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Changes of the elemental distributions in marine diatoms as a reporter of sample preparation artefacts. A nuclear microscopy application

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ABSTRACT

Studies of the elemental composition of whole marine diatoms cells have high interest as they constitute a direct measurement of environmental changes, and allow anticipating consequences of anthropogenic alterations to organisms, ecosystems and global marine geochemical cycles.

Nuclear microscopy is a powerful tool allowing direct measurement of whole cells giving qualitative imaging of distribution, and quantitative determination of intracellular concentration. Major obstacles to the analysis of marine microalgae are high medium salinity and the recurrent presence of extracellular exudates produced by algae to maintain colonies in natural media and in vitro. The objective of this paper was to optimize the methodology of sample preparation of marine unicellular algae for elemental analysis with nuclear microscopy, allowing further studies on cellular response to metals.

Primary cultures of *Coscinodiscus wailesii* maintained in vitro were used to optimize protocols for elemental analysis with nuclear microscopy techniques. Adequate cell preparation procedures to isolate the cells from media components and exudates were established. The use of chemical agents proved to be inappropriate for elemental determination and for intracellular morphological analysis. The assessment of morphology and elemental partitioning in cell compartments obtained with nuclear microscopy techniques enabled to infer their function in natural environment and imbalances in exposure condition. Exposure to metal affected *C. wailesii* morphology and internal elemental distribution.

1. Introduction

Studies of diatom elemental composition constitute a direct measurement of environmental changes, which allows anticipating consequences of anthropogenic alterations to organisms, ecosystems and global marine geochemical cycles. Moreover, the intracellular elemental distribution provides information about the physiological state of the organism and reflects biochemical demands and environmental availability [1]. *Coscinodiscus* are pollution tolerant diatoms that often dominate the phytoplankton community in coastal zones. They are efficient scavengers of trace elements being used as biomonitors of metal pollution. The study of metal uptake, accumulation and compartmentalization in whole cells of diatom *Coscinodiscus* exposed to metal overload will allow examining consequences to cellular toxicity, tolerance mechanisms, and metal fate in the environment.

Traditional bulk size-fractionation techniques used to measure phytoplankton elemental composition may present ambiguous results due to considerable detrital matter occurrence [1]. The determination of intracellular elemental distributions in isolated cells has been found challenging, involving extraction and purification of different organelles and the use of specific labeling probes, with associated risks of modifying the cell physiological state and of producing artefacts [2]. Alternatively, nuclear microprobe techniques are a powerful tool, allowing direct measurement of whole cells (without any derivatization reaction required) giving qualitative imaging of distribution, and quantitative determination of intracellular concentration. The combination of morphological images with elemental distributions is helpful since it can identify boundaries that allow distinguishing between adsorption to the border from absorption into compartment. The method is also very useful when searching for correlations between elements.

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Major obstacles to the analysis of marine diatoms are the high medium salinity and the presence of extracellular exudates produced by algae to maintain colonies in natural media and in vitro. The analysis of morphology, elemental concentrations and their distribution in individualized cell with nuclear microscopy techniques requires cellular integrity to be maintained and no interference from culture media. Cells must be completely isolated from the surrounding media components. The salt left around the cell during drying, will form large amounts of crystals around and on top of the cell severely complicating the cell identification and element analysis. However without their natural salt environment the cell will break due to the osmotic pressure, start to leak and will not be representative of the living matter anymore. The extra cellular exudates, mainly polysaccharides, involve the cells and mask cell constituents.

The objective of the study was twofold: (1) to optimize the methodology of sample preparation of marine unicellular organisms to elemental analysis with nuclear microscopy; and (2) to assess cell response to metal exposure. This paper reports and discusses results obtained in various preparation procedures and exposure conditions of marine diatom primary cultures maintained in vitro.

2. Materials and methods

2.1. Cell cultures

The diatom *Coscinodiscus wailesii* was isolated from the Tagus estuary adjacent coastal area (see Fig. 1) and grown in axenic cultures maintained in f/2 medium [3] under temperature-controlled conditions (18 ± 1 °C), in a 14 h light: 10 h dark cycle with cool white fluorescent light (Photosynthetic photon flux 80 μmol photons m^{-2} s^{-1}). Cells during the exponential phase (5 days after inoculation) were transferred to f/2 media enriched in Ni simulating natural environmental contamination levels. These cell essays were incubated in the laboratory during one-week under the same temperature and light conditions. Cells were exposed to Ni (as $NiCl_2$), final nominal elemental concentrations of 2.0 mg/l. Cells from primary cultures were used as controls.

2.2. Sample preparation

2.2.1. Removal of cell exudates

Cells were grown in petri dishes in liquid culture media. To break cell aggregates and isolate cells from involving exudates, deposited cells were collected with a micropipette and sprayed to a fix volume of fresh media. This procedure induces the mechanic separation from colonies and dilutes exudates. At least 3 passages in fresh media were performed.

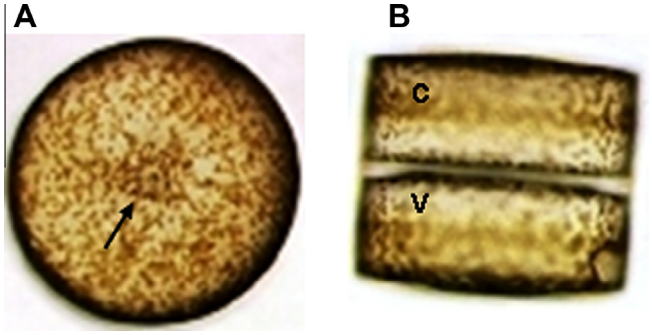

Fig. 1. Optical microphotograph images of *Coscinodiscus Waiselii* in valve (A) and girdle (B) view. Multiple pigmented bodies are chloroplasts, arrow points to central nucleus, (c) identify parietal cytoplasm and (v) vacuole. Magnification ×200.

2.2.2. Removal of salt excess

Marine environment typically has high NaCl concentrations. To avoid salt residuals and crystallization during cell aliquots drying, an additional passage was performed in distilled water instead of fresh media. This passage is critical as cell integrity must be maintained despite the changes of osmotic pressure and osmolarity. To maintain cell integrity two procedures were tested: (i) the use of cell chemical fixing agents and (ii) performing the cell passages to remove salt at low temperature. After culture passages in fresh media to remove exudates, cells were immediately fixed with 2% glutaraldehyde before the last wash in distilled water. Alternatively, a modified procedure described by Pallon et al. [4] was used. All cell manipulations were performed in ice bath, keeping temperature just above water freezing point (2–4 °C).

2.2.3. Target preparation for elemental analysis

An aliquot of 10 μl of cell suspension was deposited onto a polycarbonate foil stretched on appropriate frames and immediately quench-frozen in liquid nitrogen. Cells were allowed to dry at −25 °C in a cryostat.

2.2.4. Nuclear microscopy

The samples were examined at the proton microprobe facility of Centro Tecnológico e Nuclear/IST [5]. A proton beam of 2 MeV energy with a current of 100 pA and a resolution of 3 μm was used to scan the samples. Particle induced X-ray emission (PIXE), Rutherford backscattering spectrometry (RBS), and scanning transmission ion microscopy (STIM) were used simultaneously to obtain morphological and quantitative elemental distribution data. RBS enables the measurement of matrix composition, depth variations and sample stoichiometry to normalize PIXE spectra for calculation of elemental concentrations [6]. The off-axis STIM configuration was used to obtain additional information of areal density variations, and images of the sample morphology [7]. Acquisition and processing of results were performed using OMDAQ data acquisition system [8].

3. Results and discussion

3.1. Optimizing cell isolation procedures

To properly analyze elemental contents in marine diatoms, media removal efficiency was assessed. The presence of media residues, whether cell exudates or salts hamper identification of cell morphological details using the elemental distribution maps. Fig. 2 illustrates these two aspects.

The elements present in exudate compounds are also present in cells. Consequently, the quantification of cellular elemental contents becomes a challenge when using nuclear microscopy techniques, as discriminating between exudates and cell constituents is difficult to assess by RBS or STIM. Losses of image and spectra quality are also caused by sodium chloride crystallization. Excess of Cl hid cell morphology (Fig. 2C) and hampered accurate PIXE spectra deconvolution and quantitative analysis.

The sequential washing steps spraying cells into the new media ensured a good removal of cell exudates (Fig. 3) maintaining media osmolarity and granting cell integrity. The spraying process promotes mechanic disruption of the mucous that keeps cell aggregates while diluting the exudates. Each rinse step was rapid enough (the period of time of pipette influx and efflux) to guarantee no cell contents exchange with fresh media. In Fig. 3 the efficient removal of media is denoted by the regular cell contours