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Report of the ICES/IOC Workshop on New and Classic Techniques for the Determination of Numerical Abundance and Biovolume of HAB-Species - Evaluation of the Cost, Time-Efficiency and Intercalibration Methods (WKNCT)

22–27 August 2005

Kristineberg, Sweden

International Council for the Exploration of the Sea
Conseil International pour l'Exploration de la Mer

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H.C. Andersens Boulevard 44-46
DK-1553 Copenhagen V
Denmark
Telephone (+45) 33 38 67 00
Telefax (+45) 33 93 42 15
www.ices.dk
info@ices.dk

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Contents

1 About this report

This report from the *ICES/IOC Workshop on New and Classic Techniques for the Determination of Numerical Abundance and Biovolume of HAB-Species – Evaluation of the Cost, Time-Efficiency and Intercalibration Methods* (WKNCT) describes the events and summarises the results of the workshop that took place at Kristineberg Marine Research Station, Fiskebäckskil, Sweden 22–26 August 2005. In addition a scientific article to be submitted to the journal Harmful Algae is in preparation and also a publication in the IOC/UNESCO Manuals & Guides series is planned, describing the techniques used during the workshop. Thus the present report should be considered preliminary and inaccuracies may exist.

2 Summary

A workshop with the aim to compare classical and molecular biological techniques for quantitative phytoplankton analysis took place at Kristineberg Marine Research Station, Fiskebäckskil, Sweden, 22–26 August 2005. A total of 24 participants (appendix 1) from ten countries participated in the workshop and 15 different techniques were compared. After advice from the Scientific Steering Committee (SSC, see Annex 2) and the ICES/IOC Working Group on Harmful Algal Bloom Dynamics (WGHABD) it was decided to focus on essentially one species, *Alexandrium fundyense*. This thecate dinoflagellate *A. fundyense*, strain CA 28, was maintained in unialgal cultures and used during the workshop in four different experiments. Experiment 1 was aimed at determining in which range of cell densities the methods are applicable to. Experiment 2 was designed to test the species specificity of the methods by adding a related species, *Alexandrium ostenfeldii*, to samples already containing *A. fundyense*. Experiment 3 tested the ability of the methods to detect the target organism, *A. fundyense*, which was added to a natural phytoplankton community from the Gullmar Fjord. A range of biomasses of natural phytoplankton was tested. Experiment 4 also tested the methods ability to enumerate *A. fundyense* in field samples. It was similar to Experiment 1, although, higher concentrations of the target species with background levels of other phytoplankton species were present in the samples distributed.

The detailed results from the workshop will be presented elsewhere. In this report only an overview is presented. To summarise, the classical Utermöhl sedimentation chamber technique performed very well with similar results reported by the 2 participants who used different settling volumes to test this method. This method, however, was not as successful when the target organism *A. fundyense* was present in samples containing the morphologically similar species, *A. ostendfeldii*. In the experiment where discrimination with similar species was tested it appears that some of the *A. ostenfeldii* cells may have been misidentified as *A. fundyense.* The filtering techniques also produced good results but some tended to report lower cell numbers then the Utermöhl method. The filtering and calcofluor staining techniques performed well in the experiment that required the discrimination between *A. ostenfeldii* and *A. fundyense*. Sedgewick-Rafter and Palmer-Maloney chambers did not appear to work well when target cell range was between ~ 500–5 000 cells per Litre. These methods improved when cell concentrations increased to ranges between 25 000–100 000 cells per Litre. The Haemocytometer method was unsuccessful at recording the target cell numbers in question when compared to the other methods tested. This method is considered a quick and easy method for culture studies and during bloom situations when cell densities are exceptionally high. The Quantitative PCR method did not perform as well as expected during the workshop. It is thought that a more thorough calibration of this method would have given closer results to those reported by the other methods. The whole cell hybridisation assays with fluorescence microscope detection produced reasonable results, although cell numbers were often underestimated similar to the filtration methods above. These methods all used filtration to

concentrate the sample. The whole cell assay with ChemScan detection both over- and underestimated the cell numbers compared to other techniques. The sandwich hybridisation assay with colourimetric detection produced good results although cell numbers were often underestimated. Only a few samples were processed using the hybridisation assay with microarray fluorescent detection and the sandwhich hybridisation assay using electrochemical detection because of technical problems during the workshop. These methods are considered to be still at a development stage. After the workshop preserved samples were transported to Canada for analysis using a type of advanced particle counter called the FlowCam. Although only a subset of samples were analysed the results reported are quite good.

3 Background and aim of workshop

Investigations of the diversity, cell abundance and biomass of phytoplankton communities have a long history in marine science. In the nineteenth century the use of phytoplankton nets to collect samples was the method of choice by scientists such as Cleve and Ehrenberg. Nowadays nets are only used for qualitative and sometime semi-quantitative studies. Lohmann introduced the centrifuge for concentrating small phytoplankton in early twentieth century. It was not until Utermöhl described the sedimentation chamber technique in the thirties that quantitative analysis started to become common. The Utermöhl sedimentation chamber technique used with an inverted microscope has been the method of choice in many investigations since the fifties. During the last decades various filtering techniques and molecular biological techniques for quantitative phytoplankton analysis have been introduced. Claimed benefits with the new techniques include higher sample throughput, lower cost etc. The aim of the workshop was to compare some of the different techniques and to identify, where possible, a reference counting method against which other methods can be calibrated; (see WKNCT Terms of Reference in Annex 3).

4 Organisational history

The workshop was first discussed at the WGHABD meeting in Aberdeen in March 2002 and convenors and a term of reference was proposed. The ICES Oceanography Committee that year supported the recommendation. In 2003 the WGHABD proposed participants in the workshop and a scientific steering committee was proposed. The ICES Consultative Committee that year further endorsed the plan for the workshop to take place in 2004. Because of lack of financial support and other commitments for the convenors, the workshop was delayed to 2005. In March 2005 invitations to participants were sent out. The organisation of the workshop was further discussed during the WGHABD meeting in Norway in April 2005. The SSC met in April 2005 and held a telephone conference in mid May. Participants arrived at Kristineberg Marine Research Station 20 and 21 August and left 26–28 August.

5 Rationale for workshop experimental design

Phytoplankton communities often consist of 50–100 species and the temporal and spatial variability in composition in the sea is substantial. In understanding harmful algal bloom dynamics it is essential to have correct data on species composition, abundances and biomass. Some of the methods to be tested during the WKNCT can handle most of the phytoplankton in a sample. Because of the fact that today, several of the methods to be tested can only handle one or a few target species in a sample and are unable to estimate biovolumes (biomass), it was decided that the workshop would focus mainly on one species and disregard biovolumes. This species was to be introduced in closed containers with natural assemblages of phytoplankton at the workshop to simulate real samples as closely as possible. The dinoflagellate *Alexandrium fundyense* was chosen primarily because it is one of a few species for which the largest number of detection methods have been developed to date. *Alexandrium*

fundyense is a producer of paralytic shellfish toxins which can have devastating effects for aquaculture in the NE part of North America. Because *A. fundyense* is not found in the waters around KMRS a concerted effort was made to ensure that no viable cells were disposed in local seawater.

During the workshop four different experiments were carried out, one per day. In an effort to present and explain the results of the workshop in the best way possible, analysis of the results required the participation of a statistician. During the workshop the statistician collected the results produced at pre-set deadlines and presented a statistical report the day after each experiment. Good replication was essential because the variability of the results was expected to be substantial. It was considered important to work with a range of cell densities because some harmful algal bloom species can be harmful at densities of ca. 100 cells per Litre and may reach cell densities of >100 000 cells per Litre.

Table 1 Overview of the number of samples distributed during the workshop.

Experiment	No. of samples	No. of replicates per technique	No. of participants	No. of techniques	Total number of samples distributed
1	4	5	18	15	360
2	4	4	18	15	288
3	4	4	18	15	288
4	4	4	18	15	288

5.1 Material and methods

Altogether 15 different methods for estimating cell densities of *A. fundyense* were used during WKNCT. The methods and persons responsible are listed in Table 2. The techniques used will be described in detail in a separate publication in the IOC/UNESCO series "Manuals and Guides".

Table 2. Techniques used during the workshop and persons responsible for each technique. Volume of sample used by each method is presented in brackets. In addition a subset of samples were analysed with a type of advanced particle counter, the FlowCam. These analyses were carried out in Canada and the USA after the workshop. Jennifer Martin, was responsible for the FlowCam samples (20 mL was used to enumerate the target organism for each sample).

Type	No.	Method	Person(s) responsible
Traditional	1	Utermöhl sedimentation, Lugol (10.2-20.5 mL)	Lars Edler
	2	Utermöhl sedimentation, Formalin, Calcofluor (25 mL)	Malte Elbrächter
	3	Settlement bottle (55 mL)	Georgina McDermott
	4	Filtering and calcofluor staining (50-100 mL)	Per Andersen
	5	Filtering on semitransperent filters (50 mL)	Lars-Johan Nausvoll & Einar Dahl
	6	Filtering and freeze filter transfer (20-50 mL)	Allan Cembella
	7	Sedgewick rafter chamber (1 mL)	Georgina McDermott
	8	Palmer Maloney (0.1 mL)	Murielle LeGresley
	9	Haemocytometer (0.0018 mL)	Murielle LeGresley
Molecular	10	Quantitative PCR (100 mL)	Luca Galuluzzi & Antonella Penna
	11	Whole cell assay, fluorescence microscopy detection (50 mL)	David Kulis
	12	Whole cell assay, fluorescence microscopy detection (10 mL)	Melissa Gladstone
	13	Whole cell assay, fluorescence microscopy detection (18-100 mL)	Linda Medlin
	14	Whole cell assay, TSA enhanced (Chemscan) (18-100 mL)	Kerstin Töbe
	15	Sandwich hybridisation assay, colourimetric detection (100 mL)	Roman Marin
	16	Hybridisation assay, microarray fluorescence detection (100 mL)	Christine Gescher
	17	Sandwich hybridisation assay, electrochemical detection (93-100 mL)	Sonja Diercks

5.1.1 Experiment 1 (E1)

The purpose of E1 was to investigate the range of cell densities for which the different methods work. Replicate samples of four different cell densities were prepared using a unialgal culture of *A. fundyense*. Each method handled up to five replicates each for the different cell densities resulting in a total of almost 360 samples. The cell densities were approximately 100, 500, 1000 and 10 000 cells per Litre.

5.1.2 Experiment 2 (E2)

The aim of E2 was to investigate the species specificity for the different methods, i.e., can the target species *A. fundyense*, be discriminated from a closely related species, *A. ostenfeldii.* A range of ratios between the cell densities of the two species was created with a total abundance of approximately 10 000 cells per Litre. The ratios were 10%, 30%, 70% and 90% *A. fundyense* and the remaining cells *A. ostenfeldii.*

5.1.3 Experiment 3 (E3)

In E3, a natural phytoplankton community was collected from 5 m depth at the mouth of the Gullmar Fjord (Figure 1). Water depth at sampling location was ca. 35 m. The seawater was then spiked with varying volumes of a VPH (vertical plankton haul) sample. The aim of E3 was to investigate the influence of a variable matrix of other phytoplankton on the ability of the different methods to estimate cell numbers of *A. fundyense*. *Alexandrium ostenfeldii* was also added to the samples distributed to test for the species specificity of the methods. The total cell density of added *A. fundyense* was approximately 700 cells per Litre, whereas *A. ostenfeldii* was added to a cell density of approximately 300 cells per Litre.

5.1.4 Experiment 4 (E4)

The last experiment was essentially a repeat of E1 but with a natural phytoplankton population as a matrix for the added *A. fundyense* cells. Water was collected from 5 m depth in the Koljö Fjord (Figure 1) and transported to KMRS by car. Cell densities of approximately 500, 5000, 25 000 and 100 000 cells per Litre of *A. fundyense* were added to the sea water samples.

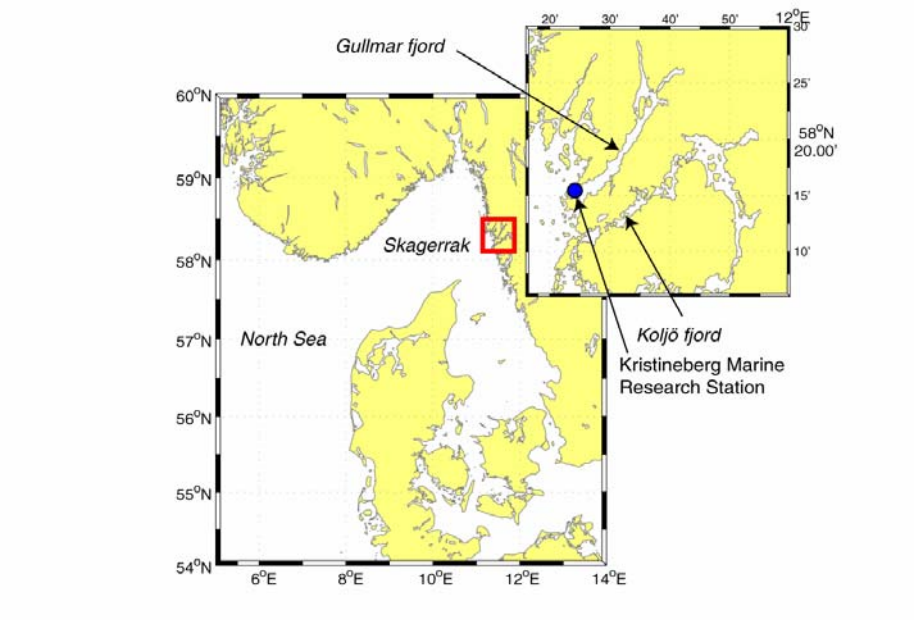

Figure 1. Map showing the area where the workshop was carried out.

6 Results

In Figures 2–5 an overview of the results from experiments 1–4 is presented.

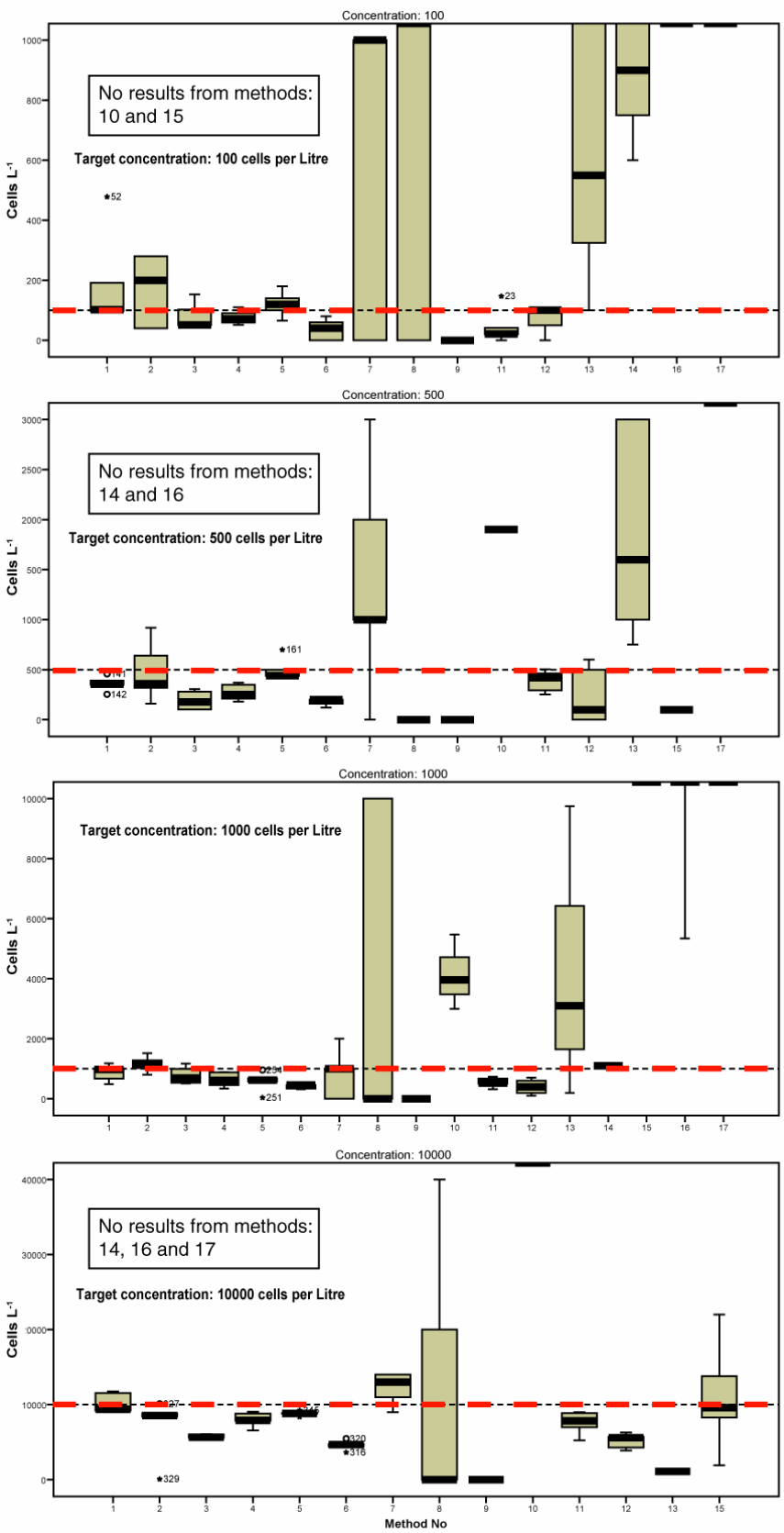

Figure 2. Results from experiment 1 where *Alexandrium fundyense* was enumerated at four different abundances as a unialgal culture. Method numbers refer to Table 2. Black bars indicates medians, the box denotes the mid 50% quartile and the whiskers denote the closest points within 1.5x the 25% and 75% percentiles. Note that no "true" abundance exists.

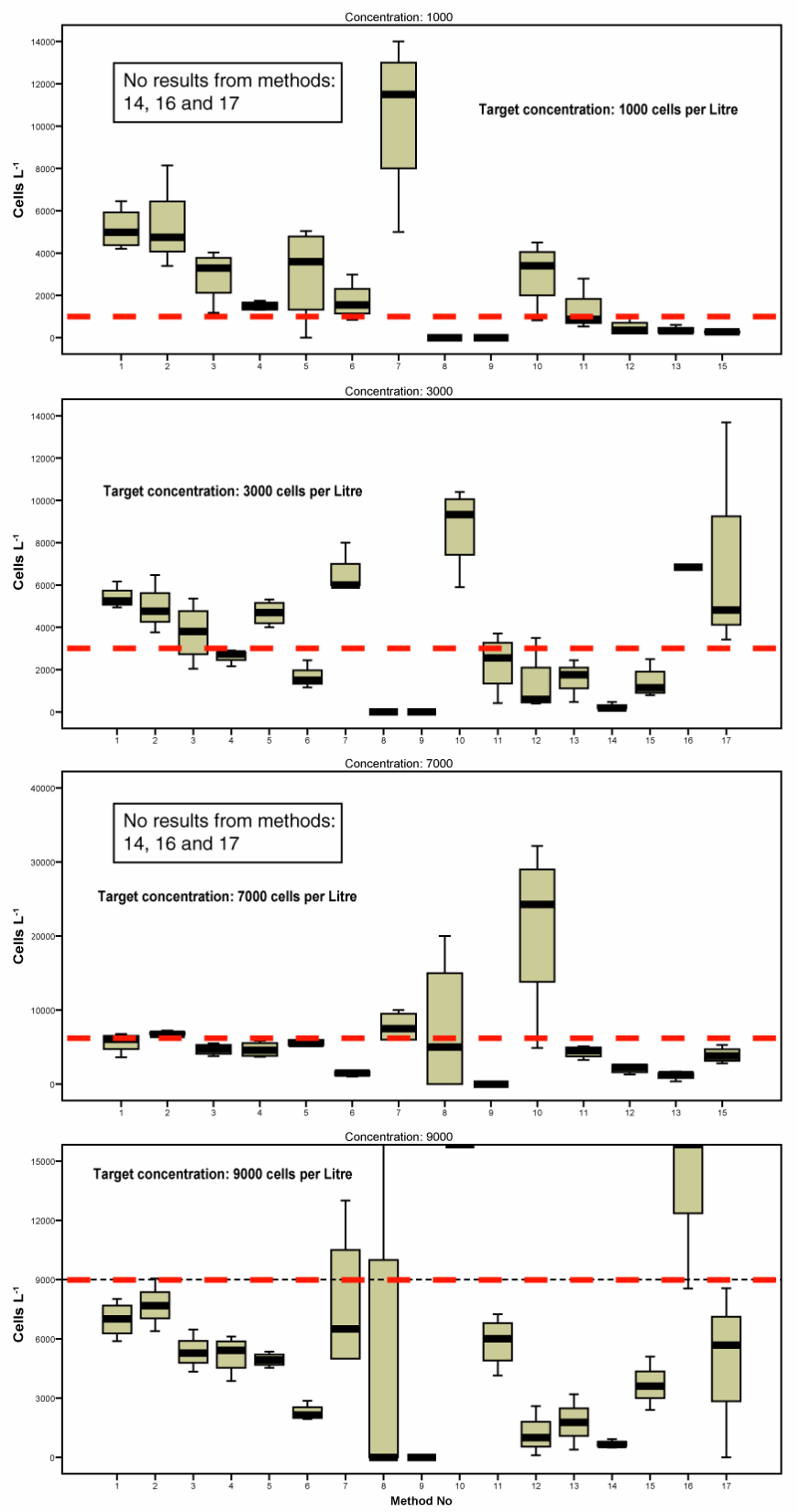

Figure 3. Results from experiment 2 where *Alexandrium fundyense* was enumerated at four different abundances in samples that also contained *Alexandrium ostenfeldii*. Total cell numbers of these two species were approximately 10 000 cells per Litre in all the samples in E2. Method numbers refer to Table 2. Black bars indicates medians, the box denotes the mid 50% quartile and the whiskers denote the closest points within 1.5x the 25% and 75% percentiles. Note that no "true" abundance exists.

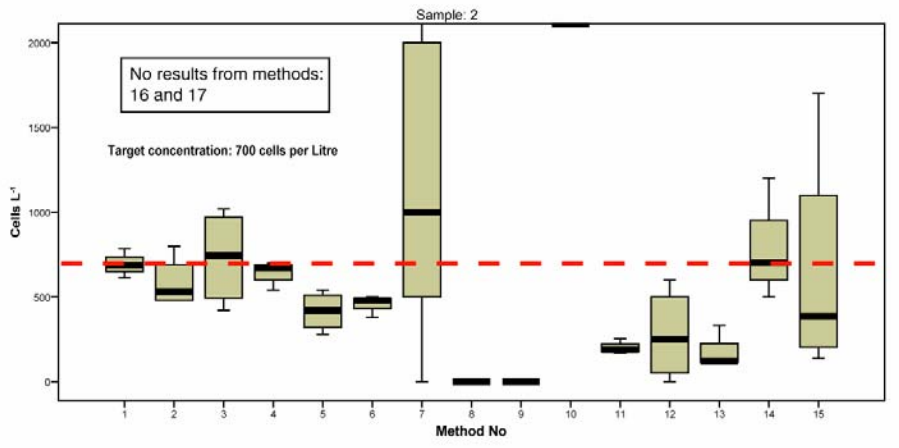

Figure 4. An example of results from experiment 3 where *Alexandrium fundyense* was enumerated in a spiked field sample from the Gullmar Fjord. The sample was also spiked with *A. ostenfeldii* to an approximate abundance of 300 cells per Litre. Additional phytoplankton from a phytoplankton net sample was added to the original sample. Method numbers refer to Table 2. Black bars indicates medians, the box denotes the mid 50% quartile and the whiskers denote the closest points within 1.5x the 25% and 75% percentiles. Note that no "true" abundance exists.

Table 3. In experiment 3, a field sample from the mouth of the Gullmar Fjord was used to introduce natural phytoplankton to the samples distributed to the participants of the workshop. Water was collected from a depth of 5 m. The table below shows chlorophyll *a* concentrations in µg per Litre which is an indicator of the biomass of phytoplankton in the sample. The concentrations recorded were generally low for the area and season. Additions of phytoplankton concentrated using a phytoplankton net was used in an attempt to increase the biomass.

Sample no.	Sample	n	Chl. *a* µg l^{-1} average	Standard deviation
1	Original sample	3	1.54	0.05
2	Original sample + addition 1	3	1.56	0.05
3	Original sample + addition 2	3	1.71	0.07
4	Original sample + addition 3	3	1.92	0.07

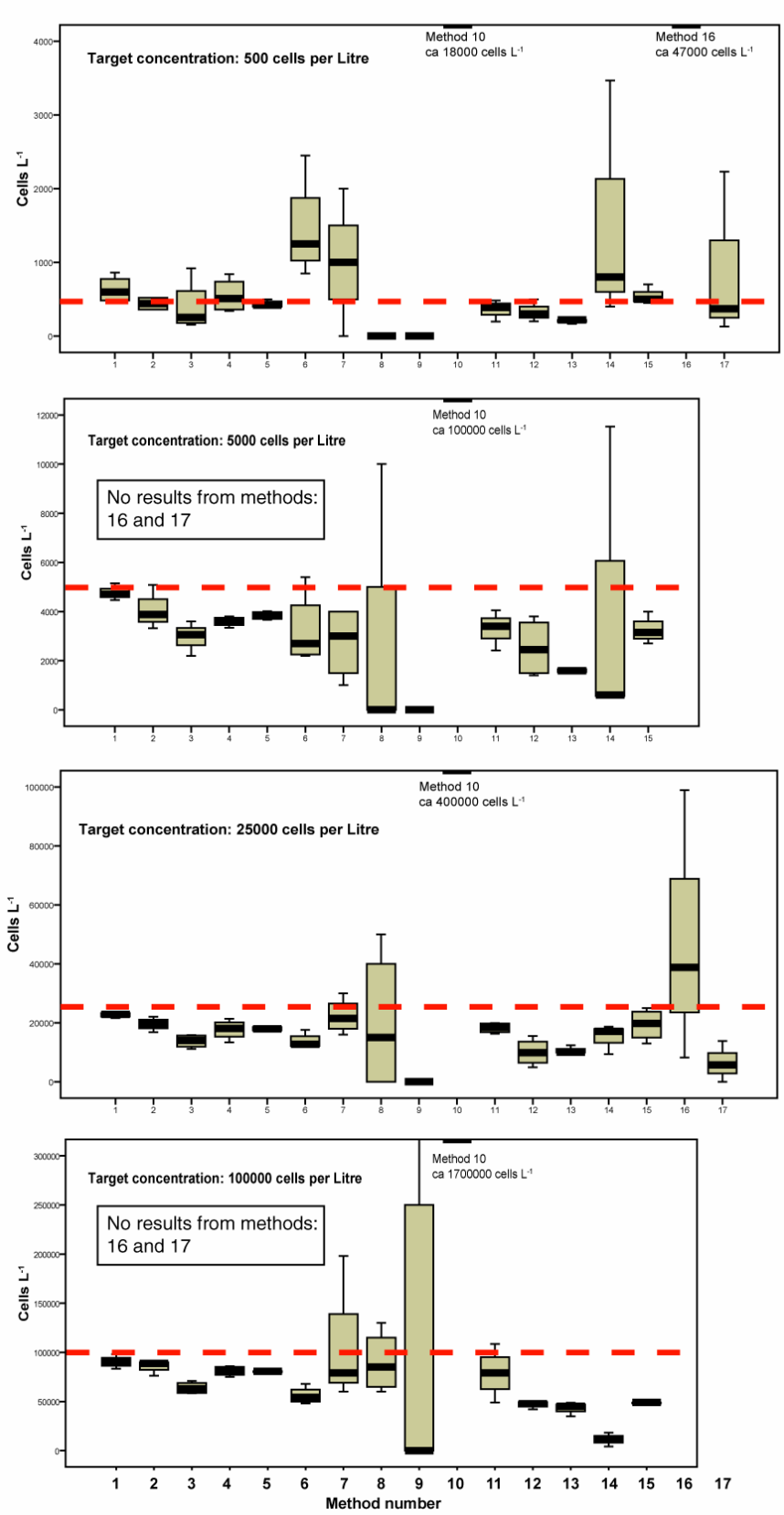

Figure 5. Results from the day 4 experiment when *Alexandrium fundyense* was enumerated at four different abundances in a spiked field sample from the Koljö Fjord. Method numbers refer to Table 2. Black bars indicates medians, the box denotes the mid 50% quartile and the whiskers denotes the closest points within 1.5x the 25% and 75% percentiles. Note that no "true" abundance exists.

6.1 Results from the FLOWCAM

Samples for analysis using the FlowCam technique were transported to Canada for analysis after the workshop. The full set of samples was not analysed but the results can still be compared to those from the workshop.

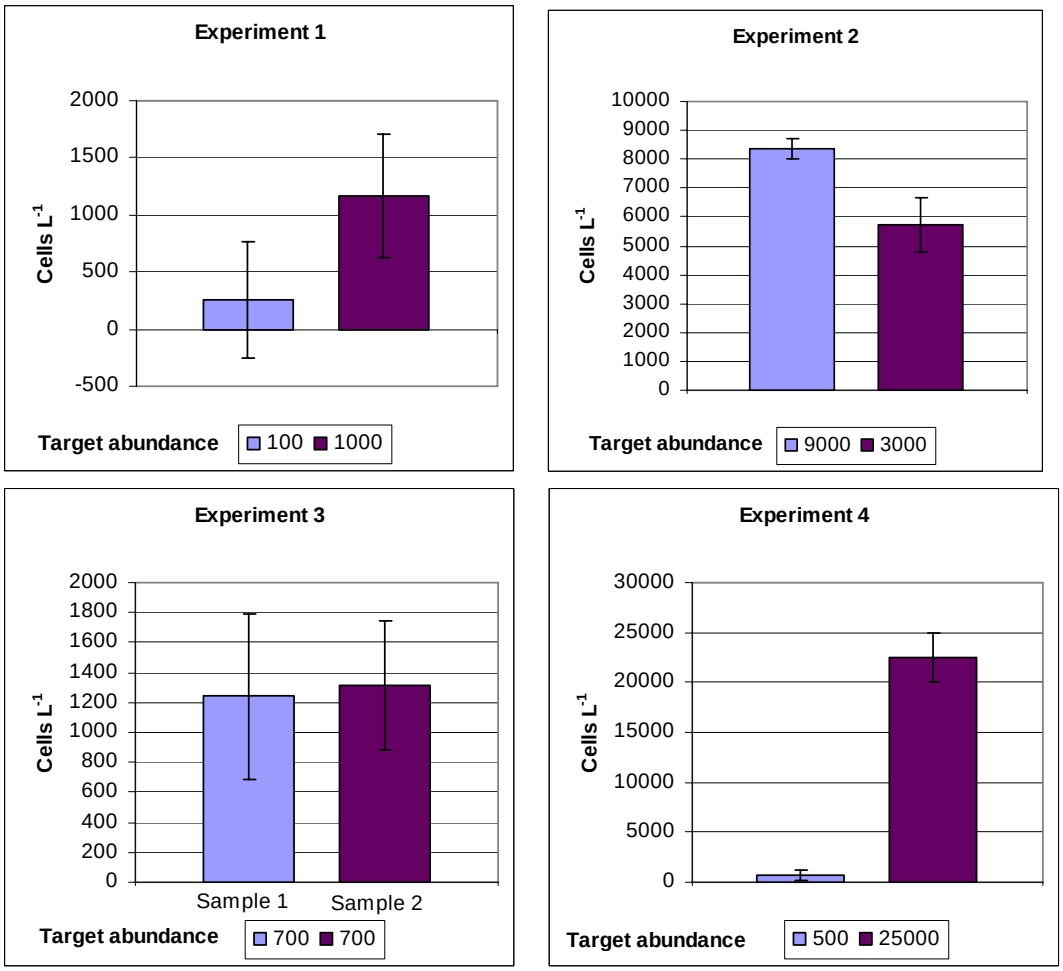

Figure 6. Results from analysis of samples using the FlowCam technique, performed after the workshop. Please refer to the text and to Figures 2–5 for to compare results with the other methods tested. The bars represent the means and whiskers denote the standard deviation. Note that no "true" abundance exists.

7 Discussion

In general the workshop was a successful test of methods for quantitative analysis of the harmful alga *Alexandrium fundyense*. Many different methods were tested and sample replication was satisfactory for statistical analysis of the results. An important question to ask, however, is do the results apply to the real world? One general aspect that was discussed extensively during the workshop was the quality of the cultures. Was the morphology of the cells similar enough to what would be expected in the field? Although some of the morphologically deviating cells made enumeration difficult for the traditional methods, this did not deter the analysts who through experience were able to overcome this difficulty. For the most part the largest proportions of deviating cells were found in samples distributed in Experiment 1. An overhead light was the only light available to the cultures in preparation for E4. Concentrated samples were siphoned from the surface of these cultures and this ensured that the samples used in this experiment contained primarily swimming cells.

The results from Experiment 1 are from unialgal samples, i.e., a pure culture of *A. fundyense*. It could be argued that this is not applicable to studies of harmful algal bloom dynamics in the sea. This experiment, however, was necessary to investigate the range of abundances for which the different methods are applicable. In a field experiment it would have been possible to change the sample volume but during the workshop this was fixed to 100 mL. This implies that in the "real world" ranges can be larger than the results indicated from E1.

Experiment 2 investigated if it is possible to discriminate two closely related species with the tested methods. In "the real world" additional information from e.g., observations using live material from net samples would have aided in the identification using microscope techniques. In addition to this, the two species tested might not co-occur in "the real world". Both traditional and molecular methods produced variable results. Methods such as scanning electron microscopy (SEM) are needed to verify the identification of closely related species.

In Experiment 3, a natural phytoplankton community from the mouth of the Gullmar fjord complicated the experiment substantially. The community had a low biomass, a higher biomass community might have given another result. Unfortunately the attempt to increase the biomass through the addition of concentrated material using a net was only partly successful. If a toxic dinoflagellate is present in a matrix of phytoplankton assemblage containing many other species the microscope techniques could run into problems. Cell densities of approximately 700 cells per Litre of *A. fundyense* and 300 of *A. ostenfeldii* are quite relevant to real world situations where the total abundance of phytoplankton can be several million cells per Litre. If the autotrophic picoplankton is included cell densities may reach 10^9 cells per Litre.

Experiment 4, was in many respects, the one that most closely resembled the "real world". A range of abundances from 500 to 100 000 cells per Litre was added to a natural phytoplankton community. The low end of this scale is in a way the most interesting because paralytic shellfish toxin producing phytoplankton can cause toxicity in shellfish to be above the regulatory limits at cell abundances as low as 100–300 cells per Litre.

Below follows a brief discussion regarding the results for the tested methods and their advantages and disadvantages. Statistical tests, such as ANOVA´s, for comparing results have not been performed on the dataset. The discussion is based on visual inspection of the graphs.

7.1 Utermöhl sedimentation, Lugol (1)

This method faired well in all the experiments except in E2 where it seems that *A. ostenfeldii* cells were misidentified as *A. fundyense*. One of the major advantages with the method is that it is possible to analyse the whole phytoplankton community. Biovolumes can also be estimated using this method. One disadvantage is the relatively long sedimentation time (e.g., 12–24 hours depending on volume) this method requires before analysis can be carried out on a sample. It is also difficult to increase the sample volume to be analysed in a simple way, although multiple chambers can be used. Another option would be to pre-concentrate the sample using large measuring cylinders prior to the settlement of the sample. The cost of investment in a high quality inverted microscope with DIC is relatively high but if the microscope is well maintained then the long term cost for the equipment used by this method is moderate. The microscopist needs to be highly trained to be able to produce high quality results. It is possible to use calcofluor staining with Lugol's as a preservative but this was not part of the intercomparison.

7.2 Utermöhl sedimentation, Formalin, Calcofluor (2)

The discussion for Method 1 also applies to this version of the method. The main difference is that the cellulose theca in thecate dinoflagellates is stained using calcofluor. In the fluorescence inverted microscope it is possible to see features that are species specific for

many toxic species. The result for E2 does not show this advantage clearly but other material might have given another results. Formalin is a greater health risk in the laboratory than Lugol's solution which essentially consists of iodine and acetic acid. The use of formalin as a preservative thus requires the use of a fume hood and the proper Health and Safety procedures for sample handling and disposal.

7.3 Settlement bottle (3)

The results indicate that this method can underestimate cell numbers when compared with the Utermöhl method. The reason for this is not clear but phytoplankton cells may adhere to the inside of the plastic cell culture bottle and thus not settle to the bottom. Time constraints mean that the proportion of the base area counted using the settlement bottles can be smaller than that when chambers are used. Given that the error of a result is proportional to the number of cells counted, this should not, however, result in any significant differences between the two methods. The advantage of the settlement bottle technique is that low cost cell culture bottles are used instead of Utermöhl-type sedimentation chambers. Thus many samples can be handled at a time and multiple bottles can be arranged for sedimentation. As samples can be directly transferred from the original sampling device directly into the cell culture bottle, errors resulting from sub sampling do not arise, and can be re-examined as many times as required without any transfer of sample. If Lugol's Iodine is used as a preservative iodine can leech into the plastic which can reduce the quality of the image. If formalin is used however the technique is safer for the analyst as sample transfers do not occur and problems relating to fumes do not arise. As for the settlement chamber technique, the microscopist needs to be highly trained to be able to produce good quality results.

7.4 Filtering and calcofluor staining (4)

This method showed really good results in the intercomparsion. Variation between replicates was very low compared to most other methods. In E2 this method excelled in discriminating between the two *Alexandrium* species. Compared to the Utermöhl methods, cell numbers were, in general, slightly lower. The main disadvantage with the method is that only part of the phytoplankton community is analysed. Harmful algal bloom species such as the diatoms, *Pseudo-nitzschia* spp., naked dinoflagellates and other flagellates such as *Chrysochromulina* spp. are not included. One advantage of the method is that calcofluor staining facilitates the observation of species specific details in thecate dinoflagellates. Other advantages are that the samples can be processed quickly using filtering for concentration and it is relatively simple to change the volume to be analysed by filtering another subsample. Thus, a larger volume can be analysed when abundance of the target HAB species is low. The cost for the microscope is slightly lower compared to that required by the sedimentation techniques because a standard upright microscope with fluorescence has a slightly lower in cost than an equivalent inverted microscope. The cost for filters and calcofluor is also low. The microscopist needs to be highly trained to be able to produce high quality results.

7.5 Filtering on semitransperent filters (5)

Results from E1–E4 showed that this method produced results consistent with the Utermöhl methods. In general, cell numbers were slightly lower. In E2 the method had some problems discriminating between the two *Alexandrium* species. A disadvantage of the method is that phytoplankton cells can sometimes be difficult to identify on the semitransparent filters. Contrast enhancement techniques do not work well when observing cells on filters. Combining this technique with observations of live cells give good results according to experiences from Norway. The main advantages of the method are that a large part of the phytoplankton community can be analysed and samples can be processed quickly using filtering for concentration. Another advantage is that it is relatively simple to change the volume to be analysed by filtering another subsample. Thus, a higher volume can be analysed

when abundance is low. The cost for the microscope is relatively low because a standard upright microscope can be used. The cost for filters is minor. The microscopist needs to be highly trained to be able to produce high quality results.

7.6 Filtering and freeze filter transfer (6)

Results from E1–E4 showed that this method often underestimated cell numbers. Experiment 2 indicated that the discrimination between the two *Alexandrium* species using this method is good. The method concentrates the sample by filtering and the filter is removed prior to observation and counting of cells in an upright microscope. It is possible to use contrast enhancement techniques. The underestimation of cell numbers by this method could have been the result of cell loss when the filter was removed. An advantage of the method is that a large part of the phytoplankton community can be analysed. Other advantages are that samples are processed quickly and the different filter volumes can be used depending on how concentrated the sample is. A low cost upright microscope with phase contrast was used during the intercomparison. The microscopist needs to be highly trained to be able to produce high quality results.

7.7 Sedgewick rafter chamber (7)

The results from E1–E4 showed a large variation for this method. When cell numbers are in the order of 10 000 cells per Litre or higher results are more consistent with the Utermöhl methods. This method is more commonly used for work with cultures and during blooms with high cell numbers. It may be possible to pre-concentrate cells using a filtering technique when the target organism is present in low concentration. An advantage of this method is that the set-up cost is relatively low because a standard upright microscope can be used. The microscopist needs to be highly trained to be able to produce high quality results.

7.8 Palmer Maloney (8)

The results from E1–E4 showed a large variation for this method. Cell numbers seemed to be frequently out of the expected range. When cell numbers were in the order of 10 000 cells per Litre or higher, results were more consistent with the Utermöhl methods. This method is mostly used for work with cultures and during blooms situations when cell numbers are exceptionally high. An advantage is that cost is relatively low because a standard upright microscope can be used. The microscopist needs to be highly trained to be able to produce high quality results.

7.9 Haemocytometer (9)

The results from E1–E4 showed large variations for this method. Cell numbers were consistently outside the expected range. This method may be useful for work with cultures and during high biomass blooms. An advantage is that cost is relatively low because a standard upright microscope can be used. The microscopist needs to be highly trained to be able to produce high quality results.

7.10 Quantitative PCR (10)

Cell numbers seem to be substantially overestimated according to results from E1–E4. The participants did not think that the method was properly calibrated for the number of copies of target LSU rDNA in the cells used during the workshop and so it is difficult to draw conclusions from the results. This molecular method can be species-specific and even strain-specific. In order to determine the specificity of the probe, however, the method need to be tested with the local population of phytoplankton in the area to be investigated. A disadvantage of the method is that only a small part of the phytoplankton community is

analysed. Equipment (Quantitative PCR-instrument) is expensive today but prices are expected to decrease. Sample throughput is partly limited by concentration of sample using filtering or centrifugation. Both were tested during the workshop and the filtering technique was considered more suitable. A disadvantage with the method could be that cells are not examined under a microscope to confirm the presence of the target organism but, once the primer species-specificity is validated in the geographical area to be investigated, this could also be an advantage in terms of gain of working time. In any case, the method could be combined with a microscopic technique in order to carry out quality checks on a subset of samples if the method is incorporated into monitoring programmes for HAB-species. This method is still at the developmental stage and persons using the method need training in advanced molecular biological laboratory work.

7.11 Whole-cell assay, fluorescence microscopy detection (11, 12, 13)

This method was performed by three scientists who used different protocols and sample volume. One of the methods (13) also used a different target-probe design then the other two. The results should therefore be treated independently. Overall, these methods (11–13) showed good results. Cell numbers were, however, frequently underestimated when compared to the Utermöhl technique. Results indicate that method no. 11 produced results most consistent with Utermöhl. In E2, all methods were able to discriminate between the two *Alexandrium* species because of the probe specificity, although Method 11 reported some cross-reactivity that could still be eliminated from the counts.

It is possible that the condition of the cultures might have influenced the results but it is not known how. Hybridisation temperature is also critical for the successful binding of the probe to its target; if the hybridisation temperature is too low then non-specific binding of the probe may occur, whereas a hybridisation temperature that is too high can result in poor labelling of the target species. During the workshop, a test on the hybridisation oven used by Method 11 revealed that the temperature selected was not accurate and had a large variance (± 5 ºC). This may explain some of the variability reported for Method 11, though the results, in general, were quite reasonable. Other unpublished studies have shown that the washing step of this method can at times result in up to 30% cell loss (Linda Medlin pers. commun.). Of the three methods, only method 12 washed cells on the filter manifold, which may have prevented cell loss. However, this method filtered a smaller volume which may have caused some underestimation in that method. The differences observed in the results of the three whole-cell hybridisation methods highlight the importance of optimising and carefully adhering to procedures. Different groups have developed different reagents and procedures for fixation and processing, and even though the same probe may be used, results can differ significantly among methods. Users are advised to read carefully the publications associated with each method under consideration to evaluate the extent of validation efforts, and to follow the protocols explicitly. Furthermore, it is clear that these molecular methods can be species- and even strain-specific. The methods and probes therefore, need to be tested with the local populations of phytoplankton in the area to be investigated.

Overall, whole-cell hybridisation methods show good promise for use in HAB cell detection and enumeration. They have a low detection limit, can readily distinguish among morphologically similar species, and are not confounded by matrix (background) effects. A disadvantage of these methods is that only a small part of the phytoplankton community is analysed – i.e., one species per probe. An advantage is that because microscopy is used for the detection of the fluorescently labelled cells, identification of the target species can be confirmed, even at low magnification, facilitating rapid counting without need for taxonomic expertise. Because the target cells remain intact and are not damaged using this method, a trained taxonomist can, if required, examine the cells morphological features as is done with

the traditional microscopy methods to confirm identification of the labelled cells. Equipment needed includes standard, fairly low cost, molecular biological equipment and an epi-fluorescent microscope. Persons using the methods need to be trained in simple molecular biological laboratory techniques.

7.12 Whole cell assay, TSA enhanced (Chemscan) (14)

Because of some technical problems, this method was not able to analyse as many samples as most of the other methods during the workshop. The results that were produced show a larger variation compared to the most of the other methods. In some of the experiments, however, results are comparable to the Utermöhl method, e.g., E2. The method is based on the same principles as Methods 11–13 but instead of the analyst counting the cells using an epi-fluorescence microscope the machine does this by scanning the filter for target-cells with the fluorescent signal of the attached probe in quick time (3 minutes). The machine applies preselected criteria to discriminate between "true" and "false" signals derived from the hybridised cells on the filter. An epi-fluorescent microscope is then used by the operator to validate each positive result and the machine. An advantage of this method is that the estimated overall time for counting a prepared sample is approximately 10 minutes. Disadvantages of this method are that at present long chain forming species such as the diatoms, *Pseudo-nitzschia* spp. cannot be detected and false positives (small fluorescent particles) need to be manually checked by the operator. When the method is performing well, this confirmation can be reduced to random checking of the positively labelled bodies in the sample. Unlike the other FISH methods which can use more then one probe per sample, this method at present can only one detect one target species/genera per sample. The technique holds great promise but may have to be considered still in development. Instrument cost is at present very high. Otherwise the same advantages and disadvantages as for 11–13 apply.

7.13 Sandwich hybridisation assay, colourimetric detection (15)

Interpretation of the results of the sandwich hybridization assay must take into consideration the following points. Sample volumes used in this study were very low. Typically, one would use sample volumes that would range from 100's of millilitres (direct filtration) to litres (sieved) when testing for low numbers of target cells such as *Alexandrium*. An additional consideration is the generation of a standard curve from which cell densities are estimated. To generate this curve, the participant required concentrated samples of the cultures used at the workshop. Generally these curves are derived from rapidly growing cultures. If the physiological state of cells in natural samples varies from that standard, then estimates of cell density may differ from what would be expected using some whole cell-base method.

In experiment 4 the results for target density of 500 cells per Litre was in good agreement with most of the traditional microscopy methods as well as the Utermöhl method. At concentrations of 5000, 25 000, and 100 000 cells per Litre the SHA system underestimated the cell target concentration. A possible explanation for this result is at these cell concentrations the standard curve used to interpret the signal is leaving the linear section of the standard curve used to estimate cell density. The best quantification in the SHA system occurs in the most linear part of the standard curve. In experiment 1 the target concentration of 100 cells per Litre was below the level of detection for the SHA system. At 500 cells per Litre in experiment 1 the SHA system under estimates the cell density even though the signal is occurring in the linear part of the standard curve. However, the traditional microscopy methods also underestimate the concentration and may reflect that the sample contained fewer cells than predicted. At the 1000 cell per Litre concentration the SHA system grossly overestimates the concentration of cells. Because it was not possible to re-run this sample no explanation can be offered for this result. For 10 000 cells per Litre in experiment 1 The SHA agrees with the results of the traditional microscopy methods. In experiment 2 the method showed a good ability to

discriminate between the two *Alexandrium* species but in all cases underestimates the target concentration of *Alexandrium fundyense*. This result was also reflected in the traditional microscopy methods. In experiment 3 the method showed a very large variability between replicates for samples. This variability may be caused by naturally occurring, sample to sample variations in target cell concentrations that are in turn reflected in results of the SHA. Overall, the SHA system worked well within its design limits. These design limits cover a wide range of conditions and concentrations of the target cell concentrations. Samples containing cell densities out of the design limits of the SHA system can be easily brought into the optimal detection range by simple adjustments to sample volume or lysate volume. The SHA system can provide a simple, high throughput screening method for the detection of HAB species for a relatively modest cost in time and money. An additional advantage of this method is that it can be easily incorporated into instruments for deployment in subsurface waters off shore. This capability has already been tested and has provided near real-time results of target species. The cost of the instruments (robotic processor and plate reader) needed to perform the SHA are moderate relative to research grade epifluorescence microscope.

7.14 Hybridisation assay, microarray fluorescense detection (16)

Only a subset of samples were analysed using this method during the workshop because of technical difficulties. Results from experiments 1–4 show that this method, in general, tended to overestimate cell numbers substantially compared to the Utermöhl method. Calibration of the method was not optimal and a recalibration might give other results. The method may be considered still in development. Otherwise the same advantages and disadvantages as for 11-13 apply. An additional advantage of this method is that a large number of species can be detected using this method provided existing probes have been developed for the target organisms, such probes, of course, are continually being developed.

7.15 Sandwich hybridisation assay, electrochemical detection (17)

Only a subset of samples were analysed using this method during the workshop because of the time required to perform the RNA isolation and the sandwich hybridisation assay. Results from experiments 1–4 show that this method both over- and underestimate cell numbers substantially compared to the Utermöhl method. Calibration of the method was not optimal and a recalibration might give other results. The method is established but the preparation of the samples, however, is complex and time consuming and requires further development in order to become more automated. The isolation of RNA from a sample is very important because the concentration and quality needs to be high. The RNA is used to determine the cell concentration in a sample, bad quality and low concentrations may in some way explain the results reported for this method during the workshop. The main advantage of the method is that a small inexpensive microchip is used as a detector. Otherwise the same advantages and disadvantages as for 11–13 apply.

7.16 FlowCam (18)

In general, the results from the FlowCam method are promising. Because samples for the FlowCam were analysed after the workshop the results cannot be fully compared to what was produced during the workshop. The samples for the FlowCam technique were stored a few months before analysis. Also time for analysis was very restricted during the workshop and this was not the case during analysis using the FlowCam technique. The results from E1 were good although variability between replicates was fairly high. It is difficult to draw conclusions from E2 because only two of the different abundances were analysed but the result were reasonable. Results from E3 showed a large variability and an overestimation of *A. fundyense* cell numbers. The means (ca. 1200 cells per Litre) are closer to the sum of *A. fundyense* (700 cells per Litre) and *A. ostenfeldii* (300 cells per Litre) indicating difficulties in discriminating

between the two *Alexandrium* species in material with natural phytoplankton present. The two samples that were analysed from E4 show very good results. All sample results are archived and can be referenced later, if required. FlowCam is a type of advanced particle counter that combines fluorescence, scattering and image analysis for identifying organisms. An advantage with the method is that a large part of the phytoplankton community can be analysed, although not always to the species level. The cost for the instrument is fairly high and sample throughput is intermediate. Combining FlowCam with microscopy is probably necessary in most studies.

7.17 General discussions

It is of importance to note what was not tested during the workshop. Here follows some examples:

1) Full phytoplankton community analysis was not tested
2) Biomass estimates were not tested
3) Identification of other species unknown to the participants in advance of the workshop was not tested

When deciding what method to use in a study of harmful algal bloom dynamics it is essential to take into account if the whole phytoplankton community needs to be taken into consideration and if the biomass of the different species is required. Sample throughput and the cost per sample and other factors have also to be considered. One of the terms of references for the workshop was to "identify, where possible, a reference counting method against which other methods can be calibrated". Because a statistical analysis of the results have not been performed it is not possible to make a recommendation.
The true target cell concentration in the samples examined at this workshop is not known, however the results would indicate that the Utermöhl sedimentation chamber provided consistent results throughout the course of experiments. An important factor when counting cells using the traditional methods is that a high quality inverted or compound microscope should be used where possible and an experienced microscopist and phytoplankton identification expert is essential.

The filtration methods were also very consistent with their results as were most of the whole cell hybridisation methods. Methods such as the Palmer Maloney, Sedgwick Rafter and Haemocytometer, although are not recommended for routine sample counting, still have a role to play especially in instances when exceptional HAB events occur and a quick estimate of cell concentrations is required before time allows for more accurate methods to be conducted.

It was agreed at the workshop that the molecular methods require more set-up time and it was acknowledged that it is difficult to transport the essential equipment required for these methods offsite from where they have been developed. Perhaps these methods may have performed better if the samples could have been sent to the participants home laboratories or if the participants arrived a week prior to the workshop to iron out any teething problems encountered by these methods. It should, however, be noted that some of these molecular methods are still at an early stage of development. These methods show great potential for future use both in research and in monitoring programmes. One must also take into consideration that these methods can be automated similar to the FlowCam method and can therefore be incorporated into offshore observation systems, which, in the future will be able to help forecast the onset of HAB events.

One question that was raised during the workshop was why did the methods using filtration to concentrate the sample underestimate the cell counts when compared to the Utermöhl sedimentation chamber technique? A possible reason for this is that the cells may have been lost during the transfer step from the filtration unit or cells may have adhered to the sides of the filtration chamber. This needs to be investigated further.

It is important to remember that when deciding on what method to use, the question that is to be addressed must be taken into account. This will help to determine what method is the most suitable approach to take.

Some examples are as follows:

1) What needs to be identified, the total identifiable phytoplankton community or just one target species
2) Is enumeration of the target organism and/or other species required
3) Is time a constraint
4) Is the method automated or does it require trained experts, please note that at present all methods discussed above need to be implemented and carried out by well experienced operators

8 Acronyms

DIC	Differential Interference Contrast, a method for enhancing contrast in light microscopy
KMRS	Kristineberg Marine Research Station, Fiskebäckskil, Sweden
SSC	Scientific Steering Committee for workshop
SEM	Scanning Electron Microscope
WGHABD	Joint ICES/IOC Working Group on Harmful Algal Bloom Dynamics.
WKNCT	ICES/IOC Workshop on New and Classic Techniques for the Determination of Numerical Abundance and Biovolume of HAB-Species – Evaluation of the Cost, Time-Efficiency and Intercalibration Methods

9 Acknowledgements

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Annex 1: List of participants

NAME	ADDRESS	TELEPHONE	FAX	E-MAIL
ANDERSEN, Per	Bio/consult A/S Johs. Ewalds Vej 42-44 DK-8230 Åbyhøj DENMARK	+45-8732-5549	+45-8625-8173	pa@bioconsult.dk
BRESNAN, Eileen Co-convener	Phytoplankton Ecology Fisheries Research Services Marine Laboratory PO Box 101 375 Victoria Road Aberdeen AB11 9DB UK	+44-(0)1224-295313 direct	+44-(0)1224-295511	E.Bresnan@marlab.ac.uk
CEMBELLA, Allan	Alfred Wegener Institute Am Handelshafen 12 D-27570 Bremerhaven GERMANY	+49-(0)471-4831-1494	+49-(0)471-4831-1425	acembella@ awi-bremerhaven.de
CUSACK, Caroline Co-convener	Marine Institute, Galway Technology Park, Parkmore, Galway IRELAND	+353-(0) 91-745205	+353-(0) 91-773907	caroline.cusack@ marine.ie
DAHL, Einar	Institute of Marine Research Flødevigen Marine Research Station N-4817 HIS NORWAY	+47 370 59040	+47 370 59001	Einar.Dahl@imr.no
DIERCKS, Sonja	Alfred Wegener Institute for Polar and Marine Research Am Handelshafen 12 D-27570 Bremerhaven GERMANY	+49-(0)471-4831-1384	+49-(0)471-4831-1425	sdiercks@ awi-bremerhaven.de
EDLER, Lars	SMHI Doktorsgatan 9 D SE-262 52 Ängelholm SWEDEN	+ 46 – (0)31-751 8906	+46-(0)431-83 167	Lars.Edler@smhi.se
ELBRÄCHTER, Malte	Deutsches Zentrum für Marine Diversitätsforschung Forschungsinstitut Senckenberg Wattenweerstation Sylt Hafenstr. 43 D-25992 Lis/Sylt GERMANY	+49-4651870408	+49-4651870408	melbraechter@ awi-bremerhaven.de

GALLUZZI, Luca	Centro di Biotecnologie Università di Urbino via Campanella 1 61032 Fano (PU) Italy	+39 0721 862832	+39 0721 862834	l.galluzzi@uniurb.it
GESCHER, Christine	Alfred Wegener Institute for Polar and Marine Research Am Handelshafen 12 D-27570 Bremerhaven GERMANY	+49-(0)471-4831-1655	+49-(0)471-4831-1425	cgescher@awi-bremerhaven.de
GLADSTONE Melissa	Cawthron Institute 98 Halifax Street East PB 2 Nelson NEW ZEALAND	+64-(0)3-5482319	+64-(0)3-5469464	Melissa.Gladstone@cawthron.org.nz
GODHE, Anna Local organiser responsible for cultures	Göteborgs universitet Avd. f. marin botanik Carl Skottbergs Gata 22 SE-413 19 Göteborg SWEDEN	+46-31-773 27 11	+46-31-773 27 27	anna.godhe@marbot.gu.se
KARLSON, Bengt Convenor	Swedish Meteorological and Hydrological Institute Oceanographic Services Nya Varvet 31 SE-426 71 Västra Frölunda SWEDEN	+46 -31-7518958	+ 46-31-7518980	bengt.karlson@smhi.se
KULIS, David	Woods Hole Oceanographic Institution Biology Department MS #32 Woods Hole MA 02543 USA	+1-508-289-2859	+1-508-457-2027	dkulis@whoi.edu
LEGRESLEY, Murielle	Fisheries & Oceans Canada Biological Station 531 Brandy Cove Road St. Andrews, NB E5B 2L9 CANADA	+ 506-529-5961	+ 506-529-5862	legresleym@mar.dfo-mpo.gc.ca
LINDAHL, Odd Local organiser	Kristineberg Marine Research Station SE-450 34 Fiskebäckskil SWEDEN	+46 523 18512	+46 523 18502	o.lindahl@kmf.gu.se
MARIN III, Roman	Monterey Bay Aquarium Research Institute (MBARI) 7700 Sandholdt Rd. Moss Landing, CA 95039-0628 USA	831-775-1779	831-775-1620	maro@mbari.org

MARTIN, Jennifer Not present at workshop, analysed samples afterwards	Fisheries & Oceans Canada Biological Station 531 Brandy Cove Road St. Andrews, NB E5B 2L9 CANADA	+ 506-529-5921	+ 506-529-5862	martinjl@ mar.dfo-mpo.gc.ca
MCDERMOTT, Georgina	Marine Institute, Galway Technology Park, Parkmore, Galway IRELAND	+353-(0)91-745202	+353-(0) 91-773907	Georgina.mcdermott@ marine.ie
MEDLIN, Linda	Alfred Wegener Institute for Polar and Marine Research Am Handelshafen 12 D-27570 Bremerhaven GERMANY	+49-(0)471-4831-1443	+49-(0)471-4831-1425	lkmedlin@awi-bremerhaven.de
NAUSTVOLL, Lars-Johan	Institute of Marine Research Flødevigen Marine Research Station N-4817 HIS NORWAY	+47 370 59040	+47 370 59001	Lars.johann.naustvoll@ imr.no
PEDERSEN, John	Statistician Bio/consult A/S Johs. Ewalds Vej 42-44 DK-8230 Åbyhøj DENMARK	+45-8732-5549	+45-8625-8173	jop@bioconsult.dk
PENNA, Antonella	Centro Biologia Ambientale Istituto di Ecologia e Biologia Ambientale University of Urbino Viale Trieste 296 61100 Pesaro ITALY	+39-(0)721-423526	+39-(0)721-423522	a.penna@uniurb.it
POULTON, Nicole Not present at workshop, analysed samples afterwards	J. J. MacIsaac Aquatic Cytometry Facility Bigelow Laboratory for Ocean Sciences 180 McKown Point Road West Boothbay Harbor, ME 04575 USA	+1 207 633-9600	+1 207 633-9641	npoulton@bigelow.org
TÖBE, Kerstin	Alfred Wegener Institute for Polar and Marine Research Biological Oceanography Am Handelshafen 12 27570 Bremerhaven GERMANY	+49-(0)471-4831-1456	+49-(0)471-4831-1425	ktoebe@ awi-bremerhaven.de

Annex 2: Scientific steering committee

NAME	ADDRESS	TELEPHONE	FAX	E-MAIL
ANDERSEN, Per	Head of Plankton Department Bio/consult A/S Johs. Ewalds Vej 42-44 DK-8230 Åbyhøj DENMARK	+45-8732-5549	+45-8625-8173	pa@bioconsult.dk
ANDERSON, Donald	Woods Hole Oceanographic Institution Biology Department MS #32 Woods Hole MA 02543 USA	+1-508-289-2351	+1-508-457-2027	danderson@whoi.edu
BRESNAN, Eileen Co-convenor	Phytoplankton Ecology Fisheries Research Services Marine Laboratory PO Box 101 375 Victoria Road Aberdeen AB11 9DB UK	+44-(0)1224-295313 direct	+44-(0)1224-295511	E.Bresnan@marlab.ac.uk
CUSACK, Caroline Co-convener	Marine Institute, Galway Technology Park, Parkmore, Galway IRELAND	+353-(0) 91-745205	+353-(0) 91-773907	caroline.cusack@marine.ie
DAHL, Einar	Institute of Marine Research Flødevigen Marine Research Station N-4817 HIS NORWAY	+47 370 59040	+47 370 59001	Einar.Dahl@imr.no
KARLSON, Bengt Convenor	Swedish Meteorological and Hydrological Institute Oceanographic Services Nya Varvet 31 SE-426 71 Västra Frölunda SWEDEN	+46 -31-7518958	+ 46-31-7518980	bengt.karlson@smhi.se
LINDAHL, Odd Local organiser	Kristineberg Marine Research Station SE-450 34 Fiskebäckskil SWEDEN	+46 523 18512	+46 523 18502	o.lindahl@kmf.gu.se

Annex 3: WKNCT Terms of Reference 2005

2C10 A **Workshop on New And Classic Techniques for the Determination of Numerical Abundance and Biovolume of HAB-Species – Evaluation of the Cost, Time-Efficiency and Intercalibration Methods** [WKNCT] (Co-Chairs: Bengt Karlson*, Sweden and Caroline Cusack*, Ireland) will be held in Kristineberg, Sweden, from 22–27 August 2005 to:

a) compare traditional methods for concentrating, preserving, and counting common HAB species using light microscope techniques;

b) compare molecular probe-based methods for cell enumeration with the traditional techniques;

c) make recommendations for further research and development efforts targeted at identified inaccuracies or deficiencies in the methods being evaluated;

d) identify, where possible, a reference counting method against which other methods can be calibrated;

e) assess the usefulness and cost efficiency of the available numerical methods in routine monitoring.

WKNCT will report by 30 August 2005 for the attention of the Oceanography Committee and the Baltic Committee.

Supporting Information

Priority:	ICES should take an active role in developing the implementation plan of the GEOHAB programme. The topic of intercalibration is relevant for GEOHAB and also fits well into the ICES profile.
Scientific Justification and relation to Action Plan:	Action Plan numbers 1.1, 1.2, 1.2.1, 5.10, 5.13.3, 5.16. Almost all HAB monitoring and dynamics studies are performed using classical techniques for determining abundance and biomass. New probe-based techniques show great potential for studying HAB dynamics and will make it possible to understand biological processes leading to HAB events. However, the validation of the new techniques is limited. Classic microscope techniques need to be compared with species and strain specific molecular probe methods as well as methods, for preserving and concentrating phytoplankton. The goal is to produce scientifically based recommendations on choice of methodology and QA/QC for HAB-monitoring programmes.
Resource Requirements:	Conveners and lecturer's work time is required. Travelling and accommodation costs are needed for meeting participants. Laboratories, appropriate equipment and convenient access to coastal waters are required during the Workshop. Technical support would be required for publication.
Participants:	Experts in relevant fields from around the world would be invited to participate.
Secretariat Facilities:	The Secretariat will be involved, as normal, in general professional and secretarial support. The Secretariat might provide web space for the proceedings.
Financial:	Travelling support is needed for participants. Sponsored by IOC, GEOHAB, and EU.
Linkages to Advisory Committees:	Harmful algal blooms are continuing issues in ACME.
Linkages to other Committees or Groups:	Support can be anticipated from the Baltic Committee, WGPE, and SGGIB.
Linkages to Other Organisations:	GEOHAB is sponsored by IOC and SCOR.

Annex 4: Images from Workshop

Figure 7. The workshop was carried out at Kristineberg Marine Research Station, Fiskebäckskil, Sweden

Figure 8. Participants Inset image: Odd Lindahl. Top row from left: David Kulis, John Pedersen, Einar Dahl, Melissa Gladstone, Murielle LeGresley, Georgina McDermott, Lars-Johan Naustvoll, Lars Edler, Middle row from left: Roman Marin, Linda Medlin, Per Andersen, Allan Cembella, Anna Godhe, Kerstin Töbe, Sonia Diercks, Eileen Bresnan, Caroline Cusack, Malte Elbrächter, Front row: Christine Gescher, Antonella Penna, Luca Galuluzzi and Bengt Karlson.

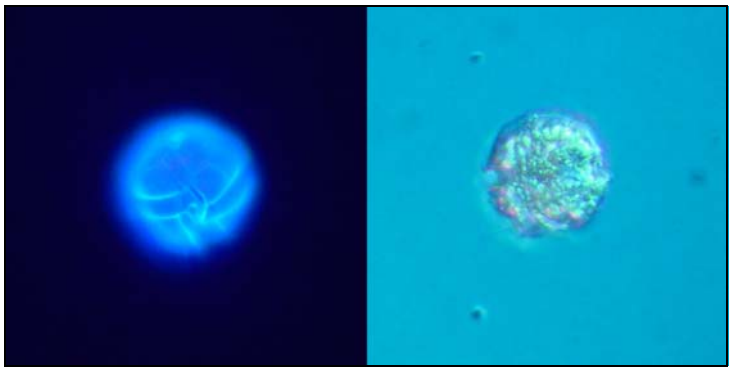

Figure 9. The target organism in the intercomparison was the autotrophic dinoflagellate *Alexandrium fundyense*. Left: Cell shown in the fluorescence microscope after staining of cellulose theca with Calcofluor, UV-excitation. Right: Cell shown in light microscope, contrast enhanced with differential interference contrast (DIC). The plate pattern is essential for identification to species level using morphology. Sample preparation: Malte Elbrächter, photo Bengt Karlson.

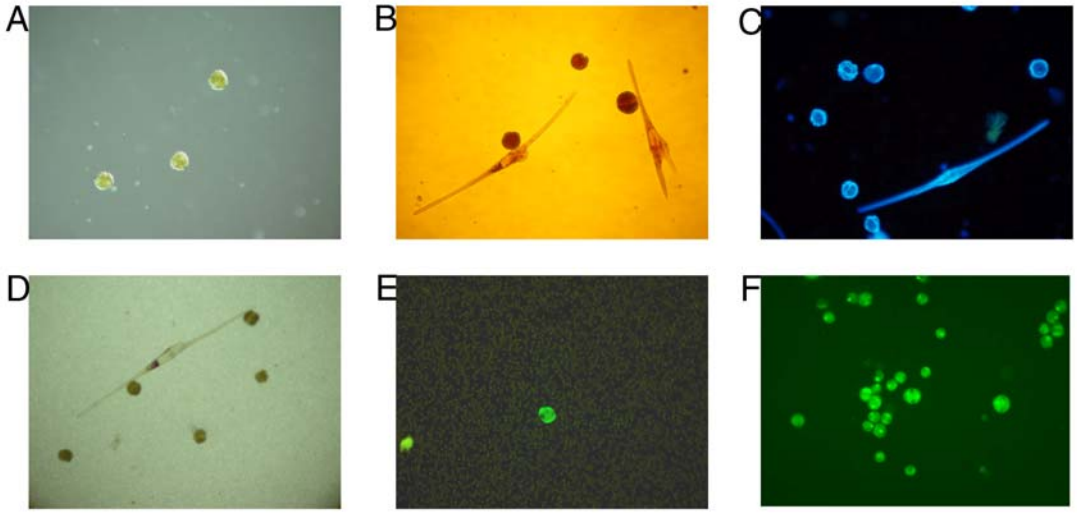

Figure 10. ***Alexandrium fundyense*** **as seen in the microscope using different techniques. A. Filter freeze transfer with contrast enhancement using DIC (appearance in Utermöhl is essentially identical), B Sedimentation flask, C Filtering + calcofluor staining, D Filtering using semitransparent filters, E.and F Whole cell hybridization assay. Sample preparation: A Allan Cembella, B, Georgina McDermott, C Per Andersen, D Einar Dahl, E Melissa Gladstone and F David Kulis. F is originally a greyscale image to which artificial colour has been added to simulate what the eye sees. Photo A-E Bengt Karlson and F David Kulis. Microscope. A-E Zeiss Axiovert 200 and F Zeiss Axioplan 40 Fl.**

Figure 11 **Image gallery from workshop. Photo: Bengt Karlson and Caroline Cusack**