**An improved approach to phytoplankton counting based on real-time statistics**

Kalevi Salonen1\*, Pauliina Salmi2, Harri Högmander3 & Jorma Keskitalo1

1*University of Helsinki, Lammi Biological Station, Pääjärventie 320, 16900 Lammi, Finland*

2*University of Jyväskylä, Department of Biological and Environmental Science, Jyväskylä, Finland*

3*University of Jyväskylä, Department of Mathematics and Statistics, Jyväskylä, Finland*

\* *Corresponding author: kalevi.salonen@jyu.fi*

**Abstract**

In microscopic counting of phytoplankton, confidence limits for mean abundance, if given at all, are generally derived from the total number of counts based on an assumption of a Poisson distribution of specimens in microscope preparations. In agreement with the literature, we show that this generally assumed complete spatial randomness is rarely met in phytoplankton samples concentrated in settling chambers or on filters. Confidence intervals calculated from counts of individual microscopic fields were generally tens of percents larger than those obtained according to the traditional approach based on total counts. Computer simulations demonstrated that, even though the original counts of individuals did not follow a normal distribution, the sampling distribution of mean phytoplankton abundance approached normality, as it should according to the Central Limit Theorem. Therefore, the calculation of confidence limits for mean abundance of phytoplankton can be based on individual counts of discrete fields (or equivalents) so that the necessary statistical information can be derived for objective and reliable interpretation of results. We argue that use of computer counting software with real-time calculation of the confidence limits based on true variation not only improves the quality of results, but could also be utilized to optimize counting and thus reduce the work load in microscopic phytoplankton counting.

Key words: Phytoplankton, microscopy, counting, distribution, confidence limits

**Introduction**

Microscopic counting of phytoplankton is one of the basic determinations used in the assessment of the ecological status of aquatic environments. Significant effort has been put into quality assurance of phytoplankton counting (e.g. Rott et al. 2007), which has led to the development of standardized procedures. In practice, many problems which affect the quality of results still exist (Rott 1981, Thackeray et al. 2013) so that results produced by similar procedures are not necessarily comparable. Further, for proper interpretation the uncertainty in counts should also be assessed and reported.

Many steps in the procedure, ranging from sampling to counting, accumulate variation which generally remains uncontrolled. Because physical, chemical and biological factors can lead to scattered vertical and horizontal distributions of phytoplankton in water bodies, sampling is no doubt one of the most important sources of variation. Subsampling further increases it, but microscopic counting generally introduces the second most important source of variation in the final results.

Utermöhl (1958) developed a settling chamber method for phytoplankton counting which has been the basis for present standards (e.g. EN 15204 2006). Lund et al. (1958) already emphasized the crucial importance of the confidence limits of the counts. At a time when electronic computers were not available, they presented a shortcut procedure to estimate the uncertainty of the expected value, based on the assumption that the distribution of specimens on a settling chamber is completely spatially random.

However, various factors can lead to over-dispersed (variance > mean) distribution of phytoplankton in settling chambers and on filters. For instance, diatom cells may be connected by polysaccharide fibrils (Svetličić et al. 2013) which may be only weakly visible under standard microscope, leading to clumped distributions of cells. Further, conditions during sample preparation and settling can affect the distribution of cells in a settling chamber. Changes in ambient temperature may cause density gradient currents in the settling chamber which may lead to lateral or radial differences in cell density (Sandgren and Robinson 1984). Similar problems can also occur with filters (Seo et al. 2010) due to over-dispersion of pores or hydrophobicity differences over the filter surface.

Count data frequently used in ecological studies regularly violate the equi-dispersion constraint imposed by the Poisson distribution (Lynch et al. 2014). Empirical observations of phytoplankton counts (Nauwerck 1963; Rott 1981; Sandgren and Robinson 1984) and their simulations (Edgar and Laird 1993) have demonstrated a common lack of complete spatial randomness. In spite of that, when confidence limits are shown in the literature, they are generally derived from the Poisson distribution (Edgar and Laird 1993) according to the recommendation of Lund et al. (1958). More often, no information is given at all on uncertainty such as confidence limits (Edgar and Laird 1993). Thus, the quality control of phytoplankton counts remains largely at the level established by the early pioneers.

Although empirical observations provide convincing evidence that the distribution phytoplankton cells rarely follows a Poisson distribution, this is not thoroughly documented, which may be one important reason why it has not been taken seriously. We collected phytoplankton counts from samples covering many different lakes and consequently with different species compositions. We hypothesized that the sampling distribution of the mean number of individuals per microscopic field is approximately normal according to the Central Limit Theorem, so that confidence limits based on a normal distribution can be satisfactorily applied with over-dispersed distributions of phytoplankton counts. We intended to provide an objective approach to assess uncertainty in microscopic phytoplankton counting.

**Materials and methods**

*Sample preparation –* Water samples were taken from several lakes in southern Finland. Samples were preserved with Lugol’s iodine (0.5–1 ml in 100 ml of water). Before settling (Utermöhl 1958), samples were acclimated to the prevailing laboratory temperature for one day and then gently mixed by turning them up and down for ca. 2 minutes. The settling chambers were kept on a table protected from direct sunlight and air flow for at least 8 h (10 ml chambers, height 2 cm) or 24 h (25 and 50 ml chambers, heights 5 and 10 cm, respectively). Fresh unpreserved picophytoplankton samples were filtered on black polycarbonate filters (Millipore, diameter 25 mm, pore size 0.2 µm). We used a 0.45 µm pore size cellulose ester filter on the top of the sintered glass support plate the Millipore filter holder to equalize water flow through the polycarbonate filter. After filtration, the filter was removed by forceps, dried in the air and mounted between a slide and a cover glass with one drop of nonfluorescent immersion oil on the top of the filter. Slides were kept frozen at -20oC until counting.

*Counting of samples –* A proprietary computer program “VersaCount” for Windows XP operating system was used during counting. Settled phytoplankton samples were counted by two persons, denoted henceforth as A (winter samples) and B (summer samples). Both persons used an inverted light microscope with phase contrast optics (person A: Wild M40, Switzerland or Olympus IX50, Japan, and person B: Wild M40). Person A used 400x or 600x magnification and person B either 300x or 600x magnification. Phytoplankton were counted from replicate microscopic fields selected pseudorandomly, whereby fields for counting were located by blindly moving the microscope stage to a new position within the area of the chamber or filter. In practice, any area close to the margin of the settling chamber was avoided. For this study, mostly species with single cells were counted; only the cyanobacterium, *Dolichospermum,* was counted as 100 µm long filaments.

Picophytoplankton were only counted by one person with an epifluorescence microscope (Olympus IX60, Japan) with 1000x magnification. Either a green (Olympus U-MWG; excitation filter 510–550 nm, dichroic mirror 570 nm, barrier filter 590 nm) or a blue (Olympus U-MWB; excitation filter 450-480 nm, dichroic mirror 500 nm, barrier filter 515 nm) filter set was used depending on whether cyanobacteria or eukaryotic algae were counted (MacIsaac and Stockner, 1993; Callieri, 2008), respectively. As for larger phytoplankton, picophytoplankton were counted from pseudorandomly selected replicate microscopic fields.

Samples were selected for this study in the order they appeared it the counting records. In the records of person A the order was temporal and in the records of person B it was alphabetical according to lake names. A total of 33 phytoplankton taxa in 200 counts, of which 40 included microscopic fields without any specimens, was used in our analysis. The total number of picophytoplankton samples on filters was 49 of which 20 included zero fields. Picophytoplankton were divided into phycocyanin-rich picocyanobacteria, phycoerythrin-rich picocyanobacteria and eukaryotic picophytoplankton. In samples with zero fields the mean number of specimens was always more than three.

*Statistical analyses and simulations –* The limits of 95% confidence intervals (±cfl% of the mean) of the results were calculated according to the standard procedure:

1. $cfl\% =\frac{100\*t0.025\*\sqrt{s^{2}/n}}{mean}$

where t0.025 is the 97.5% percentile of the t-distribution with n-1 degrees of freedom, s2 is sample variance and n is the number of replicates. These confidence limits were calculated by the “Versacount” software in real time.

Because in a Poisson distribution variance and mean are equal, Lund et al. (1958) substituted the sample variance of the above formula by the sample mean and derived the simple formula:

1. $cfl\%=\frac{200}{\sqrt{N}}$

where N = total number of counted specimens and t-value was set equal to 2 which is a practical approximation when n > 30.

Computer simulations were run to simulate the behaviour of the sampling distribution of the expected value. Samples counted by persons A and B were selected for simulations. From datasets each with 30-50 counted fields of settled phytoplankton, 15 fields were randomly selected 100 times. In the cases of filtered picophytoplankton, the original data consisted of 22 counted fields.

SPSS Statistics 20 (IBM, USA) was used for further statistical analysis. Student’s t–test was used to test differences between two independent groups. Prior to all tests Levene’s test was used to test equality of variances and Shapiro–Wilk’s test was used to test normality. If the assumptions of the t-test were not fulfilled, Mann–Whitney’s U test or Kruskal-Wallis test was chosen.

**Results**

For counts of phytoplankton in settling chambers, the coefficient of variation (CV) between replicate microscope fields was between 14-260% (median 49%, 33 taxa, n = 200). For samples including no zero fields, the respective range was 14-94% (median 43%, n=160, 25 taxa). For picophytoplankton counted on filters, median CV was 77% (range 32–432%, n = 49).

The median counts of settled phytoplankton were on average 13% (CV 222%, n = 200) lower than mean counts (Fig. 1), but the difference was not significant (Mann-Whitney U test, p = 0.49, df1 = df2 = 199). The difference was a little less (5%, CV 212%, n=160) when samples included no zeros. For filtered picophytoplankton samples the median counts were 36%, (CV 98%, n=49) lower which was not statistically significant either (Mann-Whitney U test, p = 0.099, df1 = df2 = 48, Fig. 1).



Figure. 1. Ratio of median to mean counts of settled phytoplankton (open circles – counted by person A, filled circles – counted by person B) and filtered picophytoplankton (crosses) as a function of mean cell number in the counted microscopic field. Mean cell numbers between 0 and 20 are shown in the left panel and mean cell numbers between 20 and 200 in the right panel. Numbers of settled and filtered samples are 200 and 49, respectively.

The variance to mean ratio was often greater than one (Fig. 2) indicating an over–dispersed distribution. There was a significant difference in the ratios of variances to mean counts of the settled phytoplankton between the persons A and B (Kruskal-Wallis test, p < 0.001, dfA = 52, dfB = 146). The highest variance to mean ratios were observed in the winter samples counted by person A where a diatom, *Stephanodiscus* sp., was strongly dominant. Without those samples there was no significant difference between the results of persons A and B (Mann-Whitney U test p = 0.283, dfA = 26, dfB = 146). Thus the aggregation of cells in *Stephanodiscus* samples was probably species specific. The variance to mean ratio was significantly higher in filtered than in settled samples (Mann-Whitney U test, p < 0.001, dfsettled = 199, dffiltered = 48).

Compared to the shortcut procedure of Lund et al. (1958), the 95% confidence intervals based on Student’s t-distribution for settled phytoplankton and filtered picophytoplankton were on average 1.56 (range 0.52 - 6.52) and 2.43 (range 0.02-9.99) times wider (Fig. 3), respectively and both differences were significant (independent samples t-test, p < 0.001, df1 = df2 = 199 and Mann-Whitney U test p < 0.001, df1 = df2 = 48, respectively). Variance to mean ratio explained 98% of the variation of the ratio between confidence limits calculated from original counts and those calculated according to Lund et al. (1958, Fig. 4).

Computer simulations run with counts of different phytoplankton species showed that, even though the original distribution was highly skewed or even 80% of the fields were empty, the sampling distributions approached normality when sampling was repeated (Fig. 5).



Fig. 2. The mean and variance of counts of settled phytoplankton samples (open circles – counted by person A, filled circles – counted by person B) and filtered picophytoplankton samples (crosses).



Fig. 3. Relationship between 95% confidence limits (cfl) derived from Student’s t-distribution of replicate counts and from the shorcut method of Lund et al. (1958). Above 1:1 line shortcut method yielded lower confidence limits than the method based on Student’s t-distribution. Open circles and filled circles indicate settled samples counted by persons A and B, respectively, and crosses indicate filtered picophytoplankton samples.



Fig. 4. Ratio of standard error based confidence intervals and Lund et al. (1958) based confidence intervals against the ratio of variance and mean counts of settled phytoplankton samples (open circles – counted by person A, filled circles – counted by person B) and filtered picophytoplankton samples (crosses). For combined dataset: y = 1.1029x0.4822 , R2 = 0.98.

**Discussion**

Reported data dealing with within-sample variation of settled phytoplankton counts are surprisingly scarce. However, because samples and settling conditions vary, comparison is not essential. In the absence of more detailed information of variation between counting fields (or equivalents), the few intercalibrations which have compared results between different counters may provide some context for our results. Hobro and Willen (1977) compared counts of species identified to group level, which probably eliminated difficulties in identification, between three laboratories and found coefficients of variation of 10-57%, 14-90% and 4-13% of the mean (n = 10). In another intercalibration, Vuorio et al. (2007) found CV of 9-25% (n= 9-22) for five taxa. There was inadequate background information on the original numbers of counted cells, but because within-sample variation was probably larger than variation between the results of parallel subsamples counted by different persons, we may conclude that our observed within-sample CVs were within the same range.

Despite rather good agreement between the arithmetic mean and the median of counts in our data, some spuriously high counts accompanied by skewed distributions or cases with a high frequency of empty fields often led to a smaller median than mean (Fig. 1). The median is an efficient statistic to eliminate the effect of even a significant number of outliers in data, without causing much bias to its value. Therefore it is often favoured as a robust average value. However, if we exclude accidental mistakes, such as typing errors in data input, “outliers” in phytoplankton counts are generally not real errors and hence their omission is subjective and biases the results. According to the results of Rott (1981) the elimination of outliers does not markedly affect the results. Therefore, outliers should not be rejected without good *a priori* reasons. On the other hand, because the calculation of the median does not increase work, we suggest that in phytoplankton counting both arithmetic mean and median values should be provided to assist interpretation of results.

An over-dispersed distribution of counted specimens (Fig. 2) in our results explains the high discrepancy (Figs. 3-4) between confidence limits based on Student’s t-distribution and the shortcut method of Lund et al. (1958). At the time, when personal computers were not yet available, the simplified calculation of confidence limits from total number of counted cells was helpful to estimate how much work should be allocated for counting. However, according to our results its use often leads to so dramatic underestimation of confidence limits that interpretation of results may be seriously affected. Hence, the estimation of confidence limits from total number of counted cells should be categorically discontinued.



Fig 5. Original distributions of four taxa (upper panels) and sampling distribution of the mean, when the size of the sample was 15 fields and it was repeated 100 times (computer simulation from real counting data).

Similarly, the use of average uncertainty estimates, obtained from intercalibrations, for the interpretation of results should be replaced by directly calculated confidence limits.

Our results agree with those of Edgar and Laird (1993) who on the basis of literature and simulations found that 95% confidence intervals based on Student’s t-distribution show a much greater fidelity to the expected error rate of 0.05. Our data used in computer simulations based on real counts with spatial distributions far from complete spatial randomness (Fig. 5) also suggest that confidence intervals for the mean based on normal distribution are realistic.

*Implications for phytoplankton counting* - In the European phytoplankton counting standard (EN 15204 2006) confidence limits based on Student’s t-distribution are recommended in parallel with the shortcut approach of Lund et al. (1958). However, in practice, as suggested by the almost total absence of confidence limits of phytoplankton from the literature, the recommendation remains unrealized. The necessary calculations are evidently regarded as too tedious. Therefore, there is an urgent need for phytoplankton counting software like that used in our study which provides confidence limits during counting without any additional effort.

Phytoplankton counting instructions often advise to verify that specimens are completely randomly distributed and suggest preparation of a new sample if that condition is not fulfilled. However, visual inspection of a distribution is subjective, and is of value only for the most striking deviations from complete spatial randomness. Despite we followed standard sample handling precautions, over-dispersed distribution of specimens on counting chambers or filters, as suggested by variance-to-mean ratio higher than unity, resulted in high variation of counts. Settling conditions probably have a major influence on the distribution of specimens. A stable settling temperature is recommended, but under normal laboratory conditions it can hardly be achieved, particularly in summer when there is often no compensation for diurnally fluctuating temperature.

In addition to estimating the uncertainty of the results, the distribution of phytoplankton in a settling chamber or on a filter has important ramifications for how samples are counted. Sandgren and Robinson (1984) observed that cell abundances in the periphery of the settling chamber were roughly 50% higher than around the centre. Because a possible radial abundance gradient in a settling chamber is probably continuous, this issue cannot be solved simply by stratified counting of central and peripheral parts of settling chamber. Counting of true random fields is possible, but without a computer-run stage it is impractical. A convenient alternative for both systematic and real random counting might be provided by an Archimedean spiral printed through the bottom of the sedimentation chamber. Points marked at equal distances along its length represent the whole chamber or filter area in the correct proportion.

It is commonly believed that transect counting compensates for the problem, but it is not true, because only roughly 25% of a settling chamber area is within the central 50% of the radius. Consequently, the central region often has too much weight and the traditionally used transect counting should be replaced by stripe counting which compensates for laterally or radially uneven distributions of phytoplankton in counting chambers. Interlaced replicate stripe countings can be used to calculate confidence intervals.

With filters, the prerequisites for random distribution of cells on filter are to avoid conical filtration funnel and to filter sufficient water volume to avoid bias due to the curvature of water surface at the margins of the funnel. However, possible deficiency in the quality of filters may cause similar problems as found with the settling chamber technique. Therefore similar counting procedures are recommended for both settled and filtered samples.

Generally, phytoplankton counting practices are not able to cope optimally with the variable quality of samples or different goals of counting. Because an over-dispersed distribution of specimens of settled phytoplankton samples is more the rule than the exception, and probably is technically unavoidable, its effect on the quality of the results should be compensated by implementing real-time statistics in the counting software to facilitate the selection of an appropriate counting strategy. Confidence intervals can provide a guide for counting of different taxa in a sample with different intensities according to their abundance and variation. We call such an approach dynamic counting, and have applied it successfully in phytoplankton counting (Salmi et al. 2014). It is in striking contrast with present practices where the actual counting strategy is selected *a priori* and often is the same irrespective of the quality of samples. Using counting software, such as applied in our study, real-time confidence limits can be used to reach constant confidence limits for the abundances of counted species. Respective target confidence limits can also be preset for total biomass (e.g. Salmi et al. 2014).

We anticipate that computer programs with a dynamic counting approach can increase the efficiency and quality of phytoplankton counting in a balanced way. Such programs can provide a universal and objective counting platform suitable for any samples and targets. Future standards need no longer recommend how many counts are needed but rather what level of confidence of the results should be reached. We believe that dynamic counting implemented in counting programs will help to resolve the decades old Gordian knot of phytoplankton counting (Rott et al. 2007).

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