

Physiological and morphological response of marine diatom *Cylindrotheca closterium* (Bacillariophyceae) exposed to cadmium

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ABSTRACT

The impact of the heavy metal cadmium on the lightly silicified marine diatom *Cylindrotheca closterium* was studied with regard to growth dynamics, organic matter production and morphological characteristics. *C. closterium* cultures were able to sustain positive growth in the tested range of 0–1000 $\mu\text{g Cd l}^{-1}$ with a significant decrease in specific growth rate in the exponential growth phase at the highest tested Cd concentration. The concentrations of both dissolved (DOC_{cell}) and particulate organic carbon per cell (POC_{cell}) in *C. closterium* cultures exposed to 500 and 1000 $\mu\text{g l}^{-1}$ Cd more than doubled compared with the control culture. Enhanced organic matter production as a feedback response to Cd exposure may thus contribute to cell detoxification, supported by the electrochemical data showing that Cd was not significantly accumulated by or associated with diatom cells except for the culture exposed to 1000 $\mu\text{g Cd l}^{-1}$. In order to gain more insight into the effects of cadmium on diatom morphological features, the cell surface of *C. closterium* was characterized at the nanoscale on a single cell level using atomic force microscopy (AFM). Changes induced by Cd were observed as irregular patterns of silica spheres on more silicified parts of the cell, girdle band and around the raphe. These changes clearly show that beside the physiological response, *C. closterium* also showed a morphological response which is probably due to the interference of Cd with the diatom biosilification process. This study overall contributes to better understanding of the effects of cadmium on diatoms and shows that morphological characteristics assessed by AFM may be a valuable indicator for metal contamination.

KEYWORDS Atomic force microscopy (AFM); dissolved organic carbon (DOC); cadmium bioavailability; *Cylindrotheca closterium*; growth dynamics; marine diatom; morphological changes; particulate organic carbon (POC)

Introduction

Anthropogenic pollutants in aquatic environments have significantly increased over the last few decades. Among these, heavy metals including cadmium have long been recognized as major marine pollutants (Cheung *et al.*, 2003; Ansari *et al.*, 2004; Wang & Wang, 2011; Suratno *et al.*, 2015). Cadmium (Cd) is a non-essential trace metal considered as one of the most toxic metals even at μg per litre levels (Wang & Wang, 2009). It can accumulate to hazardous levels in living organisms, posing environmental and human health risks. Cd toxicity is believed to result primarily from its interaction with sulphhydryl, carboxyl, and imidazole groups of enzymes, causing inhibition of many physiological processes, including photosynthesis and respiration (Prasad *et al.*, 1998; Wang & Wang, 2008). However, under conditions of Zn limitation, it was shown that Cd can replace Zn in the carbonic anhydrase catalytic site in the marine diatom *Thalassiosira weissflogii* which was confirmed by expression of Cd-containing carbonic anhydrase (CDCA1) (Lane & Morel, 2000; Lane *et al.*, 2005; Alterio *et al.*, 2015). Park *et al.* (2007) also observed the presence of Cd-containing carbonic anhydrase in some other diatom species.

Unicellular algae are primary producers widely used for assessing the impact of toxins in aquatic environments (Bitton & Dutka, 1986). Most of the usual tests involving microalgae have been based on population growth (OECD, 2011), respiratory processes (Pérez-García *et al.*, 1993) or ^{14}C assimilation (Kusk & Nyholm, 1991). Despite their small size, diatoms (Bacillariophyta) occupy an essential position at the base of the aquatic food web and are important primary producers (Armbrust, 2009; Ianora & Miralto, 2010), being responsible for 25–40% of the inorganic carbon fixation in oceans (Falkowski *et al.*, 2004; Granum *et al.*, 2005). Due to their cosmopolitan nature, short lifespan and quick response to environmental and anthropogenic perturbations diatoms can be used as sensors of environmental quality (De Stefano *et al.*, 2009; Rimet, 2012; Tudesque *et al.*, 2012). Morin *et al.* (2012) reviewed the response of diatoms to metal pollution from the individual level (e.g. size, growth form and morphological abnormalities) to community structure (replacement of sensitive species by tolerant ones). Although morphological abnormalities in diatoms have been reported and thoroughly studied under different types of environmental and anthropogenic perturbations (Falasco *et al.*, 2009), they have most often been reported under heavy metal stress (Morin *et al.*, 2012). The relationship between diatom frustule deformations and contamination by heavy metals including Cd (Morin *et al.*, 2007, 2008; Falasco *et al.*, 2009; Corcoll *et al.*, 2012; Pandey *et al.*, 2014) indicated that the occurrence of diatom deformities was a good indicator of high heavy metal contamination. While systematic changes in nanostructural characteristics in the aforementioned studies of diatom frustules induced by heavy metals have

been observed by electron microscopy (EM), no corresponding atomic force microscopy (AFM) measurements of diatom cells exposed to heavy metals have been reported so far, to our knowledge, although the number of AFM investigations of diatoms is growing steadily (Luís *et al.*, 2017). AFM is one of the most effective tools for studying the structure and surface properties of biological samples at the nanoscale (Binnig *et al.*, 1986; Hörber & Miles, 2003). AFM allows the examination of biological specimens in their native intact state, without the need for chemical fixation, dehydration and/or staining, associated with other high-resolution techniques such as electron microscopy. Moreover, AFM avoids the need for removal of organic matter from the diatom cells, in contrast to electron microscopy. The first AFM characterization of the diatom cell wall at the nanostructural level was made on the diatom *Navicula pelliculosa* dried with ethanol in 2001 (Almqvist *et al.*, 2001), followed by characterization of live diatom cells of *Pinnularia viridis* and *Hantzchia amphioxys* in the same year (Crawford *et al.*, 2001). In our previous studies we used AFM to characterize nanostructural and nanomechanical properties of *Cylindrotheca closterium* (Pletikapić *et al.*, 2012) and supramolecular organization of its extracellular polymers (Pletikapić *et al.*, 2011; Svetličić *et al.*, 2011, 2013). Using AFM nanomechanical measurements we also revealed that *Dunaliella tertiolecta* cells were significantly stiffer in the presence of Cd, which influenced the dynamics of the initial contact between the cell and the model charged interface (Ivošević DeNardis *et al.*, 2019).

The aim of the present study was to assess the response of the lightly silicified marine diatom *Cylindrotheca closterium* to Cd, with regard to cell physiology and cell morphology. *C. closterium* was chosen as an ecologically important cosmopolitan species, widely distributed in high and low latitude marine to brackish waters where this species can reach high densities (de Brouwer *et al.*, 2005; Najdek *et al.*, 2005). *C. closterium* has also been widely used as a diatom model system to study diatom ecophysiology, including the production and function of extracellular polymeric substances (de Brouwer *et al.*, 2005; Pletikapić *et al.*, 2011), movement (Apoya-Horton *et al.*, 2006; Araújo *et al.*, 2013) and anti-oxidative defence (Rijstenbil, 2005). Moreno-Garrido *et al.* (2003) used *C. closterium* for toxicity tests in surface sediments and showed that *C. closterium* is a suitable organism for this purpose, with a good growth rate and sensitivity to heavy metals. In a recent study by Becker & Copplestone (2019), it was established that *C. closterium* takes up a significant quantity of Cd from the sediment, as well as from the water column.

We designed an experiment for controlled laboratory conditions in which *C. closterium* was exposed to different Cd concentrations in the range 1–1000 µg l⁻¹. The level of stress caused by Cd exposure was assessed by measuring cell growth and organic matter production.

Additionally, morphological changes at the single cell level were assessed using AFM. These physiological and morphological measurements can support the prediction of diatom behaviour in different Cd contamination levels. We also introduce AFM as a method for evaluating the impact of heavy metal on nanostructural characteristics of diatom cells under ambient conditions, avoiding the drastic sample preparation procedures needed for electron microscopy, including cleaning diatom frustules from organic material and coating the samples, which could destroy the structure of lightly silicified diatoms or cell integrity of other phytoplankton species with softer cell membranes.

Materials and methods

Phytoplankton culture

The diatom *C. closterium* (Ehrenberg) Reimann & Lewin (strain CCNA1) was isolated from a seawater sample collected in the northern Adriatic Sea (offshore station SJ108, 12°45'E, 44°45.4'N, 12 m depth, 13 November 2006). One DNA sequence is deposited in GenBank under accession number HM070405 (18S (SSU) ribosomal RNA) (Pletikapić *et al.*, 2011). The diatom was grown in 250 ml conical flasks containing 100 ml f/2 medium (Guillard, 1975). Seawater for preparation of f/2 media was sampled in the Adriatic Sea (500 m offshore of Vis Island, 20 m depth) and filtered through a 0.2 µm pore size cellulose nitrate membrane filter (Whatman). Cultures were incubated in a water bath at a temperature of 20°C with constant shaking (15 rpm), 12:12 light:dark (L:D) cycle at an irradiance of 31 µmol photons m⁻² s⁻¹.

Cadmium exposure experiment

Erlenmeyer flasks containing 89 ml of f/2 medium were inoculated with 10 ml of algal culture in the exponential growth phase (5-day-old). Then, 1 ml of Cd solution with different concentrations, from 100 µg l⁻¹ (0.889 µmol l⁻¹) to 100 mg l⁻¹ (0.889 mmol l⁻¹), were added to Erlenmeyer flasks (no Cd was added to the control culture) and stoppered with cotton. Cd was applied as a cadmium nitrate (Cd(NO₃)₂) (AAS TraceCERT®, Fluka). Cadmium concentrations in the culture media tested were 1 µg l⁻¹ (0.0089 µmol l⁻¹), 10 µg l⁻¹ (0.089 µmol l⁻¹), 100 µg l⁻¹ (0.889 µmol l⁻¹), 500 µg l⁻¹ (4.449 µmol l⁻¹) and 1000 µg l⁻¹ (8.897 µmol l⁻¹). For all tested Cd concentrations an initial cellular concentration was ~10⁴ cells ml⁻¹. The cultures were grown in duplicates.

Modelling of chemical speciation

Computational simulations of chemical equilibrium were performed with Visual MINTEQ ver. 3.1. (Gustafsson, 2018) in order to calculate potentially bioavailable cadmium concentration (free ions and inorganic complexes), as well as the concentrations of Cd-EDTA complexes.

Speciation calculations were performed taking into account major, minor and trace cations and anions of the f/2 medium which are influencing cadmium speciation (Cl^- , SO_4^{2-} , CO_3^{2-} , OH^- , Na^+ , Mg^{2+} , Ca^{2+} , Fe^{3+} , Cu^{2+} , Mo^{6+} , Zn^{2+} , Co^{2+} and Mn^{2+}). For concentrations of metals Fe^{3+} , Cu^{2+} , Mo^{6+} , Zn^{2+} , Co^{2+} and Mn^{2+} concentrations in the f/2 medium were used (Guillard, 1975) and for Cl^- , SO_4^{2-} , CO_3^{2-} , OH^- , Na^+ , Mg^{2+} and Ca^{2+} average concentrations in seawater were used. The ionic strength of 0.7 M, pH of 8.15 (pH of the f/2 medium) and concentration of EDTA of 1.17×10^{-5} M (concentration of EDTA in the f/2 medium; Guillard, 1975) were used for all calculations.

Growth inhibition determination

During the experiment, 1 ml of algal cultures were sampled at the beginning of the experiment (day 0) and after 1, 2, 3, 4, 7, 10, 14 and 18 days from each test flask. Flasks were vigorously manually stirred prior to taking samples to prevent cell settling. Cells were counted using a light microscope (Olympus model BX51 equipped with DP70 Digital Camera System and operating with DP Controller and DP Manager software Olympus Corporation, Tokyo, Japan, 200× magnification) with Fuchs-Rosenthal hemocytometer. The average specific growth rate for a specific time period was calculated according to the equation described in OECD (2011):

$$\mu_{i-j} = \frac{\ln N_j - \ln N_i}{t_j - t_i} d^{-1} \quad (1)$$

where, μ_{i-j} is the average specific growth rate from time i to j; t_i is the time of the start of the period; t_j is the time of the end of the period; N_i is the cell number per volume of culture at time i; N_j is the cell number per volume of culture at time j. Growth inhibition was calculated according to the formula described by OECD (2011):

$$\%I = \frac{\mu_C - \mu_T}{\mu_C} \times 100 \quad (2)$$

where, % I is the per cent inhibition; μ_C is the average specific growth rate in the control group, and μ_T is the average specific growth rate for the treatment replicate. For statistical data analysis purposes, data values for cell density as a function of time in the area of the exponential growth (from day 0 to day 4, 7 and 10) were fitted to the following exponential function:

$$N_j = N_0 e^{\mu_{0-j}(t_j)} \quad (3)$$

to obtain fitted specific growth rates μ_{0-j} ($\mu(f)$) within tested time intervals. The fitted specific growth rate values for exposure groups and control group were compared (Supplementary figs 1-3).

Specific growth rate values differing more than ± 2 standard deviations (SD) were considered to be statistically significant.

Organic carbon analysis

Organic carbon, both dissolved and particulate, was analysed with the high-temperature catalytic oxidation method (HTCO). TOC-V_{CPH} (Shimadzu, Japan) carbon analyser with platinum/silica catalyst (Elemental Microanalysis, UK) and a non-dispersive infrared (NDIR) detector for CO₂ measurements was used for dissolved organic carbon (DOC) measurements and calibrated with potassium hydrogen phthalate. Concentration was calculated as an average of three replicates. The average instrument and Milli-Q blank corresponded to 30 $\mu\text{g C l}^{-1}$ with high reproducibility (1.5%). Particulate organic carbon (POC) was analysed with a solid sample module SSM-5000A associated with a TOC-V_{CPH} carbon analyser calibrated with glucose. A mixture of CoO and platinum/silica was used as a catalyst for the particulate phase. Prior to analysis, filtration of 50 ml of each sample was performed through 0.7 μm Whatman GF/F filters, previously combusted at 450°C for 4 h, to separate DOC and POC fractions. DOC samples were preserved with mercury chloride (10 mg l^{-1}) and stored at +4°C in the dark until analysis. POC samples were stored at -50°C until analysis.

Samples for DOC and POC analyses were taken from the cultures on day 18 of the experiment for each tested Cd concentration including the control sample and from the culture medium before inoculation. DOC and POC data were obtained by deducing measured values in the cultures from the measured value in the culturing medium. Batch cultures were not axenic; however, all precautions were taken to avoid further contamination with bacteria. During cell counting, we observed a negligible number of dead cells, lacking pigments and cell content. Therefore, analysed dissolved organic carbon is discussed as being mainly produced by *C. closterium*.

Cadmium measurements by voltammetry

Electrochemical measurements of Cd were performed using differential pulse anodic stripping voltammetry (DPASV) on a μ Autolab3 potentiostat (Autolab- Metrohm). Total Cd concentrations and dissolved concentrations of Cd present in the culture (after filtering the sample through 0.45 μm pore size cellulose nitrate filters (Sartorius, Germany)) were determined at days 7 and 18 of the growth experiment for each tested Cd concentration. Samples were digested by acidification to pH < 2 with HNO₃ (ROTIPURAN Supra 69%, Roth, Germany), and irradiated by UV-light (mercury lamp, 150 W, Hanau, Germany) for 24 hours. Prior to measurements, samples were diluted with sterilized seawater up to 2000 times, depending on the concentration of the Cd

added to the f/2 medium. Measurements were done in a three- electrode cell (663 VA Stand, Metrohm), with a hanging mercury drop (HMDE) as a working electrode, an Ag/ AgCl (sat. NaCl) as a reference electrode and a platinum wire as a counter electrode. Accumulation potential used for measurements was -0.85 V with accumulation times between 45 and 300 s, depending on the dilution factor of the samples. Concentrations of Cd were determined by a method of standard addition, and results are presented with error bars representing a 95% confidence interval.

Atomic force microscopy

For nanostructural characterization of the *C. closterium* cell surface, topography images were collected using a Multimode AFM with Nanoscope IIIa controller (Bruker) and a vertical engagement (JV) 125 μm scan- ner. Contact mode imaging was performed using silicon- nitride tips (NP-S, Bruker, nom. freq. 12–24 kHz, nom. spring constant of 0.06 N/m). The linear scanning rate was optimized between 1.5 and 2 Hz with a scan resolution of 512 samples per line. The setpoint was kept at the lowest possible value to mini- mize the interaction forces between the tip and the surface. Processing and analysis of images were carried out using NanoscopeTM software (Bruker).

Sample preparation for AFM imaging

Cells used for AFM experiments were recovered from the stationary growth phase (day 14) from the cultures with $0\ \mu\text{g Cd l}^{-1}$, $1\ \mu\text{g Cd l}^{-1}$, $10\ \mu\text{g Cd l}^{-1}$, $100\ \mu\text{g Cd l}^{-1}$, $500\ \mu\text{g Cd l}^{-1}$ and $1000\ \mu\text{g Cd l}^{-1}$. For sample prepara- tion, we used the direct drop deposition method (Mišić Radić *et al.*, 2011; Pletikapić *et al.*, 2011). Five μl of the cell culture was pipetted directly onto freshly cleaved mica. Mica sheets were placed in enclosed Petri dishes for 30–45 min to allow cells to settle and attach to the surface. Samples were then rinsed three times for 30 s in ultrapure water and placed in enclosed Petri dishes at a relative humidity of 60% to evaporate the excess of water on the mica. Rinsing of the samples with ultra- pure water was necessary to remove the excess of salt crystals that would hamper AFM imaging under ambi- ent conditions. With this procedure, the diatom cells stayed firmly attached to the mica surface, enabling stable imaging for AFM experiments.

Results

Cadmium bioavailability

Results of chemical speciation modelling at different Cd concentrations using software Visual MINTEQ ver.3.1.1 are shown in Table 1. Bioavailable (free and inorganic) Cd concentrations were far below total Cd due to the formation of strong Cd-EDTA complexes. The concentration

of free Cd is around 22× lower than the inorganic Cd fraction in which chloride complexes dominate. With increasing Cd concentration from 1 $\mu\text{g Cd l}^{-1}$ to 1000 $\mu\text{g Cd l}^{-1}$, the bioavailable Cd fraction (free and inorganic fraction) also increased from 43% to 76%. Additionally, pH measurements during a separate growth experiment showed that the initial pH of 8.1 remained unchanged for the first 4 days of the experiment, after which it started to increase, reaching 8.5 on day 7, and 9.0 on day 18 of the experiment. According to speciation calculations, the bioavailability of Cd is expected to decrease by ~20% from day 0 to day 7, for all added concentrations of Cd. By day 18, the bioavailability of Cd decreased further, dropping to 8% of bioavailable Cd for the lowest added Cd concentration of 1 $\mu\text{g l}^{-1}$, to 24% of bioavailable Cd for the 1000 $\mu\text{g l}^{-1}$ concentration.

Effect of cadmium on *Cylindrotheca closterium* growth

Growth curves of *C. closterium* exposed to different concentrations of Cd in the range 0–1000 $\mu\text{g l}^{-1}$, with optical micrographs of cultures at selected Cd concentrations, are shown in Figs 1–4. Growth curves show typical exponential growth from day 0 to day 10, followed by entering into the stationary phase from day 10 for the whole range of tested Cd concentrations (0–1000 $\mu\text{g l}^{-1}$). For all tested Cd concentrations *C. closterium* cells were able to sustain positive growth. Specific growth rates (μ) and percentage inhibition (%I) of *C. closterium* in the exponential growth phase exposed to different Cd concentrations calculated according to Eq. 1 and Eq. 2 are shown in Table 2. During the first 4 days of the exponential phase, the specific growth rate decreased from 0.48 d^{-1} (corresponding to the division rate of 0.69 div d^{-1}) in the control culture to 0.39 d^{-1} (corresponding to the division rate of 0.56 div d^{-1}) in the culture exposed to 1000 $\mu\text{g Cd l}^{-1}$. The specific growth rate for the 7-day period decreased from 0.46 d^{-1} (division rate 0.66 div d^{-1}) for the control to 0.35 d^{-1} (division rate 0.50 div d^{-1}) for the culture exposed to 1000 $\mu\text{g Cd l}^{-1}$. For the 10 day period, the specific growth rate decreased from 0.48 d^{-1} (division rate of 0.69 div d^{-1}) for the control to 0.42 d^{-1} (division rate of 0.61 div d^{-1}) for cultures exposed to 1000 $\mu\text{g Cd l}^{-1}$. The inhibitory effect of Cd on diatom growth during the exponential phase was greatest for cultures exposed to 1000 $\mu\text{g Cd l}^{-1}$ for 7 days, which resulted in a growth inhibition of 24.7% relative to the control (Table 2). Difference in specific growth rates was tested by fitting the data values for cell density as a function of time in the exponential growth phase (for first 4, 7 and 10 days) for all tested Cd concentrations and control to Eq. 3. No significant difference in specific growth rate between cultures exposed to different Cd concentrations including the control was observed during the first 4 days (SI Fig. 1). However, with prolonged exposure times (7 and 10 days), cultures exposed to 1000 $\mu\text{g Cd l}^{-1}$ did show a significant decrease of specific growth rate relative to

the control culture, as tested by fitting the data values for cell density as a function of time to Eq. 3 (SI Figs 2, 3).

Effect of cadmium on organic matter production by *C. closterium*

The effect of Cd on organic matter production was analysed by measuring dissolved (DOC) and particulate organic carbon (POC). DOC and POC were analysed at day 18 of growth in the control culture and in the cultures exposed to different Cd concentrations in order to establish whether changes in organic matter production represent a response to the presence of Cd. DOC and POC data in the cultures are reported in Table 3 and are additionally reported as DOC per cell (DOC_{cell}) and POC per cell (POC_{cell}) in order to compare the DOC and POC between the different cultures. DOC_{cell} in the control culture and in the cultures exposed to Cd concentrations in the range 1–100 µg l⁻¹ were in the range of 0.22–0.26 pg C cell⁻¹. Higher DOC_{cell} concentrations were found in cultures exposed to 500 µg Cd l⁻¹ (0.82 pg C cell⁻¹) and 1000 µg Cd l⁻¹ (0.56 pg C cell⁻¹), indicating a 3.6- and 2.4-fold increase, respectively. The lowest amount of POC_{cell} was found in the control culture (17.6 pg C cell⁻¹) and the highest amount of POC_{cell} was found in the culture exposed to a Cd concentration of 500 µg Cd l⁻¹ (46.2 pg C cell⁻¹) followed by the culture exposed to a Cd concentration of 1000 (40.0 pg C cell⁻¹), indicating a 2.6- and 2.3-fold increase, respectively.

Cadmium uptake by *Cylindrotheca closterium*

Percentage of measured Cd concentration (% Cd) in the dissolved fractions (< 0.45 µm) and total Cd in *C. closterium* cultures exposed to different Cd concentrations compared with concentrations of added Cd in the cultures are shown in Table 4. The percentage of measured dissolved Cd on day 7 ranged from 82.9 ± 6.2% to 109.0 ± 9% and of the total Cd from 91.0 ± 6.7% to 105 ± 7%. On day 18 the percentage of measured dissolved Cd ranged from 92 ± 10% to 107.5 ± 4.7% and of the total Cd from 94 ± 17% to 106.8 ± 4.8%. The obtained results show that most of the Cd was present in the dissolved fraction, indicating that Cd in the *C. closterium* culture was not significantly accumulated by or associated with diatom cells with the exception of the culture exposed to 1000 µg Cd l⁻¹ on day 7, where a significant difference between total and dissolved Cd was observed.

Effect of cadmium on nanostructural properties of *Cylindrotheca closterium*

AFM images of cells from the culture that was not exposed to cadmium, where the details of the cell surface could be observed, are shown in Figs 5–14. The cell has an elongated shape with cell length of 38–43 µm and cell width (at the centre of the cell) of 3.5–4.8 µm. The height

of the cell at the centre of the cells and in the central part where chloroplasts are situated was 600–1000 nm and 750–1500 nm, respectively and rostra height was 200–300 nm. On *C. closterium* cells, flexible rostra can be clearly distinguished from the central part of the cell (Fig. 5). Figs 6–14 show the rostra and the central part of the cell at higher resolutions, where three morphologically different parts of the frustule can be easily distinguished: the girdle band, the valve and the raphe. High-resolution images of the central part of the cell revealed a non-structured and smooth surface of the valve whereas the girdle band showed a structured surface consisting of a set of parallel bands (Figs 9–11). The longitudinal slit raphe extends along the valve (Figs 8–10, 14), and is bridged by the fibulae (Figs 8, 14). The spacing between fibulae was 250–350 nm, and fibulae arches underneath the cell wall were noticeable. On the enlarged image of the central part of the cell, vertical cross-sections were made (Figs 15, 16). The valve has a relatively smooth surface with an average roughness (R_a) of 3 nm, in contrast to the girdle band with R_a around 14 nm.

C. closterium cells exposed to the elevated concentrations of Cd ($1\text{--}1000\text{ }\mu\text{g l}^{-1}$) maintain the same overall shape of the cells. The length and height of the cells cultured in the control culture and in the cultures with elevated concentrations of Cd did not differ significantly. Cells grown exposed to 1, 10 and $100\text{ }\mu\text{g Cd l}^{-1}$ showed identical morphology to cells in the control culture with no observed nanostructural changes compared with controls (data not shown). Cells exposed to 500 and $1000\text{ }\mu\text{g Cd l}^{-1}$ showed morphological changes on the cell surface. Figs 17–26 show AFM images of *C. closterium* cells exposed to $500\text{ }\mu\text{g Cd l}^{-1}$. The cell overall morphology was similar to the morphology of cells that were not exposed to Cd, but minor changes were observed in the central part of the cell and on its more silicified parts, the girdle band and around the raphe (Figs 21–23). Clusters of smaller spherical particles (Figs 21, 22) were visible around the raphe that were not detected on *C. closterium* cells from the control culture. Also, the structure of the girdle band at the central cell part appeared not to be as smooth as in the control sample but had more pronounced underlying features (Fig. 23). The surface of the valve did not change compared with the control (Figs 21–23). The girdle band at the cell rostra appeared similar to that of *C. closterium* cells grown in control cultures (Figs 18–20, 24–26). Figs 27–36 show AFM images of *C. closterium* cells exposed to $1000\text{ }\mu\text{g Cd l}^{-1}$. Changes were visible on the entire surface of the cell in the form of irregular patterns of spherical particles. Spherical particles at the central part of the cell were mostly found on the more silicified regions, on the girdle band (Figs 31–33), and around the raphe (Figs 31, 34), while a smaller number was detected on the valve (Fig. 31). Spherical particles were also observed on the girdle band and around the raphe on the rostra (Figs 28–30, 34–36). Vertical cross sections (Figs 37–39) show that diameters of particles ranged from 50–390 nm and the

height of the particles ranged from 20–50 nm. The valve retained a relatively flat surface, but the girdle band appeared rougher due to spherical particles. R_a for the valve area was 4 nm, and R_a for the girdle band area was 25 nm.

Discussion

Heavy metals, including Cd, are important anthropogenic contaminants in aquatic ecosystems and can cause diverse negative impacts on marine ecosystems. Since phytoplankton represents the base of the trophic pyramid and the main primary producer in the marine ecosystem, research on the impact of heavy metals on phytoplankton gives an insight into their impact on the entire food chain in the marine ecosystem.

The growth dynamic results show that *C. closterium* was able to maintain a positive growth rate in the exponential phase in the whole range of tested Cd concentrations. The concentrations of bioavailable Cd (including free and inorganic forms) (Sunda, 1989; Lage *et al.*, 1994) in *C. closterium* cultures were estimated taking into account the chemical composition of the medium and the pH of the medium at the moment of Cd addition, and throughout the experiment. Lower values of bioavailable Cd compared to total Cd are due to the formation of strong Cd-EDTA complexes, which reduces the bioavailability (Lage *et al.*, 1994) and consequentially the toxicity of heavy metals (Moreno-Garrido, 1997). The highest Cd concentration of 1000 $\mu\text{g l}^{-1}$ (corresponding to 750.7 $\mu\text{g l}^{-1}$ of bioavailable Cd) induced a significant decrease in the specific growth rate compared with the control culture. The lowest specific growth rate of *C. closterium* was found in the culture with a Cd concentration of 1000 $\mu\text{g l}^{-1}$ over a 7 day period, 0.35 d^{-1} in comparison to 0.46 d^{-1} in the control culture, indicating a growth inhibition of 41.6%. Obtained results are consistent with previous studies where it was shown that exposure of *Cylindrotheca* sp. to Cd inhibited growth and reduced photosynthesis and cellular respiration (Lehman & Vasconcelos, 1979; Pistocchi *et al.*, 2000; Satoh *et al.*, 2005). Higher tolerance for toxic heavy metals compared with dinoflagellates was shown for the diatom *Cylindrotheca fusiformis*, which was not completely inhibited by Cd concentrations as high as 500 $\mu\text{g l}^{-1}$ (Pistocchi *et al.*, 2000). Higher tolerance to Cd was also shown by the diatom *Phaedactylum tri-cornutum* where the response to Cd was investigated on a molecular, metabolic and physiological level (Brembu *et al.*, 2011).

Extracellular polymeric substance (EPS) release by phytoplankton is a process mostly dependent on phytoplankton physiological state and increased production was found to be a general response to the presence of heavy metals (Pistocchi *et al.*, 1997, 2000). We found that the exposure to cadmium at concentrations of 500 and 1000 $\mu\text{g l}^{-1}$ for 18 days more than doubled both DOC per cell (DOC_{cell}) and POC per cell (POC_{cell}) compared with the control values. The increase of DOC_{cell} in the cultures exposed to heavy metal was expected and in line

with previous reports showing that *C. fusiformis* exuded a higher amount of carbohydrates when cultured in the presence of heavy metal concentrations that were not extremely toxic (Pistocchi *et al.*, 1997). The increase of POC_{cell} in the cultures exposed to Cd could be due to the higher production of extracellular polymers that stay attached to the cell surface or secretion of larger particles that remain on GF/F filters used to separate DOC and POC fractions and thus are included in the POC fraction. Furthermore, heavy metals have also been found to increase the lipid content per cell in some microalgae (Einicker-Lamas *et al.*, 2002; Yang *et al.*, 2015) thus increasing POC_{cell}. The secretion of large particles was previously demonstrated by Chin *et al.* (2004) for *Phaeocystis globosa*, which uses regulated exocytosis to secrete polysaccharide gels, including large polymers or particles. Applying high-resolution imaging by AFM (Svetličić *et al.*, 2013), we previously showed that extracellular polymers excreted by *C. closterium* were attached to the diatom cell wall or released into the surrounding seawater, and were best described as gel networks. Extracellular polymeric substances have high metal complexing capacities and could reduce metal bioavailability, thus increased EPS production may protect cells from the uptake of metals and their consequent toxicity (Decho, 1990; Pistocchi *et al.* 1997).

Our results indicate that extracellular polymer release in *C. closterium* cultures exposed to Cd could be one of the self-defence mechanisms against toxic effects. Electrochemical data show that Cd in the *C. closterium* culture was not significantly accumulated by or associated with diatom cells, being mostly present in the dissolved fraction, except for the culture exposed to 1000 µg Cd l⁻¹ on day 7 of the experiment, where the measured percentage of dissolved Cd is significantly lower than total Cd measured. The lower dissolved Cd values in this culture could be explained by bioaccumulation of Cd and/or biosorption of Cd on the cell surface or on extracellular polymers attached on the cell surface. These data are in line with previously reported data (Kim Tiam *et al.*, 2018) showing that the diatom *Nitzschia palea* exposed to Cd showed the lowest Cd dissolved values on day 7 of the experiment and increased Cd dissolved values with increased exposure time.

The addition of Cd to the cell culture of *C. closterium* did not affect the shape and size of cells. However, AFM revealed nanomorphological changes in frustule features at Cd concentrations of 500 µg l⁻¹ and 1000 µg l⁻¹

¹. The irregular patterns of spherical particles, mostly located on the more silicified regions of the cell, girdle band and around the raphe. Observed particles agreed in size (20–50 nm) with previous studies (Sumper & Kröger, 2004; Hildebrand *et al.*, 2009) showing that silicate structures of different diatom species are composed of silica spherical nanoparticles 2–200 nm in diameter. Pletikapić *et al.* (2012) showed that the nanoparticles forming the valve in *C.*

closterium were 5–15 nm in height and are incorporated into the organic material of the valve, demonstrated by removal of the organic material with sulphuric acid, after which the nanoparticles were not fused and did not form a fixed nanostructure. The observed nanoparticles withstood additional acid treatments clearly indicating that they were non-organic. Morphological abnormalities in diatoms have been reported and thoroughly studied under different types of environmental and anthropogenic perturbations (Falasco *et al.*, 2009), most often under heavy metal stress (Morin *et al.*, 2012). Morphological abnormalities of diatom cells as a consequence of exposure to high cadmium concentrations were observed by an electron microscope in smaller pennate diatoms revealing micromorphological changes in frustule features. Cd led to the development of abnormal valve outlines in *Achnanthisdium* spp., *Amphora pediculus*, *Eolimna minima*, *Gomphonema parvulum*, *Mayamaea* spp. (including *M. agrestis*) and *Nitzschia palea* (Morin *et al.*, 2008). Interruption of the raphe was noticed in *Encyonema minutum*, *Eolimna minima* and *Sellaphora seminulum*, and abnormal pore shape and pattern in *E. minima* and *G. parvulum* (Morin *et al.*, 2008). The observed morphological changes on the surface of the *C. closterium* cells are probably the consequence of the effect of Cd on the biosilification process, resulting in the appearance of irregular structures on some, more strongly silicified frustule parts since the formation of silica parts of the cell is under the control of environmental effects, such as the availability of silicon in seawater or the presence of toxic elements (Morin *et al.*, 2008). Since silicic acid uptake in diatoms is known to be regulated by sulphydryl groups on the cell surface (Lewin, 1954), metals could affect the biosilification process through the perturbation of the silicic acid uptake due to their affinity to sulphydryl groups (Falasco *et al.*, 2009). Additionally, Kim Tiam *et al.* (2018) showed that exposure of the diatom *Nitzschia palea* to Cd affected silica regulation at the genetic level with strong down-regulation of the silicon transporter, suggesting a lower uptake of silicic acid under Cd stress.

The results obtained within this study contribute to a better understanding of the impact of the heavy metal cadmium on the marine diatom *C. closterium*, providing insight into growth dynamics, organic matter production and morphological changes at the cell level. Although the obtained results apply directly to *C. closterium*, they also form the basis for further investigation of the impact of heavy metals on the nanostructural properties of other phytoplankton species by AFM, enabling the study of native intact cell surfaces at the nanoscale without drastic sample preparation procedures.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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Supplementary information

The following supplementary material is accessible via the Supplementary Content tab on the article’s online page at <http://10.1080/09670262.2020.1758347>. Supplementary figs 1–3. Results of fit to the exponential function (Eq. 3) of growth curves of *C. closterium* exposed to different cadmium concentrations in the different parts of exponential growth phase marked by red rectangles (first 4 days in Supplementary fig. 1, first 7 days in Supplementary fig. 2, first 10 days in Supplementary fig. 3).

Author contributions

T. Mišić Radić designed the study, drafted and finalized the manuscript; A. Čačković and T. Mišić Radić were responsible for *C. closterium* cultivation; A. Čačković performed AFM measurements; A. Čačković and T. Mišić Radić analysed AFM data; A. Čačković and J. Lončar analysed the growth dynamics data; A. Penezić performed electrochemical measurements and analysed the data; J. Dautović performed DOC/POC measurements, K. Jurać did statistical analysis of *C. closterium* growth data; D. Omanović did metal speciation calculation; Z. Ljubešić revised the manuscript. All authors read and approved the final manuscript.

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Table 1. Modelling of cadmium chemical speciation in the f/2 medium (pH = 8.15, c(EDTA)= $1.17 \cdot 10^{-5}$ M).

Total Cd/ $\mu\text{g l}^{-1}$	Total Cd/ μM	Free Cd/ μM	Inorganic Cd/ μM	Total Cd/ μM	bioavailable Cd-EDTA complexes/ μM	%	Cd in bioavailable fraction	% Cd in complexes
1	0.0089	0.0002	0.0036	0.0038	0.0051	42.7		57.3
10	0.089	0.0017	0.0371	0.0388	0.0502	43.6		56.4
100	0.889	0.0197	0.4383	0.458	0.4310	51.5		48.5
500	4.449	0.1300	2.891	3.021	1.428	67.9		32.1
1000	8.897	0.2900	6.445	6.735	2.162	75.7		24.3

Table 2. Specific growth rates (μ) and growth inhibition (% I) compared with control of *Cylindrotheca closterium* exposed to different cadmium concentrations in the exponential growth phase between day 4 (D4), day 7 (D7), day 10 (D10) and day 0 (D0).

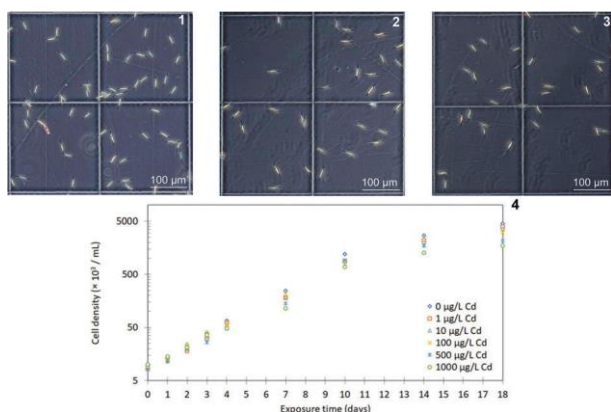
c (Cd)/ $\mu\text{g l}^{-1}$	Specific growth rates (μ/d^{-1})			Growth inhibition (% I)		
	D4	D7	D10	D4	D7	D10
0	0.48	0.46	0.48	–	–	–
1	0.49	0.44	0.46	–2.05	4.78	3.44
10	0.44	0.43	0.44	8.79	7.21	9.11
100	0.45	0.45	0.46	5.93	3.13	5.2
500	0.44	0.41	0.47	8.72	11.69	1.55
1000	0.39	0.35	0.42	18.14	24.69	11.84

Table 3. Dissolved organic carbon (DOC), dissolved organic carbon per cell (DOC_{cell}), particulate organic carbon (POC) and particulate organic carbon per cell (POC_{cell}) of *Cylindrotheca closterium* culture exposed to different cadmium concentrations at day 18 of growth. Cell density corresponds to cell density at day 18 of growth for Cd concentrations 0, 1, 10, 100, 500 and 1000 µg l⁻¹.

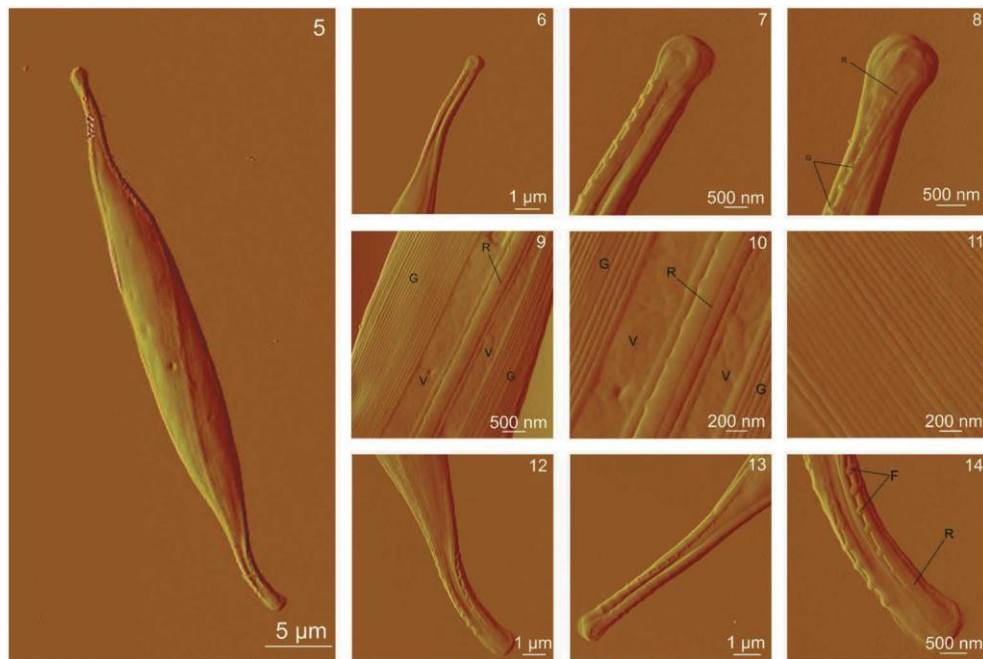
c (Cd) (µg l ⁻¹)	DOC (mg C l ⁻¹)	DOC _{cell} (pg C cell ⁻¹)	POC (mg C l ⁻¹)	POC _{cell} (pg C cell ⁻¹)	Cell density (×10 ³ cells ml ⁻¹)
0	1.053	0.23	79.9	17.6	4.528
1	0.860	0.22	101.6	25.6	3.960
10	0.901	0.25	90.2	24.6	3.656
100	0.814	0.26	109.6	35.5	3.080
500	1.747	0.82	98.1	46.2	2.120
1000	0.972	0.56	69.8	40.0	1.744

Table 4. Percentage of total Cd and dissolved Cd fraction of *Cylindrotheca closterium* cultures, compared with the concentration of Cd added in the culture measured by differential pulse anodic stripping voltammetry (DPASV). Samples were previously treated with acid (pH < 2) and UV.

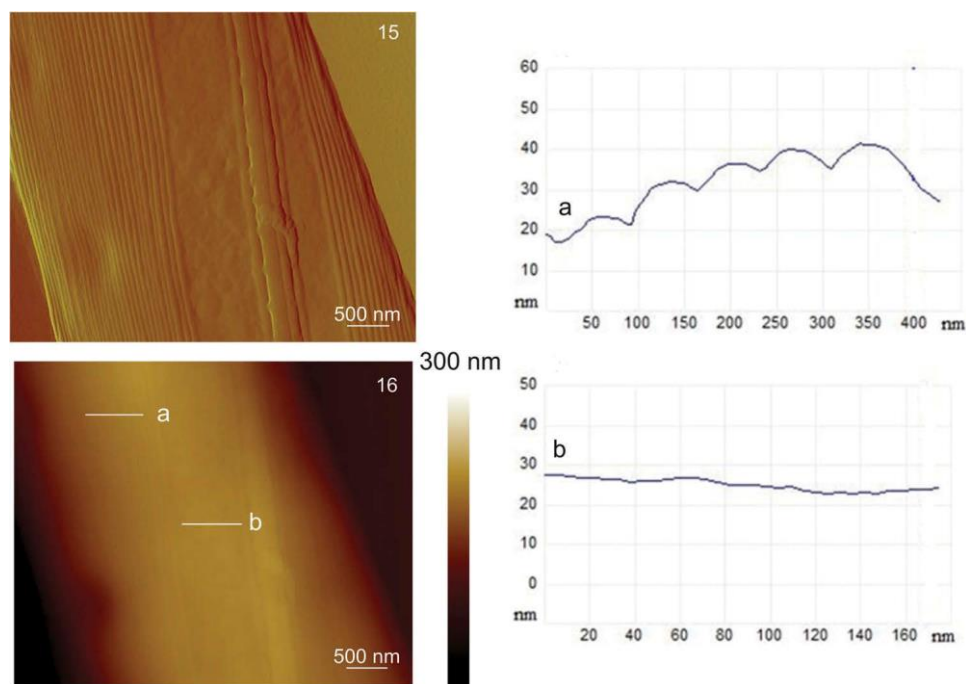
c(Cd)/ $\mu\text{g l}^{-1}$	Day 7		Day 18	
	Dissolved	Total	Dissolved	Total
1	99.0 $\pm$ 7.0	105.0 $\pm$ 7.0	92 $\pm$ 10	94 $\pm$ 17
10	109.0 $\pm$ 9.0	97.9 $\pm$ 11	99.4 $\pm$ 5.7	106.8 $\pm$ 4.8
100	106.1 $\pm$ 8.5	99.1 $\pm$ 5.3	107.5 $\pm$ 4.7	102.5 $\pm$ 8.6
500	89.8 $\pm$ 6.8	91.0 $\pm$ 6.7	106.2 $\pm$ 6.7	104.5 $\pm$ 6.2
1000	82.9 $\pm$ 6.2	95.1 $\pm$ 4.5	94.1 $\pm$ 4.4	103.7 $\pm$ 6.0



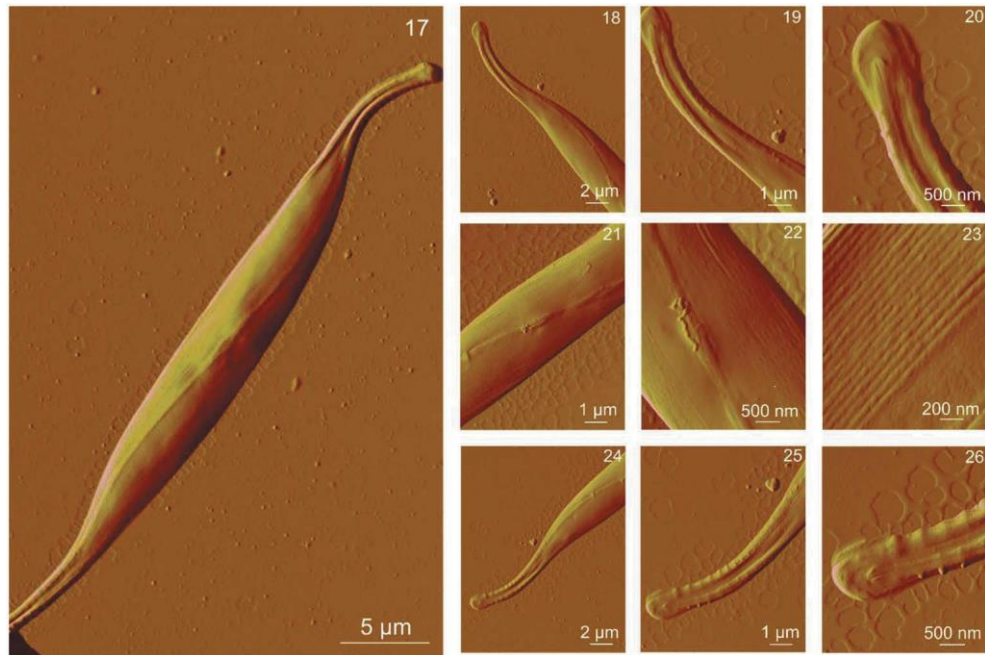
Figs 1–4. Effects of different concentrations of cadmium on the growth of *Cylandrotheca closterium*. Figs 1–3. Optical micrographs of *C. closterium* culture on day 3 of growth in the control group (Fig. 1); in culture exposed to 500 µg Cd l⁻¹ (Fig. 2); in culture exposed to 1000 µg Cd l⁻¹ (Fig. 3). Fig. 4. Growth curves of *C. closterium* exposed to different cadmium concentrations (0–1000 µg Cd l⁻¹) as measured by cell density over an 18 day period (values are the mean of duplicate determinations).



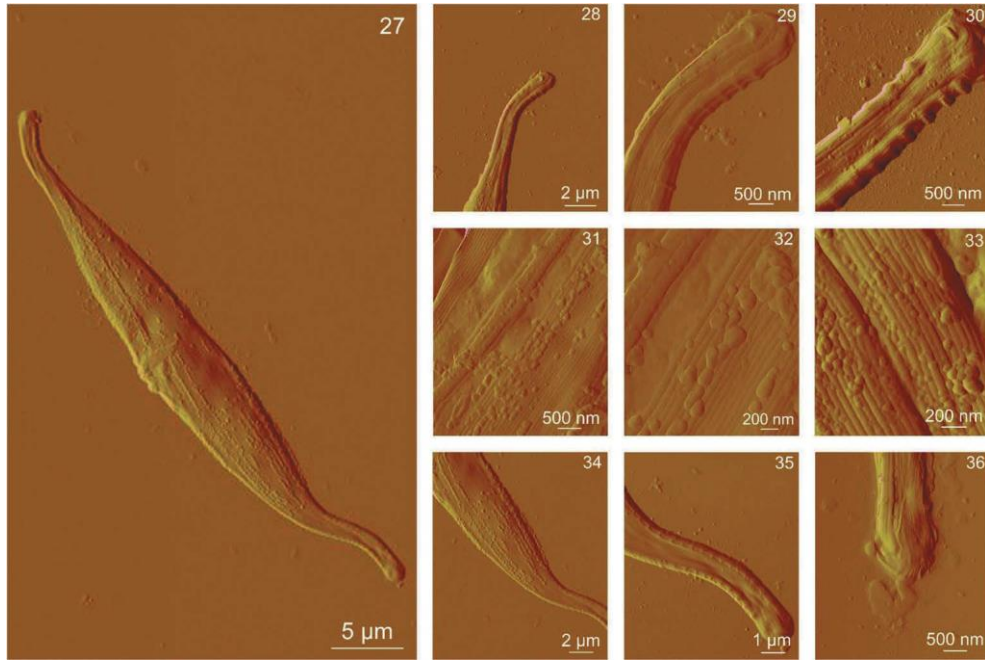
Figs 5–14. AFM images of a whole *Cylindrotheca closterium* cell and its morphological details acquired using contact mode in air. Fig. 5. The whole *C. closterium* cell. Figs 6–8. Enlarged upper rostra of the cell with raphe and fibulae. Figs. 9–11. The enlarged centre of the cell where girdle band, valve and raphe are seen in more detail. Figs. 12–14. Enlarged lower rostra of the cell. All images are deflection data with scan sizes: $45\ \mu\text{m} \times 25\ \mu\text{m}$ (Fig. 5); $8.5\ \mu\text{m} \times 8.5\ \mu\text{m}$ (Fig. 6); $5\ \mu\text{m} \times 5\ \mu\text{m}$ (Fig. 7); $4\ \mu\text{m} \times 4\ \mu\text{m}$ (Fig. 8); $4.5\ \mu\text{m} \times 4.5\ \mu\text{m}$ (Fig. 9); $2\ \mu\text{m} \times 2\ \mu\text{m}$ (Fig. 10); $2\ \mu\text{m} \times 2\ \mu\text{m}$ (Fig. 11); $8.5\ \mu\text{m} \times 8.5\ \mu\text{m}$ (Fig. 12); $8\ \mu\text{m} \times 8\ \mu\text{m}$ (Fig. 13); $4\ \mu\text{m} \times 4\ \mu\text{m}$ (Fig. 14). Labels V, G, R and F indicate the following features: valve, girdle band, raphe and fibulae.



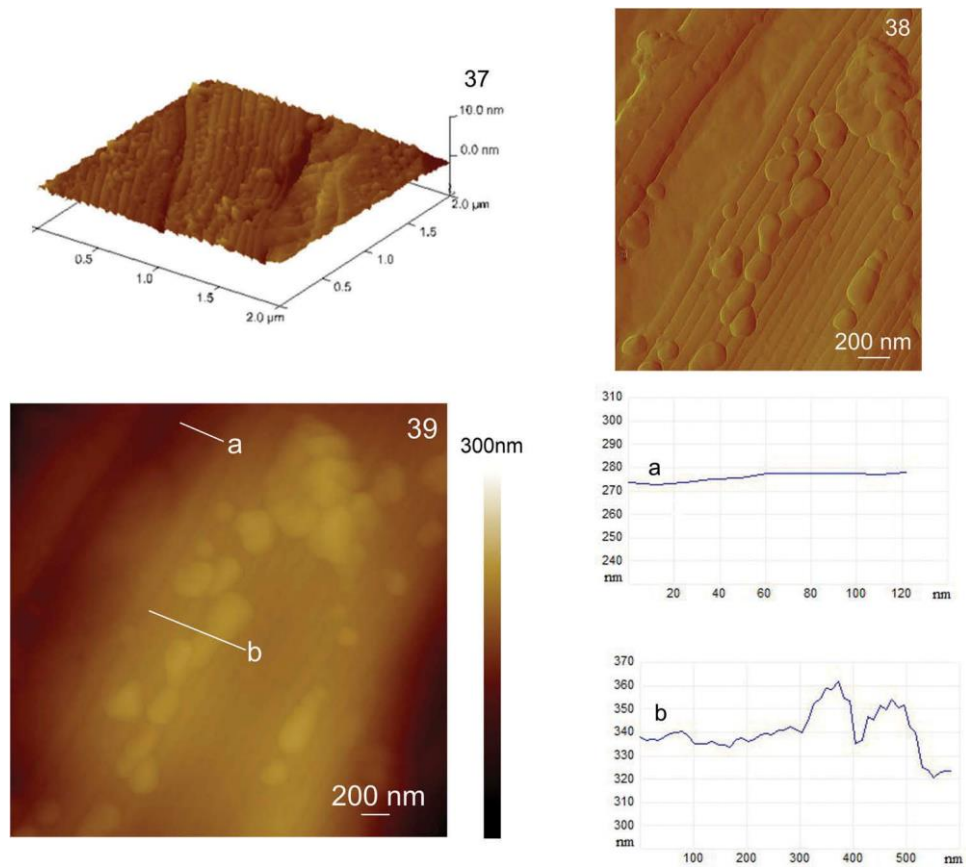
Figs 15–16. AFM images of the centre of the *Cylandrotheca closterium* cell acquired in contact mode. Fig. 15. Deflection data (scan size: $4\ \mu\text{m} \times 5\ \mu\text{m}$). Fig. 16. Height data (scan size: $4\ \mu\text{m} \times 5\ \mu\text{m}$, vertical scale: 300 nm) with analysis of vertical sections of the girdle band along the indicated line a and analysis of vertical sections of the valve along the indicated line b.



Figs 17–26. AFM images of a whole *Cylandrotheca closterium* cell grown at cadmium concentration $500 \mu\text{g l}^{-1}$ and its morphological details acquired using contact mode in air. Fig. 17. The whole *C. closterium* cell. Figs 18–20. Enlarged upper rostra of the cell. Figs 21–23. The enlarged centre of the cell where girdle band and valve are seen more detailed. Figs 24–26. Enlarged lower rostra of the cell with expressed raphe and fibulae around it. All images are deflection data with scan sizes: $35 \mu\text{m} \times 25 \mu\text{m}$ (Fig. 17); $18 \mu\text{m} \times 14 \mu\text{m}$ (Fig. 18); $9 \mu\text{m} \times 7 \mu\text{m}$ (Fig. 19); $4.5 \mu\text{m} \times 3.5 \mu\text{m}$ (Fig. 20); $9 \mu\text{m} \times 7 \mu\text{m}$ (Fig. 21); $4.5 \mu\text{m} \times 3.5 \mu\text{m}$ (Fig. 22); $2 \mu\text{m} \times 1.5 \mu\text{m}$ (Fig. 23); $18 \mu\text{m} \times 14 \mu\text{m}$ (Fig. 24); $9 \mu\text{m} \times 7 \mu\text{m}$ (Fig. 25); $4.5 \mu\text{m} \times 3.5 \mu\text{m}$ (Fig. 26).



Figs 27–36. AFM images of a whole *Cylindrotheca closterium* cell grown at cadmium concentration $1000 \mu\text{g l}^{-1}$ and its morphological details acquired using contact mode in air. Fig. 27. The whole *C. closterium* cell. Figs 28–30. Enlarged upper rostra of the cell. Figs 31–33. The enlarged centre of the cell where girdle band and valve are seen in more detail. Figs 34–36. Enlarged lower rostra of the cell. All images are deflection data with scan sizes: $50 \mu\text{m} \times 30 \mu\text{m}$ (Fig. 27); $13.5 \mu\text{m} \times 11.5 \mu\text{m}$ (Fig. 28); $4.5 \mu\text{m} \times 4 \mu\text{m}$ (Fig. 29); $4.5 \mu\text{m} \times 4 \mu\text{m}$ (Fig. 30); $4.5 \mu\text{m} \times 4 \mu\text{m}$ (Fig. 31); $2.5 \mu\text{m} \times 2 \mu\text{m}$ (Fig. 32); $2 \mu\text{m} \times 1.5 \mu\text{m}$ (Fig. 33); $18 \mu\text{m} \times 15 \mu\text{m}$ (Fig. 34); $9 \mu\text{m} \times 7.5 \mu\text{m}$ (Fig. 35); $4.5 \mu\text{m} \times 4 \mu\text{m}$ (Fig. 36).



Figs 37–39. AFM images of the centre of the *Cylindrotheca closterium* cell, grown at cadmium concentration $1000 \mu\text{g l}^{-1}$, acquired in contact mode. Fig. 37. Three-dimensional display of the girdle band (scan size $2 \mu\text{m} \times 2 \mu\text{m}$, vertical scale 10 nm). Fig. 38. Deflection data of the girdle band (scan size $2.5 \mu\text{m} \times 2 \mu\text{m}$). Fig. 39. Height data (scan size $3.5 \mu\text{m} \times 3.5 \mu\text{m}$, vertical scale 300 nm) with analysis of vertical sections section of the valve along the indicated line a and analysis of vertical sections of the girdle band along the indicated line b.