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# BIODEGRADATION OF INDUSTRIAL WASTEWATER IN A LABORATORY-SCALE CONSTRUCTED WETLAND

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# Abstract

Carbochemical industry, known as the transformation of coking coal and its derivates is of major importance as a source of energy, and an essential source of aromatic hydrocarbons for industry. Coking coke processes produce large volumes of wastewater with high loads of toxic pollutants, such as phenolic compounds that can account for almost 80% of COD of the total effluent. Substituted phenols are widespread among industrial effluents coming from petrochemical industries, petroleum refineries, herbicide production, resins, coke ovens and coking processes. Among them, dimethylphenols (DMP) or xylenols are considered toxic, poisonous and high persistent. Phenolic effluents treatment options range from physicochemical to biological, being the latter of major importance because it accomplishes a complete mineralization of the contaminants. Constructed wetlands function as a close equivalent to a natural environment, and emerge as a feasible option to understand the degradation of organic pollutants in an engineered ecosystem where biological, physical and chemical factors combine to allow this degradation. DMPs degradation in constructed wetlands is an open research field, where knowledge is still limited and more investigation is needed. This study aimed to determine the removal efficiency of two laboratory scale constructed wetlands planted with *Juncus effusus* and *Phalaris arundinaceae* fed with 3,5- and 2,6-DMP DMPs, and the effect of the plants and the physicochemical parameters on the removal performance. Also, this study evaluated the role of the aerobic bacterial communities in the degradation of 3,4- 3,5- and 2,6-DMP, by detecting the relative abundance of phenol hydroxylase (PHE) in the total bacterial community in order to evaluate possible changes in this catabolic gene. Results obtained regarding DMP and ammonium removal showed that the planted wetlands had highest removal rates compared to the unplanted wetland. A complete removal capacity of both isomers by the planted wetlands with *Juncus sp*.was accomplished, independently on the daily load. These results proved that the presence of plants had positive effect by enhancing the removal potential of the systems. The evaluation of the relative abundance of PHE showed a highly dynamic behavior of this gen in the bacterial population throughout time and suggested that PHE is a key enzyme in the aerobic degradation of 3,4-DMP, but not for 3,5-DMP and 2,6-DMP. This study highlighted the major role played with plants in constructed wetlands, as they provide oxygen, attachment and organic sources for microbial communities growth, thereby enhancing the removal capacity of isolated DMPs and ammonium. Additionally, the gathered information on the abundance of PHE and its role in the degradation of 3,4- 3,5- and 2,6-DMP, provide new insights on the dynamics of aerobic bacterial communities involved in the degradation of aromatic compounds inside constructed wetlands.

# Resumen

La industria carboquímica se conoce como la transformación de carbón mineral y sus derivados. Esta industria es de gran importancia en la generación de energía, asi como una fuente esencial de hidrocarburos aromáticos para la industria. Los procesos de transformación del carbón mineral para la obtención de coque producen grandes volúmenes de aguas residuales con altas cargas de contaminantes, como los compuestos fenólicos. El fenol y sus derivados pueden dar cuenta de casi el 80% de la DQO del efluente total de una industria carboquímica. Los fenoles sustituidos están ampliamente distribuidos en diferentes efluentes industriales como la industria petroquímica y carboquímica, refinerías de petróleo, producción de herbicidas y resinas, entre otros. Entre ellos, los dimetilfenoles (DMP) o xylenoles se consideran tóxicos, venenosos y altamente persistentes. Las opciones de tratamiento de efluentes fenólicos se extienden desde fisicoquímicos hasta biológicos, siendo este último de gran importancia, ya que logra una completa mineralización de los contaminantes. Los humedales artificiales, son ecosistemas creados a partir de bases ingenieriles, en donde factores físicos, químicos y biológicos se combinan para permitir procesos de degradación. Y, en este sentido, surgen como una opción factible para entender la degradación de contaminantes en un ecosistema equivalente a un ambiente natural. La degradación de DMPs en humedales artificiales es un campo abierto de investigación con información limitada, donde más esfuerzos de investigación son pertinentes. Este estudio tuvo como objetivo determinar la eficacia en la remoción de 3,5- y 2,6-DMP en dos humedales artificiales a escala laboratorio plantados con *Juncus effusus* y *Phalaris arundinaceae.* Asimismo se evaluó el efecto de la presencia de las plantas y los parámetros fisicoquímicos en las eficacias de remoción de los humedales. Además, este estudio evaluó el rol de las comunidades bacterianas aeróbicas en la degradación de 3,4- 3,5- y 2,6-DMP, especificamente con la deteción de la abundancia relativa de la enzima fenol hidroxilasa (PHE) en la comunidad bacteriana, con el objetivo de identificar posibles cambios en la presencia o ausencia de este gen. Los resultados obtenidos en cuanto a la remoción de DMP y amonio, mostraron que los humedales plantados fueron más eficaces en la remoción en comparación con el tratamiento control. Los humedales plantados con *Juncus effusus* lograron eliminar completamente los dimetilfenoles, indepedientemente de la carga diaria. Estos resultados demostratron que la presencia de plantas en los humedales tiene un efecto positivo en el desempeño de los mismos. Resaltando al mismo tiempo, el rol clave de las plantas al proporcionar sustrato, oxígeno y fuentes de carbono para el crecimiento de comunidades bacterianas que a su vez aumentan las capacidades de los humedales para remover contaminantes. Los resultados de la abundancia relativa de PHE mostraron un comportamiento sumamente dinámico a través del tiempo y directamente influenciado por el isómero presente en el medio. Los resultados sugieren que la enzima PHE es una enzima clave en la degradación de 3,4-DMP, no así para 3,5- y 2,6-DMP. Esta información provee de nuevas observaciones en relación con la degradación de 3,4- 3,5- y 2,6-DMP y la dinámica de las comunidades bacterianas expuestas a estos isómeros.

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# 1 Introduction

Coal as a source of energy is of major importance. For instance China has the highest share of coal worldwide, accounting for 51% of its primary energy (BP, 2015). The same applies to India, which has the second largest coal market after China. Coal is not relevant only in these cases, in fact riches countries like Britain, Italy, Germany, France and Japan have increased their coal use in 16% since 2009 and are planning to augment the construction of coal power stations (Vidal, 2015). In China for instance, as coal is their main source of energy for the last decades, the pollution caused by the emissions and wastewater has been a serious problem (Zhang et al., 1998)

Based on the current tendencies, coal is expanding their use in energy, industry and manufacturing. Together with this expansion coke industry is producing large volumes of wastewater which is highly polluted and difficult to handle (Ghose, 2002b). Due to the high amount of aromatic compounds for industry that can be obtained from coking coal (McMurry, 2008), these types of wastewater will be predominant and because of its toxicity is imperative to develop alternatives to treat them.

## 1.1 Coke industry

The petrochemical and carbochemical industries are the two main sources of aromatic hydrocarbons for industry. Carbon as raw material allowed the development of the chemical industry during the XVIII century, and remained in use for more than 100 years, later the petrochemical industry gained the advantage because of the increasing technology and lower costs. Towards the middle of the twentieth century, due to the massive increase in petroleum costs, carbochemical industry took again an important role as an option to proceed with the technologic development of our times, and also mention the fact that coal reserves considerably exceed the oil reserves (Caselles et al., 2015).

Carbochemical industry can be defined as the transformation of coking coal and its derivates. The importance of this industry was first associated with coke production for steel industry and

gas for energy (Caselles et al., 2015), although coke industry extends beyond to a variety of aromatic compounds for industrial purposes. Coke industry is the transformation of the coking coal to coke by pyrolysis while obtaining non-condensable coke gas and coal tar. Coke is a hard and porous mass, devoid of volatile matter that originates from the carbonisation of coking coal that can be also known as bituminous coal or metallurgical coal (Ghose, 2002b)

Carbonization process consists in a pyrolysis of the coking coal, in the absence of air, where it decomposes to gases, coke liquids products and coke. During the pyrolysis, as the temperature increases the fluidity of the mass reaches it maximum and finally solidifies to coke (Ghose, 2002b). As mentioned, coal tar and gas are also recovered from this process. When coking coal is coking inside the ovens, the produced gas ascend from the top on the oven and is collected as a by-product, known as coke oven gas (COG)(Razzaq, Li, & Zhang, 2013). COG gas is a non-condensable gas that can be use as fuel for its high energy capacity. Coal tar, unlike the coke oven gas, is a condensable product, that originates from cooling down the gas recovered from the ovens . After the coking process the incandescent coke is then transferred to a quenching tower where water showers are used to cool the coke. Around 90% of the coke produce in this process is used to in the steel production in blast furnaces and is known as furnace coke. Another type of coke is known as foundry coke and is used in the cupolas of foundries to produce iron (Ghose, 2002b; Razzaq et al., 2013).

In broad terms, it can be said that coking coal industries provide gas for energy, coke for metallurgic industry and several aromatic compounds for industrial purposes (Ghose, 2002a). Based on Mussatti (1998) coke production process provide two principal co-products: coke breeze and ¨other coke¨. The first is the result from crushing the coke and is the coke used in steel and iron production, since they need a specific size to use in the blast furnaces and foundry cupolas. The second one is the coke that does not fulfill the requirements to be used for steel production and instead is sold as fuel for other industries.

Additionally several by-products are recovered like coke oven gas, ammonia liquor, tar and light oil. These by-products can be further processed to obtain anhydrous ammonia, ortho cresol, phenols, anthracene, naphthalene, creosote oil, pitch, BTX fraction (benzene, toluene and

xylenes), among other compounds that are raw materials for manufacture aims, like synthetic dyes, explosives and drugs (Ghose, 2002b). A simplified diagram of selected coking coal by-products is shown below.

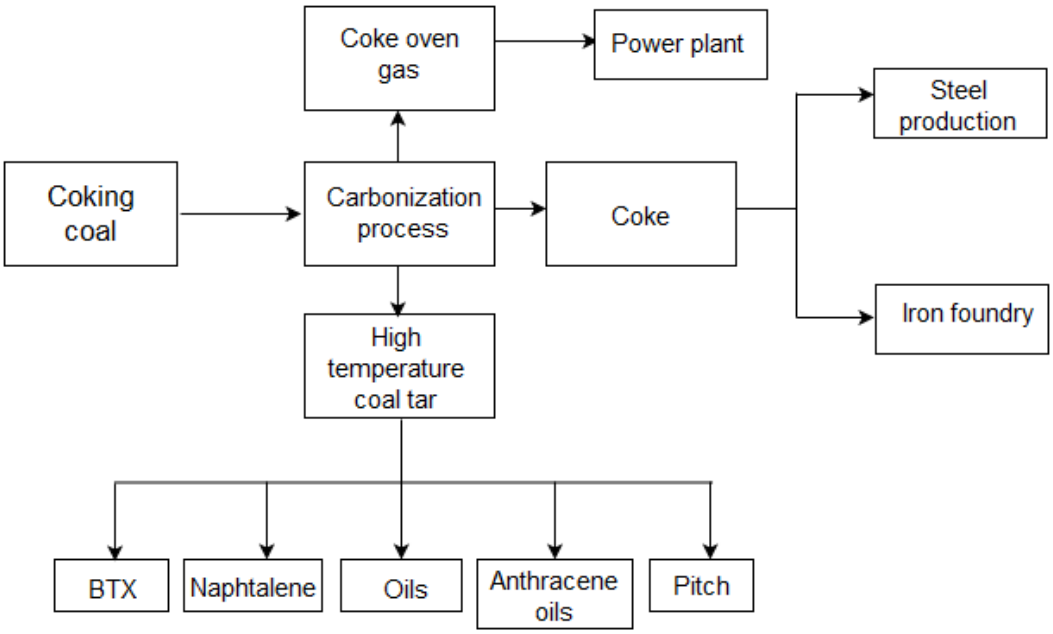



Figure 1-1. Selected coking coal by-products. Modified from NalonChem S.A website.

## 1.2 Coking coke industry wastewater

Large amounts of water are used during the coking coke processes, producing about 200-300 L per ton of coke (Wu et al., 2015). Based on Ghose (2002b), wastewater from this industry derive from three principals sources: (1) water used for quenching the coke after the ovens, which may contain coke breeze as suspended matter, (2) water to cool down and wash the gas, which is highly contaminated with residues of ammoniacal liqueur, and (3) the water used to purify byproducts. Therefore, coking coke industry wastewater is polluted with inorganic compounds such as ammonia, cyanide, thiocyanate and sulfate, and refractory, toxic, biologically inhibitory organic contaminants like nitrogenous heterocyclic compounds, phenols, PAHs, among others (Y. M. Li, Qiu, & Peng, 2003). This wastewater is highly contaminated and difficult to handle, and if discharged to natural effluents without treatment can impact severely the aquatic life and water quality, and also represents a high risk for leakages into groundwater (Broholm & Arvin,

2000). Among the different contaminants that can be found in the coke industry effluents, one kind of toxic compounds receives special attention in this investigation: the phenolic compounds.

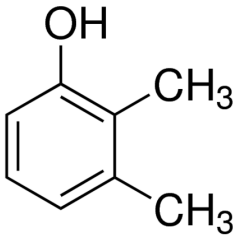
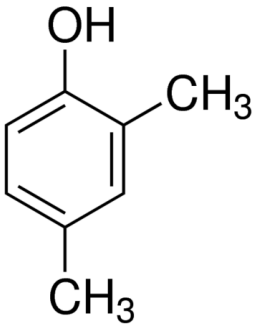
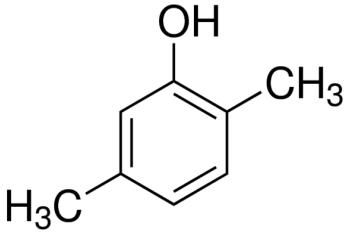
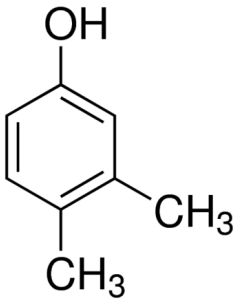
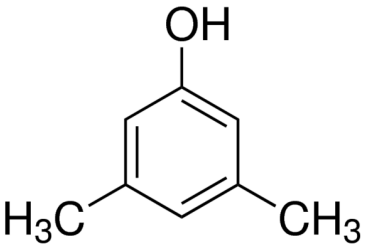
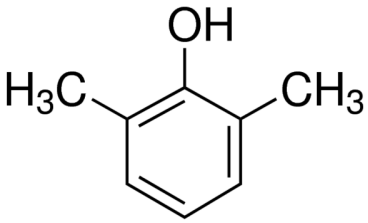
## 1.3 Phenols in coking wastewater

Phenolic effluents, or simply the mixture of phenolic compounds in wastewater can be found in petroleum refineries, synfluels production facilities, wood preserving plants, plastics, resins, dyes, pesticides and pharmaceutical manufactures, and coal industries (Beszedits & Silbert, 1990). Constituents of coal coking industry are phenol and 9 derivatives: 3 cresols (o-, m- and p-isomers) and 6 dimethylphenols (2,3-, 2,4-, 2,5-, 2,6-, 3,4-, and 3,5 isomers) (Xiao et al., 2012). Phenols, for instance, are one of the main organic components of carbochemical industry wastewater, accounting 80% of the total COD of the inffluent (Zhang et al., 1998). Phenols are toxic, carcinogenic, mutagenic and teratogenic compounds and for these characteristics, effluent discharges limits must be imposed (Veeresh, Kumar, & Mehrotra, 2005). Furthermore, phenols are included in the list of USEPA priority pollutants for their high toxicity to aquatic life and humans. Usually, a limit of less than 0,5 mg/L is imposed, since less than 1 mg/L is needed is detect negative effects in natural environments (Tomei & Annesini, 2008), and regarding drinking water a limit of 1µg/L is imposed. Several consequences in human health from phenol exposure can be seen in Mohammadi et al. (2014), which include inner organ damage and neurological disturbances.

## 1.4 Dimethylphenols

Substituted phenols are widespread among industrial effluents coming from petrochemical industries, petroleum refineries, herbicide production, resins, coke ovens and coking processes. Toxicity is considered even higher in substituted phenols and it depends on the nature and numbers of the substituted groups. They are considered to be stable in the environment, difficult to biodegradate and represent a risk of accumulation since they can penetrate into soil and groundwater (Tomei & Annesini, 2008). Among the different kinds of substituted phenolic compounds, this study focuses in dimethylphenols (DMP) or xylenols, which are organic compounds derivatives from phenol with one hydroxyl group and two methyl groups. Xylenols consist in six isomers which are similar between their physical characteristics (Fig. 1-2).

Figure 1-2. Dimethylphenols isomers

| | | |
|---|---|---|
|  |  |  |
| 2,3-DMP | 2,4-DMP | 2,5-DMP |
|  |  |  |
| 3,4-DMP | 3,5-DMP | 2,6-DMP |

Dimethylphenols can be found in by products of coking processes, disinfectants derived from coal tar, artificial resins, coal tar creosote, raw material for pesticides, and components of automobile and diesel exhaust. The six of them are considered poisonous, persistent due to their low biodegradation rates, and with high mobility due to their moderate solubility in water (Gieseler, 2014; Licha, Herfort, & Sauter, 2001).

Phenols contamination is then, a matter of public and environmental health. Along the investigations regarding the effects of exposure to phenolic effluents, scientific research have also led the search for options for removal treatments in order to positively contribute with the increasing environmental pollution.

## 1.5 Phenolic effluents treatment

### 1.5.1 Physicochemical treatments.

Among the literature several physicochemical treatment methods for phenolic effluents can be found, such as: chlorination, electrolytic oxidation, photocatalysis, ozonation, electro coagulation, flocculation, solvent extraction, adsorption (Chandana Lakshmi & Sridevi, 2009; Kulkarni & Kaware, 2013), and thermal decomposition (Jiang et al., 2003), among others. Also, biological treatment processes is well discussed and include activated sludge systems in fixed bed reactors, anaerobic stabilization ponds (Kulkarni & Kaware, 2013), sequencing batch reactors (Tomei, 2008), up flow anaerobic sludge blanket (UASB) (Veeresh et al., 2005) to name a few. Unfortunately activated sludge systems are not efficient enough to give treatment to coke process wastewater. Due to the high concentrations of pollutants and toxic substances the effluent can inhibit the activity of microorganism of the activated sludge, or in the case of refractory organics, they can be resistant to biological attack, and therefore the wastewater ends with high organic concentrations after the biological treatment (Zhang et al., 1998). Also, biological treatment for industrial wastewater need high hydraulic retention time, due to the slow biodegradability and toxicity of some substances, which can lead to great costs in exchange for a poor and inefficient treatment. In the case of biological wastewater treatment extended specially for coke process wastewater, it can be possible to remove a significant part of the pollutants, however sometimes the concentrations of pollutants like phenols are not low enough for the disposal of the wastewater (Marañón et al., 2008). Furthermore, biological treatment has the disadvantage that it needs enough space for the treatment, which is not the case for every coke plant. In these cases physical and chemical treatment can be a feasible option to treat coke wastewater (Ghose, 2002a). Nevertheless, this kind of treatment can be expensive and could need several types of reagents for an appropriate performance.

### 1.5.2 Constructed wetlands as suitable options for industrial effluents.

Activated sludge systems mentioned above for the treatment of phenolic effluents can give valuable information regarding the possibilities to treat industrial wastewater and gather

information about the community composition in the sludge. Nevertheless these kinds of systems do not behave as natural environments. Constructed wetlands can be described as artificially engineered ecosystems, and through its design and construction is possible to manipulate biological processes (Zhi & Ji, 2012). Chandana Lakshmi & Sridevi (2009) argue that in natural environments the rate of degradation depends on chemical, physical and biological factors. In this sense, systems like constructed wetlands, being semi-controlled natural systems seem to be the closest equivalent natural environments and represent an appropriate option to understand the degradation of organic pollutants in this kind of environment (Zhi & Ji, 2012).

Unlike physical treatments, that concentrate the compound in a liquid or solid phase or induce the transformation to secondary toxic intermediaries, the biological nature of constructed wetlands allows to achieve a complete mineralization, thereby reducing the risk of accumulating toxic compounds (Agarry, Durojaiye, & Solomon, 2008; Tomei & Annesini, 2008).

Constructed wetlands emerges then, as a promising, environmental friendly technology, with low external energy requirements and costs, easy to operate and to give maintenance (Chen et al., 2012; Poerschmann & Schultze-Nobre, 2014; Zhi & Ji, 2012). During the last five decades constructed wetlands have been used worldwide. Before, constructed wetlands development focused on domestic sewage treatment, nowadays the research effort relies on wetlands for industrial wastewater treatment (Schultze-Nobre et al., 2015; Wu et al., 2015).

## 1.6 Constructed wetlands

### 1.6.1 Classification

Constructed wetlands can be classified based on different criteria such as: hydrology (surface-flow or subsurface-flow), flow path (vertical or horizontal) and macrophyte type (Zhi & Ji, 2012). The following characterization, according to Vymazal & Kropfelova (2008) is based mainly on the flow regime and type of macrophyte (Fig.1-3). Beginning with Surface-flow or Free water surface systems (SF-CW, FWs), are characterized for having a free water surface and a support for vegetation if present. Likewise, SF-CW can be classified depending on the type of macrophytes: free-floating, floating-leaved, submerged, and emergent. Another type are the Sub-

surface flow systems that can be classified depending in the direction of flow in horizontal and vertical flow. Vertical sub-surface flow CWs have a layer of sand planted with macrophytes above a layer of gravel; though both layers, the water percolates when it finally reaches a drainage system. In this kind of CW arrangement, water is fed intermittently which allows good oxygenation of the filter medium. Finally, the horizontal sub-surface flow constructed wetland (HSSF-CW) arrangement, is of particular interest for this study. In this kind of wetland the flow moves horizontally because the water enters from the inlet and flows through the porous medium until it reaches the outlet, where it finally leaves the system (Fig 1-4).

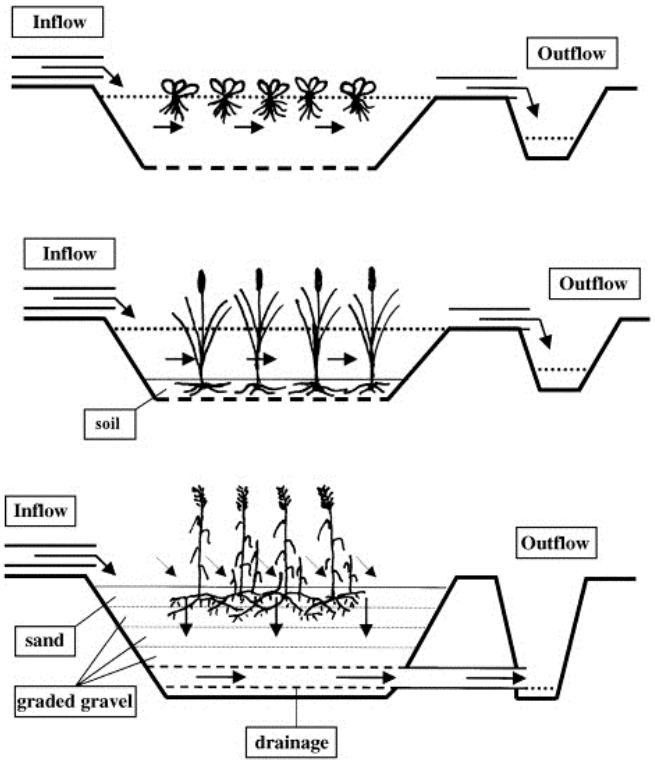



Figure 1-3. Diagram of constructed wetlands arrangements from top to bottom: Surface-flow system with free floating macrophytes, surface-flow systems with emergent macrophytes, and Vertical sub-surface flow. Taken from Vymazal & Kropfelova (2008).

Along the movement through the filter medium the water has contact with several aerobic, anoxic and anaerobic zones. Favored by these different zones, organic load in HSSF-CW can be degraded by aerobic or anaerobic bacteria inside the filter medium or attached to root surfaces, as well as sedimentation and filtration (Vymazal, 2014). The oxygen input in this arrangement

comes mainly from plants through their root system (Stottmeister et al., 2003). Additionally, transformation and elimination of nitrogen compounds can be successfully achieved with this type of wetland.

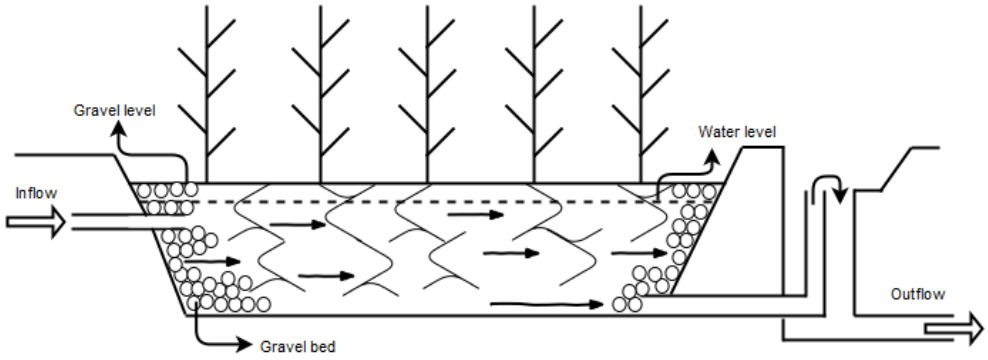



Figure 1-4. Horizontal sub-surface flow constructed wetland diagram. Modified from Vymazal & Kropfelova (2008).

### 1.6.2 Role of plants in constructed wetlands

The presence of macrophytes is one of the most distinguishing features regarding constructed wetlands. Plants used for constructed wetlands, known as helophytes, should fulfill several requirements, such as being tolerant to high nutrient and organic load, survive under anoxic or anaerobic conditions, and develop rich roots and rhizomes. Consequently, a well-developed underground system of roots adapted to anoxic conditions will provide substrate to attachment and oxygen to microbial communities. Within the root system and specifically the rhizosphere i.e the immediate soil region around the roots that is directly influenced by them, a series of physicochemical and biological processes induced by soil, plants, microorganism and pollutants interactions occur, transforming the rhizosphere into the most reactive zone within the constructed wetland (Poerschmann & Schultze-nobre, 2014; Stottmeister et al., 2003)

Plants used for wetlands by their root system are capable of release oxygen and exudates into the rhizosphere. The oxygen released can create a redox gradient suitable for chemicals transformation. The exudates of the plant, such as carbohydrates and organic acids can be a beneficial source for the microbial growth. Roots can serve as an adhesion substrate for

anaerobic and aerobic bacteria and hydrophobic organic compounds (Poerschmann & Schultze-nobre, 2014; Stottmeister et al., 2003). Is clear then, that plants metabolism affects the dynamic inside the constructed wetlands and together with physical processes occurring in the filter medium contribute to the removal of contaminants in the wastewater.

## 1.7 Removal mechanisms of contaminants in constructed wetlands

This technology and its special features and arrangements allow a manifold of processes to occur, and vary from organic load and contaminants removal to nitrogen and phosphorus transformations. Constructed wetlands exhibit several mechanisms to remove contaminants from wastewater, such as photochemical transformations, plat uptake, microbial degradation, precipitation, sedimentation and volatilization (Poerschmann & Schultze-Nobre, 2014). As a main focus of this investigation, microbial degradation will be discussed in the next sections.

### 1.7.1 Microbial degradation in constructed wetlands

Constructed wetlands can remove with high performance the organic load, nitrogen and phosphorous in wastewater due to physical, chemical and biological interactions occurring inside the wetland. It has been said also these processes are mainly driven by microorganisms, yet the knowledge on microbial communities in constructed wetlands is an open field with limited number of studies on this topic (Truu, Juhanson, & Truu, 2009).

Biological treatment extends to the exploration of a variety of microorganism capable of metabolizing aromatic pollutants like phenols (Chandana Lakshmi & Sridevi, 2009), this kind of metabolism depends on the action of enzymes, which are specific for each reaction (Agarry et al., 2008; Sridevi et al., 2012). Microbial transformation metabolism provide energy for microbial growth, which is a key determinant for an effective remediation process (Agarry et al., 2008).

Degradation, or the process of completely metabolize or mineralize organic pollutants (Agarry et al., 2008), can occur in aerobic or anaerobic conditions depending on the type of bacteria (Chandana Lakshmi & Sridevi, 2009) and require some conditions. First the microbial community taking part of the degradation must have the require enzyme or enzymes necessary

for the specific transformation in question. Transformation of one compound in many cases requires the presence of one specific single enzyme for one specific compound. In the same way degradation pathways can be only achieve by synergistic actions between different enzymes present in different species. Additionally, the compound must be available for the microorganisms, means that the compound and the bacteria should be in the same phase (e.g. liquid phase). Finally, the degrading strains can proliferate and therefore succeed in the degradation process depending on their ability to compete and have access to oxygen and organic sources (Alexander, 1985; Chandana Lakshmi & Sridevi, 2009).

A great effort has been done to describe a widely list of microorganisms capable of degrading phenols. Unfortunately, phenol degradation studies on single cultured strains do not reflect the dynamism of an engineering environment such as constructed wetlands, in which biotic and abiotic interactions are influencing the degradation of pollutants and performance of the bacterial community (Ambujom & Manilal, 1995). Is also proved, that bacteria consortium have better removal efficiency compared to its individual isolates, suggesting that the combination of different microorganisms enhance the removal rates (Ambujom & Manilal, 1995)

Studies on bacterial cultures are developed in most cases using only the bacterial strains successfully isolated and that are able to culture in the laboratory. Nevertheless, in general terms, only a small fraction of the total microorganisms in an environmental sample can be cultured. Owing to this small percentage of culturable microorganisms, these methods can undetect or underestimate the real abundance of certain microorganisms, and in this way are unable to describe accurately the microbial abundance and diversity in the natural environment (Malik et al. 2008; Truu et al., 2009). In contaminated sites, key microorganisms in the degradation of a certain compound may go undetected, thereby limiting further insights on its diversity and ecological importance (Malik et al., 2008).

Meeting the limited scope of culture methods in characterizing bacterial community composition, modern molecular technics and methods have emerged as an ideal option to address questions regarding diversity, ecology, functionality and population dynamics of these communities (Kerkhof & Häggblom 2008; Malik et al., 2008). Molecular methods are based in the characterization of cellular components such as lipids, proteins and nucleic acids, that allow

to distinguish among the different members of the bacterial community (Kerkhof & Häggblom 2008).

These culture-independent molecular methods have broadened the knowledge regarding biodegradation of chemical pollutants, as they provide valuable information regarding gene regulation of microorganisms responsible of specific biodegradation processes and pathways (Truu et al., 2009).

### 1.8 Phenol degradation

Phenols can be toxic even at low concentrations and is of major importance to develop the technology capable of degrading this pollutants with non-toxic end products (Agarry et al., 2008). In this sense, biological treatment of xenobiotics and toxic compounds, is gaining attention because it accomplishes in the best cases a complete mineralization of the compound (Tomei & Annesini, 2008). Mineralization processes can transform toxic compounds such as phenols into less toxic metabolites. Metabolites can serve as well serve as microbial substrates which can be later on embedded into microbial metabolism.

Phenol is converted by bacteria activity to carbon dioxide under aerobic conditions, and carbon dioxide and methane under anaerobic conditions. As like other aromatic hydrocarbons, phenol aerobic degradation comprise two basic steps: the activation of the aromatic ring and its subsequence cleavage, both are mainly conducted by oxygenases (Pérez-Pantoja, González, & Pieper, 2009). Activation of the aromatic ring occurs after oxygenation reactions that produce intermediates such as catechols, protocatechuates, gentisates and benzoquinols. Afterwards, these intermediates suffer a ring cleavage and the products are finally channelled to the Krebs cycle, also referred as central pathway.

Aromatic ring activation depending on the aromatic compound can be carried by members of one of three subfamilies: the Rieske non-haem iron oxygenases (Gibson & Parales, 2000), the flavoprotein monooxygenases (van Berkel, Kamerbeek, & Fraaije, 2006) and the soluble diiron multicomponent monooxygenases (Leahy, Batchelor, & Morcomb, 2003). Soluble Diiron Monooxygenases comprise enzymes capable to monooxygenate benzene/toluene to phenol/methylphenol and phenols to catechols (Leahy et al., 2003). Likewise, this subfamily is

divided in different phylogenetic groups, from which the phenol hydroxylases are the most relevant for this study.

### 1.8.1 Dimethylphenols degradation

Several factors can affect the biodegradation of phenol compounds or other toxic compounds by microorganisms, such as toxicity and chemical structure. The number, type and position of substituents in a compound affect its biodegradability in a direct way. More substituents imply less degradability, more toxicity and more recalcitrance (Agarry et al., 2008). Therefore, dimethylphenols constitute a group of substituted phenols, with alkyl groups which at first instance can be consider a drawback for their degradation.

Several possible pathways have been describe on specific bacterial strains for degradation of 2,4-DMP (Chapman & Hopper, 1968; Chen, Chao, & Zhou, 2014), 2,5-DMP (Hopper & Chapman, 1970), 3,5-DMP (Hopper & Chapman, 1970; Hopper & Kemp, 1980), 2,3-DMP (Chapman, 1972), and 2,6-DMP (Ewers et al., 1989). Degradation of phenolic effluents including dimethylphenols have been performed by Broholm and Arvin (2000), Flyvbjerg et al. (1993), and Harrison et al. (2001). These last investigations concerning DMPs mixtures give a good insight on which conditions these compounds are successfully degraded. For instance Flyvbjerg et al. (1993) performed an experiment in batch reactors using creosote wastewater containing DMPs. They reported that the position of the methyl group determines if a DMP is degraded under reducing conditions. Thus, only 2,4-DMP and 3,4-DMP which have a para-position methyl were easily degraded while the other isomers were resistant to biodegradation. Harrison et al. (2001) used a mixture of phenol, o-cresol, 2,4-DMP, 2,5-DMP and 2,6-DMP under aerobic and anaerobic conditions. Under anaerobic conditions only o-cresol was biodegraded while under aerobic conditions only 2,5-DMP was biodegraded. In this case, 2,4-DMP and 2,6-DMP were reported to have abiotic degradation. Finally, Broholm and Arvin (2000) obtained similar results with a mixture of phenolic compounds under aerobic conditions. The compounds were degraded in the next order: phenol and p-cresol, 3,4-xylenol, o-cresol and 2,5-xylenol, 2,6-xylenol, 3,5-xylenol. They propose that para-substituted phenols are firstly degrade, followed by meta-substituted phenols.

Concerning DMPs propitious degradation conditions, Licha et al. (2001) present that DMPs exhibit low degradation potentials under reductive conditions, that means, conditions where anaerobic processes are suitable. Zhang et al. (1998) evaluated the removal efficiency of an anaerobic-anoxic-oxic fixed-biofilm fed with coke plant wastewater containing DMP. They did not find degradation of DMP after the anaerobic step of the process Accordingly, Flyvbjerg et al. (1993) had negative results when analyzing the biodegradation after seven months of incubation under anaerobic conditions for five DMP isomers, concluding that anaerobic environments do not favor degradation of these compounds. Schultze-Nobre et al. (2015) reported a high removal of 2,6-DMP under aerobic conditions, but low removal under anaerobic conditions. These results can lead to the argument that dimethylphenol biologic degradation is mainly conducted by aerobic processes.

### 1.9 DMP degradation in constructed wetlands

Knowledge regarding DMP degradation in constructed wetlands is quite limited. Few efforts found in the literature can be mention, for instance Jardinier et al. (2001) evaluated the potential removal of a pilot-scale two-stage HF CW planted with *Phragmites australis* fed with coking effluents. They found a contaminant removal efficiency between 54% and 95%, concluding that this may be a valid method to treat coking wastewater (Wu et al., 2015).

A study performed using microcosms in aerobic and anaerobic conditions showed the degradation by natural bacteria consortium. Influent came from a coal tar distillation plume containing phenol, o-m- and p-cresol, and 2,4- 2,5- and 2,6-DMP. Results showed an effective biodegradation of 2,5-DMP, but reported abiotic degradation for isomers 2,4 and 2,6-DMP (Harrison et al., 2001). Important efforts concerning DMPs degradation has been developed in the Helmholtz-Zentrum für Umweltforschung (UFZ). Schultze-Nobre et al. (2015) evaluated the removal efficiency of a laboratory-scale planted fixed bed reactor planted with *Juncus effusus* containing a mixture of 2,6-, 3,4-, and 3,5-DMP, and afterwards only adding 2,6-DMP. Results showed a removal performance in the order 3,4- > 3,5- > 2,6 with 40 mg/L in the influent. An increase in 100 mg/L of DMPs caused a complete removal inhibition. This study emphasized the importance to investigate the different processes of DMPs degradation and suggest that aerobic

pathways seem to be preferred, although there is not enough information to exclude anaerobic degradation.

Additional efforts also performed in UFZ evaluated the removal performance of a Planted Fixed Bed Reactor (PFR) fed with a DMP mixture containing 3,4-, 3,5- and 2,6- DMP (Rincón, 2015; unpublished data). Results showed up to 99% of removal, being 2,6-DMP the only isomer detected in the outflow. Afterwards, DNA samples from bacterial consortium from gravel, roots, and water from a Planted Fixed Bed Reactor and a wild strain of *Delftia acidovorans* previously exposed to DMPs mixtures were analyzed with a set of degenerated primers of catabolic genes involved in aerobic biodegradation of aromatic compounds. These primers specifically targeted different sub families of monooxygenases and dioxygenases. Results obtained showed a consistent occurrence of phenol hydroxylase in the bacteria consortium.

Constructed wetlands have been commonly developed using a black box approach, in which guidelines of design and operation are used, without a complete understanding of the removal mechanisms and plant-microbes interactions between occurring inside (Fester et al., 2014; Wu et al., 2013). Despite the fact that degradation inside constructed wetlands is a process mainly driven by microorganisms, only limited studies have focus on describing these communities. Faulwetter et al. (2009) explain that microbial processes in the literature is based on assumptions and underscore the importance of studies presenting real evidence of microbial consortia working on site. Therefore, expanding the knowledge on dynamics of bacterial communities is of major importance since it can be applied to upgrade and optimize these water treatment technologies (Button et al., 2015; Truu et al., 2009). Moreover, new information can lead the way to elucidate the complex interactions and transformations occurring in constructed wetlands (Wu et al., 2013).

Despite the recent efforts on describing DMPs degradation in constructed wetlands, the knowledge still is limited and more investigation is needed (Schultze-nobre et al., 2015). Previous studies in laboratory-scale constructed wetlands have focus on degradation and behavior of DMPs mixtures with different concentrations from this particular study. Thus, this investigation provide a novel understanding of the behavior of isolated DMPs inside constructed

wetlands and the dynamics of the aerobic bacterial communities regarding their function in aromatic compounds degradation.

This study aimed to evaluate the removal efficiency of DMPs isolated isomers in two horizontal subsurface flow constructed wetland planted with *Phalaris arundinacea* and *Juncus effusus*. The aim of this study was to determine how the removal efficiencies differ according to the isomer present in the filter medium, presence of plants and physicochemical parameters.

Additionally, the objective of this study was to investigate the bacterial community in terms on its capacity to degrade aromatic compounds. Based on the assumption that DMPs degradation is mainly an aerobic process and the recurrent presence of phenol hydroxylase in bacterial communities after acclimatization with DMPs mixtures in previous studies, the present investigation focused on the detection of this enzyme to characterize the aerobic bacterial communities and evaluate possible changes in this catabolic gene.

# 2 Objectives

## 2.1 General objective

To determine the biodegradation of dimethyphenols in an horizontal subsurface flow constructed wetland and the role of the associated aerobic microorganisms.

## 2.2 Specific objectives

- To determine the removal efficiency of dimethylphenols in two planted horizontal subsurface flow constructed wetlands.
- To analyze the relationship between the different physicochemical parameters, plants species and the bacterial communities with the removal efficiencies.
- To identify changes in catabolic genes involved in dimethylphenols degradation depending on the DMP isomer present in the constructed wetland.

# 3 Materials and Methods

This study consisted of a series of experiments to determine the removal efficiency of dimethylphenol isomers by the bacterial communities using two laboratory scale HSSF-CW in two different stages. The concentrations of DMPs were monitored along the constructed wetlands as well as chemical and physical parameters. Simultaneously, DNA was extracted from samples of the bacterial communities in order to identify changes in the catabolic genes involved in DMPs degradation and evaluate the relative abundance of phenol hydroxylase.

The experiment used was performed in two HSSF-CW, during two experimental stages, for a total of four treatments (Fig. 3-1). Stage one was conducted between November 2015 and January 2016 for treatments (1) fed with 3,5-DMP and planted with *Phalaris arundinacea*, and (2) fed with 3,5-DMP and planted with *Juncus effusus*. Stage two developed between February and April 2016 for treatments (3) fed with 2,6-DMP and unplanted, and (4) fed with 2,6-DMP and planted with *Juncus effusus.* A previous stage consisting in one treatment planted with *Phalaris arundinacea* and one planted with *Juncus effusus* both fed with isomer 3,4-DMP was developed between August and October 2015.

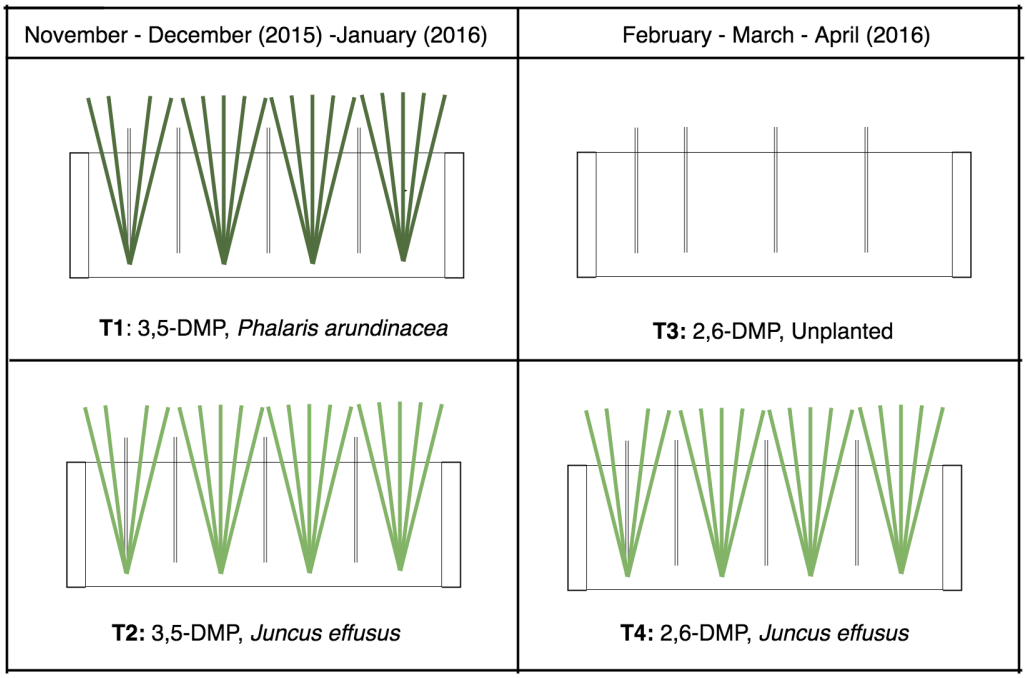



Figure 3-1. Diagram of the 4 experimental treatments.

### 3.1 Horizontal subsurface-flow laboratory-scale constructed wetland

The experiment was performed in two laboratory scale constructed wetlands, specifically horizontal subsurface-flow constructed wetlands (HSSF CW). These constructed wetlands consist in an open plastic container with the following dimensions: 100 cm length, 35 cm height and 15 cm width, and filled with gravel (diameter 2–6 mm, density 1.67 g $cm^{-3}$, porosity 40%) up to 27 cm height, and a free pore water volume of 13,8L (24,5 cm height)(Fig. 3-2). Sieves of perforated stainless steel were placed at a distance of 3 cm in front of the inflow and outflow of the gravel bed, creating equal distribution of the inflow process water and a plug flow through the gravel bed (Wiessner et al. 2010).

Figure 3-2. Photography of the laboratory scale constructed wetlands. Taken during stage two, and showing treatment 3 and 4.

Both HSSF-CW were located in the UBT/UFZ green house under controlled climatic conditions, to simulate an average summer day. The temperature between 6:00 a.m and 9:00 p.m was fixed at 22 ºC, and between 9:00 p.m and 6:00 am at 16ºC. Also, during the day, an additional source of light was reached with lamps.

### 3.2 Constructed wetlands set up and maintenance

In the first phase the wetlands were fed with artificial wastewater containing isolated 3,5-DMP (Sigma Aldrich, 98% purity) during 90 days, while in the second phase the wastewater contained isolated 2,6-DMP (Merck-Schuchardt, purity 98%) during 85 days. The artificial wastewater was composed of tap water, nutrients, salts, trace minerals and a concentration of 25 mg/L of 3,5-DMP in the first phase, and 25 mg/L of 2,6-DMP for the second phase. Exact composition of artificial wastewater can be seen in table 3-1.

Table 3-1. Artificial wastewater composition

| Component | Concentration (g/L) |
|---|---|
| 3,5-DMP | 25 mg/L |
| 2,6-DMP | 25 mg/L |
| $NaHCO_3$ | 0.252 |
| $K_2HPO_4$ | 18.35 |
| NaCl | 3.5 |
| $NH_4Cl$ | 58.88 |
| $CaCl_2 \cdot 2H_2O$ | 2 |
| $MgCl_2 \cdot 6H_2O$ | 0.825 |
| $Na_2SO_4$ | 0.443 |
| TSM (Appendix 1) | 1 mL/L |

Each CW was equipped with an inflow and outflow tank with a capacity of 30 L. Inflow tanks were filled with artificial wastewater and connected to a pump that provided a continuous flow for the wetland. Both wetlands were connected to the same pump, allowing to maintain the same inflow rate for both systems. Artificial wastewater addition was done every week on Monday and Friday to ensure a continuous flow and feeding of the wetlands during the week. Additionally, continuous maintenance of the pipes was done every month to avoid leakages and blockages. During stage 1 and 2, the stems of *Juncus effusus* were counted every month to obtain biomass related data.

### 3.3 Mass balance, water sampling and physicochemical parameters.

In parallel to the artificial wastewater addition, mass balance was conducted. Before refilling the inflow tank with new artificial wastewater, outflow tank and inflow tank were weighed in order to calculate the liters of wastewater passing through the system, the water losses and the hydraulic retention time between every measurement. Afterwards, outflow was discarded and inflow was filled with new artificial wastewater up to 30 L.

The Hydraulic Retention Times (HRTs) were: T1= 2.8±0.47 d, T2=3.04±0.44 d, T3=2.43±0.29 d, T4=3.54±0.30 d. They were calculated using the following formula and correspond to the average of the HRTs measured during the week:

$$HRT = \frac{V\ (pore\ water)[m^3]}{average\ \{Q\ (inflow)[m^3 d^{-1}] + \ Q\ (outflow)[m^3 d^{-1}]\}}$$

Pore samples were taken simultaneously with the monitoring of the physicochemical parameters within the constructed wetlands A set up including a peristaltic pump, house pipes and small stainless steel tubes at a depth of 15 cm from the gravel surface placed at sampling points 12,5 cm, 25 cm 50 cm, and 75 cm allowed us to pump the water from inside the gravel. Water was directly accessible at the inflow and outflow sampling points. The house pipes were cleaned with distilled water between every measurement to avoid contamination of water samples. Water samples were taken starting from the outflow and ending with the inflow. Physicochemical

parameters were always measured when the passing water was air bubbles free. The set up for the water sampling and physicochemical parameters monitoring can be seen in Figure 3-3.

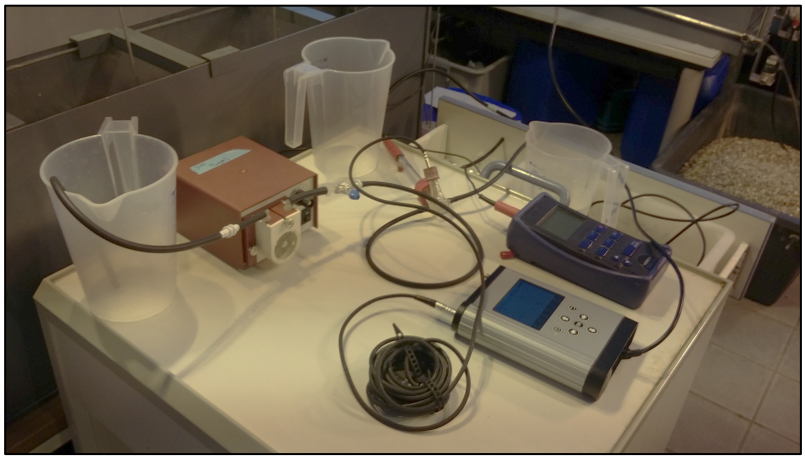


Figure 3-3. Set up for water sampling and physicochemical parameters monitoring.

Pore water samples of approximately 40 mL were taken at 0 cm, 12,5 cm, 25 cm, 50 cm, 75 cm and 100 cm (Fig. 3-4). From each water sample DMPs concentrations, dissolved organic carbon (DOC), and nitrogen species ($NH_4$, $NO_2$ and $NO_3$) were measured using their own protocol in the laboratory. The following physicochemical parameters: dissolved oxygen, temperature, redox potential and pH were measured on site at every sampling point.

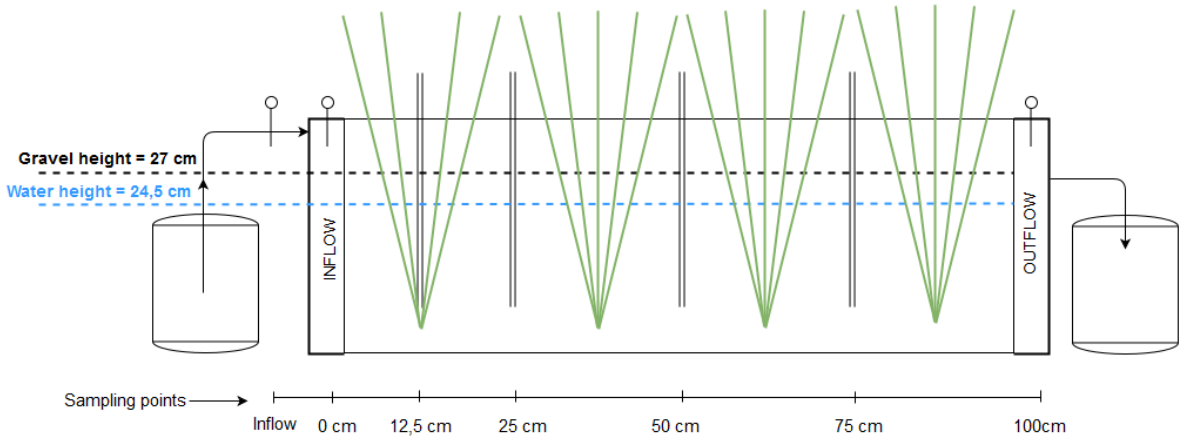



Figure 3-4. Diagram of the constructed wetland sampling points.

### 3.3.1 Dissolved oxygen

Connected to the peristaltic pump mentioned above, a flow-through cell with a sensor FTC-TOS7 was used to detect the oxygen. The sensor was exposed to the pore water stream, and the quantification of dissolved oxygen concentration was achieved using a Fibox 4 LCD trace Oxygen Meter (PreSens Precision Sensing).

### 3.3.2 Redox potential, temperature and pH

A Checktemp®1 Thermometer from Hanna Instruments cell equipped with a $Pt/Ag^{+}/AgCl/Cl^{-}$ electrode (Sentix ORP®, WTW), and connected to a flow-through cell, was used to measure temperature. Connected to the same flow-through cell a microprocessor Multi-350i handheld meter (WTW) was used to measure the redox potential. To get the standard hydrogen values, the voltage values measured by the electrodes were corrected using the following equation:

$$U_H = U_G + U_{ref} + D$$

$U_H = Redox\ potential\ [mV]$

$U_G = Measured\ voltage\ [mV]$

$U_{ref} = Standard\ voltage\ depending\ on\ temperature\ [mV]$

$D = Measured\ deviation\ to\ reference\ electrode\ [mV]$

The redox potentials were adjusted by adding +200 mV to the initial measurement.

### 3.3.3 DMPs quantification by HPLC

Around 4 mL of the water samples were filtered with acrodisc syringe filters (20 μm) inside HPLC-GC vials and stored at 10ºC until the High-performance liquid chromatography analysis was conducted. The quantification of 2,6-DMP and separation and quantification of 3,4- 3,5- and

2,6-DMP were performed with the Shimadzu High Performance Liquid Chromatography. The following equipment was used:

Column: Poroshell 120 EC-C18 (Agilent Technologies) (diameter: 3 mm, length: 100 mm, particle size: 2.7 µm)

Column temperature: 50°C (isotherm)

Flow rate: 0,2 ml/min

Eluents (mobile phase): A – 0.1% formic acid, B – 100% acetonitril

Program of elution: first minute increase of eluent B from 10% to 20%, 1 – 45 minute increase of eluent B from 20% to 40%

Injection volume: 0,3 µL

Time per analysis: 45 minutes

Detection: UV-detection at 278 nm

Chromatograms were analyzed by LCSolution's Postrun Analysis Software. A HPLC analysis of 3,5-DMP and 2,6-DMP dilutions with known concentrations (from 10 to 80 mg/L) were performed in order to make a calibration curve. DMP data in concentration (mg/L) initially obtained from HPLC analysis were transformed to loads (mg/d), by adjusting the concentrations to the water losses due to evapotranspiration for each treatment. To calculate the load two assumptions were made: 1) evapotranspiration along the flow path increases in a linear way, and 2) it exist an ideal plug flow through the wetland soil filter (Shubiao et al. 2012). This allowed to show the real contaminant load, rather than the concentration in every point, which varies depending on the water losses from the wetlands

### 3.3.4 Nitrogen species quantification

The determination of nitrogen species was conducted by colorimetric methods using a photometer Spectroquant NOVA 60® (Merck KGaA) and the test kits listed in Table 3-2. Due to the instability of nitrogen species, the analysis was performed immediately after the sampling was finished.

Table 3-2. Test kits for colorimetric analysis of nitrogen species.

| Nitrogen specie | Test kit | Standard | Wavelenght (nm) | Detection range (mg/L) |
|---|---|---|---|---|
| $NH_4^+$ -N | Merck No. 1.00683.0001 | DIN 38406 E5 | 690 | 2.0-75 |
| $NO_2^-$ -N | Merck No. 1.14776.0001 | DIN EN 26 777 D10 | 525 | 0.02-1 |
| $NO_3^-$ -N | Merck No. 1.09713.0001 | DIN 38405 D9 | 340 | 1.0-25 |

**Dissolved Organic Carbon**

Water samples were filtered with acrodisc syringe filters (20 µm) inside HPLC-GC vials and stored at 10ºC. Dissolved organic carbon was measured with the multi N/C 2100S TOC analyser (Analytik Jena). Data was analyzed with multiWin® software. Analytical quality control of the measurement system was assured by the analysis of a standard solution of potassium hydrogen phthalate at 100 mg/L.

## 3.4 Microbiological analytical methods for dimethylphenols degrading bacteria

### 3.4.1 Sampling

For each treatment, samples for DNA extraction were taken three times during the experimental stage. Pore water was collected with syringes connected to stainless steel tubes at 12.5 cm, 25 cm, 50 cm and 75 cm (Fig. 3-4). In every sampling point pore water inside the wetland was mixed with the syringes in order to resuspend the biomass around the gravel. The water sample was then placed in a filtration unit, and passed through a filter (Supor® 200, 0,2µm; PALL Life Sciences) to retain the biomass. The filter was stored in a falcon tube at -20 °C until use.

### 3.4.2 DNA extraction protocol

DNA extraction was performed using a modified protocol from DNAeasy Mini Kit (Quiagen) Blood and tissue. As a first step, the biomass that remained in the filters from the DNA sampling was washed with 800 µL lysis buffer inside the falcon tube and transferred to a 1,5 mL Eppi containing lysozyme, agitated and incubated at 37°C for 30 min. The mixture was then transferred to cryovials with two types of glass beads (<160 µm O Sigma (G4649-10G), acid washed), then 25 µL of proteinase K and 200 µL of Buffer AL were added. Cryovials were placed in the Fastprep(R)-24 (MP Biomedicals) for 30 seconds at 4 m/s speed, and after incubated at 65°C for 30 min.

After the incubation, 800 µL of Ethanol (100%) were added to the cryovials. The mixture was then transferred to DNAeasy columns provided by the kit and centrifuged (Centrifuge 5424 R, Eppendorf) at 8000 rpm for 1 minute as many times as necessary for all the sample to go through the column. Afterwards, 500 µL of Buffer AW1 was added and then centrifuged at 8000 rpm, followed by 500 µL of Buffer AW2 and again centrifuged at 14000 rpm. The collection tube was changed and again centrifuged at 14000 rpm. Finally the filter tube was placed in a new Eppi, and 60 µL of AE Buffer was added, incubated at room temperature for 10 min and centrifuged at 8000 rpm for 1 min. Samples were stored at -20°C. DNA extractions were used for qPCR.

### 3.4.3 qPCR

#### *3.4.3.1 Previos steps*

Convencional PCRs were conducted before qPCRs in order to detect the presence of the phenol hydroxylase (PHE) and 16S rRNA fragments in the extracted DNA samples from the wetlands. Preparation of standards for 16S DNA and PHE were also performed. First an over-night culture of a clon carrying the fragment for 16s rRNA of *Pseudomonas putida* and PHE of *Ralstonia* sp. was done, in 5 mL LB-medium with 5 µL of Ampicin (1:1000) for 13 hours at 36°C. Cells were harvested and centrifuged at 8000 rpm (Centrifuge 5424 R, Eppendorf) for 2 min at room temperature. The supernatant was discarded and the plasmids were extracted using a purification

protocol. A conventional PCR was conducted with the extracted plasmids. Master mix contained: 10 µl of HotStar Taq DNA Polymerase (QIAGEN), 10 µL of Nuclease-Free Water, 1 µL of Forward M13, 1 µL of Reverse M13 and 1 µL of 16s rRNA of *P.putida* or PHE of *Ralstonia* sp. extracted plasmids.

Thermal cycling conditions for these reactions included an initial activation at 95°C for 10 min, followed by 35 cycles of denaturation at 95°C for 30 sec, annealing at 55°C for 30 sec, and elongation at 72°C for 75 sec, and a final extension at 72°C for 5 min. Verification of DNA fragments was done by electrophoresis. PCR products were run in 1,2% agarose gel with 1x Tris-acetate-EDTA (TAE) buffer for 20 min at 100 V. The ladder used for identification was FastRuler DNA Low Range Ladder (50-1500 bp, Thermo Scientific). For staining nucleic acids, ethidium bromide dye was used. PCR products were purified using the GeneJET PCR purification kit (ThermoFisher Scientific). Concentrations of purified PCR products were measured by NanoDrop ND-1000 Spectrophotometer adjusted for nucleic acids using the PC based software (Thermo Fisher Scientific).
The standards chosen for qPCR were: a PCR product of 72,9 ng/µL for *P.putida*, and a PCR product of 34,3 ng/µL for *Ralstonia* sp.

Finally, the calculations for the standards for qPCR were done as follows:

$$P.putida \rightarrow 197\text{ bp} + 66\text{ bp} + 1351 = 1614 \rightarrow \text{length PCR product}$$

$$Ralstonia\text{ sp.} \rightarrow 197\text{ bp} + 66\text{ bp} + 701 = 964 \rightarrow \text{length PCR product}$$

$$\frac{\left(concentration\ PCR\ product * 10^{-9}\frac{g}{\mu l}\right)}{(length\ PCR\ product * 660)} * 6{,}022 * 10^{23} = x * 10^{10}\frac{molecules}{\mu l}$$

$$P.putida\ (16\text{s DNA}) = 4.12\times10^{10}$$

$$Ralstonia\text{ sp (PHE)} = 3.25\times10^{10}$$

From the standard of 4,12x$10^{10}$ molecules/µl for *P.putida* and 3,25x$10^{10}$ molecules/µl for *Ralstonia* sp. a dilution series of standards from $10^8$ to $10^1$ was performed before every qPCR essay (Appendix 2).

Table 3-3. List of primers for qPCR standards and essays.

| Primer | Target gen | Sequence (5'-3') | $T_a$(°C) | Template | Literature reference |
|---|---|---|---|---|---|
| M13-F<br>M13-R | 16S rRNA and PHE | GTAAAACGACGGCCAGT<br>CAGGAAACAGCTATGAC | 55 | *Escherichia coli* | Messing, 1983 |
| PHE-F<br>PHE-R | PHE | GTGCTGAC(C/G)AA(C/T)CTG (C/T)TGTTC<br>CGCCAGAACCA(C/T)TT(A/G)TC | 49 | *Ralstonia eutropha* | Baldwin et al. 2003 |
| U519-F<br>U909-R | 16S rRNA | CAGCMGCCGCGGTAATWC<br>CCGYGAATTCMTTTR | 52 | | Liaqat et al. 2014<br>Wang & Qian, 2009 |

$T_a$ Annealing temperature

#### *3.4.3.2 qPCR*

Amplifications were performed in a StepOnePlus Real Time PCR-System (Applied Biosystems). Multiple essays reaction mixtures contained for 16s rRNA: 6,25 µL of KAPA SYBR(R) FAST Universal 2xPCR Master Mix (Kapa Biosystems), 4,72µL of Nuclease-Free Water, 0,25µL of U519F forward primer, 0,25µL of U909 reverse primer , and 1 µL of DNA template. For PHE, reaction mixture contained: 6,25 µL of KAPA SYBR(R) FAST Universal 2xPCR Master Mix (Kapa Biosystems), 3,49 µL of Nuclease-Free Water, 0,63µL of PHE-F primer (5'-GTGCTGAC(C/G)AA(C/T)CTG(C/T)TGTTC), 0,63µL of PHE-R primer (5]-CGCCAGAACCA(C/T)TT(A/G)TC) (Baldwin et al 2003), and 1,5µL of DNA template. Each qPCR was performed with a triplicate of the standard dilutions (from $10^8$-$10^1$ of 16s or PHE), a triplicate of the dilution 1:10 of the sample, the same sample diluted 1:100 and 5 negative controls. Thermal cycling conditions for the reactions can be seen in Table 3-4. Data analysis was performed using the StepOne Plus Program Software (Applies Biosystems). The acceptable range of qPCR efficiency was over 80%.

Table 3-4. qPCR cycling program for 16S DNA and PHE.

| Step | Time duration | Temperature | Number of cycles |
|---|---|---|---|
| Initial activation step | 20 sec | 95 °C | 1 |
| Denaturation | 3 sec | 95 °C | 40 |
| Annealing PHE | 20 sec | 49 °C | 40 |
| Annealing 16S rRNA | 20 sec | 52 °C | 40 |
| Elongation | 20 sec | 72 °C | 40 |

Eqquipment: StepOnePlus Real Time PCR-System (Applied Biosystems)

#### *3.4.3.3 Analysis of qPCR results*

Verification of the amplification plot, standard curve, melt curve and efficiency of each qPCR essay were done to guarantee an acceptable performance of the protocol before the analysis of the data (Appendix 5). Results obtained showed the copy number of phenol hydroxylase genes and 16s rRNA genes present in the template of the reaction mixture. A normalization of this result was performed in order to obtain biologically meaningful data. Results were normalize as the copy number of genes in 1 mL for each sample. Finally, the rate of PHE copy number between 16s rRNA copy number was calculated and transformed as the percentage of the total bacterial community in which PHE was detected.

## 3.5 Data interpretation and analysis

The collected data was analyzed using graphs in order to find correlations among the physicochemical parameters and removal rates of dimethylphenols. When necessary, ANOVA tests were used to compare between treatments, with a significance level set at $p<0.05$.

# 4 Results and discussion

Experiments were developed over a period of 24 weeks. The experiment intended from the beginning to analyze the removal capacity of two constructed wetlands planted with two different plants species: *Phalaris arundinacea* and *Juncus effusus* in two stages. In the first stage the artificial wastewater would contain 3,5-DMP, while in the second stage it would contain 2,6-DMP. Due to an unexpected event after the first stage of the experiment, the wetland planted with *Phalaris arundinaceae* could not resist the toxicity of the 3,5-DMP and the plant died at the end of the treatment. In consequence, this wetland became an unplanted system (control) during the second stage. Consequently, the results and discussion regarding sections 4.1 to 4.5 of this investigation are based on four different treatments. For analysis purposes they were named as follows:

Treatment 1: 3,5-DMP *Phalaris* sp.

Treatment 2: 3,5-DMP *Juncus* sp.

Treatment 3: 2,6-DMP Unplanted

Treatment 4: 2,6-DMP *Juncus* sp.

Results and discussion of section (4.6) include a previous stage fed with isomer 3,4-DMP, named 3,4-DMP *Phalaris* sp. and 3,4-DMP *Juncus* sp. (Section 3).

## 4.1 Overall performance of experimental wetlands

Mass balance was conducted twice per week in order to determine the water losses of the systems due to evapotranspiration. Results on water losses through the whole experimental period can be seen in Figure 4-1. Since the water level is below the gravel in HSSF-CW, they are characterized by the fact that water losses are mainly due to evapotranspiration (Stottmeister et al., 2003). This can be easily noticed when comparing the three planted systems with the unplanted one. Certainly the presence of plants was the main factor causing water losses in the systems.

CW planted with *Juncus* sp. got considerably stable by the end of both stages. During stage one it exhibited a mean rate of evapotranspiration of 2,46 L/d ±0,93, whereas stage two exhibited a mean of 3,93 L/d±0,47. Changes in evapotranspiration rates between these two treatments are related with plant biomass as shown in Figure 4-2. The higher rates of evapotranspiration during stage two can be directly attributed to the higher quantity of *Juncus* sp. stems during this stage.

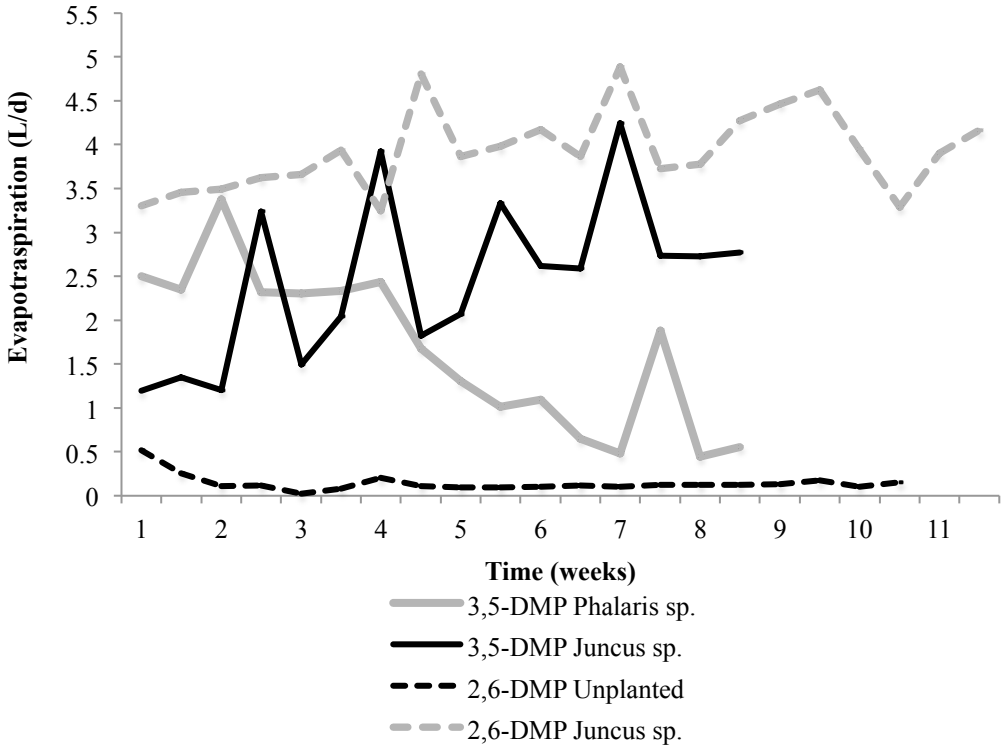



Figure 4-1. Evapotranspiration rates through experimental weeks.

Treatment planted with *Phalaris* sp. showed high evapotranspiration rates during the first weeks and decreased towards the end of the treatment (Fig.4-1). This was due to the high toxicity of 3,5-DMP for *Phalaris arundinaceae* which affected the general health of the plant and eventually led to its death. This event impacted the whole performance of this constructed wetland in many ways, and will be discussed in the next sections. Nevertheless this specie has shown to be of great performance in the accumulation of heavy metals (Brezinová & Vymazal, 2015), and due to its high amounts of belowground biomass it can release high concentrations of

organic carbon and oxygen that can enhace microbial growth and increment aerobic respiration rates, resulting in high nutrient removal (Gagnon et al., 2007; Picard et al., 2005).

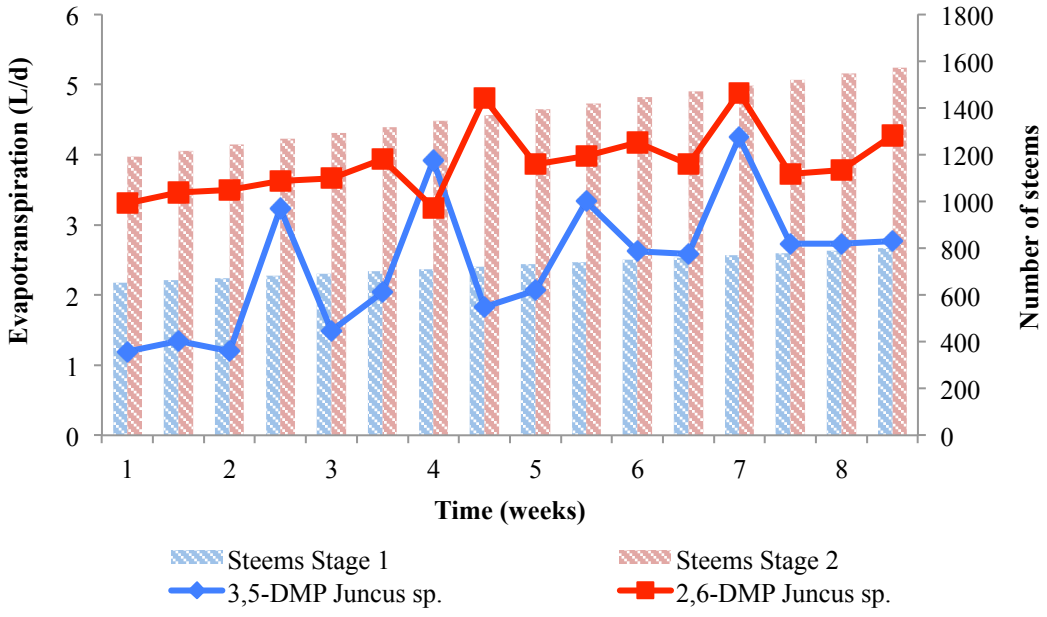



Figure 4-2. Comparison of evapotranspiration rates between CW planted with *Juncus* sp. in both stages.

## 4.2 Removal efficiency of dimethylphenols

### 4.2.1 Comparison between planted and unplanted wetlands

Concerning the removal of DMPs (Fig.4-3), the comparison of the four treatments shows that both treatments with *Juncus* sp. were the most efficient in terms of percentage of removal, reaching more than 95% of removal from the inlet. The treatment planted with *Phalaris* sp. was not as efficient as the other two planted treatments, although it reached a percentage of removal of 78% in the outlet. Finally, the unplanted wetland had the lowest percentage of removal of all treatments, with around 46% of removal.

As can be seen in Figure 4-4 the initial load in the inflow for every treatment is different, despite the fact that all of them had the same concentration of DMP in the artificial wastewater and were connected to the same peristaltic pump. Apparently the differentiated solubility of 3,5-DMP and

2,6-DMP changed the initial load for each treatment. Isomer 2,6-DMP exhibited a better solubility in the artificial wastewater, which led to inflow loads of around 100 mg/d in the second stage, whereas the first stage fed with isomer 3,5-DMP had inflow loads of around 50 mg/d.

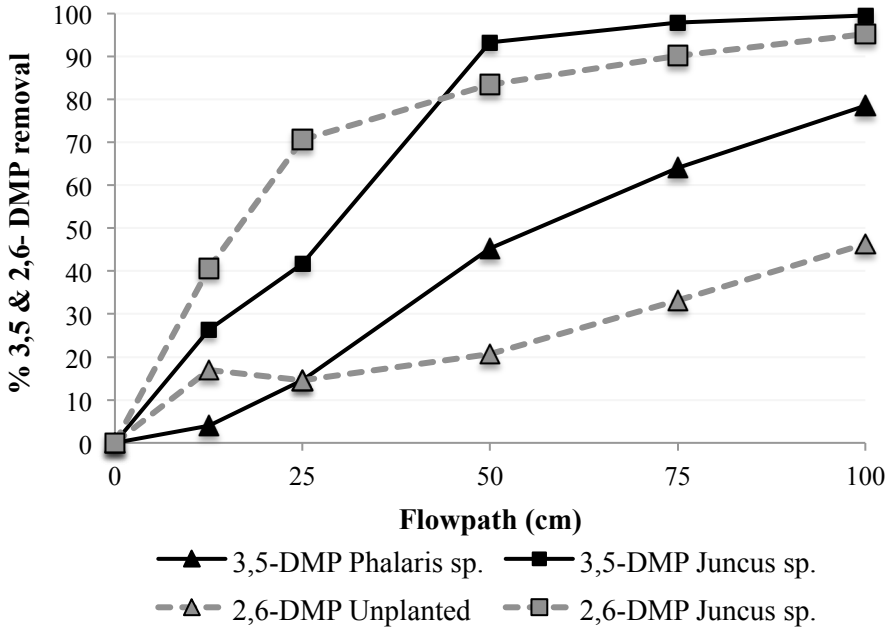
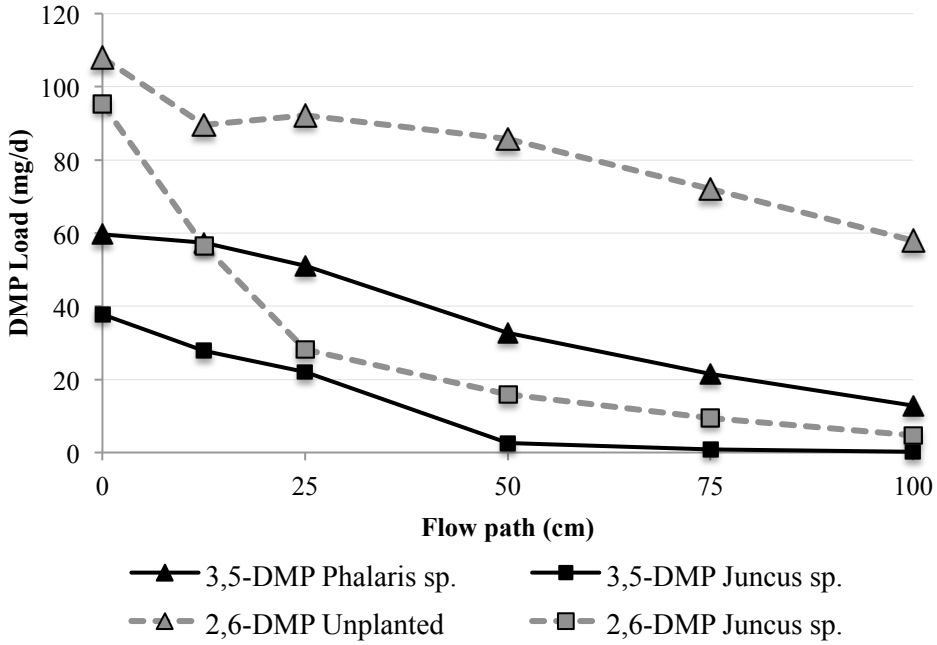



Figure 4-3 Percentage of DMPs removal along the wetland flow path for each treatment (n=8).

DMPs loads along the flow path for each treatment are shown in Figure 4-4. DMP loads behavior through all experimental weeks can be seen in Appendix 3. Both wetlands planted with *Juncus* sp. achieved a complete removal of the initial load of the DMP at 100 cm from the inlet. The wetland planted with *Phalaris* sp. failed to reach a complete removal of the DMP load. The unplanted system, although it was not as successful as the planted wetlands, it certainly had an important removal capacity. DMP loads at the outflow for each treatment showed to be significantly different ($F=19.14$, $p<0.05$). These results point out that the presence of plants had a positive impact by enhancing the removal capacity of the systems.

The presence of plants has been proved to improve the removal capacity of constructed wetlands (Faulwetter et al., 2009), for several reasons. Plants used for constructed wetlands, known as helophytes, hold a series of specific characteristics which allow them to survive in extreme

environmental conditions such as the presence of toxic pollutants in water (Stottmeister et al., 2003).




| | Standard deviations: DMP LOADS (mg/d) | | | | | |
|---|---|---|---|---|---|---|
| | inflow | 12,5 | 25 | 50 | 75 | outflow |
| 3,5-DMP *Phalaris* sp. | 5.30 | 8.06 | 7.46 | 13.96 | 14.66 | 11.95 |
| 3,5-DMP *Juncus* sp. | 20.85 | 22.41 | 16.61 | 6.18 | 1.89 | 0.56 |
| 2,6-DMP Unplanted | 19.38 | 31.71 | 28.29 | 25.02 | 27.26 | 29.87 |
| 2,6-DMP *Juncus* sp. | 26.07 | 34.65 | 39.20 | 27.54 | 17.88 | 8.74 |

Figure 4-4. DMP loads (mg/d) and standard deviations along the flow path for each experimental wetland (n=8).

At the same time, plants enhance the removal capacity of these pollutants by creating favorable conditions for the establishment of microorganisms, which have been prove to drive the main processes concerning pollutants removal inside constructed wetlands (Faulwetter et al., 2009; Truu et al., 2009). It is not completely clear what are the factors that determine microbial community dynamics inside constructed wetlands (Button et al., 2014), although several reasons are recurrently discussed in the literature. It is suggested that plants enhance microbial activity and density because their serve as an adhesion surface for microorganism. Additionally, plants can exudate carbohydrates and organics acids through their root system that can also be a beneficial source for microbial growth. Moreover, through the rhizosphere, helophytes can

release oxygen that can first create a redox gradient suitable for chemicals transformation and second, enhance microbial aerobic respiration (Gagnon et al., 2007; Poerschmann & Schultze-nobre, 2014; Stottmeister et al., 2003; Yagil et al., 2007).

It has been proposed that the support medium determine the microbial community structure in constructed wetlands. Is possible that the roots allow the development of a biofilm different from the one in the unplanted wetland that only offers gravel surfaces. In this sense, the roots could provide unique attachment conditions for specific microbial populations (Yagil et al., 2007). The root biofilm could be more capable of metabolizing the DMPs, thus showing higher removal efficiencies than gravel biofilms, and could explain the difference between planted and unplanted wetlands in the experiments.

Biofilm variations in roots between plant species can be detected, due to differences in growth rates, root morphology, types of root exudates and oxygen transfer capacities, suggesting that microbial communities are specific to the plant species (Button et al., 2016). Although this study showed differences between the removal efficiencies of *Phalaris* sp. and *Juncus* sp., this is most likely to be attributed to the deteriorated condition of *Phalaris* sp. by the end of the treatment, rather than a differentiation in both species root capacities as support medium for the bacterial communities.

### 4.2.2 Removal behavior of the different DMP isomers

The removal rates of 3,5-DMP and 2,6-DMP for CWs planted with *Juncus* sp. showed a complete removal of the DMPs in the outflow. Comparison of individual removal rates along the flow path demonstrate that both wetlands reached more than 90% removal at 50 cm from the inflow (Fig 4-5). Therefore, it can be said that the constructed wetland planted *Juncus* sp. was equally efficient on removing both DMPs isomers. These results also showed that it was able to remove them up to 100% independently on the initial load.

Isomer 3,5-DMP showed to be highly toxic for *Phalaris arundinacea*, as the plant completely died by the end of the treatment. The affectation on the plant health had an influence in the removal efficiency of this treatment, which only reached 70% of removal in the outflow. The

toxicity of this isomer also affected the plant activity in terms of evapotranspiration, as discussed previously, which started with high rates at the beginning of the treatment and finally decreased by the end of the treatment.

Previous studies concerning the same laboratory scale constructed wetlands (Gieseler, 2014; Rincón, 2015) used a mixture of 3,4; 3,5 and 2,6-DMP. Results showed different removal rates, being 3,4-DMP the first one to be removed, followed by 3,5-DMP and 2,6-DMP which had the lowest. Also, the analyzed outflow only detected 2,6-DMP. The incomplete removal of 2,6-DMP was attributed to the refractory behavior of this isomer (Janík et al., 2013; Li et al., 2010). Based on this assumption the position of the methyl groups around the hydroxyl group causes and steric impediment that could reduce the activation of the aromatic ring, a key step related of aerobic degradation. This study demonstrated that when fed isolated 3,5-DMP and 2,6-DMP can be completely removed. Is clear then, that in these cases the refractory behavior of 2,6-DMP isomer is not a factor that determines its removal. In this case, the acclimatization due to expose of 2,6-DMP led to an adaptation of the bacterial communities that allowed the complete removal of this isomer.

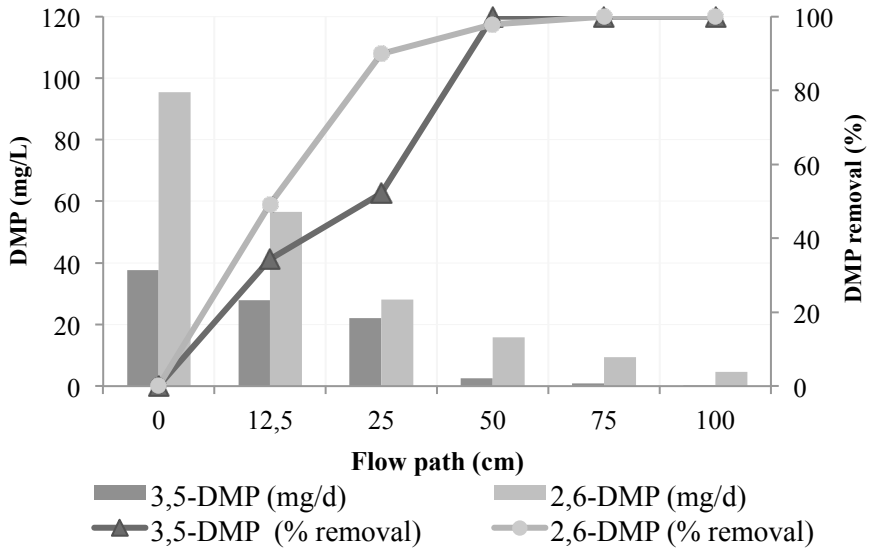



Figure 4-5. Removal efficiencies and DMP loads for wetlands planted with *Juncus effusus* (Inflow load for 3,5-DMP=37.7 mg/d & 2,6-DMP=95,4 mg/d; n=8).

Rather than chemical structure, the most likely factor influencing high DMP removal is the presence of plants in the wetland. As explained before plants can provide surface area and oxygen which turn the rhizosphere into a region suitable for microorganism attachment and growth, especially those with aerobic metabolism.

## 4.3 Physicochemical parameters effects on DMP removal

### 4.3.1 Redox potential

Plants used for wetlands by their root system are capable of release oxygen and exudates into the rhizosphere that allows them to survive in the anoxic conditions typical of waterlogged areas. Additionally, the oxygen released can first create a redox gradient suitable for chemical transformation and secondly be a source of oxygen for microbial respiration and metabolism (Stottmeister et al., 2003). The oxygen around the root creates an oxidized layer with a redox gradient of $E_h \approx +500$mv on the root surface to $E_h \approx -250$mv at 20mm from the root surface. The rhizosphere, then provides several ranges of redox potentials that enable multiple microbial processes (Faulwetter et al., 2009). The pollutants removal highly depends on the redox potentials in the wetland, where high redox potentials are associated with oxidized environments and facilitate aerobic processes, whereas low redox potentials are associated with reduced environments and facilitate anaerobic processes (Faulwetter et al., 2009).

In fact planted HSSF-CW can enhance both anaerobic and aerobic processes, being the latter mainly developed in the immediate proximity of the roots (Stottmeister et al., 2003, Faulwetter et al., 2009). A list of oxidation-reduction microbial reactions with their respective suitable range of redox potentials can be seen in Table 4-1.

Table 4-1. Microbial oxidation-reduction reactions and their respective redox potentials.

| Process | Electron acceptor | End product | Redox potential (mV) |
|---|---|---|---|
| Aerobic respiration | $O_2$ | $H_2O$ | 300 to 700 |
| Nitrate reduction | $NO_3$ | $N_2$, $NO_x$ | 100 to 350 |
| Manganese reduction | $Mn^{4-}$ | $Mn^{2-}$ | -100 to 300 |
| Iron reduction | $Fe^{3-}$ | $Fe^{2+}$ | -100 to 200 |
| Sulfate reduction | $SO_4^2$ | $S^2$ | -200 to -100 |
| Methanogenesis | $CO_2$ | $CH_4$ , $CO_2$ | -350 to -100 |

Adapted from: Faulwetter *et al.* 2009

Redox potentials measurements for all the treatments ranged between -50 mV and 400 mV. Based on these results, it can be said that aerobic degradation could be the main reason of pollution removal inside the wetlands, since their redox potentials match the ones showed in Table 4-1 that are suitable for aerobic respiration. Also, the redox potentials changed every week along the flow path, suggesting that time is an important factor to establish steady redox potentials, especially for the planted wetlands (Fig.4-6).

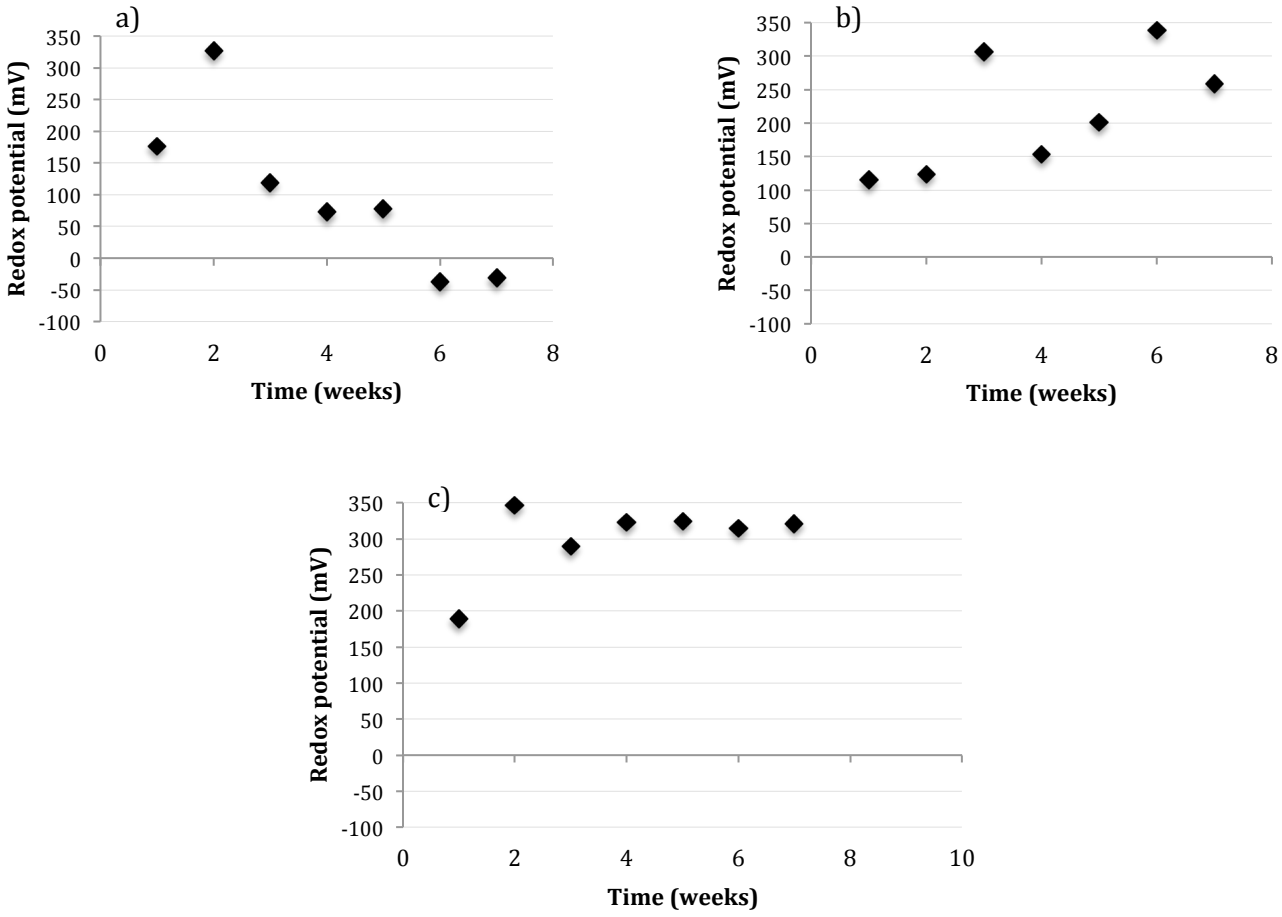



Figure 4-6. Planted wetlands redox potentials and DMP concentrations over experimental weeks at 12,5 cm for (a) 3,5-DMP *Phalaris* sp., (b) 3,5-DMP *Juncus* sp., (c) 2,6-DMP *Juncus* sp.(n=8).

During the first stage, the wetland planted with *Juncus* sp. and fed with 3,5-DMP show at 12,5 cm a progressive increase of the redox potential with every week (Fig 4-6b), and it maintained the same levels during the second stage of the experiment (Fig 4-6c). This suggests that as weeks progress the redox potentials stabilize.

Redox potential behavior through experimental weeks can be seen in Appendix 4. Some studies indicate that redox potentials in HSSF-CW usually increases from the inlet to the outlet due to progressive pollution degradation (García et al., 2003), which could explain the behavior of treatment planted with *Juncus* sp. fed with 3,5-DMP (Appendix 4.2). However, for the other three treatments the redox potentials did not change at every sampling point (Appendix 4.1, 4.3. and 4.4). Instead, within the same week, redox measurements were almost the same along the whole length of the constructed wetland independently of the sampling point. Results suggest that the factor time is the one explaining the variations in redox potentials.

Treatment planted with *Phalaris* sp. behaved in a special manner (Fig. 6-4a, Appendix 4.1). At the beginning of the first stage the plant growth nicely and strong (observation), probably with high oxygen release which explains the high redox potentials at the beginning of the treatment. As explained before at the end of the first stage the plant died. This event could explain the decrease in redox potentials in the last two weeks of sampling, probably because the plant could not transport oxygen to the rhizosphere. This strongly supports the assumption that oxygen input in the wetlands comes mainly from the plants, which is also supported by the literature on HSSF-CW (Stottmeister et al., 2003). Results also suggest that the plant performance and health have an important influence on redox potentials.

### 4.3.2 Dissolved oxygen

Regarding the measurements of dissolved oxygen inside the constructed wetlands, all the treatments showed different oxygen concentration ($F=16,73$, $p<0.05$, Fig 4-7). The low concentrations of oxygen detected indicate that the systems showed microaerophilic conditions, which are perfectly suitable for bacterial activity. Figure 4-7 shows considerable variations in the dissolved oxygen measured for both treatments with *Juncus* sp. The exact source of these

variations can be seen in Figure 4-8, which exhibit an increase in the dissolved oxygen in the sampling points closest to the outlet.

The increase in the dissolved oxygen concentration at the end of the flow path for both wetlands planted with *Juncus* sp. (Fig. 4-8) can be related with the decrease in the microbial activity and the low availability of carbon sources in these sampling points. Button et al. (2015), evaluated the microbial activity in several HSSF-CW, and found that it dramatically decreases along the flow path. Using wetlands of 100 cm length, they observed that the biggest decrease occurs between 25 and 50 cm along the flow path. Nguyen (2000) also reported in a planted gravel-bed wetland, that microbial respiration rate significantly decreased with the distance from the inlet. Assuming that microbial activity decreases near to the flow path, the increase of detected oxygen in the last sampling points can be owe to the fact that the oxygen available in the filter medium is not being used for microbial respiration, explaining the variations in our planted systems.

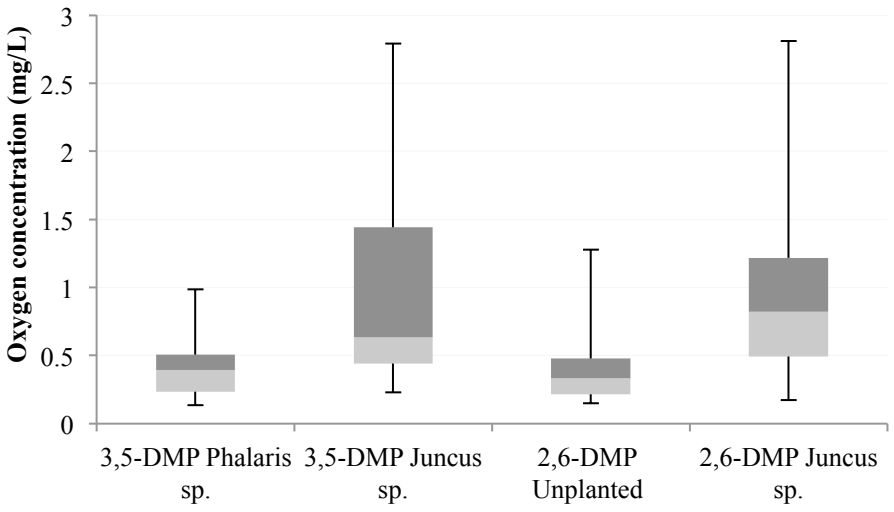



Figure 4-7. Comparison of oxygen concentration of all sampling points for each treatment (n=8).

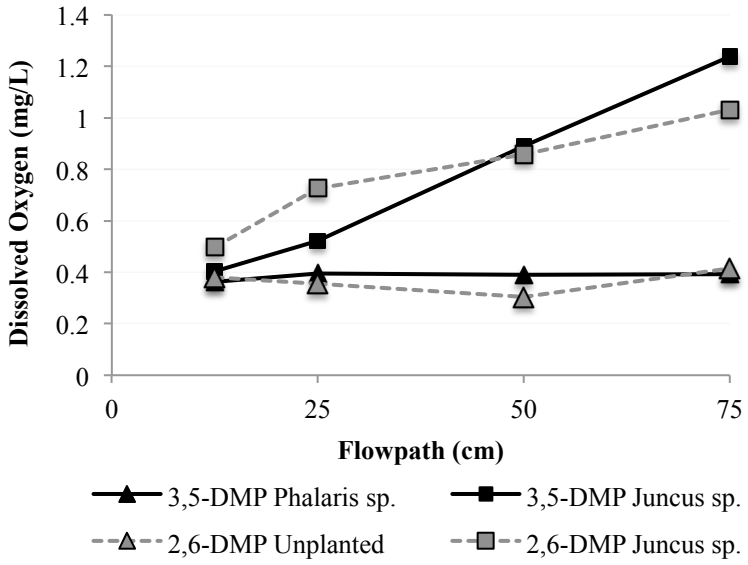



Figure 4-8. Dissolved oxygen concentration along the flow path for each experimental wetland (n=8).

### 4.3.3 Hydraulic retention time (HRT)

All the treatments operated with the same continuous flow regime, but the hydraulic retention time differed between them. Plants increased the retention time of the planted wetlands (Fig. 4-9) which can be seen as another benefit related with the presence of plants in constructed wetlands.

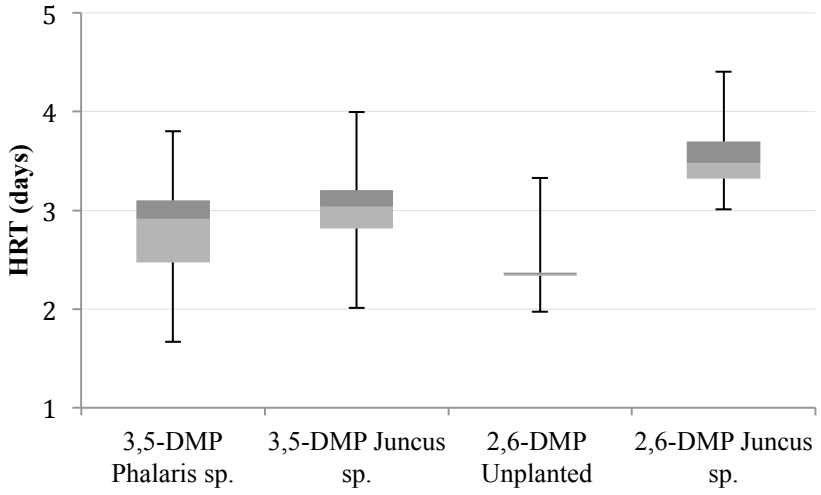



Figure 4-9. Comparison of hydraulic retention time for each treatment (n ≈22).

Vegetation distributes and reduces the velocity of the water coming from the inlet (Vymazal, 2008), and together with the gravel bed and its root system can collaborate to increase the HRT. A higher hydraulic can create higher redox potentials and increase contact time of contaminated water with biofilms in the roots, thereby enhancing the removal of pollutants (Faulwetter et al., 2009).

### 4.4 Nitrogen transformation and removal

Nitrogen species transformations and ammonium removal along the flow path for each treatment are illustrated in Figures 4-10 to 4-13. Ammonium removal was more efficient in the planted wetlands, with percentages of 98% and 99% in the two treatments planted with *Juncus* sp., followed by the planted wetland with *Phalaris* sp. with 61%. The unplanted wetland was the less efficient, with a removal of around 9%. The most obvious changes are the ones concerning ammonium concentration with less important accumulations of nitrite and nitrate. The changes in the ammonium concentration raises the question about whether this change is attributed to volatilization, adsorption, plant nitrogen uptake or microbial nitrogen transformation, to name some mechanisms.

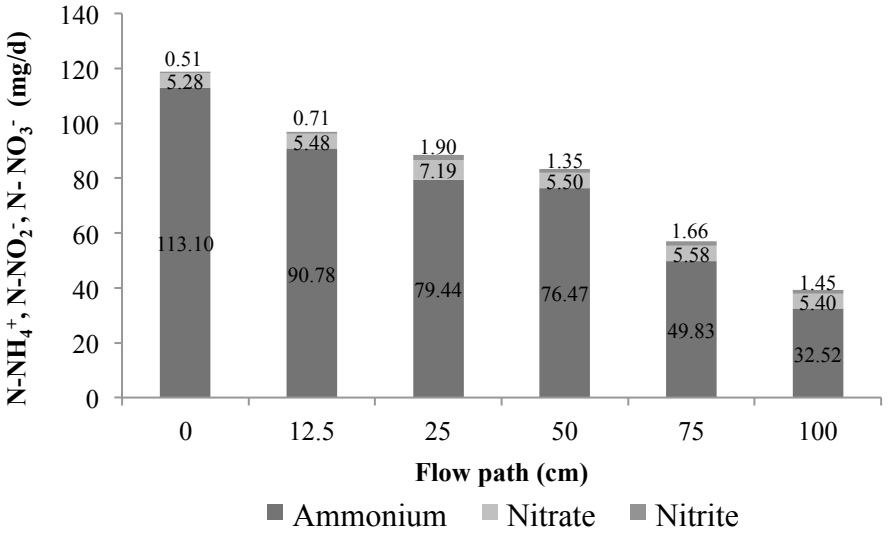



Figure 4-10. Nitrogen species loads along the flow path for the constructed wetland planted with *Phalaris* sp., fed with 3,5-DMP (n=8).

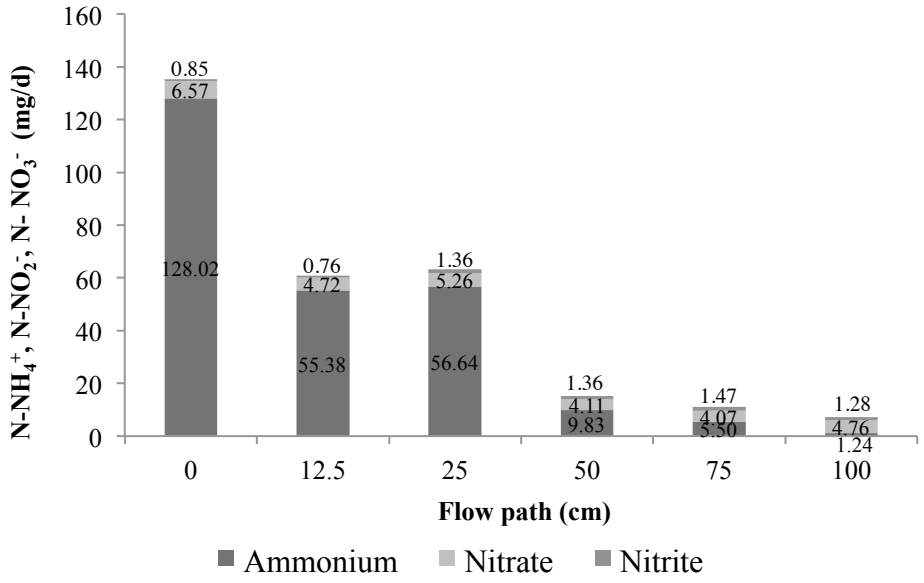



Figure 4-11. Nitrogen species loads along the flow path for the constructed wetland planted with *Juncus* sp., fed with 3,5-DMP (n=8).

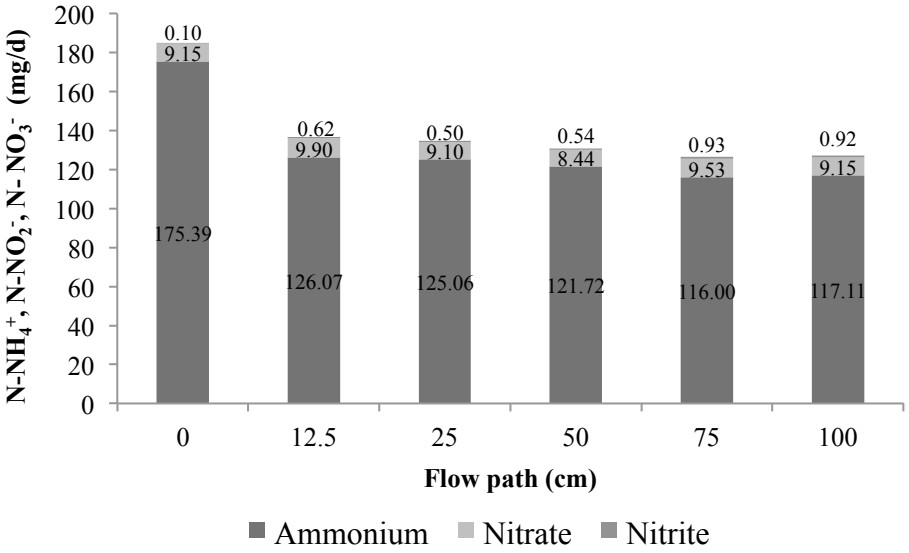



Figure 4-12. Nitrogen species loads along the flow path for the unplanted constructed wetland, fed with 2,6-DMP (n=8).

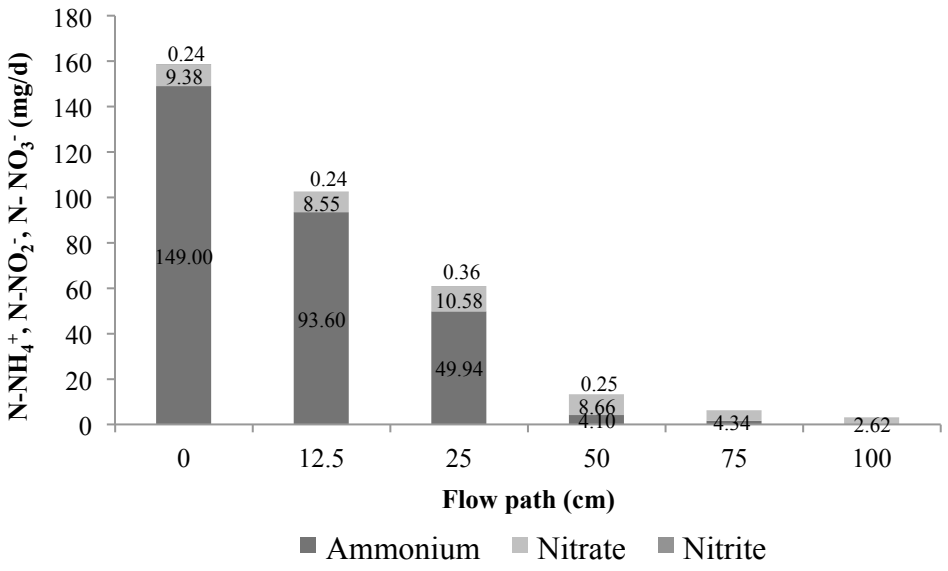



Figure 4-13. Nitrogen species loads along the flow path for the constructed wetland planted with *Juncus* sp.*,* fed with 2,6-DMP (n=8).

Vymazal & Kropfelova (2008) suggest that volatilization and adsorption play a minor role in nitrogen removal in HSSF-CW. Since there is no free water surface in this particular arrangement the volatilization is limited. In the same way, higher elimination rates due to adsorption are generally related to fine grained soils, however this soil is not particularly usefull for HSSF-CW because it offers poor hydraulic conditions. Instead, coarse soil grains are used, which do not exhibit great adsorption capacities.

Among the literature different authors emphasize the crucial role of plant uptake in nitrogen removal and refer it in some cases as the main pathway for nitrogen removal, followed by nitrification and denitrification (Scholz & Hedmark, 2010). It has been reported that the amount of plant uptake and accumulation is specific to macrophyte species (Vymazal & Kropfelova, 2008) with up to 46% decrease of nitrogen load by plant uptake (Langergraber, 2005). On the other hand, other authors confirm that plant uptake and incorporation into plant biomass is of minor importance (Chang et al., 2014; Ji et al., 2012; Stottmeister et al., 2003; Vymazal & Kropfelova, 2008). Instead, these authors propose that biological nitrification-denitrification transformation is the most important nitrogen removal mechanism, reporting percentages between 60 and 96% (Ji et al., 2012; Wang et al., 2016).

In our experiments the presence of oxidized species of nitrogen in the inflow showed an oxidation of ammonium before entering the system. The detection of these species in the inflow was not expected since nitrite nor nitrate were added to the artificial wastewater. Nevertheless, the fluctuation of nitrate values above the ones detected in the inflow provided evidence to support that nitrification processes were occurring inside the experimental wetlands. Studies report that in HSSF-CW oxidized nitrogen species are immediately reduced, in the denitrification step, thereby preventing the enrichment of nitrate or nitrite (Stottmeister et al., 2003) which could explain the low concentrations of nitrite and nitrate detected along the flow path for all treatments.

It has been reported that plant uptake rate is higher during exponential growth compared with the phase of steady growth, and because of this measurements taken in short term experiments can lead to mislead estimations of plant uptake (Vymazal & Kropfelova, 2008). Accordingly, Wiessner et al. (2013) reported a decrease in nitrogen plant uptake during plant growth stoppage, but despite this fact ammonium removal continued in the rhizosphere. In our experiments the constructed wetland planted with *Juncus* sp. was clearly in a growth stoppage phase during stage 1 and 2 of the experiments. Most of the stems were already growth by the time the measurements began with no important growth noticed during the experiment, and accordingly to this last study, in our planted wetlands ammonium removal also continued, despite the fact that plants were not in their exponential growth phase.

This strongly suggest that plant uptake was not the cause of nitrogen removal in the planted wetlands, and provide evidence to assure that microbial nitrogen transformation is the main mechanism of nitrogen removal in our experimental wetlands. Therefore, plant biomass and specifically roots, improved the nitrogen removal efficiency due to the benefits they provide to microorganisms communities. Similar results have been reported in Coban et al. (2015), they propose that microbial activity and richness is enhance in the areas with high root density owing to the oxygen provided through the plant rhizosphere.

While plants provide the oxygen required for nitrification steps around the rhizosphere, away from the rhizosphere, anaerobic zones in the filter medium provide suitable conditions for denitrification. These processes accurately exemplify the mosaic behavior of redox potentials

inside CWs (Coban et al., 2015) that facilitates an aerobic process such nitrification to occur at the same time as an anaerobic process like denitrification.

### 4.5 Carbon sources behavior: dissolved organic carbon and dimethylphenols

Design of the experiment took into account the behavior of dissolved organic carbon (DOC) measurements in order to compare them with the concentration of DMP. A transformation of the DMP values was calculated. Based on the fact that one mol of DMP contains 78, 7% of carbon the values of DMP were multiplied by a factor of 0.787, in order to obtain the contribution of DMP to the DOC values, afterwards referred as DMP-C. Results are shown in figure 4-14.

DOC and DMPs concentrations are quite similar since the experimental wetlands had the DMPs as the only input of carbon, nevertheless detected DOC values were expected to be slightly higher than DMP concentration due to rhizodeposition. Rhizodeposition is defined as the input of carbon from the plants into the rhizosphere (Stottmeister et al., 2003). These rhizodeposition products can be exudates, mucigels, dead cell material, organic acids, sugars and vitamins, although they differ from one specie to another (Braeckevelt, Kaestner, & Kuschk, 2011; Stottmeister et al., 2003). Exudates such as sugars and aminoacids can be used by microorganisms as substrates thereby augmenting the microbial biomass in the rhizosphere and enhancing the microbial degradation of xenobiotics, such as DMPs.

Besides, rhizodeposition products have shown to improve denitrification, because exudates can be an additional source of electron donors (Bialowiec et al., 2012; Chen et al., 2012; Ge et al., 2015). For instance, Lin et al. (2002) reported significantly higher nitrogen removal in planted microcosm compared with unplanted microcosm, which was attributed to the organic carbon supplied by roots. Comparable results are reported in this investigation (Section 4-4). In addition to rhizodeposition, intermediaries of DMP degradation pathways, such as citraconic acid and succinate (Ewers et al., 1989) could also promote the higher values of DOC reported in the experiments.

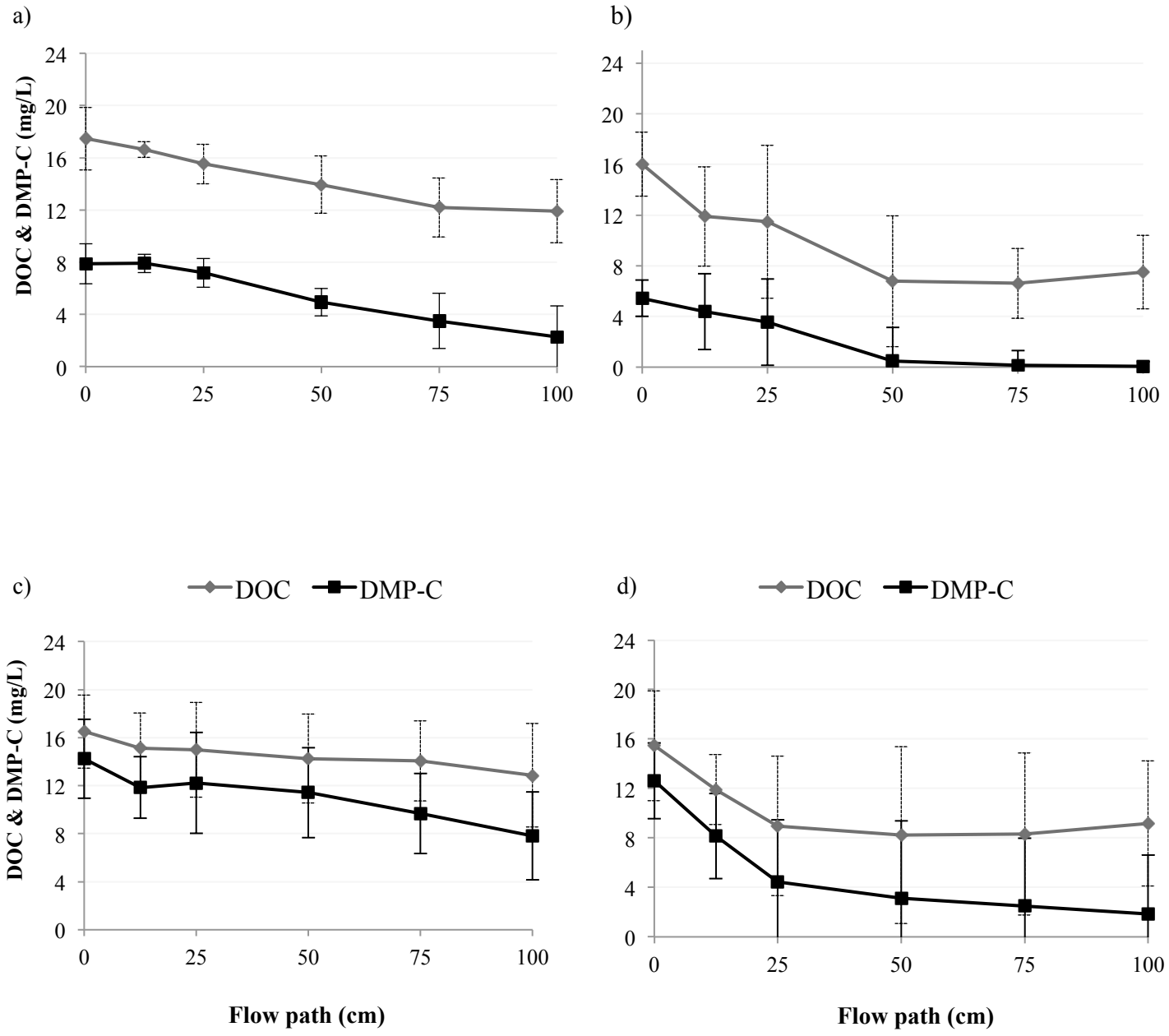



Figure 4-14. Mean concentrations of DOC and DMP-C (mg/L) along the flow path (n=8) for (a) CW planted with *Phalaris* sp, fed with 3,5-DMP, (b) CW planted with *Juncus* sp., fed with 3,5-DMP, (c) Unplanted CW, fed with 2,6-DMP and (d) CW planted with *Juncus* sp., fed with 2,6-DMP.

## 4.6 Catabolic genes involved in DMP degradation

Figures 4-15 and 4-16, show the results for phenol hydroxylase (PHE) abundance in relation with the total bacterial community. An important aspect to return on is the fact that these results came from the same wetland in different stages, first fed with 3,4-DMP followed by 3,5-DMP and 2,6-DMP. The abundance of this gene was highly dynamic in the bacterial population throughout time and the results strongly suggest that the main factor causing these changes was the isomer inside the wetland.

Acclimatization of bacterial due to phenol exposure is well documented in literature (Chandana Lakshmi & Sridevi, 2009; Tomei & Annesini, 2008). Normally in an environmental sample, the percentage of the total population capable of degrading phenol is really low, nevertheless due to phenol exposure, this percentage can increase. Certainly, this phenomenon influenced the bacterial communities in our constructed wetlands. During the adaptation time, in which no phenol was added to the artificial wastewater, the abundance of PHE is bellow 2% and increases drastically after feeding it with 3,4-DMP isomer (Fig. 4-15 & 4-16).

Figures 4-17 to 4-19 show the results for PHE copies in every treatment. Constructed wetlands planted with *Juncus* sp. and *Phalaris* sp. fed with somer 3,4-DMP showed the highest percentages of PHE presence in the bacterial community, with up to 20% of the total bacterial community holding that gene. With the beginning of isomer 3,5-DMP a progressive diminishing in PHE copies was detected for both planted wetlands. By the time wetlands were fed with 2,6-DMP the total PHE copies were bellow 2%. The unplanted wetland showed the lowest percentages, with less than 1% of the population holding that gene (Fig.4-19B). Interestingly, a correlation between the abundance of PHE along the flow path and the removal of 3,4-DMP can be noticed (Fig 4-17). For instance, 25 cm and 50 cm from the inflow show a high percentage of PHE that can also be related with the high DMP removal rate at these specific points.

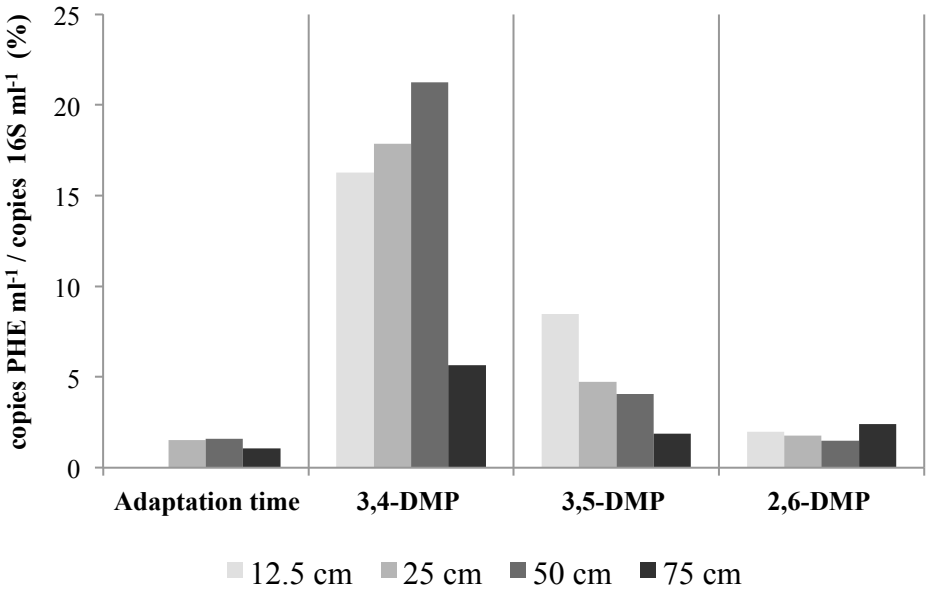



Figure 4-15. Phenol hydroxylase abundance for constructed wetlands planted with *Juncus sp.* at each sampling point.

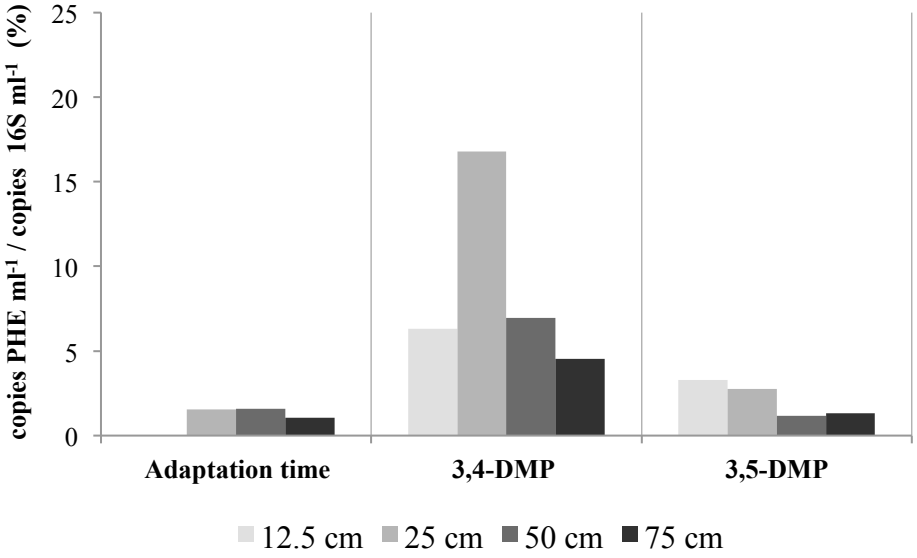
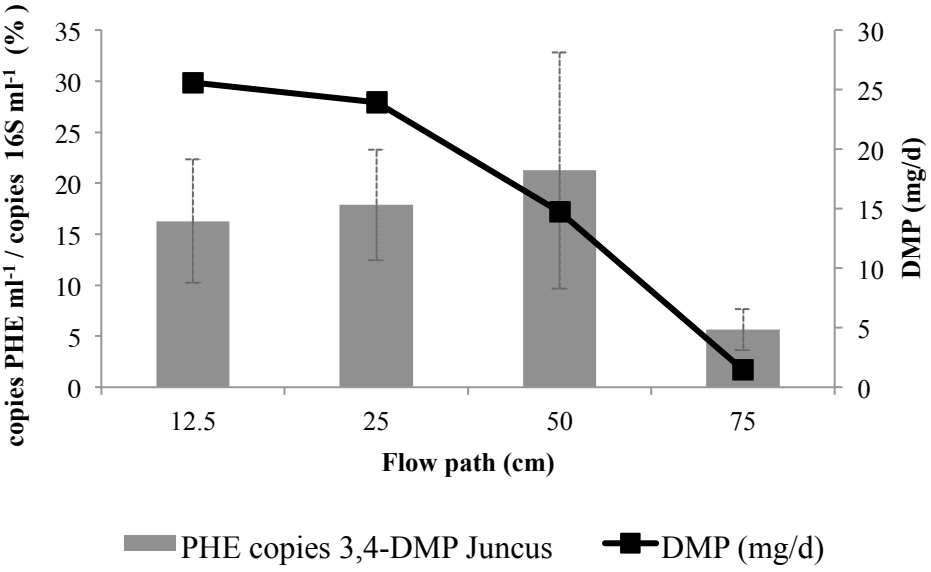
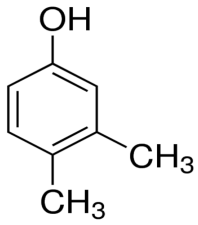



Figure 4-16. . Phenol hydroxylase abundance for constructed wetlands planted with *Phalaris sp.* at each sampling point.

A)




3,4-DMP

B)

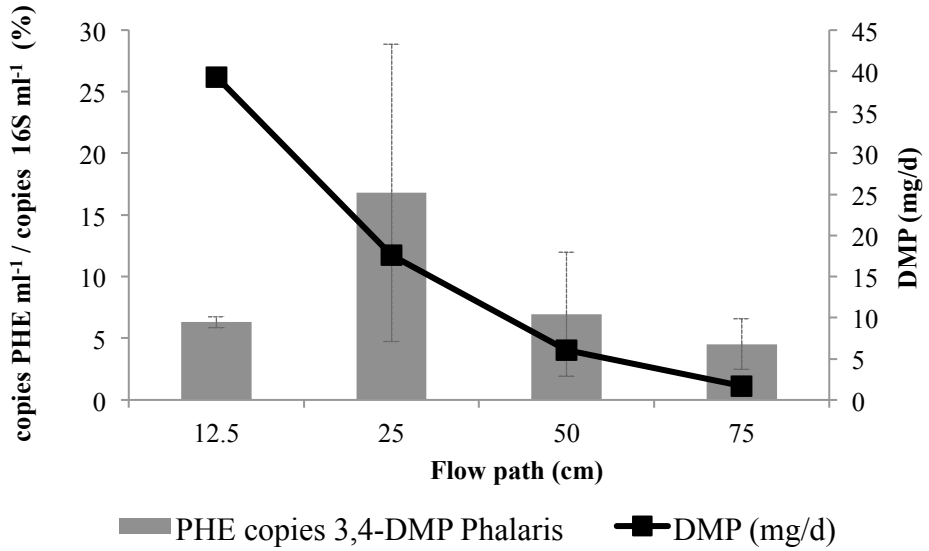
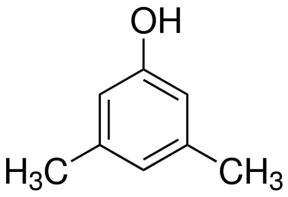
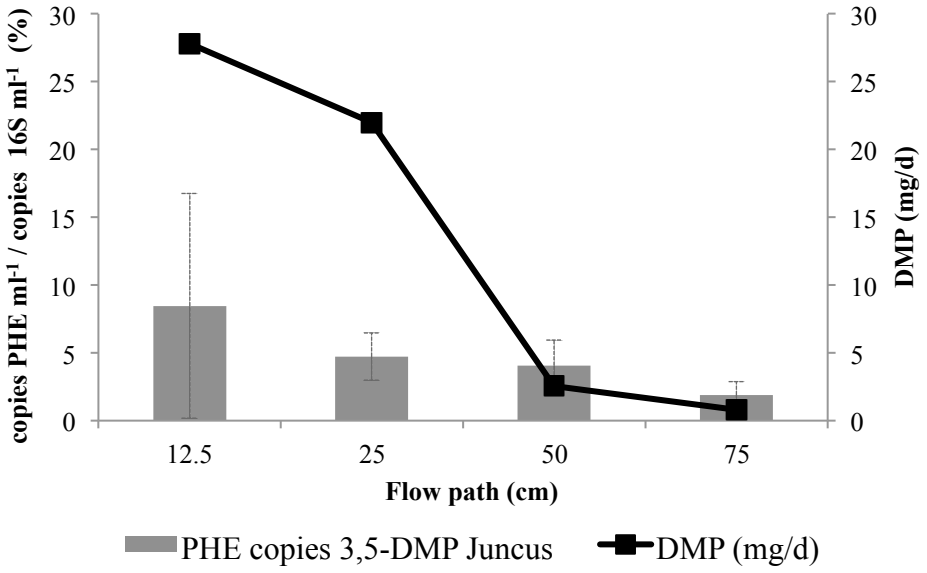



Figure 4-17. Relation between PHE copies and DMP load for treatments fed with 3,4-DMP. A) CW planted with *Juncus* sp., B) CW wetland planted with *Phalaris* sp.

A)




3,5-DMP




B)

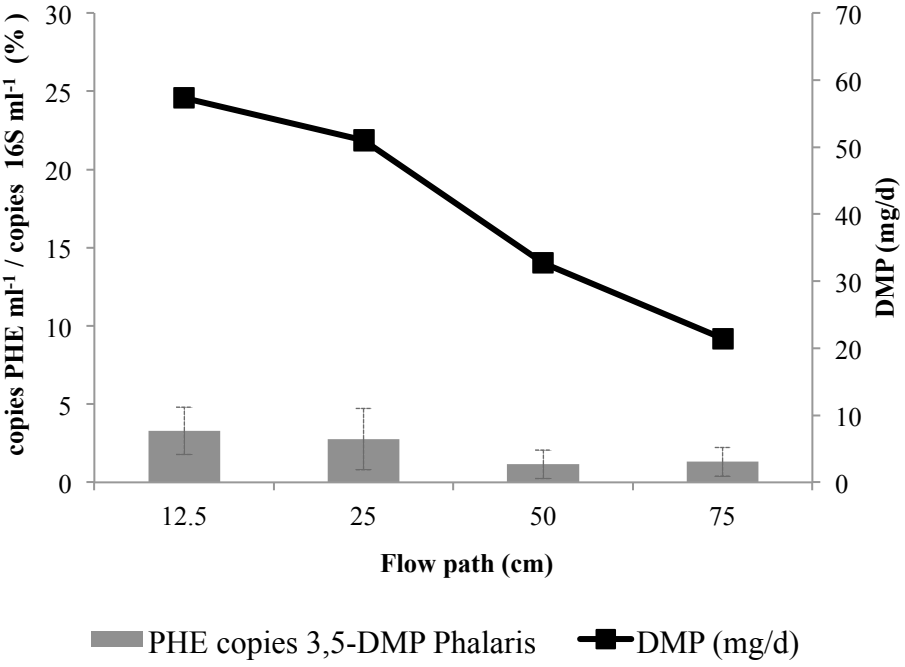
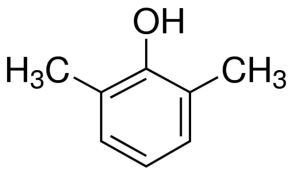
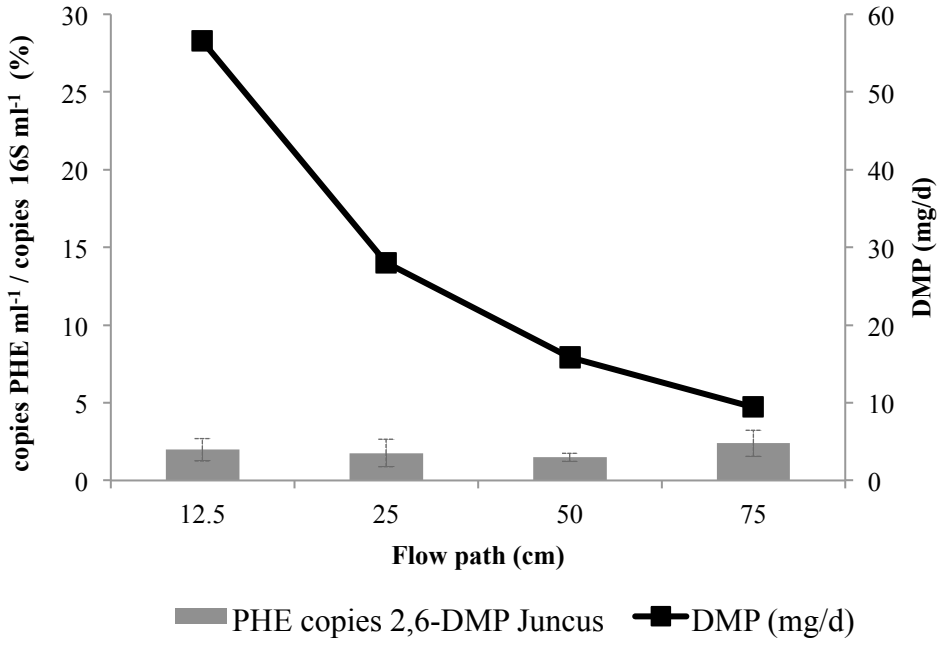



Figure 4-18. Relation between PHE copies and DMP load for treatments fed with 3,5-DMP. A) CW planted with *Juncus* sp, B) CW wetland planted with *Phalaris* sp.

A)




2,6-DMP




B)

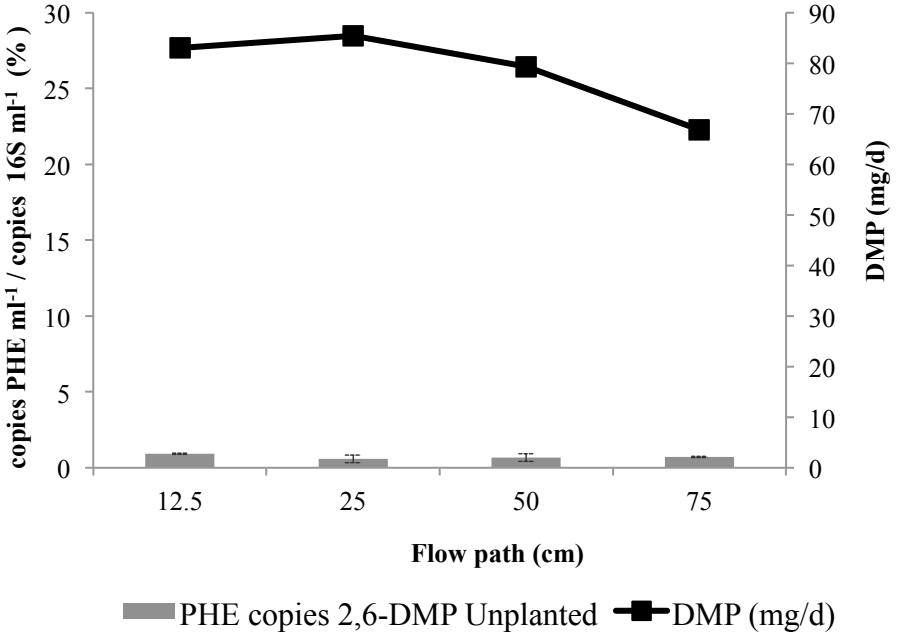



Figure 4-19. Relation between PHE copies and DMP load for treatments fed with 2,6-DMP. A) CW planted with *Juncus* sp, B) Unplanted wetland.

Constructed wetlands fed with isomer 3,4-DMP presented a drastic change in the abundance of PHE, compared with the adaptation time. This certainly shows an adaptation of the community directly influenced by the exposure to phenol. Top & Springael (2003) propose some mechanisms that occur when bacterial communities are exposed to xenobiotics, that allow them to adapt to this perturbance. They propose that usually there is an increase in the population of those members of the community better adapted to the presence of the compound. Also, is possible that during this process, some organisms can acquire genetic information decoding for xenobic removal through horizontal gene transfer (HGT). In fact, proteins and enzymes involved in aromatic degradation pathways are not linked taxonomically with the bacteria carrying them, indicating that these catabolic enzymes are implicated in gene transfer dynamics (Vilchez-Vargas, Junca, & Pieper, 2010). Eventually, independently of the mechanism of adaptation, those organisms capable of degrading or tolerate the compound will conform a bigger fraction of total the population compared to the moment before the presence of the xenobiotic. Turning now to the experimental results it can be proposed that the presence of PHE was a beneficial trait during the 3,4-DMP treatment which led eventually to a major population size of those carrying that gene, suggesting that PHE is a key enzyme in the degradation pathway of 3,4-DMP.

Our results showed for isomers 3,5- and 2,6- that there is no relationship between the total abundance of PHE and the removal efficiency of the bacterial communities (Fig. 4-18 & 4-19A). The presence of this gene seems to be of minor importance in the removal of these isomers, since both were removed up to 95% even without the presence of this gene. The lack of relationship between number of genes of PHE and phenol degradation rates has been reported in literature (Basile & Erijman, 2008).

Same study although, did find a relationship between PHE diversity (LmPH subunit of phenol hydroxylase gene) and the rate of phenol removal. In this case, the increase diversity in the subunit was related to an increase in the phenol degradation rates. Additionally, this study also reported low copies of PHE genes in relation with the total bacterial community (less that 10%), similar to our results, which also showed percentages between 2% and 8%. Apparently, the low detected number of copies does not imply poor removal of phenol, because in both cases the removal was quite efficient. It cannot be excluded then that PHE diversity is the factor causing

the high rates of 3,5 and 2,6-DMP removal in our experiments, despite the low number of copies detected within the bacterial community.

Since many of the functional genes codifying for catabolic genes are located in mobile genetic elements they can be susceptible to mutation, thus creating high variations among the enzymes (Junca & Pieper, 2004). A change of a single aminoacid in the sequence can drastically change the specificity of the enzyme, thus making the analysis of sequence diversity an analysis that should be performed hand by hand with the analysis of presence and abundance of functional genes (Vilchez-Vargas et al., 2010). Further research on PHE diversity due to changes in dimethylphenols exposure could complement the results on catabolic potential of the bacterial community within the constructed wetland and perhaps clear the fact whether the degradation of 3,5- and 2,6-DMP appertain to gene diversity rather than abundance.

A valid argument concerning the low number of copies detected in treatments with isomer 3,5 and 2,6-DMP could be that the primers used did not target all the genes involved in DMPs degradation, and results showed only the bacteria strains using a pathway that involves PHE. Thus, the low copy numbers detected for these two isomers suggest that PHE could not be a key enzyme in the initial degradation of these two isomers. Instead other enzymes not targeted in this study could be involved in the degradation.

Several DMPs suggested pathways can be found in the literature, and most of them use strains of *Pseudomonas* bacteria to describe the pathway. For instance, 3,4-DMP reported pathway by *Pseudomonas putida* begin with the hydroxylation of the aromatic ring followed by a meta cleavage of the catechol, in this case both methyl substituents remain intact (Chapman, 1972). Accordingly, Ribbons (1970) found 3,4-DMP to be substrate and inducer of the activity of PHE in *Pseudomonas aeruginosa*. Interestingly, same study reported 3,5-DMP and 2,6-DMP as inhibitors of the induction of the same enzyme.

Contrary to 3,4-DMP pathway, 3,5-DMP reported pathway in *Pseudomonas putida* is initiated by a methylhydroxylase, by adding an hydroxyl group on one of the meta methyl groups (Fig. 4-20). The follow steps include the formation of citraconic acid, pyruvate and acetyl CoA (Chapman, 1972; Hopper, Chapman, & Dagley, 1971). Isomer 2,6-DMP pathway by *Mycobacterium* sp. reported an unspecific hydroxylation that formed 2,6-dimethylhidroquinone

(Fig. 4-21), probably followed by the formation of citraconic acid, propionic acid or pyruvic acid (Ewers et al., 1989). That is, neither 3,5- or 2,6-DMP suggested pathways on the literature report the evidence of a phenol hydroxylase in the initiation of the isomer aerobic degradation.

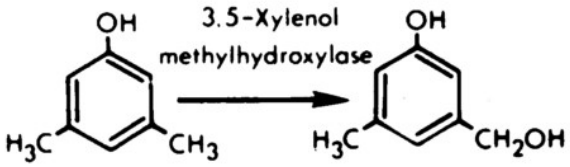



Figure 4-20. Initiation of 3,5-DMP degradation pathway reported by *Pseudomonas putida.* Taken from Hopper (1971).

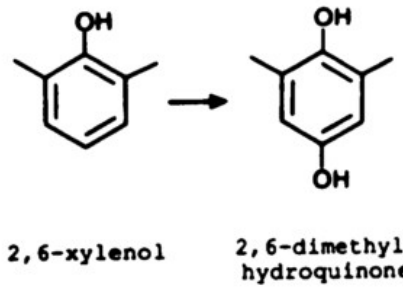



Figure 4-21. Initiation of 2,6-DMP degradation pathway reported by *Mycobacterium* sp. Taken from Ewers et al. (1989).

This deeper examination of the suggested degradation pathways on the literature show that for isomer 3,5- and 2,6-DMP the activity of PHE has not been reported of high importance. Although this information could be an appeal explanation for the low copy number of PHE detected for these isomers, this kind of generalization must be attended carefully, especially when treating with environmental samples. Microbial diversity in an environmental sample considerably exceeds the diversity of organisms isolated by cultivation methods, and it is known that catabolic genes coming from isolated species are not identical to those coming from species developing in contaminated environments (Vilchez-Vargas et al., 2010; Watanabe, Teramoto, &

Futamata, 1998). Thus, the degradation pathway of a single species is not neccessarily the same for all the members of the bacterial community.

Furthermore, is has to be taking into account that knowledge regarding aromatic compound degradation comes mainly from isolated strains, that cover only a small proportion of a more broad bacterial diversity. Thus, a great potential of catabolic pathways may still be uncover due to the limitations of the cultivation methods (Pérez-Pantoja et al., 2009).

In this study, the number of phenol hydroxylase gene is somehow replacing the measurement of phenol hydroxylase enzyme. Therefore, is important to mention that functional gene abundance does not provide information on the activity of the microorganism carrying that gene nor the expression of it. In such cases mRNA analysis must be performed (Basile & Erijman, 2008; Vilchez-Vargas et al., 2010). Nevertheless, determining these functional genes quantitative variations can provide information to develop ecological theories on microbial communities (Basile & Erijman, 2008). The results obtained also prove the presence of organisms that are harboring genes and gaining a selective advantage for their own catabolic genes. Thus, insights of this kind expand the knowledge on community interactions and functions which can be of further application in the bioremediation field (Vilchez-Vargas et al., 2010).

PHE as the enzyme in charge of the hydroxylation of the aromatic ring and activate the ring for the next steps, is considered is a rate-limiting first step and therefore a key component in the aerobic pathways for the degradation of aromatic compounds. Owing to molecular technics such as qPCR is possible to evaluate and characterize this enzyme in terms of substrate specificity, behavior and variability in semi-controled natural conditions such as constructed wetlands proving further insights on the genetic diversity of these catabolic communities (Shingler et al., 1989).

# 5 Conclusions

Overall, the presence of plants showed to improve the performance of the planted treatments, which show higher DMPs and ammonium removal compared to the unplanted wetland. *Juncus effusus* proved to be a more tolerant specie compared with *Phalaris arundinaceae* as this last one died after the treatment with 3,5-DMP.

It can be concluded that the reason of the better performance of planted wetlands is manifold. First, helophytes release oxygen through its rhizosphere creating a redox gradient around the roots which is suitable for microbial aerobic processes. At the same time, roots provide attachment areas for the growth of communities more capable of degrading DMP, which can be deduced from the comparison with the unplanted wetland. Additionally, roots exudates can enhance the pollutants removal by providing an extra organic source for the growth of microorganism. Moreover, roots can increase the hydraulic retention time, and the contact time of contaminated water with the biofilms, thereby enhancing the pollutants removal.

Planted wetlands showed a better ammonium removal compared to the unplanted wetlands. Results gave several reasons to conclude that the high removal rates were not caused by plant uptake, because the experiments were developed during a clear growth stoppage period. Instead, plants enhanced the ammonium removal by optimizing the mosaic nature of constructed wetlands that allowed a complete biological nitrogen transformation in the filter medium. First, roots provide oxygen for nitrification. Afterwards, within the anoxic areas, denitrification occurs and rapidly transforms the nitrogen-oxidized species. In our experiments is clear that plants potentiate this mosaic behavior, since the unplanted wetland had a really poor ammonium removal. Plants through their root exudates could also enhance denitrification by providing an extra electron donor in denitrification, as it has been proposed in the literature.

The evaluation of the relative abundance of PHE showed that its abundance is clearly affected by the isomer feeding the wetland. The percentage of the total community carrying the PHE gen was higher during the stage fed with 3,4-DMP, suggesting that this enzyme plays a key role in the aerobic degradation of this isomer. On the other hand, stages fed with 3,5 and 2,6-DMP reported low percentages of PHE, suggesting that 1) despite the low copy numbers of PHE, this

is still a key enzyme in the degradation pathway of these two isomer and its importance could be explained with the diversity of PHE, rather than its relative abundance. In this case further analysis addressing the diversity of this enzyme should be performed, or 2) the primers used did not target all the genes involved in the first steps in DMPs degradation, and results showed only the bacteria strains using a pathway that involves PHE. This means that other enzymes apart from PHE, are involved in the initial steps of the degradation of 3,5 and 2,6-DMP.

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# 7 Appendix

**Appendix 1.** Composition of trace mineral solution (TMS)

| Compound | Concentration (g/L) |
|---|---|
| Disodium salt (Titriplex III) | 1.0 |
| $FeSO_4$ . 7 $H_2O$ | 1.0 |
| $MnCl_2$ . 2 $H_2O$ | 0.8 |
| $CoCl_2$ . 6 $H_2O$ | 1.7 |
| $CaCl_2$. 2 $H_2O$ | 0.7 |
| $ZnCl_2$ | 1.0 |
| $CuCl_2$ . 2 $H_2O$ | 1.5 |
| $NiCl_2$ . 6 $H_2O$ | 0.3 |
| $H_3BO_3$ | 0.1 |
| $Na_2MoO_4$ . 2 $H_2O$ | 0.1 |
| $Na_2SeO_3$ . 5 $H_2O$ | 0.02 |
| HCl (concentrated solution) | 3 (mL/L) |

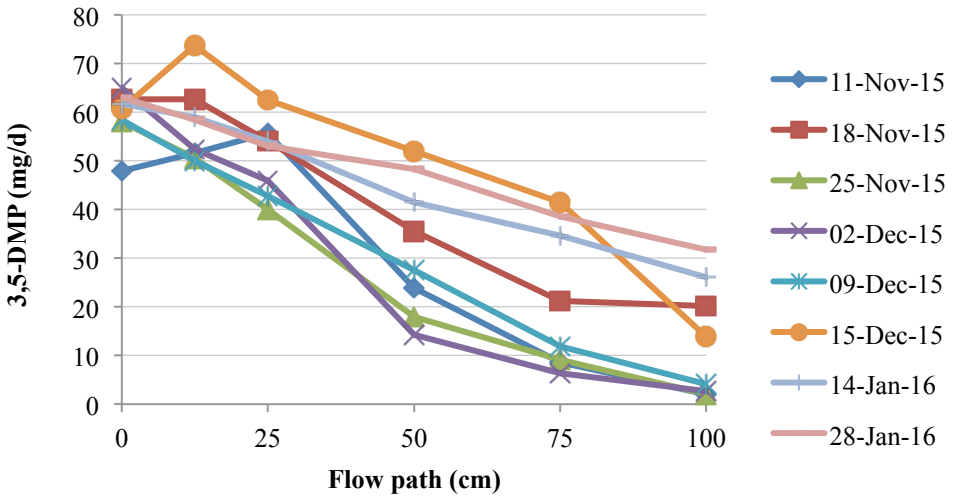
**Appendix 2.** Dilution series for qPCR standards

| | |
|---|---|
| $10^8$ | (diluted) PCR product |
| $10^7$ | 2µl of $10^8$ + 18µl $ddH_2O$ |
| $10^6$ | 2µl of $10^7$ + 18µl $ddH_2O$ |
| $10^5$ | 2µl of $10^6$ + 18µl $ddH_2O$ |
| $10^4$ | 2µl of $10^5$ + 18µl $ddH_2O$ |
| $10^3$ | 2µl of $10^4$ + 18µl $ddH_2O$ |
| $10^2$ | 2µl of $10^3$ + 18µl $ddH_2O$ |
| $10^1$ | 2µl of $10^2$ + 18µl $ddH_2O$ |

**Appendix 3**. Raw data for DMPs loads (mg/d) and behavior through experimental weeks.

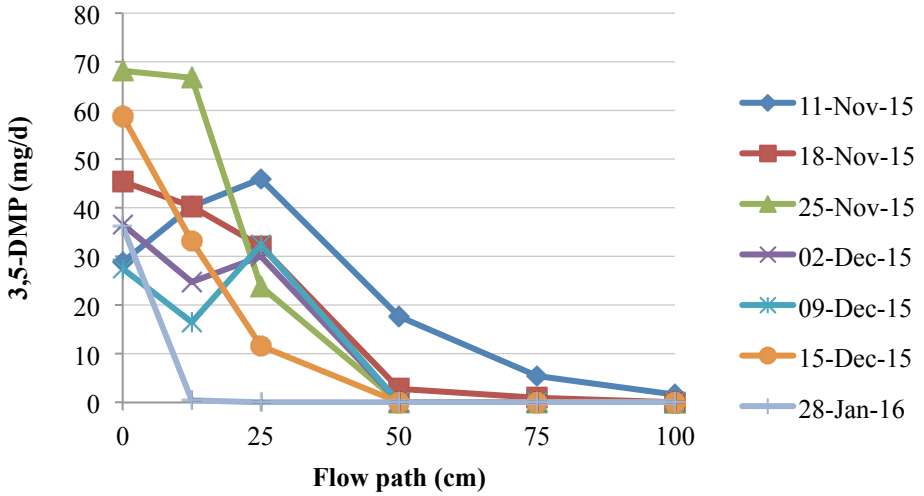
Appendix 3.1. Raw data for DMPs loads (mg/d) and behavior through weeks for treatment (1) planted with *Phalaris arundinaceae* fed with 3,5-DMP.

| | 3,5- DMP Load (mg/d) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Sampling point (cm) | 11-Nov-15 | 18-Nov-15 | 25-Nov-15 | 02-Dec-15 | 09-Dec-15 | 15-Dec-15 | 14-Jan-16 | 28-Jan-16 |
| 0 | 47.94 | 62.68 | 58.11 | 65.08 | 58.45 | 60.68 | 61.71 | 63.11 |
| 12,5 | 51.59 | 62.64 | 50.60 | 52.45 | 50.09 | 73.71 | 58.99 | 58.45 |
| 25 | 55.67 | 54.11 | 40.06 | 45.91 | 42.83 | 62.52 | 53.94 | 53.03 |
| 50 | 23.83 | 35.56 | 17.99 | 14.30 | 27.57 | 52.02 | 41.54 | 48.37 |
| 75 | 8.53 | 21.18 | 9.07 | 6.42 | 11.87 | 41.47 | 34.66 | 38.52 |
| 100 | 1.96 | 20.12 | 2.08 | 2.66 | 4.12 | 13.90 | 26.03 | 31.76 |



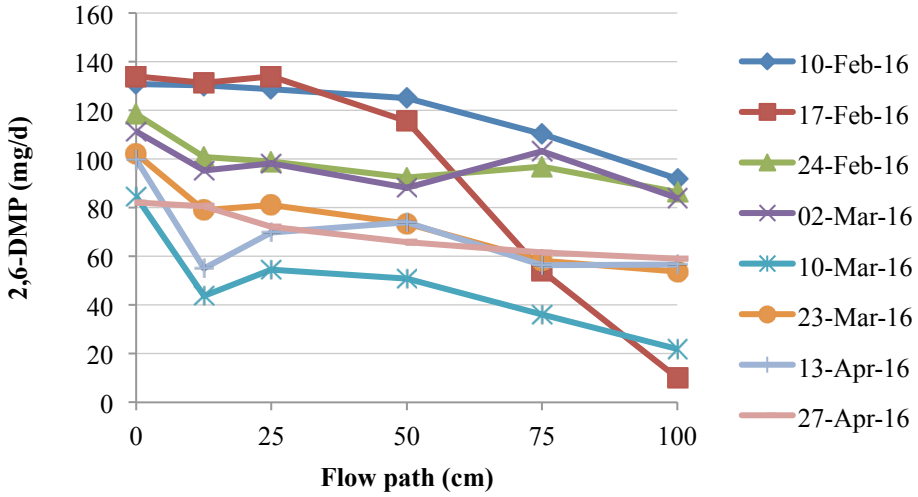

Appendix 3.2. Raw data for DMPs loads (mg/d) and behavior through weeks for treatment (2) planted with *Juncus effusus* fed with 3,5-DMP.

| | 3,5-DMP Load (mg/d) | | | | | | |
|---|---|---|---|---|---|---|---|
| Sampling point (cm) | 11-Nov-15 | 18-Nov-15 | 25-Nov-15 | 02-Dec-15 | 09-Dec-15 | 15-Dec-15 | 28-Jan-16 |
| 0 | 28.85 | 45.36 | 68.19 | 36.61 | 27.60 | 58.89 | 36.20 |
| 12,5 | 40.26 | 40.27 | 66.79 | 24.77 | 16.53 | 33.26 | 0.49 |
| 25 | 46.02 | 32.09 | 23.83 | 29.99 | 32.40 | 11.57 | 0.00 |
| 50 | 17.66 | 2.74 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| 75 | 5.40 | 0.96 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| 100 | 1.58 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |



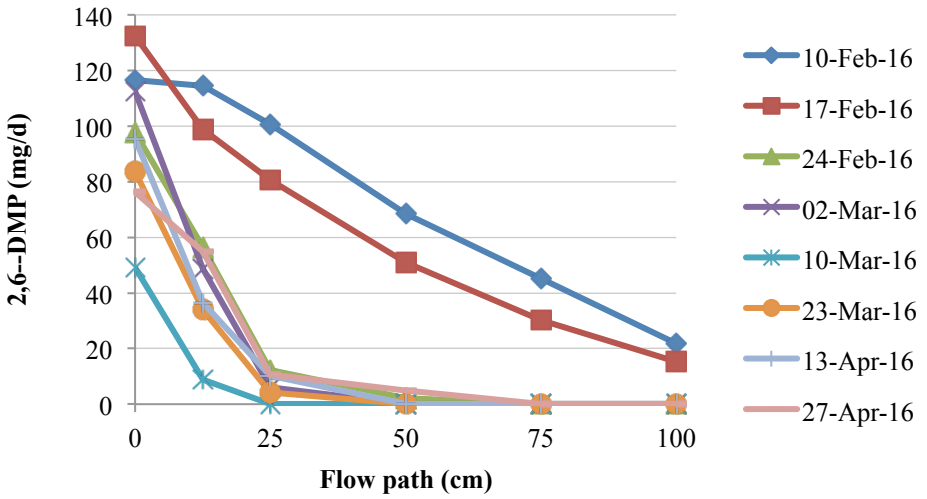
Appendix 3.3. Raw data for DMPs loads (mg/d) and behavior through weeks for treatment (3) Unplanted fed with 2,6-DMP.

| | 2,6-DMP Load (mg/d) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Sampling point (cm) | 11-Nov-15 | 18-Nov-15 | 25-Nov-15 | 02-Dec-15 | 09-Dec-15 | 15-Dec-15 | 14-Jan-16 | 28-Jan-16 |
| 0 | 130.84 | 133.97 | 118.38 | 111.34 | 84.64 | 102.11 | 99.73 | 82.19 |
| 12,5 | 130.21 | 131.31 | 100.98 | 95.31 | 43.68 | 79.13 | 55.09 | 80.70 |
| 25 | 128.63 | 133.95 | 99.03 | 98.13 | 54.47 | 81.07 | 69.72 | 72.23 |
| 50 | 125.07 | 115.46 | 92.41 | 88.14 | 50.95 | 73.39 | 73.89 | 65.77 |
| 75 | 110.35 | 54.04 | 96.81 | 103.16 | 36.17 | 58.17 | 56.41 | 61.76 |
| 100 | 91.86 | 10.04 | 86.42 | 83.91 | 21.94 | 53.70 | 56.63 | 59.06 |




Appendix 3.4. Raw data for DMPs loads (mg/d) and behavior through weeks for treatment (4) planted with *Juncus effusus* fed with 2,6-DMP.

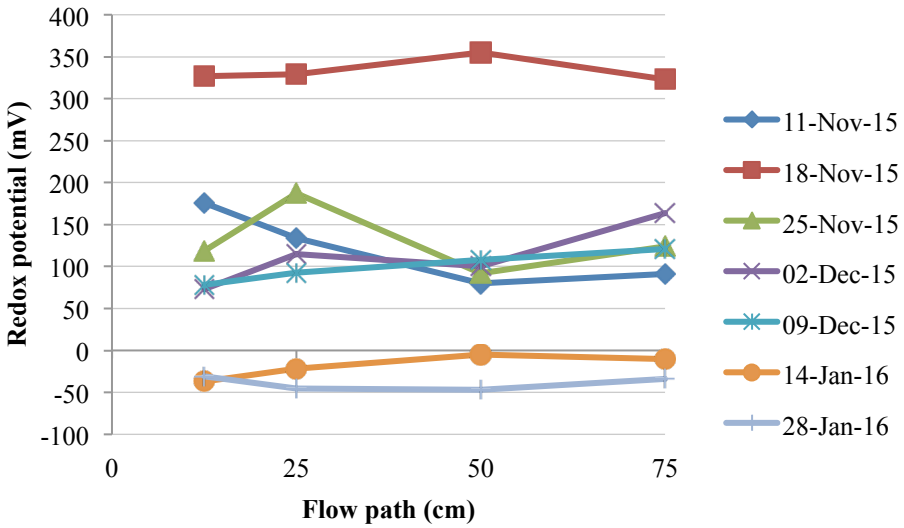
| | 2,6-DMP Load (mg/d) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Sampling point (cm) | 11-Nov-15 | 18-Nov-15 | 25-Nov-15 | 02-Dec-15 | 09-Dec-15 | 15-Dec-15 | 14-Jan-16 | 28-Jan-16 |
| 0 | 116.52 | 132.44 | 97.58 | 112.42 | 49.19 | 83.54 | 95.39 | 76.16 |
| 12,5 | 114.44 | 98.79 | 56.57 | 48.62 | 8.80 | 33.88 | 36.39 | 54.97 |
| 25 | 100.72 | 80.58 | 12.23 | 6.01 | 0.00 | 4.14 | 10.26 | 10.59 |
| 50 | 68.50 | 50.87 | 2.26 | 0.00 | 0.00 | 0.00 | 0.00 | 4.89 |
| 75 | 45.11 | 30.20 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| 100 | 21.82 | 15.17 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |




**Appendix 4.** Raw data for redox potentials (mV) behavior through experimental weeks.

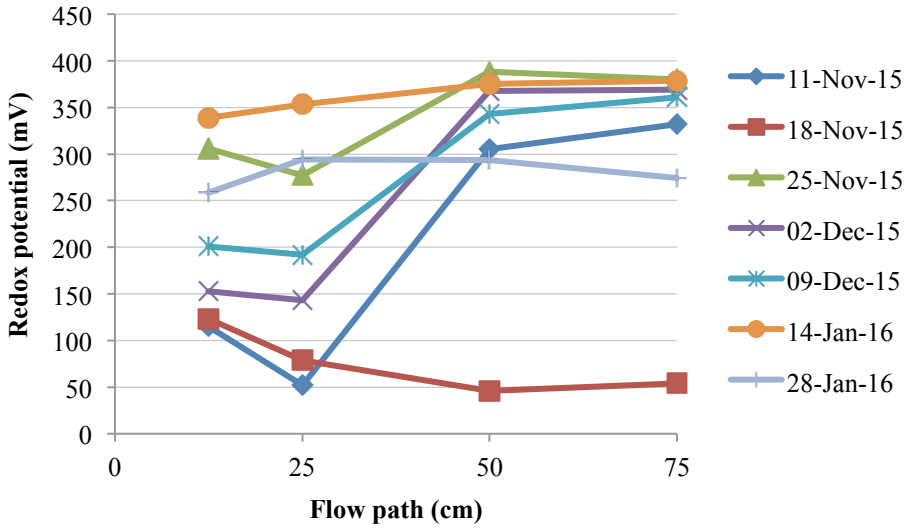
Appendix 4.1. Raw data for redox potentials (mV) behavior through weeks for treatment (1) planted with *Phalaris arundinaceae* fed with 3,5-DMP.

| | Redox potential (mV) | | | | | | |
|---|---|---|---|---|---|---|---|
| Sampling point (cm) | 11-Nov-15 | 18-Nov-15 | 25-Nov-15 | 02-Dec-15 | 09-Dec-15 | 14-Jan-16 | 28-Jan-16 |
| 12,5 | 176 | 327 | 119 | 73 | 78 | -37 | -31 |
| 25 | 134 | 329 | 187 | 115 | 93 | -22 | -45 |
| 50 | 80 | 355 | 92 | 100 | 108 | -5 | -47 |
| 75 | 91 | 323 | 124 | 164 | 121 | -10 | -34.2 |



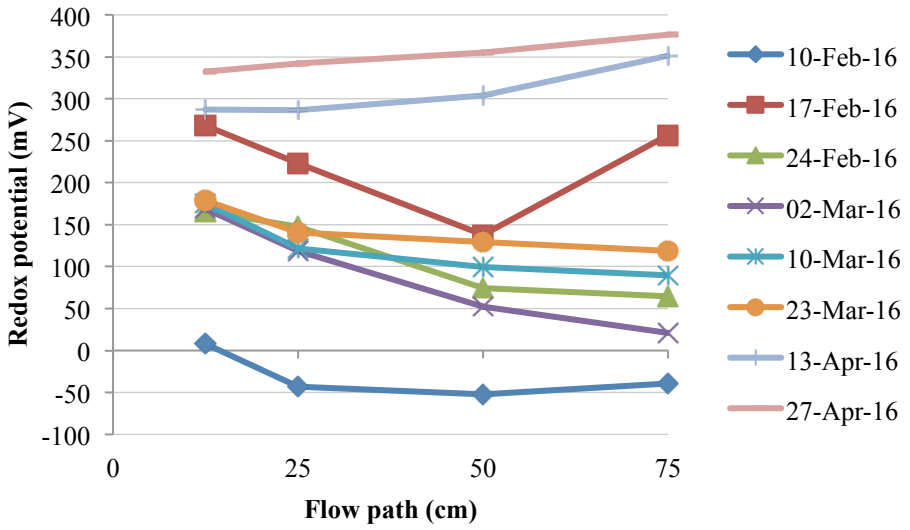
Appendix 4.2. Raw data for redox potentials (mV) behavior through weeks for treatment (2) planted with *Juncus effusus* fed with 3,5-DMP.

| | Redox potential (mV) | | | | | | |
|---|---|---|---|---|---|---|---|
| Sampling point (cm) | 11-Nov-15 | 18-Nov-15 | 25-Nov-15 | 02-Dec-15 | 09-Dec-15 | 14-Jan-16 | 28-Jan-16 |
| 12,5 | 115 | 123 | 306 | 153 | 201 | 338.9 | 258.4 |
| 25 | 52 | 79 | 277 | 143 | 192 | 353.2 | 294 |
| 50 | 305 | 46 | 388 | 368 | 343 | 375.5 | 293.6 |
| 75 | 332 | 54 | 380 | 369 | 361 | 378.7 | 274.4 |



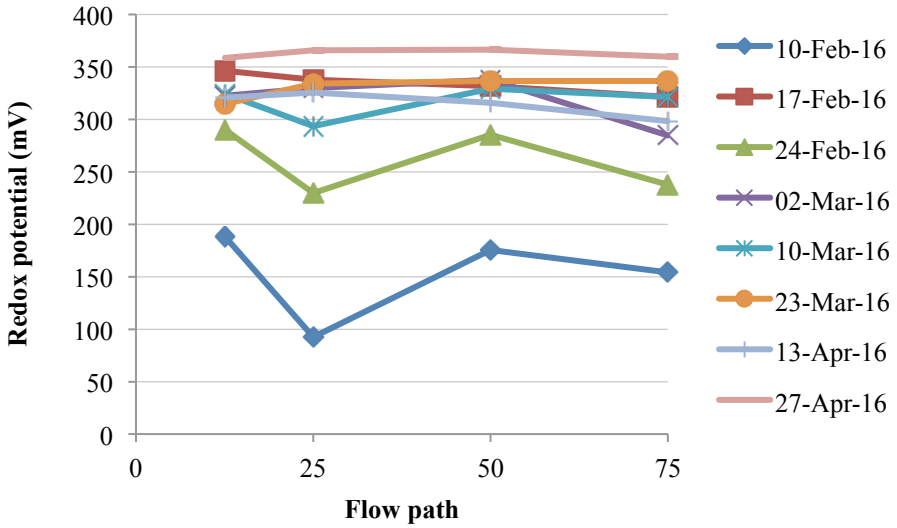

Appendix 4.3. Raw data for redox potentials (mV) behavior through weeks for treatment (3) Unplanted fed with 2,6-DMP.

| | Redox potential (mV) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Sampling point (cm) | 10-Feb-16 | 17-Feb-16 | 24-Feb-16 | 02-Mar-16 | 10-Mar-16 | 23-Mar-16 | 13-Apr-16 | 27-Apr-16 |
| 12,5 | 8.2 | 267.9 | 166 | 169.1 | 175.3 | 179.1 | 286.9 | 332.4 |
| 25 | -43.1 | 223.2 | 147.6 | 118.4 | 121.7 | 140.7 | 286.4 | 341.9 |
| 50 | -52.2 | 137.9 | 74.5 | 52.5 | 99.2 | 129 | 304.1 | 355.2 |
| 75 | -39.1 | 256.6 | 64.6 | 20.8 | 89.4 | 118.8 | 351.3 | 376.2 |




Appendix 4.4. Raw data for DMPs loads (mg/d) and behavior through weeks for treatment (4) planted with *Juncus effusus* fed with 2,6-DMP.

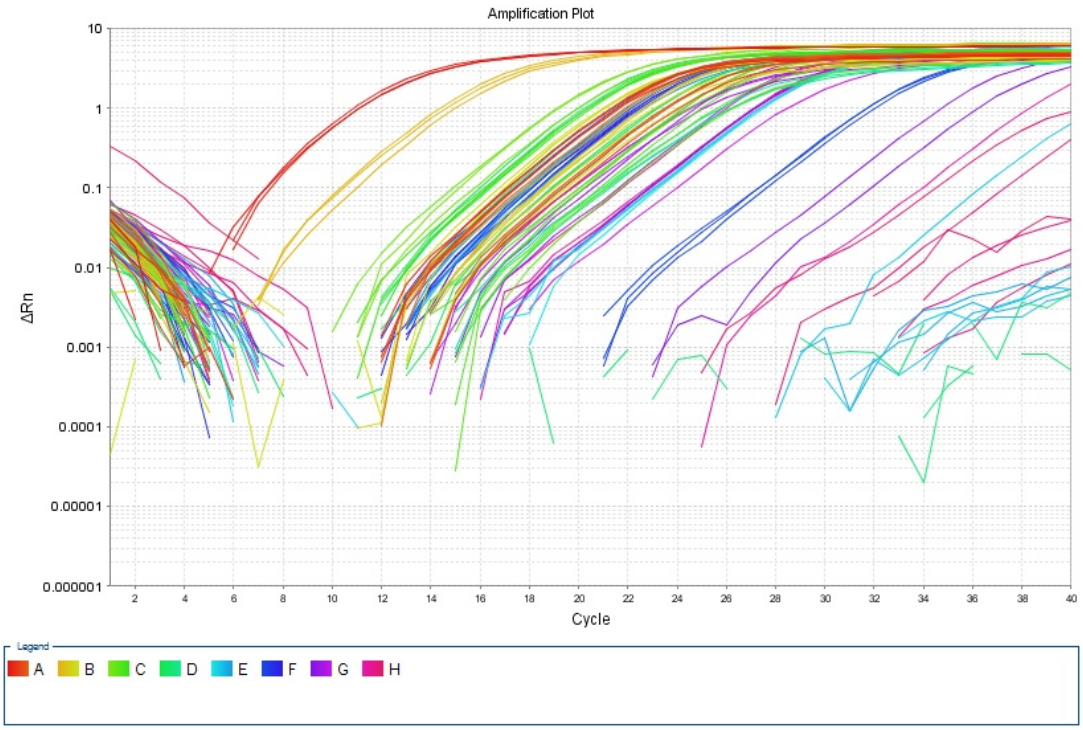
| | Redox potential (mV) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Sampling point (cm) | 10-Feb-16 | 17-Feb-16 | 24-Feb-16 | 02-Mar-16 | 10-Mar-16 | 23-Mar-16 | 13-Apr-16 | 27-Apr-16 |
| 12,5 | 188.2 | 346.6 | 290 | 322.9 | 324.2 | 314.7 | 320.5 | 358.7 |
| 25 | 92.4 | 337.6 | 229.6 | 330.1 | 293.6 | 334.1 | 325.9 | 365.9 |
| 50 | 175.6 | 331.5 | 285.5 | 337.7 | 329.4 | 336.7 | 315.7 | 366.6 |
| 75 | 154.2 | 321.5 | 237.7 | 284.7 | 321.6 | 336.5 | 298.4 | 359.7 |



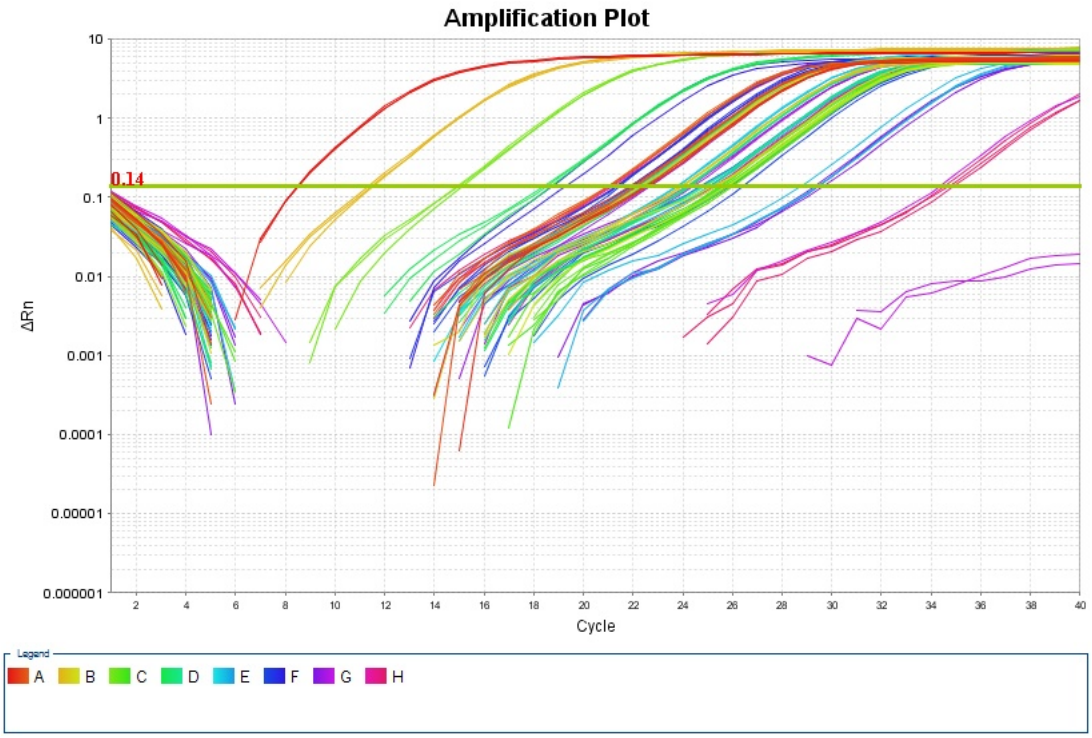
**Appendix 5**. Verification of qPCR results.

Appendix 5.1. Real time PCR amplification plot. Left curves show noise, while right curves show standard amplification curves showing the geometric, linear and plateau phases. A) amplification plot from an essay with 78% efficiency, B) amplification plot from an essay with 91% efficiency. The acceptable range efficiency was over 80% for each essay.

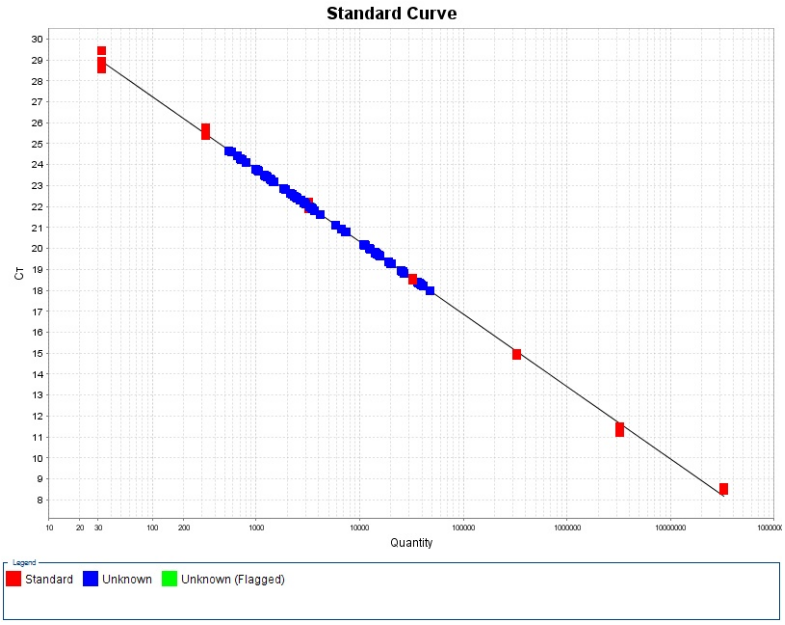
A)




B)

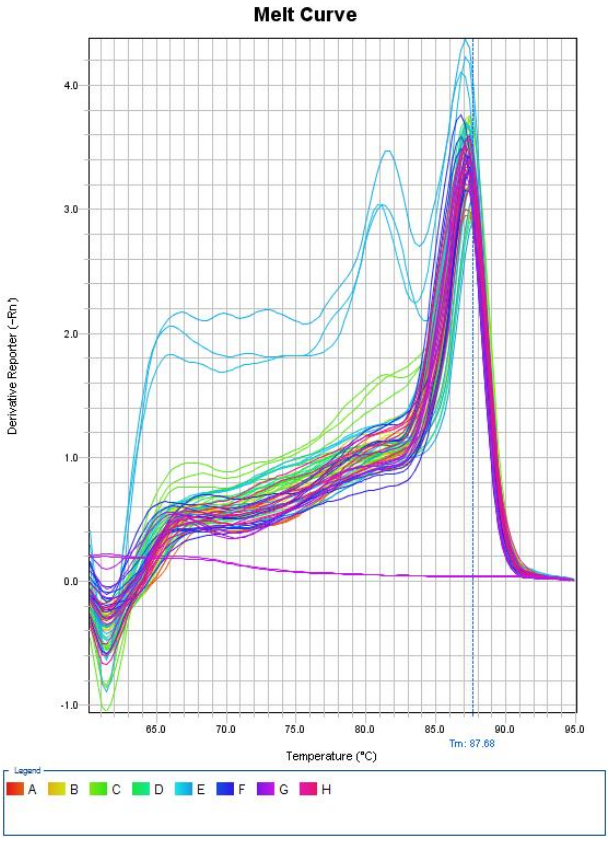


Appendix 5.2. Examples of  a qPCR standard curve based on standard dilutions of 16s and PHE (red) and analized samples (blue). All the blue points should be inside the standard curve.




Appendix 5.3. Examples of melting curves.

A) Low efficiency qPCR




B) High efficiency qPCR