needs at the international evaluation stage are still under development, and should be given high priority. However, systems for “flagging” of data are useful for the quality assurance of the input to international databases.

The following scheme for flagging of data should be used:

VALID	DATA FULFIL ALL PLAUSIBILITY CRITERIA
Estimated	Row data are suspect, but allow an estimation
Suspect	Data are doubtful because they violate defined plausibility criteria, cause unknown
Invalid	Data are inaccurate or in error, cause confessed
Missing	No Data are available

Annex 16: Proposal of a Standard Operating Procedure

HELCOM/OSPAR **COMBINE/JAMP MANUAL** ***PART L*** **ANNEX L-00**	***TITLE OF STANDARD OPERATING PROCEDURE***	**ISSUE NO.: 1** **REVISION 0: 0** **PAGE NO: 87 (19)** **ISSUE DATE: 07-02-15** **APPROVED BY:** **SIGNATURE:**

1 Introduction

1.1 *Background*

1.2 *Principle*

1.3 *Extent*

1.4 *Disturbances*

1.5 *Contamination risk*

1.6 *Safety*

2 Preparation

2.1 Cleaning and purification

2.2 *Sample identification*

2.3 *Reagents*

2.4 *Prior to sampling*

2.5 Protocol

3 Sampling

3.1 *Sampling*

3.2 *Preservation/Processing*

3.3 *Storage*

4 Method description

4.1 *Reagents*

4.2 *Calibration solutions*

4.3 *Processing*

4.4 *Calibration*

4.5 *Analysis*

5 Calculations

5.1 *Calculation function*

5.2 *Calculations*

5.3 *Precision and accuracy*

6 Quality assurance and evaluation

6.1 *Control charts*

6.2 *Evaluation*

7 Report format

8 Equipment

9 Chemicals and solutions

10 References

11 Appendices

1 Introduction

Presentation of the method and its relevance as an environmental quality factor. Relevant references are provided.

1.1 Background

General description of the basis for the method for non-experts with some detail and references.

1.2 Principle

Detiled description of the principle behind the method for e.g. technicians.

1.3 Extent

Present under what environmental conditions the method is applicable. For example present limitation due to salinity, temperature, concentrations, rates or other factors.

1.4 Disturbances

Present factors that may cause erroneous results by the method

1.5 Contamination risk

Present compounds that may contaminate the sample, distort specimens or hamper growth.

1.6 Safety

Present hazardous substances used in the method and precautions that should be applied.

2 Preparations

2.1 Cleaning and purification

2.2 Identification of sample

2.3 Reagents

2.4 Before cruise/sampling

2.5 Protocol

Identify protocols for e.g. logistic data, incubation conditions that should be used.

3 Sampling

3.1 Sampling

3.1.1 Sampling strategy

Provide advice on sampling frequency in time and spatial coverage of stations that is recommended based on statistical considerations.

3.1.2 Sampling method

3.2 Preservation/processing

Give time limits within which sample processing or analysis should have been completed.

3.3 Storage

4 Method description

4.1 Reagents

4.2 Calibration solutions

4.3 Processing

4.3.1 Preparations

4.4 Calibration

Define calibrations of instruments that should be performed routinely.

4.5 Analysis

5 Calculations

5.1 Calculation functions

5.2 Calculations

Present how calculations are best performed by e.g. manually, by data bases or distributed software.

5.3 Measurement uncertainty

Provide extended measurement uncertainty estimates of the method and detection limit according to current international standards and guidelines.

6 Quality assurance and evaluation

6.1 Control charts

.Recommend suitable control charts according to international scientific guidelines.

6.2 Evaluation

Describe quality criteria for approval of a measurement value.

7 Reporting

Present variables and other information that should be supplied with measurement values of the operating procedure.

Table 2. Primary database variables and units.

PARAMETER	SI-UNIT	VALID DIGITS	CALCULATION FUNCTION	CATEGORY	DATABASE ACRONYME	VALUE EXAMPLE

† footnote.

Define what primary (raw) data that should be reported.

Table 3. Calculated parameters.

PARAMETER	SI UNIT	VALID DIGITS	CALCULATION FUNCTION	CATEGORY	DATABASE ACRONYME	VALUE EXAMPLE

Define what secondary (calculated) data that should be possible to derive from the primary data and used in status assessment.

8 Equipment

Detailed presentation of equipment, its performance, model number and brand.

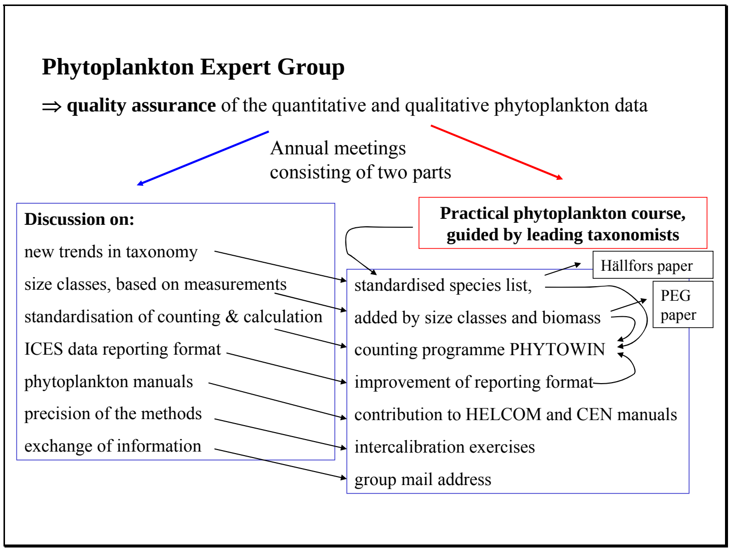
9 Chemicals and solutions

Detailed description of solutions and chemicals, their quality requirements, distributor, concentrations, preparations procedure and storage.

10 References

Give full traceable reference to scientific literature, reports or links to web sites.

Annex 17: Presentation of the activities of the HELCOM Phytoplankton Expert Group by N. Wasmund



Phytoplankton Expert Group

⇒ **quality assurance** of the quantitative and qualitative phytoplankton data

Annual meetings
consisting of two parts

Discussion on:

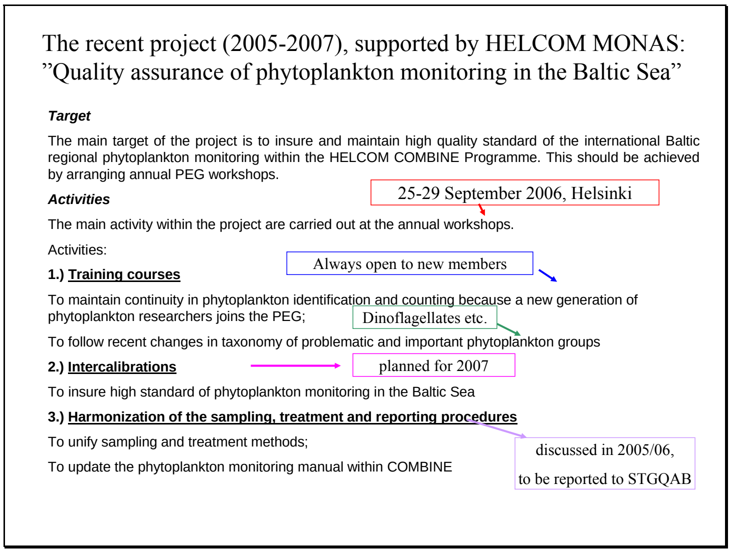
- new trends in taxonomy
- size classes, based on measurements
- standardisation of counting & calculation
- ICES data reporting format
- phytoplankton manuals
- precision of the methods
- exchange of information

Practical phytoplankton course, guided by leading taxonomists

- standardised species list,
- added by size classes and biomass
- counting programme PHYTOWIN
- improvement of reporting format
- contribution to HELCOM and CEN manuals
- intercalibration exercises
- group mail address

Hällfors paper

PEG paper



The recent project (2005-2007), supported by HELCOM MONAS:
”Quality assurance of phytoplankton monitoring in the Baltic Sea”

Target

The main target of the project is to insure and maintain high quality standard of the international Baltic regional phytoplankton monitoring within the HELCOM COMBINE Programme. This should be achieved by arranging annual PEG workshops.

Activities

25-29 September 2006, Helsinki

The main activity within the project are carried out at the annual workshops.

Activities:

1.) Training courses

Always open to new members

To maintain continuity in phytoplankton identification and counting because a new generation of phytoplankton researchers joins the PEG;

Dinoflagellates etc.

To follow recent changes in taxonomy of problematic and important phytoplankton groups

2.) Intercalibrations

planned for 2007

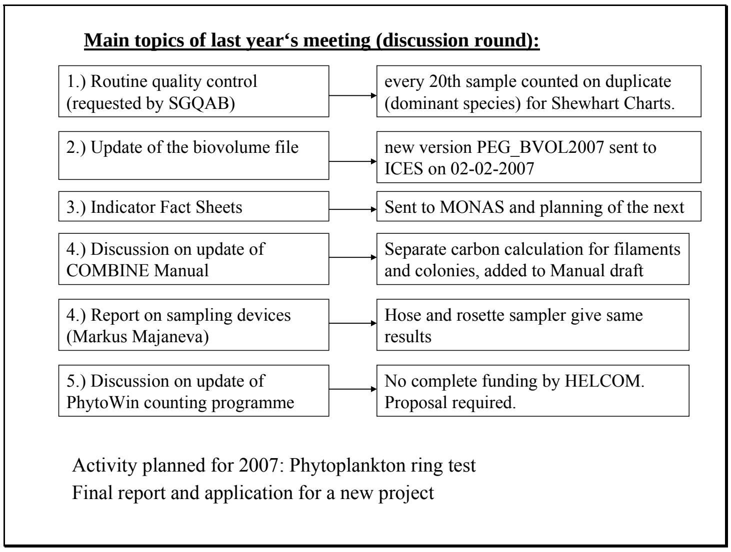
To insure high standard of phytoplankton monitoring in the Baltic Sea

3.) Harmonization of the sampling, treatment and reporting procedures

To unify sampling and treatment methods;

To update the phytoplankton monitoring manual within COMBINE

discussed in 2005/06,
to be reported to STGQAB



Main topics of last year‘s meeting (discussion round):

1.) Routine quality control (requested by SGQAB)	every 20th sample counted on duplicate (dominant species) for Shewhart Charts.
2.) Update of the biovolume file	new version PEG_BVOL2007 sent to ICES on 02-02-2007
3.) Indicator Fact Sheets	Sent to MONAS and planning of the next
4.) Discussion on update of COMBINE Manual	Separate carbon calculation for filaments and colonies, added to Manual draft
4.) Report on sampling devices (Markus Majaneva)	Hose and rosette sampler give same results
5.) Discussion on update of PhytoWin counting programme	No complete funding by HELCOM. Proposal required.

Activity planned for 2007: Phytoplankton ring test
Final report and application for a new project

Annex 18: Questionnaire on the use of guidelines for biological data collection and quality assurance by OSPAR contracting parties

1. Which type of institution are you in?

	UNIVERSITY LABORATORY
	Governmental laboratory
	Non-profit research organization
	Large commercial organization
	Commercial consultancy

2. What country are you located in (the address of your institution is optional)?

3. Do you contribute to national monitoring of?

	PHYTOBENTHOS
	Phytoplankton
	Macrobenthos
	Meiobenthos
	Coastal Fish
	Zooplankton
	Ecotoxicology
	Chemistry

4. Is your organisation taking part in any type of quality assurance/analytical quality control activity?

	NO
	Yes If Yes, at which level:

	in-house only
	between laboratories
	between countries

5. Do you use any of the following types of guidelines

	JAMP
	HELCOM Combine
	ICES Times documents
	ISO
	CEN
	National guidelines
	In house guidelines
	Other (specify)

6. Are you aware of the following guidelines:

	JAMP EUTROPHICATION MONITORING GUIDELINES - PHYTOPLANKTON SPECIES COMPOSITION, ASMO 1977
	JAMP eutrophication monitoring guidelines - chlorophyll a, ASMO 1977
	JAMP eutrophication monitoring guidelines – benthos, ASMO 1977
	JAMP guidelines on Quality Assurance for biological monitoring in the OSPAR area, ASMO 2002

	ISO 5667-9 (1992, Ed. 1): Water quality - Sampling - Part 9: Guidance on sampling from marine waters
	ISO 5667-19 (2004, Ed. 1): Water quality - Sampling - Part 19: Guidance on sampling of marine sediments
	prEN ISO 16665 (under Approval: 2005-04): Water quality - Guidelines for quantitative sampling and sample processing of marine soft-bottom macrofauna (ISO/FDIS 16665: 2005)
	prEN 15204 (under Approval: 2005-11): Water quality - Guidance standard for the routine analysis of phytoplankton abundance and composition using inverted microscopy (Utermöhl technique)
	ISO 10260 (1992): Water quality - Measurement of biochemical parameters - Spectrometric determination of the chlorophyll-a concentration

7. Is your laboratory/organisation accredited?

	NO

	YES IF YES, WHAT FORM DOES THE ACCREDITATION TAKE AND WHAT IS THE NAME OF THE ACCREDITING ORGANISATION?

	ARE YOU SEEKING ACCREDITATION

8. How do you improve and maintain the professional skills of your staff?

	SAMPLING AND SAMPLE-HANDLING WORKSHOPS (IF YES, HOW OFTEN?)
	Intercalibrations (if yes, how often?)
	Take part in ring-tests (if yes, how often?)
	Other (please specify)

9. May we refer to your institution by name in connection with any examples of 'best practice'? (*NB. We will NOT refer to named institutions/individuals under any other circumstances, and the confidential nature of your response is therefore assured*)

	YES
	No

Annex 19: Questionnaire on the use of guidelines for biological data collection and quality assurance by OSPAR contracting parties, Summary of results

WHICH TYPE OF INSTITUTION ARE YOU IN?	
University laboratory	
Governmental laboratory	9
Non-profit research organization	1
Large commercial organization	
Commercial consultancy	
Do you contribute to national monitoring of:?	
Phytobenthos	2
Phytoplankton	8
Macrobenthos	7
Meiobenthos	
Coastal Fish	4
Zooplankton	4
Ecotoxicology	4
Chemistry	9
Is your organisation taking part in any type of quality assurance/analytical quality control activity?	
No	
Yes; If Yes, at which level:	10
in-house only	2
between laboratories	10
between countries	5
Do you use any of the following types of guidelines	
JAMP	9
HELCOM Combine	1
ICES Times documents	3
ISO	4
CEN	2
National guidelines	9
In house guidelines	8
Other (specify)	
Are you aware of the following guidelines:	
JAMP eutrophication monitoring guidelines - phytoplankton species composition, ASMO 1977	7
JAMP eutrophication monitoring guidelines - chlorophyll a, ASMO 1977	8
JAMP eutrophication monitoring guidelines – benthos, ASMO 1977	6
JAMP guidelines on Quality Assurance for biological monitoring in the OSPAR area, ASMO 2002	7
ISO 5667-9 (1992, Ed. 1): Water quality - Sampling - Part 9: Guidance on sampling from marine waters	6
ISO 5667-19 (2004, Ed. 1): Water quality - Sampling - Part 19: Guidance on sampling of marine sediments	7
prEN ISO 16665 (under Approval: 2005-04): Water quality - Guidelines for quantitative sampling and sample processing of marine soft-bottom macrofauna (ISO/FDIS 16665: 2005)	6

prEN 15204 (under Approval: 2005-11): Water quality - Guidance standard for the routine analysis of phytoplankton abundance and composition using inverted microscopy (Utermöhl technique)	7
ISO 10260 (1992): Water quality - Measurement of biochemical parameters - Spectrometric determination of the chlorophyll-a concentration	7
Is your laboratory/organisation accredited?	
No	7
Yes (If Yes, what form does the accreditation take and what is the name of the accrediting organisation?)	3 (EN ISO /IEC 17025
Are you seeking accreditation	
How do you improve and maintain the professional skills of your staff?	
Sampling and sample-handling Workshops (if yes, how often?)	5 (totally)
yes	4
1x per year	1
Intercalibrations (if yes, how often?)	10 (totally)
1991, 2000 CIEM, 2004, 2005 BEQUALM	1
every 2-3 years	1
2 times a year	2
Annually, minimum	1
1x per year	1
quarterly	4
Take part in ring-tests (if yes, how often?)	9 (totally)
BEEP (european programme)	1
every 1-2 years	1
Annually, minimum	1
2 times a year (chemical par., chlorophyll-a)	1
2 times a year	1
quarterly	4
Other (please specify)	3 (totally)
Taxonomic workshops, every 2-3 years	1
several intercomparisons (ring tests) every year, usually 20 - 25 a year	1
In-house exercises	1

Annex 20: Letter from G. Martin, Estonia

Dear Johan Wikner and members of STGQAB,

Lead Laboratory on phytobenthos monitoring was etsablished in the framework of BSRP (Baltic Sea Regional Project) at Estonian Marine Institute, University of Tartu. One of the tasks of this LL was to coordinate the development of phytobenthos monitoring programmes and methodology in BSRP recipient countries (Russia, Estonia, Latvia, Lithuania, Poland). From these countries only Estonia had established phytobenthos monitoring programme before 2003. Activities of LL included supply of PB monitoring equipment to participating laboratories, conducting of set of workshops, development of monitoring methods.

During the activities of LL two training workshops were organised in 2005 and 2006 in Estonia. Results of training workshop carried out in 2005 were presented at STGQAB 2006 meeting. The aim of the workshop was:

- to establish the phytobenthos monitoring expert network in the BSRP recipient countries.
- to compare and discuss phytobenthos monitoring methods used in BSRP recipient countries at the present moment
- to propose changes/amendments to current HELCOM COMBINE Guidelines concerning phytobenthos monitoring methods

Workshop was carried out as combination of lectures and presentations and actual field excercise with intercallibration/comparison of monitoring techniques used in participating laboratories. All together 12 participants from Estonia, Russia, Latvia, Lithuania and Poland were able to attend the meeting.

Main outcome of the WS was:

1) the networking event organized in the framework of BSRP LL phytobenthos was considered as very useful and fruitful. This was a first time when phytobenthos specialists from five Baltic countries were able to communicate and test in practice the methods used in their everyday routine work. For future this form of communication and intercomparison/intercallibration seminars should be organized on a regular basis.

2) The existing methods descriptions in current HELCOM COMBINE Guidelines need to be modified and amended. The working group should be established to organize the QA related activities and also amendment of the COMBINE manual.

3) Other operational methods available for phytobenthos monitoring should be assessed and intercallibrated for future optimization of monitoring effort (remote sensing, underwater video techniques).

4) The current substrate coding system presented in the HELCOM Phytobenthos Guidelines should be revised. The system should be developed covering the full range of ecologically significant substrate types and applicable in different areas of the Baltic Sea.

5) Taxonomic knowledge of phytobenthos species should be increased in many laboratories. For this purpose international effort should be taken in form of taxonomic workshops (as an example of regular taxonomic workshops of HELCOM Phytoplankton expert group).

6) Set of phytobenthos indicators suitable for eutrophication and productivity assessment should be developed for the whole Baltic Sea area taking into account progress achieved in several ongoing and finished projects.

7) Development of reference conditions for phytobenthos parameters as well as water quality classification systems should be coordinated between neighboring countries.

Following the outcome of the 2005 workshop, next meeting was organised in May 2006 in Saaremaa Island, Estonia. At this meting the scheme used in previous meeting was used (combination of lectures and actual field excercise). The main focus of this meeting was on application of UW video techniques for monitoring and mapping of phytobenthic communities. At this meeting 16 participants from BSRP recipient countries and Finland were able to participate. During the meeting different avialable UW video techniques were studied and tested in the field against actual coverage estimations made by SCUBA divers. Main outcome of the meeting was:

- The current substrate coding system presented in the HELCOM Phytobenthos Guidelines should be revised and is not suitable for use with UW video methods. The system should be developed covering the full range of ecologically significant substrate types and applicable in different areas of the Baltic Sea.
- Taxonomic knowledge of phytobenthos species should be increased in many laboratories. For this purpose international effort should be taken in form of taxonomic workshops (as an example of regular taxonomic workshops of HELCOM Phytoplankton expert group).
- Underwater video systems presented at the workshop are useable for the purpose of habitat mapping and could be used in the combination with SCUBA and other quantitative methods.
- Further intercallibration of mapping techniques should be done actually in the field during common field work.

As the first phase of BSRP is coming to an end in June 2006 and the fate of second phase is not clear, there is a danger that activities in coordination of phytobenthos monitoring in the eastern Baltic Sea will be stoped. Recommendations from STGQAB could include:

For Helcom

- to establish the project group covering the development of methods and coordination of phytobenthos monitoring activities in the Baltic Sea area.
- to ensure further development of COMBINE Guidelines in accordance with latest developments in phytobenthos monitoring techniques and methods.
- to update the existing COMBINE Guidelines according the proposal made by BSRP LL Phytobenthos Monitoring (attached)

Sincerely,

Georg Martin

Head of Department of Marine Biology
Estonian Marine Institute, University of Tartu