# Response of the green microalga *Chlorella vulgaris* to the oxidative stress caused by some heavy metals

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**Abstract** The present work was carried out to study the response of the microalga *Chlorella vulgaris* to the oxidative stress caused by Cu, Cd and Zn on growth, total lipids, lipid peroxidation, fatty acids composition, antioxidant enzymes and ultrastructure. The lower concentrations of the three heavy metals stimulated *C. vulgaris* growth, while the higher ones inhibited the algal growth. The three tested metals could be arranged according to their toxicities to *C. vulgaris* in the following order: Cu > Cd > Zn. The three metals also induced an increase in total lipid content, lipid peroxidation and activities of peroxidase and catalase; the induction by Cu being stronger than by Cd and Zn. The fatty acids of *Chlorella vulgaris* were dominated by 16:0, 17:0, 18:1 and 18:2. The three metals caused the appearance of lauric acid , increased significantly the content of 18:0 and decreased the contents of the C14:0, C16:0, and C17:0.Considering unsaturated fatty acids, *C. vulgaris* responded to the three metals by decreasing the production of 16:1 with considerable increase in the production of 18:1. Cd and Zn increased the production of 18:2 and 18:3, however; Cu decreased their production. The overall effect of the tested metals was to increase the ratio of unsaturated to saturated fatty acids. Cellular damage was studied under transmission electron microscope. The alterations induced by Cd and Cu were invagination of cell envelop, disintegration of thylakoid membranes; increase in the size of inclusion bodies inside the vacuoles, lack of cristae in the mitochondrion, formation of mitochondrial myelin- like structure and dark dots on the cell surface. Zn induced the formation of a dark electron dense layer with an amorphous aspect on the cell surface and numerous plastoglobuli in the cytoplasm. The differences in subcellular effects induced by Cu, Cd and Zn are probably due to specific adaptation mechanisms developed by *C. vulgaris*.

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## 1. Introduction

Toxic effect of heavy metals on living systems is one of the main problems derived from environmental contamination. Microalgae, the key component of the food web in aquatic ecosystems, being seriously affected by metal pollution [1,2]. Increasing levels of heavy metals in the environment affect various physiological and biochemical processes of microalgae. It can cause adverse effects on cell division, growth, photosynthesis, respiration, uptake and assimilation of nitrate and degeneration of the main cell organelles [3,4]. Heavy metals toxicity depends on the metal concentration [5,6].

Heavy metals lead to the formation of reactive oxygen species in algal cells [7,8,9] causing lipid peroxidation [10,11]. The defense mechanisms of algae against oxidative stress can be broadly classified into two types: i) mechanisms that prevent interaction between the metal (s) and their site (s) of actions, and (ii) those that counteract the stress-induced damages. The latter include antioxidant systems consisting of enzymatic and non-enzymatic components. A large amount of convincing evidence demonstrates an increased synthesis of non-enzymatic antioxidants like glutathione and ascorbate as well as enhanced antioxidant enzymes under metal stress [12,13,8]. The amount of oxidized proteins and lipids in the algal cells thus indicates the severity of the stress in a number of microalgal species[3]. Moreover, cell resistance to growth inhibitor can over produce polyunsaturated fatty acids (PUFA) [14]. Algal tolerance to heavy metal is highly dependent upon the defense response against the probable oxidative damages [2].

In this connection, *Scenedesmus acutus* responded to nickel toxicity by higher activities of antioxidant enzymes as catalase, superoxide dismutase, glutathione reductase and glucose-6- phosphate dehydrogenase [7]. In *Cladophora glomerata* Lipoperoxides showed positive correlation to heavy metals accumulation sites indicating the tissue damage resulting from the reactive oxygen species and resulted in unbalance to cellular redox status. Also, high activities of ascorbate peroxide and superoxide dismutase, increased dehydroascorbate, decreased glutathione and soluble phenols probably counter balance of this oxidative stress [8].

The study of the ultrastructural changes induced by heavy metals is important because it enhances our understanding of the pathways of metal toxicity, and of algal possible defense mechanisms to cation stress [15]. Copper-induced structural alterations in thylakoid membranes of *Chlorella sp.* [16]. An electron dense layer on the cell surfaces and an accumulation of starch around the pyrenoids were detected in cells of *Pseudochlorococcum typicum* treated with mercury, lead and cadmium. A clear deterioration of cell organelles were recorded in Hg and Cd- treated cells more than in Pb-treated ones [17] .

In view of free radical formation by heavy metals in algae and the lack of information about the response of the antioxidant system of microalgae, this study has been undertaken to find out the response of the microalga *Chlorella vulgaris* to the oxidative stress of Cu, Cd and Zn on growth, lipids content, lipid peroxidation and fatty acids composition, cellular changes and peroxidase and catalase as antioxidant enzymes.

## 2. Materials and methods

### 1- Algal cultures

The unicellular green alga *Chlorella vulgaris*, strain (211-11b), (Sammlung von algen kulturen, Pflanzen Physiologisches Institut, Universitat Gottingen, Germany) was cultured in Kuhl medium[50]. Axenic cultures of the organisms were obtained by repeated subculturing and adding a mixture of streptomycin and tetracycline (30 ppm) to the medium for 20 minutes. The technique of mass culture [51] was applied to obtain sufficient algal cultures for the different investigations. Equal densities of algal cells (5ml of 7-day-old culture) were inoculated in 300 ml culture media. The algal suspension was grown in 400 ml cylindrical Pyrex glass vessels (50 cm in length and 4.5 cm in diameter) with narrow side tubes. The cultures were illuminated by means of fluorescent tubes (40W.F.7 day light), which gave light intensity of about 12 kilolux. The cultures were aereated with a mixture of 97% air and 3% $CO_2$. The algal growth was monitored by measuring the optical density of the cell suspension spectrophotometrically at 560 nm[52].

**2- Total lipids, Fatty acids and Lipid peroxidation**. Total lipids were determined by the method of Bligh and Dyer [53] and the fatty acids were fractioned and detected by the gas liquid chromatographic method (Radwam) [54]. Lipid peroxidation was estimated as the concentration of thiobarbituric acid-reactive substances, largely malondialdehyde, by the method of Heath and Packer[55] .

**3- Peroxidase and Catalase.** Peroxidase (EC 1.11.1.7) activity was assayed as described by Kato and Schimizu[56] . The reaction medium (3ml) consisted of 7.2 mM guaiacol , 11.8 mM $H_2O_2$ in 0.1 M sodium phosphate buffer (pH 5.8). Hundred µl of the enzyme extract was added to initiate the reaction. Total peroxidase activity was expressed as the increase in the absorbance at 470 nm per min in 100 µl of algal extract. Catalase (EC 1. 11. 1.6) activity was measured by recording the decomposition of $H_2O_2$ as expressed by a decrease in the absorbance at 240 nm according the method described by Kato and Schimizu[56]. The reaction mixture (3ml) contained 0.1 M sodium phosphate buffer (pH 7), 2mM $H_2O_2$ and 0.1 ml enzyme extract. All data were obtained from three separate cultures and were represented as mean ± SE.

**4- Electron microscopy.** For electron microscopy, cells were collected via centrifugation, washed three times with phosphate buffer (pH 7.8), fixed with 3.5% glutaraldehyde for 2 hrs, and postfixed in 1% osmium tetroxide for 2 hrs in cold phosphate buffer and rinsed. Samples were included in 2% agar blocks, dehydrated in a graded ethanol-water series to 100% ethanol and embedded in Spurr low-viscosity resin. The fixation technique of Hayat [58] was followed. Ultrathin sections were cut with a diamond knife, double stained with uranyl acetate and lead citrate, and examined under a JEX- 100SX transmission electron microscope operating at 75 KV.

## 3. Results and Discussion

**The growth**: Many heavy metal ions have a direct influence on various physiological and biochemical processes of microalgae. As the growth reflects the metabolism of the cell, it has been used as a key indicator of the toxicity of heavy metal ions in microorganisms [18]. *Chlorella vulgaris* responded differently to Cu, Cd and Zn toxicities (Figure 1). Cu stimulated *C. vulgaris* growth up to 1.5 ppm which raised the growth by 21%, further increase in copper concentration decreased the algal growth by 50% in 2.5 ppm treated culture after 7 days. In accordance with our results, Cu (16 µg /L) inhibited the growth of *Chlorella sp.* by 50% [19].

Although 0.5 ppm Cd raised the algal growth by 7%, higher Cd concentrations decreased the algal growth successively. The maximum reduction was 47% in culture treated with 5 ppm Cd (Figure1). These results indicated that *C. vulgaris* showed high tolerance to cadmium, since the $EC_{50}$ of diatoms, *Navicula incerta* and *Nitzschia closterium*, were 3.01 and 0.48 ppm, respectively, while the $EC_{50}$ of *Chlorococcum sp.* is between 2.5 and 3 ppm [20].

*Chlorella vulgaris* tolerated Zn toxicity than Cu and Cd. Thus, 30 and 50 ppm Zn reduced *C. vulgaris* growth by 30 and 46%, respectively. The three tested metals could be arranged according to their toxicities to *C. vulgaris* in the following order: Cu > Cd > Zn. In accordance with our results, the toxic effect of Cu on *Chlorella vulgaris* growth was the greatest when

compared to Pb, Cd and Zn [21]. The dose-dependent manner of growth inhibition was proved also to the effects of $Cd^{2+}$, $Cu^{2+}$, $Zn^{2+}$, $Pb^{2+}$ and $Fe^{2+}$ on the green alga *Scenedesmus quadricauda* [22]. Metals induced concentration dependent reduction in the cell count of *Scenedesmus bijuga* and *Anabaena spiroides* [23]. $Hg^{2+}$ was highly toxic than $Cd^{2+}$ and $Pb^{2+}$ to the green microalgae *Pseudo chlorococcum typicum* and *Scenedesmus quadricauda* and the lower doses of $Cd^{2+}$ and $Pb^{2+}$ were stimulatory for Chlorophyll a and proteins whereas, the higher ones were inhibitory [17].

**Total lipids and lipid peroxidation**: The total lipids content of *C. vulgaris* increased in response to Cu, Zn by 22 and 17%, respectively, whereas, decreased by 17% in response to Cd treatment (Table 1). Extensive Cd binding on lipid bodies could led to their dissolution leading to lipids reduction [24] . However, the accumulation of total lipids under heavy metal stress was reported in a number of microalgal species as a mechanism of metal detoxification [3,15].

The oxidative stress induced by $Cu^{2+}$, $Cd^{2+}$ and $Zn^{2+}$ was clearly depicted by an increase in malondialdehyde content amounted to 2.9, 2.1 and 1.7 folds, respectively (Table 1). This indicated elevated levels of the active oxygen species which in turn might disturb antioxidant balance leading to lipid peroxidation. However, $Cu^{2+}$ caused lipid peroxidation more than $Cd^{2+}$ and $Zn^{2+}$. In this connection, $Cu^{2+}$ and $Ni^{2+}$ and $Cd^{2+}$ produced significant increase in lipid peroxidation in *Anabaena doliolum*[13], *Scenedesmus acutus*[7] and *Chlamydomonas* [11], respectively.

**Peroxidase and catalase activities:** Peroxidase activity was raised in the presence of $Cu^{2+}$, $Cd^{2+}$ and $Zn^{2+}$, the induction by $Cu^{2+}$ being stronger than by $Cd^{2+}$ or $Zn^{2+}$. A similar trend was also observed for catalase with a percentage increment of 7, 4 and 1.9 folds in the above order ( Table 1). Induction of peroxidase is a general stress response and is not specific to metals [25]. Heavy metals may induce general biochemical reactions involving formation of $H_2O_2$ and/or organic peroxides [26]. Metals can break the oxidative balance of the algae, inducing antioxidant enzymes, such as superoxide dismutase (SOD), glutathione peroxidase (GPX) and ascorbate peroxidase (APX) [2]. The elevated levels of lipid peroxidation as affected by the three tested metals may explain the high activity of peroxidase and catalase. Cadmium increased peroxidase and catalase activities in *Scenedesmus armatus* [27]. $Ni^{2+}$ induced higher level of catalase in *Scenedesmus acutus* [7].$Cu^{2+}$ raised ascorbate peroxidase activity in *Selenastrum Capricornutum* [28] and peroxidase activity in *Phaeodactylum tricornutum* [29]. The antioxidant enzymes are prominent biomarkers of defense against oxidative stress. Catalase destroys the toxic $H_2O_2$. These results suggest that one major mechanism of $Cu^{2+}$, $Cd^{2+}$ and $Zn^{2+}$ resistance in *Chlorella vulgaris*, may be the ability to combat the formation of reactive oxygen species (ROS) when exposed to metals, likely by maintaining high levels of antioxidant enzymes.

**Fatty acids composition:** The fatty acids of *Chlorella vulgaris* were dominated by 16:0, 17:0, 18:1 and 18:2. Considering the saturated fatty acids, the three metals caused the appearance of lauric acid in the fatty acids profile of *C. vulgaris* which was not detected in the control cells. Cu, Cd and Zn increased significantly the content of 18:0 specially at higher concentrations, the increase reached 6.4, 4.2 and 1.8 folds, respectively. Copper at low concentration increased 14: 0 by 51%. At the same time, the three metals decreased the contents of the C14:0, C16:0, and C17:0. The most pronounced reductions were 60 and 62% in 17:0 content in cultures treated with 1.5 ppm Cu and 0.5 ppm Cd, respectively (Table 2).

Considering unsaturated fatty acids, *C. vulgaris* responded to the three metals by decreasing the production of monounsaturated 16:1 accompanied with considerable increase in the production of 18:1. In addition, low concentrations of Cd and Zn increased the production of 18:2 whereas, their higher concentrations increased the production of 18:3 by 50 and 30%, respectively (Table 2). It has been postulated that cells resistance to growth inhibitor can over produce polyunsaturated fatty acids due to the effect of the inhibitor on fatty acids desaturation [14]. On the other hand, Cu decreased the production of 18:2 and 18:3. This result was supported by the higher lipid peroxidation induced by Cu than Cd and Zn. The specific inhibition of linolenic acid under environmental stress is regarded as a monitor of lipid peroxidation [30]. In this connection, Cd caused selective decline in 18:3 in the chloroplast of Soybean[31]. The overall effect of Cu, Cd and Zn was to increase the ratio of unsaturated to saturated fatty acids, thereby increasing the fluidity of membranes[32] and membranes may become more or less fluid depending on the length of the fatty acid chain[33].

Fig. 2 shows the effects of cadmium on cell ultrastructure. *C. vulgaris* control cell, shows the normal ultrastructure (Fig 2A).The most distinct feature of the cell cytoplasm is the chloroplast (Cp) , which composed of stacks of thylakoid membranes (Ty) containing numerous starch grains. Small intrathylakoid membranes are also observed. In addition, the mitochondrion (M) with normal size and the cell contains many vacuoles with inclusion bodies inside few of them. Fig.2B-D show the cell damage caused by cadmium. Fig.2B shows that the cell envelop was often invaginated leading to the altered cell form; the thylakoid membranes are disintegrated; the protoplast of such cells appeared severely damaged; only few breakdown products of it were present in the

collapsed cells. In addition, increase in the size of inclusion bodies inside the vacuoles was observed. Fig.2C shows lack of cristae in the mitochondrion (M) which consists of only two concentric membranes with increased density in the matrix. Although Cd-induced degeneration, a begining of vacuoles formation was observed to increase their number in the surrounding cytoplasm. An increase in the number of vacuoles as well as the presence of electron dense deposits in vacuole and membrane whorls was detected in *Chlamydomonas acidophila* treated by Cd, Cu and Zn [34]. Energy-dispersive X-ray analysis revealed that vacuolar deposits inside cells treated with Cd contained Cd and phosphate. Fig.2 D shows the formation of mitochondrial Myelin- like structure (Arrow). In addition, dark dots on the cell surface (Arrow heads) were detected.

The disintegration of thylakoid membranes by cadmium was observed in *Spirulina platensis* [35]. The injury of the thylakoids by heavy metals reffered to the elevated oxygen free radicals and lipid peroxidation[36]. Our results support such interpretation, since Cu, Cd and Zn elevated lipid peroxidation. Some studies indicated that photosynthetic structures or chloroplasts of *Chlamydomonas*, *Dunaliella* and *Nostoc* are cellular targets of cadmium [15,37,17]. Copper-induced structural alterations in thylakoid membranes of *Chlorella sp* [16]. The main ultrastructural changes in algal cells following heavy metals exposure were located in the chloroplasts and mitochondria [38]. Cadmium inhibited algal photosynthesis and mostly accumulated in the chloroplasts [39].

The increase in the number of vacuoles via the formation of new ones in metal treated cells is one of the mechanisms of tolerance. Sequestering metal ions in the vacuole is a method of maintaining low cytosolic concentrations of ions. Incapacity of metal ion transport mechanisms into the vacuole may lead to cell damage [38]. The bioaccumulation of spherical electron dense bodies inside the Cd and Pb-treated *Pseudo-Chlorococcum typicum* cells or in the vacuoles was a mechanism contributed to the heavy metal tolerance by minimizing as possible the cytoplasmic metal concentrations by binding or complexing the metal ions with phytochelatin or in the form of metallo-sulfur, metallo-iron or metallo-phosphate complexes in the cytosol and carrying them into the vacuoles where the acidic pH displace the metal, allowing the peptide to return to the cytosol. In the vacuole the metal would sequestered by organic acids usually present in high concentration in the vacuoles [17]. This was performed as a cellular protection or detoxification mechanisms [40]. Also, vacuolar deposits trapping Cu and Cd were detected in *Skeletonema costatum* [41].

**Table 1. Effect of Cu, Cd and Zn on lipid content (% dry wt.), lipid peroxidation (µM. mg dry $wt^{-1}$), peroxidase (µM. mg dry $wt^{-1}.min^{-1}$) and catalase (µM. mg dry $wt^{-1}.min^{-1}$) activities of *Chlorella vulgaris* grown for 7 days.Mean ± SE.**

| | Lipid content | Lipid peroxidation | Peroxidase | Catalase |
|---|---|---|---|---|
| Cont. | 11.64 ± 0.23 | 2.3 ± 0.04 | 0.23 ± 0.03 | 0.18 ±0.01 |
| Cu (2.5ppm) | 14.20 ± 0.43 | 6.7 ±0.13 | 1.7 ± 0.01 | 0.72 ± 0.03 |
| Cd (5ppm) | 9.67 ± 0.19 | 4.8 ± 0.12 | 0.92 ±0.02 | 0.54 ± 0.02 |
| Zn (50ppm) | 13.62 ± 0.34 | 3.9 ± 0.09 | 0.44 ± 0.01 | 0.27 ± 0.01 |

**Table 2. Fatty acid composition of *Chlorella vulgaris* grown at different concentrations of Cu, Cd and Zn.**

| **Fatty acids** | | **Cont.** | **Cu** | | **Cd** | | **Zn** | |
|---|---|---|---|---|---|---|---|---|
| | | | 0.5ppm | 2.5ppm | 0.5ppm | 5ppm | 1ppm | 50ppm |
| Lauric | 12:0 | - | 0.86 | 0.80 | 0.92 | - | 1.63 | 2.14 |
| Myristic | 14:0 | 2.81 | 4.25 | 3.83 | 1.54 | 1.67 | 1.99 | 1.97 |
| Palmitic | 16:0 | 23.12 | 21.25 | 20.08 | 23.27 | 22.97 | 21.75 | 16.41 |
| Palmitoleic | 16:1 | 5.89 | 3.54 | 4.88 | 4.65 | 2.45 | 3.964 | 2.57 |
| Heptadecanoic | 17:0 | 13.72 | 10.10 | 5.42 | 10.03 | 5.27 | 9.33 | 12.71 |
| Stearic | 18:0 | 0.69 | 1.62 | 4.41 | 1.99 | 2.89 | 1.12 | 1.25 |
| Oleic | 18:1 | 12.35 | 24.41 | 27.98 | 19.10 | 24.28 | 18.66 | 22.43 |
| Linoleic | 18:2 | 33.03 | 29.35 | 29.53 | 34.58 | 27.91 | 36.43 | 29.54 |
| Linolenic | 18:3 | 8.39 | 4.60 | 3.07 | 3.92 | 12.55 | 5.123 | 10.98 |
| Total Sat. % | | 40.34 | 38.09 | 34.53 | 37.75 | 32.81 | 35.82 | 34.48 |
| Total Unsat. % | | 59.65 | 61.91 | 65.47 | 62.24 | 67.18 | 64.17 | 65.52 |
| Unsat./ Sat. ratio | | 1.48 | 1.63 | 1.90 | 1.65 | 2.05 | 1.79 | 1.9 |

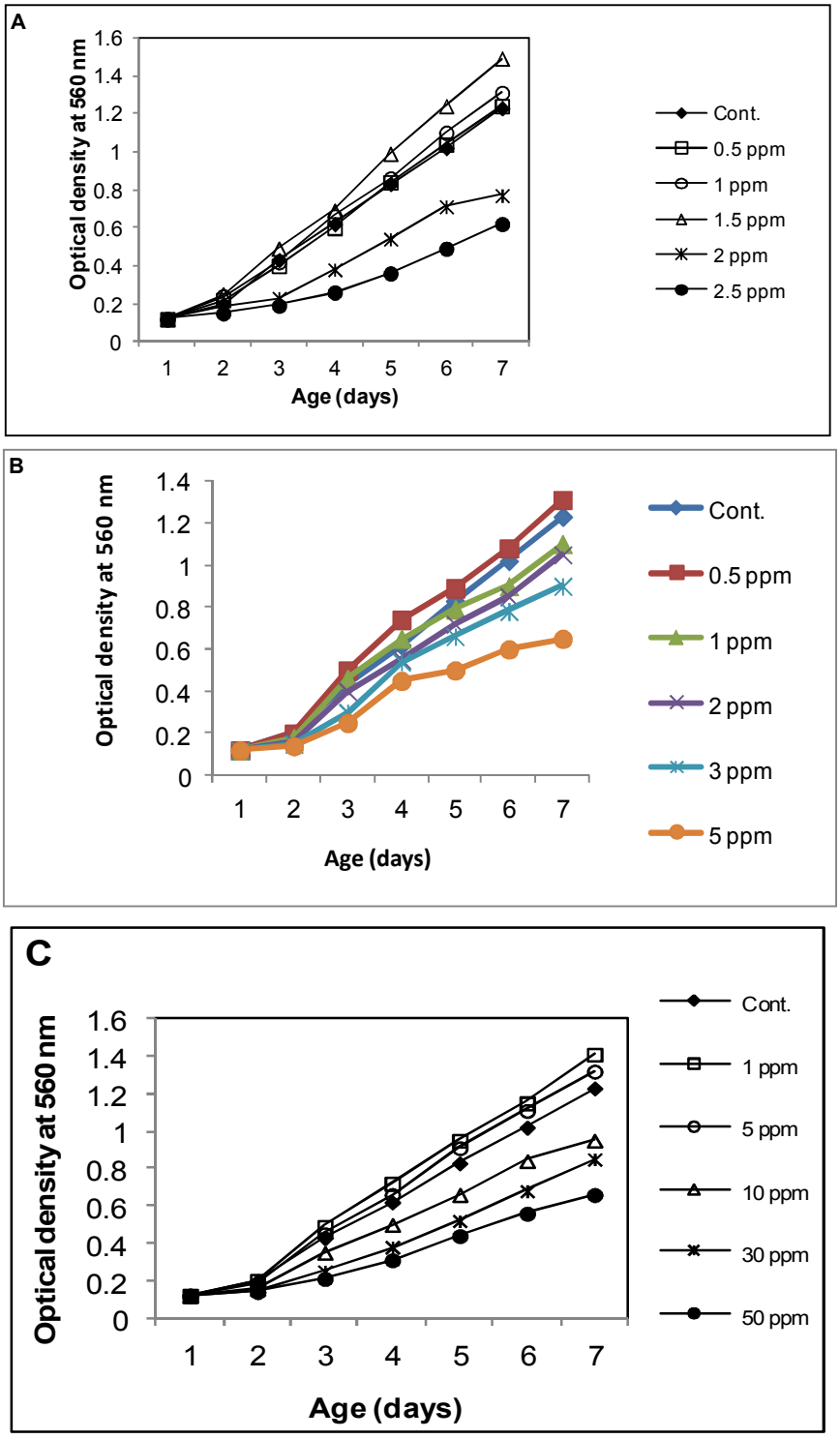


**Fig. 1 Effect of different concentrations of Cu (A), Cd (B) and Zn (C) on the growth of *Chlorella vulgaris* measured as optical density at 560 nm ( Data are means of three replicates)**

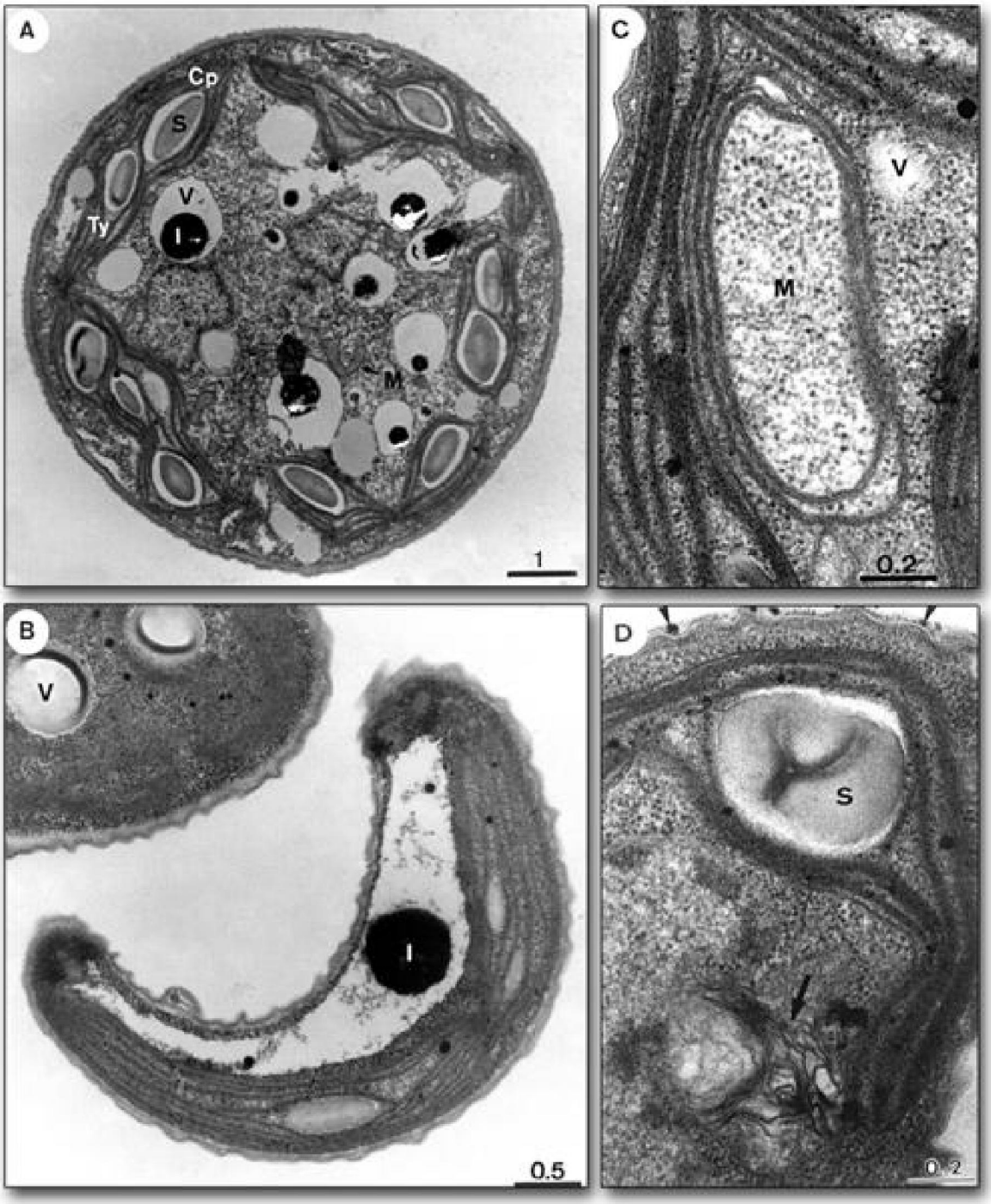


**Fig. 2** Transmission electron micrographs of *Chlorella vulgaris*. Fig.2A Control cell showing the normal ultrastructure. The chloroplast (Cp) composed of stacks of thylakoid membranes (Ty) containing numerous starch grains (S), the mitochondrium (M) with normal size and the cell contain many vacuoles (V) with inclusion bodies (I) inside few of them. Fig.2B-D Cadmium damaged cells. Fig.2B shows invagination in the cell envelop leading to altered cell form; disintegration in the thylakoid membranes; severe damage in the protoplast, increase in the size of inclusion bodies (I) inside the vacuoles. Fig.2C shows lack of cristae in the mitochondrium (M) which consists of only two concentric membranes with increased density in the matrix and bigining of vacuole (V) formation. Fig.2 D shows the formation of mitochondrial Myelin- like structure (arrow) and dark dots on the cell surface (arrow heads).

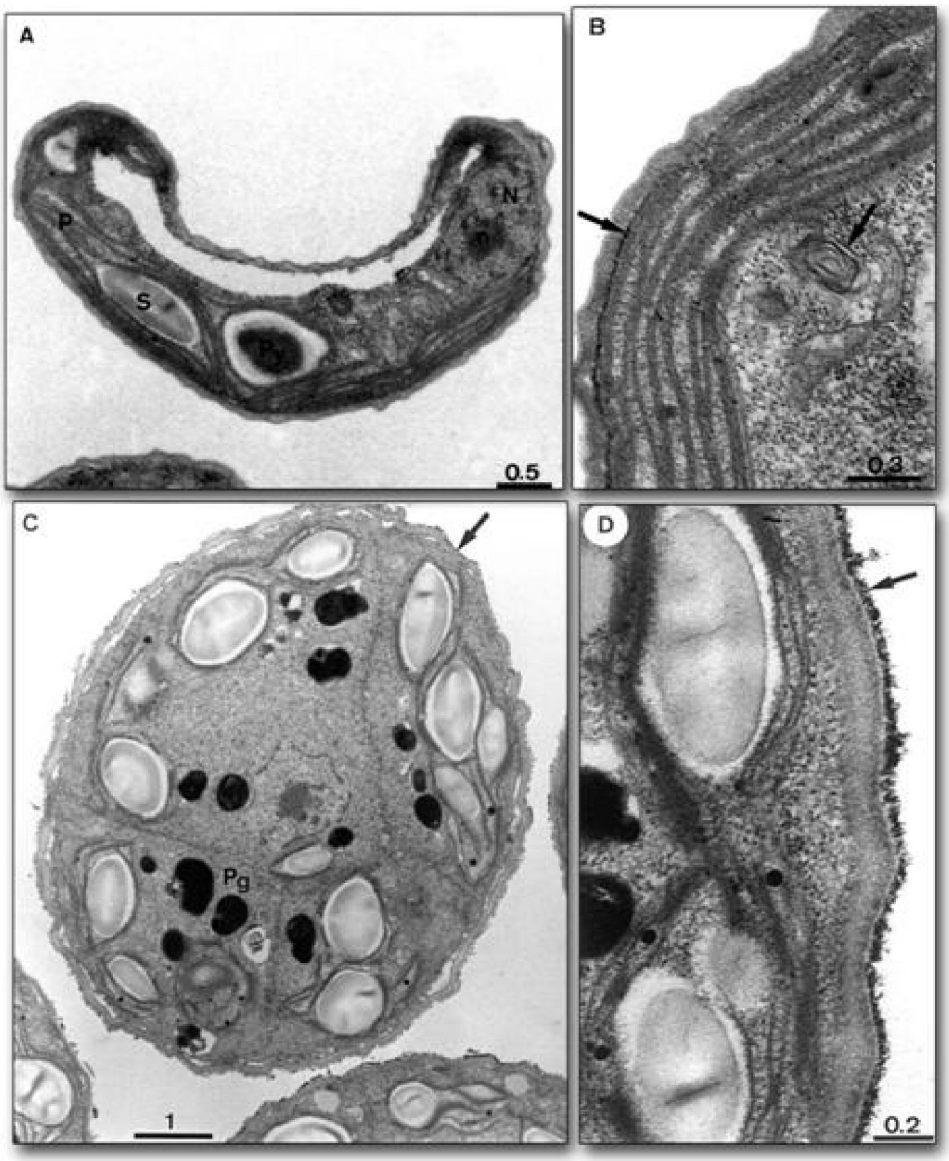


**Fig. 3** Cu and zinc damaged *C. vulgaris* cells. Fig.3A-B Cu damaged cell showing invagination in the cell envelop, cell form alteration, starch grains with pyrenoids (Py), nucleus (N) and nucleolus (n). Fig.3B shows mitochondrial myelin-like structure (arrow) and dark amorphous layer (arrow) within the cell envelop. Fig.3 C-D Zn damaged cell showing the formation of numerous plastoglobuli (Pg) of different size. Fig. 3D shows a dark electron dense layer with an amorphous aspect (arrow) on the cell surface.

Fig.3 A shows that Cu induced a similar cell form alteration to that induced by Cd (Fig.2B). Also, Cu induced mitochondrial myelin-like structure (Fig.3B). In cells treated with Cu, a dark and amorphous layer (arrow) was often observed deep within the cell envelop, most probably associated with cytoplasmic membrane. The observed electron dense layer on the algal cell surfaces after heavy metal treatments referred to the biosorbed (adsorbed) metal ions binded with different functional groups on algal cell surfaces which was considered as a protective mechanism for limiting most of the toxic ions[17]. The percentage of metal ion adsorbed fraction and insoluble fractions increased with metal concentration[42]. Lead induced the formation of similar electron dense patches consisting of disordered microfibrils in *Micrasterias* [43]. Mitochondrial myelin-like structure was detected in *Euglena* cells treated with Cd [44]. They reported that these structures are non functional phospholipid materials arise from folds and rollings of mitochondrial inner membrane which are finally ejected into the hyaloplasm and eliminated. The damage of the respiratory enzyme system is

mainly caused by Cd responsible for the formation of these structures [45].

After exposure to Zn, *C. vulgaris* cells revealed a surface layer (arrow), which at higher magnification (Fig.3D), appeared as a dark electron dense layer with an amorphous aspect. Remarkably, cell toxification with Zn lead to the formation of numerous plastoglobuli (Pg) of different size (Fig.3C). These plastoglobuli seem to have a role in binding and chelating Zn (metals) entering the cytoplasm. The presence of numerous globules with polyphenolic matrix binding Zn were detected in *Mougeotia scalaris*[46].

The appearance of dark amorphous electron dense layer in the cell envelop of cells treated with Cu and Zn reflect the external surface sorption (exclusion) which is considered as the first defense mechanism against toxic heavy metals [42,47]. Once external sorption reaches the saturated stage, internal uptake begins [48]. In this connection, lead phosphate was precipitated on the cell wall of *Anabaena cylindrica* and then inside the cell [49]. The differences in subcellular effects induced by Cu, Cd and Zn are probably due to specific adaptation mechanisms developed by *C. vulgaris*.

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