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Communication

Impact of Salinity Fluctuations on *Dunaliella salina* Biomass Production

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Abstract: The utilization of microalgae as a green carbon source for chemical production has attracted attention for its potential use in sustainable and climate-friendly solutions. This study investigates the growth of *Dunaliella salina*, a unicellular green microalga, in response to salinity variations and water and seawater addition to compensate for evaporation in open cultures. The impact of continuous and non-continuous water addition, as well as seawater addition, on the growth of *D. salina* was analyzed though tank tests. The results showed that different water-addition methods did not significantly influence cell concentrations, indicating the organism's resilience to salinity changes. Continuous water addition maintained stable salinity levels at 12%, but required continuous monitoring, while non-continuous addition reduced the intervention frequency. The overall results showed that a salinity range between 12 and 15% did not affect microalgae growth, suggesting flexibility in evaporation-loss compensation methods based on cultivation-system specifics and resource availability. Maintaining consistent biomass regardless of the water-addition method used suggests sustainable production within the tested salinity range, with seawater addition making microalgae cultivation more adaptable to regions with varying water availability. Further research, including outdoor pilot tests, is recommended to validate and extend these findings to natural environments.

Keywords: *Dunaliella salina*; salinity fluctuation; evaporation compensation

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1. Introduction

The utilization of microalgae, with their ability to efficiently convert carbon dioxide into several compounds (mainly carbohydrate, protein, and lipids) through photosynthesis, has gained significant attention for its potential role in addressing the global challenge of transitioning from a fossil fuel-dependent society to a bio-based one [1–3]. Focusing on microalgae as a sustainable raw material for fermentation and biorefinery processes, thus exploring microalgae as a green carbon source for biochemical production, opens up new possibilities for resource-efficient, sustainable, and climate-friendly solutions [4], unlike conventional discussions that often revolve around biodiesel or lipid extraction.

Biorefinery, a concept inspired by the traditional petroleum refinery, involves the integrated processing of biomass into a spectrum of products for different industries, such as food, feed, chemicals, fermentation, pharmaceuticals, etc. [5–9]. By using microalgae in biorefinery processes, industries can utilize the biomass to produce a range of bio-based materials, including bioethanol, biopolymers, biofertilizers, nutraceuticals, pigments, etc. [10,11]. This not only diversifies the bioeconomy, but also contributes to achieving a carbon-neutral society and helps to mitigate climate change [12].

Dunaliella salina, a unicellular green microalga, is known for its rapid growth rate under optimal conditions, nutrient-efficient utilization when in seawater or nutrient-rich wastewater, high content of beta-carotene and other carotenoids (such as alpha carotene, lutein, etc.), glycerol production, adaptability, toughness, and the ability to thrive in high-salinity environments, which prevents contamination [13]. Its ability to thrive in extreme conditions makes it an attractive candidate for large-scale cultivation in diverse geographical regions and in regions that are unsuitable for regular agriculture, thereby addressing

land-use concerns and reducing competition with food crops [1,14]. Moreover, the utilization of seawater for *D. salina* cultivation presents an advantage, especially considering the scarcity of freshwater resources in many regions worldwide.

The widespread adoption of microalgae-based biochemicals depends on the development of cost-effective methods for producing biomass. While outdoor open-pond systems are generally the most common and are considered more economical [15,16], evaporation from microalgae open-cultivation systems significantly impacts production costs and sustainability, especially in arid regions. Therefore, controlling salinity, particularly for species like *D. salina*, is crucial for optimal cultivation conditions. Replenishing lost water through evaporation helps to maintain optimal salinity levels, mitigating negative effects on *D. salina* growth. Strategies such as optimizing system design and implementing water-recycling technologies are essential for enhancing economic viability and environmental sustainability in microalgae-based processes.

In this study, we investigated the growth of *D. salina* in terms of biomass concentration in response to gradual salinity increases due to evaporation variations and water addition to compensate for water loss. Studying the addition of water to an outdoor, open *D. salina* culture to offset evaporation is crucial for maintaining salinity levels without compromising the growth of microalgae cells. Evaporation presents a significant challenge in outdoor, open *D. salina* cultures, as it leads to salinity increases that can slow their growth. To address this issue, we evaluated both continuous and non-continuous methods of water addition. Moreover, we investigated seawater addition instead of freshwater addition, as this can be important in regions where freshwater scarcity is a concern and offers sustainable alternatives to freshwater usage.

To the best of our knowledge, there are very limited studies, if any, that address this issue. The originality of this study lies in comparing continuous and non-continuous water and seawater addition in *D. salina* open cultures to recommend the best practices for achieving better production, ensuring the cost-effectiveness of the system, and promoting sustainable cultivation practices for microalgae biomass production. This is necessary, as we aim to maintain low prices for *D. salina* biomass when used as a raw material in various industries such as food, feed, fermentation, etc. This contributes to the utilization of a green carbon source and the shift towards a sustainable society that does not rely on fossil fuels.

2. Materials and Methods

The microalga *D. salina* (strain CS-744/01) was cultivated in f/2 medium [17,18] with a salinity of 12% under controlled laboratory conditions. The f/2 medium was prepared by combining all of the chemicals in a salt solution (Red Sea Salt, Red Sea Fish Pharm Ltd., Eilat, Israel). Tanks measuring 31.5 cm in length, 18.5 cm wide, and 24.5 cm high were used to cultivate *D. salina*. Each tank was initially filled with 3000 mL of f/2 medium and *D. salina* at a concentration of approximately 1×10^4 cells/mL. The cultures were conducted under LED lights at a light intensity of 300 µmol/m²/s, with the temperature kept constant at 25 °C Celsius. We consistently monitored the cell concentration of *D. salina* by sampling 25 µL and analyzing it using an Invitrogen Tali Image-Based Cytometer (Thermo-Fisher Scientific, Waltham, MA, USA).

Prior to conducting tank tests and in order to calculate the approximate water volume lost due to evaporation, we monitored the water evaporation rate and salinity increase using saltwater (12%) under controlled laboratory conditions. For this purpose, 3000 mL of saltwater was introduced into the tank, and the mass and salinity were periodically measured.

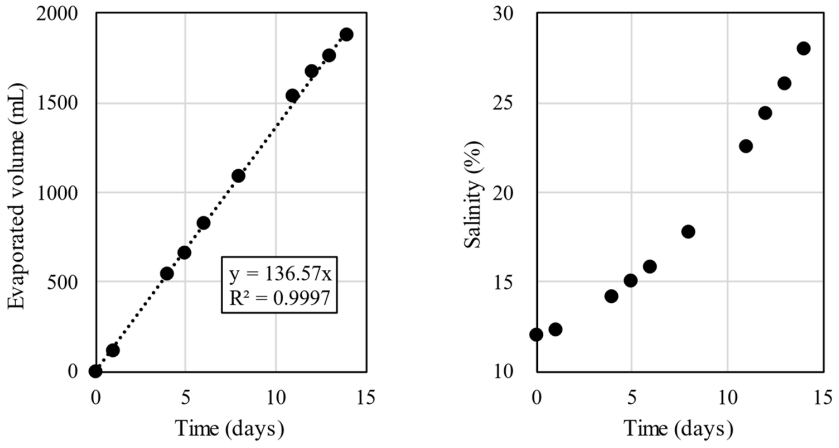
Two experimental setups were employed to assess water addition for evaporation compensation. In one tank, a continuous water (distilled water) addition system was used, employing a silicone tube connected to the tank's center bottom, along with a variable speed peristaltic pump (Fisher Scientific, Pump I, Model 3384, ultra-low flow with range of 0.005–0.900 mL/min). The silicone tube used for experiments had an inner diameter of 1.6 mm, and the speed for distilled water addition was set to the reference number 25 (which equals to 130 mL/day, approximately). In contrast, the other tank

received non-continuous or manual water addition every seven days, matching the volume added to the continuous tank or the decreased volume due to evaporation. To avoid nutrient deficiencies, a few milliliters of 1000-times-concentrated solutions containing phosphate, nitrate, and other essential elements were added to both tanks every seven days; maintaining nutrient concentrations aligned with the f/2 culture medium formulation. Moreover, tank experiments were also conducted using artificial seawater addition (3.5% salinity) instead of water to adjust salinity. In this case, the peristaltic pump set varied from references 25 to 50 (130 to 240 mL/day, approximately) to keep the salinity at 12%. The height of the culture in the tank was recorded. Growth parameters, including cell density and size, were monitored regularly, and the data were analyzed to evaluate the impact of different water and seawater addition regimes on *D. salina* culture growth and productivity. All experiments were repeated three times. Statistical differences between the sets of data compared in this study were conducted using Tukey's test.

3. Results

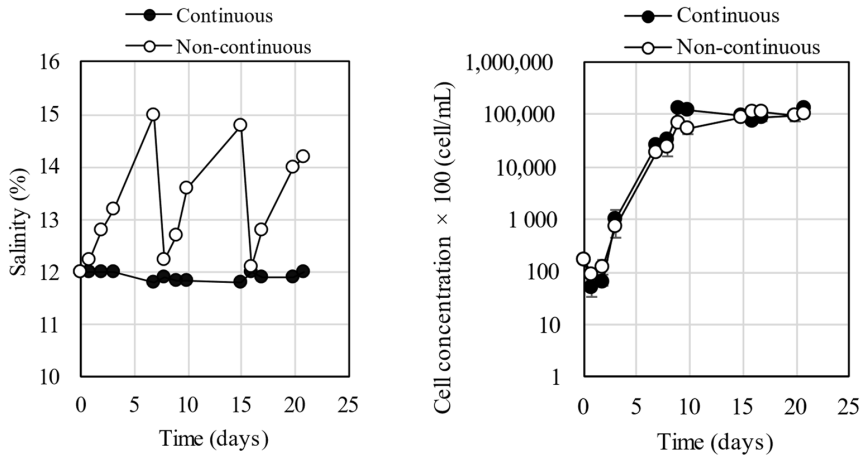
Evaporation tests were conducted to determine water loss under laboratory conditions before tank tests. The results showed an approximate water loss of 1900 mL (from an initial volume of 3000 mL) after 14 days and an increase in salinity from 12–28% (Figure 1). The impact of continuous and non-continuous water addition due to evaporation on the cell concentration of *D. salina* is shown in Figure 2. Although *D. salina* can thrive in different salinities, it was expected that salinity levels higher than the optimum (12% in our case, results not shown) or fluctuating salinity levels might lead to reduced cell concentrations due to a potential stress response within the microalgae. Decreasing biomass is not desirable, as we aim to use this as a raw material and want to maximize productivity. However, the addition of water, whether continuous or non-continuous (once a week), did not yield statistically significant changes in cell concentration (Tukey's test, $p > 0.001$), even though the salinity increased over time, rising from 12 to 15% in non-continuous cases. In continuous cases, the salinity did not change over time; it remained at 12% throughout. The cell concentration in both cases was approximately 1.7×10^4 cells/mL initially. It then entered the lag phase, followed by the log phase, and reached its maximum after around 10 days, with a cell concentration of approximately 1×10^7 cells/mL. Subsequently, it entered a stationary phase. The cell size remained constant at 6 µm throughout the experiment. This suggests that the frequency of water addition (continuous or non-continuous) in this case did not influence the growth of *D. salina* under the experimental conditions and thus did not negatively impact the biomass concentration and productivity.

Figure 3 provides insights into the response of *D. salina* to seawater addition aimed at compensating for salinity increase through evaporation. Despite an increase in the salinity (from 12–15%) and overall height level of the culture in the tank (from 5 cm to 8 cm, results not shown), no statistically significant alterations in cell concentration were observed (Tukey's test, $p > 0.001$). The salinity increased over time in the non-continuous case, reaching 15%, and then decreased to 12% with seawater addition, while it remained constant at 12% in continuous cases. The initial cell concentration in both scenarios was about 1×10^4 cells/mL. It then went through a short lag phase, moved into the log phase, and peaked at around 10 days, reaching a concentration of about 1×10^7 cells/mL. Following this, it entered a stationary phase. This suggests that while seawater addition effectively mitigated water loss due to evaporation, it did not induce notable changes in the growth of *D. salina* in terms of biomass concentration. These findings underscore the robustness and stability of *D. salina* in maintaining its cellular concentration despite variations in environmental conditions, particularly salinity and water availability; however, further studies need to be performed to assess whether this affects the production of compounds such as beta-carotene, protein, and carbohydrates, among others. It may also be necessary to evaluate cases where evaporation rates are higher due to several factors such as temperature, humidity, wind speed, etc., and where the increase in salinity is also higher.



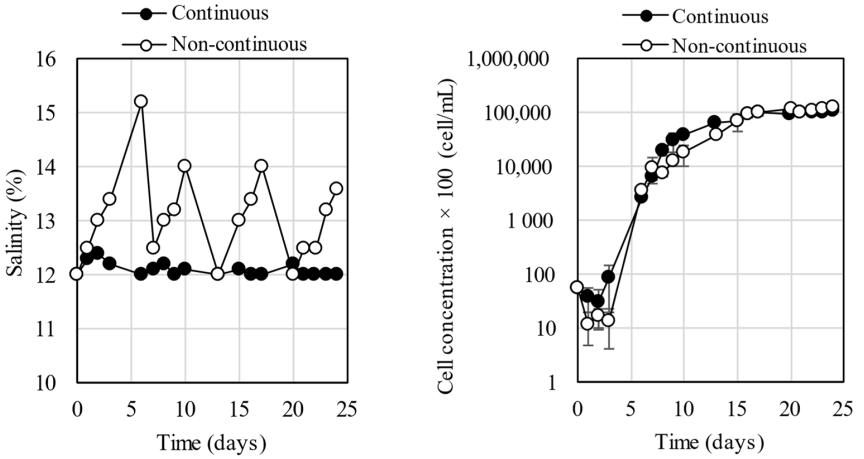
(**a**) (**b**)

Figure 1. Previous tank test using 12% saline water. (**a**) Evaporated volume over time and (**b**) salinity change over time.



(**a**) (**b**)

Figure 2. Continuous and non-continuous water addition to compensate for evaporation. (**a**) Salinity change over time and (**b**) cell concentration change over time.



(**a**) (**b**)

Figure 3. Continuous and non-continuous seawater addition to compensate for evaporation. (**a**) Salinity change over time and (**b**) cell concentration change over time.

4. Discussion

The experiments involving water addition due to evaporation highlighted the resilience or adaptive mechanisms of *D. salina* to changes in salinity. Continuous and non-continuous water additions did not significantly alter cell concentrations, indicating that the organism may have adaptive mechanisms to regulate its growth in response to salinity increases or decreases, with the latter being due to water addition. This adaptive capacity can be attributed to the osmoregulatory mechanisms present in *D. salina*, enabling it to maintain cellular homeostasis despite fluctuations in salinity levels [19]. The lack of a rigid cell wall allows for instant cell volume adjustments, expanding when environmental salinity drops or shrinking when salinity increases, along with subsequent changes in glycerol and ion concentrations [20].

Furthermore, experiments examining the effects of seawater addition to compensate for water loss through evaporation yielded promising results. Despite an increase in overall water levels within the culture tank, due to the volume of artificial seawater being added to decrease and maintain salinity at 12%, no notable changes in cell concentration were observed. This suggests that while seawater addition effectively compensates for water loss, it did not trigger substantial changes in the growth or biomass production of *D. salina*, highlighting the possibility of sustainable microalgae production in regions with limited water resources. In the case of non-continuous seawater addition, this indicates its capacity to adapt to varying environmental conditions without compromising its biomass production.

Continuous evaporation-loss compensation ensures a stable and controlled environment for the microalgae, minimizing stress by maintaining a relatively constant salinity level; however, it may require the continuous monitoring of evaporation rates and salinity levels or an automation system, which could increase the biomass cost. In that case, a pump activated by a solar panel, for example, could be considered. Non-continuous salinity adjustment reduces the frequency of interventions and monitoring required. This approach may be more practical for small-scale production or when resources or automation capabilities are limited; however, rapid and greater increases in salinity levels (more than 3%) than those tested in this research may stress the algae, potentially affecting their growth. Additionally, the microalgae may need time to acclimate to the new salinity levels, which could temporarily impact productivity.

In our experiments, we demonstrated that a salinity of between 12 and 15% may not affect the growth of the microalgae. Therefore, in general, the choice between continuous and non-continuous evaporation-loss compensation approaches depends on the specific circumstances of the cultivation system and the resources available. For large-scale commercial operations or cases where precision is required, a constant adjustment approach may be preferable. Conversely, smaller-scale operations with limited resources may find periodic adjustments more practical, despite the potential drawbacks in precision and control. The key is to maintain stable and optimal conditions for *D. salina* to produce biomass in a cost-effective manner.

In this initial study, to simplify and avoid complex parameters in outdoor experiments and to focus on the salinity impact on biomass, we utilized controlled laboratory conditions. However, to validate and extend the findings of this study, further research is recommended, including pilot tests conducted outdoors to assess the reproducibility of the results in natural settings. Outdoor pilot tests would provide valuable insights into how *D. salina* responds to environmental fluctuations, such as natural variations in sunlight, temperature, wind, and nutrient availability, which may influence its growth dynamics differently compared to controlled laboratory conditions. Moreover, comparative studies could be conducted to assess the impact of variations in pigment levels (such as carotenes and chlorophyll), beta-carotene, protein, carbohydrates, lipids, and other compounds due to fluctuations in salinity.

5. Conclusions

This study contributes to the understanding of the resilience or adaptability of *D. salina* to salinity variations and the methods of water and seawater addition in open cultures. No statistically significant difference was observed between continuous and non-continuous systems where salinity was kept constant and non-constant, respectively. The findings suggest that both continuous and non-continuous water and seawater addition approaches can be viable for maintaining stable growth conditions under varying salinity conditions ranging from 12 to 15% salinity. Further research is recommended to validate these results in an outdoor setting, consider resource management and operational preferences, and assess the impact on compound production, thereby enhancing the sustainability and cost-effectiveness of microalgae-based biorefinery processes.

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