CICIMAR OCEÁNIDES
ISSN 2448-9123
https://cicimaroceanides.mx
Centro Interdisciplinario de Ciencias Marinas
https://doi.org/10.37543/oceanides.v38i1.290

Vol.39 No. 1
Enero - Junio
2024

NITROGEN LIMITATION AND CHANGES IN SALINITY AFFECTS CHEMICAL-PROXIMATE-COMPOSITION OF *Thalassiosira weissflogii*

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ABSTRACT. Microalgae are crucial in aquaculture activities since they are used as live food for shrimp, mollusks, and fish larvae. Nevertheless, just a few microalga species are produced. In this study, we evaluate the effect of salinity at 25, 35, 45, and 55 practical salinity units (psu) in F/2 media as a control group and two nitrogen ($NaNO_3$) limited media, F/4 and F/8 on the diatom *Thalassiosira weissflogii.* To value the development of the diatom, kinetic growth curves, pH, biomass, and chemical-proximate composition were estimated. The highest growth rate and organic matter values were in media F/4 and 35 psu. Meanwhile, media F/8 at 55 psu presented the highest ash amount and a significantly lower growth rate. *T. weissflogii* is affected by high salinity concentration; however, the limitation of nitrogen with 25 and 35 psu did not affect growth considerably. Concerning the chemical-proximate composition of *T. weissflogii*, media F/4 at 25 psu presented the highest percentages of carbohydrates and proteins. In the same way, medium F2, at 25 psu, reported the highest amounts of carotenes. The results showed that high salinity was the variable that major affected the cell density, biomass and chemical-proximate composition of *T. weissflogii.*

Keywords: *Thalassiosira weissflogii,* high salinity, nitrogen limitation.

LA LIMITACION DE NITROGENO Y CAMBIOS EN LA SALINIDAD AFECTAN LA COMPOSICION QUIMICO-PROXIMAL DE *Thalassiosira weissflogii*

RESUMEN. Las microalgas son un factor crucial en las actividades acuícolas, ya que son utilizadas como alimento vivo en la etapa larvaria de camarones, moluscos y peces. Sin embargo, la producción de microalgas esta limitada a unas pocas especies. En este estudio se evaluó el efecto en la diatomea *Thalassiosira weissflogii* al ser cultivada a salinidades de 25, 35, 45 y 55 unidades prácticas de salinidad (psu), en el medio F/2 como grupo control y dos medios limitados de nitrógeno ($NaNO_3$), F/4 y F/8. Se estimaron curvas de crecimiento, monitoreo de pH, producción de biomasa y composición químico-proximal. La tasa de crecimiento y los valores de materia orgánica más altos se obtuvieron en el medio F/4 a una salinidad de 35 psu. Mientras tanto, el medio F/8 a 55 psu presentó la mayor cantidad de ceniza y una tasa de crecimiento muy baja. *T. weissflogii* se ve afectada por altas salinidades, sin embargo, la limitación de nitrógeno con 25 y 35 psu no afectó considerablemente el crecimiento. En cuanto a la composición química próxima de *T. weissflogii*, el medio F/4 a 25 psu presentó el mayor porcentaje de carbohidratos y proteínas. Así mismo, el medio F2 a una salinidad de 25 presentó una mayor concentración de carotenos. Los resultados mostraron que la alta salinidad es una variable que afecta la densidad celular, la producción de biomasa y la composición química-proximal de *T. weissflogii.*

Palabras clave: *Thalassiosira weissflogii*, altas salinidades, limitación de nitrógeno.

Fimbres-Olivarría, D., Medina-Félix, D., García-Lagunas, N., Gutierréz-Ruacho, O. G., Huerta Aldaz, N., Ortega-Rosas, C. I. (2024). Nitrogen limitation and changes in salinity affects chemical-proximate-composition of *Thalassiosira weissflogii*, *CICIMAR Oceánides* 39(1), 11-20.

INTRODUCTION

Microalgae play an important role in nature as oxygen producers and a food source for diverse aquatic animals such as mollusks, crustaceans, and fish (Sirakov et al., 2015). Microalgae are cultivated for multiple purposes like human food (Torres-Tiji et al., 2020), as an energy source for biodiesel production (Lam & Lee, 2012), heavy metal bioremediation (Leong & Chang, 2020), in the pharmaceutical industry for biomolecules production (Luo et al., 2015), and aquaculture (Shah et al., 2018). Aquaculture is the food production industry with the fastest growth rate in recent years (Garlock et al., 2020). The most common genera of microalgae cultured are *Chlorella*, *Tetraselmis*, *Scenedesmus*, *Pavlova*, *Phaeodactylum*, *Chaetoceros*, *Nannochloropsis*, *Skeletonema* and *Thalassiosira* (Sirakov et al., 2015).

The marine diatom *Thalassiosira weissflogii* has a superior ability to resist environmental factors, additionally, *T. weissflogii* is one of the most used algae for larval shrimp because of its adequate size and nutrient composition (Vella et al., 2019). In some cases, microalgae are cultured in open systems exposed to environmental conditions, including high temperatures, which causes water evaporation, increasing the salinity level that may affect the chemical-proximate composition of the algae. For the above, knowing the effects during high salinity exposure is important. Furthermore, media used for microalga provide the nutrients needed for cell growth (Peraza-Yee et al., 2022). The media most commonly used by producers is “F media”, formulated by Guillard and Ryther (1962). This media comprises macronutrients, such as nitrates, phosphates, and silicates; moreover, the micronutrients provide minerals and vitamins crucial for correct cell development (Silva-Benavides, 2016).

Fecha de recepción: 06 de octubre de 2023 Fecha de aceptación: 4 de junio de 2024

Nitrogen is the most important limiting nutrient, essential for the synthesis of proteins, nucleic acids, and enzymes in the cell; a few sources of nitrogen can be assimilated by microalgae, for example, ammonia, nitrate, nitrite, and urea (Velichkova, 2014).

Previous studies have focused on the effect of illumination and temperature on biomass production but not on the salinity effect. The present study aims to evaluate the effect of nitrogen deprivation and stress by changes in salinity in the marine diatom *T. weissflogii.*

MATERIAL AND METHODS

Microalgae culture and experimental design

The diatom *Thalassiosira weissflogii* was obtained from the University of Sonora strain collection (UNISON, México) and maintained under laboratory conditions with controlled temperature (20 ± 1 ºC) and 24 h illumination. A 4 x 3 factorial design was carried out; four salinities (25, 35, 45 and 55 psu) and three nitrogen concentrations were evaluated by modifying the $NaNO_3$ content of the F/2 medium (Guillard & Ryther, 1962). F/2 media (control group) contained 1 mL, F/4 with 0.5 mL and F/8 with 0.25 mL of $NaNO_3$.

T. weissflogii was cultivated in a static culture system using 250 mL Erlenmeyer flasks containing 150 mL of media; each replica was inoculated with 50,000 cell/mL; an aliquot of 3 mL was taken and fixed with Lugol every 24 h from each treatment, and replica for the cell count and pH measurement. The pH was measured daily with a pHmeter (pHep, Hanna ®). To determine cell density, cell counts were performed daily using a Neubauer chamber (0.1 mm deep) by applying the formula: Cell count = (Number cell counted/Number of squares counted) * 10,000. The specific growth rate was calculated according to Krzemińska et al. (2014), with the equation l= ln (N2/N1) / (t2 - t1), (N= biomass, and t= time).

Chemical-proximate composition

The dry weight was calculated by filtering 50 mL of each sample through a calibrated GFC Whatman membrane filter of (47 mm diameter), with a vacuum pump and washed with ammonium formate (3 %). Filters were dried in a conventional oven (THELCO® Laboratory oven Precision Scientific, model 130) at 80 °C for eight hours and weighed on an analytical balance (Mettler, AJ100). The filters were incinerated in a muffle (Felisa®*, model 3*60D) at 480 °C for six hours to determine the ashes. Organic matter was obtained by calculating the difference between dry weight and ash (López-Elías et al., 2003).

Additionally, *T. weissflogii* were cultured in 2 L Erlenmeyer flasks in 1.5 L media to determine the chemical-proximate composition; protein, carbohydrates, and lipids were determined following the methodology described by Fimbres-Olivarría et al. (2015). Finally, the total carotenoid content was analyzed according to (García Morales et al., 2020).

Two-way ANOVA ($p < 0.05$) and a Tukey honest significance test were performed to determine the effect of high salinity and nitrogen limitation in *T. weissflogii.* Data were analyzed in JMP© Pro 16.

RESULTS

Cell densities

The maximum cell densities were observed at day seven for all treatments (Figure 1). Media F/4 with 35 psu presented high cell density (571,250 cell/mL), followed by media F/4 with 25 psu (563,438 cell/mL) (Table 1). The nitrogen limitation did not affect the cell densities when *T. weissflogii* was cultured in lower salinities (25 and 35 psu) ($p < 0.001$; Figure 1). Nevertheless, higher salinities (45 and 55 psu) directly decrease the microalgae growth, with significant differences between lower and higher salinities ($p < 0.001$). Finally, *T. weissflogii* was not able to develop at 55 psu in all media, especially with the nitrogen limitation in media F/4 and F/8.

Differences in the specific growth rate per day were also reported among treatments (Table 1; $p < 0.001$). Nitrogen limitation did not significantly decrease the *T. weissflogii* daily division, with 0.47- 0.5 div/day in 25 and 35 psu in all media. However, a significant decrease in the specific growth rate was detected with a salinity of 55 psu in media F/8 and F/4.

pH values

Values of pH in F/2 media (without nitrogen limitation) in all salinities maintain values between 8.1 and 8.6, decreasing at day 7 (Figure 2). Media F/4 and F/8 at salinities 25 and 35 show similar values to those observed in the control media (F/2). Moreover, high salinity (55 psu) with nitrogen limitation reported inferior pH values.

Biomass composition

T. weissflogii biomass composition was affected by the nitrogen limitation and the high salinity, reporting significant differences between treatments concerning the organic matter ($p < 0.001$). Nevertheless, no significant differences were reported for dry biomass and ashes. Media F/4 and 35 psu presented a high concentration of organic matter (0.140 g/L), followed by F/4 at 25 psu and salinities of 25 and 35 in F/2 and F/4 media (Table 2).

Chemical-proximate composition of *Thalassiosira weissflogii*

No differences were observed in the protein content between experimental cultures of *T. weissflogii*, with protein average values of 17.89 % (Table 3). In the meantime, significant differences were detected ($p < 0.05$) among salinities in carbohydrates and lipids contained in *T. weissflogii*. F/4 media at 25 psu reported differences in the carbohydrate content between salinities. Moreover, F/2 media, at 25 psu, presented significant differences compared to the other treatments.

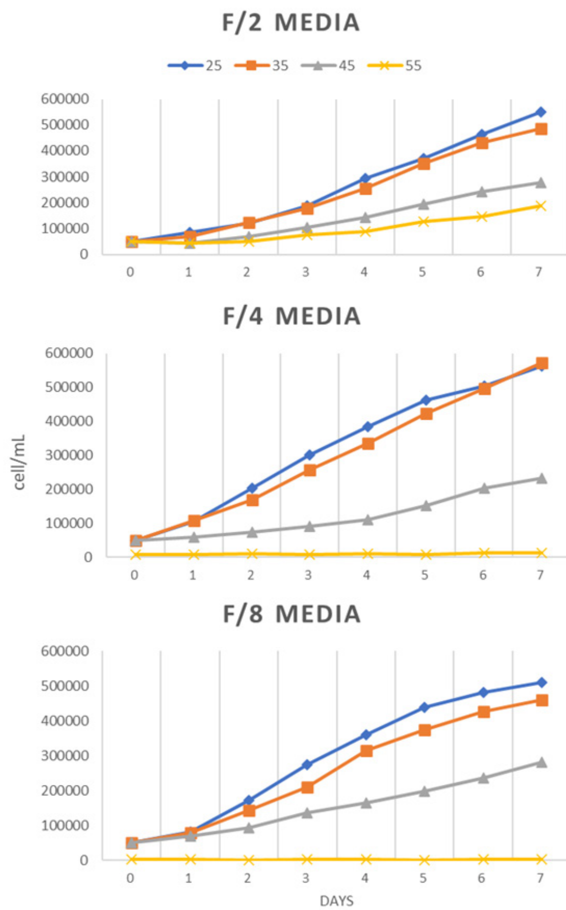

Figure 1. Kinetic growth curves of *Thalassiosira weissflogii* in media F/2, F/4 and F/8, with nitrogen limitation and salinities of 25, 35, 45 and 55 psu.

Total carotenoids in *Thalassiosira weissflogii*

Significant differences were observed in the carotenoid content of *T. weissflogii,* comparing media F/2 at 35 psu ($p < 0.001$) against the nitrogen limitation (F/4) medium at both salinities (Table 4).

DISCUSSION

The diatom *T. weissflogii* developments in all media analyzed; however, the high salinity exposure affected the cell growth. Therefore, the lower cell densities were reported at 55 psu. Additionally, media F/4 at 25 and 35 psu presented the highest cellular density; in a previous research, García et al. (2012) reported 441,000 cell/mL at 35 psu in media F/2, which is slightly lower than growing with nitrogen limitation, demonstrating that cell density can be influenced by high salinity, reaching the mineral fraction of the biomass. It is well-known that *T. weissflogii* can thrive in adverse environmental conditions, and the absence of nutrients associated with high salinity has a significant impact on the development of microalgae.

In a high salinity environment, the cells must deal with ionic imbalance and osmotic stress (Shetty et al., 2019). Diatoms are unicellular microalga enclosed in a surface from biosilica self-assembled into intricate porous shells, named frustules; these frustules provide thermal stability and mechanical and chemical resistance (Maher et al., 2018). Several species of marine microalgae can tolerate salinity fluctuations through the K^+/Na^+ pump, the production of osmolytes such as proline, sucrose, glycerol, and glycine, that contribute to the osmotic potential and at the same time repair damaged proteins, lipids membrane and nucleic acids of the cells (von Alvensleben et al., 2016). Regardless of whether the microalgae are capable of regulating high salinity through osmoregulation, high salinity is a significant stress that results in decreased biomass productivity due to the energy expended in the osmoregulation process. (Oren, 1999).

Different factors may disturb nitrogen metabolisms; for instance, it has been reported that high temperatures limit nitrogen uptake, and temperatures under 16 °C increase their ability to acquire nitrate (Berges et al., 2002). On the other hand, in a completely nitrogen-free culture, cell division was affected by the null growth rate at day four without nitrogen (De La Rocha & Passow, 2004). Specific growth rate, meaning the division of cells per day, is directly influenced by salinity. *T. weissflogii* development decreases when cultured at a high salinity of 55 psu, evidencing that salinity is an essential factor to consider to maximize cell growth.

Manipulation of microalgae media to increase growth rate is widely studied since, in most cases, a more significant number of cells means higher animal source and biomolecule production; this is related to their tolerance and adaptability to the media conditions, in which most species prefer salinities under their natural environment (Fulks & Main, 1991). Species of the genus *Thalassiosira* have a wide salinity tolerance; for instance, *T. pseudonana* presented a high cellular density at 25 psu (Baek et al., 2011).

During the microalgae growth and cell division, *T. weissflogii* consumed CO_2, promoting the production of OH^-, causing an incrementing pH value of media (Valdés et al., 2012; Wu et al., 2016). In this study, pH values reach a maximum level on day four when the diatom is growing, and the photosynthesis is higher, increasing CO_2 consumption and reacting with water to produce carbonate and bicarbonate. Consequently, pH values are higher than eight; values above nine are related to high amounts of carbonates, which alkalinize the culture medium (Richmond, 2008). On the other hand, when a light cycle applies to the microalga culture during the day, photosynthesis occurs, and consumption of CO_2 increases pH. On the contrary, during the night, respiration occurred, decreasing the pH levels (Valdés et al., 2012).

Moreover, during an outdoor cultivation system, the addition of CO_2 to the water is necessary to con-

Table 1. Maximum cellular density (mL) and specific growth rate of *T. weissflogii* after seven days of culture in nitrogen limitation media and different salinities. Different letters indicate significant differences ($p < 0.001$).

Media	Salinity (psu)	Maximum cellular density (cell/mL)	Specific growth rate (μ: div/day)
F/2	25	551,250 ± 18.0^{cd}	0.49 ± 0.01^{e}
	35	484,668 ± 63.8^{cd}	0.47 ± 0.03^{e}
	45	278,750 ± 47.90^{b}	0.35 ± 0.03^{d}
	55	189,375 ± 40.70^{b}	0.27 ± 0.04^{c}
F/4	25	563,438 ± 56.33^{d}	0.50 ± 0.02^{e}
	35	571,250 ± 25.06^{d}	0.50 ± 0.01^{e}
	45	221,563 ± 28.62±^{b}	0.31 ± 0.03^{d}
	55	12,813 ± 2.77^{a}	-2.32 ± 0.21^{b}
F/8	25	510,938 ± 19.48^{cd}	0.48 ± 0.01^{e}
	35	460,625 ± 25.66^{c}	0.46 ± 0.01^{e}
	45	280,625 ± 56.88^{b}	0.35 ± 0.04^{d}
	55	2,188 ± 1.19^{a}	-4.63 ± 0.24^{a}

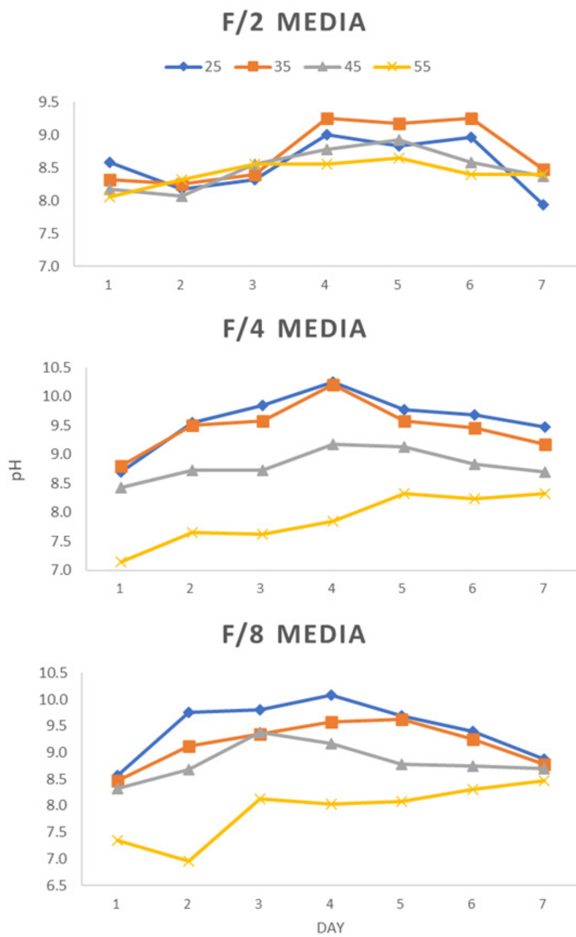

Figure 2. Daily potential of hydrogen (pH) values of *Thalassiosira weissflogii* culture in media F/2, F/4 and F/8 with nitrogen limitation and salinities of 25, 35, 45 and 55 psu.

Table 2. Dry biomass, mineral fraction (ash) and organic matter content (g/L) of *T. weissflogii* after seven days of culture in nitrogen limitation media and different salinities. Different letters indicate significant differences ($p < 0.001$).

Media	Salinity (psu)	Dry biomass (g/L)	Ash (g/L)	Organic matter (g/L)
F/2	25	0.232 ± 0.053a	0.118 ± 0.048a	0.115 ± 0.115abc
	35	0.216 ± 0.008a	0.092 ± 0.005a	0.124 ± 0.005abc
	45	0.295 ± 0.002a	0.204 ± 0.012a	0.091 ± 0.037cde
	55	0.178 ± 0.013a	0.096 ± 0.013a	0.082 ± 0.008cde
F/4	25	0.220 ± 0.012a	0.084 ± 0.22a	0.136 ± 0.015ab
	35	0.268 ± 0.060a	0.128 ± 0.043a	0.140 ± 0.017a
	45	0.201 ± 0.009a	0.118 ± 0.041a	0.083 ± 0.015cde
	55	0.244 ± 0.032a	0.119 ± 0.027a	0.125 ± 0.016e
F/8	25	0.265 ± 0.042a	0.162 ± 0.027a	0.103 ± 0.018abc
	35	0.220 ± 0.020a	0.168 ± 0.077a	0.098 ± 0.013abc
	45	0.164 ± 0.011a	0.147 ± 0.055a	0.049 ± 0.013bcd
	55	0.225 ± 0.058a	0.164 ± 0.052a	0.061 ± 0.008de

trol pH and prevent nitrogen from volatilizing from the water (Lage et al., 2021). pH is an important parameter to consider since it could modulate cell metabolism; for example, alkaline pH induces lipid accumulation in *Chlorella* sp. (Rai et al., 2015). In this study, the pH values increased in all treatments, with constant light and without CO_2 injection.

Salinity and nitrogen limitation impact the biomass composition, particularly the organic matter of *T. weissflogii*. According to previous research, *T. weissflogii* produces more organic matter when salinities are below seawater ups (García et al., 2012). Also, it has been documented that a nitrogen limitation in the microalga media affects the concentrations of carbon, chlorophyll, proteins, and amino acids (De La Rocha & Passow, 2004). Nevertheless, no differences were reported in this experiment, so more research is needed to comprehend cell metabolism during nitrogen limitation.

Microalgae are the primary food source of aquatic organisms, and they are widely cultured in the aquaculture industry to feed aquatic animals, like mollusks, echinoderms, crustaceans, and some fish larvae (Borowitzka, 1997; Torres-Tiji et al., 2020). When choosing microalgae species for commercial scale-up production, it is essential to analyze biomass, lipid content, and quantitative and qualitative lipid composition (Huerlimann et al., 2010). In this research, the chemical-proximate composition of *T. weissflogii* analysis was determined in media F/2 and F/4 with salinities of 25 and 35 psu. Carbohydrates contained in microalgae are mainly cellulose; they can be converted into fermentable sugar (Chen et al., 2013). Microalgae synthesize carbohydrates as structural components in the cell wall and as the storage component inside the cell; carbohydrates provide energy for metabolic processes (Markou et al., 2012). In this study, we report 18.8 % protein, 13.2 % carbohydrates, and 14.7 % lipids in *T. weissflogii* cultured in media F/4 at 25 psu; however, the concentration of carbohydrates and lipids decreased when *T. weissflogii* is cultured at higher salinity (35 psu) additional to a nitrogen limitation. In a similar study, the concentration of carbohydrates, lipids, and protein decreased in *T. weissflogii* when cultured in media F/2 and 50 psu (García et al., 2012).

Carotenoids are terpenoid pigments derived from tetraterpenes (C_{40}), responsible for some vegetables' colors (Cezare-Gomes et al., 2019). Microalgae produce carotenoids with two main functions: light-harvesting pigment-protein complexes and transfer light energy to chlorophyll. They also defend the cell performing essential functions during photosynthesis, acting like antioxidants by stabilizing free radicals and reactive oxygen species (Goericke & Welschmeyer, 1992; Novoveská et al., 2019). There are just a few reports of carotenoid production in *T. weissflogii*, where some pigments quantification in the microalgae were described, like fucoxanthin, diadinoxanthin, and diatoxanthin (Goericke & Welschmeyer, 1992). Additionally, some studies have been performed to understand pigment production under irradiance stress (Latasa, 1995; Zhang et al., 2017; Zudaire & Roy, 2001).

One of the advantages of microalgae culture is the possibility to moderate the growth and production of important organic compounds by manipulating several factors, mainly the nitrogen and salinity concentrations of the media. We concluded that it is possible to decrease nitrogen in media and produce a high concentration of cells per milliliter. In the same way, salinity stress was the main factor affecting microalgae development, compared to the nitrogen limitation factor. Finally, the diatom *T. weissflogii* registered high values of proteins, carbohydrates, lipids, and carotenoids, with high cell densities, even when grown in limited nitrogen concentrations.

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