The size structure and functional composition of uliplankton and nanoplankton at a frontal station in the Alboran Sea. Working Groups 2 and 3 Report

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SUMMARY: Plankton samples from the water column at an offshore station in the Alboran Sea (Western Mediterranean) were analyzed to describe the size structure of the community in the size range including pico- and nanoplankton. Three complementary techniques (Utermöhl-inverted microscopy, epifluorescence and flow cytometry) were employed in a way that allowed a large size-range overlap. We discuss the particular artifacts and methodological errors linked to these methods and particular size ranges, and illustrate how these artifacts affect the process of modelling the size-abundance spectrum. Variability associated with the frontal character of the sampling station is mainly reflected by the secondary (ecological) scaling of body size: the invasion of large cells determined a clear decrease in the negative value of the nanoplankton size-abundance slope, whereas the picoplankton spectrum remains close to the primary (physiological) scaling values. From an operative point of view, it seems more interesting to extend the size range studied through epifluorescence towards larger cell sizes than to push the Utermöhl technique down to enumerating very small cells.

Key words: Size spectrum, uliplankton, nanoplankton, pico plankton, epifluorescence, Utermöhl technique, flow cytometry.

RESUMEN: Composición funcional y estructura de tamaños del uliplankton y nanoplankton en una estación de un frente del mar de Alborán. — Se analiza la estructura de tamaños de la comunidad planktonica en una estación de carácter frontal en el Mar de Alborán. El rango de tamaños analizado incluye fundamentalmente pico y nanoplankton. Se emplean tres técnicas (Utermöhl-microscopía invertida; epifluorescencia y citometría de flujo) de forma que se consigue un solapamiento en la cobertura del rango de tamaños estudiado y se analizan los posibles artefactos y errores metodológicos así como su efecto sobre el modelado del espectro de tamaños y abundancias. La variabilidad asociada al carácter frontal de la estación se refleja de forma más acusada en la escala secundaria del espectro: la invasión de células grandes provoca un decremento del carácter negativo de la pendiente del espectro correspondiente al nanoplankton, mientras que el picoplankton permanece próximo al valor de -1.0 (escala fisiológica). Desde un punto de vista operativo, parece más rentable extender el método de epifluorescencia hacia la enumeración de células relativamente grandes, que forzar la técnica de Utermöhl para la enumeración de organismos relativamente pequeños.

Palabras clave: Espectro de tamaños, uliplankton, nanoplancton, pico plancton, epifluorescencia, técnica de Utermöhl, citometría de flujo.

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INTRODUCTION

Any size spectrum is composed of a series of overlapping size distributions that correspond to particular groups of organisms. This results in discontinuities that can be attributed to ecological interactions or that may reflect methodological problems (see Echevarría et al., 1990; Gilbert et al., 1990; Gaedke, 1992). A particular problem arises when the objective is to obtain a common size spectrum for bacterioplankton and phytoplankton (see Gasol et al., 1991) that is, for a size range covering from pico- to microplankton. This implies the combination of different microscopy techniques such as sedimentation plus inverted microscopy (Lund et al., 1958) and filtration plus epifluorescence microscopy (Hobbie et al., 1977).

There is a limit to the efficient analysis of very small cells by means of inverted microscopy simply because of optical resolution. In addition, there are other possible sources of error related to the sensitivity of cells to preservative agents, incomplete settling, etc. On the other hand, the procedure that is usually followed for epifluorescence microscopy is not an efficient method to study cells larger and less frequent than small flagellates because of the problem of passing a relatively large sample volume through small and very small nominal pore filters.

In addition to these classical techniques, flow cytometry now permits fast and precise analysis of the small phytoplankton (see Li, 1986; Platt, 1989; Yentsch and Horan, 1989), both from the quantitative and qualitative points of view. Ultraplankton, considered as composed by organisms smaller than 8-10 μm diameter (Murphy and Haugen, 1985) is the main planktonic component in the size range of transition between inverted and epifluorescence microscopy, as usually employed.

The objective of these two groups (Ultraplankton and Nanoplankton Working Groups) was to examine the full range of cell sizes potentially studied through these techniques and to try to overlap them as much as possible in a common size range. The aim was to identify the methodological limits and artifacts of each method, thus defining the ranges where quantitative data are adequately collected and useful for the mathematical description of the size-abundance spectrum.

In addition, the group aimed to examine the taxonomical and functional composition of the size range analyzed, as a necessary complement to understand the peculiarities of the planktonic size — biomass spectrum on particular time and space scales of observation.

MATERIAL AND METHODS

Sampling

The stations, sampling depths and methods are described by V. Rodriguez et al. (this volume, Water Column Structure Group Report).

Ultraplankton analysis

At the laboratory, a subsample of fresh material was analysed with a FacSort® (Becton-Dickinson) flow cytometer to characterize and estimate the abundance of ultraplanktonic populations. Fluorescence at wavelengths longer than 650 nm (488 nm excitation) was ascribed to chlorophyll a, while fluorescence at 585 nm was ascribed to phycoerythrin. These two fluorescent signals, in combination with measurements of forward and side light scatter, permitted the identification and enumeration of cyanobacteria, prochlorophytes and ultraplanktonic eukaryota (Li and Wood, 1988; Olson et al., 1990; Vaultot et al., 1990; Li et al., 1992).

For epifluorescence analyses, 2 and 10 ml of a previously preserved (glutaraldehyde 2 %) sample were stained with DAPI (Porter and Feig, 1980) and filtered through 0.2 Nuclepore® filters. In addition, 30 ml were filtered through 1.0 μm filter. The combination of volumes and filters allowed the enumeration of organisms up to a maximum cell size around 12 μm ESD (Equivalent Spherical Diameter). Filters were examined with a Leitz Dialux microscope equipped with a 2B-Ploemopack epifluorescence system (100w HBO lamp and filter sets type A and I23). Cells that autofluoresced red were noted as chlorophyll-containing and those autofluorescing yellow-orange were enumerated as phycoerythrin-containing phototrophic picoplankton.

In order to obtain the size structure of the organisms enumerated through epifluorescence microscopy, three kinds of measurements were carried out: a) random selected fields were photographed and the image processed with a Quantimet-500 (Leica-Cambridge) Image Analyzer; b) some fresh samples were directly processed through the CCD-video connection between the microscope and the Quantimet 500; c) large and scarce flagellates were measured directly with a graduated ocular under the epifluorescence microscope. Cell volumes were obtained from the ESD value directly derived from image processing. An additional effort was made to measure cyanobacteria through the inverted microscope (1000x, oil immersion), both on the settled sample as well as on
subpopulations previously sorted by means of the flow cytometer. Obviously, sinking is not the appropriate method to quantitatively collect and enumerate these very small cells; the only objective was to test the precision of the image analysis in measuring them and to compare these results with the measurements obtained from epifluorescence-based analysis.

Nanoplankton analysis

For simplicity, we shall refer to the living material analyzed through inverted microscopy as "nanoplankton", although the total size range analyzed includes some fractions of pico- and microplankton. For the analysis of this nanoplanktonic component, 25 ml subsamples were allowed to settle in 5 or 10 cm high chambers (3 h per cm height) and then analyzed following the method of Utermöhl. Cells were counted and measured at 200x, 400x and 1000x (oil immersion) on a Nikon TM2 inverted microscope assisted by a VIDS-IV (Analytical Measuring Systems) video-interactive Image Analyser. Biovolume for each individual organism was estimated as a revolution volume according to a ellipsoidal or cylindrical shape (see Rodríguez et al., 1987; Echevarría et al., 1990, in press; Gilabert et al. 1990; García, 1991). Colonial organisms were considered and measured as single particles or biomass units (Lewis, 1976).

Data presentation

We followed most of the recommendations of Platt and Denman (1977, 1978) as explained in Rodríguez and Mullin (1986) and subsequent work by the research group in the University of Málaga. After measuring the organisms and their biovolumes calculated, the data are classified into size classes to depict the size structure. Size structure has been defined on the basis of a log$_3$ (body volume) scale. Nominal volumes are defined as the equivalent of the amplitude of size class. That is, the size class between V = 1 and 2 μm$^3$ represents a ΔV = 1 μm$^3$; this is the value (log transformed) selected as the nominal volume for that size class. Obviously, it would also be adequate to use the arithmetic or geometric mean of the limits without affecting the global shape and the main parameters of the size spectrum (see Blanco et al., this volume).

RESULTS AND DISCUSSION

Size structure, taxonomic and functional composition of uliplankton

Figure 1 is a typical flow cytometer plot with the main populations identified and monitored during this study, that is, cyanobacteria, prochlorophytes and eukaryotic uliplankton. Figure 2 describes the time-integrated, vertical variability of these populations. Cyanobacteria were the dominant group, their abundances decreasing from the surface (10 m, around 10$^7$/ml) to the deepest sampling level (60 m, around 10$^3$/ml). Prochlorophytes were one order of magnitude less abundant and eukaryotic uliplankton showed intermediate abundances. Both groups (see Fig. 2) also exhibited decreasing densities with depth. (see Li and Wood, 1988; Olson et al., 1990).

Five functional groups of organisms were enumerated with epifluorescence microscopy: heterotro-
phic bacteria, cyanobacteria (phycoerythrin - photosynthetic picoplankton), small red photosynthetic cells, heterotrophic and phototrophic nanoflagellates. The abundances of cyanobacteria obtained through epifluorescence and flow cytometry were similar. The slope of the relationship between abundances obtained through these methods was 1.04 (N = 15, P (Ho vs. H1) < 0.001). All the categories showed a similar pattern, densities higher and similar at 10 and 30 m, and then decreasing at 60 m depth (Fig. 3).

There are several sources of variability in the process of measuring cell volumes with the methods employed during the workshop. Non-preserved samples of cyanobacteria have characteristic highly fluorescent cells, with a large "halo" that makes it difficult to define true cell limits on the photograph processed through image analysis, and result in overestimated cell volumes (Fig. 4). Size distributions corresponding to preserved samples show a slight displacement towards smaller cell sizes, basically because of the reduced fluorescence intensity (Fig. 4). An additional size reduction is clear when the preserved sample is directly processed (that is, without the intermediate photography) some days after preservation. Fluorescence decays and the resulting cell volumes are now closer to those usually reported in the literature (Johnson and Sieburth, 1979; Li, 1986) and to those obtained through inverted microscopy during these experiments (see Fig. 4). For reasons of coherence, all the prokaryotic cells were measured on photographs taken from preserved samples and processed by means of image analysis.

In terms of body volume, the size range studied covers from minibacteria (log V = -2.4, or 0.2 μm ESD, the nominal pore size of Nuclepore® filters) to nanoflagellates (log V = 2.7, or some 10 μm ESD). The relation between total (average) abundance and individual size can be described by the model (Fig. 5, Table 1):

\[
\log N (\text{cells/ml}) = 3.65 - 0.81 \log V (\mu \text{m}^3) \quad (1)
\]

However, there is the possibility that abundances in the smallest size classes are underestimated. Considering only sizes larger than the modal class (log V = -1.5, or 0.4 μm ESD, that is twice the nominal filter pore size), the model becomes (Fig. 5, Table 1):

\[
\log N = 3.91 - 1.01 \log V \quad (2)
\]
This model suggests that, when we restrict the regression analysis only to the supposedly unbiased data, the abundance of ultraplanktonic organisms changes as the inverse of body volume. Methodological or operative biases, hydrodynamics, trophic interactions or life-history features of component populations may result in deviations from this apparently general rule for the size distribution of particles and organisms in aquatic ecosystems (Sheldon et al., 1972; Duarte et al., 1987; Quiñones, 1992; Gaedke, 1992). Consequently, it is of fundamental importance to eliminate analytical artifacts before trying to understand physical, biological or ecological sources of variability which may affect the parameters of the size-abundance regression model.

Size structure and taxonomical composition of nanoplankton

The main taxonomic groups in the monitored size range were diatoms, dinoflagellates, ciliates and small

| TABLE 1. — Regression statistics of the model Log Bv (μm³/ml) = a + b log V (μm³) corresponding to the different analyses carried out. |
|-----------------------------------------------|---------|-----------------|-----------------|---------------|----------------|
|                                             | N      | a(Standard Error) | b(Standard Error) | r²            | F-ratio       |
| **EPIFLUORESCENCE MICROSCOPY (E.M.)**       |        |                  |                  |               |               |
| overall (1)                                  | 18     | 3.65 (0.11)      | -0.81 (0.07)     | 0.89          | 128.21        |
| reduced (2)                                  | 15     | 3.91 (0.06)      | -1.01 (0.04)     | 0.98          | 669.88        |
| **INVERTED MICROSCOPY (I.M.)**              |        |                  |                  |               |               |
| overall (3)                                  | 20     | 2.87 (0.18)      | -0.59 (0.06)     | 0.84          | 91.34         |
| 1st reduction (4)                            | 13     | 3.18 (0.12)      | -0.64 (0.06)     | 0.92          | 130.87        |
| 2nd reduction (5)                            | 10     | 3.63 (0.11)      | -0.81 (0.05)     | 0.98          | 317.78        |
| Overall (E.M. + I.M.)                        | (6)    | 3.92 (0.05)      | -0.94 (0.03)     | 0.99          | 1111.8        |
| Picoplankton                                 | (7)    | 3.98 (0.07)      | -0.94 (0.09)     | 0.95          | 119.10        |
| Nanoplankton                                 | (8)    | 3.54 (0.13)      | -0.79 (0.06)     | 0.96          | 201.97        |

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flagellates, including cryptophyceans, chrysophyceans and related organisms down to 1-2 µm ESD. Figure 6 describes the size distribution and relative contribution of these groups in terms of numerical abundance and biovolume.

Numerically, flagellates are the dominant group. The characteristic size range observed for small flagellates was 0.8 to 8 µm ESD (size classes log V = -0.6 to 2.4), and the main abundances found were at 1-4 µm ESD; however, the main contribution to biovolume was due to flagellates between 3 and 6 µm ESD (size classes log V = 1.2 to 1.8). The next group in numerical abundance are dinophyceans (Fig. 6) with a maximum density in 4-8 µm ESD cells; in terms of biovolume, however, there is a more regular distribution in size classes with a relatively more important contribution by large dinoflagellates. Diatoms cover a wide size range that extends from 2 to some 60 µm ESD (size classes log V = 0.6 to 4.8). In terms of biovolume, their size distribution is very well bell-shaped with a modal class in cells around 30 µm ESD. Small ciliates were very scarce with a biovolume size-structure very similar to that of diatoms.

The size distribution of Synecococcus cells, obtained by inverted microscopy and the video - interactive image analysis is presented in figure 4. Once a cell is identified, the measurement is quite precise and repeatable. The most abundant size classes were at 0.125 to 0.5 µm³, which correspond to ESD from 0.6 to 1 µm. Results were similar to those obtained through the image analysis of fresh samples after some fluorescence decay.

The time evolution of the depth-integrated biovolume in each taxonomic group (Fig. 7) illustrates the changes associated with the frontal character of the sampling station, that resulted in the increased abundance of diatoms on day 28 (see V. RODRÍGUEZ et al., Working Group 1 Report, this volume). The relatively large individual volume of these diatoms explains their important contribution to total biovolume in spite of their low densities. Samples taken before the water mass change mentioned, consisted mainly of small flagellates, small dinophyceans (Gyrodinium spp., Gymnodinium spp. etc.) and small and medium sized diatoms (Nitzschia spp., Chaetoceros spp.), whereas on the fourth day (January, 28) larger diatoms predominated (Rhizosolenia spp., Chaetoceros spp., Skeletonema costatum, Bacteriasstrum spp., Ditylum brightwellii, Thalassiosira spp., Thalassiothrix spp., Pseudonitzschia spp., etc.). Dinophyceans also increased their contribution to total biovolume, due mainly to the presence of larger spe-
cies (Gymnodinium catenatum). This increase seems to be related to the frontal character of the sampling station, as described by V. Rodríguez et al. (Group Report 1, this volume).

The average size-abundance spectrum (log-log plot) ranged initially from large picoplankton (size class \( \log V = -0.6; 0.8 \mu m \) ESD) to relatively small microplankton (log \( V = 5.1; \) around 60 \( \mu m \) ESD) (fig. 8A). Nominal lower limits for nano— (2 \( \mu m \)) and microplankton (20 \( \mu m \)) (Sieburth et al., 1978) are defined in the plot. Within this size range, an inevitable operational error is expected both in the smallest size classes, due to optical limitations of inverted microscopy, and in the largest size classes because of the relatively small sample volume. These expected sources of error suggest that these biases should be eliminated before any numerical analysis is carried out.

A rapid criterion (see Echevarria et al., 1990) is to eliminate those size classes that lack representation in at least one sample (Fig. 8B). This would imply eliminating, before the mathematical description, those size classes smaller than log \( V = 0 \) and larger than log \( V = 3.6 \) (this is the size class where the transition from nano— to microplankton takes place).

Another criterion is to discard those classes that could have a high error in the estimation of abundance. Taking into account operative (time invested) considerations, we decided to discard those classes whose absolute abundances were calculated from actual counts lower than 16 cells, thus accepting a 50% error in the estimate of each abundance (Lund et al., 1958). This is shown in figure 8C for the different

![Diagram](image)

**Fig. 8.** Initial (A) and final (D) size-abundance distributions of plankton as studied through inverted microscopy. (B) and (C) represent two different criteria (see text) to reduce the overall data set in (A) to that in (D); (B) representation of each size class in the 15 samples. (C) Abundances corresponding to a count of 16 cells for each magnification set (solid line) and abundances within a 50% error range (dotted lines are lower and upper limits).
magnifications employed. Only data points over the lines and within the respective size ranges are considered good estimates of abundance. Looking at the extremes of the size range, there are seven size classes whose abundances could be considered biased by this criterion.

The final, reduced size spectrum obtained through this statistical criterion (Fig. 8D) is equal to that obtained from the first, more intuitive, frequency criterion. Regression models before and after reduction of the data set (Fig. 8A and 8D, Table 1) are respectively described by the expressions:

\[
\log N = 2.87 - 0.59 \log V \tag{3}
\]

and

\[
\log N = 3.18 - 0.64 \log V \tag{4}
\]

**Analysis of the overlapping size range**

As defined in the Introduction, our objective was to extend the usual size range covered in routine analysis by epifluorescence and inverted microscopy, in order to test the efficiency of these methods in describing the size structure of plankton.

The value of the slope for the “nanoplankton” (inverted microscopy) size spectrum is now −0.81, significantly different from −1 (p < 0.05) and, consequently, from that corresponding to the “ultraplankton” (epifluorescence) spectrum. This is obviously due to the contribution of cells larger than 10 μm ESD, particularly those large diatoms which invaded the sampling station on the last day of study.

An additional consideration reverts to the mode in which the Utermöhl technique is usually applied, that is, with a 40x objective as the greatest magnification (400x total magnification with a common 10x ocular). Figure 8C shows the size classes usually monitored with 200x, 400x and 1000 (oil) magnifications. Since the small flagellates fraction has a noticeable importance in the pelagic system, we might think that routine use of 100x objectives (as has been done, e.g., by Echevarría et al., (1990), Giliáret et al., (1990) and García (1991)) would considerably improve the image of the community through inverted microscopy.
analysis. In fact, total cell abundance increases dramatically with the inclusion of these small size classes, although their importance in terms of biomass is clearly much lower. Nevertheless, the reduced efficiency of Utermöhl's method in this size range suggests caution when the scientific objective is mathematical modeling of the size-abundance spectrum. As we have seen, it is very difficult by using this method to approach the true abundance values of the smallest size classes, which ultimately have a strong influence on the computed slope of the regression model.

Variability of the primary and secondary scalings of body size

In the context of analyzing fisheries production, Dickie et al., (1987) identified two levels in the scaling of biomass to body size: the "physiological" or overall scaling that reflects the body size dependence of metabolic (physiological) rates of individuals, and a set of secondary "ecological" scales, where ecological relations control the dynamics of populations (Boudreau and Dickie, 1989). These authors suggest that this secondary allometric scaling reflects the fact that within more or less functionally homogeneous groups (Humphreys, 1979), an adjustment of population density with body size becomes necessary in order for individual organisms to satisfy their food requirements.

The integrated size-abundance spectrum for the overall range studied appears in Figure 10A (statistics in Table 1). It is adequately described by the allometric model

\[
\log N \text{ (cells/ml)} = 3.92 - 0.94 \log V (\mu m^3)
\]

The size distribution of abundance at this primary or "physiological" scaling, with a slope close to -1.0, appears to be a rather conservative feature of the planktonic community even in the context of the physical change that determined the increased presence of diatoms (see Rodriguez et al., Group Report 1, this volume). It is possible to consider secondary or "ecological" scalings that correspond to the classical definitions of picoplankton (0.2-2.0 \mu m) and nanoplankton (2.0-20 \mu m, Sieburth, 1979). These are represented in figure 10B (statistics in table 1). The size distribution of picoplankton abundance does not significantly depart from the overall, close to -1.0 slope (table 1). Physical and biological changes related to the frontal character of the sampling station are more clearly reflected at the ecological scal-

Fig. 10. - (A) Overall size-abundance spectrum as the result of combining Epifluorescence and Inverted Microscopy. (B) Identification of the secondary scalings corresponding to pico (0.2-2.0 \mu m) and nanoplankton (2.0-20 \mu m).

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stantially increase the information yield in relation to the size structure of the planktonic community.

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