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Executive summary

The STGQAB met between the 14th and 16th February 2006 at ICES headquarters in Copenhagen, Denmark. Eleven members from 8 countries participated in the meeting.

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

The group reviewed documents concerning guidelines for phyto-, zoo- and bacterioplankton of the HELCOM COMBINE manual. It was proposed that bacterioplankton guidelines should be adopted by HELCOM MONAS, while some additional work on phyto- and zooplankton guidelines were recommended before adoption.

A proposal for the revision of current data validation guidelines was also considered and work planned to be performed within the group during 2006.

A presentation of BEQUALM was made by a representative of that organization and recommendation for future interaction suggested.

No clear requests concerning OSPAR EcoQO's and OSPAR JAMP products had been presented to the group. Current group members also lacked insight into these subjects. Contribution to these items was therefore limited.

The steering group agreed in the future to take on an extended responsibility, in addition to the AQC previously considered. This included consideration of the natural variability of environmental analysis, an important source of variation in biological data.

It is recommended to recruit additional members, with engagement in the OSPAR area to the group, in order to better support quality assurance issues in this organization. Competence of marine birds, fish and biological effects measurements should be considered in this context.

1 Opening of the meeting

The meeting was opened by the chairman. The joint HELCOM/OSPAR group is chaired by Johan Wikner and co-chaired by Jørgen Nørrevang Jensen. Representatives from ICES, Estonia, Germany, United Kingdom, Sweden, Norway, Poland and The Netherlands were present at the meeting. For list of participants see ANNEX I. 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. ToR are set by HELCOM, OSPAR and ACME incorporates it to a common ToR for the group. The agenda was adopted without any major changes (see ANNEX II).

3 Appointment of a Rapporteur

Georg Martin was appointed as rapporteur with assistance from Keith Cooper and Tim Mackie.

4 Report from the Annual Science Conference of ICES 2005

Jørgen Nørrevang Jensen presented a short summary of the ICES ASC. The 2005 annual ICES Science conference was held in Aberdeen, Scotland and included 900 participants. There were 15 theme sessions, although none specifically targeted at QA. The next conference will be held in Maastricht in September 2006. The attending of the ASC is highly recommended and is a very efficient forum for all aspects of marine sciences. Theme session submissions can be made on the ICES website (deadline April 2006). Submissions relating to quality would be welcomed.

5 Report from last years OSPAR meetings

Jørgen Nørrevang Jensen gave a short explanation to of some issues concerning EcoQOs in relation to seabirds and fish communities discussed at SIME and ASMO meetings when reviewing the SGQAE report from last year. The wish was expressed to expand STGQAB with specialists in these fields.

6 Report from the last HELCOM MONAS meeting

At HELCOM MONAS 8 many QA related issues were discussed. The new monitoring and assessment scheme MONPRO was presented but decisions were postponed to enable this programme to be coordinated with the EU Marine strategy in the future. SGQAB recommendations from 2005 were discussed at the meeting but most of them were not taken into account. Reasons for this included poor specification and formulation of requests. STGQAB will endeavour to formulate more specific requests and recommendations in future communications with the HELCOM secretariat. The list of still relevant recommendations from 2005 will be resubmitted (ANNEX 4).

7 ToR a): Review and finalize the guidelines for acceptability of biological sampling and analytical practices required by monitoring programmes.

7.1 Activity of Phytoplankton expert group: New procedure to calculate phytoplankton carbon

The most important topics of the HELCOM Phytoplankton Expert Group (PEG) have been: agreement on species identification and use of species names, agreement on specific size classes and application of those in biomass calculation, standardisation of counting and calculation with development of counting software, recommendation of improvements of ICES reporting formats, development and discussions on phytoplankton manuals and intercalibration of measurements. Currently the activities are organised in the form of a HELCOM project. Activities include training courses, intercalibration exercises (in 2004 and 2006), harmonisation of counting and measuring techniques.

Norbert Wasmund gave a short report on the workshop and training course of the HELCOM Phytoplankton Expert Group (PEG), held in Helsingborg, Sweden, 26-29 of September 2005.

The training course on small dinoflagellates held by Dr Marina Montresor from the Stazione Zoologica with Anton Dohrn Napoli and Dr Anke Kremp from the Finnish Environment Institute Helsinki. Afterwards, current and future activities were discussed:

- 1) The PEG acknowledged that their recommendation to the draft of the CEN standard method was mostly considered and that CEN will decide the final version soon.
- 2) The PEG discussed the request of SGQAB to introduce internal quality control and agreed to do parallel counting of at least one dominant species at high magnification and at least one species at low magnification in every 20th sample for drawing Shewhart charts.
- 3) Ms Irina Olenia reported that the biovolume paper (Olenia et al.: Biovolumes and size classes of phytoplankton in the Baltic Sea) was accepted by the STGQAB meeting in February 2006. Meanwhile, it is also accepted by MONAS and the Heads of Delegations and is currently in press. The work on the phytoplankton biomass list will be continued.
- 4) The PEG appreciated the first direct personal contact to the ICES data centre represented by Ms Marylinn Sørensen. She presented the new Environment Reporting Format version 3.2 and got information from the group about biomass calculation and requirements on the database.
- 5) Ms Maija Huttunen informed the group about a new version of PhytoWin. Before the PEG requests HELCOM to buy the licence, the group wants to test this version, which has not yet been made available. Norbert Wasmund informed the STGQAB that the latest factors, according to the new paper on phytoplankton biovolume (see below), are still not integrated into the actual working version of the PhytoWin counting programme. The regular update (once a year) should be a matter of the licence agreement between HELCOM and the software firm KAHMA.
- 6) The PEG decided to use the equations by Menden-Deuer and Lessard (2000), separated for diatoms and non-diatoms, to calculate phytoplankton carbon. This new carbon calculation has to be inserted into the counting programme. The chairman of PEG, Norbert Wasmund, presented a text on this new carbon calculation and asked STGQAB to consider it for the HELCOM manual.
- 7) Ms Agneta Andersson suggested a project proposal on phytoplankton monitoring technique based on molecular-genetic methods (18SrDNA) as a joint project of Ms Andersson in cooperation with the PEG.

- 8) Mr Andres Jaanus reported about the influence of sampling depth on phytoplankton biomass results at two stations in the Gulf of Finland. He concluded that biomass per m³ from the 0-10 m samples are 1.3-1.4 times higher than respective data from the 0-20 m samples. Comparisons should be used with caution, as the variation may be large. Further investigations at different conditions are needed.

STGQAB discussed the progress of PEG and agreed that carbon data is a very important parameter (e.g. for modelling). Previously the factors used for plankton carbon calculation were not satisfactory. This was due to several reasons and a new strategy to determine carbon content using variable factors depending on cell size and taxonomy was proposed. STGQAB recommended the establishment of links to other similar groups, especially those involved in North East Atlantic Geographical Intercalibration Group (NEA GIG) to ensure that methodological developments are evolving in same direction.

The second recommendation from STGQAB was to also consider chlorophyll and primary production data, if available, for decisions on sampling depth.

Concerning matters with PhytoWIN programme, PEG should contact the HELCOM secretariat to find out the possibilities of regular updating of the PhytoWin programme and disseminating the programme outside the HELCOM community.

STGQAB recommended that PEG draft a new chapter on biovolume, carbon calculation and other required updates. These should be included in the COMBINE manual during the year 2006.

7.2 Review comments and decide in implementation of bacterioplankton guidelines to HELCOM COMBINE

The Chairman presented two documents which had been distributed to relevant specialists in Baltic countries and comments collated. In light of these comments several amendments were made to the documents. STGQAB adopted the revised documents and endorsed their submission to HELCOM MONAS for adoption into the COMBINE Guidelines Part-C (Annexes C-11 and C-12) (ANNEX 10 and 11).

7.3 Review data validation guidelines of the HELCOM COMBINE manual

Petra Schilling presented a first working draft of data validation guidelines (see ANNEX 5) as a basis for further development of these guidelines in cooperation with other ICES working groups. Following this work, it is recommended that a general revision of the part B of the HELCOM COMBINE Manual would be appropriate, particularly in relation to chapter B.4 which should be reviewed completely. The title of chapter "B.4 Validation of analytical methods" should be changed to "Validation guidelines". Reflecting the different levels of validation this chapter should be divided into the following three subchapters:

- 1) B.4.1: Quality checks of sampling, which should contain a reviewed and supplemented text based on the existing "Annex B-5 General remarks on sampling for chemical analysis",
- 2) B.4.2: Validation of analytical methods which should be based mainly on a reviewed version of the existing chapter "B.4.2 Validation" including some paragraphs of chapter "B.4.1 General" and general aspects of "Annex B-2 Validation of established analytical methods for chemistry",
- 3) B.4.3: Validation of data which should be a new chapter dealing with routines to develop a consistent database on known quality.

Also all existing Annexes of part B (Annex B-1 up to Annex B-17) should be reviewed and replaced by the following proposals:

- A) The existing “Annex B-1 Principal components of a quality manual” should be a new subchapter of the existing chapter “B.2.4 Documentation”: B.2.4.1 Quality manual,
- B) The existing “Annex B-1 Principal components of a quality manual” should be replaced by a reviewed version of the existing “Annex B-2 Validation of established analytical methods for chemistry” including two subchapters Annex B-1.1: Full validation procedure and Annex B-1.2: Limited validation procedure,
- C) The existing “Annex B-3 Quality audit” should be included in chapter “B.2.7 Quality Audit”,
- D) The general Parts of “Annex B-4 Technical note on measurement uncertainty” should be moved to chapter B.4.2 as a new subchapter B.4.2.6 Measurement uncertainty and the new Annex B-2 should contain only the content of attachment 1 of the existing “Annex B-4 Technical note on measurement uncertainty”,
- E) The existing “Annex B-5 General remarks on sampling for analysis” should be part of the new chapter “B.4.1: Quality checks of sampling” in a reviewed form as mentioned above,
- F) The existing “Annex B-6 Examples of reference materials for internal quality control” should replace the existing “Annex B-3 Quality audit”, see under C),
- G) The existing “Annex B-7 Units and conversions should be shifted completely to existing chapter “B.1.4 Units and conversions”
- H) All following existing Annexes of part B (Annex B-8 up to Annex B-17) are technical notes on special parameters and variables and should be shifted to part C to appropriate sections.

Finally the part B of the HELCOM COMBINE Manual should contain only the following three Annexes:

Annex B-1 Validation of an established analytical method for chemistry, examples of full and limited validation procedure (adopt as it stands but replaced the existing “Annex B-2 Validation of established analytical methods for chemistry” including the subchapters Annex B-1.1: Full validation procedure and Annex B-1.2: Limited validation procedure

Annex B-2 Protocol for evaluation of measurement uncertainty from in-house control measurements (should contain only Appendix 1 of formerly Annex B-4 but should be reviewed by STGQAC)

Annex B-3 Examples of reference materials for internal quality control (replaced Annex B-6)

In future the general aspects of validation outlined in chapter B.4 should be gradually reflected in detailed information of separate sections of part C.

STGQAB invited the following ICES working groups to review and comment on these proposal of ANNEX 5: Steering Group on Quality Assurance of Chemical Measurements in the Baltic Sea (STGQAC), Marine Chemistry Working Group (MCWG), Working Group on the Statistical Aspects of Environmental Monitoring (WGSAM), Study Group on Management of Integrated Data (SGMID), Working Group on Marine Data Management (WGMDM) and ICES data centre. For preparing an updated document before the next meeting of STGQAB these groups should provide their recommendations by 1st December 2006 to the chair of STGQAB.

STGQAB recommend a general revision of part B of the HELCOM COMBINE Manual coordinated by Petra Schilling. All members of STGQAB are invited to submit suggestions up to November, 1. 2006. A reviewed draft should be circulated in good time before the next meeting of STGQAB and should consider the proposals of the meetings of STGQAC and MCWG in 2006.

STGQAB had discussion about the definitions of “raw/primary” data and other levels of data aggregation. It was common view that there is a need for unified terminology. ICES Data Centre was asked to prepare list of definitions.

Discussion was held on possibilities of data flagging in current database and recommendation was formulated to ICES data centre to look for available data flagging standards.

7.4 Review marine zooplankton biomass factors for updating the COMBINE manual

Petra Schilling informed the group that the Federal Environmental Agency of Germany (Quality Assurance Panel of the GMMP) supports the development of biomass factors of acceptable quality concentrating on the Baltic proper by the Baltic Sea Research Institute in Warnemünde (Dr. L. Postel, H. Simon). The aim is to improve the present zooplankton biomass factors taking into account the seasonal and geographical differences in individual volume. The project was introduced to revise the HELCOM Combine Manual Annex C-7 in respect to length/biomass determination of mesozooplankton in 2006. Further details are presented in the ANNEX 8.

STGQAB recommends that the HELCOM zooplankton expert network provides a draft of the reviewed HELCOM Combine Manual Annex C-7 to STGQAB for adoption before 1st December 2006.

8 ToR b): Review and evaluate, and report on the status of implementation and the practical use of OSPAR/ICES quality assurance guidelines in marine monitoring and assessment programmes in the OSPAR/ICES/HELCOM area and provide guidance for future assessment programmes.

8.1 Status reported by national representatives

The questionnaire prepared by SGQAB 2005 was circulated by OSPAR following ASMO 2005 to laboratories and national contact points nominated by the ASMO HOD's and a compilation of the responses was sent to ICES in mid January 2006. This compilation included comments from laboratories in seven Contracting Parties. ICES had expected more comments from more partners and hence postponed the analysis in STGQAB until a more complete response had been received. After the STGQAB meeting the OSPAR Secretariat informed ICES that no further comments were expected and ICES has started an analysis of the responses intersessionally.

9 ToR c): Review and report on the quality assurance measures proposed for monitoring and assessment of OSPAR EcoQOs.

9.1 Review of the OSPAR EcoQO guidelines

The group welcomed the development of EcoQO in OSPAR region and suggested that the close collaboration should take place with the groups developing the indicators and water quality classification systems for WFD.

STGQAB is prepared to comment on the OSPAR EcoQO document if it will be available (see SGQAE recommendation 2005). STGQAB welcomes the submission of relevant documents for review in good time before December 1st 2006.

10 ToR d): Evaluate and report on the outcome of relevant workshops/intercalibration exercises/ring tests, and document future events, including progress with the implementation of phase II of the BEQUALM scheme.

10.1 Outcome of HELCOM Zooplankton workshop

The first workshop of HELCOM zooplankton expert group was held in Rostock. One important aim was to establish a common species checklist. Petra Schilling made a short presentation. Group appreciated the work and welcomed representative of the group to participate at STGQAB next meetings. The report is available www.io_warnemuende.de/research/helcom_zp/

10.2 Outcome of BSRP/HELCOM Phytobenthos workshop

G. Martin presented the results of the BSRP-HELCOM phytobenthos training workshop. The workshop was held in Estonia in May 2005. Participants from all BSRP recipient countries were participating. The programme of the workshop included theoretical as well as practical part. Practical exercises included training of phytobenthos monitoring techniques on three types of coastal habitats. The application of existing HELCOM phytobenthos monitoring guidelines was tested and results discussed. There is a need to propose changes to HELCOM phytobenthos monitoring guidelines. The activities will continue in the future and specialists from other countries are welcomed to participate. The meeting welcomed the activities in development of QA procedures in phytobenthos monitoring.

10.3 Progress of HELCOM Mon-Pro

The current status of HELCOM MONPRO project was discussed. STGQAB appreciate the work done so far and recommended to consider zooplankton as mandatory and primary production, bacterial biomass and bacterial growth as optional parameters in future monitoring programme.

10.4 Presentation of phase II of BEQUALM, Yvonne Allen

The presentation was given to the Group about the history of BEQUALM and BEQUALM II activities. BEQUALM is going to expand considerably the number of parameters. Laboratories were invited to take actively part in the schemes offered by BEQUALM. Germany have participated in recent BEQUALM scheme and the comments were that this system is rather expensive. In general group was pleased with the results of the second phase of BEQUALM.

STGQAB welcomed the report and progress made by BEQUALM in its first year as a self-funding scheme. Although progress has been made, STGQAB would also like to highlight to ICES/OSPAR that in certain areas, notably the benthic community component and for some biomarkers that the scheme has had very poor participation. Clearly such a position is not financially sustainable and it should be noted that if circumstances do not change then BEQUALM will not be able to provide the QA component for some biological effects measurements required by OSPAR (JAMP CEMP) for data submission to ICES.

Some useful feedback was received from Petra Schilling on her experience with participating of a German laboratory in the NMBQAC scheme; the main problems that were encountered, which could be applicable to a large proportion of European participants included the lack of available “own samples” for submission to the scheme for checking, unfamiliarity with some of the species that formed part of the ring test (although it was highlighted that the NMBQAC do not fail a laboratory if samples cannot be identified or are identified incorrectly; this part of the scheme is considered to be a training exercise and allows taxonomists to broaden their skills and become familiar with species that they may come across on rare occasions) and the cost of participating, not just in terms of the registration fee to be paid but the time resource required to conduct the QA. If a laboratory is participating in national, regional and international QA programmes then potentially a substantial amount of time will be taken up with these exercises and this is not sustainable. The STGQAB recommended that NMBQAC/BEQUALM further develop and take forward the questionnaire that was produced by the SGQAB in 2005, which would gather information from laboratories on their required level of participation in QA exercises and more specifically what is involved in each. This would allow NMBQAC/BEQUALM to explore ways to harmonise QA activities, in order to reduce the workload for a single laboratory and also develop mechanisms to overcome the problems of regionality.

STGQAB recommends that information is collected on QA schemes operating across Europe. Based on that information it will be possible to develop better international schemes. This is being addressed by the BEQUALM office.

11 **ToR e): Review and report on the progress with, and offer further advice on the development of QA of biological measurements in relation to OSPAR JAMP products.**

The group was not able to find a clear description of what to comment on in this case. More specific ToR or clear requests from EcoQO groups should help in this regard.

12 ToR f): Review and report on the quality assurance measures being adopted in the marine monitoring and assessment aspects of the EC Water Framework Directive

In **Estonia** and **Poland** the monitoring programmes for WFD is under development. They will be based on existing monitoring techniques and methods but the station network will be expanded considerably. QA measures will be adopted according to national legislation and will follow international standards.

Latvia. The QA issues when implementing the WFD in Latvia have not been paid any attention, as the new marine monitoring programme (planned to be adopted spring 2006) even excludes the phytobenthos from the parameters thus contradicting with WFD requirements. Marine monitoring experts did not have the possibility to comment on the programme content.

Germany. The Quality Assurance Panel of the German Marine Monitoring Programme (GMMP) requires that the laboratories of the GMMP in the coming five years develop the prerequisites to be accredited for all parameters of the GMMP to promote that laboratories follow a documented quality system according to EN ISO / IEC 17025 to ensure the comparability of measurements and minimize sources of errors. ANNEX 7 contained a summary of planned quality assurance activities of the next two years. If there are enough capacities, additional laboratories can take part upon request. A project just has started to integrate OSPAR/HELCOM/WFD- and national monitoring demands. Thus the existing monitoring programme will certainly be restructured. QA requirements are an important part within this project as well as the data flow and reporting responsibilities. To ensure a national overview of the coastal, transitional and marine status of the sea remains a primary goal.

The Netherlands. All monitoring activities are subject to regular quality assurance activities. In the Netherlands laboratories are accredited for chemical analyses, and partly for biological analyses (i.e. phytoplankton and chlorophyll-a). For chemical analyses there's also participation in international intercalibration schemes like QUASIMEME and BEQUALM for biological parameters. Data are stored in the national database, where final checks are made. This Year a database transition is planned to take place to another infrastructure, providing more facilities to holding quality information. Within the framework of WFD there is a contribution to several intercalibration exercises (NEAGIG) and the development of indicators.

Sweden. The Swedish Environmental Protection Agency and County Boards have, for several years, required contracted laboratories to be accredited to EN ISO/IEC 17025 for at least chemical variables. This ensures laboratories follow a documented quality system to ensure traceability of measurements and also minimization of sources of error.

Also, the Swedish EPA requires that laboratories participate in international QA exercises, ring tests and inter-calibration schemes like the QUASIMEME and BEQUALM.

Monitoring data is further requested to be submitted to national databases where they are subject to additional quality control measures. These data are then submitted to international databases.

The above quality assurance work is supporting monitoring work in general, and also promotes the required quality of measurements within the Water Frame Directive.

Development of parameters or parameter sets to function as indicators for phytoplankton, phytobenthos, zoobenthos and environmental toxins within the WFD have been running for several years. Indicators are expected to be ready by the end of February 2006. This includes assessment guidelines and reference levels for each indicator.

Projects are also running to determine suitable sampling strategies, in time and space, in order to provide for sufficient power for comparison with status class limits.

Norway. The Norwegian Pollution Control Authority requires contracted laboratories to be accredited. This ensures laboratories follow a documented quality system to ensure traceability of measurements and also minimization of sources of error. Monitoring data is then submitted to national databases where they are subject to additional quality control measures. Relevant data are then submitted to international databases.

Norway is implementing the new EN/ISO standard for soft bottom fauna. We have presented our national hard bottom standard to CEN and it is being adjusted based on comments from the member countries in CEN. A national standard for phytoplankton sampling has been presented for CEN and we have received comments from several countries. A new version will be presented for CEN.

Water framework directive: Norway is active in the CIS work and is contributing to the marine intercalibration work in the NEA-GIG (North East Atlantic Geographic Intercalibration Group). A plan for a WFD reference monitoring program has been developed. Norway has a 5 year deferral of the time schedule for the implementation plan for WFD due to the late signing of the EEA agreement, but will follow the EU time schedule for a representative number of water bodies. The first step of the monitoring program will start in 2007.

A new activity instruction for environmental monitoring of petroleum activities on the Norwegian continental shelf has been finished in December 2005. It came into force 1 January 2006. No major changes have been done, but it has a recommendation that reference material will be kept in a national museum collection and it also recommend that materials should be secured for DNA barcoding.

The Norwegian Biodiversity Information Centre (NBIC) is a new governmental institution reporting to the Ministry of Education and Research. It is a national information source concerning biodiversity at different levels. It is an independent institution which will collaborate with, but is not beholden to, other organisations or functions, whether management, research, or voluntary organisations. The NBIC will not itself conduct research, management, or field collection.

In close collaboration with research organisations NBIC will obtain and systematise data from databases holding primary data, conduct quality control, work with data on species and habitats and make these easily accessible to various organizations and to the general public. The data will be easily available, primarily in digital form from the web site (www.artsdatabanken.no). NBIC will ensure that collected data can be exchanged internationally in accordance with Global Biodiversity Information Facility (GBIF) standards. NBIC will produce maps, statistics, species registers, habitat registers, reports, and fact-sheets on Norwegian species.

Starting in 2005 four selected projects were prioritised: 1) red-list assessment after IUCN criteria, to be finished in 2006; 2) invasive species with a priority on a black-list; 3) a database with names of Norwegian organisms, intended to be a Norwegian standard; 4) habitats and vegetation types, a consensus on classification; 5) web map application on species information, selected organism groups will be targeted in a pilot project.

United Kingdom. The UK competent authorities have advocated that laboratories providing data for national work packages show a high level of commitment to QA and are participant in a nationally recognised QA scheme.

Development of tools and metrics to address the monitoring requirements of the WFDs biological quality elements are being developed by 3 dedicated working groups; plants for

phytoplankton & phytobenthos, benthic invertebrates for zoobenthos and fish for fish in transitional waters. These working groups have been in place for several years. It has become apparent that these groups are looking to the UK NMBAQC / BEQUALM scheme to provide QA measures and exercises to cover all the above biological quality elements, rather than just the macrozoobenthos and particle size analysis that had previously been the schemes remit. To this end a Phytoplankton Enumeration and Identification ring test was run under the schemes auspices at the end of 2005 and a fish identification ring test that is to be retained by the participating labs as reference material is to be circulated in February. QA appropriate to all the metrics are being considered within the working groups.

12.1 Proposed process to develop WFD relevant standards

STGQAB discussed the current situation. It was noted that closer links between STGQAB and CEN are needed. STGQAB recommend that a representative from ICES should take part in the meetings to establish a formal link between these organisations. The STGQAB may be used as a reference group intersessionally. Several CEN documents have been commented by SGQAE and SGQAB before. At the moment there are several documents under development. Quality assurance and marine expertise input to these documents is recommended and there are several ICES WGs who can also be called upon to provide such information.

12.2 Introduction of plankton secondary producers in the EU Water Quality Guidelines

STGQAB expresses its concern that zooplankton monitoring is not included under WFD ecosystem assessment scheme and recommends that countries continue existing monitoring schemes. New EU Marine strategy has included the secondary production in the assessment scheme, so there is a hope that these measurements will be further coordinated on an international level.

13 ToR g): Review and report on progress in the development and use of the ICES Biological Community Database

13.1 Status report by ICES data group

Hens Jensen from ICES presented the DOME (Database on Oceanography and Marine Environment) structure and development. The development of DOME is structured into three phases and phase one is currently on the way. There was concern from STGQAB group that requirements of QA information should be stated very clearly in future database. STGQAB supported the developments in ICES database and welcomed submission of any new information available for review at next meetings. ICES stated that in future they will accept data in any structured format.

13.2 Reference to species data list

It was reported to the group that in some countries there is a problem with submitting data because some species are not listed in ITIS. ICES informed the group that at the moment it is possible to use either ITIS list or any other list if reference is specified. Currently the most complete list to use in the ICES area is European Register of Marine Species (<http://www.marbef.org/data/erms.php>).

13.3 Standards for size classes and factors of zoo- and phytoplankton

There will be possibility to submit only raw data to databank together with conversion factors. Countries can submit also only calculated biomass values but in this case it is marked in database as “calculated”.

14 Proposal for a change of scope of the STGQAB and STGQAC

14.1 Proposal by Jørgen Nørrevang Jensen, Data Scientist, ICES.

A presentation was made to illustrate the existence of huge natural variability as opposed to that caused by collection, processing and analysis of samples (e.g. spatial and temporal variability). It was proposed to change the scope of the QA work undertaken by the group to include other sources of variability than analytical procedures.

Discussion in the group included the suggestion to treat analytical and other variability separately. In general STGQAB agreed the concept of addressing different sources of variability is complex, but that the requests should be made to the group in advance to be able to consult different national expert and working groups. STGQAB look to ICES to help improve interaction with other relevant ICES working groups.

15 Additional Steering group activities

15.1 Appoint a participant to the Workshop on Review of the ICES Committee and Expert Group Performance (WKREP) to be held on 15 March 2006 in Copenhagen.

Nobody from the group will be able to participate at the meeting and the suggestion was to use the online option to provide the opinion of the group.

16 Any other business.

16.1 Quality Assurance work package for the EU network of excellence MARBEF (Marine Biodiversity and Ecosystem Functioning), K. Cooper

Keith Cooper gave a short presentation on a quality assurance website that has been developed for the EU MARBEF (Marine biodiversity and ecosystem functioning) programme. MARBEF is a network of marine laboratories across Europe, all of who are engaged in biodiversity research (for further details see www.marbef.org). The website will contain a variety of information designed to raise awareness of, and participation in quality assurance issues (e.g. quality systems, quality assurance schemes, MARBEF QA framework etc). Members of the STGQAB gave their support to this initiative and agreed to work together in relation to common aims. Group suggested to include and use several materials from ICES/HELCOM/OSPAR QA schemes. STGQAB asked the project manager to keep the group informed about the future development of the webpage.

17 Date and venue of the next Steering group meeting.

STGQAB suggested the next meeting should take place at the ICES headquarters from February 13th-15th 2007.

18 Adoption of a draft report

The draft report was adopted at 15:00 on 17th February 2006.

19 Closing of the meeting

The meeting was closed at 15:05.

Annex 1: List of participants

Name	Address	Telephone no.	Fax no.	E-mail
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Annex 2: Agenda

Meeting of the ICES Steering group of quality assurance of biological measurements.

ICES Main office, Copenhagen, 14-16 February 2006.

Chair: Johan Wikner Co-chair: Jørgen Nørrevang Jensen

Agenda

- 1) Opening of the meeting
- 2) Adoption of the Agenda
- 3) Appointment of a Rapporteur
- 4) Report from the Annual Science Conference of ICES 2005
- 5) Report from last years OSPAR meetings
 - 5.1 SIME
 - 5.2 ASMO
- 6) Report from the last HELCOM MONAS meeting
 - 6.1 HELCOM MONAS 8 2005: Minutes of HELCOM MONAS 8_2005
- 7) ToR a): Review and finalize the guidelines for acceptability of biological sampling and analytical practices required by monitoring programmes.
 - 7.1 Activity of Phytoplankton expert group: New procedure to calculate phytoplankton carbon
 - 7.2 Review comments and decide in implementation of bacterioplankton guidelines to HELCOM COMBINE
 - 7.3 Review data validation guidelines of the HELCOM COMBINE manual
 - 7.4 Review marine zooplankton biomass factors for updating the COMBINE manual
- 8) ToR b): Review and evaluate, and report on the status of implementation and the practical use of OSPAR/ICES quality assurance guidelines in marine monitoring and assessment programmes in the OSPAR/ICES/HELCOM area and provide guidance for future assessment programmes.
 - 8.1 Status reported by national representatives
- 9) ToR c): Review and report on the quality assurance measures proposed for monitoring and assessment of OSPAR EcoQOs.
 - 9.1 Review of the OSPAR EcoQO guidelines
- 10) ToR d): Evaluate and report on the outcome of relevant workshops/intercalibration exercises/ring tests, and document future events, including progress with the implementation of phase II of the BEQUALM scheme.
 - 10.1 Outcome of HELCOM Zooplankton workshop
 - 10.2 Outcome of BSRP/HELCOM Phytobenthos workshop
 - 10.3 Progress of HELCOM Mon-Pro
 - 10.4 Presentation of phase II of BEQUALM, Yvonne Allen

- 11) ToR e): Review and report on the progress with, and offer further advice on the development of QA of biological measurements in relation to OSPAR JAMP products.
- 12) ToR f): Review and report on the quality assurance measures being adopted in the marine monitoring and assessment aspects of the EC Water Framework Directive
 - 12.1 Proposed process to develop WFD relevant standards
 - 12.2 Introduction of plankton secondary producers in the EU Water Quality Guidelines
- 13) ToR g): Review and report on progress in the development and use of the ICES Biological Community Database.
 - 13.1 Status report by ICES data group
 - 13.2 Reference to species data list
 - 13.3 Standards for size classes and factors of zoo- and phytoplankton
- 14) Proposal for a change of scope of the STGQAB and STGQAC
 - 14.1 Proposal by Jørgen Nørrevang Jensen, Data Scientist, ICES
- 15) Additional Steering group activities.
 - 15.1 Appoint a participant to the Workshop on Review of the ICES Committee and Expert Group Performance (WKREP) to be held on 15 March 2006 in Copenhagen.
- 16) Any other business.
 - 16.1 Quality Assurance work package for the EU network of excellence MARBEF (Marine Biodiversity and Ecosystem Functioning), K. Cooper
- 17) Date and venue of the next Steering group meeting.
- 18) Adoption of a draft report
- 19) Closing of the meeting

Annex 3: STGQAB terms of reference 2006

The **ICES/OSPAR/HELCOM Steering Group on Quality Assurance of Biological measurements (STGQAB)** (Chair: Johan Wikner, Sweden (HELCOM area,) and co-chair anonymous, country, (OSPAR area) will meet at ICES headquarters from 13th to 15th of February 2007 to:

- a) review the activities of the HELCOM Phytoplankton expert group
- b) review the outcome of the HELCOM Phytoplankton workshop
- c) review the outcome of the HELCOM Zooplankton workshop
- d) review and propose new data validation guidelines for the HELCOM manual
- e) review and propose on updated guidelines for Phytoplankton in the HELCOM COMBINE manual
- f) review and propose on updated guidelines for Zooplankton biomass calculation in the HELCOM COMBINE manual
- g) review and propose a template of standard operating procedures for the COMBINE manual
- h) review international and national intercalibration exercises and propose measures as required
- i) report on the development and data formats of the ICES database
- j) review of relevant OSPAR EcoQO's in response to specific requests
- k) Review the status of quality assurance in relation to Water Framework Monitoring Programmes
- l) Maintain a watching brief on developments of the European Marine Strategy in relation to QA

STGQAB will report on the attention of the ACME and the Marine Habitat and Oceanography Committees by ##### 2007, as well as the meeting of the OSPAR Working group on concentrations, Trends and effects of Substances in the Marine Environment.

Supporting information:

Priority:	High.
Scientific Justification and relation to action plan:	<p>Items a) – h) and j) – k) are of utmost importance for quality assurance and quality control in the HELCOM/OSPAR monitoring and assessment scheme, which is under harmonization with WFD requirements. 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.</p> <p>Item i) is in support of the development of the ICES Biological Community Database.</p> <p>Item l) watching brief on developments of the European Marine Strategy in relation to QA</p>
Resource Requirements	
Participants:	Scientists from ICES Member Countries and HELCOM/OSPAR Contracting Parties, involved in QA of monitoring of bacterioplankton, phytoplankton/primary production, phytobenthos, zooplankton or zoobenthos
Secretariat Facilities:	Meeting room and secretariat assistance is required
Financial:	
Linkages to Advisory Committee:	ACME, ACE
Linkage to other Committees or Groups:	SGQAC, WGPE, WGZE, BEWG, SGNSBP, WGECO, WGMDM, WGSAM, MNC, SGPROD, SGBFFI, Baltic Committee
Linkage to other Organisations:	OSPAR, HELCOM, ISO, CEN, EC
Cost share	OSPAR 50%, ICES 50%, HELCOM ?

Annex 4: Recommendations

RECOMMENDATION	ACTION
1. To have updated list of STGQAB members by 1 st Dec 2005.	ICES Secretariat
TO HELCOM	
2. STGQAB recommends PEG to establish contacts to phytoplankton expert groups working in EU WFD intercalibration process e.g. NEA GIG	HELCOM Phytoplankton Expert Group Chair
3. STGQAB recommends PEG to consider in future work consequences of changing the sampling depth also for chlorophyll and primary production measurements.	HELCOM Phytoplankton Expert Group Chair
4. STGQAB recommends PEG to contact HELCOM secretariat to clear the possibilities of arranging the regular update and more widespread use of the PhytoWIN software	HELCOM Phytoplankton Expert Group Chair
5. STGQAB recommends PEG to draft the new chapter for COMBINE manual concerning biovolume estimation and carbon calculation and other relevant updates.	HELCOM Phytoplankton Expert Group Chair
6. STGQAB recommends HELCOM to include chapters on bacterioplankton in COMBINE manual part C	HELCOM MONAS
7. STGQAB recommend a general revision of part B of the HELCOM COMBINE Manual coordinated by Petra Schilling	All Members of STGQAB, STGQAC, MCWG, WGS AEM, SGMID, WGMDM
8. STGQAB recommends a draft of the reviewed HELCOM Combine Manual Annex C-7 is provided after discussion within the HELCOM MONAS Zooplankton expert network.	HELCOM MONAS Zooplankton expert network
9. STGQAB recommends HELCOM to ensure participation of chairpersons of expert groups at STGQAB meetings.	ICES Secretariat and HELCOM MONAS
10. STGQAB recommends HELCOM to consider the establishment phytobenthos expert/project group.	HELCOM MONAS
11. STGQAB recommends HELCOM to consider to change the status of zooplankton to mandatory in the MONPRO scheme	HELCOM MONAS and MON- PRO
12. STGQAB recommends HELCOM to consider to change the status of bacterioplankton (biomass and growth) and also primary production to optional parameters in MONPRO scheme.	HELCOM MONAS and MONPRO
To OSPAR	
13. STGQAB recommend that JAMP guidelines be updated in relation to the new ISO/CEN standards and suggested working documents	OSPAR
14. STGQAB recommend that relevant EcoQO documents are provided to the group by 1 st December 2006 for consideration of QA aspects at their next meeting	OSPAR
To NMBAQC/BEQUALM	
15. The STGQAB recommended that NMBAQC/BEQUALM further develop and take forward the questionnaire that was produced by the STGQAB in 2005.	BEQUALM/NMBAQC
To ICES	
16. ICES should take part in the meetings of CEN and use STGQAB as reference group for that also intersessionally.	ICES Secretariat
17. ICES data centre define the flagging standards to be applied to biological data	ICES Secretariat
18. For ICES to provide draft of data validation document to relevant ICES work groups (STGQAC, MCWG, WGS AEM, SGMID, WGMDM and ICES data centre)	ICES Secretariat

Annex 5: Proposal for revision of chapter B.4 of the HELCOM COMBINE Manual

(working document delivered by Germany, status: 15. February 2006)

Proposal for a new structure of chapter B.4

B.4 Validation guidelines (*new title, until now: B.4 Validation of analytical methods*)

B.4.1 Quality checks of sampling (*replace the existing “Annex B-5 General remarks on sampling for chemical analysis”, at first it should adopt as it stands but the draft should be reviewed*)

B.4.2 Validation of analytical methods (*new title, until now: “B.4.2 Validation”, old paragraphs from “B.4.1 General” and parts of Annex B-2 should be relocate here, the whole chapter should be reviewed by STGQAC*)

B.4.2.1 Selectivity (*adopt as it stands*)

B.4.2.2 Sensitivity (*adopt as it stands*)

B.4.2.3 Range (*adopt as it stands but changes order: formerly chapter B.4.2.4*)

B.4.2.4 Limit of Detection (LOD) (*adopt as it stands but changes order: formerly chapter B.4.2.3*)

B.4.2.5 Accuracy (*adopt as it stands*)

B.4.2.5.1 Estimating random errors (*adopt as it stands*)

B.4.2.5.2 Estimating systematic errors (biases) (*adopt as it stands*)

B.4.2.6 Measurement uncertainty (*general Parts of ANNEX B-4 should relocate here without attachment 1*)

B.4.2.6.1 Procedures to estimate uncertainty

B.4.3 Validation of data (*new chapter to check and amend by qualified ICES working groups: ICES data centre, WGSAEM, SGMID, WGMDM*)

B.4.3.1 Data checks applied to individual data points and variables (*new chapter*)

B.4.3.2 Data checks applied to HELCOM database (*new chapter*)

ANNEX B-1 Validation of established analytical methods for chemistry, examples of full and limited validation procedure (*adopt as it stands but replace the existing “Annex B-2 Validation of established analytical methods for chemistry” and should be reviewed by STGQAC*)

ANNEX B-1.1 Full validation procedure

ANNEX B-1.2 Limited validation procedure

ANNEX B-2 Protocol for evaluation of measurement uncertainty from in-house control measurements (*should contain only Appendix 1 of formerly Annex B-4 and should be reviewed by STGQAC*)

ANNEX B-3 Examples of reference materials for internal quality control (*replaced ANNEX B-6*)

All following ANNEXES (from Annex B-8 up to Annex B-17) are technical notes on special parameters and should be included in appropriate sections of part C.

First proposal of a revised chapter B.4

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 Quality checks of 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
 - using sampling devices that have been found to be suitable for the particular purpose,
 - avoiding contamination of samples from the use of unclean equipment,
 - 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);
1. instructions for labelling the sample specifying its identity;
 2. 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, automatisisation, 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 and amended by STGQAC*).

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 Range

....

B.4.2.4 Limit of Detection (LOD)

....

B.4.2.5 Accuracy

....

B.4.2.5.1 Estimating random errors

....

B.4.2.5.2 Estimating systematic errors (biases)

....

B.4.2.6 Measurement uncertainty

....

B.4.2.6.1 Procedures to estimate uncertainty

....

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)

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:

- validation of “raw” data (primary and secondary data) by the originator of the data and/or the data submitter
- validation by the data centre
- validation by the end user.

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

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

- check the correctness of the defined data format
- check the expected interval range
- check the measurement units
- range checks
- check for outliers
- check for missing information/data
- check for availability of essential metadata
- 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 to database

This means the verification of data by special routines of the data centre before submitting the data to reports and assessments:

- are the correct data provided
- are the data complete (missing stations, missing intervals, completeness of accompanying parameters)
- are there new data provided (new stations, new parameter)
- check the correctness of the defined data format, expected interval range, measurement units, range checks (data range tests)
- check for outliers
- data trend controls
- incorporation of data quality flags

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 6: BEQUALM – Biological Effects Quality Assurance in Monitoring Programmes

Report for STGQAB, February 2006

A presentation on the Biological Effects Quality Assurance in Monitoring Programmes (BEQUALM) scheme was given by the BEQUALM Project Officer, Dr Yvonne Allen, from the Centre for Environment, Fisheries and Aquaculture Science (Cefas), UK. The self-funded scheme was launched in September 2004 following a 3-year research phase funded by the European Commission's Standards, Measurements and Testing programme. The costs of running the self-funded scheme are recouped entirely from registration fees paid by participants.

BEQUALM was set up to develop protocols and quality standards for a range of biological effects techniques and devise an AQC framework for monitoring compliance of laboratories generating data from these techniques for national and international monitoring programmes. The primary driver is the OSPAR JAMP CEMP, but the techniques used are also applicable to other monitoring programmes run by HELCOM and MEDPOL. All OSPAR JAMP CEMP biological effects data submitted to the ICES database by OSPAR contracting parties should have accompanying QA provided by BEQUALM, and in the case of imposex this is provided by QUASIMEME. This is necessary to allow assessment of data across the whole OSPAR area. Data submitted to the ICES database without appropriate QA may be down graded or not used in OSPAR assessments. External QA/QC is also required for biological effects techniques used for regulatory purposes, and to this end these types of assays have been included as part of the BEQUALM repertoire. The Water Framework Directive will also require QA/QC for much of the data generated as part of the quality assessments for water bodies and subsequent monitoring, and BEQUALM is in a good position to accommodate this need. Indeed, with the introduction of the EU Marine Strategy it would seem sensible that OSPAR and WFD have consistent AQC procedures for biological effects measurements.

The BEQUALM scheme comprises three components:

Whole Organism (bioassays and fish disease) - led by the Centre for Environment, Fisheries and Aquaculture Science (Cefas)

Biomarkers – led by the Norwegian Institute for Water Research (NIVA)

Community Analysis - led by the UK National Marine Biological Analytical Quality Control Scheme (NMBAQC).

The BEQUALM Project Office (Cefas) acts as the overall administrative and co-ordinating centre for the whole scheme. A website (www.bequalm.org) has been set up providing information on the activities of all components. Training CD-ROMs, developed for some assays as part of the EU funded programme are given to participants as part of the registration fee. These are updated as required to include e.g. changes in methodology and QC criteria as the scheme progresses.

Whole Organism Component Activities

The bioassays component has successfully conducted 2 rounds of intercalibrations in the first two years of the self-funded scheme. Four assays (*Tisbe battagliai*, *Daphnia magna*, *Corophium volutator*, *Arenicola marina*) were offered in Year 1 and a further two assays (*Skeletonema costatum* and *Acartia tonsa*) added to the portfolio in Year 2. For each bioassay a single intercalibration was conducted, using zinc sulphate as the reference material for the water column assays and Ivermectin-spiked sediment for the sediment assays. For fish disease assessment, 2 intercalibrations have taken place; one using a series of images sent

electronically and another using a set of slides. This will be followed up in March 2006 by a 5-day workshop to provide feedback for existing participants whilst simultaneously providing training for fish histopathologists, with the view to encouraging additional uptake to the BEQUALM fish disease programme.

The number of registrations for each assay in each year are presented in Table 1. Organisations from France, Germany, Denmark, England, Scotland, Northern Ireland, Eire, Netherlands, and Tazmania took part. In addition, the fish disease workshop is attracting participants from Canada, Spain, Norway, the Russian Federation and Switzerland. The *Acartia* was not taken forward due to lack of uptake. *Acartia*, together with *Corophium* and *Skeletonema*, is the PARCOM regulatory species for the Harmonised Offshore Chemical Notification Scheme. At the moment there is no official requirement from PARCOM for external quality control/QA for the toxicity tests that are being conducted as part of the risk assessment of chemicals used in the offshore oil and gas industry. BEQUALM has offered to give a presentation at the next meeting of EOSCA (European Offshore Speciality Chemicals Association) to promote the scheme and highlight the importance of external QA/QC for toxicity tests used within this regulatory context.

Table 1. Number of participants for each assay under the Whole Organism Component.

YEAR	TISBE ASSAY	DAPHNIA ASSAY	COROPHIUM ASSAY	ARENICOLA ASSAY	SKELETONEMA ASSAY	FISH DISEASE	TOTAL
1	7	4	10	7	--	6	34
2	5	8	7	5	8	(25)	33 (58)

(Numbers in brackets indicate participant numbers for the forthcoming fish disease workshop).

In 2005 the whole organism component was expanded further to include QA/QC for the Luminescent Bacteria Assay, with the University of Catalonia as the Lead Laboratory. An intercalibration is currently taking place with 27 participants from Spain, Netherlands, Belgium and the UK.

Biomarker Component Activities

NIVA successfully organised and conducted intercalibrations for EROD, CYP1A, VTG and protein analysis during November 2004 to November 2005. The number of participants for each assay is presented in Table 2. Organisations from South Korea, UK, Denmark, Germany, France, Croatia, Netherlands and Finland took part.

A call for expression of interest to participate in QA/QC exercises for additional assays will be made shortly. These assays will include AchE (acetyl cholinesterase), neutral red retention (lysosomal stability), the yeast oestrogen screen (YES assay) and DNA adducts.

Table 2. Number of participants for each assay under the Biomarker Component

YEAR	EROD	CYP1A	VTG	PROTEIN	TOTAL
1	13	5	5	9	32

Community Analysis Component Activities

Benthos

In 2004, the NMBAQC extended the UK scheme into Europe by inviting organisations to participate in 2 of the 5 components offered – the “Own Sample” and “Ring-Test” exercises. Uptake from Europe has been very disappointing, with only one laboratory, from Germany, participating in the first year and one from Eire in 2005. (It should be noted that 20 laboratories throughout Europe took part in the EU BEQUALM development programme).

Each year, around 20 organisations from the UK participate in this scheme. Despite the OSPAR requirement for AQC for benthic analyses across the convention, laboratories are not signing up to the scheme. The reasons for this lack of uptake are not immediately clear and need to be urgently addressed through the ICES working group STGQAB and SIME. Despite being unable to generate interest in benthos AQC outside the UK, the NMBAQC is continuing to extend its remit, taking forward AQC requirements under the WFD by holding a workshop on fish sampling and is also about to embark on the first fish ring test. In addition, workshops on epibiota and macroalgae are planned.

Phytoplankton

The Marine Institute in Dublin, under the auspices of NMBAQC/BEQUALM has recently conducted a Phytoplankton Enumeration and Identification intercalibration. The purpose of this is to compare the performance of laboratories engaged in national official or non-official phytoplankton monitoring programmes throughout Ireland and the UK. Six organizations, with a total of 16 analysts have taken part. Data is being collated and a report is due to be sent out to participants in February 2006. A wash-up meeting/workshop is planned for March where the results of the intercalibration will be discussed. It is anticipated that in the next intercalibration, organisations throughout Europe will be invited to participate. It is hoped that a high level of participation will be achieved, since this was a very successful workpackage in the EU BEQUALM development programme, with 32 participants from 13 European countries, and 27 participants from 12 European countries, taking part in the 1st and 2nd intercalibrations respectively.

Annex 7: QA activities period 2006 – 2007, Germany

Quality Assurance (QA) Panel of the German Marine Monitoring Programme of the North and Baltic Sea (GMMP) of the German Federal Environmental Agency (WG Biology)

QA activities period 2006 – 2007 (Last update: 02.02.06)

	theme	status	participants
Ringtests			
<i>Phytoplankton</i>	Intercomparison of counting and determination of biomass of cultivated algae	postpone, in preparation for 2006/2007	GMMP-Laboratories*
	Intercomparison of determination of chlorophyll- <i>a</i>	Planned for 2006/2007	GMMP-Laboratories*
<i>Macrozoo-benthos</i>	taxonomical ring test and ring tests with sediment samples (North and Baltic Sea species)	In preparation for 2006/2007	GMMP-Laboratories*
<i>Zooplankton</i>	Intercomparison of counting and determination of zooplankton species of the Baltic Sea	Planned for 2006/2007 in cooperation with Poland	HELCOM laboratories
Workshops			
<i>Phytoplankton</i>	Taxonomical Workshop: <i>Bacillariophyceae</i>	postpone, planned for 2006/2007	GMMP-Laboratories*
<i>Macrozoo-benthos</i>	Taxonomical Workshop: <i>Mollusca</i> , <i>Oligochaeta</i> , <i>Polychaeta</i> (22.03. – 26.03.2004, Kiel)	Report in preparation	GMMP-Laboratories
<i>Macrophyto-benthos</i>	Monitoring Strategies of macrophytes in relation to the Water Framework Directive and determination exercises Part 1: hard bottom (11.04. - 15.04.05, Helgoland) Part 2: soft bottom (20.06. - 24.06.05, Hiddensee)	Reports in preparation	GMMP-Laboratories*, external Experts from EG-countries
<i>Marine monitoring and Quality Assurance</i>	Workshop Marine Monitoring, Water Framework Directive and Quality Assurance, Joint session of WG Biology and Chemistry	In preparation, for 2006	Members of WG Biology and Chemistry, GMMP-Laboratories*

Further activities

- compilation of Length-biomass(carbon)-relation factors of zooplankton species of the HELCOM area
- compilation and revision of uniform phytoplankton and macrozoobenthos species lists in coordination with international species lists
- compilation and elaboration of Standard Operation Procedures (SOPs) of biological parameters determined in the GMMP (determination and counting of phytoplankton and phytoplankton biomass, determination of chlorophyll-*a* and sampling and analysis of macrozoobenthos)
- preparation of accreditation schemes for biological parameters and ring tests

* if there are enough capacities, additional laboratories can take part upon request

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Annex 8: Outcome of the German zooplankton project

The Federal Environmental Agency of Germany (Quality Assurance Panel of the GMMP) supports the development of biomass factors of acceptable quality concentrating on the Baltic proper by the Baltic Sea Research Institute in Warnemünde (Dr. L. Postel, H. Simon). The aim of the project is to improve the present zooplankton biomass factors taking into account the seasonal and geographical differences in individual volume. The project was introduced to revise the HELCOM Combine Manual Annex C-7 in respect to length/biomass determination of mesozooplankton in 2006.

The following tasks are covered by the project:

- Length (size) measurements, rapid cryoconservation and micro-carbon determination of individual mesozooplankton species
- Comparisons of the results with the cryoconservation method following Latja, R. & K. Salonen, (1978) and Salonen (1979) by parallel measurements and determination of potential shrinking due to the common preservation by 4% formalin dilution
- Development of a proposal for a revision of the HELCOM COMBINE Manual, Annex C-7, chapter 6.2 concerning Mesozooplankton biomass determination.

Two principle suggestions for the calculation of individual carbon mass are possible:

the actual carbon mass determination by the Measuring / conversion approach (length measurements and conversion to carbon) and the factor approach (approximate carbon mass determination by average factors). Currently, HELCOM recommends the factor approach (fresh mass, dry mass), but some laboratories do not follow it, and Lithuania performs the measuring / conversion approach (fresh mass). Which method will be recommended should be a matter of discussion within the HELCOM MONAS Zooplankton expert network during their 2nd workshop in August 2006.

Material was collected on 10 stations between Kiel Bight and the Northern Baltic Proper during six cruises from October 2004 until November 2005. Living individuals were narcotized by carbon dioxide, followed by taxon-/ developmental stage specific determination according to HELCOM COMBINE Manual and computer aided measurements of body size (for example cephalothoraxes length of copepods, total length of cladocerans) were carried out.

The comparison of the Rapid-Cryofixation (Köster and Postel, in prep.) versus cryofixation (Latja and Salonen, 1978 and Salonen, 1979) is not finished yet. Preliminary, the results of Philipp Köster's diploma thesis were included for this purpose. The power function basing on the formula provided by Tanskanen (1994) is close to Philipp Köster's smoothing function. Tanskanen (1994) used the procedure of Latja and Salonen (1978) and Salonen (1979) respectively. Both methods seems to provide similar results. Consequently, it might be possible to fill some gaps in our results by literature which bases on the method according to Latja and Salonen (1978) and Salonen (1979) respectively.

A photographic shrinking analysis after the common preservation by 4% formalin (formalin preserved samples versus living organisms) showed that nauplius stages of copepods apparently did not shrink, the body mass of the adults shrunk but not their chitin integument.

The following key species are covered by the project, data of the diploma thesis of P.Köster (Rostock University), and literature data:

Satisfying coverage: *Acartia bifilosa*, *Acartia longiremis*, *Acartia tonsa*, *Acartia discaudata*, *Centropages hamatus*, *Temora longicornis*, *Pseudocalanus* spp.,

Coverage with gaps: *Eurytemora affinis*, *Oithona similis*, *Cyclops* spp., *Balanus improvisus*, *Evadne nordmanni*, *Podon* spp., *Bosmina* spp., *Oikopleura dioica*, *Fritillaria borealis*, Bivalvia larvae, Gastropoda larvae, Polychaeta larvae *Synchaeta* spp.,

Coverage by literature data only: *Limnocalanus macrurus*, *Harpacticoida*, *Sagitta* spp., *Keratella* spp.

The difference between the currently applied factors (Hernroth, 1985) and our factors is partly remarkable. Generally, there is still the need to look for the reason of different results. Our results strongly support a consideration of seasonal differences in both the measuring / conversion approach and the Factor approach. Salinity does very probably not influence mesozooplankton body size. Shrink correction is not needed for organisms with chitinous integuments but for the others. The Measuring / conversion approach allow marching all developmental stages of copepods in one formula. This advantage prevents possibly difficulties in confident separating different copepodit stages.

The open tasks of the project are the final carbon determinations, especially concerning the actual comparison between the Rapid-Cryo-Fixation (Köster and Postel, in prep.) versus Cryo-Fixation (Latja and Salonen, 1978) and Salonen, 1979) and a final check of the need for regionally different factors. Not all species of the Baltic Sea are included in the German project, solutions and funding are still open at the moment (it would be best, if the same laboratory could solve the problem with the same methods). Also the contribution of contamination of the carbon content by epizoans is not estimated until now. The development of a computer aided program for counting and measuring takes much more time than expected. It is technically impossible to solve the matter within the current project.

Annex 9:

QA activities in 2006 – 2007 in HELCOM and OSPAR areas

Parameter	Subject	Organized by	Date	National/International exercise	Remarks
<i>Phytoplankton</i>	Workshop on intercalibration exercise and lectures on new changes in taxonomy	HELCOM Phytoplankton Expert Group	Workshop planned for 2006, Finland	International	Contact person: Norbert Wasmund (norbert.wasmund@io-warnemuende.de)
	Ring test of counting and determination of species and biomass of algae	German Quality Assurance Panel of GMMP in connection with HELCOM PEG	Ring test in preparation for 2006/2007	National/International potential	Contact person: Petra Schilling (petra.schilling@uba.de)
	Workshop for identification of diatoms	German Quality Assurance Panel of GMMP	Workshop planned for 2006/2007	National/International potential	Contact person: Petra Schilling (petra.schilling@uba.de)
	2 nd Phytoplankton Enumeration and Identification ring test	UK NMBAQC Scheme / BEQUALM & The Marine Institute, Galway, Ireland	Late 2006	UK/Ireland/International potential	Contact person: Myles O'Reilly (Myles.OReilly@sepa.org.uk)
<i>Chlorophyll a</i>	Chlorophyll a ring test round 45	QUASIMEME	April – September 2006	International	QUASIMEME Webpage: http://www.quasimeme.org/
	Ring test of determination of chlorophyll-a	German Quality Assurance Panel	Ring test planned for 2006/2007	National	Contact person: Petra Schilling (petra.schilling@uba.de)
<i>Macrozoobenthos</i>	Ring test of Determination of macrozoobenthos species	German Quality Assurance Panel of GMMP	Ring test in preparation for 2006/2007	National/International potential	Contact person: Petra Schilling (petra.schilling@uba.de)

Parameter	Subject	Organized by	Date	National/International exercise	Remarks
	Ring test of Determination and enumeration of macrozoobenthos species in an artificial sediment sample	German Quality Assurance Panel of GMMP	Ring test in preparation for 2006/2007	National/International potential	Contact person: Petra Schilling (petra.schilling@uba.de)
	Participant-supplied sample macrozoobenthos ("own sample")	UK NMBAQC Scheme / BEQUALM	Intercomparison in 2006/2007	National/International potential	Contact person: Myles O'Reilly (Myles.OReilly@sepa.org.uk)
	25 species sample of UK macrozoobenthos provided by NMBAQC Contractor (sample to be analysed by NMMP laboratories)	UK NMBAQC Scheme / BEQUALM	Ring test in 2006/2007	National/International potential	Contact person: Myles O'Reilly (Myles.OReilly@sepa.org.uk)
	25 species sample of UK macrozoobenthos provided by participants (Specimens from NMMP laboratories to be validated by NMBAQC Contractor)	UK NMBAQC Scheme / BEQUALM	Validation test in 2006/2007	National/International potential	Contact person: Myles O'Reilly (Myles.OReilly@sepa.org.uk)
	25 species sample of UK fish provided by NMBAQC Contractor (sample to be analysed by laboratories undertaking WFD fish monitoring – samples to be retained by labs as reference material)	UK NMBAQC Scheme / BEQUALM	Validation test in 2006	National/International potential	Contact person: Myles O'Reilly (Myles.OReilly@sepa.org.uk)

Parameter	Subject	Organized by	Date	National/International exercise	Remarks
	<i>Targeted of UK macrozoobenthos – 25 specimen ring test.</i>	UK NMBAQC Scheme / BEQUALM	Ring test in 2006/2007	National/International potential	Contact person: Myles O'Reilly (Myles.OReilly@sepa.org.uk)
	<i>Contractor-supplied sample of UK macrozoobenthos 1 sample supplied to participating laboratories)</i>	UK NMBAQC Scheme / BEQUALM	Ring test in 2006/2007	National/International potential	Contact person: Myles O'Reilly (Myles.OReilly@sepa.org.uk)
	<i>Taxonomical macrozoobenthos workshop</i>	UK NMBAQC Scheme/ BEQUALM	Workshop planned for November 2006	National/International potential	Contact person: Myles O'Reilly (Myles.OReilly@sepa.org.uk)
	<i>Epibiota, Photographic ring test (Images/video clips supplied on web for identification: http://www.nmbaqcs.org)</i>	UK NMBAQC Scheme/ BEQUALM	Ring test in 2006	National/International potential	Contact person: Jon Davies (jon.davies@jncc.gov.uk)
	<i>Training course on phytobenthos monitoring methods</i>	Estonian Marine Institute	Workshop in May 2006	International	Contact person: Georg Martin (georg.martin@ut.ee)
	<i>Macrophytobenthos intercalibration</i>	National Environmental Research Institute (Dept. of Marine Ecology)	Intercalibration 2006	National	Contact person: Peter Henriksen (pet@dmu.dk)
	<i>Zooplankton ring test</i>	Institute of Aquatic Ecology Latvia	Ring test in 2006	National	Contact person: Anda Ikaunice anda@monit.lu.lv
	<i>HELCOM Zooplankton ring test</i>	Sea Fisheries Institute Poland and German Quality Assurance Panel of GMMP	Ring test in 2006/2007	International	Contact person: Lutz Postel (lutz.postel@io-warnemuende.de)

Annex 10:

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Bacterioplankton biomass

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 Signature:

BACTERIOPLANKTON BIOMASS

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1 Introduction

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

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

1.1 Background

Bacterioplankton constitutes about half of the living mass of secondary producers in many aquatic environments. Bacteria also account for half of the secondary production in many aquatic environments, and are comparable to the productivity of phytoplankton in some environments¹.

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 ($\approx 0.5 \mu\text{m}$) 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.

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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 estimated⁵. 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. Too rapid filtration may indicate a broken filter, erroneously applied filter or too high vacuum.

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

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

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

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

1.5 Contamination risk

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

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1.6 Safety

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

2 Preparation

2.1 Cleaning and purification

Sample bottles

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

Filter equipment

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

Glass slide and cover slip

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

Microscope

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

2.2 Sample identification

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

2.3 Reagents

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

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

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2.4 Prior to sampling

Pure sample bottles (e.g. 50 ml glass bottles) are added 2.0 ml, 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 ml to a labelled sample bottle with formaldehyde added. Mix the sample by turning the bottle up-side-down 5 times.

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3.2 Preservation/processing

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

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 cells⁶. 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 ml). One tube (A) is used for rinsing the pipette between 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 seasons⁷. 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.

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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 ml), 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 ??)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 ml 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.

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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:

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

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_f) divided by the area of the camera image or the ocular grid (a) according to:

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

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. 1998⁵.

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 μm³ 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 μm³, ⁵) may provide a rough estimate of the biovolume as within year variation has been shown to be low (±SD 10%)⁵.

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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 a calculation software like Microsoft Excel (item 11.1). Calculated data can be aggregated using a pivot table procedure. Compiled data are entered into a database according to item 7.

5.3 Precision and accuracy

The detection limit of the method is estimated to 5.0×10^6 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 $3 \times \text{SD}$) when performing image analysis and 10 % at manual count (due to fewer but larger fields in the former technique). Otherwise count more fields. At high heterogeneity consider making a new slide or sonicate the sample according to item 4.3. The standard deviation between 2 homogenous samples has been estimated to $\pm 2\%$.

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

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

The measurement uncertainty has been determined according to the standard of measurement uncertainty in chemical analysis of the European Union 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 $\pm 23\%$, approximately corresponding to a 95% confidence interval (Table 1). The estimate of average number of cells per microscopic field contributes with the greatest uncertainty (i.e. variation between microscopic fields). The uncertainty is therefore similar in the unit cells dm^{-3} .

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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	$\mu\text{mol carbon dm}^{-3}$	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 [filtered area]⁻¹ ($\pm 6.4 \times 10^5$) based on image analysis estimates. The background value should be divided by the sample volume prior to subtraction from the corresponding sample. When the image analysis system finds no particles an error message may be returned. These background values should be set to zero manually.

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

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

6.2 Evaluation

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

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

For all control charts it's advised that samples differing more than $2 \times \text{SD}$ from average values are checked, and actions should be taken for samples deviating more the $3 \times \text{SD}$. 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.

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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 $70 \times 70 \mu\text{m}$, and $21 \times 21 \mu\text{m}$ for a small square.

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

Number of pixels per 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 $0.0986 \mu\text{m pixel}^{-1}$ for the system. This value should be entered in the "Calibration" cell in LabMicrobe (item 11.4.4). LabMicrobe round this value to 0.1 but use the precise value.

7 Reporting format

Make required calculation in a calculation software like Microsoft Excel and aggregate data from different images using pivot tables. Variable specific parameters are given in table 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.

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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 carbon dm ⁻³	3	5.1.5	Depth	BACTBIOM	2.02

”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) bacterivorous flagellates (cells dm⁻³) and oxygen concentration (mol O₂ 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.

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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 ml, Plastipak, Becon and Dickonson. Pincett, Millipore, Glass slides, Menzel-Gläser, 76x26 mm. Cover slip, Menzel-Gläser, 24x60 mm, #0, Sample bottles: Glass, 50 ml. Gloves: Powder 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:	<u>Acridine orange</u> : FS 09: 450-490, FT 510, LP 520 FS15: BP546, FT580, LP590 <u>DAPI</u> : 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 (P110x/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.

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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 ml 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.

Immersion 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).

Sodium pyrophosphate

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

Suspension of fluorescent beads

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

Bead standards are prepared by filtering beads at an expected concentration of 1×10^9 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 $\pm\text{CV}$ 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.

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11 Appendices

11.1 Calculation of parameters

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

11.2 Sample volume

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

Bothnian Bay

Month/m	0	1	4	8	14	15	20	40	60	80	90	100	120
Jan	5	5	5	5	5	5	5	5	7	7	7	7	7
Feb	5	5	5	5	5	5	5	5	7	7	7	7	7
Mar	5	5	5	5	5	5	5	5	7	7	7	7	7
Apr	5	5	5	5	5	5	5	5	5	5	5	5	5
May	5	5	5	5	5	5	5	5	5	5	5	5	5
Jun	5	5	5	5	5	5	5	5	5	5	5	5	5
Jul	4	4	4	4	4	4	4	4	4	4	4	4	4
Aug	4	4	4	4	4	4	4	4	4	4	4	4	4
Sep	4	4	4	4	4	4	4	5	5	5	5	5	5
Oct	4	4	4	4	4	4	4	6	6	6	6	6	6
Nov	5	5	5	5	5	5	5	6	6	6	6	6	6
Dec	5	5	5	5	5	5	5	6	6	6	6	6	6

Öre estuary

Month/m	0	1	4	8	14	20
Jan	5	5	5	5	5	5
Feb	5	5	5	5	5	5
Mar	5	5	5	5	5	5
Apr	5	5	5	5	5	5
May	4	4	4	4	4	4
Jun	4	4	4	4	4	4
Jul	3	3	3	3	3	3
Aug	4	4	4	4	4	4
Sep	4	4	4	4	4	4
Oct	4	4	4	4	4	4
Nov	4	4	4	4	4	4
Dec	5	5	5	5	5	5

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Bothnian Sea

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

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11.3 Import format

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

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

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

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11.4 Example of image analysis

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

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

11.4.1 Capture of images

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

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

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

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

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

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

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

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

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

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

11.4.2 Analysis of images

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

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

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

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

11.4.3 Processing of primary data

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

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

All files created under item 11.4.3B can should then be compiled in an calculation software like Excel to give average values per volume for all morphology types in the community.

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Calculations according to item 5 should be used. Results are reported according to item 5.2. Also the Milli-Q background is analyzed in the same way and subtracted from the sample values. Note that Milli-Q values should be divided by the same volume as the corresponding sample to be correct.

11.4.4 Image analysis instrumentation and settings

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

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

Control unit for the CCD camera

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

Buttons

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

Camera

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

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

Gain:0

Offset:0

Auto exposure (AE)

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

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

Format

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

Superpixel: 1x1

Subarray: Large

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Trigger

Camera timing: Intern

Temperature

Control the working temperature of the camera.

”Temperature” show the actual temperature of the camera.

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

Target temperature: 20°C

Software

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

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

Settings for WASABI

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

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

Resolution: Full

Bits per pixel: 16BPP

Average: 1

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

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

11.4.5 Settings for LabMicrobe

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

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

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

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The LabMicrobe window

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

Status window: Gives current activity

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

Threshold data: Min. 2 Max. 10000

Palette: Binary

Data params: Area (calibrated)

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

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

Particle: 1

Classifier tolerance: High.

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

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

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

Read raw file 1 **Read Raw file**
 16 bits signed 5 **File data type**

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

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

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

0,00 **Optional min. value**

0,00 **Optional max. value**

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

Width=1344, Height=1024.

Mark local maxima: Down

Network: 0; 0,0; 5,13

Size independency: Down

Labels: 0,?

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Reconstruct and collect window

Number of frequencies: 4

Other: 4

Plot particles: 1

Continue: Green

Parameters window

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

User Parameters: Particle# and Hemisphrod labelled

Morphology window

Minimum particle size: 1

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

Batch Microbe window

Save binary Image: Off (=down).

LabMicrobe log number: 0

Mark local maxima: Down

Data file extension: (blank)

Analyze window

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

Min. size: 0.01

Max. size: 0.40

bins (mode): 10.

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BACTERIOPLANKTON GROWTH RATE BY THYMIDINE UPTAKE

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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. BOD₇). Bacterioplankton growth rate thereby indicate 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 Azam¹.

Change in bacterial biomass production is a relatively unambiguous indicator of the flux of organic production through this food web component. Even if the relationship between the factors specific growth rate and abundance may vary, the product of biomass production reflects the substrate requirement of the bacterial community. Density limitation (i.e. competition) or other limiting factors (i.e. inorganic nutrients, temperature) do not therefore directly appear to control the bacterial community biomass production at typical environmental conditions. This agrees with empirical observations that bacterial biomass production over larger scales correlate with trophic status of a system^{2,3}.

Bacterioplankton growth rate may be complemented by bacterial biomass estimates which provide estimates of biovolumes for better precision in the biomass production values.

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.³. A positive relationship between nutrient status and bacterial growth, as well as biomass, cross 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 account for about 50% of the oxygen demand in aquatic environments⁴. This due to that oxygen constitutes the major electron acceptors in aerobic environments and that bacteria channel a large part of the carbon flux in aquatic ecosystems. Bacterioplankton

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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^{5,6}.

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^{1,7}. 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 (³H) 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 number of cells produced by empirical knowledge of the amount of thymidine per cell on average. The theoretical conversion factor agree relatively well with 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 does 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.

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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 [methyl-³H] 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 β-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 taken 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 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.

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2.3 Reagents

Isotope

Tritiated [methyl-³H] 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, ProAnalysis) with 227 ml 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

Toluene- 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.

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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 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, 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 contracting 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.

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.

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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 in room temperature and 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-³H] 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 [³H-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.

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- 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 outward the rotor. Samples should not be frozen at this stage. Centrifuge the micro-centrifuge tubes at 16 000 × g (13 000 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 bubble is 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 16 000 × g (13 000 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.

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The samples are counted in the [³H]-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

ID: 3H, 5MIN, DPM

USER	: 1	COMMENT:			
PRESET TIME	: 5.00				
DATA CALC	: SL DPM	H#:	: YES	SAMPLE REPEATS	: 1
COUNT BLANK	: NO	IC#:	: NO	REPLICATES	: 1
TWO PHASE	: NO	AQC	: NO	CYCLE REPEATS	: 1
SCINTILLATOR	: LIQUID	LUMEX	: NO	LOW SAMPLE REJ	: 0
LOW LEVEL	: NO	HALFLIFE CORRECTION			none
		DATE:			
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 ³H-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 (cm³)

Δt = incubation time (hours)

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

Bacterial growth in cells (*P_c*) is calculated as

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

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where TCF is the thymidine cell conversion factor. A conversion factor empirically determined for the Baltic Sea area of $1,4 \times 10^{18}$ cells [mol thymidine] $^{-1}$ ($n=73$, $\pm SE=0,1 \times 10^{18}$) is recommended. This factor seems independent of growth rate and is close to the theoretical factor for coastal environments⁷⁻¹².

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

5.1.2 Bacterial biomass production

Cell production is transformed to bacterial biomass production (P_b , mol carbon $\text{dm}^{-3} \text{day}^{-1}$) 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 $\mu\text{mol C cell}^{-1}$. See the standard operating procedure for bacterioplankton biomass for a definition.

5.1.3 Bacterial oxygen consumption

Bacterial oxygen consumption, ΔO_2^{bact} , may be calculated from P_b , bacterial growth efficiency, BGE , and the respiration quotient, RQ , according to:

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

Estimates of BGE are currently uncertain and varies 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 1998¹³

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

where P_b is the bacterial growth rate in $\mu\text{g C dm}^{-3} \text{h}^{-1}$. 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 bacterium¹⁵.

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:

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

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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:

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

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 $\pm 21\%$, 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	$\mu\text{mol C dm}^{-3} \text{ day}^{-1}$	0.29	± 21

The coefficient of variation ($\pm CV$) for netto dpm should stay below 20% in productive waters. During the winter season values may be somewhat higher. Values above $\pm 60\%$ 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 ($+2 \times SD$). This corresponds to $1 \times 10^7 \text{ cells dm}^{-3} \text{ day}^{-1}$ or $0.02 \mu\text{mol dm}^{-3} \text{ day}^{-1}$, approximately the same in carbon or O_2 . Typical growth rates in mesotrophic environments are 20 times higher.

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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 ($2 \times SD$) and action limits ($3 \times SD$). 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 $3 \times SD$ 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 an 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	BGTCTF	1.4x10 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.

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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	1.84×10 ⁸
Bact. Prod. ±SD	cells dm ⁻³ day ⁻¹	2	5.1.4, 5.1.5	Depth	BGCCELCV	1.12×10 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, μm³ cell⁻¹), bacterivorous 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

An 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.

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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-³H] 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-³H] thymidine is a β-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 contain large amounts of crystal water. 100% (w/v) of TCA is made by mixing 500 g of TCA powder (e.g. Merck, ProAnalyse) with 227 cm³ of Milli-Q water. Diluted solution of 50 % and 5 % are made from the stock solution by dilution with Milli-Q water.

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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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