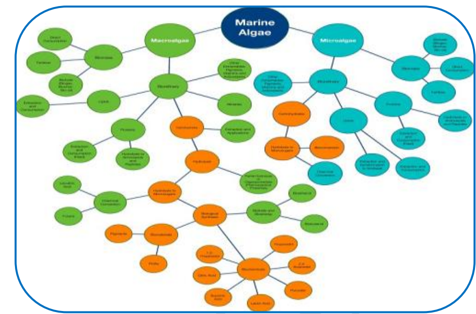
REVIEW OF RESEARCH

IMPACT FACTOR : 5.7631(UIF)   UGC APPROVED JOURNAL NO. 48514   ISSN: 2249-894X

VOLUME - 8 | ISSUE - 7 | APRIL - 2019

EFFECT OF CRUDE PETROLEUM OIL POLLUTION ON AMINO ACIDS CONTENTS, SOME ENZYMES ACTIVITIES AND ULTRASTRUCTURE OF THREE MARINE SEAWEEDSSPECIES



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ABSTRACT :

During last two decades, extensive attention has been paid on the management of environmental pollution caused by hazardous materials. In this study, we chosen three investigated species, Ulva fasciata, Sargassum hornchuchii and Pterocladia capillacea, which were collected from Abu Qir, Alexandria were subjected to concentration of 50% of water soluble fractions (WSFs) of crude petroleum oil by varying exposure time to 2,4 and 6 days. Analyses were performed to study the effect of WSFs of crude petroleum oil on the contents of amino acids, some enzymes activities and ultrastructure of the investigated algae. The results indicated that the total amino acids contents decreased significantly (at P < 0.05) in all algal samples during the exposure experiment. Treatment with WSFsof crude petroleum oil increased the activity of superoxide dismutase, ascorbate peroxidase and catalase of the three treated algal species. The study also shows that treatment with WSFs of crude petroleum oil also affect the ultrastructure of all the treated algal species.

KEYWORDS : *Algae, crude petroleum oil, amino acids, enzyme, ultrastructure.*

INTRODUCTION

Among the commonest and most noticeable pollutants of marine and estuarine waters are oil and oil byproducts (Wake, 2005; Romero- López *et al.*, 2012) and there has been a major increase in the world consumption of oil, which accompanied by rising incidences of pollution (Ndungu *et al.*, 2017). Oil pollution may be derived from barges, tankers and boats on rivers and canals, or may be derived from industrial wastes, metallurgical industries, engineering works, garages or any places using lubricating or fuel oils (Baker, 1970). Petroleum-based products are the major source of energy for industry and daily life as mentioned by Parkash and Irfan (2011). Leaks and accidental spills occur regularly during the exploration, production, refining, transport, and storage of petroleum and petroleum products (Sharma, 2008). The amount of natural crude oil seepage was predictable to be 600,000 metric tons per year with a range of uncertainty of 200,000 metric tons per year Kvenvolden and Cooper (2003).The impacts of oil spills on the marine environment are well documented (EPA, 1976; Liu *et al.*, 2016; Seidel *et al.*, 2016). When petroleum comes in water a very fast partitioning between the water, air and sediment part of the environment take place (Knap, 1982). The insoluble fraction forms a layer of 0.01 to 3.0 mm thickness on the water layer (Lichtenthaler *et al.*, 1989). Throughout the first few hours some parts evaporate and other parts are absorbed in the sediment. When the hydrocarbons are concentrated enough non-aqueous phase liquids can be made. The

remaining hydrocarbons are present in the aqueous layer or as a film on the water surface. The lighter fractions are removed within twenty-four hours by evaporation. First and most obvious, is the formation of oil slicks on the surface of the water and the cost line. Volatile fractions will evaporate causing a distinguishable odor problem have, toxic effects and causing irreversible contamination to aquatic and terrestrial habitats (Aigner *et al.*, 2010). In the mean time, the surface oil film alters the water surface viscosity, act as a barrier against air water gas exchange, e.g. oxygen, CO_2, etc., suppress evaporation and have a devastating effect on surface biota as well as birds. The water soluble fractions of the oil film, as well as oil suspensions will be incorporated in the food chain and will exert toxic effects on aquatic biota (EPA, 1976). Oil pollution is very damaging to algae and aquatic ecosystem as studied by Lewis and Pryor (2013). The damage caused by oil spill depends on different aspects, including the chemical composition of the oil, location where the contamination occurs, how much time remainders in the environment (De la Huz *et al.*, 2011). At the same time the algal cells are affected by all the constituent of crude oil including the water soluble fractions. Differences in chemical composition of test oils are actually responsible for their differential toxicity to different algae as studied by Hook and Osborn (2012). The effect of water soluble fraction of oil on algae is species dependent, which could be partially due to indirect trophic interactions, might also be related to different sensitivity of species to polycyclic aromatic hydrocarbons (PAHs) and their life cycle. (Wake, 2005; González *et al.*, 2009; De la Huz *et al.*, 2011; Ke *et al.*, 2011). The ecological impacts of oil on specific seaweed habitats has been studied by many authors, and clear differences are observed between sensitive taxa, such as *Fucus*, *Ulva* and *Pelvetia*, and resistant taxa, e.g. *Laminaria*, *Chondrus* and *Ascophylum* (Freedman, 1995).

MATERIALS AND METHODS

Algal species

Ulva fasciata, *Sargassum hornschuchii* and *Pterocladia capillacea* were collected in May 2014 from Abu Qir in Alexandria. After harvesting, whole algae were extensively washed several times with natural sea water to remove any attached sand and the rhizoidal portions were removed to avoid microbial contamination. Then the algal materials were conveyed to the laboratory in plastic bags filled with sea water.

Pollutant treatment

Experiments were conducted in 1L beakers by adding 10g of fresh algal biomass to 1L of water soluble fractions (WSFs) of crude petroleum oil of specific concentration.The WSFs concentration prepared by adequate dilution of its stock solution using seawater.The experiments were conducted for 6 days at different time intervals with aeration. All the experiments were performed at natural light condition and room temperature (29±2° C) with two replicates. Mean values were reported and controls without addition of WSF were run in parallel. The fresh biological materials were analyzed for their amino acids analyses, enzymes activities estimation and ultrastructure of algal cells.

Preparation of water soluble fractions of crude petroleum oil

The water soluble fractions (WSFs) of crude petroleum oil was prepared by using the modified method of Boylan and Tripp (1971) as follows: One part of crude oil was mixed with 20 parts of seawater in a glass stoppered bottle. The mixture is stirred by a magnetic stirrer at low speed for 12 hour. This mixture was then transferred to a separation funnel and allowed to stand for 4 hours. The aqueous phase was then drained and the remaining non-soluble fractions were discarded. The aqueous phase was designated as 100% oil extract. Dilution of this stock solution with various volumes of the medium yielded lower percentages of oil extract. The concentrations of fresh WSF was used 50% of Egyptian crude petroleum oil and prepared by using seawater for dilution in order to maintain all the WSF at the same solution constituents, the only difference is the concentration of the WSF.

Amino acids determination

The total amino acids were determined by the method described by Ya and Tunekazu (1966).

Enzymes activities estimation

The activity of superoxide dismutase was performed according to Giannopolitis and Ries (1977), Ascorbate peroxidase according to Nakano and Asada (1981), while Catalase activity was measured as described by Beers and Sizer (1952).

Ultrastructure

It was performed according to (Reynolds 1963) and Mercer and (Birbeck, 1966), then examined by Philips 400 Transmission electron microscope at 60-80 KV.

Statistical analysis

The effect of WSFs of 50% crude petroleum oil on enzymes activities was evaluated by means of a t-test before and after exposure to crude petroleum oil. While, amino acids contents were tested with analysis of variance (ANOVA-one way). Statistical analysis were performed using employed SPSS version 10.0 for testing significant of differences between treatments at the 0.05 probability level (p = 0.05). These parameters were considered to be significantly different at a level of $P < 0.05$ (Duncan, 1957).

RESULTS

Amino acids contents

Results in Table (1) showed that amino acids contents were estimated after treatment with 50% of WSFs of crude petroleum oil for different time intervals for the three algal species. Data indicated that the total amino acids content decreased in all algal samples during the exposure experiment. The total amino acids contents decreased in *U. fasciata* throughout the treatment. This decrease was quite little after two days from (0.35 to 0.32mg g^{-1} FW) and then the decrement was notably large until the last day of experiment, where recorded the lowest value (0.21mg g^{-1} FW) after six days. It was noticed that amino acids content in this alga decreased due to treatment with crude petroleum oil by approximately 40%.

The amino acids contents of treated *algae S. hornchuchii* decreased gradually during the treatment. This decrease was small until the second day, where their values decreased from (0.41 to 0.37mg g^{-1} FW) followed by large decrease throughout the next days and until the last day, where recorded its lowest value (0.24mg g^{-1} FW) after six days. The results of this alga showed that amino acids content decreased by approximately 41% after six days.In addition, *P. capillacea* showed a gradual quite little decreases of amino acids contents after 2, 4 and 6 days, where their values were (0.27, 0.24 and 0.22mg g^{-1} FW), respectively. It was noticed that the amino acids content in this alga decreased due to WSFs of crude petroleum oil by approximately 29% after six days. It was noticed that the variations of amino acids contents by the three algal species were significant (at $P < 0.05$) at each time period.

Table 1: Amino acids content of *Ulva fasciata, Sargassum hornschuchii* and *Pterocladia capillacea* before (control) and after exposure to 50% crude petroleum oil concentration for different time intervals.

Species	Amino acids content (mgg^{-1} fresh weight)			
	Time (days)			
	Control	2nd day	4th day	6th day
Ulva fasciata	0.35 ^{a}±0.07	0.32 ^{ab}± 0.02	0.25 ^{bc}±0.04	0.21 ^{cd}±0.01
Sargasum hornschuchii	0.41 ^{a}±0.08	0.37 ^{ab}± 0.04	0.31^{abc}±0.02	0.24 ^{bc} ±0.05
Pterocladia capillacea	0.31 ^{a}±0.05	0.27 ^{ab}± 0.04	0.24^{ab}±0.02	0.22 ^{b} ±0.01

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Different superscript letters indicates significant differences at $P < 0.05$ according to one way ANOVA.

ENZYMES ACTIVITIES

Enzymes activities were estimated after treatment with 50% of WSFs of crude petroleum oil for six days for the three algal species. It is clearly demonstrated that, treatment of the three algal species (*U. fasciata*, *S. hornchuchii* and *P. capillacea*) with 50% WSFs of crude petroleum oil resulted in a significant increase at $P < 0.05$ of SOD activity by approximately (51.23%, 19.23% and 26.23%), respectively compared to control (Table 2).

Results in Table (3) demonstrated that ascorbate peroxidase (APX) activity increased in the all three treated algae species (*U. fasciata*, *S. hornchuchii* and *P. capillacea)* as a result of 50% WSFs of crude petroleum oil for six days by nearly 272.87%, 165.78% and 486.02%, respectively. It was observed that the increase of enzyme activity of APX contents due to 50% WSFs of crude petroleum oil in all algal species were statistically with significant difference at $P < 0.05$ (Table 3).

Similar to SOD enzyme and APX enzyme, catalase activity (CAT) was remarkably increased after WSFs of crude petroleum oil (Table 4) in all the three algal species for six days. The increases in the three treated algae species (*U. fasciata*, *S. hornchuchii* and *P. capillacea*) were approximately 175%, 142% and 279%, respectively. It is noteworthy to mention that the increase of enzyme activity of CAT contents due to 50%WSFs of crude petroleum oil in the three treated algae was significantly different at $P < 0.05$ (Table 4).

Table 2: Superoxide dismutase activity of *Ulva fasciata, Sargassum hornschuchii* and *Pterocladia capillacea* before (control) and after exposure to 50% crude petroleum oil concentration for 6 days.

Species	Enzyme activity (unit gm^{-1} dry matter)			
	Control	Treated	Increase	Increase (%)
Ulva fasciata	4.84 ± 0.19	7.32 ± 0.28*	2.48	51.23
Sargasum hornschuchii	9.36 ± 0.22	11.16 ± 0.22*	1.80	19.23
Pterocladia capillacea	8.08 ± 0.11	10.20 ± 0.28*	2.12	26.23

(*) Marked differences are significant at $P < 0.05$.

Table 3: Ascorbate peroxidase activity of *Ulva fasciata, Sargassum hornschuchii* and *Pterocladia capillacea* before (control) and after exposure to 50% crude petroleum oil concentration for 6 days.

Species	Enzyme activity (μmol H_2O_2 min^{-1} g^{-1} d.m)			
	Control	Treated	Increase	Increase (%)
Ulva fasciata	18.47 ± 0.55	68.87 ± 0.70*	50.40	272.87
Sargasum hornschuchii	38.63 ± 0.56	156.79 ± 0.84*	118.16	165.78
Pterocladia capillacea	15.67 ± 0.28	91.83 ± 0.41*	76.16	486.02

(*) Marked differences are significant at $P < 0.05$.

Table 4: Catalase activity of *Ulva fasciata, Sargassum hornschuchii* and *Pterocladia capillacea* before (control) and after exposure to 50% crude petroleum oil concentration for 6 days.

Species	Enzyme activity (μmol H_2O_2 min^{-1} g^{-1} d. m)			
	Control	Treated	Increase	% Increase
Ulva fasciata	57.59 ± 0.49	158.39 ± 0.73*	100.8	175.03
Sargasum hornschuchii	50.39 ± 0.39	122.40 ± 0.55*	72.01	142.90
Pterocladia capillacea	28.79 ± 0.21	107.99 ± 0.38*	79.20	279.09

(*) Marked differences are significant at $P < 0.05$.

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ULTRASTRUCTURE ANALYSIS

The electronmicrograph of *U. fasciata* before exposure to 50% of WSFs of crude petroleum oil concentration showed arrangement of the cell components and clear cell wall (CW) (Plate 1). When the cells were exposed to 50% of WSFs of crude petroleum oil concentration for six days, chloroplast showed dissipation and irregularity in shape. On the other hand, the cell wall and the pyrenoids appeared unaffected (Plate 2). The electron micrograph of untreated *S. hornschuchii* showed the clear arrangement of thylakoid membranes, clear nucleus with clear nuclear envelope and clear cell wall (Plate 3). The treated cells showed some disorganization of cell components, malformation of the cell, wrinkled cell wall and appearance of some vacuoles and the nucleus is not affected (Plate 4).

The electron micrograph of untreated *P. capillacea* showed clear arrangement of thylakoids, well organized nucleus and clear cell wall (Plate 5). Meanwhile, treated cell showed disturbance of the cell inclusions, irregularity of cell wall and chloroplast structure is less clear with disorganization of thylakoids (Plate 6).

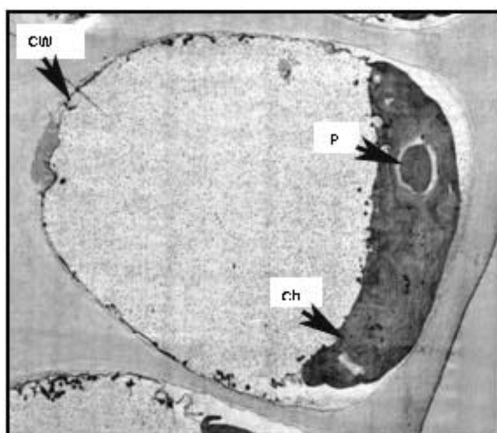

Plate 1: The electron micrograph of *Ulva fasciata* before exposure to 50% crude petroleum oil concentration showing chloroplasts (Ch), pyrenoid (P) and cell wall (CW) (2.5 × 10^3).

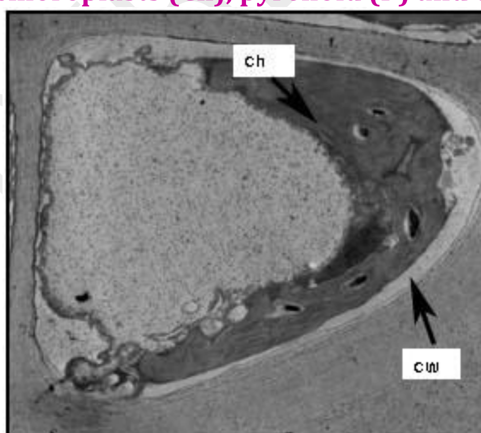

Plate 2: The electron micrograph of *Ulva fasciata* after exposure to 50% crude petroleum oil concentration for six days showing dissipation of chloroplast (C) and lamellated cell wall (CW) (2.5 × 10^3).

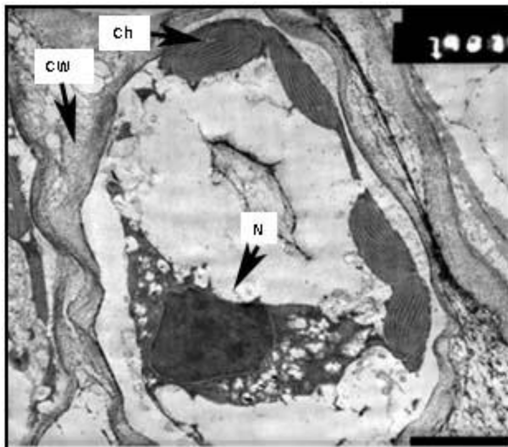

Plate 3: The electron micrograph of *Sargassum hornschuchii* before exposure to 50% crude petroleum oil concentration showing the typical chloroplast (Ch), cell wall (CW) with clear arrangement of thylakoids ($2.5×10^3$).

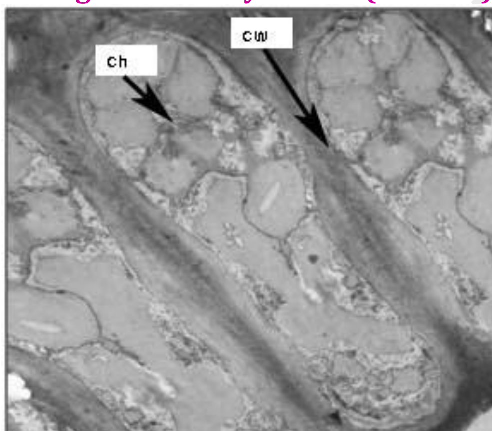

Plate 4: The electron micrograph of *Sargassum hornschuchii* after exposure to 50% crude petroleum oil concentration for six days showing dramatic disorganization of cell components of the alga and irregularity of cell wall ($2×10^3$).

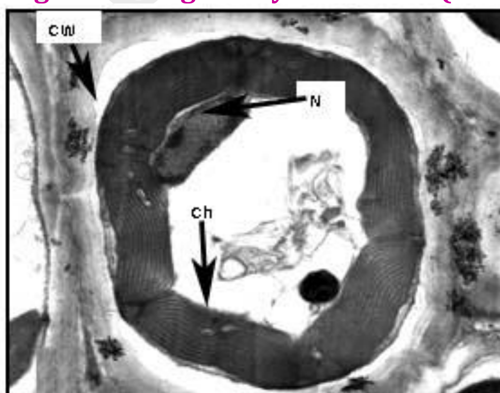

Plate 5: The electron micrograph of *Pterocladia capillacea* before exposure to 50% crude petroleum oil concentration showing the clear arrangement of thylakoids inside chloroplast (Ch), cell wall (CW) and nucleus (N) ($2.5×10^3$).

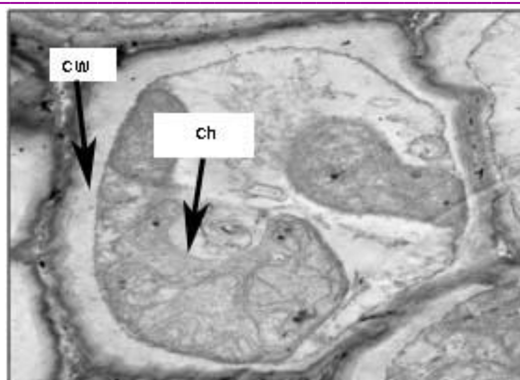

Plate 6: The electron micrograph of *Pterocladia capillacea* after exposure to 50% crude petroleum oil concentration for six days showing disturbance of the cell inclusions, irregularity of cell wall (CW) and chloroplast structure (Ch) is less clear with disorganization of thylakoids (2.5×10^3).

DISCUSSION

Amino acids contents were determined in the samples of the three algae after treated with 50% WSFs of crude petroleum oil after six days. The results indicated that the total amino acids content decreased in all algal samples during the exposure experiment. The decrease in the content of total amino acids may be due to the extreme effect of these compounds on the biochemical activities (Mona, 2000). Fortunately organisms have a variety of defenses, known as antioxidants, to combat harmful ROS and stave off oxidation and subsequent damage (Lesser, 2006). Antioxidants prevent free radical production, scavenge existing ROS and unpaired electrons and purge chain reactions. The term antioxidant applies to ‘any substance that significantly delays or inhibits oxidation’ as mentioned by Halliwell and Gutteridge (2007), this incorporates both enzymes and other soluble compounds (e.g., vitamins) (Davies, 2000).

Uptake of xenobiotic pollutants like polycyclic aromatic hydrocarbons (PAHs) increases the production of ROS, and subsequently oxidative stress, in many photosynthetic organisms including aquatic plants and algae (Mittler, 2002). Previous studies indicate PAHs are inducers of oxidative stress in humans, animals (Sun *et al.*, 2006), terrestrial plants (Paskova *et al.*, 2006), *Arabidopsis* (Alkio *et al.*, 2005) and the aquatic liverwort *Riccia fluitans* (Burritt, 2008), yet the effects of coastal PAH inputs on marine algae have not been previously investigated in depth. Hydrogen peroxide (H_2O_2) is commonly produced under stress conditions (Dummermuth *et al.*, 2003). While other ROS molecules have a comparatively short lifetime, due to their rapid reactivity, H_2O_2 itself is not particularly reactive to most biologically important molecules but has the ability to diffuse through membranes rapidly, making it a likely precursor for more reactive oxidants (Apostol *et al.*, 1989). H_2O_2 disturbs photosynthesis by inhibiting a number of enzymes in the Calvin cycle, such as fructose biophosphatase, ribulose phosphate kinase and ribulosebiophosphate carboxylase/oxygenase (Han *et al.*, 2007, 2009 and Moller *et al.*, 2007).It is clearly demonstrated that, treatment with 50% WSFs of crude petroleum oil resulted in significant increases (at $P < 0.05$) in the activities of antioxidant enzymes [superoxide dismutase (SOD) - catalase (CAT) - ascorbate peroxidase (APX)] in the three treated algal species. Enhanced activities of these enzymes indicate the induction of oxidative stress of seaweed after exposure to crude petroleum oil as studied by Wang and Zhao (2007).

Many studies have demonstrated that oxidant enzymes play a central role in degradation and detoxification of a wide range of organic contaminants, including PAHs, in many plants and micro-organisms (Juhasz & Naidu, 2000; Yang *et al.*, 2002; Lei *et al.*, 2003 and Chan *et al.*, 2006). Peroxidase (POD) and superoxide dismutase (SOD) appeared to be important oxidant enzymes for metabolism of PAHs in algae (Warshawsky *et al.*, 1995; KirsoandIrha, 1998). Wang and Zhao (2007) reported that, significant increases in the activities of antioxidant enzymes (SOD, POD) were evident, suggesting that the biotransformation of phenanthrene and pyrene in seaweed *Laminariaja ponica* was likely carried

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out by enzymatic oxidation by oxidoreductases in the plant cells. Enhanced activities of SOD and POD indicate the induction of oxidative stress of seaweed after exposure to phenanthrene and pyrene in the early stage and Polyphenol oxidase (PPO) responded relatively in the later stage of metabolism. These results coincided completely with ours, since the enhanced activities of the studied antioxidant enzymes due to the effect of PAHs indicate the induction of oxidative stress in the studied algal species.

In general, our results are in agreement with the study of Kirso and Ihra (1998), suggesting that bioaccumulation was the major process for seaweed *L. japonica* at the beginning of exposure to phenanthrene and pyrene, followed by the metabolic oxidation process, when 90% of the PAHs were removed. For the metabolities, dihydro-pyrene-diol as a major biodegradation compound extracted from the seaweed tissues, further suggesting that dioxygenase was a process for biotransformation of PAHs by seaweed *L. japonica.* As suggested by Kirso and Ihra (1998), this enzymatic process can be considered part of a detoxification mechanism for PAH metabolism by seaweed *L. japonica*. This enzymatic mechanism may be involved in the biotransformation of WSFs of crude petroleum oil in our studied species using the elevated activates of SOD, APX and CAT.

The electron micrographs, of control as well as treated cells with concentration 50% of WSFs of crude petroleum oil, clarify the great differences between control and treated cells. These differences were in shape, features of cell wall, features of chloroplast and features of the nucleus. Cell wall of the control cells appeared more or less regular compared to those of treated cells. There are some reports showing that petroleum hydrocarbons lead to membrane damage and increase membrane permeability (Kauss & Hutchinson, 1978 and Sikkema *et al.*, 1995). Meanwhile, chloroplast of treated cells appeared very lighter than untreated cells, this was true because of the reduction in chlorophyll content under the stress of high concentrations of crude petroleum oil. In addition, thylakoids are very disintegrated in treated cells, this was due to the degradation and bleaching of pigments as suggested by Zachleder and Tukaj (1993). The results in this study are in coincided with those of Lynch and Thompson (1982) and Peeler *et al.* (1989), where differences in the outer plasma membrane as well as of chloroplast and microsomal membranes under the external stress were observed.The treated cells slight changes on the outline of the chloroplasts. Clear separation of the chloroplast lamellae and some abnormalities changes in the shape of the chloroplast. Nearly all the inclusion of the nucleus began to dissociate together with gradual disappearance of the nucleus wall. The destructive effects of the lamellae structure of the chloroplast could be intern affected the photosynthesis of the cells. The same conclusion was also recorded by Vandermeulen and Ahern (1976). They revealed that crude oil extract causes modification of some physiological functions as photosynthesis and respiration. Morales Loo and Goutx (1990) concluded that the toxic influence of the WSFs disrupted the biosynthesis mechanism required for a functional photosynthetic apparatus. The effects of crude oil on the morphological characters of the green algae *Scenedesmus obliquus* and the diatom strain *Nitzschia linearis* have been observed. The most marked characteristic is that the algal cells in both strains were aggregated in clusters containing oil drops between their cells, forming an abnormal shape comparing with control culture (Gamila and Ibrahim, 2004). This is consistent with the study of Soto*et al.* (1979), who observed that aqueous crude oil extract had caused abnormalities in the cells of *Chlamydomonas angulosa*.

Kusk (1978) & Miller *et al.* (1978) have observed that algal biomass measured by chlorophyll-a, photosynthesis and nitrogen fixation in several phytoplankton like *Nitzschia palea* and *Anabaena doliolum* were inhibited by crude oil. This suggestion has the same opinion with references of (Van Putte & Patterson,1995; Nechev *et al.*, 2002) who reported that the crude oil and diesel fuel influence membrane fluidity of several phytoplankton species through membrane disruption and change of plasma membrane fatty acid and sterol composition.

The results showed that *S. hornchuchii* and *U. fasciata* were less affected than *Pterocladia capillacea* by most WSFs of crude petroleum oil concentrations. These results coincided with those of Fakhry and Abdel- Kareem (2006), where concluded that *Dunaliella salina* did not negatively affected severely by most concentrations of diesel and gasoline fuels, which suggests that *Dunaliella salina* is a diesel and gasoline-tolerant species. Thus, oil pollution may not be as adverse on certain organisms under certain conditions as it has been widely speculated, unless at very high concentration levels.

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