An improved energy-dispersive X-ray microanalysis method for analyzing simultaneously carbon, nitrogen, oxygen, phosphorus, sulfur, and other cation and anion concentrations in single natural marine microplankton cells

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Abstract

Electron-probe X-ray microanalysis (XRMA) is a technique that can be used to simultaneously quantify C, N, O, Na, Mg, Al, Si, P, S, Cl, K, and Ca present in single cells collected from the sea, with a minimal treatment of the samples. The technique has been used to determine elemental composition in plankton using scanning electron microscopes (SEM) or transmission electron microscopes (TEM). The methodologies used have shown some drawbacks. Here we present an improved methodology focusing on the analysis of the elemental concentration of marine microplankton cells, including light elements, based on the methodology of Norland et al. (1995). The most important modification is the use of a SEM microscope but reproducing TEM conditions by placing the sample grid at a distance from the holder bottom. This modification eliminates the interference of the SEM stub from the analysis of the cells, and improves the limit of detection of elements that are present in low concentrations. Experimental and theoretical data on the beam penetration depth using low incident beam energies (15 kV) are presented. The results show that a 15 kV accelerating voltage, invoked as a limitation in using XRMA for the analysis of samples thicker than 0.1 µm (Twining et al. 2008), is sufficient to analyze whole microplankton cells, and that there is no absorption of X-rays during the analysis. The method has been applied to diatoms and dinoflagellates from the Western Mediterranean Sea. The cell elemental composition results fall within the range of historical data in the literature.

Since Redfield (1934, 1958) described the link between the composition of plankton and chemistry in deep waters, the elemental composition of plankton has become a central variable for understanding ocean dynamics, and a key parameter in marine biogeochemistry, phytoplankton physiology, model formulation, paleoclimatology, evolution, and global climate change (e.g., Margalef 1998; Geider and La Roche 2002; Quigg et al. 2003; Lenton and Klausmeier 2007; Flynn 2010).

The Redfield ratio deduced from the increase in nutrient concentration with depth represents a mean value of plankton elemental composition, and can change accordingly with changes in the planktonic community (Redfield 1934). A review of the current literature indicates that this ratio spans at least one order of magnitude (Geider and La Roche 2002) and varies at two levels: differences between species and larger taxonomic groups, and phenotypic variability between populations that are acclimated to different physical or chemical environments (Quigg et al. 2003; Quigg et al. 2010). It follows that phytoplankton elemental stoichiometry is a good eco-physiological trait for modeling and tracking changes in ocean biogeochemistry and plankton dynamics but there is a need for more, and more accurate, measurements of this parameter in the natural environment.

Classical studies to determine the elemental composition of the plankton community use “bulk” analysis methods, which consist of filtering a given volume of water and analyzing the material retained on a membrane. This kind of sampling strat-
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Segura is useful for estimating the elemental composition of all particulate organic matter, but given the presence of abiotic particulate material and organic debris of other planktonic organisms (Banse 1974), it often fails to give a reliable estimate of cellular elemental stoichiometry. Furthermore, it does not provide data on the elemental composition of the functional components of the phytoplankton community, species or taxa. These components are being increasingly recognized as important players in defining the consumption and fate of all major nutrients, as well as in the dynamics of the marine trophic community and in ocean carbon export. To fill this gap, single species cultures have been analyzed in the laboratory, and a significant amount of data have been obtained. However, not all marine species are easy to culture and laboratory cultures do not always reproduce the ocean environmental conditions accurately. Apart from the mammoth task of obtaining data from all the major species, the analysis is still based on concentrating the cells in a filter, assuming a homogeneous elemental composition of all the cells in that particular culture that may not be true. In addition, dead cells and other organic debris still contribute to the final result. To overcome these problems using more recent advances in microscopy technologies, elemental analysis of individual protist cells collected from natural communities has been proposed, and its use has been encouraged (Twining et al. 2008).

One of those techniques is electron-probe X-ray microanalysis (XRMA), which uses an energy-dispersive spectrometer (EDS) detector that simultaneously identifies and quantifies not only the most important elements in marine ecology, C, N and P, but also O, Mg, Na, Al, Si, S, Cl, K, and Ca that are present on single natural cells. XRMA has been available for more than 20 years for determining elemental composition in marine and freshwater phytoplankton (Sigee et al. 1998; Krivtsov et al. 2000; Heldal et al. 2003) and bacterioplankton (e.g., Heldal et al. 1985; Booth et al. 1987). The results have contributed to our understanding of the biology and ecology of individual species in natural communities (Sicko-Goad et al. 1975; Sigee et al. 1999; Krivtsov et al. 1999). However, because of instrumental limitations, these first studies did not include measurements of critical elements such as C, N, and O.

In more recent years, with advances in XRMA technology, which makes it feasible to analyze the light elements (C, N, and O), STEM (scanning-transmission electron microscopy)-EDS has been used successfully to analyze the complete elemental composition of single marine bacteria and cyanobacteria (Norland et al. 1995; Vrede et al. 2002; Heldal et al. 2003). However, because of the small size of the TEM holder, the analysis has been restricted to cells of less than 5 µm diameter. Here we describe an improvement on the methodology, which allows us to analyze simultaneously C, N, and P, and also O, Mg, Na, Al, Si, S, Cl, K, and Ca, in individual marine plankton cells (between 4 and 200 µm length) using SEM-EDS XRMA and to overcome the problems of interference of the SEM support holder. We also discuss and analyze the beam penetration depth using low incident beam energies (15 kV), which have been invoked as a limitation in using XRMA in whole-cell analysis (Twining et al. 2008). This methodology has been developed and applied to the analysis of diatom and dinoflagellate cells collected from the NW Mediterranean Sea, and the elemental concentrations obtained (Segura-Noguera 2007) will be discussed elsewhere.

Materials and procedures

Beam penetration in microphytoplankton cells

In previous analyses of plankton cells using SEM-EDS XRMA, in which C and O were not measured, cells were collected onto polycarbonate filters (e.g., Sigee et al. 1998; Sigee and Levado 2000). Subsequently, after drying the filters, those were mounted directly on SEM stubs. Our first analysis following this method but skipping the coating of the cells to avoid contamination, showed that the C content of the filter was greater than the C inside the cells, indicating that X-rays where generated in both cell and substrate. This can be observed in Fig. 1, where the C peak of the spectrum of a diatom (dark green) is lower than the C peak of the spectrum of the nuleopore filter that supports the diatom (light green). As a result, the cell concentration of key elements—especially those that are present in low concentrations—falls below the detection limits. Consequently, we tested several supports for the samples (for more details, see Segura-Noguera 2007), and the characteristic peaks of the elements present in the supports (e.g., C, Cu, and Zn, see Fig. 1 below) could always be observed on the spectra of the microplankton cells mounted on them. From this we concluded, to our surprise, that our problem seemed to be over-penetration of the electrons into the target microplankton cells, instead of a limited depth of penetration, even using low accelerating voltages (5-20 kV).

Beam penetration and absorption of X-rays have been one of the major concerns about XRMA applied to microplankton (e.g., Krivtsov et al. 2000; Twining et al. 2008), especially when low incident energies are used. According to Sigee et al. (1998), a 10 kV probe will have a penetration distance of 6 µm in freeze-dried algal cells with a non-silica cell wall, while the penetration distance in silified organisms has been estimated to be 1 to 2 µm (Krivtsov et al. 2000; Reed 1975). Consequently, Twining et al. (2008), in their review of single-cell techniques for elemental analysis of marine protists, indicated that the typical thickness of the samples to be studied by XRMA is 0.1 µm. According to Goldstein et al. (1992), the limit among thin-film and bulk specimens for XRMA analysis is around 2 µm thick. Moreover, in bulk specimens, X-rays generated within the interaction volume are absorbed and fluorescence effects that appear during the analysis should be corrected. Microplankton cells, like the ones of the present study, measure more than 20 µm, so they should be considered as bulk specimens. However, when the cells are dried during the sample preparation, the cytoplasm shrinks and sticks onto the cell walls (Fig. 2B), so what is actually analyzed is an...
“empty container” or a thin layer if the cell collapses. The result of this process is a reduction of the total depth and hence to the electron beam over-penetration of the specimen. The selection of the working acceleration voltage in SEM XRMA must take into account that (i) characteristic peaks of all the elements of interest are generated, (ii) the characteristic peaks have enough counts to be statistically considered (Goldstein et al. 1992), (iii) the cell have been completely penetrated by the electron beam, and (iv) the image resolution is good enough so the area scanned of the unfixed and uncoated cells can be accurately measured. Higher energies guarantee the three first requirements, but lower image resolutions are obtained. Consequently, the quantification is less accurate. To fulfill the first and last requirement at least 10 kV of accelerating voltage are required. Thus, we experimentally estimated the beam penetration into microplankton cells using 10 and 15 kV accelerating voltages with latex beads of known composition of \((\text{CH}_2)_n\) and density \((1.05 \text{ g cm}^{-3})\). Beads of 1, 2, and 5 µm diameter were analyzed using 10 kV and 15 kV of incident beam energy. The results indicated that 10 kV was insufficient accelerating voltage to analyze the 5-µm-diameter beads, equivalent to latex samples larger than 1.5 pg C µm\(^{-2}\), since we obtained the same intensity (X-ray counts) for both 2 and 5-µm-diameter beads. However, linearity among the analysis of latex beads of the three different sizes was achieved when 15 kV accelerating voltage were used \((r^2 = 0.959, n = 39, P = 0.002, \text{ model II regression OLS method, Legendre 2001})\), indicating that the entire sphere of each diameter was ana-

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**Fig. 1.** Upper: X-ray spectra of a *Thalassiosira* sp. mounted on an Au covered nucleopore filter, analyzed with 20 kV of incident energy. The dark green spectrum corresponds to the analysis of the area shown in the upper right figure, and the light green spectrum corresponds to a blank analysis (only the filter is analyzed). Lower: X-ray spectra of a *Thalassiosira* sp. diatom mounted on a Cu/Zn planchet (light blue) and of a *Pleurosigma* sp. diatom mounted on a Be planchet (dark blue), both analyzed using 15 kV accelerating voltage. The areas analyzed are shown in the upper right pictures in the same color as the spectra. The presence of Au and Cu/Zn in the spectra reveals that the volume of interaction (the volume where X-rays are generated during the analysis) includes the cell and the support. Also observe the dramatic decrease in C in the cell spectra when a C-free support is used (Be and Cu/Zn planchets), indicating that the volume of interaction included a large portion of nucleopore filter.
lyzed (Fig. 3). So we chose 15 kV as the working acceleration voltage to analyze the elemental composition of microphytoplankton cells.

Sample preparation

Phytoplankton cells are concentrated onto 20-µm nylon mesh by filtration of the water sample. Once the cells are concentrated on the mesh, they are rinsed several times with 10 mL double distilled water brought to pH 8.3–8.5 with NaOH. This washing solution should be ice-cold to slow down chemical processes that may occur inside the cells during the preparation of the sample (Gisselson et al. 2001). Following, the cells are resuspended in the washing solution and centrifuged, following the protocol of Norland et al. (1995), onto a Cu finder grid for SEM specimen mounts (10 mm diameter, ref. 79050, Ted Pella Inc.) placed flat at the bottom of the centrifuge tube. The SEM grid had been previously coated with a formvar film (ref. 09823, Fluka or ref. 19222, Ted Pella Inc.). The centrifugation is done at 15°C for only 15 min and at 3000 rcf, to avoid structural damage to the cells. After removing the water, the grids are allowed to air-dry and are stored in a vacuum chamber until analysis. Coating the sample with a layer of C or Au-Pd is a usual procedure to improve the image resolution using SEM. As introduced above, in the present study the cells were not coated to prevent contamination. Consequently, the identification of the cells could be done to the genus level, but not to the species level (e.g., Protoperi-
dinium sp.), unless they had a characteristic shape (e.g., *Neoceratium furca*).

**Support holders (cell support during the analysis)**

Subsequently, instead of attaching the grid onto a regular SEM stub, the grid with the cells is placed over a Hitachi high-resolution holder customized to secure the grid at a distance of 17 mm over the holder bottom (Fig. 4). This is done to avoid the participation of the bottom holder in the X-ray spectra (affecting the background and the X-ray characteristic peaks) and the interference of the holder material with the sample elements, as explained above. With the use of this holder, the cells are analyzed under the same conditions than those used in STEM XRMA, where the sample is located in the middle of the microscope column and only the X-rays generated inside the cell, as well as in the thin C formvar layer that holds the cell, will reach the detector.

Following the method of Norland et al. (1995), an X-ray spectrum is recorded from an area, in the shape of an oval or a rectangle, which tightly circumscribed the cell. X-rays are collected for 100 s live time. It is important that the area scanned includes the whole cell to be able to calculate concentration of element per cell. Also, because once the electron beam penetrates the cell the X-rays are generated from a pear-shaped volume beneath the surface, the surroundings of the cells to be analyzed must be free of detrital particles or other cells.

To remove the contribution of the supporting formvar film, the spectra of a particle-free area, identical in size and shape, adjacent to the cell is recorded (blank analysis). Following, the X-ray counts from the blank analysis peaks are subtracted from the correspondent cell + formvar analysis peaks.

The blank analysis consisted mostly of C and O, although sometimes Al and Si peaks were visible as a consequence of X-rays generated in the vacuum chamber and holder (Al), as well as inside the detector (Si) (Goldstein et al. 1992). The holder is made of Al with traces of Fe, Cu, Cr, and Sn. Apart from Al, counts from these trace elements were not detected. The presence of these elements in the blank spectra means that they also appear and interfere with the cell analysis. This interference is eliminated when the blank is subtracted from the cell + formvar analysis. To avoid counts from the Cu grid, only cells placed entirely in the openings of the grid were analyzed.

With the use of this modified holder (Fortuño Alos and Segura-Noguera 2011), only the X-rays generated in the cell and supporting formvar film reach the detector, like in bacteria and coccoliths STEM XRMA (Norland et al. 1995; Fagerbakke et al. 1994).

**Calibration constants for quantification**

To quantify the elements in the samples, calibration constants and the error for each element (Table 1) were obtained

![Fig. 4. High-resolution holder (Hitachi) customized to fasten Cu finder grids, used as a support to analyze microphytoplankton cells. The use of those holder and grids avoids the participation of the holder and previously used supports (i.e., filters) in the analysis.](image)

### Table 1. Calibration constants for quantifying the most abundant elements present in cells, determined from different chemical compounds.

| Element | Chemical compound | Calibration constant (counts µm² pg⁻¹ s⁻¹) | Standard Error (%) |
|---------|-------------------|------------------------------------------|--------------------|
| C       | Latex beads       | 87.9                                     | 8                  |
| N       | EDTA              | 75.9                                     | 11                 |
| O       | EDTA              | 78.8                                     | 7                  |
| Mg      | MgSO₄ + ADP       | 259.9                                    | 8                  |
| Al      | Interpolation     | 243.4                                    |                    |
| Si      | Interpolation     | 226.9                                    |                    |
| P       | ADP               | 210.4                                    | 8                  |
| S       | Methylene Blue    | 198.9                                    | 7                  |
| K       | ADP               | 101.5                                    | 10                 |
| Ca      | CaCO₃             | 72.6                                     | 8                  |
following the procedure described in Norland et al. (1995), using the following compounds as standards: latex beads (Agar Scientific); ADP (adenosine diphosphate, $C_{10}H_{15}N_5O_{10}P_2K$, Sigma, A-5285); titriplex III (EDTA, $C_{10}H_{14}N_2Na_2O_8\cdot2H_2O$, Merck, 108418); MgSO$_4\cdot7H_2O$ (Merck, ref. 105886); methylene blue ($C_{16}H_{18}N_3SCl$, Merck, 457250-1GM); and calcium carbonate (CaCO$_3$, Calprec PA). To prepare the standards, latex beads were deposited onto 3-mm formvar-coated aluminum TEM grids (Agar, G2460AL). Other compounds were dissolved in double distilled water and sprayed as microdrops onto the TEM grids. Microdrops of different sizes were analyzed. Calibration constants for elements not included within those compounds (Al and Si), were calculated by interpolating among the elements with the closest atomic number (Norland et al. 1995). Fig. 5A shows a good agreement between the mass of C calculated from the volume and density of the latex spheres and the mass of C obtained from the XRMA. The analysis of ADP microdrops of different sizes shows that the stoichiometry between C and the other elements holds in all the droplets measured (Fig. 5B), proving that there has been no absorption of X-rays within the sample.

**Elemental cell analysis**

Elemental microanalysis of the cells was performed with a Hitachi S-3500N SEM equipped with an EDS Si(Li) detector (Bruker AXS). The detector processes X-rays from $Z = 3$ (includes light elements), with a resolution $\leq 129$ eV (calibrated with Mn Kα lines). As described above, X-rays were collected for 100 s (live time) with 15 kV of accelerating voltage. The nominal beam diameter was 70-80 nm. The beam current was adjusted to acquire around 1000 cps; these are optimum working conditions for the detector. The software used to acquire and analyze the spectra to obtain the total counts of each element present was QUANTAX 1.6 (Bruker AXS). The cells and scanned areas were saved with the SEM images and measured using Quartz PCI v5.1. Subsequently, the mass of each element was obtained with the equation $m_x = (I_x/A)/(C_x\cdot t)$ (Norland et al. 1995), where $m_x$ is the mass of the element x in pg; $I_x$ are the net counts of the element x (after the counts from the formvar film have been subtracted, in case that the same area and live time have been used to acquire both spectra); A is the area scanned in $\mu m^2$; $C_x$ is the calibration constant for the element x in counts $\mu m^2 pg^{-1} s^{-1}$; and t is the live time, in seconds.

**Assessment**

Individual XRMA analysis was performed on cells of dinoflagellates and diatoms, and also on nonbiogenic particles of aluminosilicates and salt crystals. The samples were collected in 5- to 8-L plastic bottles at different locations of the Catalan Sea (NW Mediterranean Sea) and at different times of the year.

The marine microplankton cells analyzed from the natural samples were a total of 136 dinoflagellates of the genera *Alexandrium*, *Scripsiella*, *Dinophysis*, *Neoceratium* (Gómez et al. 2010), *Protoperidinium* and *Prorocentrum*, and 28 diatoms of the genera *Chaetoceros*, *Pseudonitzschia*, *Rhizosolenia*, *Pleurosigma*, and *Thalassiosira*.

In Figs. 6A and 6B the differences in the spectra of diatoms and dinoflagellates can be observed: for example, the Si peak in diatoms and the greater C content in dinoflagellates than in diatoms (Geider and La Roche 2002; Segura-Noguera 2007).

To compare our XRMA results with previous studies, the mass of each element has been normalized by the cellular volume. The volume was calculated by measuring each cell during the analysis in the electron microscope, and then applying the models described in Sun and Liu (2003). C and N elemental concentrations per unit volume of each of our individual cells are compared in Fig. 7 with reported values obtained from cultured cells (except Tada et al. 2000) analyzed using

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**Fig. 5.** (A) Mass (pg) of C in latex spheres calculated using the volume and density versus the mass of C obtained by XRMA. The error bars correspond to one standard deviation of 10-20 spheres of each size. B. Mass of N, O, P, and K (pg) in relation to the mass of C (pg) present in microdrops of ADP.
Fig. 6. XRMA spectra (in black) of a *Chaetoceros* sp. cell (A), a *Protoperidinium* sp. cell (B), a crystal of sea salt formed while the grid was being dried in nonwashed samples (C), and an aluminosilicate particle (D) from the NW Mediterranean Sea. The blank spectra (in yellow) correspond to the analysis of an area of formvar film adjacent to the cell, crystal, or aluminosilicate. The white squares and ovals in the pictures represent the area of the sample and blank analyzed. Observe the lower background obtained compared with the spectra in Fig. 1.
bulk analysis methods (humic oxidation and dry combustion: Cushing and Nicholson 1958; Parsons et al. 1961; Mullin et al. 1966; Strathmann 1967; Blasco et al. 1982; Brzezinski 1985; Moal et al. 1987; Goldman et al. 1992; Verity et al. 1992; Montagnes et al. 1994; Pelegri et al. 1999; Lomas and Gibert 2000; Menden-Deuer and Lessard 2000; Tada et al. 2000; Ho et al. 2003). We have differentiated between diatoms and dinoflagellates as it is known that their mass of C per unit volume is different (Menden-Deuer and Lessard 2000), being the dinoflagellates more C dense. Our results are consistent with this observation (Fig. 7). Other elements (Mg, Si, P, S, K and Ca) are compared with published data (Brzezinski 1985; Ho et al. 2003; Gisselson et al. 2001) for the same species or genera in Table 2. Brzezinski (1985) obtained Si elemental composition following Paasche (1973). Ho et al. (2003) used high-resolution inductively coupled plasma mass spectrometry (HR-ICPMS) to obtain the amount of Mg, P, S, K and Ca of different groups of plankton.

Both Fig. 7 and Table 2 indicate that XRMA analysis data agree with the results obtained with bulk analysis methods. A statistical analysis of the equations in Fig. 7 shows that there are no statistically significant differences in the slopes between historical analysis (i.e., bulk analysis) and our single-cell analysis, both for diatoms and dinoflagellates, and for C and N content per unit volume (ANOVA, $P > 0.05$, please see $n$ in Fig. 7). However, there are statistically significant differences in the intercepts for all the bulk-single cell comparisons (ANOVA, $P < 0.01$). In fact, it can be observed in Fig. 7 that some values of C and N per unit volume of diatoms and dinoflagellates in the XRMA single-cell analysis are lower than the historical data. These results are consistent with the observations of Gisselson et al. (2001), when they compare the elemental composition of *Dinophysis norvegica* using a nuclear microprobe for single-cell analysis with other bulk analysis techniques for C, N, and P. Actually, our results for *Dinophysis punctata* (14.7-193.0 fg C µm$^{-3}$, 5.4-16.9 fg N µm$^{-3}$, and 0.4-2.2 fg P µm$^{-3}$, $n = 38$) match the single-cell results from Gisselson et al. (2001) (66.8-145.9 fg C µm$^{-3}$, 1.7-11.1 fg N µm$^{-3}$, and 0.4-
1.4 fg P µm⁻³, n = 30). Norland et al. (1995) also studied the elemental composition of E. coli by XDMA and CHN techniques. They found statistically significant differences in the N/C ratio for cells from the stationary culture, but not from cells harvested during the growing phase. The authors suggested that the difference could be due to presence of extracellular nitrogen-rich material retained in the filter. Our results also support the conclusion of Banse (1974) that bulk analysis techniques such as CHN and HR-ICPMS include, in addition to live cells, particulate organic debris rich in C and N (e.g., as observed by Wetzel and Wheeler 2007). Our concentrations of Mg, Si, P, S, and Ca also agree well with the very low concentrations of these elements reported previously (Table 2). No published data exist for O content with bulk methods, because it cannot be analyzed, but our O concentrations are consistent with those of Cyanophyceae analyzed using XDMA (Heldal et al. 2003). To the best of our knowledge, no data on Na, Al, and Cl have been reported previously for the genera we have measured, so we cannot compare our results. Sigee et al. (1998, 1999) and Krivtsov et al. (1999) show the elemental composition of single freshwater Ceratium hirundinella cells, but the reported values, in nmol per kg of dry weight, are difficult to compare with the ones reported in the present study, because their analysis did not include C, N, and O in their calculation of dry weight.

**Effect of washing on the elemental composition inside the cell**

Washing (desalting) the sample is crucial. Otherwise salt crystals are formed when the grid is air-dried, even when the maximum possible amount of water has been removed after the centrifugation. If the sample is not washed, salt crystals appear not only at the edges of the grid, which could be easily discarded, but also over the cells and inside the pores of the cells (see Fig. 2C and D). The presence of these salt crystals will not only contribute to the results of the concentration of Na, Cl, and Mg within the cell, but also, because they significantly increase the total counts of the spectra, obscure lower intensity intracellular element signals.

To determine whether the washing procedure causes a loss of the intracellular elements, we analyzed two sub-samples from an Alexandrium minutum bloom that occurred in Arenys de Mar harbor. One sub-sample was washed, and the other not. In the unwashed one only cells that seemed to be free of salt were used. A statistical study on 25 A. minutum cells (17 washed and 8 unwashed) and 9 Scripsiella sp. cells (4 washed and 5 unwashed) indicated that there were no statistically significant differences in the quantity of the elements C, N, O, P, S, K, and Ca (ANOVA, P > 0.05, n = 32), and as expected, differences were detected for Na, Cl, and Mg (ANOVA, P < 0.05, n = 32). The analysis of the individual crystal salts indicated the presence of Na, Cl, O, and Mg (34%, 58%, 6% and 1% wt wt⁻¹, respectively).

**Beam penetration and absorption of X-rays within the cells**

The beam penetration into the cells was studied both experimentally and theoretically using the Monte Carlo simulation software CASINO (Drouin et al. 2007), as well as following Reed (1975). As explained above, 15 kV working accelerating voltage was chosen because of the linearity between the counts per second versus the diameter of different spheres of known mass (Fig. 3), while this linearity was not achieved using 10 kV of accelerating voltage. A Monte Carlo simulation confirmed the lack of penetration into the 5-µm latex spheres using 10 kV of beam intensity, where the maximum penetration into a substrate of latex composition is around 2 µm. In contrast with the experimental results, the Monte Carlo theoretical deepest X-ray generated with 15 kV accelerating voltage was around 4.2 µm from the surface (Fig. 8A), corresponding to an analysis of latex with a mass thickness of 4.4 pg C µm⁻².

Dinoflagellate thecal plates possess mechanical properties comparable to softwood cell walls (Lau et al. 2007), whose oven-dried density is around 1.5 g cm⁻³ (Walker 1993): higher

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**Table 2. Comparison between the elemental composition (fg µm⁻³) of organisms of the same or close genera of diatoms and dinoflagellates measured in this study (‘XRMA’) and values reported in the literature (‘others’) when available. XRMA data are shown as the range between the minimum and maximum values measured in individual cells (between parentheses: number of cells analyzed). The data from the literature has been recalculated to fg µm⁻³.**

| Genera/species       | Method          | Si     | P       | Mg       | S       | K       | Ca       |
|----------------------|-----------------|--------|---------|----------|---------|---------|----------|
| Chaetoceros sp.      | XRMA            | 2.3-42.8 (31) |
|                      | others          | 8.4-35.8 (6)  |
| Pseudonitzschia sp.  | XRMA            | 10.2-37.8 (4)  |
|                      | others          | 6.5-7.3 (2)  |
| Nitzschia sp.        | XRMA            | 60.1-141.6 (3) |
|                      | others          | 9.8-43.6 (6)  |
| Thalassiosira sp.    | XRMA            | 0.4-1.9 (4)   |
|                      | others          | 0.7-1.3 (3)  |
| Dinophysis punctata  | XRMA            | 0.4-2.2 (38)  |
| Dinophysis norvegica | single-cell     | 0.4-1.4 (30)  |
| Dinophysis norvegica | others          | 1.8 (1)  |

*Brzezinski 1985, *Ho et al. 2003, *Guisselson et al. 2001.*
than the density of the latex beads (1.05 g cm\(^{-3}\)). A Monte Carlo simulation of the penetration of a 15 kV electron beam into cellulose shows that the interaction volume has a depth of around 3.2 µm (Fig. 8B), equivalent to a mass thickness of 4.8 pg cellulose µm\(^{-2}\). The thickness of the dinoflagellate plates is highly variable, from 0.010–0.045 µm (e.g., *Gyrodinium* sp., *Heterocapsa niei*) to 0.150–0.600 µm (e.g., *Ceratium hirundinella*, *Prorocentrum micans*) (Dodge and Crawford 1970; Morrill and Loeblich 1983). Only a few extremely thick plates have been reported for *Peridinium depressum* (1.8 µm, Dodge and Crawford 1970).

On the other hand, the cell walls of diatoms are composed of silica with different degrees of hydration (Round et al. 1990), and densities ranging from 0.5 to 2.1 g cm\(^{-3}\) (Sommer 1988). According to Reed (1975), a different sample composition, as well as a change in density, would affect the beam penetration into the specimen. To determine the beam penetration depth into diatom cells, we again modeled the interaction volume with a Monte Carlo simulation, using the lowest and the highest silica densities. The results show that a 15 kV accelerating voltage electron beam penetrates up to 11.4 µm into low-density silica (Fig. 8C), and only to 2.7 µm (Fig. 8D) in higher density silica, equivalent to a mass thickness of 5.7 pg silica µm\(^{-2}\). The thickness of diatom frustules varies within individual cells according to nutrient availability (Round et al. 1990; Takeda 1998), growth phase (e.g., Pugh 1975) and grazing pressure (Pondaven et al. 2007). According to some reported measurements (*Thalassiosira* sp., *Stephanopyxis* sp., *Staurosirella* sp., and *Coscinodiscus* sp.; Pugh 1975; Hildebrand et al. 2006; Finkel et al. 2010), diatom frustule thickness ranges from 0.06 to 0.35 µm. Therefore, based on our simulation results, 15 kV beam intensity should be sufficient to penetrate both sides of the frustules. We also calculated the electron penetration into the latex spheres, as well as cellulose and silica of different densities using Reed (1975), and the results agreed well with the Monte Carlo simulations.

To avoid relying only on bibliographic data, using SEM micrographs of cells mostly from the NW Mediterranean Sea, we measured the thickness of the cell walls of various dinoflagellates and diatoms (Table 3). With two exceptions, *Neoceratium* sp. and *Prorocentrum* sp., the total width of all the measured cells was below 3.2 µm in dinoflagellates and 2.7 µm in diatoms, which are the maximum penetration depth obtained with the Monte Carlo simulation for cellulose and high-density silica. This suggests that the beam penetration into the cells was complete. Further confirmation arises from the fact that the maximum total mass per area for all the cells analyzed in this study was lower than the maximum theoretical pene-

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**Fig. 8.** (A) Monte Carlo simulations of the interaction volume created during the analysis of latex beads using 15 kV of accelerating voltage. (B-D) Monte Carlo simulations of the interaction volume created during the analysis of cellulose \((C_6H_{10}O_5)\) of density 1.5 g cm\(^{-3}\) (B), silica \((SiO_2)\) of density 0.5 g cm\(^{-3}\) (C), and silica of density 2.1 g cm\(^{-3}\) (D). Note that axes change.
Table 3. Thickness of dinoflagellate plates and diatom frustules (average ± standard deviation, n: number of data) measured in SEM pictures of cells collected in the Mediterranean Sea, except *Thalassiosira* sp., which were collected in the Antarctic.

| Species               | Plate or frustule thickness (µm) | n  |
|-----------------------|----------------------------------|----|
| **Dinoflagellates**   |                                  |    |
| *Alexandrium* sp.     | 0.26 ± 0.10                      | 9  |
| *Scripsiella* sp.     | 0.27 ± 0.03                      | 3  |
| *Dinophysis* sp.      | 1.26 ± 0.21                      | 9  |
| *Dinophysis tripus*   | 1.07 ± 0.15                      | 4  |
| *Dinophysis acuta*    | 1.38 ± 0.16                      | 5  |
| *Dinophysis sacculus* | 0.44 ± 0.11                      | 4  |
| *Gonyaulax* sp.       | 0.65 ± 0.03                      | 2  |
| *Neoceratium* sp.     | 1.58 ± 0.66                      | 5  |
| *Prorocentrum* sp.    | 1.88 ± 0.06                      | 2  |
| *Protoperoptymium* sp.| 0.67 ± 0.38                      | 2  |
| **Diatoms**           |                                  |    |
| *Chaetoceros* sp.     | 0.18 ± 0.06                      | 2  |
| *Pseudonitzschia* sp. | 0.07 ± 0.01                      | 3  |
| *Coconeis* sp.        | 0.10 ± 0.01                      | 1  |
| *Thalassiosira* sp.   | 0.82 ± 0.73                      | 8  |

In conclusion, the results of the Monte Carlo simulations, the measurements of the cell walls and frustules, and the experimental data demonstrate that 15 kV is sufficient incident energy to analyze dried microphytoplankton using XRMA. Therefore, under our analysis conditions, the thin-film method for quantification (Hren et al. 1979; see also Norland et al. 1995) can be applied to obtain the elemental composition of microphytoplankton cells.

We also studied the effect of the use of absorption corrections, as explained in Goldstein et al. (1992), to obtain the calibration constants. This resulted in a considerable increase in the standard error of the light elements (25% for C, 40% for N, and 20% for O) of the salts used as standards (listed in Table 1), but not of the other elements, and an incorrect stoichiometry of the analysis. Therefore, we conclude that it is not necessary to apply any absorption correction to the light elements.

**Detection limit and analytical precision**

The analysis detection limit for each element depends on the quantity of counts at the element peak, the thickness of the formvar film under the cells, and the mass of the element per volume (Norland et al. 1995). We have calculated a standard deviation of the C mass thickness of 20 fg µm⁻² for the formvar film. With the detection limit of carbon defined as a relative error equal to 0.5 (Norland et al. 1995), the smallest detectable specimen mass thickness of carbon will be 40 fg µm⁻². In the case of other elements not present in the formvar film, the standard deviation of the peak has been estimated with \(\frac{P}{2(W + F)}\), where P, W, and F are the counts in the peak, in white radiation and film respectively (Norland et al. 1995). With a calculated value of 250 for (W + F) the smallest detectable peak will have 50 counts, which correspond to a detection limit of 2.5 fg µm⁻² with 100 s live time and a Cₚ value of 200 counts s⁻¹ pg⁻² µm⁻².

Typically, the detection limit of X-ray microanalysis using an EDS detector is usually 1000 µg g⁻¹ (0.1 %) (Schott 2003, marine bacteria: Norland et al. 1995). In the present work, this value was assessed for C using the results of the analysis of abiotic particles of different sizes that were present in the sample mounts, such as salt crystals (n = 8) and aluminosilicates from aeolian dust (n = 59). Theoretically, these abiotic particles should be C-free, so the C peak that appears in their spectra should originate only from the formvar film below (see Fig. 6C and D). However, when they are in contact with sea water, organic matter may be absorbed onto aeolian dust (Chester 2003). Therefore, aluminosilicate particles that had more than three times the average C content (n = 9) after the subtraction of the formvar from the whole analysis were considered contaminated, disregarded as outliers, and not used in the assessment of the detection limit. Using the abiotic particles as blanks, we calculated the C content by subtracting the formvar film analysis from the whole particle analysis, and we estimated the detection limit of the C as three times the standard deviation of the blank. Following this procedure, we obtained a detection limit of 3.08 pg for C (n = 62), which is about 0.16% (ca. 1600 µg g⁻¹) of the average total dry mass plus formvar, obtained for the dinoflagellates and diatoms analyzed. Also, the mean C content after film subtraction for all the aluminosilicates and salt particles was positive (0.97 and 1.48 pg, respectively) (Fig. 9), suggesting that those particles were not completely C-free. Therefore, one may assume that the detection limit is lower, and closer to the theoretical one described for XRMA analysis using an EDS (0.1%, Schott 2003).

The analytical precision for each element obtained during the calculation of the calibration constants and the standard error of the mean is shown in Table 1.

**Discussion**

The methodology presented can be used to simultaneously analyze carbon, nitrogen, oxygen, silicon, phosphorus, sulfur, and other cation and anion concentrations on single natural plankton cells, and to analyze most of the size ranges of the phytoplankton species present on a given phytoplankton population. SEM X-ray and TEM X-ray microanalysis (XRMA) have been used to study the elemental composition of eukaryotes from marine and freshwater systems for quite a few years (Heldal et al. 1985; Lehman 1985; Clay et al. 1991). The results have demonstrated the power of single-cell element measurements for understanding the relationships between protist biology, ecology, and biogeochemistry in natural systems.
Another cell technique that has been proposed, X-ray fluorescence spectromicroscopy (Twining et al. 2003; Diaz et al. 2009), allows some elements present in single cells to be determined with greater precision, and even at submicron resolution, but the low availability of X-ray sources and the laboriousness of the technique makes it difficult to use extensively. Electron microprobes are widely available and XRMA is currently the only method that allows the simultaneous identification of the C, N, and O plus Na, Mg, Al, Si, P, S, Cl, K, and Ca present in individual phytoplankton cells sampled from the field. However, its use has stagnated because of two major drawbacks: the interference of the support substrate and the supposed limited depth of penetration of electrons into target cells (Twining et al. 2008). The results presented herein suggest that both these drawbacks can be easily overcome, even using low accelerating voltage, and it follows that, while the conclusions of the studies do not change, analysis of phytoplankton cells done at higher voltages than the one used in the present study, might have included the analysis of both valves or frustules as well as the intracellular matrix (e.g., Krivtsov et al. 2002 analyzed Stephanodiscus rotula using 25 kV accelerating voltage, or Tien 2004 analyzed diatoms of 10-15 µm diameter using 20 kV).

The method described is a significant step forward in obtaining concentrations of the major elements (C, N, O, Na, Mg, Al, Si, P, S, Cl, K, Ca) in single cells of the species found in a natural plankton population, making it feasible for other laboratories to apply the method. The sampling protocol is easy and does not involve invasive pretreatment of the cells. The samples can be collected and prepared at sea and stored easily for later analysis. Furthermore, in one sample it is possible to analyze several individual cells of the species and genera present in the plankton at a given moment. In this article, we have presented a methodology for single-cell microplankton analysis that is relatively easy to use, and the results are in agreement with the broad range of existing data on elemental concentrations and stoichiometry of phytoplankton. As has been shown in previous studies using single-cell analysis, the methodology can also be used to explore the differences in elemental composition between species and between larger taxonomic groups, as well as the phenotypic variability between populations that are acclimated to different physical or chemical environments. These differences were observed during our study and the results will be discussed elsewhere (Segura-Noguera et al. in prep.).

**Comments and recommendations**

This method has a limited use for the study of trace metals. Although these elements can be analyzed with XRMA, they usually fall below the detection limit because the relatively high concentration of C, Si, and O, which are present in cellulose and/or silica cell walls, will obscure the lower-intensity signal of the trace elements.

Another important aspect to consider is the thickness of the formvar film. Too thin formvar films (with a formvar solution below 1%) could cause the film to break and the sample to be lost. On the other hand, too thick films (with a formvar solution above 1.5%) will generate too many C counts in the blank spectra, increasing the error of the C analysis and also having a negative effect on the detection and analysis of the other elements present in lower concentrations.

The calibration constants presented in this article depend on the instrument conditions, so they should be calculated in each laboratory. Also, they can be used only when the analysis conditions that define the amount of energy reaching the sample (beam intensity and diaphragm aperture) are constant (Norland et al. 1995). This condition may be checked with the analysis of latex beads, and the electron beam should be readjusted if deviations are detected. In our laboratory, deviations took place mainly at the beginning of the analysis session, but rarely later on, and were probably related to the stabilization of the filament of the microscope. Moreover, analysis of latex beads of known mass throughout the analysis, as described in Norland et al. (1995), verifies the accuracy of the mea-
measurements. Precaution should be taken to analyze the whole cell, adjusting the area to be scanned to the cell contour as much as possible in order to obtain more reliable results like in Norland et al. (1995).

Although we have demonstrated that an incident beam intensity of 15 kV is sufficient to analyze whole dinoflagellate and diatom cells, for the analysis of large bulky microplankton cells we recommend checking the penetration of the incident beam. This can be done easily using a Monte Carlo simulation software (e.g., CASINO, Drourin et al. 2007). Alternatively, if the density of the theca or frustule is unknown, the penetration can be checked by preparing the sample on a support containing an element that it is not a constituent of the cells, such as gold, and observing whether this element is detected in the analysis. This analysis should be done as a spot analysis or by analyzing a small area in the center of the cell or an area where it is suspected to be thicker.

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