

## The Effects of Sewage Water on Ultrastructure of *Chlorella vulgaris* and *Scenedesmus quadricauda*

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### **Abstract:**

The present work was carried out to study the effect of heavy metals from Collection station Mostorod Greater Cairo Egypt in *Chlorella vulgaris* and *Scenedesmus quadricauda* on amino acids content and ultrastructure. Results obtained revealed that the content of amino acids was increased gradually at all different concentrations of heavy metals (25%, 50%, 75% and 100%) compared to control. Cellular damage was studied by transmission electron microscope. It was observed that *Chlorella vulgaris* and *Scenedesmus quadricauda* when exposed to different concentrations of heavy metals revealed drastic changes in the overall ultrastructure such as the chloroplast separated far from the wall, the lamellae of the chloroplast appeared to be separated from each other, the pyrenoid disappeared and electro dense granular bodies identified as polyphosphate granules, appeared apparently in the cells. Damage to the algae ultrastructure was more severe with elevated heavy metal concentrations. These responses of *Chlorella vulgaris* and *Scenedesmus quadricauda* indicated their tolerance of heavy metal stress.

**Key words:** *Chlorella*, *Scenedesmus*, Heavy metals, Amino acids, Ultrastructure.

### **Introduction**

Pollution phenomenon, coming from the organic and inorganic substances which are released into the environment as a result of domestic, agricultural and industrial water activities lead to organic and inorganic pollution (**Lim et al., 2010; Muhammad et al., 2018**) High concentrations of essential metals can be toxic to living organism because of non-biodegradability, bioaccumulation and persistence in nature (**Pahlavanzadeha et al., 2010; Anbalagan and Sivakami, 2018**). Using microorganisms as biosorbent, offers alternative method to conventional processing methods, because of their strong metal binding capacity, low cost, high efficiency in dilute effluents and environment friendliness (**Philips et al., 2003; Gupta and Rastogi, 2008; Zhang et al., 2013**). Algae are ideal for

the reducing and removal the concentration of heavy metals due to high biosorption capacity, high tolerance to heavy metals, large surface area/ volume ratios, ability to grow autotrophically and heterotrophically and phytochelatin production (**Chekroun and Baghour, 2003**).

Heavy metal ions could interrupt metabolic processes by competing for the protein binding sites; activate enzymes and various biological reactive groups, causing poor or no growth. Heavy metal ions (such as  $Pb^{2+}$ ) are capable of binding to thylakoid membrane causing the alteration of the ultrastructure of thylakoids, which deteriorate the function of thylakoids and some reports in the destruction of the chloroplast of heavy metal ions at higher concentrations are reported by (**Heng et al., 2004; Lamaia et al., 2005**). A chain of metabolic events such as the photosynthesis and respiration and continuing with uptake and assimilation of nutrients, dilution of intracellular level of the heavy metal ions, *etc.* play an important role in balancing the cellular homeostasis (**Tripathi and Gaur, 2006**).

Amino acids are essential in the synthesis of proteins and in the formation of secondary metabolism molecule, participate in cell signaling, protein phosphorylation, synthesis of hormones, antioxidant capacity, gene expression and homeostasis regulation and in various physiological processes (**Cuin and Shabala, 2007; Pérez-Urria and García, 2009; Wu, 2009**).

Heavy metal resistance in microalgae may result from (i) the ability to prevent uptake (**avoidance**) by adsorption of toxic metal ions to cell-associated materials and/or cell wall components (**De Philippis and Micheletti, 2009; Naja and Volesky, 2011**) or secretion of metal-binding organic compounds to the surrounding environment. (ii) the ability to cope with high amounts of heavy metals inside tissues (**tolerance**), an active process that involves the uptake (**absorption**) and accumulation of the metal ions inside the cell (**Levy et al., 2008**).

Inside the cell, heavy metal detoxification may be achieved by binding to specific intracellular compounds to formation of metal-binding peptides or proteins such as metallothioneins (**Hassinen et al., 2011**) and phytochelatins and Gluthione (GSH) (**Hirata et al., 2005**). Organic acids i.e., malic, carboxylic, oxalic, amino acids like nicotinamine, histidine, and some phosphate derivatives are the ligands for heavy metals and are found to play a role in detoxification and tolerance. Where, the exclusion of toxic heavy metals from cells by ion- selective

metal transporters, and excretion or compartmentalization were reported by (Hu *et al.*, 2004; Gasic and Korban, 2006; Perales-Vela *et al.*, 2006). Also, polyphosphate bodies enable the fresh water algae to store and sequestered heavy metals within the cell. Polyphosphate bodies have two functions in algae, act as a detoxification mechanism and provide a storage pool for the heavy metals (Dwivedi, 2012). But polyphosphate granules have the ability to bind to many different metals (Colico *et al.*, 2010). The aim of the present work was to study the effect of heavy metals in *Chlorella vulgaris* and *Scenedesmus quadricauda* on amino acids content and ultrastructure.

## ***Materials and Methods***

### **Collection and Preparation of Samples:**

Wastewater samples were collected from collection station Mostorod Greater Cairo Egypt once during Winter (January, Daytime) 2015, they were stored in cleaned stoppered plastic bottles and refrigerated in deep-freezer at -20 °C and analyzed within few hours after arrival to the Laboratory. Physical and some chemical parameters were measured in station during the sample collection Table (1).

### **Organism and culture Methods**

Two pure algal strains (*Chlorella vulgaris* and *Scenedesmus quadricauda*) have been provided by Prof. Dr/ Gamila Hussein Ali Professor of Phycology, Water Pollution Department, National Research Centre, Egypt. The best growth conditions were at the 12days, 4000 Lux light intensity pH 7 at (22° ± 1) using BG-II 11 media (Stanier *et al.*, 1971).

**Table (1):Physical and chemical parameters of water sample collected at Mostorod plant.**

Parameters	Concentration
pH	7.3
Electric Conductivity (dsm <sup>-1</sup> )	2.04
Calcium (meq /L)	2
Magnesium (meq /L)	0.8
Sodium (meq /L)	5.77
Potassium (meq/L)	0.7
Carbonate (meq /L)	-
Bicarbonate (meq /L)	3
Chloride (meq /L)	1.5
Sulfate (meq /L)	4.77
Copper (mg/L)	0.048
Zinc (mg/L)	0.181
Manganese (mg/L)	0.116
Iron (mg/L)	0.466
Lead (mg/L)	0.282
Aluminum* (mg/L)	0.196
Barium** (mg/L)	0.054

\*Aluminum is the third most abundant element after oxygen and silicon and is a light metal; \*\*Barium is the 16<sup>th</sup> most abundant non- gaseous element of the Earth's crust (metallic alkalinity earth metal).

### Treatment

Different concentrations of wastewater contain copper, zinc, manganese, iron; lead, aluminum and barium in four concentrations of heavy metals in combination were used (25%, 50%, 75% and 100%) (v/v sterile culture medium BG-II / wastewater) total volume of 500 ml in 1000ml Erlenmeyer flasks. In 50 ml of the dilute growth cultures of *Chlorella vulgaris* and *Scenedesmus quadricauda* were transformed to 450 ml of the sterile culture medium BG-II in 1000 ml Erlenmeyer flasks. Control samples contained 500 ml of sterile culture medium BG-II only without wastewater. All treatments and control samples in triplicates were incubated at room temperature (22° ± 1) under continuous illumination (4000 lux) with a daily light dark cycle of 14-10 hrs for a period of 12 days.

**Amino acids content** were determined according to the method of (Bailey, 1967) using amino acid analyzer.

### **Electron microscopy**

The preparation of algal cell for transmission electron microscope was carried out according to the method of (Reynolds, 1963). The sections were then examined, photographed by a JEOL 1200 EXII transmission electron microscope.

## ***Results and Discussion***

It well known that algal cells exposed to heavy metals may suffer serious morphological and physiological alterations to algal cells and the stimulatory and inhibitory effects of heavy metals depend on concentration. However, mechanism of pollutant action can be described by two fundamental actions on growth processes and photosynthesis, (Visviki and Rachlin, 1994; Rocchetta *et al.*, 2006; Afkar *et al.*, 2010).

Plants synthesized a set of diverse metabolites when exposed to metals such as amino acids (proline and histidine), peptides (phytochelatin and glutathione) so the metabolism of nitrogen is central to the plant response to heavy metals. Also, amino acids are ligands for heavy metals and are found to play important role in detoxification and tolerance (Chekroun and Baghour, 2003).

It was observed from the present study Table (2) that treatment with the low and moderate concentrations of heavy metals (25%, 50% and 75%) did not cause any effect in number of amino acids but treatment with the highest concentrations of heavy metals (100 %) caused decrease in the number of amino acids in *Chlorella vulgaris* as compared with control. On the other hand, *Scenedesmus quadricauda* treatment with the lower concentrations of heavy metals (25% and 50%) cause increase in the number of amino acids and treatment with the highest concentrations of heavy metals (75% and 100 %) have no effect in the number of amino acids as compared with control. The present results agree with those observed by Omar (2002), who found that zinc at low concentrations

**Table (2): Effect of different concentrations of heavy metals on amino acids content of *Chlorella vulgaris* and *Scenedesmus quadricauda* after 12 days of incubation period**

Amino acids (µg/ml)	<i>Chlorella vulgaris</i>					<i>Scenedesmus quadricauda</i>				
	Control	25%	50%	75%	100%	Control	25%	50%	75%	100%
Aspartic	56.77	100.23	56.45	83.40	146.35	109.33	108.29	73.95	118.27	132.31
Threonine	35.15	42.47	29.38	36.45	60.45	59.00	52.66	44.98	71.22	60.54
Serine	55.63	73.13	48.08	59.73	103.37	96.99	79.54	70.92	140.41	105.62
Glutamic Acid	507.70	727.95	427.38	529.04	873.68	936.00	924.62	688.51	1341.35	952.62
Glycine	24.87	30.33	23.10	27.63	0.00	0.00	42.77	34.41	0.00	0.00
Alanine	97.53	131.27	89.93	119.06	220.09	215.55	151.28	124.83	217.79	220.53
Valine	50.52	69.51	43.55	61.85	103.87	97.05	85.81	64.47	119.10	100.31
Methionine	5.66	9.80	3.36	16.88	23.79	20.26	30.07	13.35	26.39	21.70
Isoleucine	14.44	19.09	12.57	19.51	0.00	25.56	24.93	17.97	33.32	26.08
Leucine	82.72	109.78	74.40	100.40	186.59	140.70	117.29	97.36	168.78	142.86
Tyrosine	12.77	32.29	12.62	31.31	60.60	41.87	24.34	22.14	90.03	59.01
Phenylalanine	72.56	105.97	66.50	106.58	196.27	150.03	109.34	82.71	204.17	168.10
Histidine	31.50	32.54	15.29	32.00	59.16	63.59	60.16	34.53	73.73	64.49
Lysine	45.70	66.11	30.97	56.92	110.87	101.26	85.79	69.31	115.37	106.90
NH <sub>4</sub> <sup>+</sup>	104.23	106.74	83.78	105.03	129.63	127.35	118.83	107.16	128.20	132.30
Arginine	84.47	121.63	65.52	106.97	204.84	160.99	114.53	103.94	257.36	191.40
Total number	16	16	16	16	14	15	16	16	15	15

increased total amino acid contents, however, decreased it at high concentrations. The amino acids accumulation in response to metals concentrations may lead to the assumption that suppressed protein biosynthesis encouraged accumulation of free amino acids, or may lead to some counteracting chelating mechanism against the toxicity of heavy metals (**El-Sheekh *et al.*, 2003; Osman *et al.*, 2004; Fathi *et al.*, 2005**).

Glutathione forms complexes with heavy metals and as a response to heavy metals stress, so plants induced glutathione and synthesis of cysteine. Glycine has a role in glutathione synthesis so the amount of glycine decreased in heavy metal stress (**Arya *et al.*, 2008**). Glutamic acid and glycine participate in glutathione synthesis increasing the antioxidant capacity of the plant (**Wu, 2009 and Akram *et al.*, 2011**). This accordance with present data that absence of glycine in concentration 100% in *Chlorella vulgaris* and in control, concentrations 75% and 100% in the case of *Scenedesmus quadricauda*. Isoleucine was absent in concentration 100% in *Chlorella vulgaris* because synthesis of isoleucine decreased due to their synthesis and catabolism influence by different developmental and environmental conditions which go with the line of (**Joshi *et al.*, 2010**).

**Abdel Latif and Sallam (2015)** recorded that in maize wastewater irrigation improved the accumulation of total free amino acids content. Also, **Homer *et al.* (1997)** observed amino acids accumulation in response to heavy metals pollution in three hyper accumulator species, *Phyllanthus palwanensis*, *Walsura monophylla*, and *Dechampetalum geloniodes* this is similar to present results.

The free amino acids accumulation in plants under stress have important roles in metal toxicity tolerance through detoxifying heavy metals, regulating intracellular pH and ion transport and scavenging the reactive oxygen species (**Rai, 2002**). **Miflin and Habash (2002)** reported that plants evolved some enzymes to cope with stress that convert amino acids, keto-acids, and amides to be used as carbon source when carbon deficiency becomes a limiting factor for development and growth. Total free amino acids concentration increased in broad bean plants under copper treatment and this increase indicating the role of amino acids in metal chelation (**Hall, 2002; Xiong *et al.*, 2006; Azooz *et al.*, 2012**).

Results of detailed TEM examinations of both algal species, were illustrated in **Figures (1-10)** which showed the effects of different concentrations of heavy metals (25%, 50%, 75% and 100%) as well as the control on cell ultrastructure of *Chlorella vulgaris* and *Scenedesmus quadricauda*.

*Chlorella vulgaris* at control condition **figure (1 A-C)** showed that it was characterized by spherical cells, a smooth cell wall and a parietal chloroplast. *Chlorella vulgaris* at control condition **figure (1 A-C)** showed that it was characterized by spherical cells, a smooth cell wall and a parietal chloroplast. The ultrastructure of *Chlorella vulgaris* at concentrations 25%, 50%, 75% and 100% was illustrated in **Figures (2 - 5)**. It is clear from these figures that:

-The rigged wall clearly appeared and the chloroplast separated far from the wall in (**Figure (2 A, C, D); Figure (3 A, C, D); Figure (4A-E)** and **Figure (5B, C)**).

- The lamellae of the chloroplast appeared to be separated from each other in (**Figure (2 A-E); Figure (3 A-G); Figure (4 A-E)** and **Figure (5 A-D)** and the pyrenoid disappeared in **Figure (3 A, C, D, F); Figure (4 C, D, E)** and **Figure (5 B, C, D)**.

- Electron dense granular bodies identified as polyphosphate granules, appeared apparently in the cells in (**Figure (2 B, C, D, E), Figure (3 A-G), Figure (4 A-E)** and **Figure (17 A-D)**).

- Dark electron dense layer in the cell envelop appeared in (**Figure 3D); Figure (4 A, C, E)** and **Figure (5 A, C)**.

The studied metals influenced *Chlorella vulgaris* and *Scenedesmus quadricauda* cells with the greatest ultrastructure change caused by high concentrations of heavy metals. Present data showed dark amorphous electron dense layer appeared in the cell envelop of cells after treated with heavy metals this is reflect the external surface sorption which is considered as the first defense mechanism to limit heavy metals toxicity (**Macfie et al., 1994; Tuzun et al., 2005**).**Swift and Forciniti (1997)** reported that once external sorption reaches the saturated stage, internal uptake begins in *Anabaena cylindrica* so lead phosphate was precipitated on the cell wall and then inside the cell. The differences in



subcellular effects induced by Cu, Zn and Cd are probably due to specific adaptation mechanisms developed by *Chlorella vulgaris*.

*Chlorella vulgaris* and *Scenedesmus quadricada* cells showed an alteration in ultrastructural of the organization and shape of thylakoids that confirm a damage of the photosynthetic apparatus. Chloroplasts and their arrangement represent a primary target of toxic substances in algae and higher plants.

The alternation in shape of chloroplast could be a consequence of a perturbation of cation exchange induced by heavy metal treatment while the alterations in structure of the thylakoid system could depend on heavy metals ability to bind to proteins and to interfere with their normal functions also oxidative damage induction (Nacorda *et al.*, 2007; Basile *et al.*, 2008). The accumulation of ROS, peroxides and free radical lead to lipid peroxidation, altered ultrastructure of chloroplast and distortion of thylakoid membranes (Sharma and Dubey, 2005; Islam *et al.*, 2008; Basile *et al.*, 2012).

Mitochondria are a common target of the heavy metal in freshwater green algae. From this study the starch granule increases in size due to the rapid mitochondria deterioration caused accumulation of starch grains that cells might act as energy reserve to the cell after the deterioration of organelles especially mitochondria, chloroplast, and pyrenoid. Consequently, since the respiratory activities cannot be carried out without mitochondria, accumulation of starch results in the disarrangement of the chloroplasts, as is reported by (Wong *et al.*, 1994; Miao and Wang, 2006) which agree with the present investigation.

Also, lead caused distortion of ultrastructure of chloroplast with compact grana, disrupted thylakoid membranes and small starch grains in *Vignaungui culata* (Kasim *et al.*, 2014) this agreement with our results because sewage has high amount of lead (0.282 mg/L).

While, *Scenedesmus quadricauda* was characterized at control condition by: colony containing of 2-4-8 oblong- cylindrical cells usually in one series. Outer cells with a long curved spine at each pole; inner cells without spines and a parietal chloroplast (**Figure 6**).

The ultrastructure of *Scenedesmus quadricauda* at concentrations of 25%, 50%, 75% and 100% was showed in **figures (7- 10)**. It is clear from these figures that:

-Significant increase was observed in the cell wall of *Scenedesmus quadricauda* in (**Figure (7 D, E); Figure (8 A, H); Figure (9 A, B, C, F)** and **Figure (10 A, B, C, F, G, H, I)**).

- A clear separation of the chloroplast lamellae in (**Figure (7 D); Figure 8 C, D, E, F, G, H); Figure (9 A, B, C, E, F)** and **Figure (10 A, B, C, E, G, H)** where, the pyrenoid disappeared in (**Figure (9 A, C, D); Figure (8 A, F) Figure (9 A-F)** and **Figure (10 A-I)**).

-The number of starch granules increased largely, but with a reduced size in (**Figure (7 A, B, C); Figure (8 A, F, G, H, I); Figure (9 B, C, D, E)** and **Figure (10 B, D, E, F, I)**).

-Spherical electron dense bodies were noticed within the cells in (**Figure (7 A-E); Figure (8 A, B, C, F, G, H, I); Figure (9 B, C, D, E, F)** and **Figure (10 B, D, E, F, G, I)**).

- Gradual disappearance of the nuclear membrane in **Figure (8 C, D, G, H, I) Figure (9 A)** and **Figure (10 E, F)**.

Our obtained data recorded spherical electron dense bodies bioaccumulation inside *Chlorella vulgaris* and *Scenedesmus quadricauda* cells that coincided with similar granules observed in different heavy metal treated microalgae reported in *Pseudochlorococcum typicum* by (**Aguilera and Amils, 2005**). The deposition of metal inside the cytoplasm or vacuoles was a mechanism contributed to the tolerance of heavy metal by minimizing as possible the cytoplasmic metal concentrations by complexing or binding the metal ions with phytochelatin or in the form of metallo-iron, metallo-sulfur 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, organic acids usually present in high concentration which sequestered by metals. **Perales-vela *et al.* (2006)** and **Shanab *et al.* (2012)** reported that this performed as detoxification mechanisms or a cellular protection. Also, **Rachlin *et al.* (1984)** and **Kahoko *et al.* (2003)** recorded that electron dense deposits were from polyphosphate bodies. Polyphosphate bodies have the ability to protect algal cells from metal toxicity and also to accumulate metals.

The number of vacuoles increased in *Chlorella vulgaris* due to heavy metal stress this is related to vacuolation could contribute to compartmentalization of toxic metals (**Crang and Jensen 1975**). **Niskikawa *et al.* (2003)** reported that the number of vacuoles increased as well as the presence of electron dense deposits in membrane whorls and vacuole was detected in *Chlamydomonas acidophila* treated by Zn, Cd and Cu.

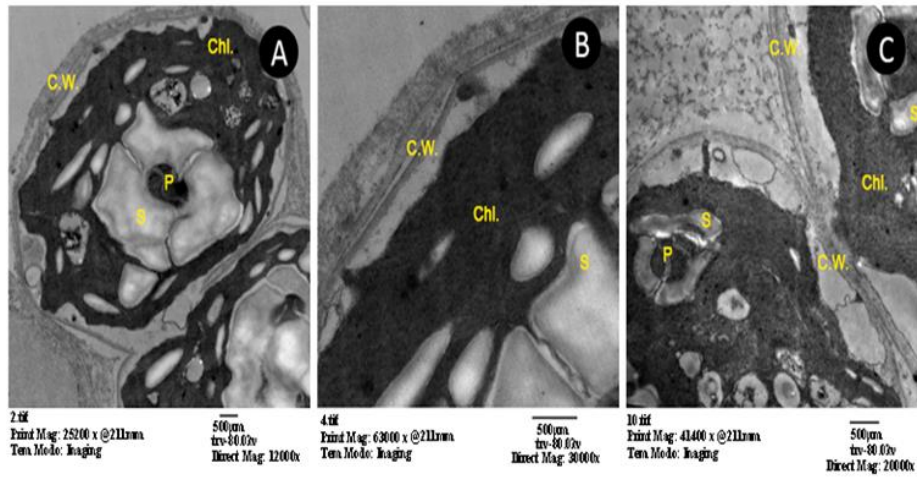


Figure (1 A-C): Ultrastructure of *Chlorella vulgaris* at Control Condition  
Abbreviations: Chl.: Chloroplast; C.W.: Cell wall; P: Pyrenoid; S: Starch

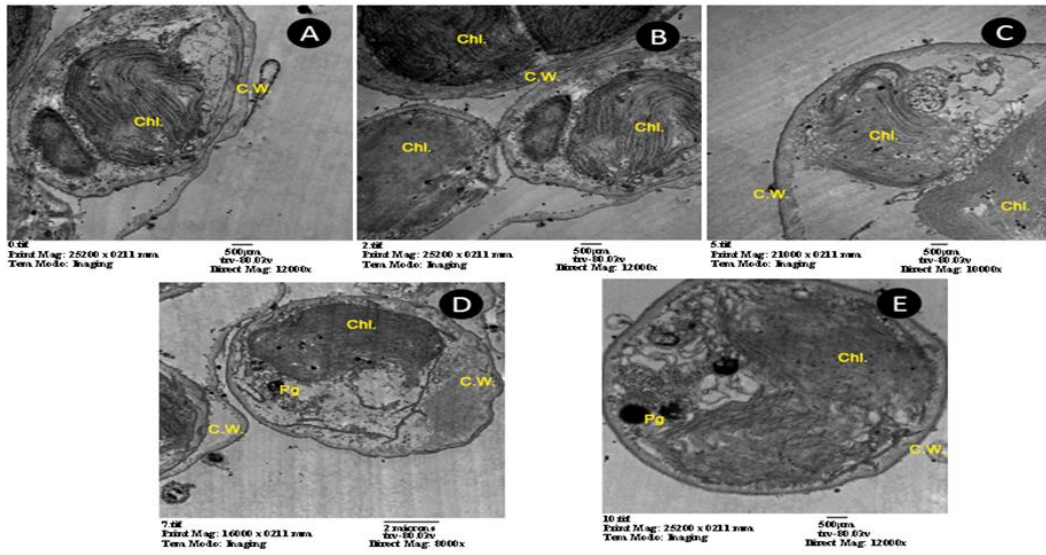


Figure (2 A-E): Ultrastructure of *Chlorella vulgaris* Treated with (25%) Sewage Concentration  
Abbreviations: Chl.: Chloroplast; C.W.: Cell wall; Pg: Polyphosphate granules

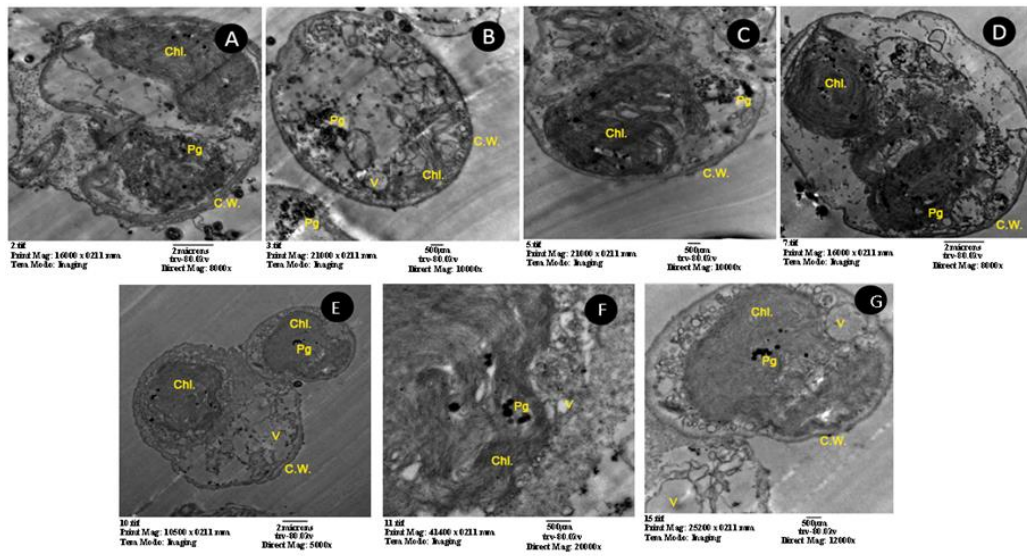


Figure (3 A-G): Ultrastructure of *Chlorella vulgaris* Treated with (50%) Sewage Concentration  
Abbreviations: Chl.: Chloroplast; C.W.: Cell wall; Pg: Polyphosphate granules; V: Vacuole

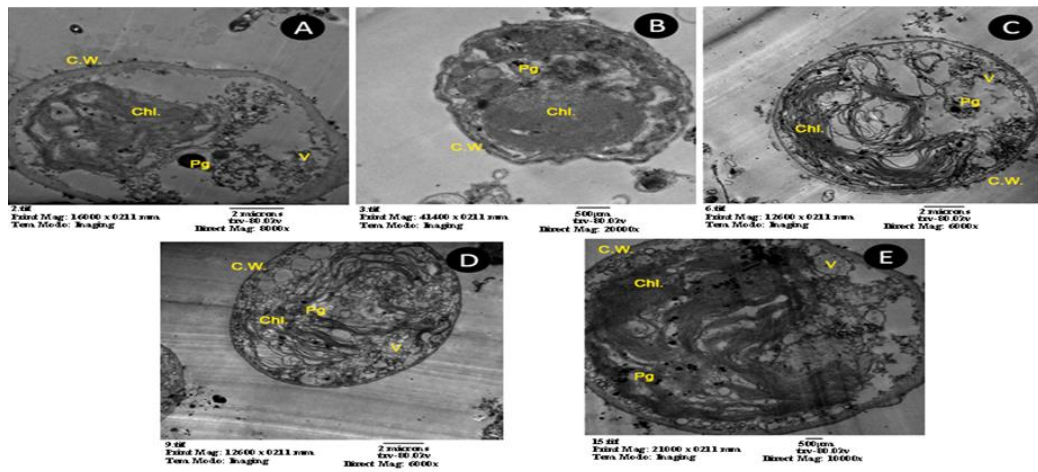


Figure (4 A-E): Ultrastructure of *Chlorella vulgaris* Treated with (75%) Sewage Concentration  
Abbreviations: Chl.: Chloroplast; C.W.: Cell wall; Pg: Polyphosphate granules; V: Vacuole

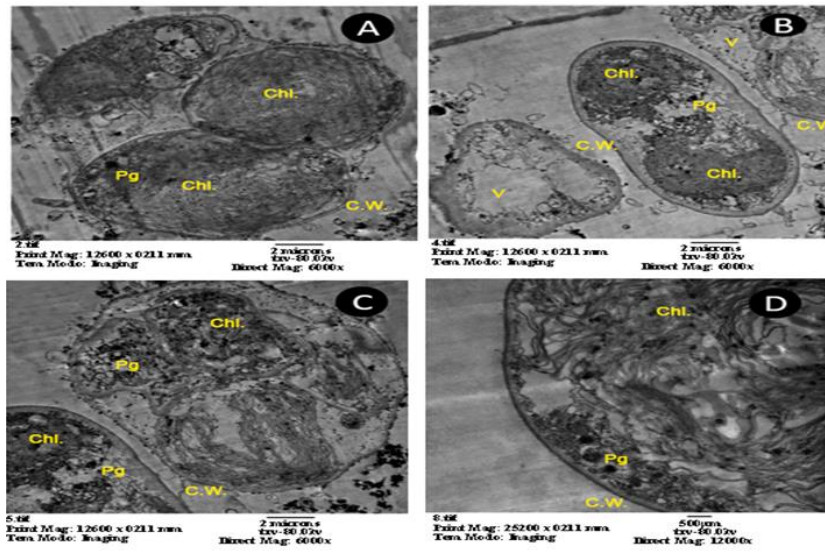


Figure (5 A-D): Ultrastructure of *Chlorella vulgaris* Treated with (100%) Sewage Concentration  
Abbreviations: Chl.: Chloroplast; C.W.: Cell wall; Pg: Polyphosphate granules; V: Vacuole

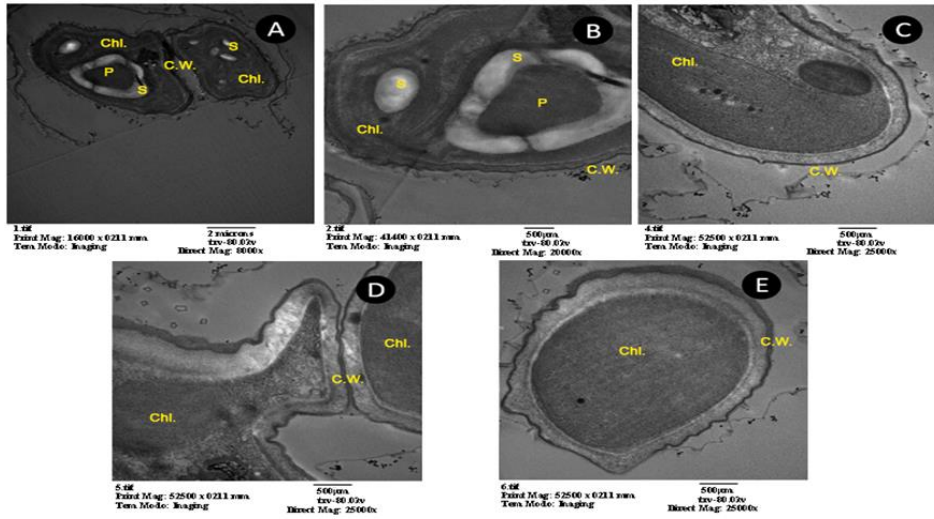


Figure (6 A-E): Ultrastructure of *Scenedesmus quadricauda* at Control Condition  
Abbreviations: Chl.: Chloroplast; C.W.: Cell wall; P: Pyrenoid; S: Starch

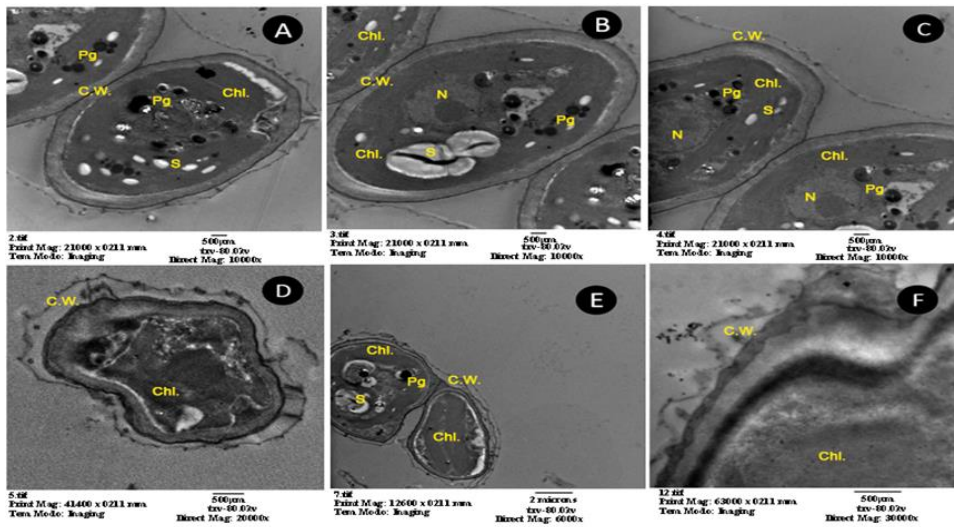


Figure 7 (A-F): Ultrastructure of *Scenedesmus quadricauda* Treated with (25%) Sewage Concentration  
Abbreviations: Chl.: Chloroplast; C.W.: Cell wall; N: Nucleus; S: Starch; Pg: Polyphosphate granules

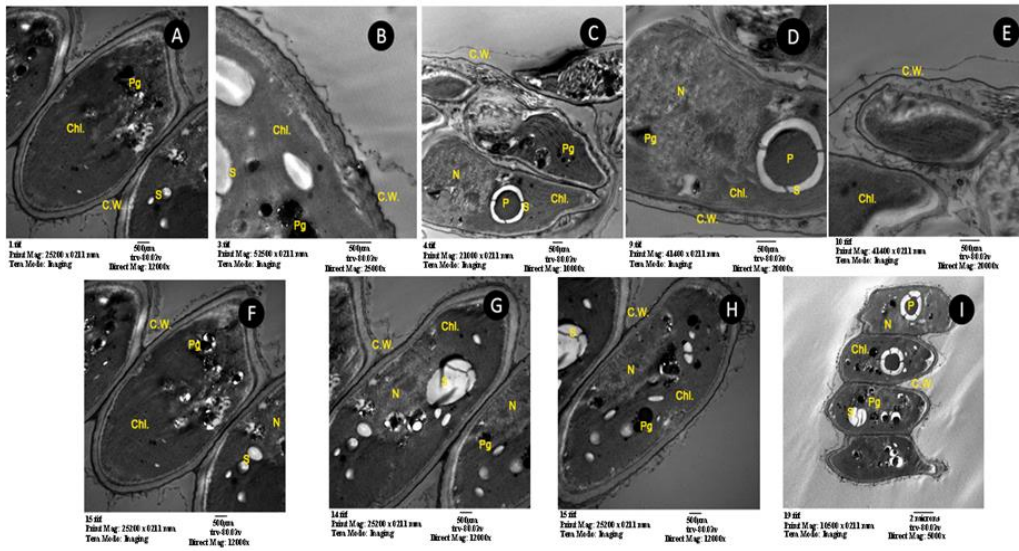


Figure 8 (A-I): Ultrastructure of *Scenedesmus quadricauda* Treated with (50%) Sewage Concentration  
Abbreviations: Chl.: Chloroplast; C.W.: Cell wall; P: Pyrenoid; S: Starch; Pg: Polyphosphate granules; N: Nucleus

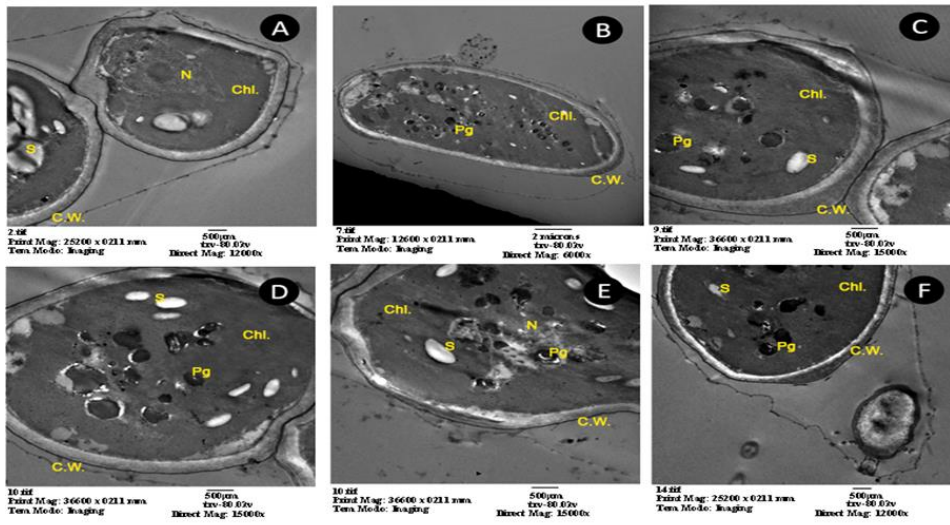


Figure (9 A-F): Ultrastructure of *Scenedesmus quadricauda* Treated with (75%) Sewage Concentration  
Abbreviations: Chl.: Chloroplast; C.W.: Cell wall; S: Starch; Pg: Polyphosphate granules; N: Nucleus

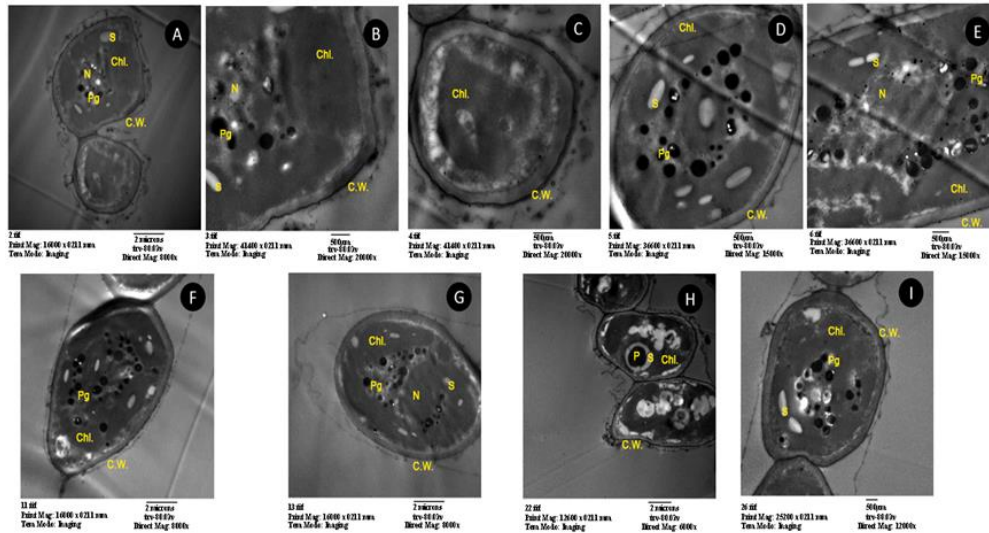


Figure (10 A-I): Ultrastructure of *Scenedesmus quadricauda* Treated with (100%) Sewage Concentration  
Abbreviations: Chl.: Chloroplast; C.W.: Cell wall; P: Pyrenoid; S: Starch; Pg: Polyphosphate granules; N: Nucleus



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## التأثيرات الناتجة عن استخدام مياه الصرف الصحي علي التركيب الدقيق *Scenedesmus quadricauda* و *Chlorella vulgaris*

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أجريت تجارب هذا العمل لدراسة تأثير المعادن الثقيلة من مياه الصرف علي التركيب الدقيق. *Chlorella vulgaris* و *Scenedesmus quadricauda* علي محتوى الأحماض الأمينية و المكونات المختلفة من المعادن الثقيلة (25%، 50%، 75%، 100%) مقارنة مع الكنترول. التركيب الالكتروني تم دراسة بالميكروسكوب الإلكتروني النافذ وقد لوحظ أن كلا من *Chlorella vulgaris* و *Scenedesmus quadricauda* عند تعرضهما لتركيزات مختلفة من مياه الصرف الصحي أدت إلي تغييرات جذرية في التركيب الدقيق لهما مثل بعد البلاستيكية عن الجدار الخلوي و انفصال صفائح البلاستيكية عن بعضها البعض و اختفاء مركز تجمع النشا من الخلية و ظهور حبيبات الفوسفات. وأظهرت الدراسة أن الأضرار التي لحقت بالتركيب الدقيق للخلية تكون أكثر شدة مع زيادة تركيزات المعادن الثقيلة. أكدت الاستجابات لطحلي *Chlorella vulgaris* و *Scenedesmus quadricauda* أنها متحملة للإجهاد بالمعادن الثقيلة .