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BIODEGRADATION OF DIMETHYLPHENOLS IN A LABORATORY SCALE CONSTRUCTED WETLAND

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ABSTRACT

Dimethylphenols (DMPs) are toxic and refractory compounds commonly constituting effluents from carbochemical industries, specifically coke-oven and creosote industry. The aim of this study was to evaluate the biodegradation of DMPs in a laboratory scaled constructed wetland planted with *Juncus effusus* by taking into account not only the removal efficiencies and performance obtained from the entire system, but also by analyzing closely the development and behavior of isolated microorganism and indigenous aerobic communities growing inside the Planted Fixed Bed Reactor (PFR). For this, previous isolated microorganisms from the PFR were tested to analyze their DMPs biodegradation potential. Further comparisons with biodegradation kinetics of xylenols using *Delftia acidovorans*, an isolated microorganism from a pilot scaled constructed wetland enabled to comprehend relevant microbiological processes and features comprising lab-scaled and pilot-scaled wetland systems. Additionally, DNA samples from the PFR and *D. acidovorans* were analyzed in order to detect key genes involved in the DMPs catabolic pathways. The PFR removed successfully 99.08% of the fed DMPs (3,4-, 3,5- and 2,6- DMP) under a regimen of increased concentrations (up to 28.86 mg/L). Nevertheless, this high removal efficiency was accompanied by negative effects on plant growth and changes in nitrogen dynamics. Differently to gravel and root microbial consortiums, isolated strains from the PFR were no capable to gain biomass and degrade a DMPs mixture (3,4-, 3,5- and 2,6- DMP) as a sole carbon and energy source. On the other hand, *D. acidovorans*, a wild strain, showed a great performance when exposed to single isomers and dual mixtures of DMP. 3,4-DMP served as a main growth substrate for this microbe and enabled cometabolic transformation of isomers 2,4-, 2,5- and 3,5- DMP and a better mineralization of 2,3-DMP in mixtures. 2,6-DMP was the only isomer not assimilated by this bacterium. Finally, a PCR screening of DNA samples from PFR and *D. acidovorans* revealed that monooxygenases might be catalyzing and fostering the activation of the aromatic ring as first step of the DMPs aerobic biodegradation. This research highlighted the importance of aerobic microbial communities to upgrade the removal of dimethylphenols by constructed wetlands.

Key words: Dimethylphenols, plant fixed bed reactor, *Delftia acidovorans*, aerobic biodegradation, monooxygenase.

RESUMEN

Los dimetilfenoles (DMFs) son compuestos tóxicos y refractarios que usualmente forman parte de los efluentes de la industria carboquímica, específicamente de la industria del coque y del creosote. El propósito de este estudio fue evaluar la biodegradación de DMFs en un humedal construido a escala de laboratorio plantado con *Juncus effusus*. Para ello se tuvo en cuenta no solo las eficiencias de remoción del sistema entero, sino también se analizó en detalle el desarrollo y comportamiento de microorganismos y comunidades aeróbicas creciendo en el interior de un reactor plantado de lecho fijo (RPLF). La primera etapa de esta investigación consintió en comprobar el potencial para degradar mezclas de DMFs de previos aislados microbianos obtenidos del RPLF y comparar su efectividad con respecto a las cinéticas de degradación de DMFs de *Delftia acidovorans*, un aislado de un humedal construido a escala piloto. Esta comparación permitió comprender características relevantes de los procesos microbiológicos que ocurren en los sistemas a escala de laboratorio y piloto. Adicionalmente, Muestras de ADN del RFLP y *D. acidovorans* fueron analizadas por PCR con el fin de detectar genes que se encuentran involucrados en las vías catabólicas de los DMFs. El RFLP removió exitosamente el 99.08% de los DMFs (3,4-, 3,5- y 2,6-) aplicados bajo un régimen de incremento de concentraciones (hasta 28.86 mg/L). Pese a ello, esta gran eficiencia de remoción estuvo acompañada por efectos negativos en el crecimiento de la planta y cambios en la dinámica de las especies de nitrógeno. A diferencia de los consorcios microbianos de las raíces y grava, las cepas aislada del RFLP no fueron capaces de ganar biomasa y degradar DMFs en mezclas (3,4-, 3.5- y 2,6) como única fuente de carbono y energía. Por otra parte, *D. acidovorans*, una cepa natural, mostró una gran desempeño cuando fue expuesta a isómeros simples y mezcla duales de DMFs. 3,4-DMF sirvió como principal sustrato de crecimiento para este microbio y permitió la transformación cometabólica de los isómeros 2,4-, 2,5- y 3,5- DMFs y una mejor mineralización del 2,3-DMF en mezcla duales. 2,6-DMFs fue el único isómero que no fue asimilado por esta bacteria. Finalmente, una selección y análisis por PCR de ADN de muestras del RFLP y *D. acidovorans* reveló que monooxigenasas podrían estar catalizando e incentivando la activación del anillo aromático como primer paso de la degradación aeróbica de DMFs. Esta investigación destacó la importancia de las comunidades microbianas aeróbicas con el fin de mejorar la remoción de dimetilfenoles por humedales construidos.

Palabras claves: Dimetilfenoles, reactor plantado de lecho fijo, *Delftia acidovorans,* degradación aeróbica, monooxigenasas.

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First of all, I want to express my infinite gratitude to God who guided me during two years of immense blessings and fruitful learning and which are finally concluded in this manuscript. Likewise, I desire to honor the memory of Dr. Peter kushck. I am certainly sure he will remain in the memory and heart of all his colleagues and friends for his kindness, commitment with sciences and companionship.

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TABLE OF CONTENT

LIST OF APPENDIX

LIST OF SCHEMES

LIST OF FIGURES

LIST OF TABLES

1. INTRODUCTION

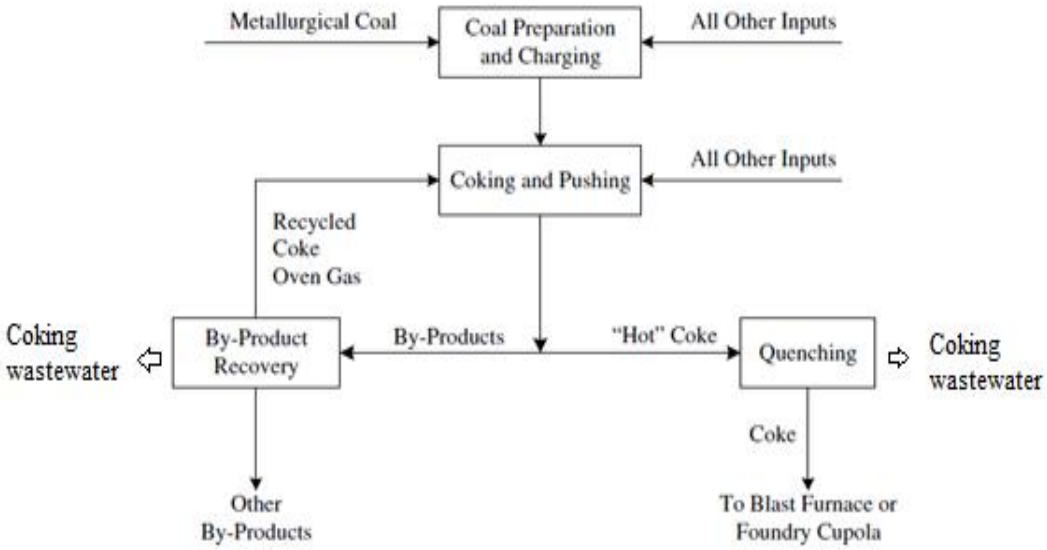
Pollution caused by coking wastewater is a serious problem all over the world, especially in China where coke is the main energy source for iron and steel production. Coking wastewater is usually generated from high temperature coal carbonization, gas purification and byproduct recovery processes. Effluents with similar composition are also produced at other industrial coal conversion processes like gasification (Xiao-Xue *et al.* 2012). According to data published on the BP Statistical Review of World Energy of June 2013, carbochemistry industry is expected to increase considerably its coal consumption over the next years, stepwise replace some oil-based energy production plants (BP, 2013) and by this, intensify the discharge of coking wastewater. This fact is of growing concern as cove-oven drainage contains high concentration of ammonia and toxic substances for ecosystems, such as phenols, cyanide (CN−), thiocyanide (SCN−), polycyclic aromatic hydrocarbons (PAHs) and heterocyclic compounds (Jin *et al.* 2012).

1.1 COKE OVEN INDUSTRY

The demand of coke is mostly linked to production of steel from blast furnaces and iron casting. Coke is metallurgical coal that has been baked into a charcoal-like substance. This substance burns more evenly than coal and also has more structural strength. Coke making implies several steps, beginning with coal pulverization and afterwards feeding of the obtained powder into an oven covered by a lid. Pulverized coal is subjected to destructive distillation for 15 and 30 hours in the in oven interior that reaches temperatures around 850 °C to 1100 °C. In order to keep the material in absence of air, the process is conducted under a slight positive pressure. Hot or incandescent coke produced inside the oven, is posterior carried through a special railroad and set inside a quench tower where is cooled with water and sieved to get right size particles. Coke

manufacturing involves generation of raw coke oven gas enriched with organic volatile compounds like benzene, toluene and xylene.

Recovery of those byproducts is generally performed onsite and the final cleaned gas is utilized for energy production or as a fuel. Pollutants emitted from coke oven industries are commonly generated during the pushing of coke from ovens, quenching of hot coke, and by-product recovery. Coking wastewater arises from the latter two aforementioned processes and its complex composition includes toxic inorganic and organic compounds (Mussatti, 1998). Scheme 1 shows the coking process step by step and underlines the primary source of coking wastewater.



Scheme 2.2. The By-Product Coke Production Process and coking wastewater generation (Adapted from Mussatti, 1998).

1.1.1 Creosote industry

Coal tar is a byproduct from coke oven and coal gasification industries and serves as a main ingredient in a distillation process that gives place to creosote production

(Rasmussen and Olsen, 2004; Breedveld and Sparrevik, 2000) Creosote is an oily liquid widely utilized in wood preservation. Dissolved chemical residues from places where wood is transformed leach into soils and induce groundwater contamination. Roughly, this polluted water contains 17% polycyclic aromatic hydrocarbons (PAHs), 38% nitrogen/sulfur/oxygen containing heterocyclic aromatic compounds (NSO-compounds) and 45% phenols (Arvin *et al.* 1988). As depicted on table 1.1, DMPs form the major fraction of drainage and runoff from creosote wood conservation industries.

Table 1.1. Chemical composition and concentration of contaminants contained in creosote polluted water (Adapted from Rasmussen and Olsen 2004).

Contaminant	Concentration (µg/L)	Percentage (%)
Phenol	370	2,16
Cresol	1046	6,12
DMPs	5411	31,67
Trimethylphenols	1919	11,23
Naphthalene	4089	23.93
PAHs	925	5,41
2 ring NSO	2940	17,20
3 ring NSO	384	2,24

1.2 DIMETHYLPHENOLS (DMPs)

Phenolic wastes are noticeably present not only in effluents related to the aforementioned coal/coke industries, but also in other sort of manufacturing activities such as petroleum refineries, resin and fiber-glass fabrication and herbicides production (Ramakrishnan and Gupta 2008). Phenols are considered as toxic, carcinogenic, mutagenic and terratogenic substances which are partially biodegraded on aquatic or soil environments when their chemical compositions are simple. Nevertheless, more structurally complex compounds as short-chained alkylated phenols have shown to be more persistent and toxic (Kahru *et al.* 2002). Dimethylphenols (DMPs) or xylenols belong to this latter group and are recognized for being poisonous, moderately soluble in water

and with possible low biodegradation rates under reducing conditions. DMPs are a relevant constituent of plumes from coal tar distillatory plants and creosote industries, and are capable to leach into the soil and groundwater (Licha *et al.* 2001). Due to their particular characteristics, this research was specifically addressed toward the study of DMPs removal with constructed wetlands (CWs).

1.2.1 General characteristics

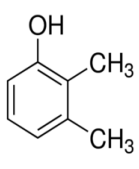
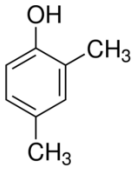
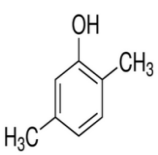
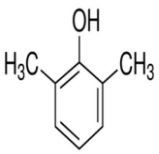
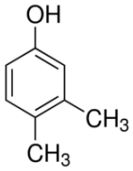
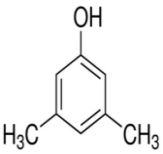
Xylenols or dimethylphenols are available as six isomeric forms. They have a molecular weight of 122,164 g/mol. These compounds are classified according to the positioning (ortho-, meta-, para-) of two methyl groups on a phenol ring and are usually referred as positional isomers. The different arisen configurations are 2,3-, 2,4-, 2,5-, 2,6-, 3,4- and 3,5- dimethylphenols (table 1.2). As would be expected the biological and physicochemical properties from each isomer vary considerably (Pilato 2010; EPA 1980). Although DMP isomers might be found separately in contaminated places, xylenols occur most of the time in mixtures with other fossil fuel byproducts like cresols, phenol and substituted phenols (EPA 1980).

1.2.2 Industrial effluents containing DMPs

Phenols are found in a large variety of industrial effluents and constitute one of the main components (quantity and number) in wastewater originated from coal carbonization processes like coke production and coal gasification (Semple and Cain 1997). Significant amounts of phenolic wastes have been also traced in drainages from petrochemical industry, pulp and paper industry, resin and fibreglass manufacturing and herbicides production (Tomei and Annesini 2008). Specifically, xylenols or dimethylphenols are considered as byproducts of coking industries and high concentrations of them are similarly released in distillatory plants of coal tar and creosote industries. A range of

phenols concentrations between 40-80% and 9-32% of the total COD have been typically observed in wastewater discharged from these industrial effluents (Tomei and Annesini 2008; Kahru *et al.* 2002).

Table 1.2. Physicochemical properties of DMPs or Xylenols (Adapted from MERISOL Co., 2008).

Isomer	2,3-Xylenol	2,4-Xylenol	2,5-Xylenol	2,6-Xylenol	3,4-xylenol	3,5-xylenol
						
MP(°C)	72.6	24.5	74.8	45.6	65.1	63.4
FP°C)	93	89	89	86	104	100
BP (°C)	216.9	210.9	211.1	201.o	227.0	221.7
p (g/mL)	0.981	1.003	0.958	0.978	0.982	0.971
TE (%ΔV/°C)	0.095	0.083	0.095	0.098	0.083	0.089
HC ($J\ mol^{-1}\ K^{-1}$)	274	281	285	246	280	296
SW (wt%)	0.47	0.61	0.49	0.64	0.50	0.49

MP=Melting point, FP=Flash point, BP=Boiling point, p=Density, TE=Thermal expansion, HC= Heat capacity, SW=Solubility in water

DMPs are of special concern due to their high toxicity, low biodegradation fate under certain REDOX conditions (reductive), notable mobility into soils and thereby groundwater contamination (Licha *et al.* 2001). A plume from a coal tar distillation plant near Wolverhampton, UK, contained principally phenol, o-, m-and p-cresols and DMPs at total combined concentrations of up to 24 g/L. Since low biodegradation was observed onsite, researches diluted water samples from the plume to test concentrations effect on soil microcosms (Harrison *et al.* 2001).

1.2.3 Health and environmental Hazards

Phenols are considered as toxic, carcinogenic, mutagenic and terratogenic substances. 2,4-DMP has not been related with cancer incidence, but mixtures and drainages that contain it are considered as extreme toxic. Therefore, any direct contact with humans must be strictly avoided (EPA 1980). The possible toxic effects of xylenols on humans were determined by using animal toxicity models. Oral toxicity in mice and rats were detected from low to moderate. The acute toxicity of a mixture containing all six isomers is moderate for the oral route and low for the dermal route (EPA 2010).

The 96 hours lethal concentration (LC50) for fish exposed to the mixed xylenols ranges from 12 mg/L to 22 mg/L. The 48 hours effective concentration (EC50) for aquatic invertebrates ranges from 7.7 mg/L to 11.2 mg/L. The 72 hours EC50 for aquatic plants from exposure to the supporting chemical is 14 mg/L for biomass and is >22 mg/L for growth rate (EPA 2010). These values reveal the severe hazards for aquatic ecosystems when low emissions of dimethylphenols are discharged to the environment.

1.2.4 Removal technologies

Several known and innovative treatment technologies have been tested to remove phenols and toxic compounds contained in coking wastewater. Most of these removal methods can be classified in regards to the nature of involved removal processes. For instance, coagulation, flocculation, membrane filtration, advanced oxidation process (AOP) and micro-electrolysis are among the most utilized physicochemical methods (Zhu *et al.* 2009; Xiong *et al.* 2011; Chen *et al.* 2009). Likewise, general sequence batch reactors (SBRs), activated sludge process and biological denitrogenation process stand for the most applied biological treatments (Chun-Hui *et al.* 2013). Some limitations of this processes, such as high cost, long operation time, and low load have encouraged a trend of

combining various technologies in order to achieve a better quality of the effluents (Wen-Wu *et al.* 2012), but this has also been reflected on the increase of operation costs. Disadvantages referred to above make the research on coking/phenolic wastewater treatment a continuous field of study that seeks for new alternatives able to enhance or replace the current methods.

Nowadays, modern and enhanced treatment processes such as electrochemical oxidation, ultrasonic/ozone combined methods, biofilm reactor with zero-valent iron process, filtration and adsorption using manganese and magnesium ores and electro-fenton oxidation, (Wen-Wu *et al.* 2012; Chun-Hui *et al.* 2013), have been investigated to remove pollutants contained in coke oven waste water. However, due to the construction investment and operating costs, it is no doubt that the biological methods are the mainstream approach for treatment of those contaminants.

Anoxic-oxic (A/O) or anaerobic-anoxic-oxic (A1/A2/O) processes are one of the most applied in biological treatment of coking wastewater treatment because of the high treatment efficiency and cost-effective advantages (Haibao *et al.* 2010; Marañón *et al.* 2008). Unfortunately, due to the refractory and inhibitory contaminants present in those effluents, the above processes are not sufficient in practice to meet the stringent environmental restrictions. The combination of this technology with other physical and chemical methods offers a good alternative in order to fulfill the regulations implemented, but this also means to raise the treatment prices.

1.2.5 Aerobic biodegradation of dimethylphenols

A key factor in aerobic biodegradation of aromatic compounds by bacteria is to count on enzymes capable to overcome the chemical stability of the aromatic ring. Complex enzymatic machinery mediates aromatic ring activation, ring cleavage and formation of

common intermediates of cell metabolism (Reineke 2001). Under aerobic conditions, those biochemical pathways employ oxygen as electron acceptor of aromatic pollutants and as activation substrate for oxygenation reactions (Cao *et al.* 2009).

Cleavage of the aromatic ring occurs in most of the cases when two hydroxyl groups are bound to the benzene ring. Therefore, substrates for ring cleavage may be 1,2-diphenolic compounds such as catechol, protocatechuate, and catechol derivatives or a 1,4-diphenolic compound, gentisate. The ring reactions to produce the aforementioned substrates are catalyzed mainly by dioxygenases that leads to the formation of a cis-dihydrodiol, which is then oxidized to the corresponding diphenolic intermediates by a dehydrogenase (like the case of benzene catabolism). Other aromatics compounds like aniline, salicylate and phenol just require dioxygenase to induce the formation of ring cleavage substrate (Figure 1). Aromatic compounds which bear alkyl substituents on the aromatic ring may undergo oxidation of the side chain before ring activation (Reineke 2001).

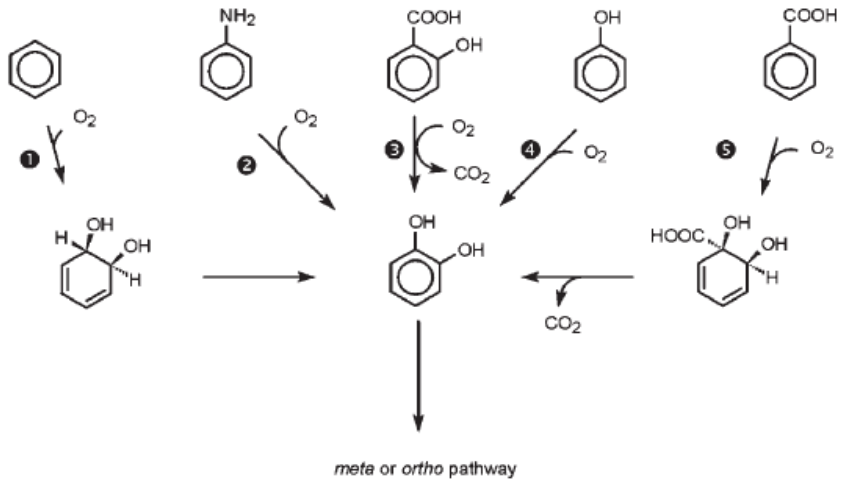


Figure 1.2. Formation of the ring cleavage substrate (Catechol) using dioxygenase and dehydrogenase ([**1**]benzene and [**5**]benzoate) and exclusively dioxygenase ([**2**]Aniline, [**3**]Salicylate and [**4**]phenol) (Reineke, 2001).

Once the aromatic ring is activated, intradiol dioxygenases (1,2 or 3,4 dioxygenase) or extradiol dioxygenases (2,3 or 4,5 dioxygenase) catalyze the ring cleavage of dihydroxy intermediates (Figure 1. 2) by using oxygen between the two hydroxyl groups (ortho-cleavage) or proximal to one of the two hydroxyl groups (meta-cleavage). The ring-cleavage intermediates are then subjected to subsequent central pathways leading to the formation of Krebs cycle intermediates (Cao *et al.* 2009).

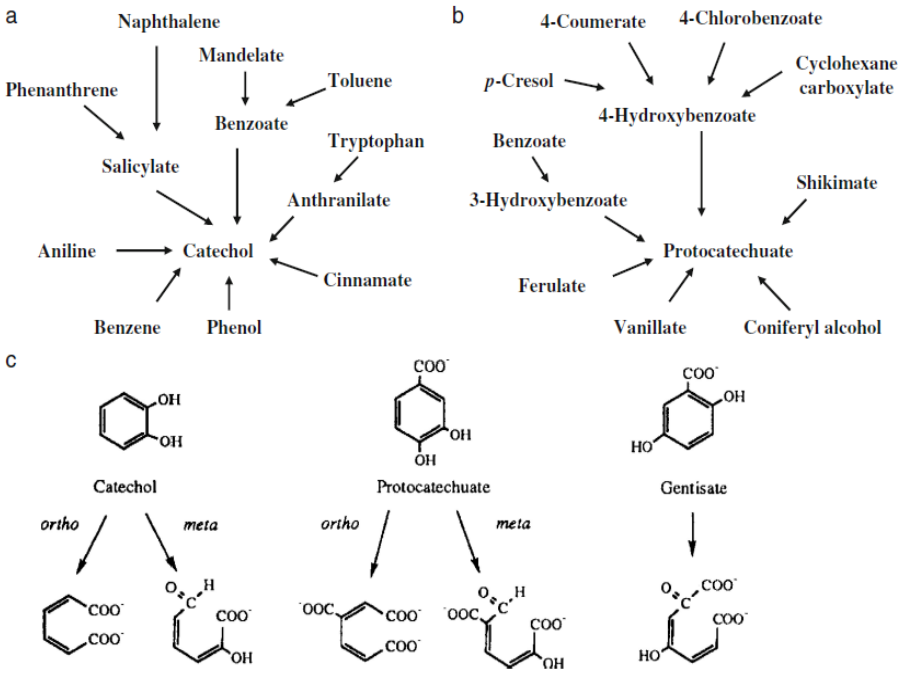


Figure 1.2. Aromatic compounds funneled to (**a**) catechol and (**b**) protocatechuate. Major aerobic routes of the aromatic ring cleavage: (**c**) ortho-, meta-, and gentisate cleavage (Cao *et al.* 2009).

Meta ring cleavage is the prevalent pathway during the degradation of methyl substituted aromatic compounds (Viggor *et al.* 2003). Studies conducted on degradation of 2,4-DMP by Pseudomonas revealed that oxidation of the methyl group para to the hydroxyl group is the first step in the removal process. Accumulation of 4-hydroxy-3-methylbenzoic acid (Ring cleavage substrate) and increase of protocatechuate 3,4-dioxygenase is then

detected (Chapman and hopper 1968). Also gentisate pathway has been identified in the catabolism of 2,5- and 3,5- DMP using Pseudomonas. According to Viggor *et al.* (2003), methyl group meta to hydroxyl group is oxidized at first and subsequently, the aromatic ring of methyl- gentisate is cleaved by gentisate 1,2 dioxygenase (Viggor *et al.* 2003).

Harrison *et al.* (2001) built 15 different artificial microcosms to evaluate the treatment of a coal tar distillate plume and proposed an aerobic degradation pathway for 2,5 DMP (figure 3) which involved the formation of 2,5-dimethyl-p-benzoquinone (Harrison *et al.* 2001). As described in figure 3, the diphenolic compound that serves as an intermediary is 2,5 dimethyl-p-benzohydroquinone. Viggor *et al.* (2003) also found that catechol meta pathway is functional in the degradation of dimethylphenols by axenic cultures of *Pseudomonas mendocina* which degrade previously p-cresol via catechol meta cleavage.

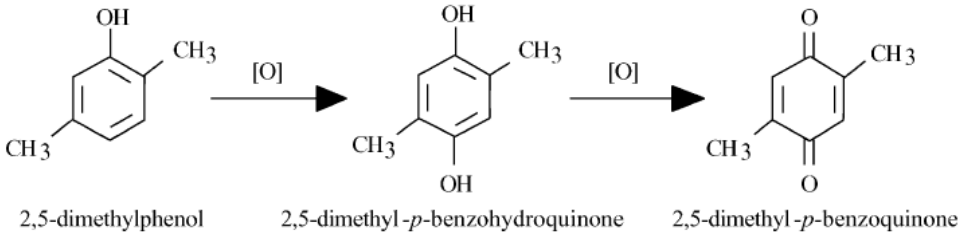


Figure 1.3. Proposed aerobic degradation pathway for 2,5-dimethylphenol (Harrison *et al.* 2001)**.**

1.2.6 *Delftia acidovorans* as a DMPs degrader

Delftia acidovorans (Delftia referring to Delft, The Netherlands, site in which this genera was discovered), formerly called as *Comamonas acidovorans*, is a gram-negative aerobic rod found ubiquitously in soil and water, and has seldom been implicated in human infections (Chun *et al.* 2009). This microbe has been isolated from soil, sediment, activated sludge, crude oil, oil brine, water and various clinical samples (Wen *et al.* 1999). To the best of our knowledge, any reports about *D. acidovorans* degrading DMPs have been

published yet. However, previous investigations carried out by Schmechta (2014), Blazquez (2013) and Curiel (2015) in our research group demonstrated for first time the biodegradation potential of this strain to remove DMPs.

1.2.6.1 Morphological and physiological characteristics

Cells associated to the genera *Delftia* are straight to slightly curved rods, 04-0.8 x 2-5-4.1 µm (occasionally up to 7 µm), which occur singly or in pairs. Motile by means of polar or bipolar tufts of one to five flagella. They are Gram-negative, oxidase and catalase-positive. Endospores are not produced, and no fluorescent pigments are produced. Specifically, *D. acidovorans* grows in the presence of 0.5 or 1-5 % NaCl at 30 °C, but it is inhibited at 4 °C and 41 °C. This microorganism does not produce any pigment on nutrient agar. Several organic compounds can be utilized as carbon and energy sources. Some of them are shown as follows: acetate, acetamide, aconitate, adipate, D- alanine, L-alanine, 2-aminobutyrate, 6- aminovalerate, L-aspartate, azelate, butanol, 2,3-butylene glycol, butyrate, caproate, citra- conate, ethanol, D-fructose, fumarate, gluconate, L-glutamate, glutarate, glycerate, glycine (See full list on Wen *et al.* 1999).

1.2.6.2 Biodegradation of toxic compounds by D. acidovorans

Phenoxypropionate and phenoxyacetate herbicides are successfully degraded by *Delftia acidovorans*. Enantioselective dioxygenases produced along this biodegradation process were isolated to test their affinity with specific herbicide enantiomers (Westendorf *et al.* 2003). Further studies on biodegradation of 2,4-dichlorophenoxyacetate (2,4-D) in a microfluidic porous medium were conducted by *Yoon et al.* (2014). Despite 2,4-D had not been reported before as an appropriate carbon source for *D. acidovorans*, the authors found out that an axenic strain supported in a porous media was capable to use this substrate after an exposure period of 35 day.

Yilmaz and Legen (2014) characterized *D. acidovorans* effectiveness to consume and remove a common anionic surfactant, sodium dodecyl sulfate (SDS). *D. acidovorans* exhibited optimum growth at SDS concentration of 1 g/L but tolerated up to 10 g /L SDS. 87 % of 1.0 g/L pure SDS was degraded after 11 days of incubation. Isolation experiments of aniline degrading microorganism enabled to indentify *D. acidovorans* as an active degrader in wastewater samples and activated sludge. DNA and deduced amino acid sequences of this strain proved the feasibility of catechol pathway when initiating the aniline ring cleavage (Urata *et al.* 2004).

1.2.6.3 Other applications

D. acidovorans turned out to become very popular in 2013 when Johnston *et al.* published their research "Gold biomineralization by a metallophore from a gold-associated microbe". As reported by them, this gold resident bacterium produces a nonribosomal peptide that creates gold nanoparticles (Johnston *et al.* 2013). Poly(3-hydroxybutyrate-co-4-hydroxybutyrate), a polymer frequently used in tissue engineering in both in vitro and in vivo studies, is also generated by *Delftia acidovorans* in fed-batch cultures from various carbon sources (Hsieh *et al.* 2009).

1.3 CONSTRUCTED WETLANDS (CWS)

In the last few decades, researchers and governments have tried to adopt an eco-technological approach to clean up or remediate wastewater using plants. This use of plants termed phytoremediation (phyto meaning plant and remediation meaning to clean or restore) actually refers to diverse collection of natural or genetically engineered plants for cleaning contaminated environments (Cunningham *et al.* 1997). CWs have come up like a well-explored and economic alternative in which phytoremediation processes and

engineering configurations are used. By definition, CWs are engineered systems that have been designed and constructed to utilize the natural processes involving wetland vegetation, soils, and their associated microbial assemblages to assist in treating wastewater. They are designed to take advantage of many of the processes that occur in natural wetlands, but do so within a more controlled environment (Vymazal and Kröpfelová 2008).

When wastewater passes through this artificial ecosystems, contained organic and inorganic pollutants are removed by a combination of physical, chemical, and biological processes including sedimentation, precipitation, adsorption to soil particles, assimilation by the plant tissue, and microbial transformations (Brix, 1994). These constructed wastewater treatments may include swamps and marshes. A large number of released CW systems are composed by marshes. They are shallow water regions dominated by emergent herbaceous vegetation including cattails, bulrush, reeds, rushes, and sedges (Idris *et al.* 2010).

1.3.1 Components

Main elements comprising constructed wetland systems are vegetation, supporting material or substrate (gravel) and water. Microorganism and aquatic invertebrates are consequently established when treatment process begins (Matamoros and Bayona 2008). Each component referred to above interacts through complex processes that enable the contaminants removal. Details and entire understanding about those removal mechanisms are still uncertain and lead to call CWs as Black box systems. CWs are of different basic designs featuring different flow characteristics and consist of saturated substrates, vegetation and microbes. Root zone (or rhizosphere) is the active reaction zone of CWs where physicochemical and biological processes take place by the interaction of plants, microorganisms, the soil and pollutants. The rhizosphere of wetland plants

provides an enriched culture zone for the microbes involved in degradation. The wetland sediment zone provides reducing conditions that are conducive to the metal removal pathway. The soil is the main supporting material for plant growth and microbial films. Hydraulic processes are influenced by soil matrix. Mixture of sand and gravel produces the best results in terms of both hydraulic conditions and the removal of contaminants. Soil physical parameters such interstitial pore spaces and effective grain sizes considerably influence the flow of wastewater in CWs and ultimately the removal of contaminants (Dhir, 2013).

1.3.2 Wetland plants

Emergent water plants are the dominant life form in wetlands and marshes, growing within a water table range from 0.5 m below the soil surface to a water depth of 1.5 m or more. In general, they produce aerial stems and leaves and an extensive root and rhizome system. Aerial stems and leaves of emergent water plant possess many similarities in both morphology and physiology to related terrestrial plants. The emergent monocotyledons, such as *Phragmites* and *Typha*, produce linear leaves from an extensive anchoring system of rhizomes and roots. The cell walls are heavily thickened with cellulose, which provides the necessary rigidity. The root and rhizome systems of these plants exist in permanently anaerobic sediments and must obtain oxygen from the aerial organs for sustained development. Similarly, the young foliage under water must be capable of respiring anaerobically for a brief period until the aerial habitat is reached, since the oxygen content of the water is extremely low in comparison to that of the air. Once the foliage has emerged into the aerial habitat, the intracellular gas channels and lacunae increase in size, thus facilitating gaseous exchange between the rooting tissues and the atmosphere. These plants are morphologically adapted to growing in a waterlogged or submersed substrate by virtue of large internal air spaces for transportation of oxygen to roots and rhizomes. Part of the oxygen may leak into the surrounding rhizosphere, creating oxidized

conditions in an otherwise anoxic environment and stimulating both decomposition of organic matter and growth of nitrifying bacteria (Vymazal and Kröpfelová 2008).

1.3.2.1 *Juncus effusus*

This macrophyte belongs to the rush family *Juncaceae*, thus it is known as soft rush. *Juncus effusus* is a rhizomatous, perennial herb with a large, tufted, cespitose growth form. *Juncus effusus* stems are stout but soft, 5-15 dm tall and 1.5-3 mm wide. Rushes provide conservation uses like erosion control, sediment accretion and stabilization, nutrient uptake and transformation, wildlife food and cover, restoration and creation of wetland ecosystems, and wastewater treatment applications. The rhizomatous nature, nitrogen fixation capabilities, dense root system, and phenotypic plasticity to flooding and drought stress provide high soil and slope stabilization capabilities, particularly in areas with flooded soils or fluctuating hydrology. The rhizomes form a matrix for many beneficial bacteria, making this plant an excellent addition for wastewater treatment. This species can have invasive characteristics in certain situations (USDA 2000). Flooded plant roots commonly form aerenchyma, which allows gas diffusion between shoots and roots. The programmed cell death involved in this induced aerenchyma formation is controlled by the plant hormone ethylene. In contrast, data published by Visser and Bögemann shows that parenchyma of *J. effusus* is highly constitutive and is not controlled by this compound (Visser and Bögemann 2006).

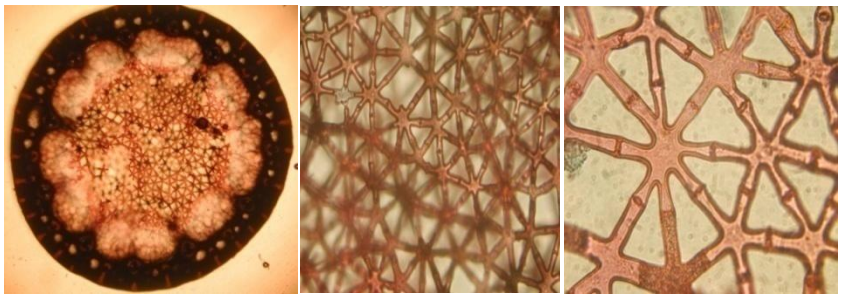


Figure 1.4. Aerenchyma of *Juncus effusus.*

1.3.3 Types of CWs

According to the life form of the dominating macrophyte, CWs may be classified into systems with free-floating, floating leaved, rooted emergent and submerged macrophytes (figure 4). Further division could be made in regards to the wetland hydrology (free water surface and subsurface systems) and subsurface flow (Töre *et al.* 2012).

The two principal classes of CWs designed for treating wastewater are the surface and subsurface flow systems, depending on whether or not the wastewater is flowing on the wetland surface. Surface flow constructed wetlands (SFCWs) consist of shallow basins or channels with planted wetland vegetation where water flows over a compacted low permeability clay liner at relatively shallow depths. Subsurface flow constructed wetlands (SSFCWs) involve shallow basins or channels with planted vegetation overlying a liner where the wastewater is treated as it flows through the gravel media and around the roots and rhizomes of planted vegetation (Matamoros and Bayona 2008). Subsurface flow wetlands are the common system design implemented in Europe for domestic wastewater treatment, while in the United States (North America), the FWS type is more common. FWS wetlands can commonly occur in communities with 1,000 or fewer people to a population greater than 1 million in cities (Dhir 2013). Attending to the water flow direction, SSFCWs can be classified as horizontal flow constructed wetlands (HFCWs) or vertical flow constructed wetlands (VFCWs). The last two configurations mentioned are the most used around the world.

(a) Horizontal surface flow systems (HFCWs) — Wastewater is maintained at a constant depth and flows horizontally below the surface of the granular medium (Vymazal and Kröpfelová, 2008). (b) Vertical flow systems (VFCWs)—Wastewater is distributed over the surface of the wetland and trickles downwards through the granular medium (Figure 1.5). Vertical systems can further be sorted in four types depending on the hydraulic regimes:

unsaturated flow, permanently saturated flow, intermittent unsaturated flow and flood and drain wetlands. Concerns over matrix clogging and the potential high cost of renovation also limit the deployment of extremely large HSSFCW wetlands (Vymazal and Kröpfelová 2008).

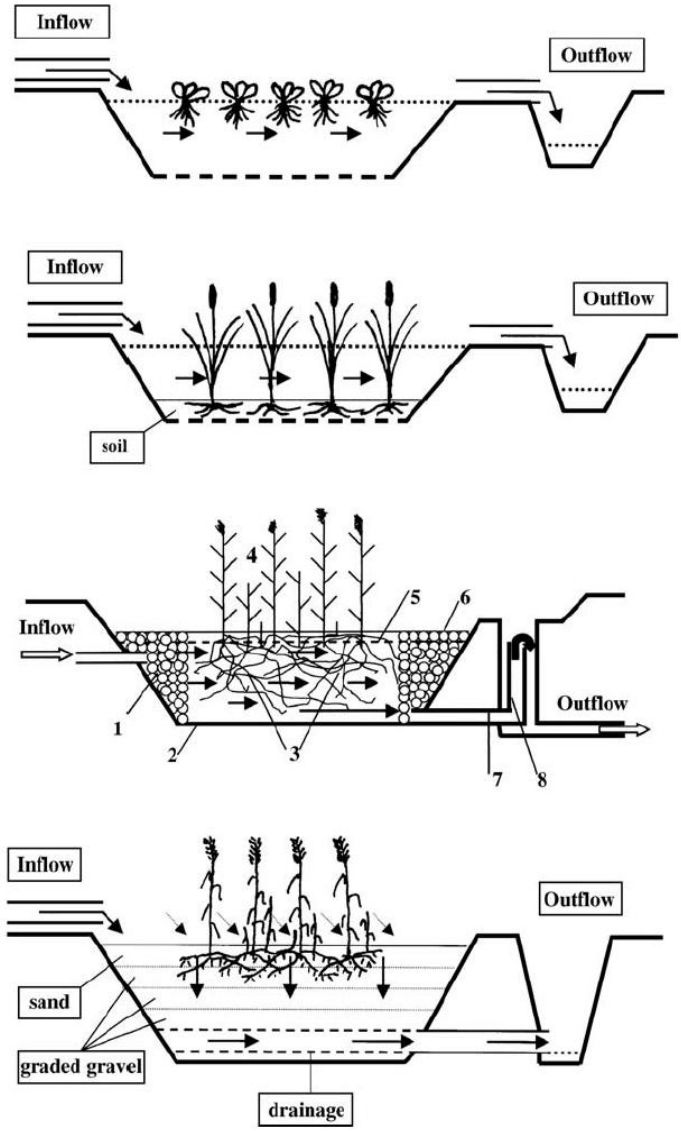


Figure 1.5. Constructed wetlands for wastewater treatment (from top to bottom): CW with free-floating plants (FFP), CW with free water surface and emergent macrophytes (FWS), CW with horizontal sub-surface flow (HSSF, HF), CW with vertical sub-surface flow (VSSF, VF) (Vymazal 2007).

1.3.4 Removal mechanism

The elimination principles are similar for all CWs systems. Raw or pre-treated waste water flows through the CW. The elimination processes take place during this passage; they are based on various complex physical, chemical and biological processes within the association of substrate, macrophytes and microorganisms. The major pollutant removal mechanisms in CWs include biological processes such as microbial metabolic activity and plant uptake as well as physico-chemical processes such as sedimentation, adsorption, and precipitation at the water–sediment, root–sediment, and plant–water interfaces (Idris *et al.* 2010). These mechanisms principally depend on: hydraulic conductivity of the substrate, types and number of microorganisms, oxygen supply for the microorganisms, and chemical conditions of the substrate (Haberl *et al.* 2003). An understanding of the treatment mechanisms is fundamental to ensure that CWs are designed effectively with enhanced treatment performances. Table 1.3 compiles a large extension of removal mechanisms and processes reported for CWs. Additionally, they are classified as physicochemical or biochemical processes in regards to the components that interact in the removal of target pollutants.

1.3.5 Treatment of industrial effluents with CWs

During the eighties the development of this technology was virtually all emphasized on the treatment of domestic and municipal wastewater (Kadlec *et al.* 2000). Nevertheless, in recent years a wide spectrum of effluents like runoff water, agriculture wastewater, industrial wastewater, urban storm water, swine wastewater, olive mill wastewater, groundwater and landfill leachate have been also treated. The target contaminants to be removed in such drainages are commonly natural organic matter (NOM), effluent organic matter (EfOM), toxic anions, nitrate, bromate, perchlorate, pharmaceutical chemicals, endocrine disrupters, aromatic organic compounds, sulphonated anthraquinones, hydro-

Table 1.3. Summary of removal mechanisms in constructed wetlands (PP: Physicochemical process, BP: Biochemical process).

Group of pollutants/ Parameter	Removal mechanism	PP	BP	References
BOD (organic matter)	Oxidation (aerobic degradation)		X	Idris *et al.* 2010; Kadlec *et al.* 2000
	Absorption	X		
	Filtration	X		
	Sedimentation	X		
	Decomposition (anaerobic degradation)		X	
Suspended Solids (SS)	Filtration	X		Idris *et al.* 2010; Kadlec *et al.* 2000
	Sedimentation	X		
Nitrogen	NH3 volatilization	X		Vymazal 2007; Tee *et al.* 2009; Kadlec *et al.* 2000
	Nitrification		X	
	Denitrification		X	
	Nitrogen fixation		X	
	Biotic uptake		X	
	Nitrate reduction to ammonium		X	
	Anaerobic ammonia oxidation		X	
	Sorption	X		
	Desorption	X		
Phosphorus	Sorption	X		Vymazal 2007; Kadlec *et al.* 2000; Vymazal and Kröpfelová 2008
	Precipitation	X		
	Plant uptake		X	
	Peat/soil accretion	X		
	Sedimentation	X		
Metals	Adsorption	X		Stottmeister *et al.* 2006; Chen, 2011
	Sedimentation	X		
	Biotic uptake		X	
	Cation exchange (REDOX)	X		
	Precipitation	X		
	Coprecipitation	X		
Pathogenic bacteria and viruses	Oxidation	X		Kuschk *et al.* 2012; Idris *et al.* 2010; Kadlec *et al.* 2000
	Exposure to UV	X		
	Sorption	X		
	Activity of root exudates		X	
	Predation		X	
	Retention in biofilms		X	
	Natural die-off		X	

Table 1.3. *Continued*

Group of pollutants/ Parameter	Removal mechanism	PP	BP	References
Emerging pollutants	Microbial Degradation		X	Kuschk *et al.* 2012; Idris *et al.* 2010
	Precipitation	X		
Sulfur	Sulfur cycle in CWs (Dissimilatory sulphate reduction)	X	X	Kuschk *et al.* 2012; Vymazal and Kröpfelová 2008

carbons, cyanides, chlorinated volatile organics and other micro-pollutants (Dhir 2003; Kadlec *et al.* 2000; Vymazal and Kröpfelová 2008). Table 1.4 provides an overview about the toxic compounds from diverse industrial effluents that have been totally or partially removed by applying this type of wastewater treatment.

1.3.6 Constructed wetlands benefits and values

The potential gains of using CWs for wastewater reclamation are considerable due to their simple operation, low implementation costs and efficient removal of wastewater-derived emerging contaminants. Previous studies estimated the use of CWs as tertiary treatment systems resulted in a similar removal efficiency for some emerging contaminants (e.g. pharmaceutical, personal care products, hormones, surfactants, pesticides and herbicides) to advanced treatment systems (Matamoros and Salvadó 2012). In comparison with a conventional wastewater treatment plant (WWTP), the construction costs for a CW are in the same range, not taking into account area requirement. Nevertheless, operation/maintenance costs are lower for CWs compared to conventional systems due to less energy demand and technical devices used. Furthermore, CWs are able to tolerate fluctuations in flow, treat wastewaters with very different constituents and concentrations, and are characterized by a high process stability and low excess sludge production (Haberl *et al.* 2003).

Table 1.4. Treatment of diverse industrial effluents with constructed wetlands.

Type of wastewater	Location	Target pollutants	Removal (%)	References
Mine effluent	Navan, Ireland	Zn	99	Dunbabin and Bowmer 1992
		Pb	60	
		Sulfate	60	
disinfection byproducts in wastewater treatment	Shanghai, China	Chlorinated compounds	>90	Chen *et al.* 2014
Chemical industry	Estarreja, Portugal	Aromatic and nitro-aromatic compounds	>97	Haberl *et al.* 2003
Acid mine drainage	eastern U.S.	Acidity	68	Wieder 1989
		Fe	67	
		Sulfate	8	
Manure Treatment	Ichtegem, Belgium.	N	95,4	Boets *et al.* 2011
		P	95,9	
		COD	79,5	
Agriculture	Hawkesbury Australia	N	44.	Bavor 2010
		P	72	
Emerging Pollutants. Pharmaceutical	-	Anti-inflammatory Drugs	>71	Matamoros and Bayona 2008
		Fragrances	>80	
		Estrogens	83-93	
Landfill Leachate	Florida, U.S.	BOD	77,4	Kadlec *et al.* 2000
		TN	61,3	
Textile industry	Slovenia	Sulfate	62	Bulc *et al.* 2006
		Anionic tensides	87	
		Color	85	
Pulp and paper industry	Chelsea Michigan, USA	BOD	80-90	Vymazal and Kröpfelová 2008
Distillery and winery	Baraha, India	COD	89	Billore *et al.* 2001
		TN	59	
Highway runoff	Newbury Bypass, UK	Cu	67	Vymazal and Kröpfelová 2008
		Cr	70	
		Ni	87	
Petrochemical industry	China	Phenols	85%	Haber *et al.* 2003

Additionally, CWs are an environmentally sensitive approach that is viewed with favour by the general public. They provide numerous benefits in addition to water quality improvement, such as wildlife habitat (wetland organism) and the aesthetic enhancement of open spaces. The capabilities of the CWs in water pollutant removal, together with other ecological services, provide a nature-based technology that supports sustainable resource management (Chen 2011).

1.4 TREATMENT OF DMPs WITH CWs

Despite their palpable benefits, the use of CWs to treat coking wastewater and dimethylphenols has been not widespread and few scientific contributions can be found on relation to that. Jardinier *et al.* (2001) reported on the use of a pilot scale horizontal flow constructed wetland (HFCW) planted with *Phragmites australis* to treat coke plant effluents in France. The authors determined that HFCW may be a valid method to substantially decrease nitrogen concentrations and also to retain some metals and polycyclic aromatic hydrocarbons (PHAs). The first attempt to employ planted sand filters to biodegrade and remove specifically DMPs was made by Rasmussen and Olsen (2004). They identified a positive influence of plants and microorganisms interaction on the removal of DMPs and other creosote contaminants, suggesting that plant uptake and microbial biodegradation act as main involved mechanisms.

Rossman *et al.* (2012) evaluated the performance of CWs cultivated with rye grass to remove nutrients and phenolic compounds from coffee processing wastewater (CPW) (*Lolium multiflorum* Lam.). Removal efficiency of 72% for phenolic compounds was attained. Other studies showed that planted and unplanted wetland systems are able to remove phenol completely (influent initially used was 300 mg/L) for an operational period of 74 days (Tee 2009).

Recently, remarkable investigations about depuration of wastewater containing DMPs with CWs have been undertaken in the UFZ. Poerschmann and Schultze-Nobre (2014) evaluated the DMPs sorption rate onto plant root from a multiphase constructed wetland. Sorption as a removal mechanism for hydrophilic phenols was encountered to be of minor significance (Juergen Poerschmann and Schultze-Nobre 2014). Schultze-Nobre *et al.* (2015) analyzed pointedly the treatment of a DMP mix of isomers (2,6-; 3,4 and 3,5) in a laboratory scaled constructed wetland (Plant Fixed Bed Reactor-PFR). The authors described removal rates for each isomer and concluded that the best system performance can be reached by applying not extreme uplifted DMPs concentrations (removal efficiencies of approximately 100 % when utilizing DMPs concentration up to 40 mg/L). Moreover, it is highlighted the role of aerobic and anaerobic communities under different REDOX regimes to obtain such efficiencies (Schultze-Nobre *et al.* 2015). In addition to these investigation, intermediates and proposed pathway schemes for xylenols degradation in CWs (J. Poerschmann and Schultze-Nobre 2014) and effects of oxidative coupling processes during the treatment of DMPs in a PFR (Poerschmann *et al.* 2014) were investigated.

Although a better understanding about the treatment of xylenols in the PFR has been attained, there is still a lack of knowledge regarding the biodegradation processes carried out inside the CWs rhizosphere. Hence the aim of this study was to evaluate the biodegradation of DMPs in a laboratory scaled constructed wetland planted with *Juncus effusus* by taking into account not only the removal efficiencies and performance obtained from the entire system, but also by analyzing closely the development and behavior of isolated microorganism and indigenous aerobic communities growing inside the PFR. For this, previous isolated microorganisms from the PFR (Curiel *et al.* 2015) were tested to analyze their potential to biodegrade DMPs mix. Further comparisons with biodegradation kinetics of xylenols using *Delftia acidovorans*, an isolated microorganism from a pilot scaled constructed wetland (Blázquez *et al.* 2013), enabled to comprehend relevant

microbiological processes and features comprising lab-scaled and pilot-scaled wetland systems. Additionally, DNA samples from the PFR and *D. acidovorans* were analyzed in order to detect key genes involved in the DMPs catabolic pathways. Upgrading existing knowledge on these biological processes will provide a broader view to appraise feasibility and possible improvements of constructed wetlands dealing with wastewater containing dimethylphenols. Finally, since *D. acidovorans* showed high DMPs removal efficiency, biodegradation tests were extended to treatment of benzene, toluene, ethyl benzene and xylene (BTEX), substances generally present in plumes from petrochemical industries and coke/coal distillatory plants (Harrison *et al.* 2001).

2. GENERAL OBJECTIVE

To evaluate the biodegradation of dimethylphenols (DMPs) in a laboratory scaled constructed wetlands.

2.1 SPECIFIC OBJECTIVES

-To determine dimethylphenols (isomers 2,6; - 3,4- and 3,5-) removal efficiency in a Plant Fixed Bed Reactor (PFR).

-To assess the effect of increased DMPs concentration on physicochemical parameters, wetland plants development and microbial communities from a PFR.

-To analyze the DMPs biodegradation potential of isolated microorganisms from a PFR and compare them with biodegradation rates and kinetic performances of *Delftia acidovorans.*

-To identify and detect catabolic genes involved in dimethylphenols degradation in samples from a PFR and *D. acidovorans.*

3. MATERIAL AND METHODS

This study consisted of two main sections. Firstly, it evaluated the removal efficiencies of dimethylphenol isomers (2,6-; 3,5- and 3,4- DMP) in a planted fixed bed reactor (PFR). The effect of increased DMPs concentrations on the bioreactor was simultaneously analyzed by monitoring its physical, chemical and biological parameters. The second part of this investigation was addressed toward the assessment of DMPs biodegradation kinetics by using microorganisms isolated from the PFR, as well as a microorganism isolated from a pilot scale constructed wetland (*Delftia acidovorans*). Additionally, BTEX biodegradation kinetics of *D. acidovorans* was taken into account due to the excellent performance of this strain to metabolize DMPs.

3.1 PLANTED FIXED BED REACTOR (PFR)

The model experiments were performed in a laboratory-scale wetland reactor under conditions of complete mixing of the pore water by a permanent circulation flow. The internal flow conditions will be comparable to conditions of a continuously stirred tank reactor and, therefore, macro-scale gradients of concentrations will be equalized and the effects of gradient changes could be determined (Wiessner *et al.* 2005). The design and the operation principles of the reactor (PFR- planted fixed bed reactor) were previously described in detail in Kappelmeyer *et al.* 2002.

The bioreactor (figure 3.1) consisted of a cylindrical glass vessel (30 cm high and a diameter of 30 cm) which enclosed a perforated steel basket (28 cm high and a diameter of 26 cm). Inside this metallic basket was placed roughly 20 kg of gravel (particle size of 2-4 mm) and a central cylinder of stainless steel from where treated water flowed directly toward a level control system (magnetic valve) regulating water level and amount of effluent discharged (outlet). The pore water forming the circulation flow was eventually

pumped by a periplastic pump into a distribution ring located outside the gravel basket and above the surface of water. The feeding inflow of the PFR was stored in a 13 L glass container (kept in dark) and mixed with nutrients and target contaminants by a syringe pump. The result affluent was then pumped by a valveless wobblen-piston pump into the inlet ring.

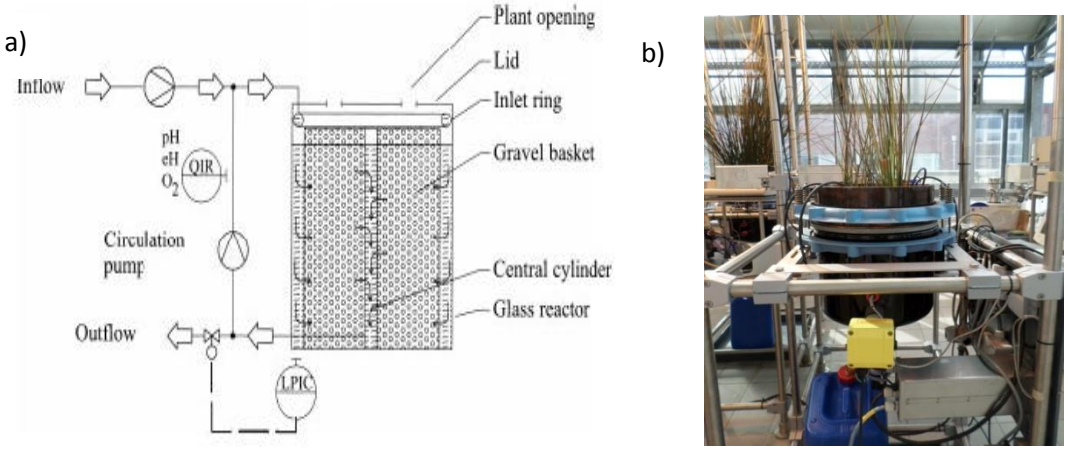


Figure 3.1. a) Components of a Planted Fixed bed reactor (PFR). LPIC= Level control wit soil pressure sensor. QIR= Display and logging of pH, REDOX potential (eH) and dissolved oxygen concentration (O_2) (Kappelmeyer *et al.* 2002). b) PFR located in the UBT/UFZ greenhouse.

3.1.1 PFR Setup

J. effusus was planted and situated according to the opening spaces from a Teflon lid that covered supporting material (in order to avoid water evaporation). The pore water volume in the planted bed was adjusted to 10 L and the hydraulic retention time was set to 5 days with a constant inflow rate of 2 Liter per day. The recirculation flow rate was approximately 20 L per day. The reactor was located in a greenhouse and operated under defined environmental conditions to simulate an average summer day in a moderate climate (Figure 3.1). The temperature will be set to 22 °C from 6 am to 9 pm to simulate daytime and to 16 °C at night. One lamp (Master SON-PIA 400 W, Phillips, Belgium) will be

switched on during daytime as an additional artificial light source whenever the natural light falls below 60 klx (Wiessner *et al*. 2007).

3.1.2 Artificial wastewater composition and experimental conditions

The artificial wastewater feeding the PFR comprised a defined concentration of salts and trace minerals (TMS, see Annex 1). Moreover, A mix of 2,6-, 3,4- and 3,5- dimethylphenols in equimolar ratio with varying concentrations was used as a sole organic carbon source to test the PFR removal performance and behavior (table 3.1). Three different experimental phases (A, B, C) of increased DMPs concentration (10, 20 and 30 mg/L respectively) were evaluated in the PFR during a running time of approximately 3 months. Owing to photo-oxidation of DMPs, measurement errors and possible microbial degradation in the feeding pipelines, the aimed concentrations of DMPs isomers in the inflow were not reached. Percentage of compound Losses and average real concentration of DMPs mix were calculated for each regime and presented in table 3.2.

Table 3.1. Composition of the artificial wastewater (Adapted from Gieseler et al. 2014)

Compound	Inflow concentration (mg/L)
DMPs	10-30
$NaHCO_3$	252
K_2HPO_4	24.8
NaCl	4.7
NH_4Cl	79.5
$CaCl_2*2H_2O$	2.7
$MgCl_2*6H_2O$	1.1
Na_2SO_4	0.6
TMS (Appendix 1)	1 (mL/L)

Bastow *et al.* (2003) compared the photo oxidation rate for five DMPs isomers exposed to sunlight and observed that xylenols concentration decreased rapidly under environmental conditions. This natural phenomenon together with human errors and possible DMPs biodegradation in the inlet pipeline could explain the substantial DPMs losses in this experiment.

Table 3.2. Experimental conditions for a PFR treating dimethylphenols.

Experimental phase	Time period (days)	Expected concentration of DMPs mix (mg/L)	Average real concentration of DMPs mix (mg/L)	Compounds losses (%)
A	March 16 to April 13, 2015 (28 days)	10	7.59 ± 0.87	24,15
B	April 13 to May 8, 2015 (26 days)	20	15.37 ± 1,99	23.15
C	May 8 to June 3, 2015(26 days)	30	26.86 ± 3.11	10.47

3.1.3 PFR maintenance, water sampling and instrumental analysis

The PFR was fed with artificial wastewater containing DMPs mix every Monday and Friday. To start with, 10 Liters of DMP synthetic water was set on circulation every Friday. Consequently, the inflow glass container was refilled up to 10 L every Monday to provide enough water to supply the CW system. Input (based on the inflow leftover) and output (effluent) were constantly measured every maintenance day in order to calculate water losses (transpiration) by mass balance. The total number of plant's shoots was counted once per week along the entire experimental period.

The sampling campaign was conducted every week (Wednesday) by taking around 8 mL of water from the inlet and outlet pipelines. As soon as pore water samples were collected from the PFR, varied analytical methods were utilized to analyze DMPs concentration,

nitrogen species content, and dissolved organic carbon. Worthwhile to mention that dissolved oxygen; REDOX potential and temperature were directly determined and read onsite.

3.1.3.1 Dimethylphenols quantification

HPLC samples were previously filtered with acrodisc syringe filters (0.2 µm), mixed with 50 µL, sodium azide to inhibit microbial growth and stored at 10 °C in a cold room. The analysis of DMPs was performed using an HPLC prominence line (Shimadzu, Japan) equipped with a Poroshell 120 column EC-C18 2.7mm 3.0mm_150mm (Agilent Technologies, USA), and a security guard Poroshell 120 EC-C18 2.7mm 3.0mm_5mm (Agilent Technologies, USA). The column was operated at a temperature of 50 °C. The mobile phase consisted of 0.1% (v/v) formic acid, and 0.1% (v/v) acetonitrile in water (Eluent A), and acetonitrile (Eluent B). The gradient program was as follows: 20% B to 40% B (45 min). The flow-rate was 0.2mL/min. Spectra were recorded from 190 to 600 nm (Schultze-Nobre *et al.* 2015). The chromatograms evaluation was made with a LCSolution's Postrun analysis software.

3.1.3.2 Nitrogen species quantification

The determination of nitrogen compounds was performed by colorimetric methods using a Photometer NOVA 60 A Spectroquant® (Merck KGaA) and appropriate test kits listed in Table 3.3. Due to unsteadiness of nitrogen species in water samples, these were immediately analyzed after sampling.

Table 3.3. 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 ES	690	2.0-75
NO_3^--N	Merck No. 1.09713.0001	DIN 38405 D9	340	1.0-25
NO_2^--N	Merck No. 1.14776.0001	DIN EN 26 777 D10	525	0.02-1

3.1.3.3 Dissolved organic carbon

Samples to be analyzed were filtered by an acrodisc syringe filters (20 µm), mixed with sodium azide and stored at 10 °C in a cold room. Dissolved organic carbon was measured by multi N/C® 2100S TOC analyzer from Analytik Jena AG (Germany). High temperature combustion up to 950°C was implemented to convert all carbon to carbon dioxide with oxygen, which served as a carrier gas. Focus radiation NDIR was the detector for CO_2 with a measuring range of 0-30,000 mg/L C; multiWin® software was used.

3.1.3.4 Dissolved oxygen, pH and redox potential

Redox potential (Pt-metal redox potential electrode; Jumo/Germany; 2ME-2G-PtK^{-1}), pH (A pH-electrode Jumo/Germany; 2GE-2-G-U) and dissolved oxygen concentration (membrane electrode, Siemens /France; Sapin 34, 7MA3100-8EF) were continuously measured in the circulation flow. PFR is equipped with such electrodes enabling to assess those physicochemical parameters onsite.

3.2 DIMETHYLPHENOLS AND BTEX BIODEGRADATION

In this section, DMPs biodegradation by microorganism isolated from the PFR and *Delftia acidovorans* were assessed over the time.

3.2.1 Enrichment, bacterial isolation and identification

Microorganism and consortiums from the PFR were obtained in previous investigations by selective enrichment (Curiel *et al.* 2015). Briefly, microorganism from roots and gravel (around 2 grams) were collected and inoculated with 50 mL of liquid medium M9 (Juretschko *et al.* 1998) (table 3.4) with added dimethylphenols mix (2,6-; 3,4- and 3,5-isomers at 70 mg/L) as a sole organic source. Inoculated medium were then incubated at 30 °C under aerobic conditions and agitation for a period of 13 days. Enrichment cultures were eventually subcultured on fresh medium in an interval of one week and isolated by serial dilutions method. Plates with M9 medium and DMPs mix (Sigma-Aldrich) were inoculated and incubated at 30 °C by using the aforementioned dissolved microbial cultures to obtain purified colonies.

Table 3.4. Components for the medium M9 (Juretschko *et al.* 1998).

Solution	Quantity for 1L
M9 10X*	100 mL
Solution A*	1,25 mL
Solution B*	650 µL
Solution C*	650 µL
DMPs mix (2,6- 3,4 and 3,5)	70 mg/L
Distillated water	Complete to 1L
Agar-Agar (Plates)	18 grams

*See appendix 2

To identify the pure strains, one to three isolated colonies were taken and dissolved separately in 60 µL of DNA water (Sigma-Aldrich) for an optimal concentration of bacteria biomass. DNA extraction was carried out by using DNeasy® Blood and Tissue kit from Qiagen, Germany. HotStarTag® DNA polymerase and HotStarTag® master mix kit (Qiagen, Germany) amplified the region 16S ribosomal DNA gen using universal primers 27f. The optimal cycling protocol for PCR was modified as shown in appendix 3 (Villarraga *et al.* 2014). After PCR, DNA was purified and quantified in order to be finally sequenced by GATC Biotech in Germany. Sequences were analyzed and compared with information found on National center of information technology.

On the other hand, *Delftia acidovorans was* isolated by the Group of Microbial Process of the Environmental Biotechnology Department at UFZ using pore water of a pilot scale constructed wetland treating effluents from one of the largest chemical industries located in Leuna, Saxony, Germany. Isolation and identification was carried out as was described above for samples from the PFR.

3.2.2 Biodegradation of dimethylphenols

As first step, isolated bacteria on the right stage of growth (log phase) were attained by culturing in nutrient broth. Growth and conservation of biodegradation activity for strains from the PFR were checked firstly using the following media with DMPs mix: M9, M9 plus yeast extract and sodium succinate. Differently, *Delftia acidovorans* was cultured in LB medium to gain microbial mass as it proved its capability to consume DMPs after having been grown in this medium. To obtain inoculums, the target microbes were inoculated in their appropriate culture medium and incubated at 30 °C under aerobic conditions and continuous stirring for 2 or more days, depending on perceived turbidity. The cells were harvested by centrifugation (7500 rpm, 10 min, 4°C) in a centrifuge HERMLE z 383 K and re-suspended two times in 2 mL of NaCl 0.85%.

Prepared inoculums for all isolates were fixed to an optical density (O.D) value of 0.05 (at 620 nm) into 50 mL of medium M9 containing different DMPs mix at 70 mg/L as sole organic carbon source. A mix of 2,6-; 3,4- and 3,5- dimethylphenols was evaluated for microorganism from the PFR (same composition that synthetic wastewater fed into the bioreactor). However, bodegradation kinetics of *Delftia acidovorans* were carried out with dual DMPs mix consisting of 3,4- dimethylphenol in combination with each DMP isomers (2,3-; 2,4-; 2,5-; 2,6- and 3,5- DMP). The purpose of this analysis was to draw conclusions about cometabolism of dimethylphenols by *D. acidovorans.* Single isomers biodegradation was formerly studied by Curiel *et al.* (2015) and Schmechta (2014). Controls were always set up to track possible non-biological degradation/transformation of DMPs.

Inoculated cultures with M9 and DMPs were incubated at 30°C with constant agitation assuring oxic conditions (air injection every 3 days). The rate of DMPs consumption was monitored by measuring bacterial growth and DMPs concentration every 24 hours or 48 hours (according to culture development). Optical density was measured by using a Perkin Elmer LAMBDA 2 UV/Vis spectrophotometer equipped with photo-diode detector and standard disposal cells of 1.5 mL. Due to photo-oxidation of DMPs and changes in cultures color, microbial growing of some samples was analyzed in a cell-counter equipment Beckman coulter DD III (number of cell per mL).

DMPs concentration was determined as described above in section 3.1.3.1. Nevertheless, the range of DMP isomers to analyze was wider than in samples from the PFR. Calibration curves that led to calculate DMP concentrations are shown in appendix 4. Each isomer separated and detected for this analytical method has a different retention time (table 3.5) that allows their identification and quantification.

Table 3.5. Retention times (RT) of dimethylphenol (DMP) isomers analyzed by HPLC method.

DMP Isomers	RT (min)
2,3	14,255
2,4	14,806
2,5	14,577
2,6	15,325
3,4	12,841
3,5	13,773

3.2.3 Biodegradation of BTEX with *D. acidovorans*

Experiments to obtain BTEX biodegradation kinetics proceed as outlined in section 4.2.1 and 4.2.2. However, benzene, toluene, ethyl-benzene and xylene were assessed separately as a sole carbon source for *D. acidovorans*. The initial concentrations to test these volatile organic compounds were 50 mg/L and 20 mg/L. They were monitored over the analysis time by gas chromatography. Furthermore, O.D was continuously measured in the inoculated samples with BTEX.

3.2.3.1 Identification of benzene by Gas Chromatography (GC) with Flame Ionization Detector (FID)

Samples of 1 mL were taken from inoculated cultures with BTEX and placed into serum vials with 50 µL of phosphoric acid 50% and sealed with rubber caps and teflon coated aluminum cover. Samples were stored at -20°C until respective measurements. BTEX concentrations were quantified by employing a HP6890 gas chromatograph (Agilent Technology) equipped with an Agilent DB-MTBE column (30 m * 450 µm and 2.55 µm film thickness). The temperature program was: 40 ° C for 2 min and then a temperature of 200 °C was reached with a ramp of 12 °C/min. FID detection temperature was 280 ° C. The carrier gas helium had a flow of 5.8 ml / min. Injection was automated using an integrator DANI HSS 86.50. The auto sampler pre-heated the samples for 30 min at 90 ° C. Proper

controls were processed like GC samples in order to prove no volatilization (false positive) of the target volatile organic compounds

3.3 ANALYSES OF GENES INVOLVED IN CATABOLISM OF AROMATICS COMPOUNDS

A set of genes encoding enzymes involved in the biodegradation pathways of aromatics were evaluated in water samples from the PFR (one per month in experimental phases A, B, C), gravel-root consortiums (Curiel *et al.* 2015) and *Delftia acidovorans* by PCR. The full list of degenerated primers, positive controls and sequences (5'-3') are presented on table 3.6. DNA extraction was carried out by using DNeasy® Blood and Tissue kit from Qiagen, Germany. HotStarTag® DNA polymerase and HotStarTag® master mix kit (Qiagen, Germany) amplified the DNA sequences initiated and marked by the degenerate primers mentioned in table 9. Base pairs sizes were checked and compared with a low range DNA ladder FastRuler® (From 50 pb up to 1500 pb) on gel-agarose 1.5 %. The optimal cycling programs varied in each PCR analysis. Cycling protocols are depicted in detail on appendix 5.

Table 3.6. Degenerate primers used to analyze genes involved in catabolism of aromatics compounds.

Control +	Primers	Target	Enzyme Substrates	Sequence (5'-3')	Size (pb)	Reference
P. putida MT-2	EXDO-D	C23O Subfamily I.2.C of extradiol dioxygenases	Clorobenzene, toluene degradation	F= AAYCCBGABCCNTGGCCNGA R=GTYTSVCCNCGBGTVADVCCRTGRCG	380	Brennerova *et al.* 2009
AETG-14	Tbu-E	TbuE (U20258) of subfamily I.2.C of catechol extradiol dioxygenases	BTEX	CTGGATCATGCCCTGTTGATG CCACAGCTTGTCTTCACTCCA	444	Hendrickx *et al.* 2006
	TmoA	Subfamily 2 of a-subunits of hydroxylase component of multi-component monooxygenases	Toluene	CGAAACCGGCTT(C/T)ACCAA(C/T)ATG ACCGGGATATTT(C/T)TCTTC(C/G)AGCCA	505	Hendrickx, Dejonghe, *et al.* 2006
	PHE (H)	Phenol monooxygenase	Toluene, methylphenols	F =5'- GAYCCBTTYCGYHTRACCATGGA R = 5'- GGCARCATGTARTCCWKCATCAT		Martínez-Lavanchy *et al.* 2015
P. putida MT-2	TOL	Xylene monooxygenase (in TOL plasmid)	Toluene, o- and p-xylene	F=TGAGGCTGAAACTTTACGTAGA R=CTCACCTGGAGTTGCGTAC	475	Baldwin *et al.* 2003
	EXDO-A	C23O genes encoding subfamily I.2.A extradiol dioxygenases	Monoaromatics	F=ATGAAVAAAGGHGTWHTGCGHCCMGG r1= GYGGCCADGCYTCNGGRTTG r2=ATRTCVAKVGADGTRTCGSTCATG	430 730	Brennerova *et al.* 2009
	C12O	C12O	Aromatic compounds	F=GCCAACGTCGACGTCTGGCA R=CGCCTTCAAAGTTGATCTGCGTGGT	20 25	Sei *et al.* 1999
P. putida F1	TodC	Subfamilies D.1.B+D.1.C+D.2.A+D.2.B+D.2.C of a-subunits of type D iron–sulfur multi-component aromatic dioxygenases		CAGTGCCGCCA(C/T)CGTGG(C/T)ATG GCCACTTCCATG(C/T)CC(A/G)CCCCA	510	Hendrickx *et al.* 2006
	EXDO-L	catechol 2,3-dioxygenase encoding genes of Proteobacteria related to TodE	Toluene	GACCAGGGSWTVGGYCACTA TTRTGNCCCCAGATGCTGAT	380	Brennerova *et al.* 2009
AETG 14	TbmD	Subfamily 1 of a-subunits of hydroxylase component of multi-component monooxygenases	Phenolic compounds	GCCTGACCATGGATGC(C/G)TACTGG- -CGCCAGAACCACTTGTC(A/G)(A/G)TCCA	640	Hendrickx, Dejonghe *et al.* 2006

P. putida: *Pseudomona putida*; AETG-14, AET6 14: *Ralstonia sp*

4. RESULTS AND DISCUSSION

As stated in previous chapters, this study was divided in two main sections: The PFR performance when dealing with synthetic water containing a mix of DMPs and aerobic degradation of DMPs by microorganisms isolated from CWs.

4.1 TREATMENT OF DMPs IN A PLANT FIXED BED REACTOR (PFR)

This experiment was carried out under a regime of increase DMPs concentrations. Bioreactor behavior in such conditions is explained in detail as follows.

4.1.1 Effect of DMPs increased concentrations on *Juncus effusus*

Transpiration is one of the main growth factors governing the development of plants. In figure 4.1, this physiological parameter was related with number of *J. effusus* shoots in order to reveal the effect of DMPs increased concentrations on wetlands plants conditions. Transition from experimental phase A to B (from 7.59 mg/L to 15.37 mg/L) did not produce any disturbance for plants growth. Experimental phase B was undergone during spring time which guaranteed the emergence of new shoots and with that a slight augmentation on the mean transpiration rate. Out of range values of transpiration (day 26) can be attributed to weather conditions such as temperature, relative humidity and level of light (Lucas *et al.* 2013). Contrary, plant growth inhibition was exhibited along the transition from 15.37 mg/L to 28.86 mg/L, reaching the lowest number of plant shoots and transpiration rate in the last week of DMPs treatment. Reduction of plant growth is certainly reflected on deficiencies of *J. effusus* to carry out a proper water management, nutrients plant uptake and decrease of radial oxygen loss (ROL) around roots (Wiessner *et al.* 2013). Helophytes transfer oxygen from the atmosphere to the rhizosphere via special

tissues (aerenchyma) controlled by the water management (Smith and Luna 2013). A stoppage in oxygen transport into the rhizosphere will definitely bring along changes in dynamics of anaerobic and aerobic microbiological processes occurring inside the PFR (Wiessner *et al.* 2013). This assumption was demonstrated later on by analyzing the trend of nitrification processes during experimental phase C when *J. effusus* growth was evidently inhibited.

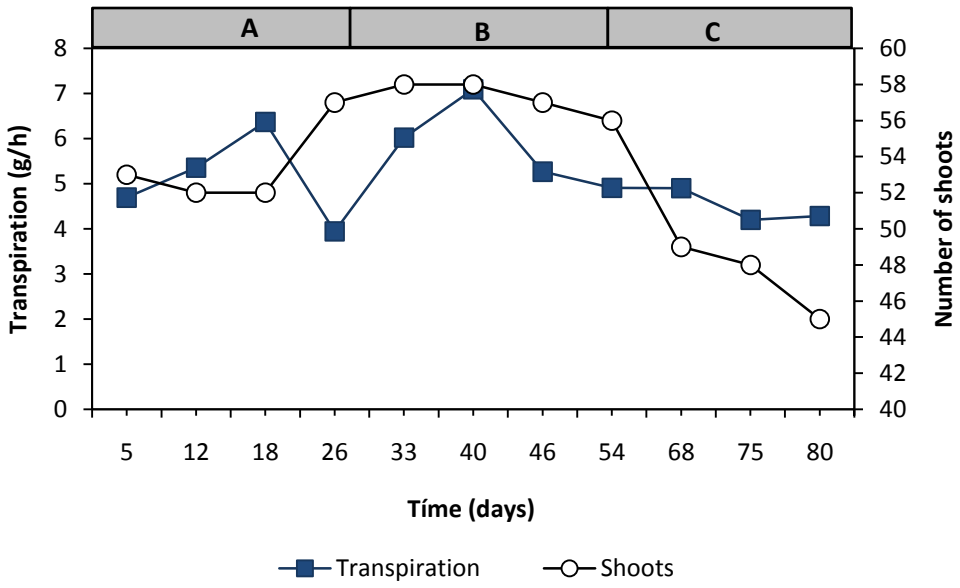


Figure 4.1. Transpiration (g/h) and number of shoots in a PFR during three experimental phases of DMPs removal treatment (A =7.59 mg/L, B= 15.37mg/L and C=26.86 mg/L).

4.1.2 Nitrogen dynamics, redox conditions and pH

In order to understand the entire PFR performance, it is worthwhile to mention that the bioreactor was under reductive conditions before this research had started. Therefore, low redox potential (Eh) values were observed at the beginning of the measurements while the system established oxidative conditions at low organic carbon load of DMPs. Along the experimental phase A (figure 4.2), recognized for having the lowest DMPs con-

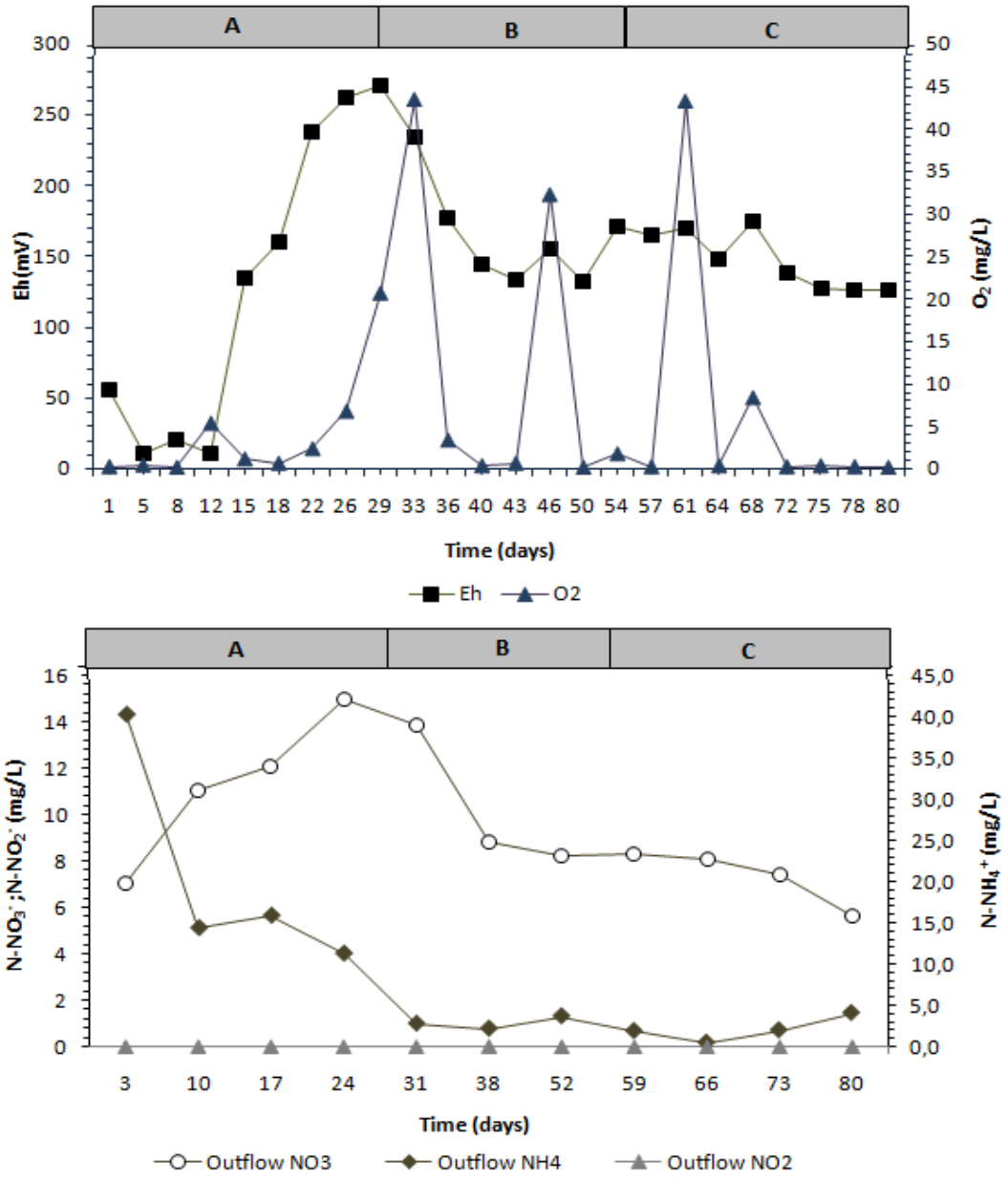


Figure 4.2. Relationship of redox potential (Eh) and dissolved oxygen (O_2) with outflow-Nitrogen species concentrations ($N\text{-}NO_3^-$=nitrate, $N\text{-}NH_4^+$=ammonium and $N\text{-}NO_2^-$=nitrite) in a PFR for 3 experimental phases of DMPs removal treatment (A =7.59 mg/L, B= 15.37 mg/L and C=26.86 mg/L).

centration (7.59 mg/L), the redox potential and oxygen dissolved tended to increase up to a maximum Eh of 270 mV and O_2 levels of 43,4 mg/L. Nevertheless, as soon as DMPs

concentration was increased (Phases B and C), those values started dropping in response to a higher oxygen consumption involved in the aerobic microbial mineralization. Simultaneously, some fluctuations in dissolved oxygen along the whole evaluation period were evidenced possibly due to the degree of light reaching the plant leaves which influences directly the gas transport and oxygen release by the roots of helophytes (Wiessner *et al.* 2005).

The pH value during the operation time was an average of 4.63 ± 0.34. Rhizosphere acidification might have been caused by a combination of several processes, including root release of protons and organic acids microbial oxidation of organic matter and a poor buffer capacity (Braeckevelt *et al.* 2011).

In general, integrated positive redox potential values were predominant over the experimental period. Moreover, a clear relationship among ammonium and nitrate concentration with redox potential was observed along the DMPs treatment process. As depicted in figure 4.2, the outflow concentrations of ammonia decreased drastically in the phase A when the system was turning out toward oxic/oxidative conditions and higher Eh values (From 55 mV up to 270 mV, see raw data information in appendix 6). This trend of ammonium removal was obviously accompanied by a subsequent increment of nitrate owing to microbial nitrification processes carried out under such an aerobic environment. Nitrification in oxygen-enriched layers of the rhizoplane seems to be relatively firmly established, including under moderately reduced overall redox conditions in the water (Eh<200 mV) (Wiessner *et al.* 2005). In parallel, the observed ammonium reduction might be also caused by NH_4^+ plant uptake that is also well known as an essential pathway for nitrogen removal in CWs using *J. effusus* (Wiessner *et al.* 2013).

Experimental phases B and C of increased DMPs concentration were followed by a significant decline of outflow nitrate levels. This phenomenon occurred possibly due to

two principal causes: denitrification and depletion of oxygen as a substrate for nitrification process. Even though denitrification is a microbial process that commonly transforms NO_3^- to N_2 in anaerobic or low oxygen concentration environments, there has been scientific evidence about denitrification arising in the presence of O_2 (Fazzolari *et al.* 1998). The so called aerobic denitrification or co-respiration implies the simultaneous use of both O_2 and NO_3^- as electron acceptors in respiration and can be performed by various genera of microorganisms. This process was formerly determined in the study of nitrogen transforming microbial communities in a HFSSCW (Coban *et al.* 2015). A second probable factor leading to the decrease of nitrate levels is the effect of DMPs on the plant health. In particular, the last part of this experiment (phase C) was recognized by a growth inhibition of *J. effusus* and reduction of transpiration rate. Helophytes supplies oxygen for ammonium oxidation by gas transport through the aerenchyma (Wiessner *et al.* 2013). Therefore, a physiological plant damage caused by DMPs concentration might be a determinant element on the abatement of nitrate due to depletion of oxygen as substrate in nitrification. Finally, the monitored low concentrations of nitrite in the outflow (0.036 ± 0.012 mg/L) were produced by the complete turnover of NH_4^+ to NO_3^- during the nitrification process.

4.1.3 Removal efficiency of dimethylphenols (DMPs) and dissolved organic carbon (DOC) in a PFR

The inflow and outflow concentrations of a DMPs mixture (3,4-; 3,5- and 2,3- DMP) and COD were measured over a period of time of 3 months and are presented in figure 1.2. The PFR was found to be successful in removing 99.08 % (taking into account the detection limit of DMPs in HPLC equipment) of DMPs fed along the different experimental phases. Despite this, the transition from B to C was characterized for having some weeks in which removal efficiency varied around 94.9 % and 98.3%. Schultze-Nobre *et al.* (2015) reached the same conclusion about high efficiency of DMP removal under low DMPs loads

(up to 40 mg/L) and an evident decrease of the effectiveness at high DMPs concentration regime. Observing more closely the composition of the effluent (Raw date information for DMP and COD concentration during the sampling campaign is shown in appendix 6) enabled to identify that 2,6-DMP is the isomer which was not completely removed. The refractory behavior of this compound could be associated with the position of methyl groups in the phenol aromatic ring which would possibly reduce the action of enzymes involved in aerobic degradation.

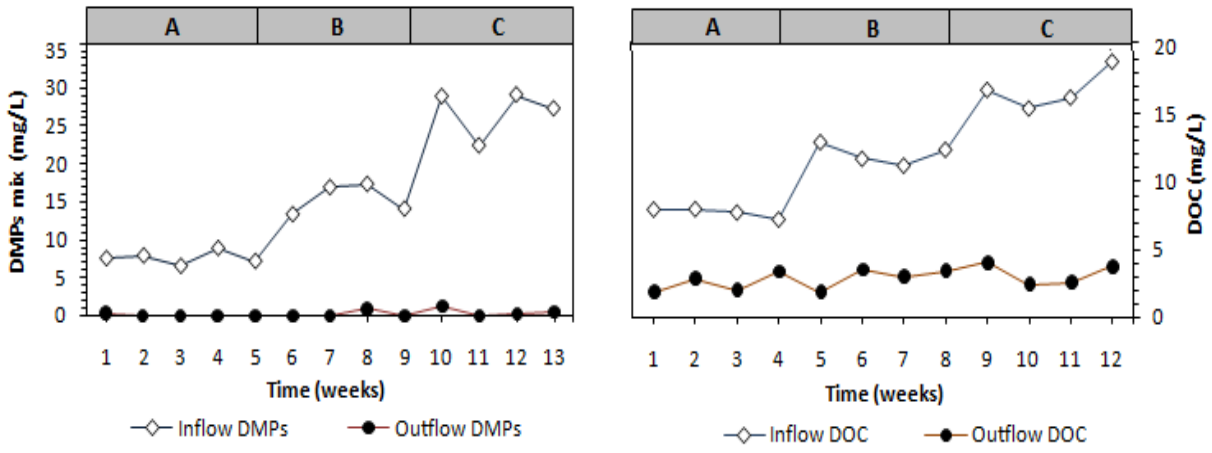


Figure 4.3. Inflow and outflow concentrations of dimethylphenols mix (2,6-; 3,4- and 3,5- isomers) and dissolved organic carbon (DOC) in a PFR during 3 experimental phases of removal treatment (A =7.59 mg/L, B= 15.37 mg/L and C=26.86 mg/L).

Oxidative conditions at low DMPs concentration prevailing during the rector operation time (section 1.2) and were ensured due to internal mixing of water pore (Kappelmeyer *et al.* 2002). Aerobic biodegradation together with phytovolatilization are likely to be the key removal mechanisms comprising the turnover of the target DMPs in the PFR. Dimethylphenols exhibit low biodegradation potential under reductive conditions (Licha *et al.* 2001) which leads to assert that aerobic biodegradation is a fundamental process for DMPs removal in the analyzed system. Volatilization of organic compound circulating inside the bioreactor is not probable to occur owing to the gravel bed that supports plants

is sealed by a Teflon lid (Braeckevelt *et al.* 2011). In contrast, previous reports indicate that plants have an important role in phenols depuration by plant uptake and root immobilization. Glass and Bohm (1971) discovered that simple phenols are able to enter into roots tissues by diffusion and consequently are transported and up taken by plants (Glass and Bohm 1971). The uptake concentrations of phenols and other organic compounds from soil or supporting material, in which plants grown, are dependent on plant variety and plant part, and they showed different uptake concentrations (Al Nasir and Batarseh 2008).

Dissolve organic carbon in the bioreactor is a fraction of the total DMPs concentration due to the PFR was exclusively fed with this organic compound. Hence carbon contain in the DMPs molecule (78.5% C) is directly correlated to the DOC concentration in the inflow. As illustrated in figure 1.3, the increase of DMPs load in each phase (A, B, C) is followed by an increment in the inflow DOC concentration. The PFR effluent contained around 2.91 ± 0.75 mg/L of COD. Considering that DMPs was almost completely removed from the system, the formation of soluble microbial products and products of plant biomass decay (Braeckevelt *et al.* 2011) could explain this source of organic carbon in the effluent. These byproducts could also benefit the DMPs removal. Rasmussen and Olsen (2004) suggested that microbial cometabolic degradation stimulated by plant exudates is important for DMPs and other organic compounds treatment (Rasmussen and Olsen 2004b; Moormann *et al.* 2002).

4.2 AEROBIC BIODEGRADATION OF DMPs BY MICROORGANISM ISOLATED FROM CWs

Pure strains from a PFR and an isolated microorganism from a pilot constructed wetland (*D. acidovorans*) were tested in order to evaluate their biodegradation performances.

4.2.1 Biodegradation of DMPs by pure strains from a PFR

Microorganism isolated from PFR root/gravel samples by selective enrichment culture and serial dilution method were initially cultured in various medium with added DMPs mix (3,4-; 3,5- and 2,6- DMP) in order to monitor their biodegradation potential under this conditions. The identified strains are listed in table 4.1 together with their biodegradation activity. As shown, none of the utilized microorganisms were able to gain microbial mass and degrade the DMPs mixture when they were inoculated in different liquid media containing DMPs as sole (minimum media M9) or complementary (sodium acetate and yeast extract) carbon source. However, Curiel *et al.* (2015) encountered that consortiums isolated from the same root/gravel samples were capable to degrade and grow in medium M9 with DMPs (figure 4.4). Both root and gravel microcosm consume around 100 % of 3,4-DMP and 3,5 DMP in only 7 days.

Table 4.1. Microorganisms isolated from the PFR and their biodegradation activity when using varied mediums enriched with DMPs.

Identity	Growth and degradation of DMPs			
	Solid	Liquid	Sodium Acetate	Yeast extract +
	MM+DMPs	MM+DMPs	MM+DMPs	DMPs
Stenotrophomonas maltophila	+	-	-	-
Mycobacterium gilivum	+	-	-	-
Microbacterium testaceum	+	-	-	-
Xanthomonas hortorum	+	-	-	-
Rhodococcus pyridinivorans	+	-	-	-
Ralstonia eutropha	+	-	-	-
Alicycliphilus denitrficans	+	-	-	-
Burkholderia sp	+	-	-	-
*Pseudomonas putida**	+	NR	NR	NR

*Microorganism S2. NR= No Result, MM=Medium M9, DMPs= dimethylphenols

Results attained for DMPs biodegradation activity by single strains and microcosms strongly suggest that each microorganism making part of the PFR consortiums has an specific role in the biodegradation of DMPs. Individual microbes can metabolize only a limited range of hydrocarbon substrates, so assemblages of mixed microbial populations with overall broad enzymatic capacities boost the removal efficiency of DMPs (Ghazali *et al.* 2004). Ambujon and Manilal (1995) agreed with this assumption on their study "Phenol degradation by a stable aerobic consortium and its bacterial isolates". They concluded that a steady microbial consortium has a better performance during phenol biodegradation than its constituent pure strains.

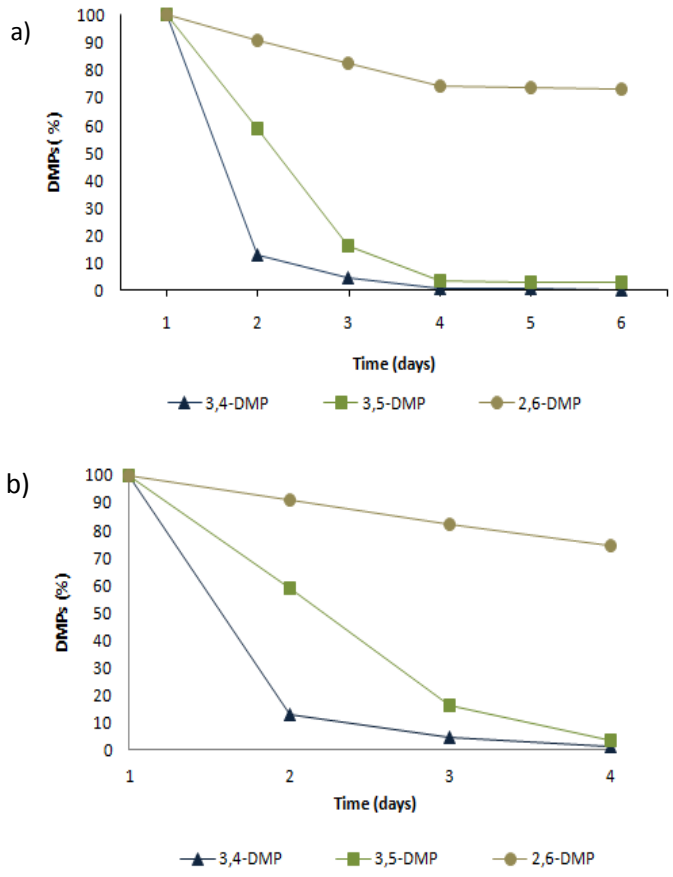


Figure 4.4. Removal efficiency of DMPs (2,6-; 3,4- and 3,5- isomers) by consortiums from PFR root(a) and gravel (b) samples.

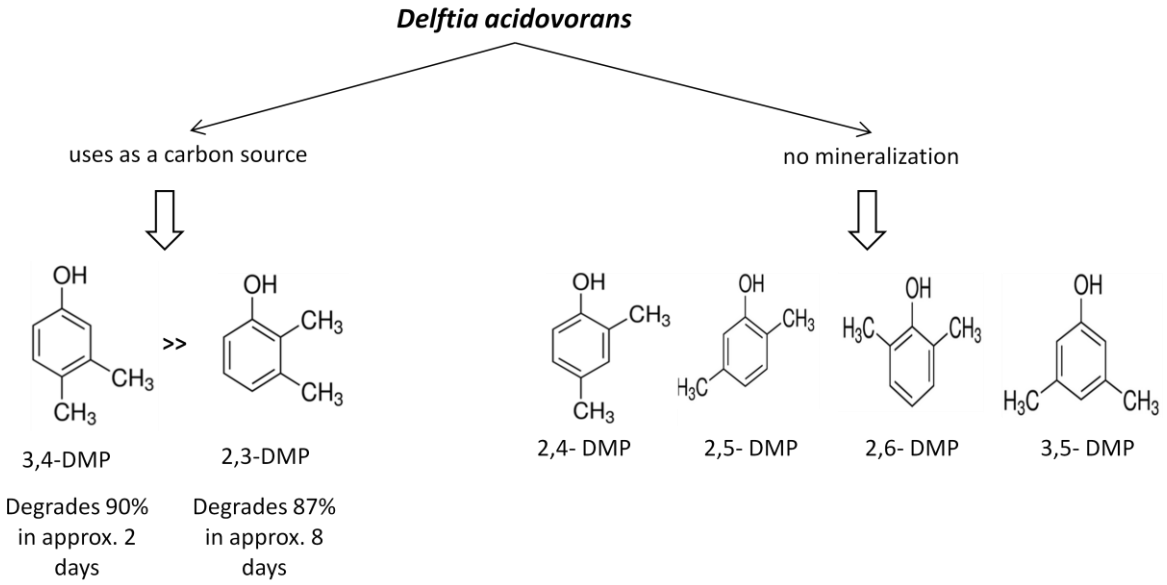
Synergistic interactions among microorganisms comprising roots and gravel microcosm could explain the effective DMPs removal by consortiums in comparison with the performance of single strains. For instance, meanwhile some microorganisms metabolize the toxic compounds and metabolites (that otherwise would reduce microbial activities), other species are acting on other compounds, such as dead-end products and intermediaries, that the formers are able to consume just partially (Ghazali *et al.* 2004). Moreover, the lack of enzymatic activity of bacterial isolates after being cultured in different media might be associated with the lost of plasmids (DNA molecules distinct to chromosome DNA) encoding aromatic degrading enzymes during replication on non-selective media (Villaraga *et al.* 2014; Hopper and Kemp 1980).

Other studies have also proved the advantageous gains of using microbial communities to degrade DMPs. Pitter (1976) evaluated the biodegradability of 123 organic compounds including the studied six dimethylphenol isomers by activated sludge under aerobic conditions at 20°C. According to this investigation, all six compounds were readily biodegrade by the activated sludge microbial community in approximately 5 days with removal rates from 89.3% up to 97.5% (Pitter 1976). Furthermore, Viggor *et al.* (2002) found that the mixed culture of strains *Pseudomonas mendocina* and *Pseudomonas fluorescens* was more effective in the decomposition of DMPs from the mixture of phenolic compounds (3,4-DMP, 2,4-DMP, cresol and phenol) as compared to the single cultures.

4.2.2 Biodegradation of single DMPs isomers by *Delftia acidovorans*

Delftia acidovorans was previously isolated as an effective degrader of DMPs from a contaminated site in Leuna (Blazquez 2013; Schmecta 2014). This bacterium was found to be able to mineralize effectively the isomers 2,3-DMP and 3,4-DMP, with the latter being the most efficient compound when added as sole energy and C-source. Nevertheless, the

other four DMP isomers were not used by this microbe as a sole source of carbon and energy (Scheme 4.1). Furthermore, in comparison with bacteria isolated from the PFR, *D. acidovorans* exhibited a great capacity to tolerate DMPs. This could be due to the adaptation of this wild strain to assimilate toxic compounds from polluted effluents (Schmechta 2014).



Scheme 4.1. Availability (descending order) of single dimethylphenols isomers as a carbon and energy source for *D. acidovorans* under aerobic conditions.

It is well known that six of the five DMPs isomers are metabolized by diverse species from the genus Pseudomonas. *Pseudomonas fluorescens* was reported as an active degrader of 2,4-DMP (Chapman and Hopper 1968). Catabolic pathways for 3,5- ; 3,4- and 2,3- xylenol have been previously described for *Pseudomonas putida* (Hopper and Kemp 1980; Ewers *et al.* 1989). *Pseudomonas alcaligenes* was also studied due to its capability to degrade 2,5-DMP (Poh and Bayly 1980). The most recalcitrant, 2,6-DMP, is mineralized by *Mycobacterium sp* and *Penicilium frequentants* (Hofrichter *et al.* 1995; Ewers *et al.* 1989). Despite all these noteworthy contributions, little has been investigated about the DMPs biodegradation potential of new isolated strains. In fact, to the best of our knowledge the

aforementioned studies carried out in our institute (UBT/UFZ) are the first ones focused on the use of *D. acidovorans* to remove dimethylphenols isomers.

4.2.3 Biodegradation of selected double mixtures of DMPs by *Delftia acidovorans*

As stated previously, 3,4-DMP was the most easily degraded compound as a single isomer followed by 2,3-DMP. Nonetheless, four of the six isomers were not used as a sole carbon or energy source. Hence the purpose of this experiment was to evaluate if *D. acidovorans* could degrade those recalcitrant isomers or boost its biodegradation potential by using mixtures consisting of the no-assimilated DMPs isomers with 3,4 DMP. Kinetics of biodegradation for a total of 5 mixtures (a, b, c, d and e) are presented in figure 4.5. Mixture a (3,4- and 2,3- DMP) is evidently featured by a rapid and high increase of the number of cells in contrast to a vertiginous depletion of the DMPs content. This biodegradation dynamic was expected as both isomers were identified to serve as source of carbon and energy for this microbe. However, it is imperative to mention that 2,3 DMP biodegradation was upgrade in the company of 3,4-DMP by reducing the time in which it is partially metabolized.

For the case of mixtures b, c and e (3,4- and 2,4- DMP [**b**]; 3,4- and 2,5- DMP [**c**]; 3,4 and 3,5 [e]) a moderate growth of the strain was attained. This growth tendency was accompanied by a partial biodegradation of isomers 3,5-, 2,5- and 2,4-DMP (Removal efficiency of 73%, 59% and 41% respectively in 2 days) that was not formerly seen with the use of single isomers. This fact could lead to suggest that those compounds are being cometabolized but not used as a source of carbon or energy to gain biomass. The lowest biodegradation rate by cometabolization was obtained for mixture b. Perhaps, 2,4-DMP exerts a slight toxic effect on *D. acidovorans*, presents a low enzymatic affinity (Blumenthal *et al.* 1986) or generates poisonous dead-end products which also affect the performance of this microorganism to degrade 3,4-DMP (yield is reduced up to 79 %).

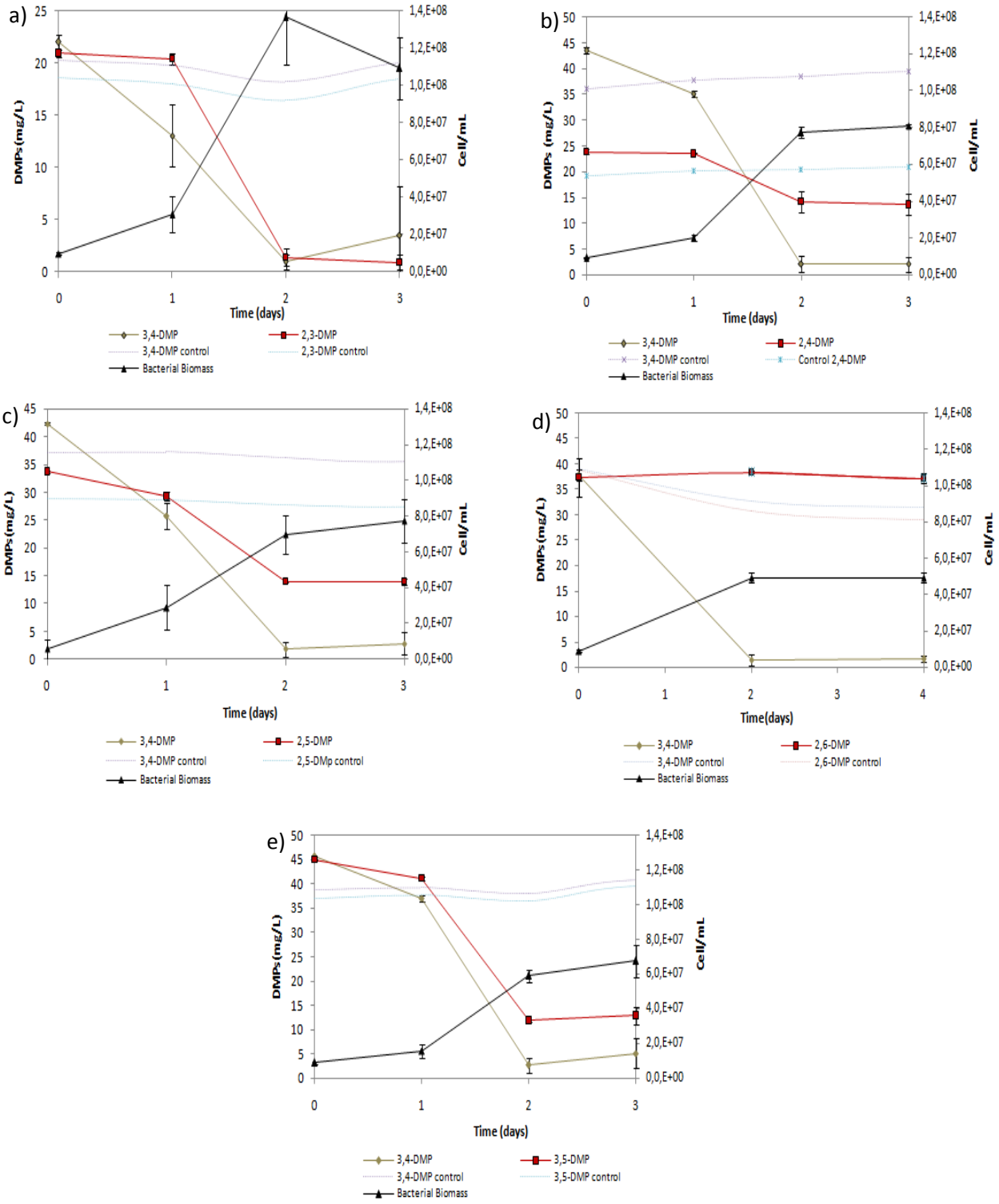


Figure 4.5. Kinetics of biodegradation for *D. acidovorans* metabolizing 3,4- DMP in combination with 2,3- (**a**), 2,4- (**b**), 2,5-(**c**),2,6-(**d**) and 3,5-(**e**) DMPs isomers.

Despite this fact, in the rest of the mixtures, 3,4- DMP was generally assimilated in 2 days with a removal efficiency around 93-97 %. A summary of these results are presented in scheme 4.2.

It is known that yield coefficient for the degradation of aromatics by bacteria is 0.5 (mg dry weight/mg carbon source) and that 10 mg/L of dry mass is equivalent to $3.5*10^7$ cells/mL. Based on these assumptions, the theoretical number of generated cells for one isomer (around 40 mg/L) can be calculated as follows:

$$40\ \frac{mg}{L} DMPs * 0.5 = 20 \frac{mg}{L}\ dry\ mass$$

$$20\ \frac{mg}{L} * \left(\frac{3.5 * 10^7 \frac{cells}{mL}}{10\ \frac{mg}{L}} \right) = 7 * 10^7 \frac{cells}{mL}$$

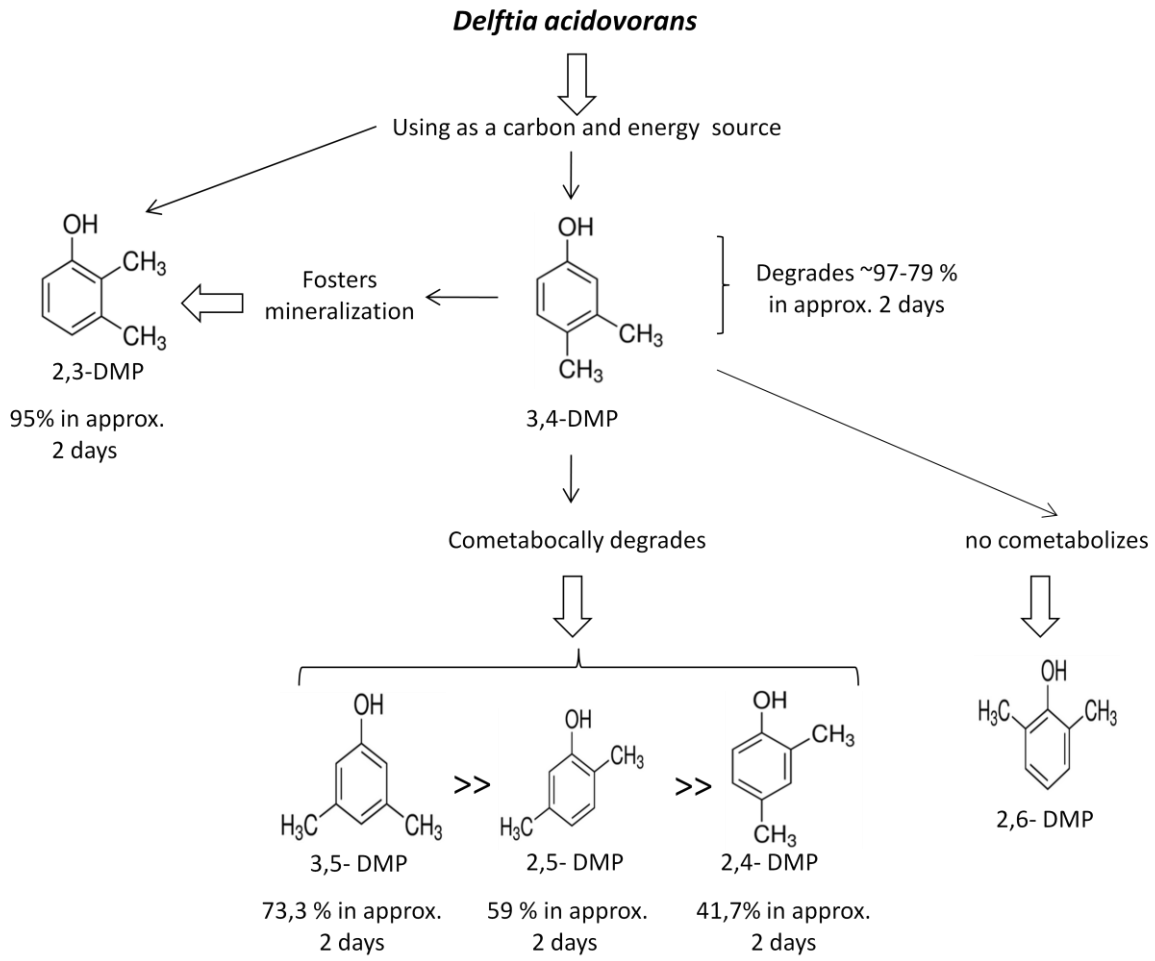
As shown in figure 4.5, mixture a (3,4- and 2,3- DMP) exhibited a microbial growth around $1.4*10^8$ cells/mL which overpasses the theoretical value calculated for mineralization of one isomer. This is clear evidence that both isomers are being used as carbon source. On the other hand, mixtures b, c and d presented a cell growth close to $7*10^7$ cells/mL meaning that just one isomer was mineralized. Since 3,4-DMP was proved to be the growth substrate, 3,5-, 2,4- and 2,5- DMP were removed from the mixtures due to cometabolic transformations.

In brief, 2,4-; 2,5- and 3,5- DMP did not support microbial growth of *D. acidovorans* but instead they underwent cometabolic transformations. This lack of growth is a reflection of the inability of this organism to use these compounds for energy generation or biosynthetic purposes and it is marked in contrast to the increase in population size or biomass when 3,4-DMP was introduced into the mixtures (Reineke 2001). The

cometabolization of the not mineralizable isomers was, therefore, carried out thanks to the enzymes and synthesized cofactors of the growing cells (Fritsche and Hofrichter 2008).

Cometabolic degradation of xylenols has been also assessed for other microorganisms and substrates. Viggorr *et al.* (2003) found out that *P. mendocina* and *P. fluorescens* (wild isolates) could not grow on 2,3-; 2,4-; 2,6-; 3,4- and 3,5-DMP as a single source of carbon and energy. Nevertheless, these strains were capable to cometabolized 2,4- and 3,4-DMP by inducing some aromatic ring cleavage key enzymes with the use of phenol and cresol as growth substrate (Viggor *et al.* 2003). *Penicillium frequentans*, a filamentous fungi, could not assimilate 2,6-dimethylphenol as sole carbon source but after precultivation on glucose and phenol, degradation was activated during a lag-phase of 24 hours (Hofrichter *et al.* 1995). Rassmusen and Olsen (2004) emphasized on the difficulty to remove DMPs in groundwater environments and highlighted the crucial role of microbial cometabolization for their removal.

2,6-DMP was the only isomer not assimilated by *D. acidovorans*, not as a sole carbon source neither in the company of 3,4-DMP. The recalcitrant nature of 2,6-DMP might be related with its chemical structure. Although there are no scientific reports explaining this peculiar behavior, it could be linked to the position of its methyl groups inside the phenol ring. The steric impediment generated around the hydroxyl group in the aromatic ring could somehow affect the bounding of enzymes involved in the biodegradation of aromatic compounds. Likewise, Ewers *et al.* (1989) realized that 2,6-DMP is a critical constituent of multicomponent phenolic wastes, because it is the first compound appearing in the effluent of the continuous culture if the function of the reactor is disturbed. This author also reported that just phenol-adapted microorganism which grew in 2,6- DMP selective media were able to metabolize 2,6-DMP (Ewers *et al.* 1989). Further research is required in order to appraise a possible adaptation of *D. acidovorans* to this substrate.



Scheme 4.2. Summary of *Delftia acidovorans* biodegradation potential (degradation rate in descending order) when using mixtures of 3,4 dimethylphenol with other DMPs isomers (2,3-; 2,4-;2,5- and 3,5- DMP) under aerobic conditions.

4.3 ANALYSES OF GENES INVOLVED IN CATABOLISM OF AROMATICS COMPOUNDS

DNA from water samples of the PFR (recirculated water), *D. acidovorans* and a gravel/root PFR consortium were analyzed by using a set of degenerated primers that enabled to amplify catabolic genes associated with aerobic degradation of aromatic compounds. Target genes encoded different sub-families and units of monooxygenases and dioxygenases. The outcome from this PCR screening is shown in table 4.2. Roughly, it was traced the presence of genes encoding three type of monooxygenases (TmoA, PHE and

TbmD). These results provided us with some clues to propose afterward a partial catabolic pathway for the aerobic degradation of 3,4-DMP by *D. acidovorans.*

To start with, genes amplified by TmoA primer were successfully determined in PFR water samples and *D. acidovorans* (figure 4.6). TmoA-like genes has been shown to be implicated in the degradation of BTEX compounds. These genes encode monooxygenases which catalyze a successive attack of the aromatic ring during aerobic degradation by adding hydroxyl groups and consequently forming catechol derivatives (Hendrickx, Junca, *et al.* 2006).

Table 4.2. Identification of catabolic genes involved in the aerobic biodegradation pathways of aromatic compounds

		Presence of genes in:		
Primers	Target*	Water samples PFR	*D. acidovorans*	Root/gravel consortium
TmoA	Hydroxylase component of toluene monooxygenase	**++**	**++**	**-**
PHE	Phenol monooxygenase	**+**	**+++**	**+++**
TOL	Xylene monooxygenase	**-**	**-**	**-**
TbmD	Hydroxylase component of toluene monooxygenase	**+++**	**+++**	**+++**
TodC	Aromatic dioxygenase	**-**	**-**	**-**
EXDO-D	Catechol 2,3 dioxygenase	**-**	**-**	**-**
EXDO-A	Catechol 2,3 dioxygenase	**-**	**-**	**-**
EXDO-L	Catechol 2,3 dioxygenase	**-**	**-**	**-**
Tbu-E	Catechol 1,2 dioxygenase	**-**	**-**	**-**
C120	Catechol 1,2 dioxygenase	**-**	**-**	**-**

+ = Light signal, **++** = fair signal, **+++** = strong signal, **-** = negative. *See further specifications on section 3.3

Furthermore, genes encoding phenol monooxygenases were favorably found in all three samples (figure 4.6). Phenol hydroxylases belong to the superfamily of 390 soluble diiron monooxygenases, and show a preference for phenol and alkyl substituted phenols as substrates. In principle, this enzyme catalyzes the monooxygenation of phenolic

compounds to converge into catecholic intermediaries that are typically cleaved by extradiol dioxygenases (Martínez-Lavanchy *et al.* 2015).

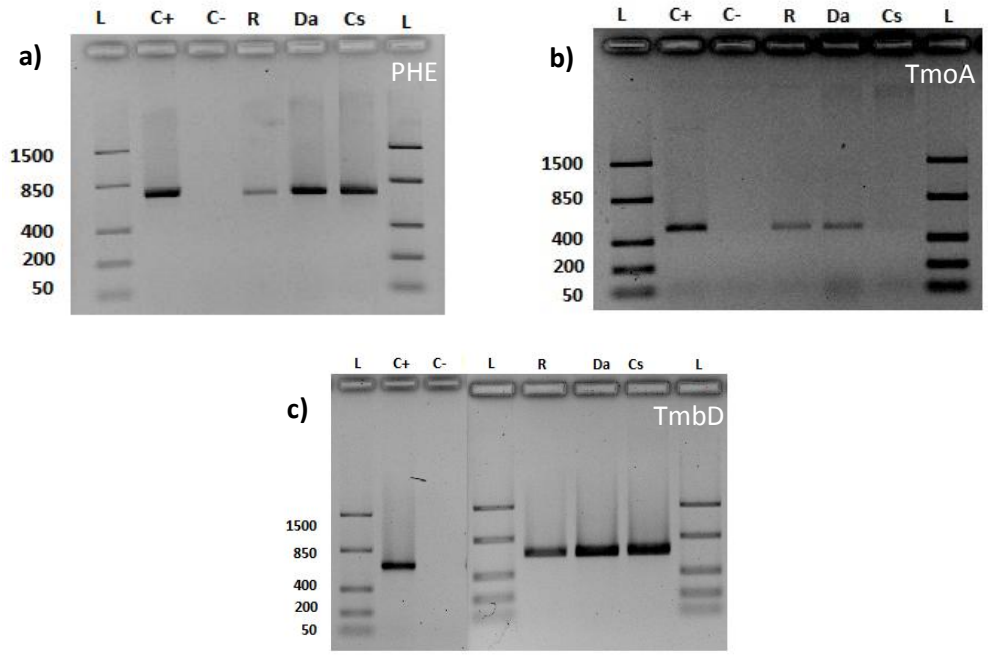


Figure 4.6. Detection by PCR of catabolic genes encoding monooxygenases PHE (**a**), TmoA(**b**) and TmbD(**c**) in water samples from the PFR (**R**), *Delftia acidovorans* (**Da**) and a gravel/root consortium (**Cs**). Ladder from 50 pb up to 1500 pb (**L**), **C+**= Positive control and **C-**= Negative control.

Finally, it was also detected in all tracked samples a monooxygenase amplified by the primer TbmD (figure 4.6). The sub-family for this specific enzyme has been related to phenol hydroxylase. This is responsible for oxidation of o-cresol produced from the initial hydroxylation of toluene, supporting the association of this family with oxidation of hydroxylated substrates (Baldwin *et al.* 2003).

Even though the identification of these genes in the DNA does not guarantee their expression, some conclusions can be drawn from this PCR screening. First, the large diversity of microbial degraders in PFR water samples and consortiums might be expressing monooxygenases as first step of the DMPs aerobic biodegradation in order to

catalyze and foster the activation of the aromatic ring. Secondly, microbial degradation of DMPs (specifically 3,4- and 2,3- DMP which served as growth substrate) by *D. acidovorans* is apparently begun with the hydroxylation of the dimethylphenols ring by monooxygenases in order to induce the establishment of catecholic derivatives.

A noticeable evidence of phenolic oxidation and catechol derivatives formation during biodegradation of 3,4-DMP with 2,5-DMP by *D. acidovorans* is the change of color in inoculated mediums (figure 4.7). A persistent brownish color is commonly linked to accumulation of poly-substituted catecholic intermediaries that are slowly mineralized when interact with the microbe. For this specific case, this change of color could have occurred owing to derivatives from biodegradation of 2,5-DMP which is not mineralized but cometabolically degraded.

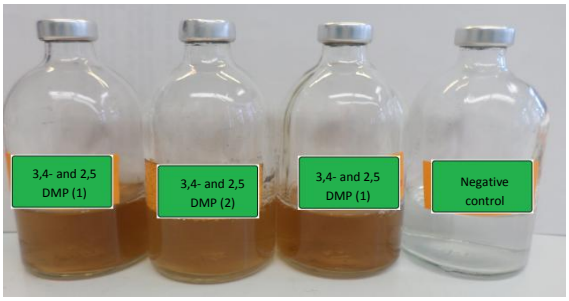


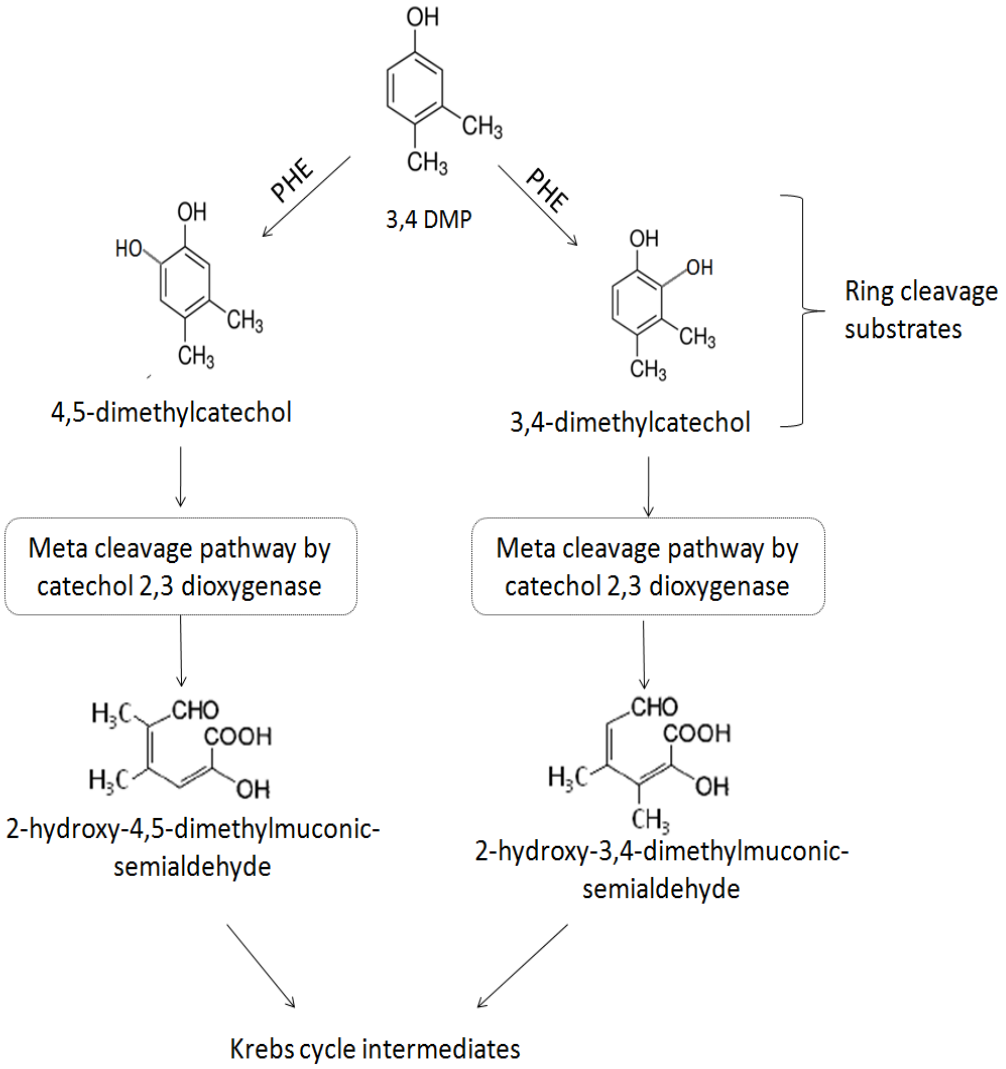
Figure 4.7. Change of color during biodegradation of mixture 3,4- and 2,5- DMP (Replicates 1,2 , 3 and negative control [C-]) by *D. acidovorans*.

Being 3,4- DMP the most readily assimilated dimethylphenol by *D. acidovorans*, we proposed a series of catabolic pathways for the aerobic biodegradation of this isomer by taking into account results and evidences gained from our PCR screening (Scheme 4.3). The mineralization of 3,4-DMP would initiate with the hydroxylation of a neighboring carbon to the hydroxyl group already presented in the DMP molecule. This reaction is catalyzed by a monooxygenase such as phenol monooxygenase-PHE (strong signal in our PCR analysis). Once the diverse catechol derivatives (4,5-dimethylcatechol and 3,4-

dimethylcatechol) are attained, the activated aromatic ring would be cleaved by dioxygenases. Considering that *D. acidovorans* has been formerly reported to undergo the catechol 2,3-dioxygenase cleavage pathway for aniline (Urata *et al.* 2004; Zhang *et al.* 2008), the intermediaries from the ring cleavage catabolism would be 2-hydroxy-4,5-dimethylmuconic-semialdehy and 2-hydroxy-4,5-dimethylmuconic-semialdehy. By last, the ring-cleavage intermediates would be then subjected to subsequent central pathways leading to the formation of Krebs cycle intermediates (Cao *et al.* 2009). Anyhow, the presence of each intermediary proposed above must be confirmed by analytical methods like HPLC. Efforts to identify such intermediaries should be taken in future experiments.

4.4 BTEX BIODEGRADATION BY *D. acidovorans*

Owing to the evident potential of *D. acidovorans* to degrade DMPs, this research was preliminarily extended toward the evaluation of BTEX removal. The gained knowledge in regards to the presence genes encoding enzymes capable to aerobically degrade aromatics led to predict that this microorganism could perform a proper role on the mineralization of single BTEX compounds. For this purpose, growth kinetics of *D. acidovorans* were conducted by using BTEX compounds at 20 mg/L as sole source of energy and carbon. Figure 4.8 depict the results of this analysis. Benzene was effectively removed at a concentration of 20 mg/L. This fact could be of special interest as benzene is known as one of the less easily degraded substance from these volatile organic compounds (Cao *et al.* 2009). Due to benzene volatility, its presence was qualitatively analyzed and monitored by GC/FID in samples and negative controls (blank) during the experiment. Chromatograms were monitored at the beginning and end of this analysis and allowed to confirm the removal of this substance in samples and not volatilization in controls (figure 4.9).



Scheme 4.3. Proposed catabolic pathways for biodegradation of 3,4- DMP with *D. acidovorans* using PHE and catechol 2,3 dioxygenase .

As shown in figure 4.8, *D. acidovorans* is able to grow on most of the BTEX compounds at 20 mg/L with the only exception of xylene. Benzene was assimilated in a shorter period of time. This preliminary work gives way to further research on BTEX degradation by *D. acidovorans* as was proved its biodegradation potential. Future studies must be accompanied by quantitative measurement of each compound (GC/FID), as well as new real-like BTEX mixtures must be evaluated.

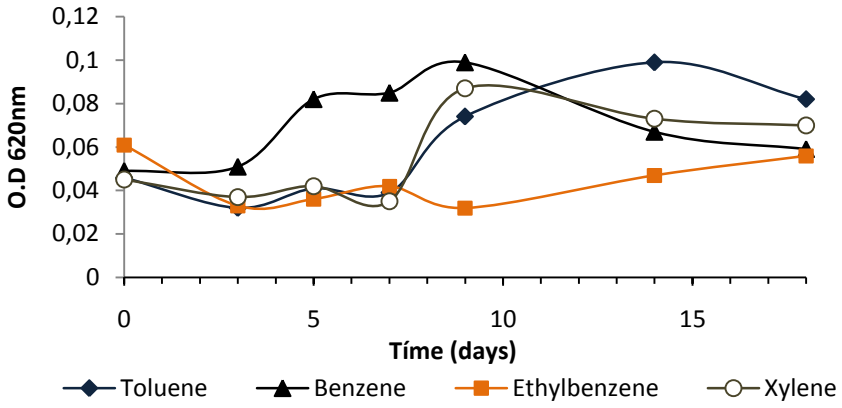


Figure 4.8. Growth Kinetics for *D. acidovorans* degrading BTEX compounds to a final concentration of 20 mg/L.

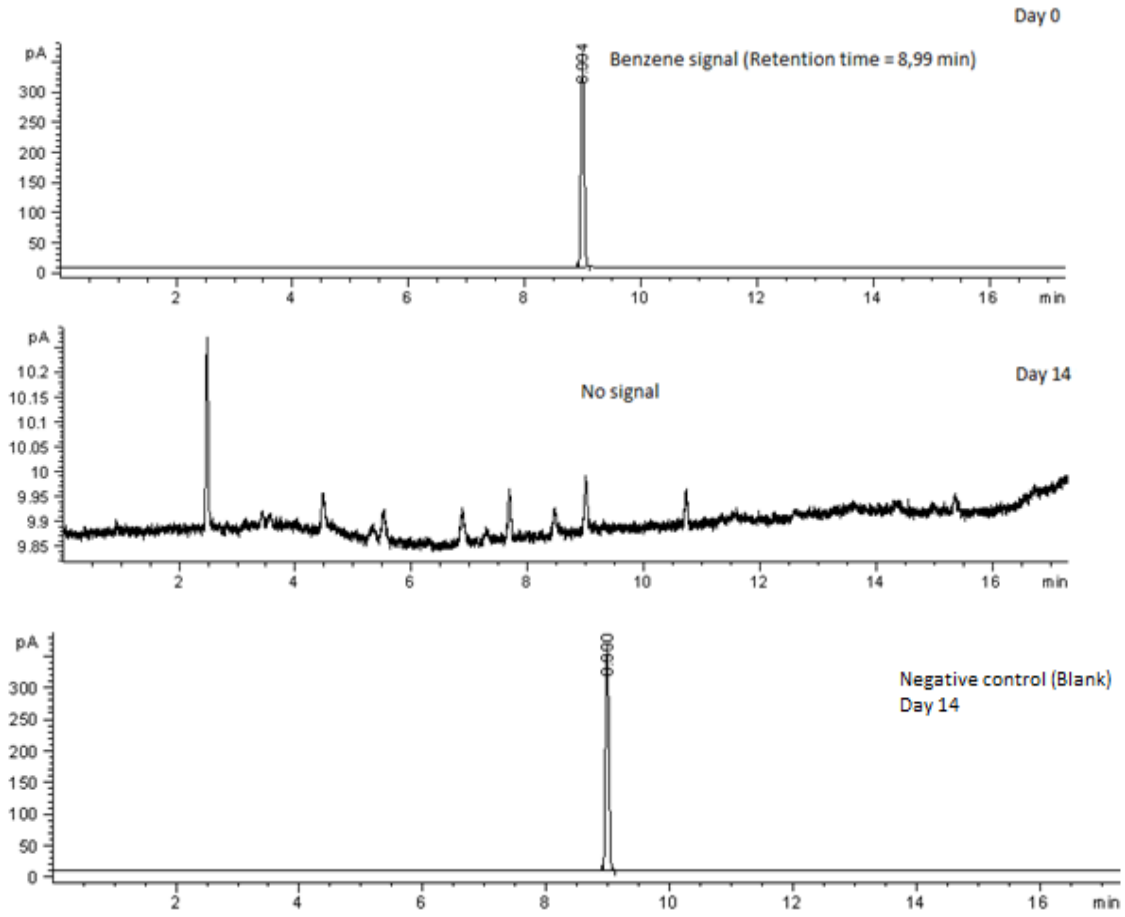


Figure 4.9. Benzene identification (GC/FID) in samples and negative control during biodegradation assay at 20 mg/L with *D. acidovorans*.

5. CONCLUSIONS

Growth plant inhibition was observed in a PFR treating DMPs during a transition of concentrations from 15.37 mg/L to 28.86 mg/L. This physiological damage was reflected on a poor water management of *J. effusus* and subsequently deficiencies in oxygen transport. The experimental phase of increased DMPs concentration was also accompanied by a slight decline of redox potential, removal of ammonium and prevalence of nitrification process. Nevertheless, the probable depletion of oxygen around the root could have altered the nitrogen dynamics (diminution of nitrate) inside the PFR and by this other parallel microbial process.

The PFR was found to be successful in removing 99.08 % of DMPs fed along the different experimental phases. However, a minor decrease of the effectiveness was experienced at high DMPs concentration regime (persistence of 2,6- DMP). On the other hand, DOC was not completely removed (74,2%) and low concentrations were detected in the outflow possibly due to the formation of soluble microbial and plant biomass decay products.

None of the isolated strains from the PFR were capable to gain biomass and degrade a DMPs mixture (3,4-, 3,5- and 2,6- DMP) as a sole carbon and energy source. In contrast, *D. acidovorans* exhibited a great capacity to deal with real-like DMPs mixtures. This could be due to the adaptation of this wild strain to mineralize toxic compounds from polluted effluents of chemical industries.

3,4-DMP served as a growth substrate for *D. acidovorans* and enabled cometabolic transformation of isomers 2,4-, 2,5- and 3,5- DMP and a better mineralization of 2,3-DMP in dual mixtures. Despite this effective performance, 2,6-DMP was the only isomer not assimilated by this bacterium, not as a sole carbon source neither in the company of 3,4-DMP. This recalcitrant nature of 2,6-DMP might be related with its chemical structure.

Bacterial degraders from PFR water samples and consortiums presented genes encoding monooxygenases that might catalyze and foster the activation of the aromatic ring as first step of the DMPs aerobic biodegradation. Likewise, microbial degradation of DMPs (specifically 3,4- and 2,3- DMP which served as growth substrate) by *D. acidovorans* could apparently be begun with the hydroxylation of the dimethylphenols by monooxygenases in order to induce the establishment of catecholic derivatives.

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7. APPENDIX

Appendix 1: ***Composition of the trace mineral solution TMS***

TMS	
Compound	Concentration (mg/L)
Disodium salt (Titriplex III)	1
$FeSO_4 7H_2O$	1
$MnCl_2 2H_2O$	0.8
$CoCl_2 6H_2O$	1.7
$CaCL_2 2H_2O$	0.7
$ZnCl_2$	1.0
$CuCl_2 2H_2O$	1.5
$NiCl_2 6H_2O$	0.3
H_3BO_3	0.1
$Na_2MoO_4 2H_2O$	0.1
$Na_2SeO_3 5H_2O$	0.02
HCl (concentrated)	3

Appendix 2: ***M9 medium component (Juretschko et al. 1980)***

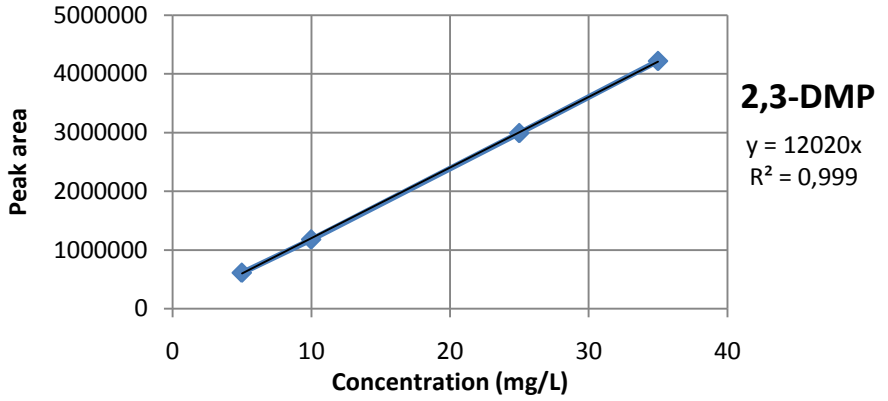
Solution A (50 mL)	
Compound	Amount (g)
$MgCl_2 .6H_2O$	0.5375
$CaCO_3$	0.100
$FeSO_4 .7H_2O$	0.2250
$ZnSO_4 .7H_2O$	0.0720
$MnSO_4 .4H_2O$	0.0424
$CuSO_4 .5H_2O$	0.0125
$CoCl_2 .6H_2O$	0.0140
H_3BO_3	0.0030
HCl	2.565 mL
Solution B	
$MgSO_4 .7H_2O$	1 M
Solution C	
$FeSO_4.7H_2O$	36mM

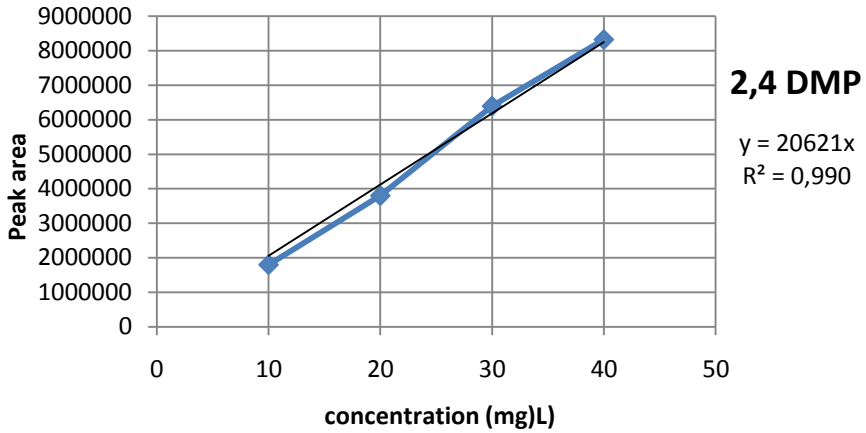
M910X (100 mL)	
Compound	Amount (g)
Na_2HPO_4	6.784
KH_2PO_4	3
NaCl	0.5
NH_4Cl	1.0

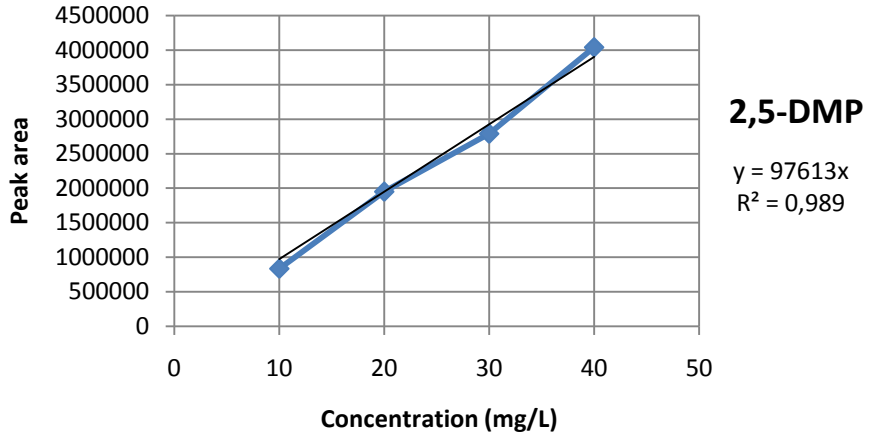
Appendix 3: ***Cycling program (32 cycles) for 16s 27f rDNA***

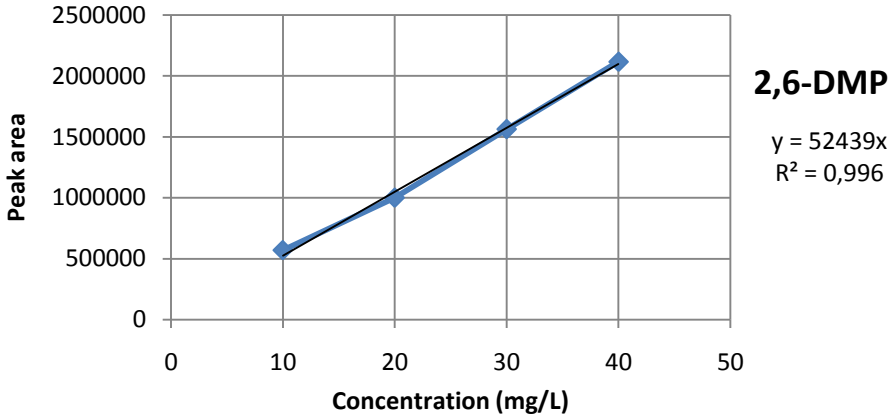
Step	Temperature (°C)	Time
Initial activation	95	15 min
3 step cycling		
Denaturation	94	30 sec
Annealing	52	45 sec
Extension	72	30 sec

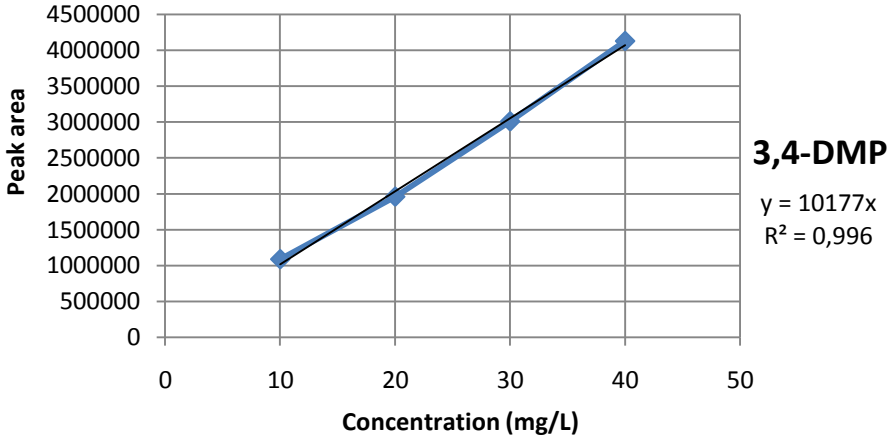
Appendix 4: ***Calibration curves for dimethylphenols quantification***

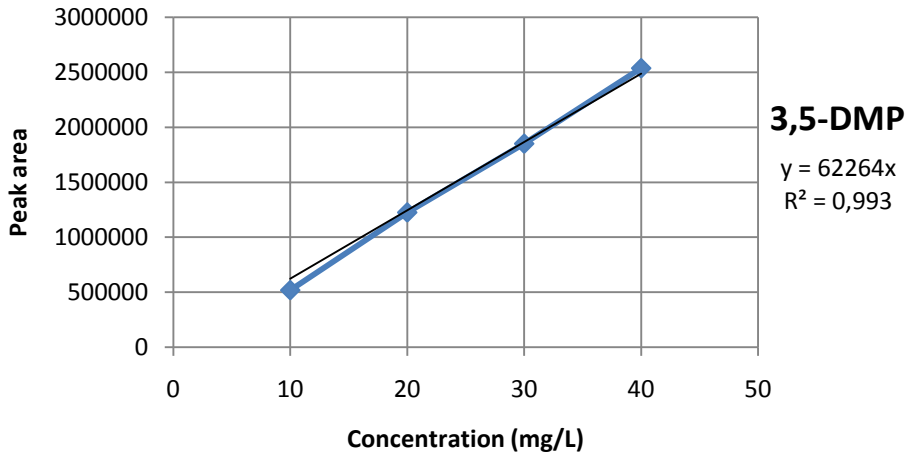





4500000
4000000
3500000
3000000
2500000
2000000
1500000
1000000
500000
0
Peak area
0
10
20
30
40
50
Concentration (mg/L)
2,5-DMP
y = 97613x
R² = 0,989


2500000
2000000
1500000
1000000
500000
0
Peak area
0
10
20
30
40
50
Concentration (mg/L)
2,6-DMP
y = 52439x
R² = 0,996


4500000
4000000
3500000
3000000
2500000
2000000
1500000
1000000
500000
0
Peak area
0
10
20
30
40
50
Concentration (mg/L)
3,4-DMP
y = 10177x
R² = 0,996



Appendix 5: ***Cycling protocols for PCR analysis of catabolic genes involved in aromatic biodegradation***

PHE		
Step	Temperature (°C)	Time
Activation	95	15 min
Denaturation	95	45 sec
Annealing	52	45 sec
Elongation	72	1 min
Final Elongation	72	8 min

TOL		
Step	Temperature (°C)	Time
Activation	95	10 min
Denaturation	95	1 min
Annealing	55	1 min
Elongation	72	2 min
30 cycles		
Final Elongation	72	10 min

EXDO-D		
Step	Temperature (°C)	Time
Activation	95	15 min
Denaturation	95	45 sec
Cycle 1	61	
Cycle 2	60	
Cycle 3	59	
Cycle 4	58	
Cycle 5	57	
Cycle 6	56	
Cycle 6-31	56	
Annealing	55	45 sec
Elongation	72	45 sec
Final Elongation	72	8 min

TOL		
Step	Temperature (°C)	Time
Activation	95	15 min
Denaturation	94	1 min
Cycles 1-10	61	30 sec
Cycles 11-26	59	30 sec
Cycles 27-40	57	30 sec
40 cyles		
Extension	72	30 sec

Tbu-E		
Step	Temperature (°C)	Time
Activation	95	15 min
Denaturation	94	1 min
Annealing	60	1 min
Elongation	72	2 min
35 cycles		
Final Elongation	72	10 min

TmoA		
Step	Temperature (°C)	Time
Activation	95	15 min
Denaturation	94	1 min
Annealing	61.2	1 min
Elongation	72	2 min
35 cycles		
Extension	72	10 min

TodC		
Step	Temperature (°C)	Time
Activation	95	15 min
Denaturation	94	1 min
Annealing	66	1 min
Elongation	72	2 min
35 cycles		
Extension	72	10 min

TmbD		
Step	Temperature (°C)	Time
Activation	95	15 min
Denaturation	94	1 min
Annealing	65.5	1 min
Elongation	72	2 min
35 cycles		
Extension	72	10 min

Appendix 6: Raw data for physicochemical parameters, DMPs and COD concentrations in a PFR during experimental period.

		DOC (mg/L)		
Experimental phase	Date	Inflow	Outflow	Removal (%)
A	18/03/2015	7,94	1,88	76,3224181
	25/03/2015	7,97	2,88	63,8644918
	01/04/2015	7,72	2,03	73,7046632
	07/04/2015	7,2	3,42	52,5
B	15/04/2015	12,91	1,86	85,5925639
	22/04/2015	11,68	3,56	69,5205479
	29/04/2015	11,18	3,02	72,9874776
	06/05/2015	12,35	3,44	72,145749
C	13/05/2015	16,77	4,08	75,6708408
	20/05/2015	15,4	2,47	83,961039
	27/05/2015	16,21	2,6	83,9605182
	03/06/2015	18,86	3,76	80,0636267

			DMPs (3,4-, 3,5- and 2,6- DMP)			
Experimental Phase	Date	Week	Infow (mg/L)	Outflow (mg/L)	Detected Isomer	Removal (%)
	11/03/15	1	7,52259637	0,30968687		95,8832449
	18_03_15	2	7,88682232	0		100
	25_03_15	3	6,51882247	0		100
	01_04_15	4	8,86417709	0		100
A	07_04_15	5	7,14932611	0		100
	15_04_15	6	13,3153704	0		100
	22_04_15	7	16,8647964	0		100
	29_04_15	8	17,2807223	0,88706647	2,6-DMP	94,866728
B	06_05_15	9	14,0342775	0		100
	13_05_15	10	28,8242824	1,21256139	2,6-DMP	95,7932643
	22_05_15	11	22,3607914	0		100
	28_05_15	12	29,0507455	0,269	2,6-DMP	99,0740341
C	02_06_15	13	27,2228874	0,437	2,6-DMP	98,3947331

Time (days)	Transpiration (g/L)	Number of shoots
5	4,69157255	53
12	5,35523027	52
18	6,37243333	52
26	3,94347683	57
33	6,02208096	58
40	7,09477897	58
46	5,26777875	57
54	4,90677134	56
68	4,90276189	49
75	4,19776119	48
80	4,28418422	45

Experimental phase	Date	Time (day)	REDOX (eH)	O_2 (mg/L)
A	16/03/2015	1	55	0,2
	20/03/2015	5	10	0,4
	23/03/2015	8	20	0,1
	27/03/2015	12	10	5,3
	30/03/2015	15	134	1,1
	02/04/2015	18	159	0,6
	06/04/2015	22	237	2,3
	10/04/2015	26	262	6,7
	13/04/2015	29	270	20,6
B	17/04/2015	33	234	43,4
	20/04/2015	36	176	3,3
	24/04/2015	40	144	0,3
	27/04/2015	43	133	0,6
	30/04/2015	46	155	32,2
	04/05/2015	50	132	0,1
	08/05/2015	54	170	1,7
C	11/05/2015	57	164	0,2
	15/05/2015	61	169	43,2
	18/05/2015	64	147	0,3
	22/05/2015	68	174	8,3
	26/05/2015	72	137	0,2
	29/05/2015	75	127	0,3
	01/06/2015	78	126	0,2
	03/06/2015	80	126	0,1

Experimental phase	Date	Time (day)	$N\text{-}NO_3^-$ (mg/L)	$N\text{-}NH_4^+$ (mg/L)	NO_2^- (mg/L)
	18/03/2015	3	7,1	40,4	0,04
	25/03/2015	10	11,1	14,5	0,03
	01/04/2015	17	12,1	16,0	0,04
A	08/04/2015	24	15	11,4	0,03
	15/04/2015	31	13,9	2,9	0,04
	22/04/2015	38	8,89	2,3	0,07
B	06/05/2015	52	8,27	3,8	0,03
	13/05/2015	59	8,37	2,0	0,03
	20/05/2015	66	8,11	0,6	0,02
	27/05/2015	73	7,43	2,1	0,04
C	03/06/2015	80	5,68	4,2	0,03