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Determination of carbon-to-nitrogen ratio in the filamentous and heterocystous cyanobacterium *Anabaena* sp. PCC 7120 with single-cell soft X-ray imaging

T Teramoto¹, M Yoshimura², C Azai³, K Terauchi³ and T Ohta²

¹College of Science & Engineering, Ritsumeikan University, Shiga, 525-8577, JAPAN
²SR center, Ritsumeikan University, Shiga, 525-8577, JAPAN
³College of Life Sciences, Ritsumeikan University, Shiga, 525-8577, JAPAN

E-mail: tteramo@fc.ritsumei.ac.jp

Abstract. Vegetative cells and heterocysts in the filamentous cyanobacterium *Anabaena* sp. PCC 7120 were observed by soft X-ray microscopy. Carbon-to-nitrogen (C/N) ratio of each cell was estimated by the difference of the absorbance of the images below and above the nitrogen K-edge absorption. It was revealed that the C/N ratios in vegetative cells and heterocysts are 4.54 and 2.46, respectively.

1. Introduction

Natural or anthropogenic conversion of unreactive N₂ to other reactive nitrogen compounds such as ammonia, is called as nitrogen fixation and this is one of the main issues for ecosystem and food production on the earth [1]. Although the chemical Haber–Bosch process produces ammonia and fills the industrial demand, a biological nitrogen fixation is expected to displace the industrial production of ammonia in terms of a sustainable and environment-friendly, low-carbon society.

Filamentous cyanobacterium *Anabaena* sp. PCC 7120 is one of its promising candidates [2]. It forms a filament of “vegetative cells”, which is responsible for oxygenic photosynthesis. When starved for reactive nitrogen compounds, one out of about ten vegetative cells differentiates to a specialized cell for nitrogen fixation, called “heterocyst”. The heterocyst expresses the nitrogenase enzyme to catalyze the nitrogen fixation reaction, while it loses photosystem II, which is the water-oxidizing enzyme of photosynthesis, to avoid degradation of the active metal center of nitrogenase [3]. The vegetative cells cannot grow without reactive nitrogen compounds and the heterocyst is not capable of photosynthetic life maintenance; the vegetative cell and heterocyst are thus mutually dependent on their metabolites. Vegetative cells provide the photosynthesis products, such as sugars or organic acids, to the neighbor heterocyst. Instead, heterocysts give amino acids to vegetative cells. The metabolites are transported through a cellular junction of the vegetable cell and heterocyst.

The C/N ratio is a nominal parameter of biological samples. This is estimated conventionally by the combustion analysis, which quantitates amounts of CO₂ and N₂ by burning dried cells. It has been revealed that the heterocyst differentiation is triggered by a high carbon-to-nitrogen (C/N) ratio of a parental vegetative cell [3, 4]. However, the C/N ratio in the single cell level *in vivo* had never been observed so far. In this study, we observed nitrogen distribution in the heterocysts and vegetative cells of *Anabaena* sp. PCC 7120 by soft X-ray microscopy at the wavelengths shorter and longer than the nitrogen K-edge energy and estimated the C/N ratio of each cell *in vivo*.
2. Experimental
The cells of *Anabaena* sp. PCC 7120 were grown on a nitrogen-depleted agar plate. The filaments including heterocysts were suspended in pure water and tightly packed between two silicon nitride membranes with the thickness of 100 nm. The absolute position of each cell in the sandwich was determined by optical and fluorescence microscopy. The soft X-ray microscopic observation was performed at the wavelengths $(\lambda)$ of 2.98 and 3.11 nm (above and below N1s absorption threshold, respectively) using the full-field transmission soft X-ray microscopy beamline (BL12) in the SR centre of Ritsumeikan University [5]. The soft X-ray microscope is composed of a SiO$_2$ plane mirror for cutting higher-order X-rays, a condenser Fresnel zone plate, an order-sorting aperture, a sample, an objective Fresnel zone plate, and a back-illuminated CCD camera. The sample was located in air, separated from the vacuum chamber by two silicon nitride membranes. The spatial resolution was 110 nm at the magnification with 600 that was estimated by knife-edge method. The effective pixel size in the image was $40\times 40$ nm$^2$. The exposure time was fixed to 3 min.

3. Results and Discussion
Figures 1 (a) and (b) are the images of heterocysts and vegetative cells of *Anabaena* sp. PCC 7120 taken by optical and fluorescence microscopy, respectively. Because heterocysts do not include photosystem II, whose chlorophylls are fluorescent even at room temperature, they do not emit the chlorophyll fluorescence (for example, the white circle in figure 1(b)). The heterocysts and vegetative cells were observed as a monolayer both in the optical and fluorescence images. Thus, their absolute positions could be determined in the silicon nitride membranes. Each cell has a barrel shape with the major and minor axes of approximately 4.5 and 3.3 $\mu$m, respectively.

The soft X-ray microscopic images corresponding to the centre part of Fig. 1 are shown in Figs. 2 (a) and (b) with below and above threshold of N K-edge absorption, respectively. From these images, we could observe clear absorbance differences between heterocysts and vegetative cells.

![Figure 1. (a) Optical and (b) fluorescence images of heterocysts and vegetative cells](image1.jpg)

![Figure 2. Soft X-ray images of heterocysts and vegetative cells at (a) $\lambda = 3.11$ nm and (b) $\lambda = 2.98$ nm](image2.jpg)
According to the Lambert-Beer law \((A = -\log (I/I_0))\), we could get the absorbance \((A)\) at each cell from the soft X-ray image. Here, \(I\) and \(I_0\) are transmitted intensities with/without the sample, respectively. The intensity of a cell image in Fig. 2 corresponds to \(I\). On the other hand, \(I_0\) could be assumed as the intensity of an area without cells, since the membrane is large enough \((250 \times 250 \, \mu m^2)\) to show several areas without cells, as shown in Fig. 2.

To statistically analyse the difference in the soft X-ray absorbance between heterocysts and vegetative cells, we collected several images of the cells. The resultant absorbances are summarized as the histograms, which is shown in Fig. 3. The absorbance used here is the area average of the cell images. The standard deviation to determine the absorbance in this way is \(\sim 15\%\). Totally, 131 vegetative cells and 54 heterocysts were sampled. The mean values and variances of the histogram are different between heterocysts and vegetative cells at both 3.11 and 2.98 nm of the illumination wavelengths. The mean values of absorbance of heterocyst and vegetative cell were 0.73 and 0.53 at \(\lambda = 3.11\) nm, respectively, while those were 0.51 and 0.44 at \(\lambda = 2.98\) nm. At each wavelength, absorbance of heterocyst is higher than vegetative cell, indicating that the contents of cellular compounds are different.

The total absorbance with different several components at specific wavelength is defined as follows:

\[
A(\lambda) = \sum_i A_i (\lambda) = \sum_i \varepsilon_i c_i \ell \tag{1}
\]

Here \(A(\lambda)\) is absorbance at wavelength \(\lambda\). \(A_i (\lambda), \varepsilon_i, c_i\) are absorbance, absorption cross section, concentration of the \(i\)-th element, respectively. \(\ell\) is the optical path length and is the thickness of the cell in the present study. The main elements contributing to the images below and above of N1s absorption-edge are C and N in the cells, because the O1s absorption threshold is far higher than N1s absorption threshold. We thus assumed the absorption of O-element as a negligible constant at the observed wavelengths and ignored it in the following analysis. From these considerations, the formula (1) can be simply rewritten as follows:

\[
A(\lambda) = (\varepsilon_C (\lambda)c_C + \varepsilon_N (\lambda)c_N) \ell \tag{2}
\]

The relationship between absorbance and concentration ratios of two elements, C and N, can be derived from absorbance at two different wavelengths \((\lambda_1, \lambda_2)\), as follows:

\[
\frac{A(\lambda_1)}{A(\lambda_2)} = \frac{\varepsilon_C (\lambda_1)c_C + \varepsilon_N (\lambda_1)c_N}{\varepsilon_C (\lambda_2)c_C + \varepsilon_N (\lambda_2)c_N} \tag{3}
\]

\[
R = \frac{c_C}{c_N} \tag{4}
\]

Figure 3. Histograms of the absorbance of heterocysts (red) and vegetative cells (black) from soft X-ray imaging at (a) \(\lambda = 3.11\)nm and (b) \(\lambda = 2.98\)nm.
Here $R$ is the same as the well-known C/N ratio. From the observed soft X-ray images, we calculated the absorbance ratio of the individual cells between 3.11 and 2.98 nm-wavelengths, and summarized as a histogram in Fig. 4(a), which exhibits a distinct difference between vegetative cells and heterocysts. We could obtain the C/N ratio by using formula (3) and reference values of absorption cross-sections of C and N in the database [6]. Figure 4(b) is the histogram of C/N ratios in each cell. The mean values of the C/N ratio in vegetative cells and heterocysts are 4.54 and 2.46, respectively. Since there is no report about the C/N ratio of Anabaena sp. PCC 7120, we compared with the same kind of photosynthetic bacteria from the reference [7]. The C/N ratio of Anabaena cylindrica, which is a closely related species of Anabaena sp. PCC 7120, is around 4.5 to 8 and this value is close to our results, indicating that our technique with soft X-ray imaging is useful to determine the cellular C/N ratio in vivo.

4. Conclusions
The filamentous and heterocystous cyanobacterium Anabaena sp. PCC 7120 was observed by soft X-ray microscopy. We derived the simple formula to estimate the carbon-to-nitrogen (C/N) ratios of the individual cells in vivo in this study and applied it to the observed images. The estimated C/N ratios of the vegetative cells and heterocysts were 4.54 and 2.46, respectively.

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