Efficiency of *Salicornia neei* in removing nitrogen and producing biomass from a hypersaline and artificial wetland to treat aquaculture effluent.

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Abstract

Background: One of the main challenges for the sustainability of land-based marine aquaculture systems is the treatment of saline effluent saturated with nitrogenous waste. In this study, we evaluated the potential of *Salicornia neei*, a halophyte plant native to South America, to remove nitrogen and produce biomass in sandy substrate with nitrogen concentrations similar to marine aquaculture effluent. Plants were collected from the natural environment and cultivated under three treatments: 1) seawater fertilized with nitrate + ammonium (Nit+Amm); 2) seawater fertilized with nitrate (Nit); and 3) seawater without fertilizer (Control).

Results: The nitrogen removal rate increased from 1.67 to 2.76 mg L^{-1} d^{-1} and from 1.95 to 2.96 mg L^{-1} d^{-1} in the Nit+Amm and Nit treatments, respectively. In the two treatments, nitrogen removal efficiency varied between 87 ± 0.39 and 92 ± 0.40%. The salinity increased from 40 to 52 g L^{-1} of NaCl during the experiment, with no observed detrimental effects on the nitrogen removal efficiency. At the end of the crop cycle, the biomass production was not significantly different between the treatments of Nit+Amm and Nit (mean Nit+Amm = 3,584 ± 249.3 g; mean Nit 3,004 ± 249.3 g) but was different with respect to the control (mean Control = 1,527 ± 70.0 g).

Conclusions: Our results demonstrate that artificial wetlands of *S. neei* can be used for wastewater treatment in marine aquaculture and for biomass production in South America.

Keywords: Aquaculture Effluents, Halophyte, Nitrogen Accumulation, Saline effluent, Sustainable Aquaculture.

Background

Aquaculture provides nearly 50% of the world's fish production, and it is expected to increase to 60% by 2030 due to the growing demand for marine fishery products [1]. Land-based marine aquaculture systems will play an important role in meeting this demand and will also do so in a more environmentally sustainable way regarding marine aquaculture in the ocean [2, 3]. However, the development of marine recirculating aquaculture systems (RAS) is limited by the ability to efficiently treat saline wastewater, which accumulates a large amount of nitrogen compounds derived from the metabolism of culture organisms [3-5]. In these RAS, the removal of nitrogen compounds, mainly ammonium (NH_4^+) and ammonia (NH3-), becomes a priority for elimination because they quickly deteriorate the water quality and cause negative effects on the culture [6, 7]. Biofilters that promote the conversion of ionized and deionized ammonium to nitrate (NO_3^-) are usually used for this purpose [8, 9]. NO_3^- is not very toxic to most cultured organisms [10, 11], with tolerable accumulated concentrations reported between 120 mg L^{-1} of NO_3^- and 150 mg L^{-1} of NO_3^- in marine RASs [12].

Recent developments of integrated systems allow the use of RAS waste products as nutrients, coupling different water loops with the main fish production water system [13]. To take advantage of these waste products, such as nitrogen compounds that accumulate in marine RAS, the use of artificial wetlands with facultative or obligate halophytes has been proposed [14-16]. Halophyte plants have the ability to absorb different forms of N, depending on different environmental factors such as the availability of CO2 [17]. For example, some species of the genus *Spartina* show a higher affinity for NH_4^+ consumption [18, 19], while others like *Juncus maritimus*, have a marked preference for NO_3^-, even in substrates that contained high availability of NH_4^+ [20]. Also, if the plants are grown in lysimeters or wetland, the interaction with soil, microorganism and plant have a higher potential to remove nitrogen compounds and produce biomass, which can be used as animal feed or human food [21, 22], and in the production of biofuels or by-products of interest to the pharmaceutical industry [2, 5, 15, 23, 24], among others. Additionally, it has been demonstrated that these systems are also efficient in removing residual phosphates from RASs [2, 15, 23, 25-27].

Salicornia neei is a succulent hydrohalophyte of herbaceous habit, native to South America and abundantly distributed on the South Pacific coast, where much of the marine aquaculture production in South America is concentrated [28]. *S. neei* is used as a gourmet food and is a type of emerging crop in the coastal zone of Chile. This plant has been described as containing high amounts of nutrients and important functional metabolites [22]. Additionally, physiological studies have been performed to observe germination patterns [29] and changes in the concentration of metabolites and antioxidants when exposed to different salinity gradients [30].

The objective of this study was to evaluate the capacity of the halophyte *S. neei* for use as a sink for dissolved nitrogen compounds in effluent from land-based marine aquaculture systems and to simultaneously evaluate the resulting biomass production. The data obtained in this study will allow us to establish whether *S. neei* is a plant suitable for treating land-based marine aquaculture effluent with the potential for use in marine recirculating aquaculture systems.

Methods

Collection of plant material and acclimatization

In July 2014, 100 *Salicornia neei* plants with fully developed roots and shoots were collected in the “Salinas de Puyalli” wetland, located in the commune of Papudo, Valparaíso Region, Chile (32° 24′ 54″ S, 71° 22′ 43″ W) and subsequently transferred to the “Laboratorio Experimental de Acuicultura” of the Pontificia Universidad Católica de Valparaíso, in Valparaíso, Chile (33° 1′ 21″ S, 71° 37′ 57″ W). Plants were sown in sand beds and irrigated with Hoagland solution once a week for 10 weeks. Once the plants adapted and recovered their vigour, they were transferred to the experimental unit.

Experimental unit

The experimental unit consisted of three RAS, each composed of three drainage lysimeters. Each lysimeter was housed in a polyethylene container measuring 0.5 m x 0.6 m x 0.6 m (length × width × depth) with a surface area of 0.9 m^2 and a total area of 2.7 m^2. A leachate collection system was installed in each lysimeter, consisting of a perforated pipe at the bottom to collect the water, followed by a layer of gravel with a diameter of 0.5 cm and height of 15 cm and polyethylene mesh with 0.3 mm pore size to cover the gravel. For the substrate, coarse sand was used until reaching 35 cm high (Fig. 1). Each RAS was connected to a nutrient storage, which in turn was fed by a main tank that contained filtered seawater. Each nutrient storage tank was equipped with an aeration pump to promote biological nitrification processes. The irrigation water supply (influent) was performed with a 0.5 HP centrifugal pump (Humboldt, TPM60). Each RAS was supplied daily with 27 litres of water through a drip irrigation system, programmed to run for 15 minutes at 09:00 and at 17:00 hrs. Drainage water (effluent) was returned to the respective collection tanks of each system to close the recirculating water loop.

Experimental design

The *S. neei* performance regarding removal of nitrogen compounds and biomass production was evaluated for 74 days under three irrigation treatments: 1) seawater fertilized with nitrate + ammonium (Nit+Amm); 2) seawater fertilized with nitrate (Nit); and 3) seawater without fertilizer (Con). The nutrient concentrations in each irrigation water supply were designed according to the typical average concentrations of ammonium (NH_4^+) and nitrate (NO_3^-) reported in land-based marine aquaculture effluent [31, 32]. The following concentrations were used: Nit+Amm = 1 mg L^{-1} of TAN (total ammonia nitrogen) and 100 mg L^{-1} of NO_3^--N; Nit = 100 mg L^{-1} of NO_3^- N; and Control (Con) = no fertilizer. The nutrient solution for each RAS was prepared directly in each collection tank and was completely renewed every 14-15 days. Nutrient removal rate (RR) was calculated as: $RR = (Ci - Co)$, Nutrient removal efficiency (RE) was calculated as: $RE = (Ci - Co) / Ci * 100$ where: Ci = concentration in the influent water; Co = concentration in the effluent water.

The physico-chemical parameters of water quality were recorded directly from the drainage water during the first eight consecutive days after nutrient addition. The estimation of NO_3^- - N concentration was performed using the cadmium reduction method. Additionally, temperature, oxygen, conductivity, salinity and pH were measured as water quality indicators. These parameters were measured using a HACH multiparameter probe (HQ40). Biomass (fresh weight) was recorded at the beginning and at the end of the experiment using a scale (Jadever, JWE-6K). The data on ambient temperature, rainfall and relative humidity were sourced from climate records of the Chilean Meteorological Office (Torquemada-Viña del Mar Station) (Fig. 2).

Statistical analysis

First, the means of nitrogen removal and biomass formation were compared using a one-way ANOVA (RStudio, Ver 3.6.0. probabilities of $p<0.05$ were considered significant. Additionally, to obtain a clearer view of the change in nitrogen concentration in the measurements, the Pearson correlation coefficient was used, and the data that showed a negative linear relationship were subsequently analyzed using the linear model (LM). Finally, the residuals were verified, determining their normality and the homogeneity of the variance (homoscedasticity). The LM analysis provided the removal rate (slope) and the initial concentration (intercept) for the proposed treatments and the control.

Results

RAS environmental conditions and parameters

During the 74 days of culture, the ambient temperature and relative humidity conditions and the temperature, pH and salinity of the cultivation system showed different levels of variability, and no rainfall was recorded during the experiment. The ambient temperature had a mean of 16 ± 4 °C but was highly variable during the day with extreme values of 9 and 31 °C, while the relative humidity was 77.8 ± 8.7%, with extreme values of 60% and 95% (Fig. 2). The temperature in the culture systems was usually higher than the ambient temperature, with a mean of 20.5 ± 1.24 °C and a range of 19.1 to 21.7 °C, with no observed differences between treatments (Table 1). The pH remained relatively constant and without differences between treatments, while the salinity had a noticeable increase from a mean of 40 g L^{-1} of NaCl on day 1 to a mean of 51.5 ± 0.19 g L^{-1} of NaCl at the end of the experiment (Table 1). No significant differences in salinity between treatments were observed ($p<0.05$).

Nitrogen removal, growth and biomass formation

Nitrate removal was high from the start of cultivation and had a clear tendency to increase as biomass production increased (Fig. 3, Fig. 4). Specifically, at the beginning of the culture, the nitrogen removal rate was between 1.67 and 1.95 mg L^{-1} d^{-1}, and at the end of the culture, it increased to 2.76 and 2.96 mg L^{-1} d^{-1} in the Nit+Amm and Nit treatments, respectively, with no significant differences observed between treatments. Consequently, the nitrogen removal efficiency was high throughout the crop and varied between 87% and 92% (Table 2).

Regarding biomass production, the treatments with Nit+Amm and Nit showed a significant increase in fresh weight from 245 ± 35 g to 896 ± 123 g and from 253 ± 7 g to 751 ± 51 g, respectively, while the control group did not show a significant increase in biomass (Fig. 4). In this way, RAS cultivation systems reached a yield between 6.6 and 8.3 kg m^{-2}, with no observed significant differences between treatments.

Discussion

This study determined that the *Salicornia neei* substrate interaction is an effective strategy for the recovery of nitrogen compounds contained in saline effluent typical of marine aquaculture. As shown in recent research, the integration of halophytes as a biofilter in recirculating systems in marine aquaculture is an adequate alternative to decontaminating waters with increased nitrogen compounds. In addition, this plant type offers characteristics that are favourable in various markets (e.g.: for pharmaceuticals, biofuel and human and animal food) [16, 33].

Effluent characteristics

Physicochemical parameters of the effluent, such as temperature and pH, showed significant differences between treatments and between inputs. For Liang et al. [34], these factors are especially important in the treatment of saline wastewater because they can affect the determinant processes in the removal of nitrogen compounds. In this study, temperature and pH were maintained within the optimal ranges (20-21 °C and 7.8-8.2) and therefore did not affect the nutrient removal processes (Table 1). This finding is consistent with Lee et al. [35], who reported that, for denitrification processes in wetland systems, the optimal temperature ranges between 20 and 40 °C and the optimal pH is approximately 8.0. Another important parameter evaluated in this study was the high effluent salinity, which reached concentrations of up to 50 g L^{-1} of NaCl. This increase was mainly due to the known environmental factor of evapotranspiration (Table 1), consistent with a study by Freedman et al. [36], who found increased salinity of treated water in artificial wetlands despite the salt uptake by plants due to soil evaporation and plant transpiration.

Nutrient removal

An extensive variety of plants adapted to salinity can be used to treat saline wastewater [37]. In this study, *S. neei* was selected to aid in nitrogen removal, mainly due to its natural occurrence throughout much of the South Pacific coast of South America [38]. The use of artificial wetland systems with *S. neei* shown that it could be an efficient procedure to eliminate of nitrogenous waste from aquaculture. Since, the daily removal rate recorded in this study was up to 2.9 mg L d^{-1} (Table 2), values higher than those reported with other halophyte species in high salinity [14]. Studies in related species have reported that they have the ability to contain nitrogenous compounds in the form of nitrate and ammonium in the vacuoles of plant cells [39], even in the presence of nitrate reductase (NR) and glutamine synthetase (GS) [40].

Furthermore, it is known that members of the Chenopodiaceae family, such as Salicornia brachiata and Sarcobatus vermiculatus, have special physiological and morphological adaptations [41] that allow them to consume, store (typically in shoots); and efficiently use a wide variety of nitrogen compounds available in the soil [42]. Therefore, it is suggested that *S. neei* due to its natural growth in saline soils with scarce nitrification processes, but with the presence of more stable forms of N such as NH4 + also should be have these adaptations [43 - 48].

Nitrogen bioaccumulation was not determined empirically in this study but can be derived from related studies. For example, in the *S. neei* Riquelme et al. [22] show, from an experimental study, that the total of N fixed in the aerial part of wild plants corresponds to 1.76 ± 0.08 g per 100 g of fresh weight. Similar results were obtained in *S. brachiata* by Rathore et al. [41] from India. Thus, we estimated that the total concentration of nitrogenous nutrients fixed in *S. neei* at the end of the trial would be between 46 and 103.9 g for the Nit treatment. While for Amm + Nit, the oscillatory fixation between 57.8 and 130.1 g of N for the total biomass formed by this treatment, indicating that *S. neei* could be assimilated most of the nitrogen available in this test. According to these results, it can also be suggested that *S. neei* could store ammonium –N, if the differences of the estimate in the two treatments are considered (approximately 20% more N with the Amm + Nit treatment). This being a reflection of the synergy produced by these two compounds when consumed at the same time [49]. However some researchers currently believe that the actual absorption may represent only a relatively small fraction of the global rate of nitrogen (N) elimination [50] and microorganisms that play the most important role in the use and transformation of nitrogen component [51].

In response to this uncertainty, other researchers have studied and obtained low removal rates by plants. Specifically, Tanner et al. [52] found that of the total nitrogen removed by planted wetland systems, only 25% corresponded to fixation in plants. Likewise, Lin et al. [32] observed that of the 73% of nitrogen removed, only 11% had been fixed in plants. Notwithstanding the above, Webb et al. [25], observed significant differences between the nitrogen removal capacity in beds planted with and without halophytes. In their study, they demonstrated a higher removal yield in planted beds (62.0 ± 34.6 mmol N m^{-2} d^{-1}) than in unplanted beds (23.0 ± 26.8 mmol N m^{-2} d^{-1}). Therefore, it can be inferred that the strong root system formed by this class of plants supports the establishment of certain microorganisms that, acting synergistically, improve the removal rate of nitrogen loads. Thus, it is not possible to determine whether plants or microorganisms have the more important role in the performance of natural removal systems, but they should be considered elements with significant functions to fulfil.

Biomass formation

The formation of *S. neei* biomass during the evaluation period reached a total net weight of 13.4 kg and 14.9 kg m^{-2} over a period of six weeks in the treatment irrigated Nit+Amm. These high yields in biomass production are comparable to those obtained by Ventura et al. [53], whose yields for *Salicornia persica* reached 16 kg m^{-2} in a span of 24 weeks. On the other hand, *S. neei* plants remained vigorous throughout the evolution period, even at high salinity concentrations close to 50 g L^{-1} of NaCl. This inherent feature of halophytes highlights the powerful response mechanisms to abiotic stress triggered by *S. neei,* reinforcing the feasibility of including this plant for Aquaculture effluent treatment, because salinity concentrations in the effluent can vary greatly in a single day due to environmental factors such as temperature and rainfall. Regarding removal of the two sources of nitrogen compounds, there was a positive interaction between the ammonium/nitrate supplied for biomass formation of *S. neei*. This positive interaction could be caused by the contribution of the nitrate ion that would act as an important osmotic anion for expansion of the foliar cells [54]. In contrast to the above, in this study, we found that irrigating with only NH_4^+ as a nutrient source (unpublished data) caused a decrease in the initial biomass in *S. neei* plants*,* indicating some toxicity. This finding agrees with Helali et al. [55], who indicate that ammonia, when supplied as the sole nitrogen source, induces toxicity in plants, evidenced by reduced growth and low biomass. Regarding the influence of the contribution of nitrate, *S. neei* was also able to use this nutrient source for biomass formation and consequently remove nitrate from the irrigation water. This again allows us to infer the ability of *S. neei* to grow and capture nitrogen nutrients from different sources. Finally, we observed that the removal times were similarly accelerated with the two nutrient sources, as indicated by the increase in biomass (Fig. 5).

Conclusions

Our results reveal that the integration of *S. neei* into artificial wetlands with recirculating aquaculture effluent would be a viable alternative for eliminating nutrient loads in saline wastewater and that this plant could be included in marine RASs. In addition, the ability of *S. neei* to thrive with both N forms is an important trait that is likely to confer high growth and yield potential in association with artificial wetlands.

S. neei is capable of using effluent similar to that produced in marine aquaculture as a nutrient source, which suggests that *S. neei* has a well-developed molecular mechanism that allows it to use different N sources and that this characteristic is due to the ability of halophytes themselves to survive in extreme conditions.

In a system where halophytic plants such as *S. neei* are used to decontaminate water from marine aquaculture, the nitrogen removal time is expected to decrease as a result of increased biomass.

Author Contributions

JAG and JO conceived and designed the study. AO performed the experiments. MRD performed the data analysis and write the manuscript with the help of JAG. JO edited and revised manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

Not applicable.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Tables

Table 1 Temperature, pH, and salinity (mean ± SE) recorded at the effluent of the culture systems (lysimeter, n=15) with *Salicornia neei*. Salinity is expressed as gram of natrium chloride per liter (g L^{-1} of NaCl). Each Input corresponds to the treatments irrigated nitrate and ammonium (Nit + Amm) and nitrate (Nit). Control: irrigated with sea water only.

Input	Treatment	Temperature (°C)	pH	Salinity (g L^{-1} of NaCl)
1	Nit + Amm	18.2 ± 4.2	8.2 ± 0.1	40.6 ± 2.2
	Nit	19.5 ± 4.7	8.2 ± 0.1	41.3 ± 1.9
	Control	19.1 ± 4.3	8.2 ± 0.1	40.0 ± 0.0
2	Nit + Amm	18.8 ± 1.6	8.1 ± 0.1	44.9 ± 2.3
	Nit	21.7 ± 3.3	8.1 ± 0.1	48.4 ± 2.2
	Control	18.6 ± 1.5	8.0± 0.1	43.6 ± 2.1
3	Nit + Amm	20.8 ± 0.6	7.9 ± 0.1	48.5 ± 2.5
	Nit	21.2 ± 0.8	7.9 ± 0.1	48.8 ± 3.2
	Control	20.8 ± 0.5	8.0 ± 0.1	43.6 ± 2.1
4	Nit + Amm	20.2 ± 1.2	8.0 ± 0.1	47.5 ± 1.9
	Nit	20.6 ± 1.4	8.0 ± 0.1	47.5 ± 2.1
	Control	20.3 ± 1.2	8.2 ± 0.1	46.5 ± 2.6
5	Nit + Amm	20.6 ± 0.6	8.0 ± 0.1	48.0 ± 2.2
	Nit	20.9 ± 0.7	7.9 ± 0.1	47.7 ± 2.4
	Control	20.7 ± 0.5	8.2 ± 0.1	46.5 ± 1.6

Table 2 Nitrate-nitrogen (NO_3^- -N) concentration at the influent (Ci) and effluent (Co), nutrient removal efficiency (RE) and daily removal rate (RR) for each treatment (lysimeter, n=15) with *Salicornia neei*. Mean values are displayed (± SE). Each Input corresponds to the treatments irrigated with nitrate and ammonium (Nit + Amm) and nitrate (Nit). Control: irrigated with sea water only.

Input	Treatment	Ci (mg L^{-1})	Co (mg L^{-1})	RE (%)	RR (mg L^{-1})
1	Nit + Amm	14.20 ± 0.75	1.9 ± 0.17	86.6	2.2 ± 0.21
	Nit	15.30 ± 0.86	1.6 ± 0.66	89.3	2.8 ± 0.37
2	Nit + Amm	12.90 ± 0.60	1.6 ± 0.15	87.3	2.2 ± 0.20
	Nit	16.49 ± 1.21	1.6 ± 0.17	90.3	3.7 ± 0.36
3	Nit + Amm	12.89 ± 0.70	1.1 ± 0.05	91.7	2.3 ± 0.28
	Nit	13.18 ± 0.66	1.2 ± 0.15	91.1	2.4 ± 0.25
4	Nit + Amm	14.28 ± 0.70	1.6 ± 0.10	88.8	2.6 ± 0.28
	Nit	16.80 ± 0.65	1.5 ± 0.05	90.9	2.7 ± 0.26
5	Nit + Amm	14.20 ± 0.69	1.5 ± 0.05	89.4	2.9 ± 0.21
	Nit	13.36 ± 0.57	1.7 ± 0.05	87.5	2.6 ± 0.15

Figures

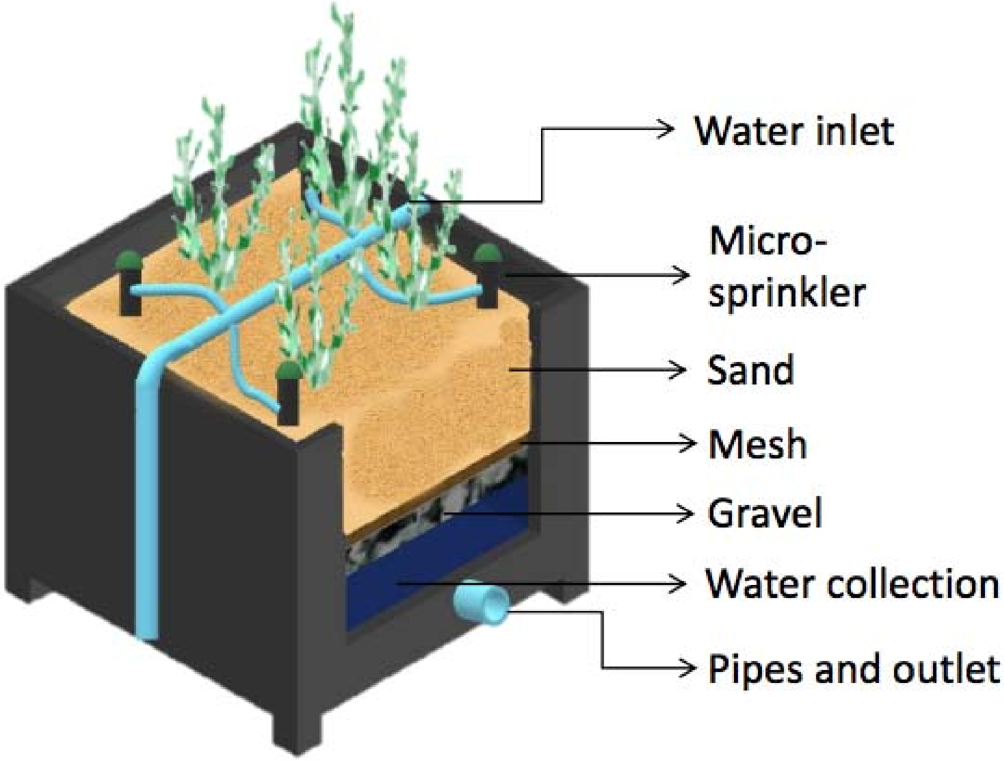

Fig. 1. The diagram shows the design of one lysimeter, depicting the overall construction, water inlet and outlet, substrate (sand and gravel separated by a mesh), and irrigation micro-sprinklers.

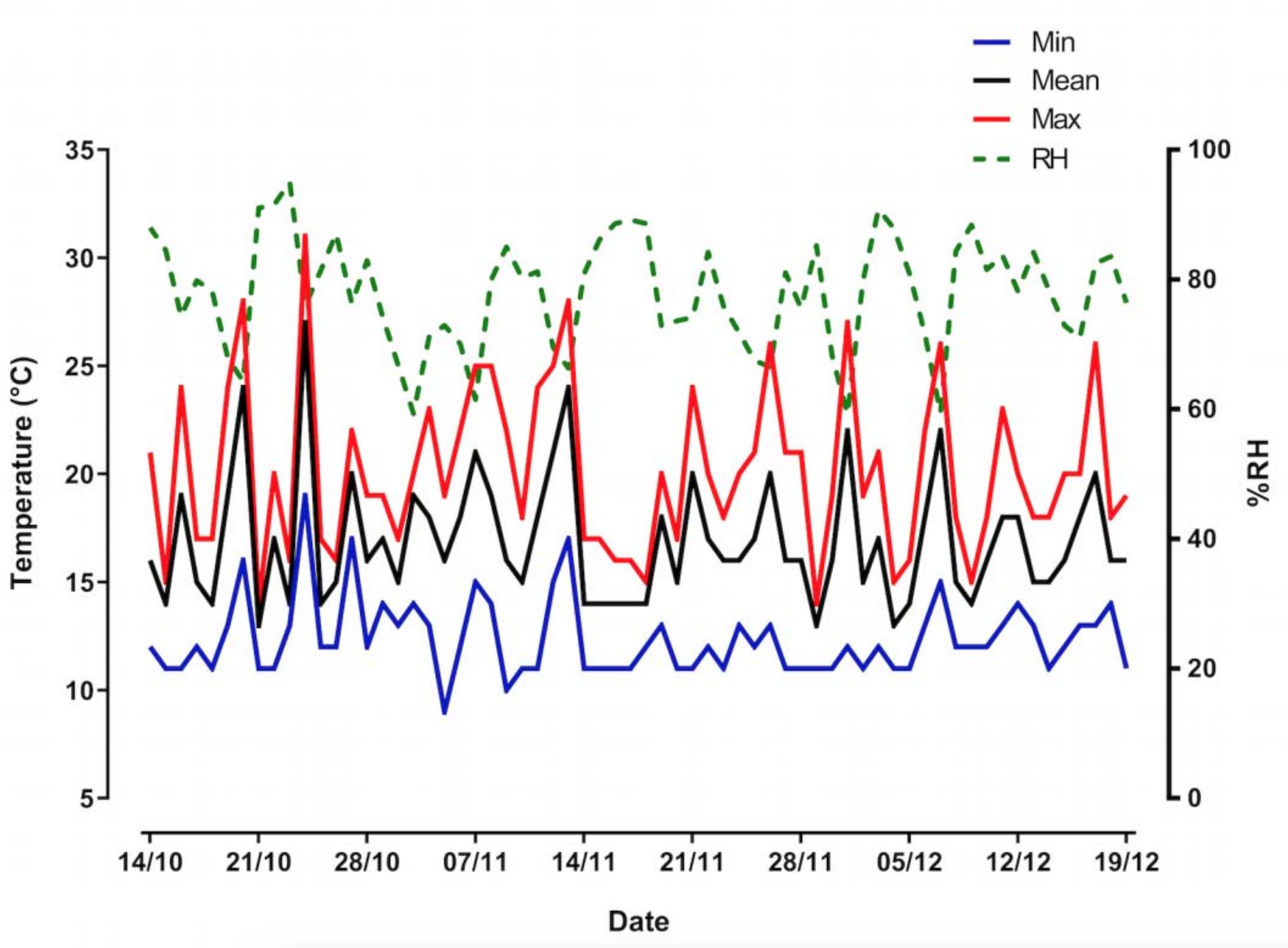

Fig. 2. Ambient temperature (°C) and relative humidity (%RH) during the date of experimentation. The graphic shows mean, maximum and minimum values for the ambient temperature, over 74 days.

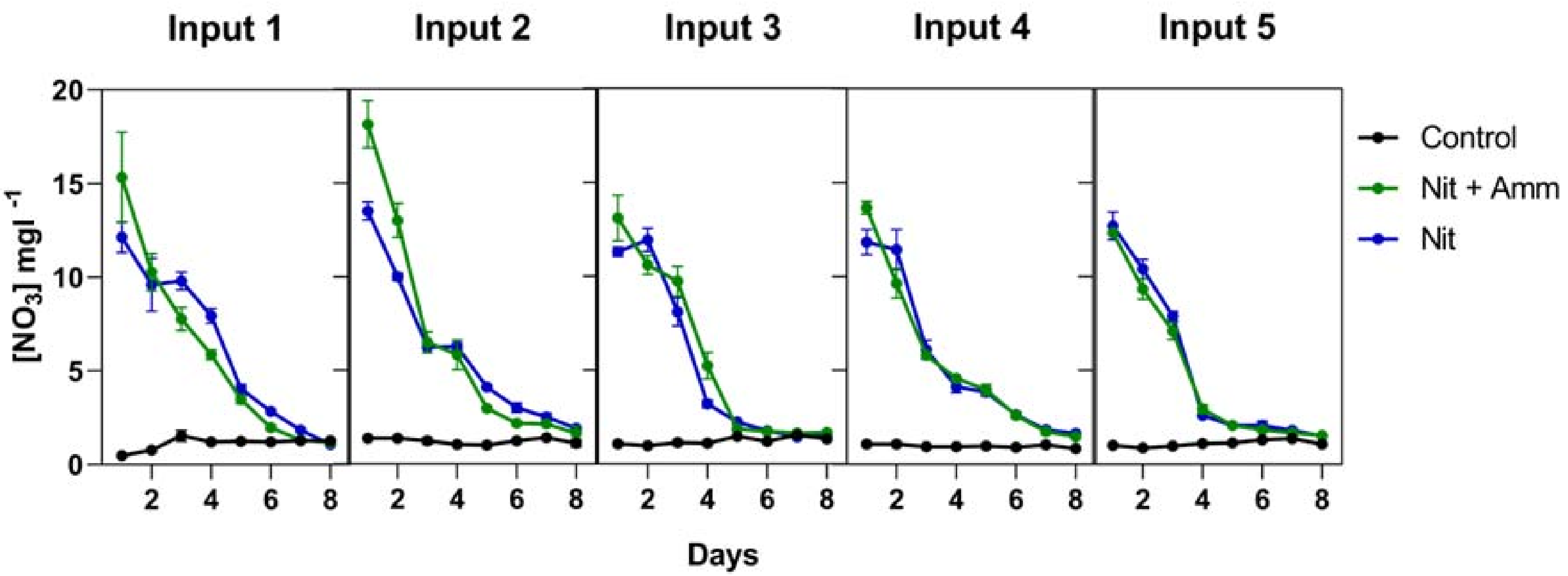

Fig. 3. Nitrate-nitrogen (NO_3^- -N) load in the lysimeters, expressed in mg L^{-1} and observed over 74 days of experimentations. Each Input corresponds to the treatments irrigated with nitrate and ammonium (Nit + Amm) and nitrate (Nit). Control: Irrigated with sea water only.

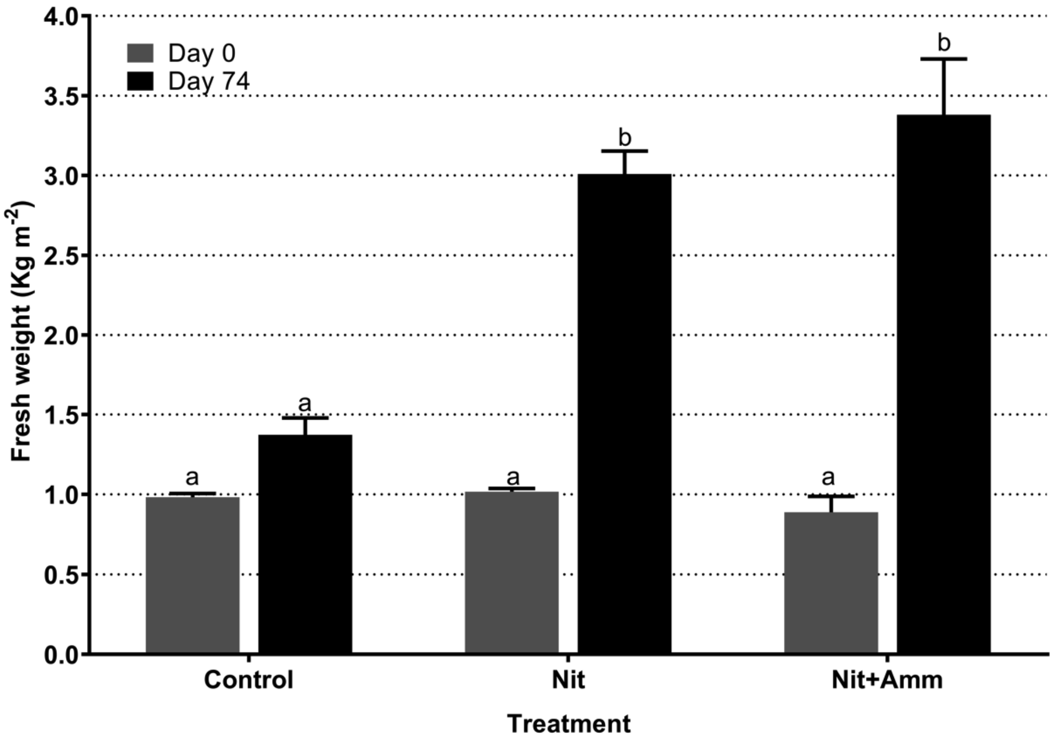

Fig. 4. Production of biomass of S*alicornia neei* expressed as yield of fresh weight (FW) per area unit (FW kg m^{-2}). Each Input corresponds to the treatments irrigated with nitrate and ammonium (Nit + Amm) and nitrate (Nit). Control: treatment with sea water only. Lower-case letters represents significant differences between treatments.

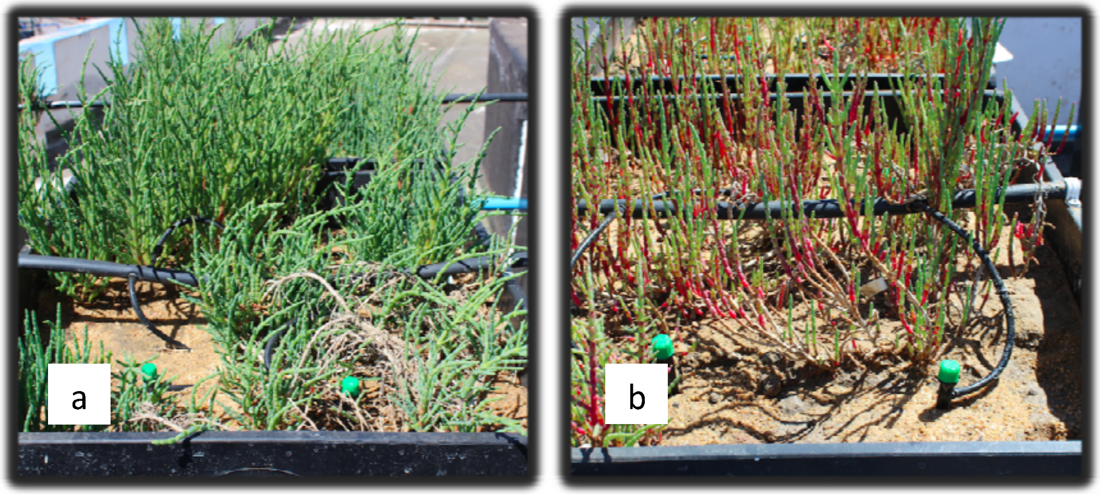

Fig. 5: Picture of two lysimeters with *Salicornia neei* at the end of the experiment (day 74). **a** irrigated with nitrate and ammonium. **b** irrigated with sea-water.

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