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Effectiveness of halophytic plants in the treatment of marine aquaculture wastewater

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Effectiveness of halophytic plants in the treatment of marine aquaculture wastewater

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A thesis submitted in fulfillment of requirements for the degree of

Philosophiae Doctor at Bangor University

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Abstract

This study aims to assess the overall efficiency of *S. europaea* and *A. tripolium* plants in wastewater treatment in saline aquaculture systems (IMTA), while generating a valuable secondary crop. Specifically plant N uptake and/or biomass production under different N concentration and forms, salinity, irradiance, temperature and cropping regimes are investigated. It is estimated that at a constant supply of ≥ 300 μmol NO_3^- l^{-1} *S. europaea* N removal of 65.3 ± 18.1 mmol N $plant^{-1}$ can be achieved over one growing season. Plant growth in a non N limited hydroponic system, with or without a repeated harvest regime, showed that uncropped plants accumulated more fresh biomass than cropped (*S. europaea* 34 kg m^{-2} and 17 kg m^{-2}, *A. tripolium* 21 kg m^{-2} and 10 kg m^{-2}, respectively). N removal is closely related to plant biomass, and therefore the repeated harvesting can reduce N removal, however it provides a regular high value product for marketing. *S. europaea* showed better growth when supplied with NO_3^- or NH_4NO_3, comparing to NH_4^+, while *A. tripolium* growth was not affected by the form of N. The measured *A. tripolium* and *S. europaea* DIN uptake, as NH_4^+ and NO_3^-, when supplied separately or in an equimolar mix were in general good fits to the Michaelis-Menten model. NH_4-^{15}N uptake rates are higher than NO_3-^{15}N uptake, except in non-starved *S. europaea* for single N forms supplied alone. Inhibition of NO_3-^{15}N uptake by the presence of NH_4^+ in solution was observed in non-starved plants. *S. europaea* responds negatively to salinity 1 compared to 10 and 30, while *A. tripolium* responds negatively to salinity 30, indicating the suitability of both plant species to be used in systems with middle range salinities, but preference for *S. europaea* over *A. tripolium* at higher salinities and *vice versa*. DIN uptake in both plant species increased linearly with increasing irradiance, and was higher at the mid-range of temperatures tested (20-40 °C). Differences in N uptake with varying environmental conditions needs to be further investigated and taken in account when designing the treatment system. *S. europaea* and *A. tripolium* were shown to uptake organic N at comparable rates to DIN uptake, and when in solution more than 68% of organic N uptake was as intact alanine and trialanine indicating the potential of these plants to directly remove DON from the wastewater. Overall the results obtained indicate that *S. europaea* and *A. tripolium* are able to perform well in IMTA, with efficient N removal and high biomass production. The data obtained provides a sound basis for system design and scaling up.

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Chapter 1 - General introduction

Introduction

Current status of aquaculture

The average yearly world *per capita* seafood consumption has been increasing in the last decades and predictions are that seafood demand *per capita* will keep growing to between 19 and 21 kg in 2030 (FAO 2002a). In parallel world population is also increasing steadily, these two trends together lead to an increase in overall seafood demand (FAO 2004). To answer that increase in demand a corresponding increase in seafood supply needs to be accomplished. However, most of the world's fishing areas have reached their maximal potential for capture fisheries; and the possibility to continue the increase the seafood production and supply arises from aquaculture (FAO 2004, 2007). Coastal aquaculture, including brackish water and mariculture, is a significant part of seafood production (GESAMP 2008). The current expansion of aquaculture is largely the result of the intensification of marine farming activities of carnivorous fish species in cages or tanks and shrimp in ponds, all of which are dependent on large inputs of resources (Folke and Kautsky 1992, Troell et al. 1999).

Though the need for brackish water aquaculture and mariculture development is unquestionable, adverse environmental consequences have to be addressed, so that aquaculture development will use technologies with environmental sustainability. Impacts of brackish and marine fish and shrimp farming depend very much on the species, culture method, stock density, feed type, hydrography of the site and husbandry practices (Pillay 1992, Wu 1995, Troell et al. 1999), independent of those factors some of aquaculture environmental impacts are of common concern (see for example the reviews: Bunting 2006, Bert 2007), and among these are the nutrients discharged in wastewater.

Aquaculture wastewater

Intensive land-based aquaculture systems can discharge more or less water according to production unit size but also according to the system used (recirculating *vs* flow-through). In open system aquacultures, flow-through systems, as the name indicates the water passes through the culture system only one time and it is then discharged. In this case the quality of the water required for production is achieved merely by dilution effect, i.e. through high water exchange

rates, implying that a very high quantity of wastewater of low contaminant concentrations is discharged in the surrounding environment. On the other hand in closed systems, recirculating aquaculture systems (RAS), the water is re-circulated and reutilised. The extent a system is named RAS can be said to be arbitrary (e.g. Piedrahita 2003) however it is widely assumed that RAS exchange 10% or less water per day (e.g. McCarthy and Gardner 2003, Park et al. 2008). In RAS, compared to flow-through systems, less water is needed and wasted, Timmons *et al.* (2001) reported that RAS can reduce the effluent waste stream by a factor of 500–1000 (Halachmi et al. 2005, Gutierrez-Wing and Malone 2006). In these systems it is essential to maintain high quality in the water being recirculated and reused for production. Also, even if the wastewater volume is reduced, RAS systems also need to discharge some water into the surrounding environment, and this can be 10 to 100 times more concentrated effluent comparing to open systems (Blancheton et al. 2007).

Nitrogen (N) is essential for animal growth since it is a fundamental component of proteins and in aquaculture is supplied to the cultured fish/shrimp, primarily, through added formulated feed. However not all added feed is assimilated into animal biomass, some being lost into the water both as particulate and dissolved forms. Wastewater from fed aquaculture contains uneaten feeds and metabolic compounds (faeces and excreta). Feed that is not consumed by the fish/shrimp, becomes solid waste (SW), part of the feed that is consumed results in undigested feed that is excreted in the faeces as SW, and in the by-products of metabolism that are excreted as dissolved wastes (DW) mostly by the gills and kidneys. N is excreted by fish mainly in the form of ammonia (NH_3), but also in organic forms such as urea, trimethylamine, creatine and creatinine (Handy and Poxton 1993). In water NH_3 and ammonium (NH_4^+) are in equilibrium depending on the pH and temperature (e.g. Colt 2006). Leaching from solid wastes further increases dissolved N in wastewater. Naturally occurring bacteria convert N excreted by fish and leached from faeces and uneaten feed, into different forms of N (namely through immobilization, mineralization, ammonification, nitrification and denitrification) and so aquaculture wastewater contains many forms of N including, NH_3, NH_4^+, NO_3^- (nitrate), NO_2^- (nitrite), creatine, creatinine, amines (including amino acids) peptides and proteins (Randall and Wright 1987, Handy and Poxton 1993, Burford and Williams 2001, Kajimura et al. 2004). The total and relative amounts of these forms vary depending, on among other things, on the production system used, aquaculture species, animal age, diet type and feed ration (Schneider et al. 2005). Usually most of dissolved N occurs in the inorganic form (DIN) and NH_4^+ is often the main DIN form occurring in wastewater (Lefebvre et al. 2001, Porrello et al. 2005, Webb et al. 2012). Even

though, dissolved organic N (DON) has been reported to be more than 20% of the total dissolved N in wastewater (Porrello et al. 2005, Konnerup et al. 2011, Webb et al. 2012).

Handy and Poxton (1993) gathered information related to N excretion in different marine fish and food wastage and presented two scenarios of N pollution in a marine fish culture system. In their minimum pollution scenario N excreted to the aquaculture water is 52.2% of the total N added to the system, a scenario with a low food wastage and high N assimilation and retention. While in the maximum pollution scenario 94.6% of the added N is excreted to the culture system, a scenario with high food wastage and low N assimilation and retention. Other authors report values of N excreted to the aquaculture water of 70–80% for fish farms and 79-94% for shrimp farms (Troell et al. 1999 and references therein, Neori et al. 2000, Schuenhoff et al. 2006). Dissolved N wastes between 34-86% of total N inputs, and N solid wastes between 6-42% have also been reported (Fernandes et al. 2007a and references therein), while Lefebvre et al. (2001) found that from total feed N 62% was released in aquaculture wastewater as dissolved N, of which 43% was NH_3+NH_4^+.

NH_3 is the most toxic form of N (Miller and Semmens 2002), with acceptable concentration of un-ionized ammonia in aquaculture systems of only 1.8 µmol l^{-1} (0.025 mg NH_3-N l^{-1}), or more frequently used < 107 µmol NH_3+NH_4-N l^{-1} (< 1.5mg NH_3+NH_4-N l^{-1}) (Neori et al. 2004, Chen et al. 2006, Crab et al. 2007). NO_2^- can also be toxic to fish, however it normally exists at low concentrations in seawater and is not excreted in significant quantities so it is not, usually, regarded as main toxic form of N in open systems, nonetheless it can be limiting to fish survival and growth in recirculating closed systems (Svobodova et al. 2005, Arredondo-Figueroa et al. 2007, Crab et al. 2007). For instance NO_2^- concentrations lower than 143 µmol l^{-1} (2 mg NO_2-N l^{-1}) are indicated for adult seabass and seabream culture (Blancheton 2000). NO_3^- is not significantly toxic to fish except at very high concentrations, 36 mmol l^{-1} (500 mg NO_3-N l^{-1}) (Pierce et al., 1993 in Colt 2006). However in the case of adult seabream and seabass culture < 7 mmol l^{-1} (100 mg NO_3-N l^{-1}) is considered the safe concentration (Blancheton 2000). NO_3^- can also be harmful from the perspective of promoting algal blooms, since N is the most important growth-limiting factor in the marine environment (Pillay 1992, Wu 1995, Troell et al. 1999), hypernutrification and eutrophication are two major processes that can result from waste discharges from aquaculture farms (Pillay 1992, Casalduero 1993, Neori et al. 2000, Chopin et al. 2001, Miller and Semmens 2002).

In order to obtain a functional RAS, very efficient water treatment techniques are needed to allow for maintaining the high water quality required for production, namely in relation to nitrogenous wastes. Additionally diverse countries and entities have regulations, best

management practices and codes of conduct regarding aquaculture effluents. In Europe the Water Framework Directive 2000/60/CE aims to prevent water pollution and sets out a control mechanism, as part of that mechanism this directive introduces pricing, so that polluters have to pay for N released in aquaculture effluents. Therefore, the need to fulfil legal requirements and reduce expenses with associated N discharges, together with the need to overcome negative effects in the cultured species, leads to a growing consensus about the importance to put in place cost effective wastewater treatment. The development of environmentally sound systems requires the removal of solid compounds and dissolved metabolites contained in wastewater, namely nitrogen.

Wastewater N removal

Providing that N essentially originates from the feed, several studies have been carried out resulting in improvements of feed characteristics and feeding strategies. Highly digestible feeds with an optimal protein/energy ratio provided for each species and each developmental stage reduce N excretion (Wu 1995), however the necessity of wastewater N removal still remains.

Solids are traditionally removed by gravitational and/or mechanical methods. Settlement is used to remove the settleable solids, while filtration (commonly screen filtration, expendable granular media filtration, and foam fractionation) is used for removing suspended and fine particles (Pillay 1992, Casalduero 1993, Buschmann et al. 1996, Van Rijn 1996, Twarowska et al. 1997, Troell et al. 1999, Blancheton 2000, Cripps and Bergheim 2000, Miller and Semmens 2002, Piedrahita 2003, Franco-Nava et al. 2004a, 2004b, Schneider et al. 2005, Gennaro et al. 2006, Van Rijn et al. 2006, Crab et al. 2007, Brambilla et al. 2008).

Removal of dissolved metabolites requires more complex and expensive processes. Troell et al. (2003) describe the two main kinds of chemical filters using activated carbon filters and ion-exchange filters, both having the problem of getting quickly biofouled, additionally ion-exchange filters gets quickly inactivated in ion-rich seawater. It is therefore essential to look for other systems to remove N, biological treatment – biofilters - can be the solution. The traditional earthen pond reservoir for water treatment comprises several naturally established biological processes, however algal collapse, anaerobioses of the sediment and unpredictable fluctuations of phytoplankton biomass and speciation often occur (Van Rijn 1996, Van Rijn et al. 2006). Consequently other biofilters that allow for more predictable performance are preferred, in RAS bacterial biofilters are the most used (Van Rijn 1996, Troell et al. 2003, Summerfelt and Sharrer

2004, Chen et al. 2006, Lyssenko and Wheaton 2006a, b, Malone and Pfeiffer 2006). Different bacteria have the capacity to convert NH_3+NH_4^+ (hereafter NH_4^+) to NO_2^- and this to NO_3^- – nitrification. Nitrification first step is the conversion of ammonia to nitrite, and is carried out by autotrophic bacteria (usually *Nitrosomonas* species) and in the second step other bacteria oxidize the nitrite to nitrate (usually *Nitrobacter* species). The bacterial biofilters consist in some type of material where those bacteria are allowed to grow in order to carry on the described transformations, maintaining low concentrations of toxic NH_4^+ and NO_2^- in the culture tanks, however do not remove N from the system. And a potential problem is the accumulation of NO_3^- in the RAS, if no treatment other than partial water exchange takes place, values as high as 21-29 mmol l^{-1} (300–400 mg NO_3-N l^{-1}) are not uncommon (Van Rijn 1996). On the other hand denitrification, a process by which facultative anaerobic bacteria convert nitrate to di-nitrogen gas, within the RAS allows for N removal without water discharge. Some naturally occurring denitrification happens within aquaculture systems but is not enough to remove all the NO_3^-. Bacterial biofilters for denitrification have been developed for the last decades (e.g. Van Rijn 1996 and references therein, Menasveta et al. 2001, Gelfand et al. 2003, McCarthy and Gardner 2003, Neori et al. 2007, Davidson et al. 2008, Sandu et al. 2008, Singer et al. 2008), however most of this technology is still under development, difficult to maintain and expensive (Neori et al. 2003, Troell et al. 2003, Neori et al. 2004, Van Rijn et al. 2006, Saliling et al. 2007, Singer et al. 2008).

One viable alternative is integrated multi-trophic aquaculture (IMTA), which is defined as a system where different culture units are set up and nutrient fluxes between them are balanced (Chopin et al. 2001, Neori et al. 2004). In IMTA the culture of fed organisms, either fish or shrimps, is integrated with extractive culture, i.e organisms that extract particulate organic matter (shellfish) and/or dissolved nutrients (algae or plants) (Chopin et al. 2001). Shellfish feed on particulate organic matter and algae/plants use light and assimilate dissolved nutrients (particularly C, N and P) to build their biomass. A balanced integration those different organisms in one system allows for the extractive organisms use the waste from the fed culture to grow and in that way to reduce the amount of pollutants left in the wastewater. Furthermore the extractive organisms cultured can provide extra commercial crops, directly providing the farm owner with additional profit and effectively removing the N in their biomass from the aquaculture system. Extractive species produced are argued to meet the standards of organic production (Diver 2006).

Different approaches using algae and plants to remove dissolved N from aquaculture wastewater have been addressed up to date and high water treatment efficiency and economic

return has been demonstrated, in fact several commercial scale IMTA exist today (Rakocy et al. , FAO 2002b, Neori et al. 2004, Diver 2006).

Use of algae for N removal

The possibility of integrating microalgae with fed culture has been shown to be successful (Troell et al. 2003, Neori et al. 2004, Schneider et al. 2005 and references therein). However it has several problems such as, difficulty in control of the microalgae in open ponds, limited water exchange since too much water exchange may wash them out, and difficulty in harvesting (Neori et al. 2004). Consequently integration of microalgal production has not been widely considered as an option.

Macroalgae on the other hand, have been widely studied in IMTA systems (e.g. Folke and Kautsky 1992, Casalduero 1993, Shpigel et al. 1993, Bodvin et al. 1996, Neori et al. 1996, Newkirk 1996, Neori et al. 2000, Chopin et al. 2001, Neori et al. 2003, Schuenhoff et al. 2003, Troell et al. 2003, Neori et al. 2004, Bunting 2006, Bunting 2008) and it has been reported that seaweeds can assimilate as much as 90% of the ammonium produced by intensive fish culture. Integration of macroalgae has been investigated both in flow through and closed systems and high N removal rates often observed, even though removal as low as 17% has also been verified (Hernandez et al. 2005). Several seaweeds (e.g. *Gracilaria*, *Porphyra*, *Pamaria*, *Chordurus*, and *Laminaria*) have shown to perform satisfactorily in such integrated systems with both high bioremediation efficiency and economic return.

Use of higher plants for N removal

Higher plants have also been used in integrated systems to treat aquaculture wastewater, plants have been cultivated either using hydroponic systems or constructed wetlands. Despite the fact that integrated aquaculture can be developed at any salinity, most of the work done up to now using higher plants in IMTA has been in carried out in freshwater or low-salinity systems, with very few exceptions.

The integration of hydroponics and aquaculture is usually referred to as aquaponics. In aquaponics the wastewater from the fed aquaculture production is used in the hydroponic culture. Fish culture (mainly tilapia but also carp, gold fish, rainbow trout and others) has been integrated with several vegetables and herbs (lettuce, basil, okra, tomatoes, spinach, arugula,

watercress, alfalfa, white clover, barley, fall rye, chives) (Rakocy et al. , McMurtry et al. 1997, Lockett, 2003 in Neori et al. 2004, Ghaly et al. 2005, Al-Hafedh et al. 2008). Aquaponic systems frequently include, besides the fish rearing tank and plant hydroponics bed, a solid removal unit and/or a nitrification bacterial biofilter (Quillere et al. 1993, FAO 2002b). In most of these systems, however, the first objective is to maximize the production of a second profitable crop rather than the wastewater treatment, and with that aim several works addressed the optimization of fish and plant ratios (Quillere et al. 1993, McMurtry et al. 1997, FAO 2002b, Al-Hafedh et al. 2008). RAS seem to be the most adequate aquaculture systems for aquaponics since the high nutrient concentration in the wastewater is needed for the plants culture (Nair et al., 1985 and Rakocy et al., 2000 in Rafiee and Saad 2005).

Also floating and submerged freshwater macrophytes, such as *Azolla filiculoides*, *Eichhornia crassipes*, *Pistia stratiotes*, *Salvinia molesta*, have been studied in integrated aquaculture systems and presented efficient nutrient removal (Redding et al. 1997, Sipauba-Tavares et al. 2002, Henry-Silva and Camargo 2006, 2008, Kovitvadhi et al. 2008).

Constructed wetlands (CWs) have traditionally been used for municipal, industrial, urban and agricultural waste management (see Vymazal 2008 for review). CWs are designed to mimic vegetation, sediment, and microbial processes found in natural wetlands. In relation the plants role, besides the direct N uptake, plants provide surface for micro-organisms, allowing for microbial assimilation, transformation and storage of nutrients (e.g. nitrification and denitrification), consequently macrophytes in CWs also play in important role in indirect nutrients removal (Weisner et al. 1994, Tanner 2001, Greenway 2007). Several studies have been carried out and demonstrated the viability of using CWs to filter aquaculture wastewater, since over all high removal rates can be achieved. CWs wetlands have been analysed to treat water both in open and closed aquaculture systems. Unlike in aquaponics often CWs primarily concern is the wastewater treatment, even though production of a second economically advantageous crop is also sought.

Numerous works have investigated N removal from freshwater aquaculture systems, and CWs with plant species such as *Phragmites australis*, *P. communis,* and *Phalaris arundinace*, providing high N removal rates, for example NH_4^+ removal between 61 % and 91 % (Schulz et al. 2003, Sindilariu et al. 2008). Some other works have looked at the treatment of brackish wastewater (salinity 3 and 5) from *Chanos chanos* and *Litopenaeus vannamei* culture, the CWs planted with either *Ipomoea aquatica*, *Paspalum vaginatum*, *Phragmites australis* or *Typha angustifolia* removed up to 95 to 98 % of total inorganic N (Lin et al. 2002, Lin et al. 2003, Lin et al. 2005). However there is little information available regarding the use of CWs in higher

salinity aquaculture wastewater treatment. Lymbery et al. (2006) used a pilot-scale CW with *Juncus kraussii* to study the removal efficiency of NaCl, total N and total P under high and low salinity/nutrients (salinities ~7 and 25, were created using sodium chloride and different nutrient loads using the filtrate from a recirculating freshwater rainbow trout culture). Nutrient loads did not affect plant growth, however high salinity was found to decrease plant growth and therefore the authors suggest that other plants with higher salinity tolerance should be investigated.

In fact, several salt tolerant plants - halophytes – have been cultured in saline environments. Miyamoto *et al.* (1996) studied the production of four halophytes (*Atriplex nummularia, Distichlis palmeri, Batis maritima*, and *Suaeda esteroa*) in brackish and seawater conditions while Noaman and El-Haddad (2000) used six other halophytes (*Spartina* sp., *Distichlis palmeri, Paspalum vaginatum, Juncus roemerianus, Salicornia bigelovii* and *Batis maritima*), results obtained indicated the potential for those halophytes to grow productively. Also Ventura et al. (2010, 2011a, 2011b, Ventura and Sagi in press) carried out experimental work regarding the culture *Salicornia* sp. and *Sarcocornia* sp. plants in relation to different aspects of halophyte cultivation for economic vegetable production (e.g. addition of molybdenum, varying salinity, varying day length, harvest regimes).

Brown et al. (1999), grew three halophytic species (*Sueda esteroa, Salicornia bigelovii, Atriplex barclayana*), the plants were irrigated using aquaculture effluent from an intensive tilapia aquaculture with added NaCl in order to make three salinity treatments (0.5, 10 and 35), overall the plant–soil system removed 98% and 94% of the applied total and inorganic N, respectively. On another study CWs planted with *Sarcocornia fructicosa* and *Arundo donax* removed 31 to 89 % of NH_4^+ and 41 to 90 % of the Total Kjeldahl Nitrogen (TKN) in highly saline tannery wastewater (Calheiros et al. 2012). And a study with *Spartina alterniflora* CW treating effluent from a *Litopeneaus vannamei* RAS presented 51 % TN, 82 % NH_4^+ removal efficiency (Sousa et al. 2011). Webb et al. (2012) carried out research work on the integration of *S. europaea* CWs with commercially-operated fish and shrimp farm. At inlet dissolved inorganic N concentrations between 93 and 439 μmol l^{-1}, the CW removed 97 to 100 % TDIN, while at higher N loading the removal efficiency was reduced to 30 to 58 %. Over the whole experimental period CWs removed in total 1.28 ± 0.05 mol N m^{-2}, from which 85% was retained in plant tissues.

N removal will depend on many factors, such as type of system (Redding et al. 1997, Cerezo et al. 2001, Vymazal 2005) microbial assemblages (Weisner et al. 1994, Redding et al. 1997, Tanner 2001, Greenway 2007), type of sediment or hydroponic technique (Hunter et al. 2001, Yang et al. 2001, Lennard and Leonard 2006), flow rates and water residence type (Knight

et al. 2000, Hunter et al. 2001, Rousseau et al. 2008 and references therein). Nevertheless, the removal of N from the system will ultimately be dependent on the capacity of the plants to produce harvestable biomass and take up N.

Plant growth and nitrogen uptake

Plants are tightly coupled to the environment, their growth is influenced by almost any environmental factor (Ting 1982). Nitrogen is a nutrient element that is central to plant growth because it is a component in substances such as proteins, enzymes, coenzymes, chlorophyll, nucleic acids, and nucleotides and nucleosides (Gauch 1972, Bannister 1976, Haynes 1986). The way in which factors (including N) influence plants growth, although widely studied, is uncertain. Liebig in 1843 first proposed the "Law of the Minimum", only the addition of the factor which is most limiting will influence growth (Gauch 1972, Ting 1982). Liebig only considered nutrients effects and stated that one limiting factor conditioned plant growth and that plant growth was directly proportional to that factor. On the other hand, in 1909, Mitsherlich's popularizes the "Law of Diminishing Returns", in which a much larger increase in growth is obtained if the factor under consideration is far from adequate, as compared with the increase if that same factor is nearly adequate.

In addition to growth, elemental tissue analysis is becoming more common to assess limiting nutrients in plants. Tissue analysis presumes that the concentration of an element in a plant is a reliable indicator of the availability of that element in the environment in which the plant grew (Gerloff and Krombholz 1966). Ridge (1991) describes the relation of external N concentrations with the N content in tissues, net N accumulation and growth rate in a typical nutrient responsive-plant. When N supply is very low N content increases as N supply increases. Then, N content reaches a plateau and remains constant even though growth can still be N-limited. Only in N-toxicity conditions is that plateau is exceeded and tissue increase in N content occurs. According to Ridge (1991) luxury uptake of major nutrients rarely occurs, that is to say, plants rarely take more N than they need when nitrogen supply is high.

Different hypotheses have been suggested for N content in plants in relation to high N supply. However, little evidence exists to support the theories in relation to N concentration above the critical value. Smart and Barko (1980) indicate an increase in N content after critical concentrations, they showed that *Spartina alterniflora* and *Distichlis spicata* have higher N content after biomass stops increasing at high N supply. Smart and Barko (1980) suggests that

that N critical concentration is exceeded when the availability of the nutrient exceeds the requirements of the plant and that luxury consumption can occur when the nutrient no longer limits growth. On the other hand Gallagher (1975), suggests that there is no increase in N content if plants (*Spartina alterniflora* and *Juncus roenerianus*) are not N limited even with increase in availability in exterior N. Some authors consider that very good growth conditions can result in a high growth rates and consequent dilution of N in plants tissues at high external N supply (e.g. Haynes 1986).

Both NH_4^+ and NO_3^- can be actively taken up by plant roots. NO_3^- taken up by the plants is reduced to NO_2^- (catalysed by nitrate reductase, NR), and from NO_2^- to NH_4^+ (catalysed nitrite reductase, NiR) (Guerrero et al. 1981, Siddiqi et al. 1990, Kronzucker et al. 1995, Forde 2000). NH_4^+, taken up by roots, or resulting from NO_3^- reduction, is converted first to glutamine by the action of glutamine synthetase (GS) and then to glutamate by the action of glutamate synthetase (GOGAT). These two amino acids are the precursors for the synthesis of the other amino acids as well as almost all nitrogenous compounds (Guerrero et al. 1981, Knoepp et al. 1993, Tischner 2000, Guo et al. 2007).

Several plant species have high N uptake rates and vigorous growth when supplied with NH_4^+ (Wang et al. 1993, Tylova-Munzarova et al. 2005, Fang et al. 2007a, Jampeetong and Brix 2009a, Konnerup and Brix 2010, Kudo and Fujiyama 2010, Jampeetong et al. 2012b). Conversely, many plant species develop symptoms of toxicity when supplied with NH_4^+, that are not detected when plants are grown with the same concentration of NO_3^- or in mixed N nutrition. NH_4^+ toxicity symptoms, observed in a variety of plant species, include high mortality, chlorosis, necrotic lesions, increased shoot : root ratio, and growth reduction (Raab and Terry 1994, Lasa et al. 2001, Britto and Kronzucker 2002, Guo et al. 2002, Houdusse et al. 2005, Kant et al. 2007, Dominguez-Valdivia et al. 2008, Garnica et al. 2009, Ventura et al. 2010, Borgognone et al. 2012).

Moreover, it has been recognized that various plant species have the capacity to take up organic N. Amino acid uptake has been observed in many plants (Kielland 1994, Schimel and Chapin 1996, Näsholm et al. 2000, 2001, Bardgett et al. 2003, Henry and Jefferies 2003b, a, Nordin et al. 2004, Warren 2006, Warren and Adams 2007, Ge et al. 2009, McFarland et al. 2010, Mozdzer et al. 2010, Hill et al. 2011a, Hill et al. 2011b, Mozdzer et al. 2011). Several amino acid transport systems have been identified in roots, both low and high affinity and with different selectivity (neutral, acidic and basic amino acids). Even though the role of some of those transporters is still unknown some other have proved to be associated with organic molecules uptake by plant roots (Fischer et al. 1998, Rentsch et al. 2007, Näsholm et al. 2009b).

Whereas lower rates of individual amino acid-N uptake, compared to both NH_4^+ and NO_3-N uptake, have been reported for some plants (Näsholm et al. 2000, Ge et al. 2009), other plants species show no difference in uptake between the inorganic and organic N forms (Näsholm et al. 1998, Näsholm et al. 2000, Henry and Jefferies 2002, Bardgett et al. 2003, Henry and Jefferies 2003b), or even have a higher amino acid-N uptake than both NH_4-N and NO_3-N is observed (Raab et al. 1996, Persson et al. 2006, Stoelken et al. 2010). And most commonly amino acid-N uptake was observed to be higher than NO_3-N and lower than NH_4-N uptake (Falkengren-Grerup et al. 2000, Öhlund and Näsholm 2001, Thornton 2001, Henry and Jefferies 2003a, Warren 2006, Warren and Adams 2007, Mozdzer et al. 2010). In some cases preferences for each N form are thought to vary accordingly to the abundance of each N form in the plants natural habitats, i.e. plants show preference for the N form typically more abundant on their habitat (Nordin et al. 2001, Bardgett et al. 2003, Kielland et al. 2006, Miller et al. 2007). Nonetheless other studies did not identify a correspondence between the more abundant N form in plants habitats and their preference (Streeter et al. 2000, Warren 2006). Also, inter-specific differences have been observed between plants from the same habitat (Miller and Bowman 2003, Miller et al. 2007, Harrison et al. 2008, Mozdzer et al. 2010, Hill et al. 2011a) that may in fact indicate advantage for some plant species in competing with other plants for N acquisition.

It has recently been demonstrated that several plants can take up N in form of small peptides (Komarova et al. 2008, Paungfoo-Lonhienne et al. 2008, Hill et al. 2011a, Hill et al. 2011b). Hill et al. (2011a) observed that *Deschampsia antarctica* in solution took up NH_4-N faster than any other form of N tested, namely alanine, dialanine, trialanine, tetralanine and NO_3^-, and uptake of tetralanine-N was faster than that of alanine, dialanine and NO_3-N. In contrast, sterile wheat took up N as L-alanine, L-trialanine and NH_4^+ at higher rates than as D-alanine, and NO_3^- at the lowest rate of all (Hill et al. 2011b).

It is argued that even though plants have the capacity to take up intact organic N, the rate they do so will greatly depend on how plants can access the N. When in soil, the rate that N is available to plants will depend in part on the N diffusion rates - spontaneous migration of N from high to low N concentration areas– to the N depleted area around the root. Diffusion rates vary depending on the N form, in general diffusion rates decrease with molecular mass but also with tendency to adsorption to the soil particle (Näsholm and Persson 2001). Also competition with microbes for the same substrate can limit N availability to plants (Owen and Jones 2001, Bardgett et al. 2003, Jones et al. 2005a, Harrison et al. 2007). Because organic molecules are good C and N sources for microbes, these may outcompete plants and only inorganic N in excess of microbial requirements would be available to plants. On the other hand, some studies

conclude that in short term, plants are outcompeted by microbes for inorganic N as well as for organic N (Hodge et al. 2000a, Lipson and Nasholm 2001, Bardgett et al. 2003, Harrison et al. 2007, 2008), and the fact that plant uptake of intact organic N has been verified in several situations while plants are in competition with soil microbes indicates that at least in some situations plants can access organic N in the presence of soil microbes (Näsholm et al. 1998, Näsholm et al. 2000, 2001, Bardgett et al. 2003, Henry and Jefferies 2003b, Ge et al. 2009, Sauheitl et al. 2009). When plant N uptake is measured in water solution the differences in availability due to dissimilarities N forms diffusion rates and microbe competition are significantly reduced.

Besides a plant's physiological capacity for N uptake, several factors are known to influence N uptake, among those are: presence of NO_3^- in solution (Crawford and Glass 1998, Cerezo et al. 2000, Glass et al. 2002); presence of NH_4^+ (De la Haba et al. 1990, Kronzucker et al. 1999, Ruan et al. 2007, Wang et al. 2010); plant N status (Morris 1980, Tylova-Munzarova et al. 2005, Fang et al. 2007a, Fang et al. 2007b, Konnerup and Brix 2010, Mozdzer et al. 2010); diurnal cycles (Clement et al. 1978, Raman et al. 1995, Macduff et al. 1997, Peuke and Jeschke 1998, Tischner 2000); light (Haynes and Goh 1978, Naik et al. 1982, Campbell 1988, Lillo 1994, Lillo 2008); and temperature (Bassirirad 2000 and references therein, Dong et al. 2001, Calatayud et al. 2008).

Halophytic plants

Unlike glycophytes, halophytes are capable of surviving and reproducing in environments where the salt concentration is around 200 mmol l^{-1} NaCl or more (salinity ≥ 12) (Flowers et al. 1986). For growth plants need to take up water, and in saline environments in order to do so halophytes maintain their water potential below that of the external water potential by taking up salt ions (Na^+, Cl^-), and use different mechanisms to avoid toxic concentrations in the cells cytoplasm where salt sensitive biochemical processes take place (Flowers 1972, Greenway and Osmond 1972, Stewart and Lee 1974, Albert 1975, Flowers et al. 1977, Koyro et al. 2008). Plant mechanisms to prevent salt ions accumulation in cell cytoplasm, include: regulation of ion uptake, ion exclusion, ion compartmentalisation and production of osmotically active compatible solutes, including betaine, proline and glutamic acid (Flowers 1972, Flowers et al. 1977, Glenn et al. 1999, Khan et al. 2001, Flowers and Colmer 2008, Koyro et al. 2008).

The salinity tolerance of halophytes makes them ideal candidates to integrate with brackish and marine aquaculture, however research needs to be focussed in halophytic plants with economic value that will perform well in such systems. *Salicornia europaea*, or samphire, and *Aster tripolium*, or sea aster, are two examples of plants that may potentially fulfil these requirements.

Salicornia spp.

Salicornia, or samphire, has a long history of human consumption as a fresh vegetable and pickled vegetable. Nowadays fresh succulent tips can be found in the UK retail market at about £2 per 100 g. And other commercial applications include the use in animal diets and feed (Glenn et al. 1991b, 1992, Attia et al. 1997, Belal and Al-Dosari 1999, Masters et al. 2007, Bechardas 2008, Murama 2008, Rozema and Flowers 2008), therapeutic applications (Im et al. 2007), use in soap, detergents and lubrication industries (Eganathan et al. 2006), and applications in the cosmetic and nutraceutical industries (Envirophyte 2009).

Several saline irrigated agriculture explorations planted with *Salicornia* have been created, and both forage and oilseed crop obtained are comparable to those obtained from conventional forage crops (Glenn and O'Leary 1985, Glenn et al. 1991b, Troyo-Diéguez et al. 1994, Glenn et al. 1999, U.S. Department of Agriculture. 1987 in Jagtap et al. 2002, Glenn et al. in press). Additionally *Salicornia* has already been shown to grow well and to be capable of high N removal in CWs treating aquaculture wastewater (Bunting and Shpigel 2009, Webb et al. 2012), and to provide regular supply of fresh succulent tips by repeated harvesting during the growing season (Cleall-Harding 2008, Bunting and Shpigel 2009, Ventura et al. 2011a, Webb et al. 2012).

Salicornia spp. (Chenopodiaceae) are succulent leafless annual (although some *Salicornia* spp. can live more than a year in the subtropics) halophytes which can tolerate highly saline environments, occurring commonly in coastal salt marshes and inland salt pans. Its taxonomy is very complex due to the great phenotypic plasticity and inbreeding (Davy et al. 2001, Kadereit et al. 2007). Nonetheless, Stace (1997) recognizes: *Salicornia pusilla* J. Woods, *S. europaea* L. agg. (*S. ramosissima* J. Woods, *S. europaea* L. and *S. obscura* P.W. Ball and Tutin) and *S. procumbens* Smith agg. (*S. nitens* P.W. Ball and Tutin, *S. fragilis* P.W. Ball and Tutin and *S. dolichostachya* Moss). It is then possible that only three species (*S. pusilla*, *S. europaea* agg. and *S. procumbens* agg.) should be recognized Stace (1997) (Davy et al. 2001).

Salicornia spp. is worldwide distributed, in the European coast the distribution can be cautiously attributed to *S. europaea* agg. (Davy et al. 2001).

In *Salicornia* plants the stem is articulate and branches are opposite, both are composed of short, cylindrical or clavate internodes, each with a succulent, photosynthetic covering (Davy et al. 2001, Kadereit et al. 2007, http://www.floraiberica.org/floraiberica /texto/pdfs/02_052_17_Salicornia.pdf). *Salicornia* has a normal root system which can be more or less extensive depending on different environmental factors (Davy et al. 2001).

Vegetative growth occurs by the addition of new stem segments and lateral branches development (Davy et al. 2001). With the addition of new succulent segments, the succulent covering of the lower internodes diminishes, turning to woody portions (Kadereit et al. 2007). *Salicornia* presents allometric vegetative growth, with growth terminating with the reproductive phase, i.e. the production of fertile segments at the ends of all branches (Davy et al. 2001, Quintã 2007, Cleall-Harding 2008). Flowering can start as early as July, and it is followed by the production of seeds after which the plants shed, usually between September and November (Davy et al. 2001, personal observation).

Seeds germination typically occurs in late winter, early spring, and it is probably triggered by lower external salinity (Ungar 1978, Pujol et al. 2000). In experimental conditions the germination of the *Salicornia* appears to be maximal in non-saline conditions (Ungar 1978, Flowers et al. 1986, Khan et al. 2000, MENTERRA 2007). Vegetative growth follows germination, and is affected by a number of environmental factors.

External salinity is a major factor influencing *Salicornia* growth, Halket (1915) reported maximum growth of *S. ramossisima* seedlings at salinity of 10, compared to plants growing in freshwater or at salinity 20-50 (Davy et al. 2001). Subsequent works using several *Salicornia* species report consistently better growth and/or survival rate of *Salicornia* plants at several external salinities comparing to freshwater(or salinities below 2) (Flowers et al. 1977, Cooper 1982, Flowers et al. 1986, Keiffer et al. 1994, Ayala and O'Leary 1995, Noaman and El-Haddad 2000, Shimizu 2000, Shimizu et al. 2003, Moghaieb et al. 2004, Ushakova et al. 2005, Shimizu et al. 2006, Ushakova et al. 2006, Cleall-Harding 2008, Balnokin et al. 2010), though Silva et al. (2007) reported maximum growth in *S. ramosissima* to be in freshwater with slightly lower growth at salinity of 12.5, and further decrease in growth rates salinities of 25 and 37.5. And although *Salicornia* can withstand salinities above that of seawater (Cleall-Harding 2008, Aghaleh et al. 2009, Balnokin et al. 2010, Sulian et al. 2012), the optimum conditions of growth are often reported to be within salinities 6 and 30 (Flowers et al. 1977, Flowers et al. 1986,

Keiffer et al. 1994, Ayala and O'Leary 1995, Noaman and El-Haddad 2000, Ushakova et al. 2005, Cleall-Harding 2008, Balnokin et al. 2010, Aghaleh et al. 2011, Sulian et al. 2012).

In relation to *Salicornia* nutrient requirements, as for vascular plants in general macronutrients including N and P, and micronutrients are necessary for growth. Valiela and Teal (1974) and Boyer et al. (2001) found that adding P (phosphorus) did not result in biomass responses in *S. virginica* concluding that plants were not limited by P. Webb (2005) examined *S. dolichostachya* response to several P concentrations (~0, 1.4 and 2.9 mmol l^{-1}) and found that P availability was not limiting plants growth. Though Alsaeedi and Elprince (2000) registered several P deficiency symptoms at P supply below 0.03 mmol l^{-1}, and optimum growth at a concentration of 0.97 mmol l^{-1}, therefore concluding that P can be limiting to *S. bigelovii* growth. Also Piggot (1969) reported some growth response of *S. europaea* to additions of P.

In contrast, several studies indicate that N availability is clearly a factor limiting *Salicornia* growth. Piggot (1969), Stewart et al. (1973) and Loveland and Ungar (1983) reported biomass increase in *S. europaea* plants fertilized with N in salt marshes. Boyer et al. (2001) have also reported biomass increase with increase of N availability in *S. virginica*, together with an increase succulent tissue biomass. Besides the fertilization in the plants natural habitat, Piggot (1969), Jefferies (1977) Webb (2005) and Kudo and Fujiyama (2010) growing *Salicornia* plants in greenhouses reported an increase in biomass with N addition. While there is a consensus in relation to N limitation to *Salicornia* plants growth the critical N supply is not yet known. Webb (2012) observed an increase in above-ground growth of *S. europaea* irrigated daily for a short period, with an increase in N concentration up to 4 mmol NH_4^+ l^{-1} (0, 2, 4 mmol l^{-1}), and no further increase at 6 mmol l^{-1}. Quintã (2007) found no consistent differences in *S. europaea* growth across different N concentrations, when plants were irrigated only during 5h per day with 2, 4, 6 or 8 mmol NO_3^- l^{-1} solution.

Besides the N amount, also the form supplied has been shown to influence *Salicornia* growth, for instance Ushakova et al. (2006) compared *S. europaea* productivity under urea-N and NO_3-N supply and found a higher biomass accumulation with urea addition. Ventura et al. (2010) compared *S. europaea* growth between NH_4^+ and NO_3^- supply (5 mmol l^{-1}) and observed lower growth when only NH_4^+ was supplied, though Jefferies (1977) observed no differences in *S. europaea* growth when supplied with NH_4^+ and NO_3^- (100 µmol l^{-1}). In contrast, Kudo and Fujiyama (2010) observed better growth of *Salicornia bigelovii* grown on NH_4^+ compared to NO_3^-, for concentration 1-3 mmol l^{-1} and no differences at 4 mmol l^{-1}.

Other factors such as plants density (Ellison 1987, Davy et al. 2001), submergence time (Cooper 1982, Boorman et al. 2001), irrigation volume (Miyamoto et al. 1996, Glenn et al. 1997,

Brown et al. 1999, Noaman and El-Haddad 2000) and light intensity (Tikhomirova et al. 2005, Ushakova et al. 2005, Ushakova et al. 2006) have been shown to also influence *Salicornia* growth.

Aster tripolium

Aster tripolium, or sea aster, can be used for food, as a fresh or cooked vegetable. In certain areas a high demand exists, and there are consumers who eat *A. tripolium* leaves 2 to 3 times a week (Beeftink et al. 1982) and this species has already been cultivated in pilot schemes in the Netherlands, Belgium, Portugal and Pakistan (Geissler et al. 2009a and references therein). Currently it is available as a fresh vegetable in UK supermarkets at a price of about £3.00 per 100g. *A. tripolium* has also the potential to be used in animal diets and feed, and as an ornamental plant (Ramani et al. 2006, Geissler et al. 2009a and references therein). Other *Aster* species are already commercially grown for cut flower production, or are of medicinal interest, being used for example in the relief of coughs and as an expectorant, furthermore the plant may possess diuretic, antitumor, antibacterial, antiviral and anti-ulcer proprieties (Wallerstein et al. 1992, Ng et al. 2003 and references therein).

The genus *Aster* (Asteraceae/Compositae) includes many different species, however only *A. tripolium* is a halophyte. *A. tripolium* is a biennial to short-lived perennial species characterized by fleshy leaves and light-purple flowers (Clapham et al. 1942, Brock et al. 2007). It grows naturally in brackish water environments, and occurs typically in the upper saltmarsh but also on cliffs and rocks near the sea, and in inland saline areas (Clapham et al. 1942, Ramani et al. 2006, Brock et al. 2007, Uno et al. 2009). It has a wide world distribution, and is frequent in most of the European coast (Clapham et al. 1942, Brock et al. 2007, Uno et al. 2009). *A. tripolium* presents large morphological differences across habitats, and different *A. tripolium* varieties, ecotypes or subspecies have been described (Clapham et al. 1942, Huiskes et al. 1985, Stienstra 1986, Sági and Erdei 2005).

In *A. tripolium* plants the leaves are alternate, and a rhizome supports numerous adventitious roots (Clapham et al. 1942). During winter leaves remain just above soil (Clapham et al. 1942). Plants flower for the first time in their second year, producing spray-type, light-purple flowers, usually between July and October (Clapham et al. 1942, Uno et al. 1996). Plants produce large numbers of fruits (achenes) that are dispersed principally by wind, and the seeds germinates from March onwards (Clapham et al. 1942, Brock et al. 2007). Germination rates

decrease with salinity (Clapham et al. 1942). Besides reproduction by seed *A. tripolium* is also able to reproduce via rhizome vegetative propagules (Clapham et al. 1942, Brock et al. 2007). External salinity is a major factor influencing *A. tripolium* growth, in fact it is sometimes referred to as a facultative halophyte, since it can grow in non saline habitats and shows optimum growth consistently at low salinities (Clapham et al. 1942, Larher et al. 1982, Shennan et al. 1987a, Lenssen et al. 1995, Kerstiens et al. 2002, Carvalho et al. 2003, Ramani et al. 2006, Brock et al. 2007, Geissler et al. 2010). Hardly any information is available in relation to A. *tripolium* nutrient requirements, with the exception of two works comparing plant performance with N supplied as NH_4^+ or NO_3^-, and in both cases NO_3^- supply resulted in higher plants growth (Jefferies 1977, Sági and Erdei 2005).

Aims

This work sets out to investigate several aspects of *Salicornia europaea* and *Aster tripolium* growth and N uptake in order to gain a better understanding of the potential to include these plants in brackish and marine IMTA. The specific objectives of this thesis are to investigate:

- Growth and inorganic N uptake in *S. europaea* and *A. tripolium* under different harvest regimes under ambient conditions

- Growth and inorganic N uptake in *S. europaea* and *A. tripolium* at different concentrations and nitrogen forms, under controlled experimental conditions

- Growth and inorganic N uptake in *S. europaea* and *A. tripolium* under response to varying environmental parameters

- The potential for uptake of organic N by *S. europaea* and *A. tripolium*.

In order to address the objectives mentioned a range of hypotheses are tested in the chapters 1 through 6 of the present report, using plant biomass, N content and stable isotopes labelling. The key hypotheses investigated are:

Chapter 2 – Plant growth rate and N content increases with increasing N concentration up to N sufficiency value, and that *S. europaea* N sufficiency value is below 2 mmol NO_3^- l^{-1} continuous supply.

Chapter 3 – *S. europaea* and *A. tripolium* plants, under non-limiting nutrient supply, will decrease biomass production with introduction of routine cropping, and therefore decrease the amount of NH_4-N and NO_3-N removed from water.

Chapter 4 – *S. europaea* and *A. tripolium* growth is lower when N is supplied as NH_4^+ comparing to NO_3^- or NH_4NO_3; plant N uptake rates at different supply concentrations follow the Michaelis-Menten model; NH_4–N uptake rates are higher than NO_3-N uptake at similar N concentrations; NO_3-N uptake is inhibited by the presence of NH_4^+ in solution, while NH_4–N uptake is not inhibited by NO_3^-; plants receiving continuous supply of N express higher N uptake rates than plants N starved for 48h.

Chapter 5 – *S. europaea* performs better at medium and high salinities, while *A. tripolium* performs better at low and medium salinities (biomass production and NH_4-N and NO_3-N uptake). Increasing irradiance and temperature, increases NH_4-N and NO_3-N uptake.

Chapter 6 – *S. europaea* and *A. tripolium* plants have the capacity to take up organic N (as amino acid and peptides) at rates comparable to that of inorganic N uptake. In a hydroponic system the amino acid and peptide N is mainly taken up as intact organic molecules, while when growing in soil the % of intact uptake will be lower due to higher competition with microbes.

Chapter 2 - Growth responses of *Salicornia europaea* to varying nitrate concentrations

Introduction

Ammonia plus ammonium, $NH_3 + NH_4^+$, (hereafter NH_4^+) is often the main DIN form occurring in aquaculture wastewater (Lefebvre et al. 2001, Porrello et al. 2005, Webb et al. 2012) and it is toxic to fish (Handy and Poxton 1993) therefore, when recirculation back to the culture tanks is required nitrification bacterial biofilters are routinely used in water treatment (Hagopian and Riley 1998, Chen et al. 2006, Malone and Pfeiffer 2006). Despite solving the immediate problem of the NH_4^+ toxicity to fish, bacterial biofilters do not remove N from the system and result in the accumulation of NO_3^- and its subsequent release to the surrounding environment. NO_3^- is only toxic at relatively high concentrations (reference concentrations between 7 and 70 mmol l^{-1} for different species) (Blancheton 2000, Lyssenko and Wheaton 2006a, b), but even at much lower concentrations it can cause eutrophication in waters influenced by discharges (Pillay 1992, Wu 1995). One approach for DIN removal from wastewater is the use of Integrated Multi-Trophic Aquaculture (IMTA). In IMTA the culture of fish or shrimps can be integrated with culture of organisms that extract dissolved inorganic nutrients (seaweed/plants) in the way of balancing the dissolved nutrients wasted in one unit with the need for nutrients input in the other (Chopin et al. 2001). With this approach the cultured algae or plant can provide extra commercial crops, directly providing the producer with product diversification and associated profit, and actively remove N from the aquaculture system.

A wide range of macroalgae and higher plants are used routinely in the treatment of freshwater aquaculture wastewater (McMurtry et al. 1997, Schulz et al. 2004, Henry-Silva and Camargo 2006, Al-Hafedh et al. 2008, Henry-Silva and Camargo 2008, Zachritz et al. 2008) and also for urban wastewater treatment (reviewed in Vymazal 2008). In the context of brackish and marine aquaculture several macroalgae have been shown to perform well in IMTA, providing high N removal while generating commercial crop (reviewed in Troell et al. 1999, Neori et al. 2004, Bunting 2008). However, comparatively less attention has been given to the use of higher plants, and halophyte plants can be included in such systems since they thrive in brackish and saline environments (Brown et al. 1999, Lin et al. 2003, 2005, Ventura et al. 2011a, Webb et al. 2012, Buhmann and Papenbrock in press).

Salicornia spp., (samphire) are annual halophytic plants with a wide geographical distribution (Davy et al. 2001) that have a long history of human consumption as both a fresh and pickled vegetable. *S. europaea* fresh succulent tips can be found in the UK retail market at about £ 2 per 100 g and it can also be used in a wide range of other commercial applications,

including the use in animal diets and feed (Glenn et al. 1991b, 1992, Attia et al. 1997, Belal and Al-Dosari 1999, Masters et al. 2007, Bechardas 2008, Murama 2008, Rozema and Flowers 2008), and applications in the cosmetic and nutraceutical industries (Envirophyte 2009). Additional to its high economic value, *S. europaea* has already been shown to grow well and to be capable of high N removal efficiency in aquaculture wastewater, and a regular supply of fresh succulent tips can be obtained by repeatedly harvested during the growing season (Ventura et al. 2011a, Webb et al. 2012), and is therefore a plant of interest to use in IMTA.

In order to build a balanced IMTA system, the performance of the selected extractive organism in terms of N removal and production, needs to be accurately predicted. Nitrogen is essential to plants as a constituent of amino acids, proteins, nucleic acids, enzymes, and other cellular constituents, and therefore plant growth is closely related to N supply (Gauch 1972, Bannister 1976, Haynes 1986). Several studies indicate that N availability can be a limiting factor for *Salicornia* sp. growth (Piggot 1969, Stewart et al. 1973, Valiela and Teal 1974, Jefferies 1977, Jefferies and Perkins 1977, Loveland and Ungar 1983, Boyer et al. 2001, Costa, 1992 in Davy et al. 2001, e.g. Boyer and Fong 2005, Webb 2005). Piggot (1969), Stewart et al. (1973) and Loveland and Ungar (1983) consistently reported biomass increase in *S. europaea* plants fertilized with N in salt marshes, indicating a N limitation in those situations. As well, Jefferies (1977) and Webb (2012) cultivated *S. europaea* receiving different N concentrations (added as NO_3^- and NH_4^+ respectively) and reported increased growth in the shoots with increase in N supply. On the other hand, Quintã (2007) found no differences both in *S. europaea* growth across different NO_3^- treatments and in the shoot:root ratio across N treatments, though it is widely accepted that although roots may increase following N additions the shoot:root ratio increases with increasing N supply. The explanation put forward by Valiela et al. (1976) is that plants produce only enough roots to process needed N.

It is also commonly assumed that the concentration of an element (such as N) in a plant is a direct result of the supply of that element to the plant while limiting, ie. the tissue concentration increases with external concentration of the element until it reaches a plateau that translates the plant sufficiency value – after which the element is no longer limiting for the plant growth (Gerloff and Krombholz 1966, Ridge 1991). However plants capable of N luxury uptake have also been observed, in which cases plant N content increases even after external N concentrations are above the of sufficiency value (Smart and Barko 1980). So far few studies have investigated N content of *S. europaea*: Loveland and Ungar (1983) found shoot N concentrations to be inversely related to the growth response to fertilization. Webb (2012) observed a lower N content in plants grown with the lower NH_4^+ supply (~0 mmol l^{-1}), in

relation to all other treatments, but no consistent differences between N content of plants grown in the 2, 4 and 6 mmol l^{-1} treatments. Quintã (2007) showed that there were no differences in respect to *S. europaea* N content across N supply treatments, and as previously described no growth differences were observed in this study either.

The present works sets out to investigate *S. europaea* responses to increasing NO_3^- continuous supply in order to better understand the use of *S. europaea* plants in IMTA systems, specifically by identifying N limiting supply, maximum plants growth and maximum N removal. Here it is hypothesized that plant final biomass, growth rate and N content increases with increasing N supply below that of N sufficiency value, and that *S. europaea* N sufficiency value is below 2 mmol NO_3^- l^{-1} continuous supply. It is further hypothesized that, following repetitive harvest, tips re-grow increases with time as well as with increasing N supply. In order to test the mentioned hypotheses, *S. europaea* growth, N content and production of succulent tips in a repetitive harvest regime is investigated under different concentrations of NO_3^- continuous supply (5 – 2000 µmol l^{-1}).

Methods

Plants

Fourth-generation *S. europaea* seeds, originating from the study by Webb et al. (2012) were stored at -20°C until used in the present experiments. Seeds were germinated in P576 plug trays with John Innes No.1 compost, continuously immersed in fresh water, in a laboratory with controlled air temperature (~30°C) and light (12h photoperiod, artificial light supply - 4 proGrow Syvania tubes) (Figure 2.1a). Two weeks after sowing seedlings were moved to a climate-controlled greenhouse at the Henfaes Research Centre, Bangor University (16h photoperiod) (Figure 2.1b).

The water supply was changed to salinity of 10 (TROPICMARIN™ artificial sea salt) and Phostrogen fertiliser (N:P:K 14:10:27 + trace elements; Bayer CropScience Ltd, Cambridge, UK) was added. The solution was frequently topped up with fresh water to compensate for evapo-transpiration, and fully replaced once a week. Five weeks after sowing plants were thinned out to one plant per plug. Seven weeks later, plants were individually transplanted from the trays (Figure 2.1 c and d) into the PVC tubes containing sand as described below.

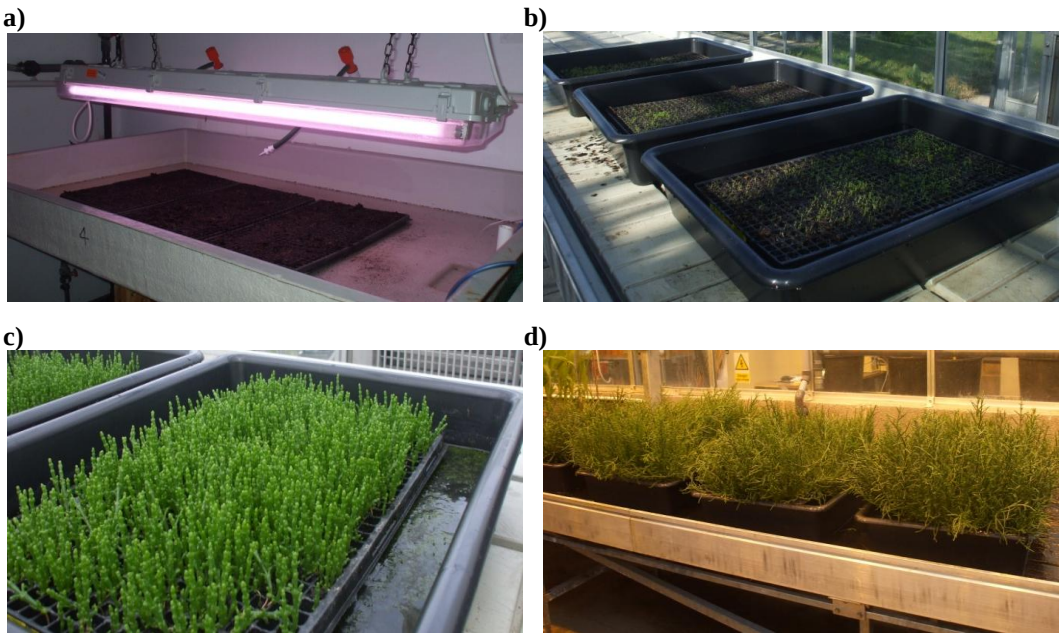


Figure 2.1 – *Salicornia europaea* plants used in the experiment, from seed to transplant. **a)** at time of sowing, **b)** 2 weeks later, **c)** 6 weeks later , **d)** 14 weeks later.

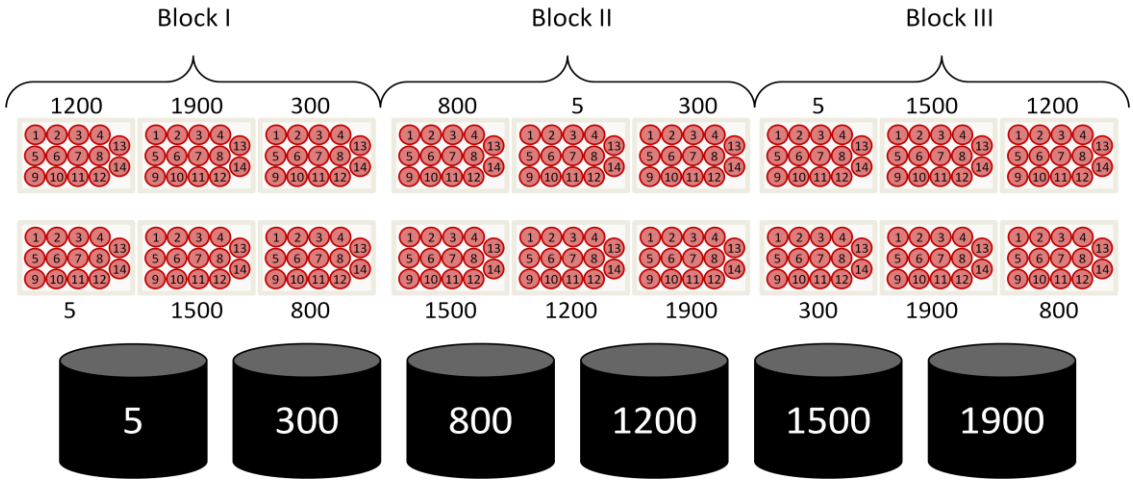
Experimental setup

For each of six N concentration treatments (5, 300, 80, 1200, 1500 and 1900 µmol l^{-1} added as $NaNO_3$), plants were water grown in sand with a continuous solution supply, from a recirculating unit, each unit comprised of one tank (capacity ~200l) connected to three grey boxes (external dimensions: 60 cm length * 40 cm width * 32.5 cm depth) (Figure 2.2). A randomized block system of three blocks (I, II, III) was used. At the beginning of the experiment, 42 plants per treatment were individually transplanted from the P576 plug trays into PVC tubes (11 cm diameter, 30 cm depth) filled with concreting sand (Figure 2.2). The PVC tubes were previously lined with geotextile at the bottom, to avoid the loss of sand and to allow water circulation, and placed on top plug trays inside the grey boxes to allow better water circulation into the sand tube.

Water was continuously pumped with a 13w Hozelock Cascade 700 submersible fountain pump, from each separate tank into the grey boxes, with an automatic siphon draining water back to the tank when the lysimeter was full so that water level varied from 15 cm to 30 cm over a cycle of approximately 45 min). The solution in grey box was continuously aerated (Pond Air 4800 pump). Irradiance, air and water temperature were recorded throughout the experiment (Onset Hobo pendant temp/light UA-002-64; Aquatic tinytag TG-3100; Temperature). Recorded light data, in lux, was converted to µmol PAR m^{-2} s^{-1}, after calibration against Li-Cor 190SA

sensor calibration was obtained by taking readings at the same time with both sensors side by side (at different times of the day and weather forecasts) at the greenhouse (r^2=0.92).

a)



b)

Figure 2.2 – Experimental setup **a)** schematic representation, **b)** picture after plants were transplanted.

Nutrient solution was made up to a salinity of 10 (TROPICMARIN^{TM} artificial sea salt), and included 0.1 ml l^{-1} Hoagland's micronutrients solution, 500 µmol P l^{-1} added as NaH_2PO_4, and six different N concentrations. The NO_3^- concentrations (added as $NaNO_3$) used were based on previous work and on known fish farm waste concentrations (Llyn Aquaculture Ltd.). A 2 mol l^{-1} solution was prepared and different volumes added to each tank at N nominal concentrations between 0 (no added N) and 2000 µmol l^{-1} (see results section for actual N concentrations). After the first two weeks, all the solutions were replaced twice a week to maintain nutrient concentrations. Filtered (0.45 µm) water samples were taken, both before and after each water

change, immediately frozen and later analysed for dissolved P and N. Dissolved inorganic phosphate (PO_4^{3-}), NO_2^- and NO_3^- were determined, in triplicate, using a 5–channel LACHAT Instruments Quick-Chem 8000 autoanalyzer after Grasshoff et al. (1983) and Hales et al. (2004). NH_4^+ was determined by fluorimetric method after Holmes et al. (1999) using HITACHI F2000 fluorescence spectrophotometer.

In order to measure plant biomass at the beginning of the experiment (11th June 2009 – day 162), 30 randomly selected plants were separated into root, shoot and tips. The roots were separated from the shoots by cutting them at the substrate level and the tips approximately 10 cm above that level (Figure 2.3). Each part of each plant was individually weighed (FW), dried at 50°C and weighed again (DW). Every 3 weeks, between 29th of July and 30th of September (days 210 and 273), 9 randomly-selected plants from each treatment (3 from each block) were removed from the system. These plants were separated into root, shoot and tips. Shoot and tips were weighed as above for both FW and DW. The roots were cleaned in order to remove any sand and then dried, and only DW was assessed for roots. The specific growth rate (g DW d^{-1}) of the above-ground portion of plants was calculated by subtracting the initial above-ground logarithm biomass (day 162) from the final above-ground biomass logarithm (which included shoot biomass at the last harvest (day 273) and the total tips biomass produced during this period), and dividing the difference by the number of days between the two harvests. Additionally, all plants left in the system were cropped every three weeks (starting on 8th July - day 190), the removed tips of each plant were weighed, FW and DW calculated as described above, and stored.

Three plants from the initial biomass samples and three plants from each treatment (one per block) from each harvest point were analysed for C and N content of root, shoot and tip tissue (Carbo Erba NA1500 Elemental Analyser CHNS-O). Tips from three plants from each treatment removed on 9th of July were also analysed for C and N content. The samples were ground to fine powder using a power mill (Cyclotec 1093 Sample Mill, with a 1mm sieve) and stored in a dry environment. Initially, five subsamples of each part of one plant were analysed without acidification (samples weighed into tin caps) and five subsamples were acidified before analysis (200 µl of 2 mol l^{-1} HCl solution added to the pre-weighed samples in silver caps, and dried at 40°C for 48h). HCl is commonly used to remove any inorganic carbonates from the plant samples before elemental analysis. However, in the present study (see the results section) N content was reduced by acidifying the samples and thus it was decided not to acidify further samples for elemental analysis.

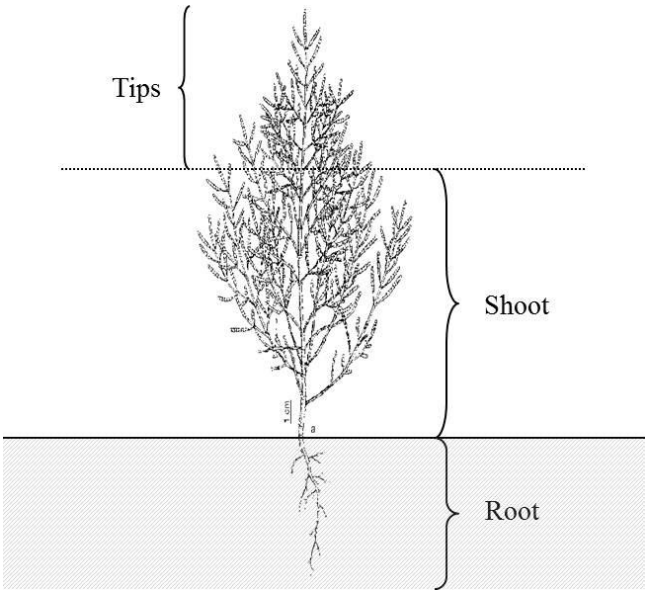


Figure 2.3 - *Salicornia europaea* schematic representation, root, shoot and tips as mentioned in this work. Adapted from http://www.floraiberica.es.

Statistical analysis

The differences in background NH_4^+ concentration and PO_4-P, in solution after and before water changes and across NO_3^- concentrations were tested using two way ANOVAs, after confirming homogeneity of variances (Levene's test). In both cases interaction between the two factors tested was not significant and so the main effects (NO_3^- concentrations and water change) are discussed. NO_3^- concentrations, after and before water changes, for each NO_3^- concentrations, were compared, using one way ANOVA test, after confirming homogeneity of variances (Levene's test). Plant biomass, shoot, root and tips, as well as the N and C plants content were compared across the three blocks using ANOVA when homogeneity of variances was confirmed (Levene's test), or Kruskal-Wallis Test when the variances were not equal. Since there were no significant differences between the 3 blocks, samples were pooled and analysed together. Correlation between the above-ground fresh and dry mass was investigated using Pearson product-moment correlation across the several NO_3^- concentrations. Following a significant correlation, the nature of the relationship was investigated; linearity was confirmed using linear regression analysis and the obtained slopes compared using t-test after Bonferroni correction. Similar analysis was performed in relation to above-ground and root dry mass. Plant specific growth rate across NO_3^- concentrations was analysed with one way ANOVA after confirming homogeneity of variances (Levene's test). In relation to biomass, N and C content and N uptake, the effect of time (harvest day) and NO_3^- concentrations were investigated using a two way

ANOVA when variances were homogeneous (Levene's test). Following the two way ANOVA, when interaction between the two factors was not significant, Tukey (or Bonferroni for unequal number of samples) pairwise comparisons were performed to investigate the main effect of each factor. If interaction was significant pairwise comparisons were performed within each factor. In cases were required assumptions for two way ANOVA were not met the differences were tested in relation to the two factors separately, using either one way ANOVA (equal variances) or Kruskal-Wallis followed, respectively, by Tukey (or Bonferroni for unequal number of samples) or LSD *post hoc* tests.

All values are reported as means ± standard deviation. The level of $p = <0.05$ was considered as significant for all the statistical analysis performed.

Results

Environmental data

Mean daily irradiance along the duration of the experiment was 280 ± 66 µmol PAR m^{-2} s^{-1} (Figure 2.4a). Mean daily air temperature for the same period was 23.4 ± 1.9°C, while daily minimum mean was 16.1 ± 1.8°C, and the daily maximum air temperature 36.5 ± 4.7°C (Figure 2.4b). Mean daily water temperature for the experimental period was 20.6 ± 1.5°C, while daily minimum was 18.8 ± 1.8°C, and the daily maximum air temperature 22.3 ± 1.6°C (Figure 2.4c). Salinity varied between 10.1 ± 0.1 after each water change and 10.3 ± 0.1 immediately before the following water change.

Water nutrients

Actual mean NO_3^- concentrations are presented in Table 2.1 in relation to each nominal target concentration. For every NO_3^- treatment, the mean NO_3^- concentrations after each water change were significantly higher than the concentrations before the water changes ($p \leq 0.004$) (Table 2.1). NO_2^- concentrations were low during the whole experiment, 1.16 ± 1.64 µmol l^{-1}, and is reported here combined with NO_3^-. NH_4^+ concentrations were low for all the samples, and there were no significant differences among treatments, but NH_4^+ concentrations after water changes, 1.16 ±

1.06 µmol l^{-1}, were significantly higher than before, 0.90 ± 0.70 µmol l^{-1} (p=0.006). Similarly, PO_4^{3-} concentrations were not significantly different across the NO_3^- treatments, but were significantly higher after water changes (509 ± 66 µmol l^{-1}) than before (480 ±49 µmol l^{-1}, p<0.001).

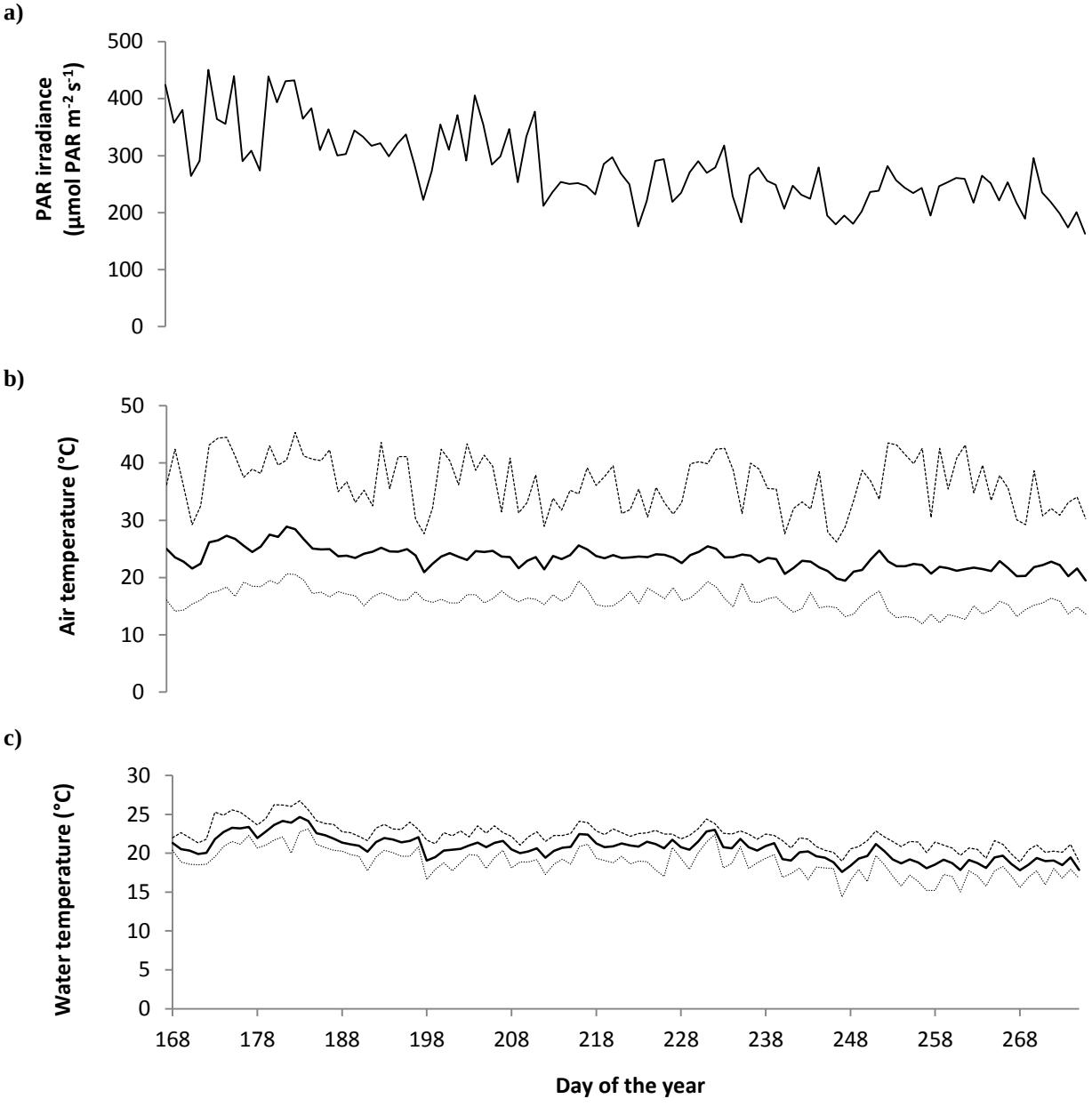


Figure 2.4 – Environmental data recorded during the experiment. **a)** Daily mean light irradiance (µmol PAR m^{-2} s^{-1}), **b)** daily mean (bold line), minimum (small dashed line) and maximum (dashed line) air temperature (°C), **c)** daily mean, minimum and maximum water temperature (°C)**.**

Table 2.1 - Measured mean (± standard deviation) NO_3^- concentration (µmol l^{-1}) in solution for each NO_3^- treatment before and after water changes and overall.

Nominal NO_3^- concentration	after change (new solution)	before change (old solution)	overall mean
5	6.24 ± 2.23	0.85 ± 1.60	3.70 ± 3.75
300	451 ± 70	171 ± 106	310 ± 166
800	918 ± 128	609 ± 194	765 ± 228
1200	1345 ± 207	1083 ± 228	1190 ± 267
1500	1733 ± 193	1412 ± 241	1541 ± 302
1900	2073 ± 286	1845 ± 254	1928 ± 317

Plant growth

Plants growing in the lower NO_3^- concentration (5 µmol l^{-1}) had 21% mortality during the experimental period, while no mortality was observed in any of the other NO_3^- treatment.

Relationships of fresh and dry weight of the above-ground biomass (Figure 2.5) were linear ($p<0.001$, $r^2>0.938$). The water content in plants from the lowest NO_3^- concentration was lower, 78.9 ± 6.4%, than plants in all other treatments ($p<0.001$), which in turn had no significantly difference between them, 91.5 ± 1.0 %, 91.7 ± 0.9 %, 91.7 ± 0.9 %, 91.3 ± 1.3 %, 91.7 ± 1.3 %, respectively for treatments 300, 800, 1200, 1500 and 1900 µmol l^{-1}.

The above-ground to root biomass relationship in plants grown in the different NO_3^- concentrations was always linear ($p<0.001$, $r^2=0.6$ concentration 5 µmol l^{-1}, $r^2>0.82$ other relationships). The slope of plants grown in 5 µmol N l^{-1}, 1.4 ± 0.3, was lower than in all other treatments ($p<0.001$), which showed no significant difference between 300 to 1900 µmol l^{-1} (Figure 2.6).

Plants grown in the 5 µmol N l^{-1} treatment exhibited a lower specific growth rate, 0.004 ± 0.002 g DW d^{-1}, than plants in all other N ($p<0.001$), while there was no significant difference between any of the other treatments (Figure 2.7).

The root biomass on each harvest date, for NO_3^- concentrations between 5 and 1900 µmol l^{-1}, is presented in Figure 2.8a. Although on the first harvest (day 210) there were no significant differences between the root biomass across NO_3^- concentrations, on day 231 root biomass of plants grown in 5 µmol l^{-1} treatment, 0.647 ± 0.161 g DW, was lower than from plants grown at 800 and 1900 µmol l^{-1}, respectively 1.59 ± 0.73 g DW ($p=0.045$) and 1.85 ± 0.62 g DW ($p=0.008$). Additionally on days 252 and 273, root biomass was lower in plants grown in

the 5 µmol l^{-1} concentration than in all other N concentrations ($p \leq 0.04$). There were no significant differences in root biomass between any of the other N concentrations. Root biomass from plants in the 5 µmol N l^{-1} concentration was not significantly different across the several harvest days. For the other N concentrations root biomass increased with time, with root biomass on day 210 significantly lower than on day 273 for every N concentration ($p<0.03$), even though the increase in two consecutive harvests wasn't always significant (Figure 2.8a).

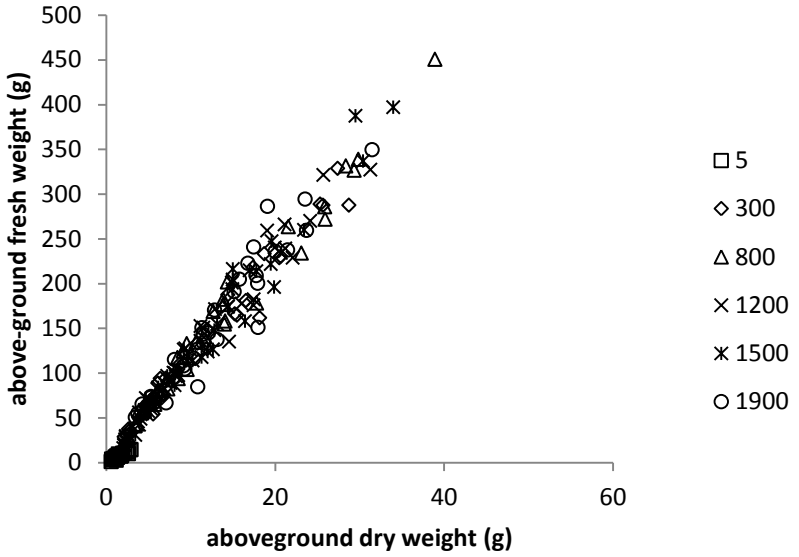


Figure 2.5 – *S. europaea* plants above-ground fresh and dry weight (g) relationships, different symbols for different solution NO_3^- concentrations (µmol l^{-1}) as in the legend.

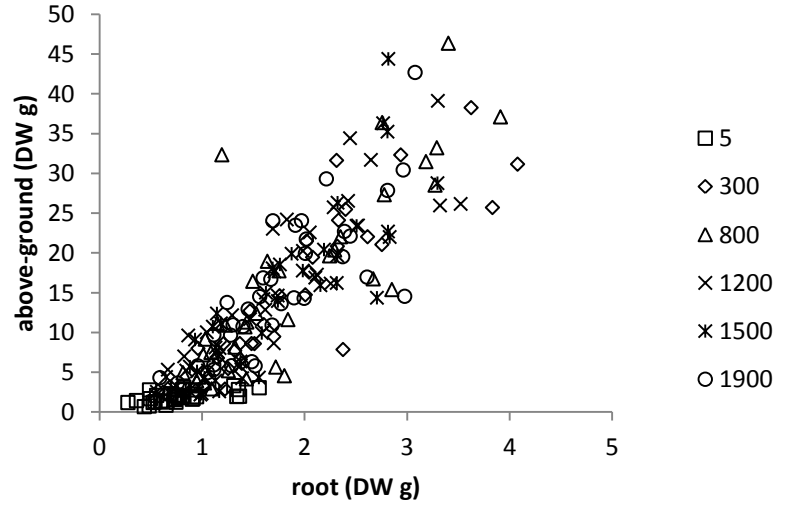


Figure 2.6 – *S. europaea* plants above-ground and root dry weight (DW g) relationships, different symbols for different solution NO_3^- concentrations (µmol l^{-1}) as in the legend.

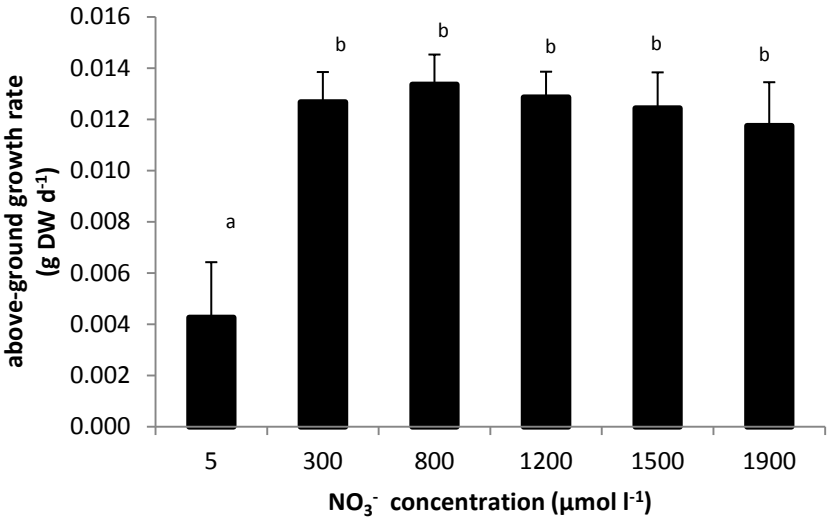


Figure 2.7 – *S. europaea* plants above-ground specific growth rate (g DW d^{-1}) in the different NO_3^- concentrations. Means sharing a letter do not differ significantly.

Shoot biomass (Figure 2.8b) was lower in plants grown at 5 µmol l^{-1}, compared to all other N concentrations at each harvest ($p<0.05$), while there was no significant differences between the other N concentrations (Figure 2.8b). The shoot biomass in 5 µmol N l^{-1} was lower on days 210 and 231 than on days 252 and 273 ($p<0.009$). For 300 to 1900 µmol N l^{-1} concentrations, shoot biomass on day 210 was lower than on days 252 and 273 ($p<0.05$). Additionally plants harvested on day 231 showed lower shoot biomass than on day 273 in case of 300, 800 and 1200 µmol N l^{-1} treatments ($p<0.05$) (Figure 2.8b).

At 5 µmol N l^{-1} no regrowth was observed in the tips after the first crop (day 189) at which point the tip biomass per plant was 0.295 ± 0.116 g DW and significantly lower than tip biomass in the 800 to 1900 µmol N l^{-1} treatments ($p<0.05$) (Figure 2.9). At this harvest, tip production at 300 µmol N l^{-1} (0.394 ± 0.161 g DW) was significantly lower than at 1900 µmol N l^{-1} (0.546 ± 0.199 g DW, $p<0.05$). In 300 to 1900 µmol N l^{-1} treatments there was no significant difference in tip biomass between N concentrations for the remaining harvests. Tip biomass increased with time for every N concentration (300-1900 µmol N l^{-1}), being lower on day 189 than in every other harvest day ($p<0.05$). However, the increase in consecutive harvests was not always significant (Figure 2.9).

Tip production over the whole experimental period, the sum of all produced tips from each plant (Figure 2.10), was not significantly different in plants grown in NO_3^- concentrations between 300 and 1900 µmol l^{-1}. However, in all these treatments the tip production was significantly higher than in plants grown in the lowest NO_3^- concentration ($p<0.001$).

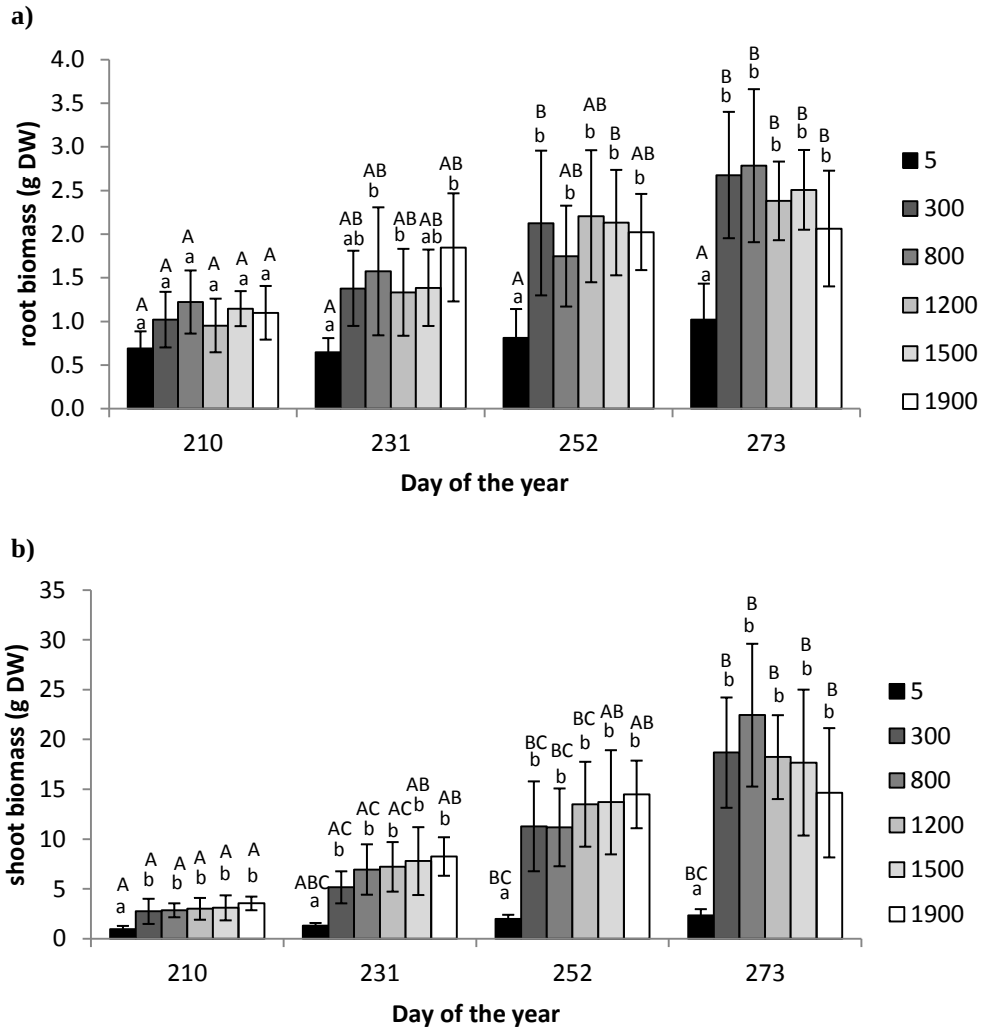


Figure 2.8 – *S. europaea* plant biomass (g DW plant^{-1}) at each harvest date, for NO_3^- concentrations between 5-1900 µmol l^{-1}. **a)** root, **b)** shoot. Within each harvest day, means sharing a lowercase letter do not differ significantly. Within each N concentration, means sharing an uppercase letter do not differ significantly.

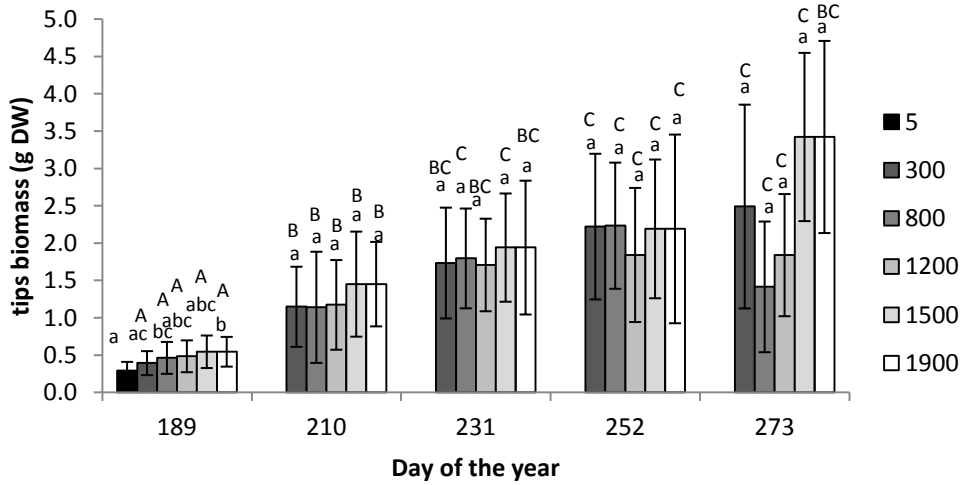


Figure 2.9 – *S. europaea* tip biomass (g DW plant^{-1}) at each harvest date, for NO_3^- concentrations between 5-1900 µmol l^{-1}. Within each harvest day, means sharing a lowercase letter do not differ significantly. Within each N concentration, means sharing an uppercase letter do not differ significantly.

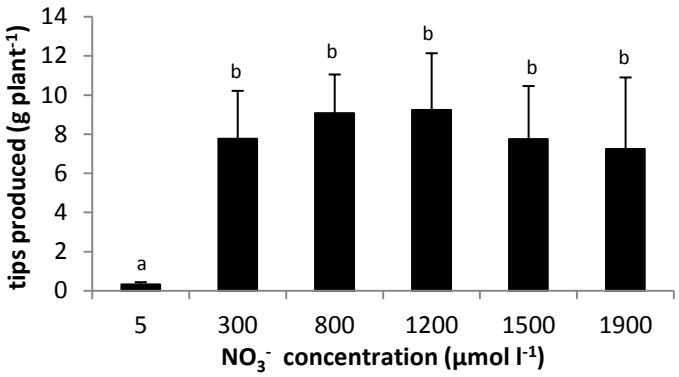


Figure 2.10 – *S. europaea* total tips biomass produced per plant (g DW plant^{-1}) during the experiment, in each NO_3^- concentration (µmol l^{-1}). Means sharing a letter do not differ significantly.

Plant carbon and nitrogen content

Initial results of C and N (mmol g^{-1} DW) content in 5 acidified and 5 non-acidifed plant samples are presented in Figure 2.11. For all tissues, tips shoot and root, the measured N content was higher when the samples were not acidified prior to analysis ($p<0.001$). There was no significant differences between C content between acidified and not acidified samples, both for tips and roots, however, C content was higher in the shoots of samples not acidified ($p<0.001$). According to these results (see discussion for explanation) it was decided to not acidify the samples before C and N content analysis, accordingly all the results following are from non-acidified samples.

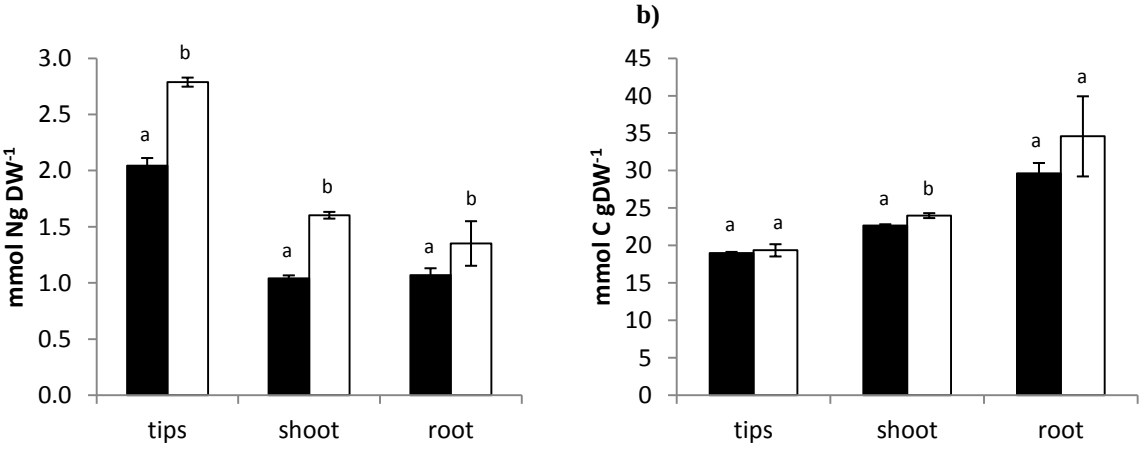


Figure 2.11 – Elemental content of *S. europaea* plants, tips shoot and root, in samples acidified (black bars) and not acidified (white bars) during preparation for analysis; **a)** nitrogen (mmol N g^{-1} DW), **b)** carbon (mmol C g^{-1} DW). For each tissue type, means sharing a letter do not differ significantly.

The N content in the plants sampled at the beginning of the experiment was higher in the tips, (4.58 ± 0.89 mmol N g^{-1} DW) than in shoots (2.54 ± 0.54 mmol N g^{-1} DW), and roots (2.33 ± 0.16 mmol N g^{-1} DW) ($p \leq 0.01$). Although N content was not significantly different between shoots and roots. In plants sampled during the experimental period (Figure 2.12), overall N content was significantly different between all plant tissue types ($p<0.001$): It was lowest in the roots (1.52 ± 0.28 mmol N g^{-1} DW), followed by shoot (2.02 ± 0.51 mmol N g^{-1} DW) and tips (3.18 ± 0.57 mmol N g^{-1}).

The N content in root tissue (Figure 2.12a) was lower in plants grown at 5 µmol N l^{-1} (1.00 ± 0.26 mmol N g^{-1} DW), than in all other concentrations ($p \leq 0.001$), while there were no significant differences between the N content in roots from concentrations 300 to 1900 µmol N l^{-1}. Root N content observed on day 252, 1.30 ± 0.29 mmol N g^{-1} DW, was significantly lower than on days 231 and 273, 1.52 ± 0.30 mmol N g^{-1} DW and 1.48 ± 0.24 mmol N g^{-1} DW, respectively ($p \leq 0.033$).

No significant differences were observed in shoot N content (Figure 2.12b) from all N treatments, between harvest days. The N contents in shoots grown at 5 µmol N l^{-1} were lower than in shoots from all other N concentrations on days 210, 231 and 252 ($p<0.001$). However, on day 273 these were significantly lower compared to shoot N contents in plants grown at 1200, 1500 and 1900 µmol N l^{-1}.

Tip N content never varied significantly across the several N treatments, neither in the case of the tips cropped on day 189 from plants in 5 - 1900 µmol N l^{-1} concentrations, nor in remaining sampling days from plants in 300-1900 µmol N l^{-1} concentrations (Figure 2.12c). Tip N content (300 - 1900 µmol N l^{-1}) was lower on day 189 than all other days ($p<0.001$), lower on day 273 than in days 210 and 231 and also lower on day 252 than on day 231 ($p \leq 0.008$).

The C contents in the plants sampled at the beginning of the experiment were significantly higher in the roots (32.9 ± 0.8 µmol C g^{-1} DW), than in shoots (27.3 ± 2.3 µmol C g^{-1} DW), and tips (23.9 ± 0.3 µmol C g^{-1} DW) ($p \leq 0.007$), while the C contents were not significantly different between shoots and tips. The C contents from plants sampled during the experimental period are presented in Figure 2.13. Overall the C contents were different between the three plant tissues types ($p<0.001$): Root C contents were the highest (31.3 ± 2.3 µmol C g^{-1}), followed by shoots (25.3 ± 2.6 µmol C g^{-1}) and the tips had the lowest C contents (21.6 ± 2.7 µmol C g^{-1}).

The C content was lower in roots from plants grown at 5 µmol N l^{-1} (29.3 ± 3.13 mmol C g^{-1} DW) than at 300 to 1200 and 1900 µmol N l^{-1} ($p \leq 0.039$), but not significantly lower than root

C content in plants grown at 1500 µmol N l^{-1} (Figure 2.13a). The C root content was lower on day 210 (29.4 ± 2.0 mmol C g^{-1} DW) compared to all other harvest days ($p \leq 0.002$).

Shoot C contents were higher in plants grown at 5 µmol N l^{-1} (30.2 ± 1.1 mmol C g^{-1} DW) compared to every other N treatment ($p<0.001$). No other differences in shoot C contents were observed between N treatments. There were no significant differences between the C contents in shoots from the several sampling days.

The difference in tip C contents between N treatments was not significant (Figure 2.14c). However, tip C for 300 - 1900 µmol N l^{-1} concentrations varied with time, with higher C contents being measured on days 189 and 273 than on the remaining harvest days ($p<0.001$).

The C:N ratio in plant tips sampled at the beginning of the experiment (5.33 ± 1.21) was significantly lower than both shoot and roots (11.2 ± 3.0 and 14.2 ± 1.3, respectively; $p \leq 0.03$). The C:N ratio in plants sampled during the experimental period (Figure 2.14) was different across the three tissues ($p<0.05$): lower in tips (7.21 ± 2.62) followed by shoots (14.4 ± 7.6) and highest in roots (22.8 ± 5.0).

The C:N ratio in root tissue (Figure 2.14a) was higher in the 5 µmol N l^{-1} treatment compared to all other N concentrations ($p<0.001$), and no further significant differences were observed in root C:N ratio between the different N concentrations. A higher root C:N ratio was measured on day 252 (26.0 ± 5.8) compared to every other sampling date ($p \leq 0.001$), while there were no significant differences between the other days.

Shoot C:N ratio (Figure 2.14b) was higher in plants grown at 5 µmol N l^{-1} treatment than in any other treatments ($p<0.001$), while there was no significant differences among the shoot C:N between the other concentrations. The C:N ratios in shoots were not significantly different between the harvest days.

The C:N ratios in tips (Figure 2.14c) were not significantly different between N treatments. Tip C:N ratios were higher at the first and last harvests, respectively 10.0 ± 2.2 and 7.45 ± 0.83, than on days 210, 231 and 252 ($p \leq 0.024$).

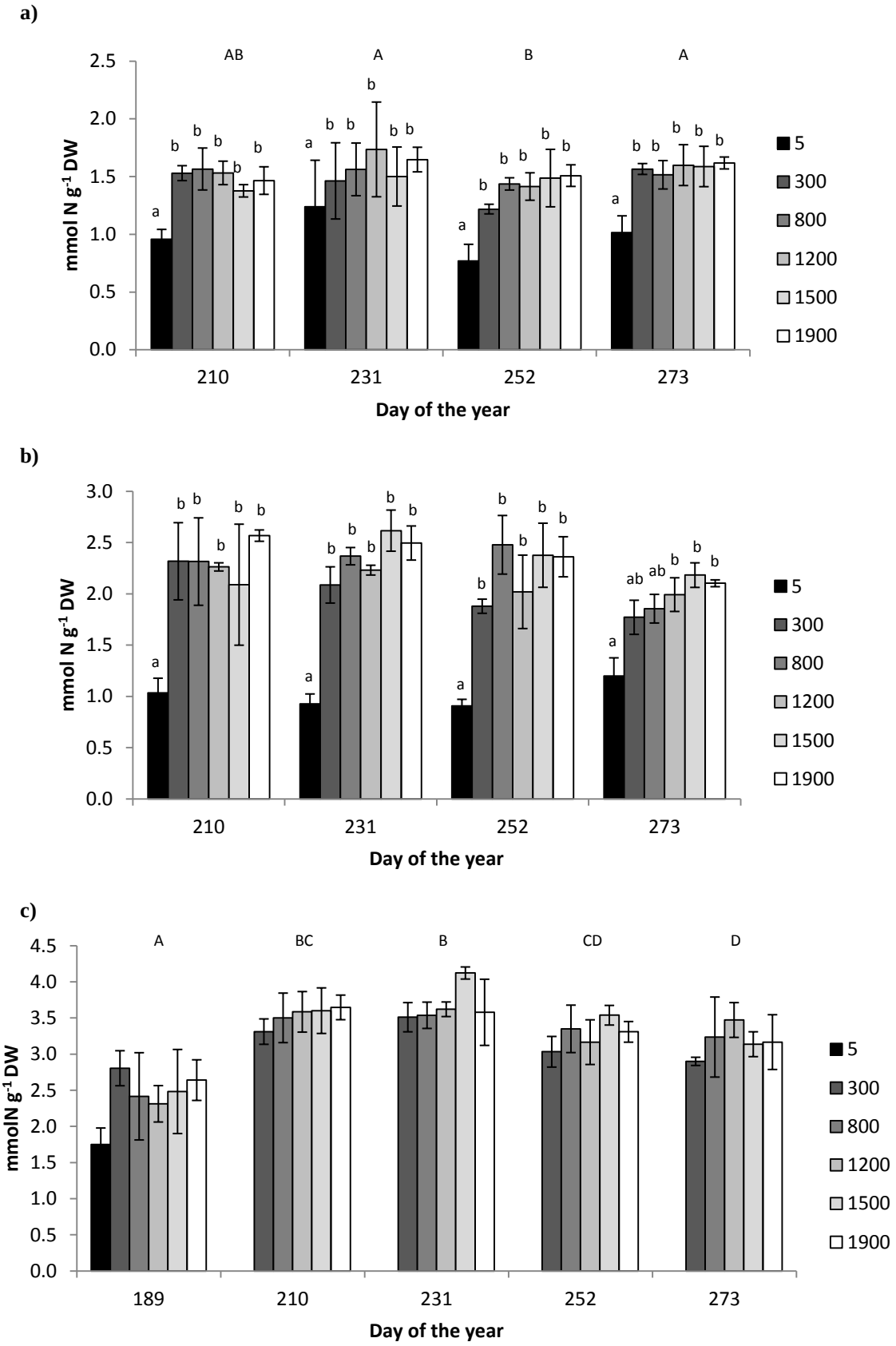


Figure 2.12 – Nitrogen content of *S. europaea* plants (mmol N g^{-1} DW), in **a)** root, **b)** shoot, **c)** tips, for plants grown at NO_3^- concentrations between 5-1900 µmol l^{-1}. Within each harvest day, means sharing a lowercase letter do not differ significantly. Within each N concentration, means sharing an uppercase letter do not differ significantly.

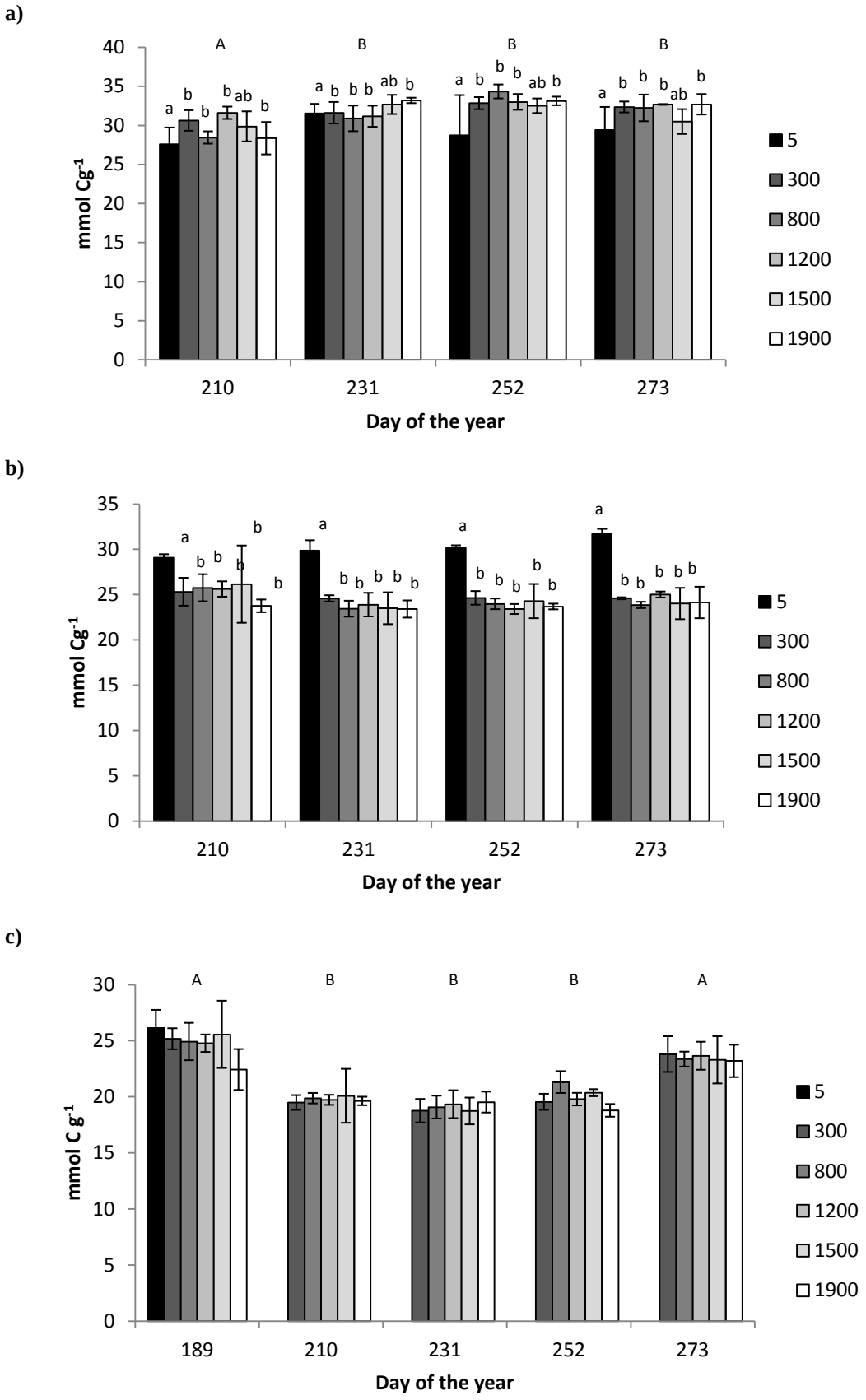


Figure 2.13 - Carbon content of *S. europaea* plants (mmol C g^{-1} DW), in **a)** root, **b)** shoot, **c)** tips, for NO_3^- concentrations between 5-1900 µmol l^{-1}. Within each harvest day, means sharing a lowercase letter do not differ significantly. Within each N concentration, means sharing an uppercase letter do not differ significantly.

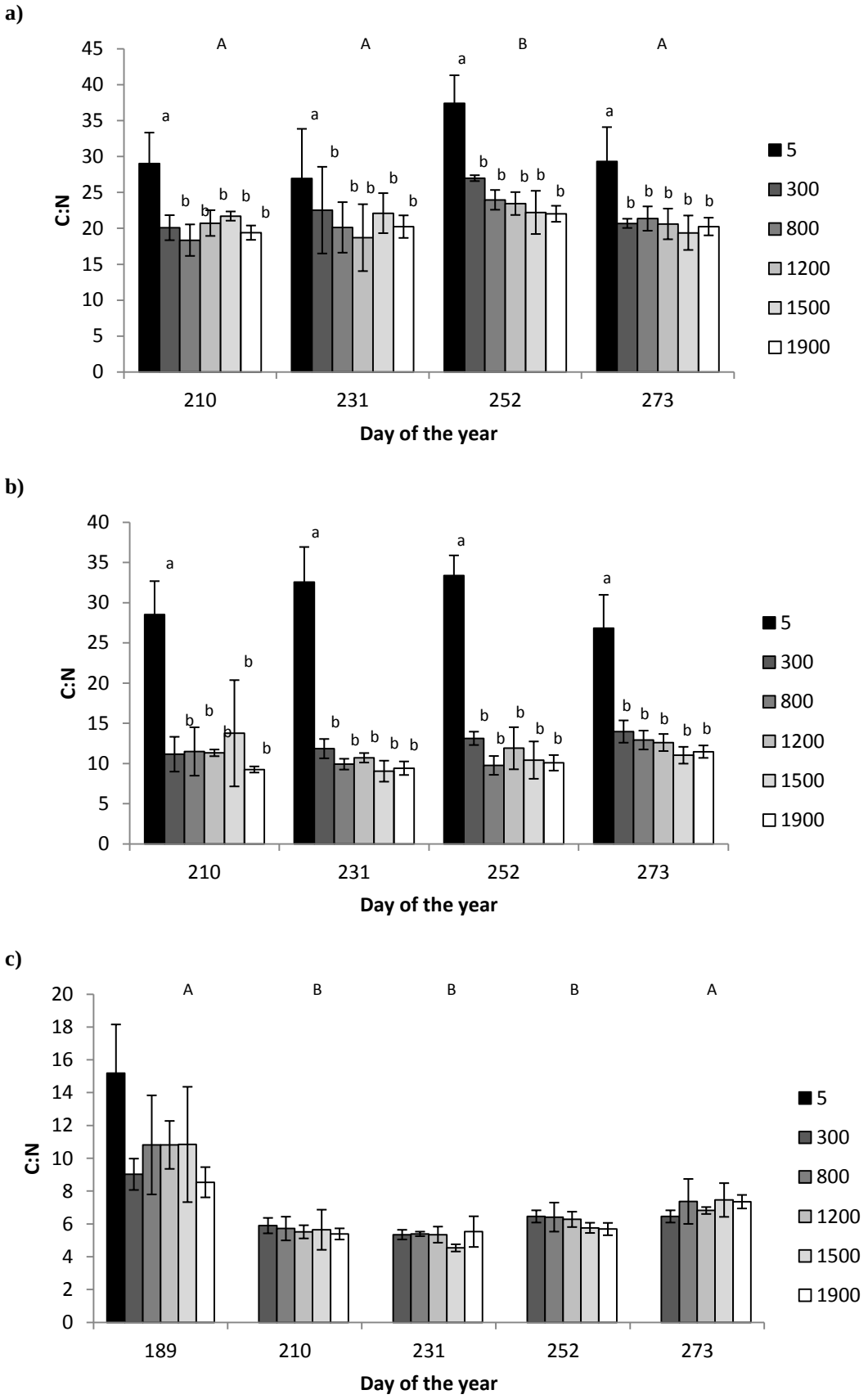


Figure 2.14 – Carbon to nitrogen ratio of *S. europaea* plants, in **a)** root, **b)** shoot, **c)** tips, for NO_3^- concentrations between 5-1900 µmol l^{-1}. Within each harvest day, means sharing a lowercase letter do not differ significantly. Within each N concentration, means sharing an uppercase letter do not differ significantly.

Nitrogen uptake

The N removed in plant tips at each crop and for the NO_3^- treatments, calculated from the cropped tips biomass and N content, is presented in Figure 2.15. N removed in tips every 3 weeks was lower at 300 µmol N l^{-1} (4.14 ± 3.03 mmol N $plant^{-1}$), than in 1900 µmol N l^{-1} treatment (5.41 ± 3.83 mmol N $plant^{-1}$, p=0.01). N removed in tips from plants in the other N concentrations was not significantly different. N removed in tips was lowest in the first and second crops (1.21 ± 0.59 and 4.33 ± 2.33 mmol N $plant^{-1}$, respectively for days 189 and 210), compared to the other days ($p<0.05$), while N concentrations removed in tips in days 231, 252 and 273 were not significantly different.

Total plant N uptake was calculated for the whole period as the plant N on day 273 minus plant N on day 162. N taken up by plants in the different NO_3^- treatments is presented in Figure 2.16a, and there was no significant difference observed between treatments. There was an overall mean uptake of 65.3 ± 18.1 mmol N $plant^{-1}$, equivalent to 24.5 ± 6.8 µmol N $plant^{-1}$ h^{-1}, assuming constant uptake during the whole period.

Figure 2.16b shows that the amount of N taken up by plants that was removed in tips, with again no significant difference between NO_3^- treatments being observed, and an overall mean of 26.6 ± 7.9 mmol N plant $tips^{-1}$. The percentage of total plant N in roots (Figure 2.17) was lower (6.00 ± 1.24 %) compared both with N in tips, 40.4 ± 7.9 %, and in shoots, 53.6 ± 7.7 % ($p<0.05$). The N retained in shoots and tips were not significantly different, nor were the proportions of N in each plant component across NO_3^- treatments.

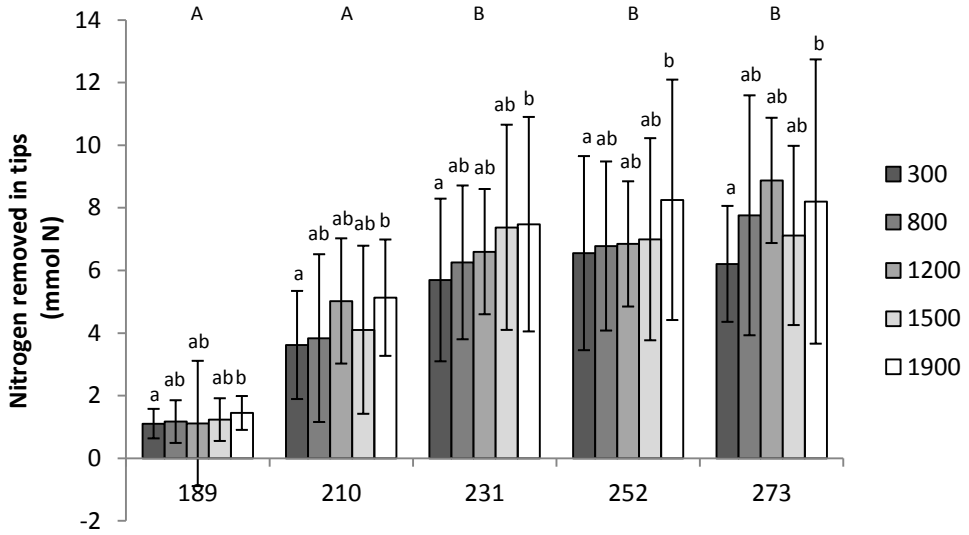


Figure 2.15 - Nitrogen removed by each plant in the tips (µmol N $plant^{-1}$) at each crop, for plants grown at different NO_3^- concentrations as in the legend (µmol l^{-1}). Within each harvest day, means sharing a lowercase letter do not differ significantly. Within each N concentration, means sharing an uppercase letter do not differ significantly.

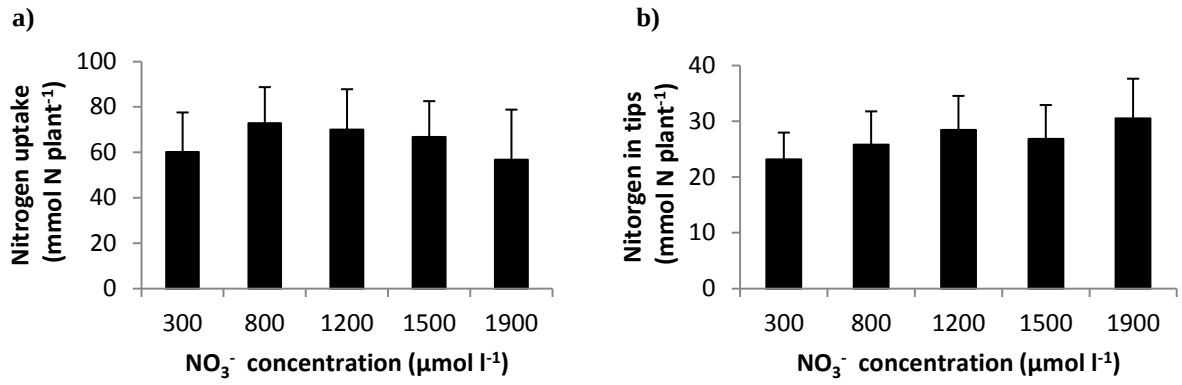


Figure 2.16 – Total nitrogen taken up **a)** by each plant, **b)** in the plant tips (µmol N plant^{-1}) during the experiment.

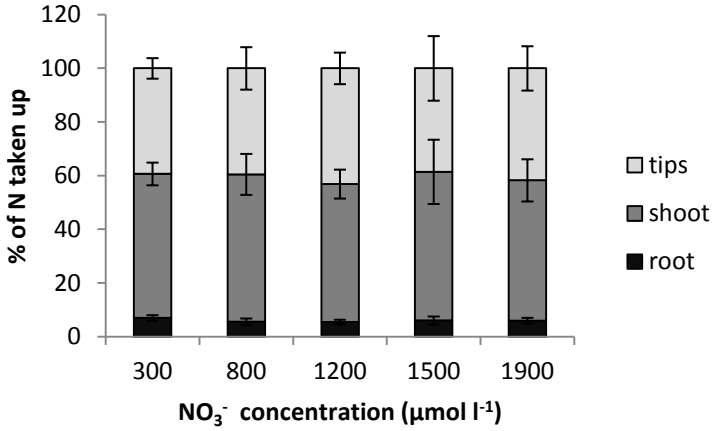


Figure 2.17 – Percentage of nitrogen taken in each part of the plant (root, shoot and tips) by the end of the experiment.

Discussion

Nitrate supply had an effect on *S. europaea* plant performance; plants grown at the lower NO_3^- concentration of 5 µmol N l^{-1} had a high mortality along with lower growth and lower shoot: root ratios. This result is in agreement with several other studies that have shown increasing *S. europaea* growth rates and/or biomass with increasing N supply, both in the field and laboratory conditions (Piggot 1969, Stewart et al. 1973, Jefferies 1977, Loveland and Ungar 1983, Webb 2012). The results are also in agreement with the theory that the shoot:root ratio should increase with increasing N supply (Valiela and Teal 1974, Hilbert 1990). Additionally, plants in the lowest N supply treatment did not produce any harvestable tips after the first harvest.

Conversely, no consistent differences were observed between plants grown at NO_3^- concentrations between 300 and 1900 µmol l^{-1}, suggesting that in these treatments the N supplied to the plants did not limit plant growth, and consequently indicating that a continuous N supply of 300 µmol NO_3^- l^{-1} is above the N sufficiency threshold in *S. europaea* under the conditions tested. Jefferies (1977) reported increased growth in *S. europaea* shoots with an increase in N supply, however, the highest tested N concentration was lower than in the present study (10, 100 and 1000 µmol NO_3^- l^{-1}). Additionally the N supply was not continuous, with N being supplied as 100 ml of solution every other day (or daily for a period of time), so the N supplied to the plants is not comparable between the two studies. Webb (2005) also reported an increase growth in *S. dolichostachya* with increasing N concentration (0, 1400 and 2800 µmol NO_3^- l^{-1}), however, N was supplied only as 1 l of N solution added 3 times a week to a group of 6 plants. Webb (2012) working with *S. europaea* plants irrigated daily, observed an increase in above-ground growth with an increase in N concentration up to 4 mmol NH_4^+ l^{-1} (0, 2, 4 mmol l^{-1}), and no further increase at 6 mmol l^{-1}. On the other hand, Quintã (2007) found no consistent differences in *S. europaea* growth across different N concentrations, when plants were irrigated only during 5h per day with 2, 4, 6 or 8 mmol NO_3^- l^{-1} solution. Kudo and Fujiyama (2010) cultivated *S. bigelovii* plants hydroponically with N supplied at different concentrations (1, 2, 3 and 4 mmol l^{-1}) and forms (NH_4^+ and NO_3^-), and reported an increase in shoot biomass with increase in N concentration for both N forms. Even though the solution was renewed every 3 days the study did not report final concentrations in solution and so it is not possible to know if the plants were kept at constant concentrations, or if depletion was significant. Because of the different methodologies used in the different studies is not possible to compare the effect of the N concentration by itself, but rather the N supply (concentration*loading rate) needs to be taken in consideration. On the other hand, to understand the effect of N concentration by itself, experiments need to be conducted with a constant N concentration and continuous supply (Cabrera et al. 1995, Caicedo et al. 2000, Horchani et al. 2010).

As mentioned above, plants grown in the lowest N concentration did not produce tips after the first harvest, whilst plants grown in the other N concentrations produced harvestable tips every 3 weeks during the period studied. While there were no differences in the biomass of tips produced by plants grown in NO_3^- treatments between 300 and 1900 µmol l^{-1}, the tips biomass regrowth increased with time.

The only consistent differences in C and N content across treatments were the lower N content and higher C contents in the 5 µmol N l^{-1} treatment compared to all other treatments. The lower N content suggests that there was N limitation in the plants grown at 5 µmol N l^{-1}, while

the lack of further differences between the other treatments indicates that in those cases plants were not N limited. These results are in agreement with the observed differences in plant growth. Additionally there was no observed accumulation of N in plant tissues resulting from N luxury uptake, in conformity with other studies in *S. europaea* (Quintã 2007, Webb 2012).

The C:N ratio is sometimes used as proxy for N differences in tissues assuming that C, being a structural component, is constant (Duarte 1990, Atkinson and Smith, 1983). In the present study C and N content varied not only with N treatment but also with time, and the differences with time are likely to be a result of plants structural changes rather than associated with nutrients supply. Jagtap et al. (2002) also observed C and N content changes with age of *S. brachiata* plants, with C content increasing with increasing age of the plant, and N content decreasing. Therefore in the case of *Salicornia* plants, the observed changes in the C:N ratio do not necessarily translate differences in N content.

Overall N content of plants in the present study (300 - 1900 µmol N l^{-1}) was higher than that reported by Quintã (2007), since in both cases lack of differences in growth suggest N supply to be above sufficiency value and in neither case luxury uptake was observed, the differences between the two studies may be a result of different methodology in the analysis (samples acidified prior to elemental analysis in Quintã (2007), but not in the present study). On the other hand, plant tissue N content in the present work is comparable to that of Webb (2012) who reported N content for above ground *S. europaea* tissues of between 2.16 and 3.28 mmol N g^{-1} and between 0.98 and 2.04 mmol N g^{-1} in roots (for N concentrations between 2 and 6 mmol NH_4^+ l^{-1}). Despite the differences in N content the trend within plants tissues was consistent in every study (Quintã 2007, Webb et al. in prep.), with lower N content in the roots, followed by the shoots and higher in the tips.

From the perspective of use of plants in IMTA systems, growth and N content data need to be combined to understand the maximum potential for N removal by *S. europaea* plants from wastewater. Using the biomass and N content obtained in the present study it is calculated that, with a continuous N supply of between 300 and 1900 µmol NO_3^- l^{-1}, a single plant can remove 65.3 ± 18.1 mmol N during one growing season. Accordingly, in a realistic scenario with 100 plants m^{-2}, the N incorporated into plant biomass would be 6.53 mol N m^{-2}. This estimate is higher than actual values obtained by Webb et al. (2012) work (1.19 and 2.39 mol N m^{-2}), and also higher than the observed in *S. europaea* N removal in constructed wetlands treating aquaculture wastewater, 1.09 mol N m^{-2} for one growing season (Webb et al. in prep.). Therefore the results of the present study indicate a significant potential to increase N removal from wastewater, providing the *S. europaea* growth and N uptake mechanism, in relation to different

factors such as N from, salinity, light and temperature, is established so that ideal growth conditions can be provided.

Despite the high potential for N removal by *S. europaea*, it is important to note that, from the N accumulated in plants only 40% was removed in the harvested tips, while 54% remained in plant shoots and 6% in the roots. These values are close to those observed previously, when between 7.9 and 19.7% were incorporated into the roots, 39.7 to 58.3% in the shoots, and 31.5 to 50.2% in the cropped tips (Quintã 2007). This draws attention to the importance of the plant shoots in N accumulation, and therefore the interest of removing the plants from the system at the end of the growing season and finding commercial applications (e.g. animals feed, fertilizer). Roots, on the other hand, account for a small proportion of N retained in plants, however, if plants are grown in a hydroponic system, roots could also easily be collected at the end of the growth season and also used for instance as animal feed or as fertilizer.

A central limitation of using *S. europaea* plants in IMTA systems is the fact that it is a annual species, and even though the growth season can be extended (Ventura et al. 2011b) there will be periods when the plants will not effectively remove N from the wastewater. As an alternative, perennial halophytic plants have the potential to remove N all year around, *Aster tripolium*, for instance, may be a plant of interest since it is also sought for human consumption.

Chapter 3 - Growth and nitrogen uptake in *Salicornia europaea* and *Aster tripolium*

Introduction

Growth in aquaculture in the coastal zone brings with it a range of environmental management issues including nitrogen enrichment of receiving waters and nitrogen toxicity for the cultured species within the aquaculture system. Since N is needed for algal and plant growth and is incorporated in their tissues, these organisms have long been used for N removal from different types of wastewater. For instance, constructed wetlands (CW) have traditionally been used for municipal, industrial, urban and agricultural waste management (see Vymazal 2008 for review). Also several pilot scale and field studies have been carried out and demonstrated the viability of using CWs to filter aquaculture wastewater (mainly freshwater and some in brackish water), since over all high removal rates can be achieved (Ng et al. 1992, Schwartz and Boyd 1995, Redding et al. 1997, Panella et al. 1999, Lin et al. 2002, Tilley et al. 2002, 2003, 2005, Zachritz et al. 2008, Webb et al. 2012).

Freshwater fish culture (mainly tilapia) has been integrated with the hydroponic culture of several vegetables and herbs of commercial interest (lettuce, basil, okra, tomatoes, spinach, arugula, watercress, alfalfa, white clover, barley, fall rye, chives) (Rakocy et al. , McMurtry et al. 1997, Lockett, 2003 in Neori et al. 2004, Ghaly et al. 2005, Al-Hafedh et al. 2008). In most of these systems, unlike in CWs, the primary objective is the production of a second crop rather than the wastewater treatment and is usually referred to as aquaponics. In brackish and marine aquaculture, macroalgae have been widely studied with the aim of N removal and production of a second crop (e.g. Buschmann et al. 1996, Troell et al. 1999, 2003, Neori et al. 2004, Schneider et al. 2005, Bunting 2008, Hayashi et al. 2008).

Besides the obvious differences, the principle behind the different techniques that include algae or plants to remove N from aquaculture wastewater is the same, and falls under the definition of Integrated Multi-Trophic Aquaculture (IMTA). Chopin et al. (2001) defines IMTA as the systems where the culture of organisms, either fish or shrimps, is integrated with extractive culture, i.e organisms that extract particulate organic matter (shellfish) and/or dissolved inorganic nutrients (seaweeds/plants). In brief, a balanced integration of various organisms in a single system allows the extractive organisms to use the waste from the fed culture for growth thereby reducing the amount of pollutants in the wastewater. Furthermore the extractive organisms can provide extra commercial crops, directly providing the producer with product diversification and additional profit. Following this principle, regarding brackish and marine aquaculture, the use of higher plants is limited to those that are able to be productive

(growth and take up N) in saline systems – halophytes (Brown et al. 1999, Lin et al. 2003, 2005, Ventura et al. 2011a, Webb et al. 2012, Buhmann and Papenbrock in press).

Research into the potential to use halophyte species as biofilters in aquaculture needs to be focussed in plants with economic value. *Salicornia europaea*, or samphire, and *Aster tripolium*, or sea aster, are sought after for human consumption as a fresh vegetable. Additionally these plants have also particular interest due to their importance as natural animal feeds and use as a component of animal diets (Glenn et al. 1991b, 1992, Attia et al. 1997, Ramani et al. 2006, Masters et al. 2007, Rozema and Flowers 2008, Geissler et al. 2009a), including in diets for fed aquaculture of both fish and shrimps (Belal and Al-Dosari 1999, Bechardas 2008, Murama 2008).

Performance of *S. europaea* in CWs has already been evaluated and results show high N removal efficiency producing a regular supply of fresh succulent tips obtained by repeatedly harvesting (cropping) during the growing season (Webb et al. 2012). Less information is available for *A. tripolium*, but some pilot studies for this species, with cultivation and repeated harvest indicate that it may perform well in a similar system. Nevertheless, Webb (2012) reported that total cumulative above-ground biomass in *S. europaea* to be lower in plants that were repeatedly cropped compared to plants were not harvested, and less N was incorporated overall into plant biomass in cropped plants. In contrast, final above-ground biomass was not different between cropped and uncropped plants in Cleall-Harding (2008) study, except for those plants grown at a higher salinity (30).

To optimize plant production (with associated economic return) and N removal efficiency, the effect of repeated cropping in both plant species needs to be further investigated, both in relation to biomass production, fresh tips production and N removal. In this study it is hypothesized that *S. europaea* and *A. tripolium* plants, under non-limiting nutrient supply, will decrease biomass production with introduction of routine cropping. It is expected that the decrease in biomass will influence the amount of N removed from water, but that the N uptake rates will not be altered. To test these hypotheses, a hydroponic culture system with *S. europaea* and *A. tripolium* plants and non-limiting N supply was assembled and maintained for several months, regular cropping regime was performed in half of the plants, plant biomass and ^{15}N (NH_4^+ and NO_3^-) uptake rates were assessed.

Methods

Plants

Salicornia europaea seeds (6th generation) were obtained from plants cultivated in a greenhouse, while *A. tripolium* seeds were collected from a local saltmarsh (Mochras, 52°81'70.29"N, 4°13'68.75"W). In both cases seeds were stored at -20C until used in the present experiment and were germinated in a climate controlled greenhouse (16h photoperiod), in P576 plug trays with John Innes No.1 compost with continuous immersion in fresh water. Approximately one week after sowing, water supply was changed to salinity 10 and Phostrogen fertiliser was added. Later, trays were thinned out to one plant per plug and nutrient solution was replaced once a week, for five weeks (Figure 3.1). Then the plants were individually removed from the compost, the roots cleaned of any substrate and transplanted into the floating systems described below.

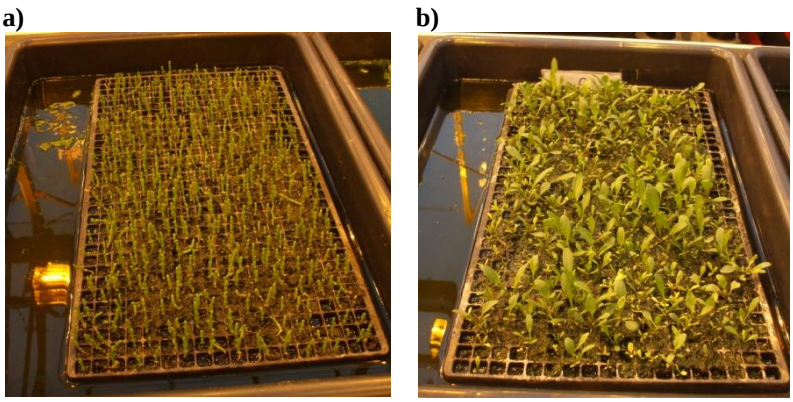


Figure 3.1 – Plants, 4 weeks after sowing, **a)** *S. europaea* and **b)** *A. tripolium*.

Experimental setup

The experiment was carried out outside, at ambient temperature and under natural light, between May and October 2011. The experimental setup consisted of three beds (Figure 3.2), each of about 450 l capacity, with floating structures (styrofoam sheets), to provide mechanical support, and covered by a poly tunnel (Giant Easy Poly Tunnel). Light intensity, air and water temperature were recorded for the duration of the experiment (Onset Hobo pendant temp/light UA-002-64). Recorded light data, in lux, was converted to µmol PAR m^{-2} s^{-1}, after calibration

against Li-Cor 190SA sensor. The calibration was obtained by taking readings at the same time with both sensors side by side (at different times of the day and weather forecasts) (r^2=0.92).

Salinity 10 irrigation water was obtained by dilution of filtered water from the Menai Strait (~34 salinity) with tap water. The irrigation water was continuously aerated and nutrients were supplied as 1ml l^{-1} Hoagland micronutrients and iron solutions, phosphorus (added as Na_2HPO_4) from 400 to 500 µmol P l^{-1}, and N (added as NH_4NO_3) from 2 to 10 mmol l^{-1} (supply increasing with time). During the experiment the solution was changed frequently to avoid nutrient depletion, and filtered water samples were collected before and after each change, immediately frozen, and later analysed for DIN and phosphorus. Dissolved inorganic phosphate (PO_4^{3-}), NO_2^- and NO_3^- were determined, using a 5–channel LACHAT Instruments Quick-Chem 8000 autoanalyzer after Grasshoff et al. (1983) and Hales et al. (2004). NH_4^+ was determined by fluorimetric method after Holmes et al. (1999) using HITACHI F2000 fluorescence spectrophotometer.

On May 28th 2011 (day 148), plants were transplanted into the beds at a density of 100 plants m^{-2}, a total of 120 *S. europaea* and 66 *A. tripolium* per bed at the beginning of the experiment. Every three weeks, between 19th of June and 24th October (days 170 and 297), half of the plants of each species were cropped. For *S. europaea*, the shoot tips above approximately 10 cm were cut, and for *A. tripolium* part of the leaves were removed (hereafter tips). The overall crop from each species was immediately weighed (fresh weight, FW) and again after oven drying at 50 °C (dry weight, DW). At the same sampling time, three cropped and three uncropped plants from each species were harvested from each bed and DW measured separately for root shoot and tips. On day 234 besides plants DW, FW was also measured. The specific growth rate (g DW d^{-1}) of the above-ground portion of plants was calculated by subtracting the logarithm of first harvest above-ground biomass (day 170) from the logarithm of above-ground biomass on the day 255 (which included shoot biomass and, for cropped plants, also the total tip biomass produced between the two sampling dates), and dividing the difference by the number of days between the two harvests.

On day 237, N uptake was estimated in selected plants that were individually incubated with ^{15}N, in 5 l solution, salinity 10, 1ml l^{-1} Hoagland micronutrient and iron solutions, and nominal concentrations of 200 µmol P l^{-1}, and 1000 µmol N l^{-1}. N was added either as NH_4Cl or as $NaNO_3$ and comprised 10% ^{15}N tracer, added as $^{15}NH_4Cl$ and $Na^{15}NO_3$, respectively in NH_4Cl and $NaNO_3$ treatments. For each of the two N treatments, three cropped and three uncropped plants of each species were incubated for one hour. Additionally, three *A. tripolium* plants in the flowering stage were also incubated. After incubation, plants were washed in distilled water and

0.1M $CaCl_2$ solution, to remove any isotope tracer adsorbed to the root surface. Above-ground biomass (tips and shoot) was separated from the root, oven-dried (50°C), weighed, ground (Cyclotec 1093 Sample Mill, with a 1 mm sieve), and subsequently analysed for total N and ^{15}N content. Additionally, 3 plants of each species, not incubated in isotope-enriched solution, but otherwise under the same experimental conditions, were analysed for ^{15}N natural abundance. Isotopic analysis of plant tissues was carried out at the University of California stable isotope facility using a PDZ Europa ANCA-GSL elemental analyser interfaced to a PDZ Europa 20-20 isotope ratio mass spectrometer (Sercon Ltd., Cheshire, UK). Excess atom per cent (at %) of the heavy isotopes was calculated as the difference between each sample heavy isotope at % (^{15}N) and the mean natural abundance ^{15}N at%. Above-ground and root dry mass, N content, and heavy isotope at% excess were used to calculate µmol excess of heavy isotopes in the plant tissues. N uptake rates were calculated per unit root dry mass (g) per unit of time (h), taking into account the percentage of N supplied in solution as ^{15}N.

Figure 3.2 – Experimental setup.

Statistical analysis

Differences in nutrient concentrations (NO_3^-, NO_2^-, NH_4^+ and PO_4^{3-}) in irrigation water between the three beds were investigated using ANOVA analysis, after confirming homogeneity of variances (Levene's test). Plant growth rates, biomass (root and above-ground and tip production) data were tested for differences between the three beds using ANOVA analysis, since no significant differences were observed for any case, all the data from the three beds were pooled. Plant above-ground growth rates were compared between cropped and uncropped plants using ANOVA or the Kruskal-Wallis test, respectively for samples with and without equal

variances (Levene's test). The effect of time (harvest day) and cropping regime on plant biomass (root and above-ground) were investigated separately using one way ANOVA where variances were equal (Levene's test) or the Kruskal-Wallis test when variances were not equal. Where significant differences were found, Tukey, Bonferroni and LSD *post hoc* tests were performed, respectively after ANOVA with equal number of samples between groups, ANOVA with unequal number of samples between groups, and the Kruskal-Wallis test. Biomass from flowering *A. tripolium* was compared to biomass from cropped and uncropped plants using ANOVA analysis or Kruskal-Wallis test, and post hoc Bonferroni or LSD, respectively. Differences in tips production across harvests were investigated using ANOVA analysis. Where significant differences were found, Tukey's *post hoc* test was performed for pairwise comparisons. The correlation between FW and DW was investigated using Pearson product-moment (cropped and uncropped, *S. europaea* and *A. tripolium*), following a significant correlation, linearity was investigated using linear regression analysis and the obtained slopes compared using t-test after Bonferroni correction. Differences in N content were analysed using ANOVA, and Tukey's test *post hoc*. Differences in N uptake rate, as NH_4^+ or NO_3^-, by cropped or uncropped plants was investigated using two way ANOVA, after confirming equal variances (Levene's test) Following the two way ANOVA, when interaction between the two factors was not significant, Tukey's pairwise comparisons were performed to investigate the main effect of significant factors. If interaction was significant ANOVA analysis were performed within each factor. Values are reported as means ± standard deviation. The significance level was considered as <0.05 for all the statistical analysis performed.

Results

Environmental data

Daily irradiance during the experiment was 617 ± 329 µmol PAR m^{-2} s^{-1} (Figure 3.3a). Mean daily air temperature for the same period was 19.1 ± 2.75 °C, while daily minimum mean was 10.3 ± 2.9 °C, and the daily maximum 40.0 ± 9.4 °C (Figure 3.3b). Mean daily water temperature for the experimental period was 16.1 ± 1.4 °C, minimum was 15.1 ± 1.8 °C, and maximum 17.4 ± 1.5 °C (Figure 3.3c). Salinity varied between 10.9 ± 0.7 after each water change and 11.8 ± 1.0 immediately before the following water change.

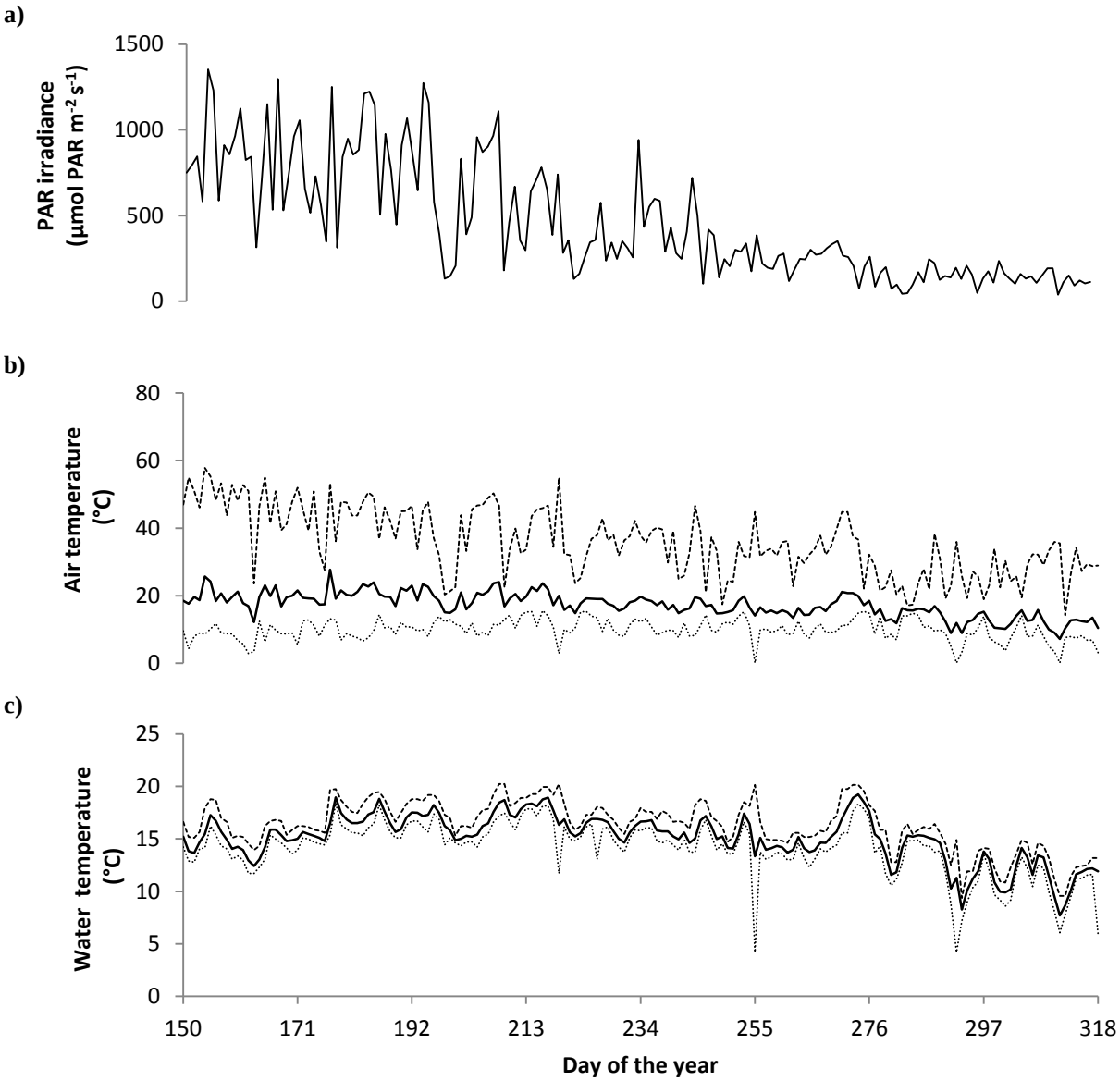


Figure 3.3 – Environmental data recorded during the experiment. **a)** Daily mean light irradiance (µmol PAR m^{-2} s^{-1}), **b)** daily mean (bold line), minimum (small dashed line) and maximum (dashed line) air temperature (°C), **c)** daily mean, minimum and maximum water temperature (°C)**.**

Water nutrients

N (as NH_4^+, NO_3^- and NO_2^-) and P as PO_4^{3-} concentration in solution varied during the experimental period, but there were no significant differences in the measured nutrient concentrations between the three experimental units. Overall mean values and standard deviation are shown in **Error! Reference source not found.**. N concentration was only below 1000 µmol l^{-1} mmediately prior to the first three water changes (199 ± 189 µmol l^{-1}), thereafter it varied between 1058 and 11636 µmol l^{-1}.

Table 3.1– Nitrogen, NH_4^+, NO_3^- and NO_2^- (µmol l^{-1}), and PO_4^{3-} (µmol l^{-1}) in irrigation water during the experimental period (mean ± standard deviation).

	Mean ± standard deviation
NH_4^+	2446 ± 1619
NO_3^-	3322 ± 2312
NO_2^-	9.67 ± 9.21
PO_4^{3-}	317 ± 163

Plant growth

The mean growth rate for uncropped plants was four times higher than for cropped plants, both for *S. europaea*, 0.541 ± 0.308 g DW d^{-1} and 0.135 ± 0.028 g DW d^{-1} ($p=0.001$) and *A. tripolium*, 0.322 ± 0.154 g DW d^{-1} and 0.082 ± 0.029 g DW d^{-1}, respectively ($p<0.05$), respectively (Figure 3.4).

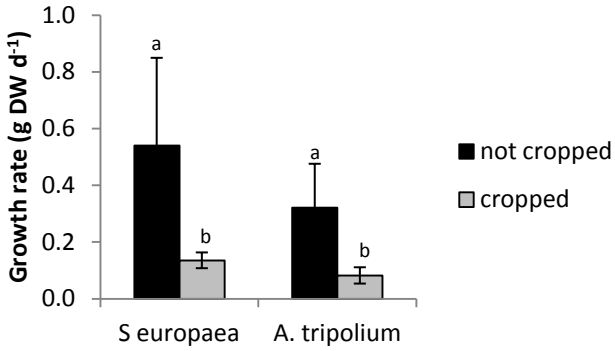


Figure 3.4 – *S. europaea* and *A. tripolium* above-ground growth rate (g DW d^{-1}) in cropped and uncropped plants. Within each species, means sharing a letter do not differ significantly.

On days 212 to 279, root biomass from *S. europaea* plants' (Figure 3.5a) was higher in uncropped plants than in cropped plants ($p<0.048$), while the difference on days 191 and 297 was not significant. Root biomass increased with time, with cropped plants root biomass being higher on days 212 to 297 than on day 170 ($p\leq0.015$), and root biomass from uncropped plants higher on days 234 to 297 than on day 170 ($p<0.05$), and also higher on days 255 and 276 comparing to day 191 ($p<0.05$).

S. europaea above-ground biomass (Figure 3.5b) was higher in uncropped than cropped plants on all sampling days ($p \leq 0.012$), except on day 191 at which point the difference was not significant. Above-ground biomass in cropped *S. europaea* plants was lower on day 170 than in all sampling days between 234 and 297, as well as on day 191 compared to days 255, 276 and 297 ($p < 0.05$). In case of uncropped plants, above-ground biomass was higher on days 255, 276 and 297 than in the remaining harvests (day 170, 191, 212 and 234) ($p \leq 0.038$).

Root biomass in *A. tripolium* (Figure 3.6a) was higher in uncropped than cropped plants from day 234 on ($p \leq 0.007$), while no significant differences were observed on days 191 and 212. For both cropped and uncropped plants, root biomass on days 276 and 297 was higher than on day 170 ($p < 0.05$), in case of plant not copped plants the root biomass on day 255 was also higher than on day 170 ($p < 0.05$).

In *A. tripolium,* above-ground biomass of uncropped plants was significantly higher than cropped plants only on days 255 and 276 ($p \leq 0.038$) (Figure 3.6b), while on the remaining sampling days the difference between the two cropping regimes was not significant. In case of cropped plants, above-ground biomass on day 170 was lower than in all other harvests ($p \leq 0.001$) except day 191, when the difference was not significant. While in case of cropped plants biomass on day 170 was only significantly lower compared to days 255, 276 and 297 ($p < 0.05$).

During the experimental period 21 % of *A. tripolium* plants flowered and seeded, independently of whether they were cropped or not. Plants that were in flower were not included in the previous analysis comparing growth in cropped and uncropped plants. However, plants in flower were also sampled and their biomass is presented in Figure 3.7, together with cropped and uncropped plants biomass at the same harvests. In relation to root biomass from flowering plants (Figure 3.6a) the only significant difference found was on day 234, when root biomass from flowering plants was higher than from cropped plants ($p = 0.003$). Above-ground biomass was higher in flowering plants comparing to cropped plants on days 191, 234 and 276 ($p < 0.05$); and also higher than uncropped plants on day 234 ($p = 0.006$).

In cropped plants, for both plant species, tips were removed between days 170 and 255, after day 255 no tips were produced (Figure 3.8 and Figure 3.9). In *S. europaea,* by day 255 all plants were flowering or producing seed and no tip regrowth was visible to the naked eye at this point. While by the same time *A. tripolium* plants become infested by Lepidoptera larvae.

Between days 170 and 255, tip dry weight production in *S. europaea* (Figure 3.8a) was lower on day 170 than in every other harvest ($p \leq 0.004$), and higher on day 212 ($p < 0.001$). Fresh weigh production presented similar trends (Figure 3.8b), except that tips biomass on day 255 was also lower comparing to days 191 and 234 ($p \leq 0.02$), mean tip production increased from 1.8 ±

0.43 g FW plant^{-1} in the first harvest to a maximum of 28.2 ± 1.48 g FW plant^{-1} on day 212, after which decreased until 7.5 ± 1.71 g FW plant^{-1} on day 255.

Dry weight tip production in *A. tripolium* plants was not significantly different between the several sampling days, with an overall mean of 1.1 ± 0.23 g DW plant^{-1} (Figure 3.9a). However when expressed as fresh weight, mean tip biomass was lower at the first harvest, 10.6 ± 2.57 g FW plant^{-1}, comparing to the following three harvest dates ($p \leq 0.06$), but not comparing to day 255, 11.6 ± 2.29 g FW plant^{-1}. In the harvests between days 191 and 234 mean tips biomass was between 17.5 ± 0.15 and 18.8 ± 2.52 FW plant^{-1}.

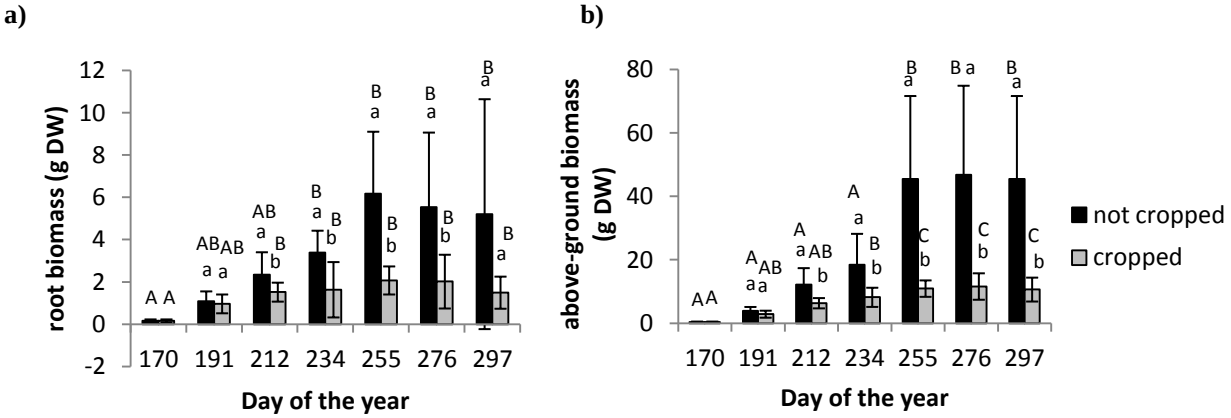


Figure 3.5 – *S. europaea* biomass (g DW) in cropped and uncropped plants at each harvest date. **a)** root **b)** above-ground. Within each harvest day, means sharing a lowercase letter do not differ significantly. Within each cropping regime, means sharing an uppercase letter do not differ significantly.

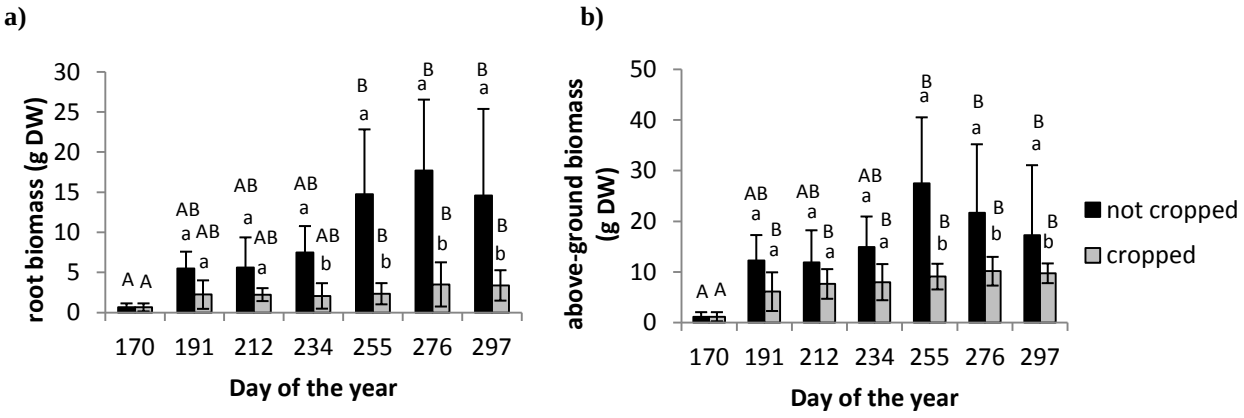


Figure 3.6 – *A. tripolium* biomass (g DW) in cropped and uncropped plants at each harvest date. **a)** root **b)** above-ground. Within each harvest day, means sharing a lowercase letter do not differ significantly. Within each cropping regime, means sharing an uppercase letter do not differ significantly.

Salicornia europaea and *A. tripolium* biomass at the time of the last crop (day 255) was as presented in Table 3.2. Above-ground and root biomass weight was measured and converted to fresh weight using the linear regressions found between FW and DW on plants harvested on day 234 ($p<0.001$, $r^2 \geq 0.95$, Table 3.3). The whole plant biomass produced over the growing season varied between 226 ± 45 g FW cropped and 510 ± 259 g FW uncropped *S. europaea* and between 124 ± 32 g FW cropped and 382 ± 175 g FW uncropped *A. tripolium*. In relation to uncropped plants root biomass represents 34 ± 9% and 46 ± 9 % of total plant biomass, respectively for *S. europaea* and *A. tripolium*. In cropped plants root biomass represents 25 ± 6 % and 21 ± 8 % of total plant biomass, respectively for *S. europaea* and *A. tripolium*.

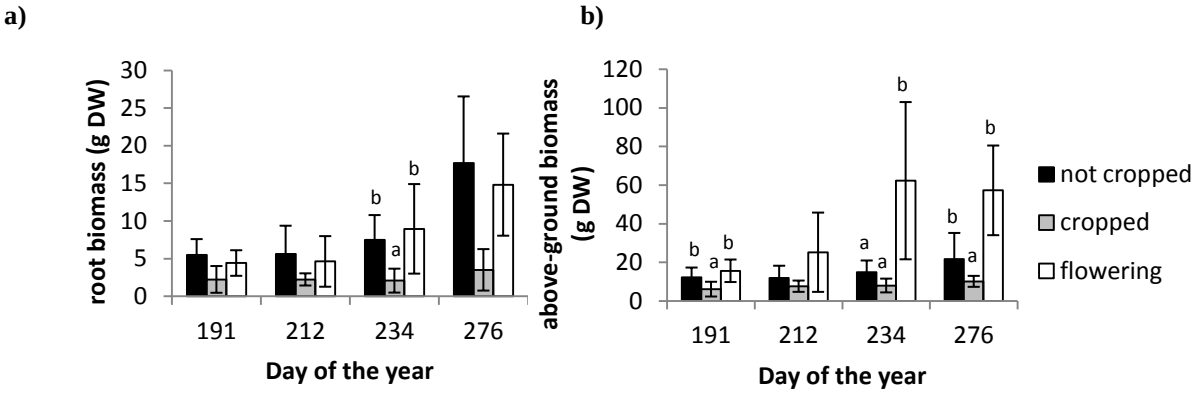


Figure 3.7 – *A. tripolium* biomass (g DW) in cropped, uncropped and flowering plants at several harvest dates. **a)** root **b)** above-ground. Within the same harvest day, means sharing a letter with flowering plants do not differ significantly.

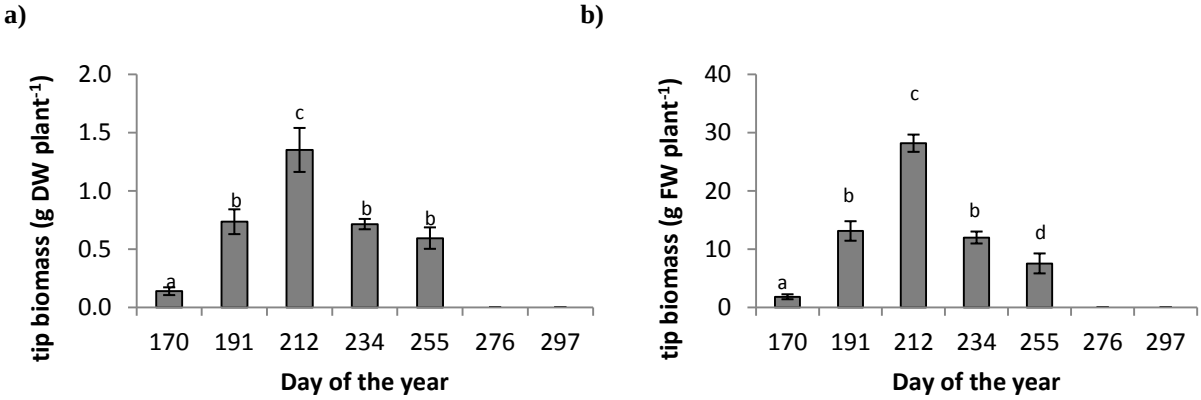


Figure 3.8 - *S. europaea* tip biomass (g plant^{-1}) at each harvest date, a**)** dry weight **b)** fresh weigh. Means sharing a letter do not differ significantly.

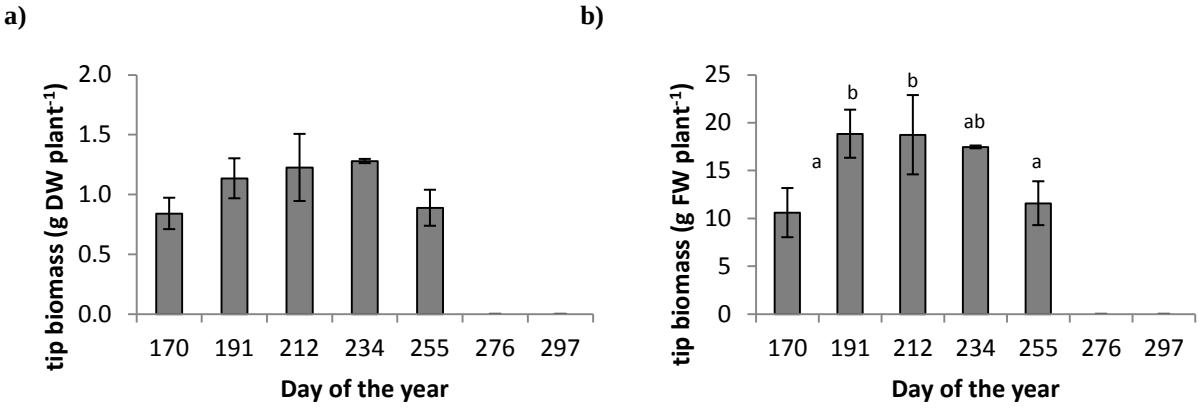


Figure 3.9 -- *A. tripolium* tip biomass (g plant^{-1}) at each harvest date, a**)** dry weight **b)** fresh weigh. Means sharing a letter do not differ significantly.

Table 3.2 – *S. europaea* and *A. tripolium* biomass produced over one growing season (g Fresh and Dry weights) (mean ± standard deviation).

		S. europaea		***A. tripolium***	
		DW (g)	**FW (g)**	**DW (g)**	**FW (g)**
Uncropped	**whole plant**	52 ± 28	510 ± 259	42 ± 19	382 ± 175
	above-ground	45 ± 26	341 ± 197	27 ± 13	206 ± 98
	root	6 ± 3	169 ± 81	15 ± 8	176 ± 96
Cropped	**whole plant**	13 ± 3	226 ± 45	10 ± 4	124 ± 32
	above-ground	11 ± 2	169 ± 33	8 ± 2	96 ± 18
	root	2 ± 0.7	57 ± 18	2 ± 1	28 ± 16

Table 3.3 – *S. europaea* and *A. tripolium* slopes of linear regression lines of FW against DW (mean ± standard deviation) for above-ground and root biomass.

	S. europaea	***A. tripolium***
above-ground	14.0 ± 0.46	7.5 ± 0.32
root	27.4 ± 9.98	11.9 ± 0.33

Plant nitrogen content

N content (mmol N g^{-1} DW) was analysed for all plants harvested on day 237, for above-ground and root tissues. In *S. europaea* N content in shoots was higher than in roots, 1.9 ± 0.14 and 1.7 ± 0.19 mmol N g^{-1} DW, respectively ($p<0.001$). In *A. tripolium* the N content in roots, 3.8 ± 0.61 mmol N g^{-1} DW, was higher than in shoots, 2.4 ± 0.51 mmol N g^{-1} DW ($p<0.001$). There were no

significant differences in tissue N content between cropped and uncropped plants, either in *S. europaea* or *A. tripolium* (Figure 3.10). However N content was lower in flowering *A. tripolium*, in both above-ground and root tissues, than in cropped and uncropped non flowering plants ($p \leq 0.007$ and $p \leq 0.002$, respectively for above ground and root tissue) (Figure 3.10).

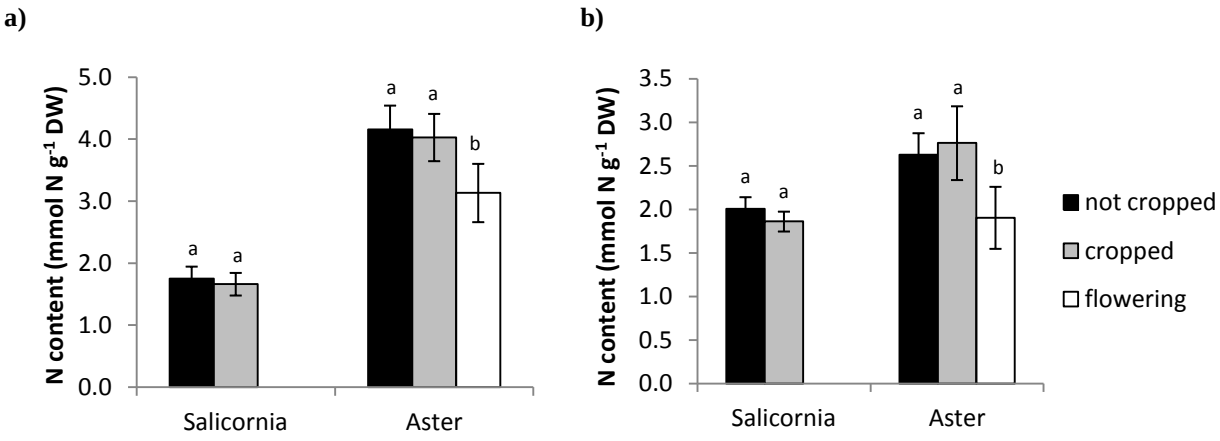


Figure 3.10 – *S. europaea* and *A. tripolium* N content (mmol N g^{-1} DW), for cropped and uncropped plants plus flowering *A. tripolium* plants **a)** root and **b)** above-ground. Within each species means sharing a letter do not differ significantly.

Nitrogen uptake

During the ^{15}N incubation, mean air temperature was 28.5 ± 4.18 °C, water temperature 21.3 ± 0.59 °C and light intensity 2356 ± 1132 µmol PAR m^{-2} s^{-1} (619 and 5425 µmol PAR m^{-2} s^{-1}, minimum and maximum light intensity respectively).

Figure 3.11 and Figure 3.12 show N uptake rates in *S. europaea* and *A. tripolium* calculated from the ^{15}N excess in plant tissues after 1h incubation with 1000 µmol NH_4NO_3, containing 10% N as ^{15}N either in the form of NH_4^+ or NO_3^-. When expressed per gram of root DW (µmol N g^{-1} root DW h^{-1}) *S. europaea* NH_4-N uptake was higher in uncropped than cropped plants, 67.2 ± 7.47 and 29.7 ± 21.03 µmol N g^{-1} root DW h^{-1}, respectively (p=0.015), while there was no significant difference in NO_3-N uptake (10.7 ± 2.24 and 8.0 ± 3.16 µmol N g^{-1} root DW h^{-1} respectively for uncropped and cropped). N uptake rate was higher as NH_4^+ than as NO_3^- in uncropped plants (p=0.001), whereas there was no significant differences in the uptake of the two forms of N by cropped plants. Looking at the whole plant N uptake rates (µmol N $plant^{-1}$ h^{-1}) similar trends are observed, except in this case the NO_3-N uptake rate in cropped plants is significantly lower than NH_4-N (p=0.007). In. *A. tripolium* plants the N uptake rate, µmol N g^{-1}

root DW h^{-1}, (Figure 3.12a) was not significantly different between cropped, uncropped or flowering plants. Overall *A. tripolium* took up NH_4-N faster, 9.7 ± 2.80 µmol N g^{-1} root DW h^{-1}, than NO_3-N, 2.0 ± 0.97 µmol N g^{-1} root DW h^{-1} ($p<0.001$). Considering the whole plant, cropping regime and flowering plants did not affect the N ($NH_4^+$and NO_3^-) uptake rate, and for every case plants took up NH_4-N at a higher rate than NO_3-N ($p\leq0.03$).

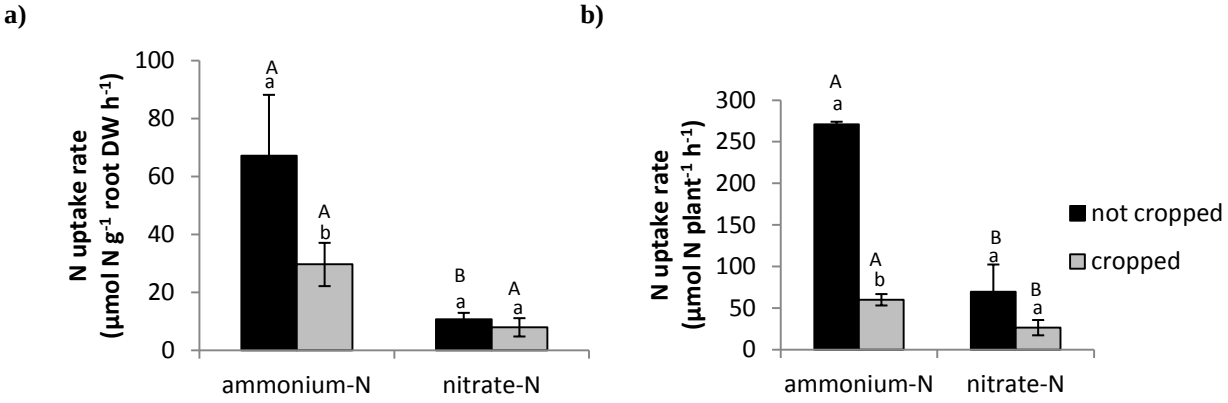


Figure 3.11 - *S. europaea* N uptake, as NH_4^+ or as NO_3^- by plants **a)** µmol N g^{-1} root DW h^{-1} **b)** µmol N $plant^{-1}$ h^{-1}. Within each N form, means sharing a lowercase letter do not differ significantly. Within each cropping regime, means sharing an uppercase letter do not differ significantly.

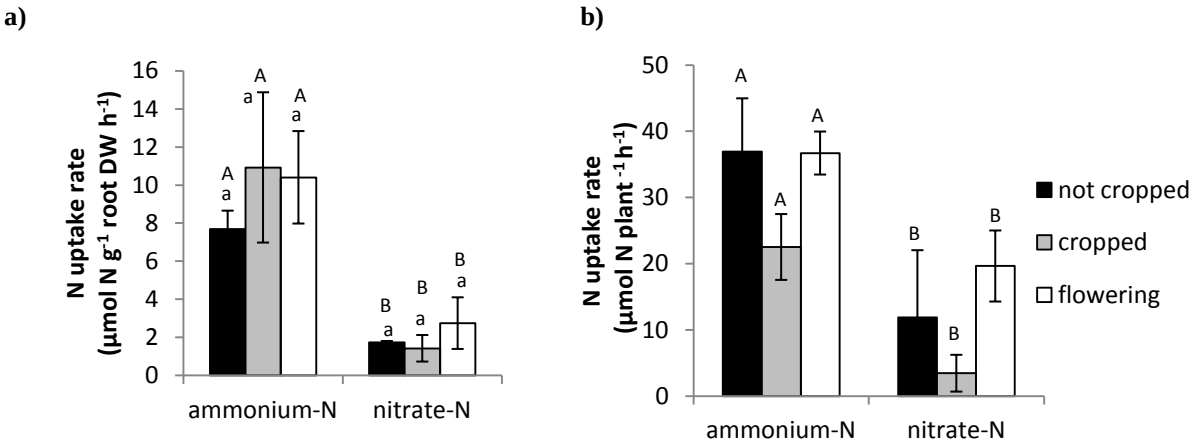


Figure 3.12 - *A. tripolium* N uptake, as NH_4^+ or as NO_3^- by plants **a)** µmol N g^{-1} root DW h^{-1} **b)** µmol N $plant^{-1}$ h^{-1}. Within each N form, means sharing a lowercase letter do not differ significantly. Within each cropping regime, means sharing an uppercase letter do not differ significantly.

Discussion

Uncropped *S. europaea* above-ground biomass by the end of the growing season, 4.5 kg DW m^{-2} (34 kg FW m^{-2}), was similar to that observed in Webb et al. (in prep.), 4.6 kg DW m^{-2}, where

plants grew in CWs receiving aquaculture wastewater. On the other hand the value obtained here is higher than the observed *S. europaea* growth reported in Oleary et al. (1985), and that of *S. bigelovii* (Glenn et al. 1997, 1999) cultivated in fields irrigated with saline water. Biomass obtained in this study is also higher than that of *S. virginica* observed in saltmarshes, which is consistently lower than 2.5 kg DW m^{-2} (Glenn et al. 1991a, Boyer et al. 2001).

Total above-ground biomass from cropped *S. europaea*, 1.1 kg DW m^{-2} (17 kg FW m^{-2}), is somewhat higher than the reported on Webb et al. (in prep.) following a similar harvest regime (0.7 kg DW m^{-2}). The removed tips biomass at each harvest varied between 0.2 and 2.9 kg FW m^{-2}, that is a wider range of that observed in Webb et al. (in prep.), 0.4 and 1.7 kg FW m^{-2}. While Ventura and Sagi (in press) reported *S. persica* crop biomass of between 0.3 and 3.6 kg FW m^{-2} at different harvests.

Ventura and Sagi (in press) mention an *A. tripolium* yield of 14 kg FW m^{-2} obtained in their ongoing work, however with the information accessible so far is not clear if this value is from cropped or uncropped above-ground biomass, or accumulated crop biomass. In the present work, uncropped *A. tripolium* above-ground biomass yield was 2.7 kg DW m^{-2} (21 kg FW m^{-2}), while cropped plants above-ground yield was less than half of that , 0.8 kg DW m^{-2} (10 kg FW m^{-2}). In cropped plants, *A. tripolium* removed biomass at each harvest was between 1 and 2 kg FW m^{-2}.

Aster tripolium is a perennial plant and even if the growth is expected to decrease during winter (Clapham et al. 1942) the observed decrease in above-ground biomass observed towards the end was due to an infestation by Lepidoptera larvae, pointing out the importance to, in the future, protect the cultures against possible pest outbreaks. Additionally in the present study 21% of the *A. tripolium* plants flowered, in contradiction with observations elsewhere that *A. tripolium* only flowers in the second year of growth (Clapham et al. 1942). However many differences have since been observed in plants growing in different habitats, leading to the description of a number of *A. tripolium* varieties, ecotypes or subspecies (Clapham et al. 1942, Huiskes et al. 1985, Stienstra 1986, Sági and Erdei 2005), and it may be possible that the time of first flowering varies, or it may simply depend on the plants growth conditions (e.g. light, temperature). Further research is needed to be able to better predict *A. tripolium* flowering patterns.

Salicornia europaea, on the other hand is an annual halophyte and growth stops during autumn, when the plants invest in reproduction. Even so, it may be possible to extend the growth period by providing artificial light to maintain the appropriate photoperiod, as demonstrated by Ventura et al. (2011b) in *S. persica*.

As the all plants established and grew well in the hydroponic system used here, in comparison to other methods, the effective use of an hydroponic system is demonstrated, with simple construction and maintenance, and the added advantage that root biomass can also easily be harvested at the end of the growth cycle. Roots, which can account as much as 46% of plant biomass, could then possibly be used for animal diets compositions or feed, and removal of the roots from the production system is important to maintain adequate growing conditions in following years.

Salicornia europaea and *A. tripolium*, uncropped plants produced more biomass compared to cropped plants. However, the use of repeated harvesting provides the grower with fresh succulent biomass every 3 weeks for the food market (retailing for around £2.00 and £3.00 per 100 g FW, respectively for *S. europaea* and *A. tripolium*) possibly providing a higher economic return compared to that for the whole plant (e.g. animal feed and diets).

Nonetheless, since N removal from the system is closely linked to plant biomass, the repeated harvesting can reduce N removal and therefore makes a less effective biofilter. Plant N uptake rates measured at the middle of the growth season, (µmol N $plant^{-1}$ h^{-1}) were lower in cropped *S. europaea* plants than uncropped, but not different in case of *A. tripolium* (NH_4-N and NO_3-N). *S. europaea* N uptake was higher than that of *A. tripolium* plants, and assuming equal N uptake throughout the day, estimated daily N uptake rates (200-818 mmol N m^{-2} d^{-1} and 62-117 mmol N m^{-2} d^{-1} respectively for *S. europaea* and *A. tripolium*) are high compared to plant N removal in planted CWs integrated with aquaculture both with or without cropping. For instance CWs planted with *Phragmites australis, Commelina communis, Penniserum purpureum, Ipomoea aquatica* and *Pistia stratiotes* removed a maximum of 39 mmol N m^{-2} d^{-1}, and in other study, CWs planted with *Typha augustifolia* and *Phragmites australis* removed about 17 mmol NH_4-N m^{-2} $d^{-1}$and 11 mmol NO_2-N m^{-2} d^{-1}, with only part of this removal being the result of plant N assimilation (Lin et al. 2002, Lin et al. 2005). *Canna indica* plants removed between 27 and 69 mmol N m^{-2} d^{-1} in harvestable biomass (Konnerup et al. 2011). In Webb (2012) CWs planted with *S. europaea*, and cropped every 3 weeks, removed 62 mmol N m^{-2} d^{-1} comparing to the removal of 20 mmol N m^{-2} d^{-1} by unplanted CWs, while in a different experiment *S. europaea* removed a maximum of 106 mmol N m^{-2} d^{-1} (Webb et al. in prep.).

Conclusion

There is a conflict between the production of fresh vegetable crops during the growing season and plant N assimilation, since the introduction of repeated cropping decreases the accumulated

plant biomass, and consequently the N removed, particularly in case of *S. europaea* plants. It is likely that apart from the cropping regime, maximising N removal efficiency and biomass production will be conflicting objectives in IMTA systems. In the present study, the situation was the one of non limiting N supply and maximum production, but when aiming to obtain higher N removal efficiency (ie % removal of N in the treated wastewater) in lower N concentrations, plant growth rates may be reduced due to the lower N supply. Thus further information is required on the relationship between N supply, plant growth and N removal rates, to enable growers to optimize IMTA systems according to their main objectives. The unexpected flowering of some *A. tripolium* plants and the larval infestation are signs of how little is still known about these plants and the importance of continuing to investigate different aspects of plant performance in different conditions. In relation to *S. europaea*, the possibility of extending the growing season needs more attention.

Chapter 4 - Growth and nitrogen uptake in *Salicornia europaea* and *Aster tripolium* at different concentrations and nitrogen forms

Introduction

Currently, close to 50% of global aquatic food production is from farmed systems and this is projected to increase to 60% by 2030 (FAO 2010). As part of this, the development of brackish and marine aquaculture will play an important contribution to meet the increasing global demand for seafood. However, the expansion of rearing of aquatic species in intensive systems needs to be accomplished while paying attention to the associated negative impacts, which includes removal of dissolved nitrogen (DN) from the wastewater. The quantity and quality of DN waste is specific to individual aquaculture units, however, DN waste has often been reported to make up more than 50 % of the total N input in the system (Troell et al. 1999, Lefebvre et al. 2001, Fernandes et al. 2007b). Most of dissolved N occurs in the inorganic form (DIN) (Porrello et al. 2005, Webb et al. 2012), DIN concentrations in aquaculture wastewater are highly variable, with concentrations of lower than 100 µmol N l^{-1} reported for some systems (Lin et al. 2003, Deviller et al. 2004) while in others concentrations higher than 1000 µmol N l^{-1} have been observed (Deviller et al. 2004, Lin et al. 2005). DIN can cause eutrophication in surrounding waters as well as be toxic to the cultured fish or shrimp (Pillay 1992, Blancheton 2000, Miller and Semmens 2002, Lyssenko and Wheaton 2006a, b), and therefore environmentally sound aquaculture systems need to include N removal processes. The integration of DIN commercially important extractive species, such as higher plants, within the aquaculture system is a developing approach for N removal (IMTA).

In the context of brackish and marine aquaculture the use of higher plants in IMTA is limited to salt tolerant plants (Webb et al. 2012). Both *Salicornia europaea* and *Aster tripolium* are halophyte plants of interest due their many commercial applications, including human consumption.

Both NH_4^+ and NO_3^- can be actively taken up by plant roots. Root transport systems catalysing NH_4^+ and NO_3^- uptake can be classified in two categories: High and low affinity transport systems, respectively HATS and LATS. While HATS operate at lower external concentrations, LATS operate at higher solute concentrations (usually > 1mM) (Siddiqi et al. 1990, Wang et al. 1993, Kronzucker et al. 1995, Cerezo et al. 1997, Cerezo et al. 2000). Two HATS and one LATS have been described for NO_3^-, one of the high affinity systems is substrate inducible (iHATS) – induced by the presence of NO_3^- in solution and the other is constitutive (cHATS) (Crawford and Glass 1998, Cerezo et al. 2000, Glass et al. 2002). In case of NH_4^+ uptake, only one HATS and one LATS have been described (Wang et al. 1993, von Wirén et al.

2000, Glass et al. 2002). For HATS the rate of root NO_3^- or NH_4^+ uptake increases with increasing ion concentration until it reaches a plateau, beyond which uptake rate is independent of ion concentration, and so they can be described by Michaelis-Menten model (Wang et al. 1993, Kronzucker et al. 1995, 1996, Bassirirad 2000). The LATS often show linear kinetics, although in few cases has also been described by the Michaelis-Menten models (Wang et al. 1993, Kronzucker et al. 1995, 1996, Crawford and Glass 1998, Cerezo et al. 2000).

NO_3^- taken up by the plants is reduced to NO_2^- (catalysed by nitrate reductase, NR), and from NO_2^- to NH_4^+ (catalysed nitrite reductase, NiR) (Guerrero et al. 1981, Siddiqi et al. 1990, Kronzucker et al. 1995, Forde 2000). NH_4^+, taken up by roots, or resulting from NO_3^- reduction, is converted first to glutamine by the action of glutamine synthetase (GS) and then to glutamate by the action of glutamate synthetase (GOGAT). These two amino acids are the precursors for the synthesis of the other amino acids as well as almost all nitrogenous compounds (Guerrero et al. 1981, Knoepp et al. 1993, Tischner 2000, Guo et al. 2007). Since NO_3^- must be reduced to NH_4^+ before it can be assimilated the uptake and assimilation of NO_3^- has higher energy costs than uptake and the assimilation of NH_4^+, and so may be a less favourable N form to plants. Several plant species prefer NH_4^+ over NO_3^- and have high uptake rates and vigorous growth when supplied with NH_4^+ (Wang et al. 1993, Tylova-Munzarova et al. 2005, Fang et al. 2007a, Jampeetong and Brix 2009a, Konnerup and Brix 2010, Kudo and Fujiyama 2010, Jampeetong et al. 2012b). On the other hand, many plant species may develop symptoms of toxicity when supplied with NH_4^+, that are not detected when plants are grown with the same concentration of NO_3^- or in mixed N nutrition. NH_4^+ toxicity symptoms, observed in a variety of plant species, include high mortality, chlorosis, necrotic lesions, increased shoot : root ratio, and growth reduction (Raab and Terry 1994, Britto and Kronzucker 2002 and references therein, Guo et al. 2002, Houdusse et al. 2005, Garnica et al. 2009, Ventura et al. 2010).

In IMTA the optimum N removal can only be achieved when the plant N uptake is accurately predicted, and in order to be able to do so it is crucial to understand plant N requirements and N uptake kinetics. And for this reason, in this study the following hypotheses are tested:

1) *S. europaea* and *A. tripolium* growth is lower when N is supplied as NH_4^+ comparing to NO_3^- or NH_4NO_3;

2) *S. europaea* and *A. tripolium* N uptake rate varies during the day;

3) *S. europaea* NH_4-N and NO_3-N uptake rates are regulated by the form of N supplied in the hours immediately before;

4) *S. europaea* and *A. tripolium* N uptake rates at different supply concentrations follow the Michaelis-Menten model;

5) *S. europaea* and *A. tripolium* NH_4-N uptake rates are higher than NO_3-N uptake at similar N concentrations;

6) *S. europaea* and *A. tripolium* NO_3-N uptake is inhibited by the presence of NH_4^+ in solution, while NH_4-N uptake is not inhibited by NO_3^-;

7) *S. europaea* and *A. tripolium* plants receiving continuous supply of N express higher N uptake rates than plants N starved for 48h.

Plants were grown with the different forms of N and biomass assessed, in other experiments ^{15}N labelled solution was used to measure N uptake rates at different times of the day, and at ^{15}N supply between ~ 0 and 2000 µmol l^{-1}, both as NH_4^+, NO_3^- and NH_4NO_3 (and additionally 1000 µmol $^{15}NO_3^-$ l^{-1} at several ratios of NH_4^+), with and without previous N starvation.

Methods

Germination of plants

Salicornia europaea seeds (6th generation from stock plants held at Bangor University) were obtained from plants cultivated in a greenhouse. *A. tripolium* seeds were collected from a local saltmarsh (Mochras, 52°81'70.29"N, 4°13'68.75"W). Seeds of both species were stored at -20°C until used in the present experiments. *S. europaea* and *A. tripolium* seeds were germinated in a climate-controlled greenhouse (16h photoperiod), in P576 plug trays with John Innes No.1 compost with continuous immersion in fresh water. Approximately two weeks after sowing, the water supply was changed to salinity 10 (TROPICMARIN^{TM} artificial sea salt) and Phostrogen fertiliser (N:P:K 14:10:27 + trace elements; Bayer CropScience Ltd, Cambridge, UK) was added. After approximately 1 week, trays were thinned out to one plant per plug. The solution was frequently topped up with fresh water to compensate for evapo-transpiration, and fully replaced once a week. Approximately 1 week later the plants were individually removed from the compost, the roots cleaned of any substrate, and transplanted into a hydroponic raft system.

Growth in different N sources

An experiment was conducted in order to investigate *S. europaea* and *A. tripolium* growth under several N from supplied (NH_4^+, NO_3^- and NH_4NO_3). On day 142 (2011), 360 *S. europaea* plants were transplanted to a hydroponic system consisting of 9 lysimeters (external dimensions: 60 cm length * 40 cm width * 32.5 cm depth), holding approximately 55 l of solution with a polystyrene floating sheet providing mechanical support for plants so that the roots were continuously immersed in water. Each lysimeter was continuously aerated (Pond Air 4800 pump). The irrigation water had a salinity of 10 (TROPICMARIN^{TM} artificial sea salt), and nutrients were added as Hoagland's micronutrient and Fe solutions (1 ml l^{-1}), 200 µmol P l^{-1} as Na_2PO_4 and 1000 µmol N l^{-1} according to three treatments, NH_4^+ (NH_4Cl), NO_3^- ($NaNO_3$) and NH_4NO_3 (NH_4NO_3). 3 lysimeters were set up per treatment. Nitrification was inhibited by the addition of dicyandiamide in every treatment at a concentration of 7.5 ppm (Ventura et al. 2010). Irrigation water was replaced weekly to maintain nutrient concentrations. Filtered (0.45 µm) water samples were taken, both before and after each water change, immediately frozen and later analysed for dissolved inorganic phosphorous (P) and N. Dissolved inorganic P (PO_4^{3-}), NO_2^- NO_3^- were determined, using a 5–channel LACHAT Instruments Quick-Chem 8000 autoanalyzer after Grasshoff et al. (1983), Hales et al. (2004). NH_4^+ was determined by fluorimetric method after Holmes et al. (1999) using HITACHI F2000 fluorescence spectrophotometer.

Aster tripolium plants transplanted to a similar system showed high mortality during the first week. The reason was not investigated, but since nitrification inhibitors have been shown to be toxic to some plants (Reddy 1964, Maftoun and Sheibany 1979), starting on day 206, the experiment was repeated without dicyandiamide in solution, and in this case mortality was highly reduced. In total 315 *A. tripolium* plants were moved into the hydroponic system, that in this case consisted of 9 plastic trays, with approximately 6 l solution each and irrigation water was replaced daily to prevent both nutrient depletion and nitrification. Water samples were taken at every second water change (before and after) and analysed for nutrient concentration as described above.

When *S. europaea* and *A. tripolium* plants were transplanted into the hydroponic systems, a subsample was taken of both above-ground (shoot) and root dry weight (DW) after oven-drying at 50 °C for one week. On days 159 and 223, for *S. europaea* and *A. tripolium* respectively, all plants were removed from the hydroponic systems and the shoot and root fresh weight (FW) and DW were measured from 9 plants from each species and N treatment. For all remaining plants, only DW was assessed.

Daily cycle of N uptake

To test if *S. europaea* and *A. tripolium* N uptake rate varies during the day, on days 159 and 223, respectively for *S. europaea* and *A. tripolium*, plants grown at all of the NH_4^+ and NO_3^- treatments were individually incubated in 200 ml of labelled nutrient solution (salinity 10), with 1 ml l^{-1} Hoagland micronutrient and iron solutions, 200 µmol P l^{-1} (Na_2PO_4), and 1000 µmol ^{15}N l^{-1} either as $^{15}NH_4Cl$ or $Na^{15}NO_3$. Pots containing the solution and plants were kept in a water bath during the incubation period in order to keep the water temperature approximately constant (Teco TR20 chiller/heater). For each N treatment, 3 plants of each species were incubated, for 1 h, every 2 h between sunrise and nightfall (10 times in total). To confirm actual nutrient concentrations, filtered water samples were collected before the beginning of the incubation period and analysed for P and N concentrations, as described above. After incubation plants were washed in distilled water and then 0.1 mol l^{-1} $CaCl_2$ solution to remove any isotope tracer adsorbed to the root surface. Plant shoots were separated from roots for DW measurement, and the whole plant was then ground (Precellys 24-Dual, Bertin Technologies), and subsequently analysed for N and ^{15}N content. Additionally, 3 plants of each species (not incubated in isotope-enriched solution) were analysed for ^{15}N natural abundance.

Isotopic analysis of plant tissues was carried out at the University of California stable isotope facility using a PDZ Europa ANCA-GSL elemental analyzer interfaced to a PDZ Europa 20-20 isotope ratio mass spectrometer (Sercon Ltd., Cheshire, UK). Excess atom per cent (at%) of the heavy isotope was calculated as the difference between each sample heavy isotope at% (^{15}N) and the mean natural abundance ^{15}N at%. Shoot and root dry mass, N content, and heavy isotope at% excess where used to calculate µmol excess of heavy isotopes in the plant tissues. N uptake rates were calculated per unit root dry mass (g) per unit of time (h).

Irradiance, air and water temperatures were recorded throughout the experiment (Onset Hobo pendant temp/light UA-002-64). The recorded light data, in lux, was converted to µmol PAR m^{-2} s^{-1}, after calibration against Li-Cor 190SA sensor. The calibration was obtained by taking readings at the same time with both sensors side by side (at different times of the day and weather forecasts) at the greenhouse (r^2=0.92).

Regulation of N uptake

In order to study the regulation of N uptake rate by the N supply during growth, some of the *S. europaea* plants grown in NH_4^+, were transferred into a solution containing NO_3^- as the sole N source, for several time periods (0, 2, 4, 6, and 8 h) before NO_3-N uptake was investigated. A control group of plants, grown with continuous NH_4^+ supply, were incubated in a solution with NH_4^+ at the same time points. Conversely, plants grown in the NO_3^- treatment were moved into NH_4^+ supply for different periods of time and then NH_4^+ uptake investigated. A control group of plants, grown with continuous NO_3^- supply, were incubated in solution with NO_3^- at the same time points. Plants were incubated in 200 ml of labelled nutrient solution (salinity 10) with 1 ml l^{-1} Hoagland micronutrient and iron solutions, 200 µmol P l^{-1} (Na_2PO_4), and 1000 µmol ^{15}N l^{-1} either as $^{15}NH_4Cl$ or $Na^{15}NO_3$. Pots containing the solution and plants were kept in a water bath during the incubation period in order to keep the water temperature approximately constant, for each N treatment, 3 plants of each species were incubated, for 1 h. At the beginning of the incubation, water samples were collected from each pot, stored and analysed for dissolved nutrients as previously described. After ^{15}N incubation plants were processed following the protocol as described above.

N uptake kinetics

A series of experiments were conducted to investigate the effects of N supply concentration on uptake rates for different forms of inorganic N (Michaelis-Menten model, and N form preference), the interactive effects of the N forms on uptake rate (N uptake when NO_3^- and NH_4^+ supplied alone or in combination) and the effect of previous nutrient supply on N uptake (48h N starvation *vs* no N starvation). On days 210 and 211 of 2011, 300 *A. tripolium* and *S. europaea* plants were transplanted into a hydroponic system similar to the one described above, except that the N supply was approximately 2000 µmol l^{-1} as NH_4NO_3 for all plants. Irrigation water was replaced weekly to maintain nutrient concentrations. Filtered (0.45 µm) water samples were taken, both before and after each water change, immediately frozen and later analysed for dissolved nutrients as described above. Plants grown in this system were then used to assess the N uptake using ^{15}N stable isotope labelling. Irradiance, air and water temperatures were recorded throughout the experiment (Onset Hobo pendant temp/light UA-002-64), and light data in lux was converted to µmol PAR m^{-2} s^{-1} as mentioned above.

Half of the plants from each species to be incubated in ^{15}N were N starved for 48h before the incubations, with irrigation water being replaced with water of salinity 10, P, micronutrients and iron concentrations as before, but without adding any form of N. These plants are from this point forward referred to as "starved", while "non-starved" refers to plants receiving the full supply of nutrients continuously until just before incubation.

For both starved and non-starved *S. europaea* and *A. tripolium*, the ^{15}N uptake kinetics were investigated when N was supplied as $^{15}NH_4Cl$, $Na^{15}NO_3$, $^{15}NH_4Cl+NaNO_3$ or $NH_4Cl+Na^{15}NO_3$ (equimolar). Nominal concentrations of ^{15}N tested were 10, 25, 50, 100, 200, 300, 400, 600, 800, 1000 and 2000 µmol l^{-1}). For each species, starvation status, N form and concentration, three plants were incubated for one hour in 200 ml of the respective labelled solution. Additionally, non starved plants were also incubated in ^{15}N solution containing varying NH_4^+ (NH_4Cl) concentrations (250, 500 and 750, 1500 and 2000 µmol l^{-1}) together with 1000 µmol ^{15}N l^{-1} as $Na^{15}NO_3$. Incubations were performed between days 228 and 231, and besides the defined N supply, the irrigation solution contained 200 µmol P l^{-1} (Na_2PO_4), 1ml l^{-1} Hoagland micronutrient and iron solutions, and the salinity was 10. Pots containing these solutions and the plants were kept in a water bath, in order to keep the water temperature approximately constant. At the beginning of the incubation, water samples were collected from each pot, stored and analysed for dissolved nutrients as previously described. After ^{15}N incubation plants were processed following the protocol as described above.

In order to investigate how N uptake rates varied with NH_4^+ and NO_3^- concentrations, both when supplied alone or in combination and depending on the starvation status of the plants, the measured N uptake rates at the different concentrations were fitted into a modified Michaelis–Menten model (Jampeetong and Brix 2009b):

$$V = \frac{V_{max} \times S - S_{min}}{K_m + S - S_{min}}$$

Where V is the N uptake rate (µmol N g $root^{-1}$ DWh^{-1}) measured at a given S, substrate concentration (µmol N l^{-1}); V_{max} is the maximum uptake rate at saturating substrate concentration; K_m is the half saturation constant, the substrate concentration where $V=V_{max}/2$; and S_{min} is the substrate concentration at which there is no net uptake (V=0).

Statistical analysis

For plants harvested on days 159 and 223, the correlation between shoot and root dry mass was investigated using Pearson product-moment correlation across the three N treatments. Following a significant correlation, the nature of the relationship was investigated; linearity was confirmed using linear regression analysis and the obtained slopes compared using t-test after Bonferroni correction. Similar analysis was performed in relation, shoot and root, fresh to dry weight. Plant biomass (shoot and root, DW) was compared, between the three N treatments, using ANOVA if variances were homogeneous (Levene's test), and Bonferroni *post hoc* test following significant effect of N treatment. When variances were not equal Kruskal-Wallis test was used instead ant the LSD *post hoc* test performed for pairwise comparisons between N treatments. Plant N content was also investigated in relation to N treatments effect using either ANOVA or Kruskal-Wallis test, respectively for data with and without equal variances. Tukey *post hoc* test was used after significant ANOVA with equal number of samples in N treatments, Bonferroni *post hoc* after ANOVA with unequal number of samples in N treatments, and LSD after significant Kruskal-Wallis. For both plant species, N uptake rate, both NH_4-N and NO_3-N, at different times of the day was compared using either ANOVA (and Tukey *post hoc*) or Kruskal-Wallis test (and LSD *post hoc*), respectively for data with and without equal variances. Also the N uptake rate by *S. europaea* plants placed in each N form for different lengths of time was compared using either ANOVA or Kruskal-Walis test. The parameters (V_{max} and K_m), obtained by fitting the measured uptake rates at different concentrations in the model (Michaelis–Menten) presented above, were compared using t-test after Bonferroni correction. N uptake rate by *S. europaea* plants in the presence and absence of nitrification inhibitor was compared using ANOVA analysis after confirming homogeneity of variances (Levene's).

Values are reported as means ± standard deviation. The level of $p = <0.05$ was considered as significant for all statistical analyses performed.

Results

Growth in different N sources

Environmental data

Between days 142 and 159, the period when *S. europaea* plants were grown with N supplied as NH_4^+, NO_3^- or NH_4NO_3, the mean daily light irradiance was 368 ± 64 μmol PAR m^{-2} s^{-1}. The mean daily air temperature for the same periods was 27.2 ± 1.2 °C, while the daily minimum mean was 16.2 ± 0.9 °C, and the daily maximum air temperature 48.5 ± 6.0 °C. The mean daily water temperature was 23.8 ± 1.1 °C, the daily minimum 20.5 ± 1.9 °C, and the maximum 26.5 ± 0.7 °C.

For the *A. tripolium* plants growth period, between days 206 and 223, mean daily light irradiance was 459 ± 58 μmol PAR m^{-2} s^{-1}. Mean daily air temperature for the same periods was 28.3 ± 2.3 °C, while daily minimum mean was 18.5 ± 5.1 °C, and the daily maximum air temperature 44.3 ± 3.7 °C. The mean daily water temperature for this period was 23.3 ± 1.4 °C, the daily minimum 18.8 ± 2.5 °C, and the daily maximum 27.4 ± 0.9 °C.

In case of *S. europaea* irrigation water, the salinity was constant at 10.2 ± 0.02 before and after each water change. In case of *A. tripolium* irrigation water, salinity varied between 10.9 ± 0.1 after each water change and 11.0 ± 0.01, before the following water change.

Water nutrients

For both plants species, in the irrigation water the target PO_4^{3-} concentration was 200 μmol l^{-1}, and N concentrations 1000 μmol l^{-1}, either as NH_4^+, NO_3^-, or NH_4 NO_3 respectively for each N treatment. The actual measured dissolved inorganic P and N concentrations in the irrigation waters during plant growth, are presented in Table 4.1**.** In case of *A. tripolium*, overall N mean concentration, for NH_4^+, NO_3^-, and NH_4NO_3 was lower than expected due to high nutrient depletion between water changes.

Plant growth and N content

The shoot to root biomass relationships for plants grown in the different N treatments were linear (r^2=0.88 and r^2=0.94, respectively for *S. europaea* and *A. tripolium* p<0.001). In case of *A. tripolium* there were no significant differences between the slopes of shoot to root biomass of the three N treatments, 1.8 ± 0.03, 1.9 ± 0.06 and 1.8 ± 0.05, respectively for treatments NH_4^+, NO_3^- and NH_4NO_3. However in *S. europaea* plants the ratios were different among all N treatments (p≤0.008): 2.1 ± 0.07, 2.6 ± 0.06 and 2.8 ± 0.05, respectively for treatments NH_4^+, NO_3^- and NH_4NO_3.

The relationships of fresh to dry weight, both for the shoots and roots, were all linear (r^2>0.76, p<0.02) and the slopes (Table 4.2) not significantly different between the three N treatments.

Table 4.1 - Overall measured mean (± standard deviation) dissolved inorganic nitrogen, NH_4^+, NO_3^- and NO_2^- (µmol l^{-1}), and dissolved inorganic phosphorus concentration, PO_4^{3-} (µmol l^{-1}) in solution for each N treatment during the plant growth period.

		NH_4^+ (µmol l^{-1})	**NO_3^- (µmol l^{-1})**	**NO_2^- (µmol l^{-1})**	**PO_4^{3-} (µmol l^{-1})**
S. europaea	**NH_4^+**	1118 ± 105	7.6 ± 4.0	0.7 ± 0.3	230 ± 12
	NO_3^-	19.8 ± 15.5	918 ± 145	3.3 ± 2.3	219 ± 5
	NH_4NO_3	527 ± 74	519 ± 142	3.0 ± 1.2	222 ± 18
A. tripolium	**NH_4^+**	607 ± 482	6.8 ± 5.2	6.5 ± 10.3	157 ± 74
	NO_3^-	8.8 ± 8.7	604 ± 424	5.4 ± 3.2	174 ± 38
	NH_4NO_3	366 ± 295	381 ± 265	9.4 ± 14.8	190 ± 76

Table 4.2 - Fresh to dry weight (g) relationships, for *S. europaea* and *A. tripolium* shoot and root, and for the different nitrogen treatments, NH_4^+, NO_3^- and NH_4NO_3 (mean ± standard deviation).

FW:DW		**Shoot**	**Root**
S. europaea	**NH_4^+**	12.1 ± 0.2	9.7 ± 1.0
	NO_3^-	13.4 ± 0.4	11.0 ± 0.5
	NH_4NO_3	11.9 ± 0.5	11.5 ± 0.3
A. tripolium	**NH_4^+**	12.1 ± 0.2	13.0 ± 0.3
	NO_3^-	13.3 ± 0.3	13.4 ± 0.3
	NH_4NO_3	11.9 ± 0.5	12.6 ± 0.3

Shoot final biomass of the *S. europaea* plants (Figure 4.1a) was lower in plants grown in the NH_4^+ treatment compared with both the plants grown in NO_3^- and NH_4NO_3 ($p<0.05$), although there was no significant difference between the shoot final biomass of plants grown in NO_3^- and NH_4NO_3 treatments. The specific growth rates of *S. europaea* shoots were 0.058 ± 0.013, 0.069 ± 0.014 and 0.070 ± 0.017 g DW^{-1} d^{-1}, respectively for plants grown in NH_4^+, NO_3^- and NH_4NO_3.

In relation to *S. europaea* root biomass (Figure 4.2a), the trends were similar to those observed in the shoots, with a lower biomass for plants in the NH_4^+ treatment compared to the other two treatments ($p<0.05$).

Shoot final biomass dry weight for *A. tripolium* was not significantly different between the plants grown with the different N source (Figure 4.1b). Specific growth rates were 0.048 ± 0.022, 0.048 ± 0.019 and 0.040 ± 0.020 g DW^{-1} d, respectively for plants grown in NH_4^+, NO_3^- and NH_4NO_3**.** *A. tripolium* root biomass DW (Figure 4.2b) was higher on plants from the NH_4^+ treatments than from NH_4NO_3 ($p=0.043$), while there were no other significant differences across the N treatments.

The N content of *S. europaea* plants grown with NH_4^+ (8.6 ± 1.2 mmol N g^{-1} DW) was lower than both plants supplied with NO_3^- and NH_4NO_3 (10.1 ± 1.6 and 10.4 ± 1.3 mmol N g^{-1} DW, respectively) ($p<0.001$). There were no other significant differences between N treatments (Figure 4.3a). Similarly, N per plant was lower in plants grown in NH_4^+, 0.4 ± 0.1 mmol N $plant^{-1}$ than in NO_3^- and NH_4NO_3, 0.6 ± 0.3 and 0.6 ± 0.2 mmol N $plant^{-1}$, respectively ($p<0.05$) (Figure 4.3b).

In *A. tripolium* the N content of the plants was different between all N treatments ($p\leq0.03$), being lower in plants from the NH_4^+ treatment, 8.6 ± 1.1 mmol N g^{-1} DW, followed by the plants in the NO_3^- treatment, 9.7 ± 2.0 mmol N g^{-1} DW, and higher in the plants grown in NH_4NO_3, 10.7 ± 1.5 mmol N g^{-1} DW (Figure 4.4a). However, when expressed per plant basis, the N content in *A. tripolium* was not significantly different between plants in the several N treatments, 1.7 ± 1.3, 1.4 ± 1.0 and 1.2 ± 1.1 mmol N $plant^{-1}$, respectively for the NH_4^+, NO_3^- and NH_4NO_3 treatments (Figure 4.4b).

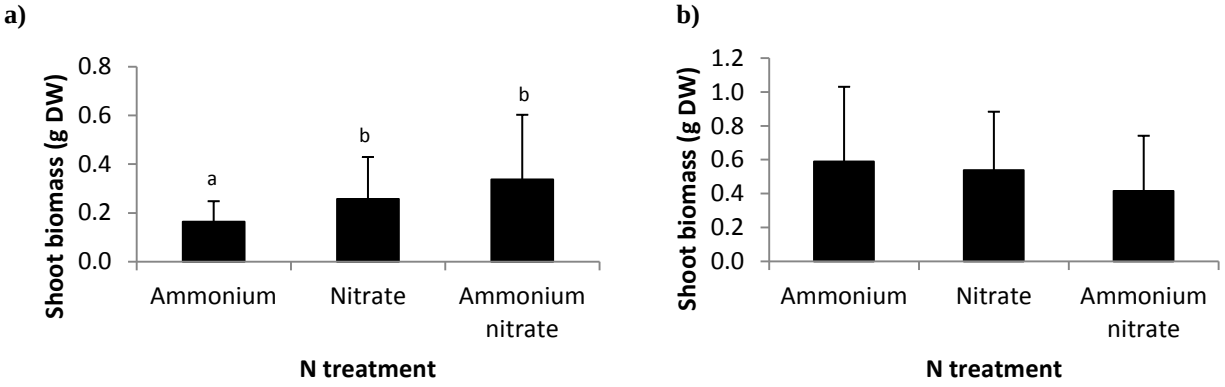


Figure 4.1 - Shoot biomass dry weight (g DW) **a)** *S. europaea* plants, **b)** A. *tripolium* plants at different nitrogen treatments. Mean ± standard deviation. Means sharing a letter do not differ significantly.

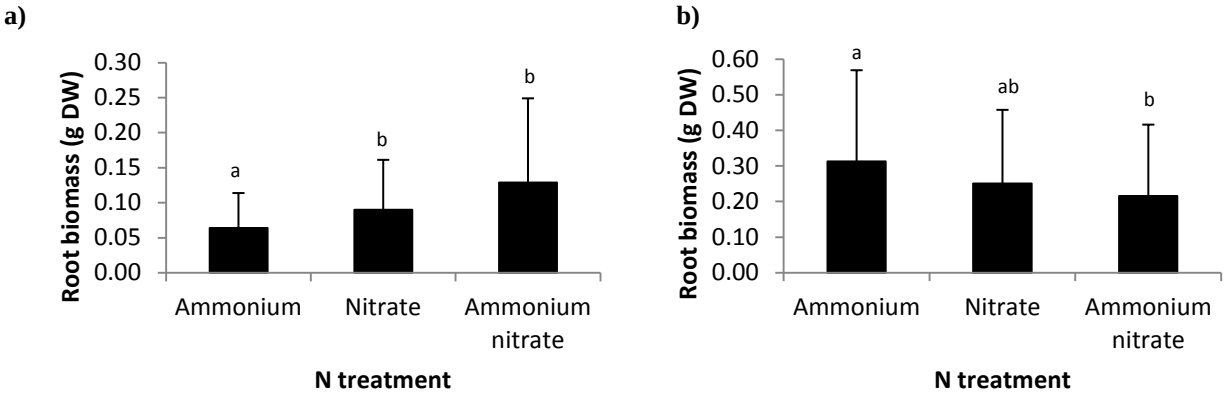


Figure 4.2 - Root biomass dry weight (g DW) **a)** *S. europaea* plants, **b)** A. *tripolium* plants at different nitrogen treatments. Mean ± standard deviation. Means sharing a letter do not differ significantly.

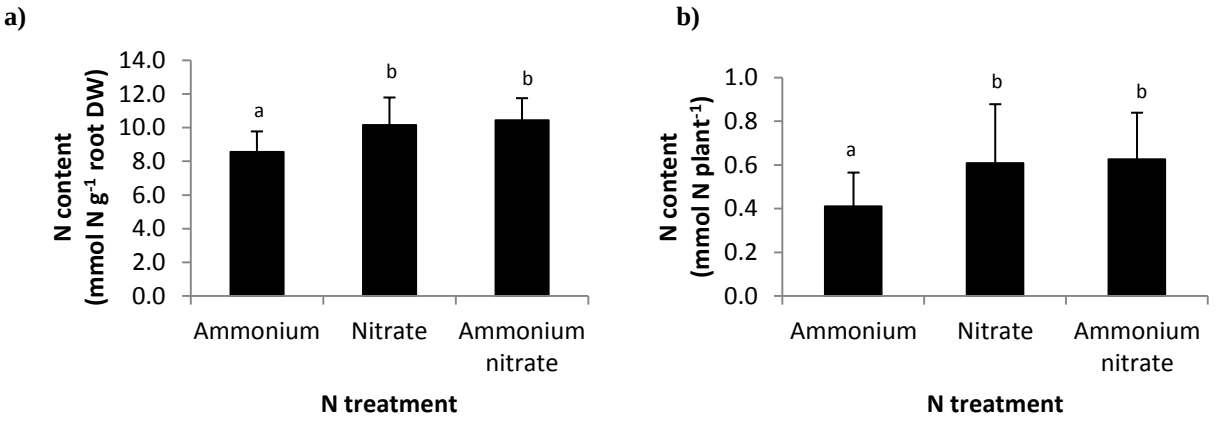


Figure 4.3 – *S. europaea* nitrogen content **a)** mmol N g^{-1} root DW **b)** mmol N $plant^{-1}$**,** for plants grown at different N treatments. Mean ± standard deviation. Means sharing a letter do not differ significantly.

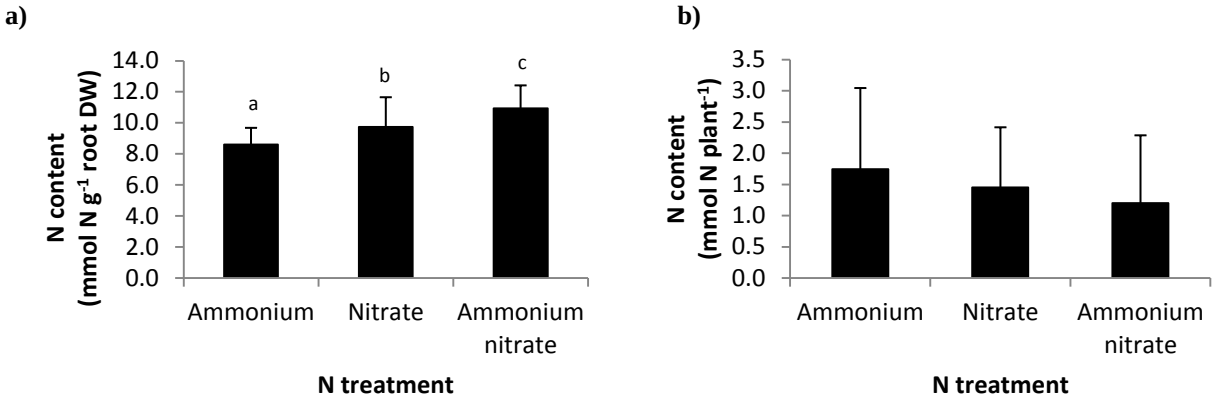


Figure 4.4 – *A. tripolium* nitrogen content **a)** mmol N g^{-1} root DW **b)** mmol N $plant^{-1}$**,** for plants grown at different N treatments. Mean ± standard deviation. Means sharing a letter do not differ significantly.

Daily cycle of N uptake

Environmental data

The environmental data recorded during the daily cycle ^{15}N incubations is presented in Figure 4.5.

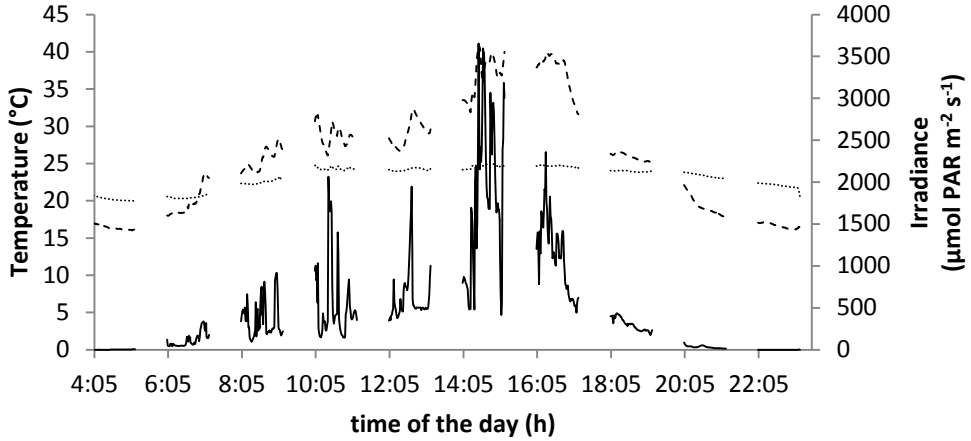
Water nutrients

During incubations in heavy isotope N solution, the mean PO_4^{3-} concentration was 183 ± 1 µmol l^{-1}, and the N concentrations were as in Table 4.3.

N uptake

The N uptake rates, assessed at different times of the day, did not vary significantly in the *S. europaea* plants, neither for NO_3-N (Figure 4.6b) nor for NH_4-N (Figure 4.6a), even though mean NH_4-N uptake rates varied from 19.5 ± 7.2 µmol N g^{-1} root DW h^{-1} between 6:05 and 7:05 h to a maximum of 44.8 ± 21.3 µmol N g^{-1} root DW h^{-1} between 20:05 and 21:05 h.

a)



b)

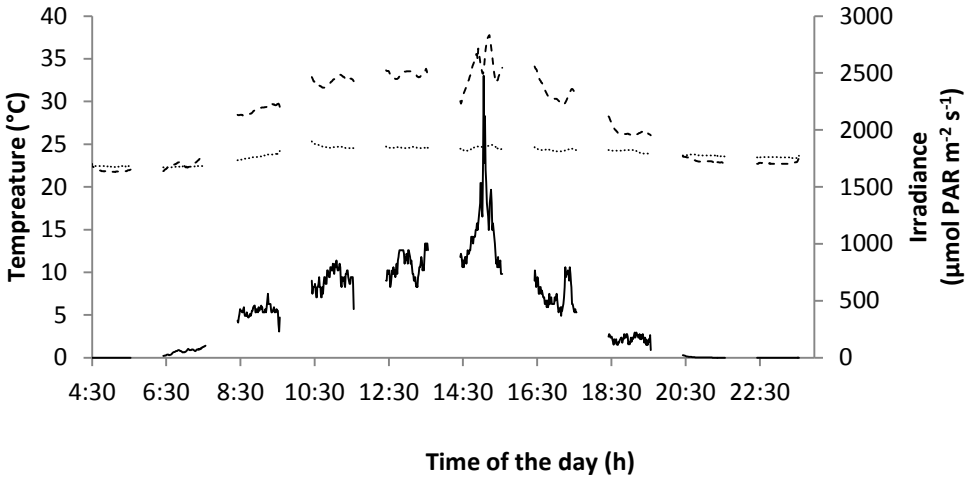


Figure 4.5 - Environmental data recorded during ^{15}N incubations for daily cycle N uptake, **a)** *S. europaea* plants, **b)** *A. tripolium*; irradiance (µmol PAR m^{-2} s^{-1}) - bold line, air temperature (°C) - dashed line, water temperature (°C) - small dashed line**.**

Table 4.3 - Measured (mean ± standard deviation) dissolved inorganic nitrogen NH_4^+, NO_3^- and NO_2^- (µmol l^{-1}) for ^{15}N incubation solutions.

		NH_4^+ (µmol l^{-1})	**NO_3^- (µmol l^{-1})**	**NO_2^- (µmol l^{-1})**
S. europaea	**NH_4^+**	927 ± 55	2.74 ± 1.11	0.349 ± 0.646
	NO_3^-	2.42 ± 0.77	1034 ± 55	1.40 ± 1.62
A. tripolium	**NH_4^+**	935 ± 27	2.2 ± 2.1	0.2 ± 0.1
	NO_3^-	2.4 ± 0.7	923 ± 48	3.1 ± 1.8

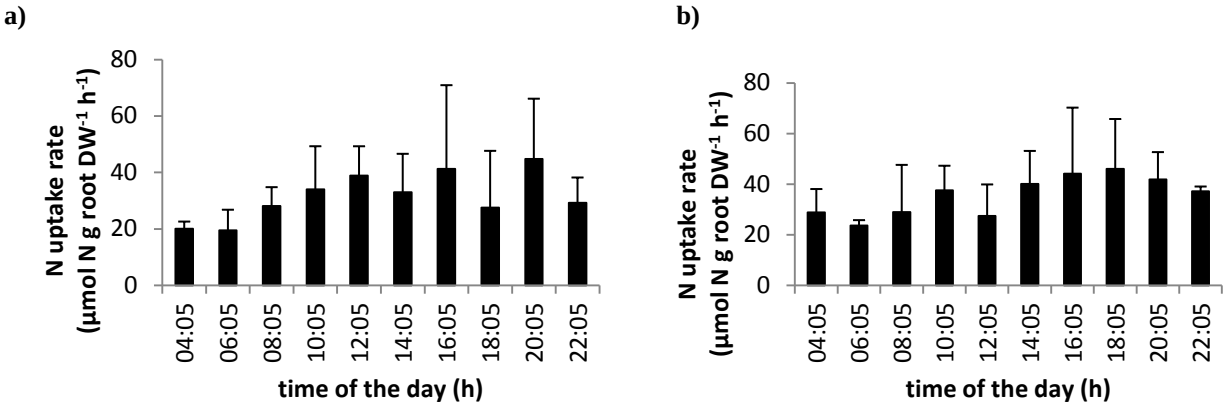


Figure 4.6 – *S. europaea* nitrogen uptake rate (µmol N g^{-1} root DW), **a)** NH_4-N uptake rate **b)** NO_3-N uptake rate. Mean ± standard deviation.

Aster tripolium NH_4-N uptake rates did not vary significantly during the day, nevertheless mean NH_4-N uptake rates varied from 22.3± 4.9 µmol N g^{-1} root DW h^{-1} between 4:30 and 5:30 h to a maximum of 53.3 ± 25.1 µmol N g^{-1} root DW h^{-1} between 20:30 and 21.30 h (Figure 4.7a). In the case of the NO_3-N uptake rates in *A. tripolium* plants (Figure 4.7b), there were significant differences between times of the day, with the uptake rate obtained from plants incubated between 20:30 and 21:30 h, being 51.1 ± 3.0 µmol N g^{-1} root DW h^{-1}, which was higher than plants incubated from 6:30 to 7:30 h, 18.1 ± 6.1 µmol N g^{-1} root DW h^{-1}, from 8:30 to 9:30 h, 20.1 ± 4.7 µmol N g root DW^{-1} h^{-1}, and from 14:30 to 15:30 h , 16.4 ± 8.2 µmol N g^{-1} root DW h^{-1} ($p \leq 0.016$). No other significant differences were observed between the several times of the day.

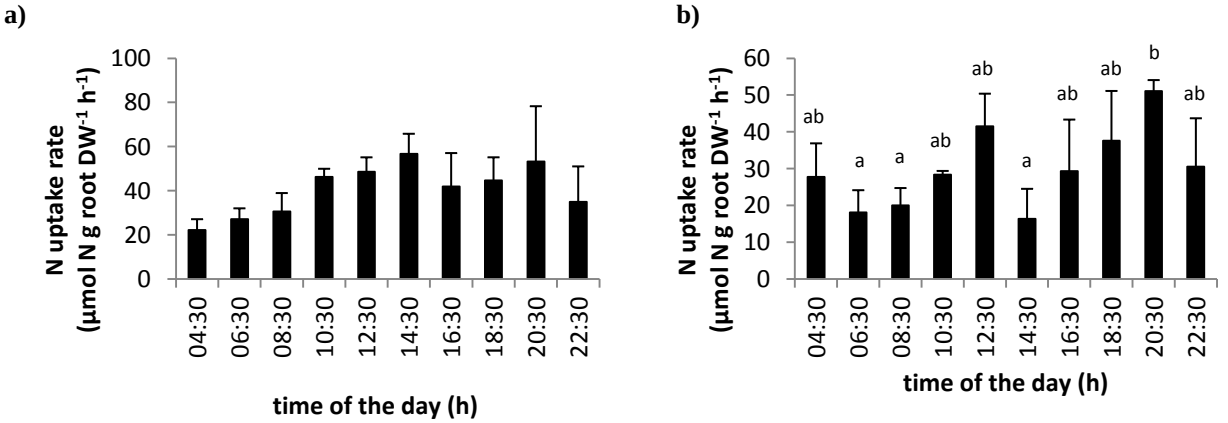


Figure 4.7 – *A. tripolium* nitrogen uptake rate (µmol N g^{-1} root DW), **a)** NH_4-N uptake rate **b)** NO_3-N uptake rate. Mean ± standard deviation. Means sharing a letter do not differ significantly.

Regulation of N uptake

Environmental data

Table 4.4 shows the mean irradiance, air and water temperatures during the ^{15}N incubations for *S. europaea* plants used to investigate the regulation of N uptake in relation to the N source during growth.

Table 4.4 - Environmental data recorded during the *S. europaea* ^{15}N incubations for induction of N uptake, light irradiance (µmol PAR m^{-2} s^{-1}), air and water temperature (°C), (mean ± standard deviation)**.**

incubation time	Irradiance (µmol PAR m^{-2} s^{-1})	Air temperature (°C)	Water temperature (°C)
0h	634 ± 284	29.7 ± 3.2	24.0 ± 0.5
2h	901 ± 557	32.6 ± 2.6	24.3 ± 0.3
4h	632 ± 528	34.0 ± 2.3	24.5 ± 0.3
6h	637 ± 350	30.1 ± 1.6	24.2 ± 0.1
8h	167 ± 62	24.0 ± 1.6	23.8 ± 0.2

Water nutrients

During incubations in heavy isotope N solution, the mean PO_4^{3-} concentration was 174 µmol l^{-1}, and the N concentrations were as in Table 4.5.

Table 4.5 - Measured (mean ± standard deviation) dissolved inorganic nitrogen NH_4^+, NO_3^- and NO_2^- (µmol l^{-1}) for ^{15}N incubation solutions.

	NH_4^+ (µmol l^{-1})	NO_3^- (µmol l^{-1})	NO_2^- (µmol l^{-1})
NH_4^+	974 ± 61	1.37 ± 0.53	0.187 ± 0.075
NO_3^-	2.52 ± 0.76	1002 ± 76	2.06 ± 1.97

N uptake

In order to test for NH_4^+ and NO_3^- induction of N uptake, *S. europaea* N uptake, as NH_4^+ and NO_3^-, was compared between plants grown in the same N source as the tested uptake and plants moved from one to the other. The NH_4-N uptake rates were not significantly different, between the two situations, at all times tested (Figure 4.8a). On the other hand the NO_3-N uptake rate was

lower when plants only received NO_3^- for the one hour of incubation: 6.7 ± 3.8 µmol N g^{-1} root DW h^{-1}, compared with plants incubated at the same time but previously supplied with NO_3^-, 42.0 ± 12.7 µmol N g^{-1} root DW h^{-1} (p=0.010). No significant differences were observed for cases where plants were supplied with NO_3^- for longer than 1h (Figure 4.8b).

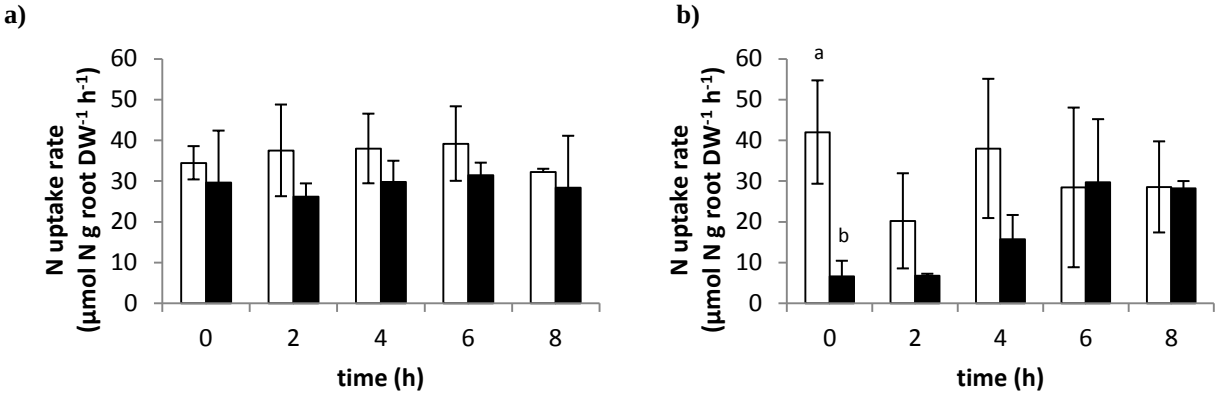


Figure 4.8 – Mean (± standard deviation) *S. europaea* nitrogen uptake rate (µmol N g^{-1} root DW) **a)** NH_4^+ **b)** NO_3^--N uptake rate. White bars represent N uptake rates by plants grown in the same N form as the measured uptake, and black bars the N uptake rates by plants for which N supply was changed to the same form as the measured N uptake at time 0 h. Means sharing a letter do not differ significantly within each time point.

Although *S. europaea* plants were grown in solution containing a nitrification inhibitor (dicyandiamide, 7.5ppm), the N uptake rate reported above was assessed in solution without the nitrification inhibitor. The effect of the presence of the nitrification inhibitor in the uptake rate of N by *S. europaea* plants was tested, and in both cases N uptake (NH_4-N and NO_3-N), even if higher in absence of nitrification inhibitor, was not significantly different in the presence or absence of nitrification inhibitor (Figure 4.9).

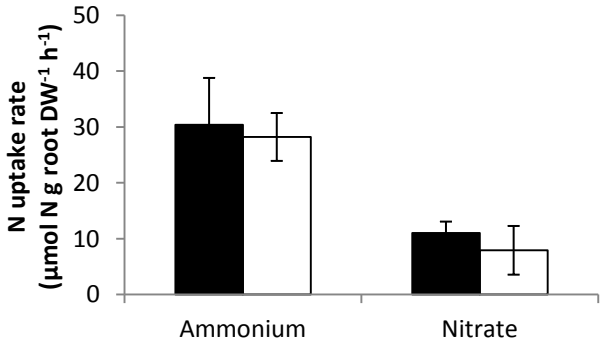


Figure 4.9 – Mean (± standard deviation) *S. europaea* nitrogen uptake rate (µmol N g^{-1} root DW). White bars in the presence of nitrification inhibitor (7.5ppm dicyandiamide), and black bars without nitrification inhibitor.

N uptake kinetics

Environmental data

Mean daily irradiance was 399 ± 46 µmol PAR m^{-2} s^{-1}. The mean daily air temperature for the same period was 29.5 ± 2.0 °C, while the daily minimum mean was 19.7 ± 4.3 ºC, and the daily maximum air temperature 45.9 ± 4.5 °C. The mean daily water temperature for the experimental period was 25.7 ± 1.6 °C, the daily minimum 22.5 ± 2.3 °C, and the daily maximum 28.5 ± 1.2 °C. In case of *S. europaea* irrigation water, the salinity did not vary more than 10.2 ± 0.2 both after each water change and immediately before the following water change. For *A. tripolium* irrigation water the salinity varied between 10.9 ± 0.12 after each water change and 11.0 ± 0.01, before the following water change.

During the ^{15}N incubations, the overall mean air and water temperatures were respectively 33.9 ± 3.3 and 24.7 ± 0.4 °C. Overall the mean irradiance was 956± 478 µmol PAR m^{-2} s^{-1}, and Figure 4.10 shows the mean irradiance for the incubation periods relative to each treatment.

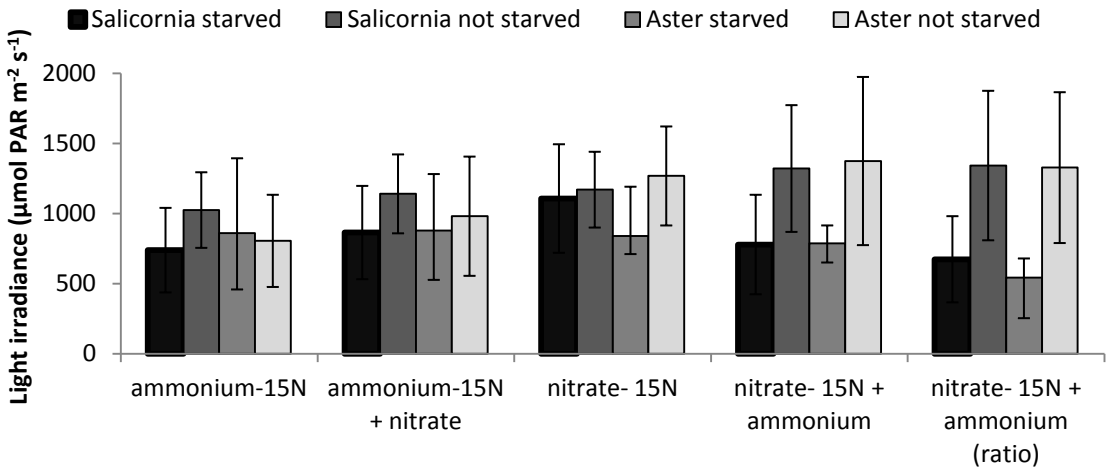


Figure 4.10- Irradiance (µmol PAR m^{-2} s^{-1}) recorded during the ^{15}N incubations, for each treatment (mean ± standard deviation)**.**

Water Nutrients

The mean total DIN was, 2163 ± 246 and 2002 ± 431 µmol N l^{-1}, respectively for *S. europae*a and *A. tripolium* irrigation waters. The mean DIP concentrations for the same period was, 228 ± 9 and 219 ± 33 µmol P l^{-1}, respectively for *S. europae*a and *A. tripolium* irrigation waters.

During incubations in ^{15}N solution, the overall mean PO_4^{3-} concentration was 166 ± 7 µmol l^{-1} and NO_2^- concentration was 1.1 ± 2.0 µmol l^{-1}. NH_4^+ + NO_3^- concentrations were as presented in Figure 4.11 and Figure 4.12.

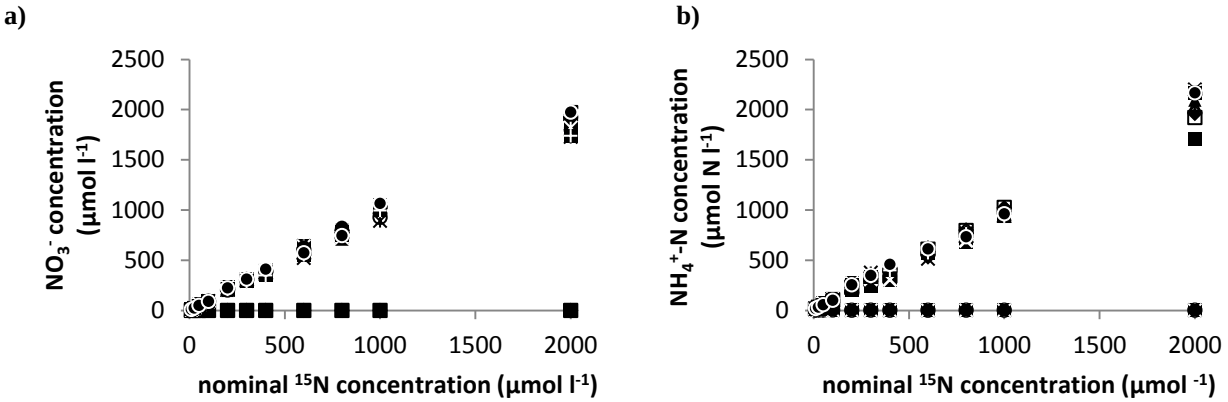


Figure 4.11 – Measured mean (± standard deviation) dissolved inorganic nitrogen concentration (µmol l^{-1}) as: a) NO_3^-, b) NH_4^+, for the several nominal ^{15}N concentrations incubation solutions. For *S. europaea* starved for the several nutrients supply combinations: ◆ $^{15}NH_4^+$, ✉ $^{15}NH_4^+$ + NO_3^-, ● $^{15}NO_3^-$, and ● NH_4^+ + $^{15}NO_3^-$; *S. europaea* not starved, ■ $^{15}NH_4^+$, ▲ $^{15}NH_4^+$ + NO_3^-, ■ $^{15}NO_3^-$, and ■ NH_4^+ + $^{15}NO_3^-$; *A. tripolium* starved, ◇ $^{15}NH_4^+$, × $^{15}NH_4^+$ + NO_3^-, ○ $^{15}NO_3^-$, and ▫ NH_4^+ + $^{15}NO_3^-$; *A. tripolium* not starved, □ $^{15}NH_4^+$, △ $^{15}NH_4^+$ + NO_3^-, ✶ $^{15}NO_3^-$, and + NH_4^+ + $^{15}NO_3^-$.

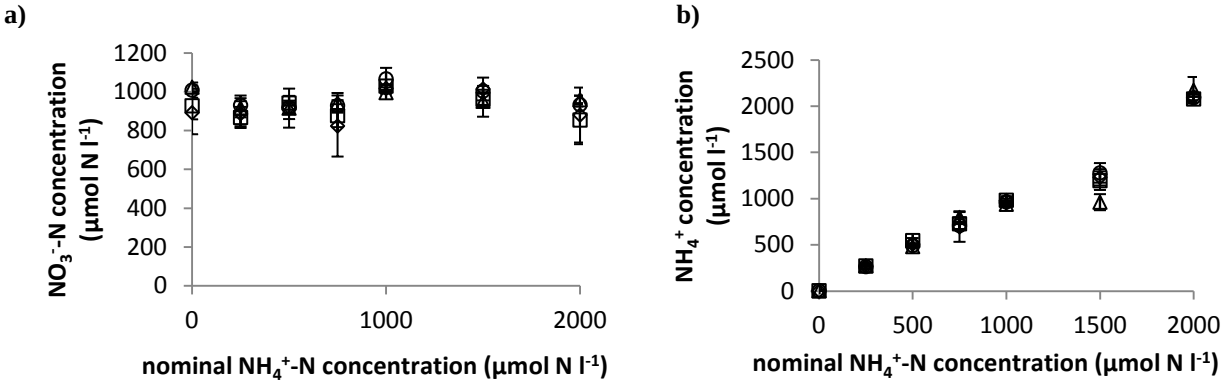


Figure 4.12 – Measured mean (± standard deviation) dissolved inorganic nitrogen concentration (µmoll^{-1}) as: a) NO_3^-, b) NH_4^+, for the several nominal NH_4^+ treatments for, ○ *S. europaea* starved and, △ non-starved plants; □ *A. tripolium* starved, ◇ and not starved plants.

Plant growth and N content

The shoot biomass (DW) of *S. europaea* plants used in ^{15}N incubations was 0.3 ± 0.1 g DW and root biomass was 0.1 ± 0.1 g DW, N content was 2.1 ± 0.2 mmol N g^{-1} DW. The shoot biomass of *A. tripolium* plants was 0.5 ± 0.2 g DW and root biomass was 0.2 ± 0.1 g DW, N content was 3.0 ± 0.3 mmol N g^{-1} DW.

N uptake

Both *S. europaea* and *A. tripolium* plants were able to take up NH_4-N and NO_3-N in all situations analysed, namely NH_4^+ and NO_3^- supplied alone or combined, and in both starved and not starved plants. All regressions showed a good fit to the model used (Michelis-Menten). The exceptions were the *A. tripolium* NO_3-N uptake rates with both NH_4^+ and NO_3^- present in solution, where the r^2 obtained were only 0.43 and 0.37, respectively in case of starved and non-starved plants (Table 4.6). All presented uptake rates were calculated from ^{15}N assimilated by plants and corrected for background non labelled N in solution.

Figure 4.13 presents the *S. europaea* N uptake rates at different N concentrations in solution, and the determined Michaelis-Menten model for each situation analysed. In every case determined S_{min} was not significantly different than 0, determined V_{max} and K_m, as well as the obtained r^2 for each fitting are presented on Table 4.6.

The NH_4^+ maximum uptake rates determined for starved *S. europaea* plants were not significantly different when N was supplied as NH_4^+ only, or together with NO_3^-. Similarly, the NO_3-N maximum uptake rates were not significantly different when the N source was NO_3^- only, or NO_3^- and NH_4^+ combined. Nonetheless, the NH_4-N maximum uptake rate was higher than the NO_3-N uptake rate, either when both forms of N were supplied separately or combined ($p<0.001$).

In the case of non-starved *S. europaea,* the NH_4-N maximum uptake rates when NH_4^+ was supplied alone were not significantly different than when NH_4^+ was supplied combined with NO_3^-, or compared to NO_3-N maximum uptake rate when the sole N source was NO_3^-. However, the NO_3-N maximum uptake was lower when both NO_3^- and NH_4^+ were present in solution, compared to NO_3-N uptake when supplied alone ($p=0.002$), and also compared to the NH_4-N maximum uptake rate when NO_3^- and NH_4^+ were present in solution ($p<0.001$).

The NH_4-N maximum uptake rates by *S. europaea* plants were not significantly different between starved and non-starved plants, neither when NH_4^+ was supplied alone nor when combined with NO_3^-. On the other hand, NO_3-N maximum uptake rate was lower in starved *S. europaea* plants than in non-starved plants when the N source was NO_3^- only ($p=0.005$), and not significantly different when both N forms were present in solution.

The K_m constant was significantly different for starved *S. europaea* in plants where NH_4^+ and NO_3^- only were supplied separately ($p=0.002$) and in non-starved plants only when NO_3^- and NH_4^+ were supplied together ($p=0.029$). No other significant differences were observed between the various K_m's determined.

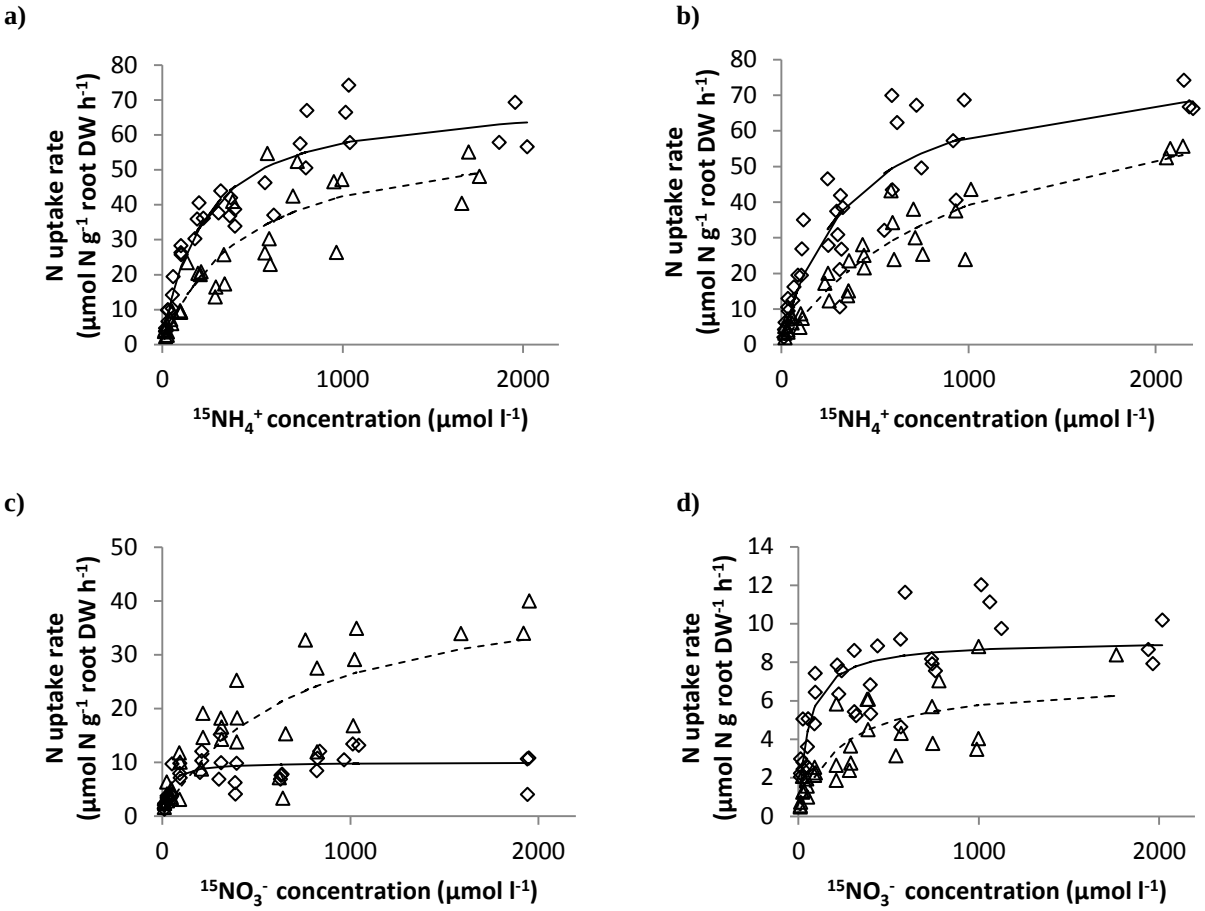


Figure 4.13 – *S. europaea* N uptake rates (µmol N g^{-1} root DW h^{-1}) at a range of nitrogen concentrations, and supplied as: **a)** $^{15}NH_4^+$ only, **b)** $^{15}NH_4^+ + NO_3^-$, **c)** NO_3^- only, **d)** $^{15}NO_3^- + NH_4^+$. Symbols represent measured uptake rates and lines the uptake rates predicted by fitting the Michaelis-Menten model. Full line (◊) represent starved plants, dashed line (Δ) represent non starved plants.

The measured uptake rates at different N concentrations for *A. tripolium* plants are presented in Figure 4.14 together with the estimated Michaelis-Menten model regression. In every case determined S_{min} was not significantly different than 0. Determined V_{max} and K_m, as well as the obtained r^2 for each regression are presented on Table 4.6.

Starved *A. tripolium* plants (as in the case of *S. europaea)* had higher NH_4-N maximum uptake rates than NO_3-N, both when the two forms of N were supplied separately or combined ($p \leq 0.002$). NH_4-N maximum uptake rates were not significantly different if N was supplied as NH_4^+ only or in combination with NO_3^-. The NO_3-N maximum uptake rates were not significantly different between NO_3^- sole supply or NO_3^- and NH_4^+ supplied together.

In non-starved *A. tripolium* plants the NH_4-N maximum uptake rates when NH_4^+ was supplied alone were not significantly different than when NH_4^+ was supplied combined with NO_3^-. However, NO_3-N maximum uptake rates were higher in plants receiving NO_3^- only, compared to plants receiving a combination of NH_4^+ and NO_3^- ($p<0.001$). And NH_4-N maximum

uptake rates were higher than NO_3-N uptake, both comparing the uptake from NH_4^+ only and NO_3^- only solutions (p=0.008), and when comparing the uptake rates from solutions containing a combination of both forms (p<0.001).

When the N supply consisted of both N forms combined, neither NH_4-N nor NO_3-N maximum uptake rates were significant different between starved and non-starved *A. tripolium* plants. When each N form was supplied alone the maximum uptake rate differed: In these cases, while NH_4-N maximum uptake rate was higher for starved plants (p=0.032), and the NO_3-N maximum uptake rate was higher in non-starved plants (p<0.001). No significant differences in the K_m constant were observed across any treatment in relation to *A. tripolium*.

Additionally, the NO_3-N uptake rate was investigated for several ratios of NH_4^+ to NO_3^- in solution, obtained by combining different concentrations of NH_4^+ and a constant NO_3^- concentration (~1000 µmol NO_3^- l^{-1}).

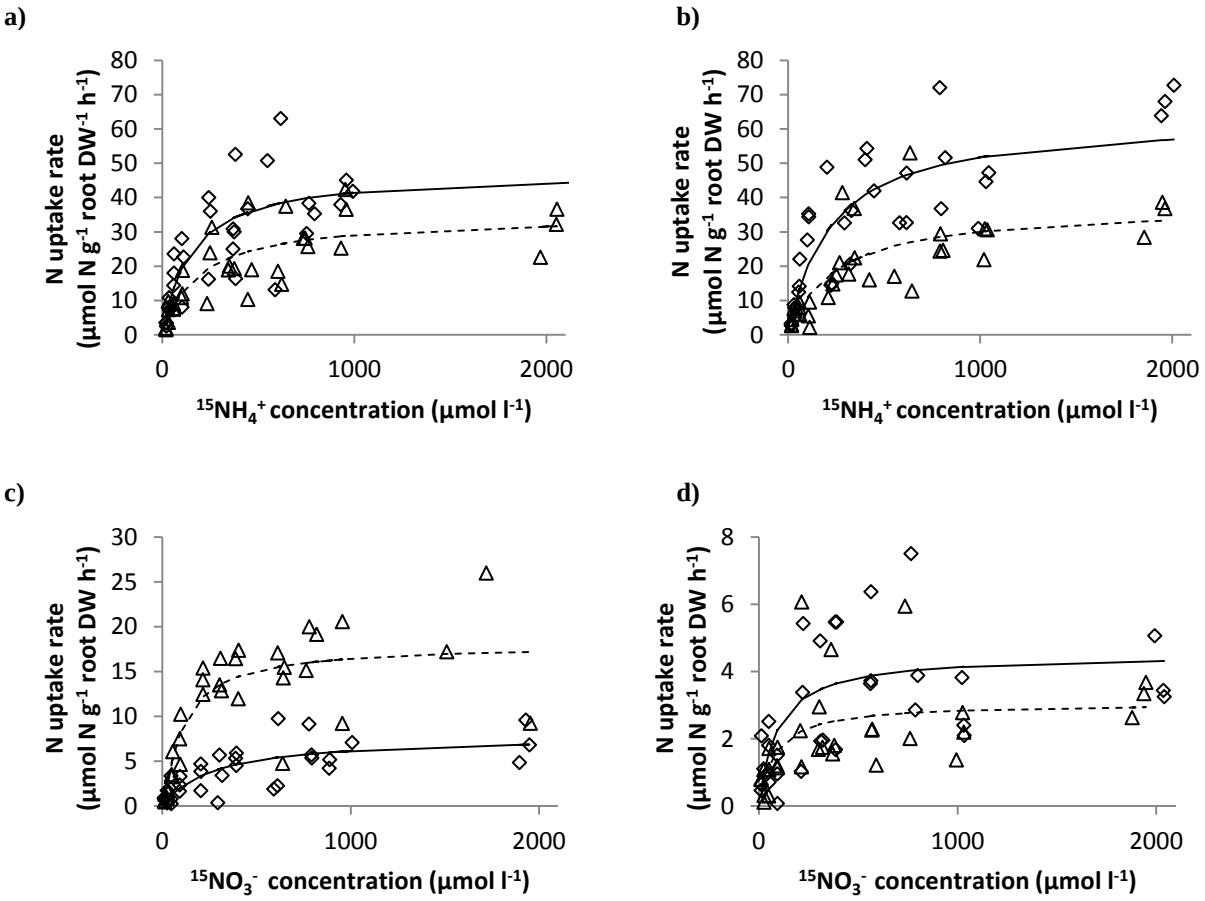


Figure 4.14 – *A. tripolium* N uptake rates (µmol N g^{-1} root DW h^{-1}) at a range of nitrogen concentrations, and supplied as: **a)** $^{15}NH_4^+$ only, **b)** $^{15}NH_4^+$ + NO_3^-, **c)** NO_3^- only, **d)** $^{15}NO_3^-$ + NH_4^+. Symbols represent measured uptake rates and lines the uptake rates predicted by fitting the Michaelis-Menten model. Full line and ◊ are for starved plants, Δ and dashed line are for not starved plants.

Above it was shown that NO_3-N maximum uptake rate was not different with or without NH_4^+ in solution for starved plants, but it was higher when NO_3^- was supplied alone in case of not starved plants. Here, the higher NO_3-N uptake rate when there was virtually no NH_4^+ in solution (0.3% of the NO_3^- supply), in comparison to uptake when N supplied as equimolar mix of NH_4^+ and NO_3^-, was confirmed for non starved plants ($p<0.001$). Additionally it is shown that as little as 30% of NH_4^+ in solution decreases the NO_3-N as much as the presence of 235% of NH_4^+, in both *S. europaea* and *A. tripolium* plants ($p<0.001$ and $p\leq0.004$, respectively) (Figure 4.15a and b).

Table 4.6 – *S. europaea* and *A. tripolium* N uptake parameters for $^{15}NH_4^+$, $^{15}NH_4^+ + NO_3^-$, $^{15}NO_3^-$ and $NH_4^+ + {}^{15}NO_3^-$, for starved and not starved plants. Maximum uptake rate (V_{max}) and half saturation constant (K_m) were obtained by fitting the observed N uptake rates to modified Michaelis-Menten model (S_{min} is not presented because it was never significantly different from 0).

			V_{max} (µmol N g^{-1} root DW h^{-1})	**K_m (µmol N l^{-1})**	**r^2**
S. europaea	**Starved**	**$^{15}NH_4^+$**	70.6 ± 4.4	225± 52	0.91
		$^{15}NH_4^+ + NO_3^-$	79.3 ± 9.0	357 ± 122	0.79
		$^{15}NO_3^-$	10.0 ± 0.8	28 ± 17	0.52
		$NH_4^+ + {}^{15}NO_3^-$	9.1 ± 0.6	55 ± 25	0.66
	Not starved	**$^{15}NH_4^+$**	61.8 ± 8.9	457 ± 178	0.81
		$^{15}NH_4^+ + NO_3^-$	77.6 ± 10.0	984 ± 266	0.90
		$^{15}NO_3^-$	44.6 ± 10.4	690 ± 376	0.68
		$NH_4^+ + {}^{15}NO_3^-$	7.0 ± 0.9	219 ± 107	0.67
A. tripolium	**Starved**	**$^{15}NH_4^+$**	63.0 ± 7.8	218 ± 103	0.70
		$^{15}NH_4^+ + NO_3^-$	47.6 ± 5.8	147 ± 85	0.64
		$^{15}NO_3^-$	7.8 ± 1.4	267 ± 164	0.58
		$NH_4^+ + {}^{15}NO_3^-$	4.5 ± 0.7	114 ± 92	0.43
	Not starved	**$^{15}NH_4^+$**	37.3 ± 5.9	243 ± 140	0.62
		$^{15}NH_4^+ + NO_3^-$	34.6 ± 4.5	189 ± 103	0.68
		$^{15}NO_3^-$	18.0 ± 1.7	98 ± 43	0.72
		$NH_4^+ + {}^{15}NO_3^-$	3.1 ± 0.5	79 ± 74	0.37

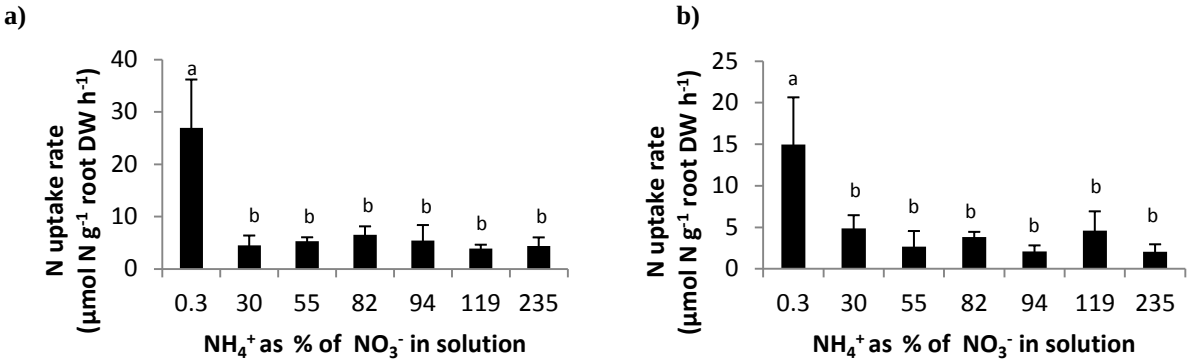


Figure 4.15 –NO_3-N uptake rate (µmol N g^{-1} root DW h^{-1}) for a range of NH_4^+ to NO_3^- ratios, **a)** *S. europaea*, **b)** *A. tripolium* non starved. Means sharing a letter do not differ significantly.

Discussion

Halophyte plants have the capacity for NH_4^+ and NO_3^- uptake comparable of those of terrestrial and aquatic plants (Stewart et al. 1973, Stewart and Rhodes 1978). In the present work it was demonstrated that *S. europaea* and *A. tripolium* can utilize both NH_4^+ and NO_3^- and grow well when either form is supplied by itself or when both forms are supplied together. Even though NH_4^+ is often the major form of N in aquaculture wastewater, NO_3^- can dominate in recirculating systems that include bacterial biofilters (Gelfand et al. 2003, McCarthy and Gardner 2003, Arredondo-Figueroa et al. 2007). Although DIN concentration in wastewater is highly variable and dependent on many factors, it usually increases as the intensity of the operations increases (Piedrahita 2003). Based on the measured plant performance in the present study it is predicted that either *S. europaea* or *A. tripolium* plants could achieve satisfactory plant production in various IMTA systems, providing high N concentration in wastewater.

A preference for a particular N source is known to vary with plant species, while in some cases N source does not affect plant growth (Martins-Loucao et al. 1993, Mahmood et al. 2005, Tylova-Munzarova et al. 2005, Dominguez-Valdivia et al. 2008, Jampeetong et al. 2012a), in other cases plant growth strongly depends on the N source. Often a combination of NH_4^+ and NO_3^- results in better plant growth than when either form is supplied separately (Shaviv and Hagin 1988, Heberer and Below 1989, Adriaanse and Human 1990, Bloom et al. 1993, Siddiqi et al. 2002, Kant et al. 2007, Nicodemus et al. 2008). Nonetheless some plant species grow better in NO_3^- (Kirkby and Mengel 1967, Lasa et al. 2001, Britto and Kronzucker 2002, Guo et al. 2002, Kant et al. 2007, Dominguez-Valdivia et al. 2008, Borgognone et al. 2012), or in NH_4^+ (Mendelssohn 1979, Martins-Loucao et al. 1993, Wang et al. 1993, Brix 1999, Tyler et al. 2003,

Mahmood et al. 2005, Tylova-Munzarova et al. 2005, Fang et al. 2007a, Jampeetong and Brix 2009b, Horchani et al. 2010, Konnerup and Brix 2010, Jampeetong et al. 2012a). Despite the fact that some plants show better growth under NH_4^+, in some other species NH_4^+ can result in toxicity when it is supplied as the sole N source (Raab and Terry 1994, Britto and Kronzucker 2002, Guo et al. 2002, Houdusse et al. 2005, Tylova-Munzarova et al. 2005, Houdusse et al. 2008, Tylová et al. 2008, Garnica et al. 2009). Sági and Erdei (2005) described signs of toxicity in *A. tripolium* plants grown in NH_4^+ only, including unhealthy and brown roots with necrotic root tips, decreases in plant growth; and necrotic lesions on stems and leaves (the N concentration was not reported). Also Jefferies (1977) observed poor growth of *A. tripolium* plants when grown in NH_4^+ comparing to NO_3^- (100 µmol l^{-1}). In the present study, however, no signs of NH_4^+ toxicity were observed in the *A. tripolium* plants, and while final shoot biomass was not different between the three N treatments investigated (~600 µmol l^{-1}), the root biomass was even higher in NH_4^+ treatment, than in NH_4NO_3. On the other hand, final biomass (shoot and root) of *S. europaea* plants grown in NH_4^+ only was lower than both in the NO_3^- only treatment, and then when both NO_3^- and NH_4^+ were supplied together. These results are in agreement with the work of Ventura et al. (2010) who compared *S. europaea* growth between NH_4^+ and NO_3^- supply (5 mmol l^{-1}), and similarly observed lower growth when only NH_4^+ was supplied. However, Jefferies (1977) observed no differences in *S. europaea* growth when supplied with NH_4^+ and NO_3^- (100 µmol l^{-1}). In contrast, Kudo and Fujiyama (2010) observed better growth of . *S. bigelovii* grown on NH_4^+ compared to NO_3^-, for concentration 1-3 mmol l^{-1} and no differences at 4 mmol l^{-1}. The differences between the *S. europaea* studies are then probably related to the tested concentrations and supply regime, and at the lower concentration NH_4^+ is tolerated. However, at higher concentrations the supply of NH_4^+ by itself is toxic to plants, and similar performance has been observed often in several plant species (Jampeetong and Brix 2009b, a, Jampeetong et al. 2012b). As has been observed in several other plant species, the presence of equimolar NO_3^- in solution alleviates NH_4^+ toxicity on *S. europaea* and the consequent decrease on growth, although the reasons for these effects are still not known (Martins-Loucao et al. 1993, Stitt 1999, Britto and Kronzucker 2002, Houdusse et al. 2005, Houdusse et al. 2008).

Besides an overall decrease in growth, NH_4^+ toxicity often results in a stronger inhibition in root growth and consequent increase in shoot:root ratios in a range of plants (Raab and Terry 1994, Britto and Kronzucker 2002 and references therein, Guo et al. 2002). However, this was not the observed in the present work; while no differences were identified in *A. tripolium* shoot

to root ratios, in *S. europaea*, the ratio was lower in the NH_4^+ treatment, intermediate in NO_3^- and higher in NH_4^+ plus NO_3^-.

In relation to the total N retained in the plants at the end of the growth experiment, in *A. tripolium* no differences were observed between plants supplied with the different N source. However, in *S. europaea*, less N was retained in the NH_4^+ treatment comparing to the two other N supply treatments. When expressing N content per unit of root biomass (g root DW), the N content in *S. europaea* was lower in the NH_4^+ treatment, suggesting a lower uptake of NH_4-N comparing to NO_3-N when both N forms are supplied separately. In *A. tripolium*, the N content (mmol N g^{-1} root DW) was lower in the NH_4^+ treatment, intermediate in NO_3^- and higher in the mixed NH_4NO_3 treatment, suggesting that NH_4-N uptake rate was lower than NO_3-N when supplied separately, and that uptake of NH_4-N, NO_3-N or both was enhanced when both are present in solution.

NH_4-N and NO_3-N uptake kinetics parameters have not previously been determined for either *A. tripolium* or *S. europaea*. Diurnal patterns for NH_4-N and NO_3-N root uptake have been observed in many plants (Clement et al. 1978, Raman et al. 1995, Macduff et al. 1997, Peuke and Jeschke 1998, Tischner 2000). In the present work, despite the lack of statistical significant differences in most cases, for both plant species and N forms the uptake was minimal in the early hours of the day and increased towards the end of day and so to decrease variability, the kinetics uptake experiments were conducted during the middle of the day. Although irradiance was not constant, the range is considerably reduced comparing to the daily fluctuations (irradiance effects on DIN uptake is investigated in the next chapter). The measured *A. tripolium* and *S. europaea* ^{15}N uptake, as NH_4^+ and NO_3^- when supplied separately or in a equimolar mix, at a range of concentrations, both in plant with continuous supply of N and plants N starved for 48h, were good fits to the Michaelis Menten model, with the exception of *A. tripolium* NO_3-N uptake rates when NH_4^+ was also present in solution. The general good fit to the Michaelis Menten model up to 2 mmol l^{-1} indicates that for these plants HATS are responsible for N uptake at the tested concentrations. Even if it different kinetics are observed at higher N concentrations, the tested range of concentration includes N concentrations likely to occur in both natural environment and aquaculture wastewater (Henry and Jefferies 2002, Lin et al. 2003, Deviller et al. 2004, Lin et al. 2005, Webb et al. 2012).

In other studies, both N starved (Romero et al. 1999, Henry and Jefferies 2003a, Jampeetong and Brix 2009b, Zhang et al. 2009, Jampeetong et al. 2012a) and non-starved (Morris 1980, Tylova-Munzarova et al. 2005, Fang et al. 2007a, Fang et al. 2007b, Konnerup and Brix 2010, Mozdzer et al. 2010) plants have been used to investigate N uptake. It was

demonstrated here that the maximum N uptake may be different depending on the immediately previous N supply and therefore some caution is recommended when extrapolating results into models and comparing results between different studies.

In relation to NO_3-N uptake, due to the existence of iHATS and cHATS, uptake of NO_3-N is expected to depend in part on the presence of NO_3^- in solution prior to the measurement of uptake (Crawford and Glass 1998, Cerezo et al. 2000, Glass et al. 2002). This is consistent with the present results, since in both plant species, when NO_3^- is supplied alone, NO_3-N uptake was higher in non-starved comparing to N-starved plants. Additionally, in the case of *S. europaea*, it was shown that after 2 h of NO_3^- supply to previously starved plants, NO_3-N uptake is similar to that of non-starved plants. In the case of NH_4-N uptake, the observations are consistent with presence of cHATS only; *S. europae*a did not exhibit differences in maximum uptake between N starved and non-starved plants. On the other hand, *A. tripolium* exhibited higher NH_4-N uptake in starved plants, which could indicate a high demand for N due to the previous lack of N supply, as described for other plants (Lee and Lewis 1994, Forde and Clarkson 1999). However, if that was the case, then higher, NH_4-N uptake would be expected if NH_4^+ is supplied together with NO_3^- to starved plants, which was not observed. When NH_4^+ was present, NO_3-N uptake by *A. tripolium* plants showed a poor fit to the Michaelis-Menten model.

Both in the case of starved and non-starved plants, there was a high variability in uptake between replicate plants. It is not clear if this reflects an inherent natural variability or is a result of the experimental design. Nevertheless, in both *S. europae*a and *A. tripolium* plants, when NH_4^+ was present in solution the maximum NO_3-N uptake was lower than when supplied alone in non-starved plants, but not different in case of starved plants. Similar inhibition of NO_3-N uptake by the presence of NH_4^+ in solution has often been observed in other plant species (De la Haba et al. 1990, Kronzucker et al. 1999, Ruan et al. 2007, Wang et al. 2010), even though the mechanism is still not understood. In the present study, NH_4-N uptake was not different when NH_4^+ was supplied alone or together with NO_3^-, and was always higher than NO_3-N uptake, with the exception of in non-starved *S. europaea* for single N forms supplied alone, where there was no significant differences between NH_4-N and NO_3-N maximum uptakes.

Several hypotheses have been proposed to explain the toxicity of NH_4^+, but none have yet been conclusively confirmed (Britto et al. 2001 and references therein, Britto and Kronzucker 2002). From the *S. europaea* plants growth and N content results, one would expect lower NH_4-N uptake rates than NO_3-N, when each is supplied separately to non-starved plants. The lack of observed differences in the measured NH_4-N and NO_3-N uptake rates may indicate that a higher proportion of the N taken up as NH_4^+ was lost from the plants after being taken up. According to

(Britto and Kronzucker 2002) plants cannot store NH_4^+ in excess, and at high external concentrations a proportion of NH_4-N is not assimilated by the plant and is returned to the solution. This futile cycle of NH_4-N requires energy and may be the reason of the lower growth observed. Also the observed higher NH_4-N uptake rate in *A. tripolium* was not translated in significantly higher growth or N content in the NH_4^+ treatment, indicating that some of the NH_4-N uptake may be redundant.

Conclusions

The results obtained in the present study indicate that, with continuous supply of N, under the concentrations tested, *A. tripolium* had no apparent preference for either of the N forms and is tolerant to NH_4^+. *S. europaea* is sensitive to NH_4^+ when supplied alone, reflected in decreased growth, and this toxicity is alleviated when NH_4^+ and NO_3^- are supplied together. Further investigation is necessary to better understand the concentrations at which NH_4^+ becomes toxic to *S. europaea* and to understand the toxicity mechanism, particularly the possibility of increased efflux from the roots. In relation to use of these plants in IMTA system, the inclusion of a bacterial biofilter before the plant-unit would reduce the negative effects of NH_4^+ on *S. europaea* and consequently provide a better growth and N removal.

Chapter 5 - Growth and nitrogen uptake in *Salicornia europaea* and *Aster tripolium* under different environmental conditions

Introduction

Both *Salicornia europaea* and *Aster tripolium* are halophyte plants of interest for inclusion in IMTA systems due their many commercial applications, including human consumption. Halophytes are plants that survive and reproduce in environments where the salt concentration is around 200 mmol l^{-1} NaCl or more (salinity ≥ 12) (Flowers et al. 1986).

In order take up water, plants need to accumulate salt ions (Na^+, Cl^-) so they maintain their water potential below that of the external water potential. However it has been shown that different enzymes isolated from halophyte plants are as sensitive to Na^+ as those from non-halophyte plants, therefore suggesting that halophyte plants have to maintain low Na^+ concentrations in the cytoplasm where biochemical processes take place (Flowers 1972, Greenway and Osmond 1972, Stewart and Lee 1974, Albert 1975, Flowers et al. 1977, Koyro et al. 2008). Halophytes use different mechanisms to prevent Na^+ accumulation in cell cytoplasm, including: regulation of ion concentrations, ion compartmentalisation and production of osmotically active compatible solutes (Flowers 1972, Flowers et al. 1977, Glenn et al. 1999, Khan et al. 2001, Flowers and Colmer 2008, Koyro et al. 2008) while maintaining adequate water potential. Some halophyte species are capable of excluding excess salt ions, either using specialized structures such as salt glands, through root membranes, or by accumulating salt in specific portions of the plant that are then discarded (eg. old leaves) (Albert 1975 and references therein, Glenn et al. 1999, Koyro et al. 2008, Balnokin et al. 2010). However most of halophytic plants do not exclude salt ions, but rather accumulate salt and use other strategies to avoid build up of toxic concentrations in the cytoplasm. Often halophyte plants can respond to salt stress by accumulating Na^+ in vacuoles, and increasing the synthesis of compatible solutes compartmentalised in the cytoplasm, with many of the compatible solutes accumulated by halophytes being nitrogenous compounds (proline, betaines, choline and polyamines) (Flowers et al. 1986, Hare et al. 1998, Glenn et al. 1999, Naidoo and Naidoo 2001, Flowers and Colmer 2008, Koyro et al. 2008).

Different species use different mechanisms, or combinations of mechanisms to achieve salt tolerance, and the optimum external salt concentration and the tolerance range of salt concentration are widely variable between halophytes. In fact, growth responses to external salinity have been used to classify halophytic plants: plants that present optimal growth at moderate salinities (6–18) and continue to grow and survive at salinities up to 40 (euhalophytes); plants that show optimal growth at very low salinities and continue to grow at reduced rates even

at higher salinities (miohalophytes); and plants which growth is greatly reduced even at moderate salinity, and salinities over 18 are lethal (Greenway and Munns, 1980 *in* Bell and O'Leary 2003).

Salicornia europaea can withstand greater than full seawater salt concentration and is one of the most salt-tolerant plant species studied (Cleall-Harding 2008, Aghaleh et al. 2009, Balnokin et al. 2010, Sulian et al. 2012), though it performs poorly at very low salinities (Keiffer et al. 1994, Moghaieb et al. 2004, Cleall-Harding 2008, Balnokin et al. 2010). *S. europaea* optimum growth has been measured at external salinities between 6 and 24 (Ushakova et al. 2006, Cleall-Harding 2008, Balnokin et al. 2010, Aghaleh et al. 2011, Sulian et al. 2012). *S. europaea* increases tissue Na^+ concentration (in vacuoles) with increasing external salinity, and Na^+ in shoots has been shown to make up to 50% of DW (Momonoki et al. 1996, Moghaieb et al. 2004, Ushakova et al. 2005, Ushakova et al. 2006, Zheng et al. 2009, Balnokin et al. 2010, Aghaleh et al. 2011, Sulian et al. 2012). Also increase in the concentration of osmolytes, such as betaine, proline and glutamic acid, with increasing external salinity has been observed in *S. europaea* (Momonoki and Kamimura 1994, Moghaieb et al. 2004, Ushakova et al. 2006, Aghaleh et al. 2011).

On the other hand, *A. tripolium* is less salt tolerant, with several studies reporting a reduction in growth with increasing salinity (Larher et al. 1982, Shennan et al. 1987a, Lenssen et al. 1995, Ramani et al. 2006), e.g. Ramani et al. (2006) demonstrated a decreased growth with increasing salt concentrations, 0, 257 and 513 mmol NaCl l^{-1}. Similar to *S. europaea*, *A. tripolium* does not possess salt excretory specialized structures, however, it does eliminate salt through the shedding of old salt-saturated leaves (Albert 1975). Na^+ concentration increases in *A. tripolium* plant tissues with increasing external salinity, as do osmolyte concentrations, particularly proline and glycine betaine (Goas et al. 1982, Larher et al. 1982, Shennan 1987, Shennan et al. 1987b, Perera et al. 1994, Ueda et al. 2003, Sági and Erdei 2005, Ramani et al. 2006, Geissler et al. 2009a).

Due to the altered uptake of salt ions with varying external salinity, the uptake of other ions present in solution can be affected, for instance K^+ and Ca^{2+} or P contents have been observed to change with salinity in some halophytes (Brown et al. 2006, Amiri et al. 2010), including *S. europaea* (Aghaleh et al. 2009, but see also Zheng et al. 2009) and *A. tripolium* (Sági and Erdei 2005). Little information is available on N uptake in relation to external salinity. In most higher plants, the NO_3^- assimilated is reduced to NO_2^- (catalysed by nitrate reductase, NR), and from NO_2^- to NH_4^+ (catalysed by nitrite reductase, NiR) (Guerrero et al. 1981, Siddiqi et al. 1990, Kronzucker et al. 1995, Forde 2000). NH_4^+, taken up by roots, or resulting from NO_3^- reduction, is converted first to glutamine by the action of glutamine synthetase (GS) and then to

glutamate by the action of glutamate synthetase (GOGAT). These two amino acids serve as the precursors for the synthesis of the other amino acids as well as almost all nitrogenous compounds (Guerrero et al. 1981, Knoepp et al. 1993, Tischner 2000, Guo et al. 2007) (see chapter 4 for *S. europaea* and *A. tripolium* DIN uptake kinetics).

As mentioned above, both *S. europaea* and *A. tripolium* accumulate more nitrogenous osmolytes with increasing salinity, suggesting an increasing in N uptake, although it can also result in preferential assimilation of N into those forms. When expressed per unit of dry weight, N content in *S. europaea* plants has been shown to be lower in plants grown in saline water, in comparison to those grown in almost freshwater (Ushakova et al. 2005, Ushakova et al. 2006, Cleall-Harding 2008). In the case of *A. tripolium* the total N content was been shown to decrease with increasing external salinity in the work carried out by Geissler et al. (2009b). On the other hand Sági and Erdei (2005) measured an increase in NR activity in *A. tripolium* with increasing salinity (between 0 and 200 mmol NaCl l^{-1}) in NO_3^--supplied plants, and increase in GS in the leaves of NH_4^+-supplied plants, suggesting that salinity may increase N uptake. Larher et al. (1982) also measured a slight increase in total N plant content with higher external salinity.

Other than salinity, additional environmental factors may influence the uptake rates of NH_4^+ and NO_3^- in *S. europaea* and *A. tripolium*. It has been repeatedly demonstrated that light stimulates plant NO_3^- uptake, as well as NR synthesis and activity in various higher plants (Haynes and Goh 1978, Naik et al. 1982, Campbell 1988, Lillo 1994, Lillo 2008). However most of the available information compares the situation of dark versus light conditions, and not between different irradiances. Also changes in N uptake during the day have often been observed (Clement et al. 1978, Raman et al. 1995, Macduff et al. 1997, Peuke and Jeschke 1998, Tischner 2000), however, the distinction between the effect of light intensity and the time elapsed since the dark period are not easily separated. However, since plant N metabolism is partially coupled to photosynthesis and it is generally accepted that photosynthesis rates increase with increasing irradiance up to a saturation point, and possibly photoinhibition, it is possible that a similar response in N uptake exists.

Temperature also affects N uptake in higher plants (Bassirirad 2000 and references therein, Dong et al. 2001, Calatayud et al. 2008). NR activity has been shown to be sensitive to high temperatures (Calatayud et al. 2008 and references therein) and studies measuring ^{15}N uptake showed increased uptake with increasing temperature in several plant species (Dong et al. 2001 and references therein).

The work described here investigated the growth and N uptake rates (NO_3-N and NH_4-N) in *S. europaea* and *A. tripolium* at different salinities, in order to find the salinity for optimum

plants performance in IMTA systems, when N is not limiting. It is hypothesised that *S. europaea* performs better at medium and high salinities, while *A. tripolium* performs better at low and medium salinities. To test this hypothesis, plants were grown at 3 different salinities, 1, 10 and 30 and biomass and ^{15}N uptake measured. The short term effects of different light intensity and temperatures on N uptake rates (NO_3-N and NH_4-N) by the two halophyte species were also investigated using ^{15}N enriched solution in order to support the better modelling of N removal from aquaculture wastewater, and the hypotheses that both, increasing irradiance and temperature, will increase N uptake are tested.

Methods

Plants

S. europaea seeds (6^{th} and 7^{th} generations of plants held at Bangor University) were obtained from plants cultivated in greenhouses, and *A. tripolium* seeds were collected from a local saltmarsh (Mochras, 52°81'70.29"N, 4°13'68.75"W). Seeds were stored at -20 °C until used in the experiments. *S. europaea* and *A. tripolium* seeds were germinated in a climate-controlled greenhouse (16h photoperiod), in P576 plug trays with John Innes No.1 compost with continuous immersion in fresh water. Approximately one week after sowing, the water supply was changed to a salinity of 10 using TROPICMARIN^{TM} artificial sea salt, and Phostrogen fertiliser (N:P:K 14:10:27 + trace elements; Bayer CropScience Ltd, Cambridge, UK) was added. After approximately 2 weeks, trays were thinned out to one plant per plug. The solution was frequently topped up with fresh water to compensate for evapo-transpiration, and fully replaced once a week. Approximately 2 weeks later the plants were individually removed from the compost, the roots cleaned of any substrate, and transplanted into a hydroponic raft system.

The hydroponic setup consisted of lysimeters (external dimensions: 60cm length * 40cm width * 32.5cm depth) holding approximately 55 l of solution and a polystyrene floating sheet, providing mechanical support for plants so that the roots were continuously immersed in water, each lysimeter was continuously aerated (Pond Air 4800 pump).

Salinity experiment

To study the effect of three salinities in plant growth and N uptake, plants from each species were grown in 9 lysimeters, three replicates per salinity, over 22 d (calendar days 179 to 201). The experimental salinities 1, 10 and 30, were made by adding different amounts of sea salt (TROPICMARIN^{TM} artificial sea salt) to tap water. Nutrients were added to the irrigation water, at approximately 2000 µmol N l^{-1} as NH_4NO_3, 200 µmol P l^{-1} as Na_2PO_4, Hoagland's micronutrient and Fe solutions (1 ml l^{-1}). Solutions were replaced weekly to maintain nutrient concentrations. Filtered (0.45 µm) water samples were taken, both before and after each water change, immediately frozen and later analysed for dissolved inorganic phosphorous (P) and nitrogen. Dissolved inorganic phosphate (PO_4^{3-}), and N in the forms of nitrite (NO_2^-) and nitrate (NO_3^-) were determined, using a 5–channel LACHAT Instruments Quick-Chem 8000 autoanalyzer after Grasshoff et al. (1983) and Hales et al. (2004). Ammonium (NH_4^+) was determined by fluorimetric method after Holmes et al. (1999) using HITACHI F2000 fluorescence spectrophotometer.

Plant biomass was assessed when plants were transplanted into the hydroponic system (day 179), after dried in the oven at 50 °C for 1 week, both above-ground (shoot) and root dry weigh (DW) were measured in a subsample of *S. europaea* and *A. tripolium* plants. Between days 200 and 202 all plants were removed from the hydroponic system, 9 plants, shoot and root, from each species and each salinity were measured for fresh weight (FW), oven dried at 50°C dried and then weighed again (DW). For all remaining plants only DW was assessed.

On day 201, plants grown at the three salinities were individually incubated in 200ml solution, salinity 10, 1ml l^{-1} Hoagland micronutrient and Fe solutions, and nominal concentrations of 200 µmol P l^{-1} (Na_2PO_4), and 2000 µmol N l^{-1} , added either as equimolar $^{15}NH_4Cl + NaNO_3$ or equimolar $NH_4Cl + Na^{15}NO_3$. Pots containing the solution and plants were kept in a water bath during the incubation period in order to keep the water temperature constant (Teco TR20 chiller/heater). For each salinity and each N treatment, 3 plants of each species were incubated for one hour. Filtered water samples were collected immediately before the incubation period and later analysed for P and N concentration, as described above. After incubation, plants were washed in distilled water and 0.1 mol l^{-1} $CaCl_2$ solution, to remove any isotope tracer adsorbed to the root surface. Plant shoot was separated from root, oven-dried at 50°C and DW measured. The whole plant was then ground (Precellys 24-Dual, Bertin Technologies) and subsequently analysed for N and ^{15}N content. Additionally, 3 plants of each species, not incubated in isotope-enriched solution, were analysed for ^{15}N natural abundance. Isotopic

analysis of plant tissues was carried out at the University of California Davis stable isotope facility using a PDZ Europa ANCA-GSL elemental analyzer interfaced to a PDZ Europa 20-20 isotope ratio mass spectrometer (Sercon Ltd., Cheshire, UK). Excess atom per cent (at%) of the heavy isotopes was calculated as the difference between each sample heavy isotope at% (^{15}N) and the mean natural abundance ^{15}N at%. Shoot and root dry mass, N content, and heavy isotope at% excess where used to calculate µmol excess of heavy isotopes in the plant tissues. N uptake rates were calculated per unit root dry mass (g) per unit of time (h).

Irradiance, air and water temperatures were recorded throughout the experiment (Onset Hobo pendant temp/light UA-002-64). The recorded light data, in lux, was converted to µmol PAR m^{-2} s^{-1}, after calibration against Li-Cor 190SA sensor. The calibration was obtained by taking readings at the same time with both sensors side by side (at different times of the day and weather forecasts) at the greenhouse (r^2=0.92).

Irradiance and temperature experiments

On day 135 of 2012, 190 *S. europaea* and *A. tripolium* plants were transplanted to a similar hydroponic system as described previously, except all plants were grown at a salinity of 10.

On day 144, 130 plants of both species were covered with shade netting to obtain 7 levels of light irradiance (100%, 73%, 66%, 30%, 23%, 12% and 3% of ambient daylight). On the following day those plants, 5 replicates from each light level, were incubated for 1 h in ^{15}N solutions (1000 µmol l^{-1} as $^{15}NH_4Cl$-N or $Na^{15}NO_3$, 200 µmol P l^{-1} as Na_2PO_4, 1 ml l^{-1} Hoagland micronutrient and Fe solutions). Pots containing the solution and plants were kept in a water bath during the incubation period in order to keep the water temperature constant (Teco TR20 chiller/heater). At the beginning of the incubation water samples were collected, stored and analysed for dissolved nutrients as previously described.

Between days 149 and 153, the remaining plants were sequentially moved from the greenhouse into a growth cabinet where air temperature was controlled. During this period the air temperature was set at 5 different levels and plants were incubated on ^{15}N solution (20, 25, 30, 35 and 40 °C), as previously described, after about 20h adaptation at each temperature. At the beginning of the incubation water samples were collected, stored and analysed for dissolved nutrients as described above.

In both cases, after ^{15}N incubation plants were processed following the same protocol as described before, except plant N content and ^{15}N at% was analysed at Mylnefield Isotope

Signatures laboratory, in a Thermo-Fisher Delta V Advantage coupled via a ConFlo IV module to a Flash Elemental Analyser (EA).

Irradiance, air and water temperatures were recorded throughout the experiment at the greenhouse (Onset Hobo pendant temp/light UA-002-64), and light data, in lux, was converted to µmol PAR m^{-2} s^{-1} as mentioned above. Irradiance in the growth cabinet, was measured with a Skye Instruments Ltd. portable quantum sensor, model SKP 200.

Statistical analysis

Differences in total N concentrations (sum of NO_3^-, NO_2^-, NH_4^+) and PO_4^{3-} concentration in irrigation water, between days 179 and 201, were compared between salinities (1, 10 and 30) using ANOVA, after confirming homogeneity of variances (Levene's test). For plants harvested on day 201, the correlation between *S. europaea* and *A. tripolium* shoot and root dry mass was investigated using Pearson product-moment correlation across the three salinities. Following a significant correlation, the nature of the relationship was investigated, linearity was confirmed using linear regression analysis and the slopes obtained compared using t-test after Bonferroni correction. Similar analysis was performed in relation, shoot and root, fresh to dry weight. Plant biomass (shoot and root, DW and FW) was compared, between the three salinity treatments, using ANOVA if variances were homogeneous (Levene's test), and Bonferroni *post hoc* test following significant effect of salinity. When variances were not equal, the Kruskal-Wallis test was used instead and the LSD *post hoc* test performed for pairwise comparisons between salinities. Plant N content and N uptake rates were also investigated in relation to salinity effect using either ANOVA or the Kruskal-Wallis test, respectively for data with and without equal variances. Tukey *post hoc* test was used after significant ANOVA, and LSD after significant Kruskal-Wallis tests. The relationship between N uptake rate and irradiance was investigated using Pearson product-moment correlation. Following a significant correlation, the nature of the relationship was investigated; linearity was confirmed using linear regression analysis and the obtained slopes and intercepts compared using t-test after Bonferroni correction. N uptake rate at five different temperatures was evaluated using ANOVA, and when temperature was shown to have a significant effect on N uptake rate, the Tukey *post hoc* test was performed.

Values are reported as means ± standard deviation. The significance level was considered as <0.05 for all the statistical analysis performed.

Results

Environmental data

Plant growth

Overall daily mean irradiance was 340 ± 122 and 306 ± 86 µmol PAR m^{-2} s^{-1}, respectively for the 2011 (days 181 to 202) and 2012 (days 137 to 151) periods the plants were grown in the hydroponic systems.

Between days 181 and 202 the mean daily water temperature was 27.4 ± 2.1 °C, and the minimum and maximum mean daily air temperatures, were, respectively, 18.1 ± 1.6 and 45.5 ± 6.4 °C. While for the period between days 137 and 151, the mean daily water temperature was 27.3 ± 1.1 °C, and the minimum and maximum mean daily air temperatures, were, respectively, 18.1 ± 0.7 and 44.8 ± 3.8 °C.

The mean daily water temperature for the experimental period between days 181 and 202 was 24.8 ± 1.3 °C, while the daily minimum was 21.9 ± 1.3 °C, and the daily maximum air temperature 28.0 ± 1.8 °C. Between days 137 and 151, the mean daily water temperature was 25.6 ± 1.3 °C, while the daily minimum and maximum was 22.4 ± 2.3 °C and 28.5 ± 1.3 °C respectively.

For the period between days 137 and 151, the solution salinity varied 10.0 ±0.1 after each water change and 10.4 ± 0.2 immediately before the following water change. For the period between days 181 and 202, plants were grown at three different solution salinities (nominal salinities1 10 and 30) and the actual salinities are presented in Table 5.1.

Table 5.1 – Measured mean (± standard deviation) salinity after and before each water change, for the three ies studied.

Nominal salinity	Measure salinity after change	Measure salinity before change
1	1.03 ± 0.05	1.13 ± 0.05
10	10.0 ± 0.2	10.6 ± 0.2
30	29.6 ± 0.8	31.1 ± 1.1

N uptake

The mean irradiance during the incubations investigating N uptake at three salinities, was 1236 ± 492 µmol PAR m^{-2} s^{-1}, air temperature 34.5 ± 2.1 °C, and water temperature 24.4 ± 0.3 °C. The mean irradiance, air and water temperature are presented in Table 5.2, for each salinity and N form tested during incubations, both for *S. europaea* and *A. tripolium*.

Table 5.2 - Environmental data recorded during the ^{15}N incubations, irradiance (µmol PAR m^{-2} s^{-1}), air and water temperature (°C), for both species, salinity and ^{15}N label (mean ± standard deviation)**.**

	Nominal salinity	**^{15}N treatment**	**Irradiance (µmol PAR m^{-2} s^{-1})**	**Air temperature (°C)**	**Water temperature (°C)**
S. europaea	**1**	**NH_4^+**	1296 ± 521	34.3 ± 2.3	24.5 ± 0.2
		NO_3^-	1315 ± 519	34.8 ± 2.3	24.5 ± 0.2
	10	**NH_4^+**	1305 ± 527	34.5 ± 2.3	24.5 ± 0.2
		NO_3^-	1359 ± 558	34.8 ± 2.3	24.5 ± 0.2
	30	**NH_4^+**	1301 ± 529	34.6 ±2.3	24.5 ± 0.2
		NO_3^-	1354 ± 566	34.7 ± 2.3	24.5 ± 0.2
A. tripolium	**1**	**NH_4^+**	1162 ± 349	33.8 ± 2.1	24.3 ± 0.2
		NO_3^-	1202 ± 354	34.1 ± 2.3	24.4 ± 0.2
	10	**NH_4^+**	1169 ± 347	34.0 ± 2.2	24.3 ± 0.2
		NO_3^-	1288 ± 525	34.2 ± 2.3	24.4 ± 0.2
	30	**NH_4^+**	1179 ± 342	34.2 ± 2.3	24.3 ± 0.2
		NO_3^-	1303 ± 527	34.2 ± 2.4	24.5 ± 0.2

The mean air temperature during the incubations investigating the N uptake at different irradiances was 36.5 ± 4.6 °C, and the water temperature was 27.1 ± 1.2 °C. The irradiance, measured during the same period, without shading, was 1518 ± 1030 µmol PAR m^{-2} s^{-1}. Table 5.3 shows the mean ambient irradiance, air and water temperatures during incubations for each plant species and N-form investigated. Table 5.4 shows the mean irradiance for each light treatment, calculated according to the shading levels applied. Salinity was 10.0 at the beginning of the ^{15}N incubation in every case.

The mean irradiance during the incubations to investigate N uptake at different temperatures was 334 ± 23 µmol PAR m^{-2} s^{-1}. Air and water mean temperatures from each temperature treatment and N-form tested during incubations are presented in Table 5.5. Salinity was 10.0 at the beginning of the ^{15}N incubation in every case.

Table 5.3 - Environmental data recorded during the ^{15}N incubations at several light levels, irradiance (µmol PAR m^{-2} s^{-1}), air and water temperature (°C), for each species and ^{15}N label (mean ± standard deviation)**.**

	^{15}N treatment	Irradiance (µmol PAR m^{-2} s^{-1})	Air temperature (°C)	Water temperature (°C)
S. europaea	NH_4^+	1966 ± 1278	39.6 ± 5.2	28.1 ± 0.4
	NO_3^-	1634 ± 955	37.6 ± 3.5	27.9 ± 0.8
A. tripolium	NH_4^+	1243 ± 663	34.4 ± 3.0	25.4 ± 0.4
	NO_3^-	1227 ± 944	34.4 ± 4.2	27.0 ± 0.3

Table 5.4 % - Measured irradiance (µmol PAR m^{-2} s^{-1}) for each light level (%), for each species and ^{15}N label (mean ± standard deviation)**.**

% Irradiance	*S. europaea*		*A. tripolium*	
	NH_4^+	NO_3^-	NH_4^+	NO_3^-
100 %	2126 ± 1329	1871 ± 919	1139 ± 600	1073 ± 723
73 %	1501 ± 961	1271 ± 687	836 ± 448	787 ± 560
66 %	1293 ± 865	1078 ± 623	727 ± 418	764 ± 578
30%	555 ± 385	458 ± 282	362 ± 196	361 ± 278
23%	430 ± 296	347 ± 219	289 ± 158	278 ± 223
12 %	221 ± 151	174 ± 109	156 ± 81	141 ± 117
3 %	55.0 ± 38.1	42.3 ± 27.2	40.8 ± 20.9	36.6 ± 31.2

Table 5.5 – Air and water temperatures (°C) for each temperature treatment, for each species and ^{15}N label (mean ± standard deviation)**.**

		S. europaea		*A. tripolium*	
Nominal temperature (°C)	^{15}N treatment	Air temperature (°C)	Water temperature (°C)	Air temperature (°C)	Water temperature (°C)
20	NH_4^+	21.3 ± 2.53	17.8 ± 1.79	21.0 ± 2.51	17.3 ± 1.61
	NO_3^-	20.7 ± 2.50	16.9 ± 1.45	20.6 ± 2.54	16.5 ± 1.33
25	NH_4^+	25.0 ± 2.14	23.1 ± 1.09	24.9 ± 2.09	23.0 ± 1.05
	NO_3^-	24.9 ± 2.05	22.9 ± 1.03	24.9 ± 2.07	22.7 ± 1.02
30	NH_4^+	29.0 ± 1.66	28.4 ± 1.48	28.9 ± 1.69	28.2 ± 1.39
	NO_3^-	28.9 ± 1.73	28.0 ± 1.31	28.9 ± 1.78	27.9 ± 1.25
35	NH_4^+	33.7 ± 0.94	31.2 ± 0.40	33.8 ± 0.98	31.2 ± 0.41
	NO_3^-	33.8 ± 1.01	31.2 ± 0.39	33.9 ± 1.02	31.2 ± 0.37
40	NH_4^+	42.0 ± 1.00	40.3 ± 2.89	42.0 ± 0.99	40.3 ± 2.94
	NO_3^-	42.1 ± 0.98	40.4 ± 3.01	42.1 ± 0.97	40.5 ± 3.10

Water inorganic nutrients

Even though the same nutrients were added at each salinity (days 179 to 201), the mean nutrient concentrations in the irrigation water during growth were not similar between the 3 salinity treatments in all cases (Table 5.6). For *S. europaea* plants at a salinity of 1, the total DIN concentration was higher than both in salinities 10 and 30 ($p \leq 0.02$). For *A. tripolium*, irrigation water the N concentration was lower at salinity 10 than both salinity treatment 1 and 30 ($p \leq 0.008$). P concentrations, for both species, were higher at salinity 1 than at salinities 10 and 30 ($p < 0.05$). However, in every case the overall mean N concentration was higher than 2000 µmol l^{-1}. In the experiment investigating N uptake at various light and temperature levels (days 135 to 152) the plants were grown at a mean N concentration of 2047 ± 441 and 2033 ± 677 µmol l^{-1}, respectively for *S. europae*a and *A. tripolium* irrigation water, while P concentrations were 230 ± 50 and 227 ± 68 µmol l^{-1}, respectively. During the incubations in the heavy isotope N solution, concentrations were as in Table 5.7.

Table 5.6 - Overall measured mean (± standard deviation) dissolved inorganic nitrogen (DIN) concentration (µmol N l^{-1}) and dissolved inorganic phosphorus (DIP) concentration (µmol P l^{-1}) in irrigation water for each salinity during the plants growth period.

	Nominal Salinity	**DIN (µmol N l^{-1})**	**DIP (µmol P l^{-1})**
S. europaea	**1**	2459 ± 188	273 ± 15
	10	2033 ± 427	237 ± 16
	30	2108 ± 467	234 ± 16
A. tripolium	**1**	2486 ± 191	277 ± 13
	10	2123 ± 260	243 ± 9
	30	2347 ± 118	235 ± 10

Table 5.7 – Measured nutrient concentrations in irrigation water during ^{15}N incubations, N as, NH_4^+, NO_3^-, and NO_2^- (µmol N l^{-1}), and–PO_4^{3-}P (µmol P l^{-1}) for each environmental factor studied and each ^{15}N label used.

Factor	**^{15}N label**	**NH_4^+ (µmol l^{-1})**	**NO_3^- (µmol l^{-1})**	**NO_2^- (µmol l^{-1})**	**PO_4^{3-} (µmol l^{-1})**
Salinity	**NH_4^+**	1022 ± 63	1018 ± 56	2.86 ± 1.54	175 ± 17
	NO_3^-	972 ± 102	1139 ± 86	1.67 ± 1.61	
Light	**NH_4^+**	999 ± 69	17.6 ± 2.7	4.08 ± 0.74	155 ± 86
	NO_3^-	12.7 ± 0.7	1106 ± 148	3.36 ± 3.07	
Temperature	**NH_4^+**	794 ± 127	21.4 ± 15.2	5.32 ± 3.14	162 ± 28
	NO_3^-	14.6 ± 1.5	957 ± 192	2.48 ± 0.84	

Effects of salinity on plant performance and N content

Salinity

S. europaea mortality was 15% at a salinity of 1, and only 2% and 5% respectively at salinities 10 and 30. In the case of *A. tripolium* plants mortality was 36% at salinity 30, 2% at salinity 1 and no plants died at salinity 10.

The shoot:root biomass relationships in plants grown at the different salinities were always linear (r^2=0.79 and r^2=0.81, respectively for *S. europaea* and *A. tripolium* $p<0.001$). In the case of *S. europaea* there was no significant difference between the slopes of the three relationships, 3.11 ± 0.08, 3.16 ± 0.03 and 2.82 ± 0.15, respectively for salinities 1, 10 and 30. While, *A. tripolium* the ratios were different between all treatments, at 3.05 ± 0.16, 1.80 ± 0.07 and 2.05 ± 0.09, respectively for salinities 1, 10 and 30 ($p<0.001$).

The relationships between FW and DW of the above-ground and root biomass were all linear ($r^2>0.90$, $p<0.001$), and the obtained slopes are presented in Table 5.8. Slopes from the FW and DW relationships for *S. europaea* shoots were all different between salinity treatments ($p\leq0.027$), while for roots the slope for plants grown at a salinity of 1 was lower than for both salinities 10 and 30 ($p<0.001$), which were not significantly different. In case of *A. tripolium*, for both shoots and roots, the slope of relationships for plants grown at salinity 10 were higher than both salinities 1 and 30 ($p\leq0.012$), which were not significantly different.

Table 5.8 - *S. europaea* and A. *tripolium*, shoot and root fresh and dry weight (g) relationship slopes, for plants grown at salinities1, 10 and 30 (mean ± standard deviation).

FW:DW slopes		**Shoot**	**Root**
S. europaea	**Salinity 1**	8.43 ± 0.24	6.65 ± 0.46
	Salinity 10	14.3 ± 0.5	10.7 ± 0.2
	Salinity 30	12.8 ± 0.3	9.73 ± 0.33
A. tripolium	**Salinity 1**	7.16 ± 0.33	8.29 ± 0.41
	Salinity 10	10.4 ± 0.2	11.2 ± 0.3
	Salinity 30	8.43 ± 0.60	7.44 ± 0.26

Final above-ground biomass in *S. europaea* plants was lower at salinity 1 than at salinities 10 and 30 ($p<0.05$), both when the final biomass was analysed as the measured DW (Figure 5.1a) and after converting to FW (Figure 5.1b) using the previously described relationships between shoot FW and DW for each salinity. Specific growth rates of *S. europaea* shoots were 0.016 ±

0.007, 0.052 ± 0.010 and 0.061 ± 0.012 g DW d^{-1}, respectively for plants grown in salinities 1, 10 and 30.

The final shoot biomass of *A. tripolium* (DW) was not significantly different between the 3 salinities (Figure 5.1c). Specific growth rates were 0.036 ± 0.016, 0.036 ± 0.016 and 0.029 ± 0.016 g DW d^{-1}, respectively for plants grown in salinities 1, 10 and 30. However, final shoot fresh weight (Figure 5.1d) was higher in plants grown in salinity 10 than for both salinities 1 and 30 ($p \leq 0.019$).

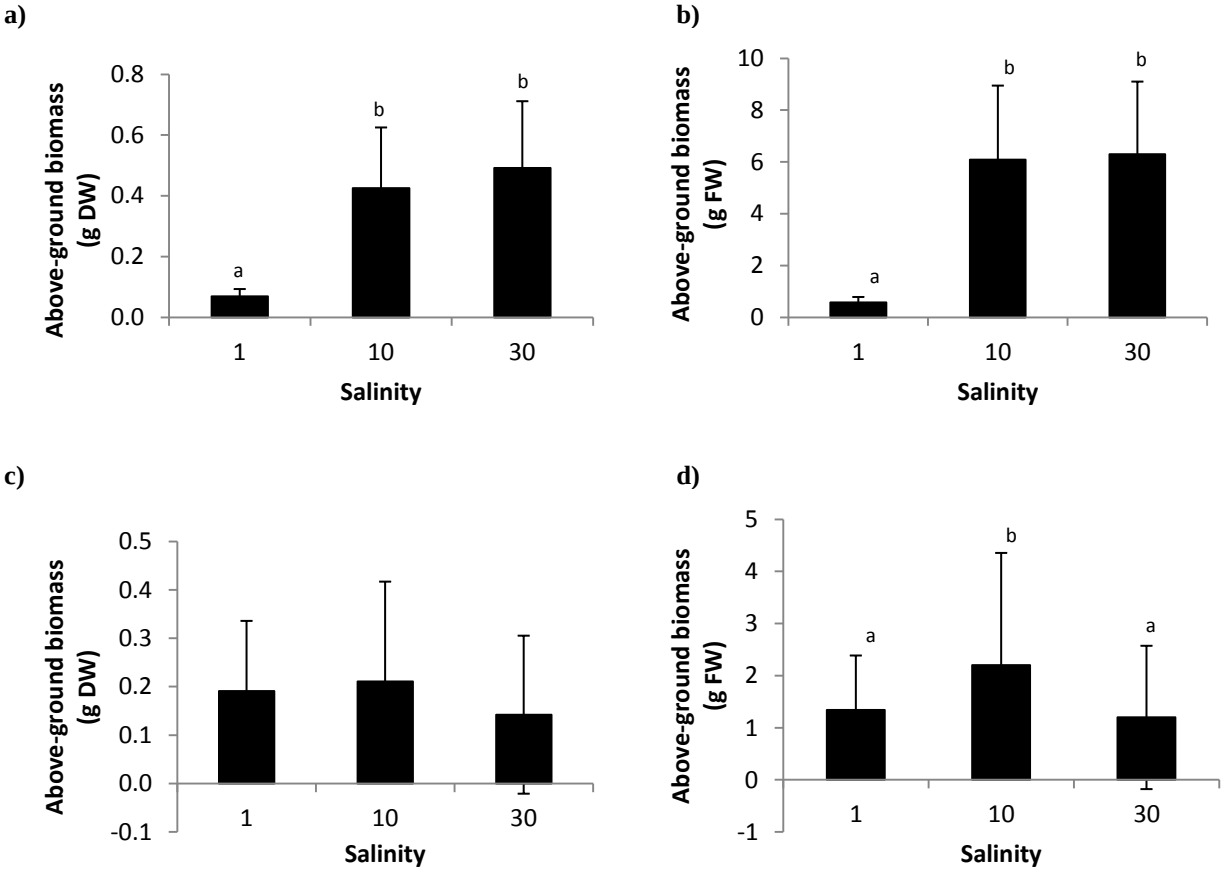


Figure 5.1 - Above-ground biomass from *S. europaea* plants **a)** dry weight (g DW) **b)** fresh weight (g FW), and from A. *tripolium* plants **c)** dry weight (g DW) **d)** fresh weight (g FW) grown at different salinities. Mean values sharing a letter do not differ significantly.

In relation to the root biomass (Figure 4.2 a and b), for *S. europaea* the trends were similar to those observed in the shoots, with both DW and FW final biomass lower in plants from salinity 1 then in salinities 10 and 30 ($p<0.05$).

In the case of *A. tripolium* root biomass DW and FW (Figure 4.2c and d), in salinity 10 was higher than both on salinities 1 and 30 ($p \leq 0.036$).

The N contents of *S. europaea* plants grown at a salinity of 30 (2.15 ± 0.16 mmol N g^{-1} DW), were lower than for salinity 1 and 10 (2.45 ± 0.34 and 2.30 ± 0.17 mmol N g^{-1} DW,

respectively). When expressed g^{-1} FW, the N contents in plants grown at salinity 1 (1.33 ± 0.26 mmol N g^{-1} FW) were higher than for salinity 10 (0.851 ± 0.101 mmol N g^{-1} FW) and 30 (0.847 ± 0.127 mmol N g^{-1} FW) ($p<0.001$ and $p<0.05$, respectively).

In *A. tripolium*, the N contents were lower in plants grown at salinity 30 (2.31 ± 0.63 mmol N g^{-1} DW) than for salinity 1 (3.05 ± 0.76 mmol N g^{-1} DW, $p=0.003$) and salinity 10 (3.23 ± 0.67 mmol N g^{-1} DW , $p<0.001$), while there was no significant difference in the N contents between plants grown at salinities 1 and 30. However, when expressed as g^{-1} FW, the N contents of *A. tripolium* was not significantly different between plants grown at salinities 10 and 30 (0.309 ± 0.064 and 0.274 ± 0.075 mmol N g^{-1} FW, respectively), but they were both lower than in plants grown at a salinity of 1 (0.425 ± 0.106 mmol N g^{-1} FW, $p<0.001$).

Irradiance and temperature

The *S. europaea* shoot biomass (DW) of plants used in ^{15}N incubations was 0.11 ± 0.12 g DW and root biomass was 0.04 ± 0.09 g DW, while the whole plant N content was 2.0 ± 0.26 mmol N g^{-1} DW. In case of *A. tripolium*, the shoot biomass was 0.17 ± 0.09 g DW, and root biomass was 0.06 ± 0.07 g DW and whole plant N content was 2.8 ± 0.30 mmol N g^{-1} DW.

Nitrogen uptake

Salinity

The N uptake rate by *S. europaea* plants (µmol N $plant^{-1}$ h^{-1}) at a salinity of 1 was lower than at salinities 10 and 30, both as NH_4^+ ($p\leq0.002$) and as NO_3^- ($p\leq0.026$) (Figure 5.4b and d). When the N uptake rate is expressed as µmol N g^{-1} root DW h^{-1} it was lower at salinity 1 than at salinities 10 and 30, both as ammonium ($p\leq0.005$) and as nitrate ($p\leq0.030$) (Figure 5.4c and c).

The NO_3-N uptake rate by *A. tripolium* plants (µmol N $plant^{-1}$ h^{-1}) was not significantly different between the three salinity treatments (Figure 5.5d), while NH_4-N uptake rate was lower at salinity 10 than salinity 30 ($p=0.018$), though there was no significantly difference in NH_4-N uptake rate between salinities 1 and 10, nor 1 and 30 (Figure 5.5b). When N uptake rate is expressed as µmol N g^{-1} root DW h^{-1}, there were no significant differences in N uptake rates either as NH_4^+ or NO_3^- (Figure 5.5a and c).

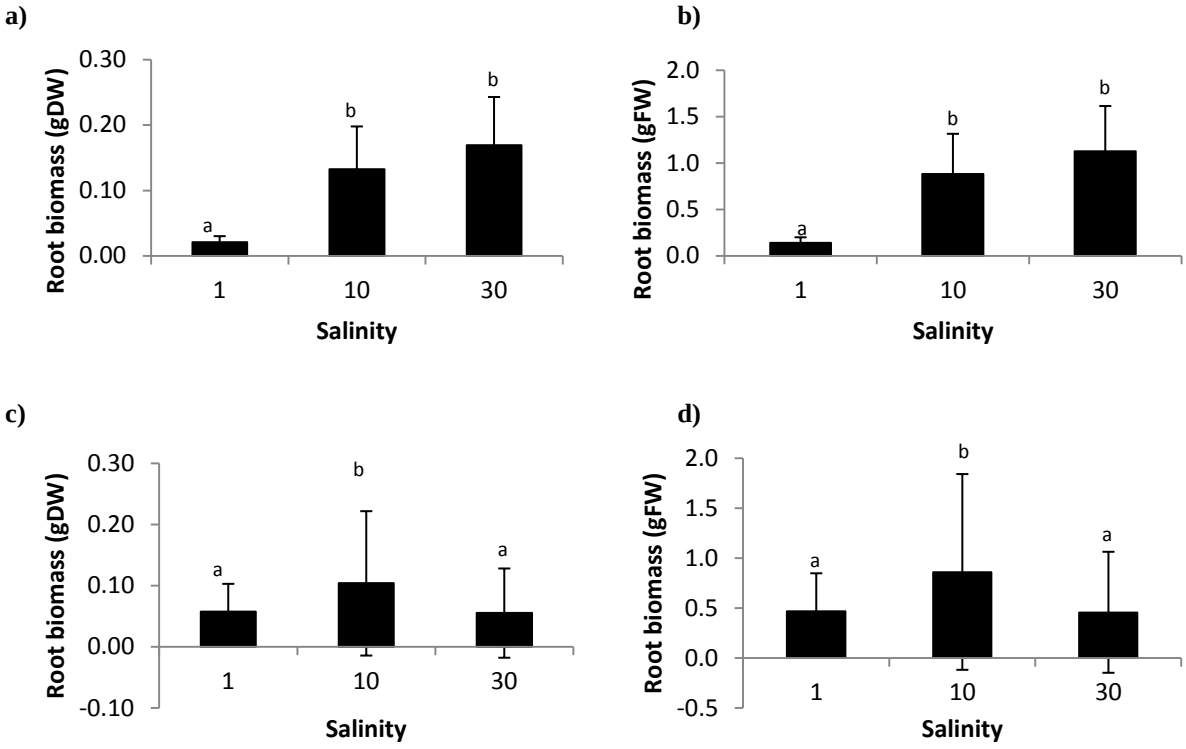


Figure 5.2 - Root biomass from *S. europaea* plants **a)** dry weight (g DW) **b)** fresh weight (g FW), and from *A. tripolium* plants **c)** dry weight (g DW) **d)** fresh weight (g FW) at different salinities. Mean values sharing a letter do not differ significantly.

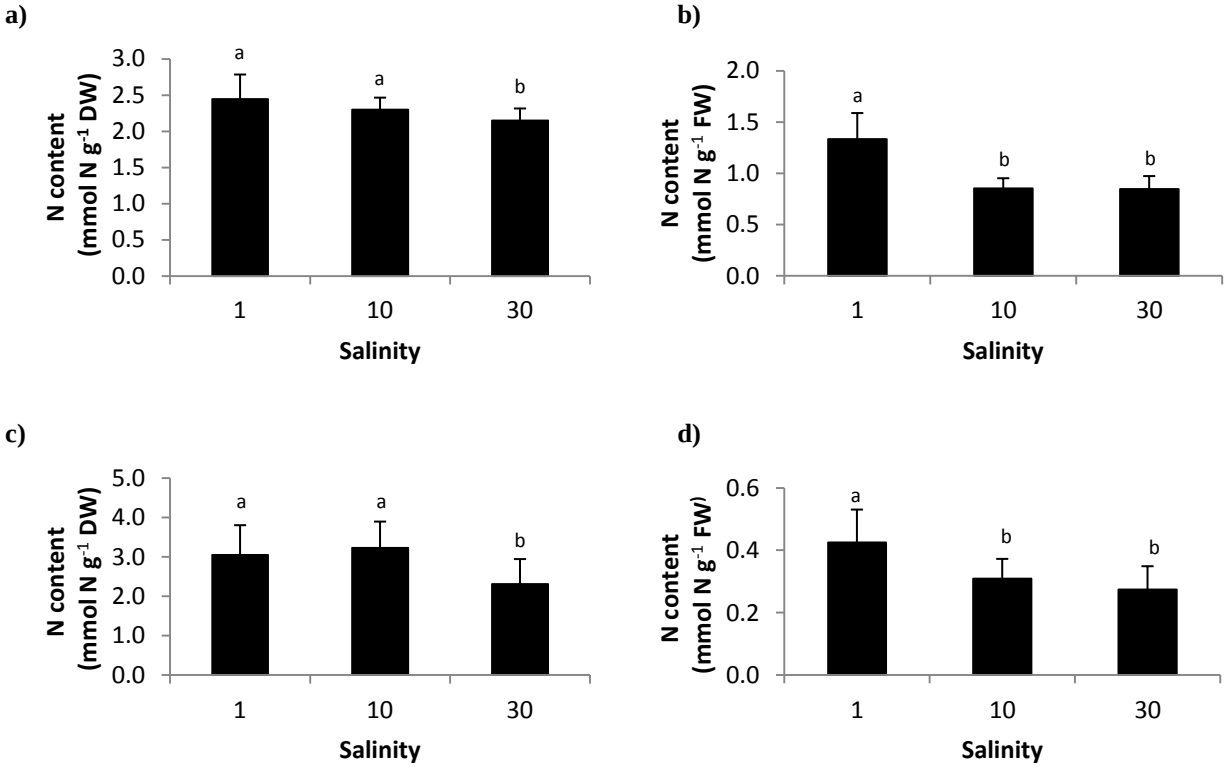


Figure 5.3 – Plant nitrogen content **a)** *S. europaea* (mmol N g^{-1} DW) **b)** *S. europaea* (mmol N g^{-1} FW), **c)** *A. tripolium* (mmol N g^{-1} DW), **d)** *A. tripolium* (mmol N g^{-1} FW), for plants grown at different salinities. Mean values sharing a letter do not differ significantly.

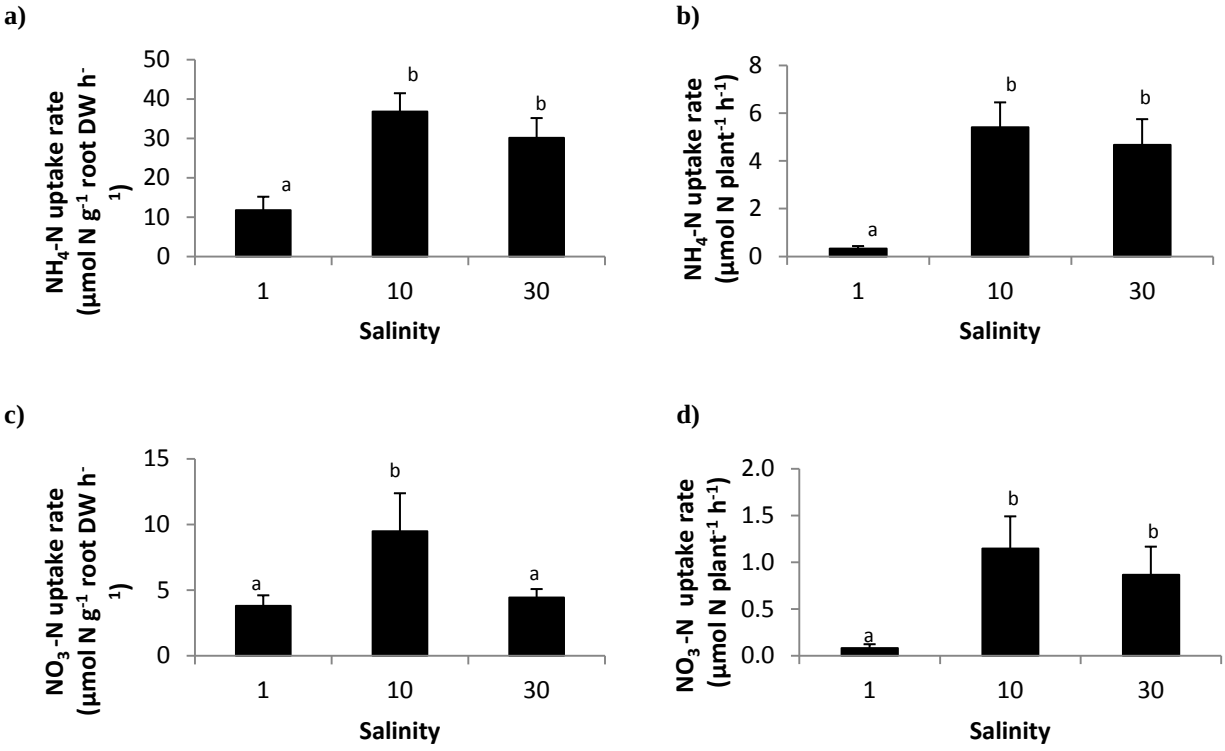


Figure 5.4 - *S. europaea* nitrogen uptake rate **a)** NH_4-N uptake rate as mmol N g^{-1} root DW, **b)** NH_4-N uptake rate as mmol N $plant^{-1}$, **c)** NO_3-N uptake rate as mmol N g^{-1} root DW, **d)** NO_3-N uptake rate as mmol N $plant^{-1}$, for plants at different salinities. Means sharing a letter do not differ significantly.

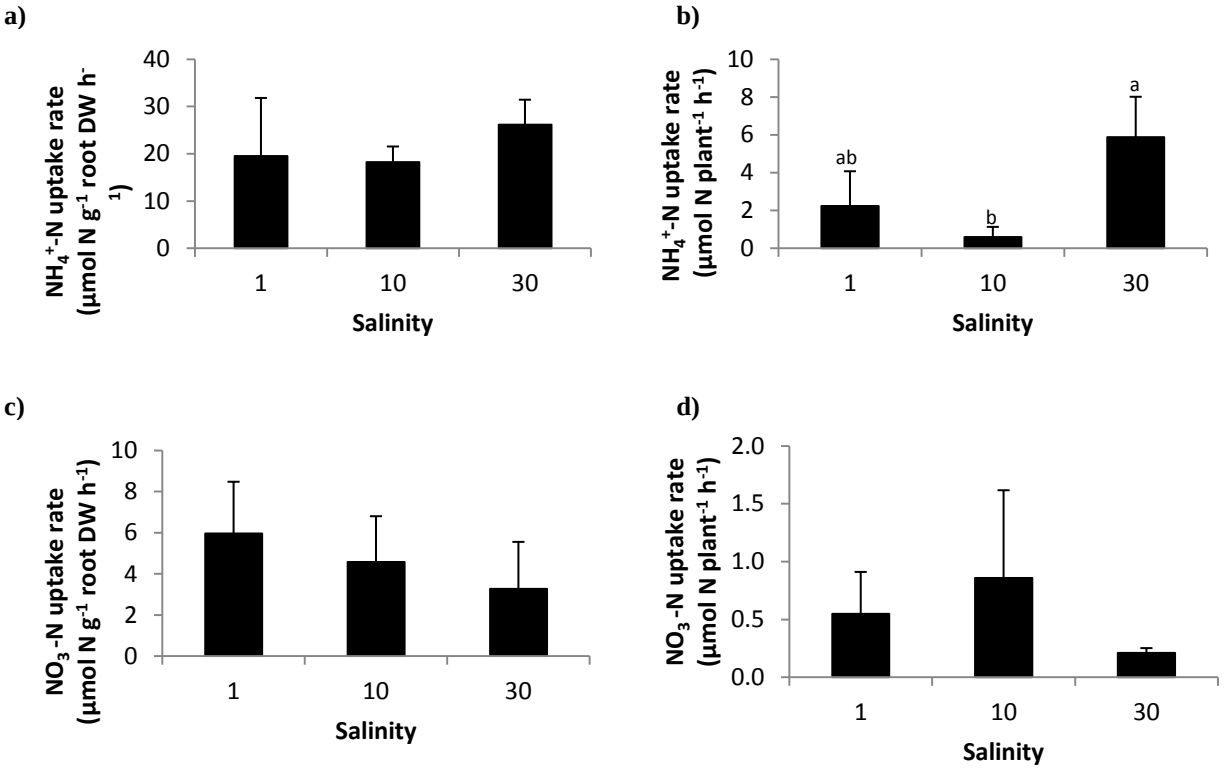


Figure 5.5 – *A. tripolium* nitrogen uptake rate **a)** NH_4-N uptake rate as mmol N g^{-1} root DW, **b)** NH_4-N uptake rate as mmol N $plant^{-1}$, **c)** NO_3-N uptake rate as mmol N g^{-1} root DW, **d)** NO_3-N uptake rate as mmol N $plant^{-1}$, for plants at different salinities. Means sharing a letter do not differ significantly.

Effects of irradiance on N uptake

The relationship between N uptake rate and irradiance was always linear (r^2>0.58 and r^2>68, respectively for *S. europaea* and *A. tripolium*, p<0.001) (Figure 5.6). The intercepts and slopes for the linear relationships are presented on Table 5.9. For both species the estimated N uptake rate in the dark was higher for NH_4-N compared to that in plants supplied with NO_3^- (p<0.001).

There were no significant differences between species, neither in respect to NH_4-N or NO_3-N uptake rate in the dark, nor in slopes of the relationships. In both species the increment in NH_4-N uptake rate was about 1 µmol N g^{-1} root DW h^{-1} for an increase in irradiance of 100 µmol PAR m^{-2} s^{-1}. For the same increase in irradiance, the increase NO_3-N uptake rate was less than 0.5 µmol N g^{-1} root DW h^{-1}.

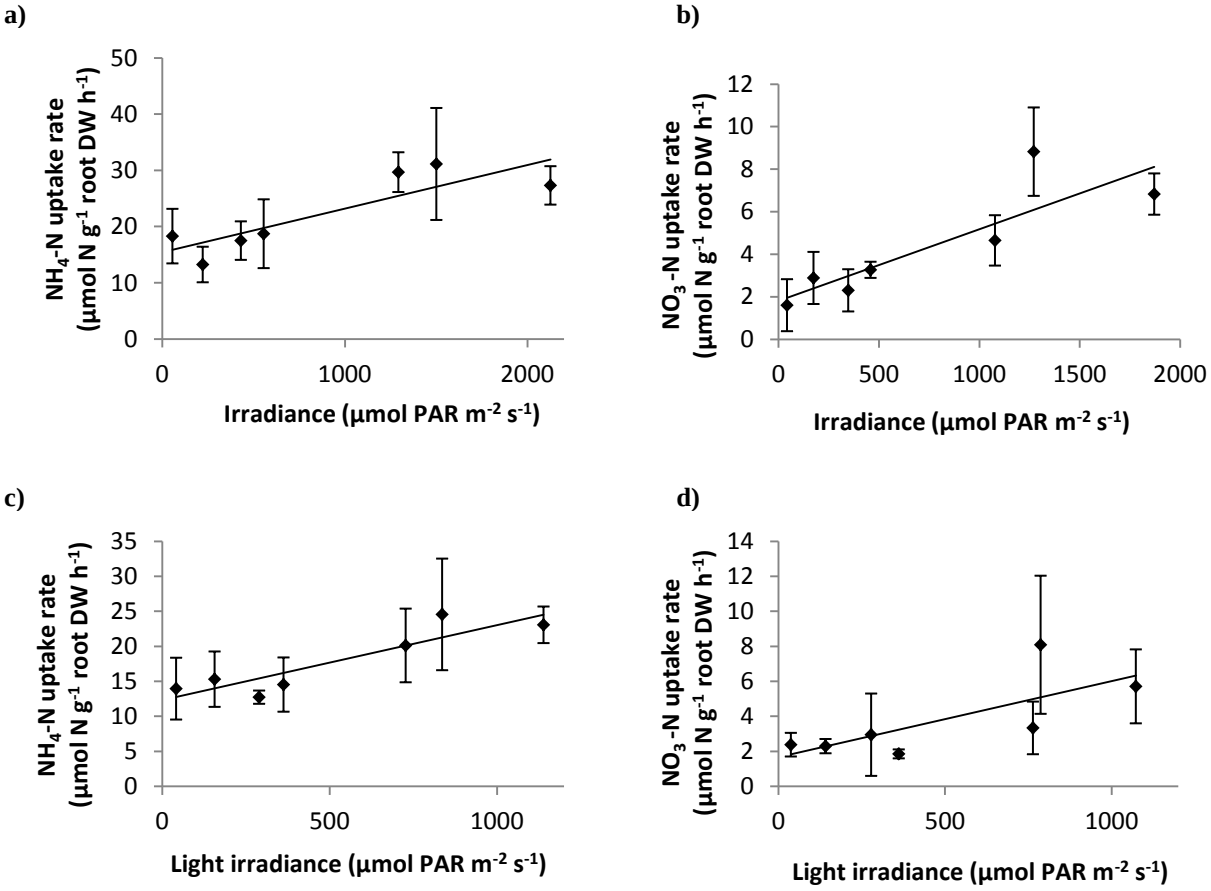


Figure 5.6 – Nitrogen uptake rate (N g^{-1} root DW) at different light levels (µmol PAR m^{-2} s^{-1}) **a)** *S. europaea* NH_4-N **b)** *S. europaea* NO_3-N, **c)** *A. tripolium* NH_4-N **d)** *A. tripolium* NO_3-N.

Table 5.9 – Slopes (µmol N g^{-1} root DWh^{-1} $µmol^{-1}$ PAR m^{-2} s^{-1}) and intercepts (µmol N g^{-1} root DWh^{-1}) (± standard deviation) for linear regressions between N uptake (µmol N g^{-1} root DWh^{-1}) and irradiance (µmol PAR m^{-2} s^{-1}), for both species and ^{15}N labels.

		Slope	Intercept
S. europaea	NH_4^+	0.008 ± 0.001	15.5 ± 1.64
	NO_3^-	0.003 ± 0.001	1.80 ± 0.45
A. tripolium	NH_4^+	0.011 ± 0.002	12.3 ± 1.35
	NO_3^-	0.004 ± 0.001	1.61 ± 0.67

Temperature

The NH_4-N uptake rates by *S. europaea* (Table 5.7a) were lower at 20 °C, compared to those measured at temperatures between 25 °C and 35 °C ($p<0.001$), but not significantly lower than the NH_4-N uptake rates at 40 °C. NH_4-N uptake was higher at 25 °C comparing to 40 °C ($p=0.002$), while there were no other significant differences between temperatures. *S. europaea* NO_3-N uptake rates (Table 5.7b) were also lower at 20 °C, compared to those at temperatures between 25 °C and 35 °C ($p\leq0.004$), but not significantly lower than at 40 °C. NO_3-N uptake rates were higher at 25 °C than at all other temperatures tested ($p<0.001$).

In *A. tripolium*, NH_4-N uptake rates were not significantly different across the temperatures tested (Figure 5.8b), but the NO_3-N uptake rates (Figure 5.8b) were lower at 20 °C compared with those measured at 25 °C and 35 °C ($p\leq0.039$), but not significantly lower than uptake rates at 30°C and 40 °C. NO_3-N uptake rates were higher at 35 °C, compared to those measured at 30 °C and 40 °C ($p\leq0.015$).

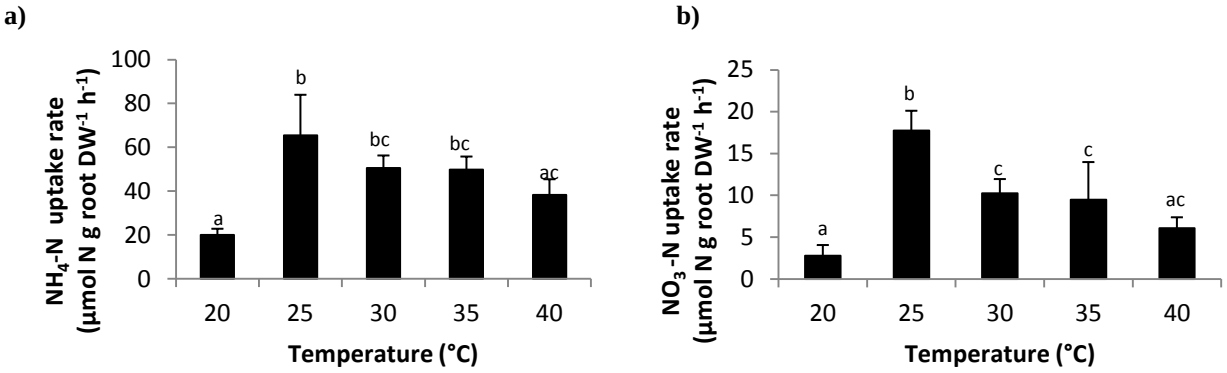


Figure 5.7 – *S. europaea* N uptake (N g^{-1} root DW) for plants at different temperatures (°C). **a)** NH_4-N, **b)** NO_3-N. Means sharing a letter do not differ significantly.

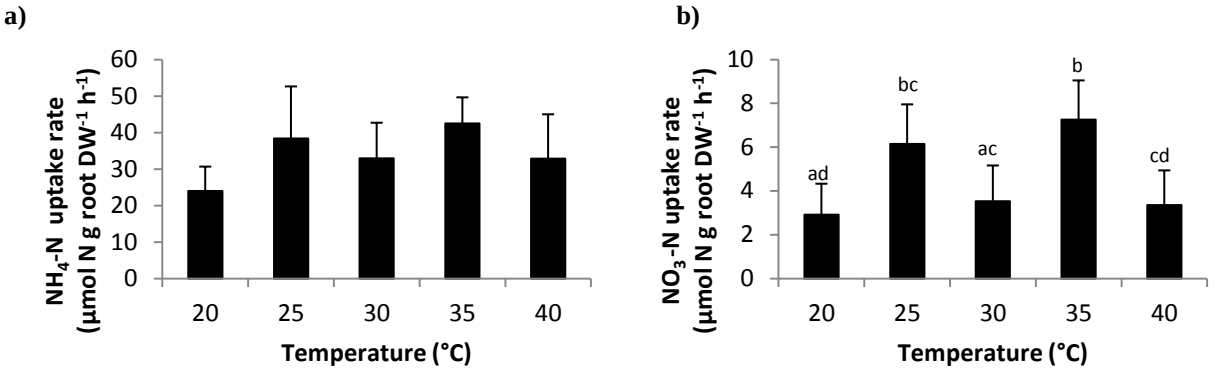


Figure 5.8 – *A. tripolium* N uptake (N g^{-1} root DW) for plants at different temperatures (°C). **a)** NH_4-N, **b)** NO_3-N. Means sharing a letter do not differ significantly.

Discussion

This study confirmed that *S. europaea* and *A. tripolium* plants have their optimal performance at different salinities. While a higher mortality of *S. europaea* (15%) was observed when plants were grown at a salinity of 1, the highest mortality of *A. tripolium* plants (36%) was observed at a salinity of 30. Besides the high mortality, growth responses to salinity were different in both species. As described in previous works (Balnokin et al. 2010, Cleall-Harding 2008, Keiffer et al. 1994, Moghaieb et al. 2004) *S. europaea* above-ground and root biomasses were lower at a salinity of 1 compared to the higher salinities investigated. On the other hand, *A. tripolium* above-ground dry weight was not different between the salinities tested, even though root biomass was higher in plants grown at salinity 10. Previous studies on *A. tripolium* have concluded that there is a preference of this species for low salinities, with biomass decreasing when salinity reaches 6 (Larher et al. 1982, Shennan et al. 1987a, Lenssen et al. 1995, Ramani et al. 2006).

It could be expected that a lower shoot:root ratio would be associated with environmental stress (Kemp and Cunningham 1981), however, allocation of dry matter between root and above-ground tissues was not different between the salinity treatments in the case of *S. europaea*. In *A. tripolium* plants, the higher shoot:root ratios of plants in a salinity of 1 may be interpreted as a further indication of the preference of this species for lower salinities. It is, however, possible that the effect of salinity is only partially translated into changes in structural dry matter, as often an increase in succulence is observed in halophytes with increasing environmental salinity, possibly indicating strategy to decrease ion toxicity by increasing water content.

In the present study, the plant water content was investigated by using the DW:FW ratio of plants grown at the different salinities. *S. europaea* water content has previously been observed to vary with salinity with an increase from about 8 ml g^{-1} DW in plants at salinity 0 to about 10 ml g^{-1} DW at salinities 5 and 10, and decreasing at higher salinities (20 and 30) to about 7 ml g^{-1} DW (Aghaleh et al. 2011). In the present study the higher water content in *S. europaea* at a salinity of 10 may indicate this species preference towards salinity 10 compared to salinity 30, and confirm the preference of both those salinities in relation to salinity 1 (with lower water content). Contradictorily, *A. tripolium* plants studied by Shennan et al. (1987a) showed a higher succulence in plants grown at lower salinities (0 and 6 comparing to 18 and 35), while in the present study water content in plants from a salinity of 10 was higher than in plants from salinities of 1 and 30. While the increase in water content from plants in the salinity treatments 1 to 10 may reflect a mechanism to cope with external salinity, the decrease in water content at higher salinity suggests salinity stress. Taking into consideration the differences in water content, when expressed as fresh weight, the effects of salinity in *S. europaea* biomass showed similar trends of that of dry weight. In *A. tripolium,* however, the above-ground biomass was higher in plants from a salinity of 10 than from salinities of 1 or 30, meaning that a higher yield of fresh crop may be obtained at a salinity of 10.

In relation to plant N content, both species had a lower N content (expressed per g dry weight) at a salinity of 30. Cleall-Harding (2008) reported a higher N content in plants grown at a salinity of 1 (about 3.3 mmol N g^{-1} DW) compared to plants treated at higher salinities (about 2.5 to 2.8 mmol N g^{-1} DW for salinities 10, 20 and 30), but no significant difference between salinities 10, 20 and 30. In that study, however, only succulent tissue was analysed which may explain the differences in relation to this study. Other studies using NO_3^- only as the N source showed decreasing N content in *S. europaea* plants with increasing salinity from 0 to 10 and to 20, from 6.0 to 4.2 and to 3.6 N % DW in case of lower irradiance and 5.9 to 3.2 and to 2.6 N % DW in case of higher irradiance treatment in one study, and in other study decreasing N content was observed both in plants shoots and roots with increasing salinities 0, 10 and 20 – with N % DW varying between 3.4 and 1.5 (Ushakova et al. 2005, Ushakova et al. 2006). Also conflicting results have been reported in case of *A. tripolium,* where total N content has been shown to decrease with increasing external salinity, Geissler et al. (2009b) reported a decrease in leaf % N DW from 5 to 4 , and in root % N DW from 3. 5 to 3, respectively for salinities 0 and 26. While Larher et al. (1982) observed a slight increase in total N plant content (from 3.63 to 3.92 N % DW) with higher external salinity (salinities of about 6 and 12). The direct comparison of these

several studies is however not possible due to the differences in the conditions that the several experiments were carried in, namely light intensity, temperature and the N form supplied.

The present study confirms the higher uptake rates of NH_4^+ comparing to NO_3^- at salinity 10, as described in chapter 4 (µmol N g^{-1} root DW h^{-1}). Furthermore, it is shown then while this this is the case at all salinities, the proportion NO_3^- taken up, relative to NH_4^+, decreased with increasing salinity. Measured NH_4-N and NO_3-N uptake rates in *S. europaea* were lower at salinity 1 than at higher salinities, even though N content was higher at salinity 30. *A. tripolium* on the other hand didn't present any difference in N uptake rates between salinities, in contrast to Sági and Erdei (2005) who found an in increase in NH_4-N and NO_3-N uptake rates with increasing salinity. Since plant biomass depends on salinity, the rate of N taken up by plant (µmol N $plant^{-1}$ h^{-1}) is also different, being lower at salinity 1 than at salinities 10 and 30, for both N forms in *S. europaea*. However, in *A. tripolium* NO_3-N uptake rate did not vary with salinity, and the NH_4-N uptake was lower at salinity 10 compared to the other salinity treatments.

In respect to N uptake (NH_4-N and NO_3-N) at different irradiances, the positive linear relationship observed between increase in uptake and irradiance, with both species presenting similar changes in NH_4-N uptake, 1 µmol N g^{-1} root DW h^{-1} for an increase in irradiance of 100 µmol PAR m^{-2} s^{-1}. For the same increase in irradiance, the increase NO3uptake rate was less than 0.5 µmol N g^{-1} root DW h^{-1}. These results confirm the dependence of N uptake and light intensity.

S. europaea N uptake was higher at the mid-range temperatures tested, being negatively affected at the lowest and (to a lesser extent) highest temperatures. Since enzymes involved in N uptake are known to be sensitive to temperatures this results are expected (Bassirirad 2000 and references therein, Dong et al. 2001, Calatayud et al. 2008). On the other hand NH_4-N uptake rate by *A. tripolium* was not affected by the tested temperatures, while NO_3-N uptake was higher at temperatures 20 °C and 30 °C, but surprisingly not at temperature 25 °C, comparing to the lowest and highest temperature. The reason for the low NO_3-N uptake at 25 °C was not expected and can't be explained in light of the information available.

Both irradiance and temperature are known to influence growth in a variety of plant species (Beinhart 1962, Chan and Mackenzie 1972, Kemp and Cunningham 1981, Pahlavanian and Silk 1988, Ball 2002, Al-Khateeb 2006, Ushio et al. 2008). Increasing irradiance has previously been shown to improve *S. europaea* biomass accumulation, Ushakova et al. (2005) reported an increase from 3.6 to 6.8 g DW $plant^{-1}$ for plants grown at salinity 10 and irradiance 100 and 150 W m^{-2} PAR respectively, while Ushakova et al. (2006) reported increase from 5.7 to

16.6 g DW plant^{-1} between plants grown at 600 and 1150 μmol PAR m^{-2} s^{-1} respectively (salinity 10). The observed differences in N uptake rates in this study indicate that irradiance and temperature within the studied ranges may also lead to changes in *S. europaea* and *A. tripolium* growth. And therefore in order to comprehend the N assimilation in plants gowning at different irradiances and temperatures, differences in N uptake rates will need to be considered together with resulting changes in biomass.

Conclusion

Measured N uptake rates together with survival and final biomass, showed *S. europaea* optimal performance at salinities 10 and 30, while *A. tripolium* shows the capacity to uptake more NH_4-N at salinity 1, and to perform equally well in NO_3-N removal at salinities 1 and 10. In relation to the suitability of these two plant species for use in an IMTA, both species could grow and remove N in the same system at a salinity of 10, however, effective plant performance at lower and higher salinities, respectively for *S. europaea* and *A. tripolium* will be decreased. Both irradiance and temperature were shown to affect N uptake in both species. However, only short term changes of both factors were analysed and to better understand plant responses longer studies, addressing growth, need to be performed. Also plant responses to the interaction of the several environmental factors need to be addressed in the future.

Chapter 6 - Organic nitrogen uptake by *Salicornia europaea* and *Aster tripolium*

Introduction

Most of the dissolved nitrogen (DN) in fed aquaculture (fish/shrimps) water is a result of leaching from uneaten food and animal faeces, as well as from animal metabolites and excretions (Handy and Poxton 1993, Burford and Williams 2001). To date only the inorganic fraction of DN has been considered to be available for plants uptake in IMTA systems, however, in the last decades it has been recognized that various plant species have the ability to take up organic N. Amino acid uptake has been measured in plants from many ecosystems, such as the Arctic tundra (Kielland 1994, Schimel and Chapin 1996, Nordin et al. 2004, Warren 2006, McFarland et al. 2010), boreal forests (Näsholm et al. 1998, Näsholm and Persson 2001, Persson and Näsholm 2001, McFarland et al. 2010) and temperate forests (Warren and Adams 2007, McFarland et al. 2010). Also a variety of agricultural plant species have been shown to have the capacity for amino acid-N uptake (Näsholm et al. 2000, 2001, Bardgett et al. 2003, Ge et al. 2009, Hill et al. 2011b), and halophytic plants have also shown to take up amino acid-N (Henry and Jefferies 2002, 2003b, a, 2010, Mozdzer et al. 2011). While lower rates of individual amino acid-N uptake, compared to both NH_4-N and NO_3-N uptake, has been reported for some plants (Näsholm et al. 2000, Ge et al. 2009), other plant species exhibit no difference in uptake between the inorganic and organic N forms (Näsholm et al. 1998, Näsholm et al. 2000, Henry and Jefferies 2002, Bardgett et al. 2003, Henry and Jefferies 2003b), or even have a higher amino acid-N uptake than both NH_4-N and NO_3-N (Raab et al. 1996, Persson et al. 2006, Stoelken et al. 2010). Amino acid-N uptake was also observed to be higher than NH_4-N and lower than NO_3-N uptake (Näsholm et al. 2000), most commonly amino acid-N uptake was observed to be higher than NO_3-N and lower than NH_4-N uptake (Falkengren-Grerup et al. 2000, Öhlund and Näsholm 2001, Thornton 2001, Henry and Jefferies 2003a, Warren 2006, Warren and Adams 2007, Mozdzer et al. 2010).

Recent studies have also demonstrated the capacity of several plants to take up N in form of small peptides (Komarova et al. 2008, Paungfoo-Lonhienne et al. 2008, Hill et al. 2011a, Hill et al. 2011b). Hill et al. (2011a) observed that *Deshampsia antartica* in solution took up NH_4-N faster than any other form of N tested, namely alanine, dialanine, trialanine, tetralanine and NO_3^-, and uptake of tetralanine-N was faster than that of alanine-N, dialanine-N and NO_3-N. Whereas, sterile wheat took up N as L-alanine, L-trialanine and NH_4^+ at higher rates than as D-alanine-N, and NO_3-N at the lowest rate of all (Hill et al. 2011b).

Even if in many case rates of organic N uptake are comparable to those of inorganic forms it is often argued that in the natural environment microbes can outcompete plants for the organic N, on short time scales at least. If that is the case, plants can only access N derived from organic supply after microbes' mineralization, instead of taking up the N as intact organic molecules (Owen and Jones 2001, Bardgett et al. 2003, Jones et al. 2005a, Harrison et al. 2007). However, intact uptake of amino acids by plants in the field and microcosms has been observed in many cases, (Näsholm et al. 1998, Näsholm et al. 2000, 2001, Bardgett et al. 2003, Henry and Jefferies 2003b, Ge et al. 2009, Sauheitl et al. 2009), and the intact uptake determined was up to 91% of plant organic derived N taken up (Näsholm et al. 1998). On the other hand, when plants are grown hydroponically the competition with microbes for N is greatly reduced, and indeed intact uptake of organic N has been demonstrated for several plants when grown hydroponically (Henry and Jefferies 2003a, Warren 2006, Warren and Adams 2007, Warren 2009, Mozdzer et al. 2010).

In the context of brackish and marine aquaculture the use of higher plants in IMTA is limited to salt tolerant plants. Both *Salicornia europaea* and *Aster tripolium* are halophyte plants of interest due their many commercial applications, including human consumption, and are currently being studied in relation to their performance in such systems (Webb et al. 2012, this thesis) *S. europaea* and *A. tripolium* naturally occur in tidal saltmarshes, and are adapted to the dynamic physical and chemical environment characteristic of such habitats. Saltmarshes are often regarded as N transformation sites (Nixon 1980), due to the very high rates of conversion between the different forms of N in solution.

In the present study it is hypothesized that both *S. europaea* and *A. tripolium* plants have the capacity to take up organic N (as amino acid and peptides) from aquaculture wastewater, and that they do so at rates comparable to that of inorganic N uptake. It is further hypothesized that when growing hydroponically the amino acid and peptide uptake is mainly taken up as intact organic molecules, while when growing in soil the % of intact uptake will be lower due to higher competition with microbes. In order to test these hypothesizes *S. europaea* and *A. tripolium* uptake of L-alanine and L-trialanine was investigated using N and carbon (C) isotopes, and compared to inorganic N uptake (NH_4-N and NO_3-N) in the same conditions. Uptake of alanine and trialanine by soil microbes was also assessed.

Methods

Measurement of dissolved nitrogen in saltmarsh porewater and aquaculture waste water

Triplicate porewater samples were taken from a sediment depth of about 2 cm using Rhizon samplers (Rhizosphere Research, Wageningen, Netherlands; pore size <0.2 µm) from two saltmarshes in North Wales (Red Wharf Bay, 53°17'46.08"N, 4°12'29.85"W; Foryd, 53°6'17.82"N, 4°19'22.05"W) in zones where *S. europaea* and *A. tripolium* occur. Filtered (<0.2 µm) water samples were also collected in triplicate from two locations in the wastewater stream of an intensive seabass marine fish farm (Anglesey Aquaculture Ltd). Both soil solution and wastewater samples were analysed for total dissolved C (TDC) and N (TDN) using a TOC-V-TN analyser (Shimadzu Corp., Kyoto, Japan); Nitrate plus nitrite (hereafter NO_3^-) was measured using a 5–channel LACHAT Instruments Quick-Chem 8000 autoanalyzer after Grasshoff et al. (1983) and Hales et al. (2004). Ammonium (NH_4^+) was measured as described by Holmes et al. (1999). Amino acids and small (<1 kDa) peptide N was analysed following the method described by Jones et al. (2002); in summary, water samples were filtered through a 1 kDa ultrafiltration membrane (Millipore, Billerica, MA, USA) and analysed fluorometrically, before and after hydrolysis in 6 M HCl at 105°C for 16 h under N_2.

Plant culture

Salicornia europaea and *A. tripolium* seeds were germinated in a climate-controlled greenhouse, in P576 plug trays with John Innes No.1 compost with continuous immersion in fresh water. When the germination rate was satisfactory, the water supply was changed to one with a salinity of 10 using TROPICMARIN^{TM} artificial sea salt and Phostrogen fertiliser (N:P:K 14:10:27 + trace elements (Bayer CropScience Ltd, Cambridge, UK). After one week, trays were thinned out to 1 plant per plug. The nutrient solution was replaced once a week. 21 days after sowing, seedlings were individually removed from the compost and roots were cleaned of any substrate. Plants were then moved to either a hydroponic floating system, or individually planted in a 7 ml container with approximately 7 g of soil (FW) and left to grow in the greenhouse for further 14 days (16 h photoperiod, air temperature 28 ±6.7°C).

Salt marsh soil was collected from the upper 10 cm at Red Wharf Bay, at the same location as the porewater sampling and immediately transported to the laboratory where subsamples were analysed for water content by comparing fresh weight (FW) and dry weight (DW) before and after drying at 80°C for 48 h.

Plants in the hydroponic system were continuously supplied with 1 mM NH_4NO_3, 0.2 mM Na_2PO_4, Hoagland's micronutrient and Fe solutions (1 ml l^{-1}). The plants growing in saltmarsh soil were watered daily, or as required to maintain the soil moist, and nutrient solution was added every 3 days. In both cases no N was supplied during the 48 h prior to the period when N uptake was determined.

All plants were moved to a growth chamber (25°C, 16h photoperiod, irradiance of ~400 µmol m^{-2} s^{-1} PAR, 70%RH) 5 days before the uptake experiments were carried out.

Plant nitrogen uptake

Plants grown hydroponically were individually placed in 15 ml centrifuge tubes containing 14 ml of labelled solution (200 µmol l^{-1} alanine ($C_3H_7NO_2$), trialanine ($C_9H_{17}N_3O_4$), ammonium chloride (NH_4Cl) and sodium nitrate ($NaNO_3$), in water of a salinity of 10 (Tropic Marine Sea Salt), with the following treatments:

$$\mathbf{^{13}C_3H_7{}^{15}NO_2} + C_9H_{17}N_3O_4 + NaNO_3 + NH_4Cl$$

$$C_3H_7NO_2 + \mathbf{^{13}C_9H_{17}{}^{15}N_3O_4} + NaNO_3 + NH_4Cl$$

$$C_3H_7NO_2 + C_9H_{17}N_3O_4 + \mathbf{Na^{15}NO_3} + NH_4Cl$$

$$C_3H_7NO_2 + C_9H_{17}N_3O_4 + NaNO_3 + \mathbf{^{15}NH_4Cl}$$

The isotope-labelled, peptide and amino acid were enriched at 98% and $^{13}C^{15}N$ dual-labelled (CK Gas Products, Hook, UK), NH_4Cl was 99% ^{15}N and $NaNO_3$ 98+%^{15}N (Cambridge Isotope Laboratories Inc., UK).

For plants grown in soil, 2 or 2.5 ml (respectively for *S. europaea* and *A. tripolium*) of individual N solution mixes were injected in the soil, according treatments listed above.

For each of the treatments, 3 plants of each species were incubated for 10, 30, 60, 120 or 240 min. After incubation plants were washed in distilled water and 0.1 M $CaCl_2$ solution, to remove any attached sediment and any isotope tracer adsorbed to the root surface. Plants shoots and root were separated, dried in the oven (80°C), weighed and ground (Precellys 24-Dual, Bertin Technologies). Subsequently, samples from the organic and inorganic labelled treatments

were analysed for $^{13}C^{15}N$ or ^{15}N, respectively. Plants incubated for 60 min in the two inorganic N treatments were also analysed for ^{13}C, in order to investigate possible re-fixation of ^{13}C, taken up and respired by microbes or plants incubated in the same growth cabinet. Additionally, 3 plants of each species, and not incubated in any isotope-enriched solution, were analysed for ^{13}C and ^{15}N natural abundance.

Isotopic analysis of plant tissues was carried out in University of California stable isotope facility using a PDZ Europa ANCA-GSL elemental analyzer interfaced to a PDZ Europa 20-20 isotope ratio mass spectrometer (Sercon Ltd., Cheshire, UK).

Excess atom per cent (at%) of the heavy isotopes was calculated as the difference between each sample heavy isotope at% (^{13}C or ^{15}N) and the mean natural abundance at% of the respective isotope. Shoot and root dry mass, N and C content, and heavy isotope at% where used to calculate µmol excess of heavy isotopes. Uptake rates were calculated per unit root dry mass (g) per unit time (h).

The use of dual-labelled organic molecules (^{13}C ^{15}N alanine and trialanine), instead of ^{15}N label only, was preferred in order to investigate differentially the uptake of ^{15}N as intact uptake of organic N, and the uptake of ^{15}N taken after external mineralization of the labelled organic N. According to Näsholm et al. (1998, 2009a) the ^{13}C in plant tissues following incubation with labelled organic molecules is primarily a result of the organic molecules uptake by the roots, although it is also possible to result in some uptake or re-fixation of mineralized ^{13}C (Rasmussen and Kuzyakov 2009). The existence of a linear relationship between excess ^{13}C and ^{15}N in plant tissue further indicates the two isotopes being taken up together in intact molecules. Additionaly, by comparing the regression slope with the expected ratio from the ^{13}C and ^{15}N in both alanine and trialanine labelled molecules, an estimate of the percentage of intact uptake can be obtained. Nevertheless, this method probably underestimates intact organic N uptake since ^{13}C taken up and respired during the incubation period is not taken in account (Näsholm et al. 1998, 2009a).

In a parallel experiment (ambient temperature), plants were incubated, for one hour, in 200 µmol l^{-1} equimolar solutions of mixed N forms:

$$\mathbf{^{14}C_3H_7NO_2} + C_9H_{17}N_3O_4 + NaNO_3 + NH_4Cl$$

$$\mathbf{^{14}C_9H_{17}N_3O_4} + C_3H_7NO_2 + NaNO_3 + NH_4Cl$$

(Label ca. 1.5 kBq ^{14}C-labelled alanine or trialanine, American Radiolabeled Chemicals, St Louis, MA, USA). 2 ml of solution was added to plants grown in soil, while hydroponic plants received 6 ml of solution. Each plant, (3 per treatment) for each species, was placed in a 500 ml

clear plastic vessel sealed immediately after label addition. Air was pumped through the vessel (600 ml min^{-1}) and the respired $^{14}CO_2$ was trapped in Oxosol scintillation fluid (National Diagnostics Ltd, Hessle, UK). The trap was changed after 5, 10, 20, 40 and 60 min, and ^{14}C activity measured by liquid scintillation counting (Wallac 1404 scintilation counter, Perkin-Elmer, Boston, MA, USA). After 60 min incubation, the plants were washed, shoots and roots were separated, dried, weighed, and then combusted in an OX400 biological oxidizer (RJ Harvey, Hillsdale, NJ, USA). $^{14}CO_2$ liberated from combustion was trapped in Oxosol scintillant, and measured by liquid scintillation counting (Wallac 1404 Scintilation counter, Wallac EG&G, Milton Keynes, UK). The oxidiser trap capture efficiency was measured as 100 ±0.4%

Microbial uptake of alanine and trialanine

The uptake of alanine and trialanine by soil microbes was assessed by measuring the ^{14}C depletion in soil solution, in either of two treatments (equimolar 140 µmol l^{-1} solution mix):

$$\mathbf{^{14}C_3H_7NO_2} + C_9H_{17}N_3O_4 + NaNO_3 + NH_4Cl$$
$$C_3H_7NO_2 + \mathbf{^{14}C_9H_{17}N_3O_4} + NaNO_3 + NH_4Cl$$

Freshly-collected soil (Red Wharf Bay saltmarsh) was weighed in batches of 1 g, in triplicate for each treatment, to which 100 µl of ^{14}C-spiked solution was added. After each incubation (1, 10, 20, 30, 60, 120 and 240 min) soil solution was extracted using 5 ml of 0.5 M Na_2SO_4 solution, shaken for 5 min, and centrifuged for 5 min at 18000 g. The ^{14}C activity was subsequently measured by scintillation counting as described above.

Statistical analysis

For both *S. europaea* and *A. tripolium*, the differences between plant C and N contents and biomass of plants grown hydroponically or in soil were assessed using the Kruskal-Wallis Test, since in all cases variance of data was not homogenous (Levene's test). Correlation between the root and shoot dry mass was investigated using Pearson product-moment correlation. Following a significant correlation, linearity was investigated using linear regression analysis (intercept 0) and the obtained slopes compared using t-test.

In the cases where values for "excess" of heavy isotope in plants tissues were negative it was assumed to be 0 i.e. no detectable excess, since these cases were the result of an uptake lower than the variability of the isotope natural abundance.

The correlation between excess ^{15}N in plant tissues and incubation time was verified using Pearson product-moment correlation. Linear regression analysis (intercept 0) was then performed, and t-test (with Bonferroni correction) used to compare the obtained slopes.

Again, to investigate the correlation between ^{13}C and ^{15}N excess in plants the Pearson product-moment correlation was used. In cases where no significant correlation was found no other statistical analyses were performed. When a significant correlation was observed the data was analysed for linearity of the investigated relationship (linear regression analysis). For cases where linearity was verified, the slopes where compared using a t-test (with Bonferroni correction). Excess ^{13}C in plants incubated with labelled inorganic N only was statistically compared to 0 using a one sample t-test.

Mean N uptake rates obtained from accumulation of excess ^{15}N from NH_4^+, NO_3^-, alanine and trialanine were compared using ANOVA analysis, if Levene's test confirmed homogeneity of variances, or the Kruskal-Wallis Test where variance was not homogenous, followed by LSD post hoc tests for significant differences between treatments. The significance level was considered as <0.05 for all the statistical analysis performed.

Results

Dissolved nitrogen in saltmarsh porewater and aquaculture

Measured DN concentrations (µmol N l^{-1}) in saltmarsh pore water and aquaculture wastewater are presented in Table 6.1. Peptide-N was 3.5 ± 0.8 times higher than amino acid-N in the saltmarsh pore water, and 2.9 ± 0.9 and 6.7 ± 4.1 times higher in wastewater, respectively for sites 1 and 2. Total N incorporated in small (<1 kDa) organic molecules represented 20.2 ± 5.4 % (mean ±standard deviation) of the TDN in the saltmarsh pore water, and only 6.0 ± 1.4% and 1.5 ± 0.5% in aquaculture wastewater, respectively for sampling sites 1 and 2. However, in wastewater site 1 the total dissolved organic N (DON) pool was close to half of the TDN, 48.4 ± 15.2 % DON (DON concentration calculated as the difference between TDN and NH_4^+ plus NO_3^-). On the other hand in site 2 of aquaculture wastewater most of the measured N was in the

inorganic form (DON calculated as -0.4 ± 4.1% of TDN). In the saltmarsh pore water 78.6 ± 15.2% of TDN was in the organic form.

Table 6.1 - Dissolved nitrogen concentration (µmol N l^{-1}) measured in pore water from 2 saltmarshes (Red Wharf Bay and Foryd) and waste water from two sites at an intensive marine aquaculture facility (Ww 1 and 2); mean ± standard deviation.

	amino acids-N	**Peptides-N**	**NH_4^+**	**NO_3^-**	**TDN**
RWB	12.5 ± 4.1	34.0 ± 0.9	25.6 ± 2.2	9.2 ± 4.4	253.7 ± 26.8
Foryd	6.0	21.6	41.4	6.5	109.2
Ww 1	19.9 ± 1.9	56.6 ± 11.4	641.0 ± 32.0	10.2 ± 3.0	1276.6 ± 137.9
Ww 2	4.6 ± 1.1	27.5 ± 11.5	67.5 ± 6.2	2051.7 ± 142.6	2109.9 ± 63.9

Plant growth and CN content

Plant biomass and C and N content at the end of the growth period are shown in Table 2. *A. tripolium* C and N content (mmol g^{-1} DW) were 2.1 and 1.1 times higher in plants grown hydroponically than in plants grown in soil ($p<0.001$). Similarly, *S. europaea* plants had a 1.2 times higher N content when grown in the hydroponic system ($p<0.001$) than when grown in soil, but there was no significantly differences in the C content of the plants in the two systems. Plants from both species had 3.3 times higher final biomass when grown in the hydroponic system than when planted in soil ($p<0.001$). Shoot and root final biomass were correlated in both growth systems and for both plant species, ($r>0.62$, $p<0.001$). In all cases the relationship was linear ($p<0.001$) and while relationship between shoot and root in *S. europaea* growing hydroponically or in soil was not significantly different, *A. tripolium* plants had a higher shoot to root ratio when grown in soil than when in hydroponics ($p<0.001$).

Table 6.2 – *S. europaea* and *A. tripolium*, carbon (mmol C g^{-1} DW) and nitrogen (mmol N g^{-1} DW) plant content, final biomass (g DW) and shoot : root (g DW), for plants grown hydroponically and in saltmarsh soil; average ± standard deviation.

		N content	**C content**	**Biomass**	**Shoot:root**
S. europaea	**Hydroponic**	1.91 ± 0.239	23.75 ± 1.081	0.141 ± 0.551	1.83 ± 0.058
	In soil	0.92 ± 0.088	23.20 ± 1.714	0.043 ± 0.113	1.99 ± 0.062
A. tripolium	**Hydroponic**	2.23 ± 0.399	29.90 ± 1.617	0.210 ± 0.089	1.68 ± 0.062
	In soil	1.86 ± 0.331	27.83 ± 1.731	0.063 ± 0.028	3.06 ± 0.138

Hydroponic plants

Nitrogen uptake by hydroponic plants

Excess ^{15}N in hydroponic plant tissue (µmol ^{15}N g^{-1} root DW), when supplied as alanine-$^{13}C^{15}N$ and as trialanine-$^{13}C^{15}N$, is shown in Figure 6.1. Excess labelled N in the plants was positively correlated with incubation time in all cases ($r>0.91$, $p<0.001$). The observed relationships were linear ($p<0.001$, $r^2>0.92$), and the obtained N uptake rates are presented in Table 6.3. The rate of trialanine-N uptake was 1.2 and 1.5 times faster (*S. europaea* and *A. tripolium*, respectively) than alanine-N uptake ($p<0.014$). Uptake rates for organic N were 1.5 and 1.9 higher (trialanine-N and alanine-N, respectively) in *S. europaea* than in *A. tripolium* ($p<0.001$).

As a result of the high uptake rates for inorganic N, significant depletion of the supplied N was observed. Hence, only samples where solution depletion of labelled molecules is calculated to be less than 25% (50 µmol l^{-1}) are presented and analysed. As well, NO_3-N uptake data was not used in any case when NH_4^+ depletion was higher than 25%, since it has been observed elsewhere (see Chapter 4) that NO_3-N uptake in both plant species is adversely influenced by the presence of NH_4^+ in solution.

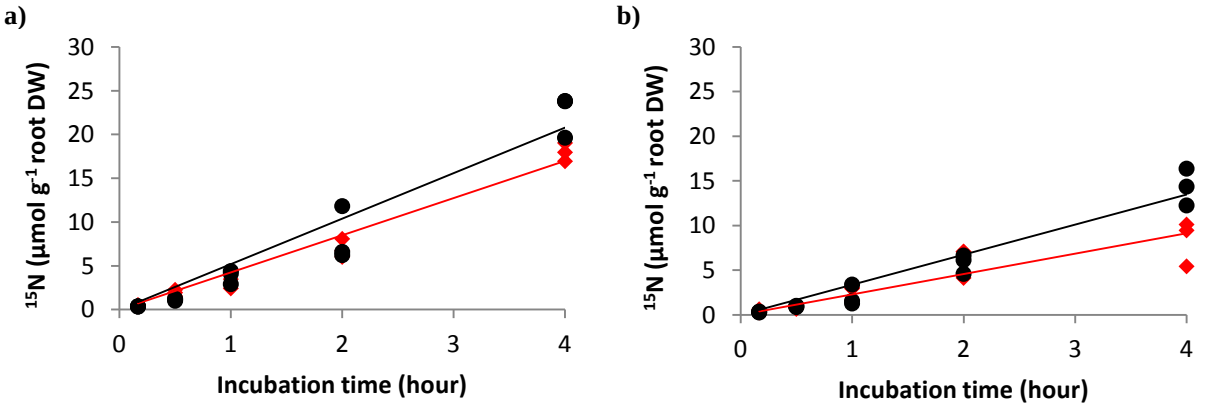


Figure 6.1 – Excess ^{15}N in plants with incubation time in **a)** *S. europaea* **b)** *A. tripolium*. Symbols and linear regression lines in red = alanine-$^{13}C^{15}N$, in black = trialanine-$^{13}C^{15}N$.

Mean uptake rates NH_4-N and NO_3-N are also presented in Table 6.3. NH_4-N uptake rates in *S. europaea* were more than 3.9 times higher than mean uptake rates of any other form of N studied ($p<0.05$), with no differences among uptake rates for NO_3-N and either alanine-N or trialanine-N ($p<0.05$). In the case of *A. tripolium*, uptake of NO_3-N was not significantly different from mean N uptake rates as NH_4^+, alanine or trialanine. Mean uptake rate of NH_4-N was more than 10 times higher than alanine-N and trialanine-N uptake rates ($p<0.05$).

Table 6.3 - Nitrogen uptake rates (µmol N g^{-1} root DW h^{-1}) as NH_4^+, NO_3^-, alanine-N and trialanine-N in *S. europaea* and *A. tripolium*; mean ± standard deviation.

	NH_4-N	**NO_3-N**	**Alanine-N**	**Trialanine-N**
S. europaea	20.4 ± 6.1	4.2 ± 2.3	4.2 ± 0.14	5.2 ± 0.2
A. tripolium	39.1 ± 23.3	6.0 ± 3.3	2.2 ± 0.16	3.3 ± 0.1

Alanine-C and trialanine-C uptake, respiration and re-fixation

Excess ^{13}C in plant tissue (µmol C g^{-1}root DW), when supplied as alanine-$^{13}C^{15}N$ and as trialanine-$^{13}C^{15}N$, is shown in Figure 6.2, and was positively correlated with time for every case studied ($r>0.93$, $p<0.001$). The observed relationships between excess ^{13}C and time were linear ($p<0.001$, $r^2>0.90$).

Excess ^{13}C in the tissue of *A. tripolium* incubated for 1h without labelled C, i.e. ^{13}C in plant tissues not taken up from the solution by the plant root, was not significant, while in *S. europaea* plants it was significant ($p=0.02$), mean value 0.69 ± 0.50 µmol ^{13}C g^{-1}root DW.

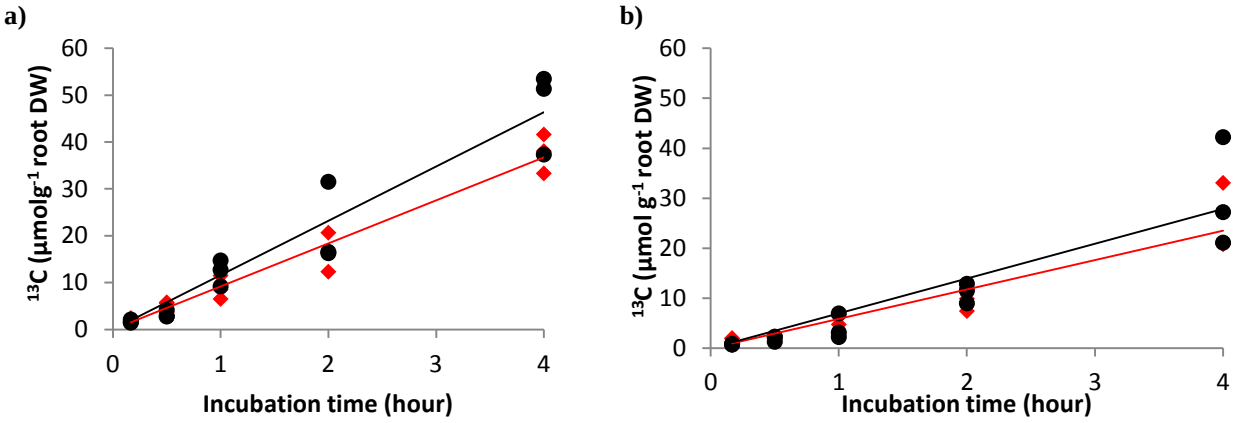


Figure 6.2 - Excess ^{13}C in plants with incubation time in **a)** *S. europaea* **b)** *A. tripolium*. Symbols and linear regression lines in red = alanine-$^{13}C^{15}N$, in black = trialanine-$^{13}C^{15}N$.

The results for C respired are presented in Figure 6.3, showing the cumulative amount of ^{14}C respired both by plants and any bacteria present in solution. However, microbial respiration in the solution is considered to be minimal and for the following analysis it will be assumed that ^{14}C is a result of plant respiration only. For the incubation times investigated, the measured $^{14}CO_2$ increase with time is best described by a second order polynomial. After one hour incubation, the respired ^{14}C measured corresponds to 1.54 ± 0.31 and 34.58 ± 6.53 % of ^{14}C in *S.*

europaea tissues, for alanine-^{14}C and trialanine-^{14}C respectively. In *A. tripolium* the percentages of respired ^{14}C are 6.27 ± 1.26 and 58.34 ± 19.56 %, for alanine-^{14}C and trialanine-^{14}C respectively.

Uptake of intact alanine-N and trialanine-N by hydroponic plants

The relationships between ^{13}C and ^{15}N in plants tissue are illustrated in Figure 6.4, with plants grown hydroponically showing a clear linear relationship between excess ^{13}C and ^{15}N ($r>0.89$, $p<0.001$). The slopes of the linear regressions are presented in Table 6.4. For both species, there were no significant differences in the ^{13}C:^{15}N ratio between alanine and trialanine treatments, within and between the two species.

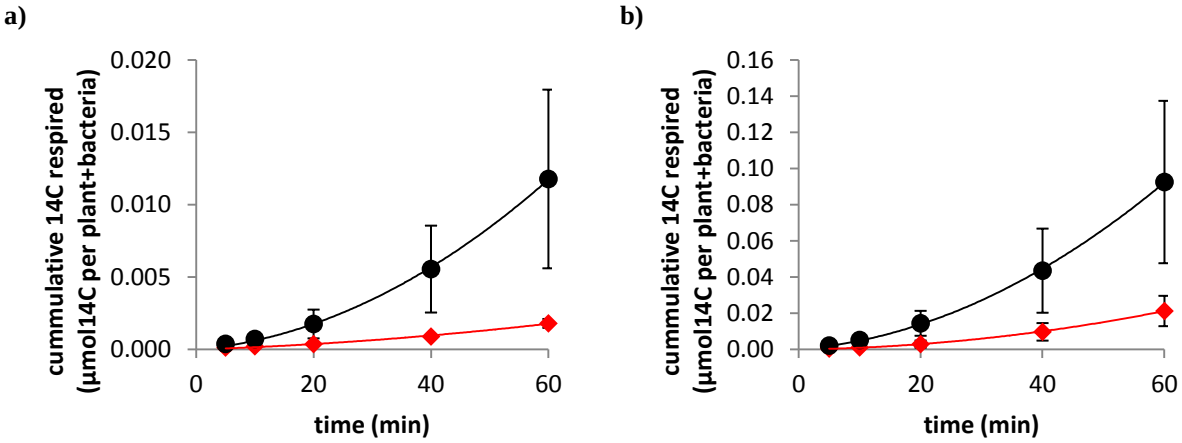


Figure 6.3 - Cumulative ^{14}C respired with time in **a)** *S. europaea* **b)** *A. tripolium*. Symbols and regression lines in red = alanine-^{13}C^{15}N, in black = trialanine-^{13}C^{15}N.

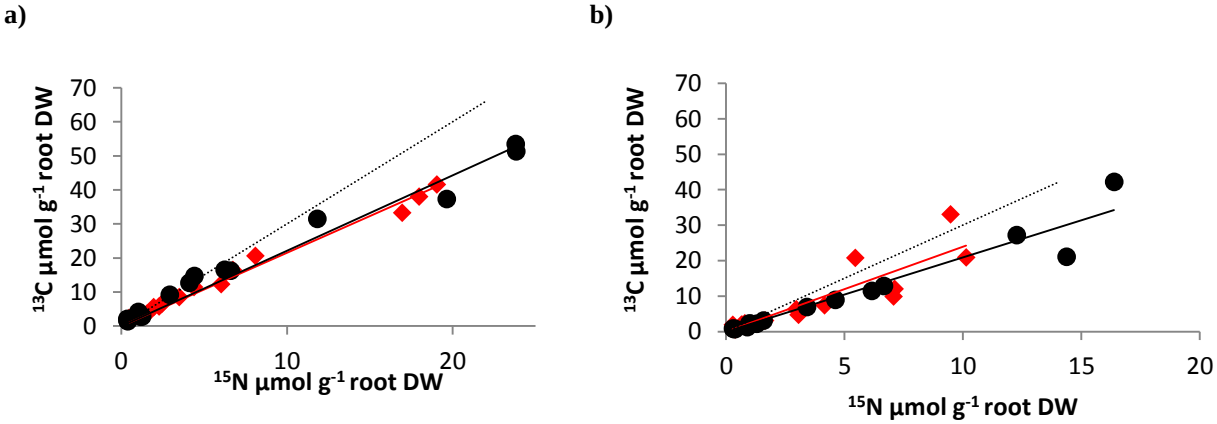


Figure 6.4 - Relationship between excess ^{13}C and ^{15}N in. **a)** *S. europaea* and **b)** *A. tripolium.* Symbols and linear regression lines in red = alanine-^{13}C^{15}N, in black = trialanine-^{13}C^{15}N. Dashed linear regression line represents the 3:1 C:N ratio.

Table 6.4 – Ratio of excess ^{13}C:^{15}N in *S. europaea* and *A. tripolium* plants, for alanine-$^{13}C^{15}N$ and trialanine-$^{13}C^{15}N$ treatments (average ±standard deviation).

		^{13}C:^{15}N
S. europaea	**Alanine**	2.16 ± 0.05
	Trialanine	2.21 ± 0.07
A. tripolium	**Alanine**	2.39 ± 0.23
	Trialanine	2.09 ± 0.12

In order to obtain a more realistic estimate intact uptake of organic N, C respiration and re-fixation were also considered. Respiration was calculated using the previously-determined relationship of $^{14}CO_2$ respired with time, together with the percentage ^{14}C respired in relation to ^{14}C in plant tissue. Since respiration measurements were only obtained for incubation periods up to one hour, only data from incubation periods up to one hour is considered in this analysis. The relationships between ^{13}C and ^{15}N are not altered and so, tissue of plants grown hydroponically still show a strong linear relationships between excess ^{13}C and ^{15}N ($p<0.001$, $r^2>0.95$), regression slopes are presented in Table 6.5. For both plants species, ^{13}C:^{15}N is higher for trialanine than alanine treatments ($p<0.001$), also organic trialanine-N uptake is higher in *S. europaea* than *A. tripolium* ($p=0.004$). There was no significant difference in the alanine treatment between the two plant species.

Table 6.5 - Ratio of excess ^{13}C:^{15}N, corrected for ^{13}C re-fixation and respiration, in *S. europaea* and *A. tripolium*, for alanine-$^{13}C^{15}N$ and trialanine-$^{13}C^{15}N$ treatments, (means ± standard deviation).

		^{13}C:^{15}N
S. europaea	**Alanine**	2.45 ± 0.08
	Trialanine	3.83 ± 0.15
A. tripolium	**Alanine**	2.03 ± 0.17
	Trialanine	3.20 ± 0.12

Plants grown in soil

Nitrogen uptake by plants in soil

In case of plants in soil, the excess ^{15}N in plant tissue (µmol ^{15}N g^{-1} root DW), supplied as alanine-$^{13}C^{15}N$ and as trialanine-$^{13}C^{15}N$ (Figure 6.5), was positively correlated with incubation

time (r^2>0.70, p<0.04). The observed relationships were linear (p<0.001, r^2>0.71), and the obtained N uptake rates are presented in Table 6.6. Trialanine N uptake was higher than for alanine in both species (p<0.005). Uptake rates of organic N, both in the case of alanine-N and trialanine-N, were not significantly different between *S. europaea* and *A. tripolium*.

Salicornia europaea exhibited a lower mean alanine-N uptake rate (p<0.05) than for both NH_4-N and NO_3-N (Table 6.6), with NH_4-N uptake double the alanine-N uptake. There was no significant difference between the two inorganic N treatments and trialanine-N uptake or between NH_4-N and NO_3-N. In case of *A. tripolium*, mean NH_4-N and NO_3-N uptake rates (Table 6.6) were not significantly different than either alanine-N or trialanine-N average uptake rates, or between them.

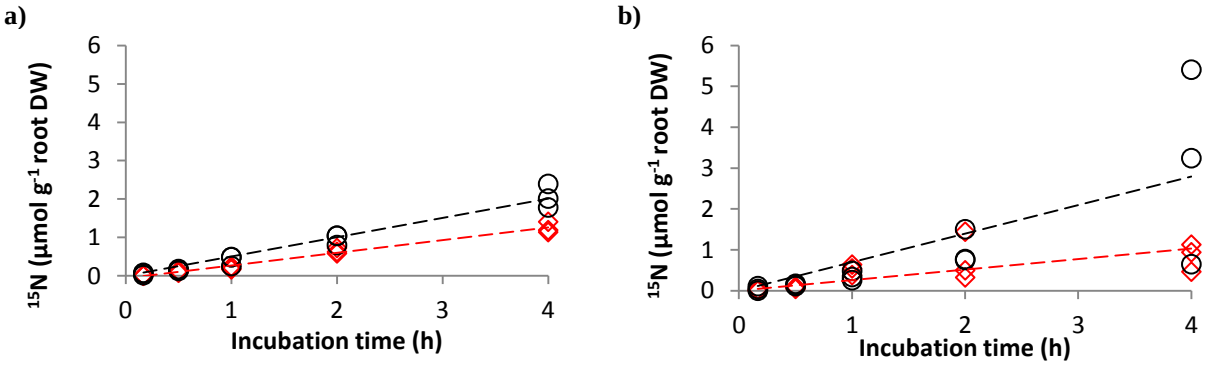


Figure 6.5 - Excess ^{15}N in plants with incubation time in **a)** *S. europaea* and **b)** *A. tripolium* in soil. Symbols and linear regression lines in red = alanine-$^{13}C^{15}N$, in black = trialanine-$^{13}C^{15}N$.

Table 6.6 - Nitrogen uptake rates (µmol N g^{-1} root DW h^{-1}) as NH_4^+, NO_3^-, alanine-N and trialanine-N in *S. europaea* and *A. tripolium* grown in soil; mean ± standard deviation.

	NH_4-N	**NO_3-N**	**Alanine-N**	**Trialanine-N**
S. europaea	0.65 ± 0.44	0.44 ± 0.11	0.31 ± 0.01	0.50 ± 0.02
A. tripolium	0.90 ± 1.07	0.46 ± 0.53	0.26 ± 0.04	0.70 ± 0.12

Alanine-C and trialanine-C uptake, respiration and re-fixation in plants grown in soil

Excess ^{13}C in plant tissue (µmol C g^{-1} root DW), when supplied as alanine-$^{13}C^{15}N$ and as trialanine-$^{13}C^{15}N$, is shown in Figure 6.2. Excess ^{13}C in the plants was positively correlated with time for every case studied (r>0.66, p<0.007) except for *S. europaea* grown in soil and supplied with labelled alanine. However, the positive correlations do not translate in linear relationships.

Excess ^{13}C in tissue of *A. tripolium* incubated for one hour without labelled C was not significant (p=0.243), while in *S. europaea* the excess ^{13}C (0.29 ± 0.22 µmol^{13}C g^{-1} root DW, was significant (p=0.023).

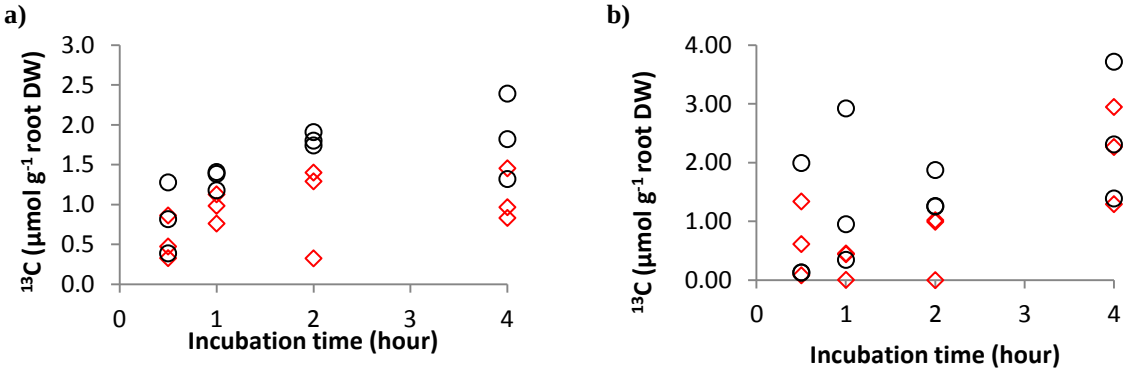


Figure 6.6 - Excess ^{13}C in plants with incubation time in **a)** *S. europaea* and **b)** *A. tripolium* in soil. Symbols in red = alanine-$^{13}C^{15}N$, in black = trialanine-$^{13}C^{15}N$.

Uptake of intact alanine and trialanine by plants in soil

The relationships between ^{13}C and ^{15}N in plant tissues are illustrated in Figure 6.7. Excess of ^{13}C and trialanine-^{15}N in plants grown in soil were positively correlated (r>0.61, p<0.017), however, the positive correlations do not translate in linear relationships. In the alanine-$^{13}C^{15}N$ treatment there was no significant correlation between excess of the two heavy isotopes.

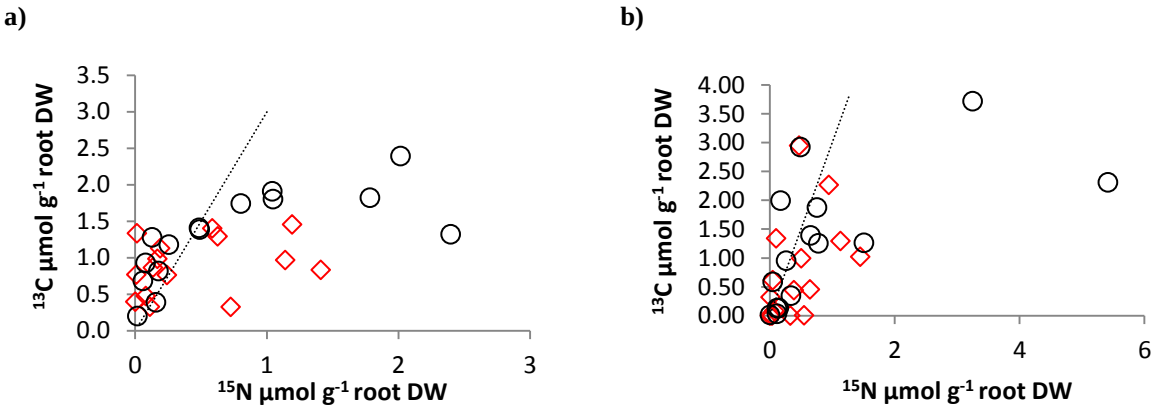


Figure 6.7 - Relationship between excess ^{13}C and ^{15}N in **a)** *S. europaea* and **b)** *A. tripolium* plants. Symbols in red = alanine-$^{13}C^{15}N$, in black = trialanine-$^{13}C^{15}N$.

Plant vs microbial uptake of alanine-C and trialanine-C

Uptake of alanine-^{14}C and trialanine-^{14}C by soil microbes was calculated from ^{14}C solution depletion, and so it includes both C incorporated into microbial biomass and that respired. Both alanine-^{14}C and trialanine-^{14}C taken up by soil microbes increased with incubation time ($r > 0.61$, $p<0.004$), (Figure 6.8a). The amount of ^{14}C taken up was close to 100% of all supplied ^{14}C in the longer incubation periods (Figure 6.8b), and so only data from the 10 min incubation period are used to calculate the ^{14}C uptake rate. Accordingly, C was taken up as alanine at a mean rate of 0.06 ± 0.03 µmol ^{14}C g soil^{-1} DW h^{-1}, and as trialanine at 0.14 ± 0.02 µmol ^{14}C g^{-1} soil DW h^{-1}.

Since each plant was grown in 2.80 ±0.04 g DW soil, and assuming a scenario where microbes can take up same amount alanine and trialanine with and without competing with plants, microbes present in each plant pot would have the capacity to take up 0.17 and 0.40 µmol ^{14}C h^{-1}, as alanine-^{14}C and trialanine respectively, representing 11.0 ± 2.2 % of added C.

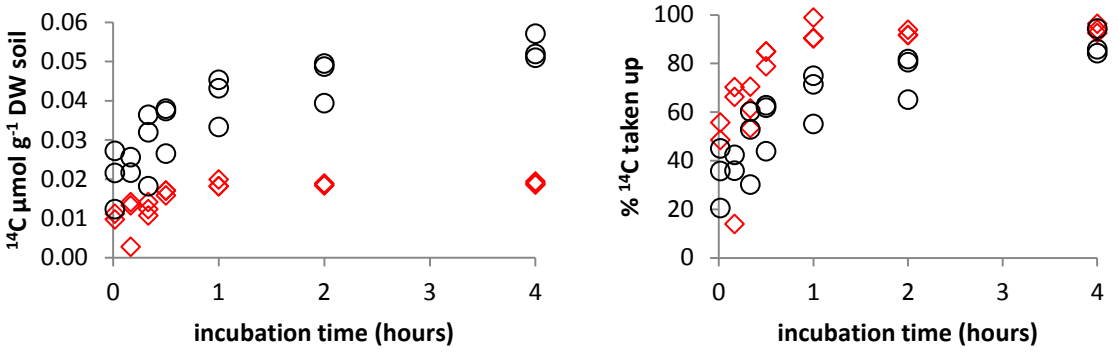


Figure 6.8 – a) Carbon taken up by soil microbes (µmol ^{14}C g soil DW^{-1}). **b)** Percentage of total supplied ^{14}C taken up by soil microbes. Red symbols- alanine-^{14}C, black – trialanine-^{14}C treatments.

Hydroponic plants *vs* plants in soil

Nitrogen was taken up by plants in water solution at a higher rate than by plants in soil, in case of all inorganic and organic N treatments ($p<0.05$). For both plant species inorganic uptake in hydroponic plants was between 10 and 43 times higher than plants in soil, while the difference in relation to organic N uptake was between 5 and 14 times.

Discussion

Porewater N analysis from a local saltmarsh, where *S. europaea* and *A. tripolium* occur naturally, revealed that most of dissolved N was organic, with low molecular weight organic N (<1 kDa) accounting for about 20 % of total dissolved N. DON has also been previously shown to be a significant fraction in other saltmarshes, and free amino acids a considerable fraction of the DON e.g. Henry and Jefferies (2002) reported amino acid concentrations in arctic saltmarsh soil ranging between 32 and 45 μmol l^{-1} during the plant growth season.

The aquaculture wastewater analysed, with samples taken from the same aquaculture system at two different sites, showed a large variation in N concentration and the proportion of the N forms analysed. Even though the site with the higher low molecular weight DON (amino acid plus peptide-N) concentration was only 6 % of total dissolved N, the determined DON fraction was close to 50 % of TDN (625 μmol N l^{-1} as DON). This value is higher than some previously reported DON fractions in wastewaters: Porrello et al. (2005) reported DON mean concentrations of 23.6 and 25.4 μmol N l^{-1}, representing respectively 25 and 27 % of TDN measured in two sites. Webb et al. (2012) measured a mean DON concentration between 45.3 ± 21.8 and 144.1 ± 6.2 μmol N l^{-1} in aquaculture wastewater before passing through a CW planted with *S. europaea* plants. This translated to about 23 % of TDN. Konnerup et al. (2011) reported DON concentrations between 418 and 639 μmol N l^{-1} in aquaculture wastewater before treatment with CW planted with *Canna generalis*, representing between 89 and 94% of TDN.

Aquaculture systems are prone to dissolved N transformations, both as result of natural occurring processes and due to the use of water treatment techniques (eg. sedimentation tanks, bacterial biofilter). Therefore even if the amino acid and peptide-N pool measured was a relatively low fraction of total dissolved N, because DON may account for up to 50 % of dissolved N it ought to be taken in consideration in the context of wastewater treatment.

In this study, *S. europaea* and *A. tripolium* were shown to take up organic N, both when supplied as an amino acid (alanine) and as a peptide (trialanine). When in solution, both *S. europaea* and *A. tripolium*, took up trialanine-N at a faster rate than alanine-N. These results are dissimilar to those described by Hill et al. (2011a, 2011b), where both for both sterile wheat and *D. antartica* plants N uptake from water solution was not different when supplied as equimolar alanine and trialanine. Besides alanine and trialanine-N uptake, Hill et al. (2011a) also investigated the uptake of organic N supplied as dialanine and tetralanine, and only tretralanine uptake by *D. antartica* plants was found to be significantly higher than alanine and dialanine-N uptake.

In both *S. europaea* and *A. tripolium*, alanine-N and trialanine-N uptake was not different to that of NO_3-N, but it was lower than NH_4-N uptake. In contrast, when investigating (L-) alanine-N and trialanine-N uptake in sterile *Triticum aestivum* Hill et al. (2011b) found that organic N uptake (about 17 μmol N g^{-1} DW root) was higher than NO_3-N (about 2 μmol N g^{-1} DW root) in both cases, but not significantly different from NH_4-N uptake (about 22 μmol N g^{-1} DW root). However, the present results are more similar to those of *D. antartica* plants that also showed lower N uptake when supplied as aminoacid (alanine), or peptide (about 50 nmol N g^{-1} DW tiller min^{-1}) compared to NH_4^+ (about 225 nmol N g^{-1} DW tiller min^{-1}), and no difference compared to NO_3-N uptake (about 40 nmol N g^{-1} DW tiller min^{-1}) (Hill et al. 2011a). Although information about other halophytic plants organic N uptake is scarce, uptake of glycine-N from water has been investigated for *Spartina alterniflora* and *Phragmites australis* (common and invasive) plants from a temperate saltmarsh (Mozdzer et al. 2010, Mozdzer et al. 2011), and *Pucinellia phryganodes* grass from an Arctic coastal marsh (Henry and Jefferies 2002, 2003a). *P. phryganodes* grew equally well when the sole N supply was glycine or NH_4^+ (Henry and Jefferies 2002) even though NH_4-N uptake was higher than glycine-N (14-15 μmol g^{-1} h^{-1}) and NO_3-N uptake was lower (Henry and Jefferies 2003a). Mozdzer et al. (2010) also observed a higher NH_4-N uptake both by *P. australis* and *S. alterniflora* than urea-N, glycine-N and glutamic acid-N uptake rates, and concluded that glycine-N alone may contribute to 17 % for N plant demand, both for *P. australis* and *S. alterniflora*.

Intact uptake of amino acids is commonly inferred from the existence of a linear relationship in ^{15}N and ^{13}C in plant tissues after incubation with ^{15}N ^{13}C labelled amino acids, that implies that both isotopes are being taken up together as organic molecules (Näsholm et al. 1998). By comparing the regression slope with 3, the expected ratio from the ^{13}C and ^{15}N in both alanine and trialanine labelled molecules, an estimate of the percentage of intact uptake can be obtained. In that way, the intact N uptake as alanine and trialanine, expressed as % of total N derived from organic molecules taken up was determined. Alanine-N intact uptake was determined as 72.0 ± 1.7 % and 79.7 ± 7.7 % for *S. europaea* and *A. tripolium* respectively, and trialanine-N, 73.7 ± 2.3 % and 69.7 ± 4.0 % for *S. europaea* and *A. tripolium* respectively. While trialanine-N was higher than alanine-N uptake both for *S. europaea* and *A. tripolium,* and *S. europaea* uptake of alanine-N and trialanine-N was higher than for *A. tripolium* plants, the intact uptake was not significantly different: Organic N uptake estimated in this mode is therefore about 30 % lower than reported. Using the ratio of excess ^{13}C to ^{15}N in tissue technique, between 65 % and 82 % intact uptakes of glycine has been reported in other plants (Warren 2006, Warren and Adams 2007, Warren 2009). In case of halophytic plants, Mozdzer et al. (2010) reported

intact glycine uptake between 60 % and 74% for *P. australis* and *S. alterniflora* plants, between 40 % and 60 % glutamic-acid-N intact uptake, while in their work urea ^{13}C was not found in the plant tissues.

However, for these estimates of amino acid-N intact uptake it was assumed that all ^{13}C excess in plant tissue was taken up incorporated in the organic molecules. It does not take in account the possibility of some of the measure ^{13}C in tissue resulted from ^{13}C re-fixation by the plants after being respired by other plants nearby or by microbes present in the plant solution system, or the possibility of inorganic uptake of ^{13}C after mineralization (Näsholm et al. 2000, Rasmussen and Kuzyakov 2009). While re-fixation of ^{13}C was quantified, by accessing ^{13}C excess in nearby plants not incubated with ^{13}C, it was not possible to account for inorganic uptake of ^{13}C ($H^{13}CO_3^-$) in this work. However, in every case gas chromatography-mass spectrometry analysis was used to validate the intact uptake of amino acid when a linear relationship between ^{13}C and ^{15}N was observed, dual labelled amino acids were identified in plant tissues (Öhlund and Näsholm 2001, Persson and Näsholm 2001, Nordin et al. 2004, Näsholm et al. 2009a).

On the other hand it can be considered a conservative estimate since ^{13}C taken uptake by the plants root from the solution can be quickly metabolised and lost via respiration (Näsholm et al. 1998, 2009b). In this study trialanine-^{14}C was respired at a higher rate than alanine-^{14}C, which is similar to results of Hill et al. (2011b), where 25 % more ^{14}C from added trialanine was respired by wheat compared to alanine. The fact that trialanine derived C was respired at a different rate of that of alanine indicates that the peptide was not cleaved prior to uptake (Hill et al. 2011a). After correcting for C respired and re-fixed after 1h incubation, the determined % of alanine-N intact uptake was 81.7 ± 2.7 % and 67.7 ± 5.7 % respectively for *S. europaea* and *A. tripolium*. While, trialanine-N intact uptake is estimated as 128 ± 5 % and 107 ± 4 % respectively for *S. europaea* and *A. tripolium*. Even though these estimations may somehow overestimate intact uptake because it is possible it includes microbes' respiration, it is evidence that most of the organic N is taken up intact.

In this study, N uptake for plants in soil was significantly lower than the similar N form uptake when plants were in solution. The biggest difference was observed in relation to NH_4-N uptake rates, 31 and 43 times lower for plants in soil comparatively to plants in solution, for *S. europaea* and *A. tripolium* respectively, while NO_3-N, alanine-N and trialanine-N uptake rates were 5 to 14 times lower for plants in soil. These results differ from Mozdzer et al. (2010), who observed proportionally similar NH_4-N and DON plant uptake when in water solution and in the field. But Hill et al. (2011a) also observed a proportionally much lower NH_4^+ in the field than in

hydroponic solution, compared to the other N forms, suggesting higher microbial competition for NH_4^+ in the soil. Also, Hodge et al. (2000a) observed higher microbial competition for NH_4^+ than NO_3^-, with microbial NH_4-N uptake being 5 times higher than for plants, and NO_3-N uptake 2 times higher in microbes than in plants. It is regularly argued that, in the short term, plants in soil are also outcompeted by microbes for organic N (Jones 1999, Jones and Hodge 1999, Hodge et al. 2000a, Hodge et al. 2000b, c, Lipson and Nasholm 2001, Owen and Jones 2001, Bardgett et al. 2003, Jones et al. 2005a, Jones et al. 2005b, Harrison et al. 2007, 2008). Because organic molecules are good C and N sources, microbes may outcompete plants for this substrate and only inorganic N in excess of microbial requirements would be available to plants. Although inorganic N uptake by soil microbes was not assessed in this work, the estimated microbial uptake observed for organic molecules could only account for small fraction of the added N, not explaining the very low plant uptake rates observed alone. Another possible reason for the lower, and unproportional observed N plant uptake, may be the fact the uptake is being limited by the rate N is delivered to roots, not due to microbe competition alone, but mainly due to N mass flow and diffusion of the several N forms in the pore water (Näsholm and Persson 2001, Owen and Jones 2001).

A linear relationship between excess ^{13}C and ^{15}N was not observed in this work, unlike in other studies with plants and soil (Näsholm et al. 1998, Näsholm et al. 2000, 2001, Bardgett et al. 2003, Henry and Jefferies 2003b, Ge et al. 2009, Sauheitl et al. 2009). Other halophytic plants have also exhibited linear relationships between ^{13}C and ^{15}N when incubated with dual labelled amino acids, even though the determined N intact uptake was only 5-11 % (Henry and Jefferies 2003b). Despite no linear relationships being measured between heavy isotopes in plant tissues, the ^{13}C in the plants increased with time suggesting some direct uptake. The high C plant content compared to N content, and relatively high ^{13}C natural abundance in plant tissue (1.08 and 0.37 at%, respectively for ^{13}C and ^{15}N), results in a much higher dilution of taken up ^{13}C in plant tissues compared to ^{15}N, and it is consequently more difficult to distinguish low uptake of ^{13}C from natural variation and analytical error (Näsholm and Persson 2001, Nordin et al. 2001). Additionaly, several other authors have considered that C heavy isotope increase in plant tissues by itself indicates intact amino acids uptake (Streeter et al. 2000, Näsholm and Persson 2001, Reeve et al. 2009).

Conclusions

Amino acids and peptides can be a important fraction of N in aquaculture wastewater, and *S. europaea* and *A. tripolium* species show capacity to take up amino acid and peptide-N at a rate comparable to that of NO_3-N uptake, although in some cases at a lower rate than NH_4-N uptake. This work strongly suggests that for *S. europaea* and *A. tripolium* in IMTA systems, direct uptake of organic N has a potentially important role in dissolved N removal from wastewater and in plant nutrition, particularly when plants are cultivated hydroponically. In the case of planted constructed wetlands for water treatment, the role of the microbial community competing for dissolved N, and the movement of each N form to the root, possibly determining the rate at which the plant can assess N, needs further investigation. Additionally these results clearly show that it is important to better describe the N composition of aquaculture wastewater, principally the DON portion and its low molecular fraction. As well as testing plant uptake of other amino acids and peptides, as as it has been shown that plants take up different amino acids at different rates, and also varying if supplied alone or in solution with other amino acids (Kielland 1994, Streeter et al. 2000, Persson and Näsholm 2001, Weigelt et al. 2005, Harrison et al. 2007).

Chapter 7 - Summary and conclusions

Summary

Plant annual biomass production and N removal

Chapter 2 work was set to test the main hypotheses: that plant growth rate and N content increases with increasing N concentration up to N sufficiency value, and that *S. europaea* N sufficiency value is below 2 mmol NO_3^- l^{-1} continuous supply, the obtained results confirm the two hypotheses. These results also support previous studies in *S. europaea* that observed growth limitation due to poor N supply (Piggot 1969, Stewart et al. 1973, Jefferies 1977, Loveland and Ungar 1983, Webb 2012), since N supply above 300 µmol NO_3^- l^{-1}, resulted in higher growth compared to the lower NO_3^- concentration of 5 µmol l^{-1}, with mortality also reduced at higher N supply. Conversely, the lack of differences in *S. europaea* plant biomass at N supply above 300 µmol l^{-1} indicates a sufficiency value below this concentration. No previous data is available in relation to continuous supply of a constant N concentration to *S. europaea*. However, the work of Kudo and Fujiyama (2010) points towards a higher sufficiency value for *S. bigelovii* since the continuous supply of N at increasing concentrations (1, 2, 3 and 4 mmol l^{-1}) resulted in increased shoot biomass.

Additionally the estimated N removal by plants with N supply above 300 µmol NO_3^- l^{-1} is constant, 6.53 mol N m^{-2}, which is comparatively high to that observed previously, between 1.09 and 2.39 mol N m^{-2} (Webb 2012, Webb et al. in prep.). No luxury N uptake was observed, which agrees with other studies in *S. europaea* (Quintã 2007, Webb 2012). These results indicate that the plants' capacity to remove N from irrigation water is directly related with biomass. Therefore as plants increase in size (later in the season), the higher will be their capacity to remove N. However, it is important to acknowledge than under the applied cropping regime, only 40% of the N accumulated in plants was removed in the harvested tips, while 54% remained in plant shoots and 6% in the roots, which are similar to proportions that have been observed before (Quintã 2007).

Understanding of plant growth and N removal in relation to N available for uptake is of great importance in designing a balanced IMTA, where optimization of biomass production and N removal efficiency is desired. If N available to the plants is less than the sufficiency value, growth will be reduced while on the other hand, if N available to plants exceeds the sufficiency value, removal efficiency will decrease. It was not possible to conduct the same experiment for

A. tripolium plants, within the scope of the present research and for that species N supply needed for optimum growth remains unknown.

The experiment presented in chapter 3, where plants where cultivated over one growth season at non-limiting N supply, at ambient light and temperature, showed a high accumulation of plant biomass. The tested hypothesis that that *S. europaea* and *A. tripolium* plants, under non-limiting nutrient supply, decrease biomass production with introduction of routine cropping, was confirmed and *S. europaea* plants produced more above-ground biomass than *A. tripolium* (*S. europaea* 4.5 kg DW m^{-2} and 1.1 kg DW m^{-2}, *A. tripolium* 2.7 kg DW m^{-2} and 0.8 kg DW m^{-2}, respectively for uncropped and cropped plants). Uncropped *S. europaea* above-ground biomass was similar to that observed by Webb et al. (in prep.) (4.6 Kg DW m^{-2}), and higher than biomass obtained in *Salicornia* fields (< 2.5 Kg DW m^{-2}) irrigated with seawater (Oleary et al. 1985, Glenn et al. 1997, 1999) that have been described as comparable to the yields of several non halophyte crops. Cropped *S. europaea* above-ground biomass, 1.1 kg DW m^{-2}, was somewhat higher than the reported by Webb et al. (in prep.) following a similar harvest regime (0.7 kg DW m^{-2}). The apparent better performance of *S. europaea* in the present study, in hydroponic culture, comparing to CWs (Webb 2012, Webb et al. in prep.), may not be solely to do with the culture system, but also a result of different N supply, however it is observed that both plants species can be successfully cultivated in hydroponically.

It was hypothesised that N uptake rates would not be affected by cropping regime, however measured plant N uptake rates (µmol N $plant^{-1}$ h^{-1}) in chapter 3 were lower in cropped *S. europaea* plants than uncropped, but not different in case of *A. tripolium* (NH_4-N and NO_3-N). *S. europaea* N uptake was always higher than that of *A. tripolium* plants, and estimated daily N uptake rates are 200-818 mmol N m^{-2} d^{-1} and 62-117 mmol N m^{-2} d^{-1} respectively for *S. europaea* and *A. tripolium*. Even if cropping reduces plant N uptake, the observed rates for both cropped and uncropped plants are higher than those observed in planted CWs treating aquaculture wastewater (Lin et al. 2002, Lin et al. 2005, Konnerup et al. 2011, Webb 2012, Webb et al. in prep.), including CWs planted with *S. europaea*, and cropped every 3 weeks, that removed 62 mmol N m^{-2} d^{-1} and 106 mmol N m^{-2} d^{-1} in two different studies.

Since the introduction of repeated cropping reduces plant biomass and consequently N removal (*S. europaea*), IMTA design will need to include decisions in relation to the harvesting regime used. This will depend on whether the main goal of each system is high N removal and high plant biomass harvested at the end of the growth period or supply of fresh plant product

(tips) every 3 weeks for the food market, at the cost of a lower N removal and accumulated biomass.

Growth and DIN uptake - N form

Chapter 4 compares *S. europaea* and *A. tripolium* growth when supplied with non limiting NH_4^+, NO_3^-, or NH_4NO_3 in order to test the hypothesis that the growth in plants from both species is lower when N is supplied as NH_4^+ comparing to NO_3^- or NH_4NO_3. *S. europaea* grown in NH_4^+ presented reduced shoot biomass comparing to plants growing in NO_3^- or NH_4NO_3. Ventura et al., (2010) also observed lower growth when only NH_4^+ was supplied compared to NO_3^-. However, different trends have been observed in other studies of *Salicornia* growth; Jefferies (1977) observed no differences in plant growth in NH_4^+ and NO_3^-, and Kudo and Fujiyama (2010) observed better growth with NH_4^+ compared to NO_3^-. As observed in many other plant species the presence of NO_3^- in solution alleviates the growth depressing effect of NH_4^+ in *S. europaea* (Martins-Loucao et al. 1993, Stitt 1999, Britto and Kronzucker 2002, Houdusse et al. 2005, Houdusse et al. 2008). On the other hand *A. tripolium* shoot biomass, in the present study, was not affected by the N form supplied, in contrast to the studies by Sági and Erdei (2005) and Jefferies (1977) where signs of NH_4^+ toxicity were observed.

N content (mmol N g^{-1} root DW) was lower in *S. europaea* grown with NH_4^+ alone, compared to plants supplied with NO_3^-, or NH_4NO_3. In *A. tripolium*, plant N content was lower in the NH_4^+ treatment, intermediate in NO_3^- and higher in NH_4NO_3. Taking in consideration N content and biomass it is predicted that NH_4-N uptake will be lower than NO_3-N in *S. europaea*. In the case of *A. tripolium*, results suggest that NH_4-N uptake rate was lower than NO_3-N when supplied separately, and that uptake of NH_4-N, NO_3-N or both was enhanced when both are supplied together.

DIN uptake - N concentration and form

NH_4-N and NO_3-N uptake kinetics was assessed when supplied separately or in an equimolar mix, at a range of concentrations (up to 2 mmol ^{15}N l^{-1}), in both in *S. europaea* and *A. tripolium* with continuous supply of N to plants previously N-starved for 48h (chapter 4) in order to

address several hypotheses, namely: *S. europaea* and *A. tripolium* N uptake rates at different supply concentrations follow the Michaelis-Menten model; plant NH_4-N uptake rates are higher than NO_3-N uptake at similar N concentrations; plant NO_3-N uptake is inhibited by the presence of NH_4^+ in solution, while NH_4-N uptake is not inhibited by NO_3^-; plants receiving continuous supply of N express higher N uptake rates than plants N starved for 48h. In fact, uptake rates fitted the Michaelis Menten model, with the exception of NO_3-N uptake by *A. tripolium* when NH_4^+ was also present in solution. And NH_4-N uptake was always higher than NO_3-N uptake, with the exception of in non-starved *S. europaea* supplied with NH_4^+ and NO_3^- separately, where there was no significant differences between NH_4-N and NO_3-N maximum uptakes. In non-starved plants NO_3-N uptake was lower when equimolar NH_4^+ was present in solution.

*S. europae*a NH_4-N uptake was not different between starved and non starved plants, however *A. tripolium* exhibited higher NH_4-N uptake in starved plants. NO_3-N uptake was higher in non-starved compared to N-starved plants, for both species. Both N starved and non starved plants are routinely used to investigate N uptake, however it was demonstrated here that the maximum N uptake may be differ significantly between starved and non starved plants, and therefore direct comparison of results between the different situations has to be avoided.

As mentioned above, considering plant N content and the biomass accumulated in plants grown in NH_4^+, NO_3^- or NH_4NO_3, NH_4-N uptake rates were expected to be lower than for those grown in NO_3^-. However that was not observed, and one possible explanation of the high measured NH_4-N uptake rates is futile cycle of NH_4-N (Britto and Kronzucker 2002), i.e. plants cannot store NH_4^+ taken up in excess, and so a proportion of NH_4-N is not assimilated by the plant and is returned to the solution (efflux). More research is still needed to corroborate this hypothesis, however it is important to be cautious when extrapolating plant N uptake from wastewater based on short term ^{15}N uptake rates, since delayed efflux may be significant.

Growth and DIN uptake - Salinity

As initially hypothesized, growth results from Chapter 5 experiment demonstrated that *S. europaea* are more salt tolerant than *A. tripolium*. The analysis of the observed mortality rates, plant biomass and water content at salinities 1, 10 and 30 indicates a poor response of *S. europaea* to the lower salinity, while *A. tripolium* responded better to lower salinities, compared to salinity 30. Plus, in a fresh weight basis, *A. tripolium* above-ground biomass was higher in

plants at salinity 10. Similar tendencies have been observed in other studies of *S. europaea* (Keiffer et al. 1994, Moghaieb et al. 2004, Cleall-Harding 2008, Balnokin et al. 2010, Aghaleh et al. 2011) and *A. tripolium* (Larher et al. 1982, Shennan et al. 1987a, Lenssen et al. 1995, Ramani et al. 2006) responses to increasing salinity.

Salicornia europaea N uptake rates (µmol N $plant^{-1}$ h^{-1}) were lower at salinity 1 compared to salinities 10 and 30, while *A. tripolium* N uptake rates were not different between salinities. In *A. tripolium*, NO_3-N uptake rate did not vary with salinity, and the NH_4-N uptake was lower at salinity 10 compared to the other salinity treatments.

DIN uptake - Diurnal cycles, irradiance and temperature

N uptake rates have been shown to follow daily cycles in several plants (Clement et al. 1978, Raman et al. 1995, Macduff et al. 1997, Peuke and Jeschke 1998, Tischner 2000), and even though in the present study (Chapter 4) the observed differences were not significant, a consistent increase in N uptake from the first hours of light was observed for both plant species and N forms (NH_4-N and NO_3-N), suggesting that the initial hypothesis of varying N uptake during the day is correct . However, since the irradiance also increases in the first hours of light is difficult to separate whether the observed trends are a result of the time elapsed since first light or also dependent on irradiance.

In Chapter 5, several irradiances were used (different shading levels) to test N uptake (NH_4-N and NO_3-N) and the hypothesis that increasing irradiance increases N uptake, and both *S. europaea* and *A. tripolium* N uptake rates increased with increasing irradiance. Both species presented similar linear relationships, with approximately a rise of 1 µmol NH_4-N g^{-1} root DW h^{-1} for 100 µmol PAR m^{-2} s^{-1} increase in irradiance. For the same increase in irradiance, the increase NO_3-N uptake rate was less than 0.5 µmol N g^{-1} root DW h^{-1}.

In relation to temperature the hypothesis that increasing temperature increases N uptake was tested (Chapter 5), however it was observed not to always be the case and as expected from the temperature susceptibility usually presented by the enzymes involved in N uptake (Bassirirad 2000 and references therein, Dong et al. 2001, Calatayud et al. 2008), the N uptake was higher at the mid-range of temperatures tested. The exception was NH_4-N uptake in *A. tripolium*, which was not significantly affected by the tested temperatures, indicating a wider optimal temperature range for NH_4-N uptake.

DON uptake

Concentration of DON (Chapter 6) in aquaculture wastewater was very different between the two sample points, what is not surprising since aquaculture systems are prone to dissolved N transformations (eg. bacterial biofilter, or naturally occurring bacteria in tanks and pipes). Thus even if the amino acid and peptide-N pool measured was a relatively low fraction of total dissolved N (up to 6%), because DON may account for up to 50 % of dissolved N it must be taken in consideration in the context of wastewater treatment.

In the present study it was hypothesized that both *S. europaea* and *A. tripolium* plants have the capacity to take up organic N (as amino acid and peptides) from aquaculture wastewater, and that they do so at rates comparable to that of inorganic N uptake. And in fact *S. europaea* and *A. tripolium* were shown to uptake of organic N, both when supplied as an amino acid (alanine) and as a peptide (trialanine). When in solution *S. europaea* and *A. tripolium,* took up trialanine-N at a faster rate than alanine-N. Alanine-N and trialanine-N uptake was not different to that of NO_3-N, but it was lower than NH_4-N uptake. This is the first work reporting peptide-N uptake by halophyte species, however amino acid-N uptake as already been observed in other halophytes. *Spartina alterniflora*, *Phragmites australis* and *Pucinellia phryganodes* glycine-N uptake was also found to be lower than NH_4-N uptake and higher than NO_3-N uptake (Henry and Jefferies 2002, 2003a, Mozdzer et al. 2010, Mozdzer et al. 2011).

After correcting for C respired and re-fixed after 1h incubation, the determined % of alanine-N intact uptake (^{13}C:^{15}N) was 81.7 ± 2.7 % and 67.7 ± 5.7 % respectively for *S. europaea* and *A. tripolium*. And trialanine-N intact uptake was estimated at 128 ± 5 % and 107 ± 4 % respectively for *S. europaea* and *A. tripolium*. It is possible that these estimates overestimate intact uptake, since some of the respired C assumed to result from plants respiration may in fact result from microbial respiration (the solution was clean but not sterilized). Nonetheless it is clear that both alanine and trialanine molecules are taken up intact by both plant species. Mozdzer et al. (2010) reported intact glycine uptake between 60 % and 74% for *P. australis* and *S. alterniflora* plants, between 40 % and 60 % glutamic-acid-N intact uptake.

Before the study was carried out it had been hypothesized that when growing hydroponically the amino acid and peptide uptake is mainly taken up as intact organic molecules, while when growing in soil the % of intact uptake would be lower due to higher competition with microbe, although this was true for the plants growing hydroponically, a direct link between lower organic intact uptake in plants grown in soil and microbes competition could not be established in the present study. N uptake by plants in soil was lower than that of plants in

solution in case of all forms, suggesting competition with microbes for N sources, however estimates of microbial N uptake do not justify all the decrease in N uptake and therefore it is suggested that the low N uptake by plants in soil is mainly due to N mass flow and diffusion of the several N forms in the pore water (Näsholm and Persson 2001, Owen and Jones 2001). No linear relationships between excess ^{13}C and ^{15}N were observed, however the ^{13}C in the plants increased with time suggesting some direct uptake (Streeter et al. 2000, Näsholm and Persson 2001, Reeve et al. 2009). Since plant tissues have a high C content compared to N content, and relatively high ^{13}C natural abundance in plant tissue, the lack of an obvious linear pattern may be a result of the elevated dilution of taken up ^{13}C and associated difficulty to distinguish low uptake of ^{13}C from natural variation and analytical error (Näsholm and Persson 2001, Nordin et al. 2001).

Conclusions

Both *S. europaea* (annual) and *A. tripolium* (short lived perennial) show growth and N uptake characteristics that indicate they can be cultivated in aquaculture wastewater. Both species have a range of commercial applications which generate interest in their production. Of these possibly the more lucrative use is as vegetable for human consumption, however only succulent fresh portions of the plant serve this purpose and in order to obtain these the plants need to be repeatedly cropped during the growing season. It was demonstrated here that introduction of cropping decreases accumulation the biomass and N assimilation into plant tissues. Therefore a compromise will need to be made between tip production and overall biomass accumulation and N removal.

Both *S. europaea* and *A. tripolium* take up a variety of N forms, including NH_4^+, NO_3, alanine and trialanine. Amino acid and peptide-N were taken up at a rate comparable to that of NO3uptake, although in some cases at a lower rate than NH_4-N uptake. Although high rates of NH_4-N uptake were observed, conflicting results from N content and biomass observations suggested the possibility of futile cycle of NH_4-N. Further work is required to confirm this and some caution is required when extrapolating plant N uptake from wastewater based in short term ^{15}N uptake rates.

Aster tripolium growth is not affected by N form supplied. However *S. europaea* growth is negatively influenced by NH_4^+ when supplied alone, comparing to NO_3^- or NH_4NO_3 supply.

Therefore the inclusion of a nitrification bacterial biofilter before the plant-unit would reduce the negative effects of NH_4^+ on *S. europaea* and consequently provide a better growth and N removal.

Salicornia europaea optimal performance was observed at salinities 10 and 30, while *A. tripolium* shows the capacity to uptake more NH_4-N at salinity 1, and to perform equally well in NO_3-N removal at salinities 1 and 10. While both species can be included in a system with wastewater salinity close to 10, for higher salinities *S. europaea* should be selected, with *A. tripolium* being better suited to lower salinities.

Nitrogen concentrations and composition in aquaculture wastewater are highly variable and specific for each system, however it is important to estimate N concentrations in wastewater in order to design a system with adequate number of plants. Further work is required in relation to *S. europaea* and *A. tripolium* growth responses to different N concentrations, however with the information available so far it can be determined that these plants have the potential to grow well at a wide range of N supply, while accumulating N in harvestable biomass.

It is also of interest to investigate other factors that may influence plants vegetative growth or reproduction induction, such as irradiance, temperature, supply of other macro and micronutrients and the interaction of those factors. As demonstrated by Ventura et al. (2011b) in *S. persica* plants, the growing season may be extended by providing artificial light.

The results obtained in this study (and available in literature), together with reasonably detailed information on the shrimp/fish production systems (waste N amount and composition, volume of wastewater, time until harvest, salinity, tanks water temperature) and knowledge of typical environmental aspects at the production location (photoperiod, temperature and irradiance changes during the year), ought to allow design and scale up of IMTA systems with *S. europaea* and *A. tripolium*. Based in all the information gathered plant performance can be predicted, and the system optimized according to the specific aims of the growers (higher plants biomass production *vs* repeated supply of fresh tips). Also an economic evaluation of the several aspects of such IMTA system need to be considered, namely in relation to the use of a hydroponic system rather than CWs, and the different possible markets for *S. europaea* and *A. tripolium* products.

References

References

Adriaanse, F. G. and J. J. Human. 1990. The effects of nitrate:ammonium ratios and nitrapyrin on the nitrogen response of *Zea mays* L. under field conditions. Plant and Soil **122**:287-293.

Aghaleh, M., V. Niknam, H. Ebrahimzadeh, and K. Razavi. 2009. Salt stress effects on growth, pigments, proteins and lipid peroxidation in *Salicornia persica* and *S. europaea*. Biologia Plantarum **53**:243-248.

Aghaleh, M., V. Niknam, H. Ebrahimzadeh, and K. Razavi. 2011. Effect of salt stress on physiological and antioxidative responses in two species of Salicornia (*S. persica* and *S. europaea*). Acta Physiologiae Plantarum **33**:1261-1270.

Al-Hafedh, Y. S., A. Alam, and M. S. Beltagi. 2008. Food production and water conservation in a recirculating aquaponic system in Saudi Arabia at different ratios of fish feed to plants. Journal of the World Aquaculture Society **39**:510-520.

Al-Khateeb, S. A. 2006. Effect of salinity and temperature on germination, growth and ion relations of Panicum turgidum Forssk. Bioresource Technology **97**:292-298.

Albert, R. 1975. Salt Regulation in Halophytes. Oecologia **21**:57-71.

Alsaeedi, A. H. and A. M. Elprince. 2000. Critical phosphorus levels for Salicornia growth. Agronomy Journal **92**:336-345.

Amiri, B., M. Assareh, M. Jafari, B. Rasuoli, H. Arzani, and A. Jafari. 2010. Effect of salinity on growth, ion content and water status of glasswort (*Salicornia herbacea* L.). Caspian J. Env. Sci **8**:79-87.

Arredondo-Figueroa, J. L., G. I. de la Mora, I. Guerrero-Legarreta, J. T. Ponce-Palafox, and I. D. Barriga-Sosa. 2007. Ammonia and nitrite removal rates in a closed recirculating-water system, under three load rates of rainbow trout *Oncorhynchus mykiss*. Revista Mexicana De Ingenieria Quimica **6**:301-308.

Attia, F. M., A. A. Alsobayel, M. S. Kriadees, M. Y. AlSaiady, and M. S. Bayoumi. 1997. Nutrient composition and feeding value of *Salicornia bigelovii* torr meal in broiler diets. Animal Feed Science and Technology **65**:257-263.

Ayala, F. and J. W. O'Leary. 1995. Growth and Physiology of *Salicornia Bigelovii* Torr at Suboptimal Salinity. International Journal of Plant Sciences **156**:197-205.

Ball, M. 2002. Interactive effects of salinity and irradiance on growth: implications for mangrove forest structure along salinity gradients. Trees **16**:126-139.

Balnokin, Y., N. Myasoedov, L. Popova, A. Tikhomirov, S. Ushakova, C. Lasseur, and J. B. Gros. 2010. Use of halophytic plants for recycling NaCl in human liquid waste in a bioregenerative life support system. Advances in Space Research **46**:768-774.

Bannister, P. 1976. Introduction to physiological plant ecology. Oxford : Blackwell Scientific.

Bardgett, R. D., T. C. Streeter, and R. Bol. 2003. Soil microbes compete effectively with plants for organic-nitrogen inputs to temperate grasslands. Ecology **84**:1277-1287.

Bassirirad, H. 2000. Kinetics of nutrient uptake by roots: responses to global change. New Phytologist **147**:155-169.

Bechardas, M. F. 2008. Evaluation of *Salicornia* as a feed ingredient for *Oreochromis niloticus* (Nile tilapia). Bangor University.

Beeftink, W. G., J. Nieuwenhuize, M. Stoeppler, and C. Mohl. 1982. Heavy-Metal Accumulation in Salt Marshes from the Western and Eastern Scheldt. Science of the Total Environment **25**:199-223.

Beinhart, G. 1962. Effects of temperature & light intensity on CO2 uptake, respiration, & growth of white clover. Plant Physiology **37**:709-715.

Belal, I. E. H. and M. Al-Dosari. 1999. Replacement of fish meal with *Salicornia* meal in feeds for Nile tilapia *Oreochromis niloticus*. Journal of the World Aquaculture Society **30**:285-289.

Bell, H. L. and J. W. O'Leary. 2003. Effects of salinity on growth and cation accumulation of *Sporobolus virginicus* (Poaceae). American Journal of Botany **90**:1416-1424.

Bert, T. M. 2007. Environmentally responsible aquaculture: Realities and possibilities. Ecological and Genetic Implications of Aquaculture Activities **6**:479-514.

Blancheton, J. P. 2000. Developments in recirculation systems for Mediterranean fish species. Aquacultural Engineering **22**:17-31.

Blancheton, J. P., R. Piedrahita, E. H. Eding, D. E. Roque d'orbcastel, G. Lemarié, A. Bergheim, and S. Fivelstad. 2007. Intensification of landbased aquaculture production in single pass and reuse systems. Pages 21-48 *in* B. Asbjorn, editor. Aquacultural Engineering and Environment. Research Signpost, T.C. 37/661(2) For P.O. Trivandrum-695, Kerala, India.

Bloom, A. J., L. E. Jackson, and D. R. Smart. 1993. Root growth as a function of ammonium and nitrate in the root zone. Plant, Cell & Environment **16**:199-206.

Bodvin, T., M. Indergaard, E. Norgaard, A. Jensen, and A. Skaar. 1996. Clean technology in aquaculture - A production without waste products? Hydrobiologia **327**:83-86.

Boorman, L. A., J. Hazelden, and M. Boorman. 2001. The effect of rates of sedimentation and tidal submersion regimes on the growth of salt marsh plants. Continental Shelf Research **21**:2155-2165.

Borgognone, D., G. Colla, Y. Rouphael, M. Cardarelli, E. Rea, and D. Schwarz. 2012. Effect of nitrogen form and nutrient solution pH on growth and mineral composition of self-grafted and grafted tomatoes. Scientia Horticulturae **149**:61–69.

Boyer, K. E. and P. Fong. 2005. Macroalgal-mediated transfers of water column nitrogen to intertidal sediments and salt marsh plants. Journal of Experimental Marine Biology and Ecology **321**:59-69.

Boyer, K. E., P. Fong, R. R. Vance, and R. F. Ambrose. 2001. *Salicornia virginica* in a Southern California salt marsh: Seasonal patterns and a nutrient-enrichment experiment. Wetlands **21**:315-326.

Brambilla, F., M. Antonini, P. Ceccuzzi, G. Terova, and M. Saroglia. 2008. Foam fractionation efficiency in particulate matter and heterotrophic bacteria removal from a recirculating seabass (*Dicentrarchus labrax*) system. Aquacultural Engineering **39**:37-42.

Britto, D. T. and H. J. Kronzucker. 2002. NH4+ toxicity in higher plants: a critical review. Journal of Plant Physiology **159**:567-584.

Britto, D. T., M. Y. Siddiqi, A. D. M. Glass, and H. J. Kronzucker. 2001. Futile transmembrane NH4+ cycling: A cellular hypothesis to explain ammonium toxicity in plants. Proceedings of the National Academy of Sciences **98**:4255-4258.

Brix, H. 1999. How 'green' are aquaculture, constructed wetlands and conventional wastewater treatment systems? Water Science and Technology **40**:45-50.

Brock, J., S. Aboling, R. Stelzer, E. Esch, and J. Papenbrock. 2007. Genetic variation among different populations of *Aster tripolium* grown on naturally and anthropogenic salt-contaminated habitats: implications for conservation strategies. Journal of Plant Research **120**:99-112.

Brown, C. E., S. R. Pezeshki, and R. D. DeLaune. 2006. The effects of salinity and soil drying on nutrient uptake and growth of *Spartina alterniflora* in a simulated tidal system. Environmental and Experimental Botany **58**:140-148.

Brown, J. J., E. P. Glenn, K. M. Fitzsimmons, and S. E. Smith. 1999. Halophytes for the treatment of saline aquaculture effluent. Aquaculture **175**:255-268.

Buhmann, A. and J. Papenbrock. in press. Biofiltering of aquaculture effluents by halophytic plants: Basic principles, current uses and future perspectives. Environmental and Experimental Botany.

Bunting, S. W. 2006. Low impact aquaculture. Centre for Environment and Society Occasional Paper 2006-3. University of Essex, UK.

Bunting, S. W. 2008. Horizontally integrated aquaculture development: Exploring consensus on constraints and opportunities with a stakeholder Delphi. Aquaculture International **16**:153-169.

Bunting, S. W. and M. Shpigel. 2009. Evaluating the economic potential of horizontally integrated land-based marine aquaculture. Aquaculture **294**:43-51.

Burford, M. A. and K. C. Williams. 2001. The fate of nitrogenous waste from shrimp feeding. Aquaculture **198**:79-93.

Buschmann, A. H., D. A. Lopez, and A. Medina. 1996. A review of the environmental effects and alternative production strategies of marine aquaculture in Chile. Aquacultural Engineering **15**:397-421.

Cabrera, R. I., R. Y. Evans, and J. L. Paul. 1995. Cyclic Nitrogen Uptake by Greenhouse Roses. Scientia Horticulturae **63**:57-66.

Caicedo, J. R., N. P. van der Steen, O. Arce, and H. J. Gijzen. 2000. Effect of total ammonia nitrogen concentration and pH on growth rates of duckweed (*Spirodela polyrrhiza*). Water Research **34**:3829-3835.

Calatayud, A., E. Gorbe, D. Roca, and P. F. Martinez. 2008. Effect of two nutrient solution temperatures on nitrate uptake, nitrate reductase activity, NH4(+) concentration and chlorophyll a fluorescence in rose plants. Environmental and Experimental Botany **64**:65-74.

Calheiros, C. S. C., P. V. B. Quiterio, G. Silva, L. F. C. Crispim, H. Brix, S. C. Moura, and P. M. L. Castro. 2012. Use of constructed wetland systems with *Arundo* and *Sarcocornia* for polishing high salinity tannery wastewater. Journal of Environmental Management **95**:66-71.

Campbell, W. H. 1988. Nitrate Reductase and Its Role in Nitrate Assimilation in Plants. Physiologia Plantarum **74**:214-219.

Carvalho, L. M., P. M. Correia, I. Cacador, and M. A. Martins-Loucao. 2003. Effects of salinity and flooding on the infectivity of salt marsh arbuscular mycorrhizal fungi in *Aster tripolium* L. Biology and Fertility of Soils **38**:137-143.

Casalduero, G. F. 1993. Integrated systems: "Environmentally clean" aquaculture.

Cerezo, M., V. Flors, F. Legaz, and P. García-Agustín. 2000. Characterization of the low affinity transport system for NO3− uptake by *Citrus* roots. Plant Science **160**:95-104.

Cerezo, M., P. García-Agustín, M. D. Serna, and E. Primo-Millo. 1997. Kinetics of nitrate uptake by Citrus seedlings and inhibitory effects of salinity. Plant Science **126**:105-112.

Cerezo, R. G., M. L. Suarez, and M. R. Vidal-Abarca. 2001. The performance of a multi-stage system of constructed wetlands for urban wastewater treatment in a semiarid region of SE Spain. Ecological Engineering **16**:501-517.

Chan, W.-T. and A. F. Mackenzie. 1972. Effects of shading and nitrogen on growth of corn (Zea mays L.) under field conditions. Plant and Soil **36**:59-70.

Chen, S. L., J. Ling, and J. P. Blancheton. 2006. Nitrification kinetics of biofilm as affected by water quality factors. Aquacultural Engineering **34**:179-197.

Chopin, T., A. H. Buschmann, C. Halling, M. Troell, N. Kautsky, A. Neori, G. P. Kraemer, J. A. Zertuche-Gonzalez, C. Yarish, and C. Neefus. 2001. Integrating seaweeds into marine aquaculture systems: A key toward sustainability. Journal of Phycology **37**:975-986.

Clapham, A. R., A. H. Pearsall, and P. W. Richards. 1942. *Aster tripolium* L. Journal of Ecology **30**:385-395.

Cleall-Harding, P. 2008. *Salicornia europaea* in integrated aquaculture : the influence of salinity on growth, yield, elemental and fatty acid composition. Bangor University.

Clement, C. R., M. J. Hopper, L. H. P. Jones, and E. L. Leafe. 1978. Uptake of Nitrate by Lolium-Perenne from Flowing Nutrient Solution .2. Effect of light, defoliation, and relatonship to CO2 flux. Journal of Experimental Botany **29**:1173-1183.

Colt, J. 2006. Water quality requirements for reuse systems. Aquacultural Engineering **34**:143-156.

Cooper, A. 1982. The Effects of Salinity and Waterlogging on the Growth and Cation Uptake of Salt-Marsh Plants. New Phytologist **90**:263-275.

Crab, R., Y. Avnimelech, T. Defoirdt, P. Bossier, and W. Verstraete. 2007. Nitrogen removal techniques in aquaculture for a sustainable production. Aquaculture **270**:1-14.

Crawford, N. M. and A. D. M. Glass. 1998. Molecular and physiological aspects of nitrate uptake in plants. Trends in Plant Science **3**:389-395.

Cripps, S. J. and A. Bergheim. 2000. Solids management and removal for intensive land-based aquaculture production systems. Aquacultural Engineering **22**:33-56.

Davidson, J., N. Helwig, and S. T. Summerfelt. 2008. Fluidized sand biofilters used to remove ammonia, biochemical oxygen demand, total coliform bacteria, and suspended solids from an intensive aquaculture effluent. Aquacultural Engineering **39**:6-15.

Davy, A. J., G. F. Bishop, and C. S. B. Costa. 2001. *Salicornia* L. (*Salicornia pusilla* J. Woods, *S. ramosissima* J. Woods, *S. europaea* L., *S. obscura* P.W. Ball & Tutin, S. nitens P.W. Ball & Tutin, S. fragilis P.W. Ball & Tutin and *S. dolichostachya* Moss). Journal of Ecology **89**:681-707.

De la Haba, P., E. Agüera, and J. Maldonado. 1990. Differential effects of ammonium and tungsten on nitrate and nitrite uptake and reduction by sunflower plants. Plant Science **70**:21-26.

Deviller, G., C. Aliaume, M. A. F. Nava, C. Casellas, and J. P. Blancheton. 2004. High-rate algal pond treatment for water reuse in an integrated marine fish recirculating system: effect on water quality and sea bass growth. Aquaculture **235**:331-344.

Diver, S. 2006. Aquaponics—Integration of Hydroponics with Aquaculture. ATTRA - National Sustainable Agriculture Information Service.

Dominguez-Valdivia, M. D., P. M. Aparicio-Tejo, C. Lamsfus, C. Cruz, M. A. Martins-Loucao, and J. F. Moran. 2008. Nitrogen nutrition and antioxidant metabolism in ammonium-tolerant and -sensitive plants. Physiol Plant. 2008 Mar;132(3):359-69.

Dong, S., C. F. Scagel, L. Cheng, L. H. Fuchigami, and P. T. Rygiewicz. 2001. Soil temperature and plant growth stage influence nitrogen uptake and amino acid concentration of apple during early spring growth. Tree Physiology **21**:541-547.

Eganathan, P., H. M. S. R. Subramanian, R. Latha, and C. S. Rao. 2006. Oil analysis in seeds of *Salicornia brachiata*. Industrial Crops and Products **23**:177-179.

Ellison, A. M. 1987. Density-Dependent Dynamics of *Salicornia Europaea* Monocultures. Ecology **68**:737-741.

Envirophyte. 2009. Improvement of the cost effectiveness of marine land-based aquaculture facilities through use of Constructed Wetlands with *Salicornia* as an environmentally friendly biofilter and a valuable by-product.

Falkengren-Grerup, U., K. F. Mansson, and M. O. Olsson. 2000. Uptake capacity of amino acids by ten grasses and forbs in relation to soil acidity and nitrogen availability. Environmental and Experimental Botany **44**:207-219.

Fang, Y. Y., O. Babourina, Z. Rengel, X. E. Yang, and P. M. Pu. 2007a. Ammonium and nitrate uptake by the floating plant *Landoltia punctata*. Annals of Botany **99**:365-370.

Fang, Y. Y., O. Babourina, Z. Rengel, X. E. Yang, and P. M. Pu. 2007b. Spatial distribution of ammonium and nitrate fluxes along roots of wetland plants. Plant Science **173**:240-246.

FAO. 2002a. The State of World Fisheries and Aquaculture. Food and Agriculture Organization of the United Nations

FAO. 2002b. Subregional workshop to promote sustainable aquaculture development in the small island developing states of the Lesser Antilles. Food and Agriculture Organization of the United Nations

FAO. 2004. The State of World Fisheries and Aquaculture. Food and Agriculture Organization of the United Nations

FAO. 2007. The State of World Fisheries and Aquaculture. Food and Agriculture Organization of the United Nations.

FAO. 2010. The State of World Fisheries and Aquaculture. Food and Agriculture Organization of the United Nations

Fernandes, M., M. Angove, T. Sedawie, and A. Cheshire. 2007a. Dissolved nutrient release from solid wastes of southern bluefin tuna (*Thunnus maccoyii*, Castelnau) aquaculture. Aquaculture Research **38**:388-397.

Fernandes, M., P. Lauer, A. Cheshire, and M. Angove. 2007b. Preliminary model of nitrogen loads from southern bluefin tuna aquaculture. Marine Pollution Bulletin **54**:1321-1332.

Fischer, W. N., B. Andre, D. Rentsch, S. Krolkiewicz, M. Tegeder, K. Breitkreuz, and W. B. Frommer. 1998. Amino acid transport in plants. Trends in Plant Science **3**:188-195.

Flowers, T. J. 1972. The effect of sodium chloride on enzyme activities from four halophyte species of chenopodiaceae. Phytochemistry **11**:1881-1886.

Flowers, T. J. and T. D. Colmer. 2008. Salinity tolerance in halophytes. New Phytologist **179**:945-963.

Flowers, T. J., M. A. Hajibagheri, and N. J. W. Clipson. 1986. Halophytes. Quarterly Review of Biology **61**:313-337.

Flowers, T. J., P. F. Troke, and A. R. Yeo. 1977. Mechanism of Salt Tolerance in Halophytes. Annual Review of Plant Physiology and Plant Molecular Biology **28**:89-121.

Folke, C. and N. Kautsky. 1992. Aquaculture with Its Environment - Prospects for Sustainability. Ocean & Coastal Management **17**:5-24.

Forde, B. G. 2000. Nitrate transporters in plants: structure, function and regulation. Biochimica et Biophysica Acta (BBA) - Biomembranes **1465**:219-235.

Forde, B. G. and D. T. Clarkson. 1999. Nitrate and ammonium nutrition of plants: Physiological and molecular perspectives. Advances in Botanical Research Incorporating Advances in Plant Pathology, Vol 30 **30**:1-90.

Franco-Nava, M. A., J. P. Blancheton, G. Deviller, A. Charrier, and J. Y. Le-Gall. 2004a. Effect of fish size and hydraulic regime on particulate organic matter dynamics in a recirculating aquaculture system: elemental carbon and nitrogen approach. Aquaculture **239**:179-198.

Franco-Nava, M. A., J. P. Blancheton, G. Deviller, and J. Y. Le-Gall. 2004b. Particulate matter dynamics and transformations in a recirculating aquaculture system: application of stable isotope tracers in seabass rearing. Aquacultural Engineering **31**:135-155.

Gallagher, J. L. 1975. Effect of an Ammonium-Nitrate Pulse on Growth and Elemental Composition of Natural Stands of *Spartina Alterniflora* and *Juncus Roemerianus*. American Journal of Botany **62**:644-648.

Garnica, M., F. Houdusse, J. Claude Yvin, and J. M. Garcia-Mina. 2009. Nitrate supply induces changes in polyamine content and ethylene production in wheat plants grown with ammonium. Journal of Plant Physiology **166**:363-374.

Gauch, H. G., editor. 1972. Inorganic plant nutrition Stroudsburg, Pa., Dowden, Hutchinson & Ross.

Ge, T. D., S. W. Song, P. Roberts, D. L. Jones, D. F. Huang, and K. Iwasaki. 2009. Amino acids as a nitrogen source for tomato seedlings: The use of dual-labeled ((13)C, (15)N) glycine to test for direct uptake by tomato seedlings. Environmental and Experimental Botany **66**:357-361.

Geissler, N., S. Hussin, and H. W. Koyro. 2009a. Elevated atmospheric CO(2) concentration ameliorates effects of NaCl salinity on photosynthesis and leaf structure of *Aster tripolium* L. Journal of Experimental Botany **60**:137-151.

Geissler, N., S. Hussin, and H. W. Koyro. 2009b. Interactive effects of NaCl salinity and elevated atmospheric CO(2) concentration on growth, photosynthesis, water relations and chemical composition of the potential cash crop halophyte *Aster tripolium* L. Environmental and Experimental Botany **65**:220-231.

Geissler, N., S. Hussin, and H. W. Koyro. 2010. Elevated atmospheric CO(2) concentration enhances salinity tolerance in *Aster tripolium* L. Planta **231**:583-594.

Gelfand, I., Y. Barak, Z. Even-Chen, E. Cytryn, J. van Rijn, M. D. Krom, and A. Neori. 2003. A novel zero discharge intensive Seawater recirculating system for the culture of marine fish. Journal of the World Aquaculture Society **34**:344-358.

Gennaro, P., M. Guidotti, E. Funari, S. Porrello, and M. Lenzi. 2006. Reduction of land based fish-fanning impact by phytotreatment pond system in a marginal lagoon area. Aquaculture **256**:246-254.

Gerloff, G. C. and P. H. Krombholz. 1966. Tissue Analysis as a Measure of Nutrient Availability for the Growth of Angiosperm Aquatic Plants. Limnology and Oceanography **11**:529-537.

GESAMP. 2008. Assessment and communication of environmental risks in coastal aquaculture. . (IMO/FAO/UNESCO-IOCI/UNIDO//WMO/IAEA/U/UNEP Joint Group of Experts on Scientific Aspects of Marine Environmental Protection)

Ghaly, A. E., A. Kamal, and N. S. Mahmoud. 2005. Phytoremediation of aquaculture wastewater for water recycling and production of fish feed. Environment International **31**:1-13.

Glass, A. D. M., D. T. Britto, B. N. Kaiser, J. R. Kinghorn, H. J. Kronzucker, A. Kumar, M. Okamoto, S. Rawat, M. Y. Siddiqi, S. E. Unkles, and J. J. Vidmar. 2002. The regulation of nitrate and ammonium transport systems in plants. Journal of Experimental Botany **53**:855-864.

Glenn, E., S. Miyamoto, D. Moore, J. J. Brown, T. L. Thompson, and P. Brown. 1997. Water requirements for cultivating Salicornia bigelovii Torr with seawater on sand in a coastal desert environment. Journal of Arid Environments **36**:711-730.

Glenn, E. P., T. Anday, R. Chaturvedi, R. Martinez-Garcia, S. Pearlstein, D. Soliz, S. G. Nelson, and R. S. Felger. in press. Three halophytes for saline-water agriculture: An oilseed, a forage and a grain crop. Environmental and Experimental Botany.

Glenn, E. P., J. J. Brown, and E. Blumwald. 1999. Salt tolerance and crop potential of halophytes. Critical Reviews in Plant Sciences **18**:227-255.

Glenn, E. P., W. E. Coates, J. J. Riley, R. O. Kuehl, and R. S. Swingle. 1992. *Salicornia-Bigelovii* Torr - a Seawater-Irrigated Forage for Goats. Animal Feed Science and Technology **40**:21-30.

Glenn, E. P., W. O'Leary J, M. C. Watson, T. L. Thompson, and R. O. Kuehl. 1991a. Salicornia bigelovii Torr.: An Oilseed Halophyte for Seawater Irrigation. Science **251**:1065-1067.

Glenn, E. P. and J. W. O'Leary. 1985. Productivity and Irrigation Requirements of Halophytes Grown with Seawater in the Sonoran Desert. Journal of Arid Environments **9**:81-91.

Glenn, E. P., J. W. Oleary, M. C. Watson, T. L. Thompson, and R. O. Kuehl. 1991b. *Salicornia-Bigelovi*i Torr - an Oilseed Halophyte for Seawater Irrigation. Science **251**:1065-1067.

Goas, G., M. Goas, and F. Larher. 1982. Accumulation of Free Proline and Glycine Betaine in *Aster-Tripolium* Subjected to a Saline Shock - a Kinetic-Study Related to Light Period. Physiologia Plantarum **55**:383-388.

Grasshoff, K., M. Ehrhardt, and K. Kremling. 1983. Methods of seawater analysis. 3rd, completely rev. and extended ed edition. Verlag Chemie, New York

Greenway, H. and C. B. Osmond. 1972. Salt responses f enzymes from species differeing in salt tolerance. Plant Physiology **49**:256-&.

Greenway, M. 2007. The Role of Macrophytes in Nutrient Removal using Constructed Wetlands. Pages 331-351 Environmental Bioremediation Technologies. Springer Berlin Heidelberg.

Guerrero, M. G., J. M. Vega, and M. Losada. 1981. The Assimilatory Nitrate-Reducing System and Its Regulation. Annual Review of Plant Physiology and Plant Molecular Biology **32**:169-204.

Guo, S., H. Bruck, and B. Sattelmacher. 2002. Effects of supplied nitrogen form on growth and water uptake of French bean (*Phaseolus vulgaris* L.) plants - Nitrogen form and water uptake. Plant and Soil **239**:267-275.

Guo, S., Y. Zhou, Q. Shen, and F. Zhang. 2007. Effect of ammonium and nitrate nutrition on some physiological processes in higher plants - Growth, photosynthesis, photorespiration, and water relations. Plant Biology **9**:21-29.

Gutierrez-Wing, M. T. and R. F. Malone. 2006. Biological filters in aquaculture: Trends and research directions for freshwater and marine applications. Aquacultural Engineering **34**:163-171.

Hagopian, D. S. and J. G. Riley. 1998. A closer look at the bacteriology of nitrification. Aquacultural Engineering **18**:223-244.

Halachmi, I., Y. Simon, R. Guetta, and E. M. Hallerman. 2005. A novel computer simulation model for design and management of re-circulating aquaculture systems. Aquacultural Engineering **32**:443-464.

Hales, B., A. van Greer, and T. Takahashi, 2004. 2004. High-frequency measurements of seawater chemistry: flow-injection analysis of macronutrients. Limnology and Oceanography **Methods 2**:91e101.

Handy, R. D. and M. G. Poxton. 1993. Nitrogen Pollution in Mariculture - Toxicity and Excretion of Nitrogenous Compounds by Marine Fish. Reviews in Fish Biology and Fisheries **3**:205-241.

Hare, P. D., W. A. Cress, and J. Van Staden. 1998. Dissecting the roles of osmolyte accumulation during stress. Plant Cell and Environment **21**:535-553.

Harrison, K. A., R. Bol, and R. D. Bardgett. 2007. Preferences for different nitrogen forms by coexisting plant species and soil microbes. Ecology **88**:989-999.

Harrison, K. A., R. Bol, and R. D. Bardgett. 2008. Do plant species with different growth strategies vary in their ability to compete with soil microbes for chemical forms of nitrogen? Soil Biology & Biochemistry **40**:228-237.

Hayashi, L., N. S. Yokoya, S. Ostini, R. T. L. Pereira, E. S. Braga, and E. C. Oliveira. 2008. Nutrients removed by *Kappaphycus alvarezii* (Rhodophyta, Solieriaceae) in integrated cultivation with fishes in re-circulating water. Aquaculture **277**:185-191.

Haynes, R. J. 1986. Mineral nitrogen in the plant-soil system. Orlando : Academic Press.

Haynes, R. J. and K. M. Goh. 1978. Ammonium and Nitrate Nutrition of Plants. Biological Reviews of the Cambridge Philosophical Society **53**:465-510.

Heberer, J. A. and F. E. Below. 1989. Mixed Nitrogen Nutrition and Productivity of Wheat Grown in Hydroponics. Annals of Botany **63**:643-649.

Henry-Silva, G. G. and A. F. M. Camargo. 2006. Efficiency of aquatic macrophytes to treat Nile tilapia pond effluents. Scientia Agricola **63**:433-438.

Henry-Silva, G. G. and A. F. M. Camargo. 2008. Treatment of shrimp effluents by free-floating aquatic macrophytes. Revista Brasileira De Zootecnia-Brazilian Journal of Animal Science **37**:181-188.

Henry, H. A. L. and R. L. Jefferies. 2002. Free amino acid, ammonium and nitrate concentrations in soil solutions of a grazed coastal marsh in relation to plant growth. Plant Cell and Environment **25**:665-675.

Henry, H. A. L. and R. L. Jefferies. 2003a. Interactions in the uptake of amino acids, ammonium and nitrate ions in the Arctic salt-marsh grass, *Puccinellia phryganodes*. Plant Cell and Environment **26**:419-428.

Henry, H. A. L. and R. L. Jefferies. 2003b. Plant amino acid uptake, soluble N turnover and microbial N capture in soils of a grazed Arctic salt marsh. Journal of Ecology **91**:627-636.

Hernandez, I., M. A. Fernandez-Engo, J. L. Perez-Llorens, and J. J. Vergara. 2005. Integrated outdoor culture of two estuarine macroalgae as biofilters for dissolved nutrients from Sparus auratus waste waters. Journal of Applied Phycology **17**:557-567.

Hilbert, D. W. 1990. Optimization of Plant-Root - Shoot Ratios and Internal Nitrogen Concentration. Annals of Botany **66**:91-99.

Hill, P. W., J. Farrar, P. Roberts, M. Farrell, H. Grant, K. K. Newsham, D. W. Hopkins, R. D. Bardgett, and D. L. Jones. 2011a. Vascular plant success in a warming Antarctic may be due to efficient nitrogen acquisition. Nature Climate Change **1**:50-53.

Hill, P. W., R. S. Quilliam, T. H. DeLuca, J. Farrar, M. Farrell, P. Roberts, K. K. Newsham, D. W. Hopkins, R. D. Bardgett, and D. L. Jones. 2011b. Acquisition and Assimilation of Nitrogen as Peptide-Bound and D-Enantiomers of Amino Acids by Wheat. Plos One **6**.

Hodge, A., D. Robinson, and A. Fitter. 2000a. Are microorganisms more effective than plants at competing for nitrogen? Trends in Plant Science **5**:304-308.

Hodge, A., J. Stewart, D. Robinson, B. S. Griffiths, and A. H. Fitter. 2000b. Competition between roots and soil micro-organisms for nutrients from nitrogen-rich patches of varying complexity. Journal of Ecology **88**:150-164.

Hodge, A., J. Stewart, D. Robinson, B. S. Griffiths, and A. H. Fitter. 2000c. Plant N capture and microfaunal dynamics from decomposing grass and earthworm residues in soil. Soil Biology & Biochemistry **32**:1763-1772.

Holmes, R. M., A. Aminot, R. Kerouel, B. A. Hooker, and B. J. Peterson. 1999. A simple and precise method for measuring ammonium in marine and freshwater ecosystems. Canadian Journal of Fisheries and Aquatic Sciences **56**:1801-1808.

Horchani, F., R. Hajri, and S. Aschi-Smiti. 2010. Effect of ammonium or nitrate nutrition on photosynthesis, growth, and nitrogen assimilation in tomato plants. Journal of Plant Nutrition and Soil Science **173**:610-617.

Houdusse, F., M. Garnica, A. M. Zamarreño, J. C. Yvin, and J. García-Mina. 2008. Possible mechanism of the nitrate action regulating free-putrescine accumulation in ammonium fed plants. Plant Science **175**:731-739.

Houdusse, F., A. M. Zamarreno, M. Garnica, and J. Garcia-Mina. 2005. The importance of nitrate in ameliorating the effects of ammonium and urea nutrition on plant development: the relationships with free polyamines and plant proline contents. Functional Plant Biology **32**:1057-1067.

Huiskes, A. H. L., J. Vansoelen, and M. M. Markusse. 1985. Field Studies on the Variability of Populations of *Aster-Tripolium* L in Relation to Salt-Marsh Zonation. Vegetatio **61**:163-169.

Hunter, R. G., D. L. Combs, and D. B. George. 2001. Nitrogen, phosphorous, and organic carbon removal in simulated wetland treatment systems. Archives of Environmental Contamination and Toxicology **41**:274-281.

Im, S. A., Y. R. Lee, Y. H. Lee, S. T. Oh, T. Gerelchuluun, B. H. Kim, Y. Kim, Y. P. Yun, S. Song, and C. K. Lee. 2007. Synergistic activation of monocytes by polysaccharides isolated from Salicomia herbacea and interferon-gamma. Journal of Ethnopharmacology **111**:365-370.

Jagtap, T. G., S. H. Bhosale, and V. L. Nagle. 2002. Ecological observations on major *Salicornia* beds from highly saline coastal wetlands of India. Wetlands **22**:443-450.

Jampeetong, A. and H. Brix. 2009a. Effects of NH4+ concentration on growth, morphology and NH4+ uptake kinetics of *Salvinia natans*. Ecological Engineering **35**:695-702.

Jampeetong, A. and H. Brix. 2009b. Nitrogen nutrition of *Salvinia natans*: Effects of inorganic nitrogen form on growth, morphology, nitrate reductase activity and uptake kinetics of ammonium and nitrate. Aquatic Botany **90**:67-73.

Jampeetong, A., H. Brix, and S. Kantawanichkul. 2012a. Effects of inorganic nitrogen forms on growth, morphology, nitrogen uptake capacity and nutrient allocation of four tropical aquatic macrophytes (*Salvinia cucullata*, *Ipomoea aquatica*, *Cyperus involucratus* and *Vetiveria zizanioides*). Aquatic Botany **97**:10-16.

Jampeetong, A., H. Brix, and S. Kantawanichkul. 2012b. Response of *Salvinia cucullata* to high NH4+ concentrations at laboratory scales. Ecotoxicology and Environmental Safety **79**:69-74.

Jefferies, R. L. 1977. Growth Responses of Coastal Halophytes to Inorganic Nitrogen. Journal of Ecology **65**:847-865.

Jefferies, R. L. and N. Perkins. 1977. Effects on Vegetation of Additions of Inorganic Nutrients to Salt-Marsh Soils at Stiffkey, Norfolk. Journal of Ecology **65**:867-882.

Jones, D. L. 1999. Amino acid biodegradation and its potential effects on organic nitrogen capture by plants. Soil Biology & Biochemistry **31**:613-622.

Jones, D. L., J. R. Healey, V. B. Willett, J. F. Farrar, and A. Hodge. 2005a. Dissolved organic nitrogen uptake by plants - an important N uptake pathway? Soil Biology & Biochemistry **37**:413-423.

Jones, D. L. and A. Hodge. 1999. Biodegradation kinetics and sorption reactions of three differently charged amino acids in soil and their effects on plant organic nitrogen availability. Soil Biology & Biochemistry **31**:1331-1342.

Jones, D. L., A. G. Owen, and J. F. Farrar. 2002. Simple method to enable the high resolution determination of total free amino acids in soil solutions and soil extracts. Soil Biology & Biochemistry **34**:1893-1902.

Jones, D. L., D. Shannon, T. Junvee-Fortune, and J. F. Farrarc. 2005b. Plant capture of free amino acids is maximized under high soil amino acid concentrations. Soil Biology & Biochemistry **37**:179-181.

Kadereit, G., P. Ball, S. Beer, L. Mucina, D. Sokoloff, P. Teege, A. E. Yaprak, and H. Freitag. 2007. A taxonomic nightmare comes true: phylogeny and biogeography of glassworts (*Salicornia* L., Chenopodiaceae). Taxon **56**:1143-1170.

Kajimura, M., S. J. Croke, C. N. Glover, and C. M. Wood. 2004. Dogmas and controversies in the handling of nitrogenous wastes: The effect of feeding and fasting on the excretion of ammonia, urea and other nitrogenous waste products in rainbow trout. Journal of Experimental Biology **207**:1993-2002.

Kant, S., P. Kant, H. Lips, and S. Barak. 2007. Partial substitution of NO3- by NH4+ fertilization increases ammonium assimilating enzyme activities and reduces the deleterious effects of salinity on the growth of barley. Journal of Plant Physiology **164**:303-311.

Keiffer, C. H., B. C. Mccarthy, and I. A. Ungar. 1994. Effect of Salinity and Waterlogging on Growth and Survival of *Salicornia-Europaea* L, an Inland Halophyte. Ohio Journal of Science **94**:70-73.

Kemp, P. R. and G. L. Cunningham. 1981. Light, Temperature and Salinity Effects on Growth, Leaf Anatomy and Photosynthesis of *Distichlis-Spicata* (L) Greene. American Journal of Botany **68**:507-516.

Kerstiens, G., W. Tych, M. F. Robinson, and T. A. Mansfield. 2002. Sodium-related partial stomatal closure and salt tolerance of *Aster tripolium*. New Phytologist **153**:509-515.

Khan, M. A., B. Gul, and D. J. Weber. 2000. Germination responses of Salicornia rubra to temperature and salinity. Journal of Arid Environments **45**:207-214.

Khan, M. A., B. Gul, and D. J. Weber. 2001. Effect of salinity on the growth and ion content of *Salicornia rubra*. Communications in Soil Science and Plant Analysis **32**:2965-2977.

Kielland, K. 1994. Amino-Acid-Absorption by Arctic Plants - Implications for Plant Nutrition and Nitrogen Cycling. Ecology **75**:2373-2383.

Kielland, K., J. McFarland, and K. Olson. 2006. Amino acid uptake in deciduous and coniferous taiga ecosystems. Plant and Soil **288**:297-307.

Kirkby, E. A. and K. Mengel. 1967. Ionic Balance in Different Tissues of the Tomato Plant in Relation to Nitrate, Urea, or Ammonium Nutrition. Plant Physiology **42**:6-14.

Knight, R. L., V. W. E. Payne, R. E. Borer, R. A. Clarke, and J. H. Pries. 2000. Constructed wetlands for livestock wastewater management. Ecological Engineering **15**:41-55.

Knoepp, J. D., D. P. Turner, and D. T. Tingey. 1993. Effects of ammonium and nitrate on nutrient uptake and activity of nitrogen assimilating enzymes in western hemlock. Forest Ecology and Management **59**:179-191.

Komarova, N. Y., K. Thor, A. Gubler, S. Meier, D. Dietrich, A. Weichert, M. S. Grotemeyer, M. Tegeder, and D. Rentsch. 2008. AtPTR1 and AtPTR5 transport dipeptides in planta. Plant Physiology **148**:856-869.

Konnerup, D. and H. Brix. 2010. Nitrogen nutrition of *Canna indica*: Effects of ammonium versus nitrate on growth, biomass allocation, photosynthesis, nitrate reductase activity and N uptake rates. Aquatic Botany **92**:142-148.

Konnerup, D., T. D. T. Ngo, and H. Brix. 2011. Treatment of fishpond water by recirculating horizontal and vertical flow constructed wetlands in the tropics. Aquaculture **313**:57-64.

Kovitvadhi, S., U. Kovitvadhi, P. Sawangwong, and J. Machado. 2008. A laboratory-scale recirculating aquaculture system for juveniles of freshwater pearl mussel Hyriopsis (Limnoscapha) myersiana (Lea, 1856). Aquaculture **275**:169-177.

Koyro, H.-W., N. Geissler, S. Hussin, and B. Huchzermeyer. 2008. Survival at extreme locations: Life strategies of halophytes - The long wag from system ecology, whole plant physiology, cell biochemistry and molecular aspects back to sustainable utilization at field sites. Birkhauser Verlag Ag, Viadukstrasse 40-44, Po Box 133, Ch-4010 Basel, Switzerland.

Kronzucker, H. J., A. D. Glass, and M. Yaeesh Siddiqi. 1999. Inhibition of nitrate uptake by ammonium in barley. Analysis Of component fluxes. Plant Physiol **120**:283-292.

Kronzucker, H. J., M. Y. Siddiqi, and A. Glass. 1995. Kinetics of NO3- Influx in Spruce. Plant Physiol **109**:319-326.

Kronzucker, H. J., M. Y. Siddiqi, and A. Glass. 1996. Kinetics of NH4+ Influx in Spruce. Plant Physiol **110**:773-779.

Kudo, N. and H. Fujiyama. 2010. Responses of Halophyte *Salicornia bigelovii* to Different Forms of Nitrogen Source. Pedosphere **20**:311-317.

Larher, F., Y. Jolivet, M. Briens, and M. Goas. 1982. Osmoregulation in Higher-Plant Halophytes - Organic Nitrogen Accumulation in Glycine Betaine and Proline during the Growth of *Aster-Tripolium* and *Suaeda-Macrocarpa* under Saline Conditions. Plant Science Letters **24**:201-210.

Lasa, B., S. Frechilla, C. Lamsfus, and P. M. Aparicio-Tejo. 2001. The sensitivity to ammonium nutrition is related to nitrogen accumulation. Scientia Horticulturae **91**:143-152.

Lee, R. B. and M. J. Lewis. 1994. Synthesis and N-15-Labeling of Glutamine and Glutamate in Maize Roots during Early Stages of N-15-Ammonium Assimilation. Journal of Experimental Botany **45**:767-777.

Lefebvre, S., C. Bacher, A. Meuret, and J. Hussenot. 2001. Modelling nitrogen cycling in a mariculture ecosystem as a tool to evaluate its outflow. Estuarine Coastal and Shelf Science **52**:305-325.

Lennard, W. A. and B. V. Leonard. 2006. A comparison of three different hydroponic sub-systems (gravel bed, floating and nutrient film technique) in an Aquaponic test system. Aquaculture International **14**:539-550.

Lenssen, G. M., W. E. Vanduin, P. Jak, and J. Rozema. 1995. The Response of *Aster-Tripolium* and *Puccinellia-Maritima* to Atmospheric Carbon-Dioxide Enrichment and Their Interactions with Flooding and Salinity. Aquatic Botany **50**:181-192.

Lillo, C. 1994. Light Regulation of Nitrate Reductase in Green Leaves of Higher-Plants. Physiologia Plantarum **90**:616-620.

Lillo, C. 2008. Signalling cascades integrating light-enhanced nitrate metabolism. Biochemical Journal **415**:11-19.

Lin, Y. F., S. R. Jing, and D. Y. Lee. 2003. The potential use of constructed wetlands in a recirculating aquaculture system for shrimp culture. Environmental Pollution **123**:107-113.

Lin, Y. F., S. R. Jing, D. Y. Lee, Y. F. Chang, Y. M. Chen, and K. C. Shih. 2005. Performance of a constructed wetland treating intensive shrimp aquaculture wastewater under high hydraulic loading rate. Environmental Pollution **134**:411-421.

Lin, Y. F., S. R. Jing, D. Y. Lee, and T. W. Wang. 2002. Nutrient removal from aquaculture wastewater using a constructed wetlands system. Aquaculture **209**:169-184.

Lipson, D. and T. Nasholm. 2001. The unexpected versatility of plants: organic nitrogen use and availability in terrestrial ecosystems. Oecologia **128**:305-316.

Loveland, D. G. and I. A. Ungar. 1983. The Effect of Nitrogen-Fertilization on the Production of Halophytes in an Inland Salt-Marsh. American Midland Naturalist **109**:346-354.

Lymbery, A. J., R. G. Doupe, T. Bennett, and M. R. Starcevich. 2006. Efficacy of a subsurface-flow wetland using the estuarine sedge *Juncus kraussii* to treat effluent from inland saline aquaculture. Aquacultural Engineering **34**:1-7.

Lyssenko, C. and F. Wheaton. 2006a. Impact of positive ramp short-term operating disturbances on ammonia removal by trickling and submerged-upflow biofilters for intensive recirculating aquaculture. Aquacultural Engineering **35**:26-37.

Lyssenko, C. and F. Wheaton. 2006b. Impact of rapid impulse operating disturbances on ammonia removal by trickling and submerged-upflow biofilters for intensive recirculating aquaculture. Aquacultural Engineering **35**:38-50.

Macduff, J. H., A. K. Bakken, and M. S. Dhanoa. 1997. An analysis of the physiological basis of commonality between diurnal patterns of NH4+, NO3- and K+ uptake by Phleum pratense and Festuca pratensis. Journal of Experimental Botany **48**:1691-1701.

Maftoun, M. and B. Sheibany. 1979. Comparative Phytotoxicity of Several Nitrification Inhibitors to Soybean Plants. Journal of Agricultural and Food Chemistry **27**:1365-1368.

Mahmood, T., W. M. Kaiser, R. Ali, M. Ashraf, A. Gulnaz, and Z. Iqbal. 2005. Ammonium versus nitrate nutrition of plants stimulates microbial activity in the rhizosphere. Plant and Soil **277**:233-243.

Malone, R. F. and T. J. Pfeiffer. 2006. Rating fixed film nitrifying biofilters used in recirculating aquaculture systems. Aquacultural Engineering **34**:389-402.

Martins-Loucao, M. A., B. Wollenweber, and J. A. Raven. 1993. Response of Salvinia spp. to Different Nitrogen Sources: The Acid-Base Regulation Approach. Oecologia **93**:524-530.

Masters, D. G., S. E. Benes, and H. C. Norman. 2007. Biosaline agriculture for forage and livestock production. Agriculture Ecosystems & Environment **119**:234-248.

McCarthy, M. J. and W. S. Gardner. 2003. An application of membrane inlet mass spectrometry to measure denitrification in a recirculating mariculture system. Aquaculture **218**:341-355.

McFarland, J. W., R. W. Ruess, K. Kielland, K. Pregitzer, R. Hendrick, and M. Allen. 2010. Cross-Ecosystem Comparisons of In Situ Plant Uptake of Amino Acid-N and NH(4) (+). Ecosystems **13**:177-193.

McMurtry, M. R., D. C. Sanders, J. D. Cure, R. G. Hodson, B. C. Haning, and P. C. St Amand. 1997. Efficiency of water use of an integrated fish/vegetable co-culture system. Journal of the World Aquaculture Society **28**:420-428.

Menasveta, P., T. Panritdam, P. Sihanonth, S. Powtongsook, B. Chuntapa, and P. Lee. 2001. Design and function of a closed, recirculating seawater system with denitrification for the culture of black tiger shrimp broodstock. Aquacultural Engineering **25**:35-49.

Mendelssohn, I. A. 1979. Influence of Nitrogen Level, Form, and Application Method on the Growth-Response of Spartina-Alterniflora in North-Carolina. Estuaries **2**:106-112.

MENTERRA. 2007. Salicornia. Bangor University.

Miller, A. E. and W. D. Bowman. 2003. Alpine plants show species-level differences in the uptake of organic and inorganic nitrogen. Plant and Soil **250**:283-292.

Miller, A. E., W. D. Bowman, and K. N. Suding. 2007. Plant uptake of inorganic and organic nitrogen: Neighbor identity matters. Ecology **88**:1832-1840.

Miller, D. and K. Semmens. 2002. Waste Management in Aquaculture. Aquaculture Information Series.

Miyamoto, S., E. P. Glenn, and M. W. Olsen. 1996. Growth, water use and salt uptake of four halophytes irrigated with highly saline water. Journal of Arid Environments **32**:141-159.

Moghaieb, R. E. A., H. Saneoka, and K. Fujita. 2004. Effect of salinity on osmotic adjustment, glycinebetaine accumulation and the betaine aldehyde dehydrogenase gene expression in two halophytic plants, Salicornia europaea and Suaeda maritima. Plant Science **166**:1345-1349.

Momonoki, Y. S. and H. Kamimura. 1994. Studies on the Mechanism of Salt Tolerance in *Salicornia-Europaea* L .1. Changes in Ph and Osmotic-Pressure in *Salicornia* Plants during the Growth Period. Japanese Journal of Crop Science **63**:518-523.

Momonoki, Y. S., S. Oguri, S. Kato, and H. Kamimura. 1996. Studies on the mechanism of salt tolerance in *Salicornia europaea* L. Japanese Journal of Crop Science **65**:693-699.

Morris, J. T. 1980. The Nitrogen Uptake Kinetics of *Spartina-Alterniflora* in Culture. Ecology **61**:1114-1121.

Mozdzer, T. J., M. Kirwan, K. J. McGlathery, and J. C. Zieman. 2011. Nitrogen uptake by the shoots of smooth cordgrass *Spartina alterniflora*. Marine Ecology-Progress Series **433**:43-52.

Mozdzer, T. J., J. C. Zieman, and K. J. McGlathery. 2010. Nitrogen Uptake by Native and Invasive Temperate Coastal Macrophytes: Importance of Dissolved Organic Nitrogen. Estuaries and Coasts **33**:784-797.

Murama, J. P. 2008. Evaluation of *Salicornia* as feed ingredient for shrimp (*Litopenaeus vannamei*). Bangor University.

Naidoo, G. and Y. Naidoo. 2001. Effects of salinity and nitrogen on growth, ion relations and proline accumulation in Triglochin bulbosa. Wetlands Ecology and Management **9**:491-497.

Naik, M. S., Y. P. Abrol, T. V. R. Nair, and C. S. Ramarao. 1982. Nitrate assimilation-its regulation and relationship to reduced nitrogen in higher plants. Phytochemistry **21**:495-504.

Näsholm, T., A. Ekblad, A. Nordin, R. Giesler, M. Hogberg, and P. Hogberg. 1998. Boreal forest plants take up organic nitrogen. Nature **392**:914-916.

Näsholm, T., M. N. Hogberg, P. Hogberg, and A. Nordin. 2009a. Carbon isotopes as proof for plant uptake of organic nitrogen: Relevance of inorganic carbon uptake Reply. Soil Biology & Biochemistry **41**:1588-1589.

Näsholm, T., K. Huss-Danell, and P. Hogberg. 2000. Uptake of organic nitrogen in the field by four agriculturally important plant species. Ecology **81**:1155-1161.

Näsholm, T., K. Huss-Danell, and P. Hogberg. 2001. Uptake of glycine by field grown wheat. New Phytologist **150**:59-63.

Näsholm, T., K. Kielland, and U. Ganeteg. 2009b. Uptake of organic nitrogen by plants. New Phytologist **182**:31-48.

Näsholm, T. and J. Persson. 2001. Plant acquisition of organic nitrogen in boreal forests. Physiologia Plantarum **111**:419-426.

Neori, A., T. Chopin, M. Troell, A. H. Buschmann, G. P. Kraemer, C. Halling, M. Shpigel, and C. Yarish. 2004. Integrated aquaculture: rationale, evolution and state of the art emphasizing seaweed biofiltration in modem mariculture. Aquaculture **231**:361-391.

Neori, A., M. D. Krom, S. P. Ellner, C. E. Boyd, D. Popper, R. Rabinovitch, P. J. Davison, O. Dvir, D. Zuber, M. Ucko, D. Angel, and H. Gordin. 1996. Seaweed biofilters as regulators of water quality in integrated fish-seaweed culture units. Aquaculture **141**:183-199.

Neori, A., M. D. Krom, and J. van Rijn. 2007. Biogeochemical processes in intensive zero-effluent marine fish culture with recirculating aerobic and anaerobic biofilters. Journal of Experimental Marine Biology and Ecology **349**:235-247.

Neori, A., F. E. Msuya, L. Shauli, A. Schuenhoff, F. Kopel, and M. Shpigel. 2003. A novel three-stage seaweed (*Ulva lactuca*) biofilter design for integrated mariculture. Journal of Applied Phycology **15**:543-553.

Neori, A., M. Shpigel, and D. Ben-Ezra. 2000. A sustainable integrated system for culture of fish, seaweed and abalone. Aquaculture **186**:279-291.

Newkirk, G. 1996. Sustainable coastal production systems: A model for integrating aquaculture and fisheries under community management. Ocean & Coastal Management **32**:69-83.

Ng, T. B., F. Liu, Y. Lu, C. H. K. Cheng, and Z. Wang. 2003. Antioxidant activity of compounds from the medicinal herb Aster tataricus. Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology **136**:109-115.

Ng, W. J., K. Kho, L. M. Ho, S. L. Ong, T. S. Sim, S. H. Tay, C. C. Goh, and L. Cheong. 1992. Water-Quality within a Recirculating System for Tropical Ornamental Fish Culture. Aquaculture **103**:123-134.

Nicodemus, M. A., K. F. Salifu, and D. F. Jacobs. 2008. Nitrate reductase activity and nitrogen compounds in xylem exudate of Juglans nigra seedlings: relation to nitrogen source and supply. Trees-Structure and Function **22**:685-695.

Nixon, S. 1980. Between coastal marshes and coastal waters - a review of twenty years of speculation and research on the role of salt marshes in estuarine productivity and water chemistry. Pages 438 - 525 *in* P. Hamilton and K. B. Macdonald, editors. Estuarine and wetland processes with emphasis on modeling. Publ. Corp., New York.

Noaman, M. N. and E. S. El-Haddad. 2000. Effects of irrigation water salinity and leaching fraction on the growth of six halophyte species. Journal of Agricultural Science **135**:279-285.

Nordin, A., P. Hogberg, and T. Nasholm. 2001. Soil nitrogen form and plant nitrogen uptake along a boreal forest productivity gradient. Oecologia **129**:125-132.

Nordin, A., I. K. Schmidt, and G. R. Shaver. 2004. Nitrogen uptake by arctic soil microbes and plants in relation to soil nitrogen supply. Ecology **85**:955-962.

Öhlund, J. and T. Näsholm. 2001. Growth of conifer seedlings on organic and inorganic nitrogen sources. Tree Physiology **21**:1319-1326.

Oleary, J. W., E. P. Glenn, and M. C. Watson. 1985. Agricultural Production of Halophytes Irrigated with Seawater. Plant and Soil **89**:311-321.

Owen, A. G. and D. L. Jones. 2001. Competition for amino acids between wheat roots and rhizosphere microorganisms and the role of amino acids in plant N acquisition. Soil Biology & Biochemistry **33**:651-657.

Pahlavanian, A. M. and W. K. Silk. 1988. Effect of Temperature on Spatial and Temporal Aspects of Growth in the Primary Maize Root. Plant Physiology **87**:529-532.

Panella, S., I. Cignini, M. Battilotti, M. Falcucci, V. Hull, N. Milone, M. Monfrinotti, G. A. Mulas, G. Pipornetti, L. Tancioni, and S. Cataudella. 1999. Ecodepuration performances of a small-scale experimental constructed wetland system treating and recycling intensive aquaculture wastewater. Tempos in Science and Nature: Structures, Relations, and Complexity **879**:427-431.

Park, J., H. B. Kim, P. K. Kim, and J. Y. Jo. 2008. The growth of disk abalone, *Haliotis discus hannai* at different culture densities in a pilot-scale recirculating aquaculture system with a baffled culture tank. Aquacultural Engineering **38**:161-170.

Paungfoo-Lonhienne, C., T. G. A. Lonhienne, D. Rentsch, N. Robinson, M. Christie, R. I. Webb, H. K. Gamage, B. J. Carroll, P. M. Schenk, and S. Schmidt. 2008. Plants can use protein as a nitrogen source without assistance from other organisms. Proceedings of the National Academy of Sciences of the United States of America **105**:4524-4529.

Perera, L. K. R. R., T. A. Mansfield, and A. J. C. Malloch. 1994. Stomatal Responses to Sodium-Ions in *Aster-Tripolium* - a New Hypothesis to Explain Salinity Regulation in Aboveground Tissues. Plant Cell and Environment **17**:335-340.

Persson, J., P. Gardestrom, and T. Nasholm. 2006. Uptake, metabolism and distribution of organic and inorganic nitrogen sources by Pinus sylvestris. Journal of Experimental Botany **57**:2651-2659.

Persson, J. and T. Näsholm. 2001. Amino acid uptake: a widespread ability among boreal forest plants. Ecology Letters **4**:434-438.

Peuke, A. D. and W. D. Jeschke. 1998. The effects of light on induction, time courses, and kinetic patterns of net nitrate uptake in barley. Plant Cell and Environment **21**:765-774.

Piedrahita, R. H. 2003. Reducing the potential environmental impact of tank aquaculture effluents through intensification and recirculation. Aquaculture **226**:35-44.

Piggot, C. D. 1969. Influence of mineral nutrition on the zonation of flowring plants in coastal salt marshes. Pages 25-35 *in* I. H. Rorison, editor. Ecological aspects of the mineral nutrition plants. Oxford Balckwell Scientific.

Pillay, T. V. R. 1992. Aquaculture and the environment. Fishing News Books Oxford. GB.

Porrello, S., M. Lenzi, G. Ferrari, E. Persia, F. Savelli, and P. Tomassetti. 2005. Loading of nutrient from a land-based fish farm (Orbetello, Italy) at different times. Aquaculture International **13**:97-108.

Pujol, J. A., J. F. Calvo, and L. Ramirez-Diaz. 2000. Recovery of germination from different osmotic conditions by four halophytes from southeastern Spain. Annals of Botany **85**:279-286.

Quillere, I., D. Marie, L. Roux, F. Gosse, and J. F. Morotgaudry. 1993. An Artificial Productive Ecosystem Based on a Fish Bacteria Plant-Association .1. Design and Management. Agriculture Ecosystems & Environment **47**:13-30.

Quintã, R. 2007. Evaluation of *Salicornia* spp. as a biofilter in intensive aquaculture: growth and nitrogen uptake in S. europaea agg. under different nitrogen regimes. Bangor University.

Raab, T. K., D. A. Lipson, and R. K. Monson. 1996. Non-mycorrhizal uptake of amino acids by roots of the alpine sedge *Kobresia myosuroides*: Implications for the alpine nitrogen cycle. Oecologia **108**:488-494.

Raab, T. K. and N. Terry. 1994. Nitrogen-source regulation of growth and photosynthesis in *Beta-vugaris* L. Plant Physiology **105**:1159-1166.

Rafiee, G. and C. R. Saad. 2005. Nutrient cycle and sludge production during different stages of red tilapia (Oreochromis sp.) growth in a recirculating aquaculture system. Aquaculture **244**:109-118.

Rakocy, J. E., D. S. Bailey, R. C. Shultz, and E. S. Thoman. Update on tilapia and vegetable production in the UVI aquaponic system. University of the Virgin Islands, Agricultural Experiment Station.

Raman, D. R., R. M. Spanswick, and L. P. Walker. 1995. The kinetics of nitrate uptake from flowing solutions by rice: Influence of pretreatment and light. Bioresource Technology **53**:125-132.

Ramani, B., T. Reeck, A. Debez, R. Stelzer, B. Huchzermeyer, A. Schmidt, and J. Papenbrock. 2006. *Aster tripolium* L. and *Sesuvium portulacastrum* L.: two halophytes, two strategies to survive in saline habitats. Plant Physiology and Biochemistry **44**:395-408.

Randall, D. J. and P. A. Wright. 1987. Ammonia Distribution and Excretion in Fish. Fish Physiology and Biochemistry **3**:107-120.

Rasmussen, J. and Y. Kuzyakov. 2009. Carbon isotopes as proof for plant uptake of organic nitrogen: Relevance of inorganic carbon uptake. Soil Biology & Biochemistry **41**:1586-1587.

Redding, T., S. Todd, and A. Midlen. 1997. The treatment of aquaculture wastewaters - A botanical approach. Journal of Environmental Management **50**:283-299.

Reddy, G. R. 1964. Effect of varying quantities of dicyandiamide on the utilization of nitrogen by several crops from sodium nitrate and ammonium sulphate. J Agr Sci **62**:35-38.

Reeve, J. R., J. L. Smith, L. Carpenter-Boggs, and J. P. Reganold. 2009. Glycine, nitrate, and ammonium uptake by classic and modern wheat varieties in a short-term microcosm study. Biology and Fertility of Soils **45**:723-732.

Rentsch, D., S. Schmidt, and M. Tegeder. 2007. Transporters for uptake and allocation of organic nitrogen compounds in plants. Febs Letters **581**:2281-2289.

Ridge, I. 1991. Plant physiology Sevenoaks : Hodder & Stoughton in association with the Open University.

Romero, J. A., H. Brix, and F. A. Comín. 1999. Interactive effects of N and P on growth, nutrient allocation and NH4 uptake kinetics by *Phragmites australis*. Aquatic Botany **64**:369-380.

Rousseau, D. P. L., E. Lesage, A. Story, P. A. Vanrolleghem, and N. De Pauw. 2008. Constructed wetlands for water reclamation. Desalination **218**:181-189.

Rozema, J. and T. Flowers. 2008. Crops for a Salinized World. Science **322**:1478-1480.

Ruan, J. Y., J. Gerendas, R. Hardter, and B. Sattelmacher. 2007. Effect of nitrogen form and root-zone pH on growth and nitrogen uptake of tea (*Camellia sinensis*) plants. Annals of Botany **99**:301-310.

Sági, B. and L. Erdei. 2005. Adaptive responses to high salinity of two subspecies of *Aster tripolium* on different nitrogen sources. Acta Biologica Szegediensis **49**:115-116.

Salilling, W. J. B., P. W. Westerman, and T. M. Losordo. 2007. Wood chips and wheat straw as alternative biofilter media for denitrification reactors treating aquaculture and other wastewaters with high nitrate concentrations. Aquacultural Engineering **37**:222-233.

Sandu, S., B. Brazil, and E. Hallerman. 2008. Efficacy of a pilot-scale wastewater treatment plant upon a commercial aquaculture effluent I. Solids and carbonaceous compounds. Aquacultural Engineering **39**:78-80.

Sauheitl, L., B. Glaser, and A. Weigelt. 2009. Uptake of intact amino acids by plants depends on soil amino acid concentrations. Environmental and Experimental Botany **66**:145-152.

Schimel, J. P. and F. S. Chapin. 1996. Tundra plant uptake of amino acid and NH4+ nitrogen in situ: Plants compete well for amino acid N. Ecology **77**:2142-2147.

Schneider, O., V. Sereti, E. H. Eding, and J. A. J. Verreth. 2005. Analysis of nutrient flows in integrated intensive aquaculture systems. Aquacultural Engineering **32**:379-401.

Schuenhoff, A., L. Mata, and R. Santos. 2006. The tetrasporophyte of *Asparagopsis armata* as a novel seaweed biofilter. Aquaculture **252**:3-11.

Schuenhoff, A., M. Shpigel, I. Lupatsch, A. Ashkenazi, F. E. Msuya, and A. Neori. 2003. A semi-recirculating, integrated system for the culture of fish and seaweed. Aquaculture **221**:167-181.

Schulz, C., J. Gelbrecht, and B. Rennert. 2003. Treatment of rainbow trout farm effluents in constructed wetland with emergent plants and subsurface horizontal water flow. Aquaculture **217**:207-221.

Schulz, C., J. Gelbrecht, and B. Rennert. 2004. Constructed wetlands with free water surface for treatment of aquaculture effluents. Journal of Applied Ichthyology **20**:64-70.

Schwartz, M. F. and C. E. Boyd. 1995. Constructed Wetlands for Treatment of Channel Catfish Pond Effluents. Progressive Fish-Culturist **57**:255-266.

Shaviv, A. and J. Hagin. 1988. Interaction of ammonium and nitrate nutrition with potassium in wheat. Fertilizer Research **17**:137-146.

Shennan, C. 1987. Salt Tolerance in *Aster-Tripolium* L .3. Na-Fluxes and K-Fluxes in Intact Seedlings. Plant Cell and Environment **10**:75-81.

Shennan, C., R. Hunt, and E. A. C. Macrobbie. 1987a. Salt Tolerance in *Aster-Tripolium* L .1. The Effect of Salinity on Growth. Plant Cell and Environment **10**:59-65.

Shennan, C., R. Hunt, and E. A. C. Macrobbie. 1987b. Salt Tolerance in *Aster-Tripolium* L .2. Ionic Regulation. Plant Cell and Environment **10**:67-74.

Shimizu, K. 2000. Effects of salt treatments on the production and chemical composition of salt wort (*Salicornia herbacea* L.), Rhodesgrass and Alfalfa. Japanese Journal of Tropical Agriculture **44**:61-67.

Shimizu, K., W. Cao, and N. Ishikawa. 2006. Comparison of ecophysiological characteristics between two types of *Salicornia* plants in Japan examined under salt treatment. Journal of Arid Land Studies **16**:25-30.

Shimizu, K., N. Ishikawa, J. Tang, S. Muranaka, and W. Cao. 2003. Changes in the crude protei, ash and glycinebataine concentrations and contents of *Salicornia herbacea* L. under salt treatment cultivation. Japanese Journal of Tropical Agriculture **47**:132-134.

Shpigel, M., A. Neori, D. M. Popper, and H. Gordin. 1993. A Proposed Model for Environmentally Clean Land-Based Culture of Fish, Bivalves and Seaweeds. Aquaculture **117**:115-128.

Siddiqi, M. Yaeesh, Malhotra, Bhupinder, M. I. N. Xiangjia, Glass, and D. M. Anthony. 2002. Effects of ammonium and inorganic carbon enrichment on growth and yield of a hydroponic tomato crop. Wiley-VCH, Weinheim, ALLEMAGNE.

Siddiqi, M. Y., A. D. Glass, T. J. Ruth, and T. W. Rufty. 1990. Studies of the Uptake of Nitrate in Barley: I. Kinetics of NO(3) Influx. Plant Physiol **93**:1426-1432.

Silva, H., G. Caldeira, and H. Freitas. 2007. *Salicornia ramosissima* population dynamics and tolerance of salinity. Ecological Research **22**:125-134.

Sindilariu, P. D., C. Wolter, and R. Reiter. 2008. Constructed wetlands as a treatment method for effluents; from intensive trout farms. Aquaculture **277**:179-184.

Singer, A., S. Parnes, A. Gross, A. Sagi, and A. Brenner. 2008. A novel approach to denitrification processes in a zero-discharge recirculating system for small-scale urban aquaculture. Aquacultural Engineering **39**:72-77.

Sipauba-Tavares, L. H., E. G. P. Favero, and F. M. S. Braga. 2002. Utilization of macrophyte biofilter in effluent from aquaculture: I.Floating plant. Braz. J. Biol. **62**:713-723.

Smart, R. M. and J. W. Barko. 1980. Nitrogen Nutrition and Salinity Tolerance of *Distichlis-Spicata* and *Spartina-Alterniflora*. Ecology **61**:630-638.

Sousa, W. T. Z., C. M. N. Panitz, and S. M. Thomaz. 2011. Performance of Pilot-scale Vertical Flow Constructed Wetlands With and Without the Emergent Macrophyte Spartina alterniflora Treating Mariculture Effluent. Brazilian Archives of Biology and Technology **54**:405-413.

Stewart, G. R. and J. A. Lee. 1974. The role of proline accumulation in halophytes. Planta **120**:279-289.

Stewart, G. R., J. A. Lee, and T. O. Orebamjo. 1973. Nitrogen-Metabolism of Halophytes .2. Nitrate Availability and Utilization. New Phytologist **72**:539-546.

Stewart, G. R. and D. Rhodes. 1978. Nitrogen-Metabolism of Halophytes .3. Enzymes of Ammonia Assimilation. New Phytologist **80**:307-316.

Stienstra, A. W. 1986. Nitrate Accumulation and Growth of *Aster-Tripolium* L with a Continuous and Intermittent Nitrogen Supply. Plant Cell and Environment **9**:307-313.

Stitt, M. 1999. Nitrate regulation of metabolism and growth. Current Opinion in Plant Biology **2**:178-186.

Stoelken, G., J. Simon, B. Ehlting, and H. Rennenberg. 2010. The presence of amino acids affects inorganic N uptake in non-mycorrhizal seedlings of European beech (Fagus sylvatica). Tree Physiology **30**:1118-1128.

Streeter, T. C., R. Bol, and R. D. Bardgett. 2000. Amino acids as a nitrogen source in temperate upland grasslands: the use of dual labelled (C-13, N-15) glycine to test for direct uptake by dominant grasses. Rapid Communications in Mass Spectrometry **14**:1351-1355.

Sulian, L., J. Ping, C. Xianyang, F. Pengxiang, W. Xuchu, and L. Yinxin. 2012. Multiple compartmentalization of sodium conferred salt tolerance in *Salicornia europaea*. Plant Physiology and Biochemistry **51**:47-52.

Summerfelt, S. T. and M. J. Sharrer. 2004. Design implication of carbon dioxide production within biofilters contained in recirculating salmonid culture systems. Aquacultural Engineering **32**:171-182.

Svobodova, Z., J. Machova, G. Poleszczuk, J. Huda, J. Hamackova, and H. Kroupova. 2005. Nitrite poisoning of fish in aquaculture facilities with water-recirculating systems. Acta Veterinaria Brno **74**:129-137.

Tanner, C. C. 2001. Plants as ecosystem engineers in subsurface-flow treatment wetlands. Water Science and Technology **44**:9-17.

Thornton, B. 2001. Uptake of glycine by non-mycorrhizal *Lolium perenne*. Journal of Experimental Botany **52**:1315-1322.

Tikhomirova, N. A., S. A. Ushakova, N. P. Kovaleva, I. V. Gribovskaya, and A. A. Tikhomirov. 2005. Influence of high concentrations of mineral salts on production process and NaCl accumulation by *Salicornia europaea* plants as a constituent of the LSS phototroph link. Space Life Sciences: Closed Ecological Systems: Earth and Space Applications **35**:1589-1593.

Tilley, D. R., H. Badrinarayanan, R. Rosati, and J. Son. 2002. Constructed wetlands as recirculation filters in large-scale shrimp aquaculture. Aquacultural Engineering **26**:81-109.

Ting, I. P., editor. 1982. Plant physiology.

Tischner, R. 2000. Nitrate uptake and reduction in higher and lower plants. Plant Cell and Environment **23**:1005-1024.

Troell, M., C. Halling, A. Neori, T. Chopin, A. H. Buschmann, N. Kautsky, and C. Yarish. 2003. Integrated mariculture: asking the right questions. Aquaculture **226**:69-90.

Troell, M., P. Ronnback, C. Halling, N. Kautsky, and A. Buschmann. 1999. Ecological engineering in aquaculture: use of seaweeds for removing nutrients from intensive mariculture. Journal of Applied Phycology **11**:89-97.

Troyo-Diéguez, E., A. Ortega-Rubio, Y. Maya, and J. L. León. 1994. The effect of environmental conditions on the growth and development of the oilseed halophyte *Salicornia bigelovii* Torr. in arid Baja California Sur, México. Journal of Arid Environments **28**:207-213.

Twarowska, J. G., P. W. Westerman, and T. M. Losordo. 1997. Water treatment and waste characterization evaluation of an intensive recirculating fish production system. Aquacultural Engineering **16**:133-147.

Tyler, A. C., T. A. Mastronicola, and K. J. McGlathery. 2003. Nitrogen fixation and nitrogen limitation of primary production along a natural marsh chronosequence. Oecologia **136**:431-438.

Tylova-Munzarova, E., B. Lorenzen, H. Brix, and O. Votrubova. 2005. The effects of NH4+ and NO3− on growth, resource allocation and nitrogen uptake kinetics of *Phragmites australis* and *Glyceria maxima*. Aquatic Botany **81**:326-342.

Tylová, E., L. Steinbachová, O. Votrubová, B. Lorenzen, and H. Brix. 2008. Different sensitivity of *Phragmites australis* and *Glyceria maxima* to high availability of ammonium-N. Aquatic Botany **88**:93-98.

Ueda, A., M. Kanechi, Y. Uno, and N. Inagaki. 2003. Photosynthetic limitations of a halophyte sea aster (*Aster tripolium* L) under water stress and NaCl stress. Journal of Plant Research **116**:65-70.

Ungar, I. A. 1978. Halophyte Seed-Germination. Botanical Review **44**:233-264.

Uno, Y., M. Kanechi, N. Inagaki, N. Taki, and S. Maekawa. 1996. Growth and protein profile responses in the halophyte sea aster (*Aster tripolium* L) suspension-cultured cells to salinity. Journal of Plant Research **109**:409-414.

Uno, Y., S. Nakao, Y. Yamai, R. Koyama, M. Kanechi, and N. Inagaki. 2009. Callus formation, plant regeneration, and transient expression in the halophyte sea aster (*Aster tripolium* L.). Plant Cell Tissue and Organ Culture **98**:303-309.

Ushakova, S. A., N. P. Kovaleva, T. V. Gribovskaya, V. A. Dolgushev, and N. A. Tikhomirova. 2005. Effect of NaCl concentration on productivity and mineral composition of *Salicornia europaea* as a potential crop for utilization NaCl in LSS. Space Life Sciences: Gravity-Related Effects on Plants and Spaceflight and Man-Made Environments on Biological Systems **36**:1349-1353.

Ushakova, S. A., N. P. Kovaleva, N. A. Tikhomirova, I. V. Gribovskaya, and A. A. Kolmakova. 2006. Effect of photosynthetically active radiation, salinization, and type of nitrogen nutrition on growth of *Salicornia europaea* plants. Russian Journal of Plant Physiology **53**:785-792.

Ushio, A., T. Mae, and A. Makino. 2008. Effects of temperature on photosynthesis and plant growth in the assimilation shoots of a rose. Soil Science & Plant Nutrition **54**:253-258.

Valiela, I. and J. M. Teal. 1974. Nutrient limitation in salt marsh vegetation. Pages 547-563 *in* R. J. Reimold and W. H. Queen, editors. Ecology of Halophytes. Academic Press, New York, USA.

Valiela, I., J. M. Teal, and N. Y. Persson. 1976. Production and Dynamics of Experimentally Enriched Salt-Marsh Vegetation - Belowground Biomass. Limnology and Oceanography **21**:245-252.

Van Rijn, J. 1996. The potential for integrated biological treatment systems in recirculating fish culture - A review. Aquaculture **139**:181-201.

Van Rijn, J., Y. Tal, and H. J. Schreier. 2006. Denitrification in recirculating systems: Theory and applications. Aquacultural Engineering **34**:364-376.

Ventura, Y. and M. Sagi. in press. Halophyte crop cultivation: The case for Salicornia and Sarcocornia. Environmental and Experimental Botany.

Ventura, Y., W. A. Wuddineh, Y. Ephrath, M. Shpigel, and M. Sagi. 2010. Molybdenum as an essential element for improving total yield in seawater-grown *Salicornia europaea* L. Scientia Horticulturae **126**:395-401.

Ventura, Y., W. A. Wuddineh, M. Myrzabayeva, Z. Alikulov, I. Khozin-Goldberg, M. Shpigel, T. M. Samocha, and M. Sagi. 2011a. Effect of seawater concentration on the productivity and nutritional value of annual *Salicornia* and perennial *Sarcocomia* halophytes as leafy vegetable crops. Scientia Horticulturae **128**:189-196.

Ventura, Y., W. A. Wuddineh, M. Shpigel, T. M. Samocha, B. C. Klim, S. Cohen, Z. Shemer, R. Santos, and M. Sagi. 2011b. Effects of day length on flowering and yield production of *Salicornia* and *Sarcocornia* species. Scientia Horticulturae **130**:510-516.

von Wirén, N., S. Gazzarrini, A. Gojont, and W. B. Frommer. 2000. The molecular physiology of ammonium uptake and retrieval. Current Opinion in Plant Biology **3**:254-261.

Vymazal, J. 2005. Horizontal sub-surface flow and hybrid constructed wetlands systems for wastewater treatment. Ecological Engineering **25**:478-490.

Vymazal, J. 2008. The use constructed wetlands with horizontal sub-surface flow for various types of wastewater. Ecological Engineering.

Wallerstein, I., A. Kadman-Zahzvi, A. Nissim, R. Stav, and S. Michal. 1992. Control by photoperiod and the rhizomatous zone over the production of basal buds and the preservation of the rosette form in *Aster* cultivars. Scientia Horticulturae **51**:237-250.

Wang, M. Y., M. Y. Siddiqi, T. J. Ruth, and A. D. M. Glass. 1993. Ammonium Uptake by Rice Roots: II. Kinetics of [image] Influx across the Plasmalemma. Plant Physiology **103**:1259-1267.

Wang, X., Y. Wang, Y. Tao, and K. Feng. 2010. Ammonium Effects on Nitrate Uptake by Roots of Upland and Paddy Rice Seedlings Related to Membrane Potential Differences. Agricultural Sciences in China **9**:799-805.

Warren, C. R. 2006. Potential organic and inorganic N uptake by six Eucalyptus species. Functional Plant Biology **33**:653-660.

Warren, C. R. 2009. Does nitrogen concentration affect relative uptake rates of nitrate, ammonium, and glycine? Journal of Plant Nutrition and Soil Science-Zeitschrift Fur Pflanzenernahrung Und Bodenkunde **172**:224-229.

Warren, C. R. and P. R. Adams. 2007. Uptake of nitrate, ammonium and glycine by plants of Tasmanian wet eucalypt forests. Tree Physiology **27**:413-419.

Webb, J. M. 2005. *Salicornia* (L.) : a potential biofilter for aquaculture? Bangor University.

Webb, J. M. 2012. A constructed wetland for treatment of marine aquaculture wastewater. PhD. Bangor University.

Webb, J. M., R. Quintã, S. Papadimitriou, L. Norman, M. Rigby, D. N. Thomas, and L. Le Vay. 2012. Halophyte filter beds for treatment of saline wastewater from aquaculture. Water Research **46**:5102-5114.

Webb, J. M., R. Quintã, S. Papadimitriou, L. Norman, M. Rigby, D. N. Thomas, and L. Le Vay. in prep. Growth and nutrient uptake in halophytes in a constructed wetland treating marine aquaculture wastewater.

Weigelt, A., R. Bol, and R. D. Bardgett. 2005. Preferential uptake of soil nitrogen forms by grassland plant species. Oecologia **142**:627-635.

Weisner, S. E. B., P. G. Eriksson, W. Graneli, and L. Leonardson. 1994. Influence of Macrophytes on Nitrate Removal in Wetlands. Ambio **23**:363-366.

Wu, R. S. S. 1995. The environmental impact of marine fish culture: Towards a sustainable future. Marine Pollution Bulletin **31**:159-166.

Yang, L., H. T. Chang, and M. N. L. Huang. 2001. Nutrient removal in gravel- and soil-based wetland microcosms with and without vegetation. Ecological Engineering **18**:91-105.

Zachritz, W. H., A. T. Hanson, J. A. Sauceda, and K. M. Fitzsimmons. 2008. Evaluation of submerged surface flow (SSF) constructed wetlands for recirculating tilapia production systems. Aquacultural Engineering **39**:16-23.

Zhang, Z., Z. Rengel, and K. Meney. 2009. Kinetics of ammonium, nitrate and phosphorus uptake by Canna indica and Schoenoplectus validus. Aquatic Botany **91**:71-74.

Zheng, Q. S., L. Liu, Z. P. Liu, J. M. Chen, and G. M. Zhao. 2009. Comparison of the response of ion distribution in the tissues and cells of the succulent plants *Aloe vera* and *Salicornia europaea* to saline stress. Journal of Plant Nutrition and Soil Science-Zeitschrift Fur Pflanzenernahrung Und Bodenkunde **172**:875-883.