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Effects of Sodium Chloride and Sodium Sulfate on *Ceratophyllum demersum* under Laboratory Controlled Conditions

Enas A. Mahdi Al-Nabhan, Dunya A. Hussain Al- Abbawy, and Nayyef M. Azeez

Department of Ecology, College of Science, University of Basrah, Basra, Iraq

*Corresponding author: dunya.hussain@uobasrah.edu.iq
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Abstract

Salinity is one of the most significant abiotic stresses for plant growth. This study aims to study how salinities on the *C. demersum* L plant, by using sodium chloride (NaCl) and sodium sulfate (Na_2SO_4) separately as well as in combination, affect fresh weight, relative plant growth, total chlorophyll and protein, and proline content. The experiments were conducted for 14 days. The result showed that the effect of Na_2SO_4 salt is more toxic than NaCl. Whereas the combined experiments with both salts show that their effects together were more harmful to the plant species at the same concentrations. The findings also revealed that high salt concentrations had a significant impact on the morphological and physiological characteristics. In the experiment assessing the impact of salt stress on plant growth and physiology, the treatment with a combination of 75 mM NaCl and 75 mM Na_2SO_4 resulted in significant reductions in several key parameters compared to the control group. Specifically, the treatment led to decreases in fresh weight (from 18.742 g to 5.723 g), dry weight (from 2.543 g to 0.845 g), relative growth rate (from 1.236 to 0.380), total chlorophyll content (from 10.212 µg/g to 2.699 µg/g), and protein content (from 42.03% to 30.180%). Additionally, this salt stress condition was associated with an increase in proline content, indicating a physiological response to the imposed salt stress. These results highlight the negative effects of elevated salt concentrations on plant growth and metabolic functions.

Keywords: Aquatic plant; Plant growth; Salinity stress; Toxicological effect

1. Introduction

Salinity is one of the critical environmental stressors affecting the aquatic ecosystem and negatively impacts water quality and biodiversity because salinity can inhibit the growth and productivity of plants worldwide (Ziarati *et al* 2019; Mahmood *et al.,* 2021; Santini, 2022). Furthermore, it affects a variety of plant morphological and biochemical processes, resulting in lower economic production (Balal *et al.,* 2022).

High concentrations of soluble salts can be found in both terrestrial and aquatic environments, and they can arise naturally or because of human activity (Gomes *et al.,* 2011; Mahmoud *et al.,* 2021). Because of anthropogenic activities, salinity is a global problem with ramifications that are not limited to a single location. It has been one of the most severe issues in Arabian countries. It has contributed to the loss of agricultural land, deteriorated crop productivity, and forced thousands of residents to migrate due to excessive salt tides and animal deaths due to a lack of freshwater. Only 10% of the world's agricultural land is unaffected by environmental stressors (Dogan *et al.,* 2020).

In an aquatic environment, salinity stress is a critical factor that affects the spread and distribution of aquatic plants (Makherana *et al.,* 2022; Abd *et al.,* 2015).

As aquatic plants are essential components of the aquatic ecosystem and play a role in protecting freshwater bodies. Their existence is evidence of the health of these ecosystems because they provide oxygen and plays a significant role in treating or accumulating various types of pollutants (Mishra *et al.,* 2022).

Plant salt tolerance is a multi-level process including molecular, organelle, cellular, tissue, and plant levels. Furthermore, salt-tolerant plants have a variety of adaptations, not only in morphological or structural aspects but also in metabolic and physiological processes, that allow them to thrive in severe saline environments (Imadi *et al.,* 2016). The concentration of salts and exposure time affect the tolerance of macrophytes to salinity. Therefore, it causes a variety of morphological, biochemical, and physiological changes in plants. It impacts practically every aspect of plant life, including germination, growth, and development (Grigore *et al.,* 2016).

Salinity produces osmotic stress in cells, which leads to intracellular ion buildup and disrupts ion homeostasis (Basu *et al.,* 2020; Munns *et al.,* 2021). High salinity impairs plant selective ion absorption at the cellular level, decreasing nutrition availability, due to the low water content (Shah *et al.,* 2022). Consequently, dehydration of cells due to a water deficiency. As a result, various morphological and physiological changes may develop (Hashem *et al.,* 2014). Changes in the functional and structural permeability of cell membranes, restrict stomata opening, causing reducing CO_2 absorption, and photosynthesis reduction. Nutrients such as potassium, calcium, magnesium, and iron are required for plant growth and the manufacturing of various plant components (Swapnil *et al.,* 2015; Aghajanzadeh *et al,* 2019). Plant imbalance occurs because of the increased salt ion in plant cells, resulting in decreased photosynthetic pigment and protein synthesis. Increased reactive oxygen species (ROS) are also a result of salt stress (Flowers, & Colmer, 2015; Swapni & Rai, 2018). As well as the plant's response to salinity is by increasing proline content, amino acid, total phenol, and ascorbic acid (Osman *et al.,* 2021).

Macrophytes use a variety of techniques in response to abiotic stresses, many of which enhance plant growth in stressful situations. Changes in morphological and developmental processes (growth plasticity), as well as physiological and biochemical processes in response to a variety of stresses, are examples of these phenomena (Saud *et al.,* 2014).

Herein, the focus is on *C. demersum*, commonly known as coontail, belonging to the family Ceratophyllaceae. It is rootless; submerged dicotyledon's seed grows well in subtropical and tropical weather regimes (Mahdi, & Al-Abbawy, 2019). Different studies were done to evaluate the effects of salt stress on aquatic plants. de Morais et al. (2019) studied the impact of salt stress on growth and physiological response. Gomes *et al.* (2017) estimated the chlorophyll content, carotenoids, proline, and nitric oxide (NO) content after exposure to different salinity concentrations. Senavirathna *et al.* (2020) improved the responses of *Myriophyllum spicatum* L. and *C. demersum* L. to salinity stress exposed the plants to different salinity concentrations.

It is noticeable that most studies focus on salt stress tolerance with NaCl because NaCl predominates and investigations using Na_2SO_4 or mixed Na_2SO_4 and NaCl salinity are rare, according to Swapnil *et al.* (2017). Therefore, this work aims to investigate the single and combined effect of NaCl and Na_2SO_4 salinity on the morphological and biochemical responses of *C. demersum*.

The study explores how NaCl and Na_2SO_4 salts, alone and combined, affect *C. demersum*, an important aquatic plant. It addresses a research gap by investigating the understudied impacts of Na_2SO_4 and combined salt exposure compared to NaCl alone. This research enhances our understanding of salt's effects on aquatic plants, aiding in strategies to mitigate salinization in aquatic habitats.

2. Methodology

2.1 Plant collection and acclimation

Samples of aquatic plants were taken from the Shatt Al-Arab River in Iraq that comply with relevant international guidelines and legislation (Al-Abbawy & Al-Zaidi, 2023). Spatial distribution and population

density of submerged aquatic vegetation in Shatt Al-Arab River. The collected samples of *C. demersum* were washed several times with river water before being put in a labeled plastic bag and transported to the laboratory. The plants were washed several times with tap water and distilled water to eliminate all solids and impurities before being placed in a 20 L plastic aquarium filled with tap water for a week to acclimate.

2.2 Salt concentration prepared

The Merck standard salts of NaCl and Na_2SO_4 were used to prepare different concentrations (25, 50, and 75 mM) separately and in combination by dissolving a known weight of the salt in 1 L deionized water.

2.3 Experimental setup

To experiment, 1.6 L plastic aquariums were filled with 1 L of known concentrations of NaCl and Na_2SO_4 salts, both individually and in combination, with three replicates for each concentration (25, 50, and 75 mM) using a randomized design. Three control aquariums were also prepared. During the experiment, 3% of Hoagland nutrition solution was added to provide plant nutrients. The pH of the solution was adjusted to be between 6.6 and 7.5 for optimal plant development. The pH was measured using as described by Chasib *et al.* (2021). Healthy plant specimens were selected and washed with tap and distilled water to remove impurities before the commencement of the experiment. Plant samples were also taken at the beginning of the experiment to establish their characteristics.

2.4 Fresh and dry weight

Weekly samples were taken to determine the fresh weight of the plant after washing it with tap water and distilled water, weighing it with a sensitive balance, and recording the weight in grams as a wet mass. The plant was then dried at 70 °C until it attained a constant weight, then recorded as a dry mass.

2.5 Relative growth rate

The relative growth rate was calculated according to Lu *et al.* (2004) as bellow

$$\text{Relative growth rate (RGR)} = \frac{final\ fresh\ weight\ (g)}{Initial\ fresh\ wright\ (g)}$$

2.6 Total chlorophyll content

The total chlorophyll content was quantified according to the method described by Arnon (1949). A sample of 0.2 g from the fresh weight of the plant was extracted with 20 ml of 80% acetone. The extract was then centrifuged for 5 minutes at 5000 rpm to obtain a clear filtrate. Absorbance measurements were taken using a spectrophotometer at two specific wavelengths: 645 nm and 663 nm. The total chlorophyll content, expressed in milligrams per gram (mg/g), was subsequently calculated utilizing the given formula.

$$\text{Total Chlorophyll mg/g} = (20.2 \times OD\ 645) + (8.02 \times OD\ 663)$$

where OD: optical Density at 645 and 663 nm.

2.7 Protein content

The plant protein was measured as a percentage, as described in Cresser and Parsons (1977). The plant was digested as 0.2 g of the dry plant was inserted in a 100 mL beaker with 5 mL of concentrated H_2SO_4, stirring for 20 minutes. The sample was heated at 120 ℃ for 5 minutes and heated again for 30 minutes at 90 ℃, then added 3 ml of the 4% acid mixture (4 mL of perchloric acid with 96 mL of condensed sulfuric acid), heated for 10 minutes to become apparent, colorless, and cooled. The volume was reduced by up to 50 mL with distilled water and transferred to a 50 mL plastic container cap until calculation using Kjeldahl method for calculation of total Nitrogen. The value was multiplied by 6.25 to calculate the protein percentage in the plant.

2.8 Proline content

The proline content is determined according to Troll and Lindsley (1955). 0.2 g of dried, crushed leaves were taken and

added to 5 mL of 95 % ethanol for an hour, centrifuged, and the transparent part taken. Evaporating the transparent part was done near dryness. A centrifugation procedure was carried out after adding 2 ml of purified water to the remaining fraction. Spectrophotometric equipment was used to measure the absorbance of 1 mL of perfume at a wavelength of 520 nm using spectrophotometric equipment. The proline content calculation as followed (Bates *et al*; 1973):

µmoles per gram tissue =
[(µg proline/ml) x ml toluene)/115.5 µg/ µmole] / [(g sample)/5]

2.9 Statistical analysis

All experiments were conducted in triplicate, and data was recorded. The GenStat v.12 program was used. The type of analysis conducted was a factorial experiment within a Randomized Complete Block Design (RCBD). The comparison was made between the means according to the least significant difference test at the probability level of 0.05. The ANOVA two-way analysis was calculated too.

3. Results and Discussion

3.1 Effect of either NaCl or Na2SO4 salts

3.1.1 Fresh weight and dry weight

Fresh weight

Observations indicate that sodium sulfate (Na_2SO_4) exerts more detrimental effects on *C. demersum* than sodium chloride (NaCl), as illustrated in Figure 1. Specifically, the fresh weight of *C. demersum* decreased by 60% following a 14-day exposure to 75 mM Na_2SO_4. In contrast, *C. demersum* demonstrated a resilience to the impact of NaCl, particularly at concentrations up to 50 mM. These findings are consistent with those reported by Al-Abbawy *et al.* (2020). Further details are presented in Figures 1 and 2.

Dry weight

The result revealed that the Na_2SO_4 salt has more adverse effects on *C. demersum* than NaCl (Figure. 2). The dry weight was decreased by 60% at 75 mM of Na_2SO_4 after 14 days' exposure.

Relative growth rate

The relative growth rate effectively measures stress levels and plant responses to various stressors since it represents the plant's life-sustaining activities. Figure 3 shows the effect of different salinity concentrations of each NaCl and Na_2SO_4 compared with the control. In single salt treatments, the lowest value was recorded for the macrophyte exposed to 25 mM of NaCl, and the most considerable value was registered at 75 mM of mixed Na_2SO_4. Furthermore, it is obvious in Figure 2 that there were more morphological changes with Na_2SO_4 treatment. As the concentration of Na_2SO_4 increased, brownish and yellowish colors were observed.

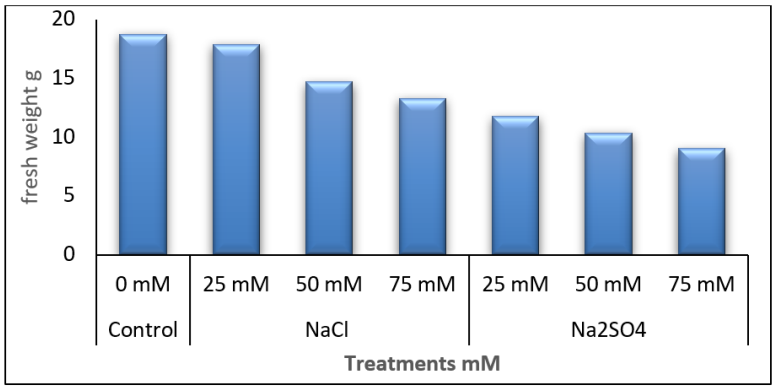

Figure 1. Variation in fresh weight of *C. demersum* as a response to different concentrations of NaCl, and Na_2SO_4

The two salts significantly affected the relative growth rate ($p \leq 0.05$) (Table 1). An increase in salt concentration for all treatments was a significant decrease in relative growth compared with the control. Salt stress has been shown in various studies to reduce plant fresh weight and growth in a range of plant species (Ahmed *et al.,* 2021; Hadia *et al.,* 2022). The findings corroborated previous observations of NaCl and Na_2SO_4 treatments inhibiting growth. The negative effects of salt on plants were most likely caused by the osmotic potential in the culture media, which restricted plant cells from absorbing the necessary water (Sarwar *et al.,* 2021). As a result, plant uptake of some mineral nutrients dissolved in water is reduced. Plant growth and development were stunted because of metabolic imbalance. According to certain research, salt ion buildup reduces development by modifying membrane permeability (Tunçtürk *et al.,* 2011). The weight of the majority of crops exposed to saline environments decreased. Water stress, ion toxicities, ion imbalance, or a combination of these factors has been blamed for salinity's deleterious consequences. This result agreed with Stoeva and Kaymakanova (2008).

Total chlorophyll and protein content

In comparison to the control treatment, the results demonstrated that exposure to the two salts affected the plant's total chlorophyll and protein content (Figures 4 and 5). The increase in salt concentration showed a more substantial reduction of both total chlorophyll and protein contents. The lowest value of total chlorophyll was when the plant was exposed to 75 mM Na_2SO_4 compared with NaCl as illustrated in Figure 4. In these experiments, exposure to the two salts separately resulted in a decline in total chlorophyll content and decreased photosynthesis activity, which was

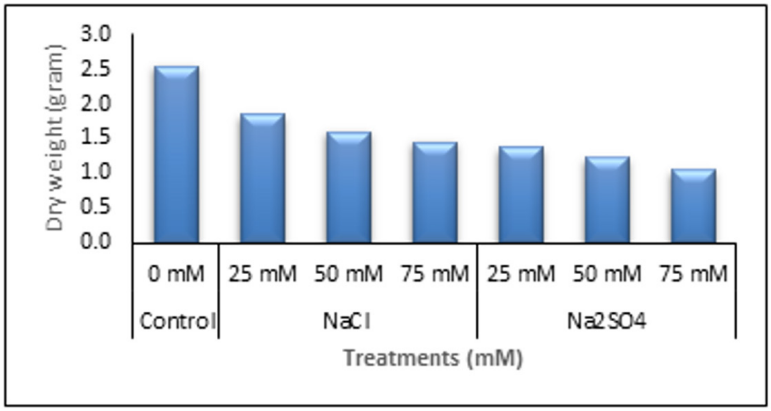

Figure 2. Variation in Dry weight of *C. demersum* as a response to different concentrations of NaCl, and Na_2SO_4

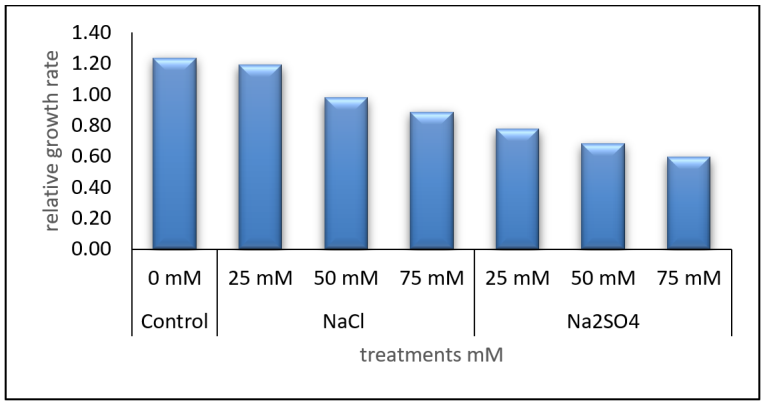

Figure 3. Variation in the relative growth rate of *C. demersum* as a response to different concentrations of NaCl, and Na_2SO_4

increased by increasing the concentration and exposure time (Akcin & Yalcin, 2016). The effect of salt accumulation on the function and structure of chloroplasts, reducing their effectiveness in pigment synthesis, could explain the decrease in chlorophyll content. Furthermore, a lack of water supply and dehydration of the structure could decrease chlorophyll concentration. This result agreed with Irakoze *et al.* (2021) . This result agreed with Al-Abbawy *et al.,* 2020. At the same time, the value of protein decreased at the same concentration of Na_2SO_4 (Figure 5). Proteins involved in the stress response have changed their sluggishness. The same observation was reported by Farooq *et al.* (2018).

Proline content

Many plants accumulate proline in response to a variety of biotic and abiotic stressors. According to Meloni *et al.* (2001), proline is a nitrogen-balanced amino acid that is a readily available source of energy and a reducing agent in plant metabolism. Under salinity conditions, researchers discovered a considerable rise in proline concentration. In this work, the proline content was considerably altered by the two studied salts ($p \leq 0.05$) (Table 1). When compared to the control, the proline content increased in all treatments. The highest value was in the presence of 75 mM Na_2SO_4, and the lowest value was 25 mM NaCl. Figure 6. Proline protects the stability of macromolecules and membranes during dehydration and maintains osmotic balance during stress. The proline content could be attributed to the expression of genes encoding essential proline synthesizing enzymes like pyrroline-5-carboxylate (P_5C) and the low activity of oxidizing enzymes such as proline dehydrogenase, which are regulated by osmotic and salt stress (Izadi *et al.,* 2014).

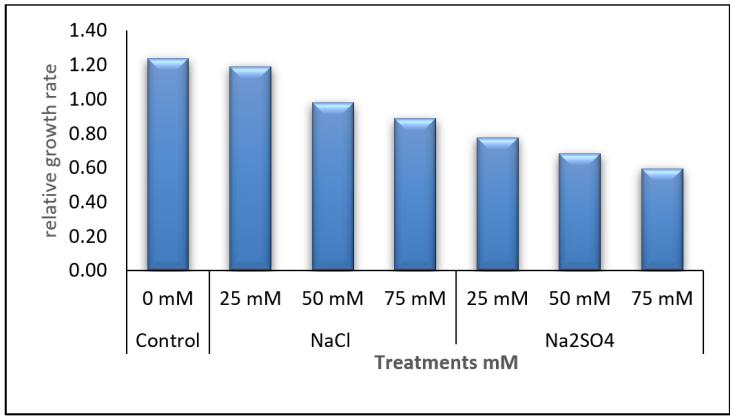

Figure 4. Variation in total chlorophyll content of *C. demersum* as a response to different concentrations of NaCl, and Na_2SO_4

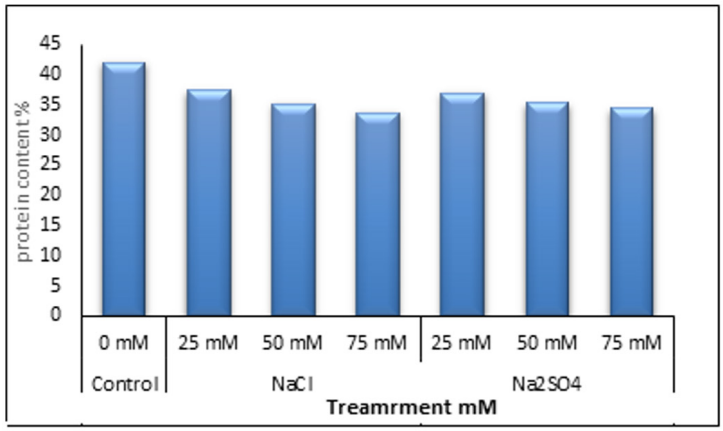

Figure 5. Variation in protein content in *C. demersum* as a response to different concentrations of NaCl, and Na_2SO_4

Table 1. Variation in fresh, dry weight, relative growth rate and some toxicological effects of a combination of salts on *C. demersum* as a response to different concentrations of NaCl, Na_2SO_4 and mixed NaCl + Na_2SO_4.

Parameters	Control	25 mM NaCl + 25 mM Na_2SO_4	50 mM NaCl + 50 mM Na_2SO_4	75 mM NaCl + 75 mM Na_2SO_4
Fresh weight (g)	18.742	8.582	7.359	5.723
Dry weight (g)	2.543	0.974	0.947	0.845
Relative growth rate	1.236	0.569	0.488	0.380
Total chlorophyll µg/g	10.212	3.708	3.055	2.699
Protein content %	42.037	35.730	34.180	30.180
Proline content mg/g	0.162	0.286	0.369	0.743

3.2 Effect of combined NaCl and Na_2SO_4 salts

In combined treatments, plant impact increased as salt concentration increased. Limited water potentials, an essential nutrient for plant growth, and ion toxicity accumulating may be responsible for salinity's negative effects on fresh weight and relative growth (Moghaieb *et al.,* 2004; Parida & Das, 2005). This result is similar to Reich *et al.,* (2017) and Al-Abbawy *et al.,* (2020).

According to the study, both salts significantly affect the growth and physiological processes of *C. demersum*, with Na_2SO_4 having a more severe impact than NaCl (Figures 1 and 2). The reduction in fresh weight and dry weight of *C. demersum* observed in this study is consistent with previous research on the effects of salt stress on various plant species (Sarwar *et al.,* 2021; Ahmed *et al.,* 2021; Hadia *et al.,* 2022).

The decrease in chlorophyll content observed in this study is also consistent with previous research, which has demonstrated that salt accumulation affects the function and structure of chloroplasts, reducing their effectiveness in pigment synthesis (Akcin & Yalcin, 2016; Irakoze *et al.,* 2020).

Interestingly, the study observed an increase in proline content in response to salt stress, which may represent a physiological response of *C. demersum* to salt stress (Meloni *et al.,* 2001; Izadi *et al.,* 2014). Proline protects the stability of macromolecules and membranes during dehydration and maintains osmotic balance during stresses, and its increase in content may indicate a plant's attempt to cope with salt stress.

Overall, the findings of this study contribute to our understanding of the impacts of salt stress on aquatic plants and the development of strategies to mitigate its negative effects on aquatic ecosystems. The results suggest that Na_2SO_4 has a more severe impact on *C. demersum* than NaCl, and that exposure to salt stress significantly affects the growth and physiological processes of the studied plant.

4. Conclusion

It can be concluded that salinity has an impact on the development, metabolic and physiological processes, chlorophyll, and protein content of *C. demersum* leaves. The high concentration of salts leads to an increase in the plant's proline level, which helps it to tolerate salt stress. The plant is better able to handle low and moderate concentrations of NaCl compared to Na_2SO_4. Moreover, Na_2SO_4 has a more significant toxic effect than NaCl in all treatments. Furthermore, the combination of the two salts has a greater impact on the plant's morphological and biochemical parameters than a single salt treatment.

Ethics declaration

Ethics approval and consent to participate

Ceratophyllum demersum is a submerged perennial macrophyte selected from Basrah, Iraq that were kindly provided by Dunya A. Hussain AL- Abbawy (Department of Ecology, College of Science, University of Basrah, Iraq).

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