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The Bioaccumulation and Toxic Effect of Pyrene and Phenanthrene in *Hydrilla verticillata (*L.F.) Royal.

التراكم الحياتي والتأثير السمي لمركبي Pyreneو Phenanthrene في النبات المائي *Hydrilla verticillata (*L.F.) Royle.

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Summary:

A laboratory experiment was conducted to investigate the effect of bioaccumulation level of Pyrene and Phenanthrene compounds on some physiological parameter of *Hydrilla vertcillata* for 10 days. The results were revealed that the bioconcentration factor (BCF) were 2.85 and 0.05 which detected under high concentration (200µg/l) while they were 1.85 and 0.03 in the control group for both compounds respectively. The concentrations of two pollutants in the leaves of the treated plants were ranged (171-2224.2 µg/g. dry weight) and (0.18-9.84 µg/g.dw) for both respectively. The results of toxicity showed that increasing of the ion permeability in plasma membrane (88%) and lipid peroxidation products as malondialdehyde (MDA) were (10.4µmol/l fresh weight) also under high concentration, whereas in control groups decreased to 28% and (1.13µmol/l) for a mixture of compounds. The ascorbic acid, chlorophyll content and lipid percentage were reduced to 4.8 mg/g.fw, 2.8mg/g and 0.04% in plants leaves while they were 18.4mg/g, 0.9mg/g and 0.026% in the control group. The statistical analysis was indicated that significant differences among stressed and control groups in experiment plants.

الخلاصة :

اجريت الدراسة مختبرياً لبيان مدى التراكم الحياتي والتأثير السمي للمركبين Pyreneو Phenanthrene وتأثيرهما على بعض المؤشرات الفسيولوجية لنبات الكطل (L.f.) Royle *Hydrilla verticillata* بعد تعرضه لتراكيز مختلفة من كلا المركبين ولمدة عشرة ايام. بينت نتائج الدراسة ان معامل التركيز الحياتي(BCF) كان (2.85 ;0.05) والتي سجلت عند أعلى تركيز (200µg/l) بينما كانت (1.85 ;0.03) في مجموعة السيطرة للمركبين على التوالي. ووصلت تراكيز المركبين في اوراق النباتات المعاملة الى (0.18-9.84 µg/g.dw) و(171-2224.2 µg/g.dw) ايضا على التوالي للمركبين. كما أشارت نَتائِجُ السمية إلى زيَاْدَة في نفاذية الايونات (88%) ونواتج أكسدة الدهون في الاغشية الحية لخلايا النبات وبلغت (10.4 µmol/l. fw) عند اعلى تركيز(200µg/l) للعاملين على التوالي. اما لمجموعة السيطرة فكانتا (28%) و((1.13µmol/l. fw لمزيج المركبين معاً. بينما انخفض محتوى حامضِ الأسكوريك والكلوروفيل الكليّ والنسبة المئويةَ للدهون الى (4.8mg/g.fw) و(2.8mg/g) و(0.04%) في اوراق النباتات المعاملة وفي مجموعة السيطرة كانت قيمهما هي (mg/g.fw) و(0.9mg/g) و(0.026%) على التوالي ايضاً. وأشار التحليل الإحصائي إلى وجود فروقات معنوية بين المجموعاتِ المجهدة والسيطرة في نباتات التجربة.

Introduction__

Pyrene and Phenanthrene compounds are from a polycyclic aromatic hydrocarbon group that were classified as hazardous pollutants and from persistent organic pollutants (POP). It has carcinogenic, mutagenic and teratogenic properties, it also causes birth defects and highly toxic to humans and animals (1, 2). Polycyclic Aromatic compounds disperse in different environmental media such as water, air, soil, plants, from their important sources of PAHS is pyrogenic origin, which is included gases and particulate matter emissions from the incomplete combustion of oil and its derivatives such as car exhaust, machinery and various industrial uses of fossil fuels, the other source is the pathogenic origin from direct effluent to oil spills and its derivatives, sewage, industrial and municipal wastewater directly into the aquatic environment (3).

The biotic and abiotic stress induces oxidation processes to produce reactive oxygen species such as oxygen superoxide radicals O_2°, hydroxyl radicals OH°, hydrogen peroxide H_2O_2 and others, which is a powerful oxidant for large molecules in the cell such as lipid, proteins and nucleic acids, then causing cell death, the increasing in superoxide dismutase (SOD) and catalase (CAT) and ascorbate (APX) peroxide and non-enzymatic antioxidant including ascorbic acid and α-Tocopherol (Vitamin E) and Glutathione (4). The Pyrene and Phenanthrene compounds are non-polar compounds, soluble in lipid and has a high affinity for organic materials. Therefore, it contribute in the induction of the generation of oxidative stress factors causing plasma membrane disruption, protein denaturation and causing genetic mutations (5) PAHs compound are interfering with the action of respiratory oxidation enzymes in mitochondria and involved enzymes in the electrons transfer through photosystem I & II processes (4), also (6) find that *H.verticillata* plant was sensitive to organic pollution such benzene-toluene- ethylene –xylene (BTEX). *Hydrilla* was identified for the first time in the Iraq in 2004 at Abo-Zerik marsh (7) and Al-Chibaish marshes (8).However, it was recorded in little Zab River within Kirkuk city as invasive species (9). The aims of the current study were investigated to the ability of bioaccumulation potential for these compounds in *Hydrilla verticillata* plant and its response to different concentrations of them by the membrane permeability, lipid oxidation and the change in MDA content as well as the changes in ascorbic acid and total chlorophyll.

Material and Methods:

1-Plant breeding and Growing conditions:

H. verticillata plant (Hydrocharitaceae) was chosen because of the long time of the growing season in the Euphrates River and its branches; the plant was classified according to (9). It has been collected during April, and grown in glass container (40 × 20 × 20 cm) with Hoaglandѕ̓ solution (1/10 strength). The acclimatization of the plant was prolonged for 20 days under a wooden canopy conditions. Then the plants were transferred to flasks 1 liter contains nutrient solutions combined with different concentrations of the Pyrene and Phenanthrene are (10, 50, 100, 150, 200 μg/l). Four plants to each treatment was distributed on three replicates with the control treatment that was without adding the compounds. Pyrene and Phenanthrene were selected as model to Polycyclic Aromatic Hydrocarbons (PAHs), it was obtained from Aldrich Chemical Co. with a purity >98%. The molecular weights of Phe and Pyr are 178.23 and 202.26 g/mol,(K_{ow} is the octanol water partition coefficient) are 4.46 and 4.88, respectively.

Different concentrations of both compounds are (10, 50, 100, 150, 200) μg/l were dissolved in small amounts of acetone then the volume was complete to one liter of distilled water. Five concentrations were used in treating plants by three replicates for each concentration except the control group, which has added to just a nutrient solution.

2– Analytical instrumentation:

To determine the concentrations of Pyrene and Phenanthrene compounds in plant tissues, High performance liquid chromatography, HPLC (Schimadzu) was used in the central laboratory /University of Kufa /College of Pharmacy.

Plant leaves was extracted after drying and grinding and extraction method according to (11, 12, 1) one gram of powder sample were twice extracted with 10 ml solution of 1:1 (v/v) acetone and hexane under ultrasonication for 1h, then centrifuged at 4,000 $r \cdot min^{-1}$, the solvent fractions were combined, cleared up with KOH saponification (removing interference from chlorophyll, xanthophylls, and other organic pigments), then organic solution passed through a chromatography column (1.5 * 40 cm) consisting of 8 g of silica gel, 4 g aluminum oxide and 2 g sodium sulfate anhydrous and 0.5 g of copper powder to reduce sulfur compounds, with elution of 1:1 (v/v) acetone and hexane. The solvents were then evaporated off by rotary evaporator (Alpha chemical) and exchanged to 2 ml with high-purity cyclohexane (for HPLC). bioconcentration factor (BCF) was calculated by dividing the concentration of compounds in solution to that in leaves (13).

3- Biochemical analysis:

The permeability of ions was used as indicator of the damage level in the cell membrane under exposure to different concentrations of PAHs (pyr. and phe.) this parameter was detected by measuring of electrical conductivity in the solution after incubated plant leaves in deionized distilled water according to (14). Lipid oxidation was estimated by malondialdehyde (MDA) according to (15) by adding chlorine tri-acetic acid to precipitate the proteins, 1ml of 2-thiobarbituric acid (TBA) was added to 1ml of supernatant, and then absorbance was read at 532 nm the results was expressed in units (MDA= µmol/g fresh weight). The changes of total ascorbic acid content in the exposed leaves of plants were estimated depending on (16), the results were expressed by (mg/g fresh weight).

Total chlorophyll content in the leaves of plants was estimated according to (17), the extraction with (Aceton 80%) was done, then absorbance of spectrophotometer was read at wavelength 645 nm and 663 nm, the results has been expressed by mg/g. Lipid contents were estimated by the method (18), the organic solvent mixture of (chloroform 2: 1methanol) was used in extraction lipid and organic layer was separated and evaporated, then taking the difference in the weight of the container after and before extraction.

4-Statistical analysis:

Treatments were designed as a Completely Randomized Design with three replicates. The means were compared by using the LSD (least significant difference) at $P \leq 0.05$ between treatments using statistical software (SPSS) version 16. data was applied by the program. (Microsoft Axcel).

3- Results and Discussion

1- Absorption and Bioconcentration:

The results of current study indicated that increasing of the bioaccumulation level of Pyrene and Phenanthrene in *H. Verticillata* plant leaves after ten days with increasing of the period exposure. Pyrene was detected high accumulative capacity reached 2224.2 ng/g. dry weight (dw), while Phenanthrene concentration was 9.84 ng/g (dw) at the highest concentration of both compounds 200 µg / l in the nutrient solution. The minimum concentration in the tissues of plants was recorded (0.18, 50.7ng/g). Statistical analysis showed significant differences between concentration of compounds in treated and control groups (LSD = (Pyrene) = 2.8; (Phenanthrene) = 0.86.

(T test) also showed significant differences between the two sets of exposure in Pyrene accumulation capacity with compared to Phenanthrene compound.

Due to the increasing of molecular weight and number of benzene rings, the ranged of bioconcentration factor (BCF) of Pyrene were increased to (1.85-2.85) while recorded (0.03-0.05) for Phenanthrene. According to chemical properties of PAHs, Pyrene has high affinity of organic matter, which increases the adsorption on the cell surfaces and increase solubility in lipids of plasma membrane and inside cell compared to Phenanthrene.

The absorption and accumulation of Phenanthrene vary with different components of plants tissues of water, lipid, protein and carbohydrates, as well as the size, shape, length and the component of roots in these plants (19). Thus, *H. Verticillata* may be tolerance low concentrations of PAHs. On the other hand, it was high accumulator for these pollutants making it one of the most promising plants in the treatment of persistent organic pollutants and phytoremediation processes.

In surrounding water and soil or by the adsorbed organic particles on the surface of roots, the plants absorb these compounds then accumulate in their roots and root hair so increasing soil content of PAHs may be increased its concentration in plant (20). (21) investigated that absorption and movement of Phenanthrene and Anthracene by *Triticum aestivum* and *Zea mays* plants in contamination soils by using the optical excitation microscope (Two-photon excitation microscope) The study pointed out that the movement of these compounds started from the root epidermis cells radial toward the cortex cells then to vascular bundles and upward to shoot. The absorption process for this type of pollutant affected by content of the lipid, surface area of the roots, and biomass, on the other hand, the availability of these contaminants in pore water and resident time in soil as well as the physical and chemical properties of each compound such as polarity, molecular weight, and solubility are the important factors in the absorbance and accumulation of this type of pollutants.

(22, 12). (23) find that the bioaccumulation of Pyrene and Phenanthrene in the tissues of the plant *Laminaria japonica* increased with increasing of the concentration and long-term of exposure, then significant damage to the tissue structure and cell death of the seaweed were observed after one week under high concentration.

Because of the *H.verticillata* as a part of aquatic food chain to aquatic organisms, therefore the bioaccumulation of this pollutants makes it more ready to pass on to the food

chain, then increasing of distribution and biomagnification of PAHs in aquatic organism to human (24).

Table (1): Concentrations (ng/g. DW) and Bioconcentration factor (BCF) of Pyrene and Phenanthrene in *H.verticillata* plant leaves exposed to different concentrations of Pyrene and Phenanthrene after 10 days.

Groups	Phenanthrene		Pyrene	
Exposure level(µg/L)	Concentration in leaves (ng/g.dw)	Bioconcnteration factors (BCF)	Concentration in leaves ng/g.dw	Bioconcnteration factors (BCF)
Control	0.18	0	50.7	0
10	0.3	0.03	171	2.85
50	0.6	0.012	418.2	1.4
100	3.6	0.036	1039.2	1.73
150	7.2	0.048	1865.4	2.07
200	9.84	0.05	2224.2	1.85
LSD	0.86		2.8	

2-Toxic effects of PAHs:

2-1**:** Permeability of perturbation

The results of present study demonstrated the ability of these compounds to change the permeability of cell membranes by measuring the change in the electrical conductivity of the leaves infusion solution after different periods for death and thermal affected cell groups with the control group (Table 2). High electrical conductivity values was detected due to increase of cations and anions in solution from 11.4 µS/cm for control group to 44.8 when plants leaves were exposed to the highest concentration of Pyrene and Phenanthrene after 24 hours, so the percentage of the permeability of cell membranes under high concentration reached to 88%, while it was 28% in the control group due to the cytoplasmic damage by pollutants under study. These findings are consistent with (25) who found that creosote substance (is made up of ten aromatic hydrocarbons) including the Pyrene and Phenanthrene were affected in the permeability of *Meriophyllum spicatum* plant leaves.

Exposure level(µg/L)	Conductivity µS/MC after 24 h	Permeability%
Control	11.4	28
10	15.4	34
50	28	62
100	34.6	73
150	42.4	87
200	44.8	88

LSD=5.2

2-2. Lipid Peroxidation and Non-Enzymatically Antioxidant:

The results indicate that Pyrene and Phenanthrene has ability to oxidize lipid of leaves in terms of malondialdhyed content (MDA) as a final product of lipid peroxidase. MDA concentrations was increased significantly to (10.4 µmol/g fresh weight) under 200µg/l while it was recorded (1.13µmol/g f.w) in control group. The correlation coefficient values of both compounds was positive with concentrations of MDA and negative with chlorophyll and lipid content values. These findings are consistent with (10) that exposed *Ceratophyllum demersum* plants to different concentrations of the Phenanthrene which were induced the generation of free radicals as reactive oxygen species (ROS) and increase the antioxidant enzymes activity such as Peroxidase, Catalase, Polyphenol oxidase (Glutathion transferase thus get oxidative damage to the plant (23) investigated that *Laminaria japonica* has ability to absorb and metabolize of Phenanthrene and Pyrene and converted to less toxic compounds under low concentration 0.1 mg/l, however the potential toxicity was appeared under 0.2 mg/l through the increase in the antioxidant enzymes effectiveness as Peroxidase (POD), Superoxide dismutase (SOD) Polyphenol oxidase (PPO).

One of detoxification mechanisms in plants is induction Glutathione- Ascorbic acid cycle to remove free radicals Therefore, the results of current study indicate that reduction in the content of ascorbic acid in treated plant leaves to (4.8 mg/g. fw) under 200 µg/l while it was recorded (18.4 mg/g) in the control group, also we can show (fig.1) the low concentrations of both compound was induced ascorbic acid generation and recorded high values at treated leaves under (10µg/l). The statistical analysis showed significant differences between the treated groups with ascorbic acid (LSD = 0.56).

Some studies (26 &27) reported a decrease in the level of antioxidants including ascorbic acid with increase in stress intensity in wheat also ascorbic acid showed a reduction under drought stress in maize and wheat, suggesting its vital participation in deciding the oxidative response.

Many vital processes such as photosynthesis, growth rate and cell division of microalgae and higher plants *Lemna gibba* were impacted by reactive oxygen species (ROS). As well as the damages on membranes by lipid oxidation or proteins associated with photosynthetic electron transport or inhibition of any cellular process downstream of photosystem II (PSII) will lead to excitation pressure on PSII (28, 29).

A similar results in the levels of MDA, ascorbic acid and permeability percentage were detected by (30) in a study of two types Potamogetonacae family under different concentrations of Pyrene and Phenanthrene.

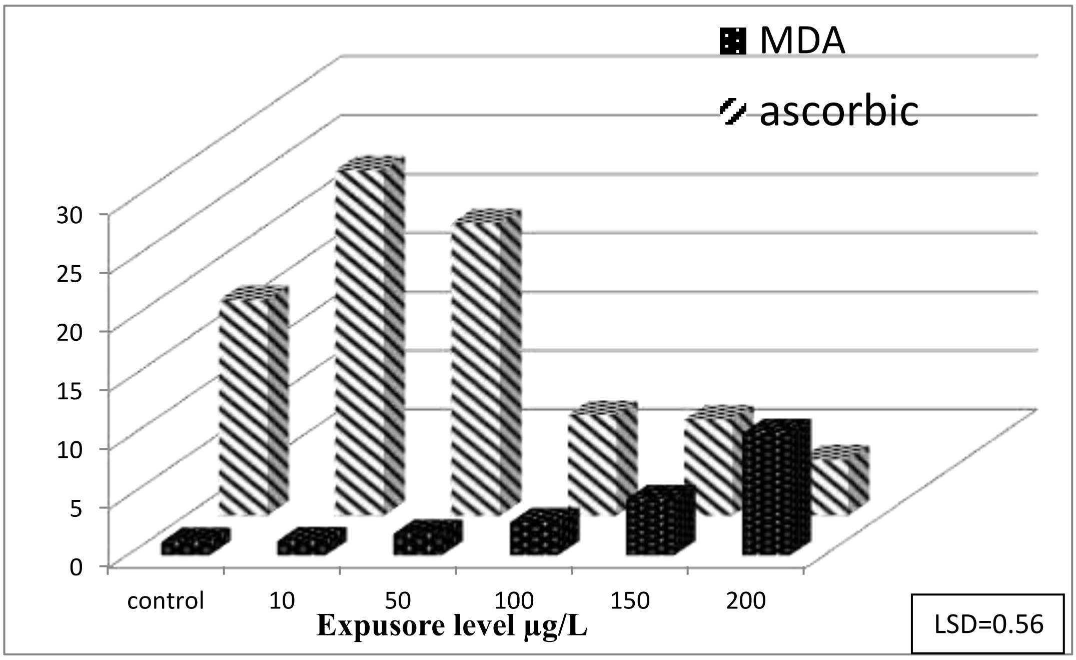

Fig. (1): The changes in the Malondyaldehyed MDA and Ascorbic Acid content in *H.verticillata* plant leaves exposed to different concentrations of Pyrene and Phenanthrene after 10 days.

2-3-The Changes of lipid and chlorophyll content:

The analysis of lipid and chlorophyll content in *H. verticillata* plant leaves after exposing to different concentrations of a mixture of Pyrene and Phenanthrene showed that decreased to 0.026% and 0.9 mg/g under higher concentration after 10 days, while it was recorded 0.04% and 2.8 mg/g in the control group respectively. This reduction is perhaps due to the plant sensitivity to Pyrene and Phenanthrene by appearance of yellowish and wilt leaves. (28) and (4) showed that these compounds has ability for lipid oxidation generating of free radicals that induced by visible light and ultraviolet radiation to produce a series of electron that may interacts with reaction chain of photosynthesis systems PI or PII these interactions called Photosenstization; or it reacts with intermediate compounds to create more toxic compounds by Photomodification reaction such as the reaction of Phenanthrene with Quinone to produce Phenanthrquinon (31, 32).

(20) indicated that total lipid content was reduced by increasing of bioconcentration factor (BCF) and there is a strong correlation relationship between them in the roots and leaves of 12 plant species was exposed to the Phenanthrene and Pyrene. (33) investigated that lipid content and thickness of the cuticle layer significantly associated with the accumulation of this type of contaminants in the internal tissues of plants. (29) stated that the pigments carotene and chlorophyll a, b were significantly reduced under high concentration of Pyrene and Phenanthrene and time duration in tomato *Solanum lycopersicum* L. cv. Hezuo plant, also that reflected on the biomass of treated plants, rate of photosynthesis and transpiration processes. (34) pointed to the same results when a tomato plant has been exposed to Fluoranthene compound as

Table (3): The percentage of lipid (%) and total chlorophyll (mg / g) in *H.verticillata* plant leaves exposed to different concentrations of Pyrene and Phenanthrene after 10 days of exposure.

Exposure level (µg/L)	Lipid percentage%	Total chlorophyll(mg/g)
Control	0.04	2.8
10	0.06	2.6
50	0.084	2.14
100	0.05	1.6
150	0.02	1.6
200	0.026	0.9

Conclusions and Recommendations:

These results demonstrated that the polycyclic aromatic hydrocarbons (PAHs), including Pyrene and Phenanthrene have high toxicity in biological systems of plants that accumulate these pollutants in their cells and tissues. However, the previous study not detected a specific mechanism in plants for metabolism of these compounds and convert them to less toxic compound, however photoinduce toxicity may occur in plant under pollutants stress such PAHs which is converts to more toxic compounds in plant body (35), also they have another mechanism to avoid toxic effect of pollutants by accumulation and concentrated this chemicals within cell wall or vacuoles. Wherever, plants vary in ability to accumulate acceptable limits by determine Bioconcntration factor BCF for each plant. These results investigated that *H. verticillata* is sensitive at high concentrations of polycyclic aromatic hydrocarbons (PAHs) by physiological indicator and induction antioxidant; it may be of promising plants in the biological treatment techniques for persistent organic pollutants.

Table (4): The correlation coefficient values between the studied parameter (PearsonCorrelation) in *H.verticillata* plant leaves exposed to different concentrations of Pyrene and Phenanthrene after 10 days.

	Pyrene conc.	Phenanthrene conc.	permeability	Malondialdehyed	Ascorbic acid	Lipid content	chlorophyll content
Pyrene conc.	1.000	.991**	1.000	.906**	-.858 *	-.696	-.943**
Phenanthrene conc.	.942**	1.000	.991 *	.941**	-.862 *	-.745	-.925**
permeability	.924**	.877	1.000	.757	-.786	-.43	-.944**
Malondialdehyed	.873 *	.941**	.906	1.000	-.745	-.613	-.891 *
Ascorbic acid	-.787 *	-.862 *	-.858	-.745	1.000	.770	.826 *
Lipid content	-.509	-.745	-.696	-.613	.770	1.000	.478
chlorophyll content	-.980**	-.925**	-.943**	-.891 *	.826 *	.478	1.000

**** Correlation is significant at the 0.01 level (2-tailed).**

*** Correlation is significant at the 0.05 level (2-tailed).**

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