UNIVERSITAT DE BARCELONA

# The functional role of emergent macrophytes in nature-based solutions (NBS) aiming to mitigate nutrient loading in freshwater ecosystems

## *El paper funcional dels macròfits emergents en solucions basades en la natura (SBN) per mitigar la càrrega de nutrients en ecosistemes d'aigua dolça*

Myrto-Georgia Nikolakopoulou

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**TESI DOCTORAL**

**Universitat de Barcelona**
**Facultat de Biologia — Departament d'Ecologia**
Programa de Doctorat en Ecologia, Ciències Ambientals i Fisiologia vegetal

# THE FUNCTIONAL ROLE OF EMERGENT MACROPHYTES IN NATURE-BASED SOLUTIONS (NBS) AIMING TO MITIGATE NUTRIENT LOADING IN FRESHWATER ECOSYSTEMS

## El paper funcional dels macròfits emergents en solucions basades en la natura (SBN) per mitigar la càrrega de nutrients en ecosistemes d'aigua dolça

Memòria presentada per Myrto - Georgia Nikolakopoulou
per optar al grau de doctora per la Universitat de Barcelona

**MYRTO – GEORGIA NIKOLAKOPOULOU**

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*Στους γονείς μου, Τρωάδα και Δημήτρη*

*To my parents*

*"I believe that there is a subtle magnetism in Nature, which, if we unconsciously yield to it, will direct us aright."*

Henry David Thoreau, 1861 "Walking"

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Warning: The "Acknowledgements" section contains inappropriate scientific language, with vague, ambiguous, and unprecise sentences. Several lengthy generalizations are also present.

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# ADVISORS’ REPORT

Dr. Francesc Sabater and Dr. Alba Argerich, advisors of the PhD thesis entitled “The functional role of emergent macrophytes in nature-based solutions (nbs) aiming to mitigate nutrient loading in freshwater ecosystems",

CERTIFY that, PhD candidate Myrto - Georgia Nikolakopoulou has carried out the present doctoral dissertation by substantially contributing to the four studies presented (Chapters 2, 3, 4, and General Discussion). Her contribution included research objectives formulation, field experiment performance, chemical analyses, data analyses, statistical analyses, interpretation of results, manuscript writing, revision and editing during the publication process.

INFORM that, none of the information contained here has been used in any other PhD theses.

Below, we detail the publication status of the chapters and indicate the impact of the journals where the chapters have been published or submitted.

**Chapter 2 (Published):**

“Emergent Macrophyte Root Architecture Controls Subsurface Solute Transport”

**Authors:** Myrto Nikolakopoulou, Alba Argerich, Jennifer D. Drummond, Esperança Gacia, Eugènia Martí, Albert Sorolla, and Francesc Sabater

**Journal:** *Water Resources Research*

**Impact factor:** 4.14

**Reference:** Nikolakopoulou, M., Argerich, A., Drummond, J. D., Gacia, E., Martí, E., Sorolla, A., & Sabater, F. (2018). Emergent macrophyte root architecture controls subsurface solute transport. Water Resources Research, 54. https://doi.org/10.1029/2017WR022381

**<u>Chapter 3 (In peer review):</u>**

“Effect of Three Emergent Macrophyte Species on Nutrient Retention in Aquatic Environments Under Excess Nutrient Loading”

**Authors:** Myrto Nikolakopoulou, Alba Argerich, Susana Bernal, Esperança Gacia, Miquel Ribot, Eugènia Martí, Albert Sorolla, and Francesc Sabater

**Journal:** *Environmental Science & Technology*

**Impact factor:** 7.86

**<u>Chapter 4 (Published):</u>**

“Enhancement of carbon and nitrogen removal by helophytes along subsurface water flowpaths receiving treated wastewater”

**Authors:** Miquel Ribot, Susana Bernal, Myrto Nikolakopoulou, Timothy N. Vaessen, Joaquín Cochero, Esperança Gacia, Albert Sorolla, Alba Argerich, Francesc Sabater, Manel Isnard, and Eugènia Martí

**Journal:** *Science of the Total Environment*

**Impact factor:** 6.55

**Reference:** Ribot, M., Bernal, S., Nikolakopoulou, M., Vaessen, T. N., Cochero, J., Gacia, E., et al. (2017). Enhancement of carbon and nitrogen removal by helophytes along subsurface water flowpaths receiving treated wastewater. Science of the Total Environment, 599-600, 1667–1676. https://doi.org/10.1016/j.scitotenv.2017.05.114

The work of the PhD candidate described above, grants her the right to defend her PhD thesis in front of a scientific committee.

*Barcelona, 29 September 2020*

Dr. Francesc Sabater

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# ABSTRACT

Freshwater degradation has been largely attributed to excess nutrient concentrations of anthropogenic origin. Freshwater degradation in combination with water scarcity are the reason of severe stress on water resources globally. As one third of the world's population does not have access to clean drinking water, nutrient pollution is imperative to be addressed. Nature-based solutions (NBS) is a recent concept to address several societal challenges, using techniques inspired by nature, or by nature itself, while preserving ecosystem sustainability. Water security is among the essential societal challenges that NBS can address, with nutrient pollution of freshwater systems being one of its primary aspects. While NBS projects aiming to address nutrient loading in freshwater systems, conspicuously include emergent macrophytes in their design, there is a lack in mechanistic understanding of how these aquatic plants enhance water treatment performance.

In the present doctoral thesis, we focused on bridging this knowledge gap by investigating the plants' physical, chemical and biological influence on the subsurface aquatic environment and the implications for nutrient mitigation. Specifically, we examined subsurface solute transport and nutrient retention under the presence of three emergent macrophyte species; *Iris pseudacorus* L., *Phragmites australis* L., and *Scirpus lacustris* L., while we also explored the influence of dissolved organic carbon (DOC) quality on nitrogen cycling. To this aim, we performed a series of three studies in a setup of 12 artificial flumes, where we used traditional methods of stream ecology (i.e. pulse additions of conservative and reactive tracers) to characterize subsurface solute transport and to estimate nutrient spiraling metrics. Likewise, we experimentally modified DOC lability in the flumes, and we characterized root system architecture of the used emergent macrophytes.

Regarding the physical effect of macrophytes, our results showed that macrophytes root architecture can substantially influence subsurface solute

transport. Specifically, a dense root system of fine roots results in increased hydraulic retention, while an architecture of thicker roots leads to inverse results, facilitating infiltration. Further, we found that the chemical and biological influence of emergent macrophytes on the subsurface aquatic environment is species specific, and depends on physiological differences between the species, and on the plants' capacity to create long residence times in the subsurface. Additionally, the presence of emergent macrophytes was found to facilitate N removal, while the availability of a labile C source mostly increased microbial respiration, rather than denitrification.

Finally, in the general discussion of the thesis, we discuss the above results and further synthesized them with information from the literature, within the context of NBS.

Overall, the present doctoral thesis elucidates some unclear aspects of the functional role of emergent macrophytes in NBS aiming nutrient mitigation, highlight the importance of suitable macrophyte species selection in NBS systems to optimize treatment performance, and provides valuable guidelines to NBS practitioners for successful macrophyte species identification.

# RESUM

La degradació dels ecosistemes aquàtics s'ha atribuït, en gran mesura, a l'excés de concentracions de nutrients d'origen antropogènic. Aquesta degradació, combinada amb l'escassetat d'aigua, és la principal causa de l'alarmant problemàtica dels recursos hídrics a nivell global. Donat que un terç de la població mundial no té accés a aigua potable, es fa imprescindible abordar la problemàtica de la contaminació per nutrients. El concepte de solució basada en la natura (SBN) ha aparegut recentment per designar aquelles tècniques inspirades en la natura que aborden diversos reptes socials tot preservant la sostenibilitat dels ecosistemes. La seguretat de poder disposar d'aigua es troba entre els desafiaments socials més importants que avui dia afronten les SBNs, essent la contaminació per nutrients en ecosistemes aquàtics un dels seus principals objectius. Si bé els SBN destinats a abordar la problemàtica de la càrrega de nutrients en sistemes aquàtics incorporen macròfits emergents en el seu disseny de forma rutinària, hi ha una manca de coneixement dels mecanismes que expliquen com aquestes plantes aquàtiques milloren el tractament de les aigües.

En aquesta tesi doctoral, ens hem proposat reduir aquesta mancança del coneixement investigant com els trets físics, químics i biològics de les plantes influeixen en el medi aquàtic subsuperficial i com afecten la mitigació de nutrients. Concretament, hem examinat el transport de soluts a la subsuperficie i la retenció de nutrients sota la presència de tres espècies de macròfits emergents; *Iris pseudacorus* L., *Phragmites australis* L. i *Scirpus lacustris* L.. Addicionalment, hem explorat la influència de la qualitat del carboni orgànic dissolt en el cicle del nitrogen. Per portar a terme aquest objectiu, es van realitzar una sèrie de tres treballs dissenyats amb una mateixa configuració de 12 canals artificials experimentals, i on es van aplicar mètodes tradicionals de l'ecologia fluvial (és a dir, addicions sobtades de traçadors conservatius i d'elements reactius) per poder caracteritzar el transport subsuperficial de soluts i estimar les mètriques de l'espiral de nutrients. Igualment, es va modificar

experimentalment el grau de labilitat del carboni en el sistema de canals, i es va caracteritzar l'arquitectura del sistema radicular d’aquests tres macròfits emergents.

Quant a l’efecte físic dels macròfits, els resultats demostren que l’arquitectura radicular dels macròfits influeix substancialment en el transport subsuperficial dels soluts. Concretament, un sistema dens d’arrels fines dóna lloc a una major retenció hidràulica, mentre que una arquitectura d’arrels més gruixudes condueix a resultats inversos, donat que faciliten la infiltració a nivell intersticial. A més, hem constatat que la influència química i biològica dels macròfits emergents en el medi subsuperficial aquàtic és específica de cada espècie, i depèn de les diferències fisiològiques entre elles, i de la capacitat de les plantes per crear major temps de residència en el medi intersticial. Addicionalment, hem provat que la presència de macròfits emergents facilita l’eliminació de nitrogen, mentre que la disponibilitat d’una font de carboni làbil fa augmentar la respiració aeròbica microbiana en lloc de la desnitrificació.

Finalment, a la discussió general de la tesi, es discuteixen els resultats obtinguts i es sintetitzen dins del context de les SBN amb informació extreta de la bibliografia.

En general, la present tesi doctoral posa de manifest aspectes poc coneguts sobre el paper funcional dels macròfits emergents quant a la reducció de nutrients en les SBNs. També posa en relleu la importància de fer una selecció adequada d’espècies de macròfits per a sistemes on s’apliquen SBN a fi d’optimitzar el tractament d’aigües; i proporciona valuoses pautes per a professionals que apliquen SBNs per tal d’identificar les espècies de macròfits més apropiades per la reducció de nutrients.

# CONTENTS

# LIST OF FIGURES

# LIST OF TABLES

# CHAPTER ONE

# General Introduction

## 1.1. Sources of nutrient loads in freshwater systems

Freshwater degradation has been largely attributed to excess nutrient concentrations of anthropogenic origin, leading to problems associated to eutrophication (Carpenter et al., 1998). Eutrophication describes the excess growth of algae, attributed to nutrient enrichment in aquatic ecosystems, with consequences in ecosystem and public health (European Commission, 2002). N and P loads in inland lotic ecosystems, primarily owing to agriculture and urbanization, are being transferred downstream expanding eutrophication problems to estuarine ecosystems (Carpenter et al., 1998). Oxygen decrease in coastal areas, or in the open ocean (Figure 1.1) is one of the primary consequences of eutrophication, threatening marine fisheries productivity, biodiversity and, global biogeochemical cycles (Breitburg et al., 2018). Predictions show that the current rate of anthropogenic nutrient loading could result to an oxygen-deficient ocean in the next thousand years (Watson, 2016).

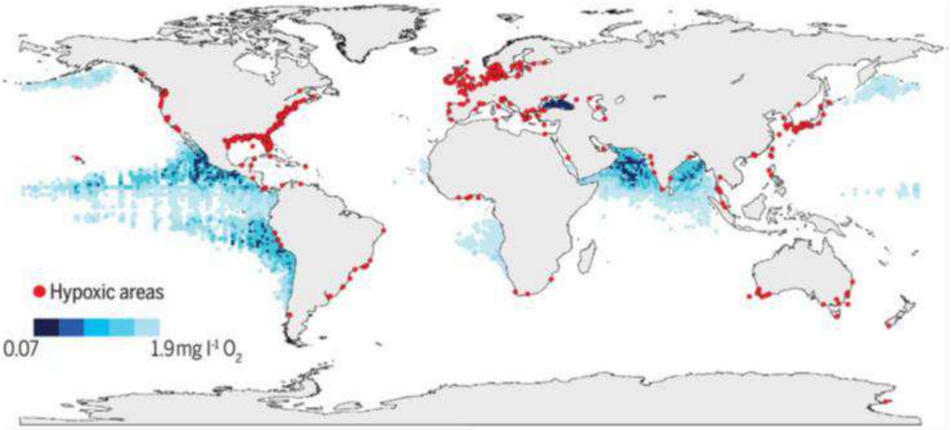


**Figure 1.1.** The global map of coastal sites, where anthropogenic nutrients have exacerbated or caused "dead zones" (i.e. hypoxic zones of oxygen concentrations < 2 mg L-1), shown as red dots (provided from Breitburg et al., 2018)

Agriculture, the largest land use on Earth occupying 37% of the Earth's surface, is considered the main source of the excess nutrient inputs to freshwater systems. Since the beginning of the Green Revolution in the 1960s, usage of N and P fertilizers increased by 1000% and 350% respectively ( FAO – Food and agriculture organization of the United Nations, 2020; Foley et al., 2011; Tilman et al., 2001), causing a major disturbance on the global N and P cycles. Fertilizer use and production, manure application, and cultivation of N fixing crops, activities that increase the bioavailable N quantity converted from abundant atmospheric N, dramatically increased N inputs to freshwater ecosystems (Matson, Parton, Power, & Swift, 1997; Vitousek, Aber, et al., 1997; Vitousek, Mooney, Lubchenco, & Melillo, 1997). Increased P inputs are mainly attributed to P-rich deposits mining, providing the raw material in fertilizer and detergent industry (Bennett, Carpenter, & Caraco, 2001; Matson et al., 1997).

Agriculture may be the predominant cause of freshwater impairment, but not the sole one. Urbanization is closely following as the second most important cause of freshwater degradation (Paul & Meyer, 2001). Despite the minor terrestrial surface that urban areas occupy as compared to agriculture, urbanization is a driver of environmental change (Grimm et al., 2008). 56% of the Earth's population (United Nations, 2020) is currently gathered in urban areas, producing, consuming, and disposing waste (Grimm et al., 2008) and this trend is projected to keep increasing (Figure 1.2). Such concentrated human activity negatively impacts local and global biogeochemical cycles by increasing nutrient concentrations in aquatic ecosystems, primarily via urban runoff and wastewater discharge (Grimm et al., 2008; Paul & Meyer, 2001).

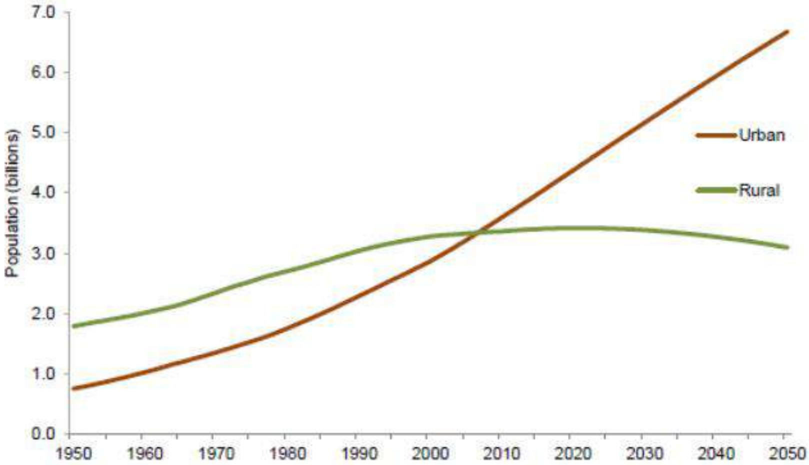


**Figure 1.2.** Urban and rural populations of the world, 1950-2050 (provided by UN – United Nations, 2019)

At the same time, agricultural, industrial, and household activities are the main consumers of water resources, contributing to physical water shortage globally (FAO – Food and agriculture organization of the United Nations, 2018). In a planet where one third of its population does not have access to clean drinking water (WHO – World Health Organization, UNICEF, 2019), the need for nutrient pollution management to protect water resources is imperative.

## 1.2. Nature-based Solutions (NBS): The concept

Water treatment is currently achieved by several conventional methods that are costly and do not promote ecosystem sustainability (Kinidi & Salleh, 2017). Alternatively, nature-based solutions is a recent concept, emerged during the last decade (IUCN - International Union for Conservation of Nature, 2016), which advocates that several societal challenges, including water quality improvement, can be addressed using techniques inspired by nature, or by nature itself. The NBS concept is young and still needs to be framed (IUCN - International Union for Conservation of Nature, 2016). However, it is viewed as the response to the United Nations 2030 Agenda for Sustainable Development call for "making cities and human settlements inclusive, safe, resilient, and sustainable" (Sustainable Development Goal [SDG] 11, (Song et al., 2019)). Therefore, not surprisingly, it has drawn a continuously increasing research interest over the years (Figure 1.3).

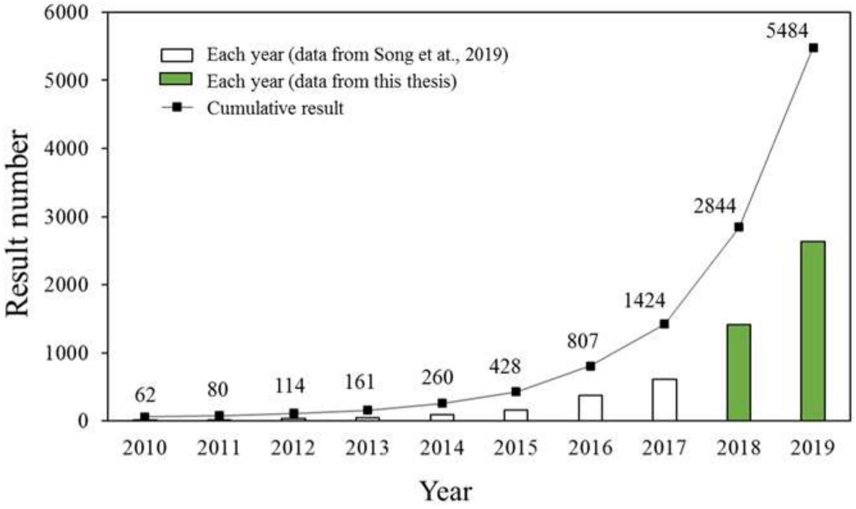


**Figure 1.3.** Temporal trend of academic literature on NBS, based on Google Scholar search of the full term "nature based solutions" (adapted from (Song et al., 2019)

The International Union for Conservation of Nature (hereafter as IUCN) defines nature-based solutions as "actions to protect, sustainably manage and restore natural or modified ecosystems that address societal challenges effectively and adaptively, simultaneously providing human well-being and biodiversity benefits" (IUCN - International Union for Conservation of Nature, 2016). A similar definition for NBS is given by the European Commission: "Living solutions inspired by, continuously supported by, and using Nature, designed to address various societal challenges in a resource efficient and adaptable manner and to provide simultaneously economic, social and environmental benefits" (Maes & Jacobs, 2017). Nevertheless, these definitions may fit to several ecosystem-related concepts and approaches preexisting to NBS. Some of these concepts are "Ecological Engineering", "Green/Blue infrastructure", "Ecosystem Approach", "Ecosystem-based Adaptation//Mitigation", "Ecosystem Services", and "Natural Capital" (Nesshover et al., 2017). Therefore, the NBS concept should be viewed as an umbrella that includes several approaches that have in common one overarching goal: to address societal challenges by relying upon nature. According to IUCN, these ecosystem-based approaches fall within five categories: 1) restoration approaches, 2) issue-specific, 3) infrastructure, 4) management, and 5) protection, which constitute the basis to achieve the following societal challenges: 1) climate change mitigation, 2) food security, 3) water security, 4) disaster risk reduction, 5) human health, and 6) economic and social development. The diagram in Figure 1.4 provided by IUCN depicts the above conceptualization of the NBS concept. Whereas approaches and concepts describe the theoretical foundation of the NBS concept, specific applications and strategies that embrace the NBS approaches represent the actions taken to achieve the set goals.

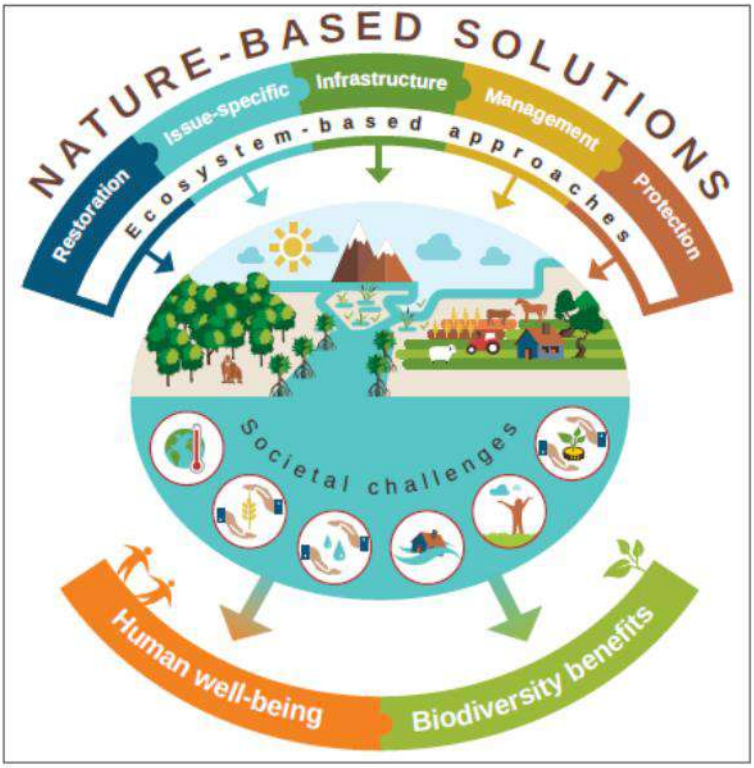


**Figure 1.4.** NBS as an umbrella term for ecosystem-related approaches, through which societal challenges are to be addressed (provided by IUCN - International Union for Conservation of Nature, 2016)

The present thesis is particularly focused on the use of NBS to achieve the crucial challenge of water security. Water storage, filtration, transportation, nutrient cycling, and flood prevention by inland water bodies are some of the known ecosystem services contributing to water security (IUCN - International Union for Conservation of Nature, 2015). Implementation of water NBS can preserve or restore these ecosystem services in natural environments or mimic them in built infrastructure.

## 1.3. The involvement of emergent macrophytes in NBS

Several ecological practices fulfilling the NBS criteria employ the use of vegetation. From stream restoration applications, to stormwater bioretention systems, constructed wetlands, and obviously, phytoremediation practices, vegetation is conspicuously used. In water-related applications either in natural, or in artificial sites, vegetation includes emergent macrophytes, shrubs, and trees as typical riparian plant types. Applications that employ the presence of vegetation, usually fall within the NBS concepts of Ecological Restoration, Green/Blue Infrastructure, and Ecosystem Services. In river restoration practices aiming to restore ecological functions (i.e. functional restoration), vegetation is considered a critical functional ecosystem feature, therefore planting emergent macrophytes or other riparian species is a common practice (Bernhardt & Palmer, 2011; Palmer, Hondula, & Koch, 2014). Green infrastructures for stormwater management and wastewater treatment (i.e. bioretention systems and constructed wetlands) rely on the presence of vegetation for their functioning and treatment performance (e.g. Payne, Fletcher, Cook, Deletic, & Hatt, 2014; Vymazal, 2011). Emergent macrophytes is the typical vegetation type used in constructed wetlands, however species adapted to only seasonal waterlogging are mostly appropriate for bioretention systems (Payne et al., 2014). Nevertheless, emergent macrophytes are used as well (Leroy et al., 2016).

Common goals of water related NBS, such as functional restoration, stormwater bioretention systems, and constructed wetlands, are water quality improvement, erosion control, flood risk reduction, preservation of biodiversity, and recreation.

These goals can be further categorized into three groups, for the improvement of the ecological quality of the ecosystems, of the physical environment, and for the provision of recreational opportunities. The involvement of emergent macrophytes in NBS with varying goals, ultimately reveals their central role in human well-being. Figure 1.5 illustrates the central role that macrophytes hold in NBS.

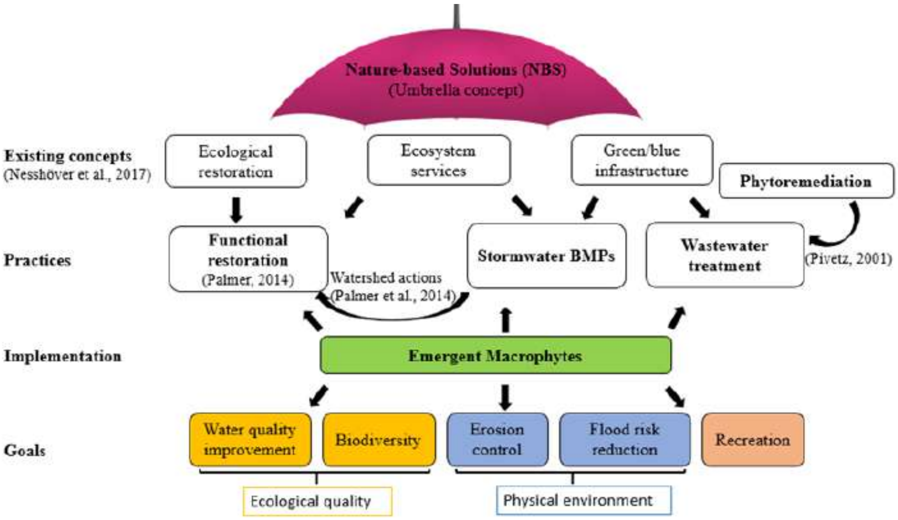


**Figure 1.5.** The involvement of macrophytes in NBS

## 1.4. Nutrient retention mechanisms

The inherent ability of healthy freshwater ecosystems, particularly of streams, to retain and remove nutrients via biogeochemical processing is generally termed nutrient retention. However, the distinction between nutrient retention and removal requires the identification of the specific processes involved. Throughout this thesis we will use the term nutrient retention, unless nutrient removal is evidenced. Nutrient retention reduces the nutrient loads to be transported to downstream ecosystems and thus, it is considered an essential ecosystem service (Grimm et al., 2005). N and P are cycled between the abiotic and the biotic environment of inland waters, with concurrent chemical transformation into various forms, resulting to temporary storage (i.e. retention), or ultimately, permanent removal from the system. Nutrient retention may occur via 1) plant and microbial assimilation, 2) microbial processes, and 3) sorption onto sediments and chemical precipitation (e.g., Hejzlar et al., 2009; von Schiller, Bernal, Sabater, & Marti, 2015).

Especially for nitrogen, denitrification, the biogeochemical process that converts nitrate into gaseous forms of nitrogen, is the principal nitrogen removal process (Gersberg, Elkins, & Goldman, 1983; Tanner, Kadlec, Gibbs, Sukias, & Nguyen, 2002). As a heterotrophic process, the availability of dissolved organic carbon (DOC) is a limiting factor for denitrification in aquatic ecosystems (e.g., Hill, Devito, Campagnolo, & Sanmugadas, 2000). Nevertheless, high DOC quality has been found capable to drive denitrification in streams and wetlands as well (Fernandez-Nava, Maranon, Soons, & Castrillon, 2010; J. P. Zarnetske, R. Haggerty, S. M. Wondzell, & M. A. Baker, 2011). Other important microbial processes leading to N retention are nitrification and anaerobic ammonium oxidation (anammox). Microbial assimilation is an important retention mechanism for both N and P, however P mainly relies on abiotic retention (Reddy, Kadlec, Flaig, & Gale, 1999).

Hydrology is among the main factors that influence microbially mediated nutrient retention. As evidenced by research in streams, hydrologic retention provides increased contact times between sediments and solutes, critical for biogeochemical processing. A hotspot of biogeochemical processing in streams is the hyporheic zone, as is termed the portion of streambed sediments where stream surface water and groundwater intersect (e.g., Bencala, 1993). Solute transport though the hyporheic zone is characterized by long residence times, leading to enhanced nutrient cycling (Boulton, Findlay, Marmonier, Stanley, & Valett, 1998).

The ability of aquatic vegetation to uptake and store N and P into their tissues is well acknowledged, as evidenced by the widespread use of rooted macrophytes for wastewater treatment (Vymazal, 2011). Plant nutrient uptake depends on the growth rate, maturity, and physiology of the plants, as well as on nutrient availability (Gacia et al., 2019; Payne et al., 2014). Nevertheless, it is generally accepted that plant uptake accounts only for a small fraction of nutrient retention, compared with microbially-mediated nutrient retention (Korboulewsky, Wang, & Baldy, 2012; Meuleman, van Logtestijn, Rijs, & Verhoeven, 2003). This question therefore arises: "Why are macrophytes employed in the design of infrastructure targeting nutrient attenuation?" The answer can be found in the indirect effects of macrophytes on nutrient retention. Emergent macrophytes root systems provide excellent habitat to microbial assemblages due to their morphological and physiological characteristics. Root surface area serves as microbial attachment site, and together with root oxygen release and labile carbon exudation, create ideal conditions for coupled nitrification-denitrification (Brix, 1994; Reddy, Patrick, & Lindau, 1989). Such plant traits vary among different plant species, causing a species-specific effect on nutrient retention.

Currently, there is a lack of mechanistic understanding about how emergent macrophytes contribute to nutrient retention, especially regarding the varying effect among different macrophyte species (Read, Fletcher, Wevill, & Deletic, 2010). Exploration of the connections between the driving factors of nutrient retention (i.e. macrophytes, hydrology, DOC lability), and identification of effective plant species based on specific traits, will contribute to optimization of NBS design for enhanced treatment performance.

# GENERAL OBJECTIVES

This doctoral thesis aims to improve the mechanistic understanding of the role of emergent macrophytes on NBS projects in freshwater ecosystems targeting nutrient pollution. As outlined in the introduction, macrophytes are extensively used in NBS, aiming to ensure water security. Nevertheless, there is a gap in knowledge about the specific macrophyte functions, which promote nutrient mitigation. Motivated to elucidate some of the unclear aspects of the topic in question, and inspired by the vision of emergent macrophytes as ecological engineers (Jones, Lawton, & Shachak, 1997), the focus of the present thesis lies on the investigation of the plants' physical, chemical and biological influence on the subsurface aquatic environment and the implications of this on nutrient loading mitigation.

Specifically, we answer the following questions:

1) Do macrophytes alter subsurface solute transport by physically harnessing the streambed, and if so, how is solute transport altered?

2) What implications would solute transport modification have in nutrient retention?

3) Would nutrient retention vary across different macrophyte species?

4) Could we easily identify the most effective species, based on plant-specific traits?

5) How would the interaction between the presence of emergent macrophytes and carbon lability (as a factor known to control denitrification) influence nutrient retention?

To answer these questions, we worked at the mesocosm scale, performing a series of three experiments in a setup of 12 artificial flumes. This experimental setup allows some control on the environmental factors facilitating hypothesis testing, while maintaining a high level of complexity similar to what one may find in nature. Therefore, the outcomes of the experiment transfer to natural environments is easier as compared to fully controlled laboratory experiments. Each experiment is an independent study, comprising a chapter of the present thesis as described below.

The first chapter answers question 1 and is entitled “Emergent macrophyte root architecture controls subsurface solute transport”. The chapter explores the role of emergent macrophyte roots in subsurface solute transport in fluvial systems. Solute transport has been characterized as a key influence on ecological processes, and thus water quality. To our knowledge, the role of emergent macrophyte roots has been neglected in subsurface solute transport models, because of the practical difficulties to collect root data. We expect that a) roots will act as structures that can create heterogeneities in the sediment (physical role); thus, root architecture will alter subsurface flow paths; b) roots will remove water via evapotranspiration (biological role), leading to slower flow velocity; and c) both scenarios will result in longer water residence times.

The second chapter answers questions 2, 3, 4, and is entitled “The effect of three emergent macrophyte species on nutrient retention in aquatic environments of excess nutrient conditions”. This chapter investigates the importance of the selection of appropriate emergent macrophyte species for the successful implementation of phytoremediation strategies aiming to address eutrophication problems in freshwater ecosystems. Additionally, the study complements the findings of Chapter 1, by exploring the relationship between subsurface hydrological patterns, attributed to different macrophyte root architectures, and nutrient retention. We hypothesize that macrophyte roots would influence pore water chemistry by altering subsurface solute transport. We expect that hydrological retention would positively influence nutrient retention.

The third chapter answers questions 2, 5, and is entitled “Enhancement of carbon and nitrogen removal by helophytes along subsurface water flowpaths receiving treated wastewater”, aims to elucidate how the presence of emergent macrophytes influences the removal of dissolved inorganic nitrogen (DIN) along subsurface flowpaths and the importance of DOC lability as a factor controlling denitrification. We expect that the presence of emergent macrophytes will stimulate DIN removal via direct assimilation and due to root exudation of labile carbon compounds that may enhance denitrification rates, and that the experimental addition of a labile C source would further enhance DIN removal.

Finally, in the general discussion of the thesis, first I explore the use of emergent macrophytes across different NBS goals, by analyzing information from implemented NBS projects. Further, I review the role of emergent macrophytes on the subsurface aquatic environment, by discussing the connections between their physical, chemical, and biological functions. Ultimately, I identify possible knowledge gaps regarding the use of emergent macrophytes in NBS, and I provide guidelines to managers for optimized NBS implementation, by synthesizing information from scientific and technical literature.

# CHAPTER TWO

# Emergent Macrophyte Root Architecture Controls Subsurface Solute Transport

Emergent macrophytes (helophytes) grow in the active channel of fluvial ecosystems. Subsurface flow beneath this area (i.e., hyporheic zone) is considered critical for ecological processes. However, little is known about the influence of helophyte roots on subsurface solute transport. We investigated the effect of three helophyte species with different root architecture (Iris pseudacorus L., Phragmites australis L., and Scirpus lacustris L.) on solute transport along subsurface flow paths. We considered both the physical and the biological roles of the roots, expecting that (1) roots will act as structures that create heterogeneities in the sediment (physical role); thus, root architecture will alter subsurface flow paths; (2) roots will remove water via evapotranspiration (biological role), leading to slower flow velocity; and (3) both scenarios will result in longer water residence times. We performed conservative tracer pulse additions in 12 flow-through flumes subjected to four treatments: absence of helophytes (Control) and presence of helophytes (Iris, Scirpus, and Phragmites). Tracer breakthrough curves were used to compare solute transport patterns between the treatments by fitting a mobile-immobile model and by applying temporal moment analysis. Results showed that helophyte roots increase subsurface water residence time by creating heterogeneities in the substrate and by removing water. Furthermore, hydraulic retention increased with the percent volume of fine roots but decreased in the presence of thicker roots. Based on these results we suggest that the root architecture of helophytes and their capacity to remove water via evapotranspiration should be considered when planning stream restoration activities aimed to improve water quality.

Nikolakopoulou, M., Argerich, A., Drummond, J. D., Gacia, E., Martí, E., Sorolla, A., & Sabater, F. (2018). Emergent macrophyte root architecture controls subsurface solute transport. Water Resources Research, 54. https://doi.org/10.1029/2017WR022381

## 2.1. Introduction

Aquatic vegetation, in the form of submerged macrophytes or emergent macrophytes (helophytes), is an inextricable element of natural fluvial ecosystems. Helophytes are characterized by at least 1 m of aerial growth (Grundwell, 1986) and are often present in the active channel of streams and rivers (i.e., the riverine area that is actively modified by average stream discharges). In base flow conditions, the active channel spatially consists of the wetted channel, the parafluvial areas, and the riverbanks (Holmes, Fisher, & Grimm, 1994). Helophytes can grow in areas of the active channel with or without surface flow (i.e., the wetted channel or the parafluvial zone and riverbanks, respectively), while their root system is located in permanently saturated sediments (Clarke, 2002). The parafluvial zone describes the dry region of the active channel adjacent to the wetted channel, with subsurface flow only, in the direction of the surface flow (Briody, Cardenas, Shuai, Knappett, & Bennett, 2016; Edwardson, Bowden, Dahm, & Morrice, 2003; Holmes et al., 1994). The portion of sediments beneath the active channel (i.e., streambed) that is permeated with surface water and groundwater constitutes the hyporheic zone. However, the spatial limits of the riverine regions are expected to expand and contract with changes in discharge. For example, during flood events the parafluvial areas will disappear and the hyporheic zone can expand beneath the riparian zone, the area bordering the active channel that supports longer-lived higher stature vegetation (Dent et al., 2000).

The hyporheic zone is considered a hot spot for ecological processes (Battin, 1999, 2000; Krause et al., 2011), where increased contact times between sediments and solutes (i.e., long water residence times) enhance biogeochemical processing (Boulton et al., 1998; Findlay, 1995; Jay P. Zarnetske, Roy Haggerty, Steven M. Wondzell, & Michelle A. Baker, 2011). Residence times in the hyporheic zone are dependent on both water exchange between the stream and the hyporheic sediments, termed hyporheic exchange, and subsurface flow paths. Hyporheic exchange with slow pore water velocities and/or long subsurface flow paths results in long residence times, which provides the maximum opportunity for solutes to interact with microbial assemblages in the substrate and promote biogeochemical processes.

Although research on subsurface flow in lotic systems has gained a lot of attention (Krause et al., 2017), most studies focus on the extent of hyporheic exchange, while less information is available on the characterization of subsurface flow paths within the hyporheic zone. Representative examples of studies aiming to characterize hyporheic flow paths include Menichino, Ward, & Hester, 2014, Ward, Gooseff, Fitzgerald, Voltz, & Singha, 2014, and Ward, Schmadel, Wondzell, Gooseff, & Singha, 2017; however, plenty of studies on flow through porous media have set the principles that govern hyporheic flow (Berkowitz, Scher, & Silliman, 2000; Dentz, Cortis, Scher, & Berkowitz, 2004).

Riparian planting is a common restoration practice for riverbank stabilization and water erosion control (Bernhardt et al., 2005; Hester & Gooseff, 2010). Presence of plant roots reinforces the soil by increasing its shear strength, resulting in increased resistance to erosion (Gyssels, Poesen, Bochet, & Li, 2005; Zuazo & Pleguezuelo, 2008). The spatial configuration of a root system in the soil, also known as root architecture, influences water erosion control (Reubens, Poesen, Danjon, Geudens, & Muys, 2007). Root volume, root density, and root-length density are parameters commonly used to describe the root system architecture and have been directly related to enhanced soil reinforcement (Reubens et al., 2007; Vannoppen, Vanmaercke, De Baets, & Poesen, 2015; Zuazo & Pleguezuelo, 2008). Further distinction between fine and coarse roots has shown that fine roots are more effective in increasing soil reinforcement than coarse roots (Reubens et al., 2007). Therefore, plant roots impact soil properties, revealing that plants can function as biological engineers (Reubens et al., 2007), with the capacity to physically modify the abiotic environment (Jones et al., 1997).

Due to the significance of fluvial ecosystem functioning associated with subsurface solute transport, hyporheic exchange is considered of vital importance in stream restoration goals (Hester & Gooseff, 2010; Krause et al., 2011). Restoration practices that modify channel morphologic features to increase habitat heterogeneity (i.e., creation of pool-rifle sequences, cross-vanes, log dams, and meander bends) have been found to enhance hyporheic exchange (Gooseff, Anderson, Wondzell, LaNier, & Haggerty, 2006; Hester & Doyle, 2008; Hester & Gooseff, 2010; Kasahara & Hill, 2006; Moren, Worman, & Riml, 2017). Additionally, subsurface interventions (i.e., modifications within the streambed) are known for promoting hyporheic exchange and for increasing subsurface water residence times but are rarely used as stream restoration techniques (Ward, Gooseff, & Johnson, 2011). Vaux (1968) was the first to propose the installation of subsurface structures to control rates of hyporheic exchange. Ward et al. (2011) revisited Vaux's models and demonstrated that hyporheic exchange may be optimized with the installation of subsurface structures to complement traditional surface restoration practices. Herzog, Higgins, & McCray (2016) modified the streambed by introducing structures of different hydraulic conductivity and suggested that this practice could be effective for storm water management in small streams. These studies are based on the concept that even small-scale streambed heterogeneities control hyporheic exchange and flow (Packman & Salehin, 2003; Salehin, Packman, & Paradis, 2004; Sawyer & Cardenas, 2009). However, limited studies on the use of subsurface interventions as restoration methods are available and, to our knowledge, have not included vegetation as a streambed (subsurface) modifier.

In the present study we explore the role of helophyte roots on subsurface solute transport during base flow conditions (i.e., the gill model, as described in Sawyer, Cardenas, Bomar, & Mackey, 2009). We seek to answer whether helophyte roots can alter subsurface flow paths and to distinguish the possible mechanisms. We view plant roots as a natural engineering system that may create heterogeneities in the substrate in a similar way as with artificial subsurface structures. We consider both the physical and the biological roles of the roots on influencing water residence time, expecting that (1) roots will act as structures that can create heterogeneities in the sediment (physical role); thus, root architecture will alter subsurface flow paths; (2) roots will remove water via evapotranspiration (biological role), leading to slower flow velocity; and (3) both scenarios will result in longer water residence times. Our objectives are (a) to characterize subsurface solute transport through bare sediment and through sediment with presence of helophytes roots and (b) to investigate the mechanism with which helophyte roots influence subsurface flow by comparing flow patterns through sediment with different root system architecture (different helophyte species) and by evaluating the relationships between flow and root architectural parameters and water loss. To address these objectives, we conducted a series of 36 solute tracer injections in 12 artificial flumes subjected to four different treatments: without vegetation (Control) and planted with three helophyte species with different root system architecture, commonly used in river restoration. Profiles of tracer concentrations over time, breakthrough curves (BTCs), were used to characterize and to contrast observed solute transport patterns between the treatments by fitting a mobile-immobile solute transport model and by applying temporal moment analysis. To our knowledge, the work presented here is the first attempt to explicitly assess how the roots of emergent aquatic vegetation (i.e., helophytes) may alter subsurface flow paths and to identify the specific root architectural traits and root functions that control subsurface flow.

## 2.2. Methods

### 2.2.1. Experimental Setup and Plant Materials

The study was conducted at the "Urban River Lab" outdoor research facility (www.urbanriverlab.com; Ribot, Bernal, et al., 2017), built on the premises of the wastewater treatment plant (WWTP) in Montornès del Vallès in Catalonia, Spain (41°32031.6″N 2°14009.2″E). The facility consists of 18 concrete flumes measuring 12 m long, 0.6 m wide, and 0.4 m deep. The channels are flat (zero slope), contain a 25-cm-thick end-to-end gravel bed of commercial coarse granitic sediment (average particle size = 40 mm), and are fed with the WWTP effluent water. At the beginning and end of each flume there are 40 cm × 60 cm sediment-free spaces (inlet and outlet basins) and a porous wall to contain the sediment (Figure 2.1). The WWTP effluent is distributed to the flumes by gravity from a 10 $m^3$ reservoir and first collected in a 50-L plastic container installed 30 cm above each flume's inlet basin, prior to entering the flume's inlet. The WWTP effluent then flows through the gravel bed of the flumes as a continuous subsurface flow, maintaining the water level at ~23 cm (approximately 2 cm below the gravel surface, with an unsaturated surface layer of the sediment). Inflow rates were adjusted at 5.016 ± 0.008 L/min, using a hydraulic valve (faucet), installed on each container to serve as a water pressure buffer and maintain similar and constant inflow rates among the flumes.

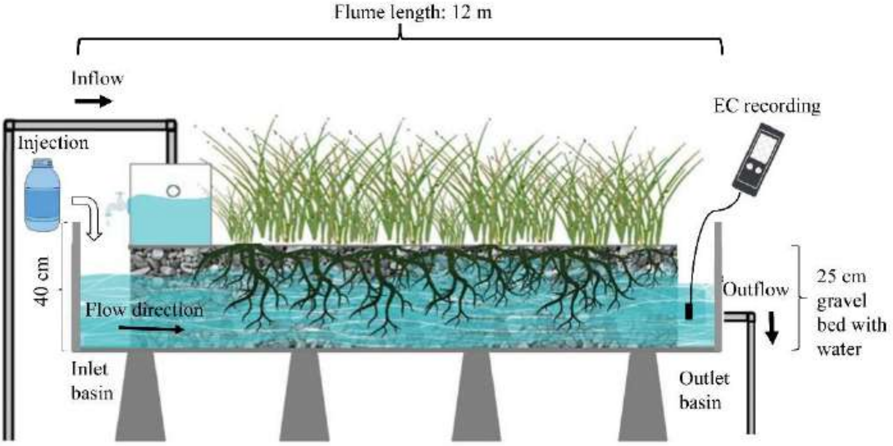


**Figure 2.1.** Longitudinal graphical representation of a vegetated flume.

The flumes were subjected to four treatments: absence of helophytes (Control), presence of Iris pseudacorus L. (common yellow flag, hereafter as Iris), presence of Phragmites australis L. (common reed, hereafter as Phragmites), and presence of Scirpus lacustris L. (common bulrush, hereafter as Scirpus). We used 3 flumes per treatment, creating 3 pseudo replicates, randomly distributed within the 18 flumes available at the facility. Therefore, 12 of the 18 artificial flumes were used for this experiment. The species chosen for this study are perennial plants commonly used for riverbank stabilization and in constructed wetlands (Vymazal, 2011), each with a different root system architecture, reflected from differences in the morphologic characteristics of roots and rhizomes. Since inherent variations between plant traits are expected in a mesocosm experiment, we prefer using the term pseudo replicates. Description of root systems of the used helophytes is summarized in Table 2.1. All flumes were planted with young plants, obtained from a nursery, in February 2015 at a density of 6.7 shoots $m^2$. By the time of the experimental period (6 months after planting), vegetation covered approximately 90% of the surface of each flume.

**Table 2.1.** Helophyte root description of Iris, Phragmites, and Scirpus[a]

| | **Iris** | **Phragmites** | **Scirpus** |
|---|---|---|---|
| **Root System General Description** | Thick roots, thick rhizomes | Fibrous roots, thin rhizomes | Fibrous roots, intermediate rhizomes |
| **Fine Root Diameter** | 2-3 mm [a] | ≤ 3 mm | ≤ 1mm |
| **Rhizome Diameter** | ~ 4 cm [a] | ~ 0.5 cm [a] | 1.5 cm |
| **Source** | Kim, Ahn, Bae, & Choi, 2009; Lai, Wang, Peng, & Chen, 2011; Laublin, Saini, & Cappadocia, 1991 | Fiala, 1976; Lai et al., 2011; Vymazal, 2011; Weisner & Strand, 1996 | Coops, vandenBrink, & vanderVelde, 1996; Lai et al., 2011 |

[a] Published values not available and values represent root diameters measured prior to the experiment.

### 2.2.2. Solute Tracer Injections

We conducted a total of 36 pulse conservative tracer (NaCl) injections in the flumes, considering temperature corrected electrical conductivity (EC, μS/cm) as a proxy for NaCl transport. We performed three injections per flume, resulting in nine injections per treatment, on a total of 12 sampling dates during 29 July until 27 August 2015. Each sampling date, we worked simultaneously on a set of three randomly selected flumes, to characterize solute transport under different environmental conditions. We expected that weather conditions would affect evapotranspiration and evaporation in the flumes and thus solute transport. All injections were conducted under rainless weather conditions, except from 13 August when two heavy rain events occurred, at 4 and 7.5 h after the start of the tracer injection. EC was measured continuously at the outlet basin of each flume and at the inlet basin of one out of the three flumes in use, at 30-s intervals, using a portable 3310 Profiline EC meter (WTW; Germany). For each pulse injection, 80 g of NaCl with 1 L of water was added to the flume. The rapid flow of water at the inlet basin ensured complete mixing of the tracer prior to transport through the gravel bed (Figure 2.1). The NaCl injections resulted in an elevated EC from 7.5 to 27.2% above background (~15% average EC elevation for all injections). The extent and range of EC elevation were the result of experimental limitations of the system, such as the high EC levels and daily fluctuations from the WWTP effluent. This percentage of elevation led to a small dynamic range of tracer data, which reflected the lack of sensitivity of the tracer measurements, resulting in truncated EC data at late times (Drummond et al., 2012); hence, solute transport parameters and temporal moments were calculated after fitting the obtained BTCs to a mobile-immobile solute transport model, to extend the tail of the BTC data (see sections 2.4.1.1 and 2.4.1.2 below).

### 2.2.3. Plant Characterization

Roots were sampled and characterized after the end of the solute tracer injection experiments. From each vegetated flume, we extracted a sample of the flume's content (sediment, roots, hereafter flume sample) in the middle of the flume (6 m from the inlet). The sample measured 20 cm long, 60 cm wide, and ~23 cm high (depth of the sediment), making a total volume of ~30 L. We manually separated roots from sediment by submerging the collected flume sample in tap water and, while agitating, collected the roots. Fine roots that still remained in the water-sediment mixture were then collected by sieving the water through a 900-µm mesh sieve. This process was repeated until all visible roots were collected. We separated the roots into fractions of rhizomes and fine roots manually, using tweezers for fine roots attached to rhizomes. Volume of each of the flume sample's fractions (sediment, rhizomes, and fine roots) was estimated by the water displacement method, using Nalgene polypropylene graduated cylinders (Thermo Fisher Scientific, USA) of 0.5 L (error ± 0.004 L) and of 2 L (error ± 0.012 L) capacity.

#### 2.2.4. Parameter Estimation

##### 2.2.4.1. Solute Transport Parameters

The EC BTCs obtained at the flume outlets were background corrected using the inlet EC (in the basin upstream from the injection) and the advective travel time in the flume, represented by the time to peak ($t_{peak}$):

$$EC_{(t)} = EC_{Out\,(t)} - EC_{In\,(t-t_{peak})} \tag{Eq. 2.1}$$

,where $EC_{(t)}$ (μS/cm) [G $L^{-1}$] is background corrected EC at time $t$, $EC_{Out\,(t)}$ (μS/cm) is EC measured at the outlet at time $t$, and $EC_{In\,(t-t_{peak})}$ (μS/cm) is EC measured at the inlet basin at time t-$t_{peak}$.

###### 2.2.4.1.1. Fractal Mobile-Immobile Model

We characterized solute subsurface transport dynamics in the flumes using a fractal mobile-immobile model (FMIM; Schumer, Benson, Meerschaert, & Baeumer, 2003), which estimates advection, dispersion, and transient storage, based on the observed background corrected EC BTCs. FMIM is a probabilistic hydrodynamic model that describes solute transport by considering the interaction between a mobile and an immobile zone. In a subsurface flow system, such as this experimental setup, we consider the mobile zone as the advective transport of preferential flow paths (i.e., free pore water) and the immobile zone as the transient storage regions with slow flow within the sediment (i.e., dead-end pore space and bound pore fluid; Ward, Gooseff, & Singha, 2010). A mobile-immobile model can appropriately characterize this system as solute is essentially immobile while retained within the transient storage regions in comparison with the transport along the preferential flow paths within the mobile zone. The governing FMIM equation that represents transport within both the mobile and immobile zone is as follows:

$$\frac{\partial C}{\partial t} + \beta \frac{\partial^\gamma C}{\partial t^\gamma} + v \frac{\partial C}{\partial x} = D \frac{\partial^2 C}{\partial x^2} \qquad \text{(Eq. 2.2)}$$

where C [M $L^{-3}$] is concentration, t is time (s) [T], x is downstream distance (m) [L], v is the average plume velocity (m $s^{-1}$) [L $T^{-1}$], D is the fractional dispersion ($m^2$ $s^{-1}$) [$L^2$ $T^{-1}$], β is the capacity coefficient ($s^{-\gamma}$) [$T^{-\gamma}$], and $\gamma$ is the time-fractional exponent (unitless). The average velocity and the dispersion represent transport within the mobile domain of the system. The capacity coefficient, $\beta$, represents the exchange between the mobile and the immobile domains. The time-fractional exponent, $0 < \gamma < 1$, describes the slope of the tail of the power-law residence time distribution, reflecting transport in the immobile domain. Values of $\gamma$ closer to 0, characterize heavier BTC tail slopes, indicating longer residence times. Model parameters were fit using FracFit, a parameter estimation tool (Kelly, Bolster, Meerschaert, Drummond, & Packman, 2017) that determines the best-fit parameters by minimizing the weighted mean square error (WMSE) function (Chakraborty, Meerschaert, & Lim, 2009). For N measurements of a breakthrough curve,

$$WMSE = \sum_{i=1}^{N} w_i (C_i - C(x, t_i))^2 \qquad \text{(Eq. 2.3)}$$

where $C_i$ is the breakthrough curve concentration at times $t_1$ to $t_N$, $C(x,t)$ is the model probability density function and the weights are given by $w_i = {}^{1}/_{C_i}$. As a result, lower concentrations receive greater weight, which is important for appropriately characterizing the tail of the breakthrough curve (Meerschaert & Sikorskii, 2011).

#### 2.2.4.1.2. BTC Analysis

A BTC provides a reach-average representation of solute transport processes (Schmadel et al., 2016). Analysis of the shape of a BTC, by calculating statistical moments and temporal metrics (Day-Lewis & Singha, 2008), allows conclusions about the dominant transport process during an injection, most commonly interpreted as advection, and processes that result in spreading of the peak (dispersion or diffusive transport) and late-time tailing (transient storage). The observed BTCs were subjected to truncation due to detection limits and background noise (Drummond et al., 2012). Therefore, we used the FMIM extended model BTCs to reach 1 μS/cm to calculate the statistical moments and water residence time (temporal metric) associated with subsurface flow through flumes of different treatments (Control, Iris, Phragmites, and Scirpus). Statistical moments have been used to describe subsurface transport in several studies (Argerich, Haggerty, Marti, Sabater, & Zarnetske, 2011; Cirpka & Kitanidis, 2000; Day-Lewis & Singha, 2008; Ward et al., 2014; Ward et al., 2016). We calculated statistical moments after Schmadel et al. (2016), by first computing a BTC normalized for the total tracer mass that has passed from the monitoring location c(t) as:

$$c(t) = \frac{C(t)}{\int C(t)dt} \qquad \text{(Eq. 2.4)}$$

where C is the observed tracer concentration. Then, we calculated the first temporal moment ($M_1$) for the BTCs as:

$$M_1 = \int tC(t)dt \qquad \text{(Eq. 2.5)}$$

which gives an estimate of mean arrival time of the tracer. Additionally, nth-order temporal moments centered about $M_1$ (central moment) were estimated as:

$$\mu_n = \int (t - M_1)^n \, C(t)dt \quad for\, n > 1 \qquad \text{(Eq. 2.6)}$$

We used the ratio $M_1/t_{peak}$, as a proxy of transient storage reflected by the comparison of the mean arrival time to the time of the preferential flowpath. If $M_1/t_{peak} = 1$, then transient storage is minimal. We calculated the dimensionless coefficient of variation (CV) and skewness of the BTCs using the 2nd and 3rd temporal moments ($\mu_2$ and $\mu_3$, respectively) as previously described in equation (2.6):

$$CV = \frac{{\mu_2}^{1/2}}{M_1}, \qquad \text{(Eq. 2.7)}$$

$$skewness = \frac{\mu_3}{{\mu_2}^{3/2}}, \qquad \text{(Eq. 2.8)}$$

Coefficient of variation provides a normalized metric of temporal variance, comparable across different mean arrival times. Skewness represents the asymmetry of the BTC. Finally, we calculated the maximum observed water residence time ($RT_{max}$) as the time elapsed from the start of the injection until the last detection of tracer at the outlet basin of each flume.

#### 2.2.4.1.3. BTC Quality Control

Due to field error (e.g., potential flow decreases due to clogging or data collection interrupted by rain), seven BTCs were truncated to omit nonsteady data. All 36 BTCs were modeled, but only BTCs with WMSE < 0.01 (i.e., less than 1% error) were included in the statistical analysis of model and moment parameters. In total, nine BTCs were excluded from the statistical analysis, specifically two from the Control, two from Iris, two from Phragmites, and three from Scirpus, resulting in a final data set of 27 BTCs.

### 2.2.4.2. Helophyte Root Architecture and Water Loss

We used % root volume to describe root architecture. We calculated the % of the volume of the flume sample occupied by fine roots (VF), rhizomes (VR), and total roots (total roots = fine roots + rhizomes; VT), as $\frac{Volume\ of\ Roots}{Volume\ of\ flume\ sample}\ x\ 100$, where Root is fine roots, rhizomes or total roots for VF, VR, and VT, respectively, and flume sample is the one extracted from each flume, consisting of roots and sediment (section 2.2.3).

We used the observed background corrected BTCs to calculate average discharge in each flume's outlet basin ($Q_{Out}$) during the time of the injections via dilution gauging (after Kilpatrick & Wilson, 1989). In the vegetated flumes, we attributed the difference in discharge between the flumes' inlet and outlet basins ($Q_{In}$-$Q_{Out}$) to water loss due to evapotranspiration (Headley, Davison, Huett, & Muller, 2012).

### 2.2.5. Statistical Analysis

To compare solute transport parameters and water loss between treatments (Control and different helophyte species) we used the log likelihood ratio test on a mixed-effects linear model, considering treatment as a fixed factor, and flume as a random effect. We used the post hoc Tukey's test for pairwise comparisons. For % volume of each fraction of roots (i.e., VF, VR, and VT), we used an analysis of variance (ANOVA) and the post hoc Tukey's test to compare them between the helophyte species. To identify the factors that control solute transport in the vegetated flumes, we built all possible linear models involving helophyte characteristics (root structure parameters and water loss), and their pairwise interactions as predictors for each of the solute transport parameters. We controlled for model complexity by limiting models to up to three predictors and by including interaction terms only if the variables involved were also included as main terms. Best performing models were identified using the Akaike information criterion corrected for small sample size (Burnham & Anderson, 2002). We automated this process using the R package "glmulti" (Calcagno & de Mazancourt, 2010). Additionally, we estimated the relative importance of each variable in each model by $r^2$ partition using the R package "relaimpo" (Gromping, 2006). All data analyses were carried out using R, version 3.3.1 (R core team, 2016).

## 2.3. Results

### 2.3.1. Helophyte Root Architecture and Water Loss

Mean values and standard error of helophyte characteristics (% root volume and water loss) are summarized in Table 2.2. Iris had the highest VR and VT, whereas samples from flumes with Scirpus had the highest VF. Samples from flumes with Phragmites had the lowest root volume, reflected by the lowest VF, VR, and VT in respect to the rest of species. However, % root volume for all root fractions was not statistically different among the species (ANOVA, $p > 0.05$) because of the high standard error due to the variability observed between flumes. Variability in % root volume may have been lower, if a larger sample size of flume samples could have been collected (i.e., >1 sample per flume). Water loss, expressed as $Q_{In}$-$Q_{Out}$, was similar between Phragmites and Scirpus and higher than Iris (log likelihood ratio test on mixed-effects model, $p < 0.0001$). Expression of water loss as evapotranspiration rates (using flume area = 7.2 $m^2$) resulted in $0.19 \pm 0.02$, $0.32 \pm 0.01$, and $0.33 \pm 0.03$ mm/min for Iris, Phragmites, and Scirpus, respectively.

**Table 2.2.** % Root volume and water loss due to evapotranspiration of helophyte species

| | | **Iris** | **Phragmites** | **Scirpus** |
|---|---|---|---|---|
| **% Root Volume (V Root /V Sample x 100)** | Fine roots (VF) (%) | 5.83 ± 1.86 | 2.62 ± 1.02 | 6.33 ± 1.98 |
| | Rhizomes (VR) (%) | 5.40 ± 2.08 | 2.07 ± 1.31 | 4.31 ± 0.82 |
| | Total roots (VT) (%) | 11.22 ± 3.93 | 4.69 ± 2.32 | 10.65 ± 2.01 |
| **Water Loss ($Q_{In}$-$Q_{Out}$)** | L min$^{-1}$ | $1.40 \pm 0.12^{A}$ | $2.28 \pm 0.08^{B}$ | $2.38 \pm 0.20^{B}$ |
| | % | $28.05 \pm 2.40^{A}$ | $45.54 \pm 1.70^{B}$ | $47.58 \pm 3.92^{B}$ |

Notes. Values given are means ± standard error. Water loss is displayed in different expressions as flow rate (L min $^{-1}$) and as percentage in respect to the inflow rate (%). The capital letters as superscripts indicate statistically different means, whereas absence of superscripts indicates no statistical differences between helophytes. Statistical differences for % root volume (VF, VR, and VT) were tested using ANOVA and the post hoc Tukey's test; $p > 0.05$, $n = 3$ for each species (three flumes per treatment and one sample per flume). Statistical differences for water loss were tested with log likelihood ratio test on mixed-effects model; fixed effects: treatment; random effects: flume and post hoc Tukey's test; $p = 0.0001$, $n_{Iris} = 7$, $n_{Phragmites} = 7$, and $n_{Scirpus} = 6$.

### 2.3.2. FMIM Model Parameters

Fractal mobile-immobile model appropriately characterized the solute transport through the flumes with WMSE between 0.17 and 0.97%. Examples of BTC data and model fits per treatment are shown in Figure 2.2. Velocity (v) did not statistically differ across the treatments, which was expected since the inlet discharge was regulated to be the same rate for all flumes (Figure 2.3a, $p = 0.091$). Scirpus showed statistically significant higher dispersion (D) than Phragmites, with the latter being the treatment with the lowest dispersion, while Control and Iris were not statistically different between each other, nor from Phragmites or Scirpus (Figure 2.3b, $p = 0.026$). However, Phragmites showed anecdotally lower dispersion than Control and Iris. The capacity coefficient ($\beta$), representing the exchange rate between the mobile and immobile zone, did not statistically differ between the treatments (Figure 2.3c, $p = 0.573$), although Phragmites and Scirpus showed slightly higher mean values than the rest of treatments. The time fractional exponent ($\gamma$) was significantly lower in Scirpus than in the Control, but similar between Iris and Phragmites without statistically significant differences between each other, or with the rest of treatments (Figure 2.3d, $p = 0.025$). It is notable though that Iris and Phragmites have intermediate mean values of time fractional exponent, between the Control and Scirpus.

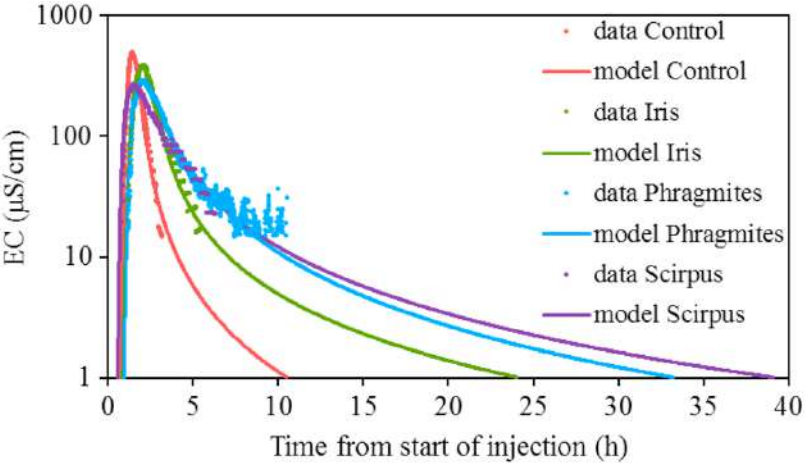


**Figure 2.2.** Examples of breakthrough curve data and the fractal mobile-immobile model fits in semilinear space, per treatment. Model fits shown are extended to 1 µS/cm.

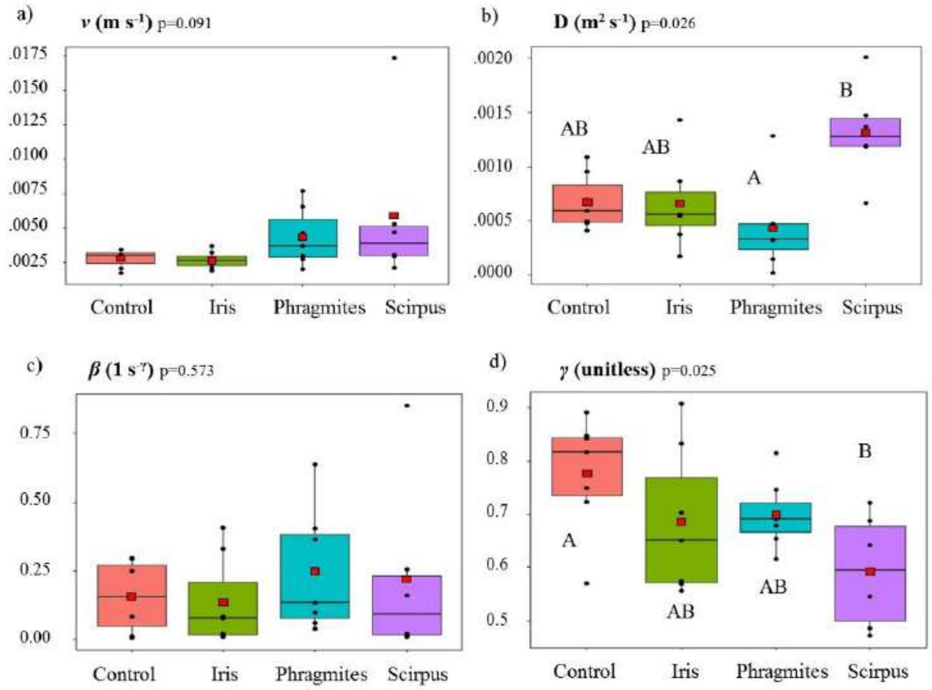


**Figure 2.3.** Box plots of the fractal mobile-immobile model parameters, across different treatments (Control, Iris, Phragmites, and Scirpus). p values result from log likelihood ratio test on mixed-effects model; fixed effects: treatment, random effects: flume; Tukey's post hoc; α = 0.05. The letters above box plots indicate statistically different means ($n_{Contol}$ = 7, $n_{Iris}$ = 7, $n_{Phragmites}$ = 7, and $n_{Scirpus}$ = 6). For each box plot, the middle line represents the median, the red square symbol indicates the mean, the box is the central 50% of the data, and the bars are the 75% quantile. (a) Velocity, (b) dispersion, (c) capacity coefficient indicating the mobile-immobile water exchange, and (d) time-fractional exponent describing the slope of the breakthrough curve tails.

### 2.3.3. BTC Analysis

$M_1/t_{peak}$, CV, and $RT_{max}$ calculated from the extended BTCs differed between Control, Iris, Phragmites, and Scirpus (Figures 2.4a, 2.4b, and 2.4d, $p < 0.05$). No statistically significant differences were found for skewness between the treatments, as all treatments were expected to be highly skewed (Figure 2.4c, p = 0.306). $M_1/t_{peak}$ was higher in Scirpus, followed by Phragmites, and then the Control. Iris had an intermediate behavior` between Phragmites and Control without statistically significant differences (Figure 2.4a, p < 0.0001). Phragmites and Scirpus demonstrated similar CV, higher than the rest of treatments. Control showed the lowest CV, and Iris mean CV was found in between, without statistically significant differences from the rest of treatments

(Figure 2.4b, p = 0.001). Scirpus showed the highest mean value for $RT_{max}$, and the lowest was found in the Control. Phragmites and Iris showed intermediate behavior, having statistically significant differences with Control and Scirpus, respectively (Figure 2.4d, p < 0.0001).

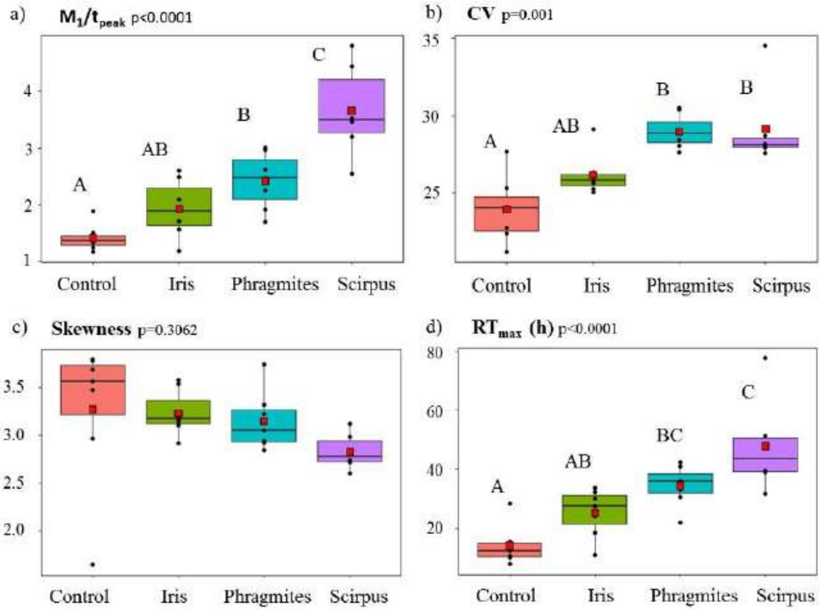


**Figure 2.4.** Box plots of statistical moments and water residence time across different treatments (Control, Iris, Phragmites, and Scirpus). p values result from log likelihood ratio test on mixed-effects model; fixed effects: treatment, random effects: flume; Tukey's post hoc; α = 0.05. The letters above box plots indicate statistically different means ($n_{Contol}$ = 7, $n_{Iris}$ = 7, $n_{Phragmites}$ = 7, and $n_{Scirpus}$ = 6). For each box plot, the middle line reflects the median, the red square symbol indicated the mean, the box is the central 50% of the data, and the bars are the 75% quantile. (a) $M_1/t_{peak}$ as a transient storage metric, (b) coefficient of variation as a metric of temporal variance, (c) skewness, and (d) maximum observed residence time.

### 2.3.4. Factors Controlling Subsurface Solute Transport

Four simple linear and nine multilinear significant relationships between solute transport parameters and the used predictors (VF, VR, VT, and water loss) were found among the five best-performing models. However, significant relationships were only found for $\gamma$, $M_1/t_{peak}$, CV, and $RT_{max}$, whereas v, D, $\beta$, and skewness did not relate with any of the predictors (Table 2.3). Three out of the four simple models included water loss as a predictor, and one included % root volume (VF), while eight out of the nine multilinear models included water loss in the predictive variables, and one of them included only % root volume (VR and VT).

The time fractional exponent was best predicted by a simple linear model, revealing a negative relationship to VF with adjusted $r^2 = 0.36$, showing that high % volume of fine roots results in heavier power law tails of the BTCs. Furthermore, one multivariate model including VT, VR, and their interaction yielded significant regression with the time fractional exponent, with an increased explanatory power of 84%. It is notable though, that VR influenced the time fractional exponent positively, showing that high % volume of rhizomes induces shorter BTC tails. $Q_{In}$-$Q_{Out}$ was not included as predictor in any of the regressions for the time fractional exponent.

The ratio $M_1/t_{peak}$ was positively related with $Q_{In}$-$Q_{Out}$, as shown by a simple linear regression identified as best-performing model (adjusted $r^2 = 0.50$), as well as with three 2-variate models including $Q_{In}$-$Q_{Out}$ and VT, VR, and VF with increased predictive power (adjusted $r^2$ of 0.60, 0.58, and 0.58, respectively). All predictors had a positive relationship with $M_1/t_{peak}$; however, the relative importance of $Q_{In}$-$Q_{Out}$ to the model was higher compared to the % root volume parameters in all three regressions.

The best-performing model for predicting CV included $Q_{In}$-$Q_{Out}$ and VF and explained 79% of the data's variance (adjusted $r^2 = 0.79$). VF showed a negative relationship with CV and contributed 36% to the model, while $Q_{In}$-$Q_{Out}$ was the main predictor with relative contribution to the model of 64%, and with a positive effect on CV. The remaining two significant regressions for the prediction of CV included $Q_{In}$-$Q_{Out}$, and $Q_{In}$-$Q_{Out}$ + VT as predictors, respectively, but did not increase the predictive power compared to the first model. In both cases the $Q_{In}$-$Q_{Out}$ was positively related to CV, whereas the % volume of total roots (VT) was negatively related. In the two-variate model, $Q_{In}$-$Q_{Out}$ was the main predictor, with higher percentage of relative importance to the model than VT.

Finally, $RT_{max}$ was best related to $Q_{In}$-$Q_{Out}$, having a positive relationship with adjusted $r^2 = 0.56$. $RT_{max}$ also yielded significant relationships with three 2-variate models including $Q_{In}$-$Q_{Out}$ and VF, VT, and VR with slightly increased or equal predictive power. In the three regressions all predictive variables were positively related to $RT_{max}$, while the main predictor was $Q_{In}$-$Q_{Out}$ with relative contribution to the model ranging from 90 to 94%, much higher than the respective contribution of % root volume parameters.

**Table 2.3.** Significant relationships ($p \leq 0.05$) between solute transport parameters and predictive variables (% root volume and water loss) found among the five best-performing linear regression models

| Parameters | Model | AICc | Weight | Adjusted $r^2$ |
|---|---|---|---|---|
| Time fractional exponent | $-0.02\ VF + 0.74$ | -14.05 | 0.35 | 0.36 |
| | $-0.04\ VT^{(42\%)} + 0.003\ VT * VR^{(37\%)} + 0.01\ VR^{(21\%)} + 0.82$ | -10.21 | 0.05 | 0.84 |
| $M_1/t_{peak}$ | $1.26\ (Q_{In}-Q_{Out}) + 0.14$ | 25.72 | 0.57 | 0.50 |
| | $1.42\ (Q_{In}-Q_{Out})^{(88\%)} + 0.06\ VT^{(12\%)} - 0.74$ | 29.59 | 0.08 | 0.60 |
| | $1.57\ (Q_{In}-Q_{Out})^{(90\%)} + 0.13\ VR^{(10\%)} - 1.00$ | 29.95 | 0.07 | 0.58 |
| | $1.28\ (Q_{In}-Q_{Out})^{(83\%)} + 0.10\ VF^{(17\%)} - 0.39$ | 29.97 | 0.07 | 0.58 |
| CV | $1.75\ (Q_{In}-Q_{Out})^{(64\%)} - 0.22\ VF^{(36\%)} + 25.34$ | 30.18 | 0.52 | 0.79 |
| | $1.78\ (Q_{In}-Q_{Out}) + 24.17$ | 32.35 | 0.18 | 0.49 |
| | $1.49\ (Q_{In}-Q_{Out})^{(59\%)} - 0.11\ VT^{(41\%)} + 25.77$ | 33.55 | 0.10 | 0.69 |
| $RT_{max}$ | $13.56\ (Q_{In}-Q_{Out}) + 7.48$ | 66.33 | 0.72 | 0.56 |
| | $13.68\ (Q_{In}-Q_{Out})^{(90\%)} + 0.81\ VF^{(10\%)} + 3.24$ | 71.57 | 0.05 | 0.59 |
| | $14.73\ (Q_{In}-Q_{Out})^{(95\%)} + 0.47\ VT^{(5\%)} + 0.94$ | 71.67 | 0.05 | 0.59 |
| | $15.62\ (Q_{In}-Q_{Out})^{(94\%)} + 0.88\ VR^{(6\%)} - 0.15$ | 72.20 | 0.04 | 0.56 |

Notes. Water loss ($Q_{In}$-$Q_{Out}$) is expressed as flow rate (L/min). The superscripts next to predictive variables indicate the relative importance in each model. The asterisk indicates interaction between variables. The velocity, dispersion, capacity coefficient, and skewness did not yield significant relationships to any of the predictive variables used. Abbreviations: VF = % volume of fine roots, VR = % volume of rhizomes, VT = % volume of total roots, AICc = Akaike information criterion corrected for small sample size.

## 2.4. Discussion

### 2.4.1. Influence of Vegetation on Subsurface Solute Transport

Differences in transport metrics between bare sediments (Control) and sediments planted with helophyte species (Iris, Phragmites, and Scirpus) provided insight into the influence of vegetation on subsurface solute transport. Transport through vegetated flumes was characterized by high transient storage ($M_1/t_{peak}$), long $RT_{max}$, and low power law exponents. Subsurface flow through vegetated flumes is therefore not well represented by the classical advection-dispersion equation but instead by anomalous transport with characteristic early first arrivals and long power law tails (Cortis, Chen, Scher, & Berkowitz, 2004). Hereafter, the term anomalous transport will be used throughout this article to describe subsurface flow with high $M_1/t_{peak}$, long $RT_{max}$, and low power law exponents. Anomalous transport results from the presence of structural heterogeneities in the substrate that commonly occurs in porous media in a wide range of scales (pore scale to basin scale). These heterogeneities lead to a range of water velocities, resulting in different flow paths (i.e., flow path heterogeneity; Berkowitz, Cortis, Dentz, & Scher, 2006; Dentz & Bolster, 2010). We observed high flow path heterogeneity in vegetated flumes, evidenced by the increased temporal variance (CV). Additionally, increased water loss results in longer water residence times (Kadlec & Wallace, 2009). Thus, the presence of vegetation influenced solute transport within the immobile zone, which led to enhanced anomalous transport, and therefore increased hydraulic retention (high transient storage and long residence time), by creating structural heterogeneities within the gravel bed and by removing water. Transport of the mobile zone of the system was not affected by the presence of helophytes, as shown by the similar velocity and dispersion between Control and vegetated flumes. Hence, the mobile and the immobile zones constitute two distinct domains, which represent different aspects of solute transport within the flumes.

### 2.4.2. Subsurface Solute Transport Dependence on Vegetation Type

Vegetation type affects subsurface solute transport as demonstrated by the observed differences in transport metrics across flumes with Iris, Phragmites, and Scirpus. Results from the models for the prediction of solute transport parameters gave us further insight into the mechanism with which helophyte roots influenced subsurface solute transport. Consistent to our hypotheses, solute transport is controlled by root architecture (described by % root volume) and water loss due to evapotranspiration. % root volume and water loss were good predictors for transport parameters that represent both the mobile and immobile zone of the system (i.e., ($M_1/t_{peak}$ and CV), while the models for the slope of the power law tails ($\gamma$), a parameter that represents explicitly the immobile zone of the system, included only % root volume. Models for the prediction of $RT_{max}$ included both % root volume and water loss. Although $RT_{max}$ is a parameter that reflects the slowest flow paths (i.e., the immobile zone), the positive relationship of it with water loss is well known (Kadlec & Wallace, 2009). Therefore, water loss has an impact on the free-pore water (i.e., mobile zone), whereas root architecture affects the physical structure of the sediments by creating structural heterogeneities. When both % root volume and water loss were included in models, the relative importance of water loss was always higher than the one of % root volume. This suggests that the BTCs were dominated by the mobile zone and that the contributions of transport processes in the immobile zone were of smaller magnitude. Dispersion, a transport parameter that describes the mobile zone, did not yield any significant linear relationships with water loss or% root volume. Yet, different dispersion between Phragmites and Scirpus (species with similar water loss) suggests that root architecture may also influence the free-pore water flow paths, although this effect requires further investigation.

Root system architecture influenced the physical structure of the gravel bed, as inferred by the exchange between mobile-immobile zones, and the power law exponents. The similar mobile-immobile water exchange coefficients across the vegetated flumes reveal a similar likelihood for solutes to be transported from the preferential flow path to slower flow regions disregarding vegetation type. However, differences across treatments in the power law exponents indicate that flow velocity through these slower flow regions differed between species, resulting in the tracer being flushed out at different times. Differences in % volume of fine roots and rhizomes across species were responsible for differences in the power law exponents, as shown by the linear models where root parameters were included as predictors for the power law exponents. Fine roots and rhizomes have different roles in affecting subsurface solute transport, as revealed by the linear models. The positive relationship between % volume of rhizomes and power law exponents reveals the role of thick roots against anomalous transport, inferring the effect of root architecture on substrate permeability. The introduction of thick rhizomes in the gravel bed may create larger free-water pores by pushing the gravel grains and thus shorter residence times. On the other hand, fine roots might be filling the pores in-between the gravels, resulting in heavier power law tails.

Therefore, % volume of fine roots, and of rhizomes, as well as a plant's capacity to remove water via evapotranspiration, should be considered as key factors in subsurface solute transport. Increased % volume of fine roots and water loss are responsible for anomalous transport, whereas a high % volume of rhizomes acts against this mechanism, resulting in shorter water residence times and lower transient storage. The interplay of these transport mechanisms defines the average solute transport process of the system. The influence of vegetation type on subsurface solute transport is conceptualized and illustrated in Figure 2.5. Helophytes of type I, such as Iris (Figure 2.5b), characterized by high % volume of rhizomes and low water loss, will create low transient storage, and short residence time, whereas helophytes of type III, such as Scirpus (Figure 2.5d), with high % volume of fine roots and high water loss, will cause heavy power law tails, high transient storage, and long water residence time. Transport metrics through sediments with helophytes type II, such as Phragmites, with low % volume of fine roots and of rhizomes, and high water loss will fall in between these two end-members. Flow parameters, such as residence time and transient storage, influence a stream's ability to process nutrients, and therefore, vegetation use and choice should be considered for restoration strategies aimed to improve water quality. Flow through sediments with presence of type III helophytes may lead to enhanced nutrient retention, as hydrology is considered a key factor influencing biogeochemical processing (e.g., Battin, 1999), which can improve water quality of impaired streams and reduce nutrient loads in estuarine ecosystems (Peterson et al., 2001).

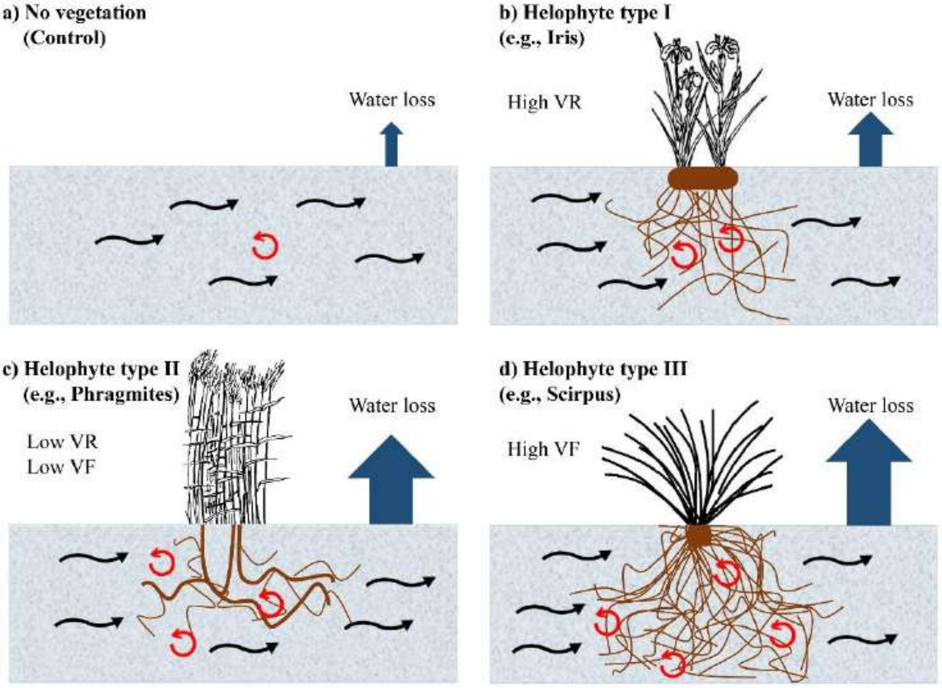


**Figure 2.5.** Conceptual diagram of subsurface solute transport through bare sediments and sediments planted with different helophyte species (Control, Iris, Phragmites, and Scirpus). The size of the blue arrows indicates the extent of water loss via evapotranspiration or evaporation in the bare sediments. The black arrows indicate the velocity of the preferential flow path, which remains the same among the treatments. The number of red arrows indicates the extent of slower flow paths, responsible for anomalous transport (more red arrows and more slow flow paths), which differs between treatments, with the Control having the fastest, and Scirpus the slowest flow paths. Average transport process results from the interplay between factors leading to longer residence times (% volume of fine roots and water loss) and factors responsible for shorter residence times (% volume of rhizomes).

### 2.4.3. Application of Findings to Natural Environments

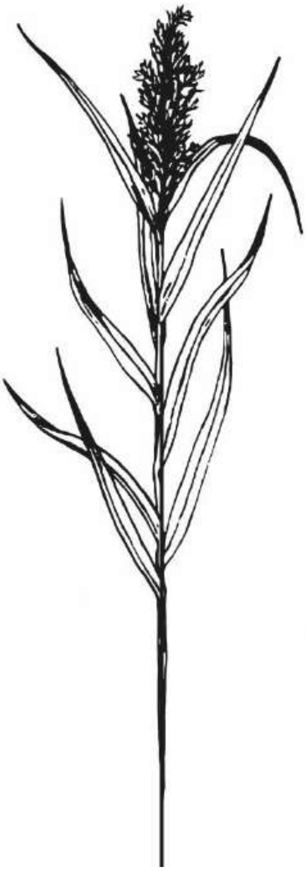
The results of this study in artificial flumes provide valuable insight into the influence of helophyte roots on subsurface solute transport in hydrologically similar natural environments, without surface flow of water, such as the parafluvial zones of arid streams, or intermittent streams during dry base flow conditions. Varying the stream environment using flume mesocosms and conducting tracer injection studies allows the isolation of factors that affect solute transport, such as root architecture and water loss within the current study. Mesocosm results can be linked to reach-scale transport through a direct comparison of model parameters since stochastic theory predicts that the slowest transport mechanism, such as transport through transient storage regions derived from root structures, will control the long-term tailing behavior of an in-stream surface water BTC (Drummond et al., 2015; Schumer et al., 2003). Therefore, model parameters obtained from the flume mesocosms can be projected to larger-scale systems to predict reach-scale transport or be compared to reach scale results to determine the controlling mechanisms of solute transport in the stream.

Our findings can be applied to stream restoration efforts that aim to improve water quality by increasing water residence times. The use of helophytes, with concurrent use of gravel in certain techniques (i.e., vegetated riprap), is usually employed in stream restoration activities for river bank stabilization and water erosion control. In the present study we show that the introduction of helophytes can have a secondary role of increasing the residence times of subsurface flow paths. A careful selection of species should consider their root architecture characteristics and their capacity to remove water via evapotranspiration. Based on our results, species with higher % volume of fine roots and with high evapotranspiration rates will increase subsurface water residence times. Substrate properties should also be considered, as substrate size and its structural heterogeneities enhance anomalous solute transport (Aubeneau, Hanrahan, Bolster, & Tank, 2014). Furthermore, the effect of substrate environment on root systems development should also be assessed, as the presence of rock fragments can restrict root growth, as reviewed in Reubens et al. (2007). On a similar basis, water chemistry should also be considered when assessing the expected root development, as it has been found to affect plant biomass allocation and root architecture. In low-nutrient environments, higher root : shoot ratios have been reported and specifically $NO_3^-$ deficiency has been shown to inhibit root branching (Forde & Lorenzo, 2001; Ning, Zhang, Cui, & Zou, 2014). Although not studied here, we expect that flow through a less permeable and more heterogeneous substrate with adequate presence of fine roots, often found in natural streams under base flow conditions, may be characterized by even longer late-time tailing than through the coarse gravel bed of the flume mesocosms. A higher proportion of fine sediments can promote more regions of slower flow velocity due to low permeability, which could decrease hyporheic exchange (Aubeneau et al., 2014). The extent of hyporheic exchange is essential to water quality improvement, as a measure of how accessible the substrate is to solutes. Since the underlying mechanism of the physical role of plant roots to affect solute transport is the modification of the structure of the sediment, root architecture might also have an influence on hyporheic exchange with surface water in streams with in-stream vegetation, on the basis that sediment heterogeneities control hyporheic exchange (Packman & Salehin, 2003; Salehin et al., 2004). Although the effect of plant roots on hyporheic exchange was not studied in the present article, it may be interesting to consider as a point for future investigation.

## 2.5 Conclusions

In the present work we investigated the effect of helophyte roots on the hydraulic parameters of subsurface solute transport. We found that roots increase subsurface residence time and transient storage, by removing water via evapotranspiration, and by creating heterogeneities in the sediment, which in turn creates diverse flow paths. Regarding the physical role of the roots, acting as obstacles to water flow, we distinguished that fine roots and rhizomes influence solute transport with opposing mechanisms. Fine roots enhance anomalous transport, while rhizomes create short residence times, concluding that a dense root system of fine roots will result in increased hydraulic retention. In general, we point out the significance of helophytes on increasing the number of subsurface flow paths (i.e., flow path heterogeneity) and suggest that thorough characterization of root system architecture should be considered in stream restoration activities targeting water quality improvement.

# CHAPTER THREE



# Effect of Three Emergent Macrophyte Species on Nutrient Retention in Aquatic Environments under Excess Nutrient Loading

Emergent macrophyte species selection is critical for the effectiveness of nature-based engineered solutions aiming to address excess nutrient concentrations in freshwater ecosystems. Yet, the mechanisms with which macrophytes enhance nutrient retention need to be further understood. Here, we compared nutrient retention among 12 artificial flumes fed with effluent from a wastewater treatment plant and subjected to four treatments: absence of macrophytes (Control), and presence of three different macrophyte species (Iris pseudacorus L., Phragmites australis L., and Scirpus lacustris L.). We estimated net and gross nutrient uptake based on longitudinal profiles of ambient concentrations and on pulse injections of ammonium ($NH_4^+$) and soluble reactive phosphorus (SRP). Further, we investigated the influence of subsurface hydrological patterns, attributed to the architectural differences in the roots of these macrophytes, on nutrient retention. Results showed a species-specific effect of macrophytes on nutrient retention and confirmed root-associated subsurface hydrological retention as a driving factor. This work contributes to improve our mechanistic understanding of the role of emergent macrophytes on nutrient retention in aquatic environments.

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## 3.1. Introduction

Urbanization and agriculture are responsible for elevated nutrient concentrations in aquatic ecosystems (Carpenter et al., 1998; Paul & Meyer, 2001). Effluents from wastewater treatment plants, as well as agricultural and stormwater runoff, deteriorate the ecological status of the receiving water by degrading water quality. Nature-based engineered practices to address problems associated with elevated nutrient concentrations use emergent macrophytes in the context of phytoremediation, as an alternative approach to conventional technologies (Brix, 1997; Vymazal, 2011).

Emergent macrophytes contribute to nutrient removal by assimilating nutrients into plant tissues, and by facilitating microbially-mediated nutrient removal (Brix, 1994; Stottmeister et al., 2003). In particular, rooted macrophytes create ideal environmental conditions in the rhizosphere for microbial assimilation and coupled nitrification-denitrification. First, because the root surface serves as microbial habitat; and second, because root-tips release oxygen and labile carbon into the interstitial water (Brix, 1994; Reddy et al., 1989). Macrophyte species selection can be critical for the successful implementation of phytoremediation strategies to improve water quality (Bratieres, Fletcher, Deletic, & Zinger, 2008; Tanner, 1996). A good understanding of how plant morphological traits influence nutrient retention from freshwater systems would lead to optimum water treatment results by considering appropriate species selection. Yet, this type of investigation is usually overlooked, and most studies only report the influence of vegetation presence on nutrient retention, or in the case of comparative studies, the difference in retention effectiveness among species (Brisson & Chazarenc, 2009; Dagenais, Brisson, & Fletcher, 2018; Shelef, Gross, & Rachmilevitch, 2013). However, some studies have confirmed the hypotheses that increased root surface area and root oxygen release can enhance microbial activity, identifying these hypotheses as mechanisms that ultimately promote nutrient retention. Such hypothesis-driven studies have examined the influence of different plant morphological traits on nutrient retention (Cheng et al., 2009; Gagnon, Chazarenc, Comeau, & Brisson, 2007; Kyambadde, Kansiime, Gumaelius, & Dalhammar, 2004; Lai et al., 2011; Read et al., 2010; Tanner, 1996).

Still, even after identification of plant traits that correlate with nutrient retention, generalization of the results to guide species selection for nature-based water treatment technologies is still constrained by methodological limitations of the studies (i.e. poor replication and high variability of experimental conditions) (Brisson & Chazarenc, 2009). Therefore, identification of the controlling factors and underlying mechanisms with which different macrophyte species contribute to nutrient retention requires further investigation. Currently, this lack of information limits the capacity to successfully restore impaired aquatic ecosystems (Read et al., 2010).

Moreover, macrophyte root architecture influences subsurface solute transport (Nikolakopoulou et al., 2018). Solute transport with increased water residence time and transient storage provides sufficient contact time between bioreactive sediments and solutes for biogeochemical processing, critical for nutrient retention (Valett, Morrice, Dahm, & Campana, 1996; Zarnetske et al., 2011). Therefore, one would expect that plant-induced changes in subsurface hydrology could influence nutrient retention. However, results about the influence of hydrology on nutrient retention are equivocal (K. S. Simon, Townsend, Biggs, & Bowden, 2005; Webster et al., 2003) most likely because the extent of transient storage is not always proportional to its metabolic activity (Argerich et al., 2011). This is a critical question for understanding the potential benefits of phytoremediation technologies, which have not been empirically investigated yet.

Here, we seek to examine whether subsurface hydrological patterns attributed to the presence of different macrophyte species can cause ecologically relevant changes in nutrient retention, and to identify the most effective species to enhance nutrient retention. To do so, we used three macrophyte species with contrasting nutrient requirements (Gacia et al., 2019) and root architecture-mediated influence on subsurface solute transport (Valett et al., 1996). We hypothesized that macrophyte roots would influence pore water chemistry by altering subsurface flow. We expected that nutrient uptake metrics, used as an index of nutrient retention, would be positively related with hydrological metrics describing hydrological retention. This work contributes to improve our mechanistic understanding of the role of emergent macrophytes in nutrient loads mitigation in aquatic environments.

## 3.2 Methods

### 3.2.1. Experimental Setting

The study was conducted at an outdoor research facility with 12 artificial flumes (length: 12 m, width: 0.6 m, depth: 0.4 m) containing a 25-cm-thick layer of granitic gravels and fed with treated municipal wastewater (http://www.urbanriverlab.com; Ribot et al., 2017). The inlet and outlet of the flumes are 40-cm-long sediment free spaces to facilitate the water inflow and outflow (Figure 3.1). Inflow rates were adjusted at 5.02 ± 0.01 L min$^{-1}$, resulting in a continuous subsurface flow through the gravel bed. To sample subsurface water along the flumes, we installed five PVC wells per flume (L: 50 cm, diameter: 2.5 cm) into the gravel at 1, 3, 5, 7, 9 m from the inlet. The wells were perforated over the last 15 cm from the well bottom.

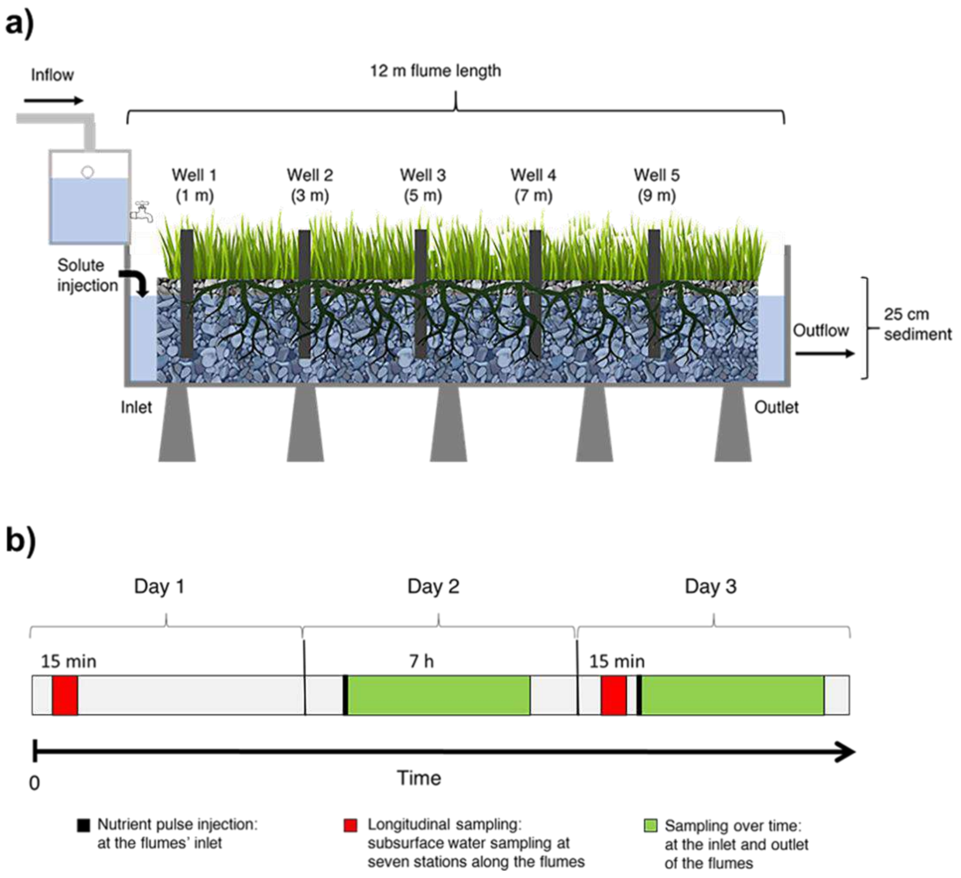


**Figure 3.1.** a) Scheme of a vegetated flume at the Urban River Lab experimental platform. Flumes were fed with WWTP effluent at an inflow rate of 5 L min$^{-1}$, resulting in a continuous subsurface flow through the gravel bed. Five PVC wells were placed along each flume to collect subsurface water. b) Diagram summarizing the sampling strategy at each flume.

The experimental setting consisted of three flumes without macrophytes (Control), and flumes with emergent macrophytes (three flumes for each of the three studied species; that is Iris pseudacorus L. (common yellow flag, hereafter as Iris), Phragmites australis L. (common reed, hereafter as Phragmites), and Scirpus lacustris L. (common bulrush, hereafter as Scirpus)). The selected macrophyte species are autochthonous, perennial, with contrasting root system architecture and evapotranspiration capacity (Nikolakopoulou et al., 2018), and are commonly used in constructed wetlands and river restoration projects (Vymazal, 2011). Iris is characterized by high % volume of rhizomes to volume of soil, Phragmites by low % volume of fine roots and rhizomes, and Scirpus by high % volume of fine roots (Nikolakopoulou et al., 2018). The three replicates per each treatment were randomly distributed among the 12 available flumes on the facility to ensure random exposure to the environmental conditions. Saplings were planted in February 2015, at a density of 6.7 shoots $m^{-2}$. By the time of the study (July 30th – August 27th, 2015) the vegetation covered approximately 90% of the surface of each flume.

In a previous study we showed that these species differentially increased hydrological retention along the flumes (Table 3.1) (Nikolakopoulou et al., 2018). Hydrological retention was described by hydrological transient storage ($M_1/t_{peak}$), which is the ratio between the mean water arrival time ($M_1$) and the time of the preferential flow path ($t_{peak}$), the maximum observed water residence time ($RT_{max}$), and the slope of the tail of the power-law residence time distribution which is described by the time-fractional exponent ($0 < \gamma < 1$). Values of $\gamma$ close to 0 correspond to stronger power-law tails and are indicative of longer residence times.

**Table 3.1.** Subsurface solute transport parameters in flumes without macrophytes (Control) and with presence of Iris, Phragmites, and Scirpus[b]

| | **$M_1/t_{peak}$ (unitless)** | **$RT_{max}$ (h)** | **$\gamma$ (unitless)** |
|---|---|---|---|
| **Control** | 1.42 ± 0.09 | 14.15 ± 2.58 | 0.78 ± 0.04 |
| **Iris** | 1.93 ± 0.19 | 25.25 ± 3.08 | 0.69 ± 0.05 |
| **Phragmites** | 2.42 ± 0.19 | 34.37 ± 2.58 | 0.70 ± 0.02 |
| **Scirpus** | 3.66 ± 0.34 | 47.78 ± 6.6 | 0.59 ± 0.04 |

[b]Values are means ± standard error. Data reported in Nikolakopoulou et al., 2018

### 3.2.2. Measurement of Nutrient Uptake

We estimated net uptake rate coefficient per unit of distance ($k_{net}$, $m^{-1}$) and gross uptake rate coefficient per unit of time ($k_{gross}$, $min^{-1}$) for ammonium ($NH_4^+$) and soluble reactive phosphorus (SRP), as indices of nutrient retention. The assessment of both $k_{net}$ and $k_{gross}$ allows a more complete understanding of the influence of each macrophyte species on N and P cycling (von Schiller et al., 2015). Sampling for nutrient retention consisted in longitudinal samplings of subsurface water from the wells and the inlet and outlet (seven locations in total), followed by solute pulse injections. We performed longitudinal samplings and solute injections in a sets of three selected flumes. Each set of flumes was sampled twice resulting in a total of 24 longitudinal samplings and 24 solute injections.

$k_{net}$ was estimated from the longitudinal samplings. Water samples were collected using a 100 ml syringe with an attached silicone tube. $k_{gross}$ was estimated using a pulse co-injection of 1 L solution containing 12 g of $NH_4Cl$, 0.875 g of $NaH_2PO_4H_2O$, and 80 g NaCl at the inlet of each flume. Temperature-corrected electrical conductivity (EC, µS/cm) was measured continuously at the outlet of each flume at 30 sec intervals. EC was considered a proxy for $Cl^-$ concentration and the change in EC during time (breakthrough curve, or BTC) was used to characterize the transport of the conservative tracer. EC measurements started 30 minutes before the injection until EC returned to background conditions (approx. 7h after the pulse injection). Simultaneously, water samples were collected at the outlet of each flume to characterize the BTC of the reactive tracers.

To account for fluctuations in the solute concentrations of the inflow water, we measured EC at 30 s intervals and collected water samples hourly at the inlet of one flume. For all EC measurements we used portable 3310 Profiline EC meters (WTW; Germany). Concentration of dissolved oxygen (DO) was monitored continuously at 10 min intervals using ProODO DO meters (YSI; USA) deployed at the outlet of each flume, and at the inlet of one flume. All water samples were immediately filtered through ashed Whatman GF/F glass-fiber filters (0.7 µm pore size) into 10 mL plastic tubes and stored frozen until nutrient analysis.

Water samples were analyzed for concentrations of elemental nitrogen as nitrate + nitrite ($NO_3^-$+$NO_2^-$) and as $NH_4^+$, and of SRP using the cadmium reduction (Keeney, & Nelson, 1982), salicylate–nitroprusside (Baethgen & Alley, 1989), and the acidic molybdate (Murphy, & Riley, 1962) methods, respectively. Water samples from longitudinal profiles were analyzed with an automatic continuous flow analyzer (Futura AMS Alliance; Italy). Samples from solute pulse injections were analyzed using a Technicon autoanalyzer (Technicon; USA) for $NO_3^-$+$NO_2^-$ and a spectrophotometer (PharmaSpec UV-1700 Shimadzu; Japan) for $NH_4^+$ and SRP.

### 3.2.3. Parameter Estimation

We estimated $k_{net}$ from longitudinal profiles of nutrient concentrations at ambient levels; and as such it reflects the balance between uptake and release processes occurring along the flumes, which determines whether a system currently acts as a net sink or source of nutrients. $k_{gross}$ was estimated from a mass balance approach to infer the capacity of the system to uptake the mass of added nutrients from the solute injections (von Schiller et al., 2015).

#### 3.2.3.1. Net Uptake Rate Coefficient

$k_{net}$ for $NH_4^+$, $NO_3^-$+$NO_2^-$, and SRP was calculated based on changes in subsurface water nutrient concentrations along the flumes following Merseburger, Marti, & Sabater, 2005:

$$C_x = C_{in}\, e^{k_{net}\, x} \qquad \text{(Eq. 3.1)}$$

where $C_x$ and $C_{in}$ are ambient concentrations of $NH_4^+$, $NO_3^-$+$NO_2^-$, or SRP (mg N or P $L^{-1}$) at distance x (m) from the inlet and at the inlet, respectively. We calculated $k_{net}$ as the statistically significant slope ($p < 0.05$) of the linear relationship between the natural logarithm of nutrient concentrations and distance along the flume. We considered flumes with $k_{net} > 0$ to act as net nutrient sinks (i.e., gross uptake > release), whereas flumes with $k_{net} < 0$ were considered to act as net nutrient sources (i.e., gross uptake < release). We assigned $k_{net} = 0$ to flumes with no significant linear regression, considering that uptake and release processes balanced each other.

#### 3.2.3.2. Gross Uptake Rate Coefficient

Three of the 24 nutrient BTCs were excluded from the analysis because of sample contamination resulting in a final dataset of 21 BTCs. We used the nutrient BTCs measured at the outlet and the temporal variation of nutrients at the inlet to compute $k_{gross}$ for each nutrient injected ($NH_4^+$, SRP) following the approach by Wilcock, Scarsbrook, Costley, & Nagels (2002). We used a mass-based approach to estimate $k_{gross}$ as it is considered more accurate than approaches using only nutrient concentration (Fraley-McNeal, Schueler, & Winer, 2007).

For each nutrient, we compared the observed mass of injected nutrient retrieved at the outlet of the flume ($M_{obs}$), with the expected mass of injected nutrient at the outlet if nutrient transport along the flume behaved as a conservative tracer ($M_{exp}$). We calculated $M_{obs}$ as:

$$M_{obs} = Q_{out} \int_0^7 C_{out} \, dt \qquad \text{(Eq. 3.2)}$$

where $Q_{out}$ is discharge at the outflow calculated from the background-corrected BTCs of EC using the dilution gauging approach (Kilpatrick, & Wilson, 1989). $C_{out}$ is the nutrient concentration measured at the outlet over the 7-hour course of the BTC (Wilcock et al., 2002). $M_{exp}$ was calculated as the sum of the mass of nutrient entering through the inflow ($M_{in}$) and the mass of nutrient injected ($M_{carboy}$):

$$M_{exp} = M_{in} + M_{carboy} \qquad \text{(Eq. 3.3)}$$

$M_{in}$ is the sum of the nutrient mass of the inflow water over the 7 hours of the solute injection:

$$M_{in} = Q_{in} \int_0^7 C_{in} \, dt \qquad \text{(Eq. 3.4)}$$

where $Q_{in}$ is the discharge of the inflow water and $C_{in}$ is the nutrient concentration at the inlet. We calculated $k_{gross}$ by linearizing the following first-order equation:

$$M_{exp} = M_{obs} \, e^{k_{gross} \, t_{peak}} \qquad \text{(Eq. 3.5)}$$

where $t_{peak}$ is the time from the start of the injection until the arrival of the peak of the conservative tracer at the outlet of the flume. Additionally, the relative $k_{gross}$ of N to P was calculated as the ratio between $k_{gross}$-$NH_4^+$ and $k_{gross}$-SRP.

### 3.2.4. Statistical Analysis

Differences in inflow water across treatments were tested using analysis of variance (ANOVA) and the post-hoc Tukey's test. To detect differences in $k_{net}$ among treatments we used the non-parametric Kruskal–Wallis test and the post-hoc Dunn's test because datasets did not meet the assumptions for normality, homoscedasticity, or both. We compared $k_{gross}$, $k_{gross}$-$NH_4^+$/$k_{gross}$-SRP ratio, and outflow DO concentration across treatments using the log likelihood ratio test in a linear mixed model. Treatment was considered a fixed factor, and flume a random effect. Post-hoc Tukey's test was used for pairwise comparisons. To explore how root-induced changes in subsurface hydrology influenced nutrient uptake rate coefficients ($k_{net}$ and $k_{gross}$) we used Spearman correlations. Finally, we used Spearman correlations to examine if variation in background nutrient concentrations of the inflow water influenced nutrient uptake in the flumes. All statistical analyses were carried out using R, version 3.3.1 (R core team, 2016) setting $\alpha = 0.05$.

## 3.3. Results

### 3.3.1. Physicochemical Characterization of Inflow Water in The Flumes

All flumes received inflow water with high EC, ranging from 2161 to 2416 µS/cm, and high temperature, ranging between 27.2 and 28.5 °C. The concentration of $NH_4^+$, $NO_3^-$+$NO_2^-$, and SRP of the inflow water differed among dates when samplings and solute injections were conducted at the different treatments (Kruskal–Wallis, $p < 0.0001$; Table 3.2). Flumes with *Iris* received the highest $NH_4^+$ concentration, while flumes with *Phragmites* the lowest (10% of the concentration that received *Iris* flumes). Inflow water of Control flumes and flumes with *Scirpus* had higher $NO_3^-$+$NO_2^-$ concentration than water of flumes with *Iris* and with *Phragmites*. Concentration of SRP in inflow water was the highest in Control flumes and flumes with *Scirpus*. No statistically significant differences were found across treatments in DO concentration in the flumes' inflow or outflow (ANOVA, linear mixed model, $p > 0.05$). DO concentration averaged 6.02 and 0.57 mg $L^{-1}$ in the inflow and outflow respectively, revealing substantial DO consumption as subsurface water moved along the flumes. No significant relationships were found between the nutrient uptake rate coefficients and the nutrient concentrations of the inflow water (Spearman correlations, $p > 0.05$).

**Table 3.2.** Chemical properties of inflow water and outflow DO concentration in the flumes for each treatment during the experiment

| | **Inflow** | | | | **Outflow** |
|---|---|---|---|---|---|
| | **$NH_4^+$ (mg N $L^{-1}$)** | **$NO_3^-$+$NO_2^-$ (mg N $L^{-1}$)** | **SRP (mg P $L^{-1}$)** | **DO (mg $L^{-1}$)** | **DO (mg $L^{-1}$)** |
| **Control** | 0.46 ± 0.03 **a** | 3.57 ± 0.08 **a** | 0.68 ± 0.02 **a** | 6.15 ± 0.04 | 0.45 ± 0.11 |
| **Iris** | 1.59 ± 0.02 **b** | 2.02 ± 0.01 **b** | 0.18 ± 0.00 **b** | 5.93 ± 0.02 | 0.58 ± 0.06 |
| **Phragmites** | 0.16 ± 0.00 **a** | 1.77 ± 0.04 **b** | 0.44 ± 0.01 **ab** | 6.11 ± 0.02 | 0.57 ± 0.01 |
| **Scirpus** | 0.63 ± 0.04 **a** | 3.78 ± 0.10 **a** | 0.77 ± 0.03 **a** | 5.89 ± 0.04 | 0.67 ± 0.03 |

Notes. Values are means ± standard error for the different days that each treatment (Control, Iris, Phragmites, Scirpus) was sampled. Data was obtained during 7 h of monitoring at each flume inlet. For each variable, lowercase letters denote statistically significant differences across treatments, whereas absence of letters denotes no statistically significant differences ($p > 0.05$).

### 3.3.2. Net Nutrient Uptake

We observed statistically significant differences across treatments for $k_{net}$-$NH_4^+$ (Figure 3.2, Kruskal–Wallis; $p < 0.05$), whereas no significant differences were found for $k_{net}$-$NO_3^-$+$NO_2^-$ and $k_{net}$-SRP (Figure 3.2, Kruskal–Wallis; $p > 0.05$). Regarding $k_{net}$-$NH_4^+$, all three vegetated treatments (*Iris, Phragmites,* and *Scirpus*) showed a positive mean value indicating that uptake predominated over release. In contrast, in Control flumes $k_{net}$ equaled to zero, indicating that uptake and release processes counterbalanced each other. Flumes with *Scirpus* showed the highest $k_{net}$-$NH_4^+$, followed by flumes with *Iris,* both treatments were statistically different from the Control. Treatment with *Phragmites* showed the lowest $k_{net}$-$NH_4^+$ among the treatments with presence of vegetation, which was not statistically different from the Control. Anecdotally, mean $k_{net}$-$NO_3^-$+$NO_2^-$ was positive in flumes with *Phragmites*, while it was negative in the rest of treatments. For SRP, $k_{net}$ was positive in flumes with *Phragmites* and *Scirpus*, and negative in Control and *Iris* flumes.

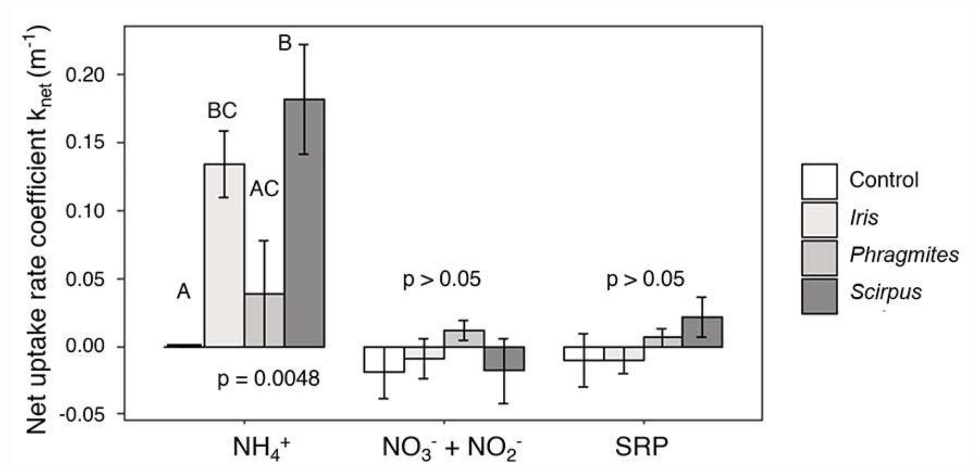


**Figure 3.2.** Mean and standard error of net uptake rate coefficients ($k_{net}$) for $NH_4^+$, $NO_3^-$ +$NO_2^-$, and SRP among different flume treatments (Control, *Iris*, *Phragmites*, and *Scirpus;* n = 6 per treatment). *p*-values result from a non-parametric Kruskal–Wallis test, and multiple comparisons from a Dunn's post hoc test; $\alpha = 0.05$. Uppercase letters denote statistically different means across treatments, whereas absence of letters denotes no statistically significant differences ($p > 0.05$). Positive and negative values of $k_{net}$ indicate whether the flumes acted as net nutrient sinks or sources, respectively. $k_{net} = 0$ indicates that uptake and release along the flumes counterbalance each other.

### 3.3.3. Gross Nutrient Uptake

Gross uptake rate coefficients statistically differed across treatments for both $NH_4^+$ and SRP (Figure 3.3, linear mixed model, $p < 0.05$). For $NH_4^+$, flumes with *Phragmites* and *Scirpus* showed higher mean $k_{gross}$ values than Control and *Iris* flumes. Particularly flumes with *Scirpus* showed the highest $k_{gross}$-$NH_4^+$. $k_{gross}$-SRP was similar in flumes with *Phragmites* and *Scirpus* and higher than in the Control flumes, which showed the lowest $k_{gross}$. In *Iris*, $k_{gross}$-SRP showed no statistically significant differences from the rest of treatments. Overall, $k_{gross}$-

$NH_4^+$ seemed to be higher than $k_{gross}$-SRP regardless of flume treatment. Additionally, the ratio between $k_{gross}$-$NH_4^+$ and $k_{gross}$-SRP was similar among all treatments (linear mixed model, $p > 0.05$).

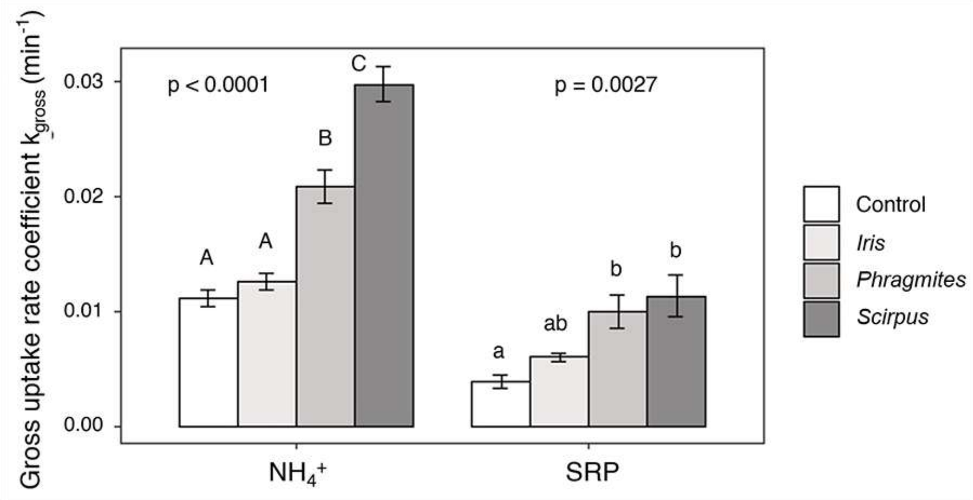


**Figure 3.3.** Mean and standard error of gross uptake rate coefficients ($k_{gross}$) for $NH_4^+$ and SRP among different flume treatments (Control, *Iris*, *Phragmites*, and *Scirpus*). *p*-values result from the log likelihood ratio test in a linear mixed model; fixed effects: treatment, random effects: flume; Tukey's post hoc; $\alpha = 0.05$. Uppercase and lowercase letters denote statistically different means for $NH_4^+$ and SRP, respectively ($n_{Control} = 6$, $n_{Iris} = 3$, $n_{Phragmites} = 6$, and $n_{Scirpus} = 6$).

### 3.3.4. Relationships Between Nutrient Uptake and Hydrological Characteristics

The relationship between subsurface hydrological characteristics and nutrient uptake considering data from all flume treatments together differed among the nutrients ($NH_4^+$, $NO_3^-$+$NO_2^-$, SRP) and the hydrological and uptake metrics considered. Both $k_{net}$ and $k_{gross}$ of $NH_4^+$ and SRP yielded positive correlations with $M_1/t_{peak}$ and $RT_{max}$, revealing monotonic associations between these variables (Figure 3.4, Spearman correlations, $p < 0.05$). However, $k_{gross}$ of each nutrient had a stronger association with the hydrological metrics than $k_{net}$, as shown by Spearman's $\varrho$ ranging from 0.80 to 0.95 for $k_{gross}$, and from 0.60 to 0.63 for $k_{net}$. $k_{gross}$ of both $NH_4^+$ and SRP yielded negative correlations with the time-fractional exponent ($\gamma$), while only $k_{net}$-$NH_4^+$ was correlated with $\gamma$. Between uptake metrics of $NH_4^+$, $k_{gross}$ had a stronger association with $\gamma$ than $k_{net}$, as indicated by higher absolute value of Spearman's ϱ. No significant correlation was found between $k_{net}$-$NO_3^-$+$NO_2^-$ and any of the considered hydrological metrics.

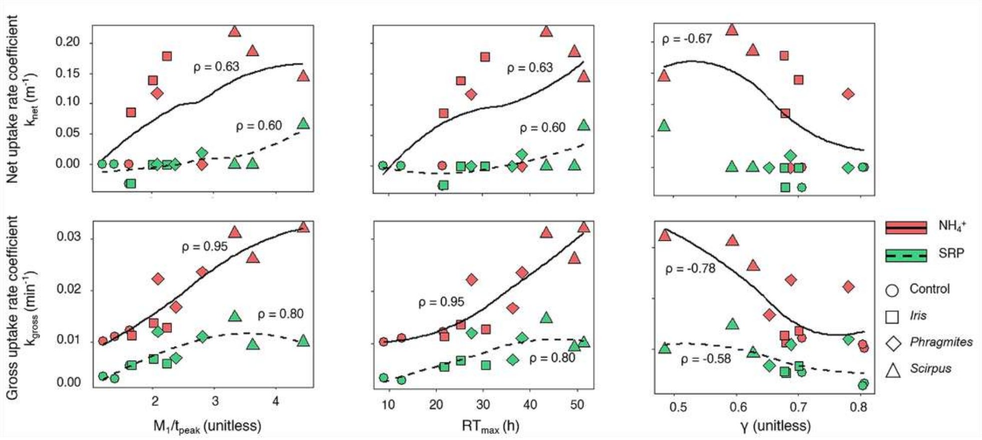


**Figure 3.4.** Relationships between uptake rate coefficients ($k_{net}$, $k_{gross}$) of $NH_4^+$ and SRP and different hydrological metrics, used as descriptors of subsurface solute transport along the flumes, considering data from all different flume treatments together (i.e, Control, Iris, Phragmites, and Scirpus). Data from hydrological metrics for each flume come from [21]. Values of $M_1/t_{peak}$ reflect extent of water transient storage along the flumes, $RT_{max}$ is the maximum water residence time, and γ describes the slope of the tail of the power law residence time distribution n = 12 in all cases (from the 12 flumes studied). The locally estimated scatterplot smoothing is showed with solid and dashed curves for $NH_4^+$ and SRP respectively, only when Spearman's correlation between hydrologic and nutrient uptake coefficients were statistically significant (α = 0.05).

## 3.4. Discussion

### 3.4.1. Influence of Macrophytes Species on Nutrient Retention

Macrophytes have a species-specific influence on the capacity for $NH_4^+$ and SRP retention from subsurface water, demonstrated by differences in $k_{gross}$ across flumes with *Iris*, *Phragmites* and *Scirpus*. Additionally, net $NH_4^+$ uptake, which accounts for both gross uptake and release processes, varied among treatments suggesting that macrophyte species also influence decomposition and mineralization processes. Nevertheless, comparison of nutrient uptake metrics between vegetated and unvegetated flumes showed that the influence of macrophytes on nutrient retention varies among species, since not all vegetated treatments were statistically different from the Control. These findings are in line with other studies reporting differences in nutrient retention among macrophyte species (Akratos & Tsihrintzis, 2007; Gacia et al., 2019; Lai, Zhang, & Chen, 2012; Wu et al., 2011) and suggest that selection of macrophyte species in water management or stream restoration planning is critical to enhance nutrient retention.

In the vegetated flumes, $k_{net}$-$NH_4^+$ was higher than $k_{net}$-$NO_3^-$+$NO_2^-$ uptake suggesting that the former N form is more actively retained than the latter supporting the idea that $NH_4^+$ is the most biologically active dissolved inorganic N species in freshwater ecosystems (Kemp & Dodds, 2002; Ribot, von Schiller, & Marti, 2017). Interestingly, flumes with *Iris* and *Scirpus* showed higher net uptake capacity to retain $NH_4^+$ than the Control, and acted as a net sink of $NH_4^+$ ($k_{net} > 0$), while no treatment was remarkably efficient for $k_{net}$-$NO_3^-$+$NO_2^-$ (in all cases $k_{net} \sim 0$). Accumulation of N in plant biomass can be substantial in the studied macrophytes (Gacia et al., 2019), yet microbial processes (assimilation, nitrification, and denitrification) associated with the rhizosphere are considered the major mechanism contributing to N uptake (Gersberg et al., 1983; Matheson, Nguyen, Cooper, Burt, & Bull, 2002). The fact that relative gross uptake (i.e., $k_{gross}$ $NH_4^+$/$k_{gross}$ SRP) was similar among all treatments, regardless the presence of macrophytes, supports the idea that gross nutrient retention is mostly attributed to microbial processes in the flumes. Hence, differences in $k_{net}$-$NH_4^+$ among vegetated treatments suggest differential extents of the relative contribution of either nitrification ($NH_4^+$ uptake) or denitrification ($NO_3^-$+$NO_2^-$ uptake) to N cycling. Macrophytes can influence the predominance of nitrification over denitrification and vice versa by root-mediated oxygen diffusion into sediments and root release of carbon exudates, respectively (Brix, 1997; Clarke, 2002). Our finding that in flumes with *Scirpus* and *Iris*, positive net $NH_4^+$ uptake co-occurs with negative net $NO_3^-$+$NO_2^-$ uptake suggests that nitrification is a relevant N cycling process. This result coincides with studies showing higher root-mediated oxygen release by *Scirpus* than by *Phragmites* roots (van der Nat &

Middelburg, 1998), conditions that favor nitrification since it is a predominantly aerobic process. However, oxygen release in the rhizosphere of *Iris* has been found lower than in *Phragmites* (Liu, Yi, Wang, Lu, & Huang, 2016), indicating that net $NH_4^+$ uptake might be ascribed to other processes than nitrification, such as microbial assimilation, or anaerobic $NH_4^+$ oxidation (anammox). In addition to high efficiency for $NH_4^+$ retention, *Scirpus* flumes showed the highest gross uptake of $NH_4^+$ as well, indicating high retention capacity for this N form. Together, these results point at *Scirpus* as a species appropriate for management of freshwater ecosystems receiving high N loads.

Combined results from net and gross uptake evidence differential pathways of nutrient retention and cycling among the studied macrophyte species. For instance, flumes with *Phragmites* showed high $k_{gross}$-$NH_4^+$ and low $k_{net}$-$NH_4^+$, revealing high release of this N form back to the water column. Similar conditions of low net nutrient retention, but high retention capacity have been previously observed in headwater streams (von Schiller et al., 2015). In contrast, flumes with *Iris* were net sinks of $NH_4^+$ despite showing low $k_{gross}$-$NH_4^+$, similar to those in Control flumes. This pattern suggests that release processes were relatively low in *Iris* flumes. Net uptake of SRP in flumes with *Iris* and *Phragmites* was close to zero, despite these two treatments showed differences in gross uptake. This result indicates that nutrient retention capacity associated with the two macrophytes species is different, yet their net influence on nutrient export is equally low. To our knowledge, there are no previous studies using nutrient spiraling metrics (e.g. $k_{net}$, $k_{gross}$) to assess the influence of macrophytes on nutrient transport along subsurface freshwater flowpaths. Therefore, we do not have previous data to compare our results with. However, some studies reporting the efficiency of these macrophyte species to retain nutrients, estimated by a mass balance approach between inflow – outflow nutrient loads, have contrasting results in relation to the relative differences in net and gross uptake among the treatments observed in our study (Korboulewsky et al., 2012; Rycewicz-Borecki, McLean, & Dupont, 2017; Wu et al., 2011). We attribute these differences to the temporal variability in nutrient uptake, due to the variability in plant and microbial activity, suggesting that an estimation of the temporal variability of net and gross uptake could contribute to better characterize the role of macrophytes species in nutrient retention in freshwater ecosystems (Feijoo, Giorgi, & Ferreiro, 2011). Yet, this study highlights that nutrient uptake processes in subsurface water associated with presence of macrophytes can be of equal importance to release processes, which would result in negligible influence of these plants on nutrient export. Therefore, it is important to consider both net and gross nutrient uptake for an accurate assessment of the contribution of macrophytes to the performance of water treatment systems.

### 3.4.2. Influence of Hydrology on Nutrient Retention

Consistent to our hypothesis, root-architecture induced differences in subsurface solute transport (Nikolakopoulou et al., 2018) had a significant influence on both net and gross nutrient retention of $NH_4^+$ and SRP. Nevertheless, solute transport had a stronger influence on gross than on net nutrient retention. This could be explained by the different biogeochemical processes involved in net and gross uptake. While gross uptake of $NH_4^+$ and SRP includes both assimilatory and dissimilatory uptake process leading to removal of nutrients from water, net uptake includes additional processes that release nutrients to water (e.g. remineralization, desorption). Each biogeochemical process involved in net nutrient uptake is controlled by complex interactions among different factors that affect the bioreactivity of the system, such as the physiology of plants and microbial assemblages, the physical and chemical characteristics of the substrate, nutrient and organic matter availability, redox conditions, light, temperature, and hydrological retention (Marce, von Schiller, Aguilera, Marti, & Bernal, 2018). Since gross uptake is not influenced by nutrient release processes that can take place over time scales beyond the duration of the solute injections (Dodds et al., 2002), the influence of hydrology on gross uptake is stronger than that observed for net uptake. However, our findings also suggest that hydrological retention associated with different macrophytes species is not a relevant factor controlling the interplay between uptake and release processes of $NO_3^-$+$NO_2^-$ along the flumes. In other words, the increase in $NO_3^-$+$NO_2^-$ uptake that hydrological retention may cause is not enough to determine the predominance of uptake over release processes; and thus, ultimately $NO_3^-+NO_2^-$ export. Overall, our results indicate that hydrological retention influence the $NH_4^+$ and SRP uptake in the study treatments, with higher uptake in flumes with higher hydrological retention. Given that species-specific root architecture can influence hydrological retention, this plant trait could be used as a surrogate to infer the potential of macrophytes to contribute to $NH_4^+$ and SRP retention from the water column.

This study highlights differential nutrient retention performance among systems with different macrophyte species and provides mechanistic understanding of differences in their performance. These findings can guide the implementation of best management practices for stormwater management aiming at water quality improvement as well as for tertiary treatment of effluent water from WWTP. In particular, biofiltration systems (e.g. wet vegetated swales), a common stormwater management practice targeting pollution retention, are expected to address the episodic nutrient loading associated with stormwater runoff. In this context, the use of macrophyte species that effectively enhance nutrient retention can contribute to buffer excess nutrient inputs to freshwater ecosystems. Additionally, in recent years, the hyporheic zone (i.e. the biogeochemically active zone of the streambed beneath or next to the main channel, where surface water mixes with groundwater), has been identified as a critical zone to be restored to address stream impairment (Hester & Gooseff, 2010). Yet, there are few restoration practices that aim at modifying the streambed to change solute transport along the hyporheic zone (Herzog et al., 2016; Koryto, Hunt, & Page, 2017). The present study complements our previous findings by showing that different macrophyte species do not only influence differently hydrological subsurface flows (Nikolakopoulou et al., 2018), but also that subsurface hydrological flowpaths, especially through rhizospheres from aquatic macrophytes, can influence $NH_4^+$ and SRP uptake. Therefore, the ability of macrophytes to harness the hyporheic zone should be considered in future restoration practices to select appropriate species that favor nutrient retention.

# CHAPTER FOUR

# Enhancement of Carbon and Nitrogen Removal by Helophytes Along Subsurface Water Flowpaths Receiving Treated Wastewater

Wastewater treatment plant (WWTP) effluents are sources of dissolved organic carbon (DOC) and inorganic nitrogen (DIN) to receiving streams, which can eventually become saturated by excess of DIN. Aquatic plants (i.e., helophytes) can modify subsurface water flowpaths as well as assimilate nutrients and enhance microbial activity in the rhizosphere, yet their ability to increase DIN transformation and removal in WWTP-influenced streams is poorly understood. We examined the influence of helophytes on DIN removal along subsurface water flowpaths and how this was associated with DOC removal and labile C availability. To do so, we used a set of 12 flow-through flumes fed with water from a WWTP effluent. The flumes contained solely sediments or sediments with helophytes. Presence of helophytes in the flumes enhanced both DIN and DOC removal. Experimental addition of a labile C source into the flumes resulted in a high removal of the added C within the first meter of the flumes. Yet, no concomitant increases in DIN removal were observed. Moreover, results from laboratory assays showed significant increases in the potential denitrifying enzyme activity of sediment biofilms from the flumes when labile C was added; suggesting denitrification was limited by C quality. Together these results suggest that lack of DIN removal response to the labile C addition in flumes was likely because potential increases in denitrification by biofilms from sediments were counterbalanced by high rates of mineralization of dissolved organic matter. Our results highlight that helophytes can enhance DIN removal in streams receiving inputs from WWTP effluents; and thus, they can become a relevant bioremediation tool in WWTP-influenced streams. However, results also suggest that the quality of DOC from the WWTP effluent can influence the N removal capacity of these systems.

Ribot, M., Bernal, S., Nikolakopoulou, M., Vaessen, T. N., Cochero, J., Gacia, E., et al. (2017). Enhancement of carbon and nitrogen removal by helophytes along subsurface water flowpaths receiving treated wastewater. Science of the Total Environment, 599-600, 1667–1676. https://doi.org/10.1016/j.scitotenv.2017.05.114

## 4.1. Introduction

During the last decades, implementation of wastewater treatment plants (WWTP) in developed countries has contributed to reduce the inputs of organic matter and nutrients derived from urban activity to natural streams (i.e., EEA – European Environmental Agency, 2010). However, despite the relevant technological advances achieved in wastewater treatments (Metcalf and Eddy, 2014) so far, the effluents from WWTPs are still important sources of dissolved organic carbon (DOC) and inorganic nutrients, such as nitrogen (N) and phosphorus (P). This excess of organic matter and nutrients can eventually cause deterioration of the water quality and ecological status of the receiving aquatic ecosystems (EEA – European Environmental Agency, 2010; Smith, Tilman, & Nekola, 1999). This problem is particularly relevant in regions with water scarcity, where inputs from WWTP effluents can account for 100% of stream flow, especially during summer (Marti, Riera, & Sabater, 2010). The process of nutrient removal within WWTP facilities has important energetic and economic constraints (Carey & Migliaccio, 2009). Therefore, it is important to explore alternative management strategies involving less cost and higher sustainability to reduce nutrient loads, such as those focused on increasing the nutrient retention capacity of receiving aquatic ecosystems (Johnson, Kaushal, Mayer, Smith, & Sivirichi, 2016).

Streams receiving WWTP effluents have a remarkable capacity to biogeochemically process the excess of dissolved inorganic nitrogen (DIN) loads from WWTP effluents (Haggard, Stanley, & Storm, 2005; Lofton, Hershey, & Whalen, 2007; Merseburger et al., 2005). In streams, DIN processing (i.e., uptake, transformation and removal) occurs along water flowpaths in both surface stream channels (Ribot et al., 2012) and subsurface hyporheic zones (Lawrence et al., 2013). These processes are mostly driven by microbial assemblages (i.e. biofilms) that develop on different streambed substrata as well as on hyporheic sediments (Battin, Kaplan, Newbold, & Hansen, 2003; Pusch et al., 1998). In addition, aquatic plants (e.g., helophytes) developing on the streambed and stream-riparian margins can also contribute to decrease DIN concentration from surface (Pastor et al., 2013; Peipoch et al., 2014) and subsurface water (Schade, Fisher, Grimm, & Seddon, 2001) because they rely on DIN to meet their N demand for assimilatory uptake. Furthermore, the root system of helophytes (i.e., rhizosphere) provide favorable habitat for biofilm development and activity in hyporheic sediments (Andrews & Harris, 2000; Ramey, Koutsoudis, von Bodman, & Fuqua, 2004) because it can provide oxygen and labile DOC (Maltais-Landry, Maranger, & Brisson, 2009; Stottmeister et al., 2003). Thus, helophytes can directly or indirectly enhance DIN uptake, transformation and removal, especially along subsurface water flowpaths and thus, they have been used as tertiary treatments of WWTP effluents (Gottschall, Boutin, Crolla, Kinsley, & Champagne, 2007; Toscano, Marzo, Milani, Cirelli, & Barbagallo, 2015). More recently, helophytes have been used as bioengineering tools in river restoration projects to stabilize river margins and reduce channel erosion (Evette et al., 2009; M. H. Li & Eddleman, 2002). However, in these restoration projects, the potential role of helophytes on DIN processing has been largely overlooked.

Denitrification is a microbial process that leads to the removal of DIN from the stream water into the atmosphere. Under low oxygen conditions, denitrifying bacteria oxidize DOC using $NO_3^-$ as an electron acceptor, which is reduced to $N_2O$, NO, and $N_2$ (Lin et al., 2009; (Seitzinger et al., 2006). However, because other biogeochemical processes associated with DIN processing co-occur in streams, the DIN removal capacity of the stream ultimately depends not only on the rate at which denitrification occurs, but also on the balance between processes contributing to DIN uptake from the water column (i.e., DIN assimilation, denitrification) and DIN release to the water column (i.e., mineralization of dissolved organic nitrogen). In this sense, studies conducted in WWTP-influenced streams have shown small downstream changes in DIN concentration, suggesting either that release and uptake processes counterbalance each other as it occurs in pristine streams (Bernal, Lupon, Ribot, Sabater, & Marti, 2015; von Schiller et al., 2015) or that rates of uptake processes, in particular denitrification, are low due to some limiting factor (Lofton et al., 2007; Merseburger et al., 2005; Ribot et al., 2012). Among other factors, such as redox and oxygen concentration, denitrification can also be limited by the availability of DOC as observed in pristine ecosystems (Hill et al., 2000) or among streams subjected to different human pressures (Mulholland et al., 2008). Given that WWTP effluents are relevant sources of DOC to receiving streams; DOC availability may not be a limiting factor for denitrification in WWTP-influenced streams (Meng et al., 2013; Saadi, Borisover, Armon, & Laor, 2006). However, the quality of DOC (i.e., the structural complexity of molecules and its lability) can also affect denitrification rates, as shown by decreases in the uptake rates when DOC sources are recalcitrant (Fernandez-Nava et al., 2010; Hagman, Nielsen, Nielsen, & Jansen, 2008; Pulou, Tournebize, Chaumont, Haury, & Laverman, 2012). Previous studies indicated that the quality of DOC from WWTP effluents mostly depends on the wastewater treatment process within the WWTP facility (Imai, Fukushima, Matsushige, Kim, & Choi, 2002; Krasner et al., 2009). For instance, the higher the nitrification efficiency during the aerobic phase of the treatment, the lower the quality of DOC at the effluent of the WWTP (Krasner et al., 2009), thus releasing more complex and less bioavailable molecules of DOC to recipient streams. Therefore, the processes occurring within the WWTP could influence denitrification rates, and ultimately the extent of DIN removal in receiving streams, because they influence the quality of DOC in these ecosystems.

In this study, we experimentally examined the influence of helophytes on DIN removal along subsurface water flowpaths and how this removal was associated with DOC removal and the availability of labile C sources. To this aim, we examined longitudinal profiles in DIN and DOC concentrations along 9 flumes containing three different species of helophytes and compared them with those observed in 3 unvegetated flumes. All flumes were continuously fed with water from a WWTP effluent and had only subsurface water flowing through. We additionally tested the effect of adding a labile C source on DOC and DIN removal in this experimental setting and on the potential denitrifying enzyme activity (DEA) associated with the microbial assemblages developed on the flume sediments. We expected that DIN removal along subsurface water flowpaths will be higher in flumes with helophytes since they assimilate DIN and their roots can release labile DOC compounds that may enhance rates of microbial denitrification. We also expected that the addition of a labile C source would further enhance DIN removal along the flumes due to the stimulation of denitrification rates associated with microbial assemblages in sediments. This study contributes to elucidate how the presence of helophytes can influence subsurface water DIN removal in stream ecosystems impacted by WWTP effluents, and provides insights on the role of DOC quality on DIN removal in these ecosystems.

## 4.2. Materials and Methods

### 4.2.1. Description of the Experimental Flumes

The study was performed at the "Urban River Lab" outdoor research facility located in the municipality of Montornès del Vallès (NE Barcelona, Spain; www.urbanriverlab.com). This facility has 12 flow-through mesocosms (flumes). Each flume consists of a cross sectional U-shaped concrete channel (length: 12 m, width: 0.6 m, depth: 0.4 m) filled to a depth of 25 cm with commercially available sediments (i.e., gravel), used in river restoration. Flumes are fed with water from the effluent of the WWTP of Montornès del Vallès, which is neither additionally treated nor diluted with any other source of water. A fraction of water from the effluent is pumped from the WWTP outlet into a tank and then distributed to all the flumes by gravity. Inflow discharge at each flume is 5 L min$^{-1}$ and water flow along the flumes is maintained at subsurface levels. Inflow water is characterized by high electrical conductivity (EC, 2.5 ± 0.02 mS cm$^{-1}$), low dissolved oxygen (DO, 4.5 ± 2% saturation), and high DOC and DIN concentrations (9.7 ± 0.6 mg C L$^{-1}$ and 5.2 ± 0.6 mg N L$^{-1}$, respectively). DIN is mostly composed by $NO_3^-$ (89 ± 3%), whereas $NH_4^+$ and $NO_2^-$ represent the 13 ± 3% and 1.3 ± 0.2% of DIN, respectively.

For this study, the experimental set up included 3 flumes only with sediments (i.e., unvegetated) and 9 flumes with sediments and 3 species of helophytes (3 flumes per species): *Iris pseudacorus L.*, *Scirpus lacustris L.* (common bulrush) and *Phragmites australis L.* (common reed). These species are autochthonous of the region and are typically used in constructed wetlands and stream restoration actions (Evette et al., 2009; Larned, Suren, Flanagan, Biggs, & Riis, 2006; Toscano et al., 2015). The plant density was set at 6.7 shoots m$^{-2}$ in each flume, which were planted in early March 2015. The 3 replicates for each treatment (i.e., no helophytes and 3 flume sets with different helophyte species) were randomly distributed across the 12 flumes. To sample subsurface water along each flume, we installed 5 PVC tubes, 50 cm long and 2,5 cm diameter, that were screened 15 cm over the bottom. PVC tubes were placed at 1, 3, 5, 7, 9 m from the inlet.

### 4.2.2. Field Experiment in the Flumes

The experiment was carried out during 4 consecutive weeks from July 29th to August 27th of 2015, when helophytes were fully developed. During this period, the weather was sunny and hot, without major rain events. The mean daily temperature was 24.1 °C, ranging from 19.4 to 27.7 °C. The mean daily relative humidity (%) was 63.4%, ranging from 52.0 to 72.0%. During the whole study period (n = 30 days), there were 7 rain events with an average of 8.6 mm per event. In any case, we sampled under raining conditions. Meteorological data was provided by the Servei Meteorològic de Catalunya (www.meteo.cat) from a meteorological station located 2.9 km from the experimental facility.

The experiment consisted of measuring longitudinal profiles of DIN and DOC concentrations as a proxy of removal capacity and then assessing the biogeochemical response of the flumes to an addition of a labile organic C source. For this purpose, we characterized longitudinal changes in DOC and DIN concentrations in the flumes previous to and during the addition of the labile organic C source (hereafter referred as the PRE and +C samplings, respectively). During each week, the experiment was conducted on a set of 3 randomly selected flumes where we collected water samples from the PVC tubes and from the inlet (0 m) and outlet (12 m) of each flume before and during the C addition (7 sampling points in total along each flume, Figure 4.1). Subsurface water samples from the PVC tubes were collected using a plastic syringe connected to a silicone tube. Samples from the inlet and outlet were directly collected using a plastic syringe. Moreover, we measured EC and DO concentration in the 7 sampling points of each flume using a WTW portable conductivity meter and an YSI portable oxygen meter, respectively. We also conducted slug additions of a conservative tracer (i.e., NaCl) into the flumes (n

= 3 per flume) to estimate the mean water residence time (WRT) in each treatment using a mass balance approach (Gordon et al. 2004).

The labile organic C source that was added into the flumes consisted of a by-product of the brewing process, which is rich in monosaccharides and oligosaccharides (see Table C.1 in APPENDIX C). We prepared a stock solution of this product (30:1 dilution in tap water) and we injected it to the inlet of the flumes at a constant rate (50 mL min$^{-1}$) during 72 h to achieve an increase in DOC concentration of 4 mg L$^{-1}$ above that measured as ambient level (see section 2.1). The +C sampling along the flumes was done one hour prior to stopping the C addition.

All water samples were immediately filtered through ashed Whatman GF/F glass fiber filters (0.7 µm pore size). A 10 mL aliquot was placed in a Falcon tube and stored frozen until the analysis of the different forms of DIN. A 25 mL aliquot was stored in acid washed glass vials with pH adjusted to 5.5-6 to analyze DOC and total dissolved N (TDN). We analyzed water samples for $NO_3^-$, $NO_2^-$ and $NH_4^+$ with standard colorimetric methods (Apha, 1995) on an Automatic Continuous Flow Futura-Alliance Analyzer at the Nutrient Analysis Service of the CEAB-CSIC. The detection limits for the used method were 13.2, 0.6 and 13.4 µg for $NO_3^-$, $NO_2^-$ and $NH_4^+$, respectively. The DIN concentration was estimated as the sum of the concentrations of the three forms of DIN. The concentration of DOC and TDN was analyzed on a Shimadzu (Tokyo, Japan) TOC-VCSH analyzer. The detection limits for the used method were 0.3 mg L$^{-1}$ for DOC and TDN, respectively. Dissolved organic nitrogen (DON) was estimated by subtracting DIN from TDN concentrations.

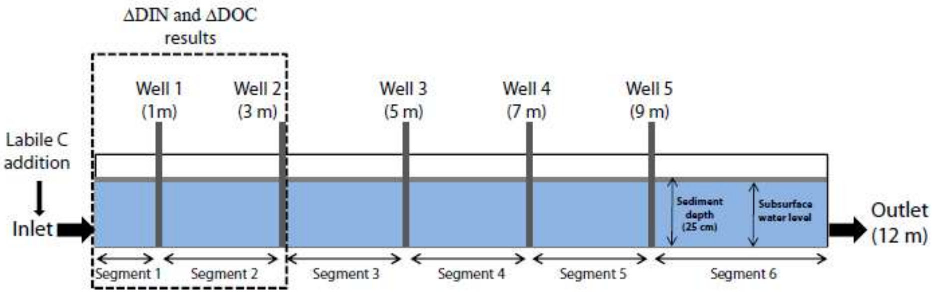


**Figure 4.1.** Scheme of the flume setting used to experimentally approach the objectives of the study. Sediment depth was 25 cm and water was set at subsurface level. We installed 5 wells at 1, 3, 5, 7 and 9 m from the inlet point. The outlet was situated at the end of the flume (12 m). We sampled the 7 points before and during labile C additions referred as PRE sampling and +C sampling, respectively. We then calculated the relative change of either DIN or DOC concentration (ΔDIN and ΔDOC respectively, both in %) between each consecutive pair of sampling points. Since the majority of DOC was consumed within the first 3 meters of the flumes, we only showed ΔDIN and ΔDOC results within segment 1 and 2 (flume section framed in bold).

### 4.2.3. Laboratory experiments with sediments from the flumes

We further assessed the influence of the labile organic C source, used in the flume experiment, on the potential denitrification enzyme activity (DEA) of biofilms that naturally grow on the sediments of the flumes. We used the acetylene ($C_2H_2$) block technique to measure the DEA as the production rate of $N_2O$ in incubation bottles following the procedure by Holmes, Jones, Fisher, & Grimm (1996). To do so, in the laboratory we incubated sediments naturally colonized in the flumes with either treated water alone or treated water plus the labile C (i.e., the by-product of the brewing process). Sediments were collected from a three different treatments (i.e, unvegetated, *Iris* and *Phragmites*) after the labile C addition. We collected ca. 300 g of sediments exposed to subsurface water flow (5-10 cm depth) at random locations along each flume (3 flumes per treatment; unvegetated, *Iris,* and *Phragmites*) and placed in a plastic bag as a composite sample for each flume Sediment samples were transported to the laboratory at 4 °C in coolers. Once in the laboratory, ca. 100 g of sediment from each flume was placed into two 250 mL glass bottles. We added 150 mL of flume water to each bottle and left the biofilm to acclimate for 12 h. After acclimation, one of the two bottles was amended with the C source (i.e., + C treatment) while the other one remained unamended (i.e., control treatment).

Incubations amended with labile C were targeted to increase DOC concentration by 4 mg $L^{-1}$ above background concentration as we did for the flume additions. The same procedure was followed for each pair of bottles for each flume treatment. The water in the incubation bottles was then made anoxic by purging helium for 10 minutes. Bottles were then sealed tight with septa-fitted screw-top lids. We added 10 mL of acetylene ($C_2H_2$) with a syringe to each incubation bottle. In DEA assays, $C_2H_2$ is used to block the transformation of nitrous oxide ($N_2O$) to nitrogen gas ($N_2$), thus the accumulation of $N_2O$ in the headspace of the incubation bottles is used to estimate denitrification rates (Holmes et al.,1996). Bottles were gently shaken for several minutes to ensure that $C_2H_2$ mixed well with the water, and were incubated in the dark at ambient laboratory temperature. Gas samples from the headspace were collected using a double needle in 10 mL vacutainers (DB Vacutainer ©), after 10 min and 18 h of the $C_2H_2$ addition. After collecting each gas sample, we added the same volume of $C_2H_2$ (i.e., 10 mL) to each bottle to maintain the gas volume constant and avoid pressure changes. The analysis of $N_2O$ concentration was conducted in the Serveis cientifico-tecnics of the University of Vic on an Agilent 7890A gas chromatography system (Agilent Technologies, Santa Clara, USA) equipped with electron-capture (ECD) and flame-ionization (FID) + methanizer detectors and three valves to obtain separately carbon dioxide ($CO_2$), methane ($CH_4$,) and $N_2O$ for every gas injection. An HP-Plot Q column (30 m x 0.32 mm x 20 µm) was used with a pre-column of the same characteristics but it was 15 m long. The injector and the oven temperature were set to 50 °C. The temperature of the FID and the ECD detectors were set to 250 and 300 °C, respectively. The methanizer temperature was set to 375 °C. For the FID detector, $H_2$ was used as a carrier gas and $N_2$ as a make-up gas at 35 and 25 mL $min^{-1}$, respectively. In the case of the ECD detector, 5% $CH_4$ in argon was used as a make-up gas at 30 mL $min^{-1}$. The detection limits for the used method was 0.02 mg $L^{-1}$ of $N_2O$.

The headspace of each bottle was measured after collection of the final $N_2O$ samples to scale up the concentrations obtained in the 10 mL vacutainers to the total $N_2O$ production in the bottle. The biomass of the biofilm in each bottle was measured as the ash-free dry mass (AFDM). Sediment in each bottle was dried at 60 °C for 12 h, cooled at room temperature and weighed to determine the dry mass. Sediment was then heated at 550 °C in a muffle furnace for 4 h and reweighed. AFDM was estimated as the difference between the weight of the dry mass and the weight of the mass after being muffled.

### 4.2.4. Data analysis

We used linear regression analysis with data from PRE-samplings to estimate DIN and DOC removal along the flumes based on the longitudinal variation of DIN and DOC concentrations for each flume treatment (i.e, unvegetated, *Iris*, *Scirpus* and *Phragmites*) before the C addition. Given that there were no additional water inputs along the flumes, we considered that longitudinal changes in DIN and DOC concentration were the result of the net balance between uptake (assimilation by biota and denitrification) and release (organic matter mineralization) processes within the flumes, and thus, could provide a good estimate of the net removal capacity of each flume. For DOC, we considered that microbial respiration was the main responsible process that contributed to the declines in concentration along the flumes (Berggren & del Giorgio, 2015; Wiegner, Kaplan, Ziegler, & Findlay, 2015). When DIN concentration increased along the flumes, we assumed that mineralization of organic matter was the main responsible of these increases (Teissier, Torre, Delmas, & Garabetian, 2007), although direct rates of mineralization were not directly measured. We also considered that root and microbial exudates could contribute to longitudinal increases in DOC concentration (Stottmeister et al., 2003), although this source was assumed to be low compared to DOC inputs from the effluent water which were high. Finally, we assumed no increases in DIN and DOC concentration along the flumes associated with evapotranspiration because longitudinal changes in EC (here used as a hydrological tracer) were minimal during the study period (i.e., <5 %; see values in section 4.2.3).

Despite longitudinal changes in concentration do not provide specific information about the magnitude of a particular biogeochemical process, this type of data analysis is useful for understanding whether the study mesocosms act either as net sinks (i.e., nutrient removal) or net sources (i.e., nutrient increase) of solutes. Longitudinal decreases in concentration for either DIN or DOC indicate that uptake processes predominate over release and thus, that the flume is acting as a net sink of these compounds and that nutrients are effectively removed along the flumes. Longitudinal increases in concentration indicate the opposite, so that release dominate over uptake processes; and thus, that the flume is acting as a net source of DIN and/or DOC along the flumes. No clear longitudinal pattern of DIN and/or DOC concentration was interpreted as an indication that uptake and release processes counterbalance each other. A similar conceptual approach has been successfully applied for inferring net nutrient uptake (i.e., nutrient removal) in more complex systems such as headwater stream reaches (Bernal et al. 2015; von Schiller et al. 2015).

To further explore the biogeochemical processes associated with longitudinal patterns of DIN concentration, we investigated longitudinal changes in the relative contribution of $NO_3^-$, $NO_2^-$ and $NH_4^+$ to total DIN. We assumed that longitudinal decreases in the proportion of $NH_4^+$ coupled to the increase in the proportion of either $NO_3^-$ or $NO_2^-$ were an indication of the occurrence of nitrification (i.e., oxidation of $NH_4^+$ to $NO_3^-$) along the flume. Notice that in this study nitrification was considered a DIN transformation process because do not influence DIN concentration and thus, the overall removal DIN capacity of each flume.

To investigate whether the addition of labile C induced changes in DIN and DOC removal along the flumes, we compared the relative change of either DIN or DOC concentration (ΔDIN and ΔDOC respectively, both in %) between data from PRE and +C samplings. Along each flume, we defined 6 segments comprised within two consecutive pair of sampling points as outlined in Figure 1. We calculated ΔDIN and ΔDOC for each segment as follows:

$$\Delta Conc_x = \left(\frac{Conc_{x-1} - Conc_x}{Conc_{x-1}}\right) x\ 100 \qquad \text{(Eq. 4.1)}$$

where $Conc_x$ and $Conc_{x-1}$ are the concentrations at a given sampling point and at the previous sampling point, respectively (mg $L^{-1}$ of either DIN or DOC). Similar to longitudinal changes in concentration, ΔDIN and ΔDOC cannot be associated to a particular biogeochemical process, but to the dominant process (i.e., uptake or release) that is characterizing the longitudinal profile of DIN and DOC at each segment. Thus, $\Delta Conc > 0$ denotes a decrease in concentration within the segment indicating uptake > release of either DIN on DOC (i.e., a removal), while $\Delta Conc < 0$ denotes the opposite. The ΔDIN and ΔDOC from the PRE- and +C samplings estimated in the different flume segments were compared using a two-way ANOVA (C addition and segments as fixed factors). The ANOVA test was run separately for each flume treatment (i.e., unvegetated, *Iris*, *Scirpus* and *Phragmites*). Post-hoc Tukey HSD tests followed significant ANOVA ($p < 0.05$).

In the laboratory assays, we focused on how the source of labile C influenced denitrification in biofilms from the flumes, which was expected to be the main biogeochemical process responsible for the permanent removal of DIN from the water column. We used data from the laboratory assays to calculate potential rates of DEA (in g $N_2O$ g $AFDM^{-1}$ $h^{-1}$) as follows:

$$DEA = \frac{M_f - M_i}{t\ x\ biomass} \qquad \text{(Eq. 4.2)}$$

where $M_f$ and $M_i$ are the $N_2O$ mass in the incubation bottle at the end and at the beginning of the incubation, respectively, $t$ is the incubation time (17.8 h), and *biomass* is the biofilm biomass in the sediments measured as AFDM (in g). Total mass of $N_2O$ in the incubation bottle was calculated using the volume of the headspace and its $N_2O$ concentration and the volume of water corrected for $N_2O$ solubility in the liquid phase with an appropriate temperature-dependent Bunsen coefficient (Knowles, 1979). We used a two-way ANOVA model to explore differences in DEA among sediments from different flume treatments (i.e., unvegetated, *Iris* and *Phragmites*) and between unamended (i.e., control) and C amended (i.e., + C treatment) incubations. Post-hoc Tukey HSD tests followed significant ANOVA ($p < 0.05$).

We ran all statistical tests with R 2.15.0 (R Foundation for Statistical Computing, Vienna, Austria, http://www.R-project.org/.). When necessary, data were log-transformed before analysis to meet assumptions of homogeneity of variance and normality (Zar 1996).

## 4.3. Results

### 4.3.1. Characterization of Subsurface Water Within the Flumes During PRE Samplings

Physicochemical conditions in subsurface water were similar for all flumes (Table 4.1). EC was high in all cases, ranging from 1.8 until 2.8 mS cm$^{-1}$. There were no changes in EC along the unvegetated flumes (<1 % increase), while EC tended to increase by 3.1, 4.6 and 2.0% along flumes with *Iris*, *Scirpus* and *Phragmites*, respectively. Oxygen saturation (in %) was consistently low in all the flumes, ranging from 3.6 to 4.5%. DIN was dominated by $NO_3^-$ and represented the 55% of TDN, whereas DON represented the remaining 45%. DOC concentration was high in all flumes, ranging from 6.8 to 11 mg C L$^{-1}$.

**Table 4.1.** Electrical conductivity (EC), dissolved oxygen saturation (DO sat) and nutrient concentrations of the subsurface water within the sediments in the unvegetated flumes and in flumes containing *Iris pseudachorus* (Iris), *Scirpus lacustris* (Scirpus) and *Phragmites australis* (Phragmites). Data shown correspond to the PRE samplings (i.e., before C addition). Values given are means ± SE. In all cases n = 21 (3 flumes per treatment and 7 sampling points per flume).

| Variable | Unvegetated | *Iris* | *Scirpus* | *Phragmites* |
|---|---|---|---|---|
| EC (mS cm$^{-1}$) | 2.8 ± 0.002 | 2.6 ± 0.01 | 2.8 ± 0.02 | 1.8 ± 0.01 |
| DO sat (%) | 4.5 ± 1.5 | 5.6 ± 2.5 | 3.6 ± 1.8 | 4.3 ± 2.0 |
| $NO_3^-$ (mg N L$^{-1}$) | 6.0 ± 0.6 | 4.3 ± 0.2 | 5.6 ± 0.5 | 2.0 ± 0.3 |
| $NO_2^-$ (mg N L$^{-1}$) | 0.1 ± 0.02 | 0.02 ± 0.001 | 0.08 ± 0.02 | 0.02 ± 0.01 |
| $NH_4^+$ (mg N L$^{-1}$) | 0.65 ± 0.4 | 0.12 ± 0.03 | 0.73 ± 0.3 | 0.09 ± 0.03 |
| DIN (mg N L$^{-1}$) | 6.7 ± 0.4 | 4.5 ± 0.2 | 6.4 ± 0.4 | 2.1 ± 0.3 |
| DON (mg N L$^{-1}$) | 4.5 ± 1.8 | 5.2 ± 0.9 | 4.4 ± 1.4 | 1.6 ± 0.3 |
| TDN (mg N L$^{-1}$) | 9.5 ± 0.6 | 8.8 ± 0.1 | 9.5 ± 0.5 | 3.7 ± 0.2 |
| DOC (mg C L$^{-1}$) | 11.3 ± 0.06 | 11.0 ± 0.05 | 10.9 ± 0.12 | 6.8 ± 0.04 |

### 4.3.2. Longitudinal Patterns of DIN and DOC Concentrations During PRE Samplings

On average, EC increased by 0.4, 3.1, 4.6 and 2.0 % between the inlet and outlet of unvegetated flumes and flumes with *Iris, Scirpus* and *Phragmites*, respectively. These results indicated that evapotranspiration had a low effect on longitudinal profiles of DIN and DOC concentrations. Mean WRT (± SE) was 3.9 ± 0.1, 5.4 ±

0.3, 7.7 ± 0.7 and 9.2 ± 0.4 h for unvegetated flumes and flumes with *Iris, Scirpus* and *Phragmites*, respectively.

Unvegetated flumes showed no longitudinal changes in DIN concentration, whereas DIN concentration significantly decreased along flumes with helophytes (Figure 4.2). On average, DIN concentration decreased by 16.5, 12.0 and 37% between the inlet and outlet of the flumes with *Iris, Scirpus* and *Phragmites* respectively.

The relative contribution of $NO_3^-$, $NO_2^-$ and $NH_4^+$ to total DIN remained constant along the unvegetated flumes (Figure 4.2A). In contrast, in flumes with helophytes the relative contribution of $NH_4^+$ decreased while the relative contribution of $NO_3^-$ increased along the flumes (Figure 4.2; B, C and D). This longitudinal shift in the relative contribution of different DIN species was more evident in the flumes with *Scirpus* and *Phragmites* than in those with *Iris*.

Longitudinal changes in DOC concentration varied among the flume treatments. Unvegetated flumes showed no longitudinal changes in DOC concentrations, whereas DOC concentration significantly declined in flumes with helophytes (Figure 4.3). On average, DOC concentration decreased by 5.3, 6.3 and 6.9% between the inlet and outlet of flumes for *Iris, Scirpus* and *Phragmites*, respectively.

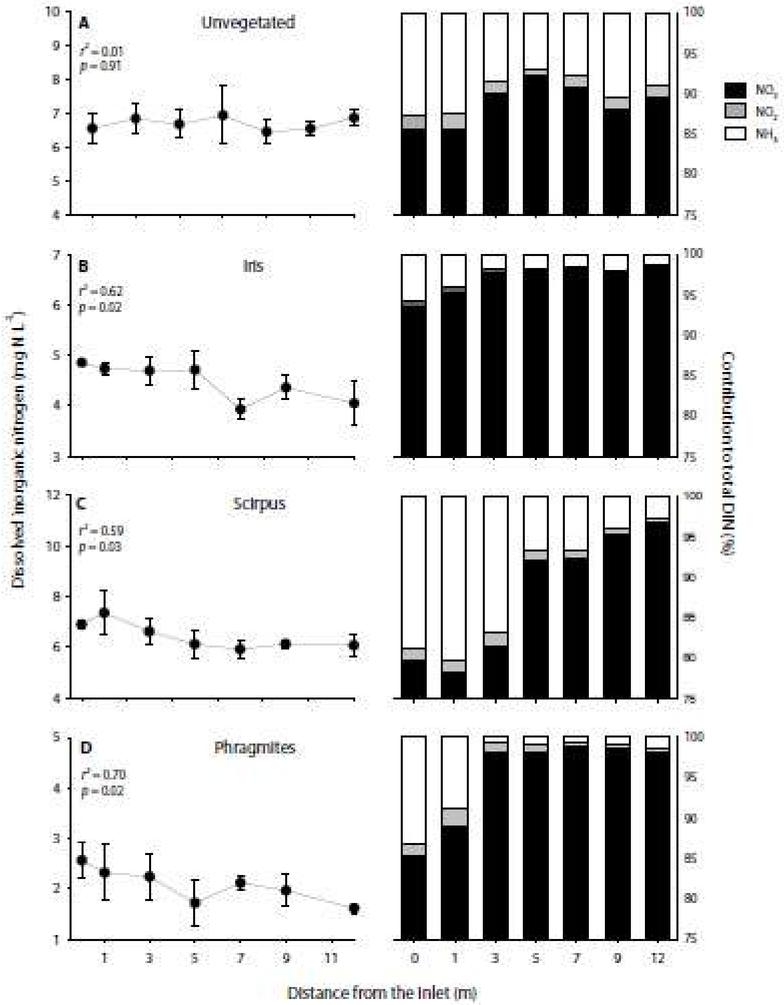


**Figure 4.2.** Longitudinal gradients of dissolved inorganic nitrogen (DIN) concentrations along the flumes (left column) and the relative contribution of nitrate ($NO_3^-$), nitrite ($NO_2^-$) and ammonium ($NH_4^+$) to total DIN concentrations (right column) before C additions (i.e., PRE samplings) in unvegetated flumes (A) and in flumes containing *Iris pseudachorus* (B; Iris), *Scirpus lacustris* (C; Scirpus) and *Phragmites australis* (D; Phragmites). For the longitudinal DIN gradients, the adjusted R-squared ($r^2$) and p-value ($p$) from the linear regression analysis are shown. For each treatment, data given are the mean ± SE of the 3 flumes.

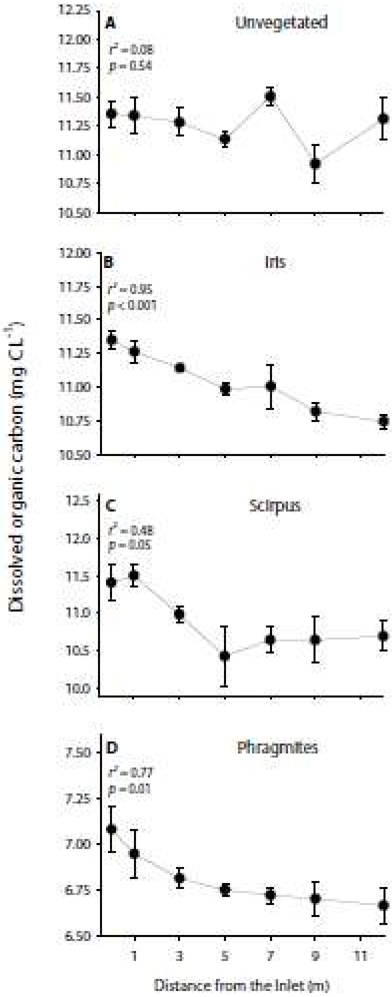


**Figure 4.3.** Longitudinal gradients of dissolved organic carbon (DOC) along the flumes before labile C addition (i.e., PRE samplings) in unvegetated flumes (A) and in flumes containing *Iris pseudachorus* (B; Iris), *Scirpus lacustris* (C; Scirpus) and *Phragmites australis* (D; Phragmites). The adjusted R-squared ($r^2$) and p-value ($p$) for the linear regression analysis are showed. For each treatment, data given are the mean ± SE of the 3 flumes.

### 4.3.3. Effect of Labile DOC Addition on DIN and DOC Concentrations

On average, the DOC addition increased DOC concentration (mean ± SE) at the inlet from 10.3 ± 0.6 to 11.5 ± 0.6 mg C L$^{-1}$, which is a 10.4% increase of background DOC concentration. Most of the DOC added was removed within the first meter of the flumes (84 ± 11%). This pattern was consistent among all flumes for all treatments. Thus, the comparison of $\Delta Conc$ between PRE and +C samplings was conducted for the first two segments of the flumes (i.e., 0-1 m and 1-3 m; Figure 4.1)

During PRE sampling conditions, there were small changes in DIN concentration for the first two segments of the flumes (Figure 4.4). Mean (± SE) $\Delta$DIN was 0.4 ± 4.0 and 3.3 ± 4.0% for the first and the second segment, respectively, with no statistically significant differences between them (two-way ANOVA, factor flume segment, $df = 1$, $F < 1.2$, p-value $\geq 0.31$). This trend was consistent among unvegetated and vegetated flumes. Similar to DIN, during PRE sampling conditions, there were small differences in DOC concentration between the two segments of the flumes (Figure 4.5). Mean (± SE) $\Delta$DOC was 0.5 ± 0.6 and 1.8 ± 0.7 % for the first and the second segment respectively with no statistically significant differences between them (two-way ANOVA, factor flume segment, $df = 1$, $F < 3.4$, p-value $\geq 0.11$). This trend was consistent among unvegetated and vegetated flumes (Figure 4.5).

During +C sampling conditions, changes in DIN concentration for the first two segments were greater than those during the PRE samplings (Figure 4.4). Mean (± SE) $\Delta$DIN was 11.0 ± 7.4 and 8.2 ± 4.7% for the first and the second segment respectively with no statistically significant differences between them (two-way ANOVA, factor flume segment, $df = 1$, $F \leq 4.4$, p-value $\geq 0.07$). During +C sampling conditions, changes in DOC concentration for the first two segments were greater than those during the PRE samplings (Figure 4.5). Mean (± SE) $\Delta$DOC was 13.1 ± 1.7 and 2.0 ± 1.1% for the first and the second segment, respectively. $\Delta$DOC was significantly higher in the first than in the second segment in all flumes except in the *Phragmites* treatment (Tukey test; p-value $\leq 0.04$).

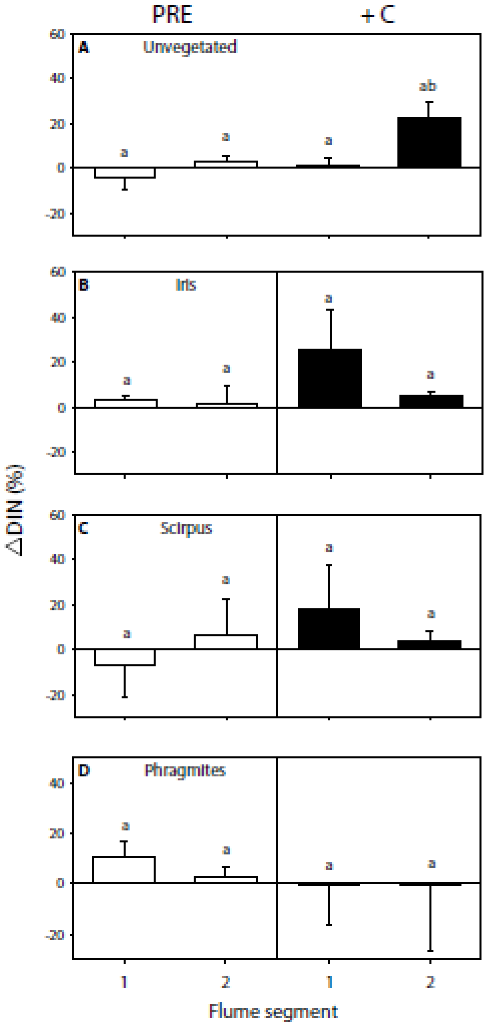


**Figure 4.4.** Percentage variation of dissolved inorganic nitrogen (ΔDIN) concentration at flume segment 1 (i.e., 0 - 1m) and 2 (i.e, 1 – 3 m) in unvegetated flumes (A) and in flumes containing *Iris pseudachorus* (B; Iris), *Scirpus lacustris* (C; Scirpus) and *Phragmites australis* (D; Phragmites). For each treatment, data given are the mean ± SE of the 3 flumes. Different letters denotes statistically significant differences ($p < 0.05$) in ΔDIN based on results from a two-way ANOVA model with flume segment (i.e., 1 and 2) and C addition (i.e., PRE in left column and + C sampling in right column) as a fixed factors and post-hoc Tukey HSD tests.

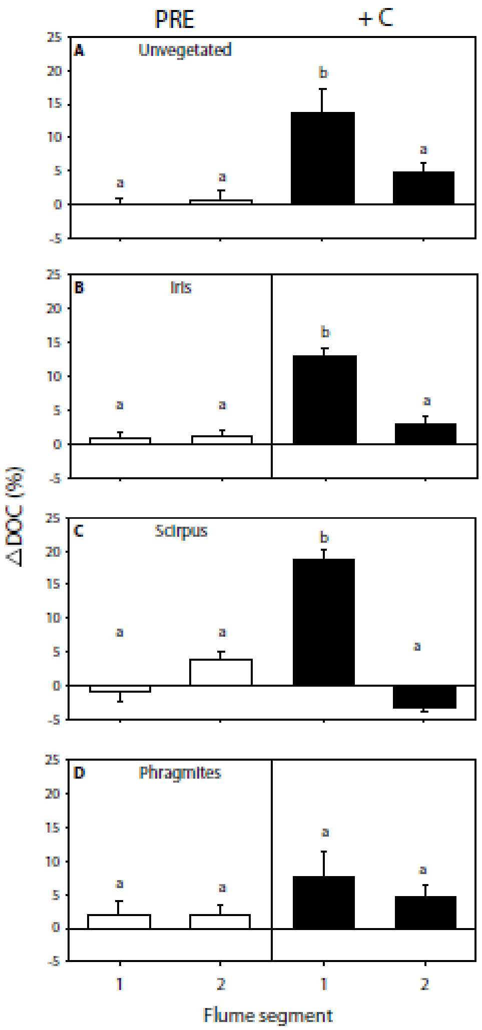


**Figure 4.5.** Percentage variation of dissolved organic carbon (ΔDOC) concentration at flume segment 1 (i.e., 0 - 1m) and 2 (i.e, 1 – 3 m) in unvegetated flumes (A) and in flumes containing *Iris pseudachorus* (B; Iris), *Scirpus lacustris* (C; Scirpus) and *Phragmites australis* (D; Phragmites). For each treatment, data given are the mean ± SE of the 3 flumes. Different letters denotes significant differences ($p < 0.05$) in ΔDOC based on results from a two-way ANOVA model with flume segment (i.e., 1 and 2) and C addition (i.e., PRE in left column and + C sampling in right column) as a fixed factors and post-hoc Tukey HSD tests.

### 4.3.4. Effect of Labile DOC Amendment on DEA In Flume Sediments

The rates of $N_2O$ production in DEA assays from unamended incubations significantly differed among sediments from different flume treatments (Figure 4.6). $N_2O$ production rates of sediments from flumes with *Phragmites* were higher than those from flume sediments with *Iris* (Tukey test; p-value = 0.003) but similar to those of sediments from unvegetated flumes (Tukey test; p-value = 0.46). DEA assays incubated with amendment of labile C showed $N_2O$ production rates 2 orders of magnitude higher than those from unamended DEA assays (Table 4.2, Figure 4.6), and $N_2O$ production rates were similar among sediment treatments (Tukey test; p-value ≥ 0.73). The interaction between the flume treatment (i.e., unvegetated, *Iris* and *Phragmites*) and the C treatment (unamended vs amended) was not significant (Table 4.2).

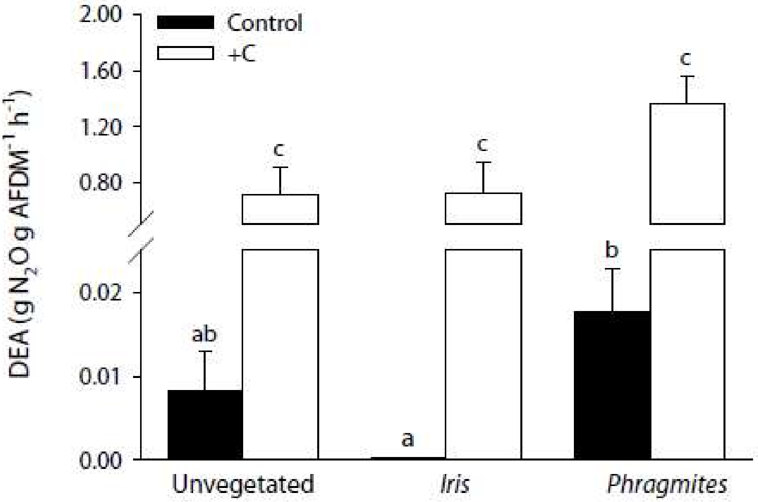


**Figure 4.6.** Comparison of potential denitrification enzyme activity (DEA; mg $N_2O$ g AFDM$^{-1}$ h$^{-1}$) between unamended (i.e., control) and C amended (+C) incubations of biofilm developed on gravels from the unvegetated flumes (A) and the flumes containing *Iris pseudachorus* (B; Iris) and *Phragmites australis* (C; Phragmites). For each treatment, data given are the mean (± SE) from the 3 different flumes. Different letters denotes significant differences ($p < 0.05$) based on results from a two-way ANOVA model with flume treatment (i.e., unvegetated, *Iris* and *Phragmites*) and C amendment (i.e., control and +C) as fixed factors and post-hoc Tukey HSD tests.

**Table 4.2.** Results from the two-way ANOVA model on N2O production rates from the potential denitrification enzyme activity (DEA) assays conducted with biofilms developed on sediments from the flumes, with flume treatment (i.e, unvegetated, Iris and Phragmites) and C amendment (i.e., control and +C) as fixed factors. Values highlighted in bold indicate significant effects ($p < 0.05$).

| *Variable* | *df* | *F* | *p-value* |
|---|---|---|---|
| $N_2O$ production | | | |
| Flume treatment | 2 | 10.33 | **0.002** |
| C amendment | 1 | 288 | **< 0.001** |
| Treatment * C amendment | 2 | 3.86 | 0.05 |

## 4.4. Discussion

Results from this study showed that WWTP effluent-receiving flumes with helophytes had a remarkable capacity to biogeochemically transform and remove DIN from the effluent. This finding was supported by two different observations: (i) the change in the contribution of different N forms to total DIN mostly along the vegetated flumes, and (ii) the significantly decrease in total DIN concentration along the flumes with helophytes. These results agreed with our expectations and were consistent with previous studies showing that aquatic plants (i.e., helophytes) promote DIN transformation and removal along subsurface water flowpaths (Nivala, Hoos, Cross, Wallace, & Parkin, 2007; Schade et al., 2001). The longitudinal shift in the relative contribution of $NH_4^+$ towards $NO_3^-$ observed in the flumes with helophytes, suggested that activity of nitrifying bacteria was enhanced in these flumes. This finding was in agreement with previous studies reporting that helophytes stimulated bacterial nitrification within the sediments by generating aerobic microenvironments during the translocation of $O_2$ from the shoots to the roots (Gersberg, Elkins, Lyon, & Goldman, 1986; Reddy et al., 1989; Williams, May, Ford, & Butler, 1994). Given the hypoxic environment in the flumes (dissolved oxygen saturation <10 %), the translocation of $O_2$ by helophytes may be essential for ensuring the occurrence of nitrification at the microsite scale. Nitrification involves the oxidation of $NH_4^+$ into $NO_3^-$; thus, this process does not directly contribute to the removal of DIN. However, in WWTP effluent-receiving systems, this is a relevant biogeochemical process that contributes to decrease high ambient $NH_4^+$ concentration and thus, decrease the harmful effects of high levels of $NH_4^+$ on aquatic biota (Camargo & Alonso, 2006; Lambert & Davy, 2011). In addition, the resulting $NO_3^-$ from nitrification can eventually be transformed into N gas by denitrifying bacteria and thus, eventually become permanently removed from the system.

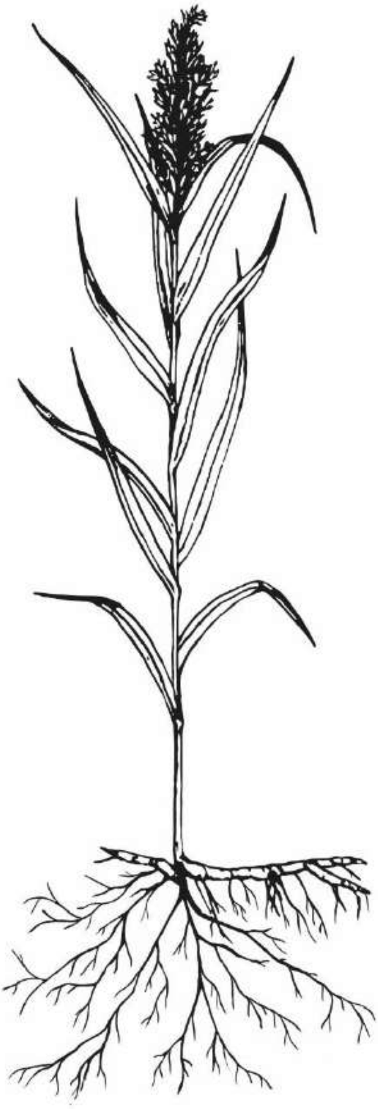
The longitudinal decrease in DIN concentration observed in vegetated flumes suggests that presence of helophytes contributed to increase removal of DIN from the water column, and further, that the magnitude of the processes responsible for DIN removal (i.e., assimilatory N uptake and denitrification) was larger than DIN production through organic matter mineralization. Previous studies have shown that helophytes rely on DIN to meet their N requirements, thus assimilatory uptake of both $NO_3^-$ and $NH_4^+$ by these plants could contribute to explain the observed pattern (Levi et al., 2015; Pastor et al., 2013; Peipoch et al., 2014). In addition, helophytes provide favorable environments for biofilm development around their root systems (Ramey et al., 2004; Stottmeister et al., 2003). Therefore, bacterial assemblages associated with the rhizosphere could additionally contribute to DIN removal through microbial assimilation. Furthermore, helophytes contributed to increase the subsurface water residence time within the flumes as suggested in previous studies (Chazarenc, Maltais-Landry, Troesch, Comeau, & Brisson, 2007; P. R. Knowles, Griffin, & Davies, 2010). This physical factor could additionally favor the interaction between DIN and biota within the flumes which may enhance biological DIN removal (Drummond, Bernal, von Schiller, & Marti, 2016; Hall, Bernhardt, & Likens, 2002).

In addition, microbial denitrification within the rhizosphere could also contribute to the observed decrease in DIN concentrations along vegetated flumes. This is especially feasible since dissolved oxygen concentration in subsurface water was low along the flumes throughout the whole study period. However, hypoxic conditions were not sufficient to explain the decline in DIN concentration, because unvegetated flumes also showed low dissolved oxygen concentration. Previous studies have proposed that helophytes burst denitrification because their root exudates are an important local source of labile DOC (Kofoed, Stief, Hauzmayer, Schramm, & Herrmann, 2012; Williams et al., 1994). However, the DEA assays in control treatments (without addition of labile C) showed that biofilms growing in vegetated flumes had the same potential for denitrification than those in unvegetated flumes. In addition, DEA assays highlighted that denitrification in sediment biofilms from the flumes was dramatically increased with the addition of the labile C source regardless of flume treatment. These results indicated that quality of DOC was a limiting factor for microbial denitrification, suggesting that quality of DOC from the WWTP effluent could constrain denitrification in the flumes (Fernandez-Nava et al., 2010; Pulou et al., 2012).

At the flume scale, addition of labile C leads to the conclusion that microbial activity was limited by the availability of labile DOC because 84 % of the added C was removed within the first meters in all the flumes. Therefore, our results highlight that the study system was strongly limited by C, despite high DOC concentrations (9.7 ± 0.6 mg C $L^{-1}$) in the WWTP effluent. Nevertheless, the high demand for labile C observed in the flumes and the concomitant high rates of DOC removal was not accompanied by declines in DIN concentration, as it would be expected if DOC would have been used as electron donor by denitrifying bacteria (Lin et al., 2009; Seitzinger 1988). This result does not necessarily mean that denitrification was not enhanced by the addition of labile DOC, in fact this burst in denitrification was suggested by the laboratory DEA experiments. Thus, we proposed that increases in the rate of denitrification may not be large enough to counterbalance DIN produced by mineralization of dissolved organic matter (Teissier et al., 2007). This result highlights that the occurrence of concomitant declines in DIN and DOC concentrations, as observed along the vegetated flumes in the PRE sampling conditions, may not necessarily be coupled to each other, and could respond to different biogeochemical processes such as assimilatory uptake (for DIN) and microbial respiration (for DOC). Despite the approach used in this study has limited power to identify particular biogeochemical processes associated with net changes in ambient nutrient concentrations, our results show that this is a helpful tool to identify contrasting patterns in DOC and DIN concentrations that emerged with the presence of helophytes in the flumes. Future studies using $^{15}N$ or $^{14}C$ additions would be helpful to disentangle the different biogeochemical processes that contribute to the observed longitudinal patterns of DIN and DOC concentration as well as to quantify the relative contribution of each biological compartment (i.e., helophytes and microbial communities) to N and C removal.

In conclusion, results from this study showed that presence of helophytes contributed to (i) remove the excess of DIN from WWTP effluents, and (ii) enhance nitrification along subsurface water flowpaths. Therefore, helophytes contribute to the transformation and removal of DIN from the WWTP effluent. Nevertheless, removal of DOC and its response to addition of labile DOC addition did not seem to be related to DIN removal at the flume scale. In contrast, laboratory DEA assays indicated that the availability of labile DOC in the WWTP effluent was a limiting factor for microbial denitrification. Considering results at both flume and sediment scale together, it seems that labile DOC additions in flumes mostly contribute to increase respiration rather than denitrification. Alternatively, considering that oxygen availability was low, results suggest that potential increases in denitrification associated with the addition of a labile C source were counterbalanced by high rates of mineralization. Altogether, this study highlights the potential role of helophytes as relevant bioremediation tool to improve water quality in WWTP effluent-influenced aquatic ecosystems. However, it also provides insights on the relevance of DOC quality from WWTP effluent and how it can contribute to deal with DIN removal in streams receiving WWTP effluents.

# CHAPTER FIVE



# General Discussion

In the present thesis we investigated physical and chemical functions of emergent macrophytes and their role in nutrient retention in freshwater ecosystems. Here, in the section of the general discussion, I consider these macrophyte functions as potential phytoremediating mechanisms for enhanced NBS treatment performance.

Specifically, in this section I 1) evaluate the use of emergent macrophytes in NBS applications, based on a survey of technical literature, 2) bring together the results of this thesis, by discussing the different influences that macrophytes exert on the rhizosphere, 3) summarize the gaps in current knowledge about NBS implementation, based on a literature analysis, and discuss the contribution of this thesis to bridge these gaps, 4) point at plant species selection as essential for NBS treatment performance and synthesize evidence from literature to link macrophyte root traits and their beneficial role in nutrient retention.

## 5.1. The Involvement of Macrophytes in NBS

Emergent macrophytes are routinely employed in NBS of varying goals. To know more about how are macrophytes used across the different NBS goals, we analyzed the Naturvation database (a case study database provided by the "NATure-based Urban innoVATION" project; https://naturvation.eu/atlas), that contains 1000 implemented NBS across Europe. The Naturvation database is a representative sample of NBS implementation in urban environments across Europe until 2017 (Almassy et al., 2018).

From the 12 global development challenges that NBS tackle according to the Naturvation database, the use of macrophytes could be involved to the first five most frequently addressed (Figure 5.1). Specifically, the provision of "green space, habitats and biodiversity" was the most frequently addressed challenge, with 87% of the projects dealing with it, while 60% of the projects addressed "regeneration, land-use, or urban development". "Health and well-being" was the third most addressed challenge, counting 56% of the projects. Almost half of the cases within the database (46%) achieved "environmental quality" improvement, and finally 38% of the cases focused and addressed water management issues. From these results it is evident that the use of macrophytes is a relevant NBS practice as it can contribute to tackle the most common NBS challenges.

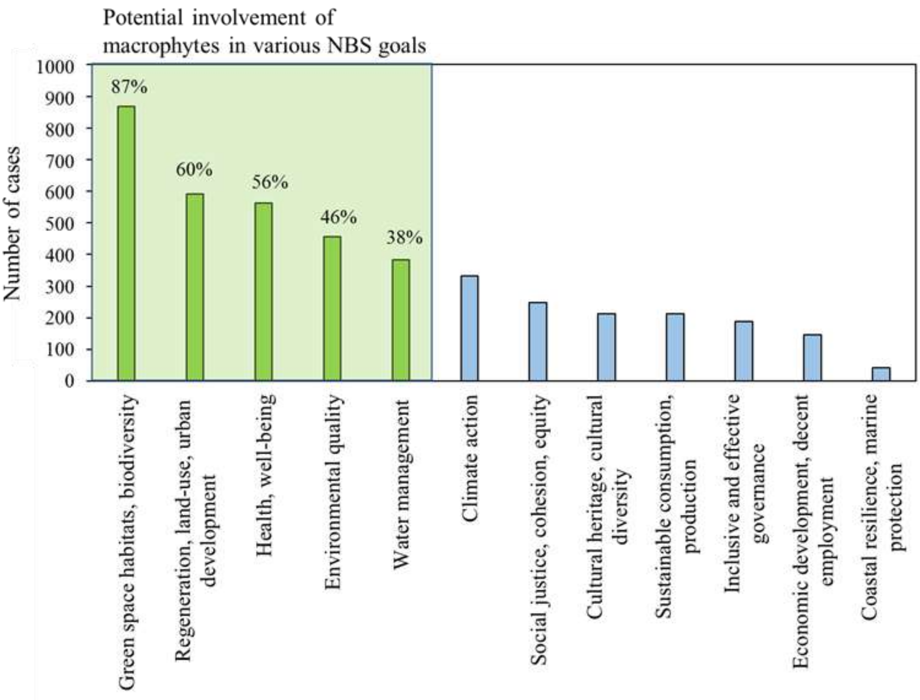


**Figure 5.1.** Number of implemented NBS cases across the different addressed challenges. Percentages above the bars denote the percentage of projects across the different addressed challenges, in respect to the total number of NBS. Each project may address more than one challenges, therefore percentages do not sum up to 100%. The use of emergent macrophytes may be involved in the five most frequently addressed NBS challenges. Data from https://naturvation.eu/

However, the nature of emergent macrophytes as aquatic plants limits their involvement to only freshwater environments. To get a sense of the potential involvement of emergent macrophytes across the different NBS goals in freshwater environments, we selected cases implemented in freshwater urban settings, labeled as “riverbank greens”, “wetland/bog/fen/marsh”, “lake/pond, river/stream/canal/estuary”, “delta”, and “other”, under the “Urban setting” field of the database. Our analysis revealed that the provision of “green space, habitats and biodiversity”, closely followed by “water management” were the most frequently addressed challenges in freshwater-related NBS cases, counting 326 and 292 cases respectively (Figure 5.2). Therefore, indicating the NBS goals where the use of macrophytes would be of most relevance.

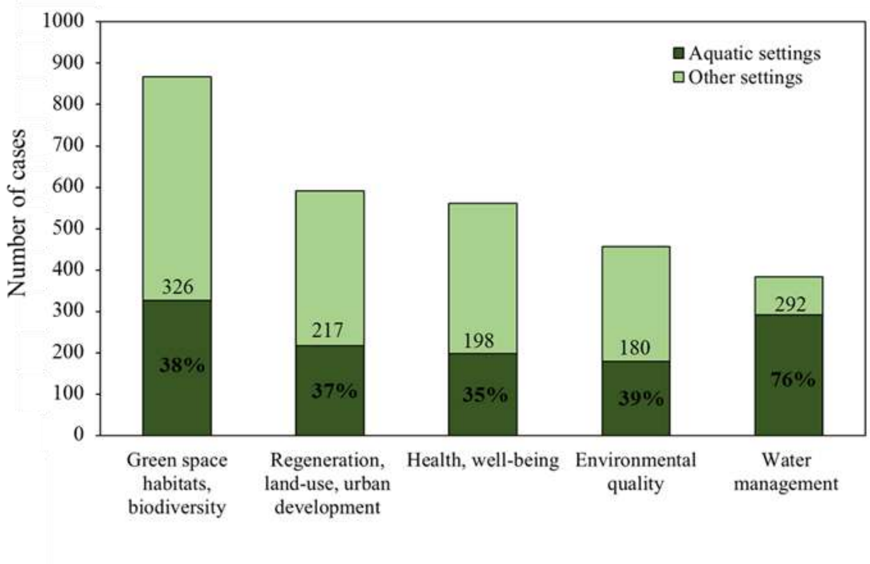


**Figure 5.2.** Proportion of NBS projects in freshwater settings, where emergent macrophytes can grow, across the addressed challenges that emergent macrophytes may contribute to. Bars indicate the number of NBS projects in all urban settings. Values on top of the dark green area of the bars, show the number of projects implemented only in freshwater settings. Percentages indicate the proportion of NBS in freshwater settings, in respect to all settings. Data from https://naturvation.eu/

Even though NBS projects span a wide range of objectives, as shown in Figure 5.1, water quality improvement is not distinguished among the NBS goals of the Naturvation database. However, water quantity and quality issues, often interconnected, are mostly represented by the "water management" challenge, as it is in line with SDG 6 for "water availability, sustainable management, and sanitation" of the United Nations 2030 Agenda for Sustainable Development (Almassy et al., 2018). Hence, the relevance of "water management" as one of the main challenges addressed by NBS in aquatic environments also reveals the great potential of emergent macrophytes as a technique that contributes to tackle water quantity and quality problems. Specifically, regarding water quality, 130 cases in the Naturvation database were reported to deliver water quality improvement (Almassy et al., 2018), representing the 34% of NBS projects in freshwater settings. This result suggests that water quality improvement holds a substantial portion of the interest of NBS projects in freshwater environments.

## 5.2 The Influence of Emergent Macrophytes on the Subsurface Aquatic Environment

Most NBS aiming to address poor water quality in aquatic ecosystems are based on the principles of phytoremediation (e.g. constructed wetlands, stormwater biofiltration systems). Enhancement of phytoremediation processes requires a good understanding of the plant-substrate-microorganisms-pollutants continuum and their interactions that take place in the rhizosphere (Cheng et al., 2009; Wenzel, 2009). Yet, the rhizosphere is considered as a "black box" and appears to be poorly studied (Cheng et al., 2009; Hinsinger, Bengough, Vetterlein, & Young, 2009; Stottmeister et al., 2003). Researchers highlight the lack of knowledge in the connection of physical, chemical and biological processes within the rhizosphere, and call for an interdisciplinary research approach to achieve an holistic view of the rhizosphere, which is considered to be one of the most dynamic interface habitats on Earth (Hinsinger et al., 2009; Philippot, Raaijmakers, Lemanceau, & van der Putten, 2013).

In response to this call, the present thesis contributes towards a holistic perception of the mechanisms taking place in the rhizosphere, by exploring the crossroads between the physical, chemical and biological influence that the presence of macrophytes exerts on the subsurface aquatic environment. In Chapter 1 we investigated the physical influence of macrophyte roots on solute transport within water saturated sediments. In Chapters 2 and 3, we explored the chemical and biological influence of macrophytes on the subsurface aquatic environment, as evidenced by variable N and P retention among different macrophyte species.

### 5.2.1 Physical Influence of Macrophytes: Hydrology

The ways in which plant roots physically modify their surrounding environment have consequences for solute transport around the roots (Gregory

& Hinsinger, 1999), and for the habitat of rhizosphere microorganisms (Hinsinger, Gobran, Gregory, & Wenzel, 2005). However, this physical effect of roots is by far the least documented, as compared to the chemical and biological effect, most likely due to methodological constraints (Hinsinger, 2005). For example, root architecture, which is one of the factors controlling the physical role of the roots into the soil, is a property difficult to maintain during sampling (Gyssels et al., 2005). The role of hydrology on the performance of NBS is rather complex, since conflicting hydrological mechanisms determine a system's success or failure (Dagenais et al., 2018). Hydraulic retention is critical in NBS aiming nutrient loading attenuation, since it provides sufficient contact time between bioreactive sediments and solutes, necessary for biogeochemical processing and thus nutrient retention (e.g., Ngo et al., 2010; Tanner, Clayton, & Upsdell, 1995; Valett et al., 1996). On the other hand, water infiltration in porous media is desired in NBS since it ensures the successful functioning of a system. Clogging is considered the main operational issue of constructed wetlands and stormwater filters, ultimately resulting to total failure of such systems (Kandra, Deletic, & McCarthy, 2014; P. Knowles, Dotro, Nivala, & Garcia, 2011). Finally, water balance is important in the design of constructed wetlands for wastewater treatment, since there is the need to ensure continuous inflow through the constructed wetlands media, and at the same time overflow prevention. Hydrological retention, and infiltration (or reversely, clogging) rely on the hydraulic properties of the NBS porous media, while water balance depends on the hydraulic loading rate, rainfall, and evapotranspiration (Pedescoll, Sidrach-Cardona, Sanchez, & Becares, 2013). Therefore, understanding the effect of macrophytes on the hydrology of NBS relies on research that explores the role of roots on sediment hydraulic properties, and on water content.

The influence of macrophytes on the hydraulic properties and water content of the sediments is studied within three different contexts: a) to provide guidelines for the optimal operation of NBS, b) to control water erosion (i.e. slope stability), and c) to investigate their impact on water resources (e.g. for irrigation).

Regarding the hydraulic properties, many studies conclude that the roots of emergent macrophytes significantly modify the amount of water that can pass through the sediments (i.e., the hydraulic capacity). The varying implications of this fact have been the stimulus of several studies within the three different contexts (a, b, c, as mentioned above). Hydraulic properties are essential within "Context a" since they drive infiltration/clogging. However, as viewed within "Context b", increased infiltration is detrimental for slope stability (A. Simon & Collison, 2002). Sediment hydraulic properties also affect water distribution and availability in the soil, as shown by studies within "Context c". The different study contexts of sediment hydraulic properties and the main parameters they influence, are summarized in Table 5.2. Particularly regarding "Context a", many studies report a positive effect on hydraulic capacity, evidenced by increased hydraulic conductivity (i.e. the ease with which water can move through porous media), which leads to enhanced water infiltration. Nevertheless, others show the adverse role of the presence of macrophytes on the hydraulic conductivity of the porous media, with clogging being the main implication. Therefore, the effect of macrophytes on the hydraulic capacity of the sediments remains unclear (Hua et al., 2014). Examples of research articles debating over this topic are listed in Table 5.1. Yet, few studies provide insights on the mechanisms with which macrophyte roots influence the hydraulic properties of the porous media, and thus, help to elucidate the reason for these controversial results (Archer, Quinton, & Hess, 2002; Hua et al., 2014; Le Coustumer, Fletcher, Deletic, Barraud, & Poelsma, 2012; Nikolakopoulou et al., 2018) Chapter 2). Archer et al., 2002, Le Coustumer et al., 2012, and Nikolakopoulou et al., 2018 agree that hydraulic conductivity of the porous media is controlled by root morphological traits, with thick roots creating preferential flowpaths owing to macropores, and thin roots being responsible for low hydraulic capacity by filling the space in-between the sediment particles. Further, Hua et al., 2014 argued for the temporal and spatial variation in plants influence on sediment hydraulic conductivity being the main cause of the controversy. The conflicting roles of macrophytes on the hydraulic capacity of the porous media, and their implications to NBS applications are summarized in Table 5.1.

**Table 5.1.** The conflicting roles of macrophytes on the hydraulic properties of the sediments, and the implications to NBS applications.

| Effect of plants | Implications | Positive/Negative Outcomes | References |
|---|---|---|---|
| Increase of hydraulic conductivity | + Infiltration | Good functioning of NBS | Archer et al., 2002; Brix, 1994, 1997; Gonzalez-Merchan, Barraud, Le Coustumer, & Fletcher, 2012; Hatt, Fletcher, & Deletic, 2009; Hua et al., 2014; Le Coustumer et al., 2012; X. Y. Li, Yang, Li, & Lin, 2009; Stottmeister et al., 2003; Z. Zhang et al., 2018 |
|  | - Low stormwater runoff retention | Flooding, overflow |  |
| Decrease of hydraulic conductivity | + High hydraulic retention | Enhanced nutrient retention | Archer et al., 2002; Le Coustumer et al., 2012; Nikolakopoulou et al., 2018; Pedescoll, Corzo, Alvarez, Garcia, & Puigagut, 2011; Stottmeister et al., 2003 |
|  | - Clogging | Flooding, overflow |  |

Note. Positive sign (+) denotes a positive implication, while negative sign (-) an adverse one. The reference list is non-exhaustive.

The effect of macrophyte roots on sediment water content via evapotranspiration is undisputed. A plethora of studies about evapotranspiration exist, spanning all three different contexts (a, b, c, as mentioned above; Table 5.2). Specifically, within "Context a" evapotranspiration is essential for the successful operation of constructed wetlands, since it affects water balance, which is a key factor in constructed wetlands design (Borin, Milani, Salvato, & Toscano, 2011). Evapotranspiration influences soil water content which is important within "Context b", since it is known to affect slope stability (e.g. Wu, 1984). Finally, regarding "Context b", evapotranspiration studies contribute knowledge to water resources research and applications, e.g. irrigation systems design e.g., Garatuza-Payan et al., 1998.

**Table 5.2.** The different contexts within which sediment hydraulic parameters and evapotranspiration are studied, and the parameters they influence.

| **Contexts** | **Sediment hydraulic parameters** | **Evapotranspiration** |
|---|---|---|
| Context a:<br>NBS design | Infiltration/clogging | Water balance |
| Context b:<br>erosion control | Infiltration | Soil moisture |
| Context c:<br>water resources | Water distribution | Water content |

Based on the revised literature, in Chapter 2 of this thesis we considered the ability of emergent macrophytes to influence subsurface hydrology by both mechanisms (i.e. changing sediment hydraulic properties and removing water content). We concluded that the joint effect of these mechanisms was responsible for increased hydraulic retention through porous media and would potentially result to enhanced nutrient retention. In that way we put together both aspects of the effect of macrophytes on rhizosphere hydrology, highlighting their synergistic role as a key factor in subsurface solute transport. However, it is generally accepted that the effect of roots on porous media hydrology has been overlooked (De Baets, Poesen, Gyssels, & Knapen, 2006; Pedescoll et al., 2011; Reubens et al., 2007) and possibly due to methodological constraints (Gyssels & Poesen, 2003). Therefore, more research is required to shed light on the physical influence that macrophytes exert on the rhizosphere.

### 5.2.2 Chemical - Biological Influence of Macrophytes

The chemical and biological effects of plant roots on the rhizosphere are interconnected. Plant roots can chemically modify their rhizosphere by depleting nutrients via uptake, thus affecting the pH, by releasing organic and inorganic root exudates which create rhizodeposits, and by releasing oxygen (Darrah, 1991; El-Shatnawi & Makhadmeh, 2001; Philippot et al., 2013). These chemical changes in turn influence the microbial communities and biodiversity in the rhizosphere (Hinsinger et al., 2009; Philippot et al., 2013). Plants exert direct biological influence on the rhizosphere as well, since roots' morphology can influence the microbial assemblages by providing substrate and habitat for attached bacteria (e.g., Vymazal, 2011). These interwoven chemical and biological effects of plant roots on the rhizosphere, describe the so-called rhizosphere effect (first recognized by Hiltner, 1904) and are indissolubly linked with the concept of phytoremediation, since it relies on root – microbial functions and their interaction, that take place in the rhizosphere.

The positive role of macrophytes in NBS aiming phytoremediation, is generally acknowledged (e.g., Brix, 1997; Stottmeister et al., 2003; Tanner, 2001; Vymazal & Kropfelova, 2009). Nevertheless, this is sometimes disputed as some authors have not seen macrophytes improving nutrient retention (e.g., da Costa, de Paoli, Seidl, & von Sperling, 2013; Scholz & Xu, 2002). These contrasting results might be attributed to different NBS designs and operation characteristics across studies, factors that as well exert significant influence on NBS treatment performance in pollutant removal (Machado, Beretta, Fragoso, & Duarte, 2017). Therefore, there is a need for further research to elucidate the role of vegetation on water quality improvement (e.g., Sultana, Akratos, Vayenas, & Pavlou, 2015). In chapters 3 and 4 of the present thesis, we discuss the chemical and biological influence of macrophytes on the subsurface environment, as evidenced by enhanced nutrient retention in the presence of emergent macrophytes.

## 5.3 Knowledge Gaps About NBS Implementation to Address Nutrient Pollution

To evaluate the role of emergent macrophytes on NBS aiming to address excess nutrient concentrations in freshwater ecosystems, we compiled a list of peer-reviewed articles from the Web of Science database by using various combinations of the following keywords: macrophyte species, nutrient uptake/retention/removal, nitrogen, phosphorus, nitrate, constructed wetlands, traits, phytoremediation, vegetation, plants, nature-based solutions, river/stream restoration, macrophyte species selection, stormwater, and biofiltration. The search was restricted to review papers centered on the role of emergent macrophytes (therefore excluding floating macrophytes or terrestrial species) in NBS targeting nutrient removal (in contrast to other pollutants) and resulted in 28 review articles published between 2003 and 2019. These 28 review papers had a predominant focus on constructed wetlands, as shown by the number of articles per NBS type (Table 5.3).

The need for further research regarding various aspects of NBS functioning was stated in 18 out of the 28 selected reviews articles. We classified the proposed knowledge gaps into six categories (Table 5.3):

1) Plant mechanisms: when it is stated that further research is needed about the mechanisms with which emergent macrophytes contribute to nutrient retention.

2) Operating factors other than vegetation: when the authors call for research about how factors other than vegetation (e.g. substrate, environmental conditions, hydrology) affect the performance of the NBS.

3) Specific pollutants other than nutrients: when the authors call for research for specific pollutants, e.g. heavy metals, pharmaceuticals, pathogens.

4) New technologies/strategies: when more research is sought to understand how new strategies (e.g. microbial augmentation, artificial aeration, using a range of supporting media, supply of additional carbon, and mixed systems) affect the performance of NBS.

5) Maintenance: when new research is needed to understand the factors driving NBS’ longevity.

6) Other/not specified: when a general need for research is stated but not being specified.

**Table 5.3.** NBS type and categories of knowledge gaps as they emerge from the 28 review articles

| Knowledge gaps categories | NBS of focus | | | | | | Number of references |
|---|---|---|---|---|---|---|---|
| | CW (14) | Bioretention (5) | Stream restoration (3) | Agricultural drainage ditches (1) | Phytoremediation (4) | Green roofs (1) | |
| **1. Plant mechanisms** | Gorgoglione, 2018<br>Malaviya, 2012<br>Li, 2018<br>Wu, 2015<br>Stottmeister, 2003 | Dagenais, 2018 Payne, 2014 Laurenson, 2013 Muerdter, 2018 | - | - | Ali, 2019<br>Wang, 2014 | - | 11 |
| **2. Operating factors other than vegetation** | Gorgoglione, 2018<br>Rehman, 2017<br>Machado, 2017<br>Sandoval, 2019<br>Li, 2018<br>Wu, 2015 | Payne, 2014<br>Laurenson, 2013 | - | Faust, 2018 | Wang, 2014 | - | 10 |
| **3. Specific pollutants other than nutrients** | Almuktar, 2018<br>Malaviya, 2012 | Dagenais, 2018 | - | - | Ali, 2019 | - | 4 |
| **4. New technologies/strategies** | Gorgoglione, 2018<br>Kumar, 2019<br>Wu, 2015 | Laurenson, 2013 | - | - | - | - | 4 |
| **5. Maintenance** | Gorgoglione, 2018 Wu, 2015 | Laurenson, 2013 | - | - | - | - | 3 |
| **6. Other/Not specified** | - | - | - | - | - | Czemiel, 2010 | 1 |

Note. CW: constructed wetlands. Value in brackets: number of references per each NBS type. The number of references (last column) refers to the amount to references per each knowledge gap category. The sum does not yield the total number of articles reviewed (28), since references may state a knowledge gap in more than one category.

From the results of our analysis (Table 5.3) is evident that the mechanisms with which emergent macrophytes contribute to nutrient removal in various NBS systems (knowledge gap category 1) is considered understudied by most reviews, counting 11 references and thus being the most commonly stated need for further research across the selected articles. Category 2 follows closely as 10 references acknowledge an important knowledge gap in the role of operating factors (other than presence of vegetation) in NBS functioning and treatment performance. Pollutants different than nutrients, new technologies/strategies, and maintenance (i.e. knowledge gap categories 3, 4, 5) are more rarely considered to require further investigation, counting four, four and three references, respectively.

Chapters 2, 3 and 4 of the present thesis provide knowledge in Category 2 (plant mechanisms), that is the most understudied area in NBS aiming nutrient attenuation, according to the reviewed literature. Specifically, in Chapter 1 we demonstrated the ability of emergent macrophytes to substantially increase hydraulic retention by their roots, and in Chapter 3 we identified root mediated increased subsurface hydraulic retention as an important macrophyte mechanism controlling nutrient retention. In Chapter 4 we showed that presence of macrophytes facilitated DIN biogeochemical transformation and retention and indicated root oxygen release as a possible underlying mechanism. The mechanistic understanding of plant-nutrient interactions is essential in NBS, since it will assist in identifying plant traits and functions that will potentially contribute to nutrient attenuation in freshwater ecosystems and will allow for transferable research findings for NBS design optimization (Brisson & Chazarenc, 2009; Muerdter et al., 2018; Nocco, Rouse, & Balster, 2016; Sultana et al., 2015; Wang et al., 2014). Additionally, in Chapter 4 we examined DOC lability as a driving factor of DIN retention via denitrification, contributing this way to knowledge gap Category 2 for investigation regarding operating factors in NBS. Therefore, the present thesis contributes to bridge the knowledge gap in the two most understudied areas in NBS functioning, and undoubtedly provides insights about the chemical-biological influence of macrophytes on the rhizosphere.

## 5.4 Macrophyte Species Selection for Effective NBS

Despite the lack of mechanistic understanding of the influence that plants exert on nutrient retention in NBS, a large amount mostly of descriptive literature has revealed differences in nutrient retention among plant species (e.g., Konnerup, Koottatep, & Brix, 2009; Kyambadde et al., 2004; Ruiz-Rueda, Hallin, & Baneras, 2009; Tanner et al., 1995). The implication of species-specific nutrient retention effectiveness on NBS is essential since the selection of suitable plant species may optimize treatment performance. This is reflected in the results of our literature analysis, as 87% of the articles providing NBS design guidance acknowledge the importance of plant species selection. However, plant species selection dictates identification of the specific plant functional traits which may contribute to nutrient mitigation; thus, the investigation of plant mechanisms appears again in the forefront.

Root traits are acknowledged in NBS design manuals as good indicators of NBS treatment performance concerning nutrient retention, while the characteristics of the aerial part of the plants are not considered equally reliable (Payne et al., 2015). Given this, in this section of the thesis we seek to elucidate the contribution of the belowground plant organs to nutrient mitigation. To this aim, we link root functional traits with nutrient retention (distinguished between plant and microbial uptake) and with the corresponding mechanisms with which they enhance it, as summarized in Table 5.4. Information presented in Table 5.4 was synthesized from a comprehensive literature review on the role of root functional traits in phytoremediation, performed by Ali et al., 2019 (discussion section and Table 3 in Ali et al., 2019). We further built upon this information by providing examples of macrophyte species per specific root trait, and by including root volume, as an additional root trait which was identified in Chapter 2 and 3 of the present thesis. Table 5.4 summarizes the current knowledge regarding macrophyte root traits and the corresponding specific functions that lead to enhancement of nutrient retention, contributing to species selection guidance for NBS treatment optimization.

Further, to help the identification of suitable plant species for nutrient retention, we created a questionnaire based on hierarchical ordering of root traits. To this aim, we restructured the information presented in Table 5.4 based on the potential of nutrient retention that each root trait may induce (Table 5.5). We consider traits of high importance the ones that may enhance both plant and microbial uptake, resulting in increased nutrient retention as compared to traits that enhance one of these retention mechanisms. Given that nutrient retention is primarily accounted to microbial uptake compared to plant uptake, we characterized of medium importance the root traits that enhance microbial activity and thus uptake, and of low importance the roots traits that influence only plant uptake. Additionally, we provide information about the way that each root trait should be assessed, by distinguishing between root traits that may be assessed by direct observation, and ones that require a background knowledge of the plant’s root physiology and anatomy. This questionnaire can be viewed as a valuable tool for practitioners allowing for easy comparison across different plant species, and subsequently the determination of the most suitable species for enhanced nutrient retention based on the number of root traits per grade of importance (i.e. high, medium, low).

**Table 5.4.** Root functional traits and proposed mechanisms with which they facilitate nutrient retention

| Categories | Root functional traits | Species examples | Plant uptake | Microbial uptake | Other | How plant traits facilitate nutrient retention: Proposed mechanisms |
|---|---|---|---|---|---|---|
| **Morphological** | Root thickness | *Canna indica* (Cheng et al., 2009) | X | | | Smaller diameter roots have greater surface area, which increases the lateral roots penetration, branching, elongation and topological connection ->plant uptake (Haling et al., 2013) |
| **Morphological** | Specific root length | *Sagittaria trifolia* (Lai et al., 2011) | X | X | | Greater specific root length enhances roots soil exploration and root-microbial interactions (Ostonen et al., 2007, Yang et al., 2017) |
| **Morphological** | Root/shoot ratio | *Acorus calamus* (Chen et al., 2007) | X | | | Plants with low root/shoot ratio may show increased bioavailability of N, P to roots (Lovelock et al., 2009) |
| **Architectural** | Root volume | *Scirpus lacustris* (Nikolakopoulou et al., 2018) | | X | | High volume of fine roots increase hydraulic retention, resulting in enhanced microbial uptake (**Chapter 2, Chapter 3)** |
| **Architectural** | Number of roots | *Canna indica* (Chen et al., 2007) | X | X | | High number of roots results to advanced root penetration and thus, increased nutrient acquisition and root-microbial interactions (Chen et al., 2007) |
| **Architectural** | Root surface area | *Canna indica* (Chen et al., 2007) | X | X | | Greater attachment area for soil organisms, higher oxygen release, and additional area for adsorption of N, P (Lai et al., 2011) |
| **Physiological** | Radial oxygen release | *Cyperus flabelliformis, Acorus calamus* (Cheng et al., 2014) | | X | | Higher oxygen availability enhances microbial activities, e.g. nitrification, microbial breakdown of organic matter, enhancing N microbial uptake, and nutrient bioavailability (Cheng et al., 2014) |
| **Physiological** | Transpiration rates | | | X | Reduced outflow in NBS | High transpiration rates lead to a decreased hydraulic retention that may enhance microbial uptake. Cause greater water removal, resulting to a reduced outflow and thus nutrient export. Aerate the rhizosphere, enhancing aerobic processes (**Chapter 2**, Kadlec and Wallace, 2009, Denman et al., 2006, Trolldenier, 1989) |
| **Physiological** | Root exudation rate | *Lupinus albus* (Watt and Evans, 1999) | | X | Nutrient bioavailability | High exudation rate enhances nutrient bioavailability and microbial activity (Mathesius et al., 2017) |
| **Anatomical** | Root porosity, aerenchyma | *Cyperus flabelliformis, Phragmites australis* (Mei et al., 2013, Armstrong et al., 1992) | | X | Nutrient bioavailability | Higher aerenchyma volume and porosity release more oxygen, which influence nutrient bioavailability and microbial activity (Gilbert and Frenzel, 1998, Armstrong et al., 1992) |
| **Anatomical** | Root epidermis | | X | | | Thin epidermis with higher permeability, facilitates nutrient uptake (Lai et al., 2011) |
| **Symbiotic** | Microbial association | | | X | | Nitrifiers, phosphate-solubilizing microbes (Kyambadde et al., 2004) |
| **Symbiotic root traits** | Mycorrhizal association | *Phragmites australis, Typha latifolia* (Cooke and Lefor, 1998 | X | | Nutrient bioavailability | Provide effective nutrient uptake by accessing a greater soil volume and enhance bioavailability of recalcitrant compounds (Payne et al., 2014) |
| **Chemical** | Fe plaque formation | *Cyperus flabelliformis* (Mei et al., 2013) | X | | | Fe plaque enhances nutrient uptake (Zhang et al., 1999, Mei et al., 2013) |

Note. Modified from Ali et.al, 2019

**Table 5.5.** Questionnaire for the identification of suitable plant species for nutrient retention, based on their root traits. Plants characterized by high number of root traits of the highest importance are considered as the most suitable ones

| | | | | | | Candidate plants to be compared | | | |
|---|---|---|---|---|---|---|---|---|---|
| **Importance** | **Categories** | **Root functional traits** | **Assessment** | **Plant uptake** | **Microbial uptake** | **Plant 1:** | **Plant 2:** | **Plant 3:** | **Plant 4:** |
| **High** | **Morphological** | High root length | Observational | X | X | | | | |
| | **Architectural** | High number of roots | Observational | X | X | | | | |
| | **Architectural** | High root surface area | Observational | X | X | | | | |
| **Medium** | **Architectural** | High volume of fine roots | Observational | | X | | | | |
| | **Physiological** | High radial oxygen release | Background knowledge | | X | | | | |
| | **Physiological** | High transpiration rates | Background knowledge | | X | | | | |
| | **Physiological** | High root exudation rate | Background knowledge | | X | | | | |
| | **Anatomical** | High root porosity, aerenchyma volume | Background knowledge | | X | | | | |
| | **Symbiotic** | Microbial association | Background knowledge | | X | | | | |
| **Low** | **Morphological** | High root thickness | Observational | X | | | | | |
| | **Morphological** | Low root/shoot ratio | Observational | X | | | | | |
| | **Anatomical** | Thin root epidermis | Background knowledge | X | | | | | |
| | **Symbiotic** | Mycorrhizal association | Both | X | | | | | |
| | **Chemical** | Fe plaque formation | Both | X | | | | | |

Nevertheless, treatment performance is only one aspect of effective NBS systems. By definition NBS embrace sustainability, as well as the preservation of the local biodiversity, therefore it is essential to consider these aspects in NBS design, additionally to treatment performance. To enhance nutrient retention performance, plant characteristics should include rapid plant growth rate (Payne et al., 2014; Osman et al., 2019), and as discussed above, several root traits (e.g. Ali, 2019, Muerdter 2018, Dagenais 2018). To ensure the system's sustainability, plant selection should be based on the plants' capacity to survive (Read, 2008), therefore long life-span (i.e. perennial species) (Almuktar et al., 2018), high tolerance to eutrophic and anoxic conditions (Almuktar et al., 2018, Wu et al., 2015), and easy adaptation to extreme weather conditions (i.e. dry and water-logged sediments) (Payne et al., 2014) are desirable plant characteristics. Finally, native species should be preferred in respect to the local plant biodiversity (Sultana et al., 2015, Wu et al., 2015). In summary, treatment performance, sustainability, and biodiversity, with respective plant characteristics should be viewed as the foundation for the successful selection of macrophyte species, as illustrated in Figure 5.3. This scheme can serve practitioners as a simple guide for basic plant species screening for effective NBS implementation.

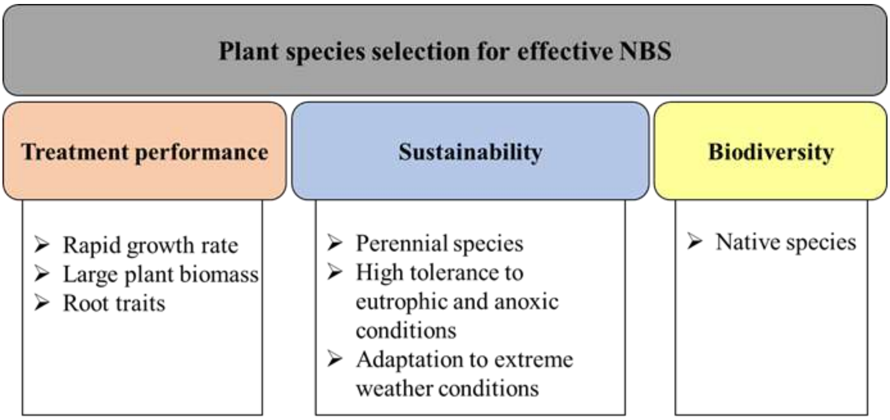


**Figure 5.3.** Treatment performance, sustainability, and biodiversity, with respective plant characteristics, as the pillars for successful plant species selection in NBS aiming nutrient pollution management. This scheme can aid NBS practitioners as a guide for plant species screening.

Our synthesis of scientific and technical literature information throughout the general discussion, revealed that despite the widespread use of emergent macrophytes in NBS, there is a knowledge gap in the causal mechanisms of macrophyte mediated enhancement of nutrient mitigation. This lack of mechanistic understanding directly impacts the identification of the specific macrophyte traits contributing to nutrient retention, thus limiting optimal NBS design by selecting suitable plant species. In the present doctoral thesis, we attempted to contribute to bridge this knowledge gap and to provide guidelines for suitable plant species selection for successful NBS implementation. Overall, we demonstrate the essential role of emergent macrophytes in NBS aiming to address nutrient pollution in freshwater systems.

# GENERAL CONCLUSIONS

**Chapter 1:** ***"Emergent macrophyte root architecture controls subsurface solute transport"***

1. Emergent macrophyte roots increase subsurface residence time and transient storage, by removing water via evapotranspiration, and by creating heterogeneities in the sediment, which in turn create diverse flow paths.
2. Fine roots and rhizomes influence solute transport with opposing mechanisms.
3. Hydraulic retention increases with high % volume of fine roots but decreases in the presence of thicker roots (rhizomes)
4. Emergent macrophytes significantly increase the number of subsurface flow paths (i.e., flow path heterogeneity).
5. Root architecture is important to consider in stream restoration activities targeting water quality improvement.

**Chapter 2:** ***"The effect of three emergent macrophyte species on nutrient retention in aquatic environments of excess nutrient conditions"***

6. Macrophytes have a species-specific influence on the capacity for $NH_4^+$ and SRP retention from subsurface water, demonstrated by differences in $k_{gross}$ across flumes with Iris, Phragmites and Scirpus.
7. Macrophyte species selection in water management or stream restoration planning is critical to enhance nutrient retention.
8. Different macrophyte species may promote different biogeochemical processes.

9. Scirpus showed both high net and gross $NH_4^+$ uptake, and therefore it is the most effective species, compared to Iris and Phragmites, and appropriate for NBS projects to address high N loads.

10. Both net and gross nutrient uptake should be considered for an accurate assessment of the contribution of macrophytes to the performance of NBS projects aiming nutrient attenuation.

11. Hydrological retention positively influences the $NH_4^+$ and SRP uptake.

12. Root architecture characterization can infer the potential of macrophytes to contribute to $NH_4^+$ and SRP retention from the water column.

**Chapter 3:** ***"Enhancement of carbon and nitrogen removal by helophytes along subsurface water flowpaths receiving treated wastewater"***

13. Emergent macrophytes contribute to the transformation and removal of DIN from WWTP effluents.

14. Labile DOC additions in flumes mostly contribute to increase respiration rather than denitrification.

15. Emergent macrophytes are potentially a relevant technique to improve water quality in WWTP effluent-influenced aquatic ecosystems.

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# SUPPORTING INFORMATION

APPENDIX A. Supplementary information of Chapter 1: General Introduction, and Chapter 5: General Discussion

APPENDIX B. Supplementary information of Chapter 2: “Emergent Macrophyte Root Architecture Controls Subsurface Solute Transport”

APPENDIX C. Supplementary information of Chapter 4: “Enhancement of Carbon and Nitrogen Removal by Helophytes Along Subsurface Water Flowpaths Receiving Treated Wastewater”

# APPENDIX A. Supplementary information of Chapter 1: General Introduction, and Chapter 5: General Discussion

**Table A.1.** Challenges addressed by NBS as reported by different sources (i.e. IUCN, Naturvation), and to which U.N. Sustainable Development Goal they contribute

| U.N. Sustainable Development Goals* | | Societal challenges addressed by NBS as reported in IUCN (2016) | Challenges addressed as reported in Naturvation database |
|---|---|---|---|
| Goal 1 | End poverty in all its forms everywhere | | |
| Goal 2 | End hunger, achieve food security and improved nutrition and promote sustainable agriculture | Food security | |
| Goal 3 | Ensure healthy lives and promote well-being for all at all ages | Human health | Health and well-being |
| Goal 4 | Ensure inclusive and equitable quality education and promote lifelong learning opportunities for all | | |
| Goal 5 | Achieve gender equality and empower all women and girls | | |
| Goal 6 | Ensure availability and sustainable management of water and sanitation for all | Water security | Water management |

| | | | |
|---|---|---|---|
| Goal 7 | Ensure access to affordable, reliable, sustainable and modern energy for all | | |
| Goal 8 | Promote sustained, inclusive and sustainable economic growth, full and productive employment and decent work for all | | Economic development and decent employment |
| Goal 9 | Build resilient infrastructure, promote inclusive and sustainable industrialization and foster innovation | | |
| Goal 10 | Reduce inequality within and among countries | | Social justice, cohesion and equity |
| Goal 11 | Make cities and human settlements inclusive, safe, resilient and sustainable | Human health,<br>Disaster risk reduction | |
| Goal 12 | Ensure sustainable consumption and production patterns | | Sustainable consumption and production |
| Goal 13 | Take urgent action to combat climate change and its impacts | Human health,<br>Disaster risk reduction,<br>Climate change mitigation | Climate action for adaptation, resilience and mitigation |
| Goal 14 | Conserve and sustainably use the oceans, seas and marine resources for sustainable development | | Coastal resilience and marine protection |

| | | | |
|---|---|---|---|
| Goal 15 | Protect, restore and promote sustainable use of terrestrial ecosystems, sustainably manage forests, combat desertification, and halt and reverse land degradation and halt biodiversity loss | | Green space, habitats and biodiversity |
| Goal 16 | Promote peaceful and inclusive societies for sustainable development, provide access to justice for all and build effective, accountable and inclusive institutions at all levels | | Inclusive and effective governance |
| Goal 17 | Strengthen the means of implementation and revitalize the Global Partnership for Sustainable Development | | |

*Sustainable development goals as reported in the United Nations 2030 Agenda for Sustainable Development

# APPENDIX B. Supplementary information of Chapter 2: “Emergent Macrophyte Root Architecture Controls Subsurface Solute Transport”

**Table B.1.** NaCl pulse injections information.

| Channel | Treatment | Injection code name | BTC code name | Date/time of injection | Volume of injection (L) | EC of injection (µS/cm) |
|---|---|---|---|---|---|---|
| 8 | Control | Hydr | Ch8Control Hydr | 8/5/2015 14:12:00 | 1 | 108600 |
| 8 | Control | Pre | Ch8Control Pre | 8/6/2015 11:01:00 | 1 | 130600 |
| 8 | Control | Dur | Ch8Control Dur | 8/10/2015 13:30:00 | 1 | 128900 |
| 13 | Control | Hydr | Ch13Control Hydr | 8/12/2015 12:42:40 | 1 | 108100 |
| 13 | Control | Pre | Ch13Control Pre | 8/13/2015 10:30:00 | 1 | 130800 |
| 13 | Control | Dur | Ch13Control Dur | 8/17/2015 13:00:00 | 1 | 131900 |
| 17 | Control | Hydr | Ch17Control Hydr | 8/12/2015 12:42:40 | 1 | 108400 |
| 17 | Control | Pre | Ch17Control Pre | 8/13/2015 10:30:00 | 1 | 131200 |
| 17 | Control | Dur | Ch17Control Dur | 8/17/2015 13:00:00 | 1 | 128900 |
| 6 | Iris | Hydr | Ch6IrisHydr | 7/29/2015 15:18:40 | 1 | 126800 |
| 6 | Iris | Pre | Ch6IrisPre | 7/30/2015 14:30:00 | 1 | 126800 |
| 6 | Iris | Dur | Ch6Iris Dur | 8/3/2015 13:30:00 | 1 | 127800 |
| 7 | Iris | Hydr | Ch7IrisHydr | 7/29/2015 15:19:50 | 1 | 131100 |

| | | | | | | |
|---|---|---|---|---|---|---|
| 7 | Iris | Pre | Ch7IrisPre | 7/30/2015 14:30:00 | 1 | 131100 |
| 7 | Iris | Dur | Ch7Iris Dur | 8/3/2015 13:30:00 | 1 | 128900 |
| 15 | Iris | Hydr | Ch15Iris Hydr | 7/29/2015 15:21:15 | 1 | 131000 |
| 15 | Iris | Pre | Ch15IrisPre | 7/30/2015 14:30:00 | 1 | 131000 |
| 15 | Iris | Dur | Ch15Iris Dur | 8/3/2015 13:30:00 | 1 | 127300 |
| 12 | Phragmites | Hydr | Ch12Phrag Hydr | 8/19/2015 13:03:00 | 1 | 111100 |
| 12 | Phragmites | Pre | Ch12Phrag Pre | 8/20/2015 11:58:00 | 1 | 130300 |
| 12 | Phragmites | Dur | Ch12Phrag Dur | 8/27/2015 13:06:00 | 1 | 130100 |
| 16 | Phragmites | Hydr | Ch16Phrag Hydr | 8/19/2015 13:03:00 | 1 | 111200 |
| 16 | Phragmites | Pre | Ch16Phrag Pre | 8/20/2015 11:59:00 | 1 | 130400 |
| 16 | Phragmites | Dur | Ch16Phrag Dur | 8/27/2015 13:05:00 | 1 | 129800 |
| 18 | Phragmites | Hydr | Ch18Phrag Hydr | 8/19/2015 13:03:00 | 1 | 109500 |
| 18 | Phragmites | Pre | Ch18Phrag Pre | 8/20/2015 12:00:00 | 1 | 128700 |
| 18 | Phragmites | Dur | Ch18Phrag Dur | 8/27/2015 13:05:00 | 1 | 127700 |
| 9 | Scirpus | Hydr | Ch9Scirpus Hydr | 8/5/2015 14:12:00 | 1 | 108300 |
| 9 | Scirpus | Pre | Ch9Scirpus Pre | 8/6/2015 11:01:00 | 1 | 130000 |
| 9 | Scirpus | Dur | Ch9Scirpus Dur | 8/10/2015 13:30:00 | 1 | 131000 |
| 10 | Scirpus | Hydr | Ch10Scirpus Hydr | 8/5/2015 14:12:00 | 1 | 110200 |
| 10 | Scirpus | Pre | Ch10Scirpus Pre | 8/6/2015 11:01:00 | 1 | 128500 |

| | | | | | | |
|---|---|---|---|---|---|---|
| 10 | Scirpus | Dur | Ch10Scirpus Dur | 8/10/2015 13:30:00 | 1 | 128200 |
| 14 | Scirpus | Hydr | Ch14Scirpus Hydr | 8/12/2015 12:42:40 | 1 | 108900 |
| 14 | Scirpus | Pre | Ch14Scirpus Pre | 8/13/2015 10:30:00 | 1 | 130600 |
| 14 | Scirpus | Dur | Ch14Scirpus Dur | 8/17/2015 13:00:00 | 1 | 131100 |

**Table B.2.** Root sampling information

| | | | | Dry biomass | | Volume | | |
|---|---|---|---|---|---|---|---|---|
| **Channel** | **Species** | **Code** | **sampling date** | **Dry weight fine roots (g)** | **Dry weight rhizomes (g)** | **Volume Sediment (mL)** | **Volume fine roots (mL)** | **Volume rhizomes (mL)** |
| 6 | Iris | sample | 10/7/2015 | | | 20466 | 1320 | 1210 |
| 6 | Iris | rootsubsample1 | | 6.851 | 23.85 | | 87 | 92 |
| 6 | Iris | rootsubsample2 | | 8.562 | 20.998 | | 93 | 89 |
| 6 | Iris | rootsubsample3 | | 10.404 | 22.624 | | 90 | 91 |
| 6 | Iris | rootsubsample4 | | 8.222 | 21.406 | | 100 | 91 |
| 6 | Iris | rootsubsample5 | | 7.078 | 24.765 | | 80 | 100 |
| 7 | Iris | sample | 10/8/2015 | | | 20866 | 580 | 410 |
| 7 | Iris | rootsubsample1 | | 6.72 | 20.023 | | 75 | 78 |
| 7 | Iris | rootsubsample2 | | 8.02 | 14.053 | | 70 | 67 |
| 7 | Iris | rootsubsample3 | | 7.244 | 25.132 | | 72 | 90 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| 7 | Iris | rootsubsample4 | | 6.272 | 16.924 | | 73 | 62 |
| 7 | Iris | rootsubsample5 | | 7.162 | 11.507 | | 77 | 70 |
| 15 | Iris | sample | 10/8/2015 | | | 20146 | 2235 | 2230 |
| 15 | Iris | rootsubsample1 | | 5.838 | 26.169 | | 71 | 95 |
| 15 | Iris | rootsubsample2 | | 8.837 | 23.311 | | 85 | 97 |
| 15 | Iris | rootsubsample3 | | 7.125 | 26.336 | | 90 | 100 |
| 15 | Iris | rootsubsample4 | | 7.91 | 31.241 | | 85 | 100 |
| 15 | Iris | rootsubsample5 | | 7.775 | 22.027 | | 84 | 90 |
| 12 | Phragmites | sample | 10/9/2015 | | | 17866 | 917 | 920 |
| 12 | Phragmites | rootsubsample1 | | 6.431 | 13.659 | | 80 | 82 |
| 12 | Phragmites | rootsubsample2 | | 9.005 | 14.661 | | 82 | 89 |
| 12 | Phragmites | rootsubsample3 | | 7.945 | 15.683 | | 89 | 79 |
| 12 | Phragmites | rootsubsample4 | | 7.036 | 14.102 | | 84 | 80 |
| 12 | Phragmites | rootsubsample5 | | 7.711 | 15.408 | | 85 | 82 |
| 16 | Phragmites | sample | 10/9/2015 | | | 17646 | 318 | 92 |
| 16 | Phragmites | rootsubsample1 | | 8.79 | 6.499 | | 70 | 28 |
| 16 | Phragmites | rootsubsample2 | | 4.788 | 4.686 | | 57 | 26 |
| 16 | Phragmites | rootsubsample3 | | 5.523 | 7.429 | | 49 | 32 |
| 16 | Phragmites | rootsubsample4 | | 5.457 | | | 67 | |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| 16 | Phragmites | rootsubsample5 | | 7.739 | | | 75 | |
| 18 | Phragmites | sample | 10/13/2015 | | | 22066 | 330 | 232 |
| 18 | Phragmites | rootsubsample1 | | 4.62 | 7.317 | | 55 | 50 |
| 18 | Phragmites | rootsubsample2 | | 5.299 | 8.509 | | 75 | 65 |
| 18 | Phragmites | rootsubsample3 | | 4.894 | 6.637 | | 60 | 57 |
| 18 | Phragmites | rootsubsample4 | | 4.493 | 9.113 | | 67 | 60 |
| 18 | Phragmites | rootsubsample5 | | 4.058 | | | 73 | |
| 9 | Scirpus | sample | 10/7/2015 | | | 16266 | 605 | 1003 |
| 9 | Scirpus | rootsubsample1 | | 11.345 | 14.737 | | 77 | 75 |
| 9 | Scirpus | rootsubsample2 | | 13.437 | 17.506 | | 92 | 83 |
| 9 | Scirpus | rootsubsample3 | | 10.525 | 17.575 | | 92 | 83 |
| 9 | Scirpus | rootsubsample4 | | 6.486 | 20.992 | | 70 | 92 |
| 9 | Scirpus | rootsubsample5 | | 10.807 | 18.01 | | 79 | 82 |
| 10 | Scirpus | sample | 10/5/2015 | | | 16266 | 977 | 495 |
| 10 | Scirpus | rootsubsample1 | | 11.967 | 9.743 | | 88 | 90 |
| 10 | Scirpus | rootsubsample2 | | 11.149 | 8.153 | | 88 | 70 |
| 10 | Scirpus | rootsubsample3 | | 9.041 | 9.917 | | 83 | 90 |
| 10 | Scirpus | rootsubsample4 | | 8.683 | 7.601 | | 81 | 79 |
| 10 | Scirpus | rootsubsample5 | | 5.932 | 8.722 | | 59 | 79 |
| 14 | Scirpus | sample | 10/5/2015 | | | 13666 | 1617 | 727 |
| 14 | Scirpus | rootsubsample1 | | 12.231 | 14.661 | | 93 | 80 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 14 | Scirpus | rootsubsample2 | 7.897 | 15.56 | | 57 | 85 |
| 14 | Scirpus | rootsubsample3 | 8.745 | 12.919 | | 65 | 90 |
| 14 | Scirpus | rootsubsample4 | 11.649 | 13.4 | | 67 | 85 |
| 14 | Scirpus | rootsubsample5 | 15.314 | 14.312 | | 87 | na |

## APPENDIX C. Supplementary information of Chapter 4: “Enhancement of Carbon and Nitrogen Removal by Helophytes Along Subsurface Water Flowpaths Receiving Treated Wastewater”

**Table C.1.** Chemical characterization of the industrial by-product of a brewery used in this study as a labile C source to assess the influence of labile C availability on nitrogen removal along the flumes.

| **Parameter** | |
|---|---|
| pH | 3.34 |
| Conductivity (μS $cm^{-1}$) | 955 |
| TC (mg $kg^{-1}$) | 13350 |
| COD (mg $L^{-1}$) | 42.6 |
| Total suspended solids (mg $L^{-1}$) | 312 |
| Nitrogen components (%) | .3-4 |
| Mineral salts (%) | .1.5-2 |
| Carbohydrates %): | .90-92 |
| Carbohydrate typology (% of total carbohydrates): | |

| | |
|---|---|
| Monosaccharides totals (pentoses, fructose and glucose): | 13 |
| Disaccharide (sucrose, maltose and other): | 69 |
| Oligosaccharides (4-11 C): | 8 |
| Polysaccharides (> 11C): | 10 |