Caspian Journal of Environmental Sciences

Online ISSN: 1735-3866
Print ISSN: 1735-3033

# Biochemical responses of some aquatic plants as indicators for the treatment of inorganic nitrogen compounds in wastewater (Case study: Domestic water treatment plant in Babil Governorate, Iraq)

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## ABSTRACT

The current study was conducted to investigate the biochemical response of two plant species including *Lemna minor* and *Ceratophyllum demersum* as indicators for the treatment and reducing the concentrations of dissolved inorganic nitrogen compounds in wastewater collected from a domestic water treatment plant in Al-Maamera area in Babil Governorate, Iraq. The experiment included the cultivation of aquatic plants in 20-liter plastic basins, which contain domestic water collected from the sedimentation basins at the station after completing the physical and chemical treatment. Some physical and chemical tests were conducted and the concentrations of some compounds of dissolved inorganic nitrogen were measured by taking the water samples every three days for a period of twenty four days. The current study showed that the pH values were raised to the basal direction when wastewater treated with *C. demersum*, while the values fluctuated when using *L. minor* with treatment between low and slight increase at other times .On the other side, the efficiency of the aquatic plants used in current study in decreasing the values of both electrical conductivity and total dissolved solids was weak and was limited to the first days of treatment. The results recorded an increased oxygen concentration when wastewater treated with *C. demersum* compared to the control which recorded higher concentrations than *L. minor*. The study showed also that the *L. minor* and *C. demersum* have very high efficiency in reducing the inorganic nitrogen compounds. So that, in the case of *L. minor*, the highest removal rate of nitrite, nitrate and ammonia were 99.36%, 88.04% and 81.25 %, respectively, while in the case of *C. demersum*, the related rates were 99.36%, 78.8% and 86.6 % respectively. As for the physiological condition of the plants, the results in the current study showed that the total chlorophyll values in *L. minor* and *C. demersum* increased after completing the phytoremediation. The MDA content decreased, while CAT enzyme was not affected by both plants. The SOD enzyme was not also affected by *L. minor*, while a rise in this enzyme was recorded by *C. demersum*.

**Key words:** *L. minor*, *C. demersum*, Phytoremediation, Defence mechanism.
**Article type:** Research Article.

## INTRODUCTION

The contamination of Water occurs mainly due to the municipal and industrial wastewaters, agricultural fertilizers and other organic and inorganic chemical compounds (Verla *et al*. 2018). The inorganic and organic contaminations are not easy to destroy environment biologically, however, they can turn from a toxic to low toxic forms (Zhang *et al*. 2017; Jiang *et al*. 2018). Growth of human populations and the increase in their activities led to alter the hydrologic cycles, landscape and the flow of nutrients that important for plant growth at an accelerating rate over the past centuries (Vitousek *et al*. 1997; Galloway & Cowling 2002). The nitrogen affecting aquatic environment include inorganic forms such as ammonium, nitrite and nitrate, many compounds of dissolved organic nitrogen such as composite dissolved organic nitrogen, urea and amino acids as well as particulate forms

Caspian Journal of Environmental Sciences, Vol. 19 No. 5 pp. 1017-1026 Received: May 18, 2021 Accepted: July 28, 2021
DOI: 10.22124/CJES.2021.5352

Publisher: University of Guilan, Iran

of nitrogen (Peierls & Paerl 1997). Wastewater from agricultural, domestic or municipal sources often contains high quantities of phosphates, ammonium and nitrate even after anaerobic decomposition of organic matter in water treatment plants (Landesman *et al*. 2011). The eutrophication of surface waters may occur by these macronutrients when present in large quantities. Thus, efficient remediation methods for controlling eutrophication and restoring the aquatic ecosystem are being pursued (Xiaoyun & Xingyuan 2015). The purification system of artificial wetland water built by aquatic plants have good purification effects, operation and convenient management, low cost, as well as ecological benefits and good landscape. Therefore, in world investgators are given more attention to the polluted water recycling by some aquatic plants (Zhang *et al*. 2014), where the aquatic plants have ability to absorb inorganic nitrogen forms such as nitrate and ammonium directly (Nordin *et al*. 2001). Assimilation of ammonia is closely linked to carbon metabolism, where the supply of organic acids which is maintained by the tricarboxylic acid cycle, is indispensable in synthesis of amino acid (Vega-Mas, 2017). Thus, availability of carbon skeleton is very essential for assimilation of ammonium in plant. Moreover, accelerated decomposition of starch is found in plant under some abiotic stress and this provides energy and more carbon skeletons for abiotic stress protection (Cao *et al*. 2009). However, acceleration of metabolism produces various reactive oxygen species and disrupts internal ROS homeostasis, then causes intracellular damage and produces secondary oxidative stress in plants (Xie 2015). Typically, cells in plant up-regulate the antioxidant enzymes activity such as superoxide dismutase (SOD) and catalase (CAT) to remove excessive ROS (Fujita 2006). This study aimed to investigate the biochemical responses of some aquatic plants and the possibility of their use in the reduction of inorganic nitrogen compounds in municipal wastewater.

## MATERIALS AND METHODS

### Experimenting the wastewater treatment with aquatic plants

Aquatic plants were collected from different sites in Hilla City and were washed well with tap water and then with warm water to remove suspended materials and placed in plastic bags (70 × 30 ×35), and left plants with tap water for a period of two weeks for the purpose of acclimatization and discarding the contaminants attached to them. Afterward, the aquatic plants were placed at a rate of 10 g $L^{-1}$ (Taha *et al*. 2011) with the addition of 20 L of wastewater collected by polyethylene containers, followed by leaving bags contained untreated wastewater as control. An amount of water was withdrawn every three days for a period of twenty four days for the purpose of conducting some physical and chemical tests with estimating the concentration of inorganic nitrogen compounds.

### Physical and Chemical analysis

Some parameters such as pH, EC (µs $cm^{-1}$) and TDS (mg $L^{-1}$) of wastewater were measured using pH-meter (multi-parameters), Oakton - USA after calibrating the device with related solutions. Dissolved oxygen was determined according to Azide- modification of Winkler method (APHA 2003). Nitrite was measured according to the method described by Parsons *et al*. (1984). Nitrate was measured according to the (Phenoldisulphonic Acid) method (APHA 2012). Ammonia was estimated as reported in the Phenate method described by APHA (2003).

### Estimation of the physiological and biochemical markers

Chlorophyll content was estimated according to method described in Mackinney (1941). The superoxide dismutase (SOD) activity was measured according to Marklund & Marklund (1974). The catalase (CAT) activity was estimated by Misra & Gupta (2006). Malondialdehyde content was measured by following the method of Zacheo *et al.* (2000).

## RESULTS AND DISCUSSION

The table below (Table 1) shows some physical and chemical parameters of municipal wastewater before phytoremediation. The pH values in water is one of the most important factors affecting the physiology and metabolism of aquatic plants, because of its effects on the availability of nutrients and other elements (Lawson 2011). In the present study, the pH values of the control and treated water elevated toward the base direction especially in *C. demersum* which recorded values higher than the control and *L. minor* (Fig. 1). The upraising pH in wastewater after phytoremediation may be due to decreased carbon dioxide by the photosynthesis (Crites & Tchobanoglous 1998), and also to increased presence of algae and aquatic plants. In these plants, due to the photosynthesis process, withdrawing carbon dioxide present in the water will increase (Thompson *et al*. 2003).

Caspian Journal of Environmental Sciences, Vol. 19 No. 5 pp. 1017-1026 Received: May 18, 2021 Accepted: July 28, 2021
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The removal of $CO_2$ tends to cause a shift in the form of alkalinity present from bicarbonate to carbonate and from carbonate to hydroxide (Kanabkaew & Puetpaiboon 2004).

**Table 1.** The concentrations of some Physical and Chemical analysis in wastewater before bioremediation

| **Measured parameters** | **values Mean ± SD** |
|---|---|
| pH | 8.30 ± 0.10 |
| TDS (mg $L^{-1}$) | 779.67 ± 26.65 |
| EC (µs $cm^{-1}$) | 1176.67 ± 28.93 |
| $O_2$ (mg $L^{-1}$) | 1.8 ± 0.28 |
| Ammonium (mg $L^{-1}$) | 11.2 ± 0.10 |
| Nitrite (mg $L^{-1}$) | 0.157 ± 0.01 |
| Nitrate (mg $L^{-1}$) | 35.385 ± 1.74 |

Oxygen is one of the most important limiting factors in aquatic ecosystems (Morgan *et al*. 1993). The solubility of oxygen depend upon many factors such as temperature, salinity, organic compounds, the partial pressure of oxygen as well as density of phytoplankton and aquatic plants (Ahangar *et al*. 2012; Wetzel & likens 2000). The results of the present study revealed increase in oxygen values when sewage wastewater treated with *C. demersum* compared to control, while *L. minor* recorded lower concentrations (Figs. 2A, B).The efficiency of *C. demersum* in water aeration throughout the study period may be due to the efficiency of aquatic plants in elevated dissolved oxygen concentrations as a result of increased effectiveness of photosynthesis (Liu *et al*. 2000). At the same time, aquatic plants differ in their role in aeration of water bodies, since some of them such as submerged plants, play a distinctive role in upraising the oxygen content in water, as evidenced by experiments with artificial swamps created in wetlands (Chimney *et al*. 2006). Decreased oxygen values observed when wastewater treating with *L. minor,* may be due to high cover of *L. minor* producing a severe under-water light attenuation and preventing circulation of water (Large *et al*. 1996) which lead to a strong depletion of oxygen in the water column (Caraco *et al*. 2006). Electrical conductivity values refer to the numerical expression of the positive and negative ions (APHA 2003). It expresses the water ability to carry electric currents and is considered as an good indicator of salts dissolved in water. It is closely connected with total dissolved solids, and its elevation occurs in areas under the influences of industrial and agricultural activities (APHA 1976). The electrical conductivity values and the percentages of their reduction are shown in Figs. 3A-B, where the plants recorded weak reductive ratios limiting to the first days of treatment. The highest reduction ratio for *L. minor* and *C. demersum* plant were 8.8 % and 13.5 % respectively, compared to the control treatment, which recorded a lower reduction rate. This is in agreement with Wendeou *et al*. (2013) who reported that the electrical conductivity (EC) in wastewater was constant at the beginning of treatment with *Lemna* sp., while thereafter, it began to increase significantly. The results of the present study also agreed with that of Al-Singri (2011), who reported that the electrical conductivity values elevated once using the reed plant in the polluted waste water treatment, due to the role of the aquatic plants in the analysis of compounds containing mineral elements by the process of phyto-degradation. The decomposition processes lead to the releasing certain metals in dissolved ionic form with the ability to transmit electricity, and this in turn increased the values of electrical conductivity depending on the many factors such as quality and concentration of the dissolved ions (Al-Wahaibi 2007). TDS are made up of positive and negative ions such as calcium and magnesium salts, sodium salts, bicarbonate, chlorides, sulphate and dissolved organic matter (Weber & Duffy 2007). Plants in this study show weak efficiency in reducing TDS values which restricted to first days of treatment, since the treatment was limited to *L. minor* and *C. demersum* on the third day, and the highest reduction rates were 1.19% (concerning to the first plant) and 6.2% (to the second one), while the control did not exhibit any reduction rate (Figs. 4A-B). The weakly-efficiency of plants in reducing TDS may be due to the fact that some ions did not play a distinct role in the plant metabolism or decomposition processes in sewage, such that, the water TDS were higher than the ability of the plants to reduce it, therefore, it is absorbed in small quantities (Patel & Kanungo 2010). Ammonia when attached by plants, is incorporated to proteins and other organic combinations by many biochemical reactions. The ammonium assimilated into the organic molecules in plants by many enzymatic processes (Masclaux- Daubresse *et al*. 2010). The plants under study demonstrated high efficiency in reducing ammonia values throughout the treatment period, as the highest reduction rate reached

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81.25 % on 9th day by *L. minor* and 86.6 % on 18th day by *C. demersum*. In the case of the control, lower reduction rate than those recorded by aquatic plants was found. So that, the highest reduction rate (50.89%) was observed on 9th day and no removal recorded in the last days of treatment (Figs. 5A-B). The rapid reduction of ammonia nitrogen may be due to the active nitrification as evidenced by an elevated $NO_3$ concentration in water (Selvarani *et al.* 2015). In addition, freely-floating plants exhibit the ability to remove nitrogen from the wastewater through denitrification processes and subsequently combining them in their biomass (Suhad *et al.* 2018). Most structures of *C. demersum* as a submerged plant found under the water surface and its photosynthetic tissues are submerged entirely in water, hence the waste water turbidity must be low, because high turbidity blocks the transmission of light to plants (USEPA 1999). So, this type of plant depletes the dissolved organic carbon and increase the dissolved oxygen leading to increment of pH value, volatilization of ammonia and chemical precipitation. High concentrations of oxygen also creates favourable condition for mineralization (Bekele 2018). Nitrite is an intermediate product when the biological conversion of ammonium to nitrate occurs by process named nitrification through two-step. The first step includes the conversion of ammonium and ammonia to nitrite which is performed by ammonia-oxidizing bacteria and then, the second step occurs by nitrite-oxidizing bacteria in completing the conversion of nitrite to nitrate (Dongke 2012). In the present study, aquatic plants showed high efficiency in reducing the nitrite concentrations throughout the treatment period, where *L. minor* and *C. demersum* recorded the highest reduction rate of 99.36% at days 6, 9 and 18 for the first plant and days 21 and 24 for the second one. As for the control treatment, lower reduction rates were recorded, and the highest reduction rate was 43.31 % on day 24 of treatment (Figs. 6A-B).The high efficiency of *L. minor* for removal of the nitrogen forms may be due to the their consumption by the new developing plant tissues and not by nutrient content increased in the old tissues (korner & Vermaat 1998). Foroughi *et al*. (2013) noted that the removal of nitrogenous forms by the plant depends on the many factors such as the growth rate and the nitrogen content of the tissue. Duckweed plant is able to take up nitrogen in the form of ammonium, nitrite, nitrate, urea or amino acids, where nitrate and ammonium consider as the most important substances for it (Landolt *et al*. 1987). Given the *C. demersum* efficiency in reducing nitrite values compared to control, this may be attributed to the fact that the submerged vascular plants are known to absorb nutrients such as nitrogen in excess of their normal metabolic requirements. Nitrate consider as the stable form of nitrogen, and always recorded high values in contrast to the other two forms of inorganic nitrogen and this high values may be due to higher rate of oxidation. However, at the same time nitrogen was found to be absorbed as nitrate, hence exhibited lower values after the phytoremediation (Patel & Kanungo 2010). Aquatic plants recorded high efficiency in nitrate reduction (Figs. 7A-B), where *L. minor* and *C. demersum* recorded high reduction rates (88.04 and 78.8) respectively at the first days of treatment.

This high treatment efficiency of floating and submerged plants is due to several reasons *L. minor* is one of the plants known to do nitrogen assimilation by roots and fronds and it seems to be the primary mechanism for nitrogen fixation in it, where nitrogen is fixed as protein in duckweed biomass (Cedergreen & Madsen 2002). In the case of *C. demersum* ,this high efficiency related to the submerged plants ability to release oxygen, and this allows nitrification processes around the plant roots, which helps absorbing nitrate or nitrate together with ammonium and consequently exhibits a good effect on the internal pH regulation in the plant cell (Christensen *et al.* 1994). Kuschk *et al* (2003) explained that the oxygen availability and temperature are considered as the main factors influencing microbiological nitrification. High oxidation level in the root zone stimulates the nitrifying bacteria growth (Williams *et al.* 1994). The nitrate produced as a result of their activity diffusing into the anaerobic regions, is transformed into the nitrogen gas and then removed from the system (Reddy *et al* 1989).

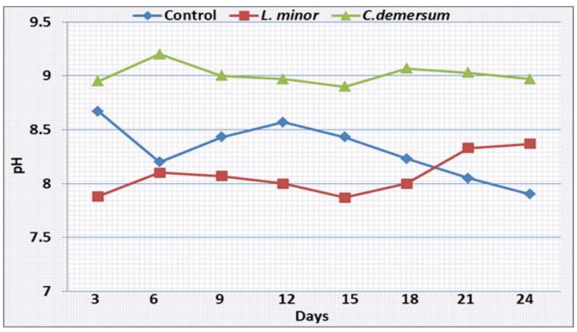


**Fig. 1.** PH values during phytoremediation.

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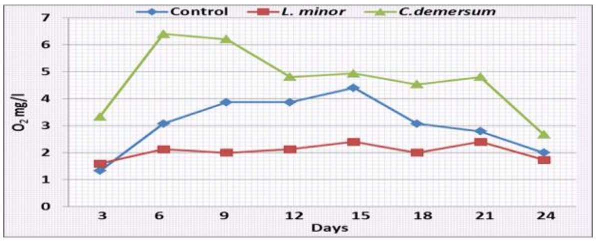
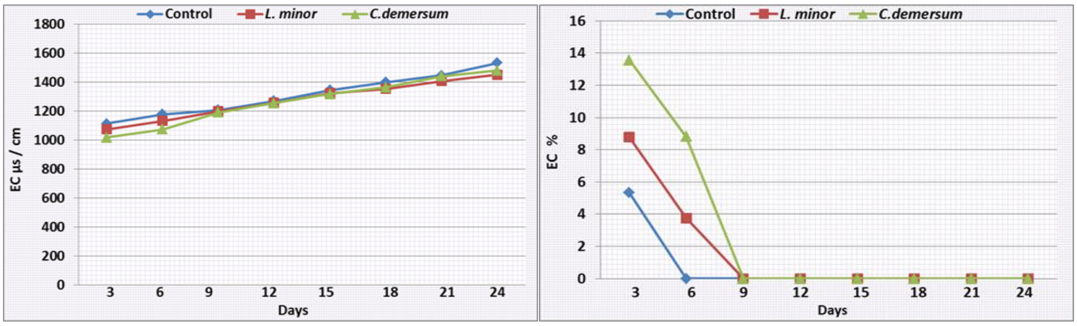
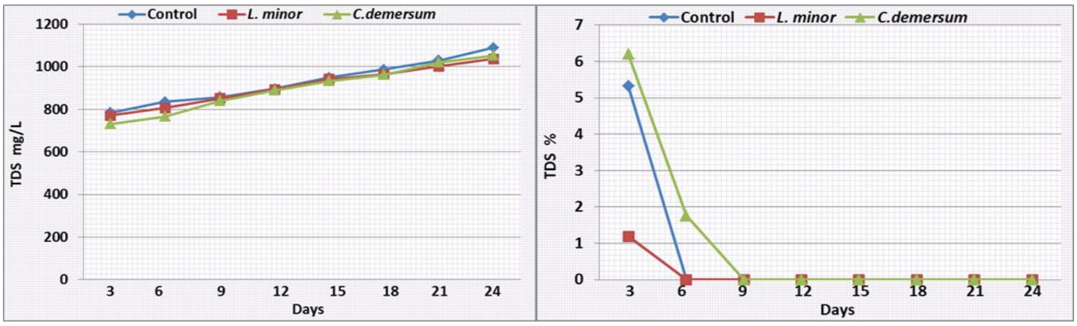


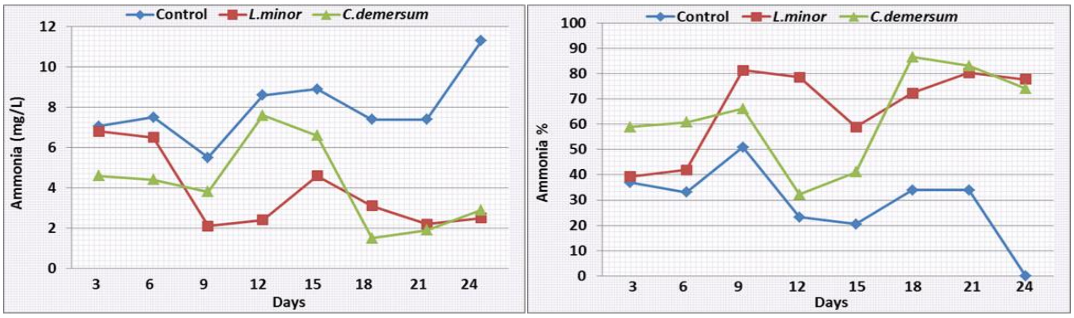

**Fig. 2.** Oxygen concentrations during phytoremediation.



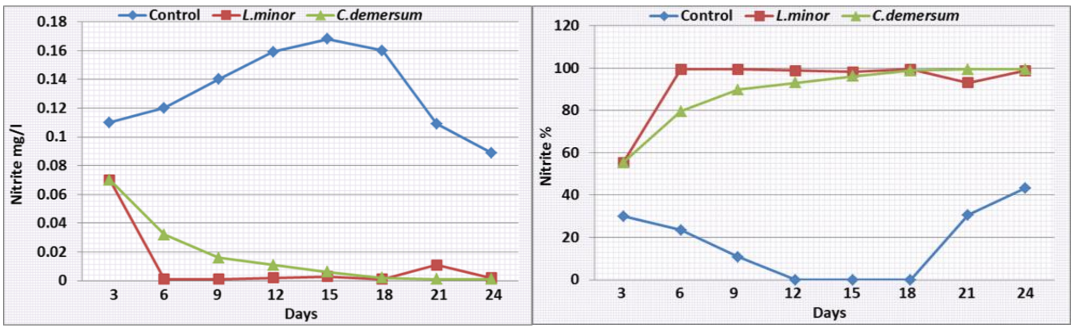

**Figs. 3.** A-B. Concentrations and rate (%) of bio-removal of EC (µs $cm^{-1}$) during remediation.




**Figs. 4 A-B.** Concentrations and rate (%) of bio-removal of TDS (mg $L^{-1}$) during remediation.



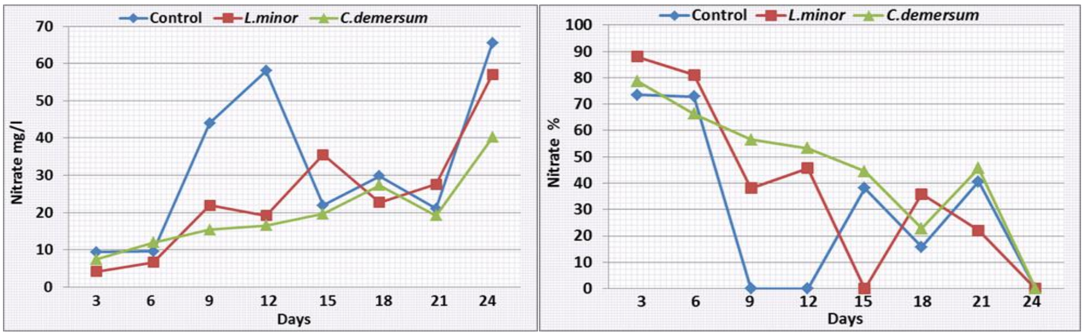

**Figs. 5 A-B.** Concentrations and rate (%) of bio-removal of ammonia (mg $L^{-1}$) during remediation.




**Figs. 6 A-B.** Concentrations and rate (%) of bio-removal of nitrite (mg $L^{-1}$) during remediation.

Caspian Journal of Environmental Sciences, Vol. 19 No. 5 pp. 1017-1026
DOI: 10.22124/CJES.2021.5352
Received: May 18, 2021 Accepted: July 28, 2021

Publisher: University of Guilan, Iran




**Figs. 7 A-B.** Concentrations and rate (%) of bio removal of nitrate (mg $L^{-1}$) during remediation.

**Physiological and biochemical indicators of aquatic plants**

Table 2 depicts the values of some biochemical indicators recorded for the aquatic plants used in the present study. Chlorophyll is one of the pigments responsible for photosynthesis and is found in plastids (Lefsrud & Kopsell 2005). This pigment needs many necessary elements such as calcium and magnesium in addition to iron (Sarvari *et al*., 2008). The total chlorophyll, chlorophyll a and chlorophyll b values in *L. minor* were within the limits of 0.177, 0.129 and 0.048 mg $g^{-1}$ respectively, compared to the plant that represents the control plant whose values were 0.127, 0.86 and 0.04 mg $g^{-1}$ respectively. So, we concluded that the chlorophyll values of *L. minor* increased after wastewater treatment compared to control plant. The same results were observed in *C. demersum* where elevated total chlorophyll content, chlorophyll a and chlorophyll b values were observed compared to control plant. *C. demersum* which represents the control, recorded a values within the limits of 0.22, 0.144 and 0.078 mg $g^{-1}$ respectively, while the values at the end of domestic water treatment were 0.315, 0.223 and 0.092 mg $g^{-1}$ respectively.The elevated chlorophyll content in plant may be due to the stimulated synthesis of chlorophyll in plant after treatment. Zengin & Munzuroglu (2005) indicated that the treated wastewater stimulated the synthesis of chlorophyll in plant. The impact of inorganic compounds on the chlorophyll contents was due to the fact that nitrogen is one of a constituent of chlorophyll molecule and considered as the main component of all amino acids in proteins which are important constituents of chloroplast. According to the statistical results, no significant difference ($p \le 0.05$) was found in the SOD values of *L. minor*. The plant which represents the control, recorded 60.78 U $mL^{-1}$, while SOD values elevated slightly (62.04 U $mL^{-1}$) after completing the treatment. In other words, the statistical results indicated that there was a significant difference ($p \le 0.05$) in the SOD values of *C. demersum*, where the control plant recorded 54.89 U $mL^{-1}$ compared to the plant after treatment (64.57 U $mL^{-1}$). The SOD enzyme values did not exhibit significant changes in *L. minor* which may be due to the fact that exposure of plants to high concentrations of some pollutants leads to synthesis of ROS in tissues and upraised rate of DNA damage, hence reducing the effectiveness of many enzyme such as SOD (Ai-jun *et al.* 2007). Based on the statistical results, the enzyme content was significantly increased in *C. demersum* after treatment, consistenting with that of Hanfeng *et al*. (2010) who explained that the changes in antioxidant enzyme activities of *C. demersum* were significantly influenced by its nutritional level under hypertrophic, eutrophic and mesotrophic conditions. On this basis the antioxidant enzyme activities such as SOD will be upraised, indicating the *C. demersum* exposure to stress under these nutritional conditions. This leads to the active oxygen elimination in the plant, which then contributes to the enhancement of the protective normal structure and function in the plant, as well as resistance to stress. The catalase values that recorded in *L. minor* is slightly lower after treatment where the control plant recorded value of 77.70 U $mL^{-1}$ compared to the plant at the end of treatment, which recorded 71.95 U $mL^{-1}$, as for *C. demersum*. The values were close for both the control plant and the plant after treatment where recorded value in control was 52.18 U $mL^{-1}$ compared to the plant at the end of treatment (53.86 U $mL^{-1}$). The statistical results indicated that no significant difference ($p \le 0.05$) appeared in the enzyme values in the studied plants. The decrease enzyme content in the studied plants may be due to high pH and high ammonium environments. The reactive oxygen increase will probably far exceed the capacity of normal enzyme in anti-oxidase system causing imbalance in internal oxidation metabolism and also a significant decline in some enzymes such as CAT content (Gao *et al.* 2012). Dian *et al.* (2018) reported that the CAT activity in the submerged plant did not exhibit significant changes at high concentrations of some pollutant and indicating that this plant may have reached the limit of its ability to synthesize CAT enzymes.

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The values of MDA in studied plants decreased in *L. minor* after treatment ,as its content in plants that representing the control reached 1.11 nmol $mg^{-1}$, but it decreased to 0.74 nmol $mg^{-1}$ after wastewater treatment. The same applies to *C. demersum*, where the control plant recorded 3.56 nmol $mg^{-1}$, decreasing by 2.79 nmol $mg^{-1}$ at the end of the treatment. The statistical results indicated that there was a significant difference ($p \leq 0.05$) in MDA values in both plants. The content of MDA was decreased in both plants after treatment, as its content in plants that representing the control was greater than treated plant, which can probably be explained by the fact that the increased anti-oxidative response alleviated or prevented lipid peroxidation (Wang *et al.* 2008; Zhang *et al.* 2013). The higher activities of antioxidants may result in lower concentrations of $O_2$– and $H_2O_2$, thereby limited cellular damages which possibly caused by ROS. It may be conversely interpreted as the increased the treatment period time, the MDA content will decrease. As Tlidjen *et al*. (2012) reported that the continuation of treatment for 21 days led to a decreased MDA content. The reason is that necrosis and plant cell death will increase over time, which will stop the production of MDA.

**Table 2.** Physiological and biochemical marker in aquatic plants (mean ± standard deviation).

| Parameters | Plant | Control | Treated | p-value |
|---|---|---|---|---|
| | | Mean ± SD | | |
| Total chlorophyll | *L. minor* | 0.127 ± 0.05 | 0.177 ± 0.04 | 0.038* |
| | *C. demersum* | 0.222 ± 0.04 | 0.315 ± 0.09 | 0.042* |
| SOD | *L. minor* | 60.78 ± 4.20 | 62.04 ± 3.01 | 0.657 |
| | *C. demersum* | 54.89 ± 4.11 | 64.57 ± 5.33 | 0.04* |
| CAT | *L. minor* | 77.70 ± 7.50 | 71.95 ± 6.33 | 0.426 |
| | *C. demersum* | 52.18 ± 4.51 | 53.86 ± 3.22 | 0.636 |
| MDA | *L. minor* | 1.11 ± 0.16 | 0.74 ± 0.03 | 0.017* |
| | *C. demersum* | 3.56 ± 0.03 | 2.79 ± 0.37 | 0.025* |

## CONCLUSION

From the work presented in this study it can be concluded that both submerged *Ceratophyllum demersum* and the floating *Lemna minor* were effective in removing inorganic nitrogen compounds from wastewater except for TDS and EC that recorded high values at the end days of wastewater treatment. In addition, the oxygen values elevated in the treatment ponds with *C. demersum*. Plants did not show an obvious increase in CAT activity and chlorophyll b for each plant and SOD activity for *L. minor* after treatment. However, significant increase was found in total chlorophyll, chlorophyll a for each plants and SOD activity for *C. demersum*. A significant decrease was obvious in MDA content in each plant after completing the phytoremediation period.

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Caspian Journal of Environmental Sciences, Vol. 19 No. 5 pp. 1017-1026 Received: May 18, 2021 Accepted: July 28, 2021
DOI: 10.22124/CJES.2021.5352

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***Bibliographic information of this paper for citing:***

Kareem, NA, Kadhim, NF 2021, Biochemical responses of some aquatic plants as indicators for the treatment of inorganic nitrogen compounds in wastewater (Case study: Domestic water treatment plant in Babil Governorate, Iraq). Caspian Journal of Environmental Sciences, 19: 1017-1026.



Caspian Journal of Environmental Sciences, Vol. 19 No. 5 pp. 1017-1026
DOI: 10.22124/CJES.2021.5352

Received: May 18, 2021 Accepted: July 28, 2021

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