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المعالجة النباتية لمركبات النيتروجين في المياه المعدومة بواسطة نبات الشمبلان *Ceratophyllum demersum* ونبات عدس الماء *Lemna minor*

رسالة مقدمة إلى
مجلس كلية العلوم / جامعة بابل
وهي جزء من متطلبات نيل درجة الماجستير في العلوم /علوم الحياة

من قبل
نورس عبد الكريم حسين علي
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2021هـ **1443 م**

بسم الله الرحمن الرحيم

« وَالْأَرْضَ مَدَدْنَاهَا وَأَلْقَيْنَا فِيهَا رَوَاسِيَ وَأَنبَتْنَا فِيهَا مِن كُلِّ شَيْءٍ مَّوْزُونٍ »

صدق الله العلي العظيم

سورة الحجر – الآية 19

Certification

I certify that this thesis was prepared under my supervision at the Department of Biology, College of Science- University of Babylon as partial fulfillment of the requirement for the degree of Master of Science in Biology – Environment.

Supervisor

Assist Prof. Dr. Nuha Falih Kadhim

College of Science -University of Babylon

Date / /2021

In view of the available recommendation, I present this thesis for evaluation by the Examining Committee.

Assist. Prof. Dr. Adi Jassim Abd AL-Rezzaq

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Date / /2021

الخلاصة :

تضمنت الدراسة الحالية تقييم كفاءة بعض النباتات المائية مثل *Lemna minor* و *Ceratophyllum demersum* في تحسين نوعية مياه الصرف الصحي التي تم اخذها بعد المعالجة النهائية لمحطة معالجة المياه في المعيميرة من خلال المعالجة البيولوجية لمركبات النيتروجين المذابة مثل مركبات النيتروجين غير العضوية والتي تشمل (الأمونيا ،النتريت والنترات) والنيتروجين العضوي المذاب في مياه الصرف الصحي ، تمت زراعة النباتات منفصلة في تلك المياه وفي ظروف بيئية طبيعية مع إجراء بعض الاختبارات الفيزيائية والكيميائية مثل قيم الأس الهيدروجيني ، الأكسجين المذاب ،التوصيلية الكهربائية و المواد الصلبة الذائبة الكلية عن طريق أخذ عينات من الماء كل ثلاثة أيام لمدة 24 يومًا.

أظهرت النتائج الحالية ان نباتي *L. minor* و *C. demersum* رفعت من قيم الاس الهيدروجيني بالاتجاه القاعدي حيث سجلا قيما تراوحت بين (7.8-8.3) و(8.9-9) للنباتين على التوالي. ارتفعت قيم الأوكسجين المذاب في احواض الماء الحاوية على نبات *C. demersum* وسجلت انخفاض في احواض نبات *L. minor* بالمقارنة مع السيطرة، حيث سجل نبات *C. demersum* اعلى قيمة للأوكسجين المذاب بلغت حوالي (6.40) في اليوم (6) من المعالجة بالمقارنة مع السيطرة، بينما سجل نبات *L. minor* اعلى قيمة للأوكسجين المذاب بلغت حوالي (2.4) في الأيام(15و21) من المعالجة بالمقارنة مع السيطرة. ازدادت قيم التوصيلية الكهربائية والمواد الصلبة الذائبة الكلية زيادة تدريجية طيلة فترة المعالجة، حيث سجل نبات *L. minor* اعلى نسبة اختزال بلغت حوالي (8.8% و 1.19%) على التوالي ، بينما سجل نبات *C. demersum* اعلى نسبة اختزال بلغت حوالي (13.5% و6.2%) على التوالي في اليوم الثالث من المعالجة.

أوضحت نتائج الدراسة الحالية كفاءة نباتي *L. minor* و *C. demersum* في تقليل قيم النيتروجين الذائب الكلي حيث سجلا أعلى معدل اختزال بنحو (24.12٪) في اليوم 9 و(29.94٪) في اليوم(18) للنباتين على التوالي مقارنة بمعاملة السيطرة. كما أظهرت النباتات الحالية كفاءة في اختزال مركبات النيتروجين غير العضوي الكلي، حيث سجل كل من *L. minor* و *C. demersum* أعلى معدل اختزال من إجمالي النيتروجين غير العضوي بلغ حوالي (76.25٪ و 73.96٪) للنباتين على التوالي في اليوم الثالث من المعالجة.

سجل كل من *L. minor* و *C. demersum* أعلى نسبة اختزال للأمونيا والتي بلغت حوالي (81.25٪) في اليوم(9) و (86.6٪) في اليوم (18) على التوالي. اما بالنسبة للنتريت، فقد سجل كل من *L. minor* و *C. demersum* أعلى نسبة اختزال بلغت (99.36٪) في الأيام (6 ،9 ،18) للنبات الأول و الايام (21 و 24) للنبات الثاني مقارنة بمجموعة التحكم .

كما سجلت النباتات المذكورة أعلاه أعلى نسبة اختزال بالنترات بلغت حوالي (88.04٪ و 78.8٪) للنباتين على التوالي في اليوم الثالث من المعالجة. كما سجلت أعلى نسبة اختزال للنيتروجين العضوي في اليوم (24) من المعالجة والتي بلغت حوالي (30.14٪ و 26.33٪) لكلا النباتين على التوالي مقارنة بمجموعة التحكم.

أما بالنسبة للحالة الفسيولوجية للنباتات الحالية ، فقد لوحظ أن كلا النباتين سجلا زيادة في محتوى الكلوروفيل الكلي ، كلوروفيل أ ، كلوروفيل ب ، الكربوهيدرات ومحتوى البرولين مقارنة بالمجموعة الضابطة ، بينما كان هناك انخفاض في قيم MDA في كلا النباتين ، بينما انخفض محتوى البروتين في نبات *L. minor*، لكنه ازداد في نبات *C. demersum*. أما بالنسبة لفعالية الإنزيمات المؤكسدة ، فقد حدثت زيادة طفيفة في إنزيم SOD في كلا النباتين وانخفاض في فعالية إنزيم CAT في نبات *L. minor* ، بينما في نبات *C. demersum* كانت فعالية إنزيم CAT قريبة من معاملة السيطرة .

Republic of Iraq
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Phytoremediation of nitrogen compounds from wastewater by *Ceratophyllum demersum* and *Lemna minor*

A thesis
Submitted to the Council of the College of Science, University of Babylon in a partial Fulfillment of the Requirements for the Degree of Master of Science in Biology

By

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2021 A.D. **1443 A.H.**

Dedication

To....

My trust, my hope and my only refuge

To....

Teacher of mankind and the source of knowledge is our Prophet Muhammad

(peace and blessings be upon him and his family)

To....

Spirits that dwelt under the soil of the beloved homeland

To those who gave their lives for our freedom .... The martyrs of Iraq

To....

The haven of safety... my dear father

To....

The sweetheart of my heart ... my mother

To....

Love and they are all love .... my brother and sister

To....

All family and friends

To....

Those who paved the way in front of me to reach the height of science … my teachers….

I Dedicate this humble effort

Nawras

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Nawras

Summary

The current study included evaluating the efficiency of some aquatic plants, such as *Lemna minor* and *Ceratophyllum demersum*, in improving the quality of the wastewater that was taken after the final treatment of the water treatment plant in Al-Muaimarah through the biological treatment of dissolved nitrogen compounds such as inorganic nitrogen compounds which include (ammonia ,nitrite and nitrate) and dissolved organic nitrogen in wastewater .The plants were cultivated separately in that water and in natural environmental conditions with conducting some physical and chemical tests for the water such as pH values, dissolved oxygen, electrical conductivity and total dissolved solids by taking water samples every three days for a period of 24 days.

The present results showed that the both of *L. minor* and *C. demersum* raised the pH values in the basal direction where the plants recorded values ranged between (7.8-8.3 and 8.9-9) respectively. The values of dissolved oxygen increased in the water basins containing *C. demersum* and a decrease was recorded in the basins of the *L. minor* compared to control, where the *C. demersum* plant recorded the highest value of dissolved oxygen which amounted to about (6.40 mg/l) on 6^{th} day of treatment, while the *L. minor* plant recorded the highest value of dissolved oxygen value of about (2.4 mg/l) on the 15^{th} and 21 days of treatment. The electrical conductivity and total dissolved solids values gradually increased throughout the treatment period, where *L. minor* recorded the highest reduction rate of about (8.8% and 1.19%) respectively, while *C. demersum* recorded the highest reduction rate which amounted to about (13.5% and 6.2%) respectively on the third day of treatment.

The results of the current study showed the efficiency of the *L. minor* and *C. demersum* plants in reducing the values of total dissolved nitrogen. Where the *L. minor* and *C. demersum* plants recorded the highest reduction rate of about (24.12%) on day (9^{th}) and (29.94%) on day 18^{th}) for the two plants respectively, in comparison with control. Also current plants showed the efficiency in reducing inorganic nitrogen compounds, where both *L. minor* and *C. demersum* recorded the highest reduction rate of total inorganic nitrogen, which was about (76.25% and 73.96%) for two plants respectively, on the third day of treatment.

Both *L. minor* and *C. demersum* recorded the highest percentage of ammonia reduction, which was about (81.25%) on the(9^{th}) day and (86.6%) on day 18^{th}) respectively. For nitrite, both *L. minor* and *C. demersum* plants recorded the highest reduction rate of about (99.36%) on days (6 ,9 and 18) for the first plant and days (21 and 24) for the second plant compared to the control. The above plants recorded highest percentage of nitrate reduction, which was about (88.04% and 78.8%) for the two plants, respectively, on the third day of treatment, and recorded the highest percentage of organic nitrogen reduction on day (24) of treatment, which amounted to about (30.14%and 26.33%) for both plants respectively compared to the control.

As for the physiological status of the current plants, it is noted that both plants recorded an increase in the content of total chlorophyll, chlorophyll a, chlorophyll b, carbohydrates and proline content compared to the control, while there was a decrease in the MDA values in each plant. While the protein content decreased in *L. minor*, but increased in *C. demersum* plant. As for the effectiveness of oxidative enzymes, a slight increase in SOD enzyme of both plants and a decrease in the effectiveness of CAT enzyme in *L. minor* plant, while in *C. demersum* plant the results of CAT enzyme were close to the control.

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Abbreviations	Key
pH	Hydrogen ion concentration
DO	Dissolved oxygen
EC	Electrical conductivity
TDS	Total dissolved solid
NTK	Nitrogen Total Kjeldhal
TDN	Total dissolved nitrogen
TIN	Total inorganic nitrogen
NH_3	Ammonia
NH_4^+	Ammonium
NO_2^-	Nitrite
NO_3^-	Nitrate
DON	Total dissolved organic nitrogen
BDON	Biodegradable dissolved organic nitrogen
SOD	Superoxide dismutase
CAT	Catalase
MDA	Malondialdehyde
PCR	Polymerase Chain Reaction

Chapter One

Introduction

1-1 Introduction

The contamination of water occurs mainly due to municipal and industrial wastewaters, agricultural fertilizers and other organic and inorganic chemical compounds (Wirnkor *et al*.,2018). The inorganic and organic contamination are not easy to destroyed biologically, but it can turn from a toxic form to low toxic forms (Zhang *et al*., 2017; Jiang *et al*., 2018). Growth of human populations and the increase in their activities led to altered 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 and Cowling, 2002). The nitrogen forms that effect on 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) and particulate forms of nitrogen (Peierls and Paerl, 1997).

Wastewater from agricultural, domestic or municipal sources often contains high quantities of phosphates, ammonium and nitrates even after anaerobic decomposition of organic matter in water treatment plants (Landesman *et al*.,2010). The eutrophication of surface waters may occur by these macronutrients when presence in large quantities in it. Thus, efficient remediation methods for controlling eutrophication and restoring the aquatic ecosystem are being pursued (Xiaoyun and Xingyuan ,2015). The purification system of artificial wetland water that 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, researchers are given more attention for polluted water recycling by using some aquatic plants (Zhang *et al.*, 2014). Where these macronutrients are readily removed by aquatic plant such as

duckweeds growing on wastewaters and polluted natural waters (Landesman *et al.*,2010).

These plant take up ammonia and nitrate through both their roots and the lower surface of their fronds (Cedergreen and Madsen,2002), and may prefer ammonia to nitrate (Fang *et al.*,2007). However, *L. minor* has been reported to take up ammonia readily and grow well at high concentrations of it (Huang *et al.*,2013). Although still higher ammonia concentrations lead to growth rate reduction and photosynthetic pigment loss, their ability to take up and tolerate relatively high levels of ammonia makes duckweeds particularly suited to the remediation of wastewater from domestic and agricultural sources that often contain considerable amounts of this ion (Ziegler *et al.* ,2016). The ability of these plants to accumulate these pollutants despite their toxicity is due to their many resistance properties which enable them to remove their toxicity by various mechanisms (Aravind and prsad, 2004; Kachout, *et al.*, 2009).

Where the aquatic plants have ability to absorbed inorganic nitrogen forms such as nitrate and ammonium directly (Nordin *et al.*,2001). Compared to nitrate, ammonium is more easily absorbed by plants as its assimilation requires less energy. 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 *et al.*, 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, and then causes intracellular damage and produces secondary oxidative stress in plants (Xie *et al.*, 2015).

Typically, plant cells up regulate the activity of antioxidant enzymes, such as peroxidase (POD), superoxide dismutase (SOD)and catalase (CAT), to remove excessive ROS (Fujita *et al*., 2006). Nevertheless, as an assistant antioxidant pathway, phenyl propanoid biosynthesis in plants provides substrates for the synthesis of many secondary metabolites, such as phenolic, flavonoids and lignin. These compounds are critical for defense responses to biotic and abiotic stresses (Wang ,2016)

1-2 The aim of the study

A study of the biochemical responses of some aquatic plants such as the submerged *Ceratophyllum demersum* and the floating *Lemna minor* and the possibility of their use in the reduction of dissolved nitrogen compounds in municipal wastewater.

Chapter Two

Literature Review

2- 2 Literature review

2-2-1 Dissolved nitrogen compounds

Nitrogen compounds are considered extremely important in wastewater management due to the effects that nitrogenous material can have on the environment. Nitrogen is a vital nutrient which is a component of a large number of organic compounds such as amino acids, proteins, coenzymes, nucleic acids and chlorophyll (Barker and Pilbeam, 2015). It is well known that nitrogen-deficiency causes many biochemical and physiological disturbances leading to the reduction in cell division rates and perturbation in process of photosynthesis (Roggatz *et al*.,1999). In addition, in the last years, the use of fertilizers has increased considerably in the objective to increase the mass of crop per land area for ensuring the increased need of world population. Nitrogen also contain various Inorganic nitrogen compounds, which are present in three compound states: ammonia, nitrite and nitrate where Some amounts of ammonia get converted to nitrate depending on the dissolved oxygen concentration in the water (Nuruzzaman *et al*.,2017).

Dissolved organic nitrogen is one of a primary nutrients that play an important role in nitrogen cycling. The wastewater effluent dissolved organic nitrogen chemical composition is complicated because its multiple forms in both refractory and bioavailable forms and biochemically is transformed by bacteria first to ammonia and nitrate and then back to atmospheric nitrogen through ammonification, nitrification, and denitrification. also, major portion of dissolved organic nitrogen consists of polymerized biological compounds (Pehlivanoglu-Mantas and Sedlak, 2006). Effluent dissolved organic nitrogen may consist of amino sugars, urea, proteins, amino acids, nucleic acids, humic acids, fulvic acids and a variety of uncharacterized components.

Ammonia is colorless gas and it is consider as the main constituent of domestic wastewater concentrations, it is very soluble in water and exists in equilibrium between (NH_3) and (NH_4^+) species in water, a number of studies indicated that the total ammonia toxicity is caused by the effect of unionized ammonia (ement and Merlin, 1995). Total ammonia is usually referred to as the summation of ammonia and ammonium concentrations (Emerson *et al*.,1975). The ammonium ions is an important element which is needed in plants for growth. Notably, ammonia enters the environment by means of the agricultural, municipal, industrial and other natural activities (Constable *et al*.,2003), other sources include gas exchange with atmosphere, degradation of organic waste matter, animal waste and nitrogen fixation (Alrumman *et al*., 2016). Ammonium is quickly absorbed by the plants , this is due to the lower energy requirement for the uptake and assimilation of ammonium ions in relation to the nitrate ions (Jampeetong and Brix ,2009).

Ammonium is transformed to nitrate by the bacterial process nitrification and nitrate or nitrite is finally reduced to gaseous end products, nitrous gas and di-nitrogen gas through the bacterial process de-nitrification. Nitrite is typically an intermediate product when ammonium is transformed in to nitrate by microscopic organisms and is therefore seldom elevated in waters for long periods of time. Nitrite is also an intermediary product as nitrate transforms to N gas through de-nitrification, the dissolution of low concentrations of NO_2 in water can result in the formation of nitrate and nitrite, both of which are used by plants during the normal process of nitrate metabolism; as such, NO_2 may act as an airborne fertilizer (Zeevaart ,1976) .Nitrate is an inorganic compound that dissolves easily in water and can be used in agricultural fields as fertilizer and applied as plant nutrients ,it consider as the most stable form of nitrogen in water and almost all sources of aqueous

nitrogen tending to be converted to nitrate .Nitrate can also be transformed to ammonium during low redox conditions (Vymazal, 2001).

2-2-2 Effect of pollution with the nitrogen compounds on ecosystem

There are two major effects of nitrogen compounds on the whole ecosystem the first one include eutrophication, where excessive nutrients such as nitrogen and phosphorus are present in water bodies lead to the growth of algae on water surface which blocking the contact of water from sunlight and air, also decrease the oxygen level in the water body, which affects the aquatic lives (Dodds and Smith, 2016).

The second effect include increase in the acidity of water bodies (Hessen *et al*.,1997). Where aquatic environment may get acidified due to the presence of surfeit ammonia forms of ammonia and ammonium sulphate leads to formation of a considerable amount of acid, as hydrogen ions are released during nitrification. Also, nitrite ions in water lead to the formation of nitric acid along with sulfate ions and consequently acidifying the stream water (Murdoch *et al*.,1992). Acidic stream water is not even suitable for reuse to satisfy human water requirements. Compared with urban areas, agricultural areas are more susceptible to health risks by the presence of nitrate-nitrogen in groundwater (Su *et al*., 2013; He and Wu, 2019).

The ammonium ions are toxic and not permitted to be stored in plants. Thus ,it is either oxidized to nitrates ions, converted to amides or assimilated to produce amino acids (Mitra ,2015).When ammonium ions take up by plants, they release in the medium solutions one hydrogen ion .Over time, the uptake of ammoniacal nitrogen by plants may decreasing the growing medium pH value by increase the hydrogen ions concentration, also, an increase of ammonium uptake in plants and

storage may result in cell damage and further leads to cell death (Mattson and Leatherwood, 2009). The nitrogen uptake by the root system depends on the nitrogen demand of the entire plant and is dependent on the nitrogen requirement for growth.

However, high concentrations of nitrite can lead to excessive accumulations of it (Okano and Totsuka,1986). Also, cell acidification which lead to negative responses such as the generation of reactive oxygen species (ROS)and inhibition of both N assimilation and plant growth, further causing acute damage to leaves, whole-plant chlorosis or even death (Schmutz *et al*.,1995). However, exposure of NO_2 to different plant species elicits different physiological responses. Therefore, the effects of NO_2 exposure on plants remain highly controversial, and a unified conclusion has not been reached, in addition, information concerning different plants that are highly tolerant to NO_2 and their natural recovery is scarce (Sheng and Zhu, 2019). Also, high nitrate concentrations in surface and ground water can stimulating the growth of algae and rooted aquatic plants and then accelerate the eutrophication, as a consequence, the aquatic ecosystem can be changed by reducing light penetration in to the water and then leading to decreases plants living in the deeper water (Niculescu *et al* ,2017).

In addition, once the concentrations of nitrate and phosphorus increase, the dissolved oxygen in water tends to decrease which affecting negatively on aquatic organisms life that need to oxygen, excessive levels of nitrate ions in drinking water may cause many health problems, especially for infants and pregnant women (Abe *et al*., 2002).When nitrate is converted to nitrite in the body system, it reduces the oxygen - carrying capacity of the blood , resulting in a condition called "methaemo-globinaemia", also known as "blue baby syndrome". The reaction between nitrite and secondary or tertiary amine from acidic

media can lead to the formation of nitroso compounds (NO_C), which are known carcinogenic, teratogenic and mutagenic (Shams,2010). Due to all the mentioned reasons, the removal of nitrate and nitrite from water is of significant importance in environment and the health. Many traditional methods have been applied to the removal of nitrogen compounds from wastewater, including physical processes (Elmidaoui *et al*, 2001), chemical processes (Cengeloglu *et al*., 2006) or biological de-nitrification processes (Abe *et al*., 2002)

2- 2-3 Phytoremediation of nitrogen compounds

2- 2-3-1 Phytoremediation

The remediation of wastewater is carried out by various physical and chemical techniques including filtration, reverse osmosis, coagulation, adsorption, flocculation, electrocoagulation and oxidation (Kabra *et al* ,2013). Most of these techniques are ineffective in the certain pollutants removal, expensive, produce secondary pollutant and cause high sludge production (Tabinda *et al* ,2019; Chandanshive *et al*.,2016). Recently, effective biological approaches have been adopted for wastewater effluent treatment along with methods to overcome the demerits of usual techniques. Phytoremediation is engineered use of selective green plants to remediate organic and inorganic pollutants from contaminated sites including waste water and contaminated soil (wu *et al*.,2015).

The word Phytoremediation comprised from the prefix Greek word phyto (meaning plant) and the suffix Latin word remedium meaning clean up (Cunningham *et al*., 1996). Phytoremediation is an ecologically benign, reliable, emerging, inexpensive and green approach that applies plants for the treatment of diverse wastewater effluents as well as

contaminated soil (Ekambaram *et al*.,2018). Where plants are involved naturally in some specific enzymatic pathways to degrade various complex compounds to non-toxic compounds which reduce the pollution effect of contaminated areas (Govindwar *et al*., 2010). Phytoremediation can be used along with conventional treatment technology (as alternatives) or in place of conventional treatment (as complementary technology) (Etim, 2012).

2- 2-3-2 Wetland plants classification

There are three groups of wetland plants (Phyto-remedial plants) that are used commonly for process in waste water treatment in proper design constructed wetland (Wu *et al*., 2015).

2- 2-3-2-1 Free floating plants:

The free floating plants including water duckweed and *Azzola* which are freely grown on the water surface in constructed wetland (Vymazal, 2011). These plants used for contaminants removing from waste water through different mechanism including adsorption and absorption by using their root system.

2- 2-3-2-2 Submerged plants :

It is include many plants that are grown with in water in constructed wetlands such as *Egeria densa* (dense water weed), *Elodea nuttallii* (Water weed) (Vymazal, 1995). Their photosynthetic tissues are submerged entirely with in water and then the turbidity must be low in waste water because high turbidity can blocks the light transmission to plants (USEPA, 1999). These plants increase the dissolved oxygen and depletes the dissolved organic carbon and this is lead to increment of pH value, ammonia volatilization and chemical precipitation, high oxygen

concentration also creates favorable condition for mineralization, the nutrients removed by these plants are retain with in root tissue of plants and by attached Micro flora (Bekele, 2018).

2- 2-3-2-3 Emergent plants :

It is include plants that grow large portion of their shoots, leaves or flowering structure out of the water such as *Phragmites, Typha latifolia* and Nut grass which are most widely used in subsurface and surface flow constructed wetlands, (Vymazal, 2011). The pollutant removal mechanisms are carried out through adsorption, absorption, filtration, rhizo-degradation, plant up taking.

2- 2-3-3 Constructed wetlands system types with its main characteristics

Many types of constructed wetlands differ in their processes which are responsible for pollution removal and the main design characteristics.

2- 2-3-3-1 Free Water Surface Constructed Wetlands (FWS CW)

Free Water Surface Constructed Wetlands with emergent macrophytes is a shallow basin or sequence of basins that containing 20–30 cm of rooting soil with a water depth of 20–40 cm, besides planted macrophytes, naturally species may be present, plants in this system are usually not harvested and provides organic carbon necessary for de-nitrification which may proceed in anaerobic pockets within the litter layer (Kadlec, 1994).

This system are efficient in organics removal through microbial degradation and colloidal particles settling, suspended solids are effectively removed via settling and filtration through the dense

vegetation, nitrogen is removed through nitrification (in water column) and subsequent de-nitrification (in the litter layer) and volatilization of ammonia under higher pH values caused by algal photosynthesis, the retention of phosphorus is usually low because of limited contact between water and soil particles which adsorb and/or or phosphorous deposits (Vymazal, 2010).

Constructed wetlands with FWS are frequently used in North America and Australia (Kadlec and Wallace,2008). In Europe, this technology has recently gained more attention, especially in Sweden and Denmark where these systems are used to eliminate nitrogen from diffuse pollution (Vymazal *et al*., 2006).

2- 2-3-3-2 Constructed wetlands with horizontal subsurface flow

Constructed wetlands with horizontal subsurface flow contain rock beds or gravel sealed by an impermeable layer and planted with wetland vegetation, the wastewater is fed at the inlet and flows through the porous medium under the surface of the bed in a more or less horizontal path until it reaches the outlet zone, where it is collected and discharged, in the filtration beds, pollution is removed by microbial degradation and chemical and physical processes in a network of aerobic and anaerobic zones with aerobic zones being restricted to the areas adjacent to roots where oxygen leaks to the substrate (Vymazal,2001).

Despite problems with this system, soil-based systems exhibited high treatment effect for organics and suspended solids (Brix and Schierup, 1989). In the late 1980s, soil material was replaced by coarse material and at present, washed gravel or rock with grain size of about 10–20 mm are commonly used (Vymazal and Kröpfelová, 2008a). The most important roles of plants in in this system are substrate provision

such as roots and rhizomes for the growth of attached bacteria, radial oxygen loss (oxygen diffusion from roots to the rhizosphere) and nutrient uptake (Brix,1994).Organic compounds are degraded effectively by microbial degradation under anaerobic conditions as the concentration of dissolved oxygen in the filtration beds is very limited (Vymazal and Kröpfelová, 2008b). Suspended solids are retained by sedimentation and filtration and the removal efficiency is usually very high (Vymazal and Kröpfelová, 2008a). The major removal mechanism for nitrogen in HF CWs is de-nitrification, removal of ammonia is limited due to lack of oxygen in the filtration bed as a consequence of permanent waterlogged conditions, phosphorus is removed primarily by ligand exchange reactions, where phosphate displaces water or hydroxyls from the surface of iron and aluminum hydrous oxides. Unless special materials are used, removal of P is usually low in this system (Vymazal,2007).

2- 2-3-3-3 Constructed Wetlands with Vertical Subsurface Flow (VF CWs)

In this system, the water is fed in large batches and percolates down through the sand medium, The new batch is fed only after all the water percolates and the bed is free of water and this enables oxygen diffusion from the air into the bed, thus, constructed wetlands with vertical subsurface flow are more aerobic than HF CWs and provide suitable conditions for nitrification and this system do not provide any de-nitrification, also very effective in organics and suspended solids removing, but removal of phosphorus is low unless media with high sorption capacity are used (Vymazal and Kröpfelová, 2008a).

In up flow vertical CWs, the wastewater is fed on the bottom of the wetland. The water percolates upward and then it is collected either near the surface or on the surface of the wetland bed (Salati ,1987). Recently,

the “fill and drain” or “tidal” CWs have been developed. In tidal flow systems the wastewater percolates upwards until the surface is flooded and the feeding is stopped and wastewater is held in the bed and at a set time later, the wastewater is drained downwards. After the water has drained from the filtration bed, the treatment cycle is complete and air can diffuse into the voids in the filtration material (Cooper, 2005).

2- 2-3-3-4 Hybrid constructed wetlands

Constructed wetlands could be combined in order to achieve a higher treatment effect by using advantages of individual systems. Most hybrid constructed wetlands combine VF and HF stages (Vymazal,2005). At present, hybrid constructed wetlands are in operation in many countries around the world and they are used especially when removal of ammonia-N and total-N is required (Vymazal and Kröpfelová, 2008a). Besides sewage, hybrid constructed wetlands have been used to treat a variety of other wastewaters for example landfill leachate, compost leaching, slaughterhouse and shrimp and fish aquaculture (Bulc, 2006; Kinsley *et al*.,2006; Reeb and Werckmann,2005).

2- 2-3-4 Phytoremediation mechanisms for nitrogen compounds

2- 2-3-4-1Biodegradation of nitrogen compounds: classic routes

The nitrogen removal mechanisms in constructed wetlands are known to involve various mechanisms such as:

2- 2-3-4-1-1 Ammonification

Is the process in which organic nitrogen is biologically converted into ammonia this process can be caused by heterotrophic bacteria and

fungi. Nitrogen are readily degraded in both aerobic and anaerobic zones of reed beds releasing inorganic ammoniacal-nitrogen, which is mainly removed by nitrification/de-nitrification processes in constructed wetlands, ammonification proceeds more rapidly than nitrification where ammonification rates are fastest in the oxygenated zone and decrease as the mineralization circuit changes from aerobic to facultative anaerobic and obligate anaerobes, the ammonification rates are influenced by available nutrients, temperature, C/N ratio and pH (Reddy and Patrick ,1984). NH_4^-N in SSF systems can be reduced by other processes, which include volatilization, adsorption and plant uptake (Vymazal ,2007). It is generally believed that the contribution of these processes to the NH4–N removal is very limited compared with nitrification/de-nitrification.

2- 2-3-4-1-2 Nitrification

The convert of a significant part of the organic nitrogen to ammonia are believed occurred due to decomposition processes in the wetlands, biological nitrification, which is performed by nitrifiers such as *Nitropira*, *Nitrosomonas*, *Nitrobacter* and *Nitrosococcus* followed by de-nitrification is believed to be the major pathway for ammonia removal in both subsurface flow systems (SSF) and surface flow systems SF constructed wetlands (Gersberg *et al*.,1985).

In traditional nitrogen treatments, the biological nitrogen removal requires a two-step process: nitrification followed by de-nitrification. Nitrification implies a chemo-lithoautotrophic oxidation of ammonia to nitrate under aerobic conditions and is performed in two oxidative stages ,the first one include convert ammonia to nitrite (ammonia oxidation) and at second stage nitrite convert to nitrate (nitrite oxidation)which is performed by different bacterial genera such as *Nitrosomonas* for the ammonia oxidation process and *Nitrobacter* for the nitrite oxidation

process, these genera use molecular oxygen as an electron acceptor and ammonia or nitrite as an energy source , while carbon dioxide is used as a carbon source (Metcalf and Eddy, 1991).

The nitrification process is very oxygen demanding, and the reduction of alkalinity by the acid made in the nitrification process can cause a deep pH reduction. The pH value is very important in the nitrification reaction since nitrification rates swiftly decline where the pH drops to lower than (7.0), thus, the appropriate chemicals such as lime should be replenished when the alkalinity in the process is reduced by the acid produced in the nitrification reaction (Ahn,2006).The rate of nitrification is also influenced by temperature, inorganic carbon source, moisture, microbial population and concentrations of ammonium–N ,the ammonia uptake rate (AUR) varies with reactor configuration, substrate type, and influent ammonium concentration (Lee *et al*.,2009).

2- 2-3-4-1-3 Denitrification

In low-oxygen environments, the biological de-nitrification mechanism makes use of nitrate as the terminal electron acceptor where denitrifying bacteria decrease inorganic nitrogen such as nitrate and nitrite into innocuous fundamental nitrogen gas (Prosnansky *et al* ,2002; Szekeres *et al*., 20092). Denitrifying bacteria can be classified into two major species, heterotrophs and autotrophs, heterotrophs are microbes such as *Psuedomonas*, *Micrococcus*, *Achromobactor* and *Bacillus* that need organic substrates to obtain their carbon source for growth and evolution, and get energy from organic matter, in contrast, autotrophs utilize inorganic substances as an energy source and CO_2 as a carbon source (Rijn *et al*., 2006). So far, the heterotrophic de-nitrification process has been mainly engaged in conventional wastewater treatment plants, while autotrophic de-nitrification has only recently been studied.

The proportion of total nitrogen removal by this process is typically 60–95%, in comparison to 1–34% assimilated by plants and algae. Heterotrophic microorganisms utilize an oxidized form of nitrogen, NO_2, NO_3, as terminal electron acceptor and organic carbon as electron donor under anoxic conditions (Ahn,2006). Consequently, the de-nitrification provides energy to denitrifiers and it is also affected by the organic matter of the electron donor. De-nitrification can only take place in the anoxic zones of the systems as the presence of dissolved oxygen suppresses the enzyme system required for this process (Ahn,2006).

In constructed wetlands, it is believed that microsites with steep oxygen gradients can be established which allow nitrification and de-nitrification to occur in sequence in very close proximity to each other and Sufficient organic carbon is needed as an electron donor for nitrate reduction, which provides an energy source for de-nitrification microorganisms (Russell *et al*., 1994). Where, in wetlands, the organic carbon is supplied mainly by the vegetation, this organic matter is used directly as a carbon and energy source for heterotrophic bacteria and the effect of vegetation on de-nitrification is dependent on the quality and the amount of the plant litter (Hume *et al.,* 2002). The decomposition rate, or the availability of the organic carbon as an energy source is linked to the initial composition of nitrogen and lignin in the plant (Hume *et al*., 2002). Submersed and free-floating plants generally have a lower initial lignin to N ratio than emergent plant species (Godshalk and Wetzel 1978; Hume *et al*., 2002). Consequently, the organic matter of a submersed plant is usually more available for decomposition, and hence for denitrifying bacteria, than the organic matter of an emergent plant. However, the primary production is higher for the emergent plants and hence, the organic matter lasts longer when emergent plants are decomposed than for the submersed macrophytes (Westlake *et al*., 1998).

Also, this carbon source can be available in reed beds from organic pollutants of wastewater or cell materials of microorganisms, this organic material can indirectly effect on the nitrifying and denitrifying bacteria, where the supply of litter on top of the sediment may limit the oxygen diffusion to lower sediment layers and then causes anaerobic sediments that is favorable for the de-nitrification process. Also, the supply of organic carbon raises the total heterotrophic activity which consumes oxygen and thus indirectly favors de-nitrification by lowering oxygen concentrations in the sediment (Nielsen *et al*.,1990).

The rate of de-nitrification is influenced by many factors such as microbial flora, nitrate concentration, type and quality of organic carbon source, different plant species residues, hydro-periods, the absence of O_2, soil moisture, redox potential, presence of denitrifiers, temperature, pH value, water level, soil type and the presence of overlying water (Sirivedhin and Gray,2006; Bastviken *et al*., 2005).

2- 2-3-4-2 Biodegradation of nitrogen compounds : Anammox Routes

The Anammox (anaerobic oxidation of ammonium) process provides alternate process for improving the removal of total nitrogen (Strous *et al*.,1999). The role of Anammox bacteria in de-nitrification is now proven to be partly responsible for the transformation of ammonia in to nitrogen gas within the nitrogen cycle, where in this process, the ammonium is autotrophically oxidized to nitrogen gas while nitrite is employed as an electron acceptor under anaerobic conditions, thus, there is no demand for aeration and addition of an external carbon source resulting in a cost saving and preventing insufficient conversion of organic substances (Ward,2003) .

The Anammox bacteria such *as Planctomycetes spp*., *Candidatus brocadia anammoxidans*, *Thiomicrospira denitrificans*, *Thiobacillus senitrificans*, *Thiosphaera ponotropha* and *Paracoccus denitrificans* are autotrophic, in contrast to classic denitrifiers which are mostly heterotrophic and thus need organic carbon for their carbon and energy supply, therefore, the stimulating of Anammox bacteria in a wastewater treatment system reduces the need for an organic carbon source, which is required in the conventional denitrification process (Sliekers , 2002). However, a strict controlled environment and reactor arrangement are needed as Anammox bacteria have a slow growth rate (Jetten *et al*., 2001). In reality, the Anammox process has been reported to produce higher removal efficiency of total nitrogen (Sun and Austin, 2007; Dong and Sun, 2007). This is save up to 90% of operation cost, due to a reduction of the input of organic matter (Chamchoi and Nitisoravut ,2007).

2- 2-3-4-3 Plant uptake

The uptake of nitrate and ammonia by macrophytes converts inorganic nitrogen forms in to organic compounds as building blocks for cells and tissues (Vymazal ,1995). Various plant species differ in their favored forms of nitrogen absorbed depending on the forms available in the wetland. The NH_4 preference is common in macrophytes living in environments with limited nitrification where NH4 is abundant (Garnett *et al*.,2001). The uptake and storage rate of nutrients by plants depend on the nutrient concentration of their tissues.

Thus, desirable features of a plant used for nutrient assimilation and storage include high tissue nutrient content, fast growth and the ability to obtain a high-standing crop, conversely, plants that have great biomass accumulation during autumn and winter may release much of

their accumulated nitrogen back into the water during the winter season (Vymazal,2007).

2- 2-3-4-4 Physicochemical processes

In newly built wetlands, the contribution of physicochemical processes to overall nitrogen removal is generally high, but decreases with time. Although many physicochemical processes can take place in constructed wetlands, the major mechanisms for nitrogen removal are ammonia adsorption and sedimentation.

2- 2-3-4-5 Ammonia adsorption

Adsorbed ammonia in constructed wetlands is bound loosely to the substrates and can be released easily when water chemistry conditions change, when the ammonia concentration in the water column is reduced as a result of nitrification, some ammonia will be adsorbed to regain equilibrium with the new concentration but If the ammonia concentration in the water column is increased, the adsorbed ammonia will also increase (Vymazal,2007). If the wetland substrates are exposed to oxygen, adsorbed ammonium may be oxidized to nitrate by periodic draining (OSun *et al*., 2005 ; Sun *et al*., 2006).The ammonium ion is generally adsorbed as an exchangeable ion on clays, and adsorbed by humic substances and the rate and extent of these reactions are reported to be influenced by several factors such as alternating submergence and drying patterns ,the type and amount of clay, submergence period characteristics of soil organic matter, and the presence of vegetation (Lee *et al*.,2009).

2- 2-3-4-6 Sedimentation

Most particulate organic nitrogen in constructed wetlands is removed by sedimentation (Taylor *et al*.,2005). Particulates may settle on

the wetland floor or may adhere to plant stems where the decomposed materials such as TN, TP and organics of low molecular weight are used by microorganisms and plants (Kadlec and Knight,1996). In wetlands, the nitrogen removal can be employed by combined physical and chemical processes and enhanced sedimentation method using magnesium-ammonium-phosphate (MAP), as added precipitation reagent has been developed for the removal of nitrogen and phosphorus in wastewater treatment and has the potential to be applied in constructed wetlands (Lee *et al*.,2009).

2-2-4 Growth and physiological responses of aquatic plants to inorganic nitrogen compounds

Nitrate and ammonium are the two inorganic nitrogen (N) forms that can be directly absorbed by plants (Nordin *et al*., 2001), but ammonium is absorbed more easily as its assimilation requires less energy. in fact, only few plants are known to be ammonium specialists, most of high plants are usually sensitive to ammonium (Kronzucker *et al* .,1997; Britto and Kronzucker,2002). Non-specialists could display toxicity symptoms such as growth suppression, leaf chlorosis, yield depressions, and even mortality in high ammonium conditions, particularly when ammonium is supplied as the sole N source (Britto and Kronzucker,2002).

Inorganic nitrogen compounds such as Ammonia-N is an important nutrient source to aquatic plants at low concentrations, but it can be toxic at higher levels (Cao *et al*,2007). Damaging concentrations of ammonia-N can inhibit photosynthesis, trigger oxidative stress, and cause water loss in plants (Cao *et al*.,2004; Neuberg *et al*.,2010). Several studies reported the inhibitory action of ammonium-N additions on plants (Gerendas *et al*.,1997; Britto and Kronzucker 2002). Which indicated that

unionized ammonia-N is the main contributor of stress and the concentration of it in the water column is not only related to the concentration of total ammonium-N but also to pH and temperature and reports regarding the stress effects of unionized ammonia on submersed macrophytes are available at the local and international scales (Caicedo and Van ,2000; Chong *et al*.,2003).

Reactive oxygen species are signals to activate the antioxidation system of a plant, where in normal conditions, the production and elimination of reactive oxygen in an organism is in a dynamic equilibrium and concentrations are maintained at low levels. under abnormal situations, the production and elimination processes of the free radicals become unbalanced and the accumulating reactive oxygen effect on biological macromolecules increase which may causing damage at the cellular, molecular and even organ levels, thereby accelerating aging of an organism (Zhao, 2001; Pflugmacher, 2004). Excess levels of inorganic nitrogen such as ammonium-N can lead to disruption of the dynamic equilibrium of the antioxidant mechanism, making reactive oxygen accumulate inside the plant cells (Wang *et al*.,2008)

The antioxidation system of plants has two components, enzymatic and nonenzymatic, both of which play important roles in eliminating internal reactive oxygen and protecting cells from peroxidation injuries, they eliminate internal H_2O_2 resulting from environmental stress or the aging process and protect membrane structure, allowing plants to resist environmental stress to a certain extent (Mishra *et al*., 2006). The level of enzyme activity and the antioxidant content inside plant cells reflect the ability of the plant to eliminate harmful reactive oxygen and resist peroxidation damage. Superoxide dismutase (SOD) catalyzes the conversion of the superoxide free radical (O_2) to H_2O_2 and O_2. However, the ability of SOD to eliminate superoxide anion is always limited. If the

production of superoxide anion exceeds the elimination capacity of SOD, the superoxide anion then causes harm to the organism. CAT can decompose toxic reactive oxygen species (H_2O_2) that produced mainly by the activity of SOD—directly into H_2O and O_2 in plant cells and play important roles in the main defense mechanism (Singh and Prasad ,2017).

The membrane system in plant cells is usually the first and main target of reactive oxygen attacks which can damage the plant's plasma membrane, disrupt electrolyte balance and causing other physiological and biochemical abnormalities. Where when stress is prolonged, the continued oxidation of the plant membrane leads to accumulation of lipid peroxidation end-products, such as malondialdehyde (MDA), and additional cell damage (Metwally *et al*., 2005).

Proteins are major workhorses of cells in living organisms and consider as the functional versatile macromolecules that constitute them . They proteins function include cellular signaling, catalysis, regulation, structural support and protection and membrane fusion (Amm *et al*., 2014). The function of a protein is determined by its structure, which is acquired following ribosomal synthesis of its amino acid chain. In addition, the conformation of a protein depends on the chemical and physical conditions of the protein environment as affected by extreme temperatures, reactive molecules and other stresses that not only disrupt the folding process of a newly synthesized protein, but also induce the mis-folding of already existing proteins (Zhou *et al*., 2016). Protein level decline may be related to Nitrate reductase activity decrease because it is believed that this enzyme to be rate limiting in the overall assimilation of nitrate (Beevers and Hageman, 1969) consequently affecting on total protein and plants growth. It is also likely that pollutants induced lipid peroxidation in plants and fragmentation of proteins due to toxic effects

of reactive oxygen species led to reduced protein content (Davies ,1987). It is because the functionality of proteins can be affected by reactive oxygen species either by oxidation of amino acid side-chains or by secondary reactions with aldehydic products of lipid peroxidation (Reinhackal *et al*.,1998). The plants can synthesis certain sets of novel proteins When subjected to pollution which plays an important role in the detoxification (Suzuki *et al*., 2002). Sugars are important in plant metabolism not only because it consider as the first complex organic compounds formed in it as a result of photosynthesis, but it also provide a major source of respiratory energy. Sugars play various ecological roles in plant protection against infection and wounds and as well as in the detoxification of foreign substances (Sativir *et al*., 2000). Depletion of disaccharides may lessen the protective effects of sugars on structural integrity of membrane in the plant cell (Crowe *et al*., 1984).

2-2-5 Use of *L. minor* and *C. demersum* plants in phytoremediation

Aquatic plants play a major role in the aquatic environment by providing them with oxygen, providing protection and food for aquatic organisms, as well as controlling the organic content and biological balance of sedimentation processes in this environment (Chevre *et al*., 2003). Aquatic organisms are greatly affected by the physical and chemical properties of the aquatic environment in which they live, as temperature ,pH, EC, Alkalinity, nutrients , etc. plays a direct role in the life cycle of plants, and therefore the presence or absence of these organisms has a strong influence within the aquatic environment (Mellina *et al*., 2002) .Various species of aquatic plants have been verified and recognized for their efficiency to accumulate inorganic and organic contaminants from waters through hydroponic or field applications

(Prasad, 2006). Numerous aquatic plant species belongs to the several families Ranunculaceae, Lemnaceae, Cyperaceae, Haloragaceae, Hydrocharitaceae, Potamogetonaceae, Typhaceae, Najadaceae, Pontederiaceae, Juncaceae and Zosterophyllaceae are the main representatives for the phytoremediation of aquatic environment (Prasad, 2006). In this technique first step is the identification and screening of plants which showed great efficacy to accumulate dissolved nutrients, metals and other contaminants (Lu, 2009). For phytoremediation technique, the selection of plants should be fast growing, easily handled and harvested (Stefani *et al*., 2011). Other biological processes of plants like growth and development and photosynthesis are vital factors for the sustainability of an aquatic system. The success of a phytoremediation system also depends on factors related with severity of the pollution (Jamuna and Noorjahan, 2009). Furthermore, various phytotechnologies such as phytodegradation, phytostabilization, rhizofiltration, rhizodegradation, and phytovolatilization have been utilized for contaminated ecosystems (Baker *et al*., 1994; Brooks, 1994; Cunningham & Ow, 1996). The reduction of contaminants present in soil occurs by the exudates of the roots of plant. These roots stabilize, demobilize and bind to the contaminants, are termed as phytostabilization process. Further, the roots of certain plant species accumulate, adsorb and precipitate contaminants in the soil and water through immobilization process. This is a significant method to eliminate organic and inorganic contaminants existing in soil, sediments and sludge media (Baker *et al*., 1994; Brooks, 1994).

Aquatic plants of all types whether emergent's, free floating or submerged or all are known for removing heavy metals by chelate these pollutants in forms that are inactive or complex in their tissues and can be stored in vacuoles away from the sensitive cytoplasm, where most

metabolic processes occur, also organic matter may be degraded in the root zone or taken up, followed by volatilization sequestration or decomposition (Wani, *et al*., 2017). Among these species Duckweed is a small floating macrophyte belonging to family Lemnaceae of monocotyledonous plants. It has 37 species belonging to 4 genera: i(*Lemna*), ii (*Spirodela*), iii(*Wolffia*) and iv(*Wolffiella*) (Cheng and Stomp ,2009). It is a simple plant having no stem or leaves. Major part of the plant comprises a thallus called "frond" which is generally composed of chlorenchymatous cells having air pockets called aerenchyma due to which duckweed floats on water. Duckweed may have no root or one or more simple roots. Roots are photosynthetically active having chloroplast in it. Roots of the duckweed plant help in nutrient uptake from water and stabilizes the plant (. Dalu and Ndamba ,2003). *Lemna minor*, belonging to the genus Lemna is the most widely spread species of duckweed which is extensively studied in wastewater treatment mainly due to its fast growth and high nutrient removal efficiency (Ozengin and Elmaci ,2007). Where the small size, simplicity of composition and rapid growth in its make the plant suitable for toxicity tests (Oecd, 2002), as well as its use in removing heavy metals and inorganic and organic pollutants (Davis *et al*., 2002).

L. minor Scientific classification according to (Reveal,2012)

Kingdom: Plantae
Clade: Tracheophytes
Clade: Angiosperms
Class: Monocots
Order: Alismatales
Family: Lemnaceae
Genus: Lemna
Species: *L. minor*

C. demersum is also one of the aquatic plant that has effective role in pollutant removal which characteristic dark green, ranging in length between (20 to 100 cm) with complex leaves once or twice a length of 1 to 2.5 cm thick leaves stack up at the end of branches to earn a scene more like the tip of guilt, which is a perennial plant with a modern and side branches This plant configuration frequently in most of the southern region of Iraq, in particular the marshes, although there are many methods used in the removal of pollutants from water, some people resort to the use of aquatic plants to remove pollutants such as heavy elements in water and sediment both (Cardwell *et al*., 2002)

Ceratophyllum demersum L Scientific classification according to (Aulback – Smith *et al*., 1996)

Kingdom: Plantae

Division: Tracheophyta

Class: Magnoliopsida

Order: Ceratophyllales

Family: Ceratophyllaceae

Genus: Ceratophyllum

Species: *C. demersum*

Picture (2-1) A- *L. minor* plant B- *C. demersum* plant

Several studies have been conducted to evaluate the role of aquatic plants in reducing the concentrations of nitrogenous compounds, such as the study was conducted in the Netherlands in 1998 to evaluate the removal of nutrients from wastewater using the *L. gibba* plant, where the plant was cultured in deep-water dishes (3.3) cm and at a constant temperature (2+26) C˚ the results showed the efficiency of total nitrogen removal (%76-82%) (Koerner *et al*.,1998). Duckweed has effective pollutants removal capacity for both municipal and industrial wastewater, since N is an essential plant nutrient, it can be removed through plant uptake of ammonium or nitrate, and stored in organic form in wetland vegetation (Debusk, 1999). Pandey (2001) reported that nitrogen removal was in the range of 50 - 75% in the discharged duckweed treatment system.

Abdel-aziz (2004) correlated the removal of ammonia with temperatures, and indicated that the percentage of ammonia removal was (73%) in summer, but decreased in winter to (44%), the removal of nitrogen by duckweed occur by newly grown tissue, not by increasing the tissue N content (Korner and Vermaat, 1998).Also, depending on the temperature, a study of (El-Shafai *et al*., 2004) showed that the ammonium removal efficiency in summer was 98% and decreased to 44% in winter, and the highest percentage of nitrogen removal was 80% through plant uptake and 5% by sedimentation and 15% of the removal mechanism is unknown (El-Shafai *et al*., 2004).

Several studies were conducted in Thailand The first study was conducted in 2004 and lasted for three months to compare the efficiency of *L. minor* plant in removing nitrogen from untreated and secondary treated sewage water, using seven ponds for each system where the pond size was (170)l, diameter (0.56)cm and depth Pond (0.70) cm at a temperature ranging between (16-32) °C and a hydraulic retention time

of three days for each pond (twenty-one days for each system), harvesting was carried out every four days with density left (700) g\m^2 after each harvest to prevent algae growth, it was found that the efficiency of removal nitrogen without treatment 68% and with treatment 64% (Bejarno, 2004). A study continued in Australia for a month in the open air in 2005, which proved that *L. minor* are effective in removing nutrients, and the efficiency of removing NO_3^-, NO_2^-, and NH_3 (98%,89% and 89%) respectively, and showing the possibility of using sewage water treated by using the plant in activities Agriculture and fish farming, while the nutrients are utilized to produce biomass from the plant and used as livestock feed and fuel production (Willett,2005).

The study of Abou el-kheir *et al*., (2007) which indicated that ammonia and nitrate showed a gradual removal by prolonged treatment periodse with *Lemna gibba* plant and recorded reduction rate of 80% and 100% for each of the ammonia and nitrate respectively.Several studies were conducted in Turkey, including the study in 2007 on the possibility of removing nutrients from wastewater using *L. minor* within two identical basins with dimensions (0.4W*0.2L*0.08D)m the effective size of the tank is (6.4)l and a temperature of (0.5±21) °C or using a light OSRAM day 18w. It was observed that at the pH of the inlet water (0.2 ±7.2 (and the outlet (0.2+8), the removal efficiency of total nitrogen was 83-87% (Ozengin and Elmaci, 2007).

The other study in 2008 lasted for seven days to find out the effect of *L. minor* on the specifications of secondary treated water in the secondary sedimentation basin. The NH_4^+removal efficiency was 15% percent higher compared to the treatment without *L. minor* (Gürtekin and Şekerdağ, 2008). Other study used the *Spirodella polyrrhiza* to treat the municipal wastewater using two ponds with the same dimensions (0.90W-1.95L), the depth of the first pond (0.80m^2) and the second (

0.70m2) at a temperature ranging between (15-38)C˚ and harvesting every four days and the flow conditions were (50%-100%) with a hydraulic retention time of sixteen days .The results showed that the total nitrogen removal rate (72% - 61%) respectively, and at a hydraulic retention time of eight days the removal rate was (32%-36%) respectively .(Benjawan,2008).

In studying the possibility of using water lentils as a plant material to compare between laboratory and field conditions to show the possibility of removing nutrients from wastewater in the Autumn season in North Carolina in 2009, at a constant temperature (23) ˚C and lighting (16) h\days in the laboratory, the plant needed four days to adapt to the sewage water, then its growth began to increase linearly with time, the rate of removal of nitrogen was about (1.3g/m^2d), while the temperature of the basin in the field study ranged between (15-25) ˚C with the rate of removal of nitrogen (2.03)g\$m^2$d). The study also indicated a good growth in spring and autumn, (Cheng and Stamp,2009). Foroughi *et al*., (2010) showed that the concentration of NH_4 in raw municipal wastewater and treated municipal wastewater decreased from 135 to 15meq/l and from 90 to 10meq/l, respectively, for each three periods of six days when wastewater purification by *Ceratophyllum demersum*.

Patel and Kanungo (2010) observed an increase in the dissolved oxygen content after the domestic wastewater phytoremediation of with *Ceratophyllum demersum* L, and indicated that all the three forms of nitrogen ammonical, nitrite and nitrate nitrogen values were decreased in domestic wastewater and the reduction rate for ammonia, nitrite and nitrate after phytoremediation was ranging from12.75-57.58, 24.74-58.35, and 35.47-65.61 % respectively. The removal of nutrients from wastewater is an important process using *L. minor*. In 2011, specifically in Iraq, at the University of Tikrit, a study was conducted on the removal

of nutrients from wastewater using *L. minor* at two different densities 5-10 g/l in non-ideal field conditions; the best plant density for nutrient uptake and reduced concentration at weight was 5 g/l and it was found that water lentil is characterized by its vital activity as it remains in a stable state when fluctuating in dissolved oxygen concentration, pH value and temperature, it was found that the process of reducing the density of the plant through harvesting increases the speed of removing nutrients in the water, and the high density of the plant in a limited space affects its growth , as it is observed that better plant growth occurs at the density of 5 g/l, and therefore greater ammonium absorption from the medium in the first three days due to the presence of high plant growth (Taha *et al*., 2011).

Wendeou *et al*., (2013) showed the reduction rate of the Nitrogen Total Kjeldhal (NTK) was 100% when grown the duckweed in an outdoor batch system under 15 different values of electrical conductivity ranging from 200 to 3000 μs/cm. Žaltauskaite *et al*.,(2014) referred that *Lemna minor* has been shown to be a potential scavenger of nutrients and heavy metals from wastewater and may be used in wastewater treatment systems where nitrates and ammonium were removed with lower efficiency, i.e., by 58.3% and 50.2–75.3%, respectively when *L. minor* exposed to untreated and biologically treated wastewater for 7 days .

Al-Asady (2014) referred to the ability of aquatic plant such as *Lemna minor* to effectively reduce the Nitrite and Nitrate concentration ,where the removal rate was (55.72%, 55.74%) for nitrite and nitrate respectively. Selvarani *et al*., (2015) used *Lemna minor* in the treatment of wastewaters and proved the efficiency of the plant in reducing the values of ammonia, nitrite and nitrate in proportions 96%, 98% and 98% respectively. Kadhim (2017) indicated the role of the *L. minor* and *C. demersum* plants in the reduction of nitrite and nitrate values in municipal

wastewater where *L. minor* recorded the highest reduction rate of nitrite 75% on day 15th, and 100% for nitrate reduction on day 25th, while the *C. demersum* plant recorded the highest reduction rate of nitrite and nitrate (95%, 87.1%) respectively, on day 25th.

Sharba (2019) recorded the competition between *C.demersum* and *H.verticillata* in reduction the nitrate and nitrite concentration , where the ratio of these two compounds reached to the lowest levels after 30 days of treatment. Maktouf *et al.,* (2018) indicated that *C. demersum* plant recorded high reduction rate of nitrate reached 87.97% when these aquatic plant used in wastewater treatment. Yaseen and AL-Azawey (2021) referred that the nitrite concentrations in the wastewater treated with *C. demersum* plant decreased after treatment and recorded high reduction rate of 89.4% at day 25. Plants also showed higher efficiency in reducing nitrate concentrations and recorded reduction rate of 96% on day 25, but its treatment was limited to days 20 and 25.

Chapter Three

Material and Methods

3- Material and methods

3-1: Description of the municipal sewage treatment plant

The central station for municipal water treatment is located in Babil Governorate in the Al Muaymirah region, which is about 5 km away from the city center on the Hilla - Diwaniyah road.

The treatment stages include several steps, starting with the mechanical or physical treatment stage, where large solid particles suspended in wastewater such as stones, cans, pieces of cloth and nylon are isolated and then sedimentation and in this process the bulk of the solid pollutants are eliminated. The second stage includes the biological treatment stage and it consists of two steps, the first includes the aeration by pumping large quantities of air into the aeration basins, thus multiplying and stimulating the aerobic bacteria that decompose the remaining organic materials in the water, then comes the sedimentation process, which aims to get rid of the aerobic bacteria and decomposing organic materials. Finally, the water leaving the basins is discharged to a drain that passes through the agricultural lands to be poured into the eastern Euphrates drainage. The water was collected for the purpose of treatment from the sedimentation basins before being discharged to a drain (Information from the station administration).

3-2 Phytoremediation of municipal wastewater

3-2-1 Aquatic plants used in phytoremediation

The *L. minor* was confirmed as a species by DNA barcoding marker Technique via specific primers for atpF-atpH noncoding spacer region. While *C. demersum* was classified in the Collage of Science / University of Babylon.

3-2-2 Experimenting the wastewater to treatment with aquatic plants

Aquatic plants were collected from different sites in Hilla city and were washed well with tap water several times and then with warm water to remove suspended materials and placed in plastic containers with dimensions of 70 (x 30 x 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 from their original origin, after which the plants were placed at a rate of 10 g / liter (Taha *et al*., 2011) with the addition of 20 liters of municipal wastewater that collected by polyethylene containers of 20 liters ,with leaving containers that contain only untreated wastewater which represented the control for phytoremediation and leaving other containers ,some of which contain tap water with *L. minor* plant, and others contain tap water with *C. demersum* plant to represent a group of control for physiological and biochemical markers measurements of aquatic plants . An amount of water was withdrawn every 3 days until the 24thday for the purpose of conducting some physical and chemical tests with estimating the concentration of some dissolved nitrogen compounds.

Picture (3-1) municipal wastewater treatment basins with aquatic plants under study

3-2-3 Physical and Chemical analysis

3-2-3-1 pH, Electrical Conductivity and Total Dissolved Solid (TDS):

pH, EC (µs/cm) and TDS (mg/l) of water is measured using pH – meter (multi - parameters), Oakton - U.S.A after calibrate the a device with solutions that used to calibrate it.

3-2-3-2 Dissolved Oxygen(DO):

Dissolved oxygen was determined according to Azide-modification of Winkler method (APHA, 2003). The results were expressed as mg\l.

3-2-4 Dissolved nitrogen compounds

3-2-4-1 Total dissolved nitrogen (TDN)

Total dissolved nitrogen was measured with the standard per-sulfate digestion method (Koroleff ,1979) and modified by (Schnetger and Lehners ,2014). In which 5 ml of each sample was taken and added to them 5 ml oxidizing reagent solution (was Prepared by dissolving potassium per-oxodisulfate and boric acid in sodium hydroxide solution) to each tubes and close the tube. Mix by swirling and then autoclaved for 30 min and allow to cooled to room temperature. Then, added 3 ml of TN mix-reagent (was prepeared by mixing vanadium chloride solution with sulphanilamide solution and N-(1-naphtyl)-ethylenediamine dihydrochloride solution.) ,mixed well by swirling and incubate for 1 h at 45°C, and allow to cool to room temperature. Measure the absorbance with a spectrophotometer at 545 nm. The calibration curve was prepared in the similar way by using standard nitrate solutions. As for the blank, distilled water was used in place of the sample.

3-2-4-2 Total inorganic nitrogen (TIN)

The total inorganic nitrogen was estimated by summing the concentrations of ammonia, nitrite and nitrate that dissolved in the sample. The results were expressed as mg\l (Seitzinger and Sanders,1997; Binkley *et al*., 1999 Bronk *et al*.,2000). DIN= [DNH_3-N + DNO_2-N + DNO_3-N]

3-2-4-2-1 Ammonia

The Phenate method that described before by the (APHA, 2003) was followed, in which 25 ml of the sample was taken, after which a Phenol solution, sodium nitro-prusside solution 0.5%, oxidizing solution

and a commercial Clorox solution were added to it, and the sample was left for an hour. The absorbance of the sample was read by using a spectrometer at 640 nm. The calibration curve was prepared by standard solutions that prepared from standard NH_4Cl in the similar way that for the sample.

3-2-4-2-2 Nitrite

Nitrite was measure according to the method described by (Parson *et al.*,1984), where 50 ml of water sample taking with adding 1-2 ml of Sulphanil-amide solution for 2 minutes and then adding 1-2 ml of N-naphthyl ethylene di-amine. The absorbance of the samples was measured at 543 nm.

3-2-4-2-3 Nitrate

Nitrate was measured according to the (Phenol di-sulphonic Acid Method) (APHA,2012), where 10 ml of water sample was taking with evaporated to dryness. 2 ml of phenol di-sulphonic acid (PDA) reagent was added to the residue from sample and then 10 ml of concentrated NH_4OH was added carefully. Finally, the content of sample was completed to 100 ml by distilled water. The absorbance of the sample was read by a spectrometer at 410 nm. The calibration curve was prepared in the similar way by using standard nitrate (10 ppm to 50 ppm solutions). As for the blank, distilled water was used in place of the sample).

3-2-4-3 Dissolved organic nitrogen (DON)

DON was calculated as the difference between the total dissolved nitrogen and the sum of inorganic nitrogen species (i.e., DNH_3-N + DNO_2-N + DNO_3-N) according to the equation below (Seitzinger and

Sanders,1997; Bronk *et al*., 2000). DON (mg /L) = TDN – [DNH_3-N + DNO_2-N + DNO_3-N]**.**

3-3 Estimation of the physiological and biochemical markers

3-3-1 Determination of total chlorophyll, chlorophyll a and chlorophyll b.

Chlorophyll estimated according to (Mackinney,1941). The results were calculated using equations below (Arnon, 1949).

Total chlorophyll (mg/g) = [A_{645}(20.2) +A_{663}(8.02)] v/w*1000

Chlorophyll A (mg/g) =[A_{663}(12.7) - A_{645}(2.69)] v/w*1000

Chlorophyll B (mg/g) =[A_{645}(22.9) - A_{663}(4.69)] v/w*1000

3-3-2 Determination of Catalase and Superoxide dismutase

3-3-2-1 solutions:

1- Phosphate Buffer Solution (PBS) 60 mM, pH 7.0: was prepared by mixing 1:1.5 of KH_2PO_4 (1 M) and $NaH_2PO_4.2H_2O$ (1 M) respectively to get PBS of 50 mM with pH value equals 7.0.

2- Pyragallol solution (0.2 mM): was prepared by dissolving 0.0252 gm of pyragallol with 10 ml of HCl and completing the volume to 100 ml with dH_2O.

3 - Trise buffer (pH 8.0): was prepared by dissolving 0.258 gm of trise

and 0.111 gm of Ethylenediaminetetraacetic acid (EDTA) in dH_2O and completing the volume to 100 ml.

4- Hydrogen peroxide 60 mM: was prepared by diluting 0.408 ml of 50% H_2O_2 (newly opened) to 100 ml of 50 mM PBS with 7.0 as pH value.

3-3-2-2 Enzyme extraction

To extract the enzyme from the plant sample, 1 gm of plant sample was grinded by manual pestle and mortar over ice with the addition of 10 ml of PBS and 0.3 gm of PVP. The extract was centrifuged on 10000 r.p.m for 15 minutes at 4 C˚. Supernatant was taken as crude enzyme extract.

3-3-2-3 Superoxide Dismutase Activity (SOD)

According to Marklund and Marklund (1974), reaction mix is consisting of 50 µl crude enzyme (was prepared by grinded 1gm of plant sample over ice with the addition of 0.3 gm of PVP and 10 ml of PBS and then centrifuged on 10000 rpm for 15 minutes at 4^0 C. Supernatant was taken as crude enzyme extract while residue was neglected), extract with 2 ml of tris buffer and 0.5 ml of pyragallol, absorption of samples was taken at 420 nm. Control solution contains dH_2O with addition the same materials. As a blank, dH_2O was used. SOD activity was calculated using the following equation:

$$\text{SOD activity (units)} = \frac{\%\,inhibition\,/\,50\% \times reaction\,volume}{total\ test\ period}$$

3-3-2-4 CAT activity

CAT activity was measured according to the method described by (Misra and Gupta ,2006). The reaction included 50 µl crude enzyme (Prepared in the same manner as reported in SOD activity determination) with adding 2ml (60 mM H_2O_2) to 1ml (50 mM Phosphate Buffer Solution (PBS) pH 7.0), the absorbance was measured during 3 min at 240 nm.

3-3-3 Malondialdehyde (MDA) content determination

The method was adopted by Zacheo *et al.* (2000) in estimating the content of Malondialdehyde. The plant extract (was prepared by crushing 0.25 gm of plant samples with 5 ml of Trichloroacetic acid (TCA) for the purpose of precipitation of proteins. Then a centrifugation process was carried out at 1000 rpm for 15 minutes. Then a volume of 3 mL of filtrate was taken and a similar volume of Thiobar butric acide (TBA) was added. It was placed in a boiling water bath for 30 minutes and then the absorbance was read at a wavelength of 532 nm, the Blank solution consists of 3ml of TCA solution and 3ml of TBA solution. The amount of MDA was calculated from the following Ber-Lambert formula, as shown below:

$$A= E*I*C$$

3-3-4 Total protein content

The Biuret method was used to estimate the protein in the plant (Bishop *et al*., 1985), where 1 gm of the plant was crushed with 6 ml of phosphate buffer solution and a centrifugation process was carried out at 1500 rpm for 15 minutes. Discard the sediment and fill the filtrate to 15 mL with a phosphate buffer solution .Then, 2 ml of the extract obtained was withdrawn and 8 ml of Biuret reagent was added to it and it was mixed well and left for half an hour, then the absorption was read by a spectrophotometer on the wavelength of 555 nanometers. The blank was prepared from 2 ml of phosphate buffer solution with 8 ml Biuret reagent. The bovine serum albumin solution was used in the preparation of the standard solution. The protein content was expressed as mg / mg vegetable tissue.

3-3-5 Total carbohydrate content

Carbohydrate content was estimated according to method of (Dubois *et al*., 1956), weighed 0.2 gm of dried and crushed plant tissue and added to them 8 mL of ethyl alcohol (80%) in a test tube and placed in a 60 ° C water bath for 30 minutes and centrifuged at 3000 cycles / minute for 15 minutes, then the clear liquid was withdrawn. The process of adding 8 ml of alcohol to the sediment was repeated twice to extract the dissolved sugars. Then collected the filtrate and Complete the volume to 25 ml by adding 80% ethyl alcohol, 1 ml of it was withdrawn and added to it 1 ml of phenol (5%) and 5 ml of concentrated H_2SO_4. As for the blank, it consisted of (1 ml (80%) ethyl alcohol, 1 ml phenol (5%) and 5 ml of concentrated H_2SO_4). The absorbance of samples was measured at 560 nm, and the Glucose solution was used in the preparation of the standard solutions.

3-3-6 Determination of proline content

Proline content was estimated according to method of (Bates *et al.*, 1973), where (0.5 g) of plant tissue was taken and crushed with (10 ml) of sulfo-salicylic acid (3%) in a ceramic mortar and filtered through (Watmann's No.1).Then 3 ml of the filtrate was mixed with 3 ml of ninhydrin solution and 3 ml of glacial acetic acid in test tubes ،and then these tubes were placed in a water bath at a temperature of (100) C for a full hour, after which they were cooled to a degree of the temperature of the laboratory and then added to it 5 ml of toluene with shaking for 20 seconds, The absorbance was taken at a wavelength of 520 nm and the concentration of proline was calculated by the standard curve and the final product was expressed in µ mole /g.

3-4 Molecular analysis

3-4-1 DNA extraction

Genomic DNA from *L. minor* plant were extracted using DNA extraction kit (WizPrep™ Plant Mini Kit), according to the following steps:

Step 1: Prepare plant tissue

One hundred of plant tissue was transferred into a mortar that contains an appropriate amount of liquid nitrogen to cover the sample. The sample was Grind with liquid nitrogen into a fine powder.

Step 2: Lysis step

- A volume of 400µl of GP1 Buffer and 5µl of RNase A (10 mg/ml) were added into the sample tube and mix by vortex.
- The Sample tube was Incubated at 65℃ for 10 minutes with inverted the tube every 5 minutes.
- A volume of 100µl of GP2 Buffer was added and mixed by vortex. Then Incubated on ice for 3 minutes.
- A Filter Column was placed in a 2ml Collection tube transferred the lysate (500µl) to Filter column and centrifuged for 1 min at 13,000 rpm.
- The Filter Column was discarded and (400µl) of supernatant was transferred carefully in Collection tube to a new micro centrifuge tube.

Step 3: Binding step

- A volume of 600µl of GP3 Buffer was added to filtrate and mixed by inverted for 5 times.

- The mixture was transferred to the Spin Column and centrifuged for 2 min. at 13,000 rpm.
- The flow-through was discarded through re-connect with Spin Column.

Step 4: Wash step

- A volume of 400µl of PW1 Buffer was added to the Spin Column and centrifuged for 30 sec. at 13,000 rpm with discarded the flow-through and re-connect with the Spin Column.
- A volume of 600µl of PW2 Buffer was added in the center of Spin Column matrix and centrifuged for 30 sec. at 13,000 rpm with discarded the flow-through and re-connect the Spin Column centrifuged for 3 min. at 13,000 rpm.

Step 5: Elution step

- A volume of 50µl of Elution Buffer was added and incubated at R/T for 1 min. Centrifuged for 1 min. at 13,000 rpm.
- The Spin Column was discarded and eluted purified DNA

3-4-2 Polymerase Chain Reaction (PCR)

3-4-2-1 Primers dilution

The primers were synthesized at (bioneer/Korea). These were provided in a lyophilized from, which were re-dissolved with deionized water according to instructions of manufacture company to reach to the final concentration (10 Pico moles/µl of suspension as a work solution.

3-4-2-2 PCR experiments

PCR amplification was done using conventional thermal cycler (Biometra - Germany) as show table (1):

Table (3-1): PCR components

Components	Volume (µl)	Concentration
PCR master mix	12.5	1X
Forward primer	1.5	10 pmol/ µl
Reverse primer	1.5	10 pmol/ µl
Template DNA	3.0	50 ng
Final volume (dH_2o)	20	

Table (3-2): PCR programme of related genes

Target gene	Steps	Temperature(C°)	Time (mint)	NO. of cycles
atpF-atpH	Initial denaturation	94	5	1
	Denaturation	94	1	35
	Annealing	53	1	
	Extension	72	2	
	Final extension	72	10	1

Table (3-3): The primers sequence of DNA barcoding marker

primer name	Sequence 5----------3	Amplicon size/bp
atpF-atpH	5- ACTCGCACACACTCCCTTTCC-3	675
atpF-atpH	5- CTTTTATGGAAGCTTTAACAAT-3	

3-4-2-3 Agarose Gel Electrophoresis Technique

The agarose gel electrophoresis was perfomed according to the method of Robinson and Lafleche (2000). This technique was used to detect genomic DNA extracts, PCR products

A. Ethidium bromide staining solution

A volume of 3 µl of Ethidium bromide at concentration 1.25 mg/ml were added to 100 ml of 0.5 X TBE buffer (Tris-Borat-EDTA

buffer); the solution was stored in a dark bottle at room temperature (Robinson and Lafleche, 2000).

B. Loading Buffer

The buffer was prepared from 0.25 % Bromophenol blue and 40% sucrose and stored at 4° C (Sambrook and Rushell, 2001).

C. TBE Buffer (1X)

To prepare 500 ml of 1X TBE buffer, 50 ml of TBE (10X) stock solution was mixed with 400 ml of dH_2O. The pH value was adjusted to 8 with concentrated HCl or 0.5 M tris base solution. Then the volume was completed to 500ml with dH_2O.

3-4-2-4 Gel electrophoresis protocol

1- Device setup: The casting gates were sited on the ends of the gel tray and locked in place firmly against casting tray. This was done by engaging the "claws" of the gate in the recess of the side wall of the tray. The comb was sited into the slots of the gel tray, (1.0 mm above the base of gel casting tray) so that the sample wells are near the cathode.

2- Gel dissolving: A1g of agarose was dissolved in 100ml of 0.5 X TBE solution by melting to 100°C to prepare 1% agarose gel for migrated genomic DNA extracts.

3- Gel casting: After agarose gel dissolving completely, it let to cooling to approximately 60°C and 2-3 µl of the ethidium bromide stock solution was added, then slowly pour the agarose into the gel- casting tray, and any air bubbles were removed. The comb was positioned at approximately 1.5 cm from one edge of the gel. The agarose was allowed to solidify at room temperature at least 30

min. After that, the claws were disengaged from the gel tray and the comb was separated gently. Then the gel was placed in the gel tank in such a way that the wells should be on end with the cathode. 1X TBE buffer was added to the buffer tank until it was about 5 mm above the top of the gel.

4- Loading the samples: Each 5µl of the genomic DNA sample was mixed with 1µl 5X loading buffer briefly and loaded into the wells. Whereas, the PCR products were loaded without loading buffer because of the PCR premix contained loading buffer.
5- Gel electrophoresis conditions: After sample loading the electric field was turned on at 5 V/cm (75V) for 60-120 min until bromophenol blue dye reached at the end edge of the gel.
6- The gel was photographed using gel documentation system (Cleaver Scientific - UK).

3-5 Statistical analysis

Data was analyzed using SPSS (version 20, SPSS Inc. Chicago, Illinois, USA). Descriptive statistics (mean ± standard deviation (SD)). Statistical analysis was carried out using t-test student test for comparing between two groups, followed factorial experiments by using least significant difference (LSD). The value of $p \leq 0.05$ was considered to be statistically significant

Chapter Four

Results

4- Results

4-1 Characteristics of municipal wastewater before treatment:

The following table shows some of the physical and chemical characteristics of the wastewater collected from the sedimentation basins of the domestic water treatment plant before the biological treatment.

Table (4-1): the physical and chemical properties of municipal wastewater before phytoremediation

Measured factors	values Mean± SD
pH	**8.30±0.10**
Dissolved Oxygen mg/l	**1.8±0.28**
EC µs/cm	**1176.67±28.93**
TDS mg/l	**779.67±26.65**
Total Dissolved Nitrogen mg/l	**131±10.66**
Total Inorganic Nitrogen mg/l	**46.742±6.42**
Ammonium mg/l	**11.2±0.10**
Nitrite mg/l	**0.157±0.01**
Nitrate mg/l	**35.385±1.74**
Dissolved Organic Nitrogen mg/l	**84.3±3.11**

4-2 Phytoremediation

4-2-1 Some physical and chemical characters

Physical and chemical properties of domestic wastewater treated with aquatic plants under study illustrated in the table (4-2) .

4-2-1-1 pH

Figure (4-1) illustrated the pH values of wastewater, where pH in *C. demersum* plant was found to be (8.9 – 9) and about (7.8 - 8.3) for *L. minor* plant and (7.9 - 8.6) for control . The statistical results showed

that there is a significant difference ($p \leq 0.05$) in the pH values in the treated water.

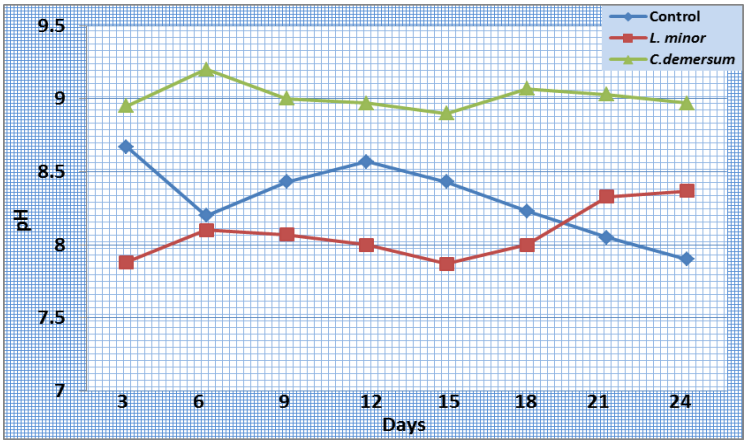


Figure (4-1): pH values during phytoremediation

4-2-1-2 Dissolved Oxygen

Figure (4-2) shows the DO concentrations in the wastewater after treatment with plants, which indicates the efficiency of *C. demersum* in water aeration throughout the study period, where the highest values were recorded on the day 6^{th} which amounted to (6.40), the highest value recorded for *L. minor* was (2.4) per days (15 and 21). Compared to the control treatment, which recorded higher concentrations than *L. minor* and the highest value recorded was (4.4) per day (15). The statistical results showed a significant difference $p \leq 0.05$ in the O_2 concentrations after treatment.

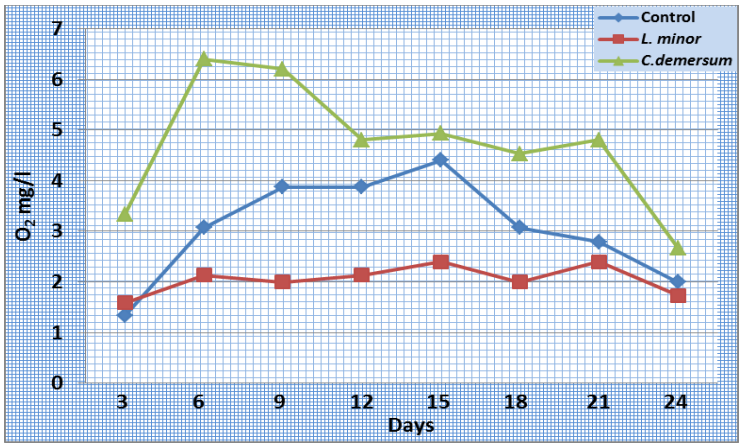


Figure (4-2): Dissolved oxygen concentrations during phytoremediation

Table (4-2) : The physical and chemical properties of municipal wastewater after phytoremediation

First line (mean ± standard deviation), second line (Range)

days	Treat.	pH	DO mg/l	EC µs/cm	TDS mg/l	Total Dissolved Nitrogen mg/l	Total Inorganic Nitrogen	Ammonium mg/l	Nitrite mg/l	Nitrate mg/l	Total Organic Nitrogen mg/l
	Mean ±S.D Range										
3	Control	8.67±0.11 (8.6-8.8)	1.33±0.23 (1.2-1.6)	1114±12.16 (1106-1128)	783.67±14.50 (769-798)	127.73±3.63 (124.09-131.36)	16.54±3.07 (13.65-19.78)	7.067±0.81 (6.60-8.00)	0.11±0.013 (0.10-0.13)	9.36±3.2700 (6.92-13.08)	111.20±6.00 (104.30-115.20)
	L. minor	7.88±14 (7.8-8.1)	1.59±0.40 (1.2-2.0)	1073.33±34.15 (1043-1111)	770.33±17.24 (755-789)	107.42±0.01 (106.42-109.45)	11.10±1.91 (9.19-13.02)	6.80±0.001 (6.60-6.80)	0.07±0.009 (0.07-0.08)	4.23±1.92 (2.31-6.15)	96.30±1.90 (94.4-98.20)
	C.demersum	8.95±0.08 (8.9-9.1)	3.33±0.23 (3.2-3.6)	1017±39.23 (972-1044)	731.33±5.03 (726-736)	101.47±6.16 (95.3-107.64)	12.17±1.56 (10.61-13.74)	4.60±0.20 (4.40-4.80)	0.07±0.021 (0.06-0.10)	7.5±1.34 (6.15-8.85)	89.30±4.60 (84.70-93.90)
6	Control	8.20±0.01 (8.1-8.2)	3.07±1.51 (2.0-4.8)	1178.33±22.67 (1161-1204)	834.67±10.78 (827-847)	103.64±1.96 101.67-105.61)	17.23±1.35 (15.74-18.39)	7.50±1.50 (6.00-9.00)	0.12±0.005 (0.11-0.12)	9.61±1.15 (8.46-10.77)	86.43±3.06 (84.10-89.90)
	L. minor	8.09±0.01 (8.08-8.1)	2.13±0.46 (1.6-2.4)	1132.33±11.59 (1119-1140)	806±2.00 (804-808)	104.55±4.36 (101.52-109.55)	13.17±2.51 (10.42-15.35)	6.50±0.70 (5.80-7.20)	0.001±0.0006 (0.0008-0.002)	6.67±2.11 (4.62-8.85)	91.37±6.70 (87.20-99.10)
	C.demersum	9.20±0.05 (9-9.2)	6.40±1.48 (5.6-7.2)	1073±7.93 (1064-1079)	766±6.55 (759-772)	101.52±1.36 100.15-102.88)	16.36±1.68 (14.43-17.51)	4.40±0.01 (4.20-4.40)	0.032±0.004 (0.03-0.04)	11.92±1.67 (10.0-13.08)	85.17±0.68 (84.40-85.70)
9	Control	8.43±0.11 (8.4-8.5)	3.87±0.92 (2.8-4.4)	1206.67±2.51 (1204-1209)	857.67±6.35 (854-865)	107.22±32.42 (75.00-139.85)	28.33±4.21 (24.24-51.11)	5.50±0.10 (5.40-5.60)	0.14±0.008 (0.13-0.14)	22.69±1.74 (21.99-45.39)	78.89±9.54 (46.11-90.20)
	L. minor	8.07±0.01 (8-8.2)	2±0.40 (1.6-2.4)	1200±15.39 (1183-1213)	851.67±7.76 (843-858)	99.39±5.60 (93.079-105.00)	24.02±1.30 (22.72-25.32)	2.10±1.30 (0.80-3.40)	0.001±0.0001 (0.0009-0.001)	21.92±0.01 (21.81-21.92)	75.40±6.35 (69.80-82.30)
	C.demersum	9±0.15 (8.8-9)	6.20±2.32 (5.6-6.8)	1189.67±12.89 (1179-1204)	839±8.18 (832-848)	100.96±3.32 (98.33-104.70)	19.20±0.41 (18.80-19.61)	3.80±0.40 (3.40-4.20)	0.016±0.005 (0.01-0.02)	15.39±0.01 (15.33-15.39)	81.77±3.44 (78.70-85.50)
12	Control	8.57±0.1 (8.4-8.7)	3.87±1.00 (2.8-4.8)	1270.33±15.17 (1254-1284)	896±10.81 (884-905)	109±22.29 (107.42-156.81)	41.76±5.11 (61.84-71.44)	8.60±0.40 (8.20-9.00)	0.159±0.001 (0.15-0.16)	33 ±5.2 (31.04-63.08)	67.24±7.41 (36.00-95.00)
	L. minor	8±0.06 (7.9-8.1)	2.13±0.23 (2-2.4)	1257.67±10.11 1 (1246-1264)	893±6.08 (886-897)	106.82±0.01 (104.82-107.82)	21.63±1.59 (20.03-23.23)	2.40±1.60 (0.80-4.00)	0.002±0.001 (0.001-0.002)	19.23±0.01 (19-19.23)	85.20±1.60 (83.60-86.80)
	C.demersum	8.97±0.23 (8.9-9)	4.8±0.40 (4.4-5.2)	1255.33±7.37 (1247-1261)	888.67±4.16 (884-892)	104.55±0.76 (103.79-105.30)	24.15±0.01 (24.14-24.15)	7.60±0.01 (7.40-7.80)	0.011±0.005 (0.005-0.014)	16.54±0.02 (16-16.54)	80.37±0.75 (79.60-81.10)

15	Control	8.43±0.23 (8.3-8.7)	4.4±0.40 (4-4.8)	1345±28.05 (1318-1374)	949.34±23.11 (925-971)	111.00±0.02 (149.76-151.67)	30.99±1.50 (29.49-32.49)	8.90±1.50 (7.40-10.40)	0.168±0.001 (0.166-0.168)	21.92±0.03 (21.2-21.92)	61.00±1.50 (118.30-121.30)
	L. minor	7.87±0.10 (7.6-8)	2.4±0.40 (2-2.8)	1323.33±9.81 (1312-1329)	942±5.19 (936-945)	99.95±8.13 (94.09-109.24)	40.18±1.70 (38.48-41.88)	4.60±0.80 (3.80-5.40)	0.003±0.0001 (0.0004-0.007)	35.58±2.50 (38-38.08)	59.77±6.91 (53.90-67.40)
	C.demersum	8.9±0.25 (8.8-9)	4.93±1.28 (4-6.4)	1318±12.52 (1305-1330)	932.67±9.50 (923-942)	100.61±10.65 (93.79-112.88)	26.22±7.09 (19.93-33.92)	6.60±1.40 (5.20-8.00)	0.006±0.0002 (0.003-0.008)	19.62±7.69 (11.92-27.31)	74.40±16.57 61.20-93.00)
18	Control	8.23±0.01 (8-8.5)	3.07±0.46 (2.8-3.6)	1398.33±16.50 (1380-1412)	987.33±10.78 (975-995)	96.36±12.27 (84.09-108.64)	37.37±0.17 (37.18-37.52)	7.40±0.01 (7.20-7.60)	0.160±0.038 (0.121-0.197)	29.81±0.19 (29.62-30.00)	59.00±12.10 (46.90-71.10)
	L. minor	8±0.05 (7.8-8)	2±0.01 (1.8-2.2)	1353.33±8.02 (1345-1361)	963.33±4.50 (959-968)	102±4.71 (100.22-111.67)	29.25±4.87 (25.25-31.15)	3.10±1.90 (1.20-5.00)	0.001±0.0001 (0.0008-0.001)	26.15±4.71 (20.15-30.15)	72.75±1.93 (79.20-83.00)
	C.demersum	9.07±0.01 (9-9.1)	4.53±0.83 (3.6-5.2)	1363.67±14.97 (1347-1376)	962.67±10.06 (952-972)	91.77±9.40 (82.58-101.36)	28.81±0.67 (28.04-29.28)	1.50±0.30 (1.20-1.80)	0.002±0.0005 (0.001-0.002)	27.31±0.76 (26.54-28.08)	62.97±10.01 (53.30-73.30)
21	Control	8.05±0.05 (8.04-8.07)	2.79±0.40 (2.4-3.2)	1446.33±1.52 (1445-1448)	1030±20.41 (1028-1030)	103.08±4.53 (98.03-106.82)	33.21±4.11 (29.43-34.90)	7.40±1.60 (5.80-9.00)	0.109±0.019 (0.088-0.126)	25.7 25.7±3.11 (16.54-30.12)	69.87±8.77 (63.10-82.00)
	L. minor	8.33±0.11 (8.3-8.4)	2.40±0.69 (2-3.2)	1407±8.88 (1397-1414)	1001.67±7.63 (995-1010)	103.43±12.12 (92.58-116.52)	29.78±3.40 (25.86-32.03)	2.20±0.20 (2.00-2.40)	0.011±0.015 (0.001-0.029)	27.56±3.57 (23.46-30.00)	73.70±15.42 (60.60-90.70)
	C.demersum	9.03±0.10 (8.9-9.1)	4.80±1.20 (3.6-6)	1440.33±14.01 (1426-1454)	1020±10.0 (1010-1030)	96 ±7.42 (93.21-111.06)	28.80±7.45 (25.66-32.82)	1.90±0.50 (1.41-2.40)	0.001±0.0001 (0.008-0.001)	26.9±3.46 (21.54-29.55)	67.20±7.05 (64.60-79.70)
24	Control	7.9±0.37 (7.8-8)	2±0.80 (1.2-2.8)	1532.67±32.71 (1498-1563)	1090±20.0 (1070-1110)	108±7.44 (90.15-110.94)	54.89±1.80 (75.14-78.75)	11.30±0.30 (11.0+11.60)	0.089±0.034 (0.062-0.128)	43.5±10.11 (40.33-67.69)	53.11±8.37 (41.40-61.40)
	L. minor	8.37±0.06 (8.1-8.8)	1.73±0.23 (1.6-2)	1451.67±8.08 (1443-1459)	1036.67±5.77 (1030-1040)	106.01±7.44 (97.42-110.76)	47.12±2.16 (43.12-55.92)	2.50±1.30 (1.20-3.80)	0.002±0.0005 (0.001-0.002)	44.6±2.50 (41.62-59.62)	58.89±7.77 (56.60-63.60)
	C.demersum	8.97±0.04 (8.9-9)	2.67±0.23 (2.4-2.8)	1480±28.51 (1451-1508)	1053.33±15.27 (1040-1070)	93.79±0.03 (91.79-95.62)	31.70±6.08 (28.90-39.79)	2.90±1.50 (1.40-4.40)	0.001±0.0003 (0.0004-0.001)	28.8±5.19 (25.10-35.39)	62.1±5.09 (54.00-65.90)
LSD (0.05)(day*treatment)		0.109	0.562	14.690	8.497	14.645	4.858	1.359	14.645	4.703	16.314

4-2-1-3 Electrical Conductivity

The electrical conductivity values and the percentages of their reduction in municipal wastewater treated with plants are shown in Figure (4-3)(A,B). Where the plants recorded weak reductive ratios that were limited to first days of treatment. The highest reduction ratio for *L. minor* was (8.8 %). While the highest reduction rate was recorded for *C. demersum* plant was (13.5 %). Compared to the control treatment, which recorded a lower reduction rate of (5.3%) which was limited to the third day. The results of the statistical analysis indicated the presence of significant differences ($p \leq 0.05$) in the reduction of electrical conductivity values in the treated water.

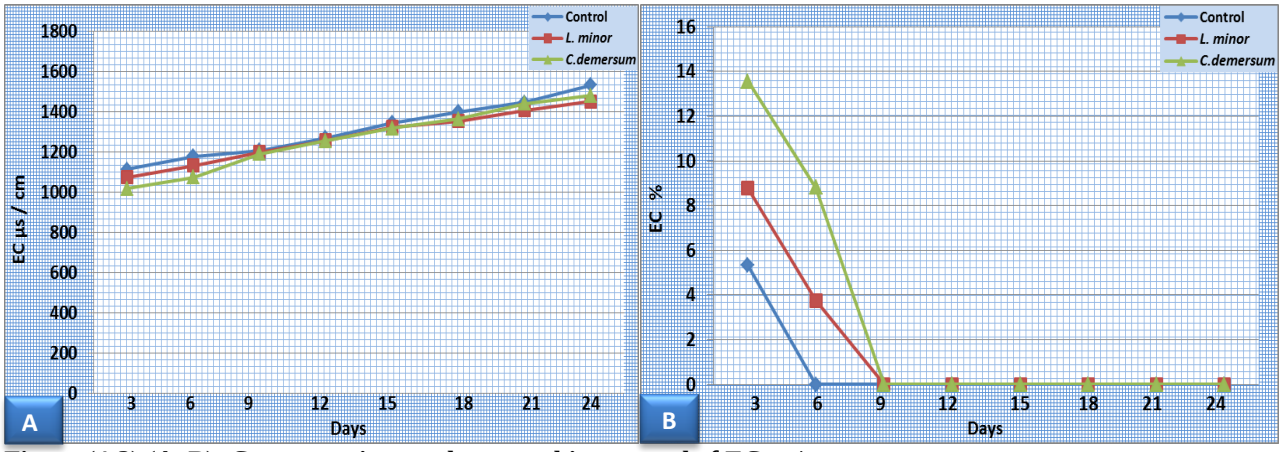


Figure (4-3) (A, B): Concentrations and percent bio removal of EC µs/cm

4-2-1- 4 TDS

Aquatic plants recorded slight reductions in the TDS values, as shown in Figure (4-4) (A, B), as the treatment was limited to *L. minor* and *C. demersum* on the third day, and the highest reduction rate was (1.19%) for the first plant and (6.2%) for the second plant. While the control treatment did not record reduction ratios throughout the treatment period and their concentrations were high compared to the treated water. The results of the statistical analysis indicated that there was a significant difference ($p \leq 0.05$) in the TDS values of treated water.

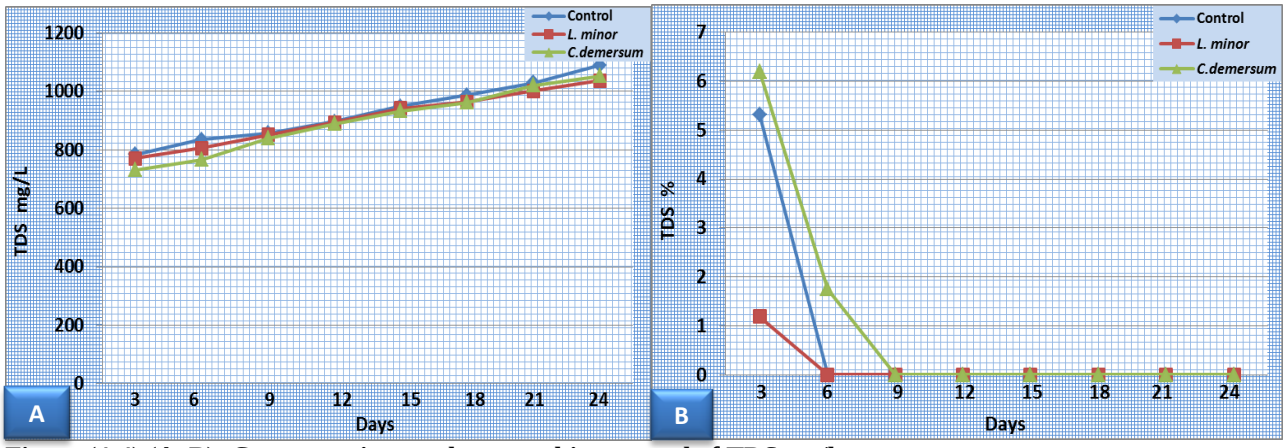


Figure (4-4) (A, B): Concentrations and percent bio removal of TDS mg/l

4-2-2 Dissolved nitrogen compounds

4-2-2-1 Total Dissolved Nitrogen (TDN)

The values of the concentrations and the percentage of removal of total dissolved nitrogen for wastewater treated by plants are shown in Figure (4-5)(A, B),which shows the *L. minor* and *C. demersum* plants recorded the highest reduction rate of about (24.12%) on day 9 and (29.94%) on day 18 for the two plants respectively, compared to the control, which recorded the highest reduction rate of about (26.44%) per day (18). The results of the statistical analysis indicated the presence of significant differences ($p \leq 0.05$) in the reduction of total dissolved nitrogen values in the treated water.

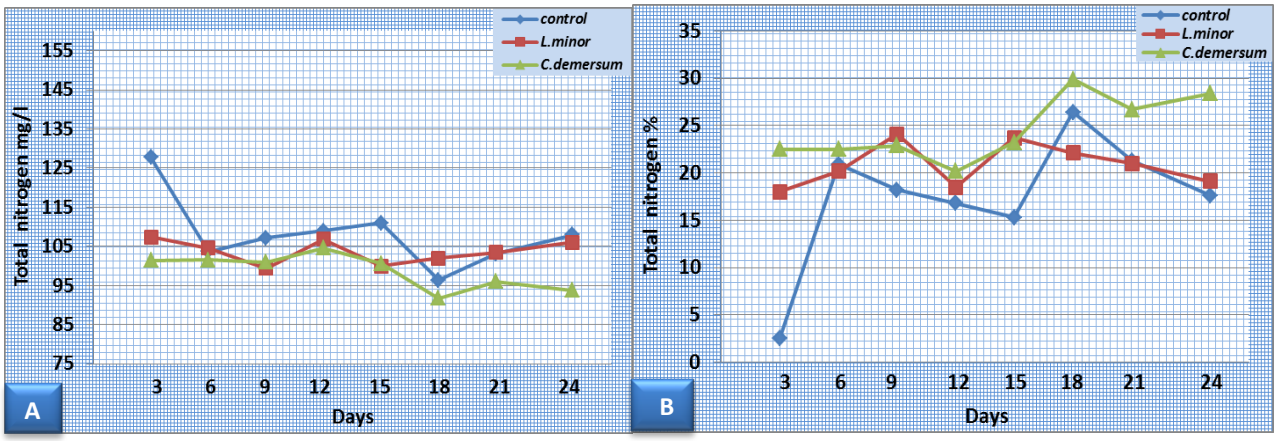


Figure (4-5) (A, B): Concentrations and percent bio removal of Total dissolved nitrogen mg/l

4-2-2-2 Inorganic nitrogen compounds

4-2-2-2-1 Total inorganic nitrogen (TIN)

Figure(4-6)(A,B)shows the values of concentrations and removal percentage of total inorganic nitrogen for wastewater treated with plants , which showed the efficiency of the current plants in reducing inorganic nitrogen compounds, where both *L. minor* and *C. demersum* recorded the highest reduction rate of total inorganic nitrogen, which was about (76.25% and 73.96%) for two plants respectively, on the third day of treatment, compared to the control which recorded the highest reduction rate of about (64.61% on the 3 day) . The results of the statistical analysis indicated the presence of significant differences ($p \leq 0.05$) in the reduction of total inorganic nitrogen values in the treated water.

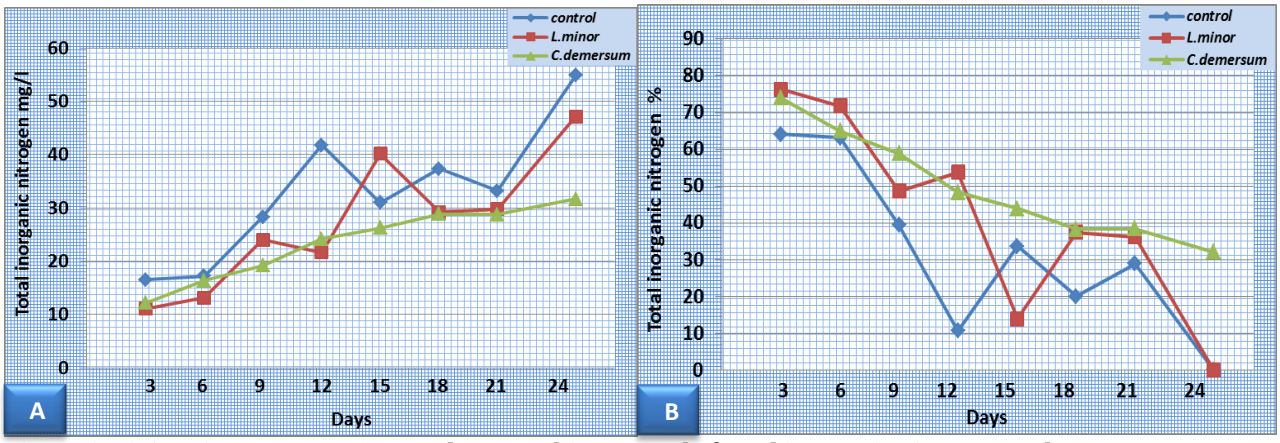


Figure (4-6) (A, B): Concentrations and percent bio removal of total inorganic nitrogen mg/l

4-2-2-2-2 Ammonium

The values of the concentrations and the percentage of removal of Ammonia for wastewater treated by plants are shown in Figure (4-7) (A, B), which showed that the plants under study demonstrated high efficiency in reducing ammonia values throughout the treatment period, as the highest reduction rate reached (81.25 %) at day (9) for the *L. minor* plants and (86.6 %) at day (18) for *C. demersum* plants. As for the control treatment, lower reduction rate was recorded than those recorded with

aquatic plants, where the highest reduction rate recorded was (50.89%) at day (9) and no removal recorded in the last days of treatment. The results of the statistical analysis indicated the presence of significant differences ($p \leq 0.05$) in the reduction of ammonia values in the treated water.

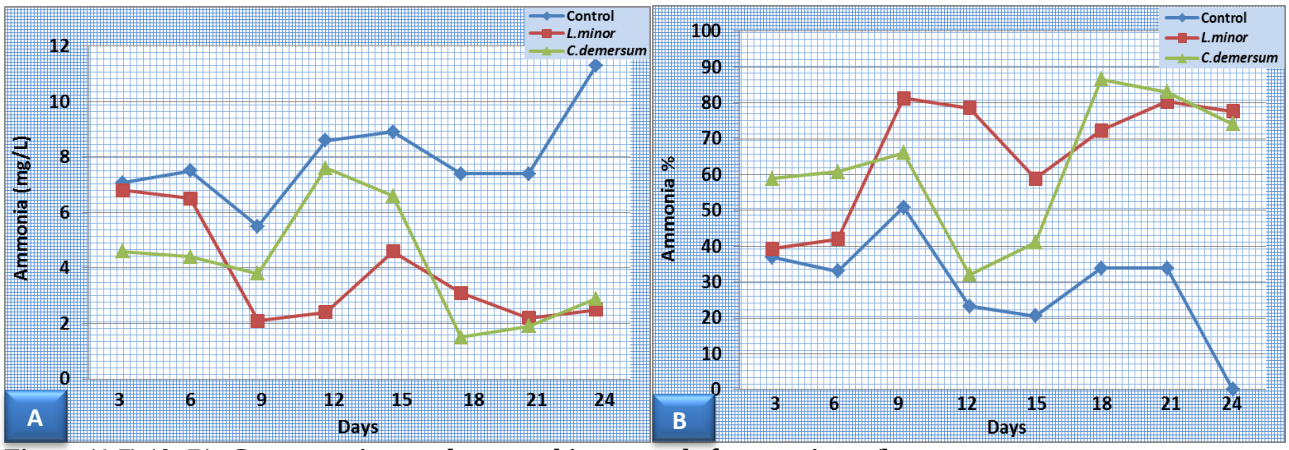


Figure (4-7) (A, B): Concentrations and percent bio removal of ammonia mg/l

4-2-2-2-3 Nitrite

In the current study, aquatic plants showed high efficiency in reducing the nitrite concentrations throughout the treatment period Figure (4-8)(A,B). 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 second plant. As for the control treatment, lower reduction rates were recorded, and the highest reduction rate was 43.31 % on day 24 of treatment. In general, *L. minor* and *C. demersum* plants showed a higher efficiency in reducing nitrite concentrations, starting from the first day until the end of the treatment period, with a clear superiority of *L. minor*. The results of the statistical analysis indicated the presence of significant differences ($p \leq 0.05$) in the reduction of nitrite values in the treated water.

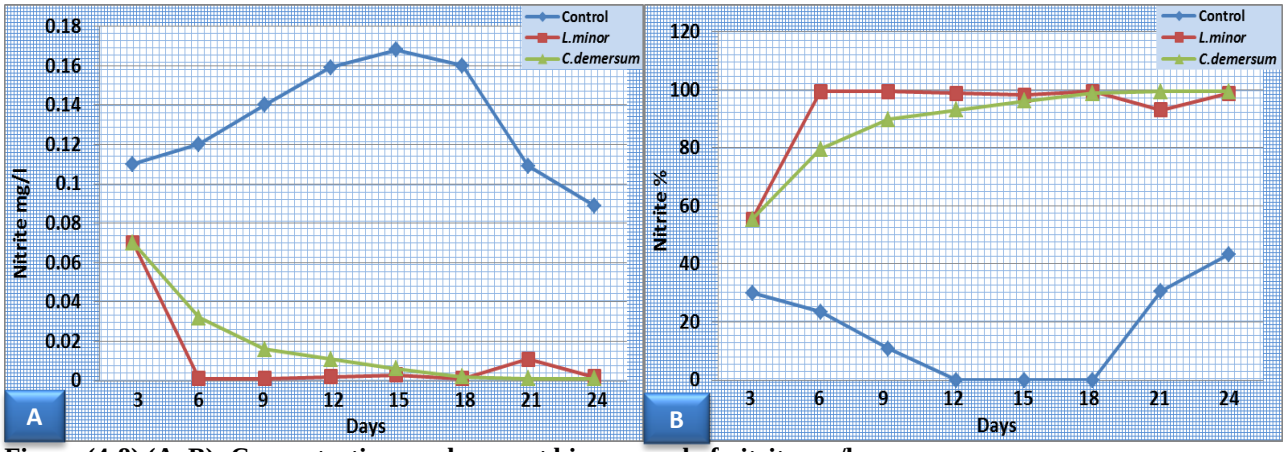


Figure (4-8) (A, B): Concentrations and percent bio removal of nitrite mg/l

4-2-2-2-4 Nitrate

Aquatic plants recorded high efficiency in nitrate reduction Figure (4-9)(A, B). Where *L. minor* plant and *C. demersum* recorded maximum reduction values (88.04 and 78.8) respectively at first days of treatment. The statistical results showed that there were significant differences ($p \leq 0.05$) in the nitrate values during treatment with plants.

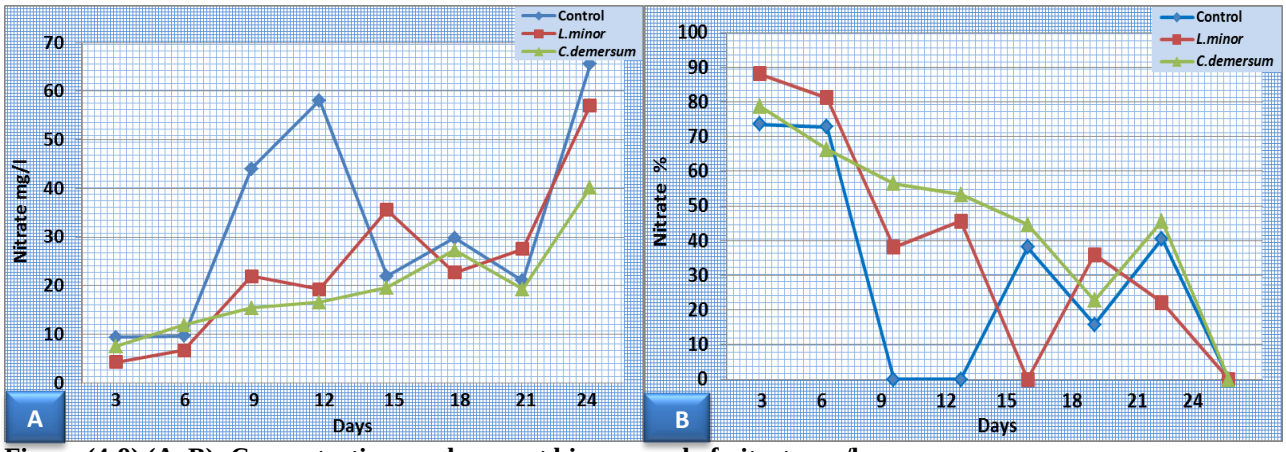


Figure (4-9) (A, B): Concentrations and percent bio removal of nitrate mg/l

4-2-2-3 Dissolved organic nitrogen(DON)

Figure (4-10) shows the values of the concentrations and the percentage of removal of total dissolved organic nitrogen for wastewater treated with plants, where the *L. minor* and *C. demersum* recorded the highest percentage of organic nitrogen reduction on day (24) of treatment, which amounted to about (30.14 % and 26.33%) for both plants

respectively, compared to the control which about (36.99%) on day 24 of treatment. The results of the statistical analysis indicated the presence of significant differences ($p \leq 0.05$) in the reduction of total dissolved organic nitrogen values in the treated water

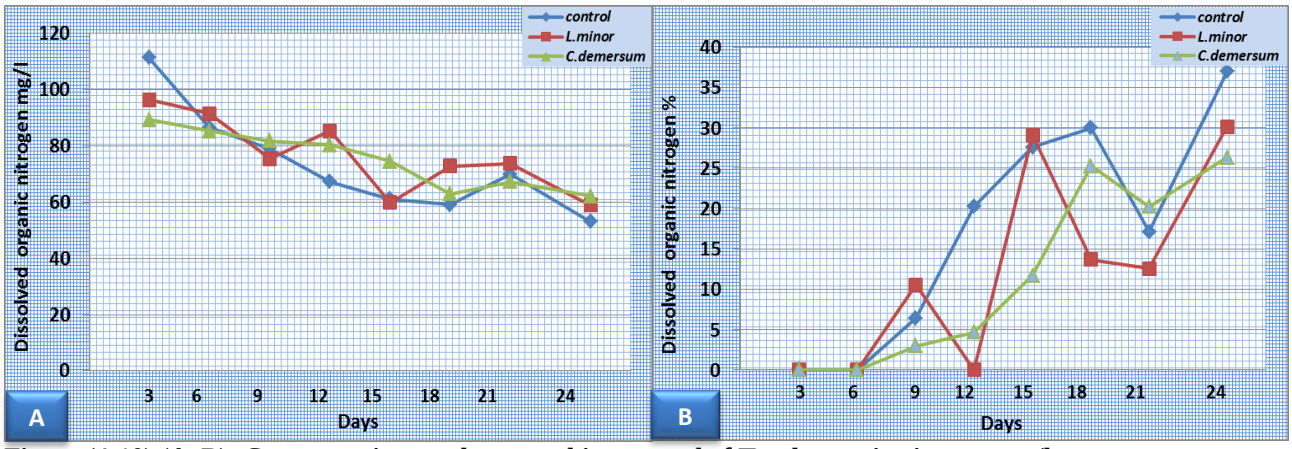


Figure (4-10) (A, B): Concentrations and percent bio removal of Total organic nitrogen mg/l

4-3 Physiological and biochemical markers of aquatic plants

Physiological and biochemical marker in aquatic plants illustrated in the table (4-3).

Table (4-3) Physiological and biochemical marker in aquatic plants (mean ± standard deviation)

Parameters	Plant	Control	Treated	P value
		Mean ±S.D		
Total chlorophyll (mg/g)	*L. minor*	0.127±0.05	0.177±0.04	0.038*
	C.demersum	0.222±0.04	0.315±0.09	0.042*
chlorophyll a (mg/g)	*L. minor*	0.086±0.03	0.129±0.05	0.030*
	C.demersum	0.144±0.02	0.223±0.03	0.033*
chlorophyll b (mg/g)	*L. minor*	0.040±0.01	0.048±0.02	0.091
	C.demersum	0.078±0.02	0.092±0.01	0.074
SOD activity (U/ml)	*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 activity (KU/l)	*L. minor*	77.70±7.50	71.95±6.33	0.426
	C.demersum	52.18±4.51	53.86±3.22	0.636
Malondialdehyde (nm/cm)	*L. minor*	1.11±0.16	0.74±0.03	0.017*
	C.demersum	3.56±0.03	2.79±0.37	0.025*

Carbohydrate content (mg/g)	***L. minor***	**54.03±7.3**	**88.62±4.5**	**0.042***
	C.demersum	**125.42±6.3**	**152.36±3.4**	**0.03***
Protein content (mg/g)	***L. minor***	**23.94±4.90**	**15.13±1.1**	**0.039***
	C.demersum	**5.44±0.05**	**14.47±1.3**	**0.005****
Proline content µ mole /g	***L. minor***	**15.41±0.68**	**24.68±5.1**	**0.036***
	C.demersum	**12.49±1.4**	**17.64±3.15**	**0.048***

4-3-1 Total chlorophyll, chlorophyll a, chlorophyll b

Figure (4-11)(4-12)(4-13)(A, B) shows the effect of domestic wastewater on the total chlorophyll content, chlorophyll a and chlorophyll b in the plants under study. The total chlorophyll values, chlorophyll a and chlorophyll b in *L. minor* were within the limits of (0.177 mg /g , 0.129 mg /g and 0.048 mg /g) respectively, compared to the plant that represents the control plant whose values were (0.127 mg/g, 0.086 mg/g and 0.04 mg/g) respectively. From this we conclude that an increase in the chlorophyll values of *L. minor* used in wastewater treatment compared to control plant. The same results were observed in *C. demersum* plant where increase in total chlorophyll content, chlorophyll a and chlorophyll b values were observed compared to control plant, Where the *C. demersum* plant which represents the control recorded a value within (0.22 mg/g , 0.144 mg/g and 0.078 mg/g) respectively ,while the values at the end of domestic water treatment were within (0.315 mg/g , 0.223 mg/g and 0.092 mg/g) respectively. The results of the statistical analysis indicate that there is a significant difference ($p \leq 0.05$) in the total chlorophyll and chlorophyll a values of the plants which representing the control and the plants used in the treatment.

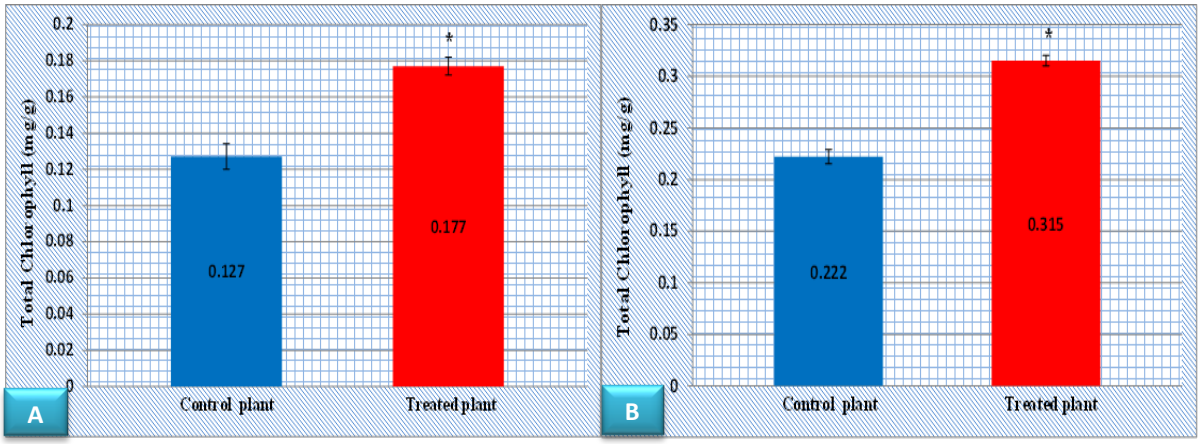


Figure (4-11): Total chlorophyll (mg/g) in (A): *L. minor*, (B): *C. demersum*

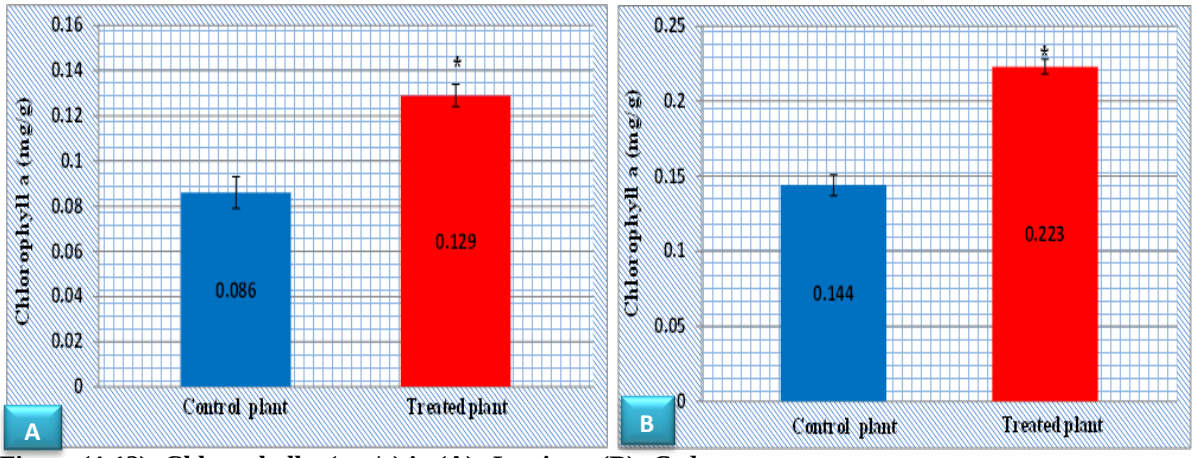


Figure (4-12): Chlorophyll a (mg/g) in (A): *L. minor*, (B): *C. demersum*

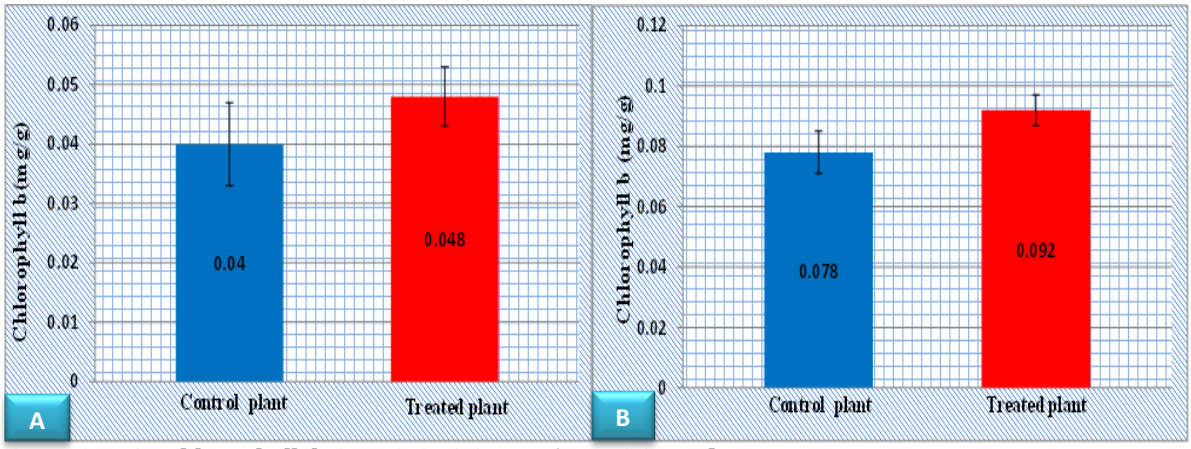


Figure (4-13): Chlorophyll b (mg/g) in (A): *L. minor* , (B): *C. demersum*

4-3-2 Enzymatic antioxidants

4-3-2-1 Superoxide Dismutase (SOD)

The statistical results indicated that a significant difference ($p \leq 0.05$) did not appear in the SOD values in *L. minor* plant, where the

plant which represents the control recorded a value within (60.78 U/ml), while SOD values increase slightly after completing the treatment which was within value of (62.04 U/ml) Figure (4-14) (A). From other side, the statistical results indicated that there was a significant difference ($p \leq 0.05$) in the SOD values in *C. demersum*, where the control plant recorded a value (54.89 U/ml) compared to the plant after treatment, which recorded a value of (64.57 U/ml) . Figure (4-14) (B)

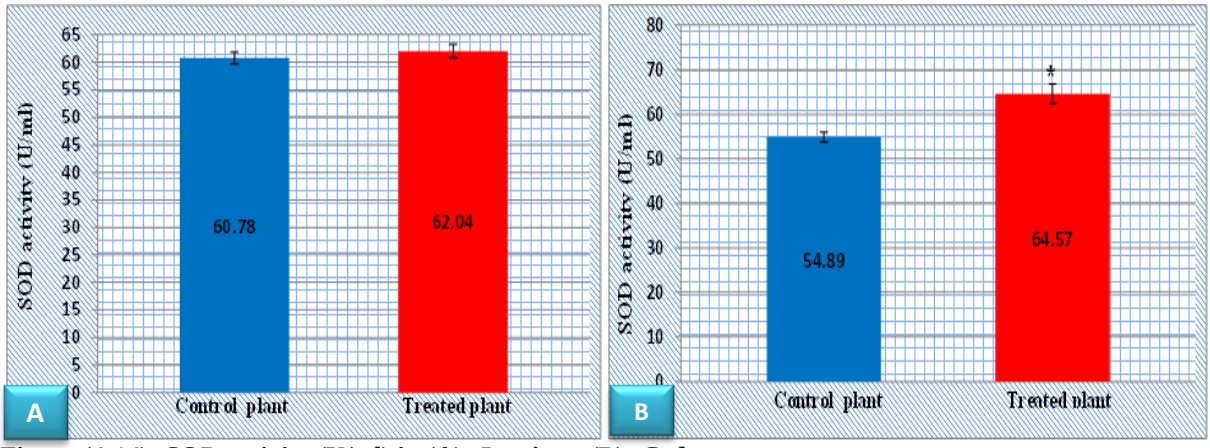


Figure (4-14): SOD activity (U/ml) in (A): *L. minor* ,(B): *C. demersum*

4-3-2-2 Catalase (CAT)

The catalase values that recorded in *L. minor* plant slightly lower after treatment where the control plant recorded value (77.70 ku/l) compared to the plant at the end of treatment, which recorded value of (71.95) figure (4-15) (A). As for *C. demersum* plants, there has been recorded close values for enzyme content in the plant used in treatment and plant which represents control where recorded value in control plant (52.18 ku/l) compared to plant at the end of treatment which recorded value (53.86 ku/l) figure (4-15) (B). The statistical results indicated that no significant difference ($p \leq 0.05$) appeared in the enzyme values in the plants under study.

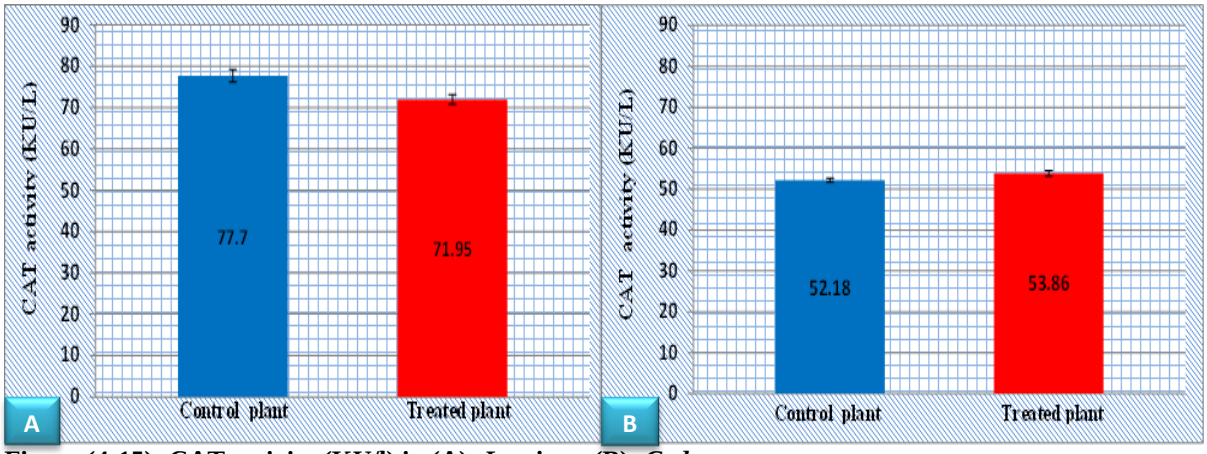


Figure (4-15): CAT activity (KU/l) in (A): *L. minor* ,(B): *C. demersum*

4-3-3 Malondialdehyde (MDA)

Figure (4-16) (A, B) shows the content of MDA in plants under study. As its values decreased in *L. minor* plants after treatment, as its content in plants that representing the control reached (1.11 nm/cm), but it decreased to (0.74 nm/cm) after completing the treatment. The same applies to *C. demersum*, where the control plant recorded its value (3.56 nm/cm) and decreased to (2.79 nm/cm) 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.

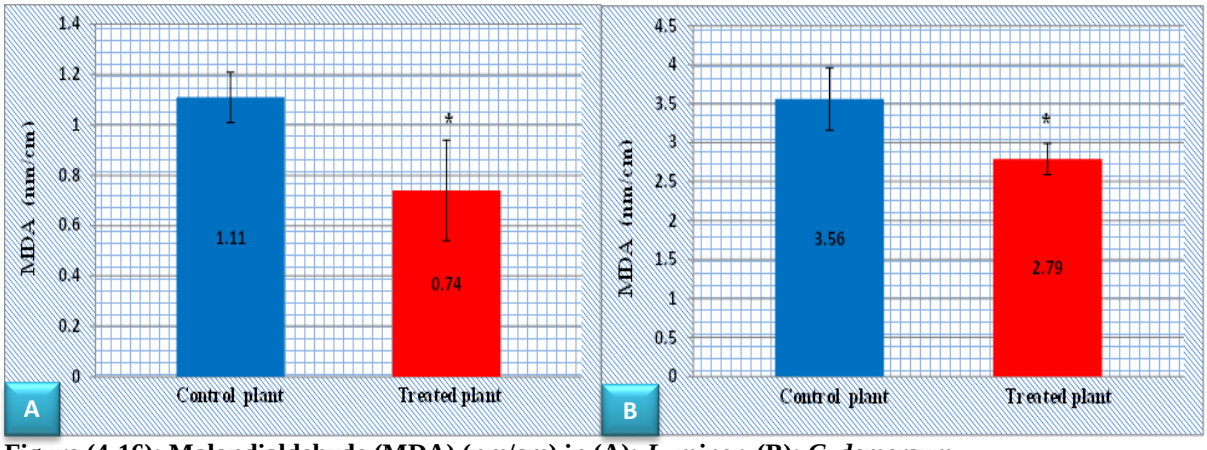


Figure (4-16): Malondialdehyde (MDA) (nm/cm) in (A): *L. minor*, (B): *C. demersum*

4-3-4 Total carbohydrate content

The concentration of carbohydrate in current study illustrated in the figure (4-17), which increased after treatment in the in both plants

compared with the control plant. Where the value of carbohydrate that recorded in the *L. minor* plant increase to (88.62 mg/g) after treatment compared to control plant that recorded (54.03 mg/g), at same time the concentration of carbohydrate in *C. demersum* plant increased after treatment and reached (152.36 mg/g) compared to the control plant that recorded (125.42 mg/g). The statistical results indicated that there was a significant difference ($p \leq 0.05$) in MDA values in *L. minor* plant.

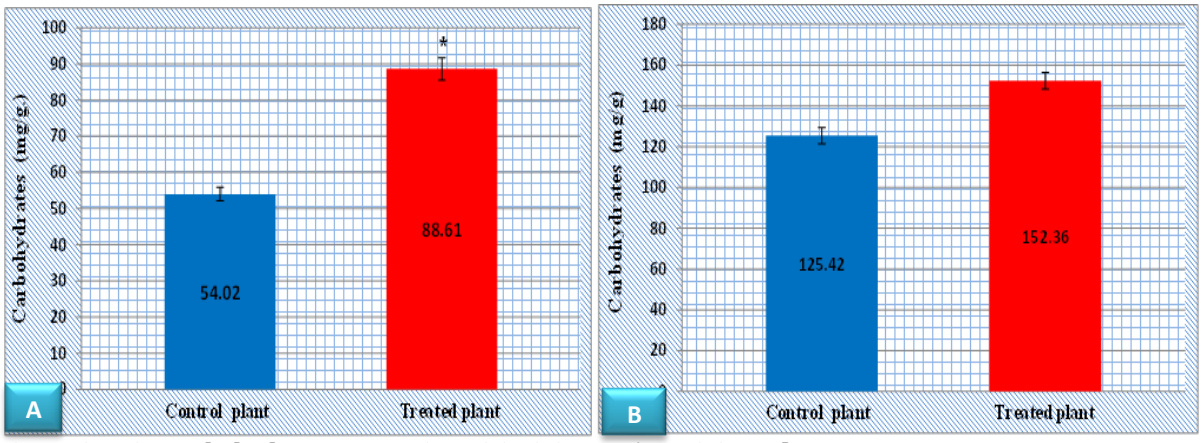


Figure (4-17): Carbohydrate content (mg/g) in (A): *L. minor*, (B*): C. demersum*

4-3-5 Total protein content

The statistical results of the current study indicated that there was a significant difference ($p \leq 0.05$) in protein values in both plants, where the results showed that the *L. minor* plant recorded a clear decrease in protein values after municipal wastewater treatment, which reached (15.13 mg/g) compared to the control plant, which recorded (23.94 mg/g) .The opposite happened with *C. demersum* plant, where the recorded value in plant that representing the control (5.44 mg/g), but it increased after wastewater treatment to (14.47 mg/g) , figure (4-18) (A,B).

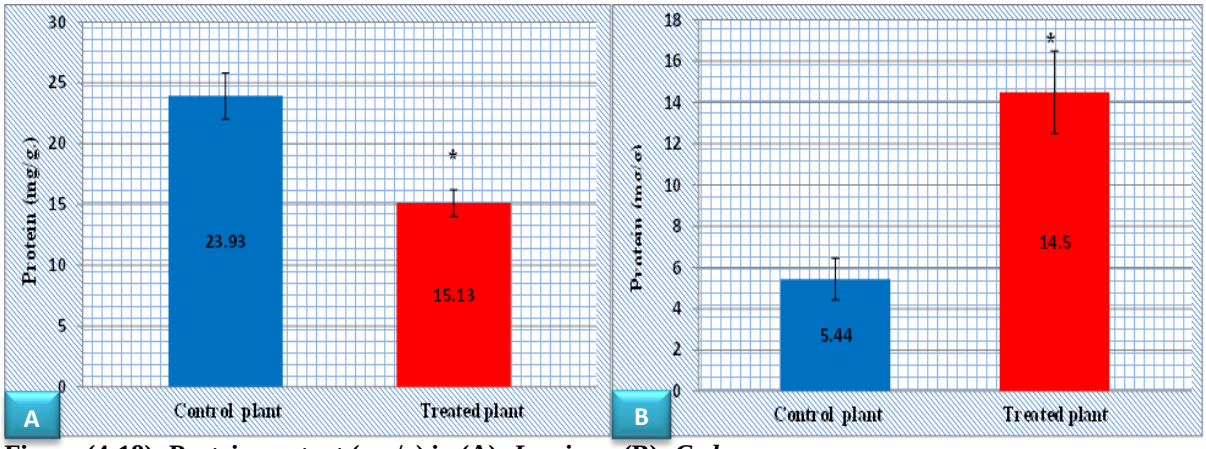


Figure (4-18): Protein content (mg/g) in (A): *L. minor*, (B): *C. demersum*

4-3-6 Proline content

The results of the current study indicate an increase in the concentration of proline in both plants compared with the control plant for each of them, this was shown through the statistical analysis that recorded a significant difference ($p \leq 0.05$) in proline values, where the value of proline that recorded in the *L. minor* plant was (15.41 µ mole /g) in the control plant, but it increased after treatment to (24.68 µ mole /g). The same is the case for the *C. demersum* plant, where the proline concentration increased after treatment and reached (17.64 µ mole /g) compared to the control plant that recorded (12.49 µ mole /g) figure (4-19) (A, B).

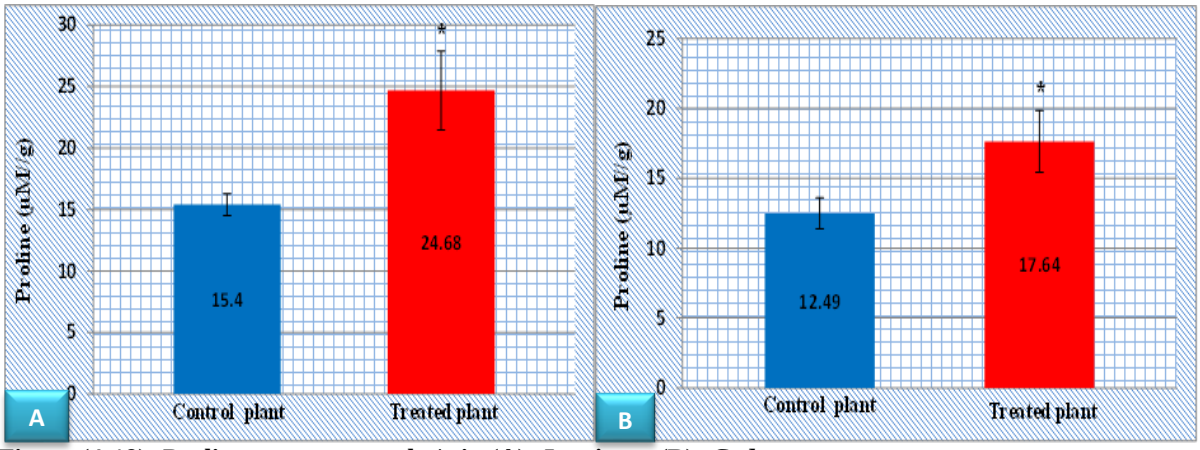


Figure (4-19): Proline content µ mole /g in (A): *L. minor* , (B): *C. demersum*

4-4 Molecular Study

Genotypes of *L. minor* using DNA barcoding marker technique

The genomic DNA was extracted from the *L. minor* samples as a first step to amplify the target region Figure (20).

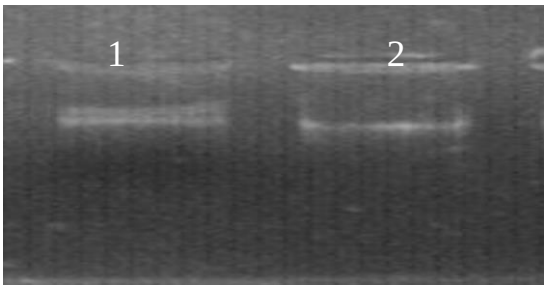
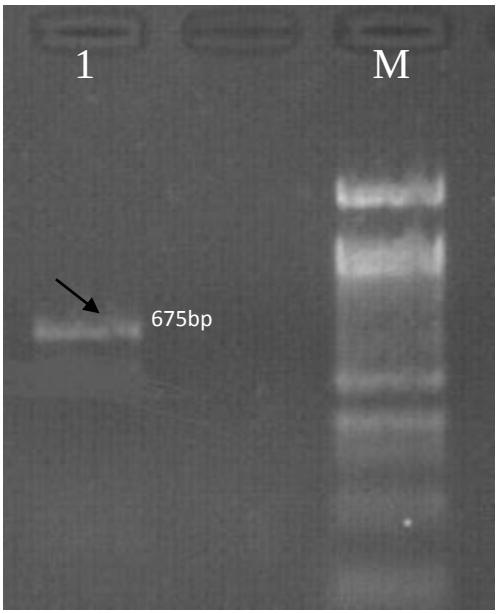


Figure (4-20): Gel electrophoresis pattern of genomic DNA extracted from *L. minor* leaves samples on 1% agarose at 70 volt for 1 hour.

Based on its reliable amplification the atpF-atpH noncoding spacer could serve as a universal DNA barcoding marker for *L. minor* The results revealed that the presence a single band (675 bp) Figure (21).



p

Figure (4-21): Agarose gel electrophoresis of atpF-atpH amplified product (675bp)
M: refers to DNA size marker; Electrophoresis conditions: 1% agarose concentration, 75 V, 20 mA for 120 min, stained with ethidium bromide.

Chapter Five

Discussion

5- Discussion

Much attention has been given to the treatment of the wastewater with the aquatic plants that having economic values and recycling of treated water. The green plants degrade, metabolize, assimilate or detoxify organic and inorganic pollutants from the environment or render them harmless, *L. minor* has been commonly used as a test organism in eco-toxicological and environmental studies due to its high sensitivity to various chemicals, small size, rapid vegetative reproduction and easy handling in laboratory conditions (Khellaf and Zerdaoui, 2010).

5-1 Phytoremediation

5-1-1 pH

The pH values in water is one of the most important factors that affecting the metabolism and physiology properties of aquatic organisms, because it affects the availability of elements and nutrients (Lawson, 2011). In the current study, the pH values of the treated water increased towered the base direction and the pH values in the *C. demersum* plant was significantly higher than that of the control and *L. minor* plant. The increasing pH in wastewater treated with *C. demersum* plant may be due to decreasing carbon dioxide from the photosynthesis (Crites and Tchobanoglous, 1998), or may be due to increasing the presence of algae and aquatic plants by which the photosynthesis process in which carbon dioxide present in the water is withdrawn will increase. Thompson *et al* ,2003). when the free CO_2 is below its equilibrium with air, an increase in pH will occur. Thus, 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 and Puetpaiboon ,2004). The pH values are also affected by the factors that regulating the acidity such as CO_2 and HCO_3,

where aquatic plants and algae have the ability to regulate the water acidity through the buffer capacity by taking and liberating positive and negative ions and achieving the balance within the surrounding aquatic environment (Ji *et al.,* 2010). The slight pH values increase was recorded by other studies such as the study by (kadhim ,2017) which showed that the pH values increased slightly when industrial and municipal wastewater treated with *C. demersum* and *L. minor*. Al-Asadi., (2014) also recorded a gradual increase in the pH values when water treated with the two plants of the Nile herb and *L. minor*. Mukherjee and Chatterjee., (2014) explain that the industrial waste water treatment with *H. verticillata* raised the pH values which macking them tended to the alkalinity. Al-Janabi., (2013) also recorded a gradual increase in the pH values when treating the industrial water for Chemical Industries with a group of plants such *C. demersum.*

5-1-2 Dissolved oxygen

Oxygen is one of the most important limiting factors in aquatic ecosystem (Morgan *et al*., 1993; Saeed *et al*., 1999). Oxygen solubility depends upon many factors such as temperature, salinity, organic compounds, the partial pressure of oxygen and density of algae and aquatic plants (Wetzel and likens., 2000; Ahangar *et al*., 2012; Sharma *et al*., 2012.)

The results of the current study showed an increase in oxygen values when sewage wastewater treated with *C. demersum* plant compared to the control . The aquatic plants efficiency to increase dissolved oxygen concentrations in the water may be as a result of increasing the effectiveness of photosynthesis (Liu *et al*.,2000). At same time aquatic plants differ in their role in aeration of water bodies, as some of them, such as submerged plants, play a distinctive role in increasing

the oxygen content in the water, as evidenced by experiments with artificial swamps that were created in wetlands (Chimney *et al*., 2006).

As for the decrease in the level of oxygen on the 25th day of treatment with *C. demersum* plant , it may be due to the decrease in the solubility of the gases at high temperatures, as well as the increase in the activity of microorganisms in the organic matter decomposition and this leads to an increase in the consumption of oxygen. (Al-Zorfi., 2010). When wastewater treating with *L. minor*, a decrease in the oxygen value was observed throughout the treatment period and this may be due to High cover of L.minor plant may produce a severe underwater light attenuation and prevent water circulation (Large *et al*., 1996) and lead to a strong oxygen depletion in the water column (Caraco *et al*.,2006). Also, the presence of plants in a manner that covers the surface led to a decrease in the chances of oxygen exchange between the water surface and the atmosphere (Beheary *et al*.,2019).

5-1-3 Electrical conductivity

Electrical conductivity values refers to the numerical expression of the positive and negative ions (APHA, 2003). It expresses the ability of water to carry electric current and is considered as an indicator of dissolved salts in water, it is closely connected with total dissolved solids, and increases in areas that fall under the influence of agricultural and industrial activity (APHA, 1976).

The current results indicate that the ability of plants to reduce electrical conductivity values was very weak and was limited to the first days of treatment. This is may be due to the presence of high concentrations of some ions such as chloride (Hutchinson, 1957). Also, may be due to the addition of some chemicals to the water such as alum, sulphates and chlorides that increase ions in water (Al-Salman and Abu

Bakr, 2003). These results are consistent with what was recorded in previous studies such as Abouel-Kheir *et al*., (2007) which showed that the electrical conductivity of water treated with aquatic plants increased to exceed the actual values at Zero time. Wendeou *et al*., (2013) explained that the electrical conductivity in wastewater was constant at the beginning of treatment with the *Lemna* sp plant but, after that, it began to increase significantly especially after the sixth day of the experiment, which attributed this to the role of plants in the analysis of compounds containing mineral elements in the process of (phyto-degradation). Where the decomposition process leads to the liberation of certain minerals in an ionic dissolved form that have the ability to transmit electricity, and this in turn increases electrical conductivity depending on the concentration and quality of dissolved ions (Al-Wahaibi, 2007).

Kadhim., (2017) showed the inability of the *L. minor*, *C. demersum and H. voticellate* to reduce the conductivity values throughout the treatment period. This may be due to the correlation effect for evapotranspiration phenomenon and increase the biomass of the plant which rise the values of electrical conductivity in water (Wendeou *et al.*, 2013).

5-1-4 TDS

TDS are made up of negative ions of compounds combined with positive ions such as sodium salts, magnesium salts, calcium salts, bicarbonate, sulfate, chlorides and dissolved organic matter (Weber and Duffy, 2007).

Plants in this study show weak efficiency in reducing TDS values which restricted to first days of treatment and recorded values lower than that of control water. The weakly efficiency of plants in reducing it may

be due to the fact that the decomposition processes in sewage were higher than the plant's ability to reduce them or that some ions did not have a distinct role in the metabolism of plant so it was absorbed in little value (Patel and kanungo,2010). The current results are in agreement with other studies such as (Abouel-Kheir *et al* ,2007; Wendeou *et al*., 2013; Kadhim ,2017 and Yaseen ,2021).

5-1-5 Dissolved nitrogen compounds

5-1-5-1 Total dissolved nitrogen (TDN)

Nitrogen is a major component in municipal wastewater, agricultural lands, storm water runoff from urban and wastewater from various types of industrial processes (DeBusk, 1999). The nitrogen is composed from different forms that can exist in water, such as particulate and dissolved organic nitrogen, ammonium, nitrite, and nitrate. These various forms can transform and serve as sources or end products for each other within the nitrogen cycle (Dotch and Gerald, 1995). The removal of large amounts of nitrogen may be due to the processes of ammonia volatilization, nitrification, denitrification, and microbial assimilation, as well as the uptake of plants such as duckweed (Vermaat and Hanif ,1998).

The current results indicate that the aquatic plants showed efficiency in reducing the total dissolved nitrogen concentrations throughout the treatment period with a closeness in the percentages of removal with the control treatment at some times of sampling This is due to the fact that organic nitrogen constitutes the largest proportion of the total concentration of nitrogen. Whereas, the control treatment was efficient in removing organic nitrogen at these times. The ability to remove nitrogen is due to the fact that aquatic plants can assimilate

nitrogen for their growth and provide a good habitat for bacteria to enhance nitrification and de-nitrification which should result in higher nitrogen removal efficiency (Thongchai and Udomphon, 2004). Where nitrogen is an essential plant nutrient, thus, it can be removed through plant uptake of ammonium or nitrate and stored in organic form in wetland vegetation, in addition, Substantial removal of nitrogen may take place through settling of N containing particulate matter in the wetland inflow (DeBusk, 1999).

5-1-5-2 Total inorganic nitrogen(TIN)

The most important inorganic nitrogen forms in wetlands are ammonium, nitrite and nitrate. Nitrogen as gaseous forms may exist as di-nitrogen, nitrous oxide, nitric oxide and ammonia. Some processes of nitrogen cycle relate to microbes activity which include nitrogen fixation, in this process ,atmospheric molecular nitrogen is reduced by cyanobacteria and bacteria to ammonium ,the main nitrogen transformation pathway is the nitrification/ de-nitrification where ammonium is oxidized first to nitrite and is then oxidized to nitrate,The de-nitrification process oxidize organic matter by using nitrate as a terminal electron acceptor with a byproduct of N_2O or N_2 by facultative anaerobic bacteria (Andrzej *et al* , 2019).

The current study showed the role of the studied plants in the reduction of total inorganic nitrogen, with a clear superiority of the *C. demersum* plant . In fact, the nitrogen losses in the constructed wetland systems are related manly to many removal mechanisms such as ammonia volatilization, ammonification (mineralization), anaerobic ammonium oxidation (anammox), di-ssimilatory reduction, autotrophic nitrification and heterotrophic de-nitrification, plant and microbial assimilation and re-mineralization during decomposition, sedimentation,

filtration, adsorption and microbial assimilation (Vymazal and Kropfelova, 2008).

In constructed wetlands, microbial processes are the main mechanisms for removal of nitrogen from wastewater (Stottmeister, 2003). As for the superiority of the *C. demersum* plant in removal, this is due to the plant's ability to withdraw large quantities of inorganic nitrogen forms, where explained Tracy *et al.* (2003) that *C. demersum* plant can tolerate high nitrogen concentrations and has a good removal effect on nitrogen in the water .

5-1-5-2-1 Ammonia

Ammonia when taken by Plants , it is incorporated into proteins and other organic combinations by many biochemical reaction, the ammonium consider as only ion that assimilated into the organic molecules in the plant tissues by means of enzymatic process (Masclaux-Daubresse *et al* ,2010).The ammonium ions can be absorbed directly by the roots of plant , it is further assimilated into the group of amide amino of glutamine by the glutamine synthetase and subsequently in to glutamic acid by the glutamate synthase, These two enzymes resulted in the assimilation of most ammonium ions (Masclaux-Daubresse *et al* ,2010. Ammonium ions can be volatilized as ammonia so that the NH^{4+} concentration in the water is reduced. Thus, at pH more than 7, the equilibrium of NH_3/NH_4^+ is shifted towards ammonia (NH_3) (Sudiarto *et al*., 2019).

The plants under study demonstrated high efficiency in reducing ammonia values at all days of treatment compared to the control. The results show the effective role of both plants in reducing ammonia where Plants prefer ammonia first, then nitrate as a nutrient source whereas nitrite is considered an intermediate state (Selvarani *et al*, 2015). The

rapid reduction of ammonia nitrogen is may be due to the active nitrification as evidenced by an increase in NO_3 concentration in water (Selvarani *et al* ,2015). Freely floating plants have the ability to remove nitrogen from the wastewater through de-nitrification processes and subsequently combine them in their biomass (Suhad *et al* ,2018).

As for *C. demersum* plant where most structures of this Submerged plants found below the water, their photosynthetic tissues are entirely submerged with in water and hence the turbidity of waste water must be low, because high turbidity blocks the transmission of light to plants (USEPA, 1999). The plants depletes the dissolved organic carbon in water and increase the dissolved oxygen, this lead to increment of pH value and volatilization of ammonia and chemical precipitation. High oxygen concentration also creates favorable condition for mineralization Bekele , 2018).

The current study agreed with several studies, such as the study by Shen *et al.*, (2006) which recorded high Percentage reduction of ammonia by duckweed plant . Muradov *et al.*, (2014) which reported that the removal efficiency for duckweed was 54.9%. Huang *et al.*, (2013), Zhang *et al.,* (2014) and Wang *et al.,* (2014) reported that the aquatic plant *Lemna minor* takes up NH_4^+ readily and grows well at concentrations of the ion of up to 84 mg/l, it takes up $NH_4^+$and NO_3^- through both roots and the lower surface of the fronds, and may prefer NH_4^+ to NO_3^- (Fang *et al.* 2007). Kutty *et al.,* (2009) noted that the maximum removal of ammonia by Duckweed was 98% in municipal wastewater. Zaltauskaite *et al.*, (2014) explained that the *Lemna minor* plant removed 50 - 75 % ammonia. Forughi *et al.*, (2013) also indicated that the *C. demersum* plant, which was used in wastewater treatment, recorded an efficiency in removing ammonia by 62%. The nutrients removal in the control, may be related to the uptake by algae and microbes that utilize nutrients during

their growth. Srivastava *et al.*, (2008) noted that the decrease in nutrient concentrations from the control was due to uptake by microorganisms and other biological activities taking place.

5-1-5-2-2 Nitrite

Nitrite is an intermediate product when the biological conversion of Ammonium to nitrate occur by process named nitrification through two-step, the first step include the conversion of ammonia and ammonium to nitrite which is performed by ammonia-oxidizing bacteria. Then, the second step occur by nitrite-oxidizing bacteria which complete the conversion of nitrite to nitrate, if the wastewater is subsequently subjected to anaerobic conditions, the de-nitrification process may occur which mean the biological reduction of nitrate to nitrogen gas by facultative heterotrophic bacteria , this process occurs when oxygen levels are depleted and nitrate becomes the primary electron acceptor for microorganisms, Some nitrogen is converted into nitrogen oxide (NO) or nitrous oxide (N_2O) and leaves the system in the gas phase (Dongke,2012).

The current results indicate that the aquatic plants showed high efficiency in reducing the nitrite concentrations throughout the treatment period which starting from the first day until the end of the treatment period, with a clear superiority of *L. minor*. The high efficiency of *L. minor* plant may be due to the removal of the nutrient forms such as nitrogen is due to their consumption by the new developing tissues and not by increasing the nutrient content in the old tissues (korner and Vermaat,.1998). Where Duckweed is able to take up nitrogen in the form of nitrate, nitrite, ammonium, urea or amino acids. However, the most important substances are nitrate and ammonium Landolt *et al.*, (1987) and Foroughi *et al.*, (2013) noted that the removal of nitrogenous forms by the

plant depends on the growth rate and the nitrogen content of the tissue. The high *L. minor* plant efficiency in nitrite reduction was recorded by other studies such as the study by Shen *et al.,* (2006) which recorded high percentage reduction of nitrite by duckweed plant

As for the efficiency of *C. demersum* plant in reducing nitrite values compared to control, this may be attributed to submerged aquatic vascular plants are known to absorb nutrients such as nitrogen and far in excess of their normal metabolic requirements (Wilson.,1972).

5-1-5-2-3 Nitrate

Nitrate consider as the stable form of nitrogen, and always record high value in contrast with the other two forms of inorganic nitrogen and this high values may be due to higher rate of oxidation but nitrogen was found to be absorbed as nitrate, hence exhibited lower values after the phytoremediation (Patel and Kanungo ,2010).

Plants in this study show high efficiency in reducing nitrate values and especially in the first days of treatment compared to the control, but the efficiency decreased in the last days of treatment. This high treatment efficiency of floating and submerged plants is due to several reasons. As for the *L. minor* plant ,it is one of the plants known to assimilation of nitrogen by fronds and roots and this is appears to be the primary mechanism of nitrogen fixation in it. Where Nitrogen is fixed as protein in duckweed biomass (Cedergreen and Madsen, 2002). Both of nitrate and ammonium consider as are the main forms of available nitrogen for duckweed. However, the absorption of ammonium is 3 to 11 times greater than nitrates .Also some portion of nitrogen is also absorbed into *L. minor* biomass through associated nitrogen fixing cyanobacteria and algae grown in duckweed ponds (Duong and Tiedje, 1985).

The poor nitrate treatment in the last days of treatment may be due to the presence of high concentrations of ammonia and nitrite, or the inhibition of the nitrification process, which includes biological oxidation of ammonia to nitrate by two types of autotrophic bacteria, these bacteria are, in fact, very sensitive to values of PH, temperature and chemical compounds in wastewater (Hockenbury and Grady, 1977). Godbold and kettner., (1991) also indicated that the absorption of some heavy metals such as lead by plants reduces the absorption and transport of some nutrients .On the other hand, Foroughi *et al.*, (2013) explained that the age of the plant plays a role in the removal, as the young plants are more efficient than the old ones in the removal process .Ayyasamy *et al.*, (2009) stated that if the plant is not harvested in a timely manner, then the nutrients can return from the plant body in the opposite direction to the water. Therefore, nitrogen removal by the plant depends on the growth rate and the tissue nitrogen content, as its content varies and depends on several factors such as plant type , age of the plant, the vegetative part where nitrogen is stored(Foroughi *et al*, 2013).

The results of the current study are in agreement with many studies, such as the Shen *et al.*, (2006) had recorded similar observations for duck weed plant and recorded percentage reduction ranging 35-65%.

Zaltauskaite *et al.*, (2014) explained that the *L. minor* plant removed 58.3% of nitrate. Forughi *et al*., (2013) indicated that the nitrate removal rate reached 41% when using the *C. demersum* plant in wastewater treatment, as the best removal rates were after the sixth day, but the removal efficiency decreased after the 18th day of treatment.

5-1-5-3 Dissolved organic nitrogen(DON)

The largest pool of fixed nitrogen in most aquatic systems is Dissolved Organic Nitrogen (DON) (Bronk, 2002). Dissolved organic

nitrogen is one of the primary nutrients that causing low dissolved oxygen conditions with discharges from wastewater treatment plants being one of the major contributors (Pehlivanoglu and Sedlak, 2004; Bronk et al., 2010; Pagilla et al., 2011). DON consists of urea, amino acids, nucleic acids, proteins, humic substances and a variety of uncharacterized compounds (Bushaw *et al*., 1996; Berman and Bronk, 2003; Pehlivanoglu-Mantas and Sedlak, 2008). Recent studies indicate that a major portion of the wastewater effluent TDN is generally in organic form(DON) which ranging from (25 - 80%) of the effluent TDN (Pehlivanoglu-Mantas and Sedlak, 2006; Sattayatewa et al., 2009.(

In the current study, aquatic plants did not show a high efficiency in reducing the values of dissolved organic nitrogen compared to the control. This may be due to the low penetration of light into the treatment basins that contain both submersible and floating plants. Where light play major role in breaking down dissolved organic nitrogen in to lower molecular weight labile substances, and ultimately makes DON biologically available to algae, bacteria and phytoplankton in the aquatic ecosystem (Moran and Zepp, 1997; Bushaw-Newton and Moran, 1999; Koopmans and Bronk, 2002; Bronk et al., 2010). Also, bacterial activity and food/microorganism ratio (depending on the characteristics of substrate and microorganisms) are crucial factors for DON removal. Where Effluent DON can be biodegraded to various compounds such as urea, free amino acids, nucleic acids and several uncharacterized labile compounds under certain environmental conditions and eventually ammonia. BDON is defined as a fraction of DON that can be ammonified by bacteria (Parkin and McCarty, 1981a).

5-2 Physiological and biochemical markers of aquatic plants

5-2-1 Total chlorophyll, chlorophyll a, chlorophyll b

Chlorophyll is one of the pigments responsible for photosynthesis and is found in plastids (Lefsrud and Kopsell, 2005) . These pigments need many necessary elements such as calcium and magnesium in addition to iron (Sarvari *et al.,* 2008). Chlorophyll pigment is a major indicator of the physiological status of plants (Steele *et al.,* 2008),Chlorophyll content in plant often measure to assess the environmental stress amount due to the fact that the changes in chlorophyll content associated with the appearance of visible symptoms on plant (Pessarakli, 2011).

The results in the current study showed that the total chlorophyll , chlorophyll a, chlorophyll b values in *L. minor and C. demersum* increased after completing the phytoremediation. The increase in chlorophyll content in plant may be due to stimulated the synthesis of chlorophyll in plant after treatment. Zengin and Munzuroglu., (2005) indicated that the treated wastewater stimulated the synthesis of chlorophyll in plant, the impact of nitrogen compounds on the chlorophyll contents was due to the fact that nitrogen is one of a constituent of chlorophyll molecule and consider as the main component of all amino acids in proteins which are important constituents of chloroplast.

5-2-2 Enzymatic antioxidants

5-2-2-1 Superoxide Dismutase (SOD)

SOD is the first defense line in the antioxidant system which can eviate the toxic effect of O_2^- in plants (Gupta *et al* ,2013). O_2^- is one of

the major ROS produced in cells in the physiological state (Yadav *et al*., 2018).

The statistical results indicated that a significant difference did not appear in the SOD values in *L. minor* plant in the plant which represents the control and treated plant. from other side, the statistical results indicated that there was a significant difference in the SOD values in control and treated plant in *C. demersum*.

The enzyme values did not exhibit significant changes in *L. minor* plant and this is may be due to the fact that exposure of plants to high concentrations of some pollutants leads to synthesis of ROS in tissues and increased rate of DNA damage, thus reducing the effectiveness of SOD (Ai-jun *et al*, 2007).

This result is consistent with many studies, such as the study of Hanfeng *et al*., (2010), who explained that the changes in antioxidant enzyme activities of *C. demersum* were significantly influenced by its nutritional level where under hypertrophic , eutrophic and mesotrophic conditions, antioxidant enzyme activities such as (SOD) will be increase ,and this is indicating the *C. demersum* exposure to stress under these nutritional conditions and this is has the effect in active oxygen elimination in the plant , which then contributes to the enhancement of the protective normal structure and the function in plant , as well as resistance to stress.

5-2-2-2 Catalase CAT

CAT can decompose H_2O_2 which consider as another toxic ROS that produced mainly by the activity of SOD directly in to H_2O and O_2 in plant cells which play important roles in the main defense mechanism (Scandalions,1993).

The statistical results indicated that a significant difference did not appear in the CAT values in both the plant that represented the control and treated plant in *L. minor and C. demersum* plant . Dian *et al.*, (2018) showed that the CAT activity in the submerged plant *H. verticillata* 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. Also, the lack of change in the activity of the enzyme may be the result of the oxidative stress of some factors such as increased salinity and drought in addition to the presence of various pollutants such as heavy metals (Karuppanapandian and Manoharan, 2008), and this in turn will reduce the efficiency of the plant to withstand environmental conditions (Karuppanapandian *et al.*, 2011).

5-2-3 Malondialdehyde (MDA) content

The most sensitive parts of cells are plasma membrane, which is show symptoms when plants are exposed to oxidative stress. MDA is a major aldehyde product of lipid peroxidation that has been used as an indicator of oxidative stress (Velikova *et al.* ,2000). MDA is the one of the end product for peroxidation that reflecting the plants grow under adverse conditions (Li, 2000).

The content of MDA was decreased in both plants under study 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).Where The higher activities of antioxidants may be resulted in lower concentrations of O_2^- and H_2O_2, thereby limited the cellular damages that possibly caused by ROS. On the other hand, the decrease in the values after treatment may be

due to the increase in the time period, as Tlidjen *et al*., (2012) was shown that the continuation of treatment for 21 days led to a decrease in the MDA content, the reason is that over time, necrosis and plant cell death will increase which will stop the production of MDA.

5-2-4 Total carbohydrate content

Soluble carbohydrates consider as important metabolites in metabolism in plant which play a crucial role in the defending system against stress as well as in the detoxification of foreign substances (Harborne and Turner,1984). The content of soluble carbohydrates in plants is liable to be effected by external stress (Costa and Spitz ,1997).

The concentration of carbohydrate in current study increased after treatment in the in both plants compared with the control plant, especially in the *L. minor* plant, where the increase was clear and the statistical results indicated that there was a significant difference in carbohydrate values in it. The increase in carbohydrate values may be due to the Soluble carbohydrates can be used as energy sources to support the complex detoxification processes, such as the removal of nitrate-N from the plant cells and assimilation to free amino acids (Touchette and Burkholder ,2000).

Soluble carbohydrates not only provide osmotic adjustment, protect macromolecules (such as proteins) and membranes, but they can also fuel carbon for energetic metabolism when photosynthesis is reduced and play pivotal roles as signaling molecules, regulating biosynthesis and sensing of plant hormones (Bartels and Sunkar,2005). Carbohydrate that distribution within plants is affected by nitrogen supply which is influences on the processes of carbon assimilation, allocation and partitioning (Kaiser,1997). Almodares *et al.,* (2008) showed that increase in sugars content occure with increasing level of N nitrogen supply

5-2-5 Total protein content

The dissolved protein content is an important indicator of the physiological state of plant (Doganlar *et al*., 2010). It has an important role in the metabolism and membrane of the cell as it regulates the processes that overlap the external and internal membrane (Kharat *et al*., 2009). The soluble proteins content changes in plants under stress conditions are responsible for adaption in metabolic pathways (Amini and Ehsanpour ,2005).The synthesis and degradation of protein respond differently to stress conditions according to the stress resistance of plants.

The results of the current study showed that the *L. minor* plant recorded a clear decrease in protein values after phytoremediation of wastewater. As for *C. demersum* plant, the results were opposite, as an increase in protein values was observed compared to control.

The soluble proteins content reduction may be caused by protein degradation under stress (Palma *et al* ,2002),or by protein fragmentation due to the toxicity of reactive oxygen species (John *et al* ,2008).Where reactive oxygen species is an oxygen-containing chemical reaction molecule such as hydrogen peroxide, superoxide anion (O_2), and hydroxyl radical (OH^-) that leading to a oxidative stress that produces these compounds as side products during metabolism that effect in plant cells and lead to their death as well as the breakdown of protein (Smirnoff, 2005). Also may be due to the toxic effects of salinity on the physiologically active parts of the tissues where high salinity influence the cell content of amino acids and reduce RNA and DNA in the plant (Izzati, 2016). Redundant ROS damage the nucleic acids, proteins and lipids in ammonia stressed cells (Zinta *et al.*,2016). Decline level of protein may be due to decrease in Nitrate reductase activity because this

type of enzyme is believed to be rate limiting in the overall assimilation of nitrate (Beevers and Hageman 1969).

From other hand ,the increase protein content in *C. demersum* plant may be as a result of absorption of nitrogen compounds such as ammonia , nitrite and nitrate where nitrate is reduced to nitrite and the last is transformed in to ammonium which is assimilated in to amino acids and other organic compounds(Kim *et al*.,2013) .Amino acids are the main substrate for protein synthesis in the cells, they can participate in coordinating the plant's carbon metabolism, becoming part of the basic process of the plant's life activities (Wang *et al* .,2013).

5-2-6 Proline

Proline is an amino acid which maintains the plant cells vitality under drought and salinity conditions because it prevents or reduces the proteins breakdown in the cell (Pessarakli ,2011). Accumulation of proline plays adaptive role in plant stress tolerance (Verbruggen and Hermans ,2008). Reactive oxygen species are easily produced in plant cells by osmotic stress and can injure the plant cells if they are not eliminated (Zhang *et al.* 2005). Thus, Proline is usually synthesized in large amounts under conditions of biotic stress and abiotic stress such as water deficiency, salt stress and heavy metal stress (Lobato *et al*., 2010; Costa *et al*.,2008; Silveira *et al*., 2003;Chen *et al*., 2001).

The results of the current study indicate an increase in the concentration of proline in both plants compared with the control plant for each of them, this increase may be due to fact that the Proline accumulation can increase dramatically in response to rising ammonia concentrations in aquatic environments (Xu *et al* ., 2012; Lee *et al.*,2013). Previous studies revealed that excess ammonia-N in plant tissues caused water imbalance in cellular and whole plant body by decreasing calcium

and potassium uptake (Roosta & Schjoerring,2007). It is well known that the large amounts of free ammonium in plant tissues cause ammonium toxicity (Britto and Kronzucker., 2002; Roosta and Schjoerring., 2007). Generally, excess ammonium decrease Ca^2 and K uptake, leading to imbalance in plants, and it thus inhibits water uptake (Britto and Kronzucker .,2002).Thus ,the accumulation of proline may prevent loss of water by maintaining membrane integrity ,inhibiting protein denaturation and sustaining cell turgor (Kim *et al*.,2004; Neuberg *et al*.,2010). Where proline accumulation is associated with osmo-protection functions (Cayley *et al.*, 1992), osmotic adjustment (Neto *et al*., 2009) and antioxidant activity (Sharma and Dietz .,2006).

Proline known works to protect plants from the influence of ROS, as it plays an important role in osmoregulation and protection of enzymes (Nikolopoulos and Manetas., 1991), stabilization of protein synthesis (Kadpal and Rao., 1985) and as scavenging of free radicals (Smirnoff and Cumbes., 1989).

5-3 Genotypes of *L. minor* using DNA barcoding marker technique

DNA barcoding is a valuable tool for taxonomists. It can be used to identify species efficiently and accurately on the basis of a standard region as a marker. To identify an ideal region in plants, which must be sufficiently variable to differentiate all the species and conserved enough to be minimally variable within species, is nevertheless a challenge (Kress *et al*., 2005; Liu *et al*.,2010) . Various loci have been tested and evaluated as DNA barcodes, and different studies have tried to define a standard barcode for plants.

Four plastid encoded genes (rpoB, rpoC1, rbcL and matK) and three noncoding spacers (atpFatpH, psbK-psbI and trnH-psbA) have been

suggested as optimal for fingerprinting genotypes of plants (Group *et al*.,2009; Hollingsworth *et al*.,2009). As expected, the coding markers (rpoB, rpoC1, rbcL and matK) were conserved in PCR product length, while the noncoding spacers (atpF-atpH, psbK-psbI and trnH-psbA) displayed more length variability. The range of amplified fragments length was 579-622 bp for atpF-atpH, 185- 576 bp for psbK-psbI and 286-504 bp for trnH-psbA among all analysed ecotypes. However, no simple correlation between amplified intergenic fragment length and the phylogenetic position of the corresponding species has been observed for any of those markers. Because of sufficient interspecific variation and relatively low intraspecific variability that was observed in duckweed, the atpF-atpH spacer was suggested to be a universal DNA barcoding marker for species identification in Lemnaceae (Wang *et al*.,2010).

Appenroth *et al* ,(2013) reported that appears to support the utility of atpF-atpH, as a more universal DNA barcoding marker for identification of duckweed species, several plastidic markers would need to be employed in order to obtain reliable conclusions for species identification of duckweeds.

Conclusions and Recommendations

Conclusions

1- From the work presented in this study it can be concluded that both submerged *C. demersum* and the floating *L. minor* were effective in removing dissolved nitrogen compounds from wastewater

2- The pollutant removal rates were efficient for all the pollutants estimated in this study except for TDS and E.C that recorded high values at end days of treatment

3- Plants did not show an obvious increase in CAT activity and chlorophyll b for each plant and SOD activity for *L. minor* plant after treatment. A significant increase was show in total chlorophyll, chlorophyll a, Carbohydrates and Proline content for each plants and the protein for *C. demersum* plant, but protein content for *L. minor* plant decreased after treatment. A significant decrease was obvious in MDA content in each plant after completed the phytoremediation period

4- Increase and decrease of some biomarkers indicates that both plants were subjected to oxidative stress during treatment. Nevertheless, both plants continued to remove pollutants until the end of the experiment

Recommendations

1. Testing the ability of wastewater treatment systems that based on floating plants in quantitative and qualitative reduction of algae by following the numbers and types of algae and study the Diversity index and richness values.
2. Study the efficiency of duckweed aquatic system in eliminating bacteria such as fecal coliform counts
3. A comparison study of the efficiency of different water treatment systems such as Free Water Surface Constructed Wetlands, Constructed Wetlands with Horizontal Subsurface Flow, Constructed Wetlands with Vertical Subsurface Flow and Hybrid Constructed Wetlands in reducing some pollutant concentrations such as nutrients and heavy metals.
4. Conducting extensive studies that include biological treatment with aquatic plants and investing the living masses of these organisms in the production of biofuels, especially with regard to the duckweed aquatic system. It also should be study the efficiency of some compounds that extracts from it as active anti-bacterial and anti-fungal substances.

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