

# Intracellular Localization of Micronutrients in Algae Cells Using Scanning Transmission Electron Microscopy–Energy-Dispersive X-ray Spectroscopy (STEM-EDX)



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**Abstract** Various elements including iron play a vital role as micronutrients in growth and development of algae cells. Understanding the process of element uptake, assimilation and utilization of these elements by algae cells are essential for optimizing growth conditions in algae ponds, for cost-effective biomass production. In addition to bulk elemental analysis methods, electron microscopy-based semi-quantitative elemental analysis methods can provide high-resolution spatial information about element distribution in algal cultures for a comprehensive and accurate understanding of the element utilization processes. Toward this goal, we standardized a protocol for localizing various elements within unicellular algae cells using scanning transmission electron microscopy–energy-dispersive X-ray spectroscopy (S/TEM–EDX). We applied this method to study iron uptake and intracellular distribution process by algae cells over a week of cultivation.

**Keywords** Algae · Iron uptake · Micronutrients · STEM-EDX

## 1 Introduction

Microalgae are rich source of lipid, protein, carbohydrates, vitamins, micronutrients, and pigments, which can be utilized in production of biofuel, nutraceuticals, dietary supplements, cosmetics, animal fodder, and many other green products [5].

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They are photosynthetic organisms that utilize atmospheric CO<sub>2</sub> to grow and require minimal other external resources. They can grow in wide range of water bodies including freshwater, seawater, and even wastewater. They can tolerate wide range of temperatures, salinities, pH, and light conditions. They have short life cycle allowing faster harvesting of desirable products compared to plants. Their productivity can be further improved by growth condition manipulation and genetic engineering. All these characteristics have made microalgae a valuable feedstock for various industries. They also are gaining interest due to their application in wastewater treatment and atmospheric CO<sub>2</sub> mitigation [5].

Algae cells utilize various elements available in different forms in the environment for their growth and development. These micronutrients are utilized directly or indirectly for several crucial processes occurring in the cells, such as photosynthesis and respiration. The cells can either utilize these elements immediately after uptake or store it for future use. Growth and biomass production by algae cells are directly dependent upon uptake, assimilation, and utilization of essential elements by these cells [4]. Presence of excess elements in the growth media can cause cell toxicity and is a waste of expensive nutrients. Understanding the process of uptake, assimilation, and utilization of essential elements by algae cells can aid in formulation of optimum dosage for these micronutrients during algae cultivation. Such knowledge will be of additional value for development of micronutrient-rich dietary supplements and animal feed products from algae.

Algae-based food and feed products are rich source of iron for the consumers. The micronutrient iron (Fe) has a major effect on the intracellular and extracellular environment of the algal species [9]. Iron is essential for cellular processes such as respiration, photosynthesis, nitrogen assimilation and fixation, and DNA synthesis, where it acts as electron donor in its ferrous state, and as electron acceptor in its ferric state [1, 3]. Iron, however, is also a limiting factor for growth of marine algae as it is highly insoluble in seawater and the dissolved iron has low bioavailability [12]. Understanding the iron uptake and utilization in a spatio-temporal manner thus becomes critical for optimizing iron requirement of the algal cells. This knowledge can aid in formulation of optimum inoculum protocol for iron in algae cultures that will be support maximum cell growth and development while controlling elemental toxicity.

Bulk elemental analysis of algal biomass is done by biochemical and spectroscopic analysis methods [6, 8, 10]. SEM-EDX is used for elemental mapping of outer surface of algae cells [6]. However, intracellular localization of iron and other elements is not possible by these techniques. Optical microscopy using iron-specific stains are used to study iron assimilation patterns within relatively large multicellular algal cells [7]. However, the limitation is that it cannot resolve finer details within unicellular algae such as *Nanochloropsis*, *Chlorella*, and *Chlamydomonas*. Transmission electron microscopy (TEM) is a method widely used by biologists to image ultrastructure of individual cells. Scanning TEM (STEM) that has better spatial resolution than conventional TEM is primarily used for analysis of materials. STEM paired with energy-dispersive X-ray spectroscopy (EDX) is used for elemental identification and mapping. EDX detector can identify individual elements

present in a sample based on their characteristic X-ray peaks produced due to their unique atomic structure and difference in energy of inner and outer electronic shells. Elements with low atomic number cannot be detected by EDX, although advances in technology in last decade has made it possible to detect boron, carbon, and nitrogen [2]. STEM-EDX is predominantly used for materials research; however, its application for biological studies is gaining interest in recent years [11, 14].

In this report, we standardized a protocol for localizing various elements within unicellular algae cells using STEM-EDX. Using this method, we mapped the various microelements in and around individual algae cells. We further focused on the elemental iron distribution pattern in algae cultures exposed to varying ionic iron content in growth media. Our study shows that STEM-EDX has great potential for simultaneous ultrastructural imaging with elemental mapping of biological samples such as cells and tissues, for studying elemental uptake physiology.

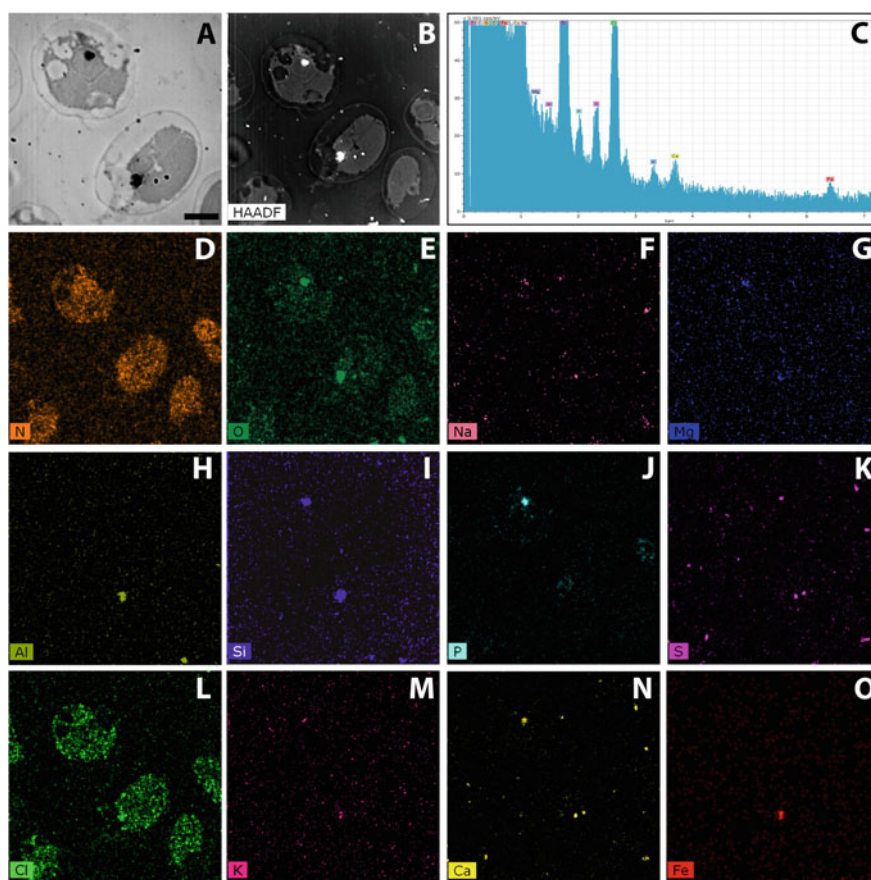
## 2 Experimental

**Cell culture:** Algae cells (*Nanochloropsis* sp.) were grown in sea water-based media with urea and phosphoric acid, in Kuhner shaker at 30 °C with 400  $\mu$ E light illumination for 12 h. Culture flasks were shaken at 100 rpm, and CO<sub>2</sub> environment was maintained inside the incubation chamber during the photosynthetic light period. Iron concentration in the culture was varied by addition of ferric chloride stock solution at (a) 2X, for a normal iron condition, and (b) 4X, for a high iron condition. Following incubation, cultures were harvested at 0, 1, 4, and 7 days, washed in normal sea water media to remove the excess iron not attached to algae cells, and processed for electron microscopy.

**STEM-EDX:** All fresh algae cells were harvested at various time points, centrifuged, and the cell pellets were immediately fixed in 2.5% glutaraldehyde and 4% paraformaldehyde in seawater media. Fixed samples were incubated at 4 °C overnight, washed thrice in seawater media, followed by a final wash in distilled water. Samples were then dehydrated in an ethanol series (25, 50, 75, 90, 100%), then infiltrated in an epon-araldite resin series (25, 50, 75, 100%). All samples were finally embedded and polymerized in 100% resin mixed with accelerator. Ultra-thin sections (70–100 nm) were cut using Leica Ultracut-7 ultra-microtome, collected on formvar-carbon-coated copper mesh TEM grids (Electron Microscopy Sciences, USA) for STEM-EDX analysis. STEM imaging was done with Titan G2 60–300 TEM (FEI Company, USA) equipped with X-FEG electron gun operating at 300 kV which was used to find well-preserved cells in multiple sections from each sample type. Nanometer-resolution *in situ* elemental maps for several individual cells and their surroundings in each sample type were then obtained using Chemi-STEM EDX detectors. The beam intensity was optimized to get a minimum 1 kcps in the energy spectrum for at least 15 min to obtain false color elemental mapping of each sample.

### 3 Results

**Detection and mapping of elements by STEM-EDX:** Presence of several elements including nitrogen (N), oxygen (O), sodium (Na), magnesium (Mg), aluminum (Al), silicon (Si), phosphorus (P), sulphur (S), chlorine (Cl), potassium (K), calcium (Ca), and iron (Fe) could be detected in variable intensity in algae cells from all the above-mentioned cultures. Location of these elements could be mapped in and around individual algae cells to the spatial resolution of few nanometers (Fig. 1).



**Fig. 1** Detection and intercellular localization of elements in algae cell cultures by STEM-EDX mapping. (a–o) Cells grown in normal Fe (2X) for 7 days. **a** STEM bright field image of cells mapped. **b** High angle annular dark field (HAADF) image of cells mapped. **c** EDX Spectrum showing elements detected in area scan of B. **d–o** STEM-EDX maps of elements detected in spectrum. Scale bar = 2  $\mu$ m

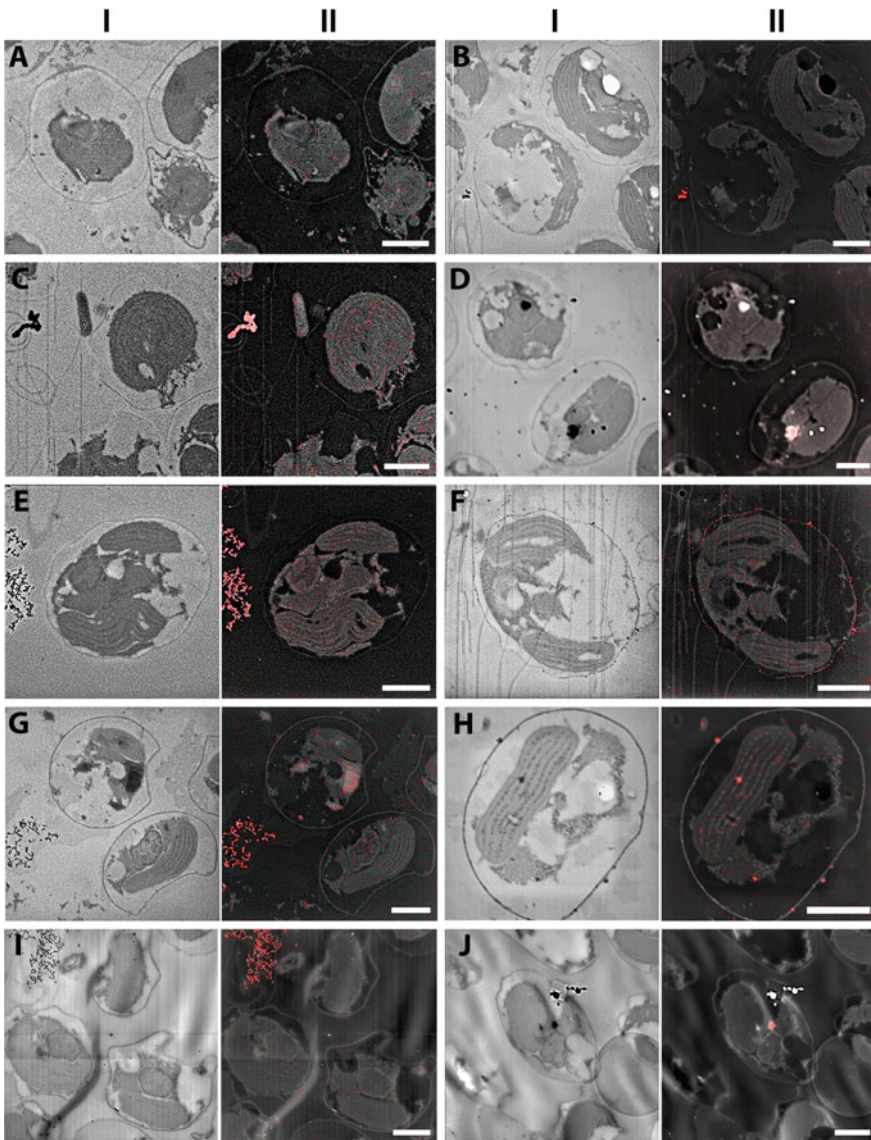
Some of these elements such as carbon, nitrogen, oxygen, and chlorine were observed to be present throughout all cells, whereas other elements were localized as tiny electron-dense particles in different parts of cells.

**Spatial localization of iron (Fe):** Variation in elemental Fe concentration in the growth media resulted in different localization patterns in and around the algae cells at various time series points (Fig. 2a–j). A very low intensity of Fe signal was detected throughout the cells in all cultures, which can be considered as basal Fe content of each cell. In cultures grown with normal iron concentration (2X), cells with only such basal Fe content was observed at Day-0 (Fig. 2a), Day-1 (Fig. 2b) and Day-4 (Fig. 2c). Such cells were also predominant in cultures grown upto 7 days. However, in few cells ( $\sim 1$ –2% of population), tiny electron-dense deposits of Fe could be observed inside the cells (Fig. 2d). Small clumps of Fe were also visible outside the cells in 2X cultures grown for 1, 4, and 7 days (Fig. 2b–d).

In cultures grown with high iron concentration (4X), cells with only basal Fe content were predominant for all time series data points as in 2X samples. However, unlike 2X samples, electron-dense Fe deposits could be seen within individual cells as early as Day-1 (Fig. 2f) and was also present in Day-4 (Fig. 2h) and Day-7 (Fig. 2j) samples. Fe deposits were detected on the plasma membrane in Day-1 cultures (Fig. 2f). By Day-4, Fe deposits could be detected in the cytoplasm as well as in the chloroplasts apart from being attached to plasma membrane (Fig. 2h). By Day-7, slightly larger deposits of Fe, compared to those seen at earlier time series points, were localized only within the chloroplasts (Fig. 2j). Large chunks of Fe precipitates were found lying outside, but in close proximity to the algae cells at all time series points with 4X iron in media (Fig. 2e, g, i). These external Fe deposits were often attached to broken pieces of membranes and other cell debris.

## 4 Discussion

These results suggest that addition of higher concentration of Fe in algae growth media can cause accumulation of Fe as dense aggregated particles within the cells, although the frequency of such cells remain extremely low ( $\sim 1$ –2% of a population) when cultures are grown for 1–7 days. Most of the extra Fe seems to get precipitated as large aggregates outside in close proximity to the cells. Above indicates possible presence of a regulated Fe uptake mechanism in these algae cells, as previously suggested by physiological studies of marine microalgae [13]. Further STEM-EDX studies combined with molecular biology experiments would be required for a clear understanding of the multiple steps and players involved in the Fe uptake, accumulation and utilization pathways of these algae cells. Similar studies can be conducted for other elements and other algal species, which will help in optimizing pond culture conditions for various algae strains based on their respective elemental requirements. The results of the current study also shows that addition of excess Fe did not bring any noticeable increase in algae cell size. Addition of excess Fe will hence be waste and could even help cohabitating



**Fig. 2** Localization of iron (Fe) in algae cell cultures by STEM-EDX mapping. **a–d** Cells grown in normal Fe (2X) for **a** 0 day, **b** 1 day, **c** 4 days, **d** 7 days, **e–j** Cells grown in high Fe (4X) for (**e**, **f**) 1 day (**g**, **h**) 4 days (**i**, **j**) 7 days. **Panel I**: STEM bright field images of cells mapped. **Panel II**: Fe maps overlaid on respective high angle annular dark field (HAADF) images. Scale bars = 2  $\mu$ m

contaminant organisms in open pond cultures. Hence, knowledge of optimum dose of Fe and other elements will also help in reducing contamination load in the ponds using inoculum-based elements input.

## 5 Conclusion

This study demonstrates that STEM-EDX can be used for cellular localization of elements of unicellular algae cells. Such studies can add valuable spatial information to elemental uptake studies that are mostly limited to bulk analysis techniques.

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