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## Redefinition of cell size classification of phytoplankton – a potential tool for improving the quality and assurance of data interpretation

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### Abstract

To date, phytoplankton cell size classification is based on linear metrics (nano: 2-20  $\mu\text{m}$ ; micro: 20-200  $\mu\text{m}$ ; macro: >200  $\mu\text{m}$ ) although three-dimensional metrics are used for cell or body size descriptions of all terrestrial and aquatic organisms. This study proposes a redefinition of phytoplankton size classification expressed in terms of cell volume ( $\mu\text{m}^3$ ) metrics by analyzing statistically a data set of 397 species from three major groups of eukaryotic taxa (diatoms, dinoflagellates, coccolithophores). The three size classes based on cell volume metrics are nanoplankton ( $10^3$ - $10^4 \mu\text{m}^3$ ), microplankton ( $10^4$ - $10^6 \mu\text{m}^3$ ) and macroplankton ( $10^6$ - $10^9 \mu\text{m}^3$ ). Size spectra of cell volume frequencies, species richness-cell size and cell abundance-cell size relationships were evaluated with the use of cell volume ( $\mu\text{m}^3$ ) metrics while fitting of the relevant equations significantly accounted for the data. The analysis of size classes versus taxonomic composition based on cell volume metrics revealed taxonomic group (diatoms, dinoflagellates, coccolithophores) differentiations in nano-micro-macro size classes, thus providing an accurate size classification within and among taxa. This classification offers more precise information on the taxon-size relationships, which are valuable for phytoplankton community structure studies and useful for further development of the remote sensing models designed for differentiating phytoplankton taxa by satellite.

**Keywords:** Phytoplankton, Cell size, Nanoplankton, Microplankton, Macroplankton, Redefinition.

### Introduction

Phytoplankton covers a wide spectrum of taxonomic groups encompassing species with different sizes (volumes) but most phytoplankton monitoring programs overlook this trait and quantify assemblages using cell density and relative abundance. Algal *in situ* studies using species-specific volume measures and taxonomic size structure of phytoplankton are less common, which is probably due to additional data analysis effort, e.g. cell dimension measurements and use of standardized shapes for species (Reavie *et al.*, 2010).

To date, the phytoplankton size classification (nano: 2-20  $\mu\text{m}$ ; micro: 20-200  $\mu\text{m}$ ; macro: >200  $\mu\text{m}$ ) introduced by Sieburth *et al.* (1978), which is based on the use of one dimension (length or equivalent spherical diameter) as a size descriptor of phytoplankton cells (Finkel *et al.*, 2010; Stemmann & Boss, 2012; Roselliet *et al.*, 2013a) has met wide acceptance as a standard procedure, although three-dimensional metrics are used for body size descriptions of all terrestrial and aquatic organisms.

Phytoplanktonic cells exhibit a wide range of shapes and, therefore, the linear dimension is inadequate as a metric of cell size. Size comparisons among organisms with different cell shapes, such as a spherical coccolithophore and a complex-shaped dinoflagellate (ellipsoid+2cones+cylinder), or comparisons of the rela-

tive contributions of different species in mixed samples, are unlikely to be accurate if their size estimation is based on linear dimensions. It is important to note that, although the cell volume parameter has been widely used in phytoplankton literature (e.g. Cermeño & Figueiras, 2008) there is no reference to the threshold values in terms of volume ( $\mu\text{m}^3$ ) for the nano-, micro-, and macroplankton cell size categories.

The use of an accurate cell size classification of phytoplankton is promising because there is an established connection between the size and the physiology of phytoplankton, including nutrient uptake (Ochoa *et al.*, 2010), carbon fixation rate (Huete-Ortega *et al.*, 2012), the degree of carbon exudation (López-Sandoval *et al.*, 2013),  $\text{N}_2$ -fixation and mixotrophy (Barton *et al.*, 2013a; Marañón *et al.*, 2013), sensitivity to toxic polycyclic aromatic hydrocarbons (Echeveste *et al.*, 2010), grazing (Naselli-Flores *et al.*, 2007; Metaxatos & Ignatiades, 2011), and sinking rates (Bach *et al.*, 2012). Cell size provides the most promising basis for modelling and tracking changes in phytoplankton community structure in response to climate change (Finkel *et al.*, 2010) for scaling communities from cellular to ecosystem level (Litchman *et al.*, 2007) and for defining universal indicators of ecological status (Petchey & Belgrano, 2010). Many ecological and biogeochemical processes are also related to cell size estimation using various types of *in*

*situ* measurements, including microscopic analysis, flow cytometry, HPLC analysis of marker pigments and DNA sequencing (Brewin *et al.*, 2011).

Satellite bio-optical methods for cell size determination are increasingly used since they have the advantage of measuring size composition over large space and time scales. These methods monitor spatial and temporal dynamics of phytoplankton median size by employing empirical models based on two remotely sensed optical variables, namely, chlorophyll-a concentration and sea surface temperature (Nair *et al.*, 2008; Barnes *et al.*, 2011; Brewin *et al.*, 2011). However, there are limitations associated with the use of an empirically-derived equation to estimate median phytoplankton size from changes across ecosystems (Polovina & Woodworth, 2012), since this procedure cannot provide information regarding the size designation of each one of the multiple species and taxa constituting the phytoplanktonic assemblages, thus resulting in the loss of knowledge on the species and/or taxa size diversity.

This study proposes a redefinition of phytoplankton cell size classification, that is, nano-, micro- and macroplankton, expressed in terms of cell volume ( $\mu\text{m}^3$ ), using a dataset of 397 species from three major groups of eukaryotic taxa (diatoms, dinoflagellates, coccolithophores) collected from a large area covering four eutrophic gulfs of the Aegean Sea, Eastern Mediterranean Sea. The hypothesis that cell volume classes have characteristic size ranges and thresholds strongly expressed in species richness and numerical abundance size spectra (Cerniño & Figueiras, 2008; Huete-Ortega *et al.*, 2010) could be tested using the novel size classification method. This paper is in accordance with the European Water Framework Directive recommending size spectrum analysis of phytoplankton as an indicator to be used for the assessment of the health status of marine ecosystems (Garmendia *et al.*, 2013).

## Materials and Methods

### *Phytoplankton data acquisition and processing*

The phytoplankton species data used in this analysis are derived from published literature (Ignatiades *et al.*, 2007), based on monthly collections (2002–2003) from a large number of stations (20 stations, maximum water depth 10–15 m) in four major eutrophic gulfs (Saronikos, Evoikos, Pagasitikos, Thermaikos Gulfs) located along the Western Aegean Sea coastline.

Phytoplankton assemblages of diatoms, dinoflagellates and coccolithophores were used for cell size classification. The cell volume of 397 species was estimated by analysing 224 samples collected from 1 m depth to minimize the effect of light limitation. Quantitative and qualitative analysis of phytoplankton to species level was performed under an inverted (Zeiss IM) microscope according to Utermöhl (1958) and the magnification used was x400. Species identification was accompanied by

measurements of their linear dimensions using an ocular micrometer, on a routine basis. Cell volume ( $\mu\text{m}^3$ ) was calculated by fitting the cellular dimensions in formulae for solid geometric shapes most closely matching the shapes of the cells (Hillebrand *et al.* 1999; Sun & Liu 2003). Average cell volume was produced from estimates of the cell volume of 20 individuals of each species per sample.

### *Statistical analyses*

The cell size classification ranges for nano-, micro- and macroplankton in terms of cell volume were established by linear regression using the log-log transformed data of the cell metrics “volume ( $\mu\text{m}^3$ )” and “maximum length ( $\mu\text{m}$ )” and the relationship was statistically validated.

Frequency distribution analysis (Álvarez *et al.*, 2011) was used for scaling relationships that describe variations of cell volume against volume-size class groups. The species numbers/cell volume and cell abundance/cell volume size spectra were determined by grouping the individual cell numbers or cell abundances into equal arithmetic size classes (nano, micro, macro). Then, cell volume histograms were constructed and the spectra were expressed on a double-logarithmic scale, without previous data normalization (Vidondo *et al.*, 1997). All statistical tests were carried out using SPSS version 20 software.

## Results

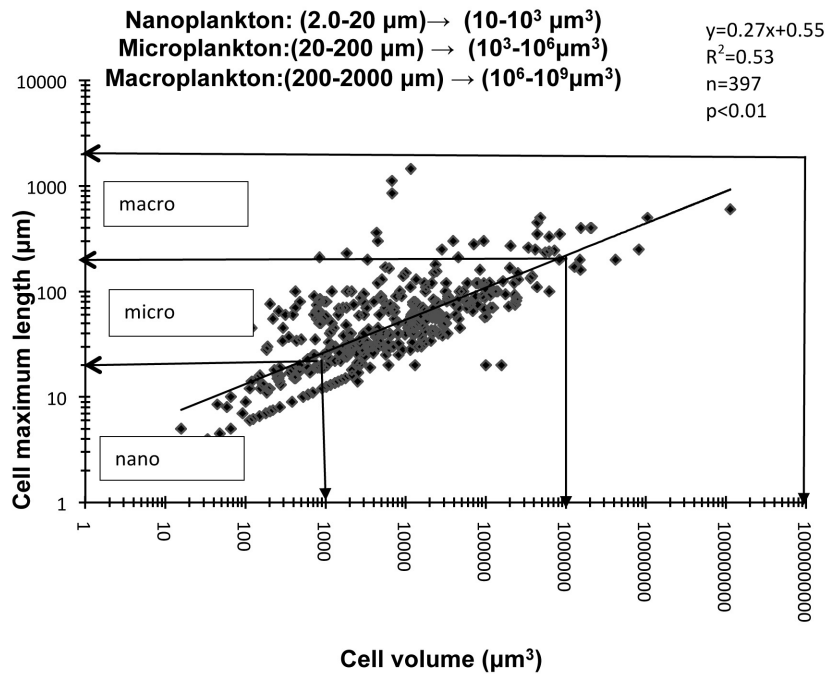
### *Cell size classification*

The comparison of cell metrics, i.e. “volume ( $\mu\text{m}^3$ )” and “maximum length ( $\mu\text{m}$ )” (Fig. 1) showed a statistically significant ( $p < 0.01$ ) positive linear curve, resulting from the log-log transformation of these parameters and allowing extrapolation of the “maximum length” metric values to the “volume” metrics. The three size classes based on the “volume” metrics had the following ranges:  $10\text{--}10^3 \mu\text{m}^3$  for nanoplankton,  $10^3\text{--}10^6 \mu\text{m}^3$  for microplankton and  $10^6\text{--}10^9 \mu\text{m}^3$  for macroplankton. The picoplankton size class has not been included in this study because of lack of the relevant data.

### *Size spectra of cell volume frequencies, species richness-cell size and cell abundance-cell size*

The distribution plots of cell volume frequency of occurrence against successive volume-size class groups are presented in Figure 2. The pattern follows a decreasing power function ( $y = 0.61x^{-1.58}$ ) and the fit of the equation ( $R^2 = 0.83$ ) is satisfactory.

The species richness versus cell-volume size spectrum based on log transformed data (Fig. 3), fitted to a negative linear function ( $y = -0.47x + 2.93$ ;  $R^2 = 0.98$ ), thus indicating that the number of species rapidly decreased with increasing cell size. Similarly, the cell abundance-cell volume size spectrum obtained by log transformed data, fitted to a negative linear function ( $y = -0.94x + 9.01$ ,  $R^2 = 0.93$ ), thus indicating a decrease in cell abundance with increasing cell size (Fig. 4).



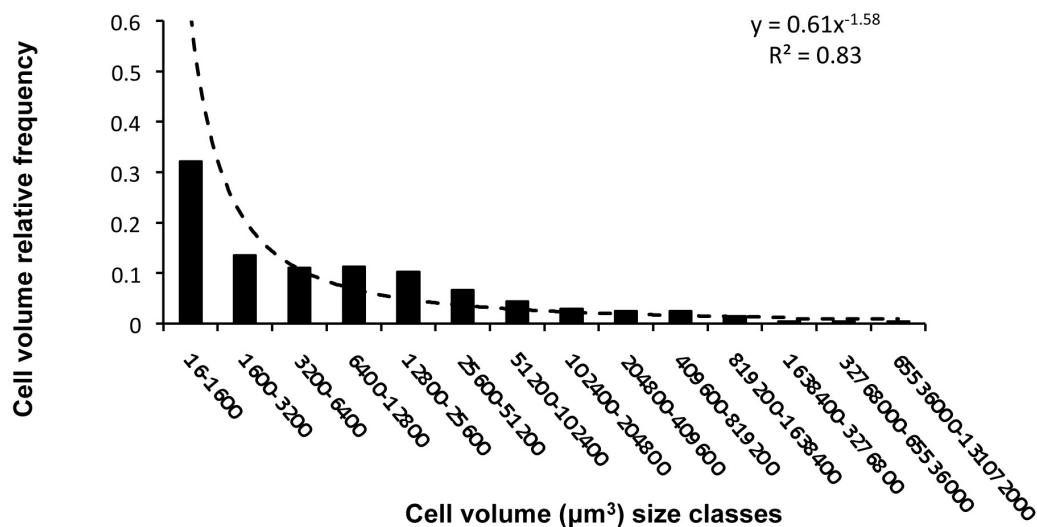
**Fig. 1:** Relationship between log cell volume and log maximum length for 397 phytoplankton taxa identified in the samples. The arrows indicate the relationship between linear metrics (nano: 2-20  $\mu\text{m}$ ; micro: 20-200  $\mu\text{m}$ ; macro: 200-2000  $\mu\text{m}$ ) and three dimensional metrics: (nano:  $10\text{-}10^3 \mu\text{m}^3$ , micro:  $10^3\text{-}10^6 \mu\text{m}^3$ , macro:  $10^6\text{-}10^9 \mu\text{m}^3$ ).

#### Size classes versus taxonomic composition

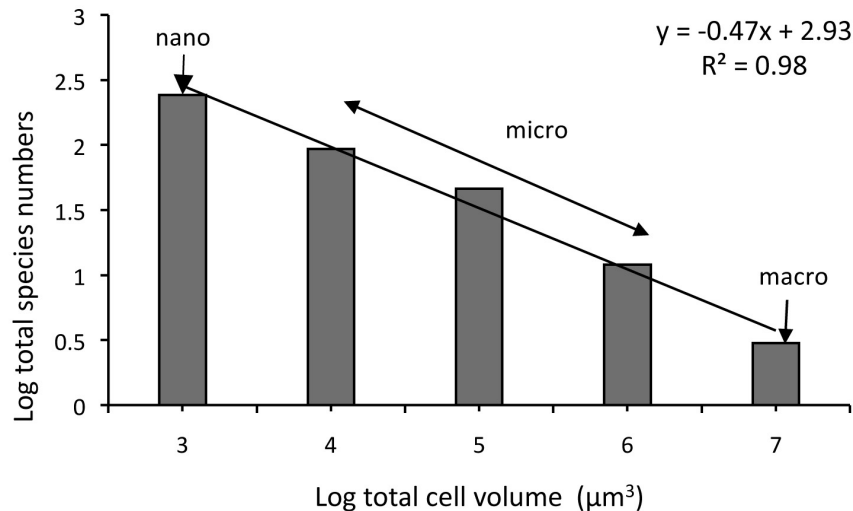
A synoptic picture (Fig. 5) of the entire community based on size-scaled total cell volume revealed the size differentiations within and among taxa. The percentage of the three different size classes, i.e. nano-, micro- and macrophytoplankton (Fig. 5A) varied within taxonomic groups; microplankton species dominated (58 %) in the diatom taxon followed by nano (23%) and macroplankton species (19%). Dinoflagellates were dominated mainly by macro-species contributing up to 67 % (nano :11%;

micro:22 %). Within coccolithophores, nano and micro-species contributed 51% and 49 % respectively to total cell volume of this taxon, whereas macro-coccolithophores are inexistent.

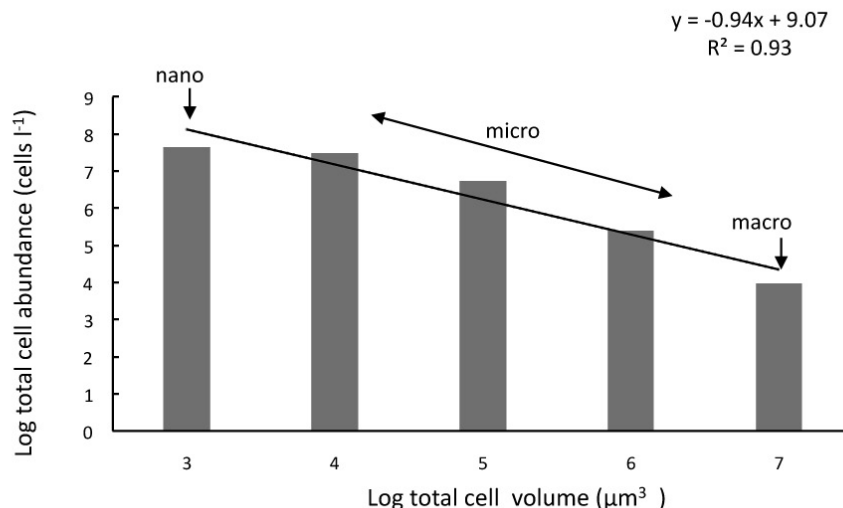
When the percentage of taxa (diatoms, dinoflagellates, coccolithophores) within each size group was considered (Fig. 5B), it was found that nanoplankton was dominated (88 %) by diatoms whereas dinoflagellates (10 %) and coccolithophores (2%) represented a very low percentage. Microplankton included 53% diatoms, 43% dinoflagel-



**Fig. 2:** Relative frequency distribution of cell volume-size classes, following a decreasing power function.



**Fig. 3:** Relationship between species richness and cell volumes based on log-transformed data of 397 phytoplankton species, fitting a negative linear function. The majority of the dataset species identified belong to microplankton.



**Fig. 4:** Distribution of cell abundances in relation to species biovolumes for the phytoplankton dataset consisting of 224 samples. The estimated negative linear function indicates a decrease in cell abundance with increasing cell size.

lates and 4% coccolithophores and macroplankton 96 % dinoflagellates (dominant taxon) and 4 % diatoms.

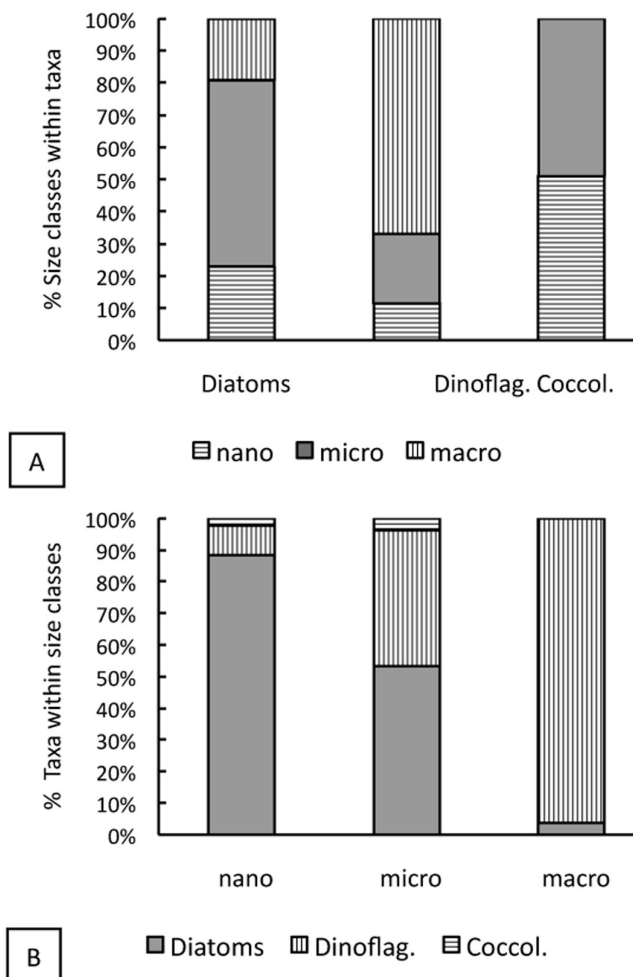
The five large macro-dinoflagellates dominating the macroplankton were *Amphisolenia bidentata* Schröder ( $1.57 \times 10^7 \mu\text{m}^3 \text{cell}^{-1}$ ), *Kofoidinium velleloides* Pavillard ( $6.54 \times 10^7 \mu\text{m}^3 \text{cell}^{-1}$ ), *Petalodinium porcelio* J. Cachon et M. Cachon ( $1.04 \times 10^7 \mu\text{m}^3 \text{cell}^{-1}$ ), *Pyrophacus steinii* (Schiller) Wall and Dale ( $1.14 \times 10^7 \mu\text{m}^3 \text{cell}^{-1}$ ) and *Noctiluca scintillans* (Macartney) Kofoid et Swezy ( $1.13 \times 10^8 \mu\text{m}^3 \text{cell}^{-1}$ ).

## Discussion

In studies of the cell size structure of phytoplankton assemblages (Gallegos *et al.*, 2010; Stemmann & Boss, 2012), diatoms and dinoflagellates are usually classified as microplankton (size: 20-200 μm) and coccolithophores as

nanoplankton (size: 2.0-20 μm). However, this classification based on linear dimensions results in loss of information in relation to species variability (Olenina *et al.*, 2006; Vadrucchi *et al.*, 2013 a) and thus, a number of investigators have used a cell-volume based but arbitrarily selected ranking for the assessment of size variations. Sommer (1985) characterized as “small size” or “medium size” those algae having volumes 30 μm³ and 700-2000 μm³ respectively while Cornet-Barthaux *et al.* (2007) ranked the key diatom volumes of the Southern Ocean arbitrarily as small (volume < 1000 μm³), intermediate (> 1000 < 10000 μm³), and large (> 10000 μm³). High performance liquid chromatography (HPLC) pigment analysis has also been employed to estimate different size classes (Vidussi *et al.*, 2001), but this method is time-consuming and some pigment groups may not strictly reflect the actual size of cells (Ras *et al.*, 2008). Recently, Marañón (2015) displayed two





**Fig. 5:** Relative contribution, in terms of number of taxa, of (A) the three different size classes (nano-, micro- and macrophytoplankton) within each taxonomic group (diatoms, dinoflagellates, coccolithophores) and (B) the different taxonomic groups within each size class.

horizontal (x) size axes in graphical presentations of phytoplankton cell size: the first expressing log cell volume ( $\mu\text{m}^3$ ) units and the second (below) expressing equivalent log spherical diameter ( $\mu\text{m}$ ) linear units, thus indicating their relationship.

This study demonstrates the positive proportional distribution of log cell maximum length as a function of log cell volume, allowing the use of simple size scaling of phytoplankton based on cell volume and specifying the thresholds of size classes. There are several advantages of using cell volume as a metric for cell size. Firstly, cell volume meets the accuracy requirements of size as a trait for the standardization of descriptors for ecological status classifications such as reproduction, resource acquisition, predator avoidance (Litchman & Klausmeier, 2008), volume scaling of physiological parameters (Irwin *et al.*, 2006), sensitivity of size classes to pollution (Vadrucci *et al.*, 2013 b) and size-based numerical modelling of pe-

lagic ecosystems (Baird & Suthers, 2007). It also allows for size classification of phytoplankton data derived from instruments designed for direct determination of cell volumes, e.g. flow cytometry, microscopic image analysis and confocal analysis (Álvarez *et al.*, 2011; Graff *et al.*, 2012; Roselli *et al.*, 2013b).

One of the approaches for the assessment of phytoplankton community structure is the analysis of phytoplankton size frequency distribution (Sabetta *et al.*, 2008). The cell volume-size spectrum of a community designates the relative proportion of sizes regardless of species taxonomy; it is defined as the probability density of volume of all organisms and it can be described by a power law (Rinaldo *et al.*, 2002). In this study, the cell volume frequency distribution per size class conforms with a power law (White *et al.*, 2008) and is in agreement with a widely recognized rule, i.e. that when sampling a certain number of cells, a large proportion is small-sized whereas only a few are large-sized (Álvarez *et al.*, 2011). This is a stochastic process (Gaston & He, 2002) and stochasticity in phytoplankton occurrences is negatively related to organism size perhaps due to efficient dispersal and fast population dynamics among the smallest taxa (Soininen *et al.*, 2013). Species with small dimensions are opportunistic and more abundant compared to larger species because they have higher growth rates and they are able to respond and adapt to environmental changes rapidly (Reynolds, 2006).

The relationship between species richness and body size is one of the most thoroughly studied subjects in animal ecology, but this relationship is largely unknown in photosynthetic organisms (Passy, 2007). Indeed, information on this relationship for marine phytoplankton does not exist, with the exception of the work of Cermeño & Figueiras (2008), who showed that the log-transformed species richness-cell volume data from different marine environments fitted inverse linear regressions with slopes ranging between -0.05 and -0.21. In this study, similar data analysis produced an analogous inverse linear regression (slope -0.47) proving that the number of species decreased from nano to macro species cell sizes. It must be noted that the slope is the most important parameter characterizing a straight line spectrum in a log-log plot (Andersen & Beyer, 2006) and the value of a slope may be determined by the size range depending on the trophic dynamics of a phytoplankton community (Zhou, 2006).

Numerous studies have focused on the cell size-cell abundance spectra (Reul *et al.*, 2006; Reul *et al.*, 2008; Huete-Ortega *et al.*, 2010) pointing to relatively consistent patterns fitted to inverse linear regressions of the log-transformed data, but without delineating the size classes across the size axis. In this study, this relationship has been investigated by setting cell size class thresholds in order to specify the endpoints of the size spectrum, from small nanoplankton to large macroplankton species, as proposed by Edwards *et al.* (2012). Size class-cell abun-

dance information is required in studies quantifying the patterns of phytoplankton size spectra variation in relation to contrasting marine environments (Cermeño *et al.*, 2006), gradients of environmental stress (Lugoli *et al.*, 2012) as well as in methods deducing information on phytoplankton size classes using ocean-color remote sensing (Devred *et al.*, 2011).

The observed slope (-0.94) of the relationship between phytoplankton abundance and cell size is near the levels (-0.91 to -0.78) of flatter slopes characterizing assemblages from inshore, eutrophic systems, whereas steeper, more negative slopes (-1.30 to -1.10) are characteristic of oligotrophic waters (Cermeño & Figureiras, 2008). There are limited estimates of size spectrum slopes for phytoplankton communities of the Mediterranean Sea for comparison with the present results. Slopes in the range of -0.51 to -0.93 have been reported for an upwelling area in the NW-Alboran Sea and in the range of -0.22 to -0.91 in the Strait of Gibraltar (Reul *et al.*, 2005; 2008). Huete-Ortega *et al.* (2010) determined a -0.96 slope of the overall size spectrum for a 10-year time series at a shelf station off the NW Iberian Peninsula (Atlantic Ocean), and size spectra slopes in a global size-structured plankton community model ranged from -0.6 to -1.2 (Barton *et al.*, 2013a).

The relationship between taxa and size variations has been the subject of many *in situ* studies (Booth, 1988; Sosik & Olson, 2007; Finkel *et al.*, 2010; Sin *et al.*, 2012) with the use of size grouping based on linear metrics ( $\mu\text{m}$ ), whereas relevant studies using size variation in terms of cell volume (Edwards *et al.*, 2012; Barton *et al.*, 2013b) did not include thresholds delimiting size categories. In recent years, the global distribution of phytoplankton functional types and size classes has been determined by remote sensing methods (Nair *et al.*, 2008; Uitz *et al.*, 2010; Hirata *et al.*, 2011; Mattia *et al.*, 2013.). Many of these methods rely on the interpretation of phytoplankton size or type from pigment data (Uitz *et al.*, 2006; Almazán-Becerril *et al.*, 2012) but this approach may introduce errors as regards the algal size classes because ceratin pigments may be shared by various phytoplankton groups and some groups can be found in more than one size class (Organelli *et al.*, 2013). Therefore, there are limitations to the use of empirically-derived equations to estimate phytoplankton size or taxonomic composition across ecosystems since they cannot provide information regarding the size designation of each one of the multiple species and taxa constituting phytoplankton assemblages, thus resulting in the loss of knowledge on the species and/or taxa size diversity (Moisan *et al.*, 2012).

In this investigation, an effort was made to present the taxonomic group (diatoms, dinoflagellates and coccolithophores) differentiation into nano, micro and macro size classes, thus providing, for the first time, an accurate size classification within and among taxa. This approach, based on data from natural populations, is more

expensive and time-consuming but offers more precise information on the taxon-size relationships required to validate regional algorithms, which are very important for further development of remote sensing models (Le Quéré *et al.*, 2005; Raitos *et al.*, 2008; Hirata *et al.*, 2012; Baird, 2010; Brotas *et al.*, 2013).

It should also be noted that the linear cell size unit metrics (nano: 2-20  $\mu\text{m}$ ; micro: 20-200  $\mu\text{m}$ ; macro: >200  $\mu\text{m}$ ) have been arbitrarily selected by Sieburth *et al.* (1978) without any statistical analysis. This investigation did not intend to devalue the huge amount of published data obtained through the application of Sieburth's unit metrics but, using these metrics as a basic tool of cell volume scaling, to upgrade Sieburth's metrics via statistical analysis and create the "three-dimensional" units concept required for correct expression of phytoplankton cell volume.

In conclusion, this investigation presents a more accurate method for quantifying taxonomic group (diatoms, dinoflagellates, coccolithophores) differentiations into nano-( $10^{-10}$  to  $10^{-3}$   $\mu\text{m}^3$ ), micro ( $10^{-3}$  to  $10^0$   $\mu\text{m}^3$ ) and macroplankton ( $10^0$  to  $10^9$   $\mu\text{m}^3$ ) size classes. Size spectra of cell volume frequencies, species richness-cell size, cell abundance-cell size relationships and cell size taxonomic composition can be expressed in cell volume ( $\mu\text{m}^3$ ) metrics allowing the definition of the nano, micro, macroplankton thresholds. Satellite data cannot provide information regarding the size designation of each one of the multiple species and taxa constituting phytoplankton assemblages, thus resulting in a loss of knowledge on species and/or taxa size diversity. It is suggested that *in situ* accurate cell size data are required to validate regional algorithms.

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