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Influence of plant species and richness on pollutant removal in treatment wetlands

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Influence of plant species and richness on pollutant removal in treatment wetlands

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Résumé

Les marais filtrants artificiels sont des écosystèmes recréés par l’homme dans le but d’optimiser l’épuration des eaux usées. Lors de la sélection d’espèces végétales pour la mise en place de ces marais filtrants, l’utilisation d’une polyculture ainsi que d’espèces indigènes non invasives est de plus en plus recommandée. Néanmoins, la plupart des marais filtrants existants sont des monocultures utilisant des plantes envahissantes, probablement à cause du manque d’évidences scientifiques sur les avantages de la diversité végétale et de la performance des espèces locales. Ainsi, les questions de recherche autour desquelles s’oriente ma thèse sont: Les polycultures présentent-elles un potentiel épuratoire aussi ou plus grand que les monocultures, et une espèce indigène est-elle aussi efficace et performante qu’une espèce exotique envahissante dans des marais filtrants ?

Trois expériences ont été conduites afin de répondre à ces questions. J’ai d’abord testé l’influence de la richesse végétale sur l’élimination des polluants en deux dispositifs expérimentaux: 1) comparant deux espèces de plantes émergentes en monoculture ou combinées séquentiellement, et 2) évaluant la performance de quatre espèces flottantes plantées en monoculture par rapport à des associations de deux (avec toutes les combinaisons possibles) et de quatre espèces. Une troisième expérience a été réalisée afin de comparer l’efficacité épuratoire de l’haplotype européen envahissant du roseau commun (*Phragmites australis*) et de la sous-espèce locale non-invasive (*P. australis* subsp*. americanus*).

La composition en espèces végétales a produit un effet notable sur la performance des marais filtrants. La comparaison des performances en mono- et en polyculture n'a pas permis de démontrer clairement les avantages de la diversité végétale pour l'élimination des polluants dans les marais filtrants. Toutefois, les marais filtrants plantés avec une combinaison d'espèces étaient aussi efficaces que les monocultures des espèces les plus performantes. La comparaison entre les deux sous-espèces de *P. australis* indiquent que la sous-espèce indigène pourrait remplacer le roseau exotique envahissant, évitant ainsi les potentiels risques environnementaux sans toutefois compromettre l'efficacité du traitement.

Les résultats prometteurs de la sous-espèce indigène de *P. australis* doivent encore être testés dans des expériences à grande échelle avant d'utiliser largement cette espèce dans les marais filtrants. Nos résultats suggèrent que, dans des conditions où la performance des macrophytes disponibles est inconnue ou ne peut être déterminée, l'utilisation d'une combinaison d'espèces présente les meilleures chances d'accomplir le plus haut niveau possible d'élimination de polluants. De plus, même si la diversité végétale ne présente pas un avantage mesurable en termes d'efficacité épuratoire, celle-ci améliore la résilience des marais filtrants et leur résistance aux stress et aux maladies.

Mots-clés : Marais filtrants, efficacité épuratoire, biodiversité, service écologique, plantes envahissantes, *Phragmites australis,* plantes flottantes.

Abstract

Treatment wetlands (TWs) are complex engineered ecosystems designed to remove pollutants from wastewater. In selecting plant species for TWs, the use of polycultures as well as the choice of local non-invasive species is increasingly recommended. However, to date, the majority of TWs have been planted in monoculture using invasive species, probably because the performance of native species and the possible advantages associated with plant diversity have not been clearly demonstrated. The research questions of my thesis are: Are polycultures as good as or better than monocultures for pollutant removal, and could a native species be as efficient as an exotic invasive species in TWs?

Three experiments were performed to answer this question. The influence of plant richness on pollutant removal was tested in two mesocosm experiments: 1) comparing two emergent plant species in monoculture and planted in sequence, and 2) evaluating four free-floating species, each in monoculture versus pairs of species (all possible combinations) and the four species planted together. A third experiment was carried out to compare the removal efficiency of the invasive European haplotype of common reed (*Phragmites australis)* and the native non-invasive subspecies (*P. australis* subsp. *americanus).*

Plant species composition influenced the performance of TWs. The comparison of the performance of monocultures versus polycultures showed that, overall, TWs planted with a combination of species were as efficient as monocultures of the best performing species. Comparing the performance of native and invasive *P. australis* strongly suggests that the native could replace the invasive species in TWs, thereby avoiding possible environmental risks without compromising treatment efficiency.

The promising results of the native *P. australis* subspecies need to be further tested in full-scale experiments in order to encourage the use of this species in TWs. The results obtained comparing plant species combinations suggest that under circumstances in which the performance of available macrophytes is unknown or cannot be determined, using a mixture of species offers the best chances of achieving the highest possible level of pollutant removal. Furthermore, it has been suggested that plant diversity contributes to TWs in terms of their resilience to stress and disease.

Keywords: Constructed wetlands, wastewater treatment, biodiversity, ecological services, invasive plants, *Phragmites australis*, free-floating plants.

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Abbreviations

COD	chemical oxygen demand
HSSTW	horizontal subsurface treatment wetland
NH_4	ammonium
NO_3	nitrate
S^{-2}	sulfide
SO_4	sulfate
SSFTW	subsurface flow treatment wetland
TN	total nitrogen
TSS	total suspended solids
TW	treatment wetland
TP	total phosphorus

To all those who truly believed…

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1. Introduction

Wetlands are transitional environments between terrestrial and aquatic ecosystems (Diakova et al., 2006). They provide various key ecosystem services, such as improving water quality and acting as biological filters that can assimilate large amounts of environmental contaminants (Sheoran, 2006). Biogeochemical processes that take place in natural wetlands inspired the design of constructed wetlands for wastewater treatment (hereafter referred as treatment wetlands or TWs).

1.1 Water quality improvement using treatment wetlands (TWs)

Treatment wetlands are complex engineered ecosystems designed to remove pollutants from wastewater by enhancing processes that occur in natural wetlands within a more controlled environment (Headley and Tanner, 2006). They have been proven to constitute a cost-effective alternative to conventional wastewater treatment mainly for small decentralized communities (Wu et al., 2014). The biological and physicochemical processes that take place between wastewater and the main components of TWs −substrate, macrophytes and microbial communities− determine their efficiency. TWs are generally classified according to their water flow regime (Vymazal and Kröpfelová, 2008):

- surface water flow TWs, in which the water is directly in contact with the atmosphere: they are commonly named free water surface TWs (FWS TW)

- or, sub-surface water flow (SSF), when the water flows through a porous media under the surface of the bed.

Free water surface TWs allow the use of a large range of macrophytes, including emergent, submerged and free-floating plants. The terms emergent, floating and submerged refer to the predominant growth form of a wetland macrophyte. “Emergent macrophytes” refer to rooted vascular plants with most aboveground part emerging above the water level. On the other hand, “free-floating plants” are non-rooted and have buoyant leaves and stems that allow them to float on the water line, and with their roots in the water column (Kadlec et al., 2000). Common free-floating plants species used in FWS TWs are diverse in their morphology and habitat; the range includes large plants with well-developed root systems such as *Pistia statiotes* (water lettuce) and *Eicchornia crassipes* (water hyacinth) to very small plants like *Wolffia* spp. and *Lemna* spp. Otherwise, sub-surface water flow TWs restricts the use of macrophytes only to emergent species. Three genera of wetland plants are commonly used both in SF TWs and SSF TWs, including the species *Phragmites australis* (the most frequent one), *Phalaris arundinacea,* and *Typha* spp. All the species mentioned in this section (both free-floating and emergent) have remarkable growth rates, tolerate TWs conditions and have shown a good pollutant removal performance (Vymazal and Kröpfelová, 2008).

The presence macrophytes plays an essential role in the performance of TWs and is important for many pollutant removal functions in TWs (Brix, 1997; Coleman et al., 2001; Kadlec and Wallace, 2009). Macrophytes uptake and store nutrients, and enhance microbial mediated processes by increasing the attachment surface area, supplying oxygen to the rhizosphere and providing organic carbon through root exudates (Coleman et al., 2001; Bais et al., 2006; Kadlec and Wallace, 2009; Vymazal, 2011). They may also improve hydraulic conductivity and prevent clogging (Brix, 1997; Chazarenc et al., 2007).

Selection of macrophyte species for TWs is an important design issue. The question whether some species are better than others, or if so, which one is the best species, has not been yet resolved (Kadlec et al., 2000). Plant species have been shown to vary in their potential for pollutant removal, and consequently species selection can influence TW efficiency (Brisson and Chazarenc, 2009). A more diverse plant community may even improve the performance of TWs, through functional complementarity (Wang et al., 2014). At the same time, given that species suitable for wastewater treatment are often considered invasive, it is important to take into account the ecological acceptability of the macrophytes selected for TWs to avoid risks to the integrity of nearby ecosystems (Tanner, 1996).

Therefore, the use of diverse macrophyte species in combination to improve the treatment efficiency of TWs and the choice of non-invasive species are increasingly recommended in TW design (EPA, 2000; Kadlec and Wallace, 2009). However, to date, the majority of TWs have been planted in monoculture using species suitable for wastewater treatment but considered invasive outside of their native range. This shows that despite the recommandations, the performance of native species and the influence of plant diversity in TWs have not been yet clearly demonstrated.

1.2 Influence of plant species richness on water treatment

1.2.1 Biodiversity and ecological services

Biodiversity plays a fundamental role sustaining ecosystem processes (Loreau et al., 2001; Quijas et al., 2010). For this reason, the current loss rate of biodiversity has created a concern regarding the role of biodiversity on the provision of the ecological services in which human society relies on (Constanza and Folke, 1997; Quijas et al., 2010). Extensive research is being done in order to identify specific relations between plant diversity and ecosystem functions. In general, positive relations have been found between plant species richness and ecosystem processes (Tilman et al., 1996; Loreau, 2010).

Most often, the relation between plant diversity and ecosystem functions was evaluated under a species richness approach (Diaz and Cabido, 2001). Species richness is defined as the number of different species present in a given ecosystem (Engelhardt and Kadlec, 2001). Previous studies found, in general, positive relations between plant species richness and certain ecosystem processes (Schläpfer and Schmid, 1999). Primary production and biomass productivity have been the ecosystem processes most evaluated –mainly in grasslands and herbaceous assemblages– and were associated with the provision of ecosystem services, such as carbon storage and erosion control (Diaz and Cabido, 2001; Balvanera et al., 2006; Loreau, 2010).

Lately, a different approach argues that plant diversity is not only important in terms of number of species per se, but furthermore on their range of functional diversity, and the complementarity between them. This approach is based on recent studies suggesting that ecosystem processes are more strongly related to functional richness, than to species richness (Diaz and Cabido, 2001). Functional richness refers to the number of different plant functional types or traits in a given ecosystem. According to Diaz and Cabido (2010), more functional traits will be present in an ecosystem with higher levels of diversity. Under the context of a wide range of functional traits, different species may present complementary effects, possibly leading to a more efficient resource use on a given ecosystem (Paquette and Messier, 2011).

1.2.2 Role of plant diversity in wetlands

The relation between biodiversity and ecosystem functioning has been evaluated mainly in the context of grassland ecosystems (Cardinale et al., 2011). Considerably fewer studies have evaluated the relationship between plant richness and the provision of ecosystem services in aquatic ecosystems, including wetlands.

Wetlands provide several ecosystem services such as: oxygen production, nutrient recycling, primary production, erosion control, carbon storage, food and material production, water supply, water pollutant removal, habitat provision and aesthetic values (Coleman et al., 2001; Chen et al., 2009). Water quality improvement is a very important ecological service provided by wetlands. Natural wetlands act as biological filters capable of assimilating large amounts of environmental contaminants (Sheoran, 2006).

Engelhardt and Ritchie (2001) and Engelhardt and Kadlec (2001) analyzed the impact of plant diversity on natural wetlands functioning. These studies found that higher plant richness enhanced wetland ecosystem processes, such as biomass productivity and phosphorus retention. In a study simulating fresh-water streams, Cardinale et al. (2011) found that higher algae species richness increased water nitrate uptake under fluctuating environmental conditions such as variable flow speed. The effect of species richness on water nutrient removal remains unclear.

Because of the importance of exploring the effect of biodiversity on a wide range of ecosystems and ecological services –as well as the relevance of water quality improvement as ecological service– TWs provide a proper model to approach this topic.

1.2.3 Previous studies on plant richness in TWs

Numerous studies have compared the performance of different plant species individually in TWs, and most have demonstrated that the choice of macrophyte species influences pollutant removal (Brisson and Chazarenc, 2009). Beyond the performance of specific species, the effect on TW performance of combining different species is still unclear. It has been hypothesized that combining different plant species in TWs maximizes treatment efficiency by means of species complementarities (Coleman et al., 2001; Fraser et al., 2004; Picard et al., 2005; Zhang et al., 2010; Zhu et al., 2010; Liang et al., 2011; Wang, 2014). Due to the limitations of TWs conditions as engineered ecosystems. Under the context of TWs, the effect of plant diversity is evaluated from a plant richness perspective, given the restrictions of these engineered ecosystems that limit the range of possible plant functional types that can be used.

Plant diversity in TWs may improve tolerance to changing conditions and stability in biogeochemical process (Eviner and Chapin, 2003). Combining plant species may contribute to optimal belowground biomass distribution and increase wetland productivity through more efficient use of available resources (Coleman et al., 2001; Cardinale et al., 2007; Zhang et al., 2010). Also, differential oxygen-root transport, root affinity for microorganism colonization, wider affinity for nutrients and organic compounds, as well as differences in plant growth associated to seasonality could together result in temporal and spatial compensation, improving TW removal efficiency (Allen et al., 2002; Sheoran, 2006; Zhang et al., 2010; Liang et al., 2011; Kumari and Tripathi, 2014).

Although there seems to be support for the hypothesis that plant diversity improves the performance of TWs, the majority of these systems have been planted in monocultures (Kadlec and Wallace, 2009) One common justification for this approach is that a TW system would require more effort to sustain several species than a single one. In addition, the contribution of increasing plant richness could make to TW efficiency has not been clearly demonstrated to date either. Previous studies of species used in combination in TWs have had ambiguous findings, and these studies are rarely replicated or they do not compare the performance of polycultures to the full range of individual species that compose it (Liang et al., 2011).

Previous studies have evaluated the effect of macrophyte species richness on biomass production, nitrogen and phosphorus removal (Table 1.1). Engelhardt and Ritchie (2001), Zhang et al. (2010), Zhu et al. (2010), Menon and Holland (2014) and Kumari and Tripathi (2014) found a positive relationship between species richness, total plant biomass and/or nutrient removal. On the other hand, results from Coleman et al. (2001), Fraser et al. (2004), Picard et al. (2005), Arrovaye (2010), Liang et al. (2011) and Dai et. al (2014) show a negative or neutral effect of plant richness. The relation between plant richness and TWs performance therefore remains unclear (Cardinale et al. 2011; Liang et al. 2011).

Table 1.1 Effect of species richness on total biomass production, nitrogen and phosphorus removal according to previous studies evaluating different macrophyte species and species richness levels (NA=No available data).

Authors	Richness levels	Number of species per treatment	Effect of species richness on:			Species Pool
			Biomass production	Nitrogen removal	Phosphorus removal	
Engelhardt and Ritchie (2001)	3	1,2,3	Positive	Positive	NA	*Potamogeton pectinatus, Potamogeton nodosus, Potamogeton crispus, Zannichellia palustris*
Coleman et al. (2001)	3	0,1,3	Negative	No effect	NA	*Typha* spp., *Scirpus* spp., *Juncus effusus*
Fraser et al. (2004)	3	0,1,4	No effect	No effect	No effect	*Carex lacustris, Scirpus validus, Phalaris arundinacea, Typha latifolia*
Picard et al. (2005)	3	0,1,4	No effect	No effect	No effect	*Carex lacustris, Scirpus validus, Phalaris arundinacea, Typha latifolia*
Arroyave (2010)	2	1, 4	No effect	No effect	No effect	*Musa velutina, Scirpus holoschoenus, Alocasia macrorrhiza, Typha latifolia.*
Zhang et al. (2010)	6	0,1,2,4,8,16	Positive	Positive	Negative	Functional groups: C3 grasses, C4 grasses, legume species, forbs.
Zhu et al. (2010)	6	0,1,2,4,8,16	Positive	Positive	NA	Functional groups: C3 grasses, C4 grasses, legume species, forbs.
Liang et al. (2011)	2	1, 5	No effect	No effect	No effect	*Canna indica, Cyperus flabelliformi, Phragmites australis, Pennistum purpureum, Hymenocallis littoralis*
Yanran et al. (2014)	2	1,2	No effect	No effect	No effect	*Ceratophyllum demersum, Myriophyllum verticillatum*
Kumari and Tripathi (2014)	2	1,2	NA	Positive	Positive	*Eichhornia crassipes, Salvinia natans*
Menon and Holland (2014)	2	1, 3	NA	NA	Positive	*Juncus effusus, Carex lurida, Dichanthelium acuminatum* var. a*cuminatum*

1.3 Invasive species in TWs

Ecological acceptability is considered one of the general requirements for plants to be suitable for use in TWs, meaning that they do not represent a threat to surrounding natural environments, as weeds do (Tanner, 1996). Nonetheless, most species commonly used in wastewater TWs are considered invasive outside of their native range. This is the case for macrophyte species like *Phragmites australis, Typha latifolia* and *Eichhornia crassipes*, to name a few.

This apparent contradiction is due in part to the fact that aquatic plants are the most likely to become natural invaders, and also because the traits highly desirable in plants used for wastewater treatment are also characteristic of invasive plant species (Sakai et al., 2001). Traits such as rapid growth, extensive root system, vegetative reproduction and high phenotypic plasticity predispose species to invasiveness and are at the same time advantageous for wastewater treatment. Total biomass production has been positively related to nutrient uptake in TWs (Tanner, 1996; Fraser et al., 2004). An extensive well-developed root system is desirable for filtration effect and for microbial attachment; belowground tissues provide a surface for biofilm formation responsible for most of the pollutant transformations mediated by microbial processes (Kadlec et al., 2000). Phenotypic plasticity allows macrophytes to survive and reproduce under TW conditions with high pollutant concentrations, hypertrophic conditions and low levels of dissolved oxygen.

While a large variety of species possess these desirable traits, in reality, only a limited number of species have been commonly used and proved a good performance and endurance in TWs conditions and are therefore commonly planted in this systems (Brisson and Chazarenc, 2009; Vymazal, 2011). Common reed (*Phragmites australis*) is the most widely used species in subsurface flow TWs (SSFTW) (Vymazal, 2011), and is also considered highly invasive outside its native range. This species, the European haplotype of common reed (referred to hereafter as “exotic *Phragmites”*), was introduced to the east coast of North America in the early 1800s and has been gradually expanding its range ever since (Saltonstall, 2002; Lelong et al., 2007). It is considered highly invasive, as it creates tall, dense monospecific stands, displacing native vegetation, reducing animal diversity and modifying environmental conditions (Chambers et al., 1999; Mal and Narine, 2004).

Due to its availability and well-established efficiency in water treatment systems, exotic *Phragmites* has been commonly planted in TWs throughout North America (Vymazal, 2011). However, with growing concerns about the threat of exotic plant invasions, government regulations increasingly require native species be used to replace invasive macrophytes in TWs. One alternative to exotic *Phragmites* in the context of TWs is the native subspecies of common reed – *P. australis* subsp. *americanus* (hereafter referred to as “native *Phragmites*”). This recently identified subspecies is much less abundant, and its decline in some parts of its range is often attributed to the spread of exotic *Phragmites* (Saltonstall, 2002).

Native *Phragmites* is broadly similar to its exotic conspecific and due to its large size, it represents a suitable candidate for TWs. Nevertheless, it has been show that native *Phragmites* produces less total biomass with shorter shoots and density than the exotic (Mozdzer et al., 2013). This suggests that native *Phragmites* may not be as efficient in pollutant removal. Therefore, its potential to replace current plantations in TWs remains to be verified.

1.4 Research questions

The use of TWs is expanding continuously, as new scientific and engineering approaches are developed to maximize their performance and range of implementation (Wu et al., 2014). From an ecological perspective, it is important to develop strategies that maximize the services provided by these ecosystems. Presently, the vast majority of TWs are planted with a single species. Moreover, invasive non-native species are also commonly used in TWs, posing a threat to local biodiversity. In this context, greater plant richness is increasingly encouraged in TW design. However, the impacts of combining different plant species on both pollutant removal and the performance of native species in TWs have not been clearly demonstrated. The aim of my research project was to evaluate the effect of plant richness in TWs (horizontal subsurface and free surface TWs) and to test the performance of a native species (native *Phragmites*) on pollutant removal in horizontal subsurface TWs. My research questions were: Are polycultures as good as, or better than, monocultures for pollutant removal, and could a native macrophyte be as efficient as exotic invasive species in TWs?

1.5 Objectives and general approach:

General objective

Compare the pollutant removal efficiency of polycultures versus monocultures and test an alternative non-invasive species in TWs.

- **Specific objectives**

Specific objective 1: Perform a comparative assessment of the pollutant removal efficiency of TWs planted in monoculture versus in polyculture (Chapters 2 and 3).

Hypothesis: Plant richness represents an advantage over monoculture for pollutant removal. Polycultures show greater removal efficiency than individual species.

In order to verify this hypothesis, I performed two experiments testing the removal efficiency of different macrophyte species individually and in combination with others. Each experiment will be presented in a different chapter of the thesis (Chapters 2 and 3). The experiment presented Chapter 2 compared two different emergent species, while in Chapter 3, I compared four free-floating macrophyte species. Aiming to maintain the constant plant richness all along the duration of the experiment, floating-plants species allowed the possibility to run an experiment in which the wastewater could freely circulate between species, having at the same time a physical barrier between them to restrict biomass growth (Chapter 3). In the case of emergent plants, it was not possible to restrict biomass growth without at the same time blocking water circulation; therefore the experiment was designed in mesocosms operated in series (Chapter 2).

The effect of combining two different emergent macrophyte species in TWs is presented in Chapter 2 and is the subject of a paper submitted to the journal *Water Research* (Title: "Does the combination of two plant species improve removal efficiency in treatment wetlands?"). This experiment was carried out in a controlled greenhouse environment at the Montreal Botanical Garden from July 2012 to July 2013. Overall removal efficiency was documented in horizontal subsurface TWs (HSSTWs) (two mesocosms connected in series) planted either with the same species or with two different species. The macrophyte species tested were *Phragmites australis* (common reed) and *Phalaris arundinacea* (reed canary grass). These species were selected because they are commonly used in TWs and efficiently remove pollutants, and also because they possess complementary seasonality and root morphology. *Phalaris'* root system is dense and superficial, while *Phragmites'* root zone is less dense but grows deeper with longer rhizomes. *Phalaris'* growth period begins early in spring, while *Phragmites* starts growing later but continues until autumn. Based on the differences between the two species, I evaluated whether the combination of macrophyte species with complementary traits influenced pollutant removal efficiency.

The effect of macrophyte richness on pollutant removal was also tested in free-floating species in TWs, and the results are presented in Chapter 2 (article in preparation: “Influence of free-floating plant species richness on water quality improvement” planned to be submitted to the journal *Ecological Applications*).

Four free-floating species were compared at three richness levels: 4 species vs. 2 species (all possible combinations) vs. 1 species (all species individually). Species selected, based on species commonly used for wastewater treatment and availability, were: *Eichhornia crassipes*, *Limnobium laevigatum, Pistia stratiotes and Salvinia molesta*. The experiment was carried out at mesocosm scale at the Montreal Botanical Garden (outdoors) from July to September 2011.

In collaboration with Dr. Kela Weber and Sonja Bissegger, from the Royal Military College of Canada, we evaluated if the presence or combination of different floating plant species influenced the catabolic diversity and activity of the microbial communities. Toward the end of the experiment, water samples were collected in the root zones of the mesocosms. Dr. Kela Weber and Sonja Bissegger performed a microbial analysis using the community level physiological profiling (CLPP). The results were published in the journal *Ecological Engineering* under the title: “Catabolic profiles of microbial communities in relation to plant identity and diversity in free-floating plant treatment mesocosms”. This paper is presented as an annex, in the end of the thesis.

Specific objective 2: Compare the removal efficiency of native non-invasive *P. australis* and exotic invasive *P. australis* (Chapter 3).

> Hypothesis: Exotic *Phragmites* will outperform native *Phragmites* in pollutant removal efficiency due to superior ecophysiological characteristics.

The comparison between native and exotic invasive *Phragmites* is presented in Chapter 4. The removal efficiency of both subspecies in horizontal subsurface TWs (HSSTWs) was tested at mesocosm scale at the Montreal Botanical Garden (outdoors). This experiment was performed for two consecutive growth seasons (summer 2010 and 2011). Both plant species and unplanted control mesocosms were tested under the same pollutant load (2010) and under high and low pollutant loads (2011).

This work was published in the journal *Ecological Engineering* under the title: "Pollutant removal efficiency of native versus exotic common reed (*Phragmites australis*) in North American treatment wetlands" (Rodriguez M. and Brisson J.).

2. Does the combination of two plant species improve removal efficiency in treatment wetlands?

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Author contributions

Mariana Rodriguez and Jacques Brisson designed the experiment; Mariana Rodriguez collected and analyzed samples and performed statistical analyses; Mariana Rodriguez wrote the first draft of the article and both authors contributed to revisions. All authors read and approved the final manuscript.

2.1 Abstract

We explored the effect of combining two plant species with complementary traits (*Phragmites australis australis* and *Phalaris arundinacea*), planted sequentially, on the performance of treatment wetlands (TWs). We performed a year-long experiment in mesocosm-scale TWs, aiming to answer the following question: will the combined removal efficiency of the two species simply equal the average efficiency of the separate monocultures, or will it outperform both monocultures, thus supporting the hypothesis that plant diversity improves pollutant removal? Overall, our results revealed that *Phragmites* was equal to or more efficient than *Phalaris* for pollutant removal, but we found no evidence that combining them would improve treatment efficiency over *Phragmites* monocultures, except for nitrate removal. Root and shoot density and morphology particular to each plant species influenced the redox conditions of the rhizosphere, affecting pollutant removal. *Phragmites* rhizosphere oxidizing conditions enhanced nitrification and ammonification processes, while possibly limiting denitrification rate. On the other hand, *Phalaris* reducing conditions seemed to restrict nitrification and enhance denitrification and sulfate reduction. Therefore, if low nitrate levels in the effluent as well as high overall pollutant removal are the objectives, combining both species may represent the best solution.

Keywords : *Phragmites australis, Phalaris arundinacea*, plant diversity, constructed wetlands, monoculture, polyculture.

2.2 Introduction

Treatment wetlands (TWs) are complex ecosystems designed for the purpose of wastewater treatment. Their removal efficiency is determined by the biological and physicochemical processes that take place between wastewater and the main components of TWs − substrate, plants and microbial communities. Plants play an important role in TW performance (Brix, 2002**;** Kadlec and Wallace, 2009). They uptake and store nutrients, and enhance microbial mediated processes by increasing the attachment surface area, supplying oxygen to the rhizosphere and providing organic carbon through root exudates (Coleman et al., 2001; Bais et al., 2006; Kadlec and Wallace, 2009; Vymazal, 2011). Plants may also improve hydraulic conductivity and help prevent clogging (Brix, 1997; Chazarenc et al., 2007). Given these influential roles, the identity and number of plant species in a TW may have an impact on its treatment efficiency. It is generally assumed that plant species should be selected for TWs based on fast growth rate; rapid establishment, usually by clonal propagation; large biomass with a well-developed belowground system and good tolerance of TW conditions. While a wide variety of species possess these traits, in reality, macrophyte species selection for TWs mostly follows established practices and commonly considers only a limited number of species, and TWs are predominantly planted with a single species (Brisson and Chazarenc, 2009; Vymazal, 2011).

It has been hypothesized that combining different plant species in TWs can improve treatment efficiency by means of functional complementarity (Coleman et al., 2001; Fraser et al., 2004; Picard et al., 2005; Zhang et al., 2010; Zhu et al., 2010; Liang et al., 2011). Plant diversity in TWs may increase tolerance to changing conditions as well as stability in biogeochemical process (Eviner and Chapin, 2003). Furthermore, differences in seasonal plant activity, root affinity for microorganism colonization and ability to take up nutrients and organic compounds could result in temporal and spatial compensation, which might improve TW removal efficiency (Coleman et al., 2001; Allen et al., 2002; Zhang et al., 2010; Liang et al., 2011). However, only a few experiments have evaluated the influence of combining plant species, often with contradictory conclusions. For example, findings by Coleman et al. (2001), Fraser et al. (2004) and Picard et al. (2005) did not support the hypothesis that polycultures would be more efficient than monocultures for nutrient removal, while Zhu et al. (2010) and Zhang et al. (2010) found a positive correlation between nitrogen removal and number of plant species in TWs.

We explored the effect of combining two plant species with complementary traits, planted sequentially, on the performance of TWs. Based on the assumption that greater morphological or temporal dissimilarities in plant species may have a better chance of producing complementary interactions, we selected *Phragmites australis* (common reed) and *Phalaris arundinacea* (reed canarygrass) for this mesocosm-scale TW. These two species are commonly used for wastewater treatment (Vymazal, 2011). Both species are from the Poaceae family, and form dense, nearly monospecific stands. Their ability to concentrate nitrogen, phosphorus and trace metal in tissues is also comparable (Vymazal et al., 2007; Vymazal and

Kröpfelová, 2008). However, prior studies comparing them in TWs revealed differences in seasonality and growth development that suggest they may show some complementarity in functions. While *Phalaris'* growing season begins early in spring, *Phragmites* starts growing later but continues until autumn (Vymazal and Kröpfelová, 2005). *Phalaris'* root system is very dense but superficial, while *Phragmites'* root zone is less dense but grows deeper with longer rhizomes (Gagnon et al., 2007). *Phragmites* has more aerenchymae than *Phalaris* (Bernard, 1999), and up to three times the cross-sectional gas space in its rhizomes (Coops et al., 1996). N_2 and N_20 gas emissions have been shown to differ between the two species; it has been reported that N gas liberation is not significant from *Phalaris* shoots, compared to *Phragmites* (Augustin et al., 2001) and that export of oxidized nitrogen measured in TWs planted with *Phalaris* was similar to unplanted wetlands and lower from the ones planted with *Phragmites* (Maltais-Landry et al., 2009). The greater capacity for internal gas transport in *Phragmites* may explain its higher tolerance to deep flooding when compared to *Phalaris* (Waring and Maricle, 2012). Root distribution and gas exchange capacity may in turn affect bacterial activity. In one microcosm experiment, bacterial density and aerobic respiration rate were significantly greater in the upper soil layer of a *Phalaris* monoculture compared to *Phragmites* (Gagnon et al., 2007).

We performed a year-long experiment in mesocosm-scale HSSF-TWs to evaluate whether planting *Phragmites* (X) and *Phalaris* (O) in four sequential combinations (i.e. XX, XO, OX, OO) affects wastewater treatment efficiency. We aimed to answer the following questions: given the removal efficiency of two macrophyte species in monoculture for a particular pollutant, how will the two species perform in combination? Will their combined removal efficiency simply equal the average efficiency of the separate monocultures, or will it outperform both monocultures, thus supporting the hypothesis that plant diversity improves pollutant removal?

2.3 Methods

2.3.1 Experimental set-up

The experiment was carried out in a controlled greenhouse at the Montreal Botanical Garden, Québec (Canada) (latitude: 45º33’43.00” N; longitude: 73º34’18.50” W). Sixteen mesocosm-scale experimental constructed wetlands were set up and evaluated over eight sampling periods between July 2012 and July 2013. The temperature of the greenhouse ranged from 35 ºC in summer to 5 ºC in winter, with an average of 15 ºC in autumn and spring. Each experimental system consisted of two coupled plastic basins (each measuring: L 70 cm W 51 cm H 36 cm) operating in series – hereafter referred to as “mesocosm 1” and “mesocosm 2”. Each mesocosm was filled with granitic river gravel (Ø = 10-15 mm), resulting in a free water volume of 24 L. The experimental systems were planted according to each of the following four plant treatments: monocultures (same species in mesocosms 1 and 2) of *Phragmites* (XX) and *Phalaris* (OO); the combination of the two plant species, with *Phragmites* in mesocosm 1 and *Phalaris* in mesocosm 2 (XO); and *Phalaris* in mesocosm 1 with *Phragmites* in mesocosm 2 (OX) (Figure 2.1). Each plant treatment was replicated four times following a randomized block design. The two species were planted in two distinct mesocosms connected in series, rather than mixing them in a single mesocosm, to prevent one macrophyte from invading the other's space, thus ensuring equal representation of both species throughout the entire experimental period. It also allowed us to monitor water quality at the frontier between the two species (passage from mesocosm 1 to mesocosm 2). *Phalaris* was planted from seeds and *Phragmites* from rhizomes collected from Îles-de-Boucherville National Park with permission from provincial authorities (Québec, Canada: 45˚35'13.19" N, 73˚29'03.33" W) in May 2009. For this experiment, we used the European *Phragmites* subspecies *(P. australis* subsp. *australis*) rather than the American native *Phragmites* subspecies *(P. australis* subsp. *americanus*).

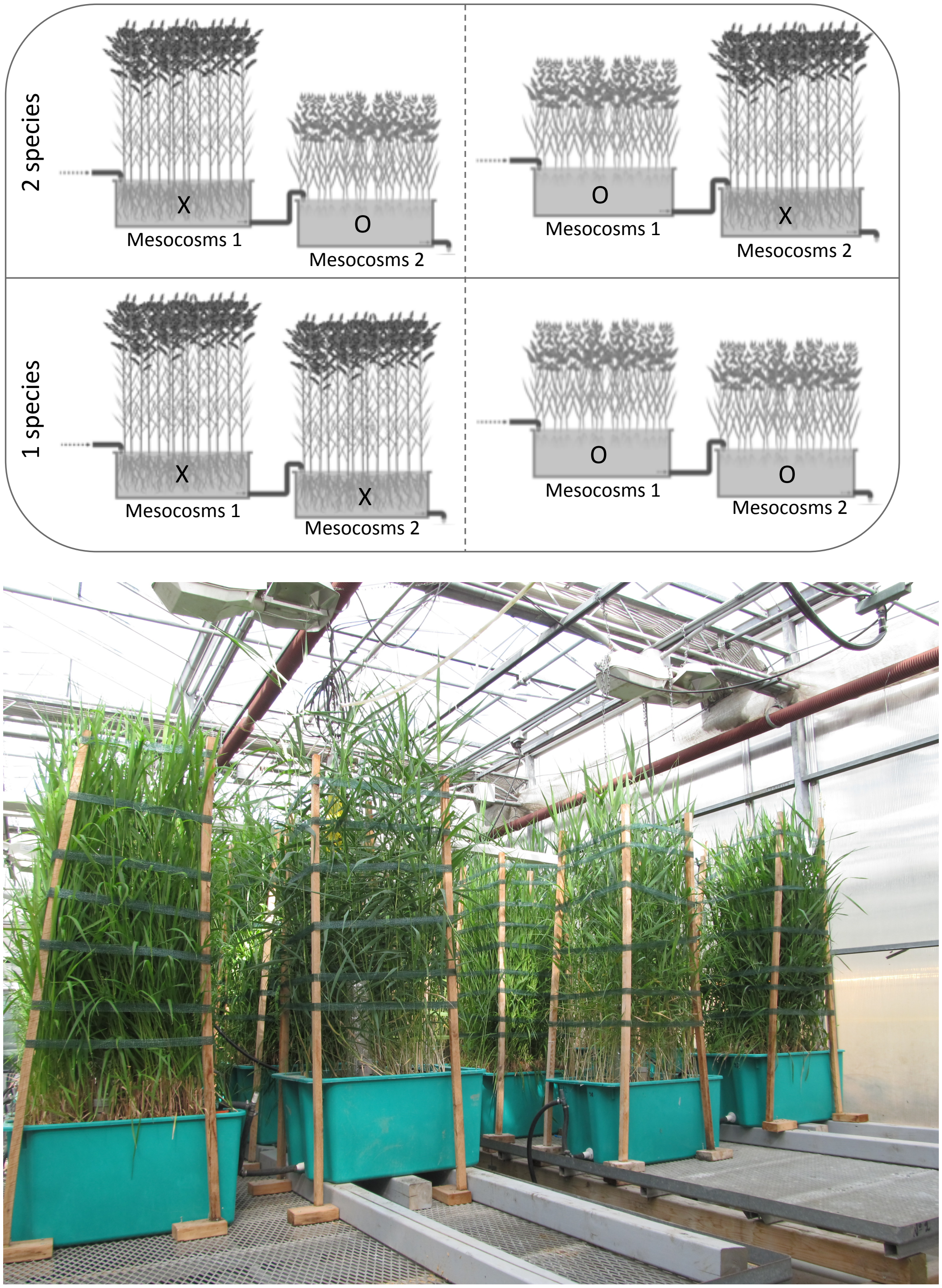

Figure 2.1. Schematic representation of the different treatments and the experimental systems (Top). Photo of the experimental set-up. Greenhouse, Montreal Botanical Garden. May 2013 (Bottom).

During the plant establishment period, from spring 2009 to spring 2012, water level was maintained constant at 2 cm below the surface, and plants were fed with a 20:20:20 nutrient solution (percentage, by weight, of nitrogen-phosphorus-potassium: N-P-K) containing microelements. During this period, occasional dieback in some of the *Phragmites* mesocosms necessitated partial replanting. Each fall, the aboveground portion of the plants was cut and removed. At the beginning of the experimental period, all units were densely colonized with mature macrophyte stands. Wooden sticks fixed to the corners of the mesocosms held strips of cloth that prevented plants from bending outside the surface area of the mesocosms (Figure 2.1). Following plant establishment, mesocosms were fed from April 2012 to the end of July 2013 with 15 Ld^{-1} with diluted fish farm sludge amended with nutrients (urea: 20.2 $mg \cdot L^{-1}$ and mono potassium phosphate: 10.1 $mg \cdot L^{-1}$) at a hydraulic loading rate of 42 $L \cdot m^{-2}d^{-1}$. Average influent concentration ($mg \cdot L^{-1}$) and (/) load ($g \cdot m^{-2} \cdot d^{-1}$) was TSS 363/11; COD 503/21; TP 37/1.5; TN 139/5.8.

2.3.2 Physico-chemical analyses

Wastewater treatment performance was monitored over eight sampling periods, three times in summer (July-Aug. 2012, July 2013), twice in autumn (Oct.-Nov. 2012) and spring (Apr.-May 2013) and once in winter (Feb. 2013). Inflow and outflow samples as well as intermediate wastewater (outflow from mesocosm 1, which also corresponded to the inflow of mesocosm 2) were collected and the following parameters were measured according to Standard Methods (APHA, 2005): TSS, TN, NH_4-N, NO_3-N, TP, S^{2-}. The outflow was collected in a container connected to the mesocosms, and its volume was measured daily during the sampling periods. Evapotranspiration was calculated as the difference between inflow and total outflow volume of the systems. Removal efficiencies were calculated based on a mass balance. The following parameters were measured *in situ* during each sampling period: pH and redox potential (Eh) (Oakton Ion Acorn series- pH/Ion C Meter Epoxy electrode), dissolved oxygen (Oakton DO 6 Acorn series), and electrical conductivity and temperature (YSI Environmental, EC 300). Measurements were taken in mesocosms 1 and 2 from interstitial water within pierced PVC tubes buried in the mesocosms.

2.3.3 Plant parameters

Root development was monitored visually following each sampling period by 360º belowground images captured with an *in situ* root imager (CI-600, CID Inc.) in transparent acrylic tubes buried in the mesocosms. Following the experimental period, at the end of July 2013, stem length and density were measured, and a portion of the plants (corresponding to ¼ of the mesocosm area) was cut and dried to estimate aboveground dry biomass. Also, a section of substrate was excavated from top to bottom (36 cm) at the center of each mesocosm using a 10 cm diameter drill. Roots and rhizomes were separated from the gravel, dried and weighed to estimate belowground dry biomass. Leaf and root samples were collected from each mesocosm and analyzed for nutrient content at the Horticulture Research Center at Laval University (Québec, Canada).

2.3.4 Data analysis

The comparison of removal performance and plant parameters among treatments was performed by one-way ANOVA analyses, after assessments of normality and homoscedasticity had been verified. Further differences between treatments were established with a post-hoc Tukey test at $p < 0.05$. Statistical analyses were performed using JMP software (JMP®, Version 6 for Mac. SAS Institute Inc.). One-way ANOVA tests were performed separately for mesocosms 1 and 2. In mesocosms 1, the performance of each species (*Phragmites* (X) and *Phalaris* (O); n=16) was compared independently, while in mesocosms 2, each combination of the two species, arranged in series, was compared (XX, OO, XO, OX; n=16). ANOVA analyses were performed for each sampling period (8), as well as for overall year-round mean removal efficiency.

2.4 Results

2.4.1 Plant parameters

We observed seasonal differences in species phenology. To summarize the parallels between *Phalaris/Phragmites* species-timing respectively: sprouting (early March/mid-March), inflorescence (May/August), maximum height reached (August/October) and senescence (October/ late November).

Phragmites and *Phalaris* showed different above and belowground biomass distributions. *Phragmites* had taller stems at a lower density compared to *Phalaris*, which had thin, shorter stems at very high density (Table 2.1). However, average dry biomass was similar for both species. Greater belowground biomass was measured for *Phragmites* than for *Phalaris,* although the difference was not significant (Table 2.1). Regardless of plant species, more belowground biomass was measured in mesocosms 2 than in 1. Both species in mesocosm 2 seemed to produce more root biomass following a mesocosm 1 planted with *Phragmites,* but the difference was not significant.

Belowground pictures showed remarkable differences between the species' root systems and their evolution over the course of each season (Figure 2.2). *Phalaris'* root systems were characterized by a very high density of fine roots, particularly in the top layer of the mesocosms. In contrast, *Phragmites* had large rhizomes and less dense fine roots, equally dispersed across depths. In spring 2012, following the addition of the wastewater inflow, mesocosms planted with *Phalaris* developed a black precipitate at the bottom layer, typical of sulfide production and sulfide iron (FeS) precipitation in anaerobic environments (Kadlec et al., 2000), which was not observed in mesocosms planted with *Phragmites* (Figure 2.2). The concentration of the black precipitate, as qualitatively indicated by the color intensity of the pictures, decreased during winter and increased during summer (Figure 2.2). For both *Phalaris* and *Phragmites* mesocosms, the pictures show a reddish color close to the tip of the roots, which is most likely due to oxidized forms of iron (Kadlec et al., 2000). The intensity of the reddish color varied according to both the season and the species; in general, it was more abundant during winter and in mesocosms planted with *Phragmites* (Figure 2.2).

Table 2.1. Plant parameters and *in situ* measurements. Different letters indicate significant differences between treatments. An ANOVA test was performed separately for each of the mesocosms 1 and 2, n=16. Plant parameters were measured in July 2013. *In situ* parameters were measured at each sampling period (8), except for the redox potential, which was measured beginning in autumn 2012 (6).

		Mesocosms 1		Mesocosms 2			
		Phragmites X	*Phalaris* O	*Phragmites* XX	*Phragmites* OX	*Phalaris* OO	*Phalaris* XO
Stem density	stems·m^{-2}	663^{p}	1762^{q}	825^{bc}	750^{c}	1675^{a}	1425^{ab}
Stem length	m	2.2^{p}	1.6^{q}	2.3^{a}	2.0^{a}	1.7^{b}	1.7^{b}
Dry biomass							
Aboveground	g·m^{-2}	2900	2707	2942^{a}	2707^{a}	2506^{ab}	1965^{b}
Belowground	g·m^{-2}	339	263	375	347	267	310
Nitrogen content							
Foliar	%	3.38	3.62	3.14	3.46	3.34	3.03
Root	%	1.47	2.05	0.85^{b}	0.98^{b}	2.04^{a}	1.31^{ab}
Phosphorus content							
Foliar	%	0.25^{p}	0.5^{q}	0.27^{a}	0.29^{a}	0.46^{b}	0.45^{b}
Root	%	0.45	0.49	0.35	0.36	0.62	0.55
***In situ* measurements**							
Evapotranspiration	mm·d^{-1}	3.3	2.4	4.2	3.3	3.0	2.6
Dissolved oxygen	mg·L^{-1}	1.56	1.51	2.19^{a}	1.94^{ab}	1.68^{b}	1.70^{ab}
Redox potential	mV	− 119.9	− 130.3	50.8^{a}	44.0^{a}	-7.5^{b}	-2.7^{b}
Electrical conductivity	mS·cm^{-1}	0.65	0.63	0.48	0.5	0.57	0.52
pH		6.40^{p}	6.75^{q}	6.12	6.16	6.3	6.17

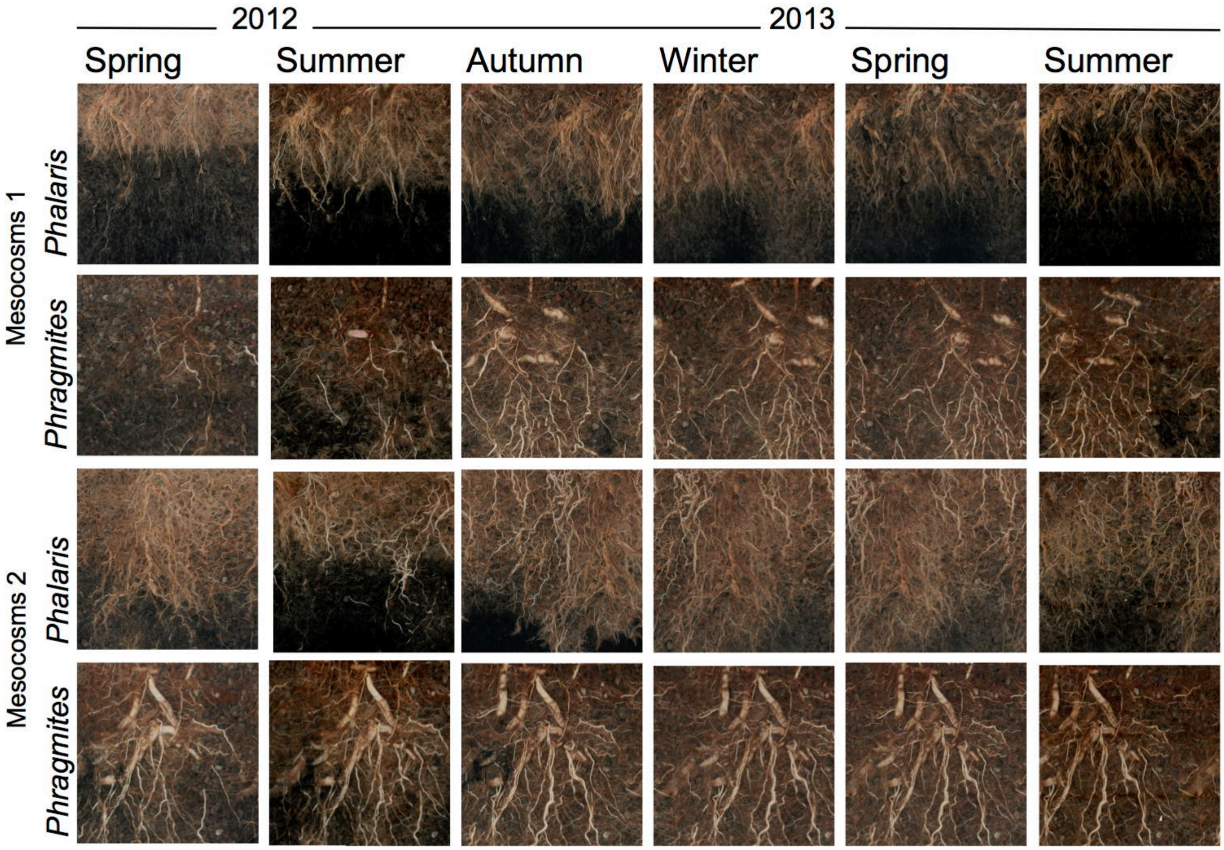

Figure 2.2. Example of root development of *Phalaris* and *Phragmites.* 360º belowground pictures (total mesocosms depth, 36 cm) captured each season with an *in situ* root scanner (CI-600, CID Inc.)

2.4.2 *In situ* measurements

Electrical conductivity ranged from 0.48 to 0.65 mS·cm^{-1} and pH from 6.1 to 6.7; in both cases higher values were measured in mesocosms 1, probably related to the higher organic and nutrient loads (Table 2.1). Evapotranspiration ranged from 2.4 to 4.2 mm·d^{-1} and was not significantly different between plant treatments, although *Phragmites* showed a slight tendency toward an evapotranspiration rate higher than *Phalaris'* (Table 2.1).

Dissolved oxygen (DO) and redox potential (Eh) increased from mesocosms 1 to 2 in all treatments, and plant species combination had an effect in mesocosms 2, but not in mesocosms 1 (Table 2.1). *Phragmites* monoculture (XX) showed significantly higher DO concentrations than *Phalaris* monoculture (OO) (Table 2.1).

Redox potential measurements (Eh) were not taken close to the vicinity of the roots: therefore they represent the anoxic/anaerobic conditions of a specific point of the mesocosm and they are not an indicator of all the processes occurring in this heterogeneous environments. We used these results in a descriptive way to discuss different patterns between treatments. Eh values in mesocosms 1 were negative and low, regardless of species, which is characteristic of strongly reducing conditions (Table 2.1). Eh values measured in mesocosms 2 differed between plant species and between seasons, showing a consistent pattern (Figure 2.3). This pattern did not seem to be affected by the combination of species (monocultures or polycultures), but only by the identity of the species present in mesocosm 2 (Figure 2.3). Therefore, the treatments: OO, XO showed a similar pattern, which was different from the treatments: XX, OX.

Both species in mesocosms 2 had the highest Eh potential during winter (Figure 2.3). *Phalaris* mesocosms (OO, XO) had lower Eh compared to *Phragmites* all year round. *Phragmites* mesocosms (XX, OX) had positive Eh values, while *Phalaris* showed overall positive Eh values in autumn and winter, but negative in spring and summer, with lowest values during summer (Figure 2.3).

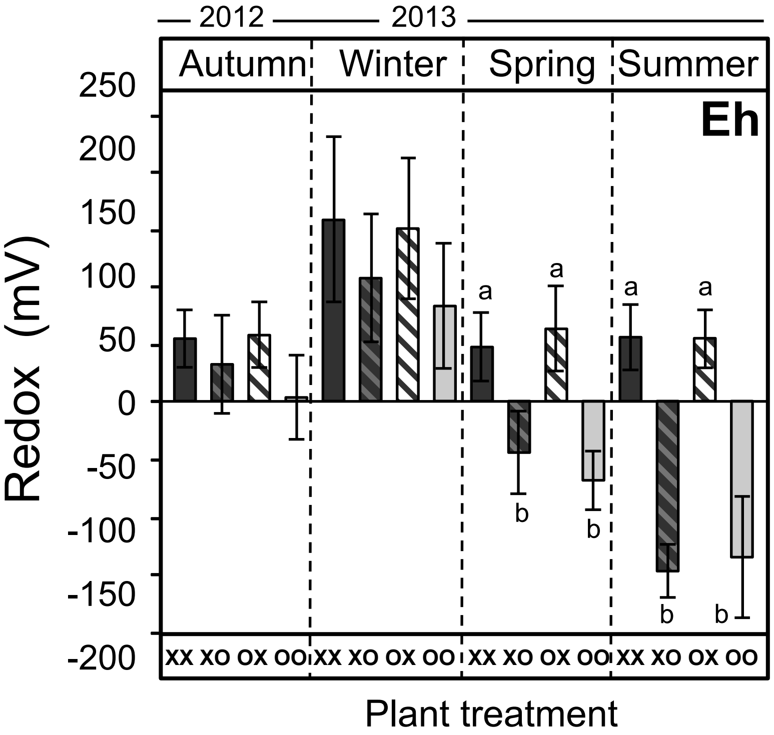

Figure 2.3. Seasonal variation of redox potential (mV) measured in mesocosms 2 for the different treatments (XX, XO, OO, OX; X for *Phragmites* and O for *Phalaris*). ANOVA test performed separately each season, n=16.

2.4.3 Pollutant removal

Both monocultures and polycultures of *Phragmites* and *Phalaris* showed high removal efficiency for all parameters tested (TSS, COD, TP, TN, NO_3, NH_4). TSS and COD removal was excellent for all treatments tested (85-95%) and there was no effect of plant species combination (Figure 2.4). Since seasonality did not seem to influence removal efficiency, results are shown only as the overall average (Figure 2.4) (Data per seasons is presented in Annex 2). Comparing mesocosms 1, plants species had a significant effect on TP and TN removal, with *Phragmites* outperforming *Phalaris* (Figure 2.4). TP removal showed the same pattern throughout the sampling year. Comparing mesocosms 2, the best combination for TP removal was *Phragmites* monoculture (XX). The combination *Phalaris-Phragmites* (OX) was more efficient at removal than *Phalaris* monoculture (OO), although the difference was not significant (Figure 2.4). *Phragmites* monoculture (XX) was also significantly more efficient than *Phalaris* monoculture (OO) for TN removal based on the year-round average; the combination of both species (XO, OX) was intermediate between the two monocultures (Figure 2.4). TN concentrations increased in winter compared to other seasons, and experimental units planted with *Phragmites* in mesocosms 1 (XX, XO) were significantly more efficient than those planted with *Phalaris* (OO, OX) (Figure 2.5). NH_4 outflow concentrations followed the same pattern as TN, *Phragmites* being significantly more efficient than *Phalaris* in mesocosms 1, as well as in monocultures (XX vs. OO) (Figure 2.4). The opposite pattern was observed for NO_3 outflow concentrations and it was the only parameter on which *Phalaris* outcompeted *Phragmites*. *Phalaris* showed lower outflow NO_3 concentrations than *Phragmites* in mesocosms 1, and a similar pattern in mesocosms 2 regardless of the species preceding it (Figure 2.4).

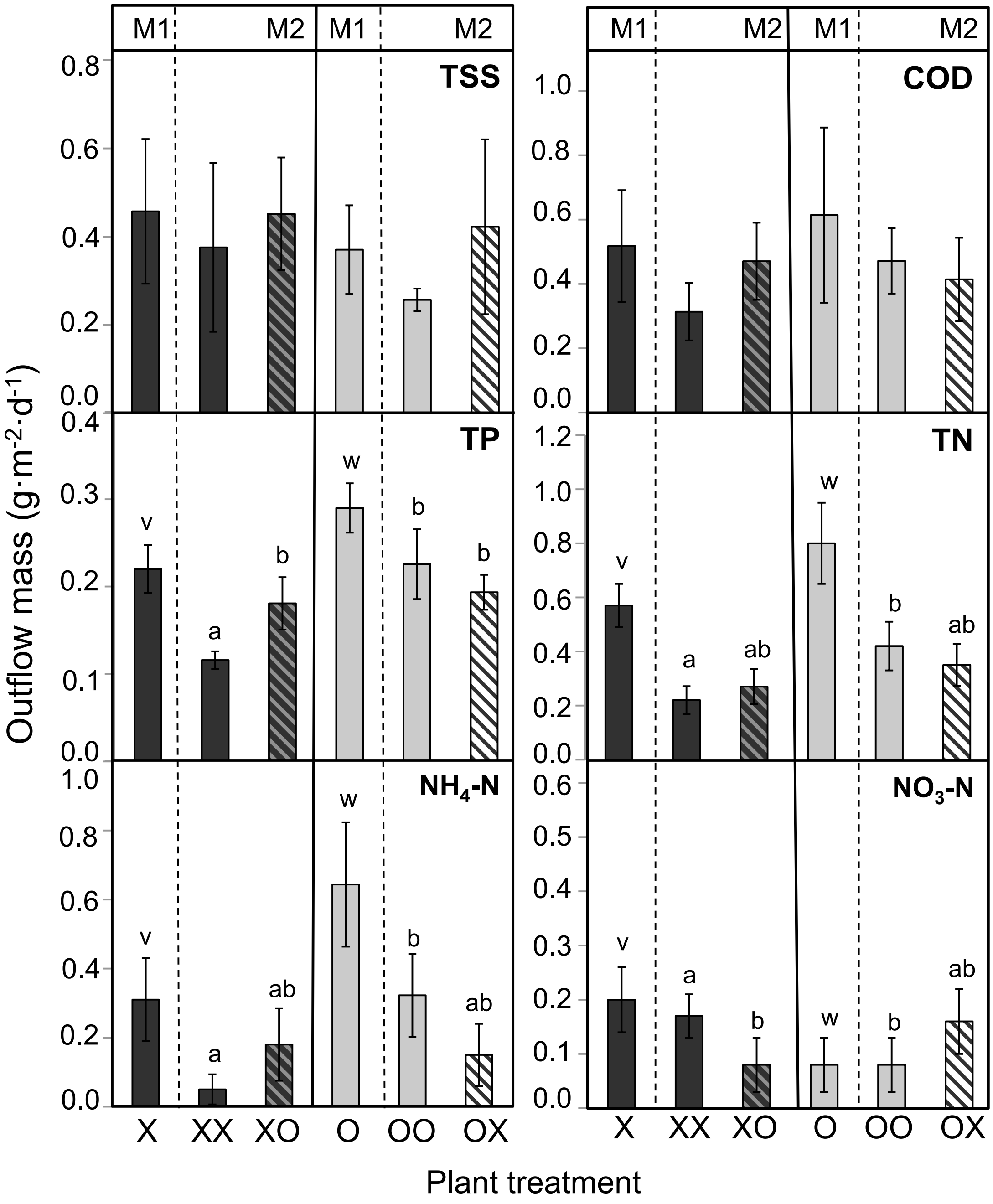

Figure 2.4. Year-round averages (8) of TSS, COD, TP, TN, NH_4-N, NO_3-N in $gm^{-2}·d^{-1}$, measured at the outflow of mesocosms 1 and 2 (XX, XO, OO, OX; X for *Phragmites* and O for *Phalaris*). Average inflow in $gm^{-2}·d^{-1}$ was 11, 21, 1.5, 5.8, 0.8, and 0.04 respectively. Different letters indicate significant differences between treatments. The ANOVA test was performed separately for mesocosms 1 and 2 (M1, M2), n=16.

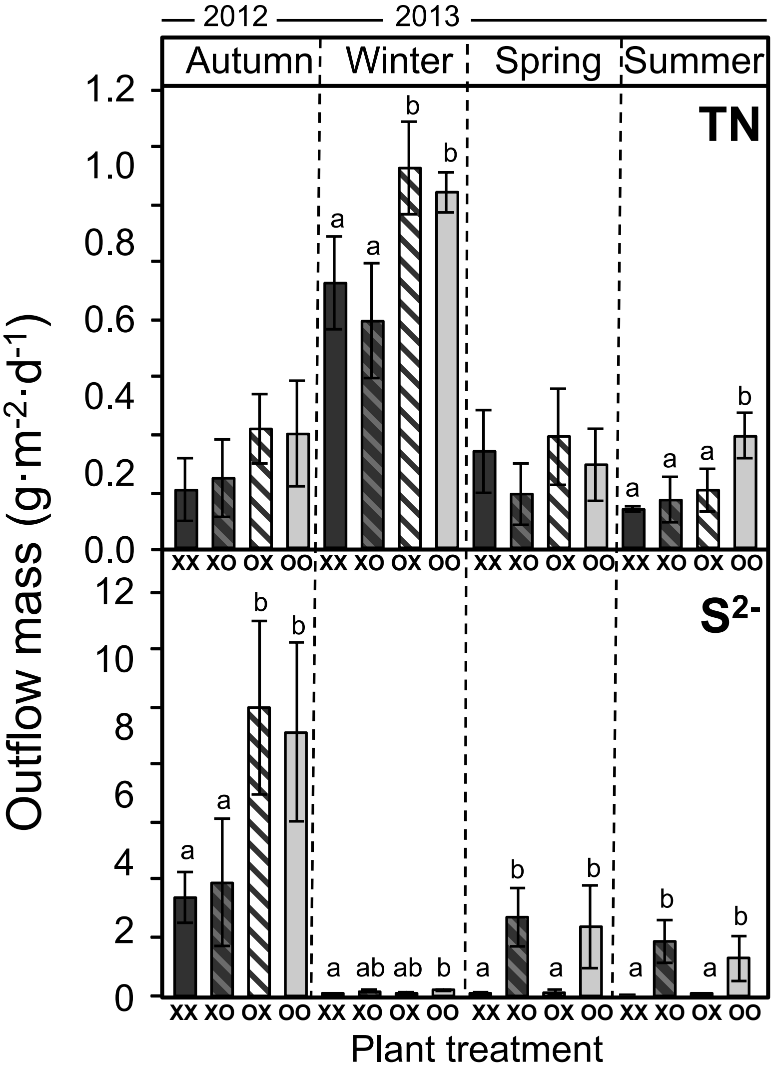

Figure 2.5. Seasonal variation of TN and S^{2-} ($gm^{-2}\cdot d^{-1}$) measured in mesocosms 2 for the different treatments (XX, XO, OO, OX; X for *Phragmites* and O for *Phalaris*). ANOVA test performed separately each season, n=16.

Sulfide (S^{2-}) outflow concentrations were higher for monoculture of *Phalaris* than for monoculture of *Phragmites* regardless of the season (Figure 2.5). In winter, S^{2-} concentrations measured at the outflow of mesocosms 2 was very low for all treatments, especially compared to levels in autumn (Figure 2.5). In spring and summer, differences were found between treatments (Figure 2.5) following a similar pattern than Eh values (Figure 2.3). S^{2-} concentrations varied depending on the plant species planted in mesocosms 2, regardless of the plant combination. Again, OO and XO showed the same pattern, and XX and OX had both values close to zero (Figure 2.5).

2.5 Discussion

Although we found differences between plant species rhizosphere that seemed to influence the chemical and physical wetland environment, in general, these differences were not translated into more efficient nutrient removal when the species were combined. Overall, our results revealed that *Phragmites* was equal to or more efficient than *Phalaris* for pollutant removal, and we found no evidence that combining them would improve treatment efficiency over *Phragmites* monocultures. However, if low nitrate levels in the effluent as well as high overall pollutant removal are the objectives, combining both species may represent the best solution.

Root and shoot density and morphology particular to each plant species influenced the oxidizing conditions of the rhizosphere. *Phragmites*' low density of large shoots and rhizomes evenly dispersed in the rhizosphere seemed to improve overall oxygen diffusion, in contrast to *Phalaris,* with its high density of fine shoots and superficial root system. Dissolved oxygen concentration was higher in *Phragmites* than in *Phalaris,* although the difference was significant only in mesocosms 2. This result suggests that the influence of plant species was only perceivable under low organic load conditions, while this effect was masked in mesocosms 1 with high organic load.

Redox values measured in our mesocosms showed in all cases anoxic/anaerobic conditions, but through the experiment *Phalaris* mesocosms showed lower redox values than *Phragmites*. During spring and summer *Phalaris* showed negative Eh values (below -100 mV) characteristic of strongly reducing environments. These results suggest an effect of plant species and season in the overall redox potential between treatments. Findings by Allen et al. (2002) and Stein et al. (2007) confirm that plant species selection in TWs is important due to its ability to influence rhizosphere redox conditions, which in turn have a strong impact on pollutant removal (Kadlec and Wallace, 2009; Wu et al., 2014). The influence of different plant species on redox potential in our experiment was more pronounced in mesocosms 2, which had a considerably lower organic load than mesocosms 1.

Due to the seasonal complementarity between *Phalaris* and *Phragmites*, it was expected that the longer period of plant activity would improve pollutant removal efficiency throughout a year-long operation. Complementary seasonal activity between *Phalaris* and *Phragmites* was indeed observed over the course of the experiment. As documented by Vymazal and Kröpfelová (2005), *Phalaris*' growth period began and finished earlier than *Phragmites'*. However, this complementary growth period did not translate into measurable greater removal efficiency. One possible explanation may be due to negligible plant uptake during the growing season compared to nutrient input load (Zhang et al., 2010). Also, bacterial activity in the rhizosphere, largely responsible for pollutant removal, may be only weakly related to seasonal plant activity, compared to other factors such as water content, temperature, oxygen level or redox potential (Stein et al., 2007; Kadlec and Wallace, 2009). It is thus not surprising that pollutant removal in TWs is also high in winter, when the plants are dormant (Stein et al., 2007).

Differences according to plant species were also observed over the course of the seasons in the belowground biomass and accompanying root pictures of each mesocosm. *Phalaris*' rhizosphere presented a layer of black metal precipitate, characteristic of strongly reducing environments where the predominant electron acceptor is sulfate. Sulfate reducing bacteria (SRB) degrade organic matter to CO_2 and H_2S, which can precipitate as metal FeS or MnS or exit the system (Kadlec et al., 2000). *Phalaris* mesocosms also showed high S^{2-} outflow concentrations, confirming SRB activity. The black precipitate layer was more pronounced and larger during summer (2012), faded in autumn and particularly in winter, and regained prominence in summer (2013). During winter, root oxygen demand decreases due to plant dormancy, and water temperature decreases, increasing oxygen solubility (Kadlec and Wallace, 2009). As a result, more oxygen is available for aerobic microbial processes (Stein et al., 2007). The higher oxygen level we found during winter may have been due to the effects of water temperature and plant dormancy or a consequence of plant harvest at the end of autumn. After harvest, the dead plant shoots enhance convective oxygen transport from the surface inducing oxidative conditions in the rhizosphere (Kadlec and Wallace, 2009). Stein et al. (2007) showed that aerobic microbial activity increases during winter, as more favorable electron acceptors are available, while SRB activity is inhibited. The low S^{2-} outflow concentrations and high redox values (ranging from +50 to 150 mV) we measured in *Phalaris* mesocosms during winter compared to the other seasons also suggest a decrease in SRB activity during this period. Lower temperature in winter also seemed to affect denitrification rates, since TN outflow concentrations were higher in winter compared to the rest of the year.

Phragmites' rhizosphere, on the other hand, exhibited overall oxidizing conditions (Eh values ranging from +50 to 230 mV) over the course of the experiment. Only a slight amount of black precipitation was evident at the very bottom of *Phragmites* mesocosms during the summer. Radial oxygen loss was particularly evident in summer root pictures, in the reddish color of Fe and Mn oxides precipitate over the roots closer to the surface (Vymazal et al., 2007). Thus, our study supports the results reported by Allen et al. (2002) and Edwards et al. (2006) showing that *Phragmites* transfers more oxygen to the rhizosphere than *Phalaris*.

This difference between species might explain their dissimilar nutrient removal efficiency. For instance, *Phragmites* advantage over *Phalaris* for TP removal may be explained by the oxic conditions in *Phragmites* units that enhance phosphorus co-precipitation with iron (Vymazal and Kröpfelová, 2008). Plant nutrient uptake does not explain this result, as both species produced similar amounts of biomass and *Phalaris* showed higher P foliar content. In terms of N, *Phragmites*' NO_3 outflow concentrations, higher than those of *Phalaris,* may also be influenced by the oxidizing conditions of its rhizosphere, which enhance nitrification and ammonification processes while possibly limiting the denitrification rate. Thus the *Phragmites*-*Phalaris* combination improved NO_3 removal compared to *Phragmites* monocultures, but the latter was more efficient for overall TN removal (Table 2.2). *Phalaris'* disadvantage for TN removal, compared to *Phragmites*, may be due to extreme reducing conditions that restrict nitrification. Consequently, perhaps increasing the *Phragmites*-*Phalaris* ratio when the two species are combined, to, for instance, ¾ *Phragmites* ¼ *Phalaris*, could further improve TN removal. Under this hypothetical scenario, the combination of the two species would be evident in TN removal, possibly retaining *Phragmites* monocultures' ability to remove TP.

Table 2.2. Advantage (+) or not (-) of combining two species, versus a monoculture (XX, XO, OO, OX; X for *Phragmites* and O for *Phalaris)* * Indicates a significant effect.

Advantage of 2 species versus 1 species?

Pollutant	**XO versus XX**	**OX versus OO**
TSS	–	–
COD	–	+
TP	–*	+
TN	–	–
NH_4-N	–	+*
NO_3-N	+*	–

Plant root systems provide mechanical support for microbial community attachment, secrete root exudates and transfer oxygen from aerial tissues into the rhizosphere (Weber and Legge, 2013). A microbial community level physiological profiling (CLPP) approach was used during the course of our study to evaluate whether the complimentary nature of *Phalaris* and *Phragmites* may lead to greater microbial functional diversity (Button et al., 2014). Plant species identity did have an influence on microbial activity. *Phalaris* planted in mesocosms 2 enhanced microbial activity and diversity compared to *Phragmites*, while in mesocosms 1 no differences were found between plant species. However, our results did not show that the combination of *Phalaris* and *Phragmites* planted sequentially increased microbial diversity and activity (Button et al., 2014).

2.6 Conclusion

Plant species seemed to influence TW efficiency, *Phragmites* outperforming *Phalaris*. However, in terms of removal efficiency, our results do not appear to support the hypothesis that plant diversity improves the performance of TWs; the best monoculture was as efficient as or more efficient than the combination of two plant species for pollutant removal. Conversely, the treatment efficiency of *Phalaris* was improved when it was combined with *Phragmites*, except with regard to NO_3 removal. Indeed, minimizing NO_3 outflow was the only situation in which the combination *Phragmites-Phalaris* outperformed *Phragmites* monocultures. In our experiment, the

TW was equally divided among the two species, but a different space allocation, such as a larger portion to *Phragmites*, may maximize NO_3 removal without compromising removal efficiency for the other pollutants. It has been documented that *Phragmites* populations tend to exclude *Phalaris* when growing together (Vymazal and Kröpfelova, 2005; Fu et al., 2011), therefore it is possible that additional investment in maintenance or design should be considered when combining the two species.

Even if combining plant species does not necessarily represent an advantage in terms of pollutant removal compared to a monoculture of the best performing species, it may provide other benefits, like improving system resilience, resistance to environmental stress or diseases, higher habitat quality and aesthetic quality (EPA, 2000; Kadlec and Wallace, 2009).

3. Influence of free-floating plant species richness on water quality improvement

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Author contributions

Mariana Rodriguez proposed the study. Mariana Rodriguez and Jacques Brisson designed the experiment; Mariana Rodriguez built the set-up, collected and analyzed samples and performed statistical analyses; Mariana Rodriguez wrote the first draft of the article and both authors contributed to revisions. All authors read and approved the final manuscript.

3.1 Abstract

We aimed to evaluate the effect of plant species richness on water quality improvement by comparing the removal efficiency of nutrients, suspended solids and organic matter in polycultures (bi-and quadricultures) to that of monocultures of each of the species composing them (*Eichhornia crassipes, Pistia stratiotes*, *Limnobium laevigatum* and *Salvinia molesta*). Floating plant treatment wetland mesocosms were established with the four different plant species either in monoculture or in polyculture. Mesocosms were fed twice weekly with 20 L of wastewater using diluted fish farm sludge effluent. We found that specific combinations performed better than others independent of the total species richness. In fact, biomass was a better predictor of removal efficiency than plant species richness. The performance of the most effective species in monoculture (*Eichhornia)* was similar to its efficiency in combination with other species, possibly due to the influence of different plant combinations on species competitive performance and biomass yield.

Our results show that increasing species richness also increases the chances of choosing the most efficient species; thus the importance of proper experimental design and results interpretation to avoid confusing the effects of plant richness with the indirect sampling effect.

3.2 Introduction

The link between species richness and ecosystem functioning is currently a central question in ecology (Bouchard et al., 2007; Balvanera et al., 2014). It has been hypothesized that a greater number of species on a given ecosystem may lead to a more efficient resource use due to functional complementarity between them (Cardinale et al., 2011). Positive relations have been found between the number of plant species and ecosystem processes such as primary production, biomass productivity, carbon storage and erosion control (Balvanera et al., 2006; Loreau, 2010). Evidence supporting this relationship comes mainly from grasslands ecosystems (Cardinale et al., 2011); a long-term grassland experiment showed that plant species richness increased plant productivity and soil carbon storage (Fornara and Tilman, 2008). Although numerous studies have recently addressed this research question in regard to different ecosystems (Cardinale et al., 2011), additional evidence is needed regarding the effect of diversity on available ecosystem services, rather than on the processes influencing ecosystem functioning.

Wetlands provide various key ecosystem services such as biodiversity support, flood and storm mitigation and water quality improvement (Mitsch et al., 2012). Considerably fewer studies have evaluated the relationship between plant diversity and the provision of ecosystem services in aquatic ecosystems, including wetlands. Findings by Engelhardt and Ritchie (2001) showed that wetland plant biomass production and phosphorus retention were positively correlated with plant species richness. Cardinale et al. (2011) simulating fresh-water streams, found that higher algae species richness increased water nitrate uptake under fluctuating environmental conditions such as flow speed variation. Though the effect of species richness on water nutrient removal remains to be determined.

Treatment wetlands (TWs), whose creation was inspired by natural wetlands, are specially designed for wastewater treatment. Further the effect of individual species on pollutants removal in TW (Brisson and Chazarenc, 2009); it has been hypothesized that TWs planted in polyculture would be more efficient than monocultures for wastewater treatment based on the principle of functional complementarity. However, the advantages plant diversity could or would provide in TWs have not been clearly demonstrated, in part because previous studies have led to ambiguous results. For instance, results from Zhang et al., (2010), Zhu et al., (2010) and Kumari and Tripathi (2014) showed a positive relationship between species richness, plant biomass and nutrient retention, while findings by Fraser et al. (2004), Picard et al. (2005) and Liang et al. (2011) did not support the hypothesis that a polyculture would be more efficient for nutrient removal than monocultures. Besides, these studies were rarely replicated and did not compare the performance of a polyculture to each of the species that compose it (Liang et al., 2011).

In this study, we aimed to evaluate the effect of plant species richness on water quality improvement by comparing the removal efficiency of nutrients (N and P), suspended solids and organic matter in polycultures (bi-and quadricultures) to that of monocultures of each of the species composing them. We evaluated the performance of each specific plant combination to test the hypothesis that increasing plant richness would improve pollutant removal beyond what is achieved by the most efficient monoculture.

3.3 Methods

3.3.1 Mesocosm system set-up

For this study, 39 mesocosm systems of 65 L each (individual measurements: L53 cm / W37 cm / D37 cm) were set up following a randomized block design in early June 2011, on an experimental site at the Montreal Botanical Garden in Quebec, Canada (Figure 3.1). Each mesocosm was divided into 4 quadrants via plastic mesh (Figure 3.1), and was either planted in monoculture (any of the 4 species x 3 replicates per species), biculture (2 different plant species; 6 possible pairings x 3 replicates) or quadriculture (all four plant species x 9 replicates) (Table 3.1) with the following free-floating macrophyte species (initial average fresh weight): *Eichhornia crassipes* (E) (350 g)*, Pistia stratiotes* (P) (350 g)*, Limnobium laevigatum* (L) (200 g) and *Salvinia molesta* (S) (80 g).

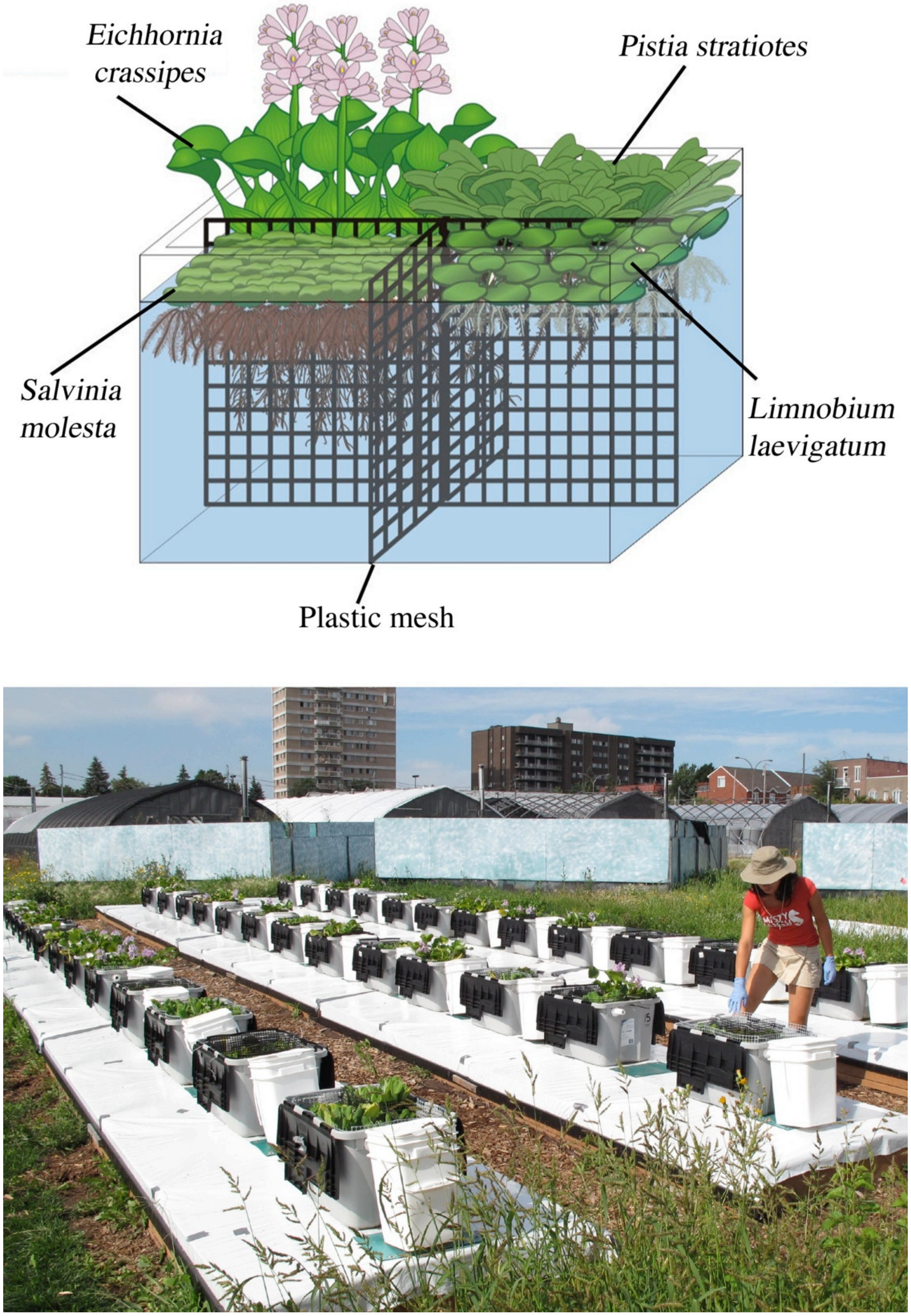


Figure 3.1. Schematic view of a quadriculture wetland mesocosm system (Drawing by V.Gagnon) and view of the experimental site at the Montreal Botanical Garden (June 2011).

Table 3.1. Experimental design description. E: *Eichhornia crassipes*; P: *Pistia statiotes*; L: *Limnobium laevigatum*; S: *Salvinia molesta*

Species richness	Plant species combinations	Replicates
1	E	3
1	L	3
1	P	3
1	S	3
2	EL	3
2	EP	3
2	ES	3
2	LP	3
2	LS	3
2	PS	3
4	ELPS	9

3.3.2 Physico-chemical analyses and biomass evaluation

After a period of two weeks for establishment of the plants, the mesocosms were fed twice weekly with 20 L of wastewater. The wastewater mixture was prepared immediately prior to feeding using diluted fish farm sludge (1:75), urea (46%) and monopotassium phosphate (23%) fertilizer to attain an average inflow concentration of ($mg \cdot L^{-1}$): TSS (188); COD (358); TP (7); TN (29); NH_4-N (4); NO_3-N (2). Prior to each feeding, the total volume of each mesocosm was set at 40 L by pumping out a volume of approximately 20 L with a manual water pump (outflow volume varied with evapotranspiration). Then, 20 L of wastewater was added manually to each wetland system. Outflow samples were collected from each mesocosm from July to September on a weekly basis (10 weeks) for physico-chemical analyses. TSS, COD, TN, NH_4-N, NO_3-N and TP were analyzed according to Standard Methods (Standard Methods, 2005). Evapotranspiration was calculated as the difference between the inflow and the total outflow volume, adding the contribution of rainfall. A plastic bucket was connected to each mesocosm to collect possible outflow in case of heavy rainfall, and its volume was measured daily. Removal efficiency was calculated based on a mass balance.

In addition, pH (Oakton Ion Acorn series- pH), dissolved oxygen (Oakton DO 6 Acorn series), electrical conductivity (YSI Environmental, EC 300) and water temperature were monitored *in situ* every week from July to September 2011.

At the end of the experiment (September 26), all plants in each mesocosm were collected. For each quadrant of each mesocosm, macrophyte root length was measured and the plant material was dried until constant weight. Root and shoot samples were analyzed for nutrient (N, P%) content at the Horticulture Research Center of Laval University (Québec, Canada).

Biomass yield was calculated as the difference between plant biomass at the time of plantation in June and the biomass of the plants collected in September. The comparison was done per species per quadrant per treatment with the purpose of evaluating the effect of plant combinations on biomass yield and possibly on pollutant removal.

3.3.3 Data analysis

One-way ANOVA analyses were performed to evaluate the effect of different plant species and plant species combinations on nutrients, TSS and COD removal rate ($g \cdot m^{-2} \cdot d^{-1}$). Assessments of normality and homoscedasticity were tested by verifying a random pattern of the residuals by predicted values plots. Further differences between plant treatments were established with a post-hoc Tukey test at $p < 0.05$. Statistical analyses were performed using JMP software (JMP®, Version 6 for Mac. SAS Institute Inc.). ANOVA analyses were performed for each sampling period (10), as well as for the overall year-round mean removal rate.

Throughout the experiment, NH_4-N and NO_3-N were evaluated; however, only TN results are presented because organic-N (estimated as TKN-N minus NH_4-N) was the main form of N (85%) measured in both inflow and outflow. NH_4-N and NO_3-N outflow concentrations were low (0.5 and 0.8 $mg \cdot L^{-1}$ respectively) and between treatments followed a pattern similar to that of TN.

3.4 Results

3.4.1 Plant parameters

Total biomass per mesocosms varied between plant species and between different plant combinations. *Eichhornia* monoculture had significantly greater dry biomass (over 300g) than all the other monocultures, more than *Pistia* and almost three times more than *Limnobium* and *Salvinia* monocultures (less than 100g) (Figure 3.2). Among the polycultures, the bicultures including *Eichhornia* had significantly more biomass than the others; the biculture EP had the most biomass, followed by ES and EL. The bicultures LP, PS and the four species together (EPLS) had similar total biomass. The combination of the smaller species, LS, had the lowest total biomass (Figure 3.2).

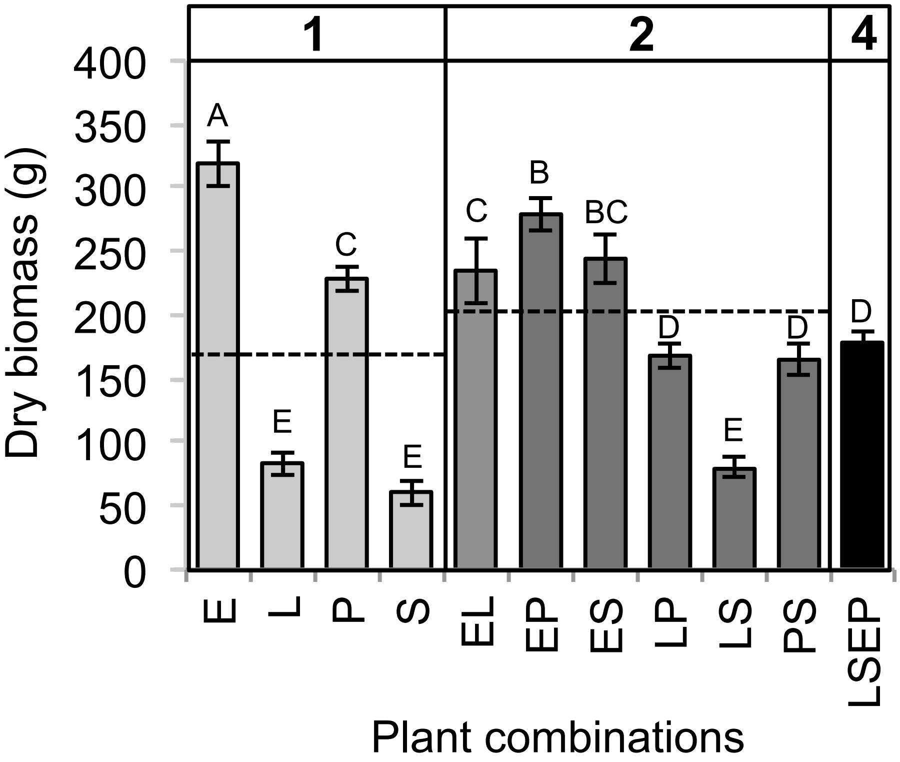


Figure 3.2. Total dry biomass per mesocosms harvested at the end of the experiment. E: *Eichhornia crassipes*; P: *Pistia statiotes*; L: *Limnobium laevigatum*; S: *Salvinia molesta.* Dashed lines represent the average dry biomass calculated per species richness level. Different letters above bars indicate significant differences between plant combinations, n=3 (Tukey test $p<0.05$).

Polycultures´ total biomass was close to the average biomass of their monoculture counterparts individually, suggesting that interspecific interactions did not influence species biomass growth rate. However, biomass per quadrant varied within species among different plant combinations, showing an effect of intra and interspecific interactions on species biomass yield (Figure 3.3). *Eichhornia* produced significantly more biomass per quadrant in polycultures than in monoculture. For example, in the biculture ES, quadrants planted with *Eichhornia* produced more biomass (average 93 g) than each of quadrants of *Eichhornia* monocultures (average 53 g). On the other hand, *Salvinia* produced almost half the biomass per quadrant growing with *Eichhornia* (ES) than in monoculture (10 g compared to 20 g). *Salvinia* was the only species that gained more biomass per quadrant in monoculture. *Eichhornia* and *Pistia's* net production in monoculture was similar, while both species together (EP) *Eichhornia* outcompeted *Pistia*. These species had more biomass in combination with *Salvinia*, and then with all species (ELPS) and in biculture with *Limnobium* (Figure 3.3). *Limnobium*'s highest yield per quadrant was in combination with *Salvinia* (LS), and it had more biomass in monoculture than in competition with *Eichhornia* and *Pistia* (Figure 3.3).

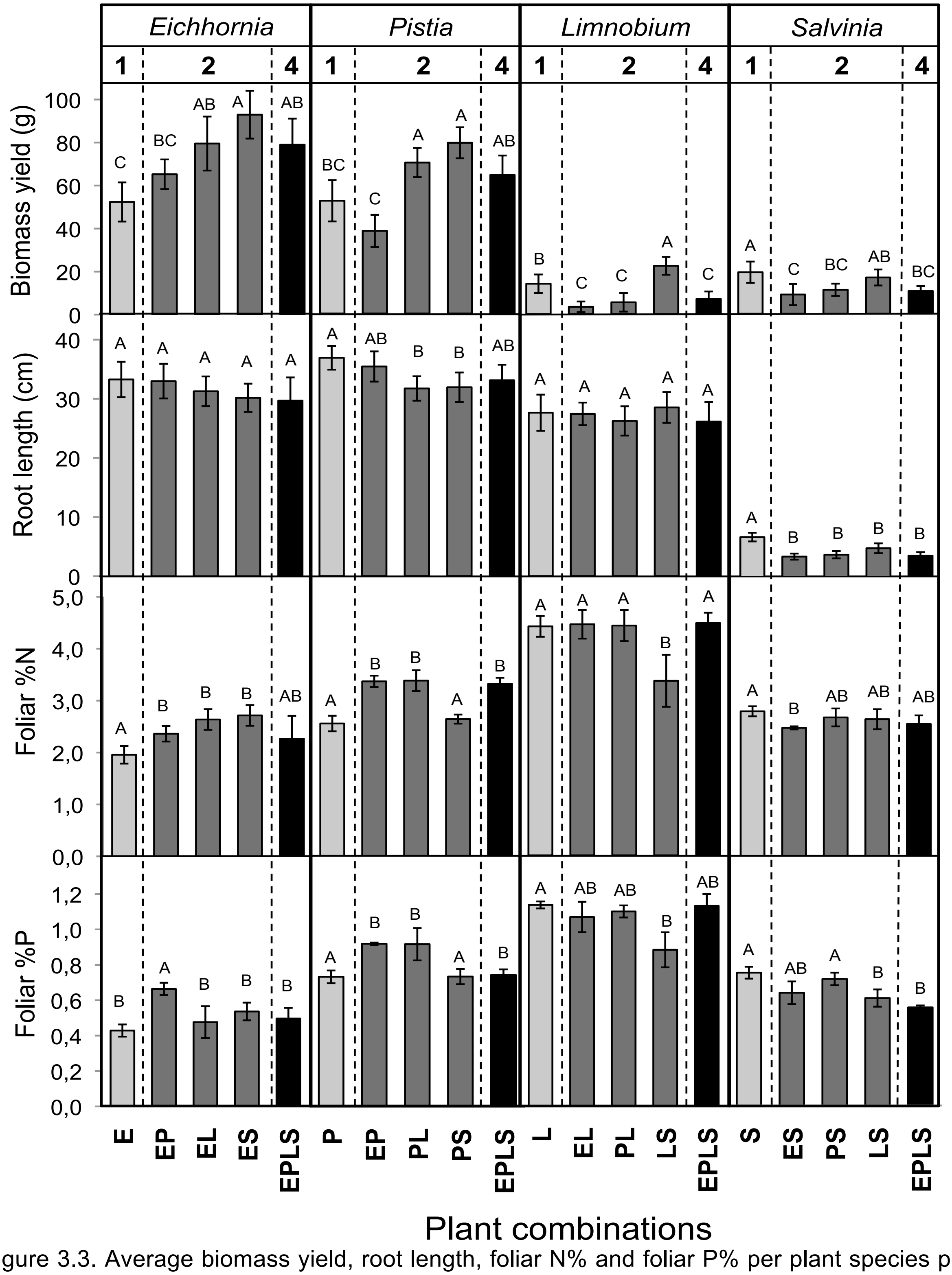


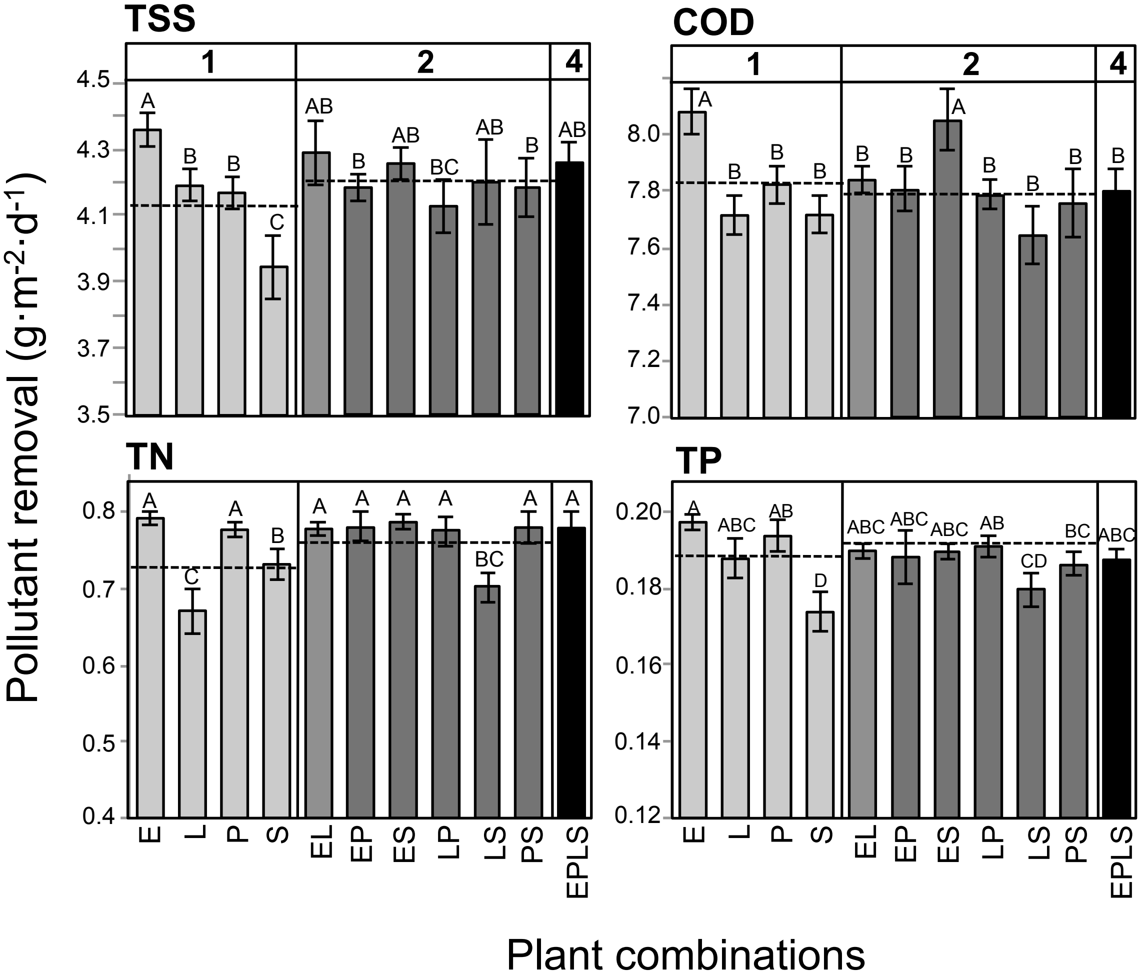
Figure 3.3. Average biomass yield, root length, foliar N% and foliar P% per plant species per quadrant for each plant combinations. E: *Eichhornia crassipes*; P: *Pistia statiotes*; L: *Limnobium laevigatum*; S: *Salvinia molesta.* Different letters above bars indicate significant differences between plant combinations, n=39 (Tukey test p<0.05).

Different plant combinations also influenced macrophyte root length (Figure 3.3), though the effect was only significant for *Salvinia.* Average root length of *Eichhornia*, *Pistia, Limnobium* and *Salvinia* was 32, 38, 24 and 5 cm respectively. *Salvinia* had significantly longer roots in monoculture than in polycultures, particularly those including *Pistia* or *Eichhornia*. *Limnobium* had slightly longer roots in combination with *Salvinia*, but shorter ones in the presence of *Pistia*. *Eichhornia* and *Pistia* had longer roots in monoculture and combined together (EP) than in the other polycultures (Figure 3.3).

Foliar N % and P % measured at the end of the experiment varied between species (Figure 3.3). *Limnobium* had the highest foliar nutrient values followed by *Pistia, Salvinia* and *Eichhornia.* Even though *Eichhornia* had the lowest foliar nutrient percentage, it still had the highest nutrient content calculated by total biomass. Foliar nutrient percentage varied within species between different plant combinations. *Eichhornia* and *Pistia* in monoculture had lower N and P % than in polycultures. *Salvinia* had higher P% in monoculture than in polycultures, but no difference in N%. *Limnobium* had less foliar N and P in combination with *Salvinia* (LS) than in the other plant treatments (Figure 3.3).

3.4.2 Pollutant removal

Average pollutant removal for the sampling season (June-Sept. 2011) was compared both among cultures differing in plant species richness (1, 2 and 4) and between each specific combination (11) (Figure 3.4). Plant species richness had a significant effect on TN removal: a one-way ANOVA followed by a Tukey test (F ratio 9.5; p=0.01*) showed that polycultures outperformed monocultures on TN removal and there was no difference between polycultures of 2 and 4 species. Species richness did not have a significant effect on the removal of the other parameters measured (COD, TP, NH_4-N and NO_3-N). TSS removal in plant polycultures appeared slightly higher compared to monocultures, but this difference was not significant (Figure 3.4).



Ranking of plant combinations on removal efficiency	
TSS	E ≥ EL = ES = ELPS = LS ≥ PS = EP = L = P = LP ≥ S
COD	E = ES > EL = P = EP = ELPS = LP = PS = L = S = LS
TN	E = P = EL = EP = ES = LP = PS = ELPS > S ≥ LS ≥ L
TP	E ≥ P = LP ≥ EL = ES = L = EP = ELPS ≥ PS ≥ LS ≥ S

Figure 3.4. TSS, COD, TN and TP average removal (g·m^{-2}·d^{-1}) from July to September 2011. Different letters indicate significant differences between plant combinations (Tukey test $p<0.05$). Dashed lines represent the average removal calculated per species richness level. Ranking table of plant combinations according to pollutant removal efficiency. E: *Eichhornia crassipes*; P: *Pistia statiotes*; L: *Limnobium laevigatum*; S: *Salvinia molesta.*

Given that the performance of each species in monoculture was distinctly different, comparing every plant combination individually versus based on the average per category of species richness led to a different perspective on the results. *Eichhornia* monoculture was the most efficient treatment for TSS, COD, TP and TN. *Limnobium* monoculture was the least efficient for TN, and *Salvinia* monoculture for TSS, COD and TP. Polycultures were either as efficient as the best performing monoculture or as the average of their monoculture counterparts; for all the parameters considered, the least efficient plant treatment was one of the monocultures, never a polyculture.

The presence of *Eichhornia* significantly improved the removal efficiency of TSS, COD and TN (Table 3.2). On the other hand, the presence or absence of the other species tested on the different plant combinations, did not significantly affect removal efficiency of the parameters evaluated (Table 3.2). TSS removal was greater for all combinations containing *Eichhornia* (with the exception of EP) and, surprisingly, also for the biculture LS; this was the only case in which a polyculture outperformed its counterparts, both *Limnobium* and *Salvinia*, as monocultures (Figure 3.4). *Salvinia* monoculture showed the lowest performance, comparable to the combination LP. *Eichhornia* monoculture and the biculture ES were the best combinations for COD removal; all other plant combinations showed a similar efficiency. In this case, the best performing species (*Eichhornia*) in combination with a less efficient species (ES) was as efficient as *Eichhornia* alone, rather than as the average of both species in monoculture. TN removal was greater in all species combinations containing the species *Eichhornia* and/or *Pistia*, and significant differences were found in the absence of either of these species. *Salvinia* and *Limnobium* monocultures were also significantly different; *Limnobium* was the least efficient species and the efficiency of the biculture LS on this measure fell between that of both monocultures. *Eichhornia* monoculture was the best treatment for TP removal, although its efficiency was comparable to all other plant combinations: it was significantly different only from *Salvinia* monoculture, PS and LS. *Salvinia* monoculture was the least performing; the presence of this species reduced P removal in all plant combinations except in ES (Figure 3.4 and Table 3.2).

Table 3.2. Differences among means when a particular species is present (Pr) versus when it is absent (Abs) in different plant combinations. Stars (*) in the Pr columns indicate statistically significant effect. E: *Eichhornia crassipes*; P: *Pistia statiotes*; L: *Limnobium laevigatum*; S: *Salvinia molesta.*

	E		P		L		S	
	Pr	Abs	Pr	Abs	Pr	Abs	Pr	Abs
TSS	4.27*	4.13	4.18	4.21	4.21	4.18	4.17	4.22
COD	7.92*	7.74	7.80	7.84	7.76	7.87	7.79	7.84
TN	0.79*	0.74	0.78	0.74	0.74	0.78	0.76	0.76
TP	0.19	0.19	0.19	0.19	0.19	0.19	0.18	0.19

A positive and significant correlation was found between biomass and TSS, COD, TN and TP removal (Figure 3.5). The correlation was stronger for COD and TP than for TN and TSS. Yet some specific species combinations performed as well as others, regardless of biomass differences, and some combinations with similar biomass performed differently. For instance, TSS removal efficiency of the biculture

LS was as good as that of other plant combinations with higher biomass such as EL, ES and EPLS (Figure 3.5). However, for TN removal, *Salvinia* and LS outperformed *Limnobium* monocultures, all with similar biomass.

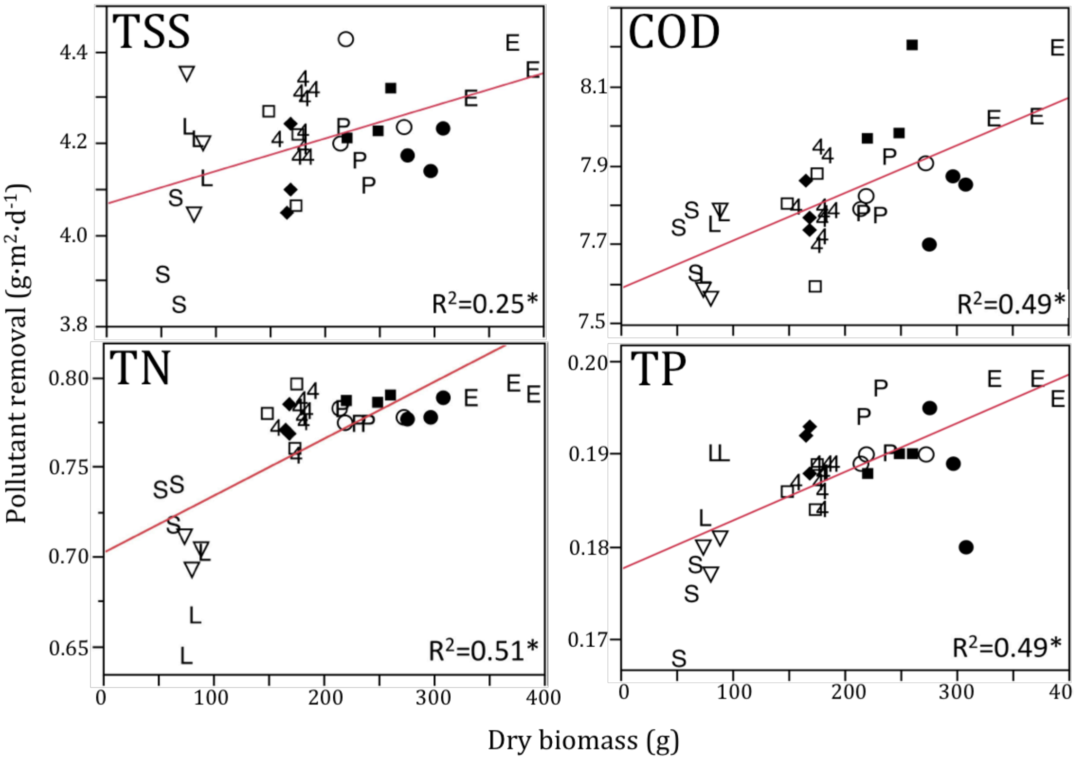


Figure 3.5. Correlation between dry biomass and pollutant removal ($g \cdot m^{-2} \cdot d^{-1}$). Monocultures: E: *Eichhornia crassipes*; P: *Pistia statiotes*; L: *Limnobium laevigatum*; S: *Salvinia molesta*. Bicultures: •:EP ;▽:LS; ■:ES; ◆:LP; O=EL;□=PS. Quadricultures: 4 (EPLS). *Indicate significant correlation ($p<0.05$).

3.5 Discussion

Our results show that plant species and the different combinations between them influence pollutant removal efficiency. The results varied among different species combinations and between the parameters evaluated; yet we found no evidence that higher plant species richness improves treatment performance over the most efficient monoculture. In only one case (*Limnobium-Salvinia*), a polyculture outperformed its monoculture counterparts. More often the performance of a polyculture was as good as the monoculture of the most efficient species that composed it, or the average performance of each species in monoculture. In both cases, the efficiency of the least effective species in monoculture improved when this species was combined with other more effective species. The effect of plant species richness must be analyzed carefully since there can be a wide variation of performance within the same richness level due to differences between individual species. In fact, biomass was a better predictor of removal efficiency than plant species richness.

Toward the end of the experiment, water samples were collected in the root zones of our mesocosms to evaluate if the presence or combination of different plant species influenced the catabolic diversity and activity of the microbial communities using the community level physiological profiling (CLPP) (Bissegger et al., 2014 and annex 1). It was found that the carbon source utilization patterns of the microbial communities from the monoculture mesocosms were distinctly different from each other, and that the polyculture communities were different from the respective monoculture counterparts. However, and similar to our findings regarding pollutant removal efficiency, their results show that increasing the number of plant species did not, on average, promote the development of microbial communities with a more active and diverse catabolic capability, but rather some specific plant selection and species interactions were important (Bissegger et al., 2014).

Our results confirm the well-known capacities of *Eichhornia* for water treatment (Gopal and Goel, 1993; Bulc et al., 2006; Malik, 2007; Munavalli and Saler, 2009; Nguyen et al., 2014): in monoculture it outperformed the other plant species and it was the only species whose presence in the different plant combinations had a positive significant effect. *Eichhornia's* greater efficiency may be due to its high biomass growth, as suggested by the positive significant correlation between biomass and pollutant removal. Also, its dense root systems may enhance suspended solids filtration and COD removal, offering suitable support media for microbial growth and organic matter degradation. Findings by Zimmels et al. (2009) and Kumari and Tripathi (2014) support our results showing greater efficiency of *Eichhornia* over *Salvinia* and *Pistia* in terms of water treatment.

Eichhornia also showed strong competitive abilities over the other species, influencing their biomass yield. Biomass yield of *Pistia, Limnobium* and *Salvinia* was reduced in the presence of *Eichhornia* (Figure 3.3). This species has a remarkable morphological plasticity and a fast growth rate; it also captures more light and available nutrients than other aquatic species. These characteristics all contribute to its strong competitive abilities (Reddy et al., 1989).

Our results show that specific combinations performed better than others independent of the total species richness (Figure 3.2). This might be explained by the variable influence of different plant combinations on the competitive performance and biomass yield of different species. Srivastava et al. (2012) showed that specific plant growth rates differ between monocultures and polycultures, particularly under stress (arsenic contamination), which can be capitalized upon to maximize pollutant removal. Biomass yield affects the potential rate of plant nutrient uptake, an important mechanism for nutrient removal by free-floating macrophytes, therefore influencing treatment efficiency (Vymazal, 2007). In our study, *Eichhornia* in biculture with *Salvinia* (the least performing species) was as efficient as *Eichhornia* monoculture for all the parameters measured. This could be associated with the competitive performance of *Eichhornia* in combination with *Salvinia; Eichhornia* produced almost twice as much biomass per quadrant grown with *Salvinia* as it did when grown in monoculture, and had significantly higher foliar N and P %. Our results suggest that *Eichhornia*'s dominant effect over *Salvinia* led to high performance of the combination ES in regard to pollutant removal, comparable to that of an *Eichhornia* monoculture. Our results differ from those of Kumari and Tripathi (2014), who found that ES outperformed both *Eichhornia* and *Salvinia* monocultures. However, it should be taken into consideration that we worked with different species, *Salvinia molesta* instead of *Salvinia natans,* and that their experimental design lacked treatment replication. *Eichhornia* removed the same quantity of pollutants as a monoculture as it did in combination with ES, while competition between *Eichhornia* and *Pistia* had a negative effect on TSS and COD removal. In the combination EP, both species showed increased biomass yield when grown together and were less efficient for TSS and COD removal compared to *Eichhornia* monoculture. Interspecific competition seemed to be dominated by *Eichhornia*, as *Pistia* produced less biomass in EP than in *Pistia* monoculture, as has been documented previously (Gopal and Goel, 1993; Bownes et al., 2010).

The only situation in which two species together outperformed their monoculture counterparts was the biculture LS. As a monoculture, *Limnobium* outperformed Salvinia for TSS and TN, while *Salvinia* outperformed *Limnobium* on TP removal. However, both species together (LS) equaled the efficiency of the best monoculture for TN and TP, and outperformed both monocultures on TSS removal. A difference in total biomass could not explain these results, since biomass was comparable between plant combinations; it is possible that differential species biomass yield played a role. *Limnobium* combined with *Salvinia* had the highest biomass, even doubling the former's as a monoculture and increasing its root length. *Salvinia* in combination with *Limnobium* had the same biomass than as a monoculture and more prolific than in combination with other species. Macrophyte morphological plasticity may contribute to complementarity between species leading to improved performance of species mixtures.

Some recent work reported higher removal efficiencies of TWs using plant mixtures; Dai et al. (2014) found higher nutrient uptake by combining two macrophyte species, *C. demersum* and *M. verticillatu*, although the differences among treatments were slight. In addition to nutrient removal, Menon and Holland (2014) showed that plant mixtures increased phosphorus retention rates in TWs, improving their efficiency. However, our results do not demonstrate an advantage of plant combinations, the only exception being the mixture LS. As suggested by Engelhardt and Ritchie (2001), strong competition among aquatic plant species may inhibit possible synergetic or complementary effects between species and therefore combination of various species in aquatic ecosystems is not necessarily translated into enhanced ecosystem functioning.

Our data also highlights the importance of proper analysis to avoid confusing the effects of diversity or plant richness with the indirect sampling effect. Engelhardt and Ritchie (2001) explain the sampling effect as increasing the probability that the best performing species would be present in a given plant combination with higher species richness. Analyzing our results by species richness rather than each specific plant combination in the case of TSS (Figure 3.3) could illustrate the sampling effect. The average performance of TSS removal increases with higher species richness. However, this is likely simply due to the significant differences among monocultures' specific performance; increasing species richness also increases the chances of choosing the most efficient species.

3.6 Conclusions

Macrophyte biomass, species richness and species identity influenced pollutant removal efficiency to some extent. The results varied among different species combinations and between the parameters evaluated; often the performance of a polyculture was as good as the monoculture of the most efficient species that composed it, or the average performance of each species in monoculture. In both cases, the efficiency of the least effective species in monoculture improved when this species was combined with other more effective species. At the same time, the efficiency of the best species in monoculture was similar to its efficiency in combination with other species. In only one case (*Limnobium-Salvinia*), a polyculture outperformed its monoculture counterparts. Overall, these results do not support the initial hypothesis that plant species richness improves the performance of TWs. Conversely, results suggest that increasing plant diversity does not compromise the efficiency of TWs, while providing the benefits of ecosystem services associated with higher plant richness in terms of habitat value for fauna, higher aesthetic values and social acceptability, among others. Furthermore, under circumstances in which the performance of available macrophyte species is unknown or cannot be determined, using a combination of species offers the best chances of achieving the highest possible level of removal efficiency.

4. Pollutant removal efficiency of native versus exotic common reed (*Phragmites australis*) in North American treatment wetlands

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Author contributions

Mariana Rodriguez and Jacques Brisson designed the experiment; Mariana Rodriguez collected and analyzed samples and performed statistical analyses; Mariana Rodriguez wrote the first draft of the article and both authors contributed to revisions. All authors read and approved the final manuscript.

4.1 Abstract

Growing concerns about the threat of invasive macrophyte species increasingly require the use of substitute native species in constructed wetlands for wastewater treatment. We conducted a mesocosm experiment at two loading rates to compare the removal efficiency of treatment wetlands planted with *Phragmites australis* from a lineage native to North America (*P. australis* subsp*. americanus*) versus the widely used but highly invasive European *P. australis*. Based on the plant's relative ecophysiological and morphological characteristics as reported in field studies, including biomass production and photosynthesis and stomatal conductance rates, we hypothesized that the native *Phragmites* would show lower pollutant removal efficiency than the exotic European subspecies. *P. australis* subsp. *americanus* was found to show potential for treatment wetlands, and there was no evidence that its removal efficiency would be inferior to that of European *P. australis*. In fact, contrary to our expectations, our results suggest that the native *Phragmites* may be the preferred subspecies, due to its slightly more effective removal of phosphorus. Further pilot or full scale experiments are needed to quantitatively assess the efficiency of treatment wetlands planted with this subspecies, as well as its resistance to diseases, before its use in treatment wetlands could be definitively recommended. Also, while plant characteristics measured under field conditions may reflect a species' potential removal efficiency, growing conditions in treatment wetlands may differently affect morphological, ecological and physiological plant attributes and, consequently, pollutant removal efficiency.

4.2 Introduction

Selection of plant species for treatment wetlands (TWs) has always been an important design issue. Tolerance to saturated substrate and high wastewater loads, as well as biological attributes including fast growth, large biomass and a well-developed root system, have been identified as desirable traits in plants used for wastewater treatment (Tanner, 1996; Kadlec and Wallace, 2009; Vymazal, 2011; Leto et al., 2013). However, although differences in removal efficiency between plant species have been widely documented, the possible correlations with specific plant attributes have been the subject of only limited detailed analysis (Brisson and Chazarenc, 2009). One exception is Tanner's (1996) pioneering comparison of pollutant removal efficiency among eight macrophyte species, showing a linear correlation between mean removal of total nitrogen and total plant biomass. A clearer understanding of the role of plant traits in treatment efficiency would allow more effective plant selection for TWs.

In addition to biological attributes, the ecological acceptability of plants selected for TWs is also important to consider, since exotic invasive species represent a threat to local biodiversity. The biological attributes considered highly desirable for plant species used in water treatment often characterize invasive plants as well. Common reed (*Phragmites australis*) is the most widely used species in subsurface flow constructed wetlands (SSFCW) (Vymazal, 2011), and it is also considered highly invasive outside its native range.

Introduced to the east coast of North America in the early 1800s, the European haplotype of common reed (referred to hereafter as “exotic *Phragmites”*) has been gradually expanding its range ever since (Saltonstall, 2002; Lelong et al. 2007). It tolerates a broad range of hydrologic conditions and disturbance regimes (Brisson et al., 2010; Taddeo and de Blois, 2012). The tall, dense monospecific stands it forms displace native vegetation, reduce animal diversity and modify environmental conditions (Chambers et al., 1999; Mal and Narine, 2004). In addition to negatively impacting biodiversity, the plant may obstruct roadside and agricultural ditches, block shoreline views and pose a fire hazard because of its dry shoots. Since its deep and dense rhizome and root systems make it highly resistant to most control methods, managing established stands is costly (Hazelton et al., 2014).

Despite these drawbacks, the exotic *Phragmites* has been commonly planted in TWs of North America due to its availability and well-established efficiency in water treatment systems (Brisson and Vincent, 2009; Vymazal, 2011). However, there are growing concerns that these TWs may be sources of propagules and invasion loci for nearby natural wetlands. Some governmental authorities are envisioning or applying regulations to prohibit *P. australis* from TWs (Wallace and Knight, 2006; MDDEP Québec, 2009), in favor of alternative native plant species such as broadleaf cattail (*Typha latifolia*) or bulrushes (*Schoenoplectus* sp.).

One alternative plant that does not appear to have been tested in TWs is the native subspecies of common reed – *P. australis* subsp. *americanus* (hereafter referred to as “native *Phragmites*”). This recently identified subspecies is much less abundant, and its decline in some parts of its range is often attributed to the spread of exotic *Phragmites* (Saltonstall, 2002). Due to its large size, it represents an excellent candidate for TWs, but while it is broadly similar to its exotic conspecific, some of its attributes suggest that it may not be as efficient in pollutant removal. In a review of the major ecophysiological differences between native and exotic *Phragmites,* Mozdzer et al. (2013) showed that the native subspecies produces less total biomass, has shorter shoots and shoot density than the exotic. Exotic *Phragmites* also has superior ecophysiological attributes, including a 50 % higher rate of photosynthesis, and up to 100 % higher rates of stomatal conductance (Mozdzer and Zieman, 2010). When grown under increased nutrient levels, both subspecies produce more biomass, but the exotic *Phragmites* outperforms the native with a significantly greater aboveground: root biomass ratio, and is more responsive to an increase in nutrients, suggesting more efficient nutrient uptake (League et al., 2007; Saltonstall and Stevenson, 2007; Price et al., 2014).

In the context of a search for alternatives to invasive exotic *Phragmites* for use in North American TWs, the aim of this study was to compare the removal efficiency of native and exotic subspecies in a mesocosm experiment and evaluate the potential of native *Phragmites* in TWs. Based on the relative plant ecophysiological and morphological characteristics reported in field studies, we hypothesized that the native *Phragmites* would represent an acceptable species for TWs, although we expected it would exhibit lower pollutant removal efficiency than the exotic subspecies.

4.3 Methods

4.3.1 Experimental set-up

The experiment was conducted on the site of the Montreal Botanical Garden, Québec, Canada (45º33’43.00” N; 73º34’18.50” W). In 2008, twenty-five mesocosms (L 107 cm, W 55 cm, H 35 cm) were filled with granite river gravel (Ø = 10-15 mm) and planted with rhizomes. Ten of the mesocosms were planted with native *Phragmites* (N), ten with exotic *Phragmites* (E), and five were left unplanted (U) (Figure 4.1). Permission was obtained to collect native *Phragmites* rhizomes from a large colony near Lac Saint-François (Québec, Canada: 45˚02’29.92”N, 74˚27’47.35”W), and exotic *Phragmites* rhizomes from Îles-de-Boucherville National Park (Québec, Canada: 45˚35’13.19” N, 73˚29’03.33” W). Plants were allowed to establish from spring 2008 to spring 2010. During this period, water level was maintained constant and plants were fed with a 20:20:20 nutrient solution with microelements. During the winters of 2008-09 and 2009-10, the mesocosms were protected with insulating textile covered with mulch. Plants had reached full maturity at the beginning of the experimental phase, in spring 2010.

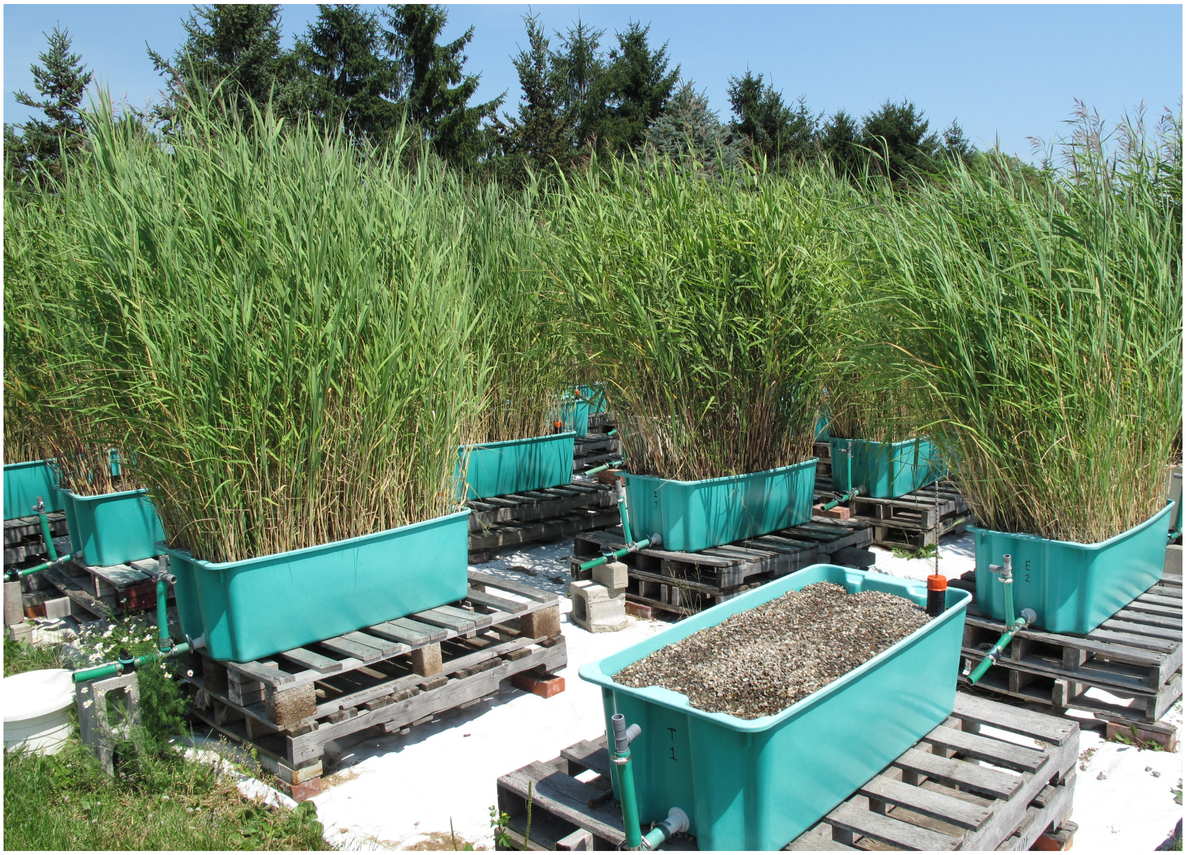


Figure 4.1. Experimental site in the Montreal Botanical Garden (Quebéc, Canada). (Photo: Jacques Brisson, July 2010)

Beginning in May 2010, mesocosms were fed with reconstituted wastewater composed of diluted fish farm sludge, urea (46 %) and mono potassium phosphate (23 %) (Table 4.1). Mesocosms were drained before batch feeding, and filled with 30L of fresh wastewater twice per week. For 10 consecutive weeks starting in July, total outflow water was sampled weekly for water quality assessment. The outflow was collected in a bucket connected to the mesocosms by an evacuation tube and its volume was measured daily. Evapotranspiration was calculated as the difference between inflow and the total outflow volume, plus rainfall.

Table 4.1. Mean inflow load and removal efficiency (± SE) for the sampling period between July and September 2010.

	TSS	COD	TP	TN	NH_4	NO_3
Inflow ($gm^{-2}d^{-1}$)	7.1 (2.2)	12.6 (3.9)	0.6 (0.08)	1.7 (0.2)	0.15 (0.05)	0.07 (0.01)
	Mean percentage removal efficiency (10-week period)					
Exotic	96 (9)	94 (7)	91 (10)	97 (4)	83 (37)	76 (23)
Native	94 (11)	90 (17)	92 (19)	97 (5)	92 (22)	79 (16)
Unplanted	75 (31)	89 (9)	61 (18)	53 (20)	-14 (50)	51 (48)

Between the 2010 and 2011 experimental periods, mesocosms were insulated for winter as described above. The protection was removed in April 2011 and wastewater batch feeding resumed in May 2011. The objective of the 2011 experimental phase was to test the three treatments, U, E and N, at two different inflow concentrations: a low load (L) similar to that of 2010, and a high load (H) (Table 4.2). Five out of the ten replicates from each planted mesocosm were randomly selected to be treated either with low or high inflow concentrations (five E and five N for each inflow load). The same procedure was followed for the unplanted mesocosms, with two mesocosms fed with low load and three with high load.

Table 4.2. Mean inflow load and concentration (± SE) for the 12-week sampling period between June and September 2011 (n=36).

	TSS	COD	TP	TN	NH_4	NO_3
	Average inflow load in $gm^{-2}d^{-1}$					
Low load	5.2 (2.1)	11.1 (1.9)	0.2 (0.1)	1.1 (0.3)	0.4 (0.1)	0.05 (0.01)
High load	18.1 (3.2)	22.6 (2.8)	0.6 (0.1)	2.5 (0.4)	0.8 (0.2)	0.07 (0.02)
	Average inflow concentration in mgL^{-1}					
Low load	198 (94)	422 (165)	7.6 (3.2)	41 (13)	15 (8)	2.1 (0.9)
High load	687 (156)	859 (215)	22.8 (4.6)	95 (21)	39 (19)	2.9 (1.6)

In 2011, batch feeding frequency was increased to three times per week. Once per week, for 12 consecutive weeks (June to October 2011), water samples were collected from the total outflow for water quality assessment after two days' retention time, and the quantity of outflow was measured daily as in 2010.

4.3.2 Plant parameters

At the end of the experimental period, both in 2010 and 2011, stem density was counted in each mesocosm and the aboveground portions were cut, dried and weighed. A section of substrate was excavated from top to bottom (36 cm) at the center of each mesocosm using a 6-inch diameter drill. Roots and rhizomes were separated from the gravel, dried and weighed to estimate belowground biomass. Leaf and root samples were collected from each planted mesocosm and analyzed for nutrient content at the Horticulture Research Center of Laval University (Québec, Canada). For purposes of comparison with plant parameters under natural conditions, shoot density and plant height were measured in three 1 m^2 plots randomly located at the sites where the rhizomes were collected: Lac Saint-François for the native *Phragmites*, and Îles-de-Boucherville National Park for the exotic *Phragmites*.

4.3.3 Data Analysis

Physico-chemical analyses (TSS, COD, NT, $N\text{-}NO_3$, $N\text{-}NH_4$ and TP) were conducted according to Standard Methods (2005). Based on a mass balance calculation, the amount of pollutants removed in 2010 and 2011 was compared between the three treatments (E, N, U). Results from 2010 (Table 4.1) generally showed the same patterns as the low treatment in 2011. Therefore, data analysis emphasizes mainly the 2011 phase of the experiment, to further explore the differences between plant species under the two different loads.

Repeated ANOVA measurements revealed that the effect of plants on pollutant removal efficiency changed over time throughout the sampling period, both in 2010 and 2011. Therefore, for the 2011 data, a two-factor ANOVA, with three treatments (E, N, U) and two loads (L, H inflow), was performed for each sampling week (n=5 for EL, EH, NL, NH; n=3 for UH and n=2 for UL). A two-factor ANOVA for the overall mean removal efficiency of the entire season (12 weeks) was performed as well (see bar graphs in Figure 4.2 and supplementary material). Plant parameters were also analyzed by a two-factor ANOVA, with two treatments (E, N) and two loads (L, H inflow) (n=5 for EL, EH, NL and NH). In case of interaction between factors, a one-way ANOVA was performed individually for each factor (see supplementary material). Assessments of normality and homoscedasticity were verified and further differences between treatments were established with the post-hoc Tukey test at $p < 0.05$. Statistical analyses were performed using JMP software (JMP®, Version 6 for Mac. SAS Institute Inc.), except for the repeated measurements ANOVA, which was performed using SAS Software (SAS Software®, Version 9.2 for Windows XP, SAS Institute Inc.)

4.4 Results

4.4.1 Pollutant removal efficiency

Experimental period - 2010

During the first sampling period in 2010, both native and exotic *Phragmites* showed excellent pollutant removal efficiency (Table 4.1). One-way ANOVA analysis with three factors (E, N, U) per week showed that planted mesocosms significantly outperformed unplanted ones in terms of TSS, COD, TN, NH_4 and NO_3 removal efficiency (see supplementary material SM 1). Few exceptions were observed, i.e. only for three out of ten sampling weeks, where removal efficiency of COD and was similar for all treatments (SM 1). There was very little difference in removal efficiency between the *Phragmites* subspecies for most pollutants. Significant differences in performance between mesocosms planted with exotic or native *Phragmites* occurred during only one week for COD, and two weeks for TSS removal efficiency, each time to the advantage of the exotic *Phragmites* (SM1).

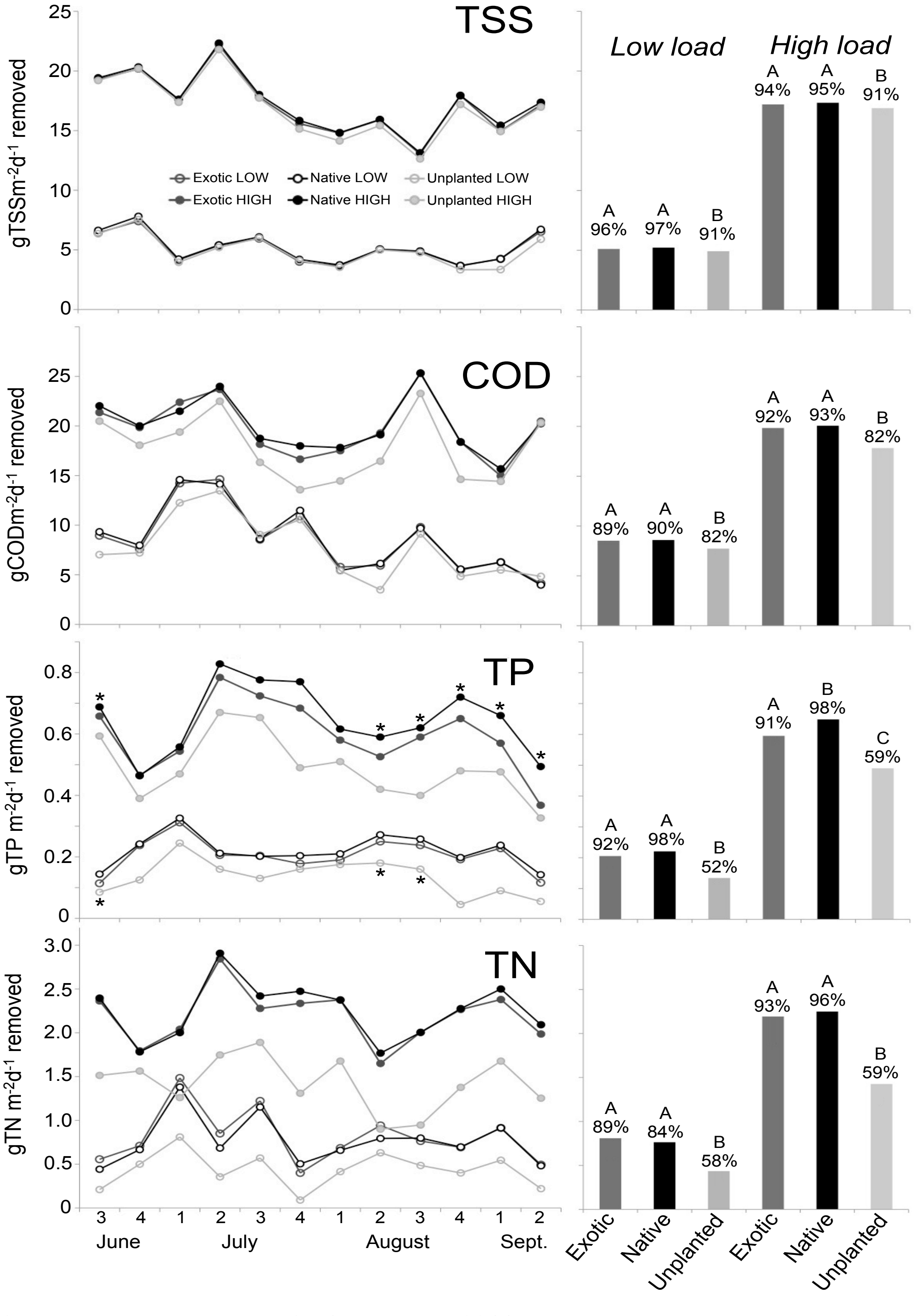


Figure 4.2. Pollutant removal in gm-2d-1 during the 2011 sampling period. Bar graphs show the overall mean of the 12 sampling weeks per treatment and the respective percentage removal efficiency. Different letters indicate a significant difference between treatments (Tukey post hoc test p<0.05). Time lines show means per treatment per week; line colors, black/dark grey/light grey, indicate native/exotic/unplanted respectively. Empty/full circles specify low/high loads respectively. *denotes weeks in which significant differences between native and exotic *Phragmites* were detected (Tukey post hoc test p<0.05).

On the other hand, TP removal was not only significantly different between planted and unplanted mesocosms, with 61 and 91% average removal efficiency respectively (Table 4.1), but also between native and exotic *Phragmites.* Although the average efficiency of both subspecies was comparable, a Tukey post-hoc test performed per week showed a significant difference in five out of ten sampling weeks (SM 1). In all cases, native *Phragmites* outperformed exotic *Phragmites,* a pattern consistent with the results of 2011 (see below).

Experimental period – 2011

As in 2010, results for 2011 showed excellent removal efficiency for all treatments, under both low and high loads, for all parameters measured (TSS, COD, TN, TP) (Figure 4.2). Repeated measurements ANOVA results showed a significant effect of load (L, H) and plant treatment (E, N, U), influenced by the effect of time, for all parameters. A two-factor ANOVA analysis per sampling week confirmed the significant effect of load and plant treatment on pollutant removal (in $gm^{-2}d^{-1}$) for all parameters throughout the sampling season (SM2). Absolute pollutant removal evolved in a very similar way under low and high inflow concentrations throughout the sampling period, with higher loads resulting in higher pollutant removal (Figure 4.2).

Percentage removal efficiency, on the other hand, was not affected by load, and was very high under both loading rates. As a general trend, more differences were found between treatments later in the season (Figure 4.2). TSS removal, for example, was significantly higher on planted versus unplanted mesocosms only after the fourth sampling week and until the end of the experiment. COD removal was also very high in all treatments; however, planted mesocosms were significantly more efficient than unplanted ones in 9 out of 12 sampling weeks (Figure 4.2). No differences for TSS and COD removal were detected between native and exotic *Phragmites*; both subspecies showed very high removal efficiency, ranging on average from 94 % to 97 % for TSS and from 89 % to 93 % for COD.

Differences between planted and unplanted wetlands were more pronounced regarding nutrient (TN, TP) removal. Total P removal efficiency in unplanted mesocosms was on average below 60 %, while planted wetlands showed around 90% efficiency (Figure 4.2). Total N removal efficiency was significantly higher in planted systems under both low and high loads (86-95 % respectively) than in unplanted mesocosms (58 %), except in one sampling week (SM2). The difference between planted and unplanted removal efficiency was greater under high N load (Figure 4.3). High ammonium (NH_4-N) outflow concentrations were detected in unplanted mesocosms, while planted mesocosms had very low outflow concentrations of both organic and inorganic N forms (Figure 4.3). Nitrate (NO_3-N) concentrations were very low for all treatments, meaning that in planted mesocosms, nitrogen was not accumulated in the form of NH_4-N or NO_3-N (Figure 4.3).

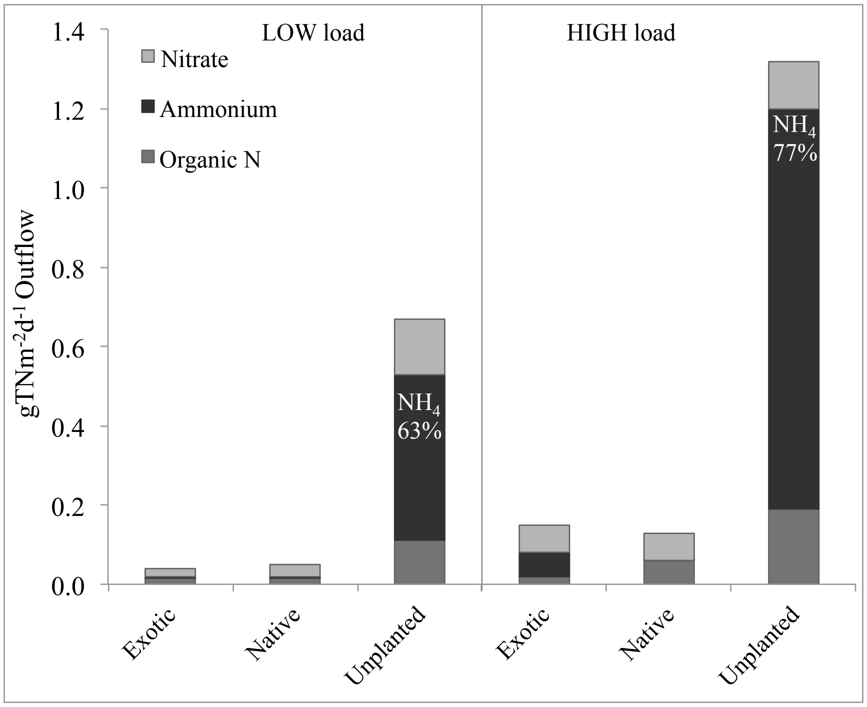


Figure 4.3. Overall average TN outflow charge (12 weeks) and proportions of the different N forms between treatments.

In terms of nutrient removal, both *Phragmites* subspecies showed excellent results (Figure 4.2). No significant differences were found between the subspecies for N removal. The most notable difference between native and exotic *Phragmites* was in terms of P removal, which was very high for both subspecies, but significantly higher for the native *Phragmites.* Load concentration also played a role, since differences between subspecies were found more frequently in mesocosms under high load (6 out of 12 sampling weeks) (SM2 and Figure 4.2).

4.4.2 Plant morphology and foliar content

Phragmites shoot density measured in the field ranged from 55 to 117 stems per m^2 respectively for native and exotic *Phragmites.* Plant stem height was around 3 m and stem diameter around 1 cm for both subspecies. These results contrast with those obtained in the mesocosm experiment, where average shoot density was approximately 20 times higher, ranging from 789 to 1366 stems per m^2 (Table 4.3). Shoots were also significantly shorter (1.8 m) and thinner (0.6 cm) compared to field measurements (Table 4.3) (See SM3 for statistical analysis).

Table 4.3. Plant parameters (± SE) measured at the experimental site (n=5) and in the fields where plants were collected: Îles-de-Boucherville National Park (Québec, Canada) and Lac Saint-François (Québec, Canada) (n=3).

		Experimental Set-up				Îles-de-Boucherville	Lac Saint-François
		High Load		Low Load			
Parameter	Unit	**Exotic**	**Native**	**Exotic**	**Native**	**Exotic**	**Native**
Stem density	stems m^{-2}	1366 (143)	789 (109)	1050 (142)	906 (167)	117 (13)	55 (4)
Stem length	m	1.8 (0.1)	1.9 (0.1)	1.6 (0.1)	1.9 (0.1)	3.3 (0.1)	2.6 (0.3)
Aboveground dry biomass	kg m^{-2}	4.1 (1.3)	3.2 (0.8)	4.0 (1.0)	4.1 (1.1)		
Belowground dry biomass	kg m^{-2}	2.3 (0.2)	2.7 (0.5)	2.8 (0.1)	3.6 (0.7)		
Nitrogen foliar content	%	2.1 (0.6)	2.1 (0.3)	1.6 (0.7)	1.4 (0.1)		
Nitrogen root content	%	0.9 (0.2)	0.9 (0.3)	0.9 (0.2)	0.8 (0.3)		
Phosphorus foliar content	%	0.12 (0.04)	0.36 (0.09)	0.10 (0.05)	0.41 (0.08)		
Phosphorus root content	%	0.25 (0.05)	0.35 (0.04)	0.27 (0.08)	0.29 (0.05)		

Morphological differences were also found between plant subspecies in the mesocosms. Although shoot density of both subspecies was considerably higher in the mesocosms than in the field, exotic *Phragmites* shoot density was significantly higher than native *Phragmites,* particularly under high input load. However, as native *Phragmites* stems were more robust*,* aboveground biomass was similar for both subspecies (Table 4.3). Native *Phragmites* had significantly greater belowground biomass than the exotic, and this difference was greater under low inflow load. Root biomass was greater under low load for both subspecies.

Although N foliar content was not affected by plant subspecies, a two-factor ANOVA showed a significant influence of load on foliar N content; under high load inflow, plant foliar N increased (Table 4.3, SM 3). Native *Phragmites* P foliar content was higher, under both low and high input load (Table 4.3, SM 3). Root P content was also significantly higher in native *Phragmites,* irrespective of load concentration*.* Evapotranspiration was significantly higher in native *Phragmites* mesocosms (Figure 4.4) and was not influenced by load inflow (SM4).

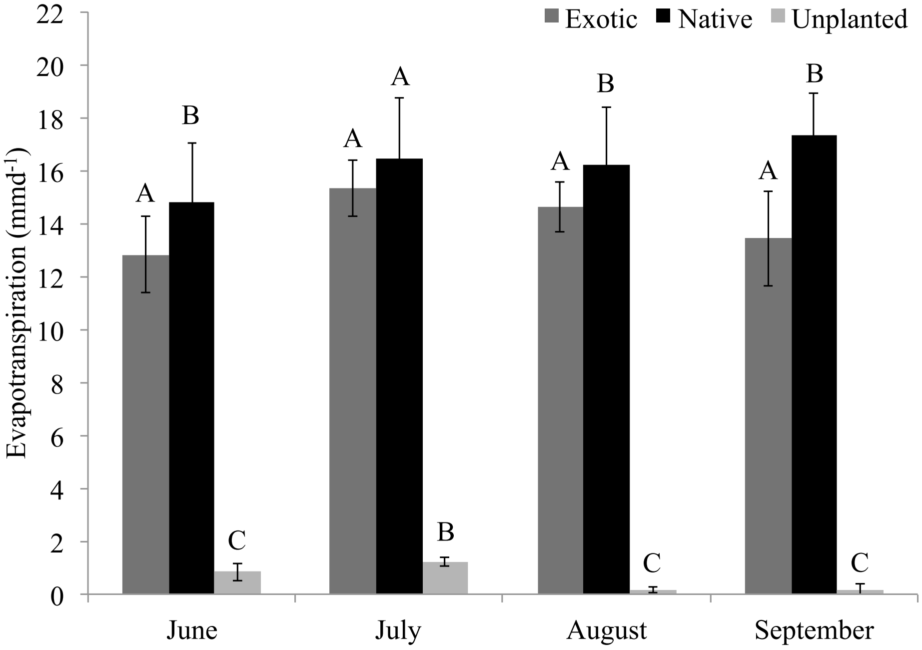


Figure 4.4. Mean evapotranspiration rate per month based on weekly measurements (± SE). Evapotranspiration was calculated as the difference between inflow and outflow volume, plus rainfall. Different letters indicate significant differences between treatments per month (Tukey post hoc test $p<0.05$).

4.5 Discussion

4.5.1 Pollutant removal efficiency

Results of our experiment suggest that *P. australis* subsp. *americanus* is indeed appropriate for use in TWs, with a level of pollutant removal efficiency comparable to that of the European subspecies.

The native *Phragmites*' efficiency in TWs is best illustrated by a comparison of our findings for planted versus unplanted mesocosms. During both phases of our experiment, the mesocosms planted with native (or exotic) *Phragmites* outperformed the unplanted mesocosms in pollutant removal, and this advantage increased under high load. Planted mesocosms were slightly more efficient at COD removal and performed significantly better for TN and TP removal. Only for suspended solids removal, which involves mainly abiotic processes, was there almost no difference in treatment efficiency between planted and unplanted mesocosms.

We attribute the greater efficiency of the planted mesocosms to several factors. The presence of plants, enhances the microbial community in the rhizosphere by offering a huge attachment surface area (Kadlec et al., 2000). Plants also provide a micro-aerobic environment through root oxygen release and a source of carbon through root exudates, which can improve aerobic degradation and nitrification (Brix, 1997; Gagnon et al., 2007; Vymazal, 2011). In addition, a high evapotranspiration rate affects retention time and treatment efficiency by increasing the concentration of pollutants in TWs (Chazarenc et al., 2003; Shelef et al., 2013). High evapotranspiration rates may also influence the adsorption of phosphates to organic particles in the media, increasing the redox potential of the system. In our experiment, native *Phragmites* tolerated TW conditions very well, showing high growth and biomass, allowing the plants to achieve their potential to improve TW efficacy.

Contrary to our hypothesis based on the ecophysiological superiority of the exotic *Phragmites* (Mozdzer and Zieman, 2010), we found no evidence that the native *Phragmites* was less efficient than its European counterpart in TWs. The two subspecies were compared under the same experimental conditions, under two different pollutant concentrations, and showed no important differences in performance. Since exotic *Phragmites* has been shown to transfer oxygen more efficiently to roots and rhizomes than the native (Tulbure et al*.,* 2012), we expected that this would translate into higher COD and TN removal efficiency. In fact, the only noticeable difference we found between the two subspecies in terms of removal was for phosphorus, for which the native *Phragmites* seemed to be more – not less – efficient than the exotic. The differences in TP removal were revealed during the two consecutive summer samplings, under both low and high loads for the second summer. Native *Phragmites* had a higher P content in leaves and roots compared to the exotic, which could partly explain its higher removal efficiency.

4.5.2 Plant morphology

The vast differences in growing conditions in subsurface TWs compared to natural wetlands may affect morphological, ecological and physiological plant attributes. In TWs, the substrate, usually composed of coarse sand or gravel, is loose, which maximizes hydraulic conductivity, thereby offering little resistance to root growth compared to more compact wetland soils. Soil fertility and nutrient supply is much higher than under most natural conditions, affecting plant growth as well as competition and density. Small systems such as microcosms or mesocosms are subject to strong edge effects, additional evapotranspiration loss and other sources of bias that may modify growing conditions (Poorter et al., 2012; Dalling et al., 2013). This was the case in our mesocosms, in which average stem density was approximately 20 times higher than in the field. Edge effect, canopy overhang and the confined, highly concentrated nutrient environment may explain the high density values obtained during the experiment.

We also identified relative morphological differences in *Phragmites* responses that were not predictable based on field observations. Under natural conditions, the exotic *Phragmites* was taller and had greater stem density than the native, a pattern reported for other locations (Mozdzer et al., 2013). In contrast, in the mesocosms, native *Phragmites* shoots were taller than the exotic. The exotic *Phragmites* had higher shoot density than the native, but the difference was much less under low pollutant load. Also, biomass production of native and exotic *Phragmites* was comparable in our mesocosms, while a review by Mozdzer et al. (2013) reported that the exotic produced on average between 151-250 % more total biomass than the native.

As expected, belowground biomass was affected by load, with both native and exotic *Phragmites* investing more in root biomass under a low load rate. However, native *Phragmites* had higher root biomass than the exotic, under both low and high inflow load, a pattern that once again contrasts with the results reported by Mozdzer et al. (2013).

4.5.3 Plant selection for treatment wetlands

While it can be reasonably assumed that plant characteristics measured under field conditions may reflect potential removal efficiency in TWs, our study shows that results are not easily transposed. Different responses by the American and European lineages of *P. australis* to TW growing conditions levelled out the differences observed in the field, so that both subspecies appeared equally efficient under our experimental conditions. In fact, contrary to our expectations, our results suggest that the native *Phragmites* may be the most suitable subpecies due to its slightly more effective removal of phosphorus.

While our mesocosm experiment suggests that native *Phragmites* have high removal efficiency, these experimental conditions resulted in an overestimation of quantitative values, and further evaluation under full-size TW conditions would be necessary. The very high evapotranspiration rate measured in the mesocosms also contributed to the high removal efficiency for all pollutants. Mesocosms have a high “edge – interior ratio”, which amplifies evapotranspiration through advection, or the so called “oasis effect” (Kadlec and Wallace, 2009; Headly et al., 2012), which would be less pronounced in full-sized TWs.

Concerns about the threat posed by invasive macrophyte species require the use of native species in treatment wetlands. The results of this comparative assessment of removal efficiency between native and exotic *Phragmites* subspecies suggest that native *Phragmites* could be an effective alternative to the exotic subspecies in North American TWs. However, while removal efficiency is the most important factor in plant selection for TWs, other characteristics should also be evaluated. Resistance to diseases and pests is particularly important, since native plants are assumed to be more susceptible (have more “local enemies”) than exotic species (Keane and Crawley, 2002), a process that has been suggested to contribute to the success of the exotic *Phragmites* in North America (Blossey, 2003). During our experiment, a fungicide treatment had to be applied to fight an infestation by *Deightionella*, a pathogenic fungus that affected both subspecies, but the native far more severely.

Finally, while most native *Phragmites* in North America have been grouped under the subspecies *P. australis* subsp. *americanus*, several different haplotypes have been recognized, as has another possible species (the so-called “Gulf Coast lineage”) in the southern United States (Saltonstall, 2002; Saltonstall et al., 2004). There may also be differences in removal efficiency between genotypes, as has been demonstrated for *P. australis* in Japan (Tomimatsu et al., 2014). Thus, our results may not apply to all other North American haplotypes of *P. australis* subsp. *americanus*.

4.6 Supplementary material

4.6.1 Supplementary material 1 (SM1)

2010: One-way ANOVA from 2010 followed by Tukey test when needed (* $p<0.05$; ** $p<0.01$) Different letters mean significant differences between treatments. n=10 for N and E and n=5 U for .

		July		August				September				
	Treatment	3	4	1	2	3	4	1	2	3	4	Mean
COD	Plant (N,E,U)	**			**	**	**	**		**	**	**
	N	A			A	A	A	A		A	AB	A
	E	A			A	A	B	AB		A	A	AB
	U	B			B	B	C	B		B	B	B
TSS	Plant (N,E,U)		**			**	**	**	**	**	**	**
	N		A			A	A	A	A	A	B	A
	E		A			B	B	A	A	A	A	A
	U		B			C	C	B	B	B	B	B
TP	Plant (N,E,U)	**	**	**	**	**	**	**	**	**	**	**
	N	A	A	A	A	A	A	A	A	A	A	A
	E	A	B	A	A	A	A	B	B	B	A	A
	U	B	C	B	B	B	B	C	C	C	B	B
TN	Plant (N,E,U)	**	**	**	**	**	**	**	**	**	**	**
	N	A	A	A	A	A	A	A	A	A	A	A
	E	A	A	A	A	A	A	A	A	A	A	A
	U	B	B	B	B	B	B	B	B	B	B	B
NH_4	Plant (N,E,U)	**	**	**	**	**		**	**	**	**	**
	N	A	A	A	A	A		A	A	A	A	A
	E	A	A	A	A	A		A	A	A	A	A
	U	B	B	B	B	B		B	B	B	B	B

4.6.2 Supplementary material 2 (SM2)

2011: Full factorial two-way ANOVA (* $p<0.05$; ** $p<0.01$) followed by Tukey test when needed (different letters mean significant differences between treatments) n=5. In case of interaction between factors, a one-way ANOVA was repeated separately for each. Load was significant throughout the entire sampling period; therefore, one-way ANOVA results are shown for the effect of plant treatment only.

				June		July				August				Sept.		
Parameter	Treatment			3	4	1	2	3	4	1	2	3	4	1	2	Mean
COD	Two-way ANOVA Full factorial	Plant (N,E,U)		**	**		**		**	**	**	**	**	**		**
		Load (H, L)		**	**	**	**	**	**	**	**	**	**	**	**	**
		Plant*Load			**		*		*	**		*				
			N	A			A				A		A	A		A
			E	A			A				A		A	A		A
			U	B			B				B		B	B		B
	One-way ANOVA	By Low load	N													
			E													
			U													
		By High load	N		A		A		A	A		A				
			E		A		A		A	A		A				
			U		B		B		B	B		B				
TSS	Two-way ANOVA Full factorial	Plant (N,E,U)					*		*	**	**	**	**	**	**	**
		Load (H, L)		**	**	**	**	**	**	**	**	**	**	**	**	**
		Plant*Load								**	*	**	**	*		
			N				A		A						A	A
			E				A		AB						AB	A
			U				B		B						B	B
	One-way ANOVA	By Low load	N							A			A	A		
			E							A			A	A		
			U							B			B	B		
		By High load	N							A	A	A	A			
			E							A	A	A	A			
			U							B	B	B	B			
TP	Two-way ANOVA Full factorial	Plant (N,E,U)		**	**	**	**	**	**	**	**	**	**	**	**	**
		Load (H, L)		**	**	**	**	**	**	**	**	**	**	**	**	**
		Plant*Load					*						*	*	*	**
			N	A	A	A		A	A	A	A	A		A		
			E	B	A	A		A	AB	A	B	B		B		
			U	C	B	B		B	B	B	C	C		C		
	One-way ANOVA	By Low load	N													A
			E													A
			U													B
		By High load	N				A						A	A	A	A
			E				A						B	B	B	B
			U				B						C	C	C	C
TN	Two-way ANOVA Full factorial	Plant (N,E,U)		**	**	**	**	**		**	**	**	**	**	**	**
		Load (H, L)		**	**	**	**	**	**	**	**	**	**	**	**	**
		Plant*Load		**						**	**	**			**	**
			N		A	A	A	A					A	A		
			E		A	A	A	A					A	A		
			U		B	B	B	B					B	B		
	One-way ANOVA	By Low load	N							A	AB	A			A	A
			E							A	A	A			A	A
			U							B	B	B			B	B
		By High load	N	A						A	A	A			A	A
			E	A						A	A	A			A	A
			U	B						B	B	B			B	B
NH_4	Two-way ANOVA Full factorial	Plant (N,E,U)		**	**		**	**	**	**	**	**	**	**		**
		Load (H, L)		**	**					**	**			**		*
		Plant*Load						**			**					
			N	A	A		A		A	A		A	A	A		A
			E	A	A		A		A	A		A	A	A		A
			U	B	B		B		B	B		B	B	B		B
	One-way ANOVA	By Low load	N					A			AB					
			E					A			A					
			U					B			B					
		By High load	N					A			A			A		
			E					A			A			B		
			U					B			B			B		

4.6.3 Supplementary material 3 (SM3)

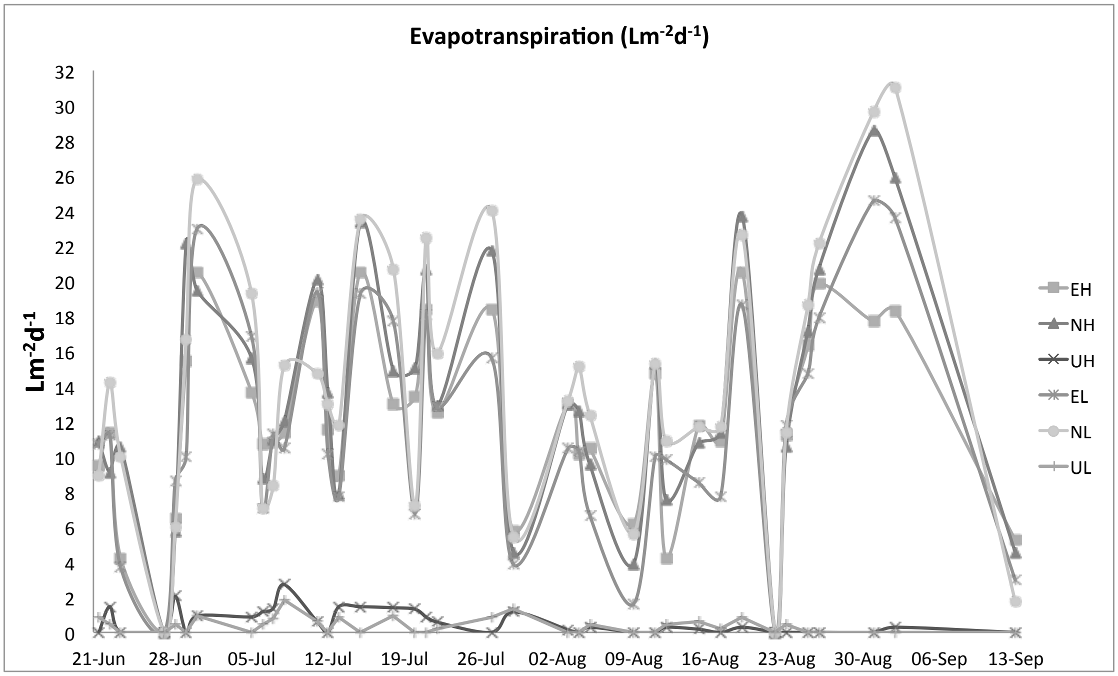
Plant parameters sampled at the experimental site in 2011. Full factorial two-way ANOVA (* p<0.05; ** p<0.01) n=5.

Two-way ANOVA

Parameter	Plant (N,E)	Load (H, L)	Plant*Load
Stem density	**		*
Aboveground biomass			
Root biomass	*	**	
Evapotranspiration	*		
Foliar N		*	
Root N			
Foliar P	**	*	*
Root P	*		

4.6.4 Supplementary material 4 (SM4)

Evapotranspiration per treatment per load throughout the sampling period 2011.



E: Exotic; N: Native; U: Unplanted; H: High load; L: Low load.

5. Discussion

In this final chapter, I summarize the results that together provide answers to the research questions of my thesis: Are polycultures as good as, or better, than monocultures for pollutant removal; and could a native species be as efficient as an exotic invasive species in TWs? Overall, the results showed that TWs planted with a combination of different species were as efficient as monocultures of the best performing species. Furthermore, comparison of native and exotic invasive *Phragmites* suggested that the native could replace the invasive species in TWs, avoiding possible environmental risks without compromising treatment efficiency. I will first discuss the influence of plant richness on the removal efficiency of TWs, then compare native and exotic *Phragmites* in TWs and conclude with the perspectives regarding possible future research.

5.1 Plant richness: Monocultures versus polycultures

Given plants' influential role in TWs, species identity and number can be expected to impact treatment efficiency. Considering two hypothetic macrophyte species (A, B) with different removal efficiencies for a specific pollutant (x), and assuming that species A outperforms species B, we can construct three possible scenarios of the comparative performance of each species in monoculture versus the combination of both species (Figure 5.1): a) AB removal efficiency is the average of the monocultures, b) AB removal equals the efficiency of the best performing monoculture and c) AB has greater pollutant removal than its monoculture counterparts. Only scenario C would support the hypothesis that plant richness presents an advantage for pollutant removal compared to a monoculture in TWs.

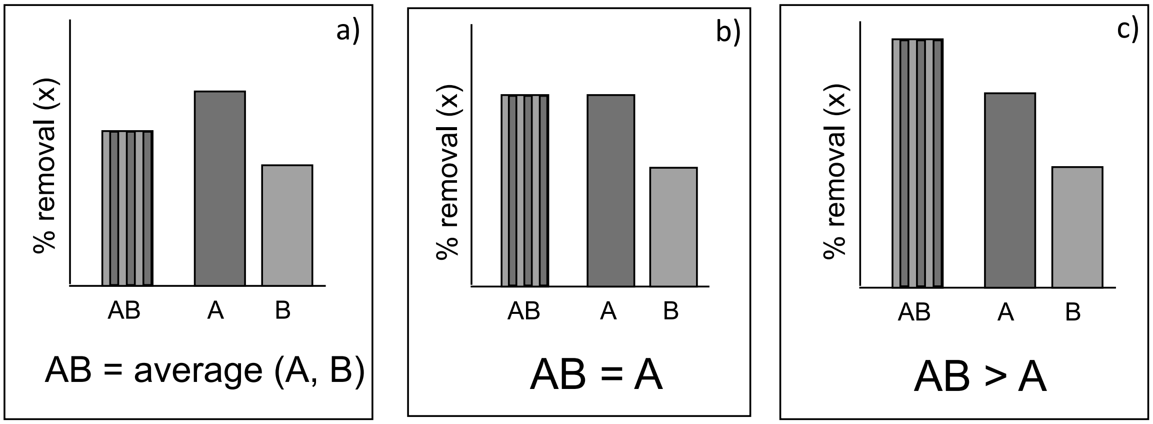
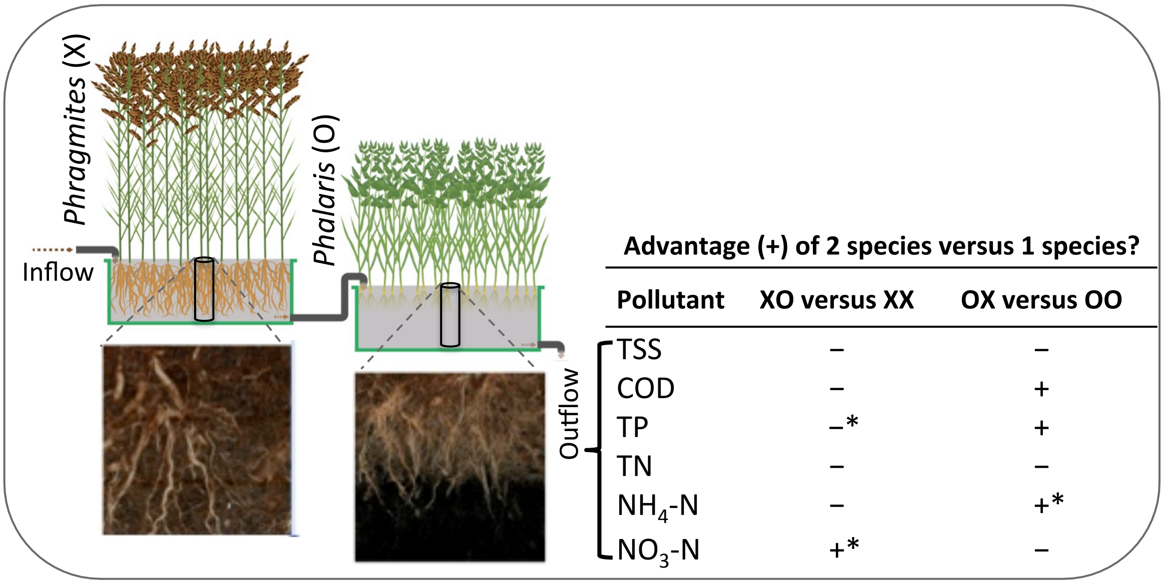


Figure 5.1. Possible scenarios (a, b, c) comparing the removal efficiency (%) of a given pollutant (X), between monocultures of species A and B versus the combination of both species. a) AB shows the average removal of both species individually; b) AB equals the best performing monoculture; c) AB outperforms the removal of both monocultures.

Two experiments were performed to address the question of the role of macrophyte richness in the removal efficiency of TWs (Chapters 1 and 2). The results varied depending on the pollutant considered or on the pair of species involved (Chapter 2). However, from a broad perspective, the results of the experiments led to similar a conclusion. Plant species identity influenced the performance of TWs, but little evidence was found to support the hypothesis that plant richness represents an advantage over monocultures for pollutant removal in TWs. However, polycultures were as efficient as the best individual species.

The results comparing monocultures of *Phalaris* (O) and *Phragmites* (X) versus the combination of both species (OX or XO) showed that the removal efficiency of plant species combinations often corresponded to the average rate of the monocultures. However, each pollutant must be considered separately in order to distinguish among different outcomes. Overall, the results of the experiment revealed that *Phragmites* performed equal to or better than *Phalaris* for pollutant removal, and we found no evidence that combining them would improve treatment efficiency over *Phragmites* monocultures, except for nitrate removal, for which the combination *Phragmites-Phalaris* (XO) outperformed both species' monocultures (XX and OO) (Figure 5.2).



Advantage (+) of 2 species versus 1 species?

Pollutant	XO versus XX	OX versus OO
TSS	–	–
COD	–	+
TP	–*	+
TN	–	–
NH_4-N	–	+*
NO_3-N	+*	–

Figure 5.2. Schematic representation of an experimental unit planted sequentially with *Phragmites* (X) and *Phalaris* (O), root pictures taken over the course of the experiment and a global summary of the results showing an advantage (+) or not (-) of combining two species, versus a monoculture. *Indicates a significant effect.

Some of the reasons it has been hypothesized that higher macrophyte richness could improve pollutant removal efficiency in TWs include: complementarity between macrophytes with regard to nutrient use and seasonal activity; better root partitioning, which could optimize nutrient uptake and increased bacterial diversity; abundance and activity. Due to the seasonal complementarity between *Phalaris* and *Phragmites*, it was expected that the longer duration of growing season would improve pollutant removal efficiency throughout a year-long experiment. However, this complementary growth period did not translate into any measurable higher removal efficiency. One possible reason may be negligible plant uptake during the growing season compared to nutrient input load (Zhang et al., 2010). Also, bacterial activity in the rhizosphere, largely responsible for pollutant removal, may be only weakly related to seasonal plant activity, compared to other factors such as water content, temperature, oxygen level or redox potential (Stottmeister et al., 2003; Edwards et al., 2006; Stein et al., 2007). It is thus not surprising that pollutant removal in TWs is also high in winter, when the plants are otherwise dormant (Allen et al., 2002).

Differences in root morphology and distribution in the two species affected the aeration conditions of the rhizosphere; *Phragmites*' large rhizomes improved oxygen diffusion, contrary to the fine roots and high shoot density of *Phalaris.* Based on these differences between species, we expected a possible benefit of planting *Phalaris* after *Phragmites* in terms of TN removal, first in terms of promoting nitrification by *Phragmites* and subsequently denitrification by *Phalaris.* However, the combination

Phragmites-*Phalaris* was less efficient for TN removal than *Phragmites* monoculture, but better for NO_3 removal, since *Phragmites*' NO_3 outflow concentrations were higher than those of *Phalaris. Phalaris'* disadvantage for TN removal, compared to *Phragmites*, may be due to its rhizosphere's extreme reducing conditions, which restrict nitrification. Consequently, perhaps increasing the *Phragmites*-*Phalaris* ratio when the two species are combined*,* to, for instance, ¾ *Phragmites* ¼ *Phalaris*, could further improve TN removal. Under this hypothetical scenario, the combination of the two species would have a positive impact on TN removal, possibly retaining *Phragmites* monocultures' ability to remove TP. *Phragmites*' advantage over *Phalaris* for TP removal may be explained by the oxic conditions in *Phragmites* units that enhance phosphorus co-precipitation with iron (Vymazal and Kröpfelová, 2008).

Overall, the best monoculture was as efficient as or more efficient than the combination of the two plant species for pollutant removal. Conversely, the treatment efficiency of *Phalaris* was improved when it was combined with *Phragmites*, except with regard to NO_3 removal. Indeed, minimizing NO_3 outflow was the only situation in which the combination *Phragmites*-*Phalaris* outperformed *Phragmites* monocultures. Our results suggest that adding a unit of *Phragmites* after a system planted with *Phalaris* could improve its treatment efficiency.

Assessing the role of plant richness in TWs is a complex question, and becomes more challenging with any increase in the number of species involved. Four free-floating macrophyte species tested independently and combined together for pollutant removal in TWs led to a similar conclusion regarding the effect of plant richness in TWs as comparing emergent species (*Phragmites* and *Phalaris)*. Overall, plant species had a significant effect on pollutant removal efficiency, while plant richness per se did not enhance removal efficiency compared to the most efficient species in monoculture. Still, care must be taken when analyzing results to determine the influence of plant species richness. TN removal, for example, was significantly different between species monocultures, showing the following pattern: *Eichhnornia* ≥ *Pistia* > *Salvinia* > *Limnobium* (Figure 5.3). When combining different species together, both in pairs and all together, the removal efficiency of each combination was as good as the most efficient species it contained, regardless of degree of plant richness (Figure 5.3). However, when comparing average TN removal per plant richness (four species together versus two species, all possible pairs, versus average of all monocultures), results suggest that increasing plant richness increases pollutant removal. Yet, this outcome is a consequence of the differences between individual species and does not actually reveal the role of plant diversity, rather representing a "sampling effect" (Loreau, 2001).

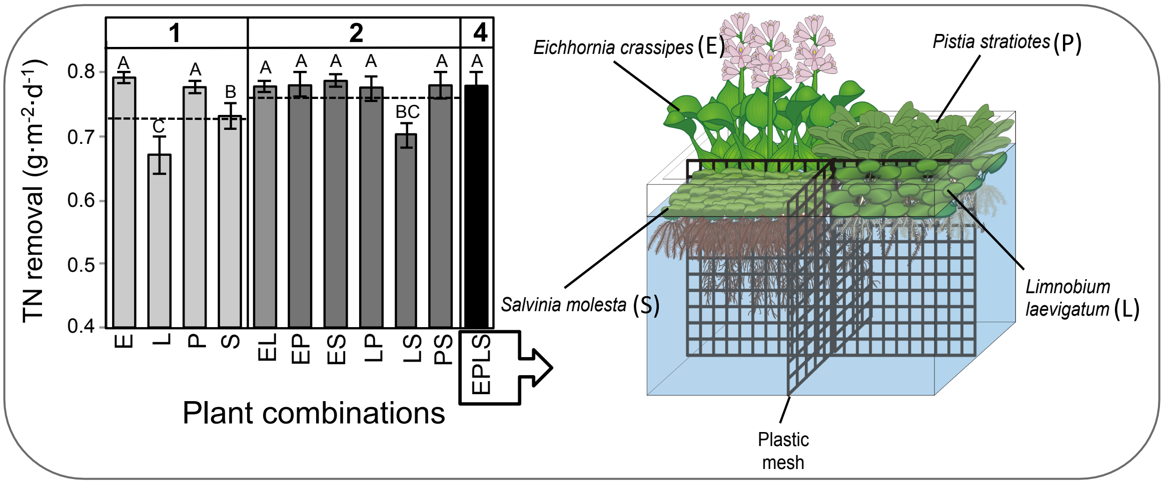


Figure 5.3. Schematic representation of a mesocosm planted with four free-floating plant species (EPLS) and average TN removal of each plant combination (July to September 2011). Different letters (ABC) indicate significant differences between plant combinations (Tukey test $p<0.05$). Dashed lines represent the average removal calculated per species richness level (four species together versus two species, all possible pairs, versus all monocultures).

The sampling effect reflects the increasing probability that the best performing species would occur in species mixtures as plant species richness rises (Engelhardt and Ritchie, 2001). Such sampling effects support the notion that individual species, rather than species richness, influence ecosystem processes (Engelhardt and Ritchie, 2001). Nonetheless, the results from our experiments evaluating the role of plant richness (Chapters 2 and 3), suggested that both individual species and species richness influence pollutant removal in TWs. The efficiency of different species together was either improved, matching the efficiency of the best performing species in the combination or corresponded to the average of both species individually. Species identity played a role, as shown by the differences between monocultures. Also, in the free-floating macrophytes experiment, it was found that the presence of

Eichhornia, whether on its own or combined with other species, had a positive significant effect on nitrogen, organic matter and suspended solids removal. Comparing pairs of species of free-floating plants, specific species combinations performed better than others regardless of plant richness. For instance, *Eichhornia* combined with *Salvinia* showed greater organic matter removal efficiency than *Eichhornia* combined with *Limnobium* or *Pistia* and all other combinations.

Total biomass (above and belowground) was a better predictor of removal efficiency than plant species richness, both for free-floating plants and in the *Phragmites* and *Phalaris* experiments. The relation between macrophyte productivity and their performance in TWs has long been recognized; therefore, high biomass production is one important desirable trait when selecting species for water treatment purposes (Tanner, 1996). The correlation between total biomass and pollutant removal was positive and significant when different combinations of free-floating macrophytes were compared. Among emergent species*, Phragmites* monocultures had greater biomass than *Phalaris* (although not significantly) and were also more efficient at pollutant removal.

Still, differences among plant combinations in terms of pollutant removal could not always be explained by differences in biomass. For instance, the biculture *Limnobium-Salvinia*, which had similar total biomass as its monoculture counterparts, achieved higher levels of suspended solids removal than both species individually, suggesting a synergetic effect among the plants in this specific combination. As well, differential removal performance between individual species with comparable biomass was observed for nitrogen (TN) and phosphorus (TP). *Salvinia* outperformed *Limnobium* for TN, while *Limnobium* outperformed *Salvinia* for TP, and the performance of both species together was in between that of the monocultures. *Limnobium*'s greater efficiency for TP removal might be related to its higher P uptake, and its lower efficiency for TN removal could be associated with the higher dissolved oxygen concentrations measured for *Limnobium* compared to the other species, possibly affecting its denitrification rate.

Macrophyte biomass, species richness and species identity influenced pollutant removal efficiency to some extent. Overall, the efficiency of the least effective species in monoculture improved when this species was combined with other more effective species. At the same time, the efficiency of the most effective species in monoculture was similar to its efficiency in combination with other species. These results do not support the initial hypothesis that plant species richness improves the performance of TWs. However, they suggest that increasing plant diversity does not compromise the efficiency of TWs. Diversity does provide benefits of ecosystem services associated with higher plant richness, including habitat value for fauna, higher aesthetic values and social acceptability. Furthermore, under circumstances in which the performance of available macrophyte species is unknown or cannot be determined, using a combination of species offers the best chances of achieving the highest possible level of pollutant removal efficiency.

Plant richness effect on TWs microbial communities was evaluated in collaboration with the Dr. Kela Weber's Environmental Sciences Group at the Royal Military College of Canada, Kingston (Ontario). Plant species and richness influence was evaluated using community level physiological profiling (CLPP) to assess the function of microbial communities. This technique was used for the experiments presented in Chapters 1 and 2. Results of the influence of free-floating plant species on microbial communities (Chapter 3) were published in the *Journal of Environmental Engineering* (2014) and are included in the appendices of the thesis. Results from the experiment comparing *Phragmites* and *Phalaris* planted sequentially as monocultures or one species after the other one (Chapter 2) are in preparation for publication, and preliminary results were presented in WETPOL 2013 and IWA 2014 (Button et al., 2014).

5.2 Effect of plant richness on microbial communities

Analysis of the microbial communities supported the results presented in Chapters 2 and 3 regarding the influence of plant identity in TWs, and also the fact that increasing the number of plant species does not increase pollutant removal or the activity and diversity of microbial communities.

Distinctly different microbial communities were associated with each free-floating plant species, and polycultures were different from their respective monoculture counterparts. The highest catabolic activity in monoculture systems was found for *Limnobium*, followed by *Pistia*, *Salvinia*, and *Eichhornia* respectively. In terms of emergent species, results varied depending on pollutant input load. Under high pollutant concentrations, microbial communities were unaffected by plant species identity, but under lower pollutant load, *Phalaris* enhanced microbial activity and diversity. Results suggested that rather than plant richness, specific plant selection and plant/plant interactions were important.

5.3 Why does plant richness have a limited effect on pollutant removal in TWs?

Compared to previous findings in the context of other ecosystems, particularly in grasslands (Cardinale et al., 2011), we found little effect of TW plant richness on ecosystem services, measured in terms of pollutant removal. The TW ecosystem's particular characteristics, the interactions between aquatic plant species and the experimental conditions are factors important to consider in interpreting these results.

An ecosystem with greater plant richness would be expected to display a wider range of functional traits, with increasing opportunities for more efficient resource use due to the "niche complementarity effect" (Diaz, 2000; Cardinale et al., 2007). Effective resource use enhances productivity, thus conceivably resulting in a positive effect on TW performance. The complementarity effect appears to be more important in nutrient limited and spatially or temporally variable environments than in nutrient rich wetlands (Cardinale et al., 2011). TWs are nutrient rich and mainly homogeneous aquatic ecosystems, characteristics that may diminish the potential complementary effect between plant species. The work of Cardinale et al. (2011) supports the argument that environmental heterogeneity enhances complementary effects between species. Evaluating the influence of algae species richness in freshwater steams, they found that nitrate uptake increased with higher algae species richness. Although these results were subject to fluctuating environmental conditions such as flow speed variation, when environmental conditions were unaltered, plant richness did not influence nutrient uptake.

Most evidence of a positive effect of richness on pollutant removal comes from terrestrial environments where nutrients are limited such as in grasslands (Cardinale et al., 2011). In nutrient rich aquatic ecosystems, strong competition for space rather than complementary or synergetic effects is likely to occur between aquatic plants (Engelhardt and Ritchie, 2001; Cardinale et al., 2011).

Overyielding is recognized as a possible consequence of the complementarity effect. It occurs when a more diversified community produces greater biomass than expected from the productivity of each individual species as a monoculture (Loreau, 2010). An extensive literature review published by Cardinale et al. (2011) found that overyielding effects are generally stronger as experiments run for longer. Results from the Cedar Creek experiment, a long-term large-scale grassland experiment testing the effect of plant diversity in ecosystem functioning, support this argument assessing overyielding effect in diverse plant species communities after ten years of experiment (Loreau, 2011). For this reason, results to date regarding the role of plant richness based on short-term experiments should be interpreted with care, and results might differ in the context of long-term experiments (Cardinale et al., 2007). Another hypothesis raised by Cardinale et al. (2011) concerns negative selection effects. The authors argue that species selection for experimental systems may prevent the expression of overyielding.

5.4 Invasive versus native species

Growing concerns about the threat of invasive macrophyte species increasingly require the substitution of native species for those with invasive potential in TWs for wastewater treatment. Among the various alternatives, the native *Phragmites (P. australis* subsp. *Americanus)* has been found promising for this context.

Given that the biological attributes considered highly desirable for plant species in TWs are often characteristic of invasive plants as well, it is not surprising that common reed (*Phragmites australis*), the species most frequently used in subsurface flow TWs (SSFTW) around the world (Vymazal, 2011), is also one of the most invasive outside its native range, and one of the weeds with the most negative impact on biodiversity in North America.

Based on the plant's relative ecophysiological and morphological characteristics reported in field studies (Mozdzer and Zieman, 2010), it was hypothesized that the native *Phragmites* would show lower pollutant removal efficiency than the invasive European *Phragmites*. In fact, no evidence was found that native *Phragmites*' performance would be inferior to that of European *Phragmites*. Otherwise, the native *Phragmites* was slightly more effective for removal of phosphorus.

As was the case for the experimental results discussed in Chapters 1 and 2 on the effect of plant richness on TWs performance, the results presented in Chapter 3 did not support the hypothesis tested. One of the factors that may explain these unexpected findings relates to certain characteristics of TW ecosystems. The greater and unlimited availability of nutrient resources in TWs strongly differs from *Phragmites*' natural growing conditions. This difference may affect morphological, ecological and physiological species attributes, and in turn their performance in TWs in terms of pollutant removal efficiency. Therefore, while the plant species characteristics measured under field conditions may reflect a species' potential removal efficiency, essential differences between the field and TWs conditions need to be considered and may influence plant attributes.

In the case of this study, a mesocosm effects known as "edge effects" or "oasis effects", also likely influenced plant development and evapotranspiration rates and therefore the extent to which the results of the experiment were influenced by the mesocosm scale needs to be further assessed by pilot or full-scale experiments. Further, native *Phragmites'* resistance to diseases must be taken into account, since during our experiment, a fungicide treatment had to be applied to fight an infestation by *Deightionella*, a pathogenic fungus that affected both subspecies, but the native far more severely.

5.5 Plant species selection for TWs

Removal efficiency is obviously one of the most important factors in plant selection for TWs, but other characteristics should also be considered. Favoring native over exotic (potentially invasive species) is strongly recommended and there is increasing evidence that a wider range of macrophyte species are suitable for water treatment, beyond the few that are commonly used (Allen et al., 2002; Guittonny-Philippe et al., 2015). Recent research by Guittonny-Philippe et al. (2015) recommends the selection of native macrophytes and suggests that root/shoot ratio, aerial height and proportion of green leaves are good indicators of plant tolerance to industrial discharge. These authors also showed that species growing near wastewater discharge are more likely to show high removal efficiency.

Plant species selection should also take into account the ecosystem services provided by TWs and specific functions that might not improve treatment efficiency but are relevant for local circumstances. Plant species diversity in TWs may provide additional ecosystem services such as providing a habitat for diverse fauna and high aesthetic value, increasing their social acceptability without compromising removal efficiency. Most TWs are designed solely for the purpose of water pollutant removal, although some others are intentionally multipurpose, potentially including wetland restoration and habitat enhancement for wildlife (Picek et al., 2008). In such contexts, ensuring that the TW is planted with high macrophyte diversity becomes in fact a priority. Even if plant diversity does not present an advantage measurable directly in terms of pollutant removal efficiency, it does contribute to TWs in terms of their resilience to stress and diseases (Kadlec and Wallace, 2009).

5.6 Possible avenues for further research

Future research could target the specific mechanisms by which plant diversity affects ecosystem processes and functioning, and influences pollutant removal in TWs. The results we obtained when evaluating the influence of plant richness in TWs suggest that interspecific plant competition affects TWs’ pollutant removal efficiency. Thus, further research on a greater number of species with the aim of assessing the specific processes that influence plant productivity and the relationship of biomass with pollutant removal could possibly lead to more effective species selection, resulting in improved TW performance. Evaluating the role of plant diversity in water treatment under full-scale studies may also contribute to understand the mechanisms in which plant richness might influence physicochemical processes in TWs. Even if full-scale studies limit the number of species and the number treatment replicates, the scale effect is important to be considered.

Estimating the additional benefits of higher plant species richness in TWs could also strengthen the arguments in favor of increasing plant diversity in TWs. For instance, the effect of species richness on the resilience of a TW system could be addressed by inducing stressful conditions likely to occur in TWs, such as drought, pollutant overload or plant diseases, under controlled experiments, ideally in long-term experiments to monitor the evolution of the system.

Finally, native non-invasive species increasingly show potential for wastewater treatment in TWs (Bonilla-Warford and Zedler, 2002; Greenway, 2005; Taylor et al., 2011; Guittonny-Philippe et al., 2015). While our experiments replacing the invasive European *Phragmites* with the native subspecies (*P. australis* subsp. *Americanus*) showed promising results, further testing in full-scale experiments is needed for such an approach to be considered for implementation in TWs across North America.

6. General conclusions

The aim of my research project was to evaluate if TWs planted in polycultures are as good as, or better than monocultures for pollutant removal, and if a native species could be as efficient as an exotic invasive species. Overall, the results showed that TWs planted with a combination of different species were as efficient as monocultures of the best performing species. Furthermore, comparison of native and exotic invasive *Phragmites* suggested that the native could replace the invasive species in TWs, avoiding possible environmental risks without compromising treatment efficiency.

Assessing the role of plant richness in TWs is a complex and challenging question depending on the number of species involved. The results from the two experiments performed to answer this question showed that both, individual species and plant species composition, influenced the performance of TWs. Species identity played a role, as shown by differences between monocultures; and the combination of species either improved the performance (matching the efficiency of the best performing species) or had the average efficiency of both species individually.

The results comparing monocultures of *Phalaris* and *Phragmites* versus the combination of both species showed that the removal efficiency of plant species combinations often corresponded to the average rate of the monocultures. However, each pollutant must be considered separately in order to distinguish among different outcomes. In general, *Phragmites* performed equal to or better than *Phalaris* for pollutant removal, and we found no evidence that combining them would improve treatment efficiency over *Phragmites* monocultures, except for nitrate removal, for which the combination *Phragmites-Phalaris* outperformed both species' monocultures.

The comparison of four free-floating macrophyte species tested independently and combined together for pollutant removal in TWs led to a similar conclusion regarding the effect of plant richness in TWs as the one from the *Phragmites* and *Phalaris* experiment. Overall, plant species had a significant effect on pollutant removal efficiency, while plant richness per se did not enhance removal efficiency compared to the most efficient species in monoculture. The results suggest that under circumstances in which the performance of available macrophytes is unknown or cannot be determined, using a mixture of species offers the best chances of achieving the highest possible level of pollutant removal.

Removal efficiency is obviously one of the most important factors in plant selection for TWs, but other characteristics should also be considered. Favoring native over exotic (potentially invasive species) is strongly recommended. Results of our experiment suggest that *P. australis* subsp. *americanus* is appropriate for use in TWs, with a level of pollutant removal efficiency comparable to that of the European subspecies, and even slightly more effective for removal of phosphorus. The promising results of the native *P. australis* subspecies need to be further tested in full-scale experiments before recommanding the use of this species in TWs.

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8. ANNEXES

8.1 ANNEX 1

Catabolic profiles of microbial communities in relation to plant identity and diversity in free-floating plant treatment mesocosms

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Catabolic profiles of microbial communities in relation to plant identity and diversity in free-floating plant treatment wetland mesocosms

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ABSTRACT

The objective of this study was to investigate if the catabolic capabilities (the overall ability to utilize a wide range of carbon sources) and catabolic profiles (the overall pattern of carbon source usage based on the carbon source types and relative usage extent) of microbial communities in free-floating plant treatment wetlands differ based on the presence or combination of different plant species. Free Floating-plant treatment wetland mesocosms were established using four different plant species: *Limnobium laevigatum* (L), *Salvinia molesta* (S), *Eichhornia crassipes* (E), and *Pistia stratiotes* (P). Mesocosms were either arranged as a monoculture (one plant species), biculture (two different plant species; all possible pairings), or quadricultures (one of each plant species). Mesocosms were fed twice weekly with 20 L of reconstituted wastewater using diluted fish farm sludge effluent. The microbial communities in each mesocosm were assessed after a 4 month operational period using the community level physiological profiling (CLPP) method. It was observed that monoculture wetland systems had different catabolic activities and catabolic richness' (number of carbon sources utilized) with the following respective trends: (L) > (P) = (S) > (E) and (L) > (E) = (P) > (S). It was also found that the carbon source utilization patterns of the microbial communities from the monoculture mesocosms were distinctly different from each other, and that the polyculture communities were different from the respective monoculture counterparts. These findings further support the hypothesis that plant type and combination plays a critical role in the development of the microbial communities present in treatment wetlands. It was also found that increasing the number of plant species did not, on average, promote the development of microbial communities with a more active and diverse catabolic capability, but rather specific plant selection and plant/plant interactions were important. In comparison to the other plant species *E. crassipes* had the largest amount of root mass available for microbial community attachment. Observations suggested that this larger root mass translated into *E. crassipes* having a dominating effect on defining the carbon source utilization patterns of microbial communities from polyculture mesocosms. Although microbial communities from the monoculture mesocosms containing *E. crassipes* had the lowest activities (on a per unit volume basis), *E. crassipes* mesocosms generally had the best COD removal rates in either monoculture or polyculture systems, potentially because of the greater amount of root mass available.

1. Introduction

Free-floating plant treatment wetlands have been widely used to improve water quality, particularly in tropical or sub-tropical climates (Costa et al., 2000; Nhapi et al., 2003; Lu et al., 2008; Nahlik and Mitsch, 2006). More recently, floating treatment wetlands have shown promising results in removing both nutrients and organics from wastewater and stormwater (Hubbard et al., 2004; Stewart et al., 2008; Tanner and Headley, 2011). As a part of these removal processes microbial communities associated with plant roots play an essential role, however this role is not completely understood (Faulwetter et al., 2009; Truu et al., 2009). Within treatment wetlands plant root systems provide mechanical support for microbial community attachment, transfer oxygen from

[redacted]

aerial tissues into the rhizosphere, and secrete exudates through their roots. It is through these processes that plants are thought to help regulate the microbial community structure and function within the surrounding rhizosphere (Weber and Legge, 2013). Plant species may differ in root morphology, in the amount and type of root exudates they produce or in the oxygen they release, which may in turn influence microbial communities. Hence, it is assumed that different plant species could provide the conditions for different microbial communities due to the differing root environments (Rothman and Bouchard, 2007; Gagnon et al., 2007). Also, ecological theory states that greater biodiversity (plant, animal, microbial) provides for a more resilient and healthy ecosystem (Cardinale, 2011). In the context of treatment wetlands it is hypothesized that through capitalizing on the complimentary nature of different plant species, greater plant diversity can lead to greater microbial functional diversity in the rhizosphere, and possibly an enhancement in the microbial community function in terms of water treatment abilities. Treatment wetlands containing more than one plant species may complement the removal efficiency demonstrated for one single species. A study performed with 16 different plant species in a vertical flow constructed wetland suggested an increase in microbial biomass carbon and nitrogen production when a greater number of plant species was used, but did not reveal any changes or differences in microbial community profiles (Zhang et al., 2010).

Community-level physiological profiling (CLPP) has been used in the past to assess ecological functions of microbial communities including their catabolic profiles (the overall pattern of carbon source usage based on the carbon source types and relative usage extent) and overall catabolic capability (the overall ability to utilize a wide range of carbon sources) in many different aquatic and terrestrial ecosystems (Weber and Legge, 2010; Faulwetter et al., 2009). The method utilizes BIOLOG^{TM} ECO plates containing 31 environmentally relevant carbon sources in triplicates. An overall community catabolic potential and activity for the specific carbon sources is evaluated giving an indication of functional abilities related to water treatment.

The objective of this study was to investigate if the catabolic capabilities and profiles of microbial communities in free-floating plant treatment wetlands differ based on macrophyte species (effect of plant identity), and on the number of plants species (effect of plant diversity).

2. Methods

2.1. Mesocosm system set-up and sampling

A total of 33 mesocosm systems of 65 L (*L* 53 cm/*W* 37 cm/*D* 37 cm) were set-up following a randomized block design in early June 2011, on the experimental site of the Botanical Garden of Montréal, Canada (Fig. 1). Each mesocosm tank was divided into four quadrants via plastic mesh and was either planted in monoculture (any one of 4 species × 3 replicates per species), biculture (2 different plant species; 6 possible pairings × 3 replicates) or quadriculture (all four plant species × 3 replicates) with the following free-floating macrophyte species: *Limnobium laevigatum* (L), *Salvinia molesta* (S), *Eichhornia crassipes* (E), and *Pistia stratiotes* (P) (Fig. 1). The quadrants were identified as a, b, c, d respectively representing the South East, South West, North West, and North East quadrants.

After a period of two weeks for establishment of the plants, wetland systems were fed twice weekly with 20 L of reconstituted wastewater using diluted fish farm sludge effluent (1:75), urea (46%) and monopotassium phosphate (23%) fertilizer to attain an average influent concentration of 188 mg L^{-1} TSS, 358 mg L^{-1} COD, 7 mg L^{-1} TP, and 29 mg L^{-1} TN.

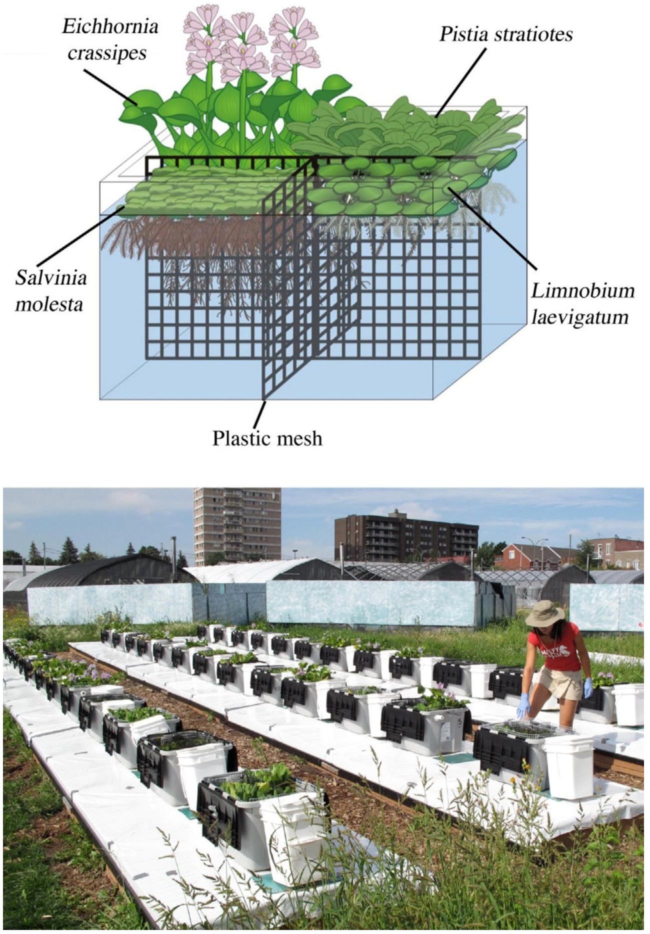

Fig. 1. Top: schematic view of a quadriculture wetland mesocosm system. Bottom: view of the experimental site.

From July to September on a weekly basis, influent and effluent samples (1 L) were collected from each mesocosm and analyzed for chemical oxygen demand (COD) according to Standard Methods (2001). Total volume of the wetland systems was set at 40 L before feeding, by pumping out approximately 20 L (outflow volume varied with evapotranspiration) with a manual water pump. Effluent samples were collected from the total outflow and analyzed. Then, 20 L of fresh wastewater was added manually to each wetland system.

In late September (after a 4 month operational period), water samples were collected from the dense root zones to evaluate the microbial communities in mono-, bi-, and quadriculture wetland systems. A sample (50 mL) from each quadrant was taken as close to the roots as possible for all monocultures and quadricultures. All biculture systems were sampled in two quadrants giving samples from the root zone of both macrophyte species.

At the end of the study (September 26th), the plant biomass in each mesocosms was collected. Root mass was differentiated from aerial mass. Samples were dried until constant weight was achieved (approximately 48 h in all cases).

2.2. Community level physiological profiling

Community level physiological profiling (CLPP) was performed on the same day as samples were taken according to the method

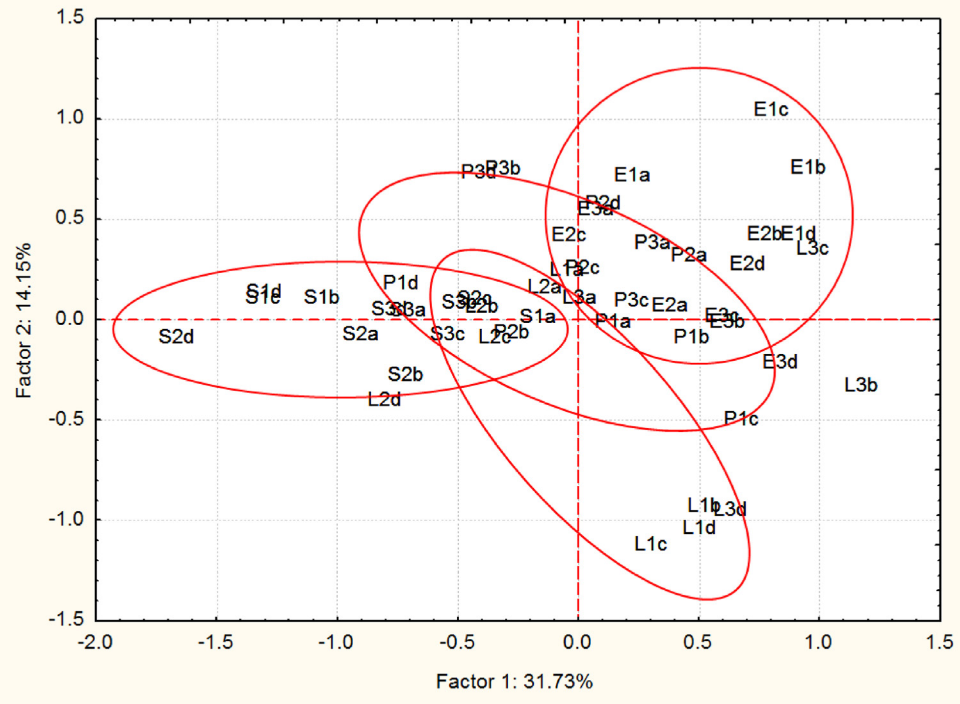

Fig. 2. PCA plot (Taylor Power Transform, slope = 0.9385) of the microbial community carbon source utilization patterns from the monoculture mesocosms. Objects represent an average of three sample analyses. 1, 2, 3 represent mesocosm replicates, a, b, c, d represent the samples from different quadrants of a specific mesocosm, and L (*Limnobium laevigatum*), S (*Salvinia molesta*), E (*Eichhornia crassipes*), P (*Pistia stratiotes*) represent the macrophyte species. (Factor 1 and factor 2 explain 46% of the overall variability.)

outlined in Weber and Legge (2010). BIOLOG^{TM} microplates are 96-well plates where each well contains a different carbon source and a redox dye indicator, tetrazolium violet. When a mixed microbial community sample is inoculated into each of the wells, the production of NADH via cell respiration reduces the tetrazolium dye to formazan, resulting in a colour change within each individual well, which can be detected photometrically. In short, each well of the BIOLOG^{TM} ECO plates (Biolog Inc., Hayward CA, USA) was inoculated with 100 μL of sample and incubated at room temperature. Plate incubations were completed in a 24 h temperature controlled laboratory at 21 °C. Plates were then read at an absorbance of 590 nm every 12 h for 84 h.

2.3. Data analysis

Analysis of the CLPP data was performed as previously described (Weber et al., 2007; Weber and Legge, 2010). To evaluate all plate data within a study, a specific incubation time point can be chosen as a metric, but choosing this time point may not be obvious. Using absorbance values taken early in the incubation time would yield little information, for at early stages (for example, 10 h) the difference between well absorbance values is too small to yield useful information (as there is very little colourimetric response). Using absorbance values taken later during the incubation can provide more information as long as the values are not above 2 (values above 2 are outside the linear absorbance range). An appropriate time point will be the time point that preserves the greatest variance between well responses while retaining the maximum number of wells within the linear absorbance range. Please see Weber and Legge (2010) for further details and an example of the selection process. For all samples in this study, absorbance readings (590 nm) at 48 h were identified as the metric for further CLPP data analysis. A separate plate was used for each quadrant of the evaluated mono-, bi-, and quadriculture mesocosm wetland systems giving a total of 96 plates. For each plate, 3 replicate carbon source utilization patterns (CSUPs) were collected giving a total of 288 objects (data sets). Each data set includes 31 variables (carbon sources) giving a total of 8928 data points for analysis.

Two different metrics were extracted from the CSUPs gathered and used for further analysis: (1) The average well colour development (AWCD), and (2) the number of carbon sources utilized (catabolic richness). The AWCD represents the average catabolic activity over all wells of the microbial community being assessed via the CLPP method, and is calculated as:

$$\mathrm{AWCD} = \frac{1}{31}\sum_{i=1}^{31}(A_i - A_0) \qquad (1)$$

where A_i represents the absorbance reading of well i and A_0 is the absorbance reading of the blank well (inoculated, but without a carbon source).

The number of carbon sources which a microbial community is able to utilize on any one plate provides a representation of the catabolic potential of a particular community. This can also be referred to as catabolic richness which is calculated as the number of wells with a corrected absorbance ($A_i - A_0$) greater than 0.25 (Weber and Legge, 2010). Where the AWCD represents overall catabolic activity, the catabolic richness metric identifies the catabolic capability range of the microbial community. A comparison of A_0 values showed no differences in blank well responses between sample types in this study (data not shown).

For the determination of a possible significant difference of AWCD and catabolic richness between mesocosm systems, a 1-way ANOVA was performed using Statistica 8 (StatSoft, Tulsa, OK) where p-values < 0.05 identified significant differences. Following ANOVA post hoc Scheffé tests were completed to identify specific differences between mesocosm types.

Principal component analysis using the covariance matrix was also performed to further assess for microbial catabolic profile

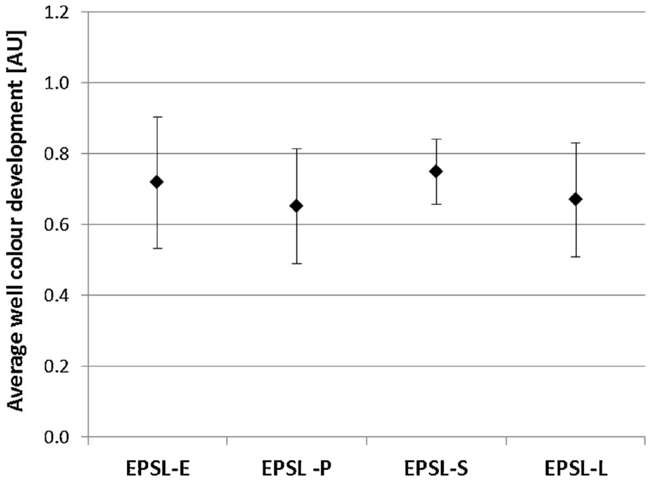

Fig. 3. Average well colour development of the microbial communities from the quadriculture wetland system analyzed from different plant quadrants (E, P, S, L). Error bars represent 1 standard deviation.

differences between mesocosm quadrants. Assessment of normality, homoscedasticity and linear correlations within the data set according to Weber et al. (2007), yielded a recommended Taylor transformation of the data in each case. PCA was completed using Statistica 8 (StatSoft, Tulsa, OK).

3. Results and discussion

Fig. 2 displays a PCA ordination of the CSUPs of the microbial communities from all monoculture mesocosms. Although an overlap of groupings occurs, it can be observed that monoculture wetland systems are generally different from each other. This finding supports the idea that specific plant properties such as root exudation rate and exudate composition, in addition to oxygen transfer rate can help in defining the function of the associated microbial communities.

Despite the observed differences between the microbial communities from the different monoculture mesocosms, when examining the activities (AWCD) of microbial communities from the different quadrants in the polyculture mesocosms no significant difference was observed. Fig. 3 summarizes the average activities (AWCD) for each plant species quadrant for all of the quadriculture mesocosms combined (i.e. from 3 separate systems). A 1-way ANOVA confirms that no significant difference can be seen between plant species within quadricultures. This suggests that water movement and diffusion drivers contributed to mixing of root exudates, and possibly microbial communities themselves, between quadrants resulting in similar microbial communities developing in the different plant species root zones. This result is interesting as water was not forcibly moved in the mesocosms. This finding was consistent across all biculture mesocosms as well (verified not only with AWCD results but also with richness, and PCA ordinations of CSUPs). In all cases no difference could be seen between the microbial community profiles developed in the different plant species quadrants. Given this general finding, further analysis was done grouping all quadrants together giving a mean for each mesocosm.

Fig. 4 displays the AWCD and richness results based on plant species number where each point represents a mesocosm type. A 1-way ANOVA analysis confirmed that there is no significant difference in either AWCD or richness based on plant species number in these mesocosm systems, it can however be seen that large variation for AWCD and richness exists for the monoculture and bi-culture systems, leading to the idea that although plant species number may not have a statistically significant effect, specific plant combinations may have an influence.

Activity (AWCD) comparisons (Fig. 5A) between monoculture mesocosms showed that *L. laevigatum* (L) had the highest catabolic activity with *P. stratiotes* (P) and *Salviniamolesta* (S) having similar catabolic activities followed by *E. crassipes* (E) with which on average had the lowest catabolic activity.

Reviewing the statistical groupings associated with Fig. 5 reveals that only the PS and ES bicultures were significantly different from each other ($p < 0.05$). The following analysis is based on mean values, further work should be completed to verify findings however preliminary analysis is given to help direct future research questions. Examining bicultures containing species E; EP and ES had similar AWCDs, with EL showing a slightly higher mean AWCD value suggesting a positive influence of species L on the biculture microbial communities. In addition, all polycultures (EL, PL, SL, EPSL) containing species L yielded similar AWCDs, which in all cases was slightly lower than the L monoculture mesocosms. L species monoculture had the greatest catabolic activity of the four monocultures. The data suggests that *L. laevigatum* (L) has a positive influence on the microbial community catabolic activity when combined with another plant species.

The PS biculture showed the greatest AWCD of all the bicultures, and was similar to the L monoculture. This was surprising as the S and P monocultures both showed lower catabolic activities than the PS biculture. This suggests that in some cases synergistic effects between plant species can help promote a healthier and more active microbial community, which will then be present for nutrient and organic removal processes in free-floating plant TWs.

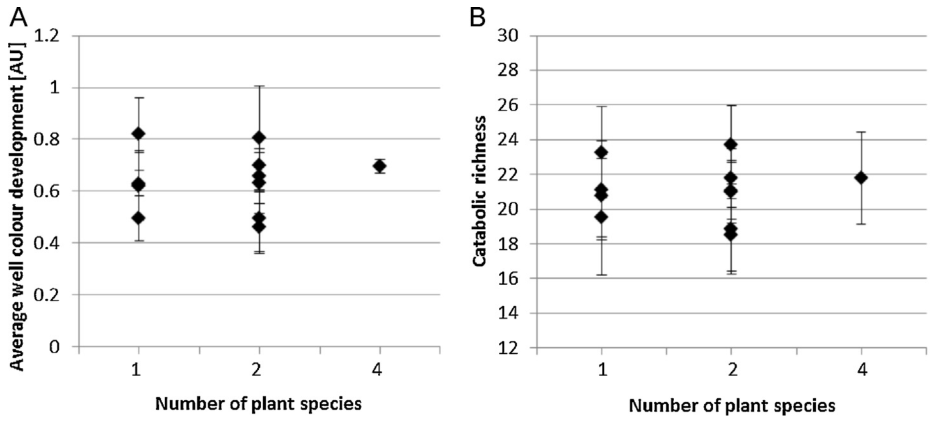

Fig. 4. Average well colour development (A) and catabolic richness (B) of the microbial communities from the monoculture, biculture and quadriculture mesocosms.

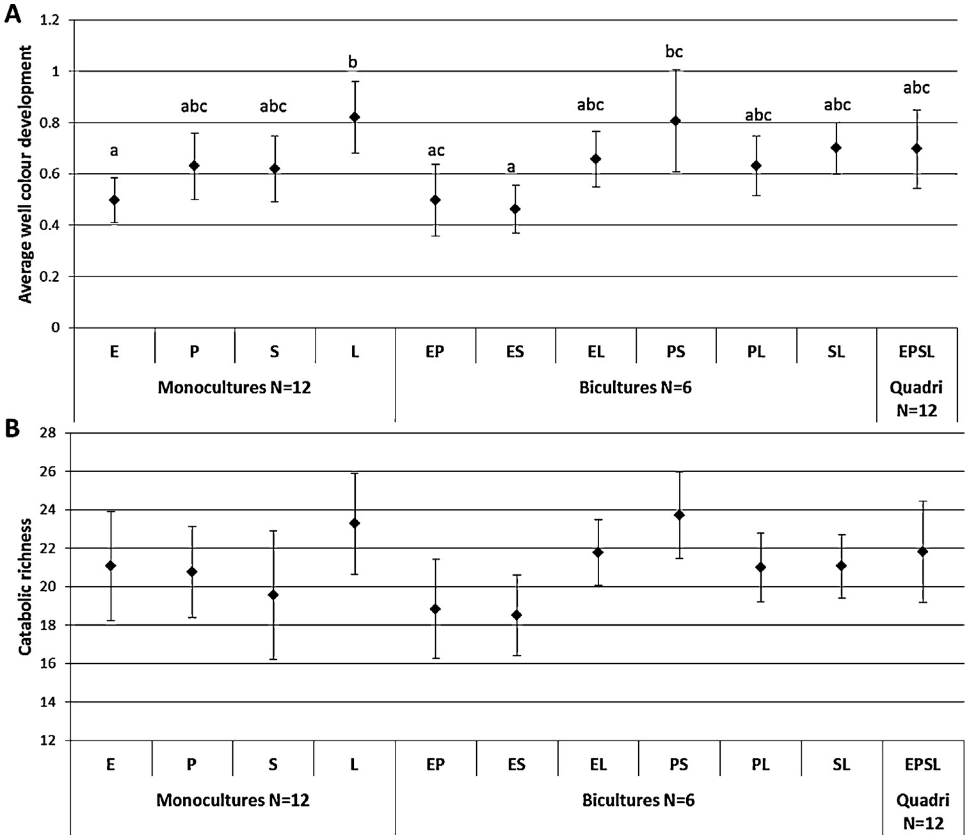

Fig. 5. Catabolic capabilities of the microbial communities from monoculture, biculture, quadriculture wetland systems. (A) Average well colour development (AWCD), and (B) richness. Error bars represent 1 standard deviation. In (A) means with different letters are significantly different (Scheffé test, $p < 0.05$), no significant differences were found in (B). Letters used for abscissa in (A) and (B) represent the plant species arrangements.

Catabolic richness measures the number of different carbon sources used by each microbial community. While AWCD is a robust overall catabolic activity term, catabolic richness is used to describe the potential for a microbial community to treat many different types of contaminants in a wastewater. Fig. 5B presents the richness comparison data for all mesocosm types. A 1-way ANOVA revealed no differences between mesocosm type ($p > 0.05$), therefore the discussion is made based on visual trends rather than significant differences and is only used as a preliminary and partial interpretation. In the investigated monocultures it was found that the richness of systems operated with *L. laevigatum* (L) was higher than systems operated with *S. molesta* (S). The other two monoculture species *P. stratiotes* (P) and *E. crassipes* (E) yielded similar richnesses.

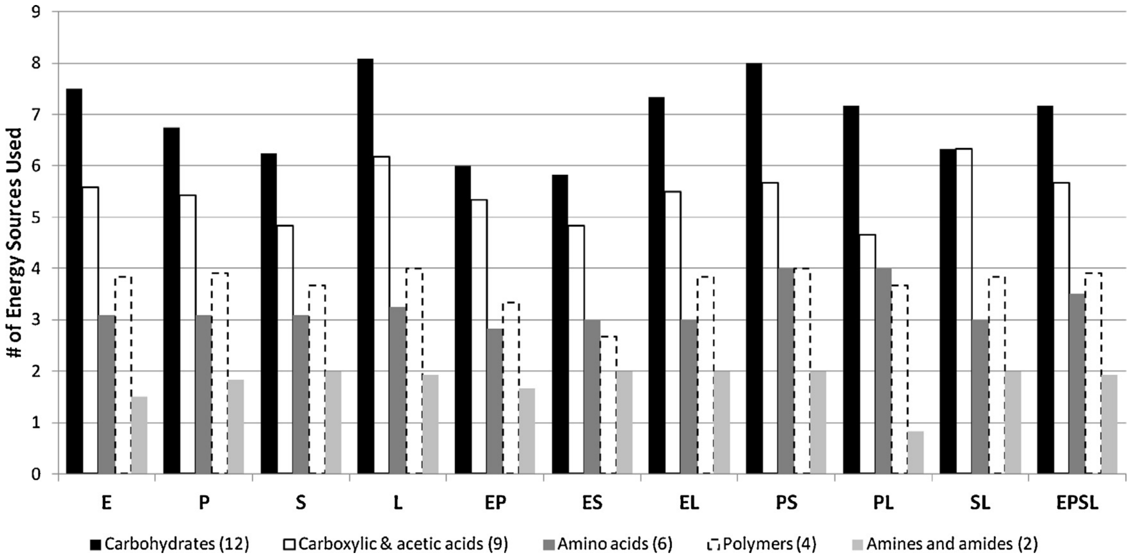

Fig. 6. Carbon source types (guilds) used in all monoculture and polyculture wetland systems ($N = 12$ for monocultures and quadricultures, $N = 6$ for bicultures). The number after the energy source group indicates the number of different sources available on the ECO plate.

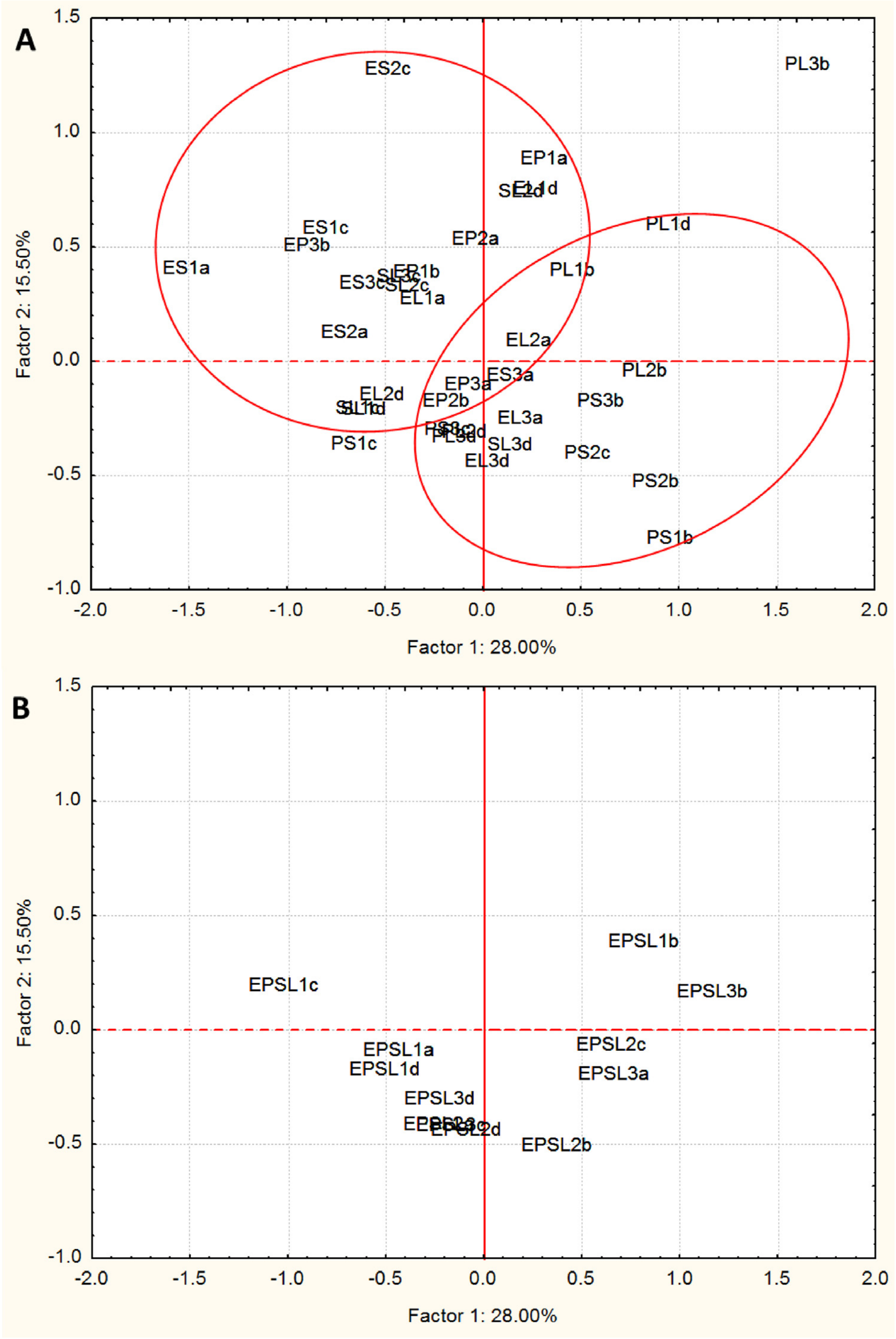

Fig. 7. PCA plot (Taylor Power Transform, slope = 0.9714 for both A and B) of the microbial community carbon source utilization patterns of (A) bicultures, and (B) quadricultures. Objects represent an average of three sample analyses. 1, 2, 3 represent mesocosm replicates, a, b, c, d represent the samples from different quadrants of a specific mesocosm, and L (*Limnobium laevigatum*), S (*Salvinia molesta*), E (*Eichhornia crassipes*), P (*Pistia stratiotes*) represent the macrophyte species. (Factor 1 and factor 2 explain 44% of the overall variability in both PCA plots.)

Similar to the AWCD results, the biculture mesocosms PS had the highest catabolic richness overall, with a further trend of PS > L, EL, EPSL > all the other mesocosms. This indicates that a different microbial community was established when *P. stratiotes* (P) and *S. molesta* (S) were paired and would suggest a broad range of pollutant removal capacity compared to other systems. This result also shows that high catabolic activity and richness can in some cases be associated with a specific combination of plants species suggesting the identity and selection of plants in free-floating plant treatment wetlands to be a relevant factor for design.

Catabolic richness evaluates the total number of carbon sources used and does not differentiate between the five groups (or guilds) of energy sources available on the ECO plate: carbohydrates, carboxylic & acetic acids, amino acids, polymers, and amines & amides (for a complete list see Weber and Legge, 2009). Fig. 6 summarizes the number of carbon sources utilized from each guild in all mono- and polyculture wetland systems and reveals that each monoculture or polyculture has its own carbon source utilization pattern. Although the number of carbon sources utilized was similar between monoculture mesocosms (Fig. 5B), it can be seen here that when comparing specific carbon source groups, the microbial communities from different mesocosms used differing proportions of carbon source types (carbohydrates and carboxylic & acetic acids specifically).

The ECO plate contains 12 carbohydrates, 9 carboxylic & acetic acids, 6 amino acids, 4 polymers, and 2 amines and amides. Fig. 6 shows that the number of carbohydrates utilized was greatest in all mesocosms, except for the biculture pairing with S and L where carbohydrate consumption was equal to carboxylic & acetic acids. However, when comparing utilization of carbohydrates and carboxylic & acetic acids on a percent basis (i.e. normalized to the total available), carboxylic & acetic acids consumption was highest in all mesocosms except in monoculture E and in biculture pairings P and S, and P and L where carbohydrate usage was highest. In a similar respect, all monoculture systems utilized around half (3/6) of the available amino acids and only bicultures with the pairings PS and PL were able to use two thirds (4/6) of the available amino acids. These findings suggest the establishment of a unique microbial community in each mesocosm.

Principal component analysis was used to further investigate potential differences in carbon source utilization patterns between mesocosm microbial communities. Fig. 7A ordinates all bicultures via a PCA using all bicultures (i.e. quadriculture mesocosms excluded here). It was observed that most bicultures containing a macrophyte species E (*E. crassipes*) are pulled to the upper left side of the plot and most bicultures containing a P (*P. stratiotes*) plant (except for EP) are pulled to the lower right. No general trend could be seen for L (*L. laevigatum*) or S (*S. molesta*) species. This suggests that perhaps both E and P have a dominating influence on the microbial community CSUP when used in conjunction with another plant species. The fact that EP was pulled to the left suggests that E dominates the CSUP not only when used in conjunction with S and L, but also when used with P. These ordinations separate the samples based on CSUPs, not catabolic activity or richness as shown in Fig. 5. Where *L. laevigatum* (L) seemed to have a positive influence on polyculture catabolic activities, *E. crassipes* (E) seems to have a dominating effect in defining the CSUP. Fig. 7B presents the quadricultures from the same PCA ordination as used for Fig. 7A. Here it can be seen that the quadricultures are generally found on the interface region between the E and P dominated bicultures, with a greater overlap seen with the P dominated bicultures. The quadricultures were not pulled into the E dominated biculture region of the plot, suggesting that although E can dominate the CSUP for biculture mesocosms once 4 species are used in a single mesocosm a greater complexity is present.

An initial attempt at directly correlating microbial activity to COD removal rate was not successful, however the activity measurements presented here via CLPP are all based on a per volume basis. To gain a full understanding of the water treatment abilities of microbial communities within a treatment wetland both microbial activity and total microbial mass (or an enumeration) is required. In the context of free-floating plant treatment wetlands microbial communities are able to attach to the roots of the macrophyte species, therefore total surface area provided by root mass should give an indication of the maximum microbial mass attainable. In this case COD removal was correlated with an adjusted activity measurement, being the product of the measured overall microbial activity (AWCD) and total root mass within a mesocosm (AWCD * grams of dry root mass). For reference, the average dry root mass for monoculture mesocosms was 365 g for *E. crassipes* (E), 229 g for *P. stratiotes* (P), 84 g for *L. laevigatum* (L), and 61 g for *S. molesta* (S). Fig. 8 presents a plot of all mesocosms studied using this relation. Linear regression ANOVA of this data yields a *p*-value of 0.03 suggesting the linear relationship to be significant. Linear regression analysis yields an R^2 value of 0.43 again suggesting a moderate relationship. It should also be noted that if sample (ES) is removed (visual outlier) from the analysis, an R^2 of 0.67 is realized. Visually, can be seen that the adjusted activity relates to an increase in the COD removal rate, with the monoculture of *E. crassipes* (E) showing the highest COD removal rate. This result was quite interesting as to the best knowledge of the authors no such relationship has ever been verified or presented in the literature. No specific pattern based on the number of plant species in a mesocosm is evident. One outlier (ES) is apparent, however as seen earlier *E. crassipes* (E) seems to have a dominating effect on other species and based on the dry root mass this is understandable where *E. crassipes* (E) develops to a greater extent (365 g for a monoculture mesocosm) than all other plant species studied, and most certainly dominates *S. molesta* (S) on a root mass basis (61 g for a monoculture mesocosm). This again re-emphasizes the importance of careful plant selection in free-floating plant treatment wetlands. It was shown here that using *E. crassipes* whether on its own or combined with *S. molesta* (S) significantly increased water treatment effectiveness, however this was not observed when *E. crassipes* was combined with *L. laevigatum* or *P. stratiotes*.

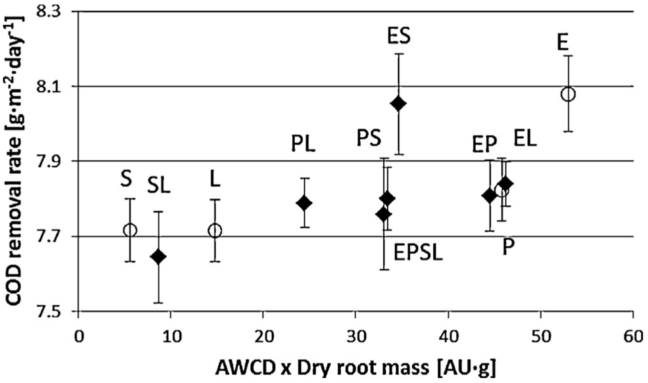

Fig. 8. Relationship between COD removal rate and adjusted microbial activity (AWCD * dry root mass). Error bars represent 1 standard deviation.

Extending the results found here to full scale systems cannot be definitively completed, however a brief extrapolation would suggest that different microbial community profiles and microbial activities could be expected in large patches of different free-floating plant types. Monoculture systems studied here showed different activities and profiles depending on plant type. Although we found the activities and profiles in the different planted quadrants of the polyculture systems to be generally similar, large monoculture patches embedded in full scale polyculture systems would most likely behave similar to the monoculture systems studied here. Larger spatial separations between plant types would reduce the possibility for root exudate or microbial community mixing and transfer. Something worthy of study in full scale polyculture systems would be the characterization of microbial communities in different large monoculture patches, but also at intersection points between monoculture patches where root exudate and microbial community mixing could occur.

4. Conclusions

This study was able to provide insight regarding the function of microbial communities in free-floating plant mesocosm wetlands setup as mono- and polycultures. When examining the monoculture mesocosm results it was found that plant selection had a significant effect on the CSUP and overall activity of the associated microbial communities. It was found that the highest catabolic activity in monoculture systems was found with *L. laevigatum* followed by *P. stratiotes*, *S. molesta*, and *E. crassipes* respectively. Biculture mesocosms containing *P. stratiotes* and *S. molesta* resulted in the highest catabolic activity and catabolic richness from all polycultures. With the exception of the *L. laevigatum* monoculture, polycultures showed either similar or increased catabolic activity and were able to utilize a broader range of carbon sources compared to their monoculture counter parts. These findings further support the hypothesis that plants play a critical role in the development of the microbial communities present in treatment wetlands. Moreover, from these findings it is suggested that polyculture TWs do not necessarily promote the development of microbial communities with a more active and diverse catabolic capability (the case of *L. laevigatum* for example). The number of plant species present was not a perfect predictor of microbial catabolic activity or diversity. Rather, synergistic effects between specific plant species (e.g. mesocosms containing *P. stratiotes* and *S. molesta*) were evident suggesting that plant species selection is an important design parameter for free-floating plant treatment wetlands.

Last, a relationship between COD removal rate and the product of AWCD and dry root mass was observed. This finding suggests that when both microbial activity and microbial community density or size is accounted for pollutant removal rates in treatment wetland mesocosms of this type can be loosely predicted. Here it was observed that the plant monoculture with the lowest activity (*E. crassipes*) provided the best COD removal rates due to the largest amount of root mass which provided a larger surface area for microbial community attachment.

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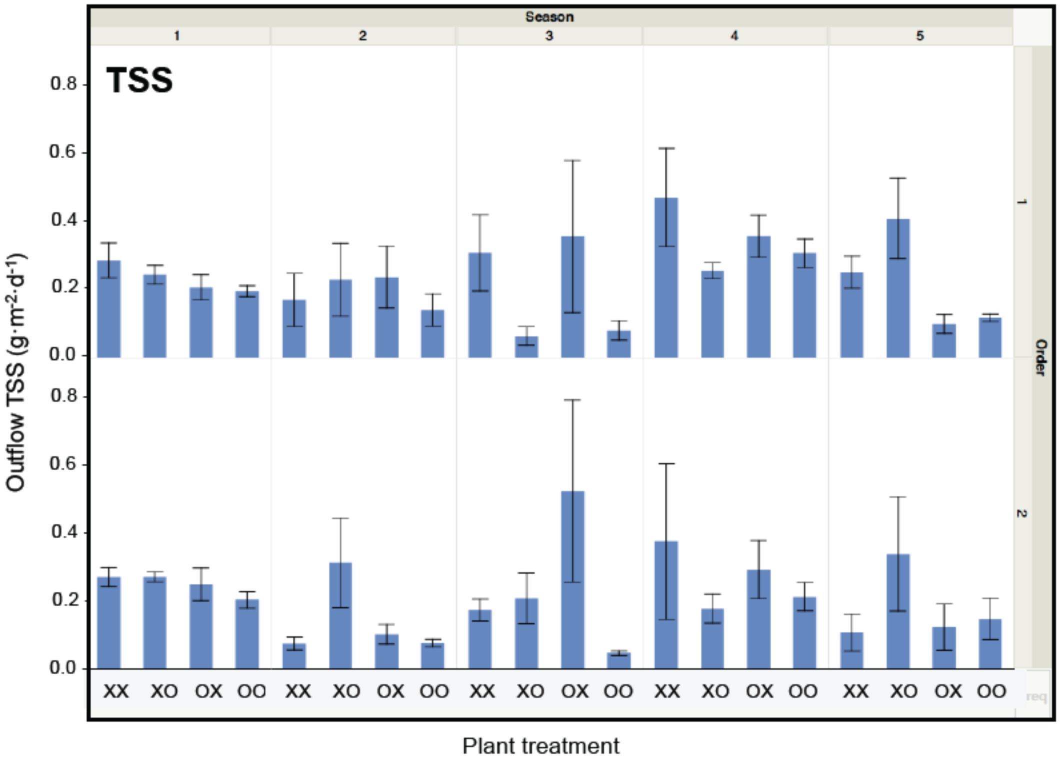
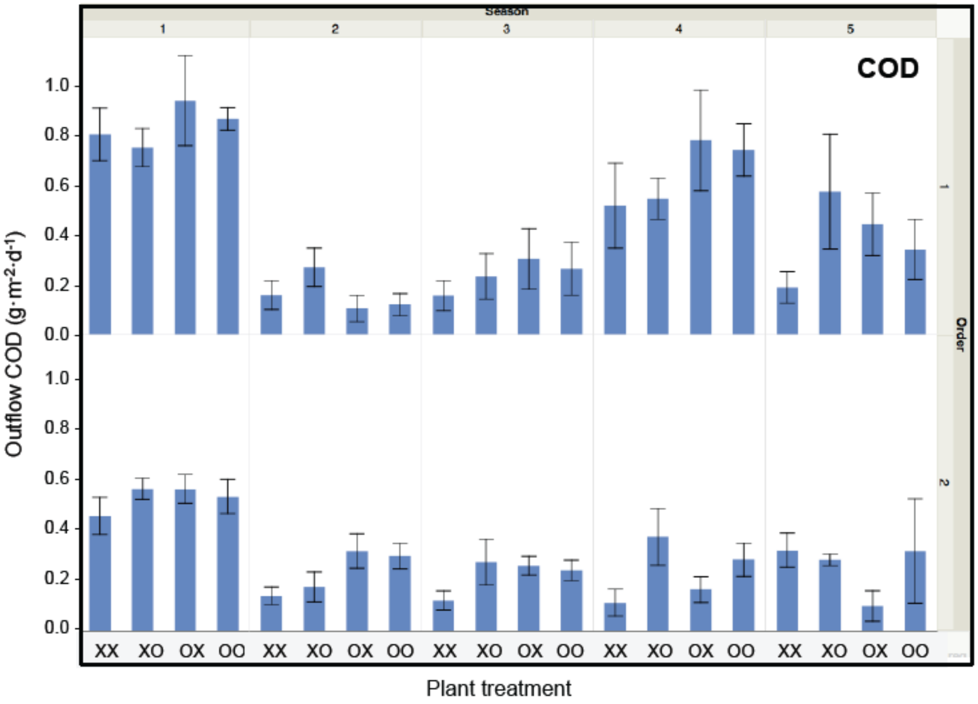
8.2 ANNEX 2

Year-round TSS, COD, TP, NH_4-N and NO_3-N in $gm^{-2} \cdot d^{-1}$, measured at the outflow of mesocosms 1 (top) and 2 (bottom).

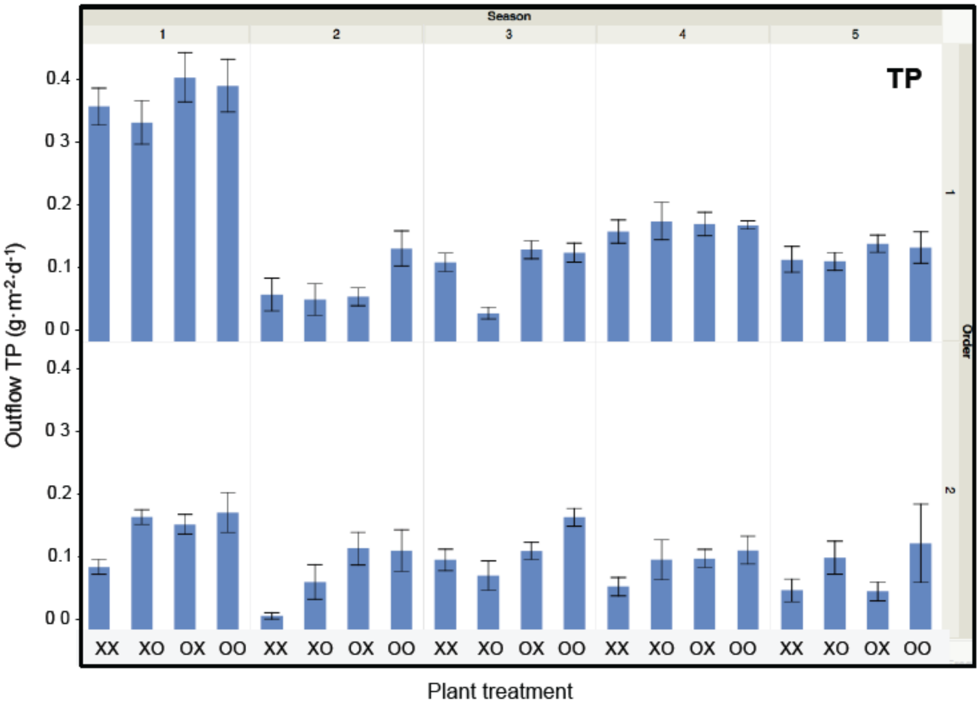
Bar graphs (error bar) showing data distribution of different plant combinations grouped per season (8 sampling periods through the year). (XX, XO, OO, OX; X for *Phragmites* and O for *Phalaris*).



1: Summer, 2012; 2: Autumn, 2012; 3: Winter, 2013 4: Spring, 2013 5: Summer, 2013



1: Summer, 2012; 2: Autumn, 2012; 3: Winter, 2013 4: Spring, 2013 5: Summer, 2013



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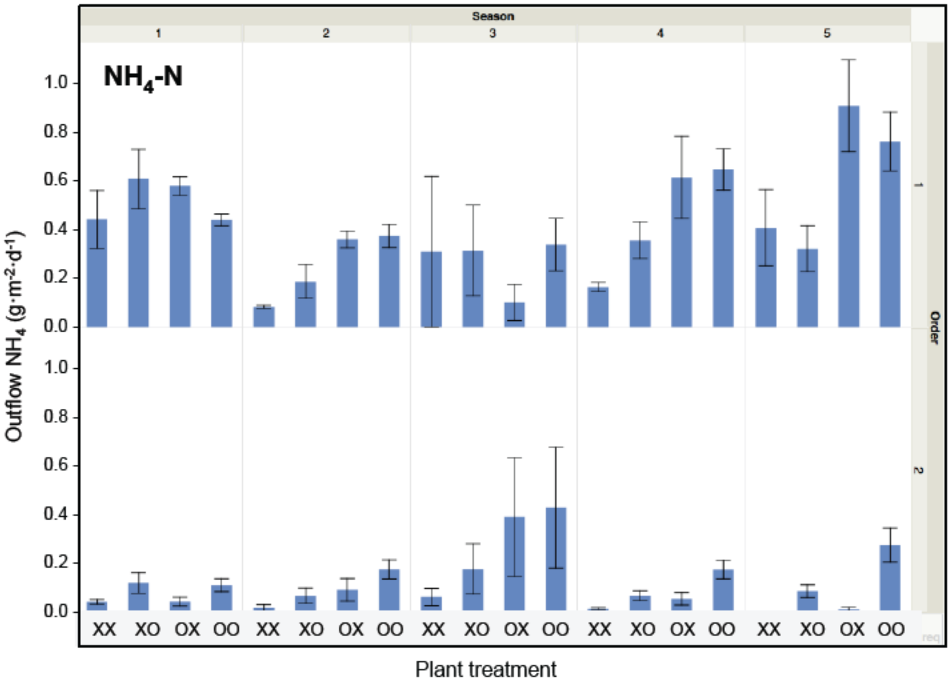
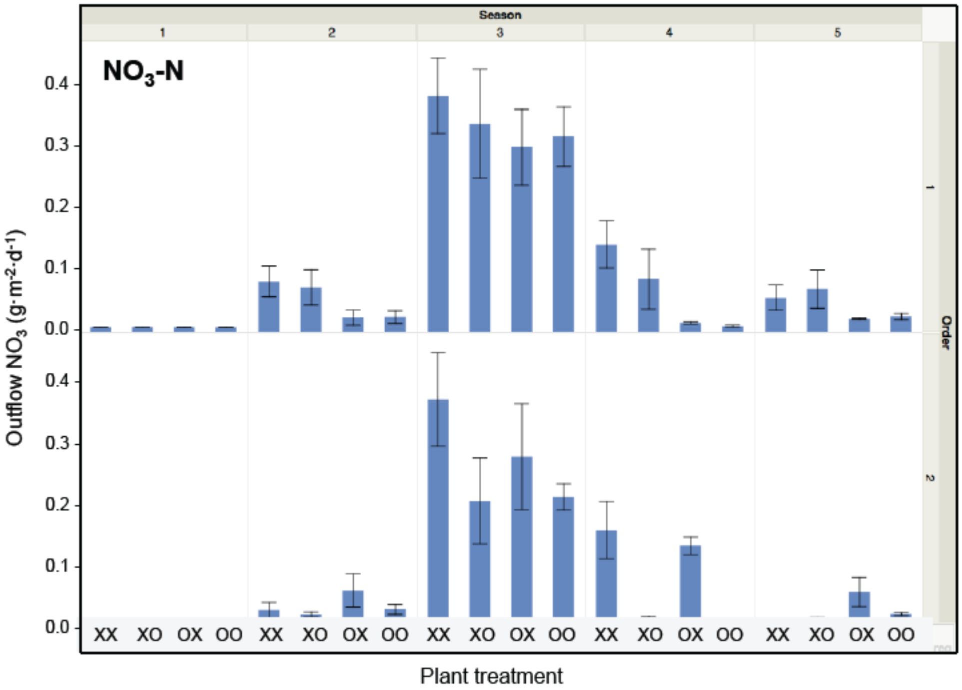


1: Summer, 2012; 2: Autumn, 2012; 3: Winter, 2013 4: Spring, 2013 5: Summer, 2013



1: Summer, 2012; 2: Autumn, 2012; 3: Winter, 2013 4: Spring, 2013 5: Summer, 2013