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SCANNING ELECTRON MICROSCOPY (SEM) OBSERVATIONS OF DEFORMITIES IN SMALL PENNATE DIATOMS EXPOSED TO HIGH CADMIUM CONCENTRATIONS[1]

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Running title: DEFORMITIES IN SMALL DIATOMS.

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Abstract: Different types of malformations are likely to affect the morphology of diatoms when exposed to particularly unstable environmental conditions, the most easily identifiable being distortion of the whole frustule. In the present study, we investigated by SEM means valve abnormalities induced by a high cadmium contamination (100 $\mu g \cdot L^{-1}$) in small Pennate diatoms. Changes in the shape of *Amphora pediculus* (Kützing) Grunow and anomalous sculpturing of the cell wall of many species like *Encyonema minutum* (Hilse *in* Rabenhorst) D.G. Mann, *Mayamaea agrestris* (Hustedt) Lange-Betralot, *Gomphonema parvulum* (Kützing) Kützing or *Eolimna minima* (Grunow) Lange-Bertalot were observed, which were not or almost not noticeable under LM. With consideration to current knowledge of diatom morphogenesis, metal uptake by the cell would induce, directly or indirectly, damages to many cytoplasmic components (e.g. microtubules, cytoskeleton, Golgi-derived vesicles) involved in the precisely organized silica deposition. This study confirms that many species, whatever their size, are likely to exhibit morphological abnormalities under cadmium stress, and that this indicator may be valuable for the biomonitoring of metal contaminations, even if SEM observations are not necessary for routine studies.

Key index words: Artificial streams; cadmium; diatoms; metals; morphological abnormalities ; SEM.

Malformed diatom cells have been observed in many field and laboratory studies (e.g. Yang and Duthie 1993; McFarland et al. 1997; Nunes et al. 2003). Deformities can be initiated or observed at many different stages throughout the life cycle, ranging from the development of the initial cell to the routine activity of mitotic cell division (Pickett-Heaps et al. 1990). As the silicate shell (frustule) is deposited after cellular functional changes, the success of frustule formation is reflected in the physiological condition of the living cell at that time and the inherent functioning of specific organelles. Abnormalities in frustule formation would presumably be evidence of negative causative agents such as physicochemical stressors in the habitat (pollution by toxics; silica starvation; extreme pH, temperature or light; etc.), or mechanical causes like crowding, parasitation or genetic causes (cells associated with minimal sizes, genetic mutations).

Most of our initial light microscopy observations, from a large sample set (n=745), lead us to believe that diatom deformities appear to be rather universal among genera and presumably species. However it is conceivable that some taxa are more easily deformed under varying levels of environmental stress. For example, specific types of malformations are more often reported in some genera than others, e.g. elongate cells belonging to the family Fragilariaceae Greville (Feldt et al. 1973; McFarland et al. 1997; Dickman 1998; Ruggiu et al. 1998; Nunes et al. 2003; Cattaneo et al. 2004; Cremer and Wagner 2004; Morin et al. 2007). Taxa within the family Naviculaceae Kützing also exhibit elongated cell forms (Gomez and Licursi 2003; Schmitt-Jansen and Altenburger 2005), while species in the Bacillariaceae Ehrenberg have entire frustules that are twisted (Subba Rao and Wohlgeschaffen 1990; Estes and Dute 1994). Deformities not only refer to distorted forms, but also to abnormal valve ornamentations (aberrations), which may or may not be associated with misshapen valves. Aberrations in the sculpturing of the valve surface of Centrics include, the wrinkled central area (Rijstenbil et al. 1994; Morin et al. 2007) or irregularily orientated areolae (Yang and Duthie 1993). Pennate

diatoms can exhibit locally and globally irregular striations (Andresen and Tuchman 1991; Dickman 1998; Gomez and Licursi 2003; Morin et al. 2007), whereas taxa within the Fragilariaceae show a displacement or undulation of the axial area (McFarland et al. 1997; Morin et al. 2007), Species in the Nitzschiaceae have many aberrations including breaks in the keel structure (Barber and Carter 1981; Morin et al. 2007), fragmented raphe aberrations (Estes and Dute 1994; Gomez and Licursi 2003), or displacement and unexpected curved branches in the raphe (Estes and Dute 1994).

Heavy metals have been suspected to be a significant factor in the appearance of diatom deformities (McFarland et al. 1997; Dickman 1998; Ruggiu et al. 1998). During an experiment conducted in freshwater microcosms exposed to high cadmium concentrations (100 $\mu g \cdot L^{-1}$), up to 30‰ of the exposed diatoms had aberrant frustules, whereas deformed valves were recorded in <10‰ of the valves observed in the non contaminated control microcosms (Morin et al. 2008b). In this particular study, morphological deformities affected 21 diatom species representing 10 genera, most of them belonging to the Raphid group (the genus *Nitzschia* in particular) and to the Araphids (*Fragilaria* spp. and *Ulnaria* spp.). Abnormalities were less abundant in Monoraphids and Centrics. According to the literature, very few small species were affected by deformities (Peres 2000). Based on these observations, we decided to investigate by SEM means whether the smaller species were affected by deformities which could not be observed under light microscopy. We also examined the relationship between obvious valve deformations and the smaller more subtle aberrations.

Microcosms were used to grow and study the diatoms under controlled conditions; they consisted of experimental channels in which biofilms developed on glass slide substrates (Morin et al. 2008b). The microcosms had continuous water circulation (velocity: 0.4 $cm \cdot s^{-1}$) and were incubated under approximately 70 $\mu mol \cdot s^{-1} \cdot m^{-2}$ illumination with a light / dark cycle

of 10 h / 14 h. A natural biofilm sample collected from the field in the Riou Mort River (South West France, 44°N / 2°E) was inoculated into the experimental systems and allowed to colonize the artificial substrates in the microcosms. In these systems, the biofilms formed are plurispecific, their specific composition corresponding to those from the stream of origin. The reservoir supplying the experimental channels was filled with 40 L of four-fold diluted Woods Hole culture medium without EDTA and supplemented with silica, according to Gold et al. (2003). Nitrate and phosphate concentrations in the system were typical nutrient levels found in the Riou Mort River during 2004 and 2005 (Morin et al. 2008a). The system was contaminated with a cadmium chloride solution ($CdCl_2$, Merck, Darmstadt, Germany) to reach a nominal test concentration of 100 $\mu gCd \cdot L^{-1}$, which approximately corresponds to extreme values recorded in the Riou Mort watershed by the GEMA team, University Bordeaux 1. We examined one sample exposed to 100 $\mu gCd \cdot L^{-1}$ for six weeks (treatment 1), a second sample that was exposed to 100 $\mu gCd \cdot L^{-1}$ for the last 4 weeks of the 6 weeks study (treatment 2), and the final sample was collected on 01 Feb, 2005 from the Riou Mort River below a former zinc ore treatment plant which created an average level of 26.2 $\mu gCd \cdot L^{-1}$ of annual exposure (treatment 3).

During the 6-week incubation, physical and chemical variables of the water (temperature, pH, electrical conductivity, dissolved oxygen concentration and saturation) were determined daily at the end of the light cycle (WTW, Weilheim, Germany). Nutrient (orthophosphate, nitrate) concentrations were analyzed weekly according to French and international standards (NF T90-023 and NF EN ISO 13395, respectively). Cadmium concentrations were determined by atomic absorption spectrophotometry (Varian AA400 – Zeeman correction, Victoria, Australia) after filtration and acidification with HNO_3 of water samples. The detection limit was 0.1 $\mu gCd \cdot L^{-1}$ and the values were consistently within the certified ranges (data not shown). Depending on the results of the analyses, additions of culture medium or cadmium

were realized as required to compensate for the decrease of nutrient or metal concentrations due to algal scavenging.

After a six-week colonization period, the glass slides were removed from the artificial streams. Both faces of the glass substrates were scraped and analyzed for metal concentrations and microscope observation of diatoms. Cadmium uptake was assayed following two protocols: (i) one aliquot was used for the determination of total metal content in the biofilm, (ii) the other aliquot was washed for 10 minutes with 4 nM EDTA at pH=8 to determine the intracellular metal content of the periphyton (Behra et al. 2002). After filtration and mineralization of each sample (after washing with EDTA or not), cadmium concentrations were measured by atomic absorption spectrophotometry (Varian AA400 – Zeeman correction, Victoria, Australia) and by autosampler, with a 0.1 $\mu gCd \cdot L^{-1}$ detection limit.

The sample fraction devoted to SEM observation of diatoms was preserved in a buffered formaldehyde solution and prepared according to Charles et al. (2002). After digestion of the organic content of the cells in boiling hydrogen peroxide (30% H_2O_2) and hydrochloric acid (35% HCl) followed by three cycles of centrifugation of the sample and pellet rinsing with distilled water, the cleaned material was analyzed for cadmium determination – to ensure that cadmium uptake corresponded to cadmium accumulated in the cell and not bound to or associated with the frustules. Cleaned subsamples for scanning electron microscopy study were either (a) dried onto aluminium foil or (b) filtered through 8 and 0.22 µm Nucleopore filter paper (1 cm diameter; Whatman, Kent, ME, England), then mounted on aluminum stubs using double sided carbon tape. The stubs were subsequently gold coated with ~500 Å of gold and examined using an FEI XL30 tungsten filament environmental SEM (FEI Company, Hillsboro, OR, USA). Samples were surveyed and photographed under high vacuum with accelerating voltages ranging from 5-25 kV and a working distance of ~10 mm. Small taxa (<50 µm) were selectively chosen for study. Transect surveys were initiated and numerical

counts with photomicrographs for the common taxa were collected. Ninety specimens per sample were evaluated. When necessary, samples were tilted between 10-45 degrees to enhance surface structure or improve secondary electron recovery. Digital images were stored under a TIFF format and the image aspect ratio was corrected from 1.1:1 ratio produced by this SEM, to 1:1 using Xlstretch conversion software (M.T. Otten, FEI Company). On occasion Adobe Photoshop® (Adobe, San Jose, CA, USA) was also used to correct the aspect ratio for individual images. The photomicrographs collected from this study are stored in the Diatom image collection at the Canadian Museum of Nature (CANA-I-28047-28317). Cadmium content in the biofilm as measured with both protocols revealed that living diatoms were exposed to high metal concentrations: the periphyton sequestered ~3200 µgCd·g, dry weight $^{-1}$, of which more than one third was accumulated in the living cells, not in the cell wall. The illustrations (Fig. 1) outline a few of the abnormalities that were recorded in this study. The shape of frustules for the small species *Amphora pediculus* (Kützing) Grunow was affected by the exposure to high cadmium concentrations: Fig. 1 A-B illustrates differences in symmetry between normal and impacted valves. SEM observations also revealed unsuspected abnormalities in the raphe structure of many diatoms exhibiting a normal outline of the valve. Valve thickness and interruptions of the raphe were observed for many species (*Encyonema minutum* (Hilse *in* Rabenhorst) D.G. Mann, *Eolimna minima* (Grunow) Lange-Bertalot or *Naviculadicta seminulum* (Grunow) Lange-Bertalot; not shown). Fig. 1 C-D illustrates one individual of *E. minutum* with one pole normal and the other formed with a break in the apical raphe fissure. The ends of the raphe fissure of *Gomphonema parvulum* (Kützing) Kützing (Fig. 1 E-F) display an unusual pattern of abnormalities. In the enlargement of the foot pole of *G. parvulum* (Fig. 1 F), many of the poroids of the apical pore field seem to be deformed or amalgamated. Local perturbations of the pore structure and arrangement of striae were also observed in small species like *Eolimna minima* (Fig. 1 H), with irregular striation (for

instance, doubling in striae) as well as deformed pores probably resulting in a fusion of abnormal pores. These anomalous patterns of local striae abnormalities were also observed in individuals of other small species, such as *Achnanthidium minutissimum* (Kützing) Czarnecki (not shown).

To quantify differences in abnormality after Cd exposure, we compared types and numbers of deformities in Cd exposed microcosms (treatments 1 & 2) and one river station (treatment 3) downstream of a former Zn ore treatment plant (Table 1). The deformities in small diatoms were most abundant (16%) in the highest Cd exposure (treatment 1) with the lowest number of deformities in the river site (4%) with a lower exposures to Cd (18.1 $\mu gCd \cdot L^{-1}$ in Feb, 2005). Morin et al. (2008b) have previously reported < 10‰ deformities in control mesocosms. *Mayamaea* spp. (including *M. agrestis* (Hustedt) Lange-Bertalot) and *Eolimna minima* sensu lato had the most pronounced deformities with modifications in valve form and the distal raphe fissures. It was sometimes difficult to evaluate distal raphe deformities in the genus *Mayamaea*, due to the variation in distal fissures typically observed under ephemeral environmental conditions (Lange-Bertalot et al. 2003). In contrast, *Nitzschia palea* consistently had valve deformities at one apex, although the distal fissures at these deformed apices could not be observed and evaluated. *Achnanthidium* spp. (including *A. minutissimum*), along with *Gomphonema parvulum* also displayed valve deformities at the apices. Although the raphe was often observed to be deformed, variations in shape were the most common deformity after Cd exposure.

Valve formation is under precise genetic control (Volcani 1981; Pickett-Heaps et al. 1990; Schmid 1994). Both membrane-mediated morphogenetic and macromorphogenetic mechanisms mould the wall features (for a review, Pickett-Heaps et al. 1990) in a selected

manner, creating a species-specific valve which constitutes the basis for diatom taxonomy. This modelling is the result of a precisely timed sequential interaction between the plasmalemma, cytoskeleton, endoplasmic reticulum, Golgi-derived vesicles and mitochondria (Schmid 1994). However, silicification in diatoms is also controlled by mass action effects, such as the amount of available soluble silicon in the environment during the time of a cell cycle (Claquin et al. 2002) as well as other environmental factors such as the presence of toxicants (Cohn et al. 1989; Spurck and Pickett-Heaps 1994).

In Pennates the initial area to develop is the sternum for araphid and raphid diatoms (Round et al. 1990). Mayama and Kobayasi (1989) and Mayama and Kuriyama (2002) have studied and proposed a diagrammatic representation of sequential valve formation: the deposition of the primary side of the sternum is followed by the reflexing of its ends at the pole and the centrifugal extension of the secondary arms from the central nodule, then raphe formation is completed by the fusion of the primary rib with the second arm. These processes involve microtubules running along the raphe (Pickett-Heaps et al. 1990); it is also the first area to show abnormalities in the teratological forms observed here. The administration of anti-microtubule drugs during early valve formation has induced teratology around the raphe area in some pennate diatoms, depending on the drug concentration (Schmid 1980; Cohn et al. 1989; van de Meene and Pickett-Heaps 2004). Oxidative stress, as a consequence of metal accumulation in the cytoplasm (elevated intracellular concentrations were measured in this study), has recently been shown to result in depolymerization of microtubules (Lee et al. 2005) and also in abnormal silica deposits within silica deposition vesicles (Safonova et al. 2007). This would have deleterious effects on macromorphogenesis of the forming valve whose characteristic shape is absolutely dependant on the location and structure of the raphe as well as on a “correct” silica supply and polymerization to quartz glass. Damage to the cytoskeleton causing malformation of the raphe might compromise the nanoscale uniformity

of the pore architecture: according to Mayama and Kuriyama (2002), deformation of areolae or striae patterns as well as other abnormalities occur later in development. These later forming abnormalities may be secondary effects controlled by oxidative stress-induced damage to cytoplasmic components involved in macromorphogenesis (Schmid 1994). The occasional appearance of abnormalities in the shape of frustules could be interpreted as damage by the metal and possibly a side effect of defence mechanisms against uptake of the toxicant (as shown for ultrastructural alterations by Torres et al. 2000), but these diatoms were still able to sustain positive growth, which has been interpreted by Schmitt-Jansen and Altenburger (2005) as a physiological adaptation of these species to the potential toxicant. This leads us to reflect on the morphological variants and the plasticity of diatoms: have these entities originated from a sample containing no deformed diatoms? If these are truly reproductively isolated populations, then these forms may merit a specific status. Towards this perspective, it would be important to distinguish whether the cytoskeletal oxidative damages are responsible for the abnormalities or whether a genetic effect (such as mutation) is also occurring, which inhibits or alters valve formation. Cadmium genotoxicity has been well documented (q.v. review in Bertin and Averbeck 2006), however it is still difficult to link or infer that the deformities would be a direct consequence of mutagenic effects, nor that the anomalous Cd-treated diatoms would be reproductively isolated.

It has been suggested that diatom deformities may be used as indicators for specific types of pollutions like metal contamination (Dickman 1998; Stevenson and Bahls 1999; Peres 2000; Fore and Grafe 2002), however this study leads us to reflect on what should be considered, for biomonitoring purposes, as a deformity. Adshead-Simonsen et al. (1981) observed morphological changes in populations of *Tabellaria flocculosa* (Roth) Kützing exposed to cadmium contaminations. More specifically, these authors observed *T. flocculosa* forming

straight chains instead of the common zigzag configuration. At the species level, many light microscopy observations have described abnormal individuals, differing from the normal forms in global shape or in rough features. The present SEM investigations outline that the manifestations of cadmium toxicity via the condition of the valve (shell) can be observed at various levels, from colonial population formation to individual valve morphology. Many mechanisms known to play a crucial role in cell division and cell wall formation are likely to be affected by high intracellular concentrations of Cd, and this work stresses that deformities are not limited to any one size class, although selected taxa appear to be differentially affected after exposure to differing metal concentrations.

Nevertheless the use of deformities for routine biomonitoring of metal contaminations necessitates selecting a tool that is easily applied to routine research. From the literature and results of this study, LM observations are appropriate for the evaluation of deformities created by metal pollution. However, the degree of overlap between distinct deformities and subtle aberrations on each valve must be considered, and SEM investigations would be required when subtle valve aberrations do not correlate with the larger valve deformations.

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1 Table 1. LM and SEM enumerations for three treatment exposures to Cd. Numbers presented with a separating slash display the # of observed

2 deformities / possible # of additional deformities. Identifications of possible deformities are based on minor aberrations which could easily be

3 considered part of the variation observed in a typical population.

	LM			SEM				
	Valves examined	# of deformed valves	# of small diatoms concerned	Valves examined	# of deformed valves	*A. minutissimum complex*	*E. minima* *M*. spp.	*N. palea*
Treatment 1	410	15	5	92	15/2	2/1	4	7/1
Treatment 2	420	14	1	90	6/2	0	6/2	0
River site Treatment 3	499	7	2	92	4/ 5	1	1/2	
Common deformity		valve shape, disrupted ornamentation	valve shape			deformed apices	valve shape, distal raphe	deformed apices

4

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Figure captions

Figure 1. SEM microphotographs of normal (nf) and abnormal (af) forms of the diatoms *Amphora pediculus*: A(nf)-B(af), *Encyonema minutum*: C-D(af), *Gomphonema parvulum*: E-F(af) and *Eolimna minima*: G(nf)-H(af).