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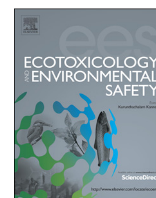

Ultrastructural alterations in *Phacus brachykentron* (Euglenophyta) due to excess of organic matter in the culture medium

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ABSTRACT

Morphological and ultrastructural changes induced by exposure to excess of organic matter were analyzed in *Phacus brachykentron* (Pochm.). The cells were isolated from sites in Matanza River, Buenos Aires, Argentina, which have a high degree of organic matter contamination coming from waste waters discharges of the meat industry. Master strains were cultured on soil water medium and a toxicity bioassay was performed. As a result of the enriched medium, several morphological and ultrastructural cellular alterations were observed by optical, scanning, and transmission electron microscopy. Among these, we can point out changes in cell dimensions, remarkable widening of some pellicle bands, increased number and volume of paramylon grains, displacement of the nucleus from the central to the lateral position, some chloroplasts with their thylakoids disordered, and cell lysis. The response to organic enrichment was very fast, i.e. during the 48 h of the bioassay. Therefore, any significant increase of organic matter would rapidly affect wild euglenoids. Our results suggest that the alterations observed, such as the presence of large intracellular paramylon bodies or the deformation of euglenoid cells in natural samples, have the potential to be used as environmental bioindicators.

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1. Introduction

Anthropogenic degradation of the aquatic ecosystem has drastically changed its chemical and physical properties, especially nutrient concentration (Paul and Meyer, 2001; Meyer et al., 2005). Excessive enrichment of freshwater bodies with organic matter is mainly caused by food, industrial, domestic, and agricultural processes. The increase of organic matter discharged into fresh water bodies alters nutrient levels, changing the concentration of compounds such as phosphates, nitrates, and chlorides, inducing modifications in the biota (Conforti et al., 1995; Dokulil, 1996; Conforti, 1998; O'Farrell et al., 2002; Paul and Meyer, 2008). As the world human population increases exponentially (Cohen, 2003), so does its environmental requirements, and the demand for indicators to determine status and trends of environmental conditions.

Due to their ecological importance as primary producers and high sensitivity to environmental changes, algae are aquatic organisms that are commonly used in different ecological and paleoecological investigations (Stoermer and Smol, 1999; Ács et al., 2003; Torrisi and Dell' Uomo, 2006). The ability to culture them in the laboratory and their high reproduction rates makes them suitable as test organisms for environmental studies on abundance, morphology, and ecophysiological effects of various pollutants (Dewez et al., 2005). To infer water quality many indexes and systems have been created based on algae as bioindicators (Chapman, 1996; McCormick and Cairns, 1994).

Many euglenoids are commonly found in places that are rich in organic matter (Lackey, 1968; Munawar, 1972; Wolowski, 1998, 1992, 1988; Rosowski, 2003). Morphological alterations have been previously reported in species with rigid pellicle, and those changes were related to high concentration of organic matter (Conforti, 1991, 1998; Conforti et al., 1995; Bauer et al., 2012). To further investigate this response, specimens of *Phacus brachykentron* (Pochm.) were isolated from the Matanza River, Buenos Aires, Argentina, which presents high organic matter contamination caused by the discharges of the local meat industry. Here we studied the response of this species to different organic matter concentrations in the medium. Alterations in population growth, cell morphology and ultrastructure, pellicle, chloroplasts, paramylon grains, and the nucleus were investigated. We discuss the systematic and ecological implications.

2. Materials and methods

2.1. Cell culture

Phacus brachykentron (Pochm.) was isolated by one of the authors (Visitación Conforti) from the Matanza River, Buenos Aires, Argentina. Samples were filtered

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with a plankton net (mesh size 20 μm). A unialgal clonal culture was obtained via single cell isolation using Pasteur pipettes. Single cells were washed twice with physiological solution (0.9 percent NaCl, pH 7), and poured into glass tubes (100 mL) containing Soil Water Medium (SWM, Pringsheim, 1946), which was stabilized with $CaCO_3$. A stable culture developed after 2 months; culture growth was verified with an optical microscope. Stock cultures were maintained in SWM at 24 ± 1 °C on a 12 h/12 h dark/light cycle with 25–35 μE/m^2/s illumination provided by 40-W, cool white, fluorescent tubes.

2.2. Assays

When algal growth was verified, 24 tubes containing 5 mL of soil–water–$CaCO_3$ medium were prepared. Four tubes were separated as control; the remainders were enriched with sterile, Bacteriological Peptone OXOID® (Typical analysis: Total Nitrogen fourteen percent w/w, Amino Nitrogen 2.6 percent w/w, Sodium chloride 1.6 percent, pH (one percent solution) 6.3 at 25 °C), to the following concentration: 3.3, 6.6, 10, 13 and 17 mg/ml. The tubes were inoculated with 10^4 cell/mL and incubated for 48 h in the same conditions as the stock cultures.

2.3. Cell counting

Counts of regular and altered morphs of *P. brachykentron* were performed with an Olympus CK40 inverted microscope at 400× magnification using 2 mL Utermöhl sedimentation chambers with an estimated error (α=0.05). It was considered altered morphs when cells had its margins with notches, an increased dorsal–ventral thickness, and the presence of large paramylon bodies.

To obtain a quantitative estimate of cell growth, cell number was determined using a Neubauer chamber in an optical microscope. Cell counts on a sample (cells/ml) were repeated until the standard error of the mean was lower than ten percent (Venrick, 1978).The growth rate was estimated as exponential growth equation μ= (ln Nf−ln Ni)/day, where Nf=final cell number and Ni=initial cell number (Maclauso et al., 2009).

2.4. Morphometric determination

Images of the treated cells with the highest concentration of peptone were taken with an Olympus BX50 microscope to measure length, width, and thickness with the ImageJ software.

2.5. Light microscopy and TEM

An aliquot of the cellular suspension was fixed with three percent formaldehyde solution for light microscopy and the rest was used for TEM observations. Light microscopy studies were performed with an Olympus BX50 microscope on control and treated specimens. At least 300 control and altered cells were measured. Photographs were recorded with a digital camera Olympus C-7070 Wide Zoom. They were analyzed using ImageJ software. Three parameters were measured for each cell: length, width and thickness.

Cells for TEM were collected via centrifugation at 3500 × *g* for 20 min. They were fixed overnight at 4 °C in 2.5 percent glutaraldehyde and 0.1 M cacodylate buffer pH 7.4. In both cases, material was post-fixed for 2 h in one percent osmium tetroxide and buffer phosphate 0.1 M pH 7.4. The cells were washed three times in phosphate buffer 0.1 M pH 7.4, dehydrated in acetone series (30 percent, 50 percent, 70 percent, 95 percent, and two of 100 percent) and embedded in Spurr resin low density (Spurr, 1969). Sections were cut with a diamond knife and stained with uranyl acetate two percent in aqueous phase for 45 min. and lead citrate five percent for 5 min. (Reynolds, 1963). The grids class used was 200 copper mesh. Then they were examined using a ZEISS EM 10 A/B electron microscope from the Electron Microscopy Service of FCEN, UBA.

2.6. Paramylon extraction and SEM

Paramylon (ß-1, 3-glucans, storage carbohydrates in euglenenoids) was extracted and purified according to Kiss et al. (1988). Before extraction, cells were washed three times with buffer phosphate 50 mM pH 7. Then, 100 ml of the culture medium was centrifuged at 3700 × *g*. Pellets were frozen at −21 °C overnight and

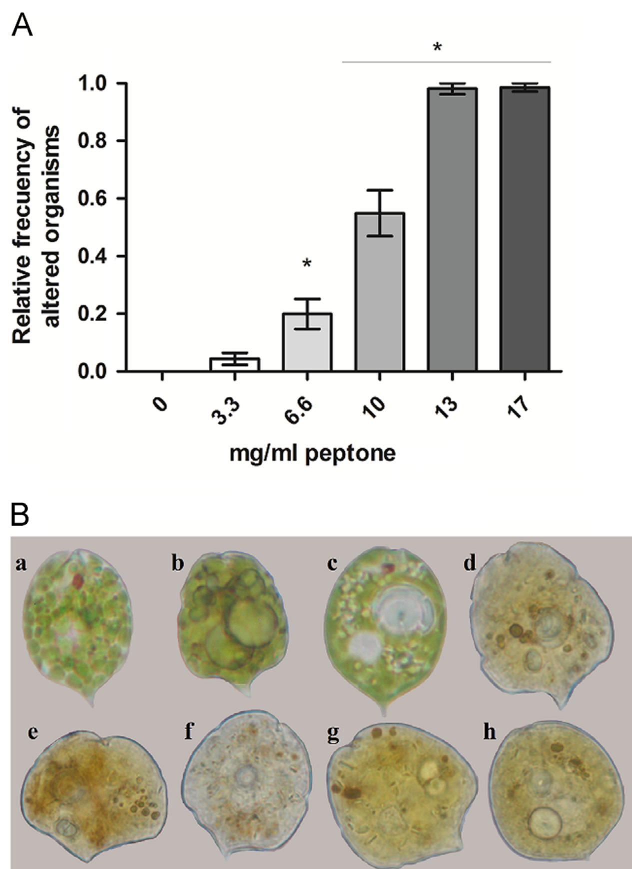

Fig. 1. (A) Relative frequency of deformed cells observed at different peptone concentrations (* $p<0.05$), n=4. (B) Optical micrographs of control and treated cells of *P. brachykentron*. (a) Control cells; (b–c) Treated cells showing notches in their contours and large paramylon bodies. (d–h) Treated cells of *P. brachykentron* that could be confused with the following species; (d) *P. unguis*, (e) *P. acuminatus*, (f) *P. onyx*, (g) *P. brevicaudatus*, and (h) *P. stokesii*.

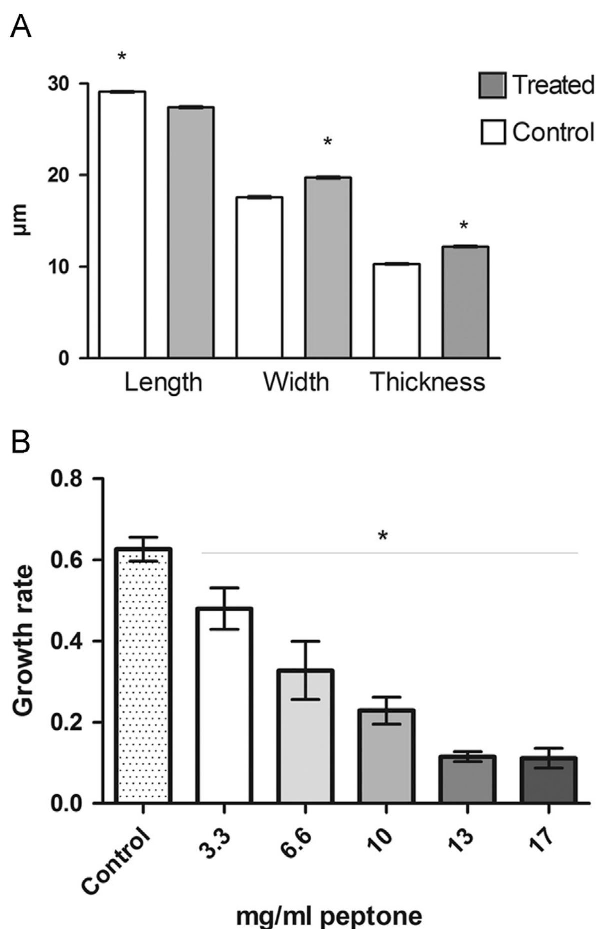

Fig. 2. (A) Analysis of the length, width and thickness of treatment cells with the highest concentration of peptone (* $p<0.050$), n=301. (B) Growth rate of control and treated cells (* $p<0.050$), n=4.

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then resuspended in two percent SDS (w/v) in 0.125 M Tris buffer (pH 6.8). The cell suspension was disrupted by sonication employing 10 s successive cycles with a maximum frequency of 20 KHz with 50 s intervals in ice bath and then incubated for 60 min at 37 °C. Paramylon grains were recovered by centrifugation for 30 min at 1014 × *g*. Treatment was repeated until a translucent supernatant was obtained. Then they were washed twice with hot glass-distilled water (70 °C), filtered through Millipore filters GSWP 04700 (0.20 μm pore). The filters were attached to the stubs with double sticky tape carbon and air-dried to be subsequently coated with gold/palladium. Specimens were examined and photographed in a ZEISS SUPRA 40 Scanning Electron Microscopy from the Service of FCEN, UBA.

2.7. Statistical analysis

Each treatment was carried out in quadruplicate. Data for cellular density and length, width, and thickness were evaluated by one factor analysis of variance (ANOVA). Values of $p < 0.050$ were considered significant.

3. Results

Fig. 1A shows the relative frequency of altered cells obtained in each treatment. As peptone concentration increases, so does the frequency of altered organisms up to a point where all the cells were affected. These cells were considered altered when they had a contour with notches (e.g., Fig. 1B(b, d, f and g)), an increased thickness, and/or the presence of large paramylon bodies (e.g., Fig. 1B(b, c)).

In individuals treated with the highest concentration, three morphometric parameters were measured (length, width, and thickness; Fig. 2A). The exposed algae significantly decreased their length, while their width and thickness significantly increased. Organic enrichment decreased the growth rate of *P. brachykentron* significantly in all assayed concentrations (Fig. 2B).

When comparing TEM micrographs, we observed ultrastructural differences between exposed cells (Fig. 3A–C, E) and the control (Fig. 3D, F). Cells treated with the highest concentration of organic matter showed a marked change in their apical view due to an increase in size of the paramylon grains (Fig. 3A, B). The lateral view also changed from very flat to globose (Fig. 3C).

With regard to the pellicle, we did not observe strip replication, but some bands showed a remarkable widening (Fig. 3B, E see arrows) with respect to the control (Fig. 3D, see arrows), allowing cell deformation. The pellicle band size measured in the controls ranged from 0.260 ± 0.01 μm to 0.416 ± 0.01 μm, whereas in the treated cells with the highest peptone concentration the range was from 0.613 ± 0.01 μm to 1.654 ± 0.01 μm.

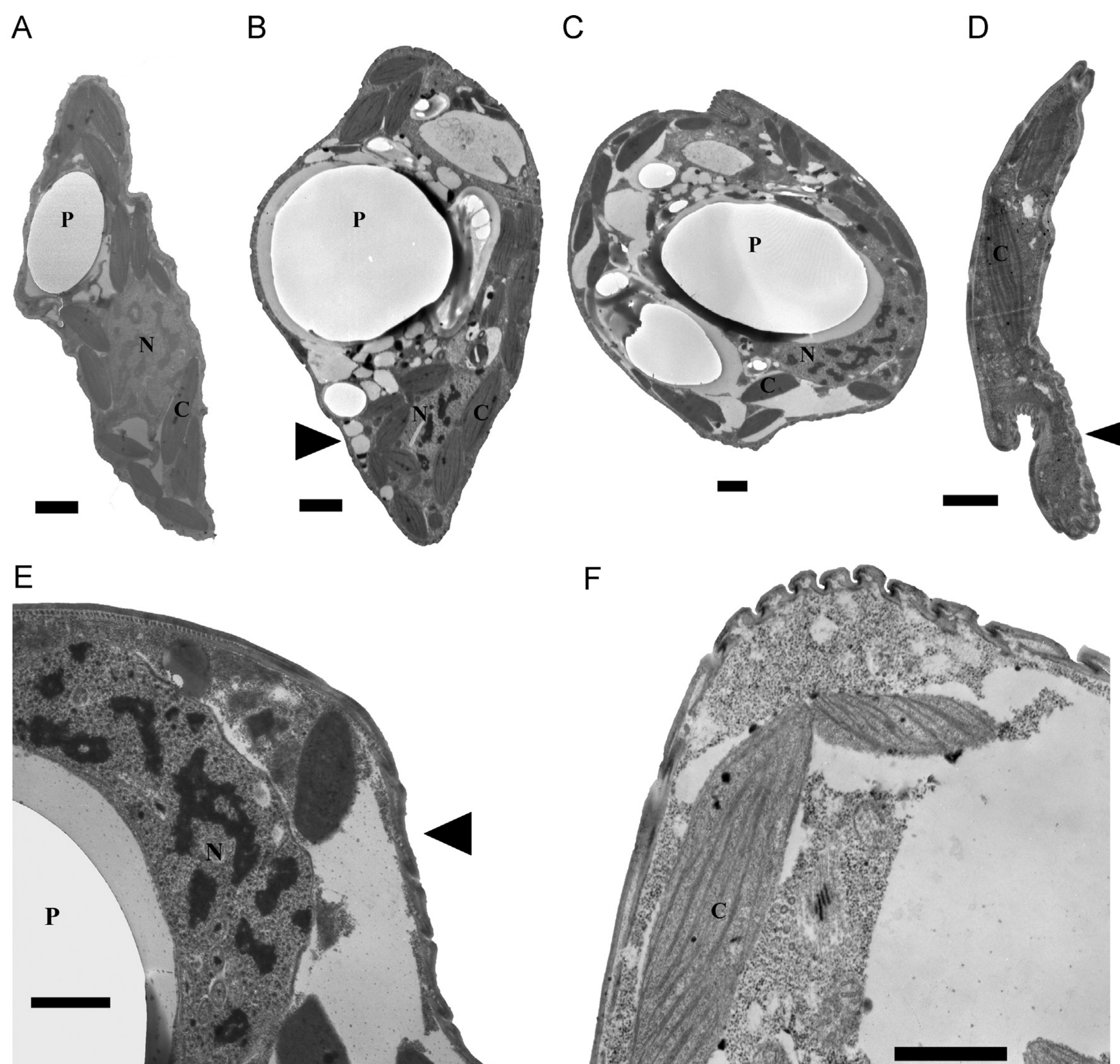

Fig. 3. TEM micrographs of cells treated with the highest concentration of organic matter: (A) apical view. (B) apical view: arrows point to the enlarged bands. (C) lateral view showing a globose contour. (E) detail of the cell showing the nucleus displaced by the paramylon; body and arrows point to the enlarged bands. TEM micrographs of control cells: (D) Apical view, the arrows point to the normal bands. (F) Chloroplasts detail with normal organization of thylakoids. Scale barrs: 1 μm. C, chloroplast; N, nucleus; P, paramylon.

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The nucleus was displaced from the central to the lateral position by the paramylon body (Fig. 3E). Some treated cells showed chloroplasts with their thylakoids disordered (Fig. 4B) respect to those observed in the control cell (Fig. 4A).

SEM analysis of the treated cells showed a great increase in volume and shape alterations in the paramylon bodies relative to those observed at the control cells. These grains presented a high increase in their thickness and a reduction of the light paramylon ring (Fig. 5A, B).

Finally, in the organic-rich cultures we observed that deformation caused cellular disruption, with a release to the medium of large number of paramylon grains.

4. Discussion

Most of the information on the ecology of euglenoids comes from floristic studies where the ecological aspect was not the main topic, and specialty on research performed on the genus *Euglena*. Here we studied a species that was isolated from a river that is polluted with organic matter. Growing *P. brachykentron* with increasing amounts of organic matter offered us the possibility of assessing the contaminant effects on the cell.

In contrast to that observed in others euglenoids exposed to different contaminants, including various heavy metals (Rocchetta et al., 2007; Bauer et. al., 2012), *P. brachykentron* showed changes in cell morphology when cultured with different concentrations of peptone. Exposed cells suffered significant modifications in their dimensions and shape, presenting notches in their contour.

Our results have important systematic implications. The globose morphology of treated cells does not coincide with the original description of the genus *Phacus* made by Dujardin (1841), who described these cells as rigid and more or less flattened, nor with the posterior description of Marin et al. (2003), who amended this diagnosis, specifying once again that the cells are laterally compressed. Only recently, on the basis of molecular evidences, Linton et al. (2010) modified the diagnosis, which included the possibility that cells could be spindle-shaped or ovoid, allowing the inclusion of altered cells in the genus.

When DNA sequencing is unavailable, in asexual organisms such as euglenoids systematic determination is only based on morphological parameters. According to Huber–Pestalozzi (1955), some cells in the treated cultures could be considered to be by their morphology as *P. stokesii* (Pl. XXXIII, fig. 194), *P. onyx* (Pl. XLVII, fig. 289), *P. unguis* (Pl. XLVII, fig. 291), *P. brevicaudatus* (Pl. XXXV, fig. 205), or *P. acuminatus* (Pl. XXXVIII, fig. 228), instead of *P. brachykentron* (Fig. 1B(d–h)). Based on these results, many new taxa described from bodies rich in organic matter might only be different morphs of the same species.

The SEM analysis of the treated cells showed a great increase in volume and change in shape of the paramylon grains. These are the ß-1, 3 glucan cell storage granules. Historically, number, shape and external morphology of these bodies have been widely employed as a diagnostic character among euglenoid species (Gojdics, 1953; Conforti, 1998; Brown et al., 2003; Shin and Triemer, 2004; Ciugulea et al., 2008; Monfils et al., 2011). In addition, large grain types can be used at the generic level to support major clades and generic relationships, and may provide insight into taxonomic placement of euglenoids currently unavailable for sequencing (Monfils et al., 2011). On the basis of the changes detected on paramylon grains in this study and in previous ones (Conforti, 1998), we conclude that these bodies have little diagnostic value as a species systematic character in euglenoids.

The response observed in *P. brachykentron* does not differ from those observed in other species of the euglenophytes such as *P. tortus* and *L. acus* isolated from the Matanza river (Conforti, 1998). The TEM analysis showed that some bands of the cell pellicle suffered a widening which allowed an increase in cell volume. Therefore, cellular deformation in euglenoids may be facilitated at least by two mechanisms: an increase in the number of bands as was observed in *Lepocinclis acus* (Conforti et al., unpublished results) or by a widening of the strips, without any

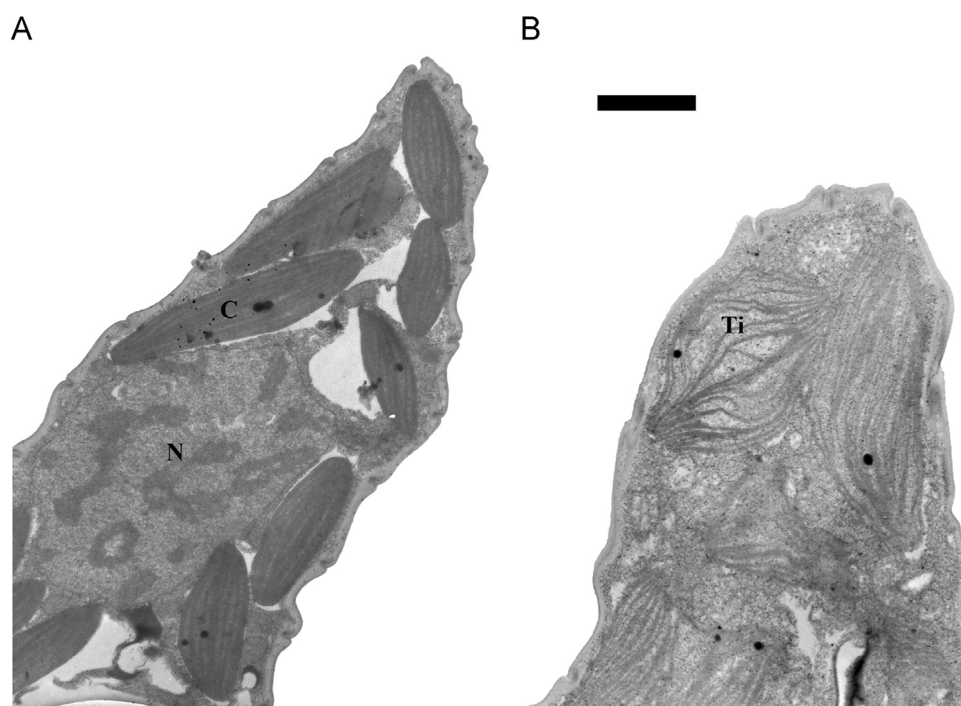

Fig. 4. (A) TEM micrographs control cell showing normal thylakoids. (B) TEM micrographs control cell showing treated cell showing disorder thylakoids. Scale bar 1 μm. C, chloroplast; N, nucleus; Ti, tylakoids.

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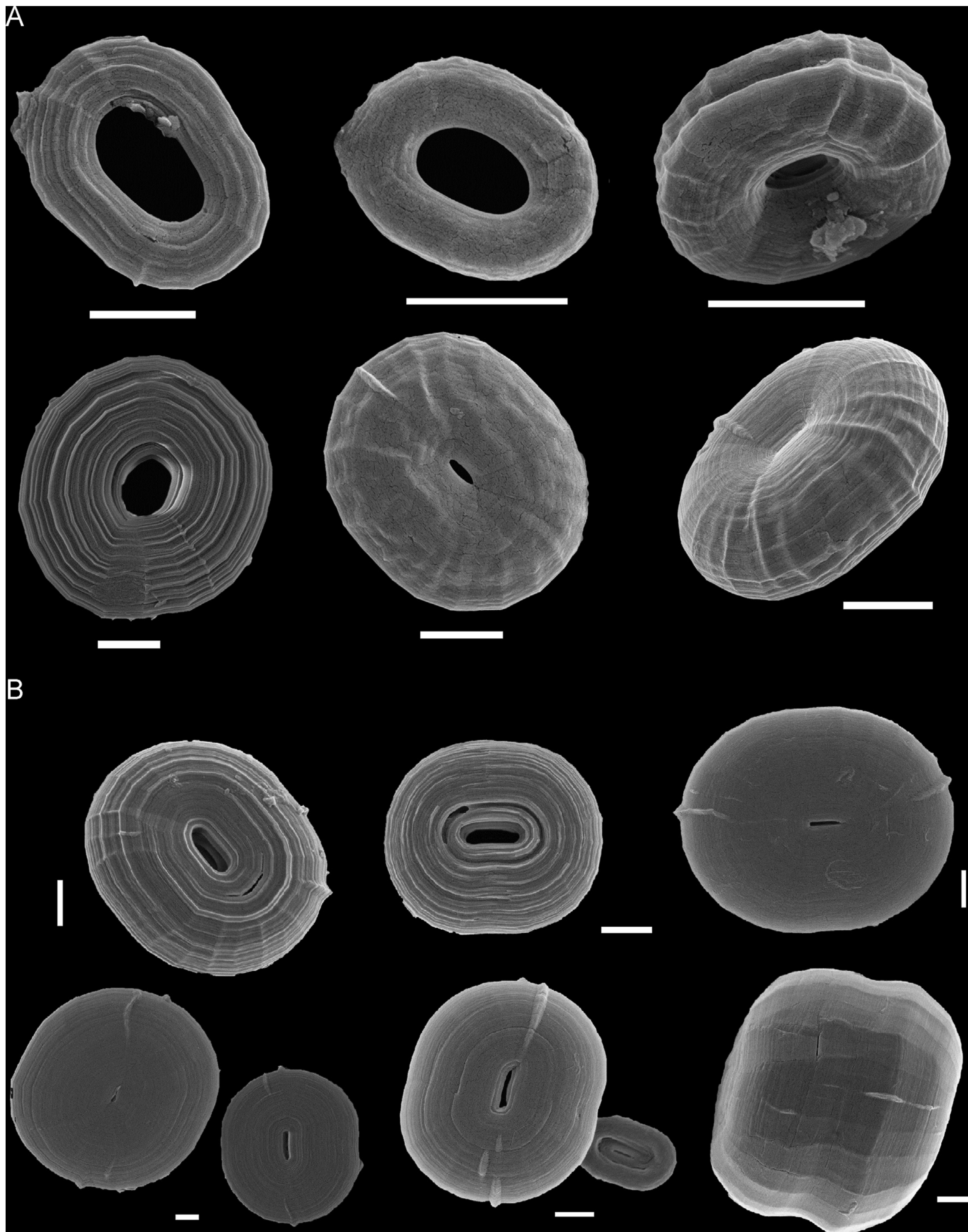

Fig. 5. (A) SEM micrographs of paramylon bodies of control cells. (B) SEM micrographs of paramylon bodies of treated cells with the highest concentration of peptone. Scale bars: 1 μm.

alteration in its normal ultrastructure. In *P. brachykentron* cell deformation did not change the striae orientation as observed in other species such as *L. acus* and *L. spirogyra* (Conforti, 1998).

The organic enrichment significantly decreased the growth rate of *P. brachykentron* at all assayed concentrations, which could mean that in natural environments this type of contamination might reduce markedly its abundance. At the end of the bioassays, we observed that deformation caused cell lysis, releasing to the medium a large number of paramylon bodies, suggesting that cells might support deformation only to a certain extent.

5. Conclusions

In *P. brachykentron*, cell dimensions and paramylon bodies, which are usually used in systematic determination, were strongly affected by the enriched medium, suggesting that organisms that are identified as different species may turn out to be just ecomorphs of the same taxon.

The morphological response of this alga to different levels of organic matter was fast, within 48 h of exposition to the pollutant. Therefore, we think this species has the potential to be an excellent tool for biological monitoring of this type of pollution.

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The algae survived to the adverse conditions for some time, allowing the observation of cell changes up to a point where some of the cells broke down and released their content to the medium.

On the basis of our results we conclude that the presence of deformed cells in natural samples and/or bulky intracellular paramylon bodies might be used as environmental bioindicators of freshwater bodies with organic matter enrichment.

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