Biological purification of nutrient-rich saline water by halophytes and their potential as valuable co-product in aquaculture systems

Von der Naturwissenschaftlichen Fakultät

der Gottfried Wilhelm Leibniz Universität Hannover

zur Erlangung des Grades

Doktorin der Naturwissenschaften

Dr. rer. nat.

genehmigte Dissertation

von

Dipl.-Biol. Anne Kathrin Buhmann

geboren am 04.06.1982 in Hemer

2014

Referentin: Prof. Dr. rer. nat. Jutta Papenbrock

Korreferent: Prof. Dr. rer. hort. Manfred Schenk

Tag der Promotion: 01.04.2014

Erklärung kumulative Dissertation

aus:

Gemeinsame Ordnung für die Promotion zur Doktorin der Naturwissenschaften oder zum Doktor der Naturwissenschaften (Dr. rer. nat.) an der Gottfried Wilhelm Leibniz Universität Hannover (25.03.2013), § 8 Dissertation, (3):

A:

"[…] [2]Es ist eine ausführliche Darstellung voranzustellen, die eine kritische Einordnung der Forschungsthemen und wichtigsten Erkenntnisse aus den Publikationen in den Kontext der wissenschaftlichen Literatur zum Thema vornimmt […]"

Die voranzustellende ausführliche Darstellung ist in dieser Arbeit aufgeteilt in die Kapitel 1 und 6.

B:

"[…] sowie die individuellen eigenen Beiträge und ggf. die Beiträge weiterer Autoren an den jeweiligen Publikationen darlegt."

Publication (Chapter 2)

Buhmann, A., Papenbrock, J., 2013a. Biofiltering of aquaculture effluents by halophytic plants: Basic principles, current uses and future perspectives. Environmental and Experimental Botany 92, 122–133.

The literature research on the current use of halophytes as biofilter for aquaculture effluents and the literature research on the important factors that influence the capacity of a biofilter with halophytes were done by A. Buhmann. The literature research on technical and economic feasibility of halophytes as biofilter for aquaculture effluents and the writing of the manuscript was done by J. Papenbrock and A. Buhmann.

Publication (Chapter 3)

Buhmann, A., Papenbrock, J., 2013b. An economic point of view of secondary compounds in halophytes. Functional Plant Biology 40, 952–967.

The literature research on the range of studies that exists for secondary metabolites was done by A. Buhmann. The selection and evaluation of the studies that reach application in different

fields, the literature research on currently used methodology in the field of secondary metabolites in halophytes and the writing of the manuscript by J. Papenbrock and A. Buhmann.

Publication (Chapter 4)

Buhmann, A., Waller, U., Wecker, B., Papenbrock, J., Optimization of culturing conditions and selection of species for the use of halophytes as biofilter for nutrient-rich saline waters. (Submitted to "Agricultural Water Management" in January 2014).

The overall idea for the experiments was developed by J. Papenbrock, A. Buhmann, U. Waller and B. Wecker. The more detailed design of the experiment was done by J. Papenbrock and A. Buhmann. The experiments were conducted by A. Buhmann as well as the analysis of the water samples and the plant material. The manuscript was written by J. Papenbrock and A. Buhmann.

Publication (Chapter 5)

Waller, U., Buhmann, A., Hanke, V., Kulakowski, A., Wecker, B., Papenbrock, J., Integrated multi-trophic aquaculture in a zero-exchange recirculation system for marine fish combined with hydroponic halophyte production. (In preparation for submission to "Aquaculture International").

The overall idea for the experiment was developed by U. Waller, B. Wecker, A. Kulakowsi and J. Papenbrock. The marine recirculating aquaculture system used in the study was assembled and maintained by U. Waller, V. Hanke and A. Kulakowski. The experiment was conducted by A. Buhmann and U. Waller. The analysis of the plant material was done by A. Buhmann. U. Waller was responsible for the analysis of the water samples, fish feed, fish weight and all the measurement of all data on experimental conditions regarding the aquaculture system and the greenhouse. The manuscript was written by U. Waller, A. Buhmann and J. Papenbrock.

Summary

Large amounts of nutrient-rich wastewater are produced every day which partly contain salts. The use of halophytes to purify saline effluents is a more sustainable solution than direct release to the environment and a low cost alternative to conventional treatment plants. Salt-tolerant plants can also be used as crop plants in saline agriculture which is enforced by soil salinization and depletion of freshwater resources. This thesis investigates relevant questions on the development of an efficient halophyte biofilter and on the development of halophytic crop plants. Special emphasis lays on the combination of both approaches. A promising possibility for such a combination is the production of halophytic crop plants using marine aquaculture effluents. The thesis includes literature reviews as well as experimental work.

In chapter 2 current use and performance of halophytes as biofilter for marine aquaculture effluents are reviewed. Halophytes are used as biofilter for open and recirculating aquaculture systems, in natural or constructed wetlands, in temperate regions as well as in the tropics. Salinity, flooding, nutrient level, root characteristics and technical applications influence the efficacy of a halophyte biofilter. Another literature review (chapter 3) discusses the diversity, application potential and economic feasibility of secondary metabolites found in salt-tolerant plants. Several compounds found in halophytes are interesting for various fields such as pharmacognosy, functional foods, nutraceutical and technical implementation. The definition of appropriate culture conditions and the selection of suitable species are still necessary regarding both reviewed fields of research and application.

The aim of the experimental work of this thesis was to determine optimal culture conditions and select suitable species for the applications of halophytes as biofilter and their use as valuable co-product (chapter 4). Greenhouse experiments simulating application by the use of artificial seawater were conducted with the halophyte species *Tripolium pannonicum* (Jacq.) Dobrocz.. Plant growth, removal of nitrogen and phosphorus, and physiological parameters at different culturing conditions were evaluated. Optimal conditions were used to test the utility of different halophyte species. Afterwards three halophyte species were integrated into a pilot scale marine recirculating aquaculture system to study their applicability as biofilter and feasibility as valuable co-product (chapter 5). Results are promising but further optimization on culturing conditions and fish feed to plant biomass relation are necessary to enhance the efficiency of the halophyte biofilter.

Keywords: biofilter, halophytes, marine aquaculture, nutrient uptake, saline agriculture, secondary metabolites.

Zusammenfassung

Täglich werden große Mengen an nährstoffhaltigem Abwasser produziert, ein Teil davon ist salzhaltig. Die Nutzung von Halophyten zur Aufbereitung dieser salzhaltigen Abwässer ist nachhaltiger als die direkte Abgabe in die Umwelt und eine kostengünstige Alternative zu konventionellen Aufbereitungsanlagen. Zusätzlich finden salztolerante Pflanzen als landwirtschaftliche Nutzpflanzen in der salinen Landwirtschaft Verwendung. Dieser Bedarf wird durch Bodenversalzung und Verknappung von Frischwasserressourcen verschärft. Die vorliegende Arbeit untersucht relevante Fragen in Bezug auf die Entwicklung eines effizienten Halophyten-Biofilters sowie die Entwicklung salztoleranter Nutzpflanzen. Der Schwerpunkt liegt hierbei besonders auf einer Kombination beider Ansätze. Eine vielversprechende Möglichkeit für eine solche Kombination ist die Verwendung von Prozesswässern aus der marinen Aquakultur für den Anbau von salztoleranten Nutzpflanzen. Die vorliegende Arbeit enthält sowohl Literaturauswertungen als auch experimentell durchgeführte Arbeiten.

In Kapitel 2 wird die gegenwärtige Nutzung und Eignung von Halophyten als Biofilter für Prozesswässer aus der marinen Aquakultur mit Hilfe aktueller Publikationen ausgewertet. Halophyten werden als Biofilter für offene und geschlossene Aquakultursysteme, in natürlichen oder künstlichen Feuchtgebieten und sowohl in der gemäßigten Klimazone als auch in den Tropen verwendet. Salzgehalt, Überflutung, Nährstoffgehalt, Wurzeleigenschaften und technische Aspekte beeinflussen die Effizienz eines Halophyten-Biofilters. Ein weiterer Literaturüberblick (Kapitel 3) diskutiert die Vielfältigkeit, die Eignung für die Anwendung und das wirtschaftliche Potential von sekundären Pflanzenstoffen salztoleranter Pflanzen. Viele Verbindungen, die in Halophyten vorkommen, sind interessant für Bereiche wie Pharmakognosie, funktionelle Nahrungsmittel, Nahrungsergänzungsmittel und technische Anwendungen. Die Auswahl geeigneter Arten und Optimierung ihrer Kulturbedingungen ist für beide anhand von Literaturrecherchen untersuchten Bereiche der Anwendung von Halophyten notwendig.

Das Ziel des experimentellen Teils dieser Arbeit war die Bestimmung von optimalen Kulturbedingungen und die Auswahl von geeigneten Arten für die Nutzung von Halophyten als Biofilter sowie ihre Verwendung als wertvolles Nebenprodukt (Kapitel 4). Mit der salztoleranten Art *Tripolium pannonicum* (Jacq.) Dobrocz. wurden eine Anwendung simulierende Gewächshausversuche unter Verwendung von künstlichem Meerwasser durchgeführt. Hierbei wurden das Pflanzenwachstum, die Nährstoffaufnahme aus dem Wasser und pflanzenphysiologische Parameter bei verschiedenen Kulturbedingungen bewertet. Dann

wurden optimale Bedingungen verwendet, um die Eignung verschiedener Halophyten-Arten zu untersuchen. Anschließend wurden drei Halophyten-Arten in ein marines rezirkulierendes Aquakultursystem in Pilotversuchsgröße integriert und ihre Eignung als Biofilter und wertvolles Nebenprodukt untersucht (Kapitel 5). Die Ergebnisse sind vielversprechend. Um den Nutzwert des Halophyten-Biofilters zu steigern, ist jedoch eine weitere Optimierung in Bezug auf die Kulturbedingungen in der Anwendung und das Verhältnis von Fischfutterzugabe zur Pflanzenbiomasseproduktion notwendig.

Schlüsselwörter: Biofilter, Halophyten, marine Aquakultur, Nährstoffaufnahme, saline Landwirtschaft, Sekundärmetabolite.

Content

Chapter 1

General introduction

Salt tolerant plants

Salt is a stress factor for plants. In many plant species saline soil causes growth reduction, affects ontogeny or is even lethal. There are different ways how salinity causes stress in plants. Osmotic stress is caused by a low water potential due to a high ion concentration. Additionally, salinity can cause ion toxicity in the plant tissue and nutrient deficiencies due to ion competition (Flowers et al., 1989; Flowers and Colmer, 2008; Munns and Tester, 2008). Glycophytic plant species are very sensitive to salt. Contrarily, salt tolerant plant species, called halophytes, are able to grow and develop in a saline environment.

Halophytes are defined as "plants that complete their life cycle in a salt concentration of at least 200 mM NaCl under conditions similar to those that might be encountered in the natural environment" in Flowers et al. (1986). They are naturally abundant in coastal ecosystems like salt marshes and mangrove forests and also in inland salt-affected sites like salt lakes. Additionally, man-made saline areas like potash mines get colonized by certain halophyte species (Brock et al., 2007; Prinz et al., 2009). Common adaptation mechanisms of halophytes to deal with low water potential and ion toxicity caused by salinity are exclusion of salts from the root, accumulation of ions in the vacuole (compartmentalization) and plasmatic accumulation of osmolytes. An additional mechanism occurring in some species is the excretion of ions by salt glands (Flowers and Colmer, 2008; Munns and Tester, 2008). Figure 1 summarizes different strategies for salt tolerance in plants.

Salt tolerance has evolved secondarily and several times independently in angiosperm plants. It is not a conserved characteristic from ancestral marine algae but was developed during different geologic ages and in different regions of the world (Flowers et al., 2010). Therefore, halophytic plant species occur in different plant families and show different kinds of adaptations to saline environments. Comparing different halophyte species the degree of salt tolerance differs a lot. This is a consequence of the multiple development of salt tolerance and the variety of morphological and physiological adaptations to deal with a saline environment (Flowers and Colmer, 2008). Generally monocotyledons seem to be less salt-tolerant than dicotyledons (Flowers and Colmer, 2008). The most salt-tolerant plant species occur in the subfamilies Chenopodioideae, Salicornioidae and Suaedoideae of the plant family Amaranthaceae (Rozema and Schat, 2013). Those highly salt-tolerant species not only tolerate

salt concentrations up to sea water salinity but growth is even stimulated by certain salt concentrations. Figure 2 shows the different effects of salt on different halophyte species.

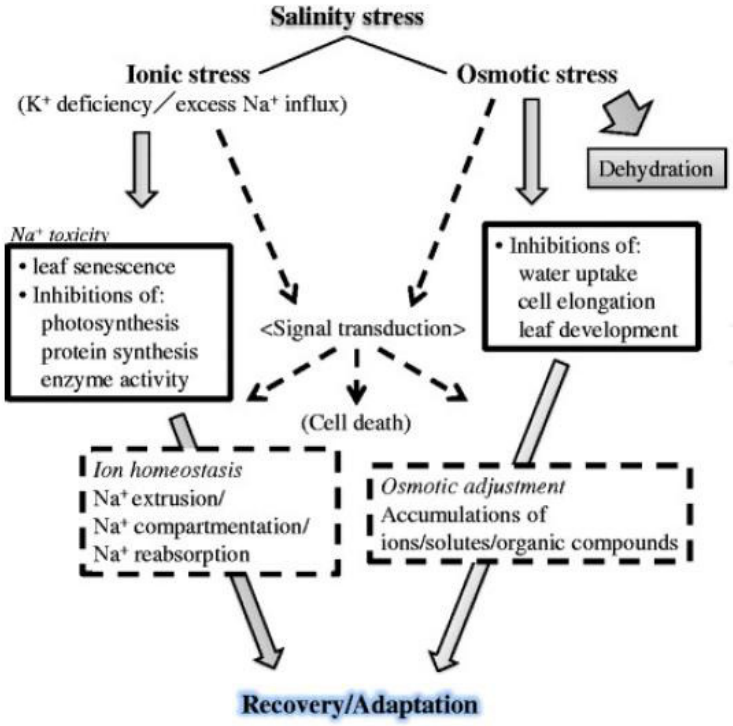

Figure 1. Adaptive responses of plants to salt stress, from de Oliveira et al. (2013).

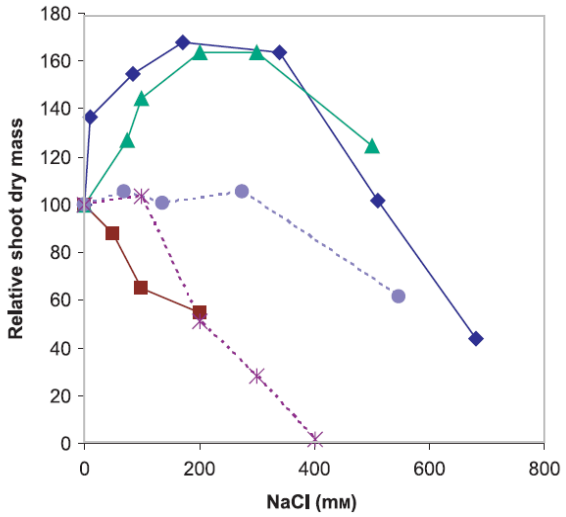

Figure 2. Effect of salinity on growth of different halophyte species (relative to growth under absence or low NaCl), solid lines: dicotyledons, broken lines: monocotyledons, blue: *Suaeda maritima* (L.) Durmot., green: *Disphyma australe* (Sol. ex Aiton) J.M. Black, violet (cycles): *Distichlis spicata* (L.) Greene, violet (stars): *Puccinellia peisonis* (Beck) Jáv., red: *Thellungiella halophila* (C.A.Mey.) O.E.Schulz; from Flowers and Colmer (2008).

Salt tolerance is complex not only from an evolutionary point of view with the different parallel trails of development but also when looking at the response to saline conditions of one single halophytic plant species. In one species salt tolerance is caused by several different morphological and metabolic adaptations. Salt tolerance mechanisms can be induced by saline conditions in some plant species; in others they are always active independently from the environmental conditions (Rozema and Schat, 2013). Many different genes play a role in the successful stress response of a halophytic plant species in a salt-influenced environment (Flowers and Colmer, 2008; Munns and Tester, 2008). There has been progress in the identification of genes and understanding the mechanisms underlying a successful response to salt stress, but still many open questions remain due to the complexity of salt tolerance (Flowers and Colmer, 2008; Munns and Tester, 2008).

The need for saline agriculture

Knowledge about halophytes can help to supply a growing world population with sufficient food due to their characteristic of growing in saline conditions compared to most of the conventional crop plants. Today and future food production is endangered by soil salinization and depletion of freshwater resources in many regions of the world. Salinization is defined as an accumulation of salts in the upper part of the soil that causes a negative impact on agricultural production, environmental health, and economic welfare (Rengazamy, 2006). Szabolcs (1994) published a map showing that salt affected areas already covered 7% of the total land area worldwide in the early 90s (Figure 3). Until now this area certainly increased exacerbating the agricultural, environmental and economical problems that arise. Soil salinization is dramatically accelerated by irrigation, especially in arid areas. Every year 1-2% of irrigated agricultural land is lost due to salinization (FAO, 2002). Because irrigated agriculture produces 40% of the food worldwide (FAO, 2002) this means a serious threat to future alimentation of people.

Additionally to salinization, global food production is seriously affected by a decrease of freshwater resources (Kundzewicz, 2009). Irrigated agriculture is very vulnerable to a decline in freshwater availability because it covers 70% of the freshwater withdrawals worldwide (FAO, 2013). Global population is growing with about 80 million people a year (UN, 2009) increasing rapidly the demand for food. By 2050 world population will need 70 to 100% more food than today (Godfrey et al., 2010). Increasing salinization and decreasing availability of freshwater strongly reduce agricultural productivity of the affected areas and threaten actual

and future food supply. Because salinization as well as freshwater depletion frequently occurs in regions of the world with high population growth, already existing problems like hunger and poverty are enforced (UN, 2009; FAO, 2011).

Figure 3. Salt affected areas of the world, from Pessarakli and Szabolcs (1994).

Growth of salt-tolerant crops on saline soils or irrigated with saline water could reduce the problems caused by a decrease of agricultural production due to soil salinization and reduced freshwater availability. A soil is defined as saline if the electrical conductivity of a saturated soil extract is above 4 mS m^{-1}, which equals 3 g NaCl l^{-1} or 50 mM NaCl (Richards, 1954). A NaCl concentration of only 40 mM causes a strong decrease of plant growth in glycophytes, higher concentrations are lethal (Munns and Tester, 2008). Some conventional crops like wheat or tomato are defined as moderately salt-tolerant and grow at salt concentrations up to 5-6 g l^{-1} (Maas and Hoffman, 1977; Fooland, 2004; Munns et al., 2006). Only a few crop plants such as barley and cotton can be grown at concentrations up to 10 g l^{-1} and are defined as highly salt tolerant (Maas and Hoffman, 1977). But most of the conventional crops are glycophytes and can neither be farmed on salt-affected soils nor be irrigated with saline water (Maas and Hoffman, 1977; Munns and Termaat, 1986). In contrast to conventional glycophyte crop species, halophytes tolerate salt concentrations up to sea water salinity as described above. This beneficial characteristic of halophytes can be used to enhance saline agriculture in two ways (Rozema and Schat, 2013). One is the modification of conventional crop species by the insertion of genes from halophytes responsible for salt tolerance. Another

approach is the development of crops from already salt-tolerant plant species. This thesis will focus on the second approach.

Halophytes as crop plants

Attempts to increase salt tolerance for example in tomato, tobacco and wheat have been partly successful, but the insertion of salt stress tolerance genes to conventional crop plant species remains a laborious and protracted task (Munns et al., 2002; Wang et al., 2004; Xue et al., 2004; Cuartero et al., 2006). This is probably due to the complex mechanisms and the multigenic traits responsible for salt tolerance in different plant species. The development of crops from already salt-tolerant plant species might be a less intricate approach.

Even at higher salinity levels halophytes show growth rates comparable to conventional crop plants (Flowers and Colmer, 2008) and domestication of halophytes is suggested to be promising (Fowers and Colmer, 2008; Rozema and Schat, 2013). The approach is not new but until now the use of halophytes as crop plants has rather a traditional and regional importance than a large-scale agricultural and market relevance. For example, *Crithmum maritimum* L., *Tripolium pannonicum* (Jacq.) Dobrocz. and *Plantago coronopus* L. are regionally consumed as salad or vegetable (Koyro, 2006; Meot-Duros and Magné, 2009; Ventura et al., 2013). Halophytic crop species with a broader publicity and agricultural application are *Diplotaxis tenuifolia* L. and species from the genus *Salicornia* (deVos et al. 2013; Ventura and Sagi, 2013, Shpigel et al., 2013). Nevertheless, there is still a strong need for research for the identification of suitable halophyte species and suitable genotypes for the use as crop plants in large-scale agriculture and for the determination of the necessary culturing conditions (Rozema and Schat, 2013; de Vos et al., 2013).

Apart from the use for human consumption various studies also indicate other potential products derived from halophyte species. Those potential products comprise forages (e.g. Norman et al., 2013), material for industry (Reddy et al., 2008), oil or biomass for energy production (Abideen et al., 2011) and plant products with traditional medicinal use (Liebezeit, 2008; Ksouri et al., 2011). Halophytes are a source of valuable secondary metabolites probably due to their variate development of metabolic adaptations to cope with a stressful environment (Bouftira et al., 2007, Chung et al., 2005). Research in this field follows several different methodological approaches, for example studying the ethnobotanical background of a species, bioactivity of a plant extract or chemical structure of a new compound (Zhu and Row et al., 2010; Boughalleb et al., 2009; Ksouri et al., 2011; Yu et al., 2012). A more

systematic approach to find the active components in plant extracts and determine their application potential is needed. To develop an appropriate procedure it is important to draw conclusions from research work done so far.

Common crop plants often have a long history of variety development. Selection regarding taste, appearance, structure, productivity, efficient use of nutrients, defence against pathogens and herbivores and low sensitivity against several environmental factors resulted in a broad spectrum of seed material used for agriculture (Allard, 1999). De Vos et al. (2013) suggest the selection of suitable varieties, for example regarding taste and salt tolerance, as important for the development of halophytic crops. For halophyte species with high potential for the use as crop plants the selection of different ecotypes will build an important base for the breeding of suitable varieties for large-scale agriculture and different applications.

Wetlands for the treatment of effluents

Beside their potential as crop plants halophytes can be used as biofilter to treat different kinds of effluents. Worldwide approximately 1,500 km³ of wastewater are produced every day (UN, 2003). The most important contaminants contained in wastewater are microbial pathogens, nutrients, heavy metals, persistent organic matter, as well as suspended sediments, pesticides and oxygen-consuming substances (UN, 2009). Eutrophication of water bodies due to high phosphorus and nitrogen concentrations in untreated wastewater is the predominant water quality problem worldwide (UN, 2009). Over 80% of the wastewater worldwide is not collected or treated (UN, 2012). The treatment of wastewater by sewage plants is costly and requires advanced technology. The application of wetlands for the treatment of wastewater is suggested as a low cost alternative (Hammer, 1989).

A wetland consists of plants, water and a medium (Kadlec and Wallace, 2009). Plants take up nutrients for their growth and development and reduce the amount of nitrogen and phosphorus in an effluent. Several plant species accumulate heavy metals and even organic compounds can be reduced in an effluent due to plant uptake or degradation by plants (Weis and Weis, 2004; Reboreda and Cacador, 2007; Carvalho et al., 2010). Macrophytes also provide a trap for suspended solids with their root system and shoot standing (Cronk and Fennessy, 2001). Settled organic material is rapidly decomposed because the plants enhance bacteria growth by providing a large surface area on their roots. The degradation of organic material has a high demand of oxygen and is enhanced through oxygenation of the soil by the plant roots, which reduces the biological oxygen demand of an effluent (Cronk and Fennessy, 2001). The roots

of wetland plants often have an aerenchyma which facilitates the oxygen transport (Cronk and Fennessy, 2001). The occurrence of both, anaerobic and aerobic zones in a wetland enhances the nitrogen removal by ammonification, nitrification and denitrification (Richardson and Vepraskas, 2001). The medium of a wetland also contributes to the filtering effect of a wetland depending on its size and structure. For example, suspended solids are trapped, anaerobic zones enhance denitrification and certain elements and compounds adsorb to substrate particles (Richardson and Vepraskas, 2001).

Natural as well as constructed wetlands are used for the purification of effluents. All over the world natural wetlands such as coastal tidal and salt marshes, riverine marshes and mangrove swamps are used as filter for various kinds of effluents (Mitch and Gosselink, 2007). For a more controlled treatment the use of constructed wetlands has been developed (Vymazal and Kopfelova, 2008). There are different types of constructed wetlands: free water surface (FWS), horizontal subsurface flow (HSSF) and vertical flow (VF) wetlands (Kadlec and Wallace, 2009). FWS wetlands simulate natural wetlands and are characterized by an open water surface, emergent or floating vegetation. Vymazal (2007) classifies an FWS with floating vegetation and an FWS with emergent vegetation as two different types of wetland. A HSSF wetland consists of gravel or soil planted with wetland vegetation and the water is lead horizontally below the surface from an inlet to an outlet side. In a VF the water is led vertically along the root zone. Different types of wetlands are shown in Figure 4. They are characterized by different removal mechanisms and capacities, for example for nutrients (Vymazal, 2007).

Wetlands are applied for the purification of different kinds of wastewaters, comprising municipal wastewater, domestic wastewater from single households, animal wastewater, minewater, industrial wastewater, urban stormwater, field runoffs and different kinds of leachates for example from solid wastes (Kadlec and Wallace, 2009). Some wastewaters such as effluents from industry of field runoffs have a high salinity and the plantation of halophytes to a constructed wetland applied to treat these effluents becomes mandatory (Almeida et al., 2009; Jordan et al., 2009; Manousaki and Kalogerakis, 2011; Diaz et al., 2013). Differently constituted effluents might have different effects on plants and some halophyte species will be more suitable for certain applications than others. More detailed research is needed to answer open questions and establish a data base for large-scale application.

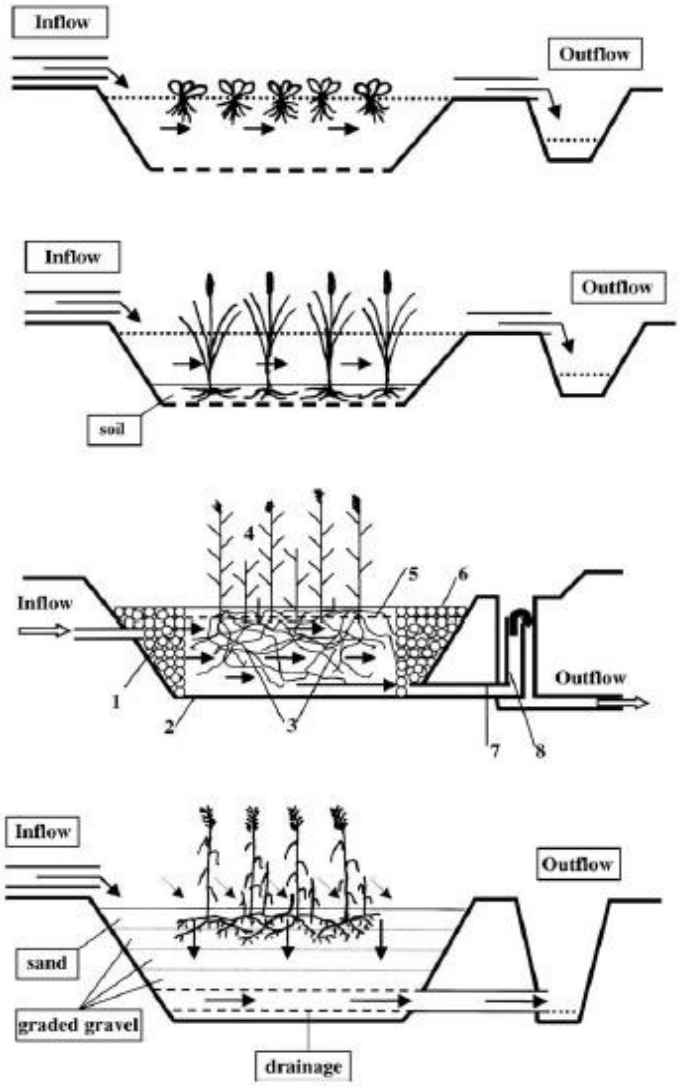

Figure 4. Different types of constructed wetlands (CWs), from top to bottom: CW with floating plants on free water surface, CW with free water surface and emergent plants, CW with horizontal subsurface flow, and CW with vertical flow**,** from Vymazal (2007).

Purification of marine aquaculture effluents by plants

A promising field for the application of halophyte biofilters is the treatment of effluents from marine aquaculture. Global fish supply from capture was at the same quantitative level during the last 20 years (Figure 5). At the same time the importance of aquaculture to cover worldwide demand for fish increased strongly (Figure 5). Marine fisheries accounted for 64% of worldwide fish supply with 78.9 and 19.3 million tonnes coming from capture and aquaculture, respectively (FAO, 2012). Effluents from aquaculture are often released untreated to the environment. Because they show high concentrations of nitrogen and phosphorus due to excess fish feed and excretion serious problems of eutrophication can be

caused at affected sites. Additionally, the effluents often contain high amounts of suspended solids and show a high biological oxygen demand (Dierberg and Kiattisimkul, 1996; Páez-Osuna, 2002). The increasing importance of marine aquaculture for the supply with marine fish makes the development of adequate solutions for the treatment of its effluents urgent.

Plants in a (constructed) wetland are suitable for the application as a biofilter for aquaculture effluents because they take up nitrogen and phosphorus and also contribute to the reduction of suspended solids and biological oxygen demand, as described before. There is a broad range of studies conducted on the application of planted wetlands for the treatment effluents from freshwater aquaculture since the 1980s (Watten and Busch, 1984; Schwarz and Boyd, 1995; Schulz et al., 2003). Regarding the use of halophytes for the treatment of effluents from marine aquaculture literature is scarcer, difficult to compare and conclusions for future research and application remain unclear. For instance, it has to be clarified which factors influence the filter capacity of a halophyte biofilter and which plant species are suitable to determine necessary conditions for the setup of a halophyte biofilter.

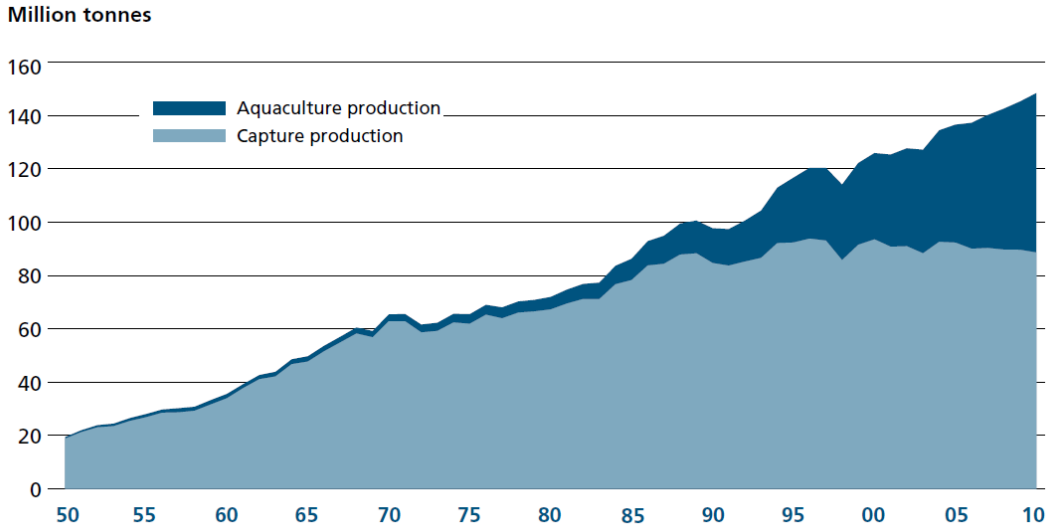

Figure 5. World capture fisheries and aquaculture production, from FAO (2012).

Saline water treatment by halophytes combined with saline agriculture

The potential of halophytes as crop plants and their applicability as plant biofilter for nutrient-rich saline effluents suggest the possibility to combine both qualities. Then, the nutrients are not just removed from the water but a recycling of nitrogen and phosphorus takes place due to the incorporation of the nutrients into valuable biomass. A possible combination is the

application of a constructed wetland planted with halophytes to treat effluents from saline aquaculture before their release to the environment or municipal water treatment and the simultaneous production of valuable plant biomass marketable as vegetables (Webb et al., 2012; Shpigel, et al., 2013). The composition of marine aquaculture effluents seems to be very promising for the application of a halophyte biofilter with simultaneous production of a valuable co-product due to the presence of plant-available nitrogen and phosphorus as described above. Additionally, there is no contamination of the effluents with heavy metals and harmful organic compounds, at least in various recirculating aquaculture systems (RAS). This is advantageous because substances taken up by the plant as biofilter could possibly be harmful for consumption if plant parts are also used as valuable co-product (Rattan et al., 2005; Muchuweti et al., 2006).

Beside the treatment of effluents from open aquaculture systems that release effluents after a certain time of water retention in the system, there is also the possibility to include a plant culture as biofilter and generation of a valuable co-product into RAS. In a RAS water circulates between the culture basin and different system-dependent mechanical, physical and microbiological filters reducing the amount of suspended solids, nutrients and microbes resulting in a low water exchange rate (Orellana et al., , 2013). This low water exchange makes an RAS more environmentally friendly than land-based open systems or cage aquaculture (Martins et al., 2010). A phototrophic component with algae or higher plants can be included into a RAS as additional filter to supplement or even replace other filters in the system (Schneider et al., 2005). Hydroponic plant culture is frequently used for the growth of crop plants in RAS on the base of a setup described by Watten and Busch (1984). The combination of aquaculture with hydroponic crop culture is named aquaponics (Roosta and Hamidpour, 2011). Until now, this approach has only been investigated for freshwater aquaculture (Watten and Busch, 1984; Seawright et al., 1998; Trang and Brix, 2012). Low exchange RAS enhance the sustainability of aquaculture (Martins et al., 2010) and are an important future technology for the growing marine aquaculture sector as well. This makes research on the reuse of resources such as water and nutrients by aquaponic production of halophytic crops in marine RAS important for future sustainable food production.

Although the constitution of marine aquaculture effluents seems to be promising their suitability for halophytic crop production has to be studied in more detail. Additionally, research on the efficiency of halophyte biofilters and the simultaneous production of a valuable product should be also open to the application of other types of saline wastewater apart from marine aquaculture effluents, for example saline field runoffs. In general, for a

combination of halophytic crop production and the use of the plants as biofilter it is important to investigate, (i) appropriate culture conditions to produce valuable biomass, (ii) appropriate culture conditions for an efficient biofilter performance, (iii) the suitability of different potential halophytic crop species for the use as biofilter for nutrient-rich saline water and for the production of a valuable co-product.

Outline of the thesis

This thesis includes literature reviews (Chapter 2 and 3) as well as experimental work (Chapter 4 and 5).

Literature review:

- Identification of factors that influence the capacity of a halophyte biofilter used for the purification of marine aquaculture effluents (Chapter 2).
- Definition of open research questions concerning the use of halophytic plants as biofilter for aquaculture effluents (Chapter 2).
- Outline of actual applications for halophytes as biofilter for aquaculture effluents (Chapter 2).
- Analysis of secondary metabolites found so far in halophytic plants with interest for different economic applications (Chapter 3).
- Determination of different methodological approaches in research on secondary metabolites in halophytes to derive conclusions for future work (Chapter 3).

Experimental work:

- Identification of species and ecotypes with high potential for the combined use as biofilter for nutrient rich saline waters and halophytic crop plants (Chapter 4).
- Identification of optimal culture conditions for the combined use of halophytes as biofilter for nutrient rich saline waters and crop plants (Chapter 4).
- Determination and comparison of the efficiency of selected halophyte species as biofilter for nitrogen and phosphorus in a pilot scale zero exchange marine recirculating aquaculture facility (Chapter 5).
- Evaluation of the potential of selected halophyte species as valuable co-product for marine aquaculture (Chapter 5).

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Chapter 2

Buhmann, A., Papenbrock, J., 2013a. Biofiltering of aquaculture effluents by halophytic plants: Basic principles, current uses and future perspectives. Environmental and Experimental Botany 92, 122–133.

Environmental and Experimental Botany 92 (2013) 122–133

Contents lists available at SciVerse ScienceDirect

Environmental and Experimental Botany

journal homepage: www.elsevier.com/locate/envexpbot

Biofiltering of aquaculture effluents by halophytic plants: Basic principles, current uses and future perspectives☆

Anne Buhmann, Jutta Papenbrock*

Institut für Botanik, Herrenhäuserstr. 2, D-30419 Hannover, Germany

ARTICLE INFO

Article history:
Received 7 February 2012
Received in revised form 14 June 2012
Accepted 11 July 2012

Keywords:
Constructed wetlands
Halophytes
Hydroponic systems
Marine aquaculture effluents
Nutrients

ABSTRACT

Halophytes comprise a promising group of plants for different applications due to their special physiological characteristics and biochemical composition. Their ability to grow in salt-affected habitats makes them useful for recycling the nutrient-containing effluents from saline aquacultures. The potential of different halophytes for nutrient uptake and remediation has been investigated in several laboratory and field studies and the application of natural and constructed wetlands. Various factors influence the filtration capacity of a halophyte biofilter for aquaculture effluents, such as salinity, flooding, nutrient level, root characteristics and technical applications. Those effects studied so far are characterized and those in need of further study are outlined. Technical aspects in artificial wetlands such as water flow direction, water level, hydraulic retention time and hydraulic loading rate, influence the transformation of the nutrients within the wetland and their uptake by the plants. Open as well as re-circulating systems are considered. Because soil processes are lacking, the application of hydroponic culture shifts the importance of nutrient removal toward plant uptake. This is important when besides the pure nutrient removal the recycling of the nutrients become a focus in terms of sustainability. The economic feasibility, including different utilization possibilities, of selected halophytes with filtering capacities is delineated. The economic attractiveness of a halophytic biofilter can also be upgraded by the use of salt-tolerant species with a commercial value. Modularized versions of waste water treatments by plants in temperate and tropic regions could help to reduce the nutrient load in the bodies of water and to recycle the nutrients. More effort is needed to determine the specific nutrient removal mechanisms within different types of wetlands planted with halophytes and to point out appropriate halophyte species and wetland conditions for different applications.

1. Introduction

Aquaculture effluents contain various substances that can, on the one hand, be very harmful for the cultured organism, environment and mankind but on the other hand valuable when used adequately. Because of excess feed supply and excrements of the cultured organism aquaculture effluents contain a lot of nitrogen (N) and phosphorus (P) compounds in organic and inorganic form (Briggs and Funge-Smith, 1994; Hargreaves, 1998). Ammonia and nitrite are toxic for the cultured marine fish and organisms in the surrounding environment, with LC_{50} values starting at 0.5 mg NH_3–N l^{-1} (Hargreaves, 1998; Tilley et al., 2002). Nitrate is toxic for aquatic organisms only at higher concentrations, with maximum values of 2 mg NO_3–N l^{-1} and 20 mg NO_3–N l^{-1} suggested for sensitive freshwater species and marine organisms, respectively (Camargo et al., 2005). If the effluents leave the aquaculture facilities unfiltered, nitrate and phosphate cause hypertrophication of adjacent ecosystems. Nitrate is assumed to be the limiting nutrient in most aquatic ecosystem (Day et al., 1989); in marine aquatic ecosystems phosphate is thought to be the limiting nutrient (Sundareshwar et al., 2003). Therefore high nitrate as well as phosphate concentrations can cause algal blooms in ecosystems affected by aquaculture effluents. Apart from dissolved inorganic nutrients, aquaculture effluent often contains high amounts of organic solids in settled, suspended and dissolved form. Suspended solids damage the gills of cultured organisms and ammonia is produced during mineralization (Chen et al., 1993). The degradation of organic solids results in a high biological oxygen demand and together with

☆ *Annotation:* All vascular plant species mentioned in this review are classified into one of four different categories of salt tolerance and marked with superscript numbers from 1 to 4: [1]Category 1: 0–25% seawater, 0–150 mM NaCl, [2]Category 2: 26–50% seawater, 151–299 mM NaCl, [3]Category 3: 51–75% seawater, 300–449 mM NaCl, [4]Category 4: 76 to ≥100% seawater, 450 to ≥599 mM NaCl. The categories allow estimating the salt tolerance of a certain species and do not express the physiological or ecological salt.

* Corresponding author at: Institut für Botanik, Leibniz Universität Hannover, Herrenhäuserstr. 2, D-30419 Hannover, Germany. Tel.: +49 511 762 3788; fax: +49 511 762 19262.
E-mail address: Jutta.Papenbrock@botanik.uni-hannover.de (J. Papenbrock).

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http://dx.doi.org/10.1016/j.envexpbot.2012.07.005

the oxidation of inorganic compounds in a high chemical oxygen demand (Cao et al., 2007). Other critical groups of aquaculture effluents are toxic organic compounds, heavy metals and antibiotics which will not be discussed here.

The identification of environmental problems caused by aquaculture effluents and the implementation of governmental restrictions encouraged engineers to develop various types of technical filters and treatments for aquaculture effluents. However, these applications are cost intensive and often require special knowhow for correct utilization. As an alternative, natural and constructed wetlands have been suggested to be a cost and work effective possibility to filter aquaculture effluents (Brown and Glenn, 1999; Lin et al., 2003). For municipal and industrial wastewater as well as freshwater aquaculture, the use of biofilters and constructed wetlands planted with glycophytes has been established (Kadlec and Knight, 1996). Less work has been done on the application of halophytes and saline wetlands as filter for saline aquaculture effluents. There is an increasing importance of aquaculture in supplying mankind with seafood-derived proteins worldwide (FAO, 2010). The rising importance of marine aquaculture challenges a sustainable handling of the generated effluents and its constituents.

2. Nutrients in aquaculture ponds and wetlands

To understand the possible contributions of halophytic macrophytes to filter the process water of marine aquaculture systems it is important to understand the biogeochemistry of nutrients such as N and P in aquaculture ponds and wetlands. In aquaculture production systems there is an accumulation of nutrients, especially in semi-closed or closed recirculating systems where little or no water is exchanged. Most of the accumulated nutrients such as compounds of N and P reach the water due to unmetabolised or excess feed (Lemarié et al., 1998; Hargreaves, 1998). The cultured organisms convert about 10–35% of the N and P into biomass; the rest is excluded as dissolved organic or inorganic as well as particulate nutrients into the aquaculture pond (Holby and Hall, 1991; Briggs and Funge-Smith, 1994; Shimoda et al., 2007). Concerning N, a substantial load is excreted into the water in the form of ammonium, as the end product of protein catabolism of the cultured organism. Another considerable load is organically bound N as constituent of feces and excess feed which sink to the bottom of the pond (Hall et al., 1992; Hargreaves, 1998).

In natural wetlands the most abundant form of N is sediment organic N accounting for 80–90%. Plant-incorporated N and inorganic N represent another significant fraction (Vepraskas and Faulkner, 2001). The main transformation processes concerning N in wetland soils include ammonification followed by nitrification and denitrification (Maltais-Landry et al., 2009). Because wetland soils are typically predominated by anaerobic conditions apart from a thin upper layer of a few centimeters denitrification is promoted in the presence of nitrate. Ammonification and nitrification are bound to a thin upper layer of the wetland, where oxygen from the atmosphere or produced by microalgae diffuses into the covering water and the pore water of the soil, and oxygenated zones around roots, where oxygen generated by the plants is released to the soil in the presence of aerenchyma. Ammonification also takes place under anoxic conditions, but with a lower conversion rate (Vepraskas and Faulkner, 2001).

P gets into aquaculture ponds mainly as organically bound phosphate as constituent of feces and excess feed where it is dissolved in the pond water or sinks to the ground (Holby and Hall, 1991). The percentage of organic matter accumulation, adsorption to the sediment, dissolving and precipitation in the fate of phosphate compounds depends on the type of the culture, the constitution of the water and type of the sediment. In a study of phosphate mass balances in a cage culture, P compounds released as solutes to the water column was 25–30%, sedimentation 50–56%, accumulation in the sediment 47–54% and release from the sediment 2–4% (Holby and Hall, 1991). Ashraf and Pillai (2003) studied different pond cultures of shrimp and found that all added phosphates were adsorbed within the soil (to clays, organic and inorganic complexes) building soluble and insoluble complexes with Fe-, Al-, and Ca-containing compounds. Concerning the application of halophytes as biofilter for aquaculture effluents it is necessary to predict the plant available phosphate in a certain system. This is not jet possible on the base of published data. Therefore there is a need to study the fate of P in different types of marine aquaculture ponds in more detail.

Most of the P (80–90%) in wetland soils occurs in the form of organic phosphate in the sediment and in the form of fixed mineral phosphate (Vepraskas and Faulkner, 2001). Another part of the P is incorporated in plants and just a small part is present in the form of orthophosphate in the pore water of the soil. Because, contrary to the case with N, the valence of P does not change in the wetland soil its cycling is rather dependent on the pH and the form that phosphate enters the wetland rather than on the redox potential (Nguyen, 2000). However, the redox potential accounts for the dissolution of phosphate from bonding to certain inorganic or organic substances, such as to the ferric iron Fe^{3+} (Vepraskas and Faulkner, 2001; Álvarez-Rogel et al., 2007).

3. Biofiltration of aquaculture effluents by halophytes

Commonly, biofilters, including natural and constructed wetlands, are used for the treatment of municipal wastewaters. The application of treatment wetlands for wastewaters from industry (such as petrochemical, textile, paper, food and mining industry), agricultural wastewaters like dairy and fish farm effluents and runoff waters (for example from highways, airports, landfills and storms), gained in importance over the recent decades (Vymazal and Köpfelová, 2008). Schwartz and Boyd (1995) analyzed a constructed wetland planted with *Schoenoplectus californicus*[1] (C.A.Mey.) Soják, syn. *Scirpus californicus* (C.A.Mey.) Steud., *Zizaniopsis miliacea*[1] (Michx.) Döll & Asch. and *Panicum hemitomon*[2] Schult. which removed the nutrients from a catfish pond successfully. Schulz et al. (2003) found a constructed wetland planted with *Phragmites australis*[2] (Cav.) Trin. ex Steud. to purify the effluents from a rainbow trout culture sufficiently. Because there is an increasing demand for seafood with a simultaneous decrease of natural stocks and eligible coastal areas, inland marine aquaculture and therefore the treatment of generated saline effluents becomes more important. A possibility to meet this trend is the development of applicable constructed wetlands planted with halophytic macrophytes. Besides uptake of the nutrients, macrophytes contribute to the filtering effect of a wetland by slowing down the water providing a trap for suspended solids with their root and shoot system (Cronk and Fennessy, 2001). The biological oxygen demand decreases because of the microbial decomposition of the settled organic material. The plants enhance growth of bacteria by providing a large surface area on their widespread roots and a large source of carbohydrates for bacterial consumption.

Different factors influence the capacity of nutrient removal from effluents from marine aquaculture by halophytes. Those factors include salinity, flooding, nutrient level, root system and technical applications as shown in various studies summarized in Table 1. Salinity is a stress factor for many plant species and might inhibit plant growth. Therefore nutrient uptake by the plant may dependent on the salt tolerance of the species. Besides salinity, growth (and therefore plant nutrient uptake) of many species is inhibited by flooding. The nutrient level might influence the filtering effect of

Table 1
Selected publications concerning halophytes as biofilter for aquaculture effluents with different conditions and nutrient uptake rates. [1–4]Salt tolerance of a plant species ([1]no salt tolerance: 0–1% seawater, 6 mM NaCl, [2]low salt tolerance: 2–49% seawater, 6–299 mM NaCl, [3]medium salt tolerance: 50–99% seawater, 300–598 mM NaCl, [4]high salt tolerance: ≥100% seawater, ≥599 mM NaCl), CW: constructed wetland, TN: total nitrogen, IN: inorganic nitrogen, P: phosphate, TP: total phosphate, RAS: recirculating aquaculture system. The salinity of the effluent differs partly from the salinity at the outflow of the wetland due to evapotranspiration and precipitation, but is not named in all studies; given outflow salinities: Sansanayuth et al. (1996): 39.7–41.2 g l^{-1}, Brown et al. (1999): 1.6, 28.6 and 84.7 g l^{-1}, Sousa et al. (2011): 21.3 and 22.5 g l^{-1}.

Publication	Place	Species	Type of CW	Substrate	Type of effluent (culture)	Salinity in % seawater	Concentration in the effluent in mg l^{-1}			Removal efficiency in %		
							TN	IN	P	TN	IN	P
Sansanayuth et al. (1996)	Thailand	*Acrostichum aureum*[4]	Subsurface flow CW	Gravel	Shrimp	100[b]	4.42–4.83	–	0.15–0.17	53.80–61.30	–	43.80–76.50
Brown et al. (1999)	Arizona (greenhouse)	*Suaeda esteroa*[4] *Salicornia bigelovii*[4] *Atriplex barclayana*[2]	Drainage lysimeters	Washed river sand	Tilapia (intensive)	1, 29, 100[b]	77.22	13.02[c]	25.00 (TP)	94.40–99.09	45.40–97.94	98.95–99.74 (TP)
Rivera-Monroy et al. (1999)	Colombia	–	Natural mangrove	–	Shrimp	65[b]	–	0.10[c] ($NH_4 + NO_2 + NO_3$)	–	–	37.04[c] ($NH_4 + NO_2 + NO_3$)	–
Fancy (1999)	Thailand	*Avicennia marina*[4] *Rhizophora mangle*[4]	Replanted natural mangrove	–	Shrimp	–	–	1.41[c]	–	–	46.71[c]	–
Lin et al. (2003)	Taiwan	*Phragmites australis*[2]	Free surface flow CW, subsurface flow CW	Local soil, river gravel	Shrimp (RAS)	–	–	0.67[c]	8.45 (PO_4–P)	–	68.06[c]	5.40 (PO_4–P)
Klomjek and Nitisoravut (2005)	Thailand	*Typha angustifolia*[2] *Cyperus corymbosus*[1] *Brachiaria mutica*[2] *Digitaria bicornis*[2] *Chrysopogon zizanioides*, syn. *Vetiveria zizanioides*[3] *Spartina patents*[4] *Leptochloa fusca*[2] *Echinodorus cordifolius*[2]	Free surface flow CW[a] (5 d water maintenance, 2 d drying)	Mixture of soil and sand	Municipal wastewater (addition of NaCl)	6–26[b]	–	18.79–19.51 (NH_3–N)	3.93–4.40 (TP)	–	67.40–76.50 (NH_3–N)	28.90–44.90 (TP)

Lymbery et al. (2006)	Australia	*Juncus kraussii*[3]	Horizontal flow CW	Sand	Rainbow trout (addition of NaCl)	19–71[b]	7.30–29.67	–		1.60–12.70	69.00	–	88.50
Primavera et al. (2007)	Philippines	*Avicennia*[4] spp.	Natural mangrove with additionally planted mangrove seedlings	–	Shrimp	43–91[b]	–	3.22[c]		–	–	19.93[c]	–
		Bruguiera cylindrica[4] *Ceriops decandra*[3] *Ceriops tagal*[4] *Rhizophora mucronata*[4] *Xylocarpus granatum*[4] *Nypa fruticans*[4]											
Sousa et al. (2011)	Brazil	*Spartina alterniflora*[4]	Vertical flow CW	Crushed oister shells and beach sand	Shrimp (postlarvae)	57[b]	5.20	3.37[c]		1.57 (PO_4–P)	51.00	71.94[c]	64.00 (PO_4–P)

[a] Type of wetland defined by authors of the review.
[b] Unit different from the published one.
[c] Calculated from published data.

a plant because different species might have different optimal concentrations for the nutrients influencing the quantity and efficiency (quantity per time unit) of nutrient uptake. The root characteristics of different plant species in a wetland can influence the filter capacity by providing a trap for suspended solids and a large surface area for microorganisms in different qualities on the one hand. On the other, halophyte species differ in presence and specificity of aerenchyma which can influence the presence of oxygenated zones within the soil and therefore the growth of certain bacteria and processes such as ammonification and nitrification. Technical applications such as the volume of effluent applied to the wetland in a certain time influence factors such as flooding and nutrient level (see Table 1 for references). Table 1 shows some of the results published concerning halophytes (including mangroves) as biofilters for aquaculture effluents naming nutrient uptake rates and some selected factors of importance. However, Table 1 is more an overview than a basis for the quantitative comparison of the results of the different publications as the studies differ greatly in the conditions applied. Often just one system or the influence of one parameter on nutrient removal is investigated in one constructed wetland system which makes it difficult to determine the important factors for the nutrient removal in general.

Brown et al. (1999) found that salt inhibited the nutrient removal capacity of *Suaeda esteroa*[4] Ferren & S.A. Whitmore, *Salicornia bigelovii*[4] Torr., and *Atriplex barclayana*[2] (Benth.) D.Dietr. In the experimental set up with irrigated drainage lysimeters using effluents from intensive tilapia culture 9, 171 and 599 mM NaCl (0.5, 10 and 35 ppt) were applied. The nutrient uptake was reduced to about one half at 171 mM NaCl and one third at 599 mM NaCl in comparison to 9 mM NaCl indicating that the removal of N and P in the soil–plant system was more effective at lower salinities (9 and 171 mM NaCl). In a study investigating the behavior of *Juncus kraussii*[3] Hochst. in a subsurface-flow constructed wetland (Lymbery et al., 2006) salinity had a negative effect on the removal of phosphate but not on the removal of nitrate. Despite the reduction of effectiveness, nutrient removal was evident even at high salinities for both constructed wetland systems. Removal of 62% of total N and 76.5% of total phosphate (Lymbery et al., 2006) and 99% of total N and total P (Brown et al., 1999) were reached at salinities of 411 mM NaCl (24 mS/cm) and 599 mM NaCl (35 ppt), respectively. Constructed wetlands exposed to higher salt concentrations showed a lower nitrate removal but a higher plant N content, maybe a physiological adaption to salt stress by storing N-containing osmoregulative compounds (Brown and Glenn, 1999). The results of the studies show that even though a halophyte species is tolerant to the salinity level in an effluent, the filter capacity can be reduced. Halophytes might tolerate certain salinities, but their optimal salinity in terms of biomass production often lies far below (Flowers and Colmer, 2008). Plant growth directly influences nutrient uptake. Therefore nutrient uptake can be negatively correlated with salinity even for halophytes being tolerant to seawater salinity. Halophytes show different sensitivity toward salinity depending on the plant species. Therefore we assigned all plant species mentioned in this review to four different categories of salt tolerance (see annotation at the beginning of the review and Table 2). Salinity and the sensitivity of the plant species used are important factors influencing the filtering effect of a wetland.

Salt tolerance could be an important factor for the choice of the right plant species as biofilter for a specific marine organism in culture. Marine aquaculture covers a wide range of fish and shrimp species and developmental stages that have different requirements for the salt concentration in the culturing water concerning inland and low-salinity aquaculture (Forsberg and Neill, 1997; Atwood et al., 2003). There again different species applied as biofilter for saline effluents show different preferences for salt concentrations. Brown et al. (1999) found that *Atriplex barclayana*[2] showed less biomass production and nutrient uptake at high salinities than *Suaeda esteroa*[4] and *Salicornia bigelovii*[4] as it was less salt tolerant than the two succulent salt-marsh species, although *Atriplex barclayana*[2] and *Suaeda esteroa*[4] performed much better than *Salicornia bigelovii*[4] at lower salinities. Lymbery et al. (2006) found that the application range of *Juncus kraussii*[3] is restricted to effluent salinities below 342 mM NaCl (20 g l^{-1}). Therefore, it is crucial to decide for the adequate species for a certain salt concentration in the aquaculture effluent. Also dilution due to precipitation can play a role in the formation of species composition in a wetland serving as biofilter. In a mesohaline wetland with a seawater salinity of 9–23% (3–8 ppt) constructed to filter effluents from a shrimp farm despite the plantation of various and more salt tolerant species the less salt tolerant species *Typha latifolia*[2] became the most abundant species due to low salinities in the wetland (Tilley et al., 2002).

Besides salinity, nutrient level and irrigation volume can have an important effect on the filtering capacity of halophytes. Lymbery et al. (2006) generated two different nutrient levels using the filtrate of a rainbow trout farm with the high levels of N and P in the water about five times above the low level. Removal of total N was 69.0% and 12.5% and removal of total phosphate was 88.5% and 53.3% for high and low nutrient level respectively. Therefore total N and total P were removed more effectively at higher nutrient levels. Brown and Glenn (1999) found that the growth of *Suaeda esteroa*[4] increased with increasing irrigation volume. The inorganic N in soil and water leaving the lysimeters decreased with increasing irrigation volume whereas the concentration of soluble reactive P in the leaching water increased with increasing irrigation volume. Brown and Glenn (1999) suggest that these contrary results for N and P are explicable with plants playing a lesser role in the removal of P than in the removal of N, with most of the P removal in the wetland being due to binding processes in the soil.

Hegedűs et al. (2010) conducted an experiment using a gravel-bed hydroponic mesocosm, simulating parts of the natural environment under controlled conditions, supplied with effluent from a catfish farm with a seawater salinity of 3% (1357 μS cm^{-1}). They found that *Phragmites australis*[2], *Typha angustifolia*[2] L., *Glyceria maxima*[1] (Hartm.) Holmb. and *Schoenoplectus tabernaemontani* (C.C.Gmel.) Palla, syn. *Scirpus lacustris* subsp. *tabernaemontani* (C.C.Gmel.) Syme performed much better in terms of growth, health and uptake of N and P than *Tripolium pannonicum*[3] (Jacq.) Dobrocz., syn. *Aster tripolium* L., *Bolboschoenus maritimus*[2] (L.) Palla, syn. *Scirpus maritimus, Triglochin palustris*[2] L. and *Carex vulpina*[2] L. All species studied are naturally inhabitants of saline areas. The better-performing species were not only assumed to have a higher tolerance toward salt but also toward prolonged flood condition and deposition of organic waste. Flooded soils are hypoxic or anoxic depending on the soil depth concerned and the length of the inundation. Plants tolerant to flooding show various morphological adaptations such as aerenchyma and adventives roots as well as metabolic adaptations. Metabolic adaptations related to anaerobic metabolism where less ATP is produced, the pH in the cytoplasm declines and ethanol is produced which usually leads to a drastic reduction of cell metabolism and destruction of cells due to acidification and intoxication (Cronk and Fennessy, 2001). In a series of studies to evaluate constructed wetlands with different macrophytes in their efficiency to purify saline wastewater *Typha angustifolia*[2] and *Digitaria bicornis*[2] (Lam.) Roem. & Schult. were the best performing species (Klomjek and Nitisoravut, 2005). But *Cyperus corymbosus*[1] Rottb., *Brachiaria mutica*[2] (Forssk.) Stapf, *Spartina patens*[4] (Aiton.) Muhl. and *Leptochloa fusca*[2] (L.) Kunth also survived and enhanced the treatment performance of the wetland supplied with municipal wastewater. *Echinodorus cordifolius*[2] (L.) Grieseb. and *Chrysopogon zizanioides*[3] (L.) Roberty, syn. *Vetiveria zizaniodes* (L.) Nash died during the experiment presumably due to stress caused by prolonged high saline flooding.

Table 2
Species and their level of salt tolerance. All vascular plant species mentioned in this review are classified into one of four different categories of salt tolerance and marked with superscript numbers from 1 to 4: [1]Category 1: 0–25% seawater, 0–150 mM NaCl, [2]Category 2: 26–50% seawater, 151–299 mM NaCl, [3]Category 3: 51–75% seawater, 300–449 mM NaCl, [4]Category 4: 76 to ≥100% seawater, 450 to ≥599 mM NaCl. The categories allow estimating the salt tolerance of a certain species and do not express the physiological or ecological salt optima.

Species, level of salt tolerance and publication	
Category 1: 0–25% seawater, 0–150 mM NaCl	
Atriplex triangulars, syn. *Atriplex prostrata*	Loveland and Ungar (1983)
Cyperus corymbosus	Cantero et al. (1998)
Glyceria maxima	Engels et al. (2011)
Kosteletzkya virginica	Blits and Gallagher (1990)
Schoenoplectus californicus, syn. *Scirpus californicus*	Watson and Byrne (2009)
Schoenoplectus tabernaemontani, syn. *Scirpus lacustris* spp. *tabernaemontani*	Bouzillé et al. (2001)
Zizaniopsis miliacea	Welch and Kitchens (2006)
Category 2: 26–50% seawater, 151–299 mM NaCl	
Atriplex barclayana	Nerd and Pasternak (1992)
Bolboschoenus maritimus, syn. *Scripus maritimus*	Lillebø et al. (2003)
Brachiaria mutica	Dangar (2005)
Carex vulpina	Grigore and Constantin (2010)
Crithmum maritimum	Hamed et al. (2004)
Digitaria bicornis	Klomjek and Nitisoravut (2005)
Distichlis spicata	Kemp and Cunningham (1981)
Echinodorus cordifolius	Calheiros et al. (2012)
Excoecaria agallocha	Joshi and Ghose (2003)
Leptochloa fusca	Bhatti et al. (1983)
Panicum hemitomon	Hester et al. (1998)
Phragmites australis	Lissner and Schierup (1997)
Plantago coronopus	Koyro (2006)
Portulaca oleracea	Grieve and Suarez (1997)
Sporobolus virginicus	Marcum and Murdoch (1992)
Triglochin palustris	Chang et al. (2001)
Typha angustifolia	McMillan (1959)
Typha latifolia	McMillan (1959)
Category 3: 51–75% seawater, 300–449 mM NaCl	
Bruguiera cylindrica	Parida and Das (2004)
Ceriops decandra	Ball (2001)
Chrysopogon zizanioides, syn. *Vetiveria zizaniodes*	Nanakorn et al. (1998)
Juncus kraussii	Naidoo and Kift (2006)
Lumnitzera racemosa	Wakushima et al. (1994)
Tripolium pannonicum subsp. *tripolium*, syn. *Aster tripolium*	Shennan et al. (1987)
Category 4: 76 to ≥100% seawater, 450 to ≥599 mM NaCl	
Acrostichum aureum	Medina et al. (1990)
Avicennia marina	Clough (1984)
Nypa fruticans	Ukpong (1991)
Rhizophora mangle	Ukpong (1991)
Rhizophora mucronata	Khan and Aziz (2001)
Salicornia bigelovii	Glenn et al. (1991)
Salicornia europaea	Moghaieb et al. (2004)
Spartina alterniflora	Hester et al. (1998)
Spartina anglica	Rozema and Van Diggelen (1991)
Spartina patens	Hester et al. (1996)
Suaeda esteroa	Sleimi and Abdelly (2002)
Xylocarpus granatum	Paliyavuth et al. (2004)

There is also the possibility of using established crop plants as a biofilter; species that are not halophytes but show some tolerance to low salinity conditions. For example, Jerusalem artichoke has been successfully irrigated with saline aquaculture wastewater mixed with brackish groundwater water with a seawater salinity of 23% (11.4 dS m^{-1}). Also melon has been successfully irrigated with low salinity shrimp farm effluents without any impact on yield and fruit quality (Miranda et al., 2008; Gengmao et al., 2010). The disadvantage of the use of those crops as biofilter for saline aquaculture effluents is the limitation to effluents with low salinities or the need to dilute highly saline effluents.

With their roots spreading into the wetland sediment, some halophytes can contribute to both surface enlargement and oxygen supply to otherwise anoxic surroundings. Maltais-Landry et al. (2009) investigated the influence of constructed aeration and plantation of macrophytes on the treatment of the effluent from a trout farm, N uptake by plants was increased by 48–128% probably due to oxygenation of the soil. Sousa et al. (2011) also showed that oxygenation of the soil increased N removal but contrarily to Maltais-Landry et al. (2009) wetlands planted with the halophyte *Spartina alterniflora*[4] Loisel. had a lower oxygen level than unplanted ones. This result might be due to a larger atmospheric diffusion at the atmosphere-water-interface for the unplanted treatments and algae-growth elevating oxygen concentration by photosynthetic activity and that shading of the surface in the plant-treatments lowered this oxygen donating influences. A slightly higher removal rate for phosphate in the planted as in the unplanted treatments was explained by the uptake of phosphate by the plants and by the growth enhancement of anaerobic heterotrophic bacteria consuming phosphate. Therefore the abundance of oxygen in the pore water of a wetland soil plays an important role in nutrient removal.

Planted wetlands can contribute to the removal of suspended solids and biological oxygen demand with removal rates of 72–79% for biological oxygen demand and 43–56% for suspended solids for the different plant species mentioned before, with *Digitaria bicornis*[2] performing best (Klomjek and Nitisoravut, 2005). A wetland planted with *Phragmites australis*[2] filtering effluents from a shrimp aquaculture removed 24% of the biological oxygen demand and 71% of the suspended solids (Lin et al., 2003). Sousa et al. (2011) found removal rates of 89% and 71% for inorganic solids and of 82% and 96% for organic solids for wetlands planted with *Spartina alterniflora*[4] and unplanted wetlands, respectively. The authors explain the higher removal rates of organic solids with a higher level of oxygen in the unplanted treatment and therefore a higher rate of organic matter mineralization. They suggest the higher levels of oxygen in the unplanted wetlands to be due to a larger atmosphere–water interface allowing gas diffusion and to a stronger growth of algae and generation of oxygen by photosynthesis. These results challenge the role of halophytes within a wetland in the removal of biological oxygen demand and suspended solids and further research is needed.

The results for the filtering effect of different halophytes could be due to different technical applications in the experiments such as types of wetland, hydraulic retention times or types of sediment. Natural wetlands often have a higher degree of species diversity, plant density, plant age and spatial diversity of soil characteristics (Cronk and Fennessy, 2001). In the application of a constructed wetland the individual demand for performance characteristics can be controlled more easily (Vymazal and Köpfelová, 2008). Vymazal (2007) compared different types of constructed wetlands concerning their potential to remove nutrients. Free water surface constructed wetlands showed a higher ammonia volatilization due to a higher surface–atmosphere-interface

whereas subsurface-flow constructed wetlands showed a higher ammonia adsorption and phosphate sorption due to a more efficient effluent-to-substrate contact. Due to a high oxygenation subsurface constructed wetlands with vertical flow showed high conversion rates of ammonia to nitrate but low removal of nitrate (due to inhibition of denitrification). Free water surface constructed wetlands and subsurface constructed wetlands with horizontal flow showed higher removal rates for nitrate (due to anaerobic conditions in the soil enhancing denitrification). Nutrient uptake of plants was proportionately more important in free-floating plants constructed wetlands because there was a lack of the soil processes. Vymazal (2007) came to the conclusion that a combination of different types of constructed wetlands is more effective because the amount of nutrient removing processes in a single type of constructed wetland is limited. Lin et al. (2002a,b, 2003, 2005, 2010) successfully combined a free water surface and a subsurface flow constructed wetland for the treatment of effluents from milkfish and from shrimp aquaculture. The efficiency of different wetlands as biofilter depends on the type of substrate because it influences adsorption and precipitation abilities and in particular sorption of phosphate, oxygenation and pH of the soil. Also the hydraulic loading rate and the hydraulic retention time have to be taken into account. The hydraulic loading rate is commonly expressed as the amount of effluent applied to a wetland per square meter per day. Gao et al. (2011) found the removal rate of chemical oxygen demand, ammonia N and total N of a constructed wetland with emergent plants to be reduced when the hydraulic loading rate exceeded 1 $m^2\,day^{-1}$. Also the pollutant loading rate defined by the hydraulic loading rate multiplied by influent concentration is an important parameter (Lin et al., 2003). The length of time that a pollutant remains in a constructed wetland is specified by the hydraulic retention time.

In the application of wetlands as biofilter for aquaculture effluents open systems where pond water is exchanged regularly and recirculating aquaculture systems where process water of the aquaculture passes a filtering system and then goes back to the culturing pond need to be differentiated. Disposal of water is less (semi-closed system) or close to zero (closed system) in recirculating aquaculture compared to open systems which might result in higher hydraulic loading rates, higher pollutant loading rates and lower hydraulic loading time due to higher stocking densities resulting in higher nutrient concentrations and the necessity to reuse the water.

We assume based on the results summarized in Table 1 and on our own results (see chapter 5) that there are two crucial points for designing a productive, well functioning wetland with good biofiltering activities and gain in economically meaningful biomass: (a) selection of suitable well characterized plant species (ecotype, climatic adaptation, plant age, plant health, salinity tolerance and others) and (b) optimal technical construction of the wetland for this particular plant species to assure, perfect supply of growth-influencing parameters (light, temperature, watering, macro- and micronutrients and others). Substrate of the wetlands and the fish or shrimp species in the primary circuit including their feces do not seem to influence the gain in biomass significantly. Many approaches to construct wetlands appear too much directed by and focusing on engineering aspects and too less on providing conditions for optimal plant growth and health.

4. Mangroves as biofilter for aquaculture effluents in the tropics

In tropical countries the main activity in marine aquaculture is shrimp culturing with an increasing importance (Robertson and Phillips, 1995; de Graaf and Xuan, 1998). Usually shrimp aquaculture at tropical coastlines comes along with extensive mangroves clearance (Robertson and Phillips, 1995; de Graaf and Xuan, 1998; Valiela et al., 2001), as mangroves are the natural vegetation of many tropical coastlines (Gautier et al., 2001). Despite the intensification of the culture systems over the last decades (Robertson and Phillips, 1995; Bachére, 2000) many tropical shrimp farms suffer severe yield losses due to diseases and some sites have to be abandoned because of diseases, salinization and contamination of the soil (Stevenson, 1997; de Graaf and Xuan, 1998; Gräslund et al., 2003). The nutrient-rich effluents from shrimp aquaculture cause hypertrophication of adjacent ecosystems such as seagrass meadows and coral reefs (Páez-Osuna, 2001). Ironically, many problems linked with shrimp production are related to the clearances of mangroves (Gautier et al., 2001; Lewis et al., 2003). Besides traditional usage such as collection of wood, cutting of nutritional and medicinal parts of plants and capture of crustaceans and fish, mangroves provide service in coast protection against erosion, flooding and storms (Mazda et al., 2002; Kathiresan and Rajendran, 2005; Alongi, 2008), have a high importance in the preservation of biodiversity, and play an important role in climate protection because of their high productivity (Clough et al., 1983). Therefore the idea of a sustainable shrimp aquaculture with the integration of mangroves as natural biofilter has been developed. Traditionally the mangrove ecosystem has been considered as tolerant dumping side for wastes and effluents and some studies about the use of mangrove sides as natural filter for municipal wastewater have been carried out (Nedwell, 1975; Clough et al., 1983; Boto and Wellington, 1988; Tam and Wong, 1993; Wong et al., 1995; Chu et al., 1998). Enhanced growth of mangroves was observed due to the addition of nutrients to the ecosystem (Clough et al., 1983; Gautier et al., 2001).

A series of studies on the purification of recirculating aquaculture process water by constructed mangrove wetlands was conducted resulting in a faster growth of shrimp in the treatments with water exchange to ponds planted with mangroves (*Rhizophora*[4] spp.). A decrease of N in the treatments with mangrove wetlands was observed but an increase of phosphate in sediment and in the water in all treatments (Shimoda et al., 2005, 2007). In a study connecting a shrimp culture with a constructed wetland planted with the mangrove fern *Acrostichum aureum*[4] L. the decrease of suspended solids, biological oxygen demand, total organic carbon, total N and total P in this planted wetland was much higher than in control wetland (Sansanayuth et al., 1996). A study with constructed wetlands for the purification of high saline mariculture effluents showed an improved removal of suspended solids, biological oxygen demand and total P due to the plantation of mangrove seedlings (Su et al., 2011). Higher removal efficiencies were achieved at the highest hydraulic retention times (2 days) but higher mass removal was achieved at the lowest hydraulic retention times (0.5 days).

Gautier et al. (2001) found a natural, non-constructed mangrove at the Caribbean coast of Colombia to be sufficient for the removal of suspended solids while water was recirculated between a commercial shrimp farm and the mangrove. Nutrient contents did not increase in the shrimp ponds but increased in the mangrove area due to a bird community in the mangrove. McKinnon et al. (2002) found a higher concentration of bacteria and microalgae in the effluent-influenced part of a mangrove, finally consumed by micro- and mesozooplankton and fish as parts of the natural mangrove community. Both examples show that the fauna can be important for the nutrient budget in a mangrove used as biofilter. For wetlands used as biofilter for aquaculture effluents in temperate zones their function as wildlife habitat is also mentioned (Schwartz and Boyd, 1995). But to our knowledge there is a lack of studies on the related nutrient fluxes. This might be because wetlands in temperate zones are less productive and biodiverse than natural mangroves

and therefore the influence of fauna on the nutrient budget is secondary.

Most of the studies conducted on mangroves as biofilter for shrimp aquaculture effluents deal with open natural wetland systems without recirculation. A natural mangrove biofilter in Thailand consisting of *Avicennia marina*[4] (Forssk.) Vierh. (95%) and *Rhizophora mangle*[4] L. (5%) was found to mineralize organic N and reduce inorganic N compounds derived from a shrimp farm in a satisfying manner (Fancy, 1999). A natural mangrove in the Philippines dominated by *Avicennia*[4] spp. also showed a high removal capacity for nitrate when flushed with shrimp aquaculture effluent at daytime but not at night time (Primavera et al., 2007). They found longer leaflets of *Nypa fruticans*[4] Wurmb and faster growth of mangrove seedlings as support for nitrate uptake of the macroflora within the mangrove biofilter, a loss of N because of denitrification or microfauna uptake was discussed. Rivera-Monroy et al. (1999) investigated the N budget of a natural mangrove in Columbia receiving effluents form three shrimp farms and found it to be an efficient sink for inorganic N. More than 60% of the loss of dissolved inorganic N was found to be due to denitrification. The results on the importance of the denitrification and micro- and macrofauna-uptake as contributors to the biofiltering effect of a mangrove is controversially discussed and might be dependent on the mangrove ecosystem and the constitution of the aquaculture effluent (Shimoda et al., 2005). Rivera-Monroy et al. (1999) related a high ammonia input with high denitrification rates and suggest ammonia enrichment studies on mangroves.

Shrimp pond effluents often contain high amounts of suspended solids (Tilley et al., 2002). Due to their wide root systems mangroves are an effective trap for suspended solids contained in aquaculture effluent (Halide et al., 2003). Gautier et al. (2001) observed a removal rate of suspended solids greater than 90%. But the load of suspended solids from shrimp farms can exceed the tolerance level of the mangrove plants (Ellison, 1998). Besides the discharge of effluents containing suspended solids, the soil of shrimp ponds is often dredged after harvest to avoid contamination of the water in the following culture and dumped into the wetlands nearby. Vaiphasa et al. (2007) related a reduction of plant growth and a higher mortality in a natural mangrove in Thailand to excess sedimentation caused by aquaculture activities. The five-year-record based on quantification of mangrove growth and mortality, remote sensing and sedimentation also found that mangrove species differ in their tolerance toward sedimentation with *Avicennia marina*[4] being more tolerant than *Excoecaria agallocha*[2] L., *Lumnitzera racemosa*[3] Willd. and *Bruguiera cylindrica*[3] (L.) Blume.

Several laboratory studies have been accomplished for different mangrove species to determine nutrient requirements, N source preference and effect of different salinities on nutrient uptake (Naidoo, 1987, 1990; Boto and Wellington, 1988; Kao et al., 2001; Yates et al., 2002; Parida and Das, 2004; Buhmann, 2009). Besides their importance for the understanding of mangrove physiology and the possible application in the fields of mangrove conservation and reforestation, those studies also demonstrate the role of mangrove plants as biofilter. However, laboratory studies often work with mangrove seedlings or young plants and long-term studies are scarce. Mangrove species react differently to nutritional conditions (Yates et al., 2002) probably because mangroves are an ecological not a phylogenetic group and evolved different adaptations to the same environment.

There are several studies on biofilters based on organisms with potential to purify aquaculture effluents (polycultures). These systems demonstrate the possibility to exploit different organisms such as bivalves and seaweed to keep a closed recirculating system stable (Shpigel et al., 1993; Sandifer and Hopkins, 1996; Neori et al., 2004; Neori, 2008), however, acceptance within the fish farmer community is rather low.

Table 3
Recommended wetland to pond area ratios for efficient purification and the basis for their calculation for some studies with mangroves as biofilters for aquaculture effluents, original value published in brackets; N: nitrogen, P: phosphate, IN: inorganic nitrogen, TN: total nitrogen.

Publication	Wetland to pond area ratio	Basis for the calculation
Robertson and Phillips (1995)	2.00–22.00	N- and P-removal
Rivera-Monroy et al. (1999)	0.04–0.12	IN-removal
Fancy (1999)	0.07 (1:14)	IN-removal
Shimoda et al. (2005)	6.20–8.90	P-removal
Shimoda et al. (2007)	2.10–5.20	TN-removal
Primavera et al. (2007)	1.80–5.40	NO_3^--removal

5. Technical and economic feasibility of halophytic biofilters for marine aquaculture effluents in temperate zones

Even though most studies have been carried out on experimental systems there are some investigations on the integration of halophytes into recirculating aquaculture systems at a commercial scale. Lin et al. (2005) coupled a 64 m^2 size commercial-scale recirculating aquaculture system for intensive shrimp culture with a 32 m^2 size wetland. The wetland was a combination of a free water surface and subsurface flow constructed wetland and was planted with *Typha angustifolia*[2] and *Phragmites australis*[2]. The constructed wetland was challenged with high hydraulic loading rates (1.57–1.95 m day^{-1}) but was effective in removing suspended solids, biological oxygen demand, total ammonia and nitrite. Tilley et al. (2002) demonstrated that a two-year-operating 7.7 ha size constructed wetland dominated by *Typha latifolia*[2] L. could effectively reduce total P and suspended solids and maintain biological oxygen demand, ammonia and nitrate at low levels receiving effluents from a 8.2 ha size intensive shrimp farm. The required wetland-to-culture-pond-area ratio was calculated by a model following Kadlec and Knight (1996), giving emphasis on different parameters such as hydraulic loading rate and nutrient removal. From Lin et al. (2005) can be deduced that the wetland-to-shrimp-pond-area ratio needed to treat the effluent from an intensive aquaculture would be 0.096 and Tilley et al. (2002) stated that wetland-to-shrimp-pond-area ratio needed is 0.083. Both studies came to the conclusion that a constructed wetland would be an appropriate filter within a recirculating system of a large-scale intensive aquaculture. Because mangroves as biofilter became interesting for the economically important sector of intensive aquaculture in the tropics there are many calculations on the area of mangrove needed to purify aquaculture pond effluents, summarized in Table 3. The differences between the calculated ratios are probably due to diverse culture practices, constituents in the effluent, characteristics of the biofilter and calculation parameters. More research is needed to generalize conclusions about the optimal wetland-to-pond-area ratio for aquaculture effluent purification. Cardoch et al. (2000) performed a cost economic analysis comparing a conventional on-site treatment with a wetland treatment for a commercial seafood processor. They came to the conclusion that the wetland treatment causes around 75% less of the costs generated by the conventional treatment with savings of over US$1.5 million per year.

The economic attractiveness of a halophytic biofilter can also be upgraded by the use of salt-tolerant species with a commercial value. Brown et al. (1999) tested the feasibility of different halophytes (*Suaeda esteroa*[4], *Salicornia bigelovii*[4] and *Atriplex barclayana*[2]) with potential as forage or oilseed crops as biofilter for saline aquaculture effluents. Halophytes are cultivated as vegetable plants, forage plants and oilseed crops. Comparable with

conventional crops the yield of the most productive halophytes is 10–20 tons ha^{-1} (Glenn and Brown, 1999). Grieve and Suarez (1997) found *Portulaca oleracea*[2] L. to be tolerant for chloride- and sulfate-dominated salinities and a valuable, nutritive crop. *Plantago coronopus*[2] L. has been reported to be a potential cash crop for human consumption. It contains valuable substances such as vitamin A, C and K as well as calcium (Koyro, 2006). Gallagher (1985) tested various halophyte species for their potential as cash crops and picked a few species for further study, such as *Atriplex triangularis*[1] as a spinach-like vegetable, *Kosteletzkya virginica*[1] (L.) C. Presl ex A. Gray as a grain crop and *Sporobolus virginicus*[2] (L.) Kunth, *Distichlis spicata*[2] (L.) Greene and *Spartina patens*[4] as a forage crop for hay production. Swingle et al. (1996) demonstrated that *Suaeda esteroa*[4], *Atriplex barclayana*[2] and *Salicornia bigelovii*[4] can be used as ingredients of lamb diet. O'Leary et al. (1985) and Watson (1990) studied the agricultural production of several *Atriplex*[1–2] species as forage crops. *Salicornia bigelovii*[4] has been proposed as salt-tolerant oilseed crop (Glenn et al., 1991) and studies on the progression of the yield by N-fixing bacteria (Rueda-Puente et al., 2003) and plant-growth promoting bacteria (Bashan et al., 2000) have been accomplished.

Currently, there are various approaches to tap the market for halophytes especially for *Salicornia*[4] spp. as vegetable as well as using halophytes as biofilter and valuable side product for aquaculture wastewater in Europe. The company Serra Maris bvba (Intellicrops, 2009) located in Belgium works on the development of saline crops into profitable crops. Those saline crops include species like *Salicornia*[4] spp. and *Tripolium pannonicum*[3]. In Israel a group of researchers developed a concept to grow and export *Salicornia*[2-4] spp. to the European market (The PERES Center for Peace, 2007). They found a higher biomass production, a higher content of vitamin C, a better taste and less water loss during export occurring at elevated salinity levels in the soil. In the four-years-project they established a year-round production of native *Salicornia*[4] species in semi-commercial conditions producing 9 tons of fresh products on an area of 700 m^2. A study on the effect of seawater concentration on the biomass production and nutritional value of two ecotypes of *Salicornia*[4] and *Sarcocornia*[4] species each, used as crops in Israel foud a deacreasing yield when seawater concentration was increased above 50% (Ventura et al., 2011). But they also found a higher content of antioxidants with increasing seawater concentration and an uneffectedly high fatty acid content in *Salicornia*[4]. In a cooperation project between researchers from Israel and Texas the applicability of *Salicornia bigelovii*[4] as "tool for nutrient removal and source of valuable byproduct" is investigated (Samocha and Klim, 2012). Various investigations are planned for example on the ingredients of the vegetable oil, the possible production of biofuels, the applicability of the plant as biofilter for shrimp culture effluents, the utilization of the seed meal as fodder for shrimp and cattle, selection of ecotypes for enhanced growth, seed production and resistance to insects. The Llyn Aquaculture Ltd in North Wales (United Kingdom) offers design, supply and management of recirculating aquaculture facilities having a pilot commercial farm to test their systems (Llyn Aquaculture, 2008). One of their approaches is to use the effluents from the culture of various marine fish and crustacean species to grow *Salicornia*[4] spp. in constructed wetlands with a maximal output of 10 kg per m^2 per year. They established the scientific basis together with the University of Bangor (Wales, UK) including growth performance of different varieties, techniques for seed collection and germination, appropriate salinity and nutrient levels, and protein and fatty acid content of the product. A report of Alterra (a research institute being part of the Wageningen UR in the Netherlands) discusses the use of halophyte filters to treat saline wastewater and develops a theoretical model of a recirculating system containing constructed wetlands planted with vascular plants and macro-algae species

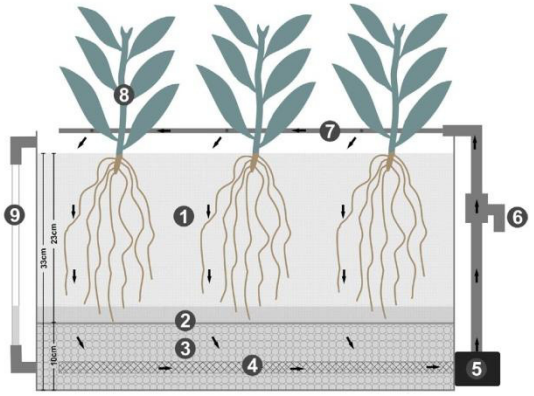

Fig. 1. Diagram of the lysimeters used to test the biofilter capacity of different halophytes and appropriate growth conditions in a greenhouse at the Institute of Botany of the Leibniz University in Hannover, Germany (dimensions: length 60 cm, width 40 cm, height 41 cm). The circulation of the water is indicated by arrows. (1) Layer of sand, (2) cloth to separate the layer of sand from the layer of gravel, (3) layer of gravel, (4) drainage pipe, (5) pump, (6) lockable outlet for water sampling, (7) pipe with holes for the irrigation of the plants, (8) plants, and (9) pipe serving as water-level-indicator and overflow.

(Van der Gaag et al., 2010). They strongly recommend using indigenous species such as *Phragmites australis*[2], *Typha*[2] spp. and *Ulva lactuca* L. to avoid the establishment and further spreading of alien and invasive species. GrovisCo, a company in Tholen (Netherlands), grow turbot (*Scophthalmus maximus*) at a commercial scale (70 t per year, 200 t per year are planned). To meet the governmental restrictions for the wastewater they started to plant the halophyte species *Salicornia europaea*[4] and *Tripolium pannonicum*[3] and successfully sell them as vegetables by now, though the harvesting by hand is expensive (Fischmagazin, 2010). At the port in Rotterdam (Netherlands) the "Happy Shrimp Farm" produces *Penaeus vannamei* using the residual heat of a close-by power station to provide the optimal temperatures for the culturing of the tropical shrimp species (Van der Kaaij, 2007). Besides they grow *Salicornia*[4] spp. using the effluents from the shrimp culture.

Several marine fish species such as *Seriola lalandi* (Yellowtail amberjack), *Dicentrarchus labrax* (European seabass), or *Sparus aurata* (Gilt-head seabream) have potential to be cultivated at a commercial scale. Dependant on the fish species the optimal salt concentration of the process water is different. In a current project we collaborate with three project partners from research institutions and companies with experience in the field of aquaculture and engineering. The aim of the conducted studies is to select the adequate halophyte species of economic value depending on the fish species in culture. The halophytes are to recycle the nutrients generated in the fish culture in terms of biomass production and to contribute to maintain appropriate quality in the process water of the recirculating aquaculture system. To permanently remove nutrients from the system plant biomass is to be harvested frequently and can be used for example as food or base for biofuel production. We first test the biofilter capacity and growth requirements in lysimeters at controlled conditions in a green house at the Institute of Botany, Leibniz University Hannover, Germany (Fig. 1). Then our project partners apply the promising species in a small scale recirculating system. None of the saline water ought to be released to the environment and less than one percent of the water is lost by filtration procedures and evaporation and needs to be replaced by drinking water. The system consists of a 7.1 m^3 indoor basin with *Dicentrarchus labrax*, technical and biological filters of high standard and a 10 m^2-greenhouse with planted lysimeters. The connection of the primary (fish

culture) and the secondary (halophyte culture) circuits was already successfully done. Both fish and plants survive and grow since several months. *Tripolium pannonicum*[3] grows very well at sea water concentrations and could be used as biofilter in *Seriola lalandi* cultures. *Plantago coronopus*[2] is less salt-tolerant. It tolerates salinities up to 1.75% seawater salinity and could be planted in wetlands to treat aquaculture effluents of species such as *Dicentrarchus labrax* that has an optimum of about 1.5% seawater salinity. Those two halophyte species already perform well in small scale recirculating system whereas other species such as *Crithmum maritimum*[2] L., *Spartina anglica*[4] C.E.Hubb. and *Salicornia*[4] spp. are still being tested in preliminary experiments. For some of the halophytes there is already a European market established; other species might need some promotion to become commonly accepted as vegetable, salad or biomass for gas and fuel production(Böer, 2006) For example, *Salicornia*[2-4] spp. is served in several European countries as vegetable (Ventura et al., 2011). *Tripolium pannonicum*[3] is an attractive leafy vegetable (Geissler et al., 2009), *Plantago coronopus*[2] has tasty leaves for the preparation of salad (Koyro, 2006) and *Crithmum maritimum*[2] has ingredients such as essential oils with potential for nutritional and medicinal applications (Hamed et al., 2004). All three species are used and accepted in certain regions but more general popularity and acceptance is missing yet. The potential use of *Spartina anglica*[4] is as provider of biomass for the application in a fermentation plant to produce synthetic gas or fuel (Scott et al., 1990). Our project is still in its experimental phase and needs to be upgraded. The final ambition is an up-scaling of the small scale recirculating system with a recirculating aquaculture complex comprising about four basins with 1500 m^3 of water used for the culture of different marine fish species. Currently, we are planning to construct about 300 m^2 of wetland planted with different halophyte species. However, more solid data need to be collected to make reliable calculations for the size of the constructed wetland or hydrobotanical system in relation to the size of the aquaculture system.

Both fish and plants serve as food and strong regulations will be applied before selling them to the consumers. There are some aspects which need to be investigated in more detail before establishing in a commercial circulating aquaculture system. The influence of both types of organisms on each other needs to be carefully investigated. For example, roots of plants sometimes produce exudates which might be harmful to fish. The quality and composition of feed might affect growth and composition of the plant species. These kinds of investigations are currently designed and might help to judge the uncertainties and risks of recirculating aquaculture systems. In some aquaculture systems especially in the tropics, toxic organic compounds, antibiotics and heavy metals might be important to investigate regarding the possibility of phytoremediation and the risk for human consumption of the applied halophytes as vegetables.

6. Future perspectives

The effluents from marine aquaculture contain various substances harmful for the cultured organism and the environment. Microorganisms, sediment properties and macrophytes can contribute to the removal of such substances. Natural and constructed wetlands combine the filtering effect of substrates, microorganisms and halophytes and show a great potential as biofilter for saline aquaculture effluents in temperate and tropical regions. The capacity of a wetland to filter aquaculture effluents depends on diverse factors. Most studies note the cycling of the nutrient within the biofilter but the accurate removal mechanism often remains unclear. More detailed research in this field is needed to enable recommendations for the application of certain halophyte biofilters for certain saline aquaculture systems. Cycling of P could be a prospective application of constructed wetlands due to the global phosphate crisis. More studies are needed with respect to properties of different substrates in combination with plant species, water levels, hydraulic loading rates and hydraulic retention times, long term applications of the biofilter, seasonal and spatial variations, contribution of the halophytes to the filtering effect of a wetland and the role of plant-associated microorganisms. An interesting application is hydroponics with macrophytes as biofilter connected to an aquaculture system. Floating plant constructed wetlands facilitate control of the system, harvest of plant material and make the plant nutrient uptake the most important process. Also a biological filtering cascade consisting of different halophytes, including mangroves in the tropics, and other organisms such as seaweed, microalgae, seagrass or mussels could be used to reduce the nutrient load. To generate applicable results, the cooperation of different disciplines such as botany, ecology, biogeochemistry and engineering sciences is indispensible.

Acknowledgements

Research in our laboratory was supported financially by the DBU (AZ27708/1-3) and by two COST Short-Term Scientific Missions (FA0901-300811-008754 and FA0901-041011-011415).

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Chapter 3

Buhmann, A., Papenbrock, J., 2013b. An economic point of view of secondary compounds in halophytes. Functional Plant Biology 40, 952–967.

CSIRO PUBLISHING

Functional Plant Biology, 2013, **40**, 952–967
http://dx.doi.org/10.1071/FP12342

Review

An economic point of view of secondary compounds in halophytes

Anne Buhmann[A] *and Jutta Papenbrock*[A,B]

[A]Institute of Botany, Leibniz University Hannover, 30419 Hannover, Germany.
[B]Corresponding author. Email: jutta.papenbrock@botanik.uni-hannover.de

This paper originates from a presentation at the COST WG2 Meeting 'Putting halophytes to work – genetics, biochemistry and physiology' Hannover, Germany, 28–31 August 2012.

Abstract. Salt tolerance of halophytes relies on several strategies, among them, the production of species-specific secondary metabolites. Chemically, a broad variety of secondary compounds of economic interest is present in halophytes. Several of these secondary compounds are restricted to halophytic species or are found in higher concentrations than in glycophytes. For their exploitation, optimal plant cultivation conditions and extraction, fractionation and isolation processes need to be identified. On the one hand, the function of single compounds can be more precisely determined and controlled; on the other hand the mixture of compounds in crude extracts might have synergistic effects. Also, different plant organs and plants in different developmental stages contain highly varying amounts and compositions of secondary compounds. Secondary compounds from halophytes have potential uses in various fields such as pharmacognosy, functional foods, nutraceuticals and technical implementations. Many of the potential applications are still in the research and development phase; some products are already on the market. We describe and evaluate the economic potential of several halophytes such as *Salicornia* spp. and *Crithmum maritimum* containing valuable compounds used in different applications.

Additional keywords: Nutraceuticals, oxidative stress, pharmacology, *Salicornia*, salinity, technical applications.

Received 11 November 2012, accepted 22 February 2013, published online 21 March 2013

Introduction

Owing to global climate change, salinisation of soils is becoming a more and more serious threat for agriculture. Therefore salt-tolerant plants could serve as the crop plants of the future (Rozema and Flowers 2008). So far, few halophytes have been exploited for their potential to be grown as crop plants. According to Aronson (1989) there are more than 1560 plant species worldwide in 550 genera belonging to 117 different plant families showing salt tolerance, i.e. they grow well at salinity levels in the irrigation water of 85 mM NaCl or 5 g L^{-1} total dissolved solids. Of the species in the Aronson list, more than 50% come from just 13 families. These evolved probably through radiative evolution into diverse niches, including saline habitats, during the early evolution of angiosperms (Glenn *et al.* 1999). Halophytes occur throughout the phylogenetic tree of angiosperms, both in primitive (e.g. Laurales, Nymphales) and advanced (Asterales, Orchidales) orders (Flowers *et al.* 1977; Flowers and Colmer 2008). Therefore one can expect a broad range of metabolites and secondary compounds in halophytes.

Several secondary compounds are restricted to halophytic species or have not been found in glycophytic species in reasonable amounts. We will focus here on the metabolite patterns typically found in halophytes. From the comparison of the salt tolerant species *Thellungiella salsuginea* and the closely related less salt-tolerant species *Arabidopsis thaliana*, we have learned that transcript intensity analyses and metabolite profiles point towards a stress-anticipatory preparedness in *T. salsuginea* (Gong *et al.* 2005). In the primary metabolism, the concentrations of sugars, free amino acids and the number of double bonds in fatty acids are higher in *T. salsuginea* in non-saline and saline conditions than in *A. thaliana*. In other species, the synthesis of the lipophilic carotenoids and sterols is induced as well under saline conditions. The amounts of unusual polysaccharides and several glycosides are upregulated in saline conditions and may play a role in salt tolerance mechanisms (Aquino *et al.* 2011). Many members of the large group of phenolic compounds, some of them acting as antioxidants, can be found in high concentration, for example in mangroves. Some compounds are present in non-saline conditions in glycophytes and halophytes, such as the osmoprotective betaine glycosides, but are increasing in halophytes with increasing saline conditions. Many examples are reported from the Amaranthaceae family and the increase in glycinebetaine concentration plays an adaptive role to increasing saline conditions (Khan *et al.* 1998; Flowers and Colmer 2008).

Our review focuses on the analysis of metabolites found in halophytes that might be of interest for pharmaceutical, cosmetic, nutraceutical or technical applications. Before making use of

the metabolites several biological and technical problems need to be solved, such as constant production of the respective compounds, establishment of suitable extraction methods, transfer of preliminary promising results to application and regulatory aspects of bringing new products on the markets as summarised in Fig. 1.

Metabolic adaptations of halophytes to salinity

Halophytes use different strategies in their adaptations to the salty environment, mainly to regulate their salt content (Flowers and Colmer 2008). Different ions, such as potassium, and their respective transporters play an important role in osmotic homeostasis during changing environmental conditions (Flowers *et al*. 1977). At the cytosolic level, salt tolerance of halophytes is based on species-specific metabolite patterns. Osmolytes are one group of salt-induced organic compounds that accumulate in the cytoplasm in response to osmotic stress. The accumulation of osmolytes in the cytosol plays a key role in the osmotic adjustment of the cells, especially in reducing osmotic potential and contributing to maintain water homeostasis. They also prevent the misfolding/denaturation of proteins and ensure that they maintain their native structure. Thus, osmolytes are often termed 'chemical chaperones'. Most of the organic osmolytes bear no net charge at physiological pH, and even at high concentrations do not affect cytoplasmic functions such as protein catalysis. These properties of osmolytes have enabled their application in biotechnology and medicine (see 'Products on the Western market', below) (Hagihara *et al*. 2012).

Salt-induced effects apparently depend on the plant species, salt concentration and plant developmental stage. Metabolomic studies show that increased salinity leads to a change of conserved and divergent metabolic responses in glycophytes and halophytes. A change in the balance between amino acids and organic acids may be a conserved metabolic response of plants to salt stress whereas each species shows specific adaptations to changing salinity (Sanchez *et al*. 2008). Most notable is the observation that the steady-state pools of many stress-related metabolites were already enhanced in halophytes compared with glycophytes before exposure to salinity (Sanchez *et al*. 2008). In glycophytes, the metabolic profile changed after the exposure to salinity, whereas in halophytes the levels of salinity-related metabolites are already increased before the exposure to salinity suggesting a constitutive adaptation mechanism in halophytic species (Sanchez *et al*. 2008). Therefore the potential of cultivating halophytes in moderate salinity to obtain high contents of secondary metabolites seems to be promising and a systematic investigation of each crop species is required.

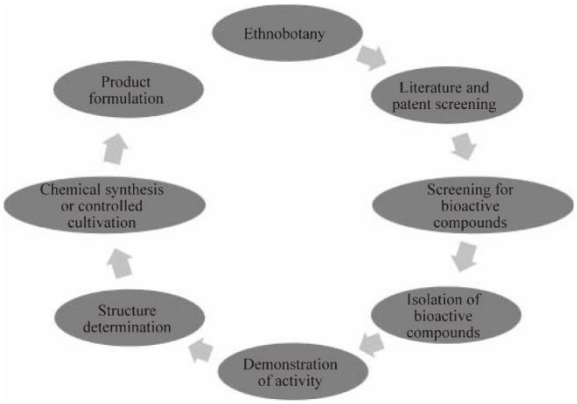

Fig. 1. General approach from the identification to the application of secondary compounds of economic value.

Despite of these adaptive mechanisms, unusually high salt concentrations in the soil and in the irrigation water and sudden changes thereof create stress for a plant. Abiotic stress has been found to increase the production of reactive oxygen species (ROS). Plants are capable of quenching ROS and it has been shown that this capacity, synthesis of antioxidants, for instance, is stress responsive (Hafsi *et al*. 2010). Hafsi *et al*. (2010) found a significant increase in the enzymatic and non-enzymatic antioxidant values of *Hordeum maritimum* plants exposed to salinity (100 mM NaCl with or without 3 mM K^+) in comparison to non-saline controls with or without 3 mM K^+. For example, the ascorbate content of plants increased with exposure to salt in the absence of K^+.

In many halophytes a positive correlation among increasing salt concentration in the medium and increasing contents of secondary metabolite production hast been observed. *Nitaria retusa* showed an increasing phenol and flavonoid content with increasing salinity (0 to 800 mM NaCl) whereas the leaf phenol content of *Atriplex halimus* was maximal in presence of 100 mM NaCl and subsequently decreased with a further increase of applied salt concentration (Boughalleb and Denden 2011). Both species grew well at low salt concentrations but showed a decrease in growth at higher salt concentrations (400–800 mM NaCl), especially *A. halimus*. These results suggest that it is possible to manipulate the content of phenols and flavonoids per gram plant material by increasing the salinity in the watering solution or in the medium depending on the halophyte species; but the yield of plant material will decrease if the chosen salinity level is too high. The content of polyphenols can be different between different species within the same genus and also between different ecotypes. Ksouri *et al*. (2008) found higher contents of phenolic compounds, flavonoids and condensed tannins in *Mesembryanthemum edule* compared with *Mesembryanthemum crystallinum* harvested from the same region within the same environmental conditions. Ksouri *et al*. (2007) showed that *Cakile maritima* plants grown from seeds collected at an arid site reacted with better growth, higher polyphenol content and higher antioxidative activity towards increasing salinity (100 and 400 mM NaCl) compared with plants from seeds collected at a humid site.

Besides salinity, other environmental stress factors can influence the content of secondary metabolites in halophytes. Meot-Duros and Magné (2009) found a much higher content of chlorogenic acid for *Crithmum maritimum* in plants growing on sand hills than in plants growing below cliffs. As an explanation for the higher concentration, the authors suggested a higher need for radical scavenging as protection against different environmental stress factors including nutrient deficiency, water stress and ionic stress caused by sea spray, that are

present at the sand hills and absent below the cliffs. Water stress also caused a higher phenolic content and antioxidative activity in *C. maritima* (Ksouri *et al*. 2008) and in *M. edule* (Falleh *et al*. 2012). A higher content of antioxidants in *Mesembryanthemum* species was also related to high irradiance (Falleh *et al*. 2012) and UV-radiance (Ibdah *et al*. 2002).

Flowering can cause drastic changes to metabolism because it often induces senescence of the organism. Therefore the light regime plays an important role in growing plants for secondary compounds. The influence of daylength and salinity on the flowering of members of the Salicornieae was investigated. Daylength had a strong influence whereas moderate salinity did not change the onset of flowers (Ventura *et al*. 2011).

Leaving environmental factors aside other studies pay attention to the influence of plant growth stage and plant organ on the content of antioxidative substances. Several studies with different halophytic species, *M. crystallinum*, *C. maritimum*, and *Limonium densiflorum*, found that the concentration of phenolic compounds and flavonoids is highest in the flowering period (Bouftira *et al*. 2010; Medini *et al*. 2011; Jallali *et al*. 2012). A study comparing the content of polyphenols in leaves and flowers showed that methanolic extracts from flowers showed higher contents than from leaves (Ksouri *et al*. 2008). However, from an economic point of view, when comparing the biomass ratio of flowers to leaves, it only makes sense to collect the flowers when they contain very valuable compounds, such as the stigmas of saffron crocus (*Crocus sativa*) (Molina *et al*. 2005).

Analysis of metabolites: extraction methods and identification of valuable compounds

There are many (ethno-) botanical reports on the use of halophytes and extracts thereof in traditional medicine, biotechnology and foodstuffs (summarised recently by Ksouri *et al*. 2011). This long standing knowledge may provide indications for further research. However, the descriptions for extraction methods are sometimes imprecise. Often the effects of crude aqueous or organic extracts are described. The use of different solvents can increase the yield of certain chemically related substances. These crude extracts have been used for the analysis of a compound group, such as the determination of total phenolics by the Folin-Ciocalteau method (Singleton and Rossi 1965; Ainsworth and Gillespie 2007), total flavonoids (Dewanto *et al*. 2002) and condensed tannins (Sun *et al*. 1998). The antioxidative capacity of the extracts has been determined by measuring the 2-diphenyl-1-picryhydrazyl (DPPH) free radical scavenging activity or the oxygen radical absorbance capacity (ORAC) (Dudonné *et al*. 2009).

The influence of growth conditions on the biosynthesis of compound groups can be readily tested by cultivating halophytes under different environmental conditions (temperature, salinity, water availability, light intensity). Also the importance of biological factors (genotype, organ and ontogeny) needs to be investigated for optimal yields of secondary compounds. Our own results (data not shown) show high ORAC in different organs of halophytes, such as *Salicornia* spp. and *C. maritimum* cultivated under moderate salt stress, in comparison to fruits and vegetables generally sold on the market (Ninfali *et al*. 2005), demonstrating a potential of halophytes as functional food or nutraceutical.

As several studies show, extraction method has a strong effect on the results. Maceration extracts from *C. maritimum* shoots contained greater amounts of phenolic compounds than soxhlet extracts (Jallali *et al*. 2012). For *M. crystallinum* leaves, methanolic and ethanolic extracts contained higher amounts of polyphenols and anthocyanins than water extracts (Bouftira *et al*. 2010). Ksouri *et al*. (2008) found the highest polyphenol and flavonoid contents in pure methanol extracts of *Limoniastrum monopetalum* leaves, with less in an acetone extract and least in water, ethanol and hexane extracts. But for the same species, a pure acetone extract provided the highest results for leaf tannin content. Therefore, the appropriate extraction method depends on the plant species, the compound sought and as Medini *et al*. (2011) and Falleh *et al*. (2013) also showed on the plant organ. These results demonstrate the importance of choosing the right extraction method to gain an optimal and reproducible yield of certain secondary metabolites for commercial use.

For the identification of the putatively active substance of interest fractionation and further screening needs to be conducted. HPLC analysis can be used for separation and, dependent on the detectors, for identification. Usually GC-MS and LC-MS are finally used to identify already known compounds. Structure elucidation can be performed by MS-MS or NMR methods. After the effective compound has been identified and its structure elucidated methods to provide large amounts of the respective substance have to be established. This up-scaling process of the purification in a reproducible way is usually done by process engineers to solve technical aspects of extraction, such as efficiency and solvent-to-plant material ratio. In the presence of high salt concentrations modification of existing methods for chemically similar substances from glycophytes need to be optimised (Kassing *et al*. 2010; Kassing and Strube 2012).

Another option is to develop a chemical synthesis of the natural compound if the structure is not too complicated. We will illustrate this option by a success story of a compound originally isolated from the seagrass *Zostera marina*: it was observed that rates of decomposition of *Zostera* flotsam are generally low compared with other vascular macrophyte sources of detritus, but are influenced by many variables. The seagrass detritus undergoes an initial period of leaching, leaving a poor substrate for bacteria because what soluble material remains is deficient in inorganic nutrients, contains inhibitory phenolic compounds, and is protected by cellulose and lignin (Harrison 1982, 1989). One inhibitory compound was identified in bioactivity tests, zosteric acid (Todd *et al*. 1993). Recently, it was shown that large amounts of zosteric acid can be synthesised in a relatively simple procedure (Villa *et al*. 2010). Antifouling effectiveness of zosteric acid has been demonstrated both in static laboratory assays and with zosteric acid directly dispersed in marine water (Barrios *et al*. 2005). Zosteric acid seems to be an ideal antifouling compound because it has low general toxicity but specifically inhibits biofilm formation at early stages by hindering the bacteria to change from the planktonic motile stage to the sessile stage (Villa *et al*. 2010).

Product development and zosteric acid formulation are underway, for example, as anti-fouling paints. In this example one secondary compound showing bioactivity was isolated and due to the chemical synthesis the time-consuming and expensive establishment of cultivation conditions for the plant species was not necessary.

Crude plant extracts, fractions of an extract or isolated compounds have all been used in further investigations of their effects. A mixture of compounds in extracts might have synergistic effects whereas the function of individual compounds can be more precisely determined and controlled. But, as discussed by Falleh *et al*. (2012), one should also keep in mind that the most abundant compound is not necessarily the most active and that within an extract or a fraction compounds might have antagonistic effects covering individual activity of single compounds. Therefore investigation of bioactivity both at the single compound level as well as at the level of mixed compounds (different kinds of crude extracts and their fractions) are of importance. The activity of a compound or extract can be screened with different types of tests. These tests include screening for inhibition of certain bacterial and fungal strains on Petri dishes, inhibition of virus growth and determination of antioxidant or anti-carcinogen activity in cell cultures containing cells with induced oxidative stress or cancer cells, amongst others. Finally, the activity of a substance or extract has to be tested in the system where it is to be applied, for example, food that needs to be protected against certain pathogens to be conserved for consumption.

For commercial use, the species and cultivar needs to be clearly identified and recognisable to establish a standardised production using defined plant material. Otherwise production of secondary metabolite contents in a reproducible way is impossible. For example, often the species *Salicornia herbacea* is mentioned in commercial applications. However, from a botanical point of view this species does not exist. Due to the reduced morphology of *Salicornia* ssp., only genetic methods can identify *Salicornia* taxa (Kadereit *et al*. 2007; Liebezeit 2008). Individuals classified as *S. herbacea* belong now to the *Salicornia europaea* clade (Kadereit *et al*. 2007). Therefore, *S. herbacea* is re-named in the text and tables of this review as *S. europaea*.

From promising preliminary results to applications

Secondary compounds of halophytes used in pharmacognosy

Pharmacology is concerned with the study of drug action. A drug can be broadly defined as any man-made, natural, or endogenous (within the cell) molecule that exerts a biochemical or physiological effect on the cell, tissue, organ or organism. If substances have demonstrated medicinal properties, they are considered pharmaceuticals. This needs to be demonstrated by laboratory tests and finally by clinical trials, prospective biomedical or behavioural research studies of human subjects that is designed to answer specific questions about biomedical or behavioural interventions. Pharmacognosy is a branch of pharmacology which deals especially with the composition, use, and development of medicinal substances of biological origin and especially medicinal substances obtained from plants (Flückiger and Tschirch 1885).

There are several traditional uses of plant parts and extracts of halophytes for the treatment of various diseases and ailments such as diabetes, cancer, inflammation and gastrointestinal disorders. The study of those ethnobotanical uses of halophytes forms a basis to evaluate the potential of certain halophytes for economic use and to search for compounds responsible for the traditionally known curative or health promoting effects (Liebezeit *et al*. 1999; Kefu *et al*. 2002; Liebezeit 2008; Ksouri *et al*. 2011; Zhao *et al*. 2011). The long-term objective should be to form a reliable base for pharmacological application of secondary compounds of halophytes by discovering the specific effects of extracts or particular compounds against a specific disease or health-related problem in laboratory tests and finally in clinical studies.

S. europaea is a good of a species with widespread applications in traditional medicine on the one hand, and for the availability of several detailed analytical studies that cover not only one but many applications of this species in the medical field on the other. Rhee *et al*. (2009) reviewed botany, chemistry and pharmacology of *S. europaea* and reported several secondary metabolites such as tungtungmadic acid and ferulic acid as its bioactive compounds with anti-hyperlipidemic, anticancerous, anti-inflammatory and antioxidative effects. Sung *et al*. (2009) reported that an aqueous extract of *S. europaea* protected human dermal fibroblast cells from induced oxidative stress, acted as a potential tyrosinase inhibitor and decreased the synthesis of melanin in B16 melanoma cells. They concluded that the aqueous extract of *S. europaea* can be applied in agents aimed to have skin whitening and rejuvenating effects. For other halophytes just singular effects or compounds have been studied: we summarise them in Table 1.

Different halophytic species are in the focus of research for plant-derived substances to prevent and treat metabolic diseases. For example, an extract of *M. crystallinum* was tested for the treatment or prevention of obesity and shown to have different effects on adipocytes such as reduction of fat accumulation and reduction of gene expression related to cell differentiation (Kurosu and Kazuichi 2011). The differentiation of adipocytes also was strongly reduced by the application of a flavonoid glucopyranoside isolated from a *S. europaea* extract (Kong *et al*. 2012). Jung *et al*. (2012) found an extract of *Artemisia capillaries* with various key active compounds to prevent subsequent damage caused by substances known as advanced glycation end products (AGEs) that are generated during diabetes and cardiovascular diseases. Other studies have focussed on the treatment of gastrointestinal disorders by applying extracts from halophytes. Endale *et al*. (2011) found that an extract of *Suaeda asparagoides* inhibited spontaneous contraction of muscle strips from rat gastric antrum. Crude extracts of *Tamarix indica* showed anti-diarrhoeal activity in mice (Habiba *et al*. 2010).

Besides metabolic diseases, another important field of application is the treatment of cancer by halophyte-derived substances. For example, extracts from *S. europaea*, *Glehnia littoralis* and *Suaeda fruticosa* were shown to have anti-proliferative and toxicity effects on human colon cancer cells (Ryu *et al*. 2009; Um *et al*. 2010; Kang *et al*. 2011; Oueslati *et al*.

Table 1. Examples of extracts and secondary metabolites from halophytes with application potential for pharmacology (in alphabetical order of references)

Abbreviations: AEG, advanced glycation end products; DPPH, quantification of the antioxidative activity by measuring the 2-diphenyl-1-picryhydrazyl (DPPH) free radical scavenging activity; MTS and MTT, colourimetric assay for measuring the activity of cellular enzymes that reduce the tetrazolium dye MTS or MTT, respectively; SRB, sulforhodamine B (SRB) assay is used for cell density determination based on the measurement of cellular protein content

Effect	Application potential	Class of compound	Secondary metabolite	Solvent	Demonstration of activity : tests and assays	Demonstration of activity : cell culture and clinical tests	Species	Reference
Against metabolic diseases								
Inhibition of spontaneous gastric muscle contraction	Treatment of functional gastrointestinal disorders			Aqueous fraction of an ethanolic extract and subsequent fractionation by different solvents		*In vitro* motility studies with muscle strips from rat gastric antrum (organ bath)	*Suaeda asparagoides*	Endale *et al.* (2011)
Anti-diarrhoeal activity, inhibition of writhing	Use in traditional medicine, further pharmaceutical potential	Polyphenols flavonoids alkaloids saponins	Tannins	Methanolic extract		Mice with induced diarrhoea or writhing	*Tamarix indica*	Habiba *et al.* (2010)
Inhibition of advanced glycation end products (AGE) formation	Therapeutic or preventive agents for diabetic complications and oxidative stress-related diseases	Phenols	4,5-di-*O*-Caffeoylquinic acid, umbelliferone, esculetin, esculin, scopoletin	Different fractions and subfractions of a methanolic extract	AGE inhibitory activity, rat lens aldose reductase inhibition DPPH radical scavenging activity trolox equivalent antioxidant activity $ONOO^-$ scavenging activity $ONOO^-$-mediated tyrosine nitration		*Artemisia capillaries*	Jung *et al.* (2012)
Reduction of adipogenic differentiation	Anti-obesity agent	Flavonoids	Quercetin 3-O-β-d-glucopyranoside	*n*-Butanol fraction	Oil-red O staining	Mouse 3T3-L1 cells	*Salicornia europaea*[A]	Kong *et al.* (2012)
Reduction of fat accumulation at an early stage of differentiation	Treatment or prevention of obesity and other metabolic syndromes			Ethanolic extract	Glycerol-3-phosphate dehydrogenase assay luciferase assay oil-red O staining cell viability	Mouse 3T3-L1 cells	*Mesembryanthemum crystallinum*	Kurosu and Kazuichi (2011)
Against cancer								
Toxicity against colon cancer cells antioxidative activity	Dietary source for medicinal application	Phenols, flavonoids		Ethyl ether fraction of methanolic extract ethyl acetate fraction of methanolic extract	Cell viability radical scavenging activity effect on lipid peroxidation metal chelating activity hydrogen peroxide level	Colon cancer cells(HCT 16 and HT-29) normal immortalised intestinal cells (INT-40)	*Salicornia europaea*[A]	Kang *et al.* (2011)

Anti-proliferative effects on human colon cancer cells	Anti-proliferative substances for treatment of colon cancer	Polysaccharides		Extraction of crude and fine polysaccharides	MTS assay apoptosis analysis	Colon cancer cells (HT-29)	*Salicornia europaea*[A]	Ryu *et al.* (2009)
Anti-cancer activity against human lung carcinoma and colon adenocarcinoma cells, specificity against DLD-1	Source of antioxidants which exhibit anticancer capacities			Dichlormethan extract	Cytotoxicity assay	Human lung carcinoma (A-549) and colon carcinoma cell lines (DLD-1, Caco-2 and HT-29)	*Suaeda fruticosa*	Oueslati *et al.* (2012)
Anti-proliferative effect and induced apoptosis on colon cancer cells	Cancer chemopreventive food	Furanocoumarines polyacetylenes	Bergapten isopimpinellin xanthotoxin imperatorin panaxydiol falcaindiol falcarinol	85% Aqueous MeOH fraction *n*-hexane fraction	MTT assay	Colon cancer cells (HT-29)	*Glehnia littoralis*	Um *et al.* (2010)
Cytotoxicity against several cancer cell lines, especially human monoblastic leukemia U937 cells	Natural cytotoxic product	Sesquiterpenoid	Bigelovin	Ethanolic fraction of petroleum ether extract	MTT assay SRB assay cell growth and morphology	Cancer cell lines (A549, SGC-7901, BEL-7402, U251, B16)	*Inula helianthus–aquatica*	Zeng *et al.* (2009)
Against pathogenic microorganisms								
Growth inhibition of *Staphylococcus aureus* and *Escherichia coli*	Food additives replacing synthetic ones in food industry (also technical application)	Phenolic acids	Syringic acid, transcinnamic acid	Methanolic extract	Antibacterial activity tests		*Cynara cardunculus*	Falleh *et al.* (2008)
Growth inhibition of different pathogenic bacteria, fungi and filamentous fungi	Potent source for natural antibiotics			Several fractions of methanolic extract after hexane extraction	Microplate bioassay		*Mesembryanthemum edule*	Falleh *et al.* (2013)
Growth inhibition of *Staphylococcus epidermidis*, *Staphylococcus aureus*, *Micrococcus luteus*, *Escherichia coli*, *Pseudomonas aeruginosa*	Antioxidants for therapeutic or nutraceutical industries and for food manufactures (also technical application)	Polyphenols phenolic acids flavonoids		Methanolic extract	Screening for antimicrobial activity		*Tamarix gallica*	Ksouri *et al.* (2009)
Growth inhibition of *Micrococcus luteus* and *Bacillus cereus*	In food manufactures and cosmetology as preservative agents and biopesticides, or in medicine as new antibiotics (also technical application)	Polyacetylene	Falcarindiol	Extraction by chloroformic acid, purification by hexane and fractionation by methanol	Screening microplate bioassay		*Crithmum maritimum*	Meot-Duros *et al.* (2010)

(*continued next page*)

Table 1. (*continued*)

Effect	Application potential	Class of compound	Secondary metabolite	Solvent	Demonstration of activity: tests and assays	Demonstration of activity: cell culture and clinical tests	Species	Reference
Growth inhibition of *Escherichia coli* and *Saccharomyces cerevisiae*	Natural food additives (also technical application)			Ethanolic extract	Antibacterial assay		*Salicornia europaea*[A]	Yu *et al.* (2012)
Growth inhibition of different food-born human pathogens such as *Staphylococcus aureus*, *Micrococcus luteus* and *Candida holmii*	Natural food additives (also technical application)			Different pure solvents with increasing polarity and mixtures thereof (hexane, ethanol, acetone, methanol, water)	Disc diffusion method		*Limoniastrum monopetalum*	Trabelsi *et al.* (2010)

[A]Formerly known and in literature stated as *Salicornia herbacea*.

2012). Zeng *et al.* (2009) tested the cytoxicity of several sesquiterpene lactones from *Inula helianthus–aquatica* against several cancer cell lines and determined bigelovin as the most potent one with the potential to induce apoptosis and inhibit proliferation of cancer cells.

There are several studies on the antimicrobial activity of extracts of several halophytes against human pathogenic bacteria and fungi such as *Bacillus subtilis*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Aspergillus fumigates* and *Candida albicans* (Orfali 2005; Saidana *et al.* 2008; Ivanova *et al.* 2009; Manikandan *et al.* 2009; Chandrasekaran *et al.* 2011; Megdiche et al. 2011; Nostro *et al.* 2011; Samiullah *et al.* 2011; Sunita *et al.* 2012). Bioassay-guided fractionation of a chloroformic extract obtained from leaves of *C. maritimum* led to the chemical isolation of falcarindiol, which was responsible for a strong growth inhibition of *Micrococcus luteus* and *Bacillus cereus* (Meot-Duros *et al.* 2010). Some studies investigated the antimicrobial and antioxidative activity of halophyte extracts in parallel. This approach implicates a possible relationship between antioxidative and antimicrobial effects of an extract suggesting that the same compounds could be responsible for different useful activities. Both antioxidative activity and antimicrobial activity were found, for example, in extracts from *S. europaea* (Yu *et al.* 2012), *Tamarix gallica* (Ksouri *et al.* 2009) and *Cynara cardunculus* (Falleh *et al.* 2008). But there is also an indication that antioxidative activity and antimicrobial activity are based on different compounds within the extracts or are evoked by the same group of secondary metabolites but different specific substances. Trabelsi *et al.* (2010) assessed the antimicrobial activity of a *L. monopetalum* extract with the highest antioxidative activity but they only found 'a slight antimicrobial activity' against different human pathogen strains. Falleh *et al.* (2013) investigated the antioxidative and antimicrobial activity of methanolic aqueous extracts (v/v; 20/80, 40/60 and 60/40) from leaves, stems and roots of *M. edule*. The antioxidative and antimicrobial activities were very diverse in extracts of different plant organs and different kinds of extract.

For the application of extracts and compounds extracted from different halophyte species in the field of pharmacology and nutraceuticals, tests to ensure safety and consistent quality are essential. Jallali *et al.* (2012) state the necessity to test the 'safety, edibility and in vivo efficacy' of the compounds being evaluated as bioactive in investigated halophytes. Chaudhary *et al.* (2012) applied quality control parameters to ensure a constant quality of *Cressa cretica* plant material for industrial use in the medical field. Habiba *et al.* (2010) tested the applied extract for cytotoxic activity using the brine shrimp *Artemia salina*. Bouftira *et al.* (2008) determined the physical stability of a cream formulation containing an extract of *M. crystallinum*. Siracusa *et al.* (2011) found several flavonoids extracted from *Capparis spinosa* and a chlorogenic acid obtained from *C. maritimum* to have dose-dependent activity but it was not stable under simulated gastrointestinal conditions. They stated that the 'results question the validity of sole measurements of the antioxidative activity of a plant extract or a substance contained because if medically applied the degree of uptake of a certain substance matters'.

Promising research with halophytes being used in functional food and nutraceuticals

The boundaries between functional foods and nutraceuticals are indistinct but can be recognised based on the way in which they are consumed: functional foods are consumed as regular food whereas nutraceuticals are consumed as capsules, pills and tablets (Espín *et al*. 2007). Plant oils that have a high content of mono- and polyunsaturated fatty acids and a low content of saturated fatty acids are beneficial for human health and can be used in valuable nutraceuticals (Williams 2000; Iso *et al*. 2002; Williams and Burdge 2006; Dubois *et al*. 2007). Weber *et al*. (2007) compared the oil quantity and fatty acid composition of different halophyte species (*Arthrocnemum indicum*, *Alhaji maurorum*, *C. cretica*, *Halopyrum mucronatum*, *Haloxylon stocksii* and *S. fruticosa*). They all contained high amounts of linoleic acid. In seeds of *S. aralocaspica* linoleic acid and oleic acid were the most abundant fatty acids (Wang *et al*. 2012). *Arthrocnemum macrostachyum* leaves showed high contents of linoleic and α-linolenic acid (Custódio *et al*. 2012). *C. maritimum* leaves and seeds have been found to be a valuable source for mono- and polyunsaturated fatty acids (Guil-Guerrero and Rodríguez-García 1999; Atia *et al*. 2010). All those studies show that oils extracted from halophytes can be useful sources for valuable fatty acids in their composition comparable to other valuable type of oils already on the market such as olive, canola, sunflower and cotton oil (Weber *et al*. 2007; Atia *et al*. 2010). Besides their value as source for essential oils, oils from halophytes also have antioxidative and antibacterial properties (Atia *et al*. 2011; Custódio *et al*. 2012).

Antioxidants have high potential for use in nutraceuticals because of their protective and health-promoting effect concerning damage and diseases caused by ROS-induced stress in the body (Bouayed and Bohn 2010). Again ethnobotanical studies provide an informative basis for the existence of compounds with antioxidative activity in certain halophyte species (e.g. Kefu *et al*. 2002). The continuative steps are studies that determine the antioxidative activity of crude plant extracts of different halophytes (e.g. Bouftira *et al*. 2010; Thirunavukkarasu *et al*. 2010) and studies that determine groups of substances within the extract that are known to show antioxidative properties, such as phenolic acids, flavonoids and tannins (e.g. Benhammou *et al*. 2009). Finally, it is essential to know which substances exactly are responsible for the antioxidative activity of a plant extract, which are the main active compounds and to evaluate the potential of these substances to play a health promoting, protective and healing role in the human body. We list some of the studies with this aim in Table 2.

Several studies assess the antioxidative activity of a crude plant extract and determine substances within the extract that are already known to have antioxidative properties. Accordingly compounds like syringic acid, catechin and rutin hydrate were found as the main phenolic compounds in extracts of different halophytes and were related to the antioxidative activity of the extract (Falleh *et al*. 2008; Ksouri *et al*. 2009; Oueslati *et al*. 2012). A more detailed approach is to use different types, phases or fractions of extracts, assess the one with the highest antioxidative activity and determine its constituents with known antioxidative properties. Correspondingly a series of studies on *M. edule* found this species to have higher antioxidative activity compared with other *Mesembryanthemum* species (Falleh *et al*. 2009). The main flavonoid compounds such as quercitrin have known antioxidant properties (Falleh *et al*. 2011*a*) and the flavonoids with the highest antioxidative activity are procyanidins and propelargonidins (Falleh *et al*. 2011*b*). High antioxidative activity was also found in extracts of *M. crystallinum* and 2,6-bis (1,1-dimethylethyl)-4-methylphenol was determined to be the major component of the fraction of a methanolic extract with the highest antioxidative activity (Bouftira *et al*. 2007). Another example is the detection of various interesting compounds with known antioxidative, antimicrobial, antiviral, anti-inflammatory, antitumoral and immune activating properties in *C. maritimum*. Meot-Duros and Magné (2009) detected the strong antioxidant chlorogenic acid in higher concentrations than in other species of the Apiaceae and Jallali *et al*. (2012) determined substances such as epigallocatechin, vanilic acid and quercetin-3-galactoside. Trabelsi *et al*. (2012) describe epigallocatechin-3-O-galate found in *Limoniastrum guyonianum* to have high potential for industrial use because its antioxidative activity and protective effects in the treatment of various diseases such as neuronal disorders, diabetes and cancer have already been investigated to the point of studies in animal models.

Despite the body of work reported above, there are only a few studies that determine and prove a particular substance in an extract to be the main active component. Kim *et al*. (2000) reported esculetin and luteolin 7-O-rutinoside as main compounds with antioxidative activity in extracts of *Artemisia montana*, with an antioxidative activity comparable to L-ascorbic acid. Kim *et al*. (2012) determined different saponins in a *S. europaea* extract and especially 30-norhederagenin 3-O-β-D-glucuronopyranosyl-28-O-β-D-glucopyranoside showed strong antioxidative activity. Consecutive tests to prove the intracellular effect of such main active components of an extract are rare. After isolating a new chlorogenic acid derivate named tungtungmadic acid (3-caffeoyl, 4-dihydrocaffeoyl quinic acid) from *S. europaea* and determining its protective effect against induced liver injury in rats (Chung *et al*. 2005, 2006), Hwang *et al*. (2009) tried to assess if and how the substance prevents hepatic injury induced by oxidative stress. They stressed treated and untreated hepatocytes and found that the protective effect of tungtungmadic acid is at least partly due to its ability to scavenge ROS and regulate the antioxidative enzyme HO-1. Lee *et al*. (2011) determined four known flavonol glycosides as the main active antioxidative compounds in a fractionated extract of *Limonium tetragonum*. Additionally they investigated their intracellular effects. The compounds had no toxic effect up to concentrations of 100 μM, showed intracellular ROS scavenging activity and inhibited lipid peroxidation and DNA-damage. The range of scavenging activity of the compounds differed partly between the direct and the intracellular measurement. Similar tests were conducted by Kong *et al*. (2009), they found isorhamnetin 3-O-β-D-glucopyranoside extracted from *S. europaea* to have dose-dependent intracellular scavenging activities.

As discussed in many of the studies mentioned above and in Table 2, antioxidants are of interest not only in the field of nutraceuticals but also in the field of medicine. They have

Table 2. Examples of extracts and secondary metabolites from halophytes with antioxidative activity (in alphabetical order of references)

Abbreviations: ABTS, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) is used in its radical form in the TEAC (Trolox equivalent antioxidant capacity) assay; DPPH, quantification of the antioxidative activity by measuring the 2-diphenyl-1-picryhydrazyl (DPPH) free radical scavenging activity; GSH, reduced glutathione; MTT, colourimetric assay for measuring the activity of cellular enzymes that reduce the tetrazolium dye MTT, respectively; ORAC, determination of the oxygen radical absorbance capacity

Application potential	Class of compound	Secondary metabolite	Solvent	Demonstration of activity	Halophyte species	Reference
Medical, cosmetic and industrial purposes(e.g. as food additive or against viruses)	Phenol	2,6-Bis (1,1-dimethylethyl)-4-methylphenol	Methanolic extract	DPPH-scavenging activity	*Mesembryanthemum crystallinum*	Bouftira *et al.* (2007)
	Phenolic acid	Tungtungmadic acid	Methanolic extract	DPPH-scavenging activity inhibition of lipid oxidation assay of oxidative DNA single strand breaks	*Salicornia europaea*[1]	Chung *et al.* (2005)
	Phenolic acid	Tungtungmadic acid	Methanolic extract	Protection effect against induced liver injury (test with rats)	*Salicornia europaea*[1]	Chung *et al.* (2006)
Source of health-promoting polyphenols	Phenolic acids flavonoids	Syringic acid trans-cinnamic acid epicatechin quercetrin	Methanolic extract	Superoxide anion radical-scavenging activity DPPH-scavenging activity	*Cynara cardunculus*	Falleh *et al.* (2008)
Source of functional phenolic compounds	Flavonoids	Phloretin quercitrin avicularin	Methanolic extract	Total antioxidant capacity (reduction of Mo) DPPH-scavenging activity ABTS assay superoxide anion radical-scavenging activity chelating effect on ferrous ions iron reducing power β-carotene bleaching test inhibition of lipid oxidation	*Mesembryanthemum edule*	Falleh *et al.* (2009)
Functional or nutraceutical food to prevent or moderate oxidative stress-related diseases	Flavonoids	Quercitrin (in leaves) avicularin (in leaves)catechin (in stems) procyanidin B2 (in stems)	Methanolic extract	Total antioxidant capacity (reduction of Mo)DPPH-scavenging activity iron reducing power β-carotene bleaching test	*Mesembryanthemum edule*	Falleh *et al.* (2011*a*)
Nutraceutical in the pharmaceutical industry	Condensed tannins	Procyanidins propelargonidins	Different methanolic extracts	DPPH-scavenging activity ABTS assay β-carotene bleaching test	*Mesembryanthemum edule*	Falleh *et al.* (2011*b*)
Chemopreventive and therapeutic applications (treatment of liver disorders)	Phenolic acid	Tungtungmadic acid	Methanolic extract	Protection effect against induced hepatotoxicity (Hepa1c1c7 cells)	*Salicornia europaea*[1]	Hwang *et al.* (2009)

Use in several fields, such as nutraceuticals, cosmetics and agro-food industry	Flavonoids phenolic acid	Epigallocatechin quercetin-3-galactoside vanillic acid	Different acetonic extracts	DPPH-scavenging activity iron reducing power	*Crithmum maritimum*	Jallali *et al.* (2012)
Treatment of diseases associated with oxidative damage		Esculetin luteolin 7-O-rutinoside	Methanolic extract	DPPH-scavenging activity in cell culture (AC2F hepatocytes): free radical generation	*Artemisia montana*	Kim *et al.* (2000)
Uses as natural antioxidants	Saponins	Four saponins with different antioxidant activity (for chemical details see publication)	*n*-Butanol extract	DPPH-scavenging activity peroxynitrite-scavenging activity	*Salicornia europaea*[1]	Kim *et al.* (2012)
Prevention of radical-mediated cellular damage; natural antioxidant	Flavonoid	Isorhamnetin 3-O-β-D-glucopyranoside	Fractionation process by dichlormethane, methanol, hexane, butanol	DPPH-scavenging activity hydroxyl radical assay alkyl radical assay in cell culture (HAT-1080, HL-60 and U-973 cells): MTT assay intracellular scavenging of ROS extent of oxidative damage of genomic DNA myeloperoxidase activity assay measurement of intracellular GSH level	*Salicornia europaea*[1]	Kong *et al.* (2009)
Interesting source of antioxidants for therapeutic or nutraceutical industries	Phenolic acid flavonoids	Syringic acid isoquercitin catechin	Methanolic extract	Total antioxidant capacity (reduction of Mo) DPPH-scavenging activity ABTS assay superoxide anion radical-scavenging activity chelating effect on ferrous ions iron reducing power β-carotene bleaching test inhibition of lipid oxidation	*Tamarix gallica*	Ksouri *et al.* (2009)
	Flavonoids	Myricetin-3-*O*-β-D-galactopyranoside myricetin-3-*O*-α-L-rhamnopyranoside quercetin-3-*O*-β-D-glucopyranoside quercetin-3-*O*-β-D-galactopyranoside	Fractionation process by dichlormethane, methanol, hexane, butanol	DPPH-scavenging activity hydroxyl radical assay in cell culture (HAT-1080 cells): MTT assay intracellular scavenging of ROS intracellular lipid peroxidation extent of oxidative damage of genomic DNA	*Limonium tetragonum*	Lee *et al.* (2011)
Valuable source of antioxidant products	Phenolic acid	Chlorogenic acid	Methanolic extract	Total antioxidant capacity (reduction of Mo) DPPH-scavenging activity ABTS assay	*Crithmum maritimum*	Meot-Duros and Magné (2009)

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Table 2. (*continued*)

Application potential	Class of compound	Secondary metabolite	Solvent	Demonstration of activity	Halophyte species	Reference
Valuable source of natural bioactive molecules	Phenolic acid	2,5-Dihydroxybenzoic acid rutin hydrate	Acetone extract	Total antioxidant capacity (reduction of Mo) DPPH-scavenging activity iron reducing power β-carotene bleaching test ORAC assay in cell culture (human skin fibroblast cells): antioxidant cell assay	*Suaeda mollis*	Oueslati *et al.* (2012)
Valuable sources of natural antioxidants in nutraceutical and food industries	Flavonoids	Gallocatechin epigallocatechin epigallocatechin-3-*O*-galate	Water/acetone, subfractions of heptane, ethyl acetate and methanol separations	Total antioxidant capacity (reduction of Mo) DPPH-scavenging activity iron reducing power	*Limoniastrum guyonianum*	Trabelsi *et al.* (2012)

[A]Formerly known and in literature stated as *Salicornia herbacea*.

potential to be applied in the treatment of diseases such as neuronal disorders, diabetes, cancer and viral infections such as herpes and HIV due to their property to reduce ROS-caused stress and damages.

Technical implementations

Several possibilities of using extracts or compounds from halophytes for technical applications have been investigated, including agriculture, animal production and within the food industry. Boughalleb *et al.* (2009) tested the activity of different extracts from Tunisian halophytes against several plant pathogenic fungi and found *Atriplex inflata*, *Atriplex semibaccata*, *Atriplex portulacoides* and *Salicornia fruticosa* to have promising effects. Rele *et al.* (2003) describes *Sesuvium portulacastrum* as source of 20-hydroxyectisone, a phytosteroid which can be used as an insecticide causing moulting in several insects. Saidana *et al.* (2007) tested the insecticidal activity of different extracts from different halophytes against *Tribolium confusum*, an insect responsible for severe harvest losses. The active extracts from *Frankenia laevis*, *Statice echioides*, and *Tamarix boveana* had a higher effectiveness against larvae than against adults with a mortality of 80–97% if added to the diet of the beetles. Also Thirunavukkarasu *et al.* (2011) consider extracts from the bark of *Excoecaria agallocha* as a potent anti-insecticidal agent if applied against the larvae of several mosquitos.

Kumar *et al.* (2009) tested the activity of crude extracts from different halophytes against different bacterial and fungal species which cause silkworm disease and are the major harm to economic production of silk in Asia. Extracts from the mangrove species *Rhizophora mucronata*, the seagrass *Syringodium isoetifolium* and the seaweed *Padina tetrastomatica* showed promising results in their activity against different pathogens, which opens up an alternative towards chemical treatments of silkworm disease that are expensive and harmful for the environment.

Bouftira *et al.* (2007) suggested 2,6-bis (1,1-dimethylethyl)-4-methylphenol found in a fraction of a methanolic extract of purple *M. crystallinum* leaves as an alternative to the synthetic antioxidant butylated hydroxytoluene (BHT), which is used to slow down autoxidation related changes in the colour and taste of food. Yu *et al.* (2012) tested the antioxidative effect of *S. europaea* extracts within peanut oil and evaluated them as potential food additive as substitute for synthetic antioxidants in food industry. Several other authors who investigated the antioxidative and antibacterial activity of different extracts from halophytes such as *C. maritimum* and *M. edule* suggest the application potential in food industry or environmental pesticides (Falleh *et al.* 2008; Meot-Duros *et al.* 2010; Trabelsi *et al.* 2010; Falleh *et al.* 2013). Many of the potential applications are still in the research process and need to be evaluated in more detail; others are already on the market.

Falcarindiol is notable for food manufacturers and the cosmetic industry as a preservative agent and biopesticide or in medicine as a new antibiotic (Meot-Duros *et al.* 2010). Compounds and extracts of *C. maritimum* seem to have a high potential for further detailed studies and should be investigated

by interdisciplinary researchers in more detail to obtain valid data for application as pharmaceuticals.

Another interesting field of technical application of compounds or extracts from halophytes is the growth inhibition of bacteria, fungi and algae – to protect specific surface areas. Besides their activity against different fungal, yeast and bacterial species Lellau and Liebezeit (2003) tested the anti-algal activity of extracts from different halophyte species. They found the highest activity against algae in extracts from *Artemisia maritima* and *S. europaea*.

Products on the Western market

Registration and notification of new products

Before any plant extracts or isolated products can be sold on the market several regulations need to be followed. These are different depending upon whether they are going to be sold on the national market or worldwide. There are specific registrations and notifications for pharmaceuticals, compounds with pharmacological effects, cosmetic ingredients, nutraceuticals and for technical applications that need to be carefully implemented. Some of these are very expensive and associated with considerable administrative work.

Pharmaceutical applications

There are several pharmaceutical products already on the market. *Salicornia* spp. capsules with pharmacological activity are sold mainly on the US and Chinese market with promised anti-inflammatory, anti-hyperlipidemic, anti-diabetic and anti-carcinogenic effects (e.g. RockWellNutrition, Alibaba). In China more than 100 species of Chinese halophytes are used in traditional medicine, including *Lycium chinese*, *Glycerrhiza uralensis*, *Apocynum venetum* and *Nitria tanguatorum* (Zhao *et al*. 2011). These plants are cultivated in the saline soils of north-western China and are marketed domestically. On the German homeopathy market *Salicornia* spp. *globuli* (C 30) and tincture are offered (Archea Pharma GmbH Dr. Makosi, Bensheim, Germany). *Salicornia* spp. iodine powder is sold by the companies Bayer (Leverkusen, Germany) and Dr Pandalis (Glendorf, Germany).

The principal bioactive compound of methanolic extracts of *T. gallica* is the alkaloid tamarixin, along with traces of its aglucone tamarixetin. The plant also contains high levels of tannins and quercitol (IUCN 2005). Syrups of *T. gallica* characterised mainly by its hypercoagulant action are sold by RockWellNutrition. It is promised that this remedy plays an important role in the erythropoesis and thrombolytic series by activating iron and cholesterol metabolism and correcting a deficit in the regeneration of the thrombin. Like many other plants extracts, it also acts as an anti-thromboplastic element. *T. gallica* is indicated for conditions such as hypochromic anaemia, mononucleosis and hypocoagulation. *T. gallica* extracts are also offered for various purposes in Ayurveda medicine, such as immunodulator, and for children with carminative and digestive properties with the product Bonnisan by Himalaya Herbal Health Care (Riga, Latvia). In the case of the product Bonnisan the effects are based on 29 clinical case studies but no experimental papers are published (http://www.himalayahealthcare.com/products/bonnisan.htm#f, accessed 29 September 2012).

Application in cosmetics

There are cosmetic products including *S. europaea* extracts on the market as skin cream or beauty cream, also used in the Thalasso therapy. However, it is not described how the extracts were produced and tested for their effects (http://www.lessonia.com/default.aspx, accessed 1 November 2012). Extracts of *S. europaea* are also mentioned for being included in anti-aging creams rich in polyphenols as activator of filaggrin and profilaggrin (German patent DE102010017151A1). Again the exact method of production of the extract is not explained in a peer-reviewed publication.

Use as functional food and nutraceuticals

Phytochemicals from halophytes are found in both, functional food and nutraceutical products. Based on their specific properties osmolytes are used in a broad range of applications in biotechnology and medicine and also in nutraceuticals (Hagihara *et al*. 2012). They are found in products such as FoldRight (Life Enhancement products, Petaluma, CA, USA) offered with the promise that the product corrects misfolds of proteins when aging and contains betaine, creatine, glycine, inositol, proline, taurine, trehalose and β-alanine. It needs to be mentioned that the statements have not been evaluated by the Food and Drug administration and that these products are not intended to diagnose, treat, cure or prevent any disease.

It was already suggested by Charnoc (1988) that plants grown in salty environment could be used as herbal salts. Later herbal salts from *Salicornia brachiata* und *Suaeda nudiflora* were patented and offered as nutraceuticals. Plants are dried and milled and directly sold as herbal salt. Next to NaCl they contain many other mineral salts in high amounts and accumulate iodine (German patent DE60208082T2).

Technical application

Extracts from different plant species containing high amounts of phenolic acids are generally known to inhibit the growth of algae. Especially the phenols cinnamic acid, *p*-coumaric acid, caffeic acid and ferulic acid show algicidal effects (German patent DE19602664A1). As discussed above, many halophytes contain high amounts of phenolic acids. *Grindelia camporum* appears to be salt tolerant; populations are found in saline flats and near salt lakes and springs. It exudes large amounts of aromatic resins that cover the surface of the plant. The resins are nonvolatile mixtures of bicyclic terpene acids, esters, and related structures that are insoluble in water but soluble in organic solvents. The amount of resin produced ranges from 5 to 18% of dried biomass. *Grindelia* resins have properties similar to the terpenoids in wood and gum colophony, which are used commercially in adhesives, varnishes, soaps, and numerous other industrial applications. With increasing costs and declining supplies of these wood-based materials, substitutions with *Grindelia* resins in this market (700 000 tons per year) may become practical (BOSTID 1990).

Future perspectives

The distribution of halophytes in many families leading to the diversity of chemical compounds and the stress-adapted metabolism has resulted in a high potential to extract compounds of a wide chemical diversity and effect range. Both traditionally used halophytes and as yet unexplored salt-tolerant species have the potential to contribute chemicals, even of hitherto unknown structures. The search for valuable salt-tolerant crop plants to act against problems caused by the salinisation of arable land and scarcity of fresh water and the already existing knowledge of a strong application potential in many sectors makes a use-orientated research in this field necessary. The economic application of secondary compounds of halophytes relies on the provision of reproducible plant material regarding quality and quantity of the desired metabolites. The provision of genetically defined plant material and the determination of culture conditions, plant organ and developmental status that guarantee both satisfactory biomass production and metabolite concentration within the plant material are compulsory for future breeding programs. For this purpose, agronomic techniques using seawater or brackish water irrigation need to be optimised. Furthermore, a reproducible extraction feasible at an economic scale, structure determination of new compounds and definition of the dose-dependence of an effect are equally important in confirming the applicability of a given extract or compound *in vitro* and *in vivo* and in the situation of implementation. Some of the research studies published cover several aspects of this approach but most projects are far from the transfer of knowledge to application and industrial production. We believe that the research community should concentrate first on a few species, analyse them in detail and build a reliable basis for concrete economic application. Screening a broader variety of species for potentially applicable compounds should be the next step. There are still a large number of potentially useful halophytic plants that are yet to be investigated.

Acknowledgements

The research in our laboratory and travelling is funded by the Deutsche Bundesstiftung Umwelt (AZ 27708), the DAAD (Vigoni project 54644032) and by the COST (action FA0901). We would like to thank Bernard Huchzermeyer and Tim Flowers for critically reading the manuscript and valuable suggestions for improving it.

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Chapter 4

Buhmann, A., Waller, U., Wecker, B., Papenbrock, J., Optimization of culturing conditions and selection of species for the use of halophytes as biofilter for nutrient-rich saline waters. (Submitted to “Agricultural Water Management” in January 2014).

Optimization of culturing conditions and selection of species for the use of halophytes as biofilter for nutrient-rich saline waters

Anne Buhmann

Leibniz University Hannover, Institute of Botany, Herrenhäuserstr. 2, D-30419 Hannover, Germany, anne.buhmann@botanik.uni-hannover.de

Uwe Waller

Hochschule für Technik und Wirtschaft des Saarlandes, Göbenstr. 40, D-66117 Saarbrücken, Germany, uwe.waller@htwsaar.de

Bert Wecker

neomar GmbH, Am Osterberg 22, D-31311 Uetze-Eltze, Germany, bert.wecker@neomar.de

Jutta Papenbrock

Leibniz University Hannover, Institute of Botany, Herrenhäuserstr. 2, D-30419 Hannover, Germany, Jutta.Papenbrock@botanik.uni-hannover.de

Corresponding author:

Jutta Papenbrock, Leibniz University Hannover, Institute of Botany, Herrenhäuserstr. 2, D-30419 Hannover, Germany, Tel. +49 511 762 3788, Fax +49 511 762 19262, Jutta.Papenbrock@botanik.uni-hannover.de

Abstract

Salt-tolerant plants can be used as biofilter for nutrient-rich saline waters such as aquaculture process water. The efficiency of a plant biofilter is influenced by various factors that need to be investigated in order to improve the suitability for different applications. *Tripolium pannonicum* (Jacq.) Dobrocz. was used as one model plant to test different culturing conditions and to determine biofilter performance. This performance was evaluated by taking different parameters into account, such as biomass production, plant nitrogen and phosphorus uptake as well as physiological parameters and decrease of nitrate-N and phosphate-P concentrations in the experimental fluid. After optimization of culturing conditions, additional plant species known as edible were studied to follow the idea of generating a valuable co-product beside the use as biofilter. It was shown that a nitrate-N concentration of at least 10 mg l^{-1} is necessary for reasonable biomass production. A phosphate-P concentration of 0.3 mg l^{-1} is sufficient, but higher concentrations promote the uptake of phosphate-P. The addition of iron in chelated form is inevitable for the growth of healthy plant biomass; the addition of manganese is beneficial but not implicitly necessary. Salt concentrations lower than sea water salinity such as 15 psu promote biomass production and nutrient uptake. The use of a hydroponic culture system is more suitable than sand or expanded clay culture if controlled conditions and nutrient recycling are aimed. The comparison of different halophyte species in 0.24 m² tanks with nine plants each resulted in above ground fresh weight of 185 to 398 g and total uptake of nitrogen and phosphorus of 0.6 to 2.1 g and 0.1 to 0.4 g, respectively, during five weeks of experiment. All tested species have potential to serve as biofilter and valuable co-product. A promising application is the growth of halophytic vegetable plants in marine aquaponic systems.

Keywords: Aquaculture; Nutrient deficiency; Nutrient recycling; Salt tolerance; Wetlands.

1. Introduction

Different plant species and types of natural and constructed wetlands are successfully applied for phytoremediation and biofiltration of municipal and industrial wastewater or affected soil and effluents from agroindustry and aquaculture (Kadlec and Knight, 1996; Verhoeven and Meulemann, 1999; Vymazal, 2010). Operating those plant biofilters is a low cost opportunity to mitigate the impact of effluents on the environment: the load of nutrients, heavy metals and organic substances in wastewater that would otherwise cause hypertrophication and toxification of surrounding ecosystems is reduced by the plants. Some of the applied plant species have a market value, for example as vegetable and fodder or due to valuable metabolites or suitability of the biomass as material for fuel or gas production. Those marketable properties represent an added value beside the utility of the plants as biofilter.

An interesting application of plants as secondary biofilter and valuable co-product appears to be aquaculture. Fish and invertebrate animals produced in aquaculture retain only some of the carbon, nitrogen, and phosphorus administered with the feed. There are several studies that combine fresh water aquaculture with plants grown in hydroponic culture. Watten and Busch (1984) published results from experimental trials on fish and tomato culture. The system used was composed of a fish tank, a settling tank, a trickling biofilter, and the hydroponic bed for the culture of tomatoes. Since then, this basic layout was used in many other investigations. The conversion of nitrogenous excretory products is conducted in nitrifying biofilters and the remaining nitrate-N and other nutrients in the process water serves as base for plant growth. In several studies plants are applied as a secondary biofilter and are concurrently used for the production of valuable vegetables, such as lettuce, spinach, tomato, cucumber, and pepper (Lennard and Leonard, 2006; Graber and Junge, 2009; Roosta and Mohsenian, 2012; Petrea et al., 2013).

Some industrial, agricultural or municipal waste waters are saline. Plant species often used in filter beds are very sensitive to salt and the use of salt-tolerant plant species becomes mandatory. Those halophytes tolerate salinities up to above sea water salinity, depending on the species. Several studies investigated the use of salt-tolerant plants as biofilter and nutrient sink for different nutrient-rich saline effluents (Grieve and Suarez, 1997; Klomjek and Nitisoravut, 2005; Wu et al., 2008). Recently, Dias et al. (2013) proofed a good performance of *Salicornia bigelovii* Torr., *Atriplex lentiformis* (Torr.) S. Watson, *Distichlis spicata* (L.) Greene, *Spartina gracilis* Trin., *Allenrolfea occidentalis* (S. Watson) Kuntze and *Brassica hyssopifolia* (Pall.) Kuntze in a saline drainage water reuse system.

Marine aquaculture is a novel source of saline effluents with rising importance because of the increasing demand of sea food. The FAO (2012) stated: “As production from capture fisheries has remained virtually constant, further aquaculture growth will be needed to meet the rising global demand for seafood.” Studies on the application of halophytes as biofilter for aquaculture process water are summarized by Buhmann and Papenbrock (2013a), including the two aspects i) nutrient recycling in recirculating systems and ii) reduction of environmental impact in open installations.

There are several studies describing the potential of halophytes as vegetable or as raw material for fodder and to provide secondary metabolites that can be used for pharmaceuticals, functional foods, nutraceuticals, and technical implementations (Ksouri et al., 2011; Buhmann and Papenbrock, 2013b). The production of salt-tolerant plants with market value that serve as secondary biofilter for marine aquaculture process water at once, is promising. Recently, species of the edible salt tolerant plant genus *Salicornia* have been studied in constructed wetlands for the purification of marine aquaculture effluents (Webb et al., 2012; Shpigel et al., 2013). Both studies showed high yields of marketable biomass and effective nitrogen and phosphorus removal.

An additional challenge for the combination of nutrient-rich saline wastewater purification by halophytes with the production of a valuable co-product is the limited knowledge about the cultivation of halophytes. For the cultivation of the edible halophyte genera *Salicornia* and *Sarcocornia* different aspects of cultivation have been described recently (Ventura et al., 2010, 2011a and b; Katschnig et al., 2013; Ventura and Sagi, 2013). Other halophyte species also have potential as saline vegetable crops such as *Tripolium pannonicum* (Jacq.) Dobrocz., *Plantago coronopus* L. and *Crithmum maritimum* L. (Ben Amor et al., 2005; Koyro, 2006; Koyro et al., 2011, Ventura et al., 2013). Beside the potential as biofilter and valuable co-product it is highly beneficial to foster the use of saline water for agricultural production because freshwater is or becomes a short running resource in many regions of the world (FAO, 2008, 2012; Rozema and Schat, 2013). In this context it is also important to establish suitable setups to avoid soil salinization and saltwater intrusion into the watershed.

The efficiency of a plant biofilter is influenced by various factors (Kadlec and Knight, 1996; Verhoeven and Meulemann, 1999; Vymazal, 2010; Buhmann and Papenbrock, 2013a), such as the influence of salinity on nutrient uptake, light conditions and availability of micronutrients. In our opinion those factors remain unclear if plants are studied within an aquaponic system with specific properties. Due to the limited information about the culture of

halophytes it is substantial to determine the factors that influence the performance of halophytes as biofilter before application. Besides this, it is necessary to investigate different halophyte species and their biofilter capacity before integrating them into an aquaponic system.

In this study, the word biofilter is used for the function of the plants to reduce the concentration of nitrogen and phosphorus in an effluent by uptake into the plant tissue. We conducted greenhouse experiments with artificial sea water as a base for plant nutrient solution. Only one factor was investigated in each experiment using *T. pannonicum* as a model plant to determine conditions that are favourable for the performance of a halophyte biofilter. The influence of substrate, salinity, and the effect of nitrate-N and phosphate-P concentration was tested as well as the beneficial effect of an addition of iron and manganese. Finally, the biofilter capacity of different species was compared under optimized conditions.

2. Material and Methods

2.1 Plant material

For some of the species seeds were collected at the North Sea, Jade Bay, Germany:, *Atriplex portulacoides* L. (53°29‘13N; 8°03‘16“O) and *Salicornia dolichostachya* Moss (53°29‘13N; 8°03‘16“O). Two different seed collections were used in the case of *Tripolium pannonicum* (Jacq.) Dobrocz., one from the coordinates 53°29‘13N; 8°03‘16“O, named as ecotype 1 (et1) and one from the coordinates 53°26‘19‘‘N; 8°09‘49‘‘O, named as ecotype 2 (et2) in the following. *Salicornia* plants were identified as *S. dolichostachya* on the base of ETS sequence analysis (Singh, 2013). Seeds of *Plantago coronopus* L. were ordered at Jelitto Staudensamen GmbH (Schwarmstedt, Germany). For the species *Lepidium latifolium* L. and *Atriplex halimus* L. one specimen was ordered at Rühlemann´s Kräuter & Duftpflanzen (Horstedt, Germany). Seeds were produced from the *L. latifolium* plant and cuttings were obtained from the *A. halimus* plant.

The plants for the experiment were grown in a greenhouse at a temperature of 22°C. Artificial light was given in a 14 h/10 h light/dark rhythm (sodium vapor lamps, SON-T Agro 400, Philips, Amsterdam, Netherlands). Seeds were sown on propagation soil (Einheitserde, Einheitserdewerk Hameln-Tündern, Germany) and irrigated with tap water according demand. When seedlings had a height of 1 to 2 cm they were transplanted to pots filled with sand (0 to 2 mm grain size, Hornbach, Hannover, Germany), watered as needed and supplied twice a week with a modified Hoagland solution according to Epstein (1972) with the

following modification: $MoNa_2O_4$ instead of MoO_3 and NaFe-EDTA instead of NaFe-DTPA. The final elemental nutrient concentration in the solution was 160 mg Ca l^{-1}, 237 mg K l^{-1}, 24 mg Mg l^{-1}, 225 mg N l^{-1}, 62 mg P l^{-1}, 32 mg S l^{-1} for macronutrients and 4.37 mg B l^{-1}, 0.03 mg Cu l^{-1}, 1.06 mg Fe l^{-1}, 0.12 mg Mn l^{-1}, 0.05 mg Mo l^{-1}, 0.45 mg Zn l^{-1} for micronutrients. Plants transferred to the experimental setup had approximately the same size. The time between sowing and start of the experiment was 3, 4-5, 5, 6 and 7 weeks for *P. coronopus*, *T. pannonicum*, *L. latifolium*, *S. dolichostachya* and A. *portulacoides,* respectively. The cuttings were planted on sand (0 to 2 mm grain size, Hornbach), watered as needed and supplied with the modified Hoagland solution described above twice a week. One week before the experiment, plants were adapted stepwise to salinity by adding 0.5, 1.0 and 1.5% of NaCl to the irrigation water (every second day the next higher salt concentration).

2.2 Experimental setup

The experiments were conducted in another greenhouse with temperatures of around 20/15°C during day/night. From October till May artificial light was provided on at a 12 h light/dark rhythm (sodium vapor lamps, SON-T Agro 400, Philips). Light intensity ranged from 65 µmol m^{-2} s^{-1} (only artificial light in the dark in winter) to 850 µmol m^{-2} s^{-1} (midday in summer without clouds) depending on the time of the year, the time of the day and the cloudiness. For *S. dolichostachya* day length was artificially elongated to 18 h by applying artificial light early in the morning (from 4 to 8 a.m.) and at night (from 6 to 10 p.m.) (sodium vapor lamps, SON-T Agro 400, Philips) to prevent flowering of the plants (Ventura et al., 2011b).

Polypropylene tanks (L x W x H, 600 x 400 x 425 mm) (RAKO-Container, Utz-Gruppe, Schüttorf, Germany) were used for the experiments. The experimental tanks were designed and modified by Erwin Sander Elektroapparatebau GmbH, Uetze-Eltze, Germany to allow hydroponic culture as well as culturing on substrate (Buhmann and Papenbrock, 2013a).

For hydroponic culture the tanks were filled with 70 L of artificial seawater (salt mixture from Seequasal GmbH, Münster, Germany) and aerated constantly by small compressors and one air stone in the middle of each tank (Eheim, Deizisau, Germany). In each tank a floating styrofoam board (15 mm thick) was used to keep the plants 1 to 2 cm above the water surface. The hypocotyl was fixed with soft foam in 35 mm holes. Each board provided space for nine plants.

For culture with sand substrate, the experimental tanks were filled with a gravel layer of 10 cm (8 to 16 mm grain size, Hornbach) at the bottom. A sand layer of 23 cm (0 to 2 mm grain

size, Hornbach) was filled up which was separated from the gravel by a fleece (Freudenberg Vliesstoffe SE & Co.KG, Weinheim, Germany). The tanks were filled with 24 to 26 L of artificial sea water (Seequasal GmbH) in order to reach the necessary water level for recirculation. The artificial sea water contained Cl^-, Na^+, $SO4^-$, Mg^{2+}, K^+ and Ca^{2+} in standard seawater proportions (Turekian, 1968) and the major elements apart from Cl were measured with inductively coupled plasma optical emission spectrometry in a 15 psu solution (ICP-OES) (iCAP 6000 ICP Spectrometer, Thermo Fisher Scientific Corporation), showing concentrations of 600 mg Na l^{-1}, 530 g S l^{-1}, 530 g Mg l^{-1}, 240 g K l^{-1} and 200 g Ca l^{-1} (method for analysis was not accurate jet, data are only an approximation of the composition). The water inlet to the pump was a drainage pipe manifold fixed underneath the gravel layer. Water circulation was driven by a centrifugal pump (Aquabee UP 300, Aquabee Aquarientechnik, Zerbst, Germany) delivering the culturing fluid to a sprinkling system. The sprinkling system was combined of two parallel pipes with holes fixed some centimeters above the sand layer. The water circulation was automatically activated for 12 h during a day. For cultures with expanded clay (8 to 16 mm, Hydrokultur Spezialist, Paderborn, Germany) was filled in the experimental tanks. The tanks were filled with 30 to 35 L artificial seawater. Water surface leveled at the 70 L-mark used for the hydroponic culture. The substrate surface was kept 1 to 2 cm above the water surface. The expanded clay culture was aerated in the same way as the hydroponic culture.

Tanks were refilled with tap water daily up to a marked water level for compensating evapotranspiration to keep salinity and nutrient concentration in the solution stable. Salinity and nutrient concentration were monitored weekly. In all experiments nine plants were planted into each experimental tank. The control group and every treatment group consisted of 27 plants distributed in three randomly selected experimental tanks.

2.3 Specific experimental conditions

A first experiment was conducted to investigate the influence of different culture modes (hydroponic culture, substrate culture) and different types of substrate (sand, expanded clay). This experiment is abbreviated as $E_{SUBSTRATES}$. In $E_{SUBSTRATES}$ micronutrients were added to artificial sea water with a salinity of around 15 psu (practical salinity unit) as in the modified Hoagland solution described above, macronutrients were added as follows: 120 mg $MgSO_4$ l^{-1}, 447 mg KCl l^{-1}, 685 mg $NaNO_3$ l^{-1}, 63 H_2NaPO_4 mg l^{-1} (237 mg K l^{-1}, 24 mg Mg l^{-1}, 114 mg N l^{-1}, 13 mg P l^{-1}, 32 mg S l^{-1}).

In a proximate experiment manganese and iron were investigated as possible reason for the chlorosis occurring in the hydroponic culture and expanded clay culture of $E_{SUBSTRATES}$. In this experiment (named $E_{MICRONUTRIENTS}$ in the following) a control with no addition of micronutrients to the artificial seawater (15 psu) was compared to a treatment with the addition of iron as NaFe-EDTA (7.34 mg l^{-1}, 1.10 mg Fe l^{-1}, Fluka, Taufkirchen, Germany), one with the addition of iron as Fe-EDDHA (18.31 mg l^{-1}, 1.12 mg Fe l^{-1}, Duchefa Biochemie, Haarlem, The Netherlands), one with the addition of manganese (as 0.25 mg $MnCl_2$ l^{-1}, 0.11 mg Mn l^{-1}) and one treatment with the addition of the two micronutrients together, iron as Fe-EDDHA (18.31 mg l^{-1}, 1.12 mg Fe l^{-1}) and manganese (as 0.25 mg $MnCl_2$ l^{-1}, 0.11 mg Mn l^{-1}). Moreover, the performances of the two different seed collections of *T. pannonicum* (et1 and et2) were compared within the treatment with addition of iron as NaFe-EDTA (7.34 mg l^{-1}, 1.10 mg Fe l^{-1}). In $E_{MICRONUTRIENTS}$ apart from the micronutrients 685 mg $NaNO_3$ l^{-1} (114 mg N l^{-1}) and 63 mg H_2NaPO_4 l^{-1} (13 mg P l^{-1}) were added.

Additionally, an experiment to test the impact of different salinities (15.0, 22.5 and 30.0 psu) on plant growth, nutrient uptake and photosynthesis was carried out ($E_{SALINITY}$). As nutrients 685 mg $NaNO_3$ l^{-1} (114 mg N l^{-1}), 63 mg H_2NaPO_4 l^{-1} (13 mg P l^{-1}) and 9.16 mg Fe-EDDHA l^{-1} (0.56 mg Fe l^{-1}) were added to the artificial sea water of different salinities.

In an experiment named $E_{NITRATE}$ we used different nitrate-N concentrations (1, 10, 15, 25, 50 and 100 mg NO_3-N l^{-1}, applied as $NaNO_3$) in the culturing solutions to find out which nitrate-N concentration in the water might be limiting for the growth of halophytes. Apart from the different nitrate-N concentrations, phosphate-P (38 mg H_2NaPO_4 l^{-1}, 9 mg P l^{-1}) and iron (9.16 mg Fe-EDDHA l^{-1}, 0.56 mg Fe l^{-1}) were added to the artificial seawater (15 psu).

A similar experiment was carried out to identify the influence of phosphate-P concentration on the biofilter capacity, using different treatments with phosphate-P concentrations of 0.3, 1.6, 3.3, 5.0, 9.8, 16.3 mg phosphate-P as H_2NaPO_4. In this experiment, named $E_{PHOSPHATE}$, nitrate-N (304 mg $NaNO_3$ l^{-1}, 50 mg N l^{-1}) and iron (9.16 mg Fe-EDDHA l^{-1}, 0.56 mg Fe l^{-1}) were added to the artificial seawater (15 psu) as well. In a final experiment named $E_{SPECIES}$ we compared the different halophyte species *T. pannonicum* (et1 and et2), *P. coronopus*, *A. portulacoides*, *A. halimus*, *L. latifolium* and *S. dolichostachya* under application of those conditions which were figured out as best with respect to plant health, nitrate-N and phosphate-P uptake and biomass formation in the preceding experiments: hydroponic culture, artificial seawater with a salinity of 15 psu, 304 mg $NaNO_3$ l^{-1} (50 mg NO_3-N l^{-1}), 38 mg H_2NaPO_4 l^{-1} (9 mg P l^{-1}) and 9.16 mg Fe-EDDHA l^{-1} (0.56 mg Fe l^{-1}).

All experiments were conducted one after another between February and September 2013. Only the substrate experiment took place in May till June 2012. Each experiment lasted for five weeks (35 days).

2.4 Determination of plant growth

At the beginning of each experiment a set of nine plants of comparable size were randomly selected. The total wet weight of roots and shoots of all nine plants was determined. The dry weight of roots and shoots were determined after drying the tissues at 110°C until stable weight was reached. At the end of an experiment the fresh weight and the dry weight of total shoot and root biomass of each tank was determined as described for the beginning of the experiment.

2.5 Water parameters

Water samples were taken weekly with the first sampling at the day of planting and the last sampling at the day of harvesting at the end of the experiment. Water sampling in sand culture was done by using a discharge pipe. Water samples in the expanded clay and hydroponic culture systems were taken by a pipe released into the water at different places. Water samples were filtered (0.22 µm pore size, Carl Roth, Karlsruhe, Germany) and stored at -60°C. All water samples were taken in triplicates.

The colorimetric assays for the determination of nutrients in water samples (phosphate-P, nitrate-N, nitrite-N and ammonium-N) were conducted in 96-well microplates (Sarstedt AG & Co., Nümbrecht, Germany) and measured in a microplate reader (Synergy Mx, BioTek Germany, Bad Friedrichshall, Germany). Standard curves were on each microplate by using a blank and five calibration standards made from a stock standard (IC-Standards, Carl Roth, Karlsruhe, Germany) and treating them like the samples. For the preparation of samples, blanks, and standards ultrapure water was used (Purelab Ultra, ELGA LabWater Deutschland GmbH, Celle, Germany).

For the determination of nitrate-N concentrations in water samples the method of Zhang and Fischer (2006) was modified for microplate reading. Calibration standards with concentrations between 0.5 and 10 mg NO_3^- l^{-1} were prepared. For colorimetric reaction 10 µl of 10% sulfaminic acid was added to 80 µl of the water. The plate was incubated in a shaking water bath (1083, GFL Gesellschaft für Labortechnik GmbH, Burgwedel, Germany) at 80°C

for 30 min to allow nitrite-N to precipitate. Afterwards 10 µl of 2.5% resorcinol solution was added to each well and the microplate was again shaken for 1 min at a frequency of 850 min^{-1} (Thermomixer compact, Eppendorf, Wesseling-Berzdorf, Germany). Then 150 µl of 36 N sulphuric acid was added and again incubated in a shaking water bath at 80°C for 60 min. The microplates were cooled to 15°C in a water bath. For all incubation steps the microplate was covered with polypropylene sealing foil (HJ-Bioanalytik GmbH, Mönchengladbach, Germany). After incubations the microplates were centrifuged at 200 *g* for 1 min (Jouan CB3i, Thermo Electron Cooperation, Waltham, Massachusetts, USA) and wiped dry carefully. The absorption was measured at 505 nm.

Ammonium-N was determined according to DIN 38406-5 modified for the use of microplates. To each 250 µl sample 25 µl of a solution containing 0.81 M sodium salicylate, 0.44 M trisodium citrate dehydrate and 3.25 M sodium nitroprusside dehydrate was added. Afterwards a solution containing 9 mM sodium dichloroisocyanurate dissolved in 0.4 M NaOH was added and the plate was kept for 1 h at room temperature. The absorption was measured at 655 nm.

Phosphate-P and nitrite-N were determined exactly as described in Hérnandez-López and Vargas-Albores (2003).

The results for the nutrient concentrations are expressed as NO_3-N, NO_2-N, NH_4-N and PO_4-P in the following text parts, tables and figures. The pH value was determined by a pH meter (Multi 350i pH/ISE-/Sauerstoffleitfähigkeits-Messgerät, WTW Technische Werkstätten GmbH, Germany).

2.6 Analysis of elements

For the determination of phosphorous, iron and manganese the samples of dried plant material used for the determination of plant growth were homogenized (roots and shoots separately) and a subsample taken from each homogenate was grinded to fine powder (MM 400, Retsch GmbH, Haan, Germany). About 38 mg of powder from each sample was incinerated for a minimum of 8 h in a muffle furnace (M104, Thermo Fisher Scientific Corporation, Waltham, Massachusetts, USA). After cooling the samples to room temperature 1.5 ml of 66% nitric acid was added. After 10 min 13.5 ml of ultrapure water was added. The solution was filtered (0.45 µm pore size, Carl Roth) and stored in vials before final analysis at -60°C. For every sample preparation an empty vial was treated parallel to the samples and later used as a blank. The samples were analysed by inductively coupled plasma optical emission spectrometry

(ICP-OES) (iCAP 6000 ICP Spectrometer, Thermo Fisher Scientific Corporation). For the determination of iron and manganese only young leaves were used. In the dry plant powder nitrogen was determined using a C-N-S elemental analyser (Vario EL III, Elementar Analysensysteme, Hanau, Germany) instructions.

2.7 Determination of chlorophyll and carotenoids

For the extract 400 µl of ice-cold 80% acetone was added to a sample of frozen and grounded leaf material (50 mg). The sample was kept on ice for 10 min, mixed every 2 min and centrifuged at 14,000 x g for 5 min. The supernatant was collected and stored on ice in the fridge. The pellet was re-extracted three times with 200 µl ice-cold 80% acetone and centrifuged as described above. All supernatants were pooled for pigment determination. Absorption was measured at 750.0, 663.2, 646.8 nm and 470.0 nm using a spectrophotometer (Uvikon XS, Biotech instruments, Germany) and total chlorophyll and carotenoid contents were calculated according to Lichtenthaler (1987).

2.8 Chlorophyll fluorescence measurements

A pulse modulated chlorophyll fluorescence meter (Imaging PAM M, Walz, Effeltrich, Germany) was used to measure photosynthetic activity. A fully expanded young leaf was dark adapted for 30 min and then cut off from the plant for the measurement. Minimal fluorescence (F_0) and maximal fluorescence (F_m) were measured after dark adaption. Maximum quantum efficiency of photosystem II (PSII) photochemistry (F_v/F_m) was calculated according to Baker (2008). Measurements were conducted on three leaves from different plants for every treatment and the arithmetic mean was calculated for further evaluation.

2.9 Statistical analysis

All statistical analyses were conducted using R software version 3.0.2 (www.r-project.org) in combination with R Studio (RStudio, Boston, USA). Pairwise comparison between the treatments was conducted with a Tukey-type multiple contrast test according to Hothorn et al. (2008) with alpha = 0.05. Principal component analysis (PCA) was performed on all the parameters of $E_{NITRATE}$ and $E_{PHOSPHATE}$ using the ade4 package (Dray and Dafour, 2007) and subsequently of the first two components pairwise comparison was conducted between the treatments according to Hothorn et al. (2008).

3. Results

Different experiments were carried out to define optimal culturing conditions for the use of halophytes as biofilter for the removal of nitrate-N and phosphate-P from process water by *T. pannonicum*: (i) the influence of substrate types ($E_{SUBSTRATES}$), (ii) the effectiveness of micronutrients in dependence from the kind of chemical combination and concentration ($E_{MICRONUTRIENTS}$), (iii) the influence of process water salinity ($E_{SALINITY}$), (iv) the influence of the nitrate-N concentration in the process water ($E_{NITRATE}$), and the influence of the phosphate-P concentration in the process water ($E_{PHOSPHATE}$). The results of experiments are compiled in Table 1.

In $E_{SUBSTRATES}$, plants cultured in expanded clay and hydroponic culture showed a higher gain of biomass and uptake of nitrogen than those cultured in sand, but not significantly (Table 1). The hydroponic culture treatment displayed a significantly higher uptake of phosphorus than the other two treatments ($p<0.001$, Table 1). Although in terms of growth and nutrient uptake plants grown in hydroponic culture and in expanded clay performed better than those cultured in sand, they had a much lower chlorophyll and carotenoid content. Plants cultured in expanded clay and hydroponic culture had a content of just 525 and 252 $\mu g\ g^{-1}$, whereas plants grown in sand contained 812 $\mu g\ g^{-1}$ (Table 1).

$E_{MICRONUTRIENTS}$ was conducted to find out if prevention of chlorosis is possible by adding manganese or iron in form of Fe-EDDHA instead of iron as Fe-EDTA to the solution. The pH in the nutrient solution was in the range of 7.9 and 8.5 in all the experiments, due to high $CaCO_3$ content in the artificial sea water (data not shown). Fe-EDDHA is more stable at higher pH than Fe-EDTA and therefore possibly more suitable. Plants grown with Fe-EDDHA showed significantly higher chlorophyll and carotenoid contents compared to plants grown with Fe-EDTA ($p<0.001$, Table 1). The lowest chlorophyll and carotenoid content was exhibited by plants grown without the addition of iron. The addition of manganese did not increase the chlorophyll and carotenoid content. However, manganese content in young leaves was higher in the treatments with manganese than in those without (Table 1). On the contrary, iron content in young leaves was higher in the treatments without iron than in the treatment with addition of iron to the solution (Table 1). Maximum quantum efficiency of PSII photochemistry did not show any differences between the two iron sources, but values were lower for the treatment without iron and manganese and for the treatment with sole addition of manganese (Table 1).

Table 1. Results of the experiments to determine the influence of different factors on the biofilter capacity of *T. pannonicum* (arithmetic mean and standard deviation). Each experiment lasted for five weeks (35 days), for each treatment three tanks (0.24 m) with nine plants each were used as replicates. Mean values with different letters indicate a significant difference between treatments in the according parameter ($p<0.05$). $E_{SUBSTRATES}$: application of different substrates, $E_{MICRONUTRIENTS}$: application of micronutrients, $E_{NITRATE}$: addition of different NO_3-N concentrations to the culturing solution, $E_{PHOSPHATE}$: addition of different PO_4-P concentrations to the culturing solution, $E_{SALINITY}$: different concentrations of artificial sea water, FW: fresh weight, DW: dry weight, N: Nitrogen, P: Phosphorus, et1 and et2: ecotype 1 and 2 of *T. pannonicum*, *data for young leaves.

		Gain of biomass		Nutrient uptake by plants		F_v/F_m	Chlorophyll	Carotenoids	Iron*	Manganese*	Nitrogen	Phosphorus
Experiment	Treatment	FW in g	DW in g	N in g	P in mg		in µg g^{-1} FW	in µg g^{-1} FW	in mg g^{-1} DW	in mg g^{-1} DW	in mg g^{-1} DW	in mg g^{-1} DW
$E_{SUBSTRATES}$	Sand culture	$1024^{a} \pm 161$	$61^{a} \pm 8$	$2.06^{a} \pm 0.14$	$146^{a} \pm 12$		$812^{b} \pm 314$	$123^{b} \pm 39$				
	Hydroponic culture	$1272^{a} \pm 180$	$79^{ab} \pm 12$	$2.37^{a} \pm 0.53$	$381^{b} \pm 145$		$252^{a} \pm 44$	$42^{a} \pm 7$				
	Extended clay	$1357^{a} \pm 95$	$94^{b} \pm 2$	$2.25^{a} \pm 0.28$	$103^{a} \pm 22$		$525^{ab} \pm 115$	$78^{ab} \pm 14$				
$E_{MIKRONUTRIENTS}$	without addition	$20^{a} \pm 4$	$4^{a} \pm 1$	$0.08^{a} \pm 0.01$	$23^{a} \pm 3$	$0.57^{a} \pm 0.07$	$49^{a} \pm 11$	$21^{a} \pm 3$	$0.38^{b} \pm 0.13$	$0.05^{ab} \pm 0.01$		
	Fe-EDTA, et1	$118^{b} \pm 33$	$11^{b} \pm 3$	$0.44^{b} \pm 0.18$	$77^{ab} \pm 35$	$0.79^{b} \pm 0.02$	$235^{ab} \pm 153$	$53^{ab} \pm 25$	$0.24^{ab} \pm 0.06$	$0.04^{ab} \pm 0.02$		
	Fe-EDTA, et2	$214^{c} \pm 52$	$18^{c} \pm 4$	$0.82^{c} \pm 0.19$	$112^{b} \pm 61$	$0.82^{b} \pm 0.01$	$388^{b} \pm 61$	$79^{b} \pm 9$	$0.13^{a} \pm 0.04$	$0.03^{a} \pm 0.00$		
	Fe-EDDHA	$114^{b} \pm 17$	$13^{bc} \pm 2$	$0.50^{bc} \pm 0.09$	$80^{ab} \pm 19$	$0.84^{b} \pm 0.01$	$875^{c} \pm 104$	$159^{c} \pm 15$	$0.16^{ab} \pm 0.00$	$0.03^{a} \pm 0.01$		
	Mn	$16^{a} \pm 1$	$3^{a} \pm 0$	$0.08^{a} \pm 0.02$	$19^{a} \pm 4$	$0.61^{a} \pm 0.04$	$68^{a} \pm 28$	$23^{a} \pm 5$	$0.32^{ab} \pm 0.13$	$0.08^{b} \pm 0.03$		
	Fe-EDDHA+Mn	$144^{bc} \pm 22$	$16^{bc} \pm 3$	$0.61^{bc} \pm 0.14$	$95^{ab} \pm 21$	$0.84^{b} \pm 0.00$	$757^{c} \pm 36$	$139^{c} \pm 6$	$0.19^{ab} \pm 0.06$	$0.07^{a} \pm 0.00$		
$E_{NITRATE}$	100 mg l^{-1} NO_3-N	$742^{b} \pm 96$	$63^{b} \pm 8$	$2.94^{c} \pm 0.35$	$401^{b} \pm 87$	$0.71^{ab} \pm 0.05$	$720^{a} \pm 39$	$141^{a} \pm 10$			$45^{c} \pm 3$	
	50 mg l^{-1} NO_3-N	$875^{b} \pm 20$	$60^{b} \pm 5$	$2.49^{c} \pm 0.34$	$379^{b} \pm 95$	$0.70^{ab} \pm 0.05$	$823^{a} \pm 187$	$160^{a} \pm 29$			$41^{c} \pm 1$	
	25 mg l^{-1} NO_3-N	$743^{b} \pm 6$	$57^{b} \pm 2$	$2.12^{b} \pm 0.20$	$304^{b} \pm 60$	$0.69^{a} \pm 0.08$	$758^{a} \pm 104$	$151^{a} \pm 18$			$35^{bc} \pm 4$	
	15 mg l^{-1} NO_3-N	$911^{b} \pm 105$	$64^{b} \pm 15$	$1.84^{b} \pm 0.40$	$385^{b} \pm 142$	$0.78^{a} \pm 0.04$	$866^{a} \pm 175$	$156^{ab} \pm 29$			$29^{b} \pm 8$	
	10 mg l^{-1} NO_3-N	$958^{b} \pm 132$	$74^{b} \pm 9$	$2.05^{b} \pm 0.20$	$444^{b} \pm 75$	$0.81^{ab} \pm 0.02$	$839^{a} \pm 281$	$152^{a} \pm 46$			$29^{b} \pm 1$	
	1 mg l^{-1} NO_3-N	$265^{a} \pm 36$	$31^{a} \pm 5$	$0.23^{a} \pm 0.06$	$80^{a} \pm 12$	$0.83^{b} \pm 0.00$	$483^{a} \pm 178$	$99^{a} \pm 30$			$14^{a} \pm 2$	
$E_{PHOSPHATE}$	16.3 mg l^{-1} PO_4-P	$634^{a} \pm 33$	$47^{a} \pm 2$	$1.84^{a} \pm 0.09$	$267^{d} \pm 13$	$0.78^{a} \pm 0.06$	$759^{a} \pm 158$	$141^{a} \pm 23$				$4.67^{c} \pm 0.33$
	9.8 mg l^{-1} PO_4-P	$634^{a} \pm 79$	$45^{a} \pm 5$	$1.68^{a} \pm 0.26$	$218^{cd} \pm 26$	$0.78^{a} \pm 0.05$	$790^{a} \pm 19$	$140^{a} \pm 7$				$4.14^{bc} \pm 0.37$
	5.0 mg l^{-1} PO_4-P	$569^{a} \pm 153$	$42^{a} \pm 10$	$1.62^{a} \pm 0.60$	$168^{bc} \pm 48$	$0.75^{a} \pm 0.08$	$781^{a} \pm 100$	$150^{a} \pm 16$				$3.90^{bc} \pm 0.07$
	3.3 mg l^{-1} PO_4-P	$655^{a} \pm 59$	$47^{a} \pm 3$	$2.09^{a} \pm 0.27$	$213^{bc} \pm 24$	$0.81^{a} \pm 0.01$	$654^{a} \pm 137$	$117^{a} \pm 27$				$4.28^{bc} \pm 0.22$
	1.6 mg l^{-1} PO_4-P	$574^{a} \pm 39$	$42^{a} \pm 3$	$1.64^{a} \pm 0.28$	$118^{ab} \pm 22$	$0.80^{a} \pm 0.03$	$676^{a} \pm 105$	$124^{a} \pm 18$				$3.03^{ab} \pm 0.33$
	0.3 mg l^{-1} PO_4-P	$567^{a} \pm 53$	$42^{a} \pm 3$	$1.49^{a} \pm 0.22$	$73^{a} \pm 27$	$0.79^{a} \pm 0.03$	$609^{a} \pm 131$	$117^{a} \pm 24$				$1.94^{a} \pm 0.62$
$E_{SALINITY}$	15.0 psu	$664^{b} \pm 154$	$50^{b} \pm 10$	$1.57^{a} \pm 0.43$	$221^{a} \pm 43$	$0.80^{a} \pm 0.01$	$834^{a} \pm 131$	$155^{a} \pm 20$				
	22.5 psu	$383^{a} \pm 84$	$33^{ab} \pm 8$	$1.34^{a} \pm 0.31$	$158^{a} \pm 47$	$0.81^{a} \pm 0.01$	$875^{a} \pm 157$	$162^{a} \pm 25$				
	30.0 psu	$236^{a} \pm 43$	$25^{a} \pm 5$	$1.06^{a} \pm 0.49$	$130^{a} \pm 61$	$0.80^{a} \pm 0.01$	$798^{a} \pm 99$	$148^{a} \pm 17$				

Plants grown with Fe-EDDHA showed little gain of biomass compared to the plants in $E_{SUBSTRATES}$ (Table 1) and visual rating indicated comparatively dark green leaves. This lead to the conclusion that iron was overdosed and half of the concentration of Fe-EDDHA was added in the following experiments. In those experiments gain of biomass was higher, the colour of the leaves appeared normally green and chlorophyll and carotenoid content was comparable to the highest values in $E_{MICRONUTRIENTS}$ (Table 1).

In $E_{MICRONUTRIENTS}$ plants cultured without addition of iron and manganese and plants cultured with sole addition of manganese showed a significantly lower gain of biomass and nitrogen and phosphorus uptake than plants cultured with an addition of iron ($p<0.001$, Table 1). The addition of manganese and Fe-EDDHA caused a higher gain of biomass and nitrogen and phosphorus uptake than the sole addition of Fe-EDDHA. The highest gain of biomass, nitrogen and phosphorus uptake occurred in the treatment with addition of Fe-EDTA and *T. pannonicum* et2 (Table 1). This indicated that *T. pannonicum* et2 grew better under our experimental conditions than et1 and therefore et2 was used for the following experiments $E_{SALINITY}$, $E_{NITRATE}$ and $E_{PHOSPHATE}$.

$E_{SALINITY}$ was performed to investigate the influence of salinity on the biofilter capacity and to determine a suitable salt concentration between 15 and 30 psu. Results in Table 1 show that gain of biomass was significantly higher at the lowest salt concentration ($p<0.001$). Uptake of nitrogen and phosphorus declined with increasing salt concentration, but not significantly. There was no difference in maximum quantum efficiency of PSII photochemistry between the treatments.

$E_{NITRATE}$ and $E_{PHOSPHATE}$ were carried out to determine appropriate nitrate-N and phosphate-P concentrations in the solution for an effective biofilter performance. $E_{NITRATE}$ did not result in big differences among the treatments with 10 to 100 mg NO_3-N l^{-1} in the parameters shown in Table 1. Only nitrogen content in leaves declined with decreasing nitrogen concentration in the culturing solution and plant uptake of nitrogen exhibited the same tendency, but without strong differences. But in the treatment with 1 mg NO_3-N l^{-1} differed significantly from the others: gain of biomass was less, plants took up less nitrogen and phosphorus, chlorophyll and carotenoid content and also nitrogen content was much lower than in the other treatments ($p<0.001$, Table 1). Contrarily to the other results, maximum quantum efficiency of PSII photochemistry was slightly higher at lower nitrate-N concentrations in the culturing solution (Table 1). A biplot including all the measured parameters (Figure 1) and a corresponding all pair comparison of the principal component analysis (PCA) (Table S1) reveal the significant

difference in the biofilter capacity of plants grown at 1 mg NO_3-N l^{-1} and those grown at higher NO_3-N concentrations ($p<0.001$). The first two principal components explain 73.23% of the differences in the data set.

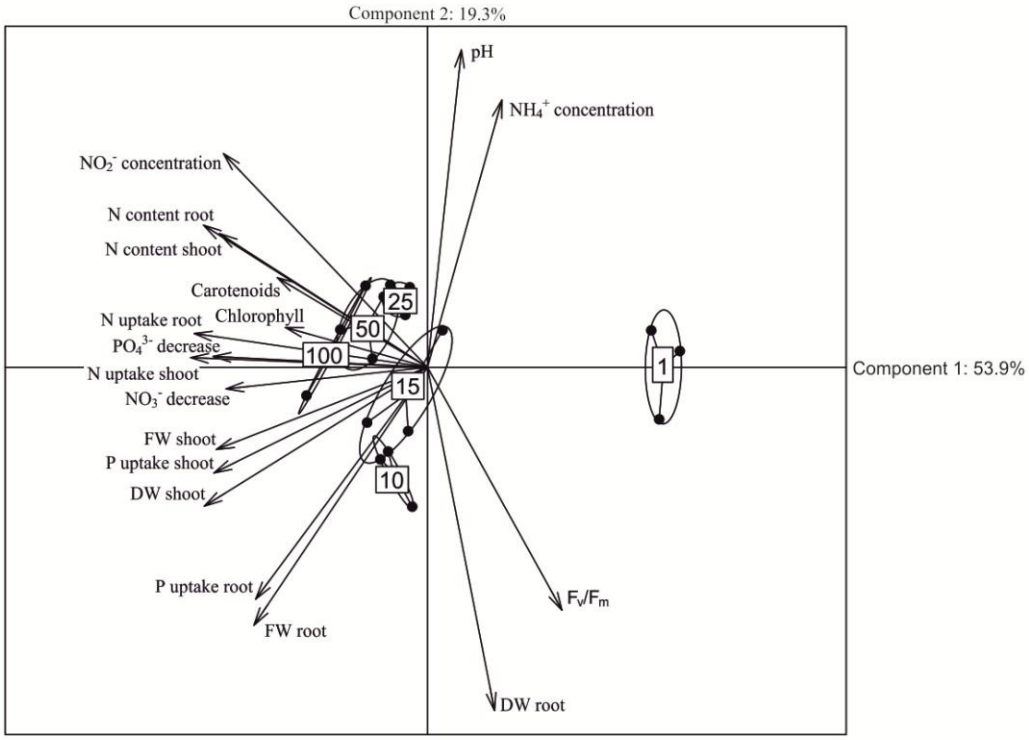

Figure 1. Biplot of the first two components of a principal component analysis (PCA) including all measured parameters in $E_{NITRATE}$. The numbers 1, 10, 15, 25, 50, 100 indicate the NO_3-N concentrations of the treatments in the experiment. Loops indicate the dispersion of the replicates from one treatment. FW: fresh weight, DW: dry weight, N: Nitrogen, P: Phosphorus, F_v/F_m: maximum quantum efficiency of PSII photochemistry, et1 and et2: ecotype 1 and 2 of *T. pannonicum*.

In $E_{PHOSPHATE}$ the results for gain of biomass, uptake of nitrogen and maximum quantum efficiency of PSII photochemistry did not show any differences between the treatments (Table 1). But uptake of phosphorus and phosphorus content of the plants declined significantly with decreasing phosphate-P concentration in the solution ($p<0.001$, Table 1). Additionally there was a lower chlorophyll and carotenoid content in the treatments with 0.3-3.3 mg l^{-1} phosphate-P compared to those with 5.0-16.3 mg l^{-1} phosphate-P in the solution. An all pair comparison of the PCA including all the measured parameters reveals a significant difference ($p<0.001$) between the biofilter capacity of plants grown at 0.3 and 1.6 mg PO_4-P l^{-1} and plants grown at 9.8 and 16.3 mg PO_4-P l^{-1} and between plants grown at 3.3 mg PO_4-P l^{-1} and

plants grown at 16.3 mg PO_4-P l^{-1} (Table S2). The first two principal components explain 62.30% of the differences in the data set.

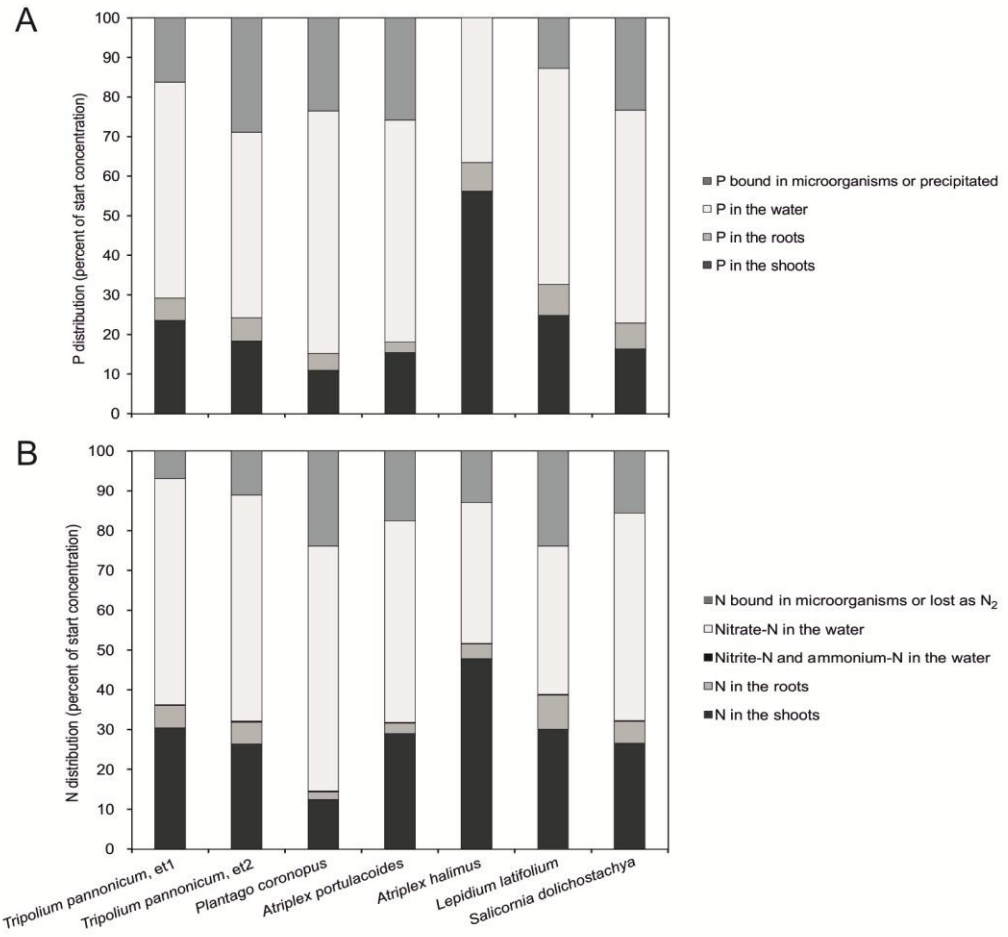

Figure 2. Fate of nitrogen (A) and phosphorus (B) during the experiment $E_{SPECIES}$ stated as nitrogen and phosphorus distribution in the system at the end of the experiment in percent of the start concentration (means for the different species). Total nitrogen (between 4.1 and 4.3 g; NO_3-N, NH_4-N and NO_2-N in the water and N in the plants) and phosphorus (between 0.68 and 0.69 g; PO_4-P in the water and P in the plants) in the tanks at the beginning of the experiment served as hundred percent for the calculations. N: Nitrogen, P: Phosphorus.

In $E_{SPECIES}$ the conditions determined as appropriate in the preceding experiments were applied to compare the biofilter capacity of different halophyte species. Figure 2 shows the percentage distributions of nitrogen and phosphorus in the system at the end of the experiment, indicating differences between the halophyte species. The starting concentrations of nitrogen and phosphorus at the beginning of the experiments were nearly the same which makes the percentage distributions at the end of the experiment comparable. In the tanks planted with *A. halimus* 47.8% of nitrogen and 56.2% of phosphorus in the system were found

in the shoot at the end of the experiment. In the tanks planted with *P. coronopus* only 12.4% of nitrogen and 10.9% of phosphorus were found in the shoot. For all species between 2.0 and 8.6% of the nitrogen and between 2.6 and 7.8% of the phosphorus was found in the roots. The percentage of nitrogen lost as N_2 or bound in microorganisms differed between different species with the lowest average value in tanks planted with *T. pannonicum*, et1 (7%) and the highest average value in tanks planted with *P. coronopus* and *L. latifolium* (23.9%). The percentage of phosphorus bound in microorganisms or precipitated was also different between different species with the lowest average value in tanks planted with *A. halimus* (0%) and the highest average value in tanks planted with *T. pannonicum*, et2 (29%). The rest of the nitrogen in the tanks remained as nitrate-N (between 35.4% and 61.6%) or to a very low percentage as nitrite-N or ammonia-N (<0.2%). Between 36.6 and 61.3 % of the phosphorus remained as solved orthophosphate in the solution.

Figure 3 shows the nitrate-N, phosphate-P, ammonium-N and nitrite-N concentration during the species comparison experiment. There was a decrease of nitrate-N and phosphate-P in the tanks for all species: the average decrease of nitrate-N was 29 mg l^{-1} and the average decrease of phosphate-P was 5 mg l^{-1} over 5 weeks. The highest differences in nitrate-N and phosphate-P concentrations in tanks planted with different species occurred at the end of the experiment with 30 mg l^{-1} and 4 mg l^{-1} for nitrate-N and phosphate-P, respectively. Ammonium-N concentration remained constant between 0.01 and 0.16 mg l^{-1} and the nitrite-N concentration increased over the first 3 weeks and then evened out at values between 0.03 and 0.12 mg l^{-1}. Both nutrients showed slight differences in concentrations between the different plant species.

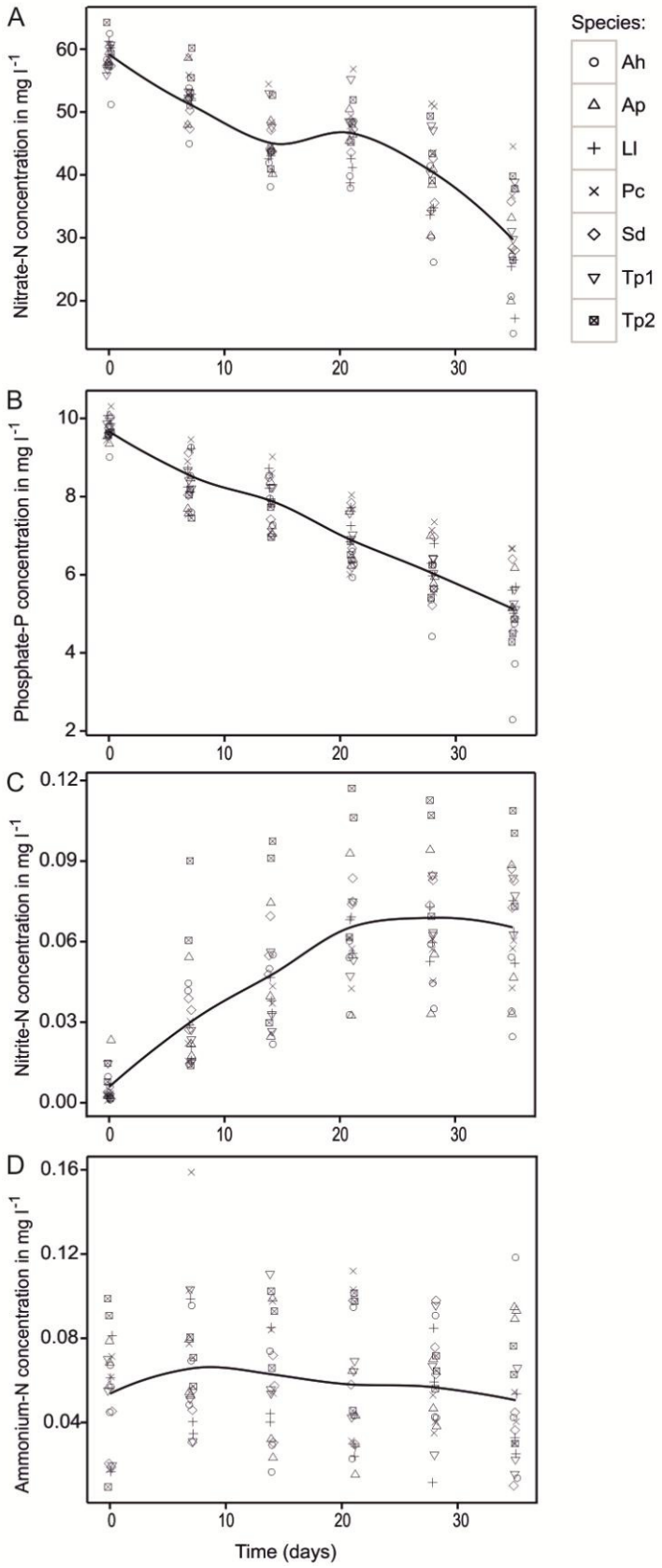

Figure 3. Change of nutrient concentrations (A: nitrate-N, B: phosphate-P, C: nitrite-N and D: ammonium-N) during the five weeks (35 days) of the experiment $E_{SPECIES}$. Values for all three replicates of the different treatments (species) are shown.

Gain of biomass (fresh and dry weight) and nitrogen and phosphorus uptake by the plants in $E_{SPECIES}$ are shown in Figure 4. The total gain of fresh weight differed between the species with 233 g per tank planted with *P. coronopus* and 505 g per tank planted with *T. pannonicum*, et1. However, *S. dolicostachya*, *A. halimus* and *T. pannonicum*, et1, also displayed a comparably high biomass production. Only *P. coronopus* showed a significantly lower growth (gain of total dry weight) compared to the other species ($p<0.05$). Shoot fresh weight production of the different species was between of 185 g (*P. coronopus*) to 398 g (*T. pannonicum*, et1) per tank. Five week plant nitrogen uptake ranged between 0.5 and 2.0 g per tank for the different species. Uptake of nitrogen (total and shoot uptake of nitrogen) was also significantly less in *P. coronopus* than in the other species ($p<0.05$). Five week plant phosphorus uptake ranged between 0.07 and 0.37 g per tank for the different species. Phosphorus uptake was significantly higher in *A. halimus* than in the other species ($p<0.05$). *Atriplex halimus* also displayed a higher shoot to root ratio for gain of biomass and nutrient uptake than the other species, followed by *A. portulacoides*. *Atriplex halimus*, *A. portulacoides.* and *L. latifolium* exhibit a smaller difference between gain of fresh weight and gain of dry weight indicating a lower water content of the plants of these species. *T. pannonicum* et1 showed a slightly but not significantly higher gain of biomass and nitrogen and phosphorus uptake than *T. pannoniucm* et2.

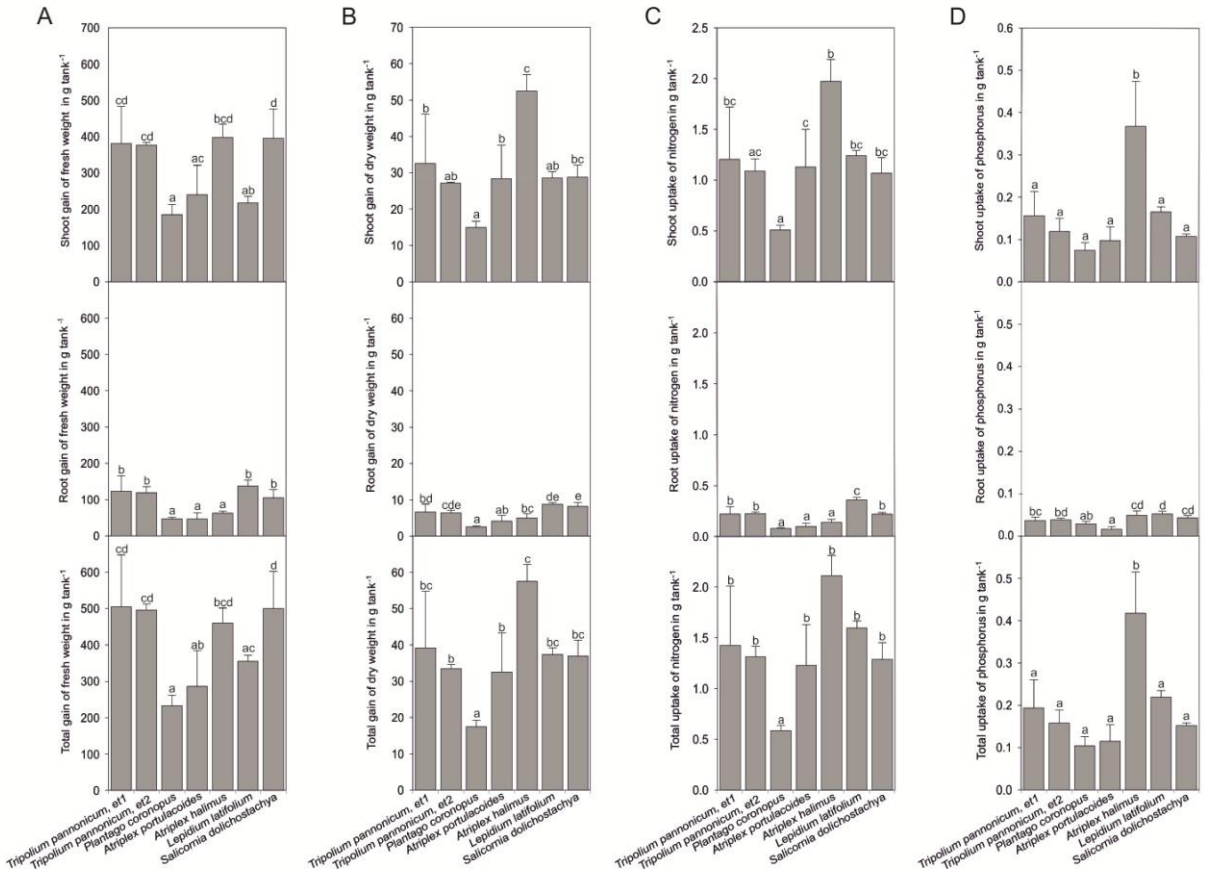

Figure 4. Gain of biomass and nitrogen and phosphorus uptake by the plants of the different species in the experiment $E_{SPECIES}$ (arithmetic mean and standard deviation). Each experiment lasted for five weeks (35 days), for each species three tanks (0.24 m²) with nine plants each were used as replicates. A: gain of fresh weight (FW), B: gain of dry weight (DW), C: Plant nitrogen uptake and D: Plant phosphorus uptake. For each parameter values for shoot, root and total plant are shown separately. Mean values with different letters indicate a significant difference between treatments (species) in the according parameter ($p<0.05$).

4. Discussion

4.1 Salt-tolerant *Tripolium pannonicum* grows well in hydroponic culture

The growth and nutrient uptake of *T. pannonicum* was dependent on culture conditions. $E_{SUBSTRATES}$ revealed hydroponic culture as the most suitable mode of culture. Regarding growth and nitrogen uptake plant performed similarly well in expanded clay culture and hydroponic culture. But phosphate-P uptake was much higher in hydroponic culture than in both substrate cultures. The lower phosphate-P uptake in expanded clay and sand culture could be due to adsorption of phosphate-P to substrate particles and precipitation. This might have caused a lower plant availability of phosphate-P in the substrate cultures even though the amount of phosphate-P added to the tanks at the beginning of the experiment was the same for

all treatments. Although substrate culture leads to a higher reduction of total phosphate-P in the water (results not shown) hydroponic culture should be favoured if the aim is nutrient recycling. In terms of nutrient recycling the often declared advantage of using substrate in a plant biofilter to provide habitat for nitrate-reducing bacteria that crucially increase the reduction of nitrogen in the water is also adverse. True nutrient recycling in a plant biofilter can only be achieved by plant uptake of nitrate-N and phosphate-P resulting in valuable biomass and not by loosing nitrogen in gaseous form or adsorption and precipitation of phosphate-P. Besides, the total loss of nitrate-N was comparable in hydroponic culture and in substrate cultures in this study, probably due to constantly aerobic conditions in all treatments.

Additionally to those results, working with hydroponic culture (water culture) has several other advantages compared to the use of expanded clay or sand culture: there are no weeds or soil pests, harvest is more hygienic, it is possible to easily harvest the root biomass for further use (e.g. biogas production) and there is no problem of intricate disposal or reprocessing of the used substrate that contains salt and organic material. Contrarily to our results Lennard and Leonard (2006) and Sikawa and Yakupitiyage (2010) showed that lettuce grown to filter effluents from freshwater aquaculture demonstrates a higher yield grown in sand or gravel culture than grown in water culture. This suggests that the suitability of the mode of culture in terms of biomass production is species-dependent.

4.2 Chelated iron stable at high pH needs to be added to hydroponic cultures to prevent chlorosis

The advantage of using sea water for the culture of halophytes is that it naturally contains most of the nutrients important for plant growth. For example, calcium, potassium, magnesium and sulphur are similar or much higher concentrated in seawater than in conventional nutrient solutions. However, the composition of seawater is not optimal for the culture of land plants. Beside the insufficient amount of nitrogen and phosphorus in seawater, iron and some other microelements might be limiting. Another unfavourable characteristic of seawater for the culture of plants is a high pH of 7.5 to 8.4 which can reinforce the problem of low concentrated micronutrients. The optimal pH for hydroponic plant culture is 5.5 to 6.5 (Lindl, 2002). A slightly acidic solution prevents important nutrients from precipitating into insoluble and plant unavailable salts and facilitates nutrient uptake for plants (Marschner, 2012).

In $E_{SUBSTRATES}$ all nutrients commonly abundant in plant nutrient solutions (micro- and macronutrients) were added to the artificial seawater to ensure best conditions for healthy plant growth. Nevertheless, plants grown in hydroponic culture and expanded clay culture exhibited chlorotic leaves. The high pH in the experimental fluid (7.9-8.5) probably caused insufficient iron accessibility for the plants. Iron was sufficiently abundant in the solution: 20 µg Fe l^{-1} were contained in the artificial seawater and 1.1 mg Fe l^{-1} was added in form of Fe-EDTA. But free iron ions precipitate in the form of Fe(III) and become hardly available for plants at high pH. Additionally, Fe-EDTA is not stable at high pH and the EDTA builds chelates with other ions abundant in the solution. The chlorosis could be prevented by adding Fe-EDDHA to the solution in $E_{MICORNUTRIENTS}$ and all the following experiments. Fe-EDDHA is more stable than Fe-EDTA at high pH and can prevent plants from iron deficiency in a alkaline soil or culturing solutions with high pH (Lucena and Chaney, 2006). Ventura et al. (2013) also demonstrated a successful elimination of chlorosis in *T. pannonicum* in a saline agricultural system on sand dune soils by the application of Fe-EDDHA.

In $E_{SUBSTRATES}$ plants grown in sand and expanded clay culture showed much higher chlorophyll and carotenoid contents than plants grown in hydroponic culture, however, the plants in all treatments were grown in the same solution with the same pH and the same iron source (Fe-EDTA). A possible reason for the difference could be that in substrate rather than in hydroponic culture, plants have the chance to establish an acidified micro-environment around the roots to facilitate the iron uptake.

Manganese deficiency can also cause chlorosis similar to iron deficiency and its uptake is also affected by high pH. But solely addition of manganese to the artificial seawater did not improve growth, nutrient uptake or physiological status of the plants compared to the control. Only the addition of manganese together with Fe-EDDHA to the artificial seawater caused a slight increase of growth and nutrient uptake which might indicate a weak manganese deficiency in plants not supplied with additional manganese.

The results suggest that plants grown in seawater need an additional Fe source that is stable at a high pH. The addition of manganese is beneficial but not implicitly necessary. Other micronutrients might also become limiting if the seawater is used in a batch culture without exchange or low exchange rates. This problem might occur when integrating halophytes to a recirculating aquaculture system with low water exchange rates if fish food does not contain the necessary micronutrients, only in insufficient concentrations or bound to organic material in a form unavailable for plants. An alternative to the addition of micronutrients to the culture

medium is the additional supply of the plants with certain nutrients by foliar spray. This approach has already been studied for crops grown on calcareous soil (Fernández and Ebert, 2005; Fageria et al., 2009) and freshwater aquaponics at high pH (Tyson et al., 2008; Roosta and Hamidpourb, 2011; Roosta and Mohsenian, 2012) and should be tested for marine aquaponic systems. For large-scale commercial applications foliar application of micronutrients might be too labour-intensive.

The main function and occurrence of iron in the plant is the biosynthesis of chlorophyll and in proteins of electron transport chains (Marschner, 2012). In $E_{MICRONUTRIENTS}$ the measurement of maximum quantum efficiency of PSII photochemistry (F_v/F_m) in young leaves showed a difference between no addition of iron and the addition of iron in general. But a difference in F_v/F_m between the iron sources Fe-EDTA and Fe-EDDHA could not be detected. The iron content of PSII is much lower than the iron content of PSI. Therefore PSII is less by iron deficiency or only in an advanced state (Marschner, 2012). Iron content of leaves neither was a good indicator for iron deficiency. The iron content of young leaves was higher in deficient than in healthy plants. This is in accordance to the "chlorosis paradoxon" described for plants grown on calcareous soils by Römheld (2000). A possible explanation is that iron is less diluted in young leaves of iron-deficient plants due to growth depletion (Römheld, 2000).

4.3 Higher salinity up to seawater level decrease performance as biofilter

The decline of biomass production and uptake of nitrogen and phosphorus with increasing salt concentration in $E_{SALINITY}$ shows that aquaponic growth of vegetables at full strength sea water may harm biofilter performance and biomass production. The influence of salinity on mineral nutrition of plants is complex. Reduction of crop yield, biomass production or crop quality due to salinity can be caused by reduced nutrient availability, uptake and transport competition of Na^+ and Cl^- with nutrients or partitioning within the plant (Grattan and Grieve 1999). For example, the reduction of nitrogen accumulation in plants by salinity has been related to Cl^- antagonism of NO_3^--uptake or reduced water uptake and is dependent on the form or nitrogen and concentrations of other constituents in the nutrient or soil solution (Grattan and Grieve, 1999; Hu and Schmidhalter, 2005). In general, the impact of salinity on mineral nutrition of plants is not only dependent on the strength of the salinity and the plant species but also on the composition of the salt (Grattan and Grieve, 1999). Therefore saline waters mainly containing NaCl might have a different effect on biomass production and

biofilter performance than seawater-based effluents were salinity is also caused by other ions such as Ca^{2+}, K^{+} and Mg^{2+}.

The impact of salinity is species-dependent because halophyte species differ much in their tolerance towards high salt concentrations (Flowers and Colmer, 2008). That a comparatively highly salt-tolerant species like *T. pannonicum* shows a growth reduction of 65% with an accompanied decline in nutrient uptake of > 30% underlines the importance of salinity influencing the performance of a biofilter implemented with halophytes. That the plants took up less nitrogen in the treatments with a higher salinity was partly due to a reduced nitrogen and phosphorus content of the plants (data not shown). On the other hand, a decrease in biomass production resulted in a reduced amount of plant nitrogen and phosphorus uptake.

Our results support the outcome of previous studies. In an experimental set up with irrigated drainage lysimeters using effluents from intensive tilapia culture salinities of 0.5, 10 and 35 psu were applied. The nutrient uptake was reduced to about one half at 10 psu and one third at 35 psu in comparison to 0.5 psu indicating that the removal of NO_3-N and PO_4-P in the soil-plant system was more effective at lower salinities (Brown et al., 1999). In a study investigating the behaviour of *Juncus kraussii* Hochst. in a subsurface-flow constructed wetland, salinity had a negative effect on the removal of PO_4-P but not on the removal of NO_3-N (Lymbery et al., 2006). However, removal of 62% of total N and 76.5% of PO_4-P (Lymbery et al., 2006) and 99% of total N and total P (Brown et al., 1999) were still reached at salinities of 24 psu and 35 psu, respectively. The results of the studies show that even though a halophyte species is tolerant to the salinity level in an effluent, the filter capacity can be reduced and also plant growth directly influences nutrient uptake.

Nutrient-rich saline waters that are to be purified by halophyte biofilters might show salinities lower than full strength seawater. This holds the chance to choose appropriate halophyte species for different conditions and applications. For example several marine organisms or their developmental stages can be cultured at lower salinities than full strength seawater (Forsberg and Neill, 1997; Atwood et al., 2003). Hence, the salinity of the culture water and the halophyte species should be chosen carefully for a maximum benefit from both, fish and plant culture (Buhmann and Papenbrock, 2013a).

4.4 Nitrate concentration as low as 10 mg l^{-1} even at high NaCl concentration does not limit biofilter capacities

The concentration of nitrogen and phosphorus is very low in sea water and insufficient for the growth of land-plants (Sleimi and Abdelly, 2002). The composition of the artificial seawater used in this study was similar to natural sea water and did not contain any nitrogen or phosphorus. In the experiments of this study, nitrate-N and phosphate-P were added to the artificial sea water to simulate conditions that might occur in the effluents to be treated by halophytic plants. In effluents considered as nutrient-rich regarding environmental impact or fish health, nitrate-N concentrations might not be that excessive from a crop plant nutritional point of view. For example, in marine aquaculture a nitrate-N concentration of 125 mg l^{-1} can already affect the growth of the cultured fish, at higher concentrations health and feed efficiency are affected (van Bussel et al., 2012). In modern aquaculture systems that include biofilters containing nitrifying and denitrifying bacteria to guarantee health of the fish, nitrate-N concentrations are often kept below 50 mg l^{-1} (van Rijn et al., 2006). Those low nitrogen concentrations might already be limiting for the growth of certain plant species, but $E_{NITROGEN}$ showed that nitrate-N concentrations as low as 10 mg l^{-1} did not limit biomass production and biofilter performance.

In $E_{NITROGEN}$ plant growth and physiology were not affected at nitrate-N concentrations between 10 and 100 mg NO_3-N l^{-1}. Therefore for saline agriculture with seawater as suggested by Rozema and Schat (2013) or with brackish water (Ventura et al., 2011a) a nitrate-N availability in this concentration range has to be ensured. Nitrate-N concentrations normally occurring in marine aquaculture facilities (Schneider et al., 2005; Orellana et al., 2013) are suitable for normal growth rates and healthy development of the plants and therefore suitable for aquaponic vegetable production. As already mentioned, nitrate-N uptake by plants is affected by salinity. If nitrogen concentration in the soil or culturing solution is below optimal concentrations, negative effects of salinity can often be reduced by the addition of nitrogen (Grattan and Grieve, 1999; Hu and Schmidhalter, 2005). In $E_{NITROGEN}$ biomass production could not be increased by higher NO_3-N concentrations indicating that with 10 mg l^{-1} already an optimal NO_3-N level is reached. Results suggest that halophyte crop culture with effluents containing 10 mg NO_3-N l^{-1} guarantees maximal growth and nutrient uptake and no further increase is expected at higher NO_3-N concentrations. However, if the nitrogen concentration of an effluent used for the growth of halophytic crop plants is low (around 10 mg l^{-1}) plants might use up the nitrogen down to a growth limiting concentration. Therefore high flow rates

through the biofilter (short hydraulic retention time) should be applied when nitrate-N concentrations in the effluent are low.

A low concentration of 1 mg NO_3-N l^{-1} affects plant growth, plant nitrogen and phosphorus uptake and chlorophyll and carotenoid content and therefore biofilter capacity and production of viable plant material, as shown in $E_{NITROGEN}$. Therefore, limiting nitrogen concentration is reached somewhere between 1 and 10 mg NO_3-N l^{-1}, indicating those low nitrate-N concentrations to be insufficient for the application of a halophyte biofilter. Maximum quantum efficiency of PSII photochemistry was not affected at 1 mg NO_3-N l^{-1} (Table 1). This suggests that phase two of the plants reaction towards N deficiency was not reached which is characterized by a decrease of photosynthetic capacity and leaf senescence. Plants in $E_{NITROGEN}$ were still able to react to the low N concentration by morphological and physiological adaptations (Marschner, 2012), reflected in low yield and low shoot to root ratio (data not shown).

4.5 Low phosphate concentrations do not influence nitrate up-take

In the same way as for nitrogen it is important to investigate the influence of different phosphate-P concentrations that occur in effluents to be purified by halophyte crop plants. Phosphate-P concentrations evaluated as high with regard to their negative environmental impact or harming effect on a cultured organism in marine aquaculture might be limiting for plant growth. In marine aquaculture facilities typical phosphate-P concentrations are between 1 and 15 mg l-1 (Deviller et al., 2004; Tal et al., 2009; Orellana et al., 2013), depending on the system.

In $E_{PHOPHATE}$ there were no differences in gain of biomass, uptake of nitrogen and photosynthesis between plants grown at different phosphate-P concentrations in the culturing solution between 0.3 and 16.3 mg l^{-1}. But there was a decline of plant phosphorus uptake and phosphorus content with decreasing phosphate-P concentration in the solution and slightly lower chlorophyll and carotenoid content in the treatments with 0.3-3.3 mg l^{-1} phosphate-P compared to the other treatments. Additionally PCA revealed a significant difference between 0.3 and 1.6 mg l^{-1} treatments and higher phosphate-P concentrations. It results that phosphate-P concentrations of 1.6-3.3 mg l^{-1} are favourable for phosphate-P uptake but lower phosphate-P concentrations do not harm biomass production and uptake of nitrogen. This is in accordance with Chen et al. (1997) successfully growing lettuce in a hydroponic nutrient film technology (NFT) system with a phosphorus concentration of 0.3 mg l^{-1}. Therefore, there

should not be any serious limitation of biofilter capacity phosphate-P concentrations as low as 0.3 mg l^{-1} in recirculating marine aquaponics. Alder et al. (2003) even managed to reduce phosphorus concentrations down to <0.01 mg l^{-1} exploiting different growth stages of lettuce and luxury consumption in a NFT system. This approach would be interesting for the reduction of phosphorus concentration of effluents from open aquaculture systems to maximal reduce environmental impact.

4.6 Several salt-tolerant plant species can act as biofilter and valuable co-product in different applications

Tripolium pannonicum, *P. coronopus*, *A. halimus*, *A. portolacoides*, *L. latifolium* and S. *dolicostachya* are halophyte species that are known as edible with at least regional importance or show valuable secondary metabolites with market potential (Koyro, 2006; Liebezeit, 2008; Koyro et al., 2011; Buhmann and Papenbrock, 2013b; Kaur et al., 2013; Ventura and Sagi, 2013; Ventura et al., 2013). Therefore all of them have potential to be used as crop plants in saline agriculture or valuable co-product of a biofilter for nutrient-rich saline effluents. The examination of the species in this study ($E_{SPECIES}$) suggests that all the tested halophyte species are suitable for the production of valuable biomass at a salinity of 15 psu. Ventura and Sagi (2013) list the results of various studies for yields obtained from different halophyte species. Species from the genera *Atriplex*, *Batis*, *Salicornia* and *Sarcocornia* grown under field conditions showed yields expressed in fresh weight between 14 and 28 kg m^{-2} $year^{-1}$ (38 to 77 g m^{-2} d^{-1}) and yields expressed in dry weight between 1.5 and 1.8 kg m^{-2} $year^{-1}$ (4 to 5 g m^{-2} d^{-1}). These values are well comparable with our results from $E_{SPECIES}$ were the different species exhibited a shoot gain of fresh weight between 22 and 47 g m^{-2} d^{-1} and shoot gain of dry weight between 1.8 and 6.2 g m^{-2} d^{-1}.

Results from hydroponic culture experiments in a greenhouse might be better comparable to our results than field studies on soil. De Vos et al. (2013) investigated the influence of salinity on growth of two potential halophyte crop species in greenhouse hydroponic culture. They determined an approximate gain of total fresh weight (root and shoot) of 6 and 3 g per plant in 20 days for *D. tenuifolia* at salinities of 200 mM and 300 mM NaCl, respectively (11 and 6 g m^{-2} d^{-1}). *Cochlearia officinalis* L. showed an approximate gain of total fresh weight of 15 and 4 g per plant in 35 days at salinities of 200 mM and 400 mM NaCl, respectively (16 and 4 g m^{-2} d^{-1}). The salinity of 15 psu in $E_{SPECIES}$ equals 250 mM NaCl. For easier comparison the values from de Vos et al. (2013) were recalculated into values in g m^2 d^{-1} assuming the same

plantation density as in our study (38 plants per m²). The gain of total fresh weight for both species investigated in de Vos et al. (2013) was below the gain of shoot fresh weight for all species in our study even at a lower salinity.

Beside the crop potential of different halophyte species their potential as biofilter for nitrogen and phosphorus for nutrient-rich saline waters was determined in $E_{SPECIES}$. The different species showed a plant uptake of nitrogen between 0.06 and 0.23 g m^{-2} d^{-1} and plant uptake of phosphorus between 0.009 and 0.044 g m^{-2} d^{-1}. These values for nutrient uptake are well comparable with those from two applications of *Salicornia* planted in constructed wetlands as biofilter for marine aquaculture effluents. Using *Salicornia europaea* to purify effluents for the culture of different marine organisms Webb et al. (2012) determined a total plant nitrogen and phosphorus uptake of 0.17 and 0.028 g m^{-2} d^{-1}, respectively. In Shpigel et al. (2013) the application of *Salicornia persica* for the purification of a sea beam resulted in a total plant nitrogen uptake of 0.04 and 0.08 g m^{-2} d^{-1}. Results for plant nutrient uptake in $E_{SPECIES}$ were also comparable with results on the potential of fruit production for the recycling of nutrients in a fresh water aquaponic system (Graber and Junge, 2009). Nutrient removal by harvesting of cucumber, aubergine and tomato was between 0.08 and 0.43 g m^{-2} d^{-1} for nitrogen and between 0.02 and 0.07 g m^{-2} d^{-1} for phosphorus. Therefore, all tested species in $E_{SPECIES}$ showed promising biofilter efficiency for nitrogen and phosphorus removal.

Regarding a recycling of nutrients, it is important to retain high amounts of the nitrogen and phosphorus in a biofilter system with halophyte crop plants in harvestable valuable biomass. High retention of nitrogen and phosphorus in the roots, loss of nitrogen as N_2, precipitation of phosphate-P and nitrogen-N and phosphorus adsorbed by microorganisms are adverse. In our system around 10 to 60% of the nitrogen and phosphorus of the system were retained in harvestable shoot biomass and around 35 to 60% remained as nitrogen and phosphorus in the water. Therefore, only small amounts of nitrogen and phosphorus were retained in roots and lost due to microorganisms and precipitation. A high shoot to root ratio as for *A. halimus* indicates the favourable high retention of nutrients in the shoots and low retention of nutrients in the roots. *Plantago coronopus* results to be less suitable than other tested species due to lower gain of biomass, nutrient uptake and higher loss of nitrogen in the tanks due to microorganisms.

With respect to the application as biofilter in aquaponic systems, the concentrations of nitrite-N and ammonium-N (Fig. 4) were always below toxic concentrations for fish (Orellana et al.,

2013). Therefore well aerated hydroponic systems are well suited as biofilter even in a recirculating system consisting of primary (fish) and secondary (plants) circulations.

Seeds of *T. pannonicum* et1 and et2 were collected at two different sites. The higher growth and nutrient uptake of *T. pannonicum* et2 compared to et1 in $E_{MICRONUTRIENTS}$ under insufficient iron supply indicate differences between those two collections. Different factors at the two collection sites such as salinity, nutrient availability and flooding probably caused different adaptations of the *T. pannonicum* plants to their environment suggesting that they can be classified as different ecotypes of the same species. $E_{SPECIES}$ could not confirm the difference between et1 and et2 under optimal growth conditions. However, for the application of halophytes as crop plants and as biofilter for nutrient-rich saline waters it is important to select suitable ecotypes and to breed varieties with favourable characteristics regarding taste, appearance, growth rate, nutrient uptake and salt tolerance, amongst others. De Vos et al. (2013) also suggest this approach.

5. Conclusions

Halophytes have a high potential for their use as new crop plants for saline agriculture and as biofilters in different applications. In this study, basic cultivation techniques and identification of putative growth limitations have been identified and optimized. *Tripolium pannonicum* was used to conduct experiments on culture conditions and their impact on biomass production and biofilter efficiency. Optimal conditions found were applicable to grow different halophyte species with crop potential. Therefore general assumptions can be drawn and increase the knowledge about successful cultivation of new and salt-tolerant crop plants.

This study revealed that the use of hydroponic culture is the favourable culture mode in terms of nutrient recycling and controlled conditions. Saline effluents used for the culture of halophytes should contain nitrate-N concentration of at least 10 mg l^{-1}, a phosphate-P concentration of 0.3 mg l^{-1} is sufficient. In sea water based effluents iron has to be added in a pH stable chelated form, the addition of manganese is beneficial but not implicitly necessary. Salt concentrations lower than sea water salinity are favourable for biomass production and biofilter performance, even for highly salt tolerant species. All tested halophyte species have potential to serve as biofilter for nutrient-rich saline effluents and valuable co-product. The selection of suitable ecotypes and breeding of varieties is mandatory for future applications.

Our results form a basis for future breeding activities of valuable, salt-tolerant crops and can be transferred to apply halophytes as biofilter for nutrient-rich saline municipal, agricultural

and industrial wastewater. An interesting field for future application of our results is the aquaponic growth of halophytic crop plants using effluents from marine aquaculture (open systems) or integrating the halophyte culture into a recirculating aquaculture facility (closed system).

Acknowledgements

We would like to thank the gardeners Yvonne Leye and Lutz Krüger for taking care of the plants and Rebecca Hosang, Pamela von Trzebiatowski and Julia Volker for valuable technical assistance. The project was financially supported by the Deutsche Bundesstiftung Umwelt (DBU) (AZ27708/1-3).

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Table S1

Tests of hypothesis for the main component analysis in $E_{NITROGEN}$. Of the two main components the smaller p-value for each test of hypothesis was chosen for indication of significant differences between treatments. The numbers 1, 10, 15, 25, 50, 100 in the column named "Hypothesis" indicate the NO_3-N concentrations of the treatments in the experiment.

Hypothesis	p-value
10 - 1 ≠ 0	<0.01
15 - 1 ≠ 0	<0.01
25 - 1 ≠ 0	<0.01
50 - 1 ≠ 0	<0.01
100 - 1 ≠ 0	<0.01
15 - 10 ≠ 0	0.28
25 - 10 ≠ 0	<0.01
50 - 10 ≠ 0	<0.01
100 - 10 ≠ 0	<0.01
25 - 15 ≠ 0	0.04
50 - 15 ≠ 0	0.48
100 - 15 ≠ 0	0.12
50 - 25 ≠ 0	0.87
100 - 25 ≠ 0	0.11
100 - 50 ≠ 0	0.91

Table S2

Tests of hypothesis for the main component analysis in $E_{PHOSPHORUS}$. Of the two main components the smaller p-value for each test of hypothesis was chosen for indication of significant differences between treatments. The numbers 0.3, 1.6, 3.3, 5.0, 9.8, 16.3 PO_4-P in the column named "Hypothesis" indicate the PO_4 concentrations of the treatments in the experiment.

Hypothesis	p-value
1.6 - 0.3 ≠ 0	0.75
3.3 - 0.3 ≠ 0	<0.01
5.0 - 0.3 ≠ 0	0.02
9.8 - 0.3 ≠ 0	<0.01
16.3 - 0.3 ≠ 0	<0.01
3.3 - 1.6 ≠ 0	<0.01
5.0 - 1.6 ≠ 0	0.79
9.8 - 1.6 ≠ 0	<0.01
16.3 - 1.6 ≠ 0	<0.01
5.0 - 3.3 ≠ 0	0.85
9.8 - 3.3 ≠ 0	0.98
16.3 - 3.3 ≠ 0	<0.01
9.8 - 5.0 ≠ 0	0.67
16.3 - 5.0 ≠ 0	0.58
16.3 - 9.8 ≠ 0	0.15

Chapter 5

Waller, U., Buhmann, A., Ernst, A., Hanke, V., Kulakowski, A., Wecker, B., Orellana, J., Papenbrock, J., Integrated multi-trophic aquaculture in a zero-exchange recirculation system for marine fish combined with hydroponic halophyte production. (In preparation for submission to “Aquaculture International”).

Integrated multi-trophic aquaculture in a zero-exchange recirculation system for marine fish combined with hydroponic halophyte production

Uwe Waller, Anne Buhmann, Anneliese Ernst, Verena Hanke, Andreas Kulakowski, Bert Wecker, Jaime Orellana, Jutta Papenbrock

Abstract

Salt tolerant plants can be used as a biofilter for effluents from marine aquaculture. Three salt tolerant plant species, *Tripolium pannonicum* (Jacq.) Dobrocz., *Plantago coronopus* L. and *Salicornia dolichostachya* Moss have been integrated into a modern 7 m³ pilot scale recirculating aquaculture system (RAS). The RAS used for this study is a highly engineered system equipped with mechanical and microbiological water treatment resulting in a low water exchange rate. The RAS was stocked with juvenile European sea bass (*Dicentrarchus labrax*, L.). The reason for including a halophyte biofilter into the RAS was the recycling of nutrients and the production of a valuable co-product in addition to the fish. After 35 days 248 fishes had gained altogether 5.6 kg of fresh weight. At the same time total plant biomass production was 21.8 kg in three hydroponic culture tanks (15 m² surface area per unit). Gain of shoot biomass was 17, 25 and 61 g m^{-2} d^{-1} for *T. pannonicum*, *P. coronopus* and *S. dolichostachya*, respectively. The plants retained 7 g phosphorus and 46 g nitrogen. This represents 9% of the nitrogen and 10% of the phosphorus introduced with the fish feed and partly dissolved in the water. The micronutrients supplied to the hydroponic culture tanks were sufficient for *T. pannonicum* and *P. coronopus,* however a higher amount of ferric acid is probably necessary for *S. dolichostachya*. Gain of harvestable biomass and nutrient retention in plants are promising. The hydroponic system can be further optimized to increase productivity per unit area and water volume. A potential production of 16 to 20 kg of plant material for the production of 1 kg of European sea bass was calculated for the system, depending on the plant species. Harvested plant material was free from harmful microorganisms and is suitable for human consumption.

Introduction

The sustainable expansion of aquaculture worldwide requires the development of technologies which allow for the recycling of matter and energy. Presently aquaculture is still operating in mono-species systems. The system-immanent loss of nutrients, organic compounds and energy are a cause for concern; having a potentially negative ecological effect on the environment. Beyond that, the bio-economy of mono-species aquaculture is weak. A new approach, integrated multi-trophic aquaculture (Chopin et al., 2008), combines the production of fish with filter feeders and plants or algae. This concept is applicable to many standard aquaculture installations, such as pond or net cages. Another trend in global aquaculture is towards recirculating aquaculture systems (RAS) (Martins et al., 2010; Daalsgard et al., 2013) which allows the production of almost every aquaculture species regardless of their natural distributional range (Orellana et al., 2013).

Recirculating aquaculture systems are operating independently from the environment, however, within their system borders substantial amounts of organic and inorganic matter (i.e. non-retained feed and metabolites) are accumulating. The accumulation of matter needs to be thoroughly controlled by means of bio-process technology in order to maintain adequate living conditions and to maximize carrying capacity and productivity. Orellana et al. (2013) highlighted that a functional RAS for marine fish is highly engineered. The production process needs to be accurately controlled by process automation.

Modern, highly engineered RAS are designed to meet the needs of the cultured species. Animal welfare is not solely an ethical aspect but also safeguards production by avoiding the limitation of living conditions during the course of production. In order to minimize energy consumption and to avoid vectors for the introduction of pathogens or contaminants the process water is kept within the RAS. Water losses resulting from evaporation and water treatment processes are replenished. A modern RAS can be operated with a water consumption rate of 0.01 of the system volume per day or even less (Orellana et al., 2013).

A central part of water treatment in RAS is the conversion and removal of dissolved nutrients. Ammonium is converted to nitrate by a nitrifying biofilter and nitrate is removed from the system by a denitrifying biofilter. Low levels of phosphorous are removed during the denitrification process because of the large surface area of the granulate material in the filter and by sedimentation. The technology of the system is complex but still does not include any components for a recycling of matter, especially dissolved nutrients. The removal of nutrients through technical components described above is costly and does not improve the

environmental balance. Thus, it is desirable to include a component that is capable of using and removing dissolved nutrients from the process water and thus, enhance the bio-economy and the environmental compatibility of the RAS.

Accumulated matter in RAS consists as a first approximation of inorganic nitrogenous, phosphorous and carbon compounds as well as organic compounds that are mostly not identified. The inorganic compounds comply to a large extent with the nutrient requirements of plants and algae. Thus, the potential of process water from RAS for plant cultivation is obvious. Approaches are dated back to 1978 and 1984, when Lewis et al. (1978) and Watten and Busch (1984) combined the production of tilapia and tomatoes. Many other trials have been published from freshwater aquaculture in the past decades. More recently Sikawa and Yakapitiyage (2010) published results from experiments combining hybrid catfish (*Clarias macrocephalus* × *C. gariepinus*) and lettuce (*Lactuca sativa* L.) in a pond/hydroponic system. Their results proved the feasibility but also showed constraints. The high particle load in the process water of the employed low-tech RAS turned out to be a major drawback for the integration. Modern RAS technology includes several process steps for particle removal (Orellana et al., 2013) and provides the necessary process water quality for hydroponic plant production. For marine aquaculture, examples of integrating the culture of fish and plants are less common. Webb et al. (2012) investigated the feasibility of constructed wetlands recycling nutrients from a commercial RAS operation. They grew *Salicornia europaea* successfully with water effluents from a shrimp, sole, and turbot farm. The system was able to remove a large amount of nutrients before the water was discharged. However, the wastewater flow reported by the authors clearly indicates that the commercial RAS was exchanging water during the production process. This was likely to maintain water quality. Any water exchange, however, is in conflict with the concept of closed RAS aquaculture, an aim of our study.

The aquaponic production of halophytes as marketable co-product and for the recycling of nutrients in an integrated zero-exchange RAS has not yet been investigated. In RAS low dissolved phosphorus and nitrogen concentrations in the process water are maintained in order to ensure well-being of the cultured organism. Typical concentrations are <100 mg l^{-1} nitrate-N and between 1 and 15 mg l^{-1} for phosphate-P (Deviller et al., 2004; Tal et al., 2009; Bussel et al., 2012; Orellana et al., 2013). These are far below usual concentrations for hydroponic plant growth (Park et al., 2009). Most plant nutrient solutions contain excess nutrient to supply the plants for several days in batch culture. Contrarily, there is a permanent input of nutrients by addition of fish feed and excretion to the process water of an RAS which can meet the nutrient requirements of the plants. A high flow rate can ensure a sufficient nutrient

supply of the plants in the hydroponic culture even at low nitrate and phosphate concentrations in the process water of an RAS. Apart from nitrate and phosphate, micronutrients may become a limiting factor for halophyte growth in the system due to insufficient abundance in the process water or limited plant availability caused by high pH and precipitation (Tyson et al., 2008; Buhmann et al., submitted).

The integration of secondary photoautotrophic production, in the form of a halophyte biofilter, into a modern, highly engineered RAS will add value to the system as many halophytes have market value. In order to integrate halophytes successfully into a RAS production process, it is necessary to synchronize both primary fish and secondary halophyte production. The aim of this study was: i) to develop the necessary parameters for the design and construction of an integrated marine recirculating aquaculture system (IMRAS) using different halophyte species, ii) to investigate the efficiency of different halophyte species and nutrient uptake cultured in process water from marine fish production and iii) to evaluate the potential of halophyte biomass production as a valuable co-product in the system.

In this study, a RAS stocked with European sea bass (*Dicentrarchus labrax*) was operated to provide the necessary flow of nutrients to the secondary hydroponic biofilters. Experiments were carried out under typical aquaculture conditions to allow an evaluation of the efficiency of a secondary biological filtration of process water from marine fish aquaculture. The terms secondary biological filtration or halophyte biofilter are used in this study for the function of the halophytic plants to reduce the amount of nitrogen (N) and phosphorus (P) in the process water of the RAS by uptake into the plant tissue. The feasibility of three halophyte species, *Tripolium pannonicum* (Jacq.) Dobrocz., *Plantago coronopus* L. and *Salicornia dolichostachya* Moss, was investigated. For this purpose, process water from the RAS was passed onto three hydroponic culture tanks that were maintained in a greenhouse aside the RAS. The production of valuable biomass and the uptake and recycling of N and P by the plants was evaluated. Finally, results were used to calculate the necessary plant biomass production for retention of the N and P in the process water.

Material and Methods

Experimental fluid circuits

The experimental circuit consisted of a 10 m^3 experimental indoor recirculating aquaculture system (RAS), a sand bed as seedling nursery and three 1.7 m³ hydroponic culture tanks for experimental plant production. Seedling nursery and hydroponic culture tanks were setup in two separate greenhouses of 12 and 40 m² base areas, respectively (Figure 1).

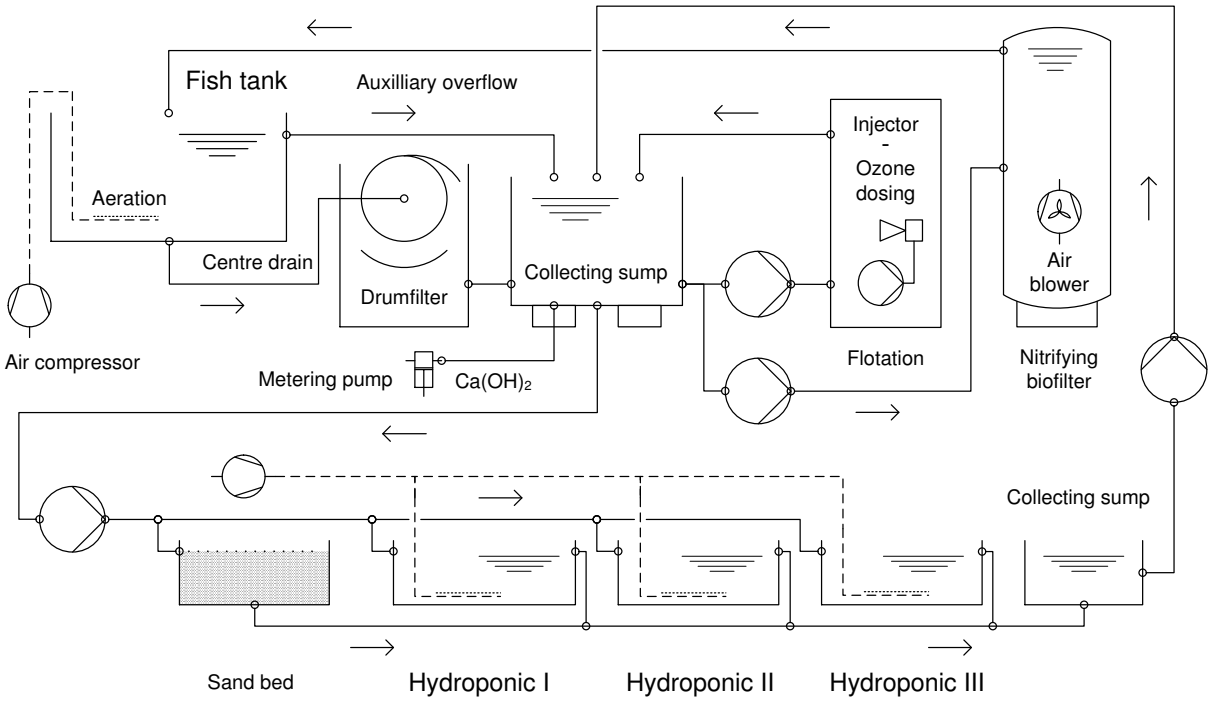

Figure 1. Experimental set up of the RAS (primary circuit) and hydroponics (secondary circuit).

The nutrients used in the hydroponic experiment came from a European sea bass culture which was maintained in a 7 m^3 fish tank having an approximate length, width, and depth of 4.9, 1.9, and 0.8 m, respectively. Water level was kept at 0.7 m. The process water was passed over to the water treatment through a centre drain. An auxiliary surface skimmer controlling water level in the fish tank was installed for safety reasons. The water treatment of the experimental RAS included a two step solid separation. Large particles were removed by drum filtration (Hydrotech 501, 0.35 m² filter area, 40 µm screen, Veolia, France) before the process water entered into the collecting sump. In immediate proximity to the inlet pipe, $Ca(OH)_2$ was dosed by a metering pump to adjust the pH. Fine solids were subjected to a flotation process in a protein skimmer. The protein skimmer was a standard device (Helgoland 500, 0.27 m³ reaction space, Erwin Sander Elektroapparatebau GmbH) operated at

a process water flow rate of around 3 $m^3 \cdot h^{-1}$, and an air flow rate of around 5 $m^3 \cdot h^{-1}$. The degradation of dissolved organic matter, which forms the separating layer of the foam bubbles in the flotation process, was enhanced by oxidation through ozone generated in a 2 $g \cdot h^{-1}$ device (Schroeder et al., 2011) (Erwin Sander Elektroapparatebau GmbH). The dosing of ozone was carefully controlled by Redox sensors and kept in safe limits below 0.05 $mg \cdot l^{-1}$ residual oxidant concentration or 400 mV (Sander, 1998; Reiser et al., 2010). Dissolved organic matter was removed along with particles and microorganisms during the flotation process. Dissolved ammonia/ammonium excreted by fish and dissolved nitrite was removed by a nitrifying biofilter. The biofilter outlet was the hydraulic pressure head (2.2 m) of the RAS from which the process water was passively flowing back to the fish tank. A small volume of water (0.2 $m^3 \cdot h^{-1}$) was continuously passed on to a sand bed filter used as a seedling nursery in a small greenhouse. Denitrifying microbial processes as well as plant biomass production in the sand bed filter kept the total N and P concentration of the process water within desired limits for the fish under culture.

Process water quality was continuously monitored by sensors. Nutrient concentrations as well as total N and carbon concentrations were determined in discrete process water samples taken daily in fish tank and in each of the hydroponic culture basins. The concentrations of total ammonium-N, nitrite-N, nitrate-N, and P were measured with an autoanalyzer (AA3, Seal Analytical GmbH, Norderstedt, Germany). Carbon and N were determined using an automated CN analyzer (multi N/C 3100, Analytik Jena, Jena, Germany). Ammonium-N and nitrite-N remained below detectable concentrations of 0.8 and 0.2 mg l^{-1}, respectively. More sensitive manual photometric tests were used to detect the ammonium-N and nitrite-N concentration. Total inorganic carbon concentration in the process water was mainly determined by the concentration of hydrogen carbonate.

System control

System control was by a programmable logical controller (Siemens SPC 200). The programmable controller was mainly used in a three-step controller mode. Data acquisition was by means of probes which were, in most cases, installed in the collecting sump (pH, redox, conductivity, temperature). An oxygen sensor in the fish tank was used to verify the necessary oxygen flow (aeration) into the process water. The redox potential was read within the reaction space of the protein skimmer. Crosschecking of Redox readings was achieved through secondary Redox sensors in the collecting sump. The control of ozone concentration

in the protein skimmer and receiving components was safeguarded by an additional time control. The control of pH through calcium hydroxide dosing was based on empirical algorithms to improve the steady-state control accuracy and protected against overdosing by time control. Calcium was supplied as hydroxide to stabilize the pH (8.1 ± 0.1) in the process water. The salinity of the process water was maintained at approximately 15.8 psu.

Flow rate towards the fish tank was maintained at around 17 $m^3 \cdot h^{-1}$. The average retention time (t_r) of the process water in the fish tank was around 0.4 h ($t_r = V_t \, F_w^{-1}$, t_r = average retention time [h], V_t = fish tank volume [m^3], F_w = process water flow [$m^3 \, h^{-1}$]). A total exchange of process water in the fish tank was computed to be completed every 3 h (EIFAC, 1986). This comparably high flow rate was necessary to maintain appropriate living conditions for the fish species under culture, European sea bass.

RAS fish production

The RAS was stocked with juveniles of European sea bass obtained from a commercial marine inland RAS (Meeresfischzucht Völklingen GmbH, Völklingen, Germany). At the beginning of the experiment, 248 fish having an average individual weight of 31.8 g were stocked. Fish were fed to satiation with a commercial pellet feed (1.2 to 1.5 mm pellet size, Coppens international Marico Apex, Helmond, The Netherlands). The amount of N and P in the given feed was calculated from the specification of the manufacturer (N: 9.3%, P: 1.3%).

Plant material

Seeds of *Tripolium pannonicum* (Jacq.) Dobrocz. and *Salicornia dolichostachya* Moss were collected at the North Sea, Jade Bay, Germany (53°29‘13N; 8°03‘16“O). *Plantago coronopus* L. seeds were obtained from Jelitto Staudensamen GmbH (Schwarmstedt, Germany). Seeds were sown in propagation soil (Einheitserde, Einheitserdewerk Hameln-Tündern, Germany). Prior to the experiment, plants were grown to seedling size in a greenhouse at the University of Hannover (Germany). The greenhouse was maintained at a temperature of 22°C. During daytime the natural light was supplemented for 14 hours with artificial light (sodium vapor lamps, SONT Agro 400, Philips, Amsterdam, The Netherlands). Tap water was used for irrigation. When a shoot length of 1 to 2 cm was reached the seedlings were transplanted individually to pots filled with sand of 2 mm grain size (Hornbach, Hannover, Germany). Twice a week a modified Hoagland solution was supplied to the plants (Epstein, 1972).

Nursery periods were 3, 4, and 7 weeks for *P. coronopus*, *T. pannonicum* and *S. dolichostachya*, respectively. One week before the start of the experiment the plants were adapted to the experimental salinity by adding sodium chloride to the irrigation water. Salinity was increased by 5 psu every second day.

Hydroponic setup

The hydroponic culture tanks were located in a greenhouse. The ambient weather conditions determined the process water temperature within the hydroponic culture tanks, which was recorded during the course of the experiment. Plants under culture were illuminated by solar radiation. The daily light period followed the seasonal astronomical sunshine duration for *T. pannonicum* and *P. coronopus.* The hydroponic culture tank used for the cultivation of *S. dolichostachya* was separated from the other two tanks by a non-transparent curtain and was illuminated for 18 h by two high pressure sodium lamps to suppress flowering under short-day conditions (Ventura et al., 2011b).

The hydroponic culture tanks were constructed as longitudinal raceways having an approximate length, width, and depth of 6.0 x 0.8 x 0.5 m, respectively. Water level within the hydroponic culture tanks was maintained at 0.4 m. Every hydroponic culture tank was supplied with process water from the primary RAS at a flow rate of 0.2 $m^3 \cdot h^{-1}$. Average retention time was 8.3 h (see above). A total exchange of the water within every hydroponic culture tank was computed to be completed every 72 h. During the experiment a trace element solution was added to every hydroponic culture tank three times a week (7 ml per tank); including ferric citrate ($1.79 \cdot 10^{-1}$ $g \cdot d^{-1}$, Lebosol®-Dünger GmbH, Elmstein, Germany), zinc ($ZnSO_4 \cdot 7xH_2O$, $2.05 \cdot 10^{-5}$ $g \cdot d^{-1}$), molybdenum ($(NH_4)_6Mo_7O_{24} \cdot 4xH_2O$, $7.54 \cdot 10^{-7}$ $g \cdot d^{-1}$), cobalt ($CoCl_2 \cdot 6$ x H_2O, $4.48 \cdot 10^{-6}$ $g \cdot d^{-1}$), copper ($CuSO_4 \cdot 5xH_2O$, $4.73 \cdot 10^{-8}$ $g \cdot d^{-1}$), and manganese ($MnSO_4 \cdot 7xH_2O$, $3.45 \cdot 10^{-5}$ $g \cdot d^{-1}$). The hydroponic culture tanks were aerated to equilibrate dissolved gas concentrations. An air compressor and diffuser were used. The ascending air bubbles were simultaneously circulating the process water.

Halophytic biomass production

The three different species of halophyte plants were investigated in separate hydroponic culture tanks. At the beginning of the experiment, for each species 185 plants of similar size were distributed equal distant over the surface plane of one hydroponic culture tank. Start

biomass was determined in a pooled sample of 15 randomly selected plants. During the experiment typical horticultural maintenance was carried out. Non-vital plants were removed and counted. Plants were treated with beneficial organisms to minimize pest infestation.

At the end of the experiment a second set of data was obtained for every halophyte species from three groups of 15 plants. The three samples of plants were harvested in 1, 3, and 5 m distance from the head end of the hydroponic culture tank. Biomass gain was determined as fresh and dry weight. Fresh weight and dry weight of the plants were determined separately for shoots and roots. Afterwards the samples were dried at 110°C until constant weight was reached and dry weight was determined. Gain of biomass was calculated by subtracting the biomass at the beginning of the experiment from the biomass at the end of the experiment.

Of each sample, a subsample of fresh material was used for the determination of chlorophyll and carotenoids and a subsample of died material was used for the determination of N and P content.

Nutrient uptake of the plants

Each subsample of dried material of 15 pooled plants (roots and shoots separately) was homogenized and a subsample was grinded to a fine powder (MM 400, Retsch GmbH, Haan, Germany). The dried powder was used for N determination with a CNS elemental analyser (Vario EL III, Elementar Analysensysteme, Hanau, Germany). Phosphorous determination required a further preparation of samples. Approximately 38 mg of powder was incinerated for at least 8 h in a muffle furnace (M104, Thermo Fisher Scientific Corporation, Waltham, Massachusetts, USA). The incinerated samples were cooled to room temperature and 1.5 ml of 66% nitric acid was added. After 10 min incubation, 13.5 ml of ultrapure water was added and the solution was filtered (0.45 µm pore size, Carl Roth) and stored at -60°C. A blank was prepared for each incineration process by treating an empty vial like the samples. Phosphorus was analysed in the filtrate by inductively coupled plasma optical emission spectrometry (ICP-OES) (iCAP 6000 ICP Spectrometer, Thermo Fisher Scientific Corporation). Plant uptake of N and P during the experiment was calculated with the help of the data for dry weight gain of biomass and the determined N and P content.

Chlorophyll and carotenoids

For the extraction of chlorophyll and carotenoids, ice-cold 80% acetone (400µl) was added to a sample of frozen and grounded fresh leaf material (50 mg). First, the sample was kept on ice for 10 min, mixed every 2 min and centrifuged at 14,000 x g for 5 min. Afterwards the supernatant was collected and stored on ice in the fridge. Repeating this procedure the pellet was re-extracted three times with 200 µl ice-cold 80% acetone. All supernatants were pooled for pigment determination and absorption was measured at 750.0, 663.2, 646.8 nm and 470.0 nm using a spectrophotometer (Uvikon XS, Biotech instruments, Germany). Total chlorophyll and carotenoid contents were calculated according to Lichtenthaler (1987).

Plant data calculation

All data for biomass and nutrient uptake of the plants was first calculated for the pooled sample and then expressed as values per plant for each of the three replicates per species by dividing the values by 15, because the pooled samples consisted of 15 plants. Arithmetic mean and standard deviations were calculated from the subsequent three values for each parameter. All data referring to the total plant are calculated from shoot and root data and discrepancies can occur due to rounding of numbers from the original data.

Quality of harvested plant material

At the end of the experiment three shoots of every halophyte species was randomly harvested and pooled. The fresh material was analysed in an accredited microbiological laboratory (MikroBiologie Krämer, Dillingen, Germany). The samples were analysed for microbial counts of pathogens relevant for the marketability of vegetables and fish (*Escherichia coli*, *Salmonella* spp., *Lysteria monocytogenes*, Enterobacteriaceae, *Pseudomonas* spp., *Vibrio* spp.).The total counts of mesophilic bacteria were also determined. The values were compared to guidance and critical values from literature.

Results

Environmental conditions during the experiments in the primary and secondary circuit

Several parameters were monitored during the course of the experiment, such as temperature, light conditions and nutrient concentrations. The air temperature in the greenhouse dropped below 20°C during the night and increased to nearly 50°C during the day (Figure 2, upper graph). Due to the large water volume in the hydroponics process water temperature never exceeded 28°C (Figure 2, lower graph). The temperature differed from 2 to 4°C between day and night. During the course of the experiment, process water temperature slowly decreased from 24 to 26°C in August and from 20 to 22°C in September.

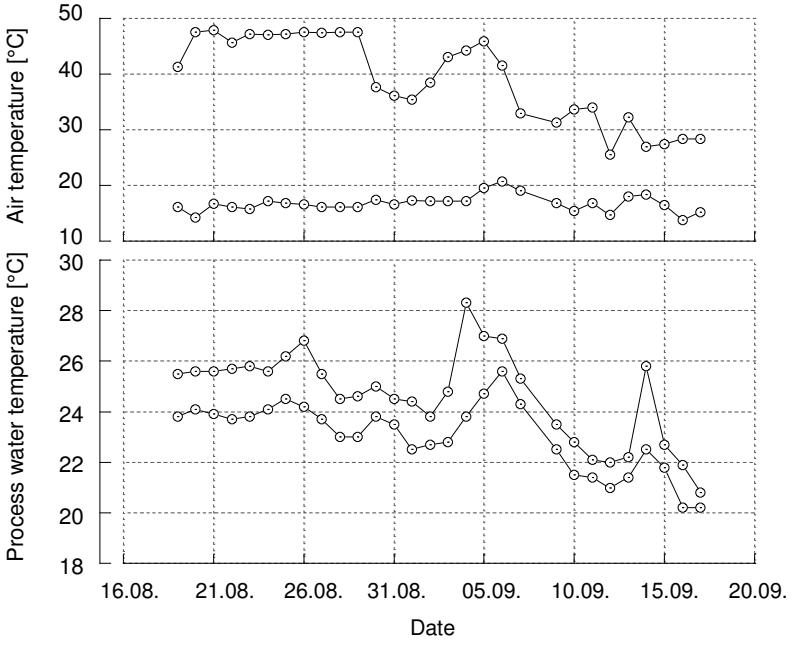

Figure 2. Air and process water temperature in the greenhouse and hydroponics during the course of the experiment (upper line maximal and lower line minimal value for each day).

The photosynthetic active radiation (PAR) was measured in the middle of the greenhouse 1 m above the surface of the hydroponic culture tanks (Figure 3). During the day, PAR reached approximately 900 $\mu mol \cdot m^2 \cdot s^{-1}$ at the beginning of the experiment (August) and dropped to about 500 $\mu mol \cdot m^2 \cdot s^{-1}$ at the end of the experiment (September). When precipitation occurred, for example at 26th of August 2013, the PAR dropped to 250 $\mu mol \cdot m^2 \cdot s^{-1}$. To obtain a constant PAR in a greenhouse during different seasons, additional light sources need to be installed.

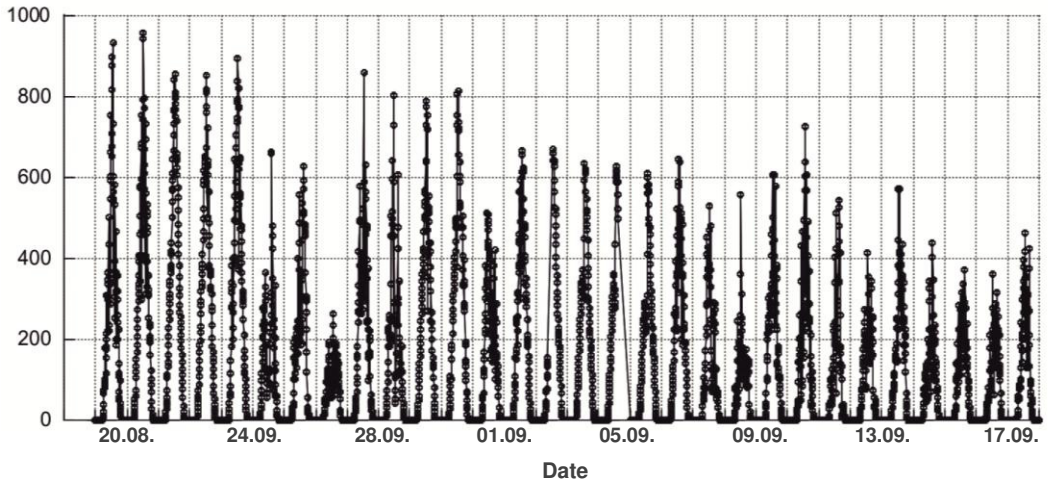

Figure 3. The photosynthetic active radiation in $\mu mol \cdot m^{-2} \cdot s^{-1}$ in the middle of the greenhouse 1 m above the surface of the hydroponic culture tanks during the course of the experiments.

The intensity of the additional light received by the *S. dolichostachya* plants was dependent upon the position within the hydroponic culture tank. Photosynthetic active radiation varied considerably at the surface plane (Figure 4). Maximum PAR reached 250 $\mu mol \cdot m^{2} \cdot s^{-1}$ below the two high pressure sodium lamps and dropped to less than 20 $\mu mol \cdot m^{2} \cdot s^{-1}$ in the space between.

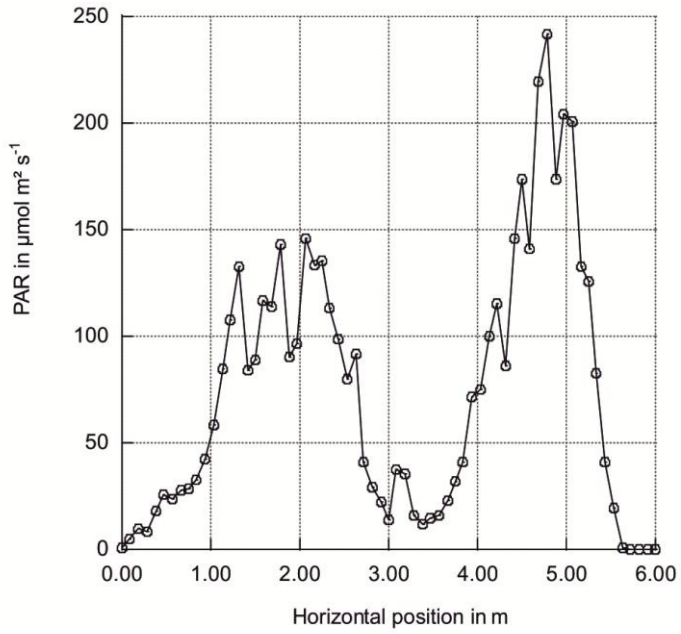

Figure 4. Photosynthetic active radiation in $\mu mol \cdot m^{-2} \cdot s^{-1}$ above the hydroponic culture tank planted with *S. dolichostachya*; determined at the surface plane of the hydroponic culture, at the level of the hypocotyl of the plants at various points along the 6 m length of the hydroponic culture tank (horizontal position).

Regular monitoring of the nutrients

Due to aeration of the hydroponic process water a mixed water column was assumed. Therefore, water samples for nutrient determination taken at the outlet are representing the nutrient regime within hydroponics Total ammonium-N and nitrite-N concentration were below the detection level of 0.07 and 0.007 $mg \cdot l^{-1}$ throughout the experiment. The nitrate-N concentration of 18.73 $mg \cdot l^{-1}$ in the process water was in accord with the total N concentration of 19.42 $mg \cdot l^{-1}$, phosphate-P concentration was 2.79 $mg\ l^{-1}$ (Table 1). Most of the dissolved inorganic carbon (IC) in the process water was hydrogen carbonate because of the carbon dioxide released by fish that were maintained in the RAS. The organic carbon fraction, which is the difference between total carbon and IC, was below detectable limits. This indicates an immediate removal of particles from the process water. Otherwise, the leaching of particles would lead to much higher concentrations. The ozone enhanced floatation process contributed to the decay of organic molecules and removal through an internal microbial loop.

Table 1. Nutrients determined in the RAS process water and in the hydroponic culture tanks. TN, total nitrogen; TC, total carbon; IC, inorganic carbon.

			Process water in the hydroponics					
Parameter	**RAS process water**		***T. pannonicum***		***P. coronopus***		***S. dolichostachya***	
	Average	Stdev	Average	Stdev	Average	Stdev	Average	Stdev
TN [mg l^{-1}]	19.42	1.44	21.02	2.43	21.57	1.84	16.24	2.17
NO_3^--N [mg l^{-1}]	18.73	3.01	22.04	2.91	21.29	3.46	16.03	1.91
PO_4^{3-}-P [mg l^{-1}]	2.78	0.59	3.00	0.50	2.77	0.79	2.85	0.72
TC [mg l^{-1}]	19.20	3.22	19.01	4.67	20.18	5.09	19.03	2.59
IC [mg l^{-1}]	18.73	3.01	19.77	3.27	19.59	4.26	19.10	2.27

Growth of fish and nutrients available from fish feed

At the beginning of the experiment 248 stocked fish had an average individual weight of 31.8 g. After 35 days the fish had gained 5605 g (Table 2). Individual weight at the end of the experiment averaged 54.4 g. Growth of fish is exponential (Ricker, 1975) and can be

expressed as $w_1 = w_0 \cdot e^{G \cdot t}$ where w_0 and w_t are the individual weights of animals at the beginning and the end of the growth period (t). G denotes the instantaneous growth rate which can be calculated from $G = (log_e(w_1) - log_e(w_0))/(t_1 - t_0)$. An instantaneous growth rate of 0.015 corresponds to a specific growth rate of 1.5% body weight per day (commonly computed in aquaculture production research). The feed conversion rate (FCR) was calculated as a quotient of the feed given and the weight gained. During the 35 days experiment the overall FCR amounted to 0.93 (Table 2).

Table 2. Summary of all fish culture parameters.

	Feed [g]	**Feed N [g]**	**Feed P [g]**	**Number of fish**	**Fish biomass [g]**	**Individual body weight [g]**	**Weight gain [g]**	**Instanttanenous growth rate (G)**	**Feed conversion ratio (FCR)**
Start of the experiment	5200	484	68	248	7886	31.8	5605	0.0153	0.93
End of the experiment					13491	54.4			

During the experiment 484 g N and 68 g P was fed to the fish. The amounts of N and P available for plant growth can be estimated: Lemarie et al. (1998) report a N and P content in adult European sea bass of 11.7 and 6.4 $mg \cdot g^{-1}$ body weight, respectively. In consideration of the total gained weight of 5605 g, the amounts of N and P retained in the body mass (biomass) equal 65.6 and 35.9 g, respectively. Thus, 14% of the feed N and 53% of the feed P was deposited in body tissues. It can be assumed that the remaining 86% of the feed N and 46% of the feed P were released into the process water as dissolved or particulate matter. Particulate matter was subjected to drum filtration and flotation and quickly removed. However, leaching is a significant process in aquaculture (Lupatsch and Kissil, 1998). In view of the tank retention time, it can be assumed that most of the N and P got dissolved in the process water (Table 1) and was available for halophytic plant growth.

Growth and nutrient uptake of plants

Biomass production was based on nutrients from the marine RAS. Table 3 shows the number of plants that survived the 35 days of experiment, plant biomass, nutrient content in plants and chlorophyll and carotenoid content in the leaves for the beginning and the end of the experiment for all three species. The plants of *T. pannonicum* and *P. coronopus* planted into the hydroponic culture tanks at the beginning of the experiments had similar weights; the plants of *S. dolichostachya* were much bigger, being nearly 4 times heavier than the other two species (Table 3). At the end of the experiment 170, 181 and 181 of the 185 plants at the beginning of the experiment survived in the hydroponic culture tanks for *S. dolichostachya*, *T. pannonicum* and *P. coronopus*, respectively. As Figure 5 indicates, the three halophyte species showed a different quantity of growth and nutrient uptake during the five weeks of experiment. The fresh weight gain for *S. dolichostachya* was much higher (74 g) than for *T. pannonicum* and *P. coronopus* with 30 and 20 g per plant, respectively (Figure 5). Gain of shoot biomass was 25, 16 and 60 g per plant for *T. pannonicum*, *P. coronopus* and *S. dolicostachya*, respectively (Figure 5). For all species shoot biomass production accounted for about 80% (Figure 5). For all species dry weight was about 6% of fresh weight. Only the percentage of root dry weight related to root fresh weight was higher (8%) for *S. dolichostachya* and lower (5%) for *P. coronopus*, indicating a higher water content in roots of *P. coronopus* and a lower content in *S. dolichostachya* than in the other two species.

Table 3. Number (No.) of plants in one hydroponic culture tank, plant biomass per plant (fresh weight and dry weight in g), nutrient content in plants (N and P in mg g^{-1}dry weight), chlorophyll and carotenoid content in leaves (in µg g^{-1} fresh weight) for the three halophyte species *T. pannonicum*, *P. coronopus* and *S. dolichostachya* at the start and end (after five weeks) of the experiment. Standard deviation is only shown for the values at the end of the experiment because for the start of the experiment measurements were performed with only one sample per species.

		Plant biomass						Nutrient content in plants						Chlorophyll	Carotenoid
	No. of plants in one tank	Fresh weight in g			Dry weight in g			N in mg g^{-1} dry weight			P in mg g^{-1} dry weight			content	content
Plant species		shoot	root	total	shoot	root	total	shoot	root	total	shoot	root	total	µg g^{-1} fresh weight	
Start of experiment															
Tripolium pannonicum	185	0.74	0.22	0.97	0.099	0.025	0.123	55.682	40.422	52.625	7.762	6.844	7.578	1093	191
Plantago coronopus	185	0.74	0.16	0.90	0.083	0.018	0.101	42.420	30.234	40.260	4.525	3.106	4.274	672	128
Salicornia dolichostachya	185	2.66	0.56	3.22	0.366	0.114	0.479	41.030	25.811	37.422	3.722	3.269	3.614	301	61
End of experiment															
Tripolium pannonicum	181	25.46 ± 2.58	5.93 ± 0.25	31.39 ± 2.41	1.564 ± 0.186	0.357 ± 0.027	1.921 ± 0.180	35.008 ± 3.698	35.214 ± 4.089	35.031 ± 3.685	4.503 ± 0.552	7.501 ± 1.966	5.045 ± 0.751	262 ± 55	52 ± 12
Plantago coronopus	181	17.20 ± 4.09	4.17 ± 1.07	21.37 ± 5.15	1.021 ± 0.210	0.211 ± 0.047	1.232 ± 0.255	30.994 ± 4.350	29.998 ± 1.455	30.818 ± 3.790	4.705 ± 0.565	12.886 ± 1.074	6.110 ± 0.690	419 ± 37	78 ± 7
Salicornia dolichostachya	170	62.34 ± 10.15	14.85 ± 3.01	77.19 ± 13.13	3.834 ± 0.622	1.250 ± 0.171	5.084 ± 0.791	39.749 ± 2.500	26.467 ± 0.466	36.470 ± 1.915	4.538 ± 0.865	6.163 ± 0.754	4.939 ± 0.811	246 ± 58	61 ± 2

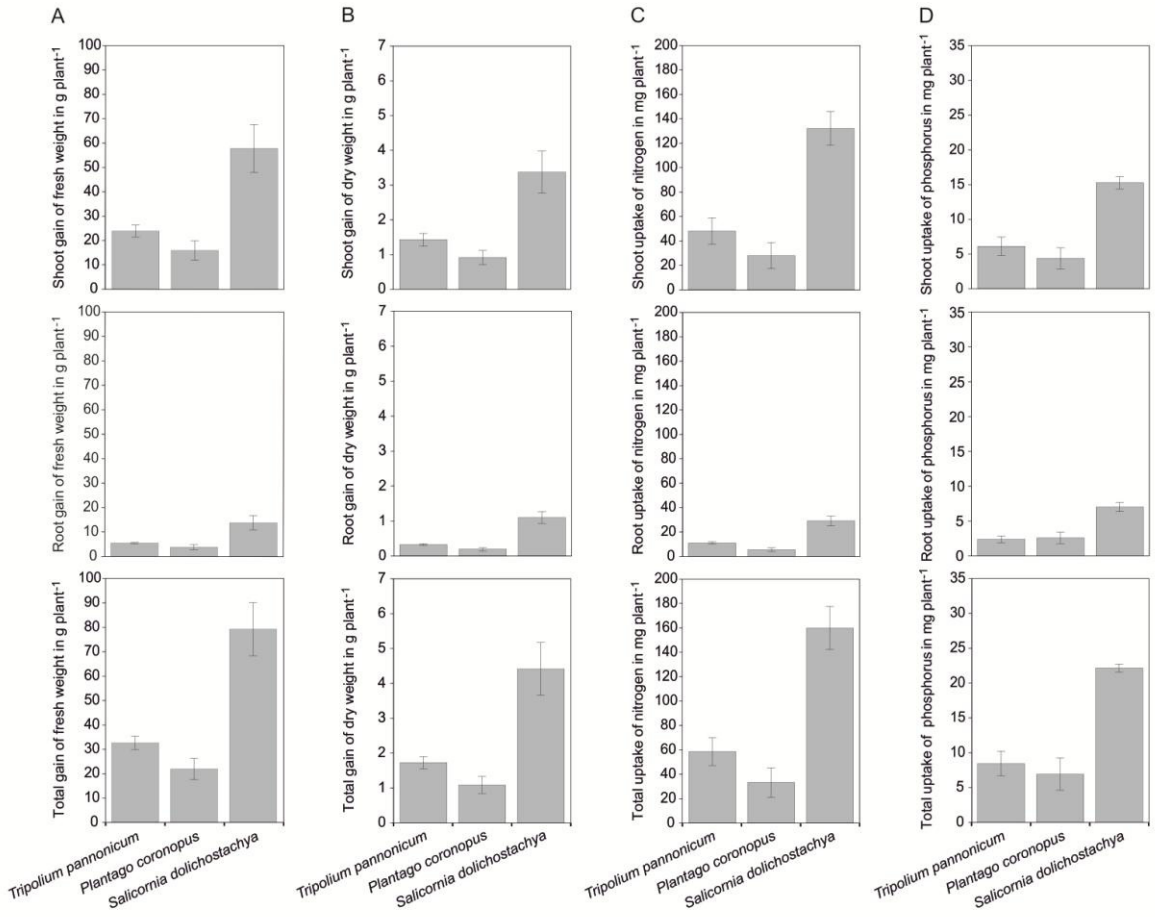

Figure 5. Gain of biomass (fresh weight and dry weight) and nitrogen and phosphorus per plant for the three halophyte species *T. pannoniucum*, *P. coronopus* and *S. dolichostachya*. For each parameter values for shoot, root and total plant are shown separately.

The higher biomass production of *S. dolichostachya* compared to the other two species (approximately three times higher) and the slightly lower biomass production of *P. coronopus* compared to *T. pannonicum* is also reflected in the nutrient uptake by the plant species. *Salicornia dolichostachya* took up 168 mg of N and 23 mg of P per plant during the 35 days of experiment. Total plant nutrient uptake of *T. pannonicum* and *P. coronopus* was lower with 61 and 34 mg for N and 9 and 7 mg for P, respectively (Figure 5). Shoots accounted for about 60 to 70% of total plant P uptake and for about 80% of total plant uptake of N.

Nutrient content of the plants or plant parts differed partially between the beginning and end of the experiment. For *T. pannonicum* and *P. coronopus* P content in the shoot was lower at the end of the experiment, whereas both values were similar for *S. dolichostachya* (Table 3). Phosphorus content in the root of *P. coronopus* was much higher at the end of the experiment. There was also a difference in chlorophyll and carotenoid content of the leaves for all plant species at the beginning and the end of the experiment. For *T. pannonicum* the chlorophyll

content was 76% lower at the end of the experiment (Table 3). Chlorophyll content at the end of the experiment was also lower for *T. pannonicum* and *P. coronopus* with 38% and 18% (Table 3). The carotenoid contents were lower at the end of the experiment with equal percentages of difference than the chlorophyll content (Table 3). Only leaves of *S. dolichostachya* showed the same carotenoid content at the start and the end of the experiment (Table 3).

Microorganisms harmful for human consumption on the leaves

Table 4 summarizes the results from the microbial counts of pathogens on the shoot material of the three species harvested at the end of the experiment and also shows guidelines and critical values for salads and marine fish (DGHM, 2004 and 2007). Microbial counts show that none of the pathogens were abundant in a quantity harmful for human consumption.

Table 4. Microbial counts for different pathogens relevant for marketability of vegetables and fish in colony-forming units per g of fresh plant material (CFU g^{-1}) on the freshly harvested shoot material of the three halophyte species; *T. pannonicum*, *P. coronopus* and *S. dolichostachya*. GV: guideline value, CV: critical value. *According to DGHM (2004, 2007).

Tested pathogen	***Tripolium pannonicum***	***Plantago coronopus***	***Salicornia dolichostachya***	**Guideline and critical values***
Aerobic mesophilic bacteria	1.5×10^4	4.7×10^5	2.0×10^4	GV: 5×10^7
Echerichia coli	< 10	< 10	< 10	GV: 1×10^2 CV: 1×10^3
Salmonella spp.	no detection in 25 g plant material	no detection in 25 g plant material	no detection in 25 g plant material	CV: no detection in 25 g plant material
Listeria monocytogenes	< 10	< 10	< 10	CV: 1×10^2
Enterobacteriaceae	2.1×10^3	2.3×10^3	6.0×10^2	GV: 1×10^4 CV: 1×10^5
Pseudomonas spp.	$< 1.0 \times 10^5$	$< 1.0 \times 10^5$	$< 1.0 \times 10^5$	GV: 1×10^6
Vibrio spp.	no detection in 25 g plant material	no detection in 25 g plant material	no detection in 25 g plant material	CV: no detection in 25 g plant material

Discussion

Performance of the RAS in terms of controlled and steady conditions

Compared to various open aquaculture systems that release the water after a certain time, the nutrient levels in this system were kept fairly low. This is desired in modern RAS production. Marine fish aquaculture strives for dissolved nitrate-N concentrations far below 100 mg l^{-1}. Van Bussel et al.(2012) showed a steady decline of specific growth rate in turbot (*Psetta maxima*) along with increasing nitrate-N concentrations (0 to 500 mg l^{-1}). The feed conversion rate was affected above 200 mg l^{-1}. Operating companies keep nitrate-N concentrations even lower. Maximum concentration is usually maintained below 50 mg l^{-1}, which is usually attained by means of denitrification (van Rijn et al., 2006). Dissolved N concentrations in this study were kept at around 20 mg l^{-1} during the course of the experiment. The N sink in the experimental RAS was the sand bed nursery, which was operated parallel to the hydroponic culture tanks. The hydroponic plant area would have been too small to keep nutrients at the required low concentrations. Concentration of phosphate-P was constantly around 3 mg l^{-1}. This is typical for RAS, where system dependent concentrations between 1 and 15 mg l^{-1} are found (Deviller et al., 2004; Tal et al., 2009; Orellana et al., 2013). Ammonium-N and nitrite-N concentrations remained below the detection levels of 0.07 and 0.007 mg l^{-1} throughout the experiments and therefore in a range that can be considered not to be harmful for the fish (Orellana et al., 2013).

Performance of fish culture

The experimental conditions for the fish were favourable for growth, health and survival. Fish grew at an average instantaneous growth rate of 0.015 or a specific growth rate of 1.5 % d^{-1}, which appears to be moderate in view of the growth reported in other experimental investigations. Thetmeyer et al. (1999) and Eroldogan et al. (2004) reported slightly lower specific growth rates (1.0% d^{-1}, 0.8% d^{-1}). Papoutsoglou et al. (1998) found growth rates between 1.7 and 3.2% d^{-1}, depending on feeding levels. The feed conversion ratio (0.9) found in this experiment was higher compared to reports (0.6) from Thetmeyer et al. (1999). Eroldogan et al. (2004) confirm this high feed conversion ratio (0.64) in fish fed to satiation but found a lower feed conversion (0.9) at restricted feeding rates. It can be concluded that growth and feed conversion was average in the experiment but reproduced the situation in real aquaculture operations.

Performance of plant culture

The experimental setup allowed for investigation of halophytic plant growth under steady conditions. The nitrate-N concentration of the modern RAS used in this study was levelling around 20 mg l^{-1}. It was therefore far below concentrations of approximately 300 mg l^{-1} commonly used for tomato and cucumber hydroponic culture (Park et al., 2009). Investigations by Buhmann et al. (submitted) proved unlimited growth of halophytes at nitrate-N levels of 10 mg l^{-1} and phosphate-P levels of 1.6-3.3 mg l^{-1} in a controlled hydroponic culture experiment simulating aquaponic conditions. Of seedlings planted at the beginning of the experiment, 92 to 98% survived and did not show any obvious signs of N or P deficiency. The 35 days of experiment resulted in a total gain of plant biomass of 21.8 kg for all three species together. Therefore, the dissolved nutrient concentrations of approximately 20 mg l^{-1} nitrate-N and 3 mg l^{-1} phosphate-P were sufficient to sustain growth of the halophytes. Only leaves of *T. pannonicum* became chlorotic during the 35 days of the experiment. The chlorophyll and carotenoid content at the end of the experiment were 76% and 68% less than at the beginning, respectively. The chlorophyll and carotenoid contents were also lower than that reported for healthy plants at the same age in previous experiments (Buhmann et al., 2014, submitted). The chlorosis was probably caused by iron deficiency due to a high pH as described in Ventura et al. (2013) and confirmed by Buhmann et al. (submitted). Ferric citrate was added to the plant culture to prevent iron chlorosis. The water was transported through the tank at a flow rate of 0.2 m^2 h^{-1} and therefore the ferric citrate added to the hydroponic culture tanks was slowly but constantly removed after addition. The ozone treatment of the water in the RAS leads to a destruction of the iron citrate and therefore a removal from the system. The chlorosis in *T. pannonicum* could probably be prevented by a higher concentration of iron citrate or a more frequent application. For *P. coronopus* and *S. dolichostachya* the iron concentration seemed to be sufficient, even though the chlorophyll and carotenoid concentrations at the end of the experiment were also below concentration at the beginning.

Plant performance in terms of valuable biomass and nutrient removal

Several studies describe the use of halophytes as a biofilter for marine aquaculture effluents (reviewed in Buhmann and Papenbrock, 2013), but the combination of a marine RAS with the aquaponic growth of halophytic crops is new. Therefore, we compare our results with freshwater aquaponics with conventional crops to evaluate the biomass production and

nutrient uptake. In our study, salt-tolerant plant species with potential to be used as leafy vegetables were investigated. Therefore, only studies with leafy vegetable species, like lettuce and spinach, were chosen for comparison. Additionally, we compare the results of this study to results from two studies that use a sand culture of *Salicornia* to produce vegetable biomass and to treat effluents from open marine aquaculture systems.

In terms of the production of a valuable co-product it is important to evaluate the harvestable shoot biomass production as it is marketable as vegetable. In this study, gain of shoot fresh weight was 17, 25 and 61 g m^{-2} d^{-1} for *P. coronopus*, *T. pannonicum* and *S. dolichostachya*, respectively (35 days of experiment, 36 plants per m² at the beginning of the experiment). Some freshwater aquaponic systems are much more productive, some show a similar productivity. Lennard and Leonard (2006) grew *Lactuca sativa* at a plant density of 38 plants per m² in a freshwater aquaponic system culturing Murray Cod (*Maccullochella peelii*). They reached 197 to 240 g m^{-2} d^{-1}, a much higher gain of shoot fresh weight compared to our study. Licamele (2009) had, with 134 g m^{-2} d^{-1}, also a higher productivity of *L. sativa* in freshwater aquaponic systems culturing Nile tilapia (plant density of 32 plants per m²). But, other combinations of freshwater aquaculture with the different leafy vegetable species (*Ipomoea aquatica*, *L. sativa*, *Brassica rapa* at a plant density of 30 plants per m²) resulted in a much lower shoot fresh weight gain of between 1 and 41 g m^{-2} d^{-1} (Endut et al., 2010; Trang et al., 2010). Compared to those values, productivity was partly higher in this study. The large differences between the studies in crop biomass production, although using comparable plant densities, are probably due to different systems, different duration of the culturing period and different culturing conditions.

For the application as biofilter it is also important to evaluate nutrient uptake of the plants in this study. Plant N uptake was 35, 63 and 172 mg m^{-2} d^{-1} and plant P uptake was 7, 9 and 24 g m^{-2} d-1 for *P. coronopus*, *T. pannonicum* and *S. dolichostachya*, respectively (35 days of experiment, 36 plants per m² at the beginning of the experiment). The species in the freshwater aquaponic system described in Trang et al. (2010) exhibited a plant uptake of N between 35 and 93 mg m^{-2} d^{-1} and plant uptake of P between 7 and 30 mg m^{-2} d^{-1}. Therefore, similar to biomass production, the nutrient uptake values in our study were comparable to those in a fresh water aquaponic system.

Recently, results of two applications of *Salicornia* planted in constructed wetlands as biofilter for different marine organisms cultured at full strength seawater salinity were published. Webb et al. (2012) grew *Salicornia europaea* at a plant density of 90 plants per m² and

harvested 8.85 g m^{-2} d^{-1} total plant dry weight. The gain of total dry weight for *S. dolichostachya* in this study was only 3.8 g m^{-2} d^{-1} and therefore only half as high. But the shoot fresh weight gain of 72 g m^{-2} d^{-1} for *S. dolichostachya* in our study was similar to the gain seen in the study by Shpigel et al. (2013). They planted 100 plants of *S. persica* per m² and harvested 48 to 71 g m^{-2} d^{-1} fresh shoot material. In Webb et al. (2012) nutrient uptake per plant was 170 and 28 mg m^{-2} d^{-1} for N and P, respectively. Shpigel et al. (2013) found an N uptake of 40 to 80 mg m^{-2} d^{-1}. Compared to those values, the plant nutrient uptake of this study was similar for N uptake and P uptake of *S. dolichostachya*, but lower for P uptake of *T. pannonicum* and *P. coronopus*. The partly higher productivity and nutrient removal efficiency of Webb et al. (2012) and Shpigel et al. (2013) can probably be explained by the 2 to 3 fold higher planting density.

Recycling of nutrients from a marine aquaculture system implies the conversion of the nutrients abundant in the system to a valuable product. This is implemented in this study by the aquaponic production of valuable halophyte shoot biomass marketable as vegetable or salad. For the evaluation of the species-specific nutrient recycling not only the total plant uptake of N and P but also shoot to root ratio of nutrient uptake is of interest. Shoots produced four times more biomass than roots and took up 2 to 3 times more P and 4 to 5 times more N. *Plantago coronopus* seems to be less suitable than the other two species in terms of biomass production and N uptake. Nevertheless, results for all species are promising in terms of nutrient recycling. A future task is to find a utilization of the remaining 20-30% root biomass. A possible application is the use of the root biomass for biogas production in a fermentation process.

The interpretation of the higher biomass production and nutrient uptake of *S. dolichostachya* in this study compared to the other two species has to be done carefully. The hydroponic culture tanks of *S. dolichostachya* were illuminated for 18 h to prevent plants from flowering. This elongation of day length probably caused the higher biomass production of this species. In future experiments other illumination regimes to prevent flowering should be tested; such as elongation of day length by giving just 4 h additional light in the morning and in the evening (Buhmann et al., submitted) or interrupting the dark period by a short period of artificial illumination. This would improve the comparability to the culture of other species without artificial illumination and save energy costs in a commercial large-scale application.

The harvested leaves are free from microorganisms harmful for human consumption

It is important to examine the potential food safety concerns associated with the applied process water used to produce the vegetable product (Blidariu and Grozea, 2011). Although in modern RAS the outbreak of diseases and parasites are successfully avoided (Orellana et al., 2013), the halophytes grown in the aquaponic culture tanks could potentially be contaminated with bacteria and viruses that may be present in the fish culture; such as species from the genera *Salmonella*, *Vibrio* and *Escherichia*. The harvested plant material of this study did not show any of the tested pathogens in concentrations classified as harmful for human consumption. The RAS system is generally low in bacterial counts due to the ozone treatment of the process water and the fact that the shoots of the plants do not have any contact with the process water. These conditions probably accounted for the positive result and can be stated as appropriate for the aquaponic production of valuable plant biomass.

Relation of fish to plant biomass and future implications

In this study the total weight gain of the fish was 5.6 kg and of plants 5.5, 3.7 and 12.6 for *T. pannonicum*, *P. coronopus* and *S. dolichostachya*, respectively. Therefore, plant weight gain is similar to fish weight gain or only about 2 times higher for *S. dolichostachya*. The amount of feed applied to the system per m² was 347 g. Endut et al. (2010) suggest an amount of 15 to 42 g fish feed per m² of plant growing area. In our study, the amount of cultured fish, here expressed as amount of fish feed used, was far too high for the small plantation area if the aim is the total removal of the excess nutrients from the feed. But the aim of this study was to investigate the possibility of nutrient recycling by aquaponic production of halophytes and not the total replacement of other filters in the RAS. Nevertheless, it is favourable for the valuable plant biomass to retain as much of the nutrients generated in the RAS as possible.

For future application and optimization, the plant biomass production necessary to retain all the nutrients generated by the weight gain of 1 kg fish, was calculated. The calculations are relevant for the culture of sea bass in a juvenile state in a modern marine RAS. The calculation has been done on the basis of total weight gain of fish in the system, N and P that were not retained in the fish, and N and P content of the plants. To retain the abundance of N in the process water at 1 kg fish production, 37, 45, 33 kg fresh plant material (total plant) is necessary for *T. pannonicum*, *P. coronopus* and *S. dolichostachya*, respectively. On the basis of total P in the system, calculations result in a lower plant to fish biomass ratio. To retain the

abundance of P in the process water at 1 kg fish production, 20, 16 and 18 kg fresh plant material (total plant) is necessary for *T. pannonicum*, *P. coronopus* and *S. dolichostachya*, respectively. Therefore, the total amount of possible plant biomass production should be calculated based on total P in the system. *Plantago coronopus* is less effective in terms of N removal and more effective in terms of P removal than the other two species. *Tripolium pannonicum* and *S. dolichostachya* show similar values.

In the process water of the system, 418 g N and the 32 g P were abundant due to excess fish feed and excretion during the 35 days of experiment (calculated on the basis of total N and P in the fish feed and retention by the fish). Nutrient retention in plant material was 46 g N and 7 g P for all three plant species together (532 plants on a planting area of 15 m²). This means that 11 % of the N in the water (9% of total feed N) and 22% of the P in the water (10% of total feed P) was retained in the plants. Macrophytes, as described in Schneider et al. (2005), retained 57% of the feed N and 17% of the feed P. Further optimization to increase the nutrient retention of plants in the system, described in this study, is necessary. A higher planting density and a bigger plantation area would lead to a higher nutrient retention in the plants in relation to the nutrient abundance in the water due to fish production.

Suitable plant density in a hydroponic culture system depends very much on the species in culture and on the size of the plant at the time of harvest. For example, comparatively low plant densities of 2 to 3 plants per m² are applied for fruit vegetables such as tomato and zucchini (Auerswald et al., 1999; Rouphael and Colla, 2005; Incrocci et al., 2006). Plant densities for baby leaf vegetables, harvested early for the production and sell of mixed salads, are much higher (e.g. 1857 plants per m²) (Fallovo et al., 2009). Vegetables of an intermediate size, like radish, spinach and lettuce, are planted at densities of about 30 to 100 plant per m² (de Pinheiro Henrique and Marcellis, 2000; Yorio et al., 2001; Frantz et al., 2004). The plant species used for this study can be accounted to vegetables of intermediate size, therefore, a plantation density of 36 plants per m² seemed appropriate. However, plants still had space between them at the time of the harvest, especially *P. coronopus*. This suggests that a higher plantation density is possible. Webb et al. (2012) and Shpigel et al. (2013) successfully applied a plantation density of 90 to 100 plants per m² for species from the genus *Salicornia* in sand culture. A higher plantation density is probably possible for all three species used in this study. Investigation on the optimal plantation density of the halophyte species suitable for application in aquaponics should be performed.

This study demonstrated that an integrated halophyte culture for the recycling of nutrients in a modern zero-exchange RAS is possible. Nutrient recycling by halophyte culture is characterized by: i) the plant uptake of N and P from the process water resulting in the production of plant biomass and ii) the generation of valuable halophytic crop biomass in an aquaponic system. Also, the laborious and expensive denitrification process usually applied in RAS can likely be replaced by the aquaponic production of halophytes installed into a secondary loop of the RAS. For better nutrient recycling, the efficiency of the plant culture and the production of healthy plant material can be further optimized. Halophytic plant production needs to be adjusted in terms of culture conditions (i.e. the ratio of plant and fish weight gain to plant biomass production).For commercial applications, an economic and ecologic balance calculation is necessary, including the use of recourses such as energy and water and the added value of valuable plant biomass production and a reduction in the environmental impact of aquaculture production.

Acknowledgements

We would like to thank the gardeners Yvonne Leye and Lutz Krüger for taking care of the plants and Rebecca Hosang, Pamela von Trzebiatowski and Julia Volker for valuable technical assistance and Christian Boestfleisch for support in harvesting the plants. The project was financially supported by the Deutsche Bundesstiftung Umwelt (DBU) (AZ27708/1-3) and the Erwin Sander Elektroapparatebau GmbH, Germany.

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Chapter 6

General discussion

Salt-tolerant crops for the future

Glycophytic crop species have a long history of variety breeding. Based on wild types of the respective species a lot of effort resulted in a broad spectrum of crop plants with high yield and economic value. In contrast, for halophytic crop species domestication, selection of suitable ecotypes and variety breeding is a relatively new approach (Yensen, 2008). Halophytes have a long history of traditional and regional use of various wild-grown halophytes or small-scale cultivation for nutritional or medicinal purposes (Liebezeit, 2008; Ksouri et al., 2011). But they still lack importance in large-scale agriculture worldwide (de Vos et al., 2013). One possible reason is that during the history of conventional crop development freshwater was sufficiently abundant in most regions of the world and arable land and the range of conventional crops could cover the demand. But depletion of freshwater and loss of arable land due to salinization endangers future food supply worldwide and the research on and application of new agricultural approaches become necessary. This might enhance the development and importance of halophytic crop plants in the future.

An important reason for the slow development of halophytic crop plants is that their economic potential is widely unknown to farmers and consumers (de Vos et al., 2013). But the long history of traditional and regional use of halophytes builds a base of knowledge about many edible species beside those with use in traditional medicine or with utility as forage plants. Species with broader market acceptance are from the genus *Salicornia*. There is a wider spectrum of studies on species from the genus *Salicornia* than for other halophyte species. There are studies on germination (Khan et al., 2000), cultivation (Rueda-Puente et al., 2003; Ventura and Sagi, 2013), influence of salt (Aghaleh et al., 2009; Ventura et al., 2011a) and valuable metabolites (Chung et al., 2005; Kim et al., 2012), beside others. The results from these investigations and making them public might have led to a higher degree of recognition of species from the genus *Salicornia* as crop plants worldwide. This leads to a higher acceptance regarding farmers and consumers and to a higher market value of species from this genus and the derived products (de Vos et al., 2013). Therefore, research on halophytes and different aspects regarding their utility as crop plants is an eminent prerequisite for their application in large-scale agriculture.

Culture conditions for the production of halophytic crops

One important reason for the slow development of halophytic crop plants might be a lack of knowledge on appropriate culturing conditions and techniques. Only recently more detailed studies on promising cash crop halophyte species shed some light on important factors for cultivation. Factors which influence the successful culture of halophytic crop plants, such as light regimen and addition of micronutrients, for example molybdenum and iron, have been studied (Ventura et al., 2010, 2011b, 2013). Results in this thesis (chapter 4) also showed that the addition of iron in an appropriate form is an important factor for the production of healthy biomass of salt-tolerant species. Also the type of culture and substrate and the nitrate concentration in the culturing solution could be determined as important factors influencing the biomass production.

Several studies focus on the influence of salinity. The effect of different NaCl or seawater concentrations on biomass production, physiology and nutritional value were studied for different potential halophyte crop species (Koyro et al., 2006; Geissler et al., 2009; de Vos et al., 2010; Ventura et al., 2011a; de Vos et al., 2013). An experiment described in this thesis (chapter 4) revealed that growth of *Tripolium pannonicum* (Jacq.) Dobrocz. is reduced drastically between salinities of 15 and 30 psu (43 and 86% seawater salinity, if seawater salinity is defined as equal to 599 mM NaCl like in chapter 2). This is in concordance with Koyro et al. (2006) and Geissler et al. (2009) who found an eminent growth reduction at salinities above 50% seawater salinity, for *Plantago coronopus* L. as well as for *T. pannonicum* (the authors defined seawater salinity as equal to 500 mM NaCl). De Vos et al. (2013) showed that salinity also caused growth reduction for *Diplotaxis tenuifolia* (L.) DC. and *Cochlearia officinalis* L. with a reduction of maximum yield at around 150 mM NaCl and 100 mM NaCl, respectively (25% and 17% seawater salinity, respectively, if seawater salinity is defined as equal to 599 mM NaCl like in chapter 2). In a field experiment conducted by Ventura et al. (2011a) with *Salicornia* and *Sarcocornia* species growth only started to decline at 50% seawater salinity (33 g l^{-1} of a commercial sea salt was defined as 100% sea water salinity), which shows the higher salt tolerance of the species from these genera.

The extensive work on the effect of salinity on growth and physiology of potential halophytic crop species reflects the interest in understanding the mechanisms of salt tolerance of the different species. Beside the gain of basic knowledge on the physiology of halophytes these studies also contribute to a base for application (Huchzermeyer and Flowers, 2013). But for a successful development of halophytic crop plants in the future and the optimization of

culturing conditions an understanding of the physiological response of halophytes to various factors is necessary.

Breeding of halophytic crop plants

Another important point for the successful introduction of halophytic crop plants to large-scale agriculture is to catch up with the breeding process that is already in an advanced state regarding conventional crop plants. De Vos et al. (2013) found a variety of *C. officinalis* with a less bitter taste than usually known for this species. They suggest taste as one future breeding criterion, beside a higher tolerance towards salinity. However, Yensen (2008) describes the difficult and costly breeding and patenting process for a variety of the cereal grain halophyte *Distichlis palmeri* (Vasey) Fasset ex. I.M.Johnst. and for a variety of *Salicornia bigelovii* Torr., which is also described in Zerai et al. (2010).

In this thesis it was shown that different individuals of *T. pannonicum* behaved differently towards iron-deficient culture conditions. In the experiment described in chapter 5 various plants exhibited chlorotic leaves probably due to low iron availability at a high pH. But other individuals cultured under the same conditions exhibited normal green leaves. Additionally, plants from seed material collected at one location showed a higher chlorophyll content and therefore performed better under iron-deficient conditions than plants from seed material collected at a different location in an experiment described in chapter 4 ($E_{MICRONUTRIENTS}$). These observations suggest that the conditions in the natural habitat of *T. pannonicum* are highly variable and different populations are adapted to specific environmental conditions. Another example for differences within the same species was shown in experiments with *Salicornia dolichostachya* Moss described in chapter 4 ($E_{SPECIES}$) and chapter 5, where phenotypic differences between individual plants were observed. Due to their long, slim as well as succulent internodes the *S. dolichostachya* plants generally had an attractive appearance regarding their market potential as vegetable. But some individuals developed short and thick internodes making them less attractive as marketable product. Both types of plants were identified to belong to the same species on the base of ETS sequence analysis (data not shown). Either the marker system is not suitable to reveal different *Salicornia* taxa or the two phenotypes belong to different ecotypes of the species *S. dolichostachya*.

The results suggest that future research should include the identification of different ecotypes of a potential halophytic crop species and the selection of certain ecotypes suitable for a specific application. This selected ecotypes could build the base for further breeding activity.

Market potential of halophytes due to their valuable secondary metabolites

Apart from their utility as food crop plants, various halophytes have a high market potential because they contain valuable compounds. Contrary to glycophytes many halophyte species show a high amount of different secondary metabolites, for example phenolic acids, flavonoids and saponins with high antioxidative capacity (Chung et al., 2005; Benhammou et al., 2009; Meot-Duros et al., 2009; Kim et al., 2012). The development of diverse secondary metabolites is probably due to a necessity to develop a strong response against salt stress. For example, saline condition can cause the formation of free radicals due to oxidative stress and antioxidative compounds help the plant to defend itself against them (Flowers and Colmer, 2008).

It is important to close the gap between research on secondary metabolites in halophytes and introduction to successful application. Chapter 3 reveals pharmacognosy, functional foods, nutraceuticals and technical implementations as promising fields for application. Lubbe and Verpoorte (2011) state important prerequisites buyers of medical, aromatic and cosmetic plant material usually expect. Those comprise a reproducible effect of the compound or extract outside the laboratory, safety of the material for the consumer and/or environment, traceability of the production process of the plant material, sufficient and constant supply, and given demand for the product. These aspects are consistent with important future research tasks that are determined in chapter 3. Here, the provision of genetically defined plant material, determination of culture conditions that are most suitable for the production of a certain compound, and defined culture conditions and extraction methods for the provision of the desired metabolite in reproducible quality and quantity are determined as important aspects for implementation.

Today, research on secondary metabolites screens many different halophyte species for example from the genera *Salicornia*, *Atriplex*, *Mesembryanthemum* and *Crithmum* and results in a huge bunch of potentially interesting compounds (Kim et al., 2000; Benhammou et al., 2009; Meot-Duros et al., 2009; Falleh et al., 2011; Kim et al., 2012). As stated in chapter 3 future research has to proceed in a more strategic way and focus on the examination of interesting metabolites of a few promising species to establish a sound base of knowledge. This makes application of the scientific knowledge more likely. Only after gaining this basic knowledge and experience the screening for interesting compounds in a large number of halophyte species that are still unexplored should be performed step by step. *Salicornia herbacea* L. would be a promising species for enforced research to reveal interesting

secondary compounds and establish methodology because a lot of investigation has already been done (Chung et al., 2005 and 2006; Hwang et al., 2009; Kong et al., 2009; Rhee et al., 2009; Ryu et al., 2009; Kang et al., 2011; Kim et al., 2012; Kong et al., 2012; Yu et al., 2012). However, in the case of *Salicornia* it is important to clarify taxonomic difficulties (Kadereit et al., 2007). Research on interesting secondary compounds found in halophytes helps to generate a better knowledge on the utility and value of potential halophytic crop plants.

Efficacy of halophytes as biofilter for nutrients

The identification of optimal culture conditions is not only important for the growth of halophytes as crop plants but also for their utility as biofilter. The efficacy of halophytes as biofilter for nutrients depends on various factors. In chapter 2 several of those factors are determined for the use of halophytes as biofilter for marine aquaculture effluents. Accordingly, salinity, flooding, nutrient level, root characteristics and technical applications can influence the nitrogen and phosphorus removal by a wetland planted with halophytes. As shown in chapter 4, nutrient uptake capacity of *T. pannonicum* declined with increasing salinity. Also a low nitrogen concentration of 1 mg l^{-1} nitrate-N drastically reduced plant growth and uptake of nitrogen and phosphorus. Different phosphorus concentrations had a less obvious influence on plant growth and nitrogen uptake, thus phosphate uptake was reduced at phosphate-P concentrations as low as 0.3 and 1.6 mg l^{-1}. Results on irrigation time in sand culture showed that preferred conditions are species-specific (data not shown). These results on appropriate culture conditions for halophyte species in terms of efficient nitrogen and phosphorus uptake under simulated conditions build a base to assess the important parameters for application.

Results gained under simulated conditions might not always be compliant with reality. An experiment described in chapter 4 ($E_{MICRONUTRIENTS}$) and experiments described in Ventura et al. (2013) revealed that the addition of iron in form of a highly stable complex such as Fe-EDDHA is necessary to prevent iron chlorosis in leaves of *T. pannonicum* caused by the high pH of (artificial) seawater. Chlorosis was also observed in *S. dolichostachya* when cultured in artificial seawater without the addition of iron as Fe-EDDHA (data not shown). For the application of *T. pannonicum*, *S. dolichostachya* and *P. coronopus* as biofilter for nutrients in a marine RAS the use of Fe-EDDHA was not possible (chapter 5). Unknown effects due to accumulation of EDDHA in the process water and possible harmful effects on the cultured fish should be prevented. Therefore, ferric citrate was added to the aquaponic culture of the

plants which is degradable in the ozone treatment of the RAS but less stable at high pH (Lucena, 2003). But due to the carbon dioxide of the fish respiration the pH of the system decreases and ferric citrate was a useful iron source in the experiment, at least for two of the three applied halophyte species. The optimal pH for the nitrification process important in a RAS to prevent fish from ammonia toxication is a high pH, such as 8.5 (Tyson et al., 2004). Therefore a pH balance for the optimal culture of plants, fish and bacteria in the filters as well as the problem of iron deficiency in plants is also a problem in freshwater aquaponic systems (Tyson et al., 2008a and b). One promising approach to solve the problem is foliar application of micronutrients (Tyson et al., 2008a, Roosta and Mohesian, 2012). Beside iron other essential micronutrients such as manganese, copper and zinc are also less available at high pH and according deficiency in plants can also become a problem (Rackoy et al, 2006; Roosta and Hamidpour, 2011).

In the case of iron, simulated conditions gave a suitable hint to a problem that might occur in application. But the specific solution to the problem had to be adjusted to the specific conditions of the application. Differences in results under simulated and applied conditions can also occur if salinity is due to different constituents. For a halophytic plant species the osmotic stress caused by a nutrient solution containing NaCl and a solution containing the constituents of seawater is the same if the conductivity of the culturing solution is the same. But in terms of ionic stress plants react differently to different elements that cause the salinity of a culturing solution (Rozema and Schat, 2013). Therefore, the tolerance level of a halophyte species determined under environmental conditions or by experiments using NaCl might be different from the salt tolerance level of the same halophyte species grown in seawater or a specific effluent. This is important for application because the salt tolerance level of a halophyte species possibly has to be determined for the specific composition of the effluent to be treated by the halophyte biofilter, for example agricultural runoffs, industrial effluents or effluents from marine aquaculture.

Beneficial combination of halophyte and marine fish culture

Marine fish can only be cultured in salty water similar to the constitution of the seawater that forms their natural environment. Therefore, the characteristic of halophytes of being tolerant to saline conditions is an important prerequisite for the combined marine fish and plant culture. But several marine aquaculture species or their developmental stages can be cultured at salinity levels far below seawater salinity (Forsberg and Neill, 1997; Atwood et al., 2003).

This is an advantage for the integrated culture of marine fish and halophytes because most halophyte species grow better at salinity levels below seawater salinity which also enhances nutrient uptake.

The combination of halophytic crop culture and marine fish culture described in this study has two advantages. On the one hand, the halophytes can be used as biofilter by exploiting plant uptake of the nitrogen and phosphorus abundant in the effluent of the fish culture due to excess feed and excretion. The second benefit of combining marine fish culture and halophyte culture is the added-value due to the production of a valuable co-product.

Operating a RAS is costly (Gutierrez-Wing and Malone, 2006). An integrated halophyte biofilter applied for the removal of nutrients can save costs by partly replacing more expensive technical filters (Schneider et al., 2005). It can also contribute to the economy of an aquaculture system due to the generation of a marketable product beside the produced fish (Blidariu and Grozea, 2011). The results of chapter 5 show that a modern marine RAS is suitable for the integrated culture of halophytic crops with a high market potential and that they take part in the nutrient removal process within the system. Therefore, both concepts, partly replacement of costly technical filters in their function as biofilter and generation of a marketable product, are valid for the integration of halophytic crop culture in a marine RAS.

An additional advantage of the combination of fish and plant culture and the sustainable use of resources in integrated marine RAS is that it might enhance the acceptance of the consumer. In public, intensive aquaculture production partly has a bad image due to frequent reports on its unpleasant effects on the environment and on the cultured organisms. Controlled marine RAS is an environmental friendly technology and with beneficial conditions for the cultured organisms and the sustainable use of resources due to a recycling of nutrients in an integrated culture of halophytic crop plants could possibly change the impression of the consumer. This reassessment of aquaculture could enhance the progress of sustainable food production for the future.

On the base of the data of chapter 5, 18 kg plant biomass of *S. dolichostachya* can be produced from the nutrients in the process water from 1 kg fish production. If shoot accounts for 80% of the plant biomass, 14.3 kg harvestable plant biomass can be produced. Menterra (2007) reported a price of 10 £ kg^{-1} fresh *Salicornia* from wild harvest which equals 12€ kg^{-1}. Therefore the estimated value for the marketable biomass of *S. dolichostachya* potentially producible from the nutrients of the production of 1 kg fish in the system described in chapter 5 would be 172 €. The plants applied in the experimental design investigated in chapter 5

covered an area of 15 m² and only retained 11% of the nitrogen and 22% of the phosphorus potentially plant available in the process water of the fish culture. To use all the phosphorus of the fish culture for the production of the crop halophytes a plantation area of 68 m² instead of 15 m² would have been necessary. For the use of all the nitrogen in the system a plantation area of 136 m² would have been necessary and also the addition of phosphorus, because in relation to the abundance in the water a higher percentage of phosphorus than nitrogen is used by the plants making it the limiting factor for crop production. Further optimization for example a higher planting density as suggested in chapter 5 can reduce the plantation area needed. If the halophyte culture is used only partly to replace technical filters in a RAS and mainly to produce a valuable co-product a smaller plantation area can be used.

In the integrated culture of marine fish and halophytes both cultures can possibly have negative effects on each other. None of those effects could be observed in the experiment described in chapter 5. There was no contamination of the harvested plant material with pathogens possibly derived from the fish culture. But substances possibly abundant in the effluent due to the fish feed or medication could harm plant growth and could have a negative effect on the biomass quality for consumption. The other way round, plant exudates could harm the cultured fish. Investigation is needed regarding these questions. Another challenge is crop protection without the use of chemicals that are harmful for the fish, only using beneficial organisms and natural products (Dayan et al., 2009; Blidariu and Grozea, 2011).

Sustainable utilization of resources

Sustainable development was defined as “development that meets the needs of the present without compromising the ability of future generations to meet their own needs” (WCED, 1987). Many common production processes are not sustainable. They release substances such as nutrients and heavy metals with negative impact on water resources and environment and even if conventional water treatment is applied many valuable substances are wasted instead of recycling them in another production process. But depletion of freshwater resources leads to an upward trend in the reuse of water (WHO, 2014).

Several practices in marine aquaculture have a high environmental impact due to the release of effluents that can contain various substances such as nutrients, organic material and medication (Read and Fernandes, 2003). Also transfer of diseases, mixing of domesticated and wild stocks as well as capture of wild fish for feed and wild seedstock collection have a negative impact on the environment (Naylor et al., 2000). The development of RAS is stated

as a perspective on environmental sustainability of aquaculture, covering the following aspects: reduced water consumption, opportunities for waste management and nutrient recycling, better hygiene and disease management, and biological pollution control (Martins et al., 2010).

The sustainability of a RAS can be increased by the aquaponic production of vegetables (Blidariu and Grozea, 2011). The sustainability is increased because resources abundant due to a production process are used for another production process. In this study this is reflected by the re-use of nitrogen and phosphorus in the process water due to the production of marine fish by the production of valuable plant biomass in the aquaponic halophyte culture. As stated in chapter 2 and chapter 4 hydroponic culture is more suitable than substrate culture in terms of nutrient recycling because in this system plants are the important components of the biofilter. In hydroponic culture plant nutrient uptake is the most important process causing nutrient removal instead of microbial nutrient uptake, bacterial conversion, precipitation and adsorption abundant in substrate containing culture systems. A depletion of phosphate availability for fertilizer production is predicted within the next 50 to 100 years (Cordelli et al., 2009). Therefore, the reuse of phosphorus abundant as waste in a production system such as marine aquaculture for the growth of crops can become quite an important recycling process in the future.

The approach of integrated multi-trophic aquaculture combines the production of fed aquaculture species (e.g. shrimp or fish) with the production of inorganic extractive species (e.g. algae or higher plants) and the production of organic extractive species (e.g. bivalve molluscs) (Neori et al., 2004; Schneider et al., 2005; Troell et al., 2009). In addition to an integrated halophyte culture applied as biofilter for nutrients and to generate a valuable co-product, several different organisms as filter components and also as different valuable co-products can probably enhance the sustainability of an aquaponic system. Beside halophytes there are other potential biotic components with market potential that are applicable in an integrated multi-trophic aquaculture, for example several macro- and micro-algae species as well as mollusks and also bacteria that can be produced by using the bio-floc technology (Neori et al., 2004; Schneider et al., 2005; de Schryver et al., 2008; Sará et al., 2009).

Future implications

The combined use of halophytes as biofilter for nutrient-rich saline waters and halophytic crop plants is promising. Regarding the development of halophytic crop plants important

tasks for research in the near future are ecotype selection and breeding of suitable varieties, determination of the nutritive value of potential halophytic food crop plants and detailed research on valuable secondary compounds contained in a few promising halophyte species. Regarding the application of halophytic crop plants as biofilter for nutrient-rich saline waters the determination of species-specific requirements for a few promising halophyte species and the improvement of the productivity of the halophyte culture in a marine aquaculture system are future research tasks. Additionally, the applicability of results from marine aquaponics to the treatment of other saline effluents such as agricultural runoffs and industrial effluents should be investigated.

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Danksagung

An dieser Stelle möchte ich mich bei allen bedanken, die mich bei der Durchführung und Fertigstellung der vorliegenden Arbeit unterstützt haben.

Mein besonderer Dank gilt **Prof. Dr. Jutta Papenbrock** für das Ermöglichen der Arbeit und die zahlreichen Diskussionen und Vorschläge zu den Versuchen und den Ergebnissen. Außerdem möchte ich mich ganz herzlich bei **Prof. Dr. Manfred Schenk** dafür bedanken, dass ich Messungen im Institut für Pflanzenernährung durchführen konnte und für die Übernahme des Korreferats, sowie bei **Prof. Dr. Bernhard Huchzermeyer** für die Übernahme des Vorsitzes der Promotionskommission.

Ganz besonders möchte ich mich auch bei **Yvonne Leye** und **Lutz Krüger** für die Unterstützung in Sachen Saatgut und Pflanzenanzucht bedanken, sowie für die vielen kreativen Lösungsvorschläge, das mit Anpacken zu jeder Zeit, die Gespräche und offenen Ohren und das gemütliche Frühstücken an Pflanztagen.

Bei **Julia Volker**, **Pamela von Trzebiatowski** und **Birgit Lippmann** möchte ich mich für die Unterstützung im Labor und für die Tipps zu zahlreichen kleinen Details bedanken, bei **Dr. Daniel Gerhard** für die hilfreiche Statistikberatung.

Ich danke meiner gesamten Arbeitsgruppe für die tolle Arbeitsatmosphäre, für die Unterstützung und den Teamgeist. Besonders möchte ich mich bei **Sebastian Guretzki**, **Christian Boestfleisch**, **Felix Hirschmann, Anja Riemenschneider** und **Ariel Turcios** für die Klärung zahlreicher kleiner Fragen und die Mensapausen bedanken.

Prof. Dr. Waller möchte ich für das Ermöglichen der Versuche im Saarland und die Beratung in vielen inhaltlichen Fragen danken. Auch bei **Dr. Bert Wecker**, **Verena Hanke**, **Dr. Anneliese Ernst** und **Rebecca Hosang** möchte ich mich für die gute Zusammenarbeit im Projekt bedanken. Dem ganzen Team der Forschungshalle Völklingen möchte ich für die Unterstützung beim letzen Versuch und die Gastfreundschaft danken.

Mein Dank gilt auch **Prof. Dr. Rath** für die Ermöglichung der Gewächshausversuche und **Werner Hock** und **Andreas Meyer** für die Unterstützung bei zahlreichen technischen Fragen. Dem ganzen BGT-Team möchte ich für die angenehme Arbeitsatmosphäre danken, besonders **Gökhan Akyazi** und **Sandra Haase**.

Meinen Freunden, meinem Mitbewohner und meinem Freund möchte ich für die Unterstützung, das Verständnis in stressigen Zeiten und das Interesse an Pflanze, Salz und Fisch in den letzten 3,5 Jahren und ganz besonders in der Endphase danken.

Curriculum vitae

Anne Kathrin Buhmann

*04. Juni 1982, Hemer, Germany

Education and career history

08.2010 – 01.2014	Graduate studies at the Institute of Botany, Gottfried Wilhelm Leibniz University Hannover, Germany; financially supported by the Deutsche Bundesstiftung Umwelt (DBU) (AZ27708/1-3); Topic of the thesis: "Biological purification of nutrient-rich saline water by halophytes and their potential as valuable co-product in aquaculture systems"
07.2009 – 08.2010	Scientist in the research and development project "High PUFA Algae" at the Leibniz Center for Tropical Marine Ecology, Bremen, Germany
09.2008 – 06.2009	Diploma thesis at the Leibniz Center for Tropical Marine Ecology, Bremen, Germany and the Gottfried Wilhelm Leibniz University; Title of the diploma thesis: "Untersuchungen zum Phytoremediations-potential zweier Mangrovenarten"
10.2003 – 06.2009	Studies of biology at the Gottfried Wilhelm Leibniz University Hannover, Germany; Major: ecology, minor: plant physiology, molecular biology
10.2002 – 10.2003	Studies of earth sciences at the Gottfried Wilhelm Leibniz University Hannover, Germany
08.2000 – 06.2002	Gymnasium Schule Marienau, Dahlem-Marienau, Germany (Secondary school)

Conference participation and presentations

08.2012	COST WG2 Meeting 2012: Putting halophytes into work: genetics, biochemistry, physiology; poster presentation: Singh, D., Buhmann, A., Seal, C., Papenbrock, J., Influence of salinity and temperature on the germination of *Salicornia*.
02.2012	International workshop “Sustainable cultivation and exploitation of halophyte crops in a salinizing world” of the COST action FA0901 (Putting halophytes into work); presentation with the title: “Filtering of aquaculture effluents by halophytic plants: Basic principles, current uses and future perspectives”.
06.2011	Seminar of the initiative for sustainable aquaculture of the Deutsche Bundesstiftung Umwelt (DBU), presentation with the title "Biologische Abwasserreinigung durch integrierte Kultur von Halophyten in landbasierten marinen Kreislaufanlagen für die Fischzucht (erste Phase)".

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