



RESEARCH ARTICLE

Methodological aspects of phytoplankton analysis in transitional waters.

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Abstract

- 1 - The methodological issues in the analysis of phytoplankton guilds in transitional waters, using inverted microscopy (Utermöhl technique), will showing and discuss the four steps (sampling, conservation, sedimentation and counting).
- 2 - The importance of inter- and intra- laboratory comparison tests to avoid or minimize discrepancies in identification and counting among analysts was also emphasized.

Introduction

The incorrect application of a method, especially in the field of biology, can cause the operator to make a series of errors, which may have pronounced effects on the final result. This type of errors becomes all the more serious when the analysis involves microscopic objects (such as phytoplankton microalgae), since the method entails using expedients in the counting strategy.

With the aim of improving the quality of analytical results in laboratories, and bearing in mind the requirements of the European Water Framework Directive (2000/60/EEC), the need for uniform procedures to assess the ecological quality of transitional waters by analysing of structural characteristics of phytoplankton has now gained importance.

The necessity of having common procedures regards sampling and sample treatments as well as the determination of phytoplankton descriptors. Moreover, since many of the procedures indexes and indicators, proposed for these ecosystems come from the acquired knowledge in the field of the ecology of fresh water and marine ecosystems; it is necessary to identify limits and any problems in the

methodological application that can reduce the effectiveness as descriptors of ecosystem health. This paper aims to discuss methodological issues in the analysis of abundance and composition of phytoplankton guilds in transitional water ecosystems. In particular, this topic is analyzed in four steps: sampling, conservation, sedimentation and counting, with reference to EN 15204 “Water quality. Guidance standard on the enumeration of phytoplankton using inverted microscopy (Utermöhl technique)”. Currently this European Standard is the only method which is complete and provides detailed guidelines.

Material and methods

Sampling

Sampling is performed with Niskin or Ruttner bottles. If the waters are shallow, sampling can be performed directly, by hand. In the case of oligotrophic conditions, it is better to sample greater quantities of water and concentrate them in the laboratory afterwards. Sampling with net is not advise in transitional waters ecosystems. After taking the samples, the water is poured into the sample bottles. Glass bottles are preferable.

Preservation

Samples can be preserved with various fixatives. The most used fixatives are: alkaline Lugol's iodine solution and neutral 20% formaldehyde solution. Samples preserved with Lugol's can be stored in a dark bottle in a cold room (5-15 °C) for up to 1 year. 3 ml of Alkaline Lugol's solution per litre of sample is standard; however, the ideal quantity depends on algal density.

Formaldehyde (HCHO) can also be used as a fixative. 40% formaldehyde solution is diluted to 20% with distilled water, and 20-40 ml is then added to each litre of sample. Samples fixed in this way can be analysed by epifluorescence microscopy or SEM.

Sedimentation

It is divided in two step:

Sample homogenisation: re-suspension and separation of particles can be achieved by

shaking the sample as gently as possible. This can be performed manually with a combination of horizontal rolling and vertical inversion of the sample bottle for a specific number of times (about 100) or for about 1 min.

Sub-sample preparation: after homogenisation, a known volume of the sample should be used to fill the counting chamber: the appropriate volume depends on the phytoplankton density and the quantity of non-living suspended particles (detritus) in the sample.

Place the chamber on a horizontal surface and cover the chamber with a glass lid, taking care not to leave any air inside. The sedimentation should take place in the dark at a constant temperature which is similar to the temperature of the sub-sample. Vibration must be avoided and counting must be performed when all the cells have settled to the bottom. For Lugol-preserved seawater samples see Table 1.

Table 1 – Settling times for Lugol-preserved seawater (Helcom 2001)

Volume of chamber ml	Height of chamber cm	Settling time h
2	1	3
10	2	8
25	5	12
50	10	24
100	20	48

Counting procedure

There are three alternative counting strategies when using sedimentation chambers:

1. CASUAL FIELD, counting a number of randomly selected fields
2. TRANSECT, counting transects
3. BOTTOM, counting the whole chamber

How many fields or algal objects to count?

The number of fields or algal objects to count can be set according to the level of precision or

detection required, since the precision/detection limit depends on the number of algal objects/fields counted. The precision (D) of a count can be expressed as either the standard error or as a proportion of the mean.

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

Where

n is the number of fields counted;

\bar{x} is the mean number of objects per field;

$\sum x$ is the total number of algal objects counted.

Intercalibration

Inter- and intra- laboratory comparison tests should be performed on a regular basis to avoid or minimize discrepancies in identification of phytoplankton taxa and cell counting among analysts.

Discussions

Sampling

In transitional waters the use of plankton nets for sampling is generally not appropriate. It is mainly due to the limited depth and the concentration of organic matter. Furthermore, sampling with nets does not yield values of phytoplankton biomass or abundance per unit volume of water, and is thus only a semi-quantitative method.

It is preferable therefore to perform sampling with Niskin or Ruttner bottles and by hand. Regarding the choice of sample bottles, bottles in polyethylene (PE) and in polyvinyl chloride (PVC) are not fragile but have disadvantages. The material tends to absorb the fixative, affecting the sample. Glass bottles are therefore preferable.

Preservation

Concerning the choice of preservatives, we prefer Alkaline Lugol's iodine solution. We advise to limit formaldehyde solution use to a minimum, because it is highly toxic, causes allergic reactions and is a probable human carcinogen (Safety Directory.com).

For advantages and disadvantages of Iodine and Formaldehyde fixation, see Unesco, 1978, Hällfors *et al.*, 1979, EN 15204, Magaletti *et al.*, 2001. For their shrinking and swelling effects on phytoplankton cells see (Menden-Deuer *et al.*, 2001). For storage, samples should be kept in the dark and at low temperature (between 1°C and 5°C). Before the beginning of

sedimentation, the sample must be acclimatized to the appropriate temperature and homogenised.

Sedimentation

The ideal volume is dependent on the judgment of the analyst, who must take account of the relative size of the algae (visibility) and the number of non-algae particles (detritus). Dense samples require small volumes, in order to allow for the independent settling of particles – if there are too many particles, agglutination may occur, resulting in non-random distribution. In addition, the particles (including algae) may pile up on the bottom of the chamber, forming a thick layer which will subsequently be difficult to read under the microscope. On the other hand, less dense samples, for example taken from oligotrophic waters, require larger volumes, otherwise the number of algae per field may be too low, and there will either be a large error when counting random fields and transects, or counting will be inefficient, since many fields or counting grids will be required in order to eliminate the errors.

Counting procedure

A combination of the three counting strategies should be used for each sample. Strategy 3 is appropriate for detecting rare species or for counting large species whose distribution in the chamber may not be random. Coenobia should be treated as a single unit and not counted as individual cells. If the cells which comprise a colony are not easily distinguishable, the colony should also be considered a single unit.

Intercalibration

As part of the TWReferenceNET Project, we organized an inter-calibration exercise for phytoplankton in Athens with operators from partner institutions. Participants were given a single unknown sample (a natural water sample) which they each had to analyse in turn, referring to a list of species and photographs. All the analysts chose to count the sample using the TRANSECT counting strategy, because among other reasons, it was considered to be the

quickest. The individual results were compared and evaluated (Table 2). There was some similarity among the operators concerning the

total taxa found but there were differences in the total number of cells counted.

Table 2 – Some results from inter-calibration exercise from TWReferenceNET Project

	Operator 1	Operator 2	Operator 3	Operator 4	Mean	SD	C.V.
Total cells counted	222	121	211	167	180.25	46.1	0.25
Total taxon founded	24	24	28	24	25	2	0.08

It also emerged that in order to limit errors, it is often advisable for the operator to limit him/herself to the genus level without proceeding with the classification into species unless he or she is able to recognise them with certainty. Taxa should never be identified beyond the level at which the analyst feels confident.

Conclusions

In conclusion, this paper had the purpose to introduce some problematic aspects regarding the Utermöhl method applicability in transitional water ecosystems, which obviously needs a bigger widening. However, this paper represents one of the first attempts aiming to focus the aspects about which the phytoplankton specialists should talk in order to provide methodologies specific for the transition water ecosystems.

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