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Cadmium Bioaccumulation in *Tetraselmis suecica*: An Electron Energy Loss Spectroscopy (EELS) Study

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Abstract. Electron energy loss spectroscopy (EELS) was used to study the distribution of cadmium within the microalga *Tetraselmis suecica* when submitted to cadmium contamination. This analytical technique, which is associated to transmission electron microscopes, demonstrated that cadmium was stored specifically in the osmiophilic vesicles of *T. suecica*. The EELS study of the oxidation states revealed that cadmium was stored as Cd^{2+}. In addition, the EELS quantification showed a significant relationship between cadmium, nitrogen, and sulfur concentrations. The toxic element is probably bounded to organic molecules via S-Cd bounds.

Cadmium is a very toxic element, particularly for humans (Goering *et al.* 1995). It has also been proven to be harmful in aquatic systems for numerous organisms, such as macroalgae, mollusks, and crustaceans (Phillips 1990). Even at low concentrations, microorganisms (in particular microalgae) are very sensitive to the toxic effects of this pollutant (Berland *et al.* 1976; Rachlin *et al.* 1983; Smith 1983; Mazurek *et al.* 1992; Rachlin and Grosso 1993), which disturbs the equilibrium of ecosystems. In addition, the ecotoxicologic risks are increased by the large accumulation of this pollutant by the phytoplankton (Lin *et al.* 1991; Wikfors *et al.* 1994). This last process contributes to the transfer of cadmium to organisms of higher trophic levels. It is therefore of interest to study the accumulation and chemical forms of storage of this metal in microorganisms.

Global analysis can establish the accumulation of cadmium by microalgae. However, knowledge of the subcellular location of cadmium will provide essential information on metal toxicity and bioaccumulation mechanisms. X-ray microanalysis has been widely used to study such mechanisms. However, for several microalgae, intracellular metal detection was unsuccessful, probably due to a lack of sensitivity of the X-ray microanalysis. To palliate this problem, electron energy loss spectroscopy (EELS) can be used as an alternative method of cadmium detection and quantification in biological specimens. This technique, although not very often used in ecotoxicologic studies, offers many advantages. First, EELS presents a better energetic resolution and a higher sensitivity than X-ray microanalysis (Castaing 1987). Second, it allows detection and quantification of light elements (Egerton 1986; Budd and Goodhew 1988), which generally are implicated in the storage of heavy metals. Finally, EELS permits the determination of the oxidation states of ionized elements (Leapman *et al.* 1982; Mansot *et al.* 1994). However, since EELS spectra are more complicated than those of X-ray microanalysis, great care must be taken, especially in the case of cadmium-$M_{4,5}$ edges detection. These edges are partially overlapped by nitrogen-K edge, allowing the cadmium presence to pass unobserved.

For this study we used EELS to detect and quantify cadmium and other elements implicated in the metal storage in *Tetraselmis suecia*. This marine microalga is able to accumulate large amounts of cadmium from the contaminated medium (Nassiri *et al*. 1996).

Materials and Methods

Culture Condition and Metal Contamination

T. suecica was obtained as a pure isolate from Laboratoire de Biologie Marine, collection of algae, Université de Nantes. This strain was isolated from Bouin Bay (France). It grows on filtered (0.45 µm) underground salt water, which is widely used for the mass production of algae (Baud and Bacher 1990; Pirastru 1994). The Cd background concentration in the filtered salt water was very low (0.0001 mg/L). It was determined immediately after collection from the Bouin station (France) by means of a Hitachi Z 8200 atomic absorption spectrometer, following the method described by Amiard *et al.* (1991). Various graded concentrations (0, 0.05, 0.1, 0.2, 0.5, 1, 2, 5, 10 mg/L) of cadmium were tested on duplicate cultures. The cells were grown under the conditions described by Nassiri *et al.* (1996). Initially, the number of cells per ml was 10,000. After 4 days of exposure, growth inhibition

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was observed from a concentration of 2 mg/L, which causes 10% reduction, while 5 and 10 mg/L induce 30 and 53% growth inhibition, respectively. Samples of each 4-day culture were taken, filtered on 3-µm Millipore membrane, and prepared for conventional and analytical transmission electron microscopy.

Ultrastructural Examination and EELS Analyses

In order to reveal resultant ultrastructural change or damage following cadmium exposure, samples were pre-fixed using 3% glutaraldehyde in underground salt water and then washed with a cacodylate (0.2 M) buffer. Cells were post-fixed with 1% osmium tetroxyde. After dehydration, *T. suecica* cells were embedded in Spurr's medium. Ultrathin sections (30–40 nm) were obtained using a Reichert MU3 ultramicrotome fitted with a diamond knife. The sections were placed on uncoated 300-mesh grids and were stained with an alcoholic solution of uranyl acetate and an alkaline bismuth solution (Knight 1977). Sections were examined using a JEOL 100 CX transmission electron microscope.

For electron energy loss spectroscopy investigations, the samples were prepared without post-fixation and staining. Experiments were carried out on a Philips CM 30 electron microscope running at 200 kV with an undersaturated LaB_6 cathode. The EEL spectra were recorded in the diffraction coupling mode by means of a Gatan 666 parallel spectrometer. The condenser, objective, and spectrometer entry aperture diameters were, respectively, 100 µm, 50 µm, and 2 mm, leading to a convergence half-angle of 0.3 mrad, a collection half-angle of 12 mrad, and an energy resolution of 1 eV (FWHM of the zero loss peak). The EEL spectra were recorded with an energy dispersion of 0.5 eV per channel on areas of about 100-nm diameter. In these experimental conditions, the limit of detection of analyzed elements is estimated to be 0.5%, according to the study of Castaing (1987) on the EELS technique.

The quantitative analyses were performed using a Gatan EL/P software. The concentrations of cadmium and other elements (sulfur, chlorine, calcium, nitrogen, and oxygen) involved in pollutant storage are estimated as an element/carbon atomic ratio, following the procedure of Egerton (1986). In this case, the background is fitted with a power-law function ($A \cdot E^{-r}$, where E is the energy loss, and A and r are extrapolated constants). The atomic fraction is obtained by the following expression:

$$\frac{N_X}{N_C} = \frac{I_X(\Omega, \Delta E) \cdot \sigma_C(\Omega, \Delta E)}{I_C(\Omega, \Delta E) \cdot \sigma_X(\Omega, \Delta E)}, \quad (1)$$

where N is the number of atoms of an element (Cd, N, S, etc.) in the area analyzed, I (Ω, ΔE) is the intensity of a characteristic edge of an element with an integration window ΔE for a spectrometer collection angle Ω, and σ is the tabulated inelastic cross section of an element partially integrated on the energy window ΔE and in the collection angle Ω. Since the inelastic cross section of cadmium ($M_{4,5}$ edges) is theoretically unknown, it was experimentally determined using cadmium sulfide (CdS) as a standard.

According to equation (1),

$$I_{Cd}/I_S = N_{Cd}\, \sigma(\Omega, \Delta E)_{Cd}/N_s\, \sigma(\Omega, \Delta E)_S \quad (2)$$

$$= A(\Omega, \Delta E)_{Cd}/A(\Omega, \Delta E)_S,$$

where A represents the integral of a characteristic edge of an element, and $A(\Omega, \Delta E)_{Cd}$ and $A(\Omega, \Delta E)_S$ represent the areas obtained by integration of the edges of Cd and S on the same energy windows (here 60 eV). In the standard CdS, $N_{Cd}/N_S = 1$,

$$\rightarrow A_{Cd}/A_S = \sigma_{Cd}/\sigma_S = 1/7.9 \qquad \text{(determined experimentally)}$$

$$N_{Cd}/N_C = N_{Cd}N_S/N_SN_C, \qquad \text{from equation (2)}$$

$$\rightarrow N_{Cd}/N_C = A_{Cd}\sigma_S A_S \sigma_C / A_S \sigma_{Cd} A_C \sigma_S$$

since $\sigma_S/\sigma_{Cd} = 7.9$ and $\sigma_C/\sigma_S = 0.157$ (calculated from EL/P)

$$\boxed{\rightarrow N_{Cd}/N_C = A_{Cd}/A_C \times 1.24.}$$

The integral under the Cd-$M_{4,5}$ edges and the integral under the C-K edge permit calculation of the Cd/C ratio. The precision estimation for atomic fractions was determined experimentally from spectra (ten for each element) recorded on the analyzed area. This precision was about 5% for O/C, N/C, S/C, Cl/C, and Ca/C determinations. In the case of cadmium (Cd/C), it reached 10% due to the supplementary experimental determination of the inelastic cross-section (σ_S/σ_{Cd}) ratio from spectra (10) recorded on standard CdS.

Results

The fine structure of control cells was described by Manton and Parke (1965). Figure 1 shows an electron micrograph of a control cell from a culture in its exponential growth phase (4 days). Up to a cadmium concentration of 1 mg/L, growth was unaffected during the test period. The ultrastructure of contaminated *T. suecica* remains equivalent to that of the control. From a cadmium concentration of 2 mg/L, the growth was inhibited when compared with the control culture. At an ultrastructural level, contaminated cells do not present any damage, as reported in Nassiri *et al*. (1996). For a cadmium concentration of 5 mg/L, the fine structure is also well preserved, and only the osmiophilic vesicles are changed; the number and volume appear to increase as a consequence of cadmium contamination (Figures 2 and 3). These changes were observed in 80% of the analyzed cells ($n = 50$). In the case of Cd 10-mg/L contamination, such vesicle evolution was observed at the same frequency (80%) in analyzed cells.

EELS investigations are mainly concerned with osmiophilic vesicles. The evolution of such structures appears to be a specific response to cadmium contamination. With the objective to study the accumulation of cadmium within the "whole" cells, our investigations were concerned with cadmium (5 mg/L) contaminated cells, rather than cadmium (10 mg/L) exposed cells. Indeed, in the case of 10-mg/L cadmium contamination, *Tetraselmis suecica* developed another detoxification process that induced the release of organic material which constitutes an adsorbing area to cadmium, reducing metal-free ion concentration in the medium (Nassiri *et al*. 1996).

A typical spectrum (Figure 4) recorded on the osmiophilic vesicles of control cells shows that these structures are only constituted of the common light elements (C, N, O). It is important to notice that, after background subtraction before the N-K edge, the last one decreases monotonously as a function of energy. After cadmium (5 mg/L) contamination, osmiophilic vesicles present strong enrichment in chlorine and sulfur (Figure 4). Calcium is occasionally stored in these vesicles. This was revealed in 8% of the analyzed vesicles ($n = 50$), while in the control cells this element was never detected. In the low and high loss regions ($N_{2,3}$, $N_{4,5}$ minor edges and $L_{2,3}$

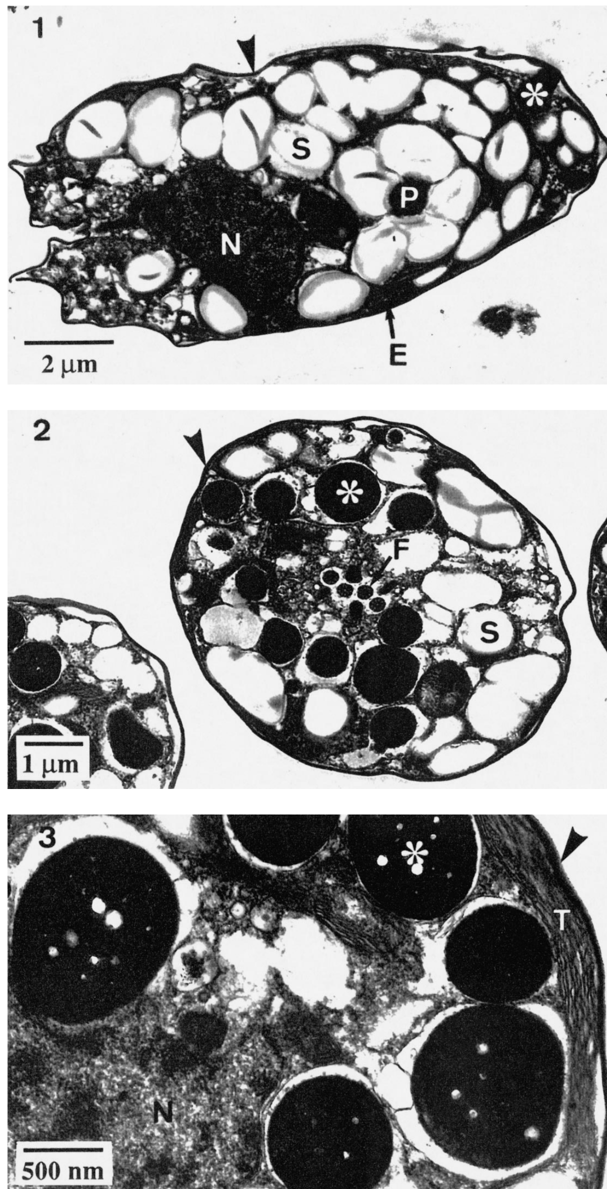

Fig. 1. Longitudinal median section of control cell. Arrowhead = cell wall, asterisk = osmiophilic vesicle, E = eyespot, N = nucleus, P = pyrenoid, S = starch vesicle

Fig. 2. Anterior median section of cadmium (5 mg/L) contaminated cells. The organelles are well preserved. Note the increase in number and volume of osmiophilic vesicles (asterisk). Arrowhead = cell wall, F = flagella, N = nucleus, S = starch vesicle

Fig. 3. Detail of osmiophilic vesicles (asterisk) of cadmium (5 mg/L) treated cells. These structures become electron-dense and finely granulated. Arrowhead = cell wall, N = nucleus, S = starch vesicle, T = thylakoids

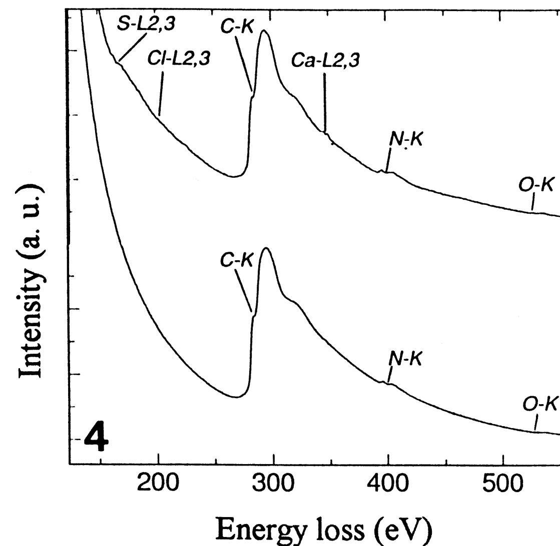

Fig. 4. EEL spectra recorded on osmiophilic vesicles of Cd (5 mg/L) treated cells (above) and also on control cells (below). After cadmium contamination, vesicles are enriched by calcium, chlorine, nitrogen, and sulfur

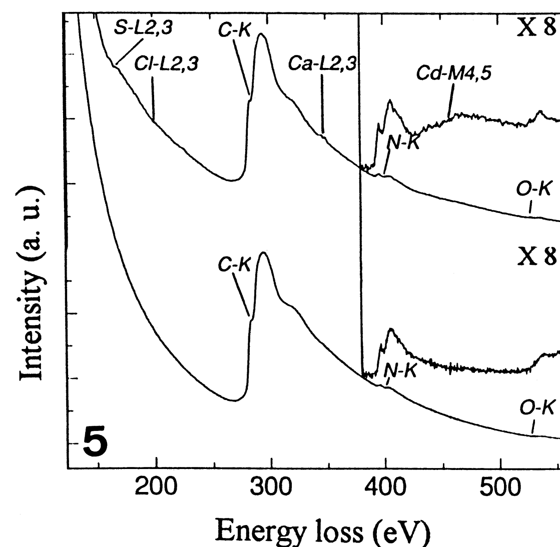

Fig. 5. After background subtraction before the N-K edge from spectra presented in Figure 4, Cd-$M_{4,5}$ edges are clearly visible in the EEL spectrum (above) recorded on cadmium (5 mg/L) treated cells. In the osmiophilic vesicles of control cells (below), only the N-K edge is recorded in this energy loss region. The magnification of potential regions for the detection of Cd-$M_{4,5}$ edges is superimposed on to the original spectrum

edges), no cadmium signals were easily evidenced; therefore, Cd-$M_{4,5}$ edges were specifically investigated. After the subtraction of the background before the N-K edge, the resulting spectrum clearly showed the presence of the Cd-$M_{4,5}$ edges, demonstrating the existence of cadmium in osmiophilic vesicles (Figure 5). In order to quantify unambiguously the cadmium in contaminated samples, the N-K and Cd-$M_{4,5}$ edges contributions were separated. For this purpose, subtraction of the background in an energy window of about 15 eV in the decay region of the N-K edge (Figure 6) allowed us to obtain the Cd-$M_{4,5}$ contribution. The comparison of Cd-$M_{4,5}$ edge shapes obtained for the standard (cadmium oxide) and cadmium in osmiophilic vesicles demonstrated clearly the presence of cadmium as Cd^{2+} in these structures (Figure 7). EELS examination of the nucleus and the other cytoplasmic organelles revealed that these structures are devoid of cadmium. From

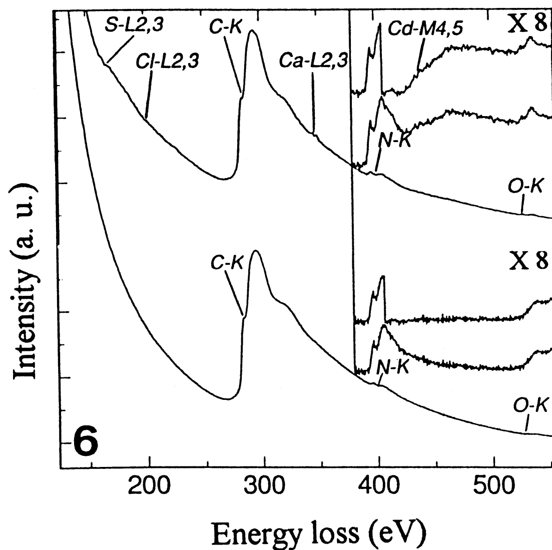

Fig. 6. Subtraction of background in the N-K edge regions from the preceding spectra (Figure 5) reveals a clear signal solely due to cadmium. The measurement of the integral beneath the Cd-$M_{4,5}$ edges in an energy region of 50 eV allows quantification of the cadmium concentration in the Cd (5 mg/L) contaminated cells. Analyses of control samples demonstrated an absence of cadmium, as shown in the lower spectrum. The magnification of potential regions for the detection of Cd-$M_{4,5}$ edges is superimposed on to the original spectrum

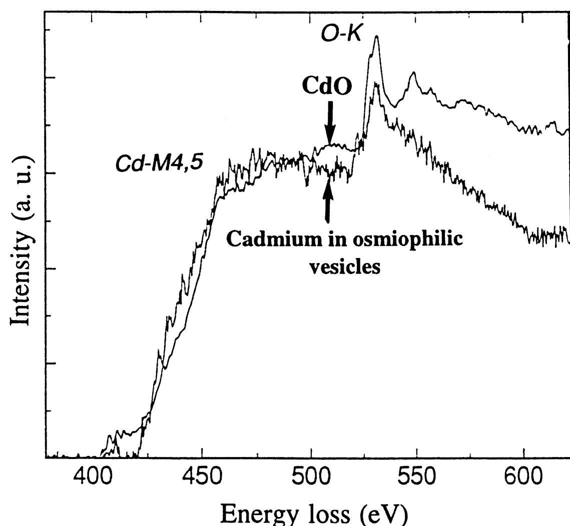

Fig. 7. Cd-$M_{4,5}$ edges recorded on standard (CdO) and on osmiophilic vesicles of cadmium (5 mg/L) treated cells. The similarity of the Cd-$M_{4,5}$ edges indicates that cadmium is stored as Cd^{2+} within *T. suecica*

these observations osmiophilic vesicles of *T. suecica* can be considered as the intracellular sites of metal storage.

After the background subtraction (Figure 6), the integral under Cd-$M_{4,5}$ was determined, allowing us to quantify the Cd/C ratio as described above (Materials and Methods). The Cd/C, Cl/C, N/C, O/C, and S/C atomic ratios in osmiophilic vesicles of treated samples (5 mg/L) obtained after analyses of different cells ($n = 50$) are presented in Figure 8. As evidenced by the *T*-test, there is significant correlation only between the Cd/C and S/C and between the Cd/C and N/C in osmiophilic vesicles. This suggests that nitrogen and sulfur are involved in cadmium storage. The concentration of Cd represents 1.5 to 3.8% of that of C in analyzed areas, while N and S represent, respectively, 11.5 to 24.1% and 2.9 to 6.1%.

Discussion

In previous work (Nassiri 1996), we established that the effect of cadmium on cell growth, estimated after 4 days of exposure, becomes evident from a metal concentration of 2 mg/L. The inhibition of growth occurs without any discernible ultrastructural damages. This phenomenon, which was reported for many other algae (Henry *et al.* 1991; Visviki and Rachlin 1991), pointed out that growth is a highly sensitive indicator of cadmium toxicity. Nevertheless, cadmium exposure induces evolution of osmiophilic vesicles, which both number and volume seem to increase in response to cadmium contamination. The resulting low cytotoxicity of cadmium involves the existence or the development of detoxification processes, which are characterized by the evolution of osmiophilic vesicles in contaminated cells. In fact, from a metal concentration of 5 mg/L, intracellular cadmium sequestration is observed only in these structures. The shape and the energy position of the Cd-$M_{4,5}$ edges indicates that the metal is stored as Cd^{2+}. The incorporation of cadmium within the cells suggests that some toxic effects of this heavy metal are a result of the interaction with endogenous cellular constituents.

After 5-mg/L cadmium exposure, osmiophilic vesicles are also enriched in chlorine, nitrogen, and sulfur; calcium is also occasionally stored, while in the osmiophilic vesicles of control cells this element is absent. After cadmium contamination, calcium is probably stored specifically in a few specialized vesicles. Such behavior is comparable to that observed in the granules or lysosomes of many invertebrates that store specifically essential elements. It was suggested that the toxic cation which accumulates in the cells may alter Ca^{2+} metabolism, inducing an increase of free Ca^{2+} in the cytosol. The excess of Ca^{2+} could be sequestered in membrane limited vesicles (George and Pirie 1980; Viarengo and Nicotera 1991; Viarengo and Nott 1993). After copper contamination, osmiophilic vesicles can also contain phosphorus (Ballan-Dufrançais *et al.* 1991). Copenetration of pollutants and other essential elements or pollutants was reported for *Chlorella ellipsoidea*, *Dunaliella salina*, *T. suecica*, and *Scenedesmus* (Stokes 1975; Ballan-Dufrançais *et al.* 1991; Okamura and Aoyama 1994; Visviki and Rachlin 1994), suggesting that metals affect the permeability of the plasma membrane. In the case of contaminated *T. suecica*, EELS quantification demonstrated a significant correlation between the concentration of Cd^{2+} and those of nitrogen and sulfur. These common elements are probably due to heavy metal–binding polypeptides, which are known to chelate cadmium in many microalgae (Robinson 1989; Wikfors *et al.* 1991). These cysteine-rich polypeptides can trap pollutants, reducing the concentration of cytosolic, free-metal ions. If that is the case, the combination metal-polypeptides are metabolized within the vesicles. These latter can be compared to lysosomal system vesicles. Indeed, it was suggested that such molecules involved in metal storage can be sequestrated in a lysosomal or vacuolar system (Heuillet *et al.* 1986; Viarengo and Nott 1993).

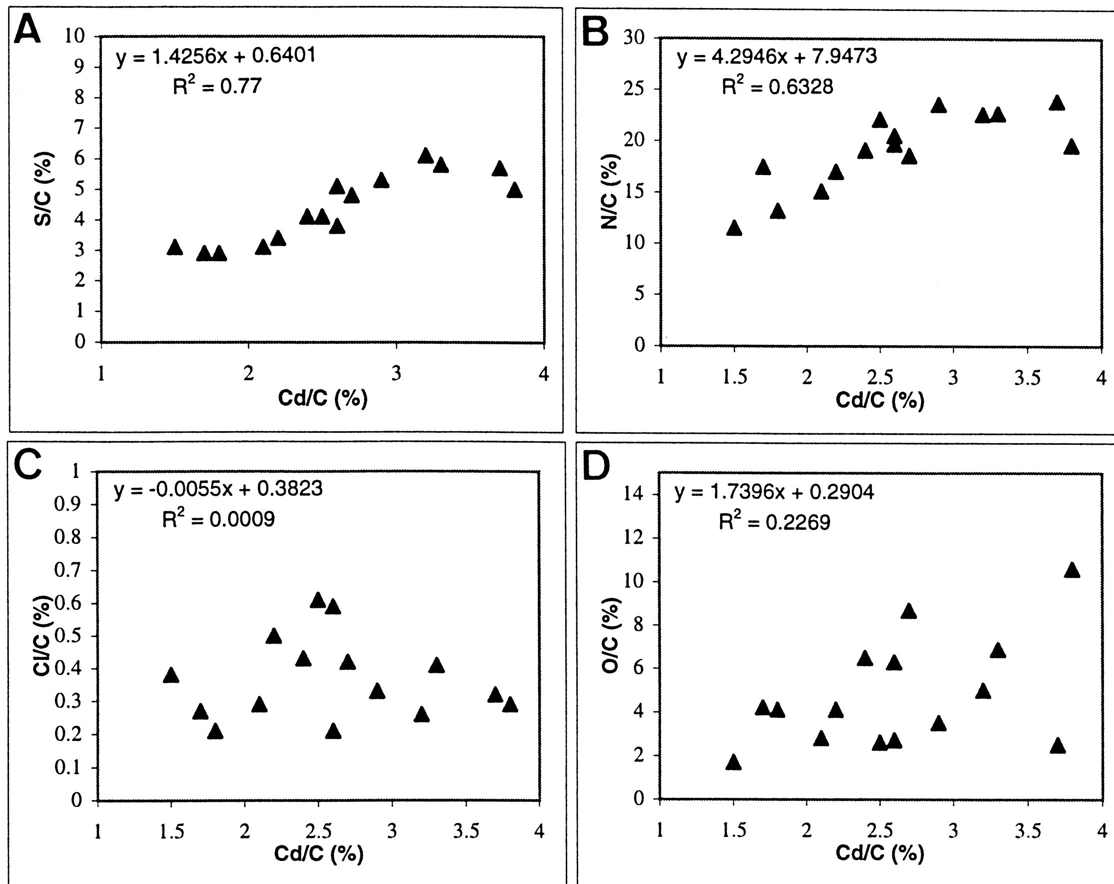

Fig. 8. ELLS quantification of cadmium concentrations (Cd/C) and elements detected in osmiophilic vesicles of Cd (5 mg/L) contaminated *T. suecica*. (A) Variation of S/C atomic ratios as a function of cadmium concentrations (Cd/C). There is a significant relationship between cadmium and sulfur concentrations ($R = 0.88$; T-test, $P < 0.05$). (B) Variation of N/C atomic ratios as a function of cadmium concentrations (Cd/C). There is a significant relationship between cadmium and nitrogen concentrations ($R = 0.8$; T-test, $P < 0.05$). (C) Variation of Cl/C atomic ratios as a function of cadmium concentrations (Cd/C). There is no significant relationship between cadmium and chlorine concentrations ($R = 0.03$; T-test, $P > 0.05$). (D) Variation of O/C atomic ratios as a function of cadmium concentrations (Cd/C). There is no significant relationship between cadmium and oxygen concentrations ($R = 0.47$; T-test, $P > 0.05$)

As a conclusion, EELS can be used to detect and quantify efficiently cadmium in biological specimens, even when partially masked by nitrogen, which "unfortunately" evolves in concentrations similar to that of cadmium in analyzed sites. In addition, the oxidation states of stored pollutants can be determined using electron energy loss near-edge structures, which would be of interest in the evaluation of metal toxicity to organisms of a higher trophic level.

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