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REMOVAL OF SELECTED CONSTITUENTS OF COAL PYROLYSIS EFFLUENTS IN CONSTRUCTED WETLANDS LABORATORY SCALE

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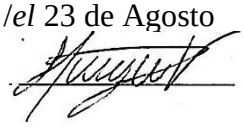
Unterschrift / *Firma*: 

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Abstract

This study aimed to describe the performance of laboratory scale constructed wetlands run with a dimethylphenol isomer mixture (DMPs) containing artificial industrial wastewater and to identify associated bacteria degrading DMPs as pollutants in carbochemical industrial wastewater. The two constructed wetland systems studied were a horizontal subsurface flow constructed wetland (HSSF CW) and a planted fix bed reactor (PFBR). The artificial wastewater contained a mixture of DMPs isomers (2,6-DMP, 3,4-DMP and 3,5-DMP) in equimolar ratio but different concentrations. The investigations were carried out for three months.

The DMPs removals were 96% for the HSSF CW and 100% for the PFBR.

Samples of microbial cenosis were taken from both systems. Selection of microorganism was performed by isolation with DMPs as sole carbon source. Bacteria able to grow in selective medium were identified by 16 rDNA sequencing. Eight different bacterial strains were identified and one unidentified fungus was isolated. The bacteria strains were studied for DMPs aerobic degradation activity whereby complex microbial consortiums outperform single strains. DMP removal in liquid batch cultures and in wetland systems caused at low concentration the formation of stable metabolites with low bioavailability.

Results in this work demonstrated that constructed wetlands are capable to treat DMPs as carbochemical wastewater pollutants whereby the microbial consortia have the ability to degrade DMPs. Further research should focus on the formation of the organic residues of low bioavailability to further improve treatment performance of constructed wetlands.

Key words: Dimethylphenols degradation, constructed wetlands, bacteria.

Resumen

La presente investigación tuvo como propósito describir la eficacia de humedales artificiales a escala laboratorio en el tratamiento de aguas residual de la industria carboquímica e identificar microorganismos capaces de degradar dimetilfenoles (DMPs). Se estudiaron dos sistemas de humedales artificiales: Horizontal de flujo subsuperficial (HSSF CWs) y reactor plantado de cama fija (PFBR), los cuales fueron alimentados continuamente con una mescla equimolar de tres isómeros de DMPs (2,6-DMP, 3,4-DMP y 3,5-DMP). La investigación se llevó a cabo durante tres meses, durante los cuales el porcentaje de remoción de DMPs fue de 96% para HSSF CW y 100% para PFBR.

Para ambos tipos de humedales se tomaron muestras de cenosis microbiana cultivadas en medio líquido con DMPs como única fuente de carbono para seleccionar e aislar microorganismos. Se encontraron ocho cepas bacterianas diferentes, identificadas por medio de secuenciación de la porción 16 rDNA. Las cepas bacterianas aisladas fueron objeto de estudio como degradadores aerobios de DMPs, se observó que los consorcios microbianos superaron la capacidad degradadora en comparación al de las cepas bacterianas por separado. En los cultivos líquidos y humedales durante la remoción de DMPs se formaron de metabolitos estables con poca biodisponibilidad.

En conclusión la investigación demostró que los humedales artificiales son capaces de tratar DMPs como contaminantes de efluentes de la industria carboquimica y la capacidad de consorcios microbianos de degradar DMPs. La formación de residuos orgánicos persistentes requiere de mayor investigación para mejorar el rendimiento de los humedales artificiales.

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Introduction

The concern about limited oil resources is unavoidable. Is the petrochemical industry able to support the present and future demand under the same production scheme? So it appears that carbochemical industry may be able to compete with petrochemical industry.

The International Energy Outlook 2013 has projected a faster grow of coal use over petroleum, due the sustained petroleum high prices and increases on its consumption (EIA, 2013). Nevertheless, carbochemical industry also supports around 70% of steel production with 721 million tons of metallurgic coal in 2010 (World Coal Association, 2012).

Coke, gas and tar are the main commodities from coal thermal decomposition but along with these products, wastewater with high content on toxic substances such phenols, ammonium, cyanide and thiocyanate are released (Chen et al., 2011; Ghose, 2002; Vázquez et al., 2006; Zhang et al., 1998). Dimethylphenols (DMPs) are typical components from carbochemical industry wastewater; toxic by ingestion and skin absorption (Watson et al. 1986). Outstanding DMPs as recalcitrant components, it is worth to recognize the risk of water reservoirs contamination and the urgent need to treat efficiently carbochemical effluents with low cost and maintenance.

As a result physicochemical and biological treatment methods have being developed, although major issues that prevail: concentrates disposal (after effluent treatment) and stability of removal. Since Constructed Wetlands (CWs) are emerging as a low cost industrial wastewater treatment method; they are able to remove considerable organic pollutants load (Dordio and Carvalho, 2013). CWs application to coal effluents is an opportunity to take advantage of the complex physicochemical and biological interactions in a near-nature system for the treatment of industrial effluents.

Although the implementation of CWs for industrial effluents treatment has been widely studied the scientific literature regarding carbochemical effluents is very limited (Huang et al., 2012; Jardinier et al., 2001), and the bacterial population involved in the degradation

process is not well known. The understanding of the biological degradation of DMPs in CWs will help to optimize their design and performance (Faulwetter et al., 2009).

The aim of this study was to describe the degradation processes of DMPs in two CW systems and to identify and characterize DMPs degrading bacteria. The experimental design consisted of two types of CWs at laboratory-scale placed in a greenhouse under relatively stable environmental conditions. They were feed continuously with artificial wastewater containing selected DMPs as the main organic pollutants. Physicochemical parameters (DMPs, dissolved organic carbon, redox potential and oxygen) were measured. Microbiological samples were taken for further bacteria isolation, DNA extraction, PCR and sequencing in order to identify DMPs degrading bacteria.

1. Carbochemical industry effluents

1.1 Carbochemical industry

Carbochemical industry is based on pyrolysis, thermochemical decomposition of organic material at elevated temperatures without oxygen input. In the process large organic molecules, like carbohydrates, are broken into smaller ones resulting in liquid, gas and solid products. The resulting solid particles have high carbon concentration of up to 95% and less impurities such hydrogen, sulfur, nitrogen and oxygen (Equation 1). Pyrolysis precedes combustion or gasification depending on the desired outputs: gas, coke and coal tar (Castells and García, 2012).

$$C_nH_mO_p + Heat \rightarrow \sum_{liquit} C_a H_b O_c + \sum_{gas} C_x H_y O_z + \sum_{solid} C$$

Equation 1: Pyrolysis generic reaction (Basu, 2010)

From the operational point of view there are three types of pyrolysis depending on temperature, time of exposure to carbonization and desired products. Conventional pyrolysis occurs at 500°C with a residence time of 5 seconds for gas generation and hours for solid products (coke). Fast pyrolysis happens at temperatures from 400°C to 800°C, residence time of up to 2 seconds were condensates are generated. Flash pyrolysis reaches temperatures higher that 600°C in less than 0.5 seconds producing gas and light hydrocarbons (Castells and García, 2012).

Coal production has shown a significant increase in the last decade, faster than petroleum or other fossil fuels. Triggers such the energy security, have lead coal market to expand in countries such China and India and increased exportations from USA and Indonesia (World Coal Association, 2012).

The carbochemical industry plans to cover the growing energy demand by improvement of coal plants efficiency from 40% to 46% and carbon capture and storage programs (IEA,

2013). Technological solution such coal to liquid, implemented in China, is one example of the already direct competence of coal and diesel by price and reduction on CO_2 emissions.

1.2 Effluent composition from carbochemical industry

Carbochemical industries generate wastewater mainly from quenching of coke discharge from ovens, cooling and gas washing and purification of products; caring out toxic compounds such phenols, cyanide, and sulfide among other less-toxic (Table1).

Table 1: Examples of coke wastewater composition after primary treatment

Literature reference	Parameter (mg/L)			
	DOC	Ammonia	Nitrate	Phenols
Ghose 2002	629.1	454.9	49.3	371.9
Zhang et al 1998	1496	300 - 700	16.106	78.2
Vázquez et al 2006	807 - 3275	504 - 2340	-	110 – 350
Li et al 2003	600 - 900	200 - 300	38 – 49	47.5
Jin et al 2013	940 - 2730	93 - 790	9.9 - 18.2	18 – 290

Pollutants concentration in coke industries wastewater depend on its particular production chain (coal used, products and technologies) and locations as shown by Marañón et al. (2008) in Table 2.

Table 2: Typical pollutant concentrations in the coke wastewater

Parameter mg/L	**Australia**	**Germany**	**China**	**Spain**
BOD	450 – 720	1600 – 2600	200 - 380	500 - 1000
DOC	1800 – 2200	4000 – 6500	630 - 860	800 - 3000
TSS	40 – 50	2 – 10	-	25 - 50
NH_4^+-N	200 - 275	50 – 150	220 - 280	200 - 400
Phenols	60 – 330	400 – 1200	50 – 80	180 - 300
SCN^-	180 - 200	200 – 500	-	180 - 450
CN-	70 – 95	4 – 15	-	15 - 40

Phenolic compounds are common pollutants in most of coke industry wastewater and have proven to reach concentration up to 1900 mg/L at raw wastewater (Chu et al., 2012) in

different forms such methylphenols, dimethylphenols and ethylphenols (Wei et al., 2012; Zhang et al., 1998), see Table 3.

Table 3: Concentration of phenolic compounds in coke wastewater

Studies	Phenol (mg/L)	Methylphenols (mg/L)	Dimethylphenols (mg/L)
Zhang et al. 1998	78.2*	64.9	11.8
Li et al. 2003	9.3	36.0	26.9

* Total phenols

Due phenolic compounds toxicity, the council of European Communities has set a maximum admissible concentration of phenols in water for human consumption at 0.5 μg/L. The European Commission and the Environmental Protection Agency (EPA) considers the following phenolic compounds at the priority pollutant list: 2,4-dimethylphenol, 2-chlorophenol, 2,4-dichlorophenol, 2-nitrophenol, 4-nitrophenol, 2,4-dinitrophenol, pentachlorophenol, phenol and 2,4,6-trichlorophenol (US EPA, 2013).

1.3 Methods to treat carbochemical effluents

Treatment of coal wastewater is a required practice in attempt to meliorate the impact of pollutants in aquatic environments. Pre-treatment methods are used to reduce solids, unsolved components and high ammonium concentrations, followed by physical and biological treatment.

At pre-treatment stage insoluble constituents such oil, tar and grease are exposing to mechanical separation followed by thermic distillation (Kuschk, 1991). For high phenolic loads liquid-liquid extraction is implemented. This extraction procedure can recover phenols up to 99% at optimal conditions (Jiang et al., 2003). Ammonia removal requires stripping process, fizzing wastewater under alkaline conditions to enhance ammonia state change from liquid to gas (Marañón et al., 2008).

Physicochemical methods involve activated carbon adsorption, ion exchange, chemical precipitation, membrane separation, coagulation and treatment with zero-valent iron. Most of them are connected with considerable operation costs due final concentrates disposal (Lai et al., 2007).

Membranes can be integrated to biological degradation treatment to reach high wastewater standards for final disposal. In model experiments for a coking wastewater the contaminants were removed by nano-filtration and reverse osmosis, reaching a total COD removal of 82.5% and 99.9% for phenol, therefore the concentrates from this method were highly polluted and a further treatment was needed (Jin et al., 2013).

Biological treatment is based on living organism's catabolism, in which complex molecules are broken down. Simple molecules are subsequently used in anabolic processes to generate biomass and energy. For the coking wastewater treatment several biological processes are conventional: activated sludge, biofilm and batch reactors.

Activated sludge process is a wide used method for different composed wastewaters by its efficiency on organic components removal by combination of microbial aerobic and anaerobic zones. In a case of coking wastewater Vázquez et al. (2006) proved removal efficiencies of 75% for COD, 98% removal for phenolic compounds and a nitrification efficiency of 71%.

Biofilm reactor systems are based on aerobic or anaerobic metabolic degradation/transformations of organic and inorganic compounds by microorganism fixed on a solid matrix. This method was described for a coal gasification wastewater treatment where phenols were removed up to 89% of efficiency with a maximal removal of COD of 81% (Hui-qiang et al., 2011). In contrast, methanogenic fermentation showed an efficiency of COD reduction of 72%, at low energy consumption and less formation of recalcitrant humic-like products in comparison toh oxygen dependent methods (Kuschk et al., 2010).

Investigators have affirmed that microorganism, as the key factor for organic compound removal, should be acclimated to wastewater in order to achieve acceptable degradation rates and overcome threshold concentrations. In coking effluents the mayor toxic components for microorganism are phenols, cyanides and thiocyanate, which cause inhibitory effects on microbial activity (Park et al.,2008 and Yamagishi et al., 2001).

Although biological treatment often is performed to acceptable extent, there are different opinions about toxic limitations caused by phenolic compounds (Zhou et al., 2013). Studies have revealed phenols as toxic for nitrifying microorganism affecting ammonium removal rate in activated sludge systems (Yamagishi et al., 2001). In contrast Vázquez et al. (2006)

reported about significant percentage of nitrogen removal while phenols degradation occurred, probably because of reduction in phenols concentration within the system and organism tolerance developed.

Whereas constructed wetlands have been used to treat different kind of wastewater, little is known about their performance for carbochemical effluents. Two examples of constructed wetlands for coke industry effluents are published. (Jardinier et al., 2001) showed efficiency of nitrogen removal from 54% to 96%, and high variable DOC removal from 5% to 86%. This weak biodegradability was attributed to the toxicity and recalcitrance of the carbon pollutants. Huang et al. (2012) presented the performance of an iron-carbon substrate constructed wetland reaching COD removal of 20% and meeting quality standards to recirculate the final effluent.

Constructed wetlands are feasible technological systems to treat carbochemical industry effluents. No description of phenolic compounds degradation has been deeply studied yet. Therefore an extended knowledge about phenol compounds degradation and its correlation with other parameters in constructed wetlands would give the chance for better process control and further treatment optimization.

2. Constructed wetlands

Constructed wetlands (CWs) are channels filled with soil that works as a support system for the vegetation. As the water flows through the rooted soil system, biological reactions take place in the root zone of the wetland were are degraded the pollutants. CWs are suitable due their low operation and maintenance costs for different effluents as for instance domestic, agricultural and industrial (Vymazal, 2007). According their hydraulic design three CW groups can be identified: free water surface, horizontal subsurface and vertical subsurface flow (Kadlec and Wallace, 2008).

2.1 Free water surface constructed wetland (FWS CW)

This group of CWs has wide open water areas, similar to natural marshes capable to attract significant amount of biota. A FWS CW contains a shallow substrate from sand or gravel which functions as a filter and supports plants (figure 2). This group of CWs is usually used for urban storm water treatment due the ability of handle variable flow levels. Owing the risk of human exposure to pathogenic microorganisms, FWS CWs are implemented as a secondary or tertiary treatment. They are competitive in operation and maintenance costs (Kadlec and Wallace, 2008).

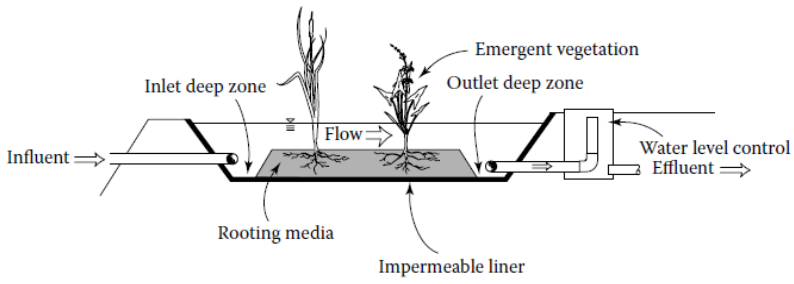

Figure 2: Free water surface CW (Kadlec & wallace, 2008)

2.2 Horizontal subsurface flow constructed wetland (HSSF CW)

Subsurface flow CWs are characterized for a horizontal flow through the pores of the substrate beneath the substrate surface (figure 3) (Kadlec and Wallace, 2008). As the water is going through the system it is in contact with the rhizospheric zone where microorganism degrade/transform substances by biochemical processes (Verhoeven and Meuleman, 1999). HSSF CWs may have oxidized and reduced zones depending on dimensions, type of soil and wastewater and retention time.

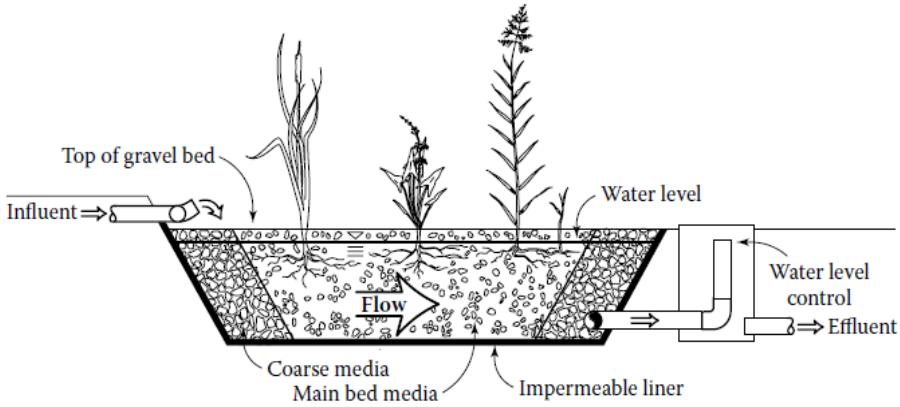

Figure 3: Horizontal subsurface flow CW (kadlec & Wallace, 2008)

In a subsurface system, redox p**o**tential usually increases from inflow to outflow due to the organic pollutants degradation (Headley et al., 2005), it evidence the favorable conditions for aerobic anoxic and anaerobic microbial population able to degrade pollutants by different metabolic pathways (Faulwetter et al., 2009).

HSSF CWs are usually designed to treat primary effluents due low pathogenic risk for human by the controlled subsurface flow. Eventually they are more expensive than FWS CWs due its operation characteristics (constant flow), but in contrast they can operate under colder climatic conditions (Kadlec and Wallace, 2008).

2.3 Vertical subsurface flow constructed wetland (VSSF CW)

This group of wetlands is characterized by an intermittent vertical water flood with flood and dry periods. In the dry period the substrate is exposed to the atmosphere ensuring enough oxygen inflow to degrade/transform pollutants, like ammonium indicated by Behrends et al., (1996) in Kadlec & Wallace (2008).

Combinations between VF with the other types of CWs are common due HSSF and FWS CWs limitation on ammonia oxidation, creating nitrification-denitrification environments. Despite its advantages this group was not wide accepted because of high operational costs and maintenance requirements (Vymazal, 2007).

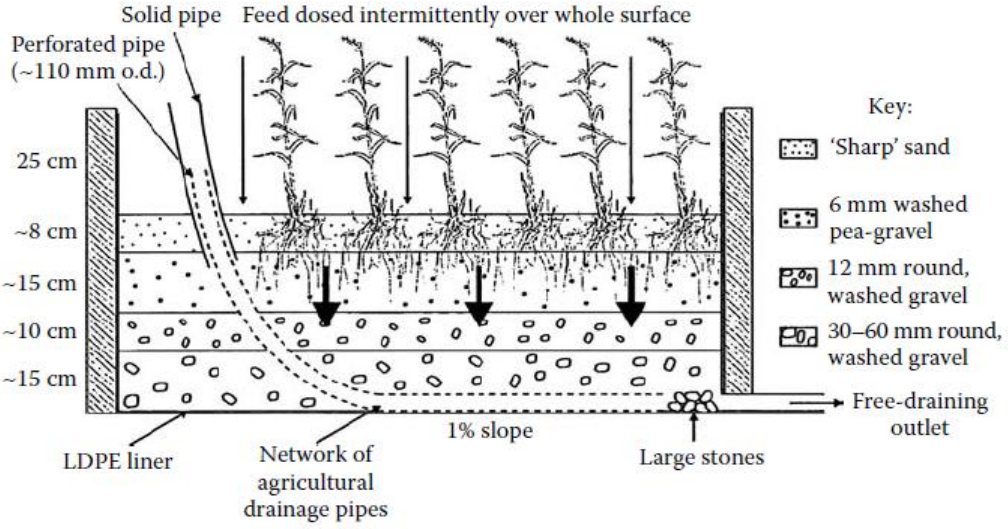

Figure 4: Vertical flow CW (Kadlec &Wallace, 2008)

With the aim to optimize removal rates in CWs, several mechanism have been documented, but it is a fact that removal of most pollutants is depend greatly on microbial metabolism (Knight et al., 2002). For example the nitrogen removal: despite plants which have an important uptake of nitrogen species, microorganisms are the mayor factor responsible for nitrogen species transformation.

Most studies on CWs track removal performance based on physicochemical aspects, assuming microorganism metabolism as the main base of removal processes. Therefore

microorganism direct removal activity has been a subject of research over the past ten years (Faulwetter et al., 2009).

Owing the complexity of CWs behavior variables such as oxygen concentration, redox potential, pH, temperature, plants, etc. microorganisms as the key players are highly impacted by all changes in the CW matrix. Oxygen levels can give an overview of catabolic circumstances, respiration or fermentation. At roots profundity in horizontal flow CWs the plants are able to transport certain amount of oxygen; therefore even when there is a relatively deep zone, oxygen concentrations are detectable by plant release or diffusion (Allen et al., 2002).

Redox potential indicates oxidative or reductive environments, giving a glance on biotransformation of pollutants. High redox potential is usually associated with an oxidative environment in which aerobic processes are taking place mainly for removal of COD, BOD and NH_3 (García et al., 2004; Headley et al., 2005). Therefore it can be expected that in HSSF CWs redox potential increases from the inlet to the outlet due to degradation process.

Microbial population can be affected in density, activity and diversity by rhizospheric changes. Roots are the preferred substrate surface for biofilms and a change of vegetation will cause a microcosms variation (Collins et al., 2005).

Seasonal changes influence microorganism particular in cold climate where microbial activity depends on temperature. This explains why wetland efficiency tend to decrease in winter (U.S EPA, 1999) although some publications have shown that low temperatures do not always affect the DOC removal in a significant way (Mæhlum et al., 1995).

3. Dimethylphenols (DMPs) as pollutants in industrial effluents

The alkylphenols dimethylphenols (DMPs) are also known as xylenols. These organic components are phenolic derivates that contain two methyl groups and one hydroxyl group. There are six isomers of DMP depending on the methyl group position (figure 5).

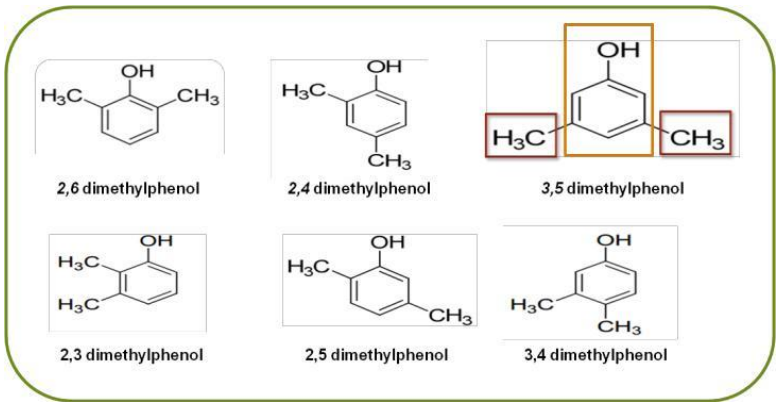

Figure 5: Dimethylphenol isomeres structure

These components are formed by the methylation of phenol in presence of distinct metal oxide catalyzers and can exist as single isomers. DMPs have a high water solubility, moderate vapor pressure, insignificant hydrolysis rate, moderate photo-oxidation, low persistence and low bioaccumulation potential and moderate volatilization (table 2).

DMPs have a moderate mobility considering their Henry's Law constants from 9.51×10^{-7} to 1.12×10^{-6} atm-m/mole. They is a risk of infiltration to water sources through soil. These compounds are biodegradable up to 94.3% of COD in 5 days (2,6-DMP) in aerobic conditions at activated sludge laboratory test at 20°C (EPA 2010).

DMPs do not hydrolyze since they have not labile groups to be hydrolyzed under environmental conditions, even though considerable quantities can dissolve in water.

Table 4: Physicochemical properties and environmental characteristics of dimethylphenols (EPA 2010)

Property	2,3-DMP	2,4-DMP	2,5-DMP	2,6-DMP	3,4-DMP	3,5-DMP
Water solubility at 25°C (mg/L)	4750	7870	3540	6050	4760	4880
Henry´s Law Constant	7.3×10^{-7}	9.5×10^{-7}	1.1×10^{-6}	6.6×10^{-6}	4.1×10^{-7}	6.1×10^{-7}
Photodegradation (half-life)	4.8 h	5.3 h	4.8 h	5.8 h	4.7 h	3.4 h
Hydrolysis (half-life)	stable	stable	stable	stable	Stable	stable
Biodegradation (5 days)	95.5%	94.5%	94.5%	94.3%	97.5%	89.3%*
Persistence	low	low	low	low	Low	low
Bioaccumulation	low	low	low	low	Low	low

*After 5 days

The exposition to DMPs can occur by inhalation and skin contact. They present different lethal doses depending on the isomer and organism (rat, mouse or rabbit). The impacts of DMPs in this study will be focus on 2,6, 3,4 and 3,5-DMP.

In general DMPs are toxic by ingestion and skin absorption and some can be carcinogens as 2,4-DMP (Bruze and Zimerson, 1997) promoting papilloma in mice (Patty et al., 1982). The only one case of ingestion (all isomers solution) evidence bowl sounds, vomiting; severe metabolic acidosis, and cardiac and renal failure to a fatal end (Watson et al., 1986).

Medical test on Albino mice were studied for 2,4-DMP shown clinical signs of lethargy, prostration, ataxia and hematological changes with a dose of 250 mg/kg day as lowest observed adverse effect level (LOAEL) in a 90 day trial and 50 mg/kg day as no observed adverse effect level (NOAEL). From the studies is suspected that DMPs are cardiovascular, gastrointestinal, kidney, respiratory and skin toxicant (EPA, 2010).

2,6-DMP was described as non-mutagenic in strains of the *Salmonella typhimurium* (Epler et al., 1979), although 2,4-DMP at concentrations of 0.33, 1.0, 3.3, 10, 33 and 500 µg/plate showed the highest ineffective dose for mutagenic reactions at 33 µg/plate (NTP, 2012)

For aquatic toxicity a table with half lethal dose (LD_{50}) depending on isomer and organisms is shown below (table 5). The data of other isomers are not adequate or have not yet been studied.

Table 5: Aquatic toxicity of dimethylphenols (EPA 2010)

DMP isomer	Toxic concentration	Organism	Time of exposure
Fish			
2,4	16.6 mg/L +	Fathead minnows	96 h
3,5	22 mg/L +	Goldfish	96 h
2,6	>27 mg/L +	Fathead minnows	96 h
	12 mg/L +	Winter flounder	96 h
Invertebrates			
2,6	11.2 mg/L *	Water fleas (*Daphnia magna*)	48 h
all isomers	7.7 mg/L *	Water fleas (*Daphnia magna*)	48 h
Aquatic Plants (Biomass)			
all isomers	14 mg/L *	Freshwater green algae	72 h

+ LD50: is the concentration of the chemical that cause the death of half of the population in certain time. * $EC_{50:}$ refers to half maximal effective concentration in which the component induces a response halfway in respect to the base state, after specific time.

Phenolic compounds have shown toxicity for humans and aquatic life. In the microcosms, DMPs toxicity has been described by Hartnik et al. (2007) identifying toxic compounds in creosote contaminated ground water, where phenols along with benzenes and quinolones represent the 80% of the overall toxicity. For DMPs, all isomers were toxic at concentrations of 12.5 mg/L.

4. Microbial degradation of phenolic compounds

Phenolic compounds health and environmental hazard risk implies an imperative need to remove them from wastewater. Physicochemical and biological removal methods have being studied although biological treatment is preferred owing to less toxic intermediates and competitive cost (Klein and Lee, 1978).

Biological degradation of phenolic compounds depends mainly on microorganism capacity of assimilation and generation of specific enzymes able to breakdown aromatic compounds in to more simple molecules. Phenol biodegradation can be realized under aerobic or anaerobic conditions depending on microorganism's oxygen requirements.

Aerobic biodegradation initiates with the oxygenation of the aromatic ring by a mono oxygenase, phenol hydroxylase enzyme in different position in respect to the hydroxyl group forming dihydroxy metabolites: catechol, gentisate, protocatechuate and others. Three aromatic cleavage pathways are known in dependence of the metabolite and position of cleavage metabolite (meta, ortho or para). Most of the pathways lead intermediate products able to enter into the tricarboxylic acid cycle – TCA cycle (Mahiudddin et al., 2012).

Catechol ortho cleavage initiates at the ortho position, which is the link between two hydroxyl groups by the catechol 1,2-dioxygenase leading to the formation of succinyl Co-A and acetyl Co-A. Meta cleavage of catechol occurs in the position meta between one hydroxyl group and the proximate non-hydroxyl carbon resulting in pyruvate and acetaldehyde (figure 6). Catechol meta pathway has been described in *Pseudomonas putida, Pseudomonas cepacia, Pseudomonas picketti* and *Alcaligened eutrophus* (Leonard and Lindley, 1998), *Pseudomona stutzeri* for 2,3- and 3,4-DMP (Baggi et al., 1996) and *Pseudomona XQ23* group (Xiao et al., 2012) for 2,3-DMP; while ortho cleavage was described for *Trichosporon cutaneum*, *Rhodotorula rubura* and Acineto*bacter calcoacetium* (Paller et al., 1995).

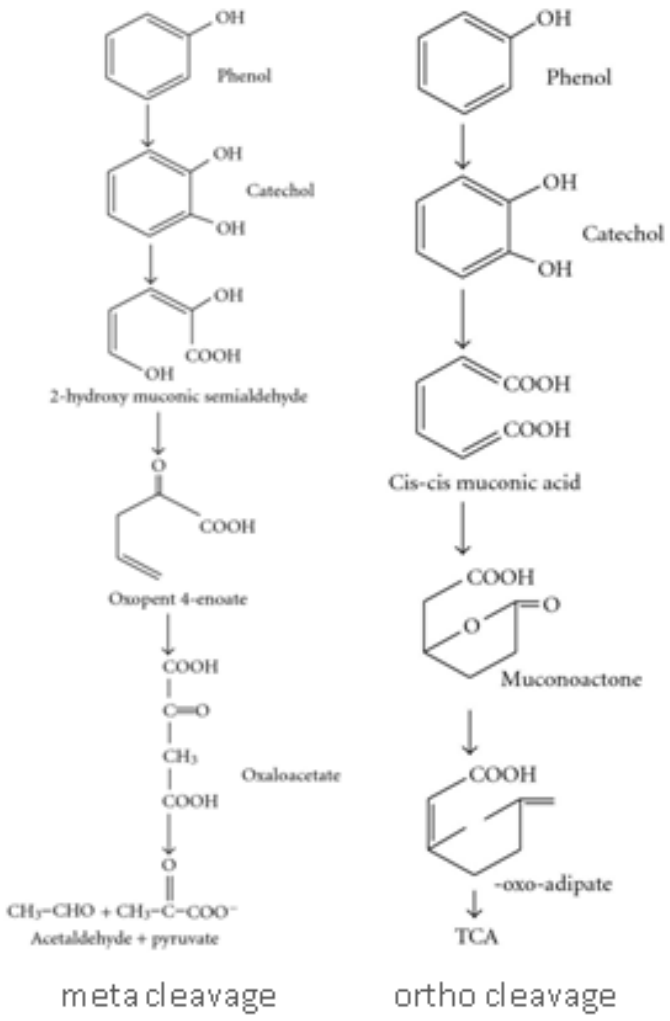

Figure 6: Phenol degradation pathways (Mahiudddin et al., 2012)

Gentisate pathay, common for aromatic compounds, initiates with a gentisate oxygenase enzyme to form maleylpyruvate. Its final products are fumarate and pyruvate. This pathway was described for *Pseudomonas putida* carrying the plasmid pRA500 which encodes the degradation of 3,5-DMP (Jain et al., 1984) and *Pseudomonas alcaligenes* P25X for 2,5 and 3,5-DMP (Gao et al., 2005) (figure 7).

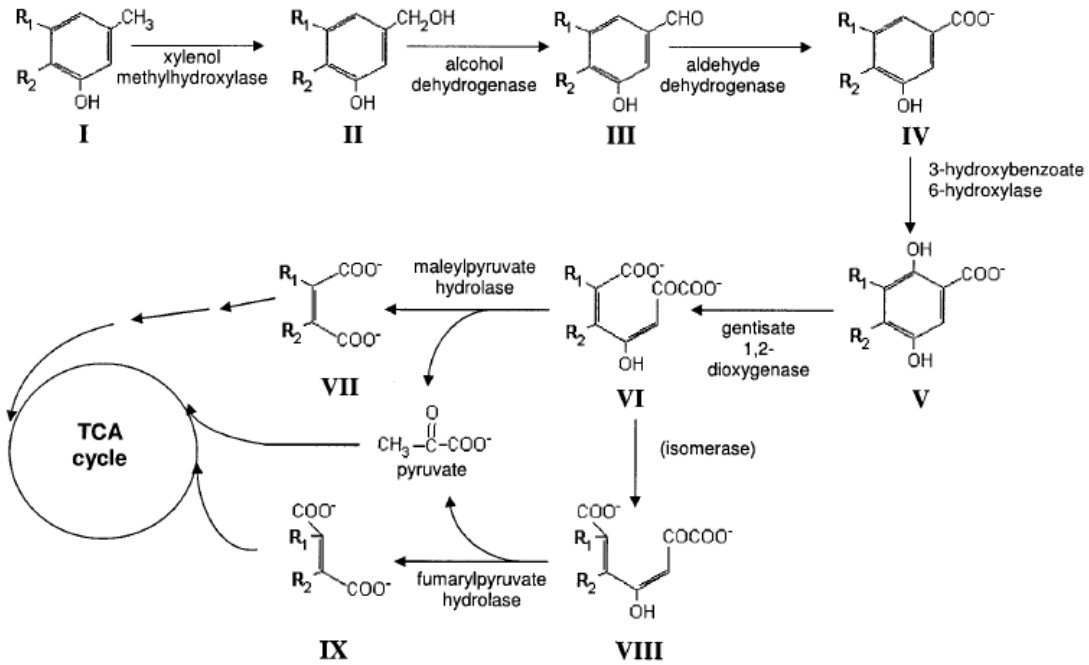

Figure 7: Gentisate pathway for the degradation of 2,5 and 3,5-DMP (Gao et al., 2005)

I, 2,5-xylenol; **II**, 3-hydroxy-4-methylbenzylalcohol; **III**, 3-hydroxy-4-methylbenzaldehyde; **IV**, 3-hydroxy-4-methylbenzoate; **V**, 4-methylgentisate; **VI**, 5-methylmaleylpyruvate; **VII**, citraconate.

One less common pathway is the protocatechuate where the protocatechuate ring is splitted via ortho, meta or para cleavage depending on the enzyme. Intermediate products are 3-oxoadipate, pyruvate and 2-oxopent-4enoate respective the cleavage pathway. *Pseudomonas fluorescence* CP18 and CP24 were described to degrade 2,4 and 3,4-DMP by the protocatechuate ortho pathway (Viggor et al., 2002).

The anaerobic degradation pathway of phenol is initiated by carboxylation of the aromatic ring where first a phosphorylation is mediated by a phenyl phosphate synthase forming phenyl phosphate. Later the phenyl phosphate is carboxylated to form 4-hydroxybenzoate in requirement of Mn^{2+}. The complete process leads to Acetyl Co-A and Succinyl Co-A. Although the existence of microorganism able to use DMPs as a carbon source is documented the fermentation process remains in study.

5. Material and methods

The experimental investigations analyzed dimethylphenols (DMPs) degrading bacteria inherent in constructed wetlands. Following both systems were used: a horizontal subsurface-flow constructed wetland (HSSF CW) and a planted fix bed reactor (PFBR), which both simulates the rhizospheric processes in CWs.

5.1 Laboratory-scale constructed wetlands

The experimental design was similar to that already described by Wu et al., (2013). CWs of horizontal sub-surface flow - HSSF CWs consisted of open containers of 100 cm length, 35 cm height and 15 cm of width, filled with gravel of 4-8 mm diameter. Two CWs were studied, one planted (PCW) with *Juncus effusus* and the other remained unplanted (UCW), for reference.

CW water flow design was adjusted to a saturated zone, 3 cm below the gravel surface, with a capacity of 13.8 L and a theoretical retention time of about 2.8 days for the PCW and 2.4 days for the UCW (figure7 and 8). Both CWs were constantly feed with artificial wastewater consisting on deionized water, buffer, ammonium, phosphate and trace minerals, containing in equimolar ratio 3,4-, 3,5- and 2,6-DMP with a total concentration of 40 mg/L (Table 6).

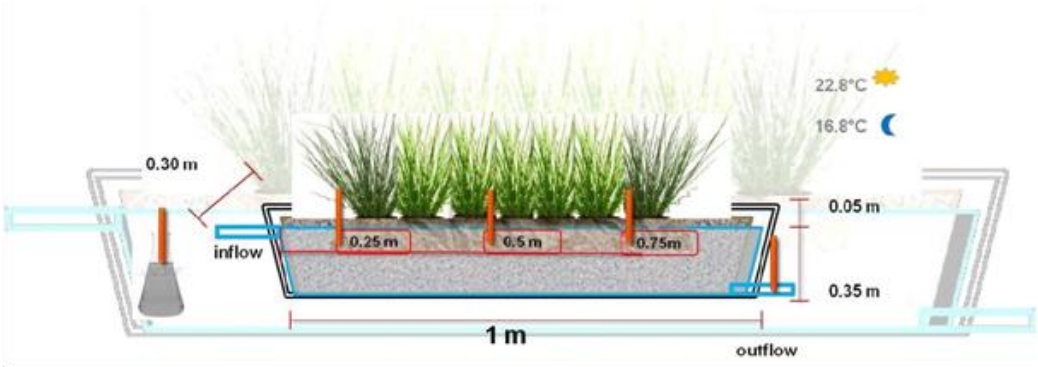

Figure 7: Planted constructed wetland scheme

The experimental laboratory-scale CWs were place in a greenhouse under controlled climatic conditions simulating an average summer day. The temperature was 22 °C from 6:00 a.m. to 9:00 p.m. and 16 °C from 9:00 p.m. to 6:00 a.m. Lamps were used as an additional source of light during day time.

Figure 8: Photograph of the planted experimental laboratory-scale constructed wetland

Table 6: Constructed wetlands artificial wastewater composition

Component	Concentration [mg/L]
DMPs (2,6, 3,4 and 3,5)	0 – 40 (total)
$NaHCO_3$	252
K_2HPO_4	24.8
NaCl	4.7
NH_4Cl	79.5
$CaCl_2 . 2 H_2O$	2.7
$MgCl_2 . 6 H_2O$	1.1
Na_2SO_4	0.6
TMS3 (appendix 1)	1 mL/L

The storage tank of the artificial wastewater of 30 L was cleaned and refilled every Friday with fresh artificial wastewater for each wetland. On Monday 18 L of fresh artificial wastewater were added to ensure continuous feeding. The samples for physicochemical parameters were taken usually on the Wednesdays.

5.2 Laboratory scale reactor

The laboratory assembling for the planted fix bed reactor was similar to that described by (Fischer et al., 2010). A glass vessel, 30 cm in diameter and 30 cm height, was filled with gravel (4-8 mm) and planted with *Juncus effusus.* By this a hydraulic retention time of about

5 days and a pore water volume of 12 L in a permanently fluid mix conditions was realized as presented in the figure 9.

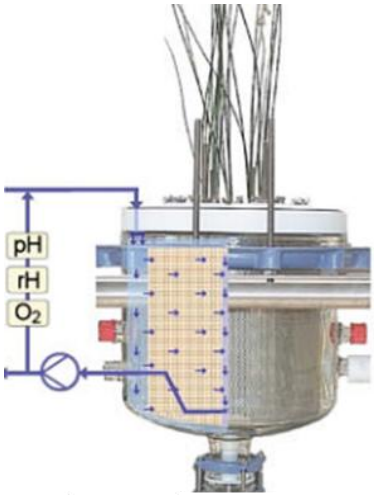

Figure 9: Planted fix bed reactor scheme (Helmholtz-Zentrum für Umweltforschung-UFZ, 2011)

The reactor was constantly fed with an artificial wastewater consisted of deionized water, buffer, ammonium, phosphate and trace minerals, furthermore contained in an equimolar ratio 3,4-, 3,5- and 2,6-DMP for a final concentration of 40 mg/L (Table 7).

Table 6: Artificial wastewater composition of the reactor

Component	Concentration [mg/L]
DMPs (2,6-, 3,4- and 3,5-)	0 – 40
$NaHCO_3$	252
K_2HPO_4	24.8
NaCl	4.7
NH_4Cl	79.5
$CaCl_2 . 2 H_2O$	2.7
$MgCl_2 . 6 H_2O$	1.1
Na_2SO_4	0.6
TMS3 (appendix 1)	1 mL/L

The wastewater storage tank of 10 L was cleaned and refilled every Friday with fresh artificial wastewater for the reactor. On Monday 6 L of fresh artificial wastewater was added to ensure continuous feeding. The samples for physicochemical parameters were taken ones per day for five days.

5.3 Physicochemical analytical methods

The experimental phase was executed from March to May 2014. For the experimental CWs the samples were collected from five points at the distance of 25, 50 and 75 cm from the inflow through small stainless steel pipes at a depth of 15 cm from gravel surface level, including in- and outflow as shown in figure 7. The set up for water sampling is shown in figure 10. Redox potential, oxygen and temperature were measured by a flow-through cell.

The reactor samples were collected from the inflow and the outflow. Inflow samples were analyzed for DMPs and DOC, while outflow samples were analyzed for all parameters. The redox potential and the oxygen concentration were measured continuously in the circulation flow of the planted fixed bed reactor. Water pH analyses were performed immediately after taking the samples. All samples were taken in duplicates storing them at minus 16 °C to analyze DMPs and DOC conditional on the availability of the analytical devices.

Figure 10: Set -up for water sampling for the constructed wetlands

5.3.1 Dimethylphenols analysis by HPLC

Separation and quantification of the three DMP isomers (2,6-, 3,4- and 3,5-DMP) were performed with the Shimadzu High Performance Liquid Chromatograph (HPLC). The separation of compounds was achieved by the analytical column Poroshell 120 EC-C18 Agilent Technologies (diameter: 3 mm, length: 100 mm, particle size: 2.7 µm), 50°C (isotherm). The elution was carried out at the flow rate of 0.2 ml/min using a mobile phase of A: 0.1% formic acid, 0.1% acetonitril in distilled water; B: 99.9% acetonitril. The DMP

isomers were quantified by calibration with standards at 278 nm; injection volume was 30 µL. Chromatograms were analyzed by LCSolution's Postrun Analysis software.

5.3.2 Dissolved organic carbon analysis (DOC)

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.

5.3.3 Redox potential

For CW samples, a stream of pore water was pumped through a flow-through cell equipped with: Pt/Ag^+/AgCl/Cl^--electrode (Sentix ORP®, WTW) connected to a microprocessor pH-Meter pH 539 (WTW) and a Checktemp®1 Thermometer from Hanna Instruments to determine temperature. To get the standard hydrogen values the electrodes measured voltage values were corrected using the following equation:

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

U_H - Redox potential [mV]

U_G - Measured voltage [mV]

U_{ref} - Standard voltage depending on temperature [mV]

D - Measured deviation to reference electrode [mV]

For the planted fixed bed reactor in the continuous recirculation flow the redox potential measurements were performed with a metal (platinium) electrode (2ME-2G-PtK^{-1} from Jumo, Germany).

5.3.4 Oxygen

Fibox 3 LCD trace Oxygen Meter (PreSens - Precision Sensing GmbH) was used to measure oxygen concentration in the pore water of the HSSF CWs. The oxygen sensor FTC-TOS7 was exposed to water sample stream in a flow through cell. Values were displayed by the OxyView-PSt3-V5.41 software. In the planted fixed bed reactor oxygen was measured continuously by membrane electrode (Siemens/France 34 oxygen sensor 7MA3100-8EF).

5.4 Microbiological analytical methods

5.4.1 Sampling

Bacteria in this study were isolated from the planted horizontal sub-surface flow constructed wetland (PCW) and the planted fixed bed reactor (PFBR). Pore water composite samples were collected from several points from the rhizospheric zone of the systems (PCW and PFBR), about 15 cm below the gravel bed surface. Water samples were immediately concentrated by centrifugation at 8000 rpm for 20 minutes to a final volume of 0.5 ml for the PFBR and 2.5 ml for PCW samples. An additional composite sampling of gravel was performed for the planted PCW from a depth of about 15 cm below the gravel surface.

5.4.2 Media and microbial culture conditions

To select DMPs degrading bacteria, PFBR and PCW water samples were inoculated by duplicates in M9 liquid medium (table 7) supplemented with DMPs mix solution which contained 40 mg/l of each isomer (2,6-, 3,4- and 3,5-DMP) as a sole carbon source. All liquid cultures were incubated in aerobic conditions at 30°C on a rotatory shaker at 150 rpm.

Table 7: Components for the M9 medium

Solution	Quantity for 1 L
M9 10x *	100 Ml

Sol. A *	1.25 Ml
Sol. B *	650 µL
Sol. C *	650 µL
Carbon source	40 mg/L or 70 mg/L
Destilled water	completed to 1 L
Agar-agar (only for plates)	18 g

* See appendix 2

After 8 days of incubation 1 mL of each PFBR and PCW M9 cultures were transferred to M9 liquid medium with 40 mg/L of only one of the DMP isomers (2,6-, 3,4- and 3,5-DMP) for a total of six M9 liquid cultures. After 3 days DMPs concentration was increased (desirable of 70 mg/L).

Subsequently of 10 days incubation time, three volumes were taken from M9 liquid cultures (100 µL, 50 µL and 20 µL) spreading onto M9-DMP mix agar plates (70 mg/L of each 2,6-, 3,4- and 3,5-DMP) as a sole carbon source. Plates were incubated at 30°C for 6 days. After colonies were visible at stereoscope, single colonies were transferred on M9-DMPmix agar plates and incubated for 5 days at 30°C. Once the cultures were pure, isolations were transferred on LB agar plates (Table 8).

Table 8: LB medium composition

Solution	**Quantity for 1 L**
Yeast extract	5 g
Tryptone	10 g
NaCl	10 g
Agar-agar (only for plates)	20 g

PCW gravel samples were inoculated in minimum liquid medium MM (table 7) supplemented with 70 mg/L of each DMP isomer (2,3-, 2,4-, 2,5-, 2,6-, 3,4- and 3,5-DMP) as the sole carbon source for a final volume of 100 mL. MM liquid cultures were incubated under aerobic conditions at 30°C for 13 days in continuous mixing. DMPs used in the study were purchased from Sigma-Aldrich.

Table 9: Minimum medium MM composition

Component	Quantity for 100 mL
Mineral salts (appendix 3)	10% (1 mL)
Trace elements (appendix 4)	0.2% (0.02 mL)
Carbon source (DMPs)	70 mg/L
Distilled water	completed to 100 mL

Morphological analysis was performed using a light microscope Axioskop 2 (Zeiss- Germany) with a measuring scale on 100 µm supported by the microscope software Axiovision 4.8. Colonies morphology analysis was realized by stereomicroscope (Zeiss Stemi DRC, Germany) from M9-DMPmix agar plates.

5.4.3 DNA manipulation, sequencing and sequence data analysis

From one to three colonies were taken from pure isolations to dissolve them separately in 60 µL of DNA water (Sigma-Aldrich) in a 1.5 mL Eppendorf tube for an optimal concentration of bacteria biomass. DNA extraction was carried out by using DNeasy® Blood & Tissue kit from Qiagen, Germany.

HotStarTag® DNA Polymerase and HotStarTag® Master mix kit (Qiagen, Germany) were implemented to amplify the region 16S ribosomal DNA gen using universal primers 27f. The optimal cycling protocol for PCR was modified as shown in table 10.

Table 10: 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

After PCR, the DNA was purified by QIAquick Purification kit (Qiagen, Germany) and quantified by NanoDrop™ 1000 spectrophotometer in a spectrum range of 220 - 750 nm for

nucleic acids with PC base software (Thermo Fisher Scientific) and detector 2048-element linear silicon CCD array. In order to verify the correct function of PCR process and the state of DNA fragments samples and controls PCR products were exposed to electrophoresis in agarose gel 1%.

DNA sequencing was carried out by GATC Biotech, Germany. Comparison of 16S DNA gene sequences obtained were analyzed by using BLAST at the National Center for Biotechnology information Web site (http://www.ncbi.nlm.nih.gov/blast).

5.4.4 Degradation analysis

To ensure an inoculum of bacteria in exponential growth phase an inoculum of 3 to 5 colonies from LB agar plates were cultivated in 3 mL LB liquid medium for two days under aerobic conditions at 30°C on a rotatory shaker at 150 rpm. The cells were harvested by centrifugation (8000 rpm, 10 min, 4°C) and re-suspended two times in 2 mL of buffer NaH_2PO_4, pH 7.0.

Cells were grown in 50 mL M9 liquid medium with 70 mg/L DMP mix (2,6-, 3,4- and 3,5-DMP) as a sole carbon source for 8 days. Initial inoculum for all isolates was fixed to a value of 0.05 for optical density at 620 nm (OD 620 nm). A control was set up to follow possible non-biological degradation/transformation of DMPs.

5.4.5 Analytical measurements

Growth in M9-DMP was monitored by optical measurements at 620 nm (OD_{620nm}) with a Perkin Elmer LAMBDA 2 UV/Vis spectrophotometer equipped with photo-diode detector and standard disposal cells of 1.5 mL. DMPs were detected by two systems depending on the sample composition. The DMP mix was analyzed by HPLC (see 6.3.1) and the separated DMPs were analyzed by an UV Cary® 300 UV-Vis spectrophotometer (Agilent Technologies) (wave range from 200 to 400 nm, light source of Tungsten – Halogen and detector R928 PMT).

5.5 Statistical analysis

The research was based on a descriptive analysis of data from HSSF CWs which treated an artificial coal pyrolysis effluent. The time series of physico-chemical parameters (pH, redox potential, dissolved oxygen, dissolved organic carbon and dimethylphenols) were presented in graphics showing month's means and standard deviations for correlating them with the degradation of DMPs.

The results on degradation rate and identifications of the bacteria as well as the track of main phenol-related physicochemical aspects will support the improvement of a constructed wetland model for carbochemical industry wastewater treatment.

6. Results and discussion

The chapter presents the physicochemical and microbiological results of two constructed wetland systems: Horizontal Subsurface Flow Constructed Wetlands (CW) and Plated Fixed Bed Reactor (PFBR) concerning removal of dimethylphenols DMPs in a weekly sampling campaign from March to May 2014. The CW has six sampling dates while PFBR has five sampling dates. The HSSFCW and PFBR removal performance of DMPs (loads, DMP isomers relation, DMPs and DOC relation) and degraders microorganism related will be discussed.

6.1 Treatment of dimethylphenols in horizontal flow constructed wetland

The dynamics removal of dimethylphenols in two HSSF CWs: planted (PCW) and unplanted (UPCW) were studied in a period of time of three months from March to May 2014. Both CWs were continuously supplied with an artificial wastewater containing following dimethylphenols: 2,3-DMP, 3,4-DMP and 3,5-DMP in a mean inflow concentration of 9.6 ± 0.8, 9.3 ± 1.9 and 11.5 ± 1.2 mg/L respectively, for a final DMPs total mean inflow concentration of 30.4 ± 3.7 mg/l.

DMPs concentrations were analyzed in following five points for the PCW in the flow path direction: inflow, 25 cm, 50 cm, 75 cm and outflow; while the UPCW DMPs concentration was measured in the inflow and outflow, as a reference system.

DMPs loads are presented in figure 11. Both CWs present a decrease on DMPs concentration through the flow path; a higher efficient DMPs removal was observed for the PCW. The outflow DMP loads for the PCW were below 12 mg/d in comparison with 63 mg/day for the UPCW.

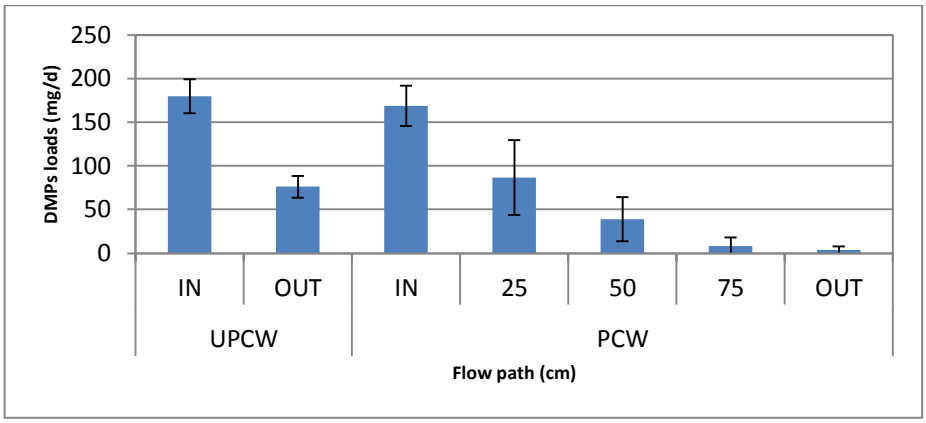

Figure 11: DMPs load trough flow path in UPCW and PCW

The overall mean efficiency for both systems is displayed in figure 12 for all sampling dates. The PCW reveals higher load removal efficiency, not lower than 98% while the UPCW presents only a mean load removal of 58%. Both PCW and UPCW were continuously feed with artificial wastewater since December 2013.

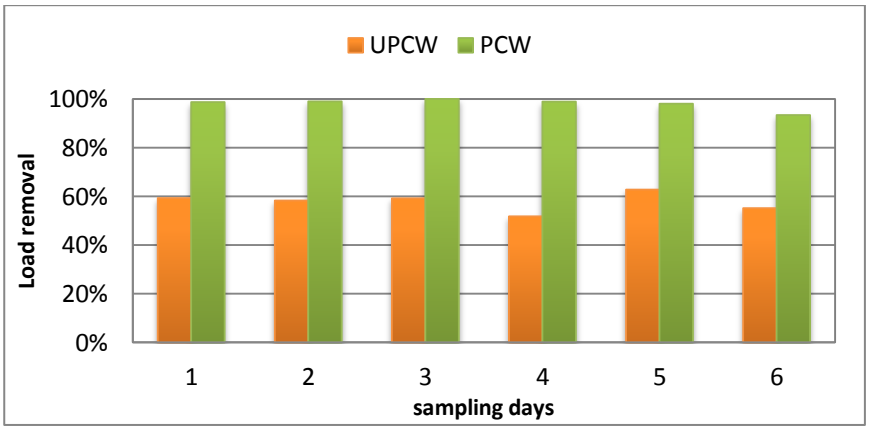

Figure 12: DMPs removal efficiency of laboratory scale horizontal constructed wetlands (mean outflow load values for all sampling days)

These results indicate the positive impact of plants in a CW for DMPs removal. Better performance of PCW over UPCW was also demonstrated for nitrogen and phosphorus removal by Picard et al., (2005). *Juncus effusus* as well as other macrophytes enrich the solid wetland matrix with root nets that form the desirable conditions for microbiological growth and biofilm constitution.

Juncus effusus has been reported to release oxygen in soil matrix at rates of 0.69 mg/h per plant (Wießner et al., 2002) supporting an oxygenated environment. This explains the DMPs removal outperformance of PCW over UPCW in aerobic conditions. This result conforms the observations of Chapman (1968) and Fedorak (1984), who found that DMPs degradation is less efficient under anaerobic conditions.

The mayor DMP removal for the PCW was in the first half of the flow path of the system; there was realized a mean load removal of about 77%. This pattern may be due to the grater disposition of oxygen, minerals and trace elements creating a rich zone for microorganisms, with high diversity and therefore higher removal rates. This suggests that for further applications and upscaling the system, longer CWs in length are would not imperative to achieve high removal rates.

Figure 13 shows a degradation pattern for the DMPs isomers. In the first half of the system 3,4-DMP and 3,5-DMP reached more than 75% of load removal while 2,6-DMP achived just 68%. DMPs isomers tendency suggests an influence of DMPs isomers chemical structure on their degradation/removal. 3,4-DMP has the methyl groups in position meta and para respect the hydroxyl group; 3,5-DMP has meta position, and 2,6-DMP has an ortho position. In this last case there is less available positions for conventional aromatic substitution and further ring cleavage, making 2,6-DMP to one of the most recalcitrant isomers. Longer degradation time of 2,6-DMP is also due to the low oxygen concentration on the last half of the PCW.

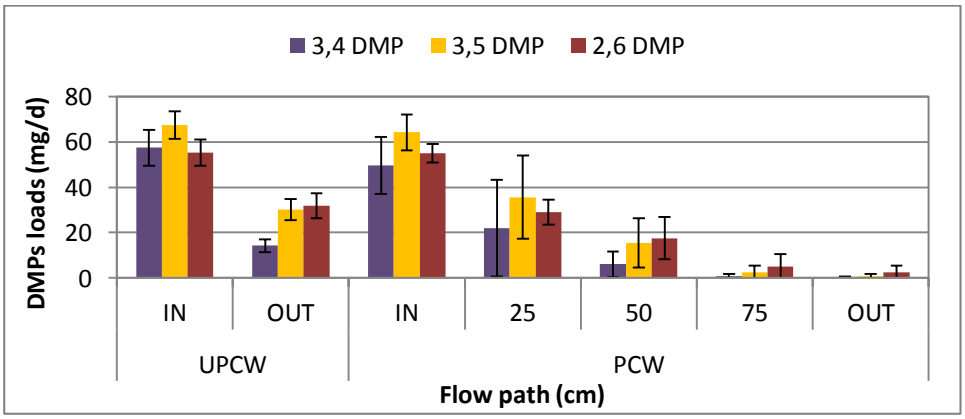

Figure 13: DMPs isomers loads thought the UPCW and PCW flow path

The relation between DMPs concentrations and dissolved organic carbon (DOC) is given by the carbon concentration contained in DMPs molecules: 1 mol DMP contains 78.7% of carbon. Therefore the DMPs concentrations were converted to a factor of 0,787 to be compared.

Figure 14 shows the DOC and DMP-C values through the flow path in the UPCW and PCW. For both wetlands the DOC in the inflow was about 23 mg/L higher than the corelated DMP-C. For the UPCW the disolved carbon was mainly due to the DMP-C concentration with a slight decrease in the outflow. This could be due physical and biological degradation of DMPs through the flow path.

There is a positive correlation between DOC and DMP-C, along the flow path. However, this correlation is closer in the first half of flow path. It suggests that later in the second half of the flow path persists a left carbon (different from DMP-C), that could come from the accumulation of intermediate metabolites from DMP decomposition, and or rhizodeposition containing root exudates and microbial biomass, in sites closer to outflow.

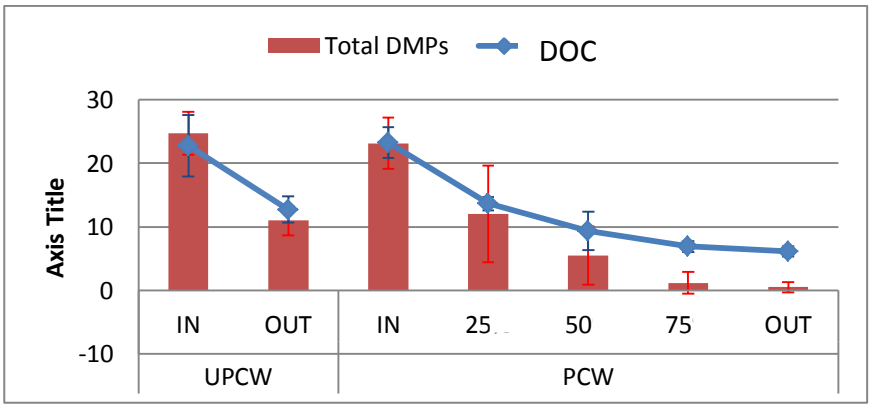

Figure 14: Relation between cod and DMPs-C along the flow path (mean of all samples)

Figure 15 illustrates the comparison of pH and oxygen for both CWs. p H in UPCW did not show any important fluctuations through the flow path in a range from 7.4 to 7.5; while in PCW pH reached low values as 6.3; caused by DMPs oxidation and formation of acid metabolites intermediates. Poerschmann and Schultze-Nobre (2014), demonstrated strong positive correlation between decreasing pH values and degradation/removal of DMPs concentration. The redox potential for PCW and UPCW were

below 300 mV which indicates a reducing environment with low oxygen concentrations typically for horizontal subsurface constructed wetlands (Faulwetter et al., 2009).

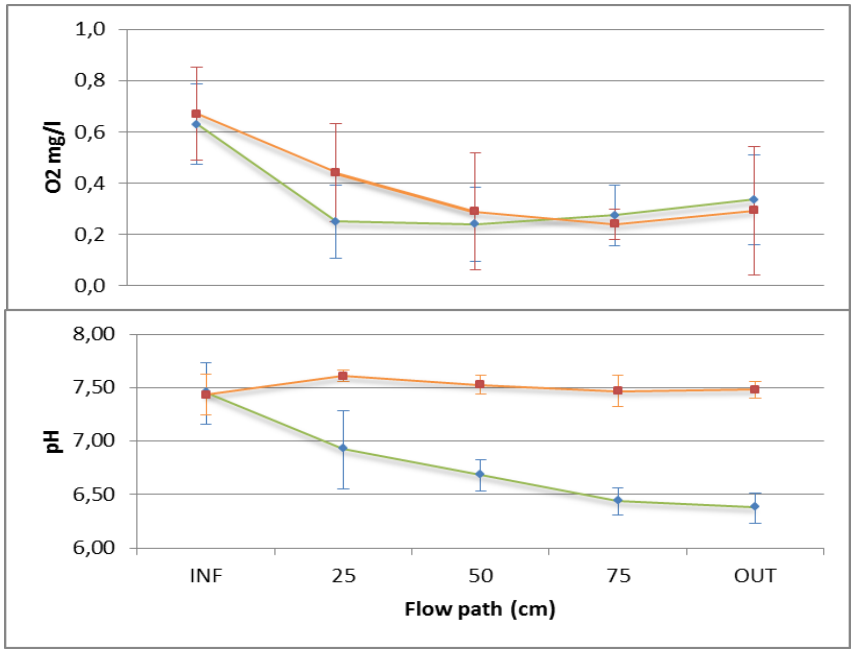

Figure 15: Comparison of oxygen and pH for PCW and UPCW thought the flow path (mean of all sample days)

6.2 Removal of dimethylphenols in the Planted Fixed Bed Reactor (PFBR)

The removal dynamics of the DMPs in the PFBR was studied in the period from 19 May to 23 May 2014. The PFBR was continuously supplied with an artificial wastewater containing following DMPs: 2,3-DMP, 3,4-DMP and 3,5-DMP in a mean inflow concentration of 7.2 ± 0.7; 8.6 ± 0.9 and 9.3 ± 1.3 mg/L respectively, with a final total DMP mean inflow concentration of 25.6 ± 2.8 mg/L.

DMPs concentrations were analyzed in the inflow and outflow. Evapotranspiration was neglected due to the reactor properties (Kappelmeyer et al., 2002). Redox potential, oxygen and pH were measured in the recirculation flow. The inflow was estimated with 2.04 L per day.

DMPs load are shown in figure 16. The total DMPs load was 51.5 ± 5.3 mg/day with an overall removal efficiency of the PFBR of 100% with DMPs concentration under the

detection limit. This can be explained from the nature of continuously mixing of the pore water which increased availability of dissolved oxygen for DMPs biodegradation.

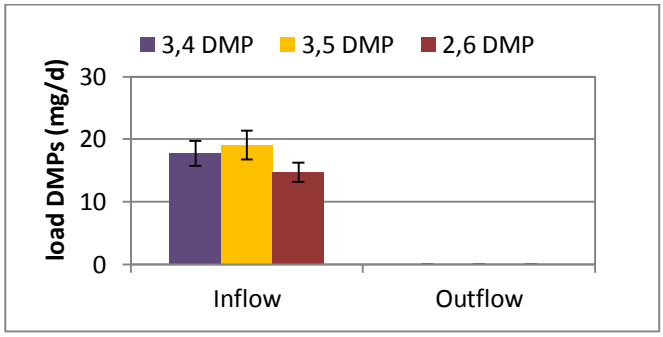

Figure 16: DMPs isomer loads in the PFBR (mean of all sampling days)

The relation between DMPs concentrations and disssolved organic carbon (DOC) is given by the carbon concentration in the DMP molecule. One molecule of DMP contains 78.7% of carbon; therefore the DMPs concentration was multiplied with a factor of 0.787 to compare carbon concentrations. At inflow the concentrations of DOC correspond allmost complete to concentrations of DMP carbon fraction (DOC-C) as shown in figure 17.

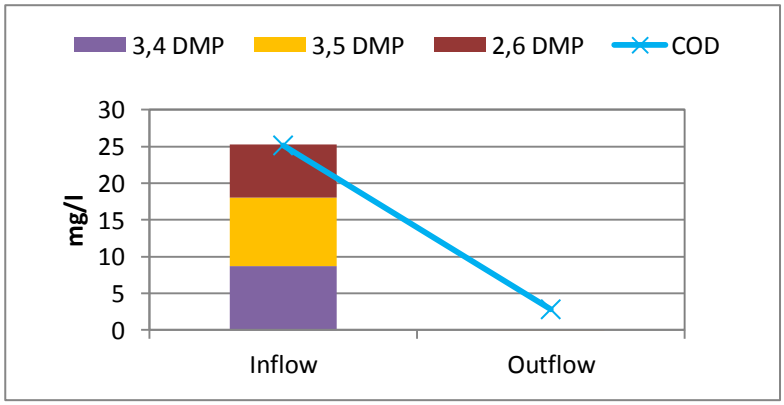

Figure 17: Relation between doc and DMPs-C in the outflow (mean of all sampling days)

Inflow DOC was about 23 mg/L with correlation at the outflow with decreasing concentrations of DMP-C. This can be due to carbon sources different that from the DMPs: metabolites from DMPs degradation and components from rhizodeposition containing root exudates and microbial biomass.

Redox conditions in the reactor exhibited oxidative conditions with a mean of 196,6 ± 16.1 mV and oxygen concentrations with a mean of 0,19 ± 0,15 mg/L. Strong correlation was observed between pH and oxygen indicating the oxidation of DMPs, consuming oxygen generating acidic intermediate metabolites and CO_2, influencing the acidity of the media.

Carbon deficient conditions in the PFBRs have exhibited redox potential about 800 mV in a study of (Wießner et al., 2005), indicating that continuously mixing ensures oxidative conditions. That can change to reductive conditions in the presence of organic material.

6.3 Identification of microorganism

Water and gravel samples from PCW and PFBR were collected to identify DMPs degrading bacteria and their degradation potential. The first two batch cultures were aimed for isolation and identification, selecting bacteria able to use DMPs as carbon and energy source. The third batch culture was performed to detect single bacteria's ability of to degrade DMPs. It was assumed that the natural selection of microorganisms adapted to grow in presence of a xenobiotic entails potential degradation of the compound (Ascon-Cabrera and Lebeault, 1993).

Following two DMPs formulas were used: a mixture with 2,6, 3,4 and 3,5-DMP, hereafter named DMP mix and the single isomers (2,3-, 2,4-, 2,5-, 2,6-, 3,4- and 3,5-DMP). For the DMP mix the analytical method used was HPLC while single isomers were measured by UV-Vis spectrophotometer to avoid overlapping of absorbance curves.

DMP degradation was estimated by the residual DMPs concentration in the medium. Microbial ability to grow in DMP enriched medium was evaluated by measuring their biomass in terms of optical density (OD) at 620 nm.

6.3.1 Isolation from water samples

Composite water samples of PCW and PFBR from approximately 15 cm depth were taken and concentrated by centrifugation at 8000 rpm for 8 minutes. 1 mL of the concentrated biomass was inoculated to a final volume of 100 mL in M9 medium and DMPs mix as a sole carbon source for 8 days. The test was carried out by duplicate.

DMPs residual concentrations are shown in figure 18, evidencing degradation of all tree isomers after 8 days. For PCW samples the removal efficiency was 92% for 3,4-DMP, 77% for 3,5-DMP and 24.8 % for 2,6-DMP. PFBR samples removal efficiency was 98% for 3,4-DMP, 75% for 3,5-DMP and 35% for 2,6-DMP. These results are consistent with the findings in aerobic degradation of DMPs in microcosms where 3,4-DMP was degraded faster than 3,5-DMP and 2,6-DMP (Broholm and Arvin, 2000).

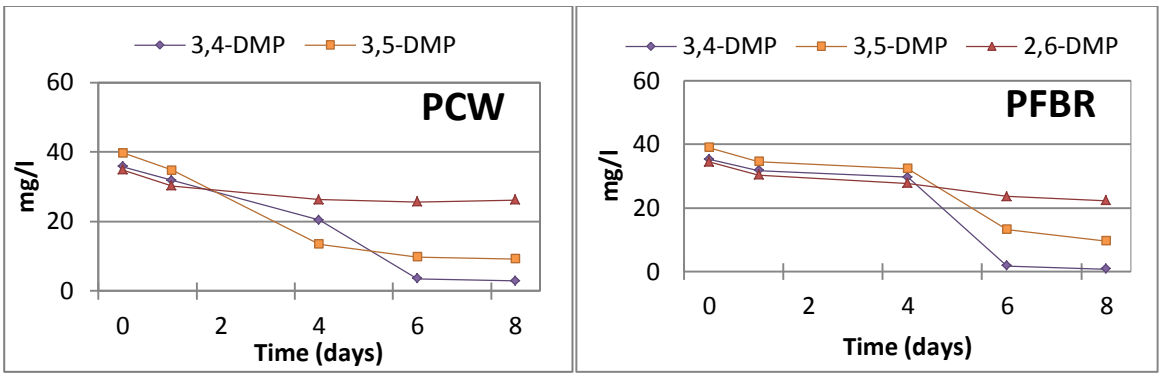

Figure 18: DMPs mix degradation in batch cultures from PCW and PFBR water samples. Incubation time of 8 days at 30°c, DMPs initial concentration was about 40mg/l (each ismomer)

During cultivation period, cultures in M9 medium and the DMPs mix (2,6-DMP, 3,4-DMP and 3,5-DMP) evidenced a change in culture color after 4 days (figure 19). This medium coloration may be due to oxidation of phenols forming humic-like compounds that are further polymerized causing the persisted brownish color (Field et al., 1989). This color pattern is similar to that obtained by Kuschk et al. (2010) in abiotic air-pretreatment of coke plant effluent, indicating oxidation of phenolic compounds.

Figure 19: M9 batch cultures after 4 days

From DMP mix batch cultures 1 mL was transferred to a new M9 medium with separated DMPs isomers (2,6-DMP, 3,4-DMP and 3,5- DMP) for an overall of 6 M9 flask. DMPs concentrations were measured by UV-VIS spectrophotometer for 10 days, obtaining the following results (see figure 20).

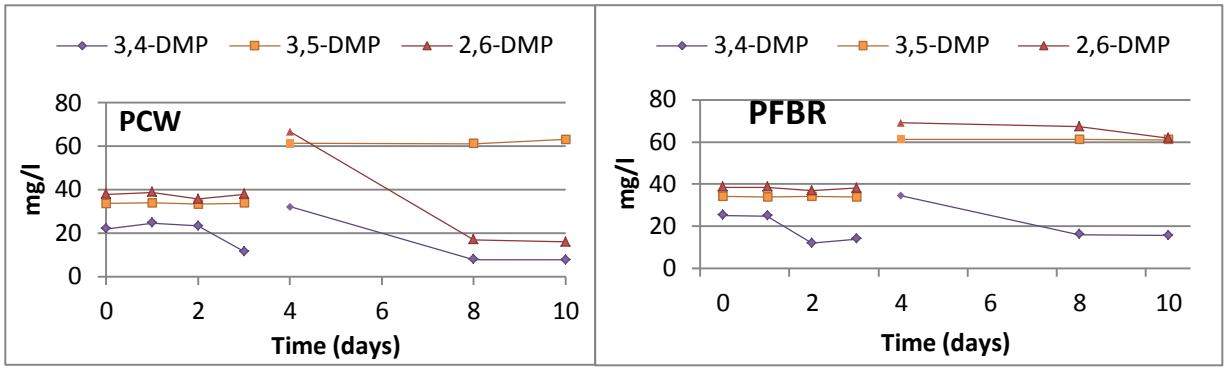

Figure 20: DMP isomers degradation in batch cultures from PCW and PFBR water samples. Incubation time of 10 days at 30°C, after 3 days DMPs were incremented to about 70 mg/L.

After evidencing a DMP residual concentration below 15 mg/L cultures were feed whit 1 mL concentrate solution of each DMP isomer. Concentrations on day 4 were taken as initial concentration to calculate the overall degradation. After 10 days 76% of 2,6-DMP was removed on PCW batch and 11% in PFBR batch culture. 3,4-DMP presented removal efficiency of 76% in PCW culture and 55% in PFB culture. 3,5-DMP was not degraded in PCW or PFBR cultures.

Changes in color with single 3,4-DMP cultures were evident at day 3 of incubation with a light yellow color followed by brownish color only in PFBR culture at 8 days. 2,6-DMP cultures presented a yellowish color since day 8. The colors were persistent for the complete test period (figure 21).

Figure 21: M9 medium with single DMP isomers, from right to left PCW and PFBR, day 10 of incubation period.

The color changes can be related to DMP degradation/transformation activity. For PCW and PFBR the change of color was presented to correspond to the decrease on 3,4-DMP concentration. As well as slight change in color for 2,6-DMP for the PCW. Therefore non change of color for 3,5-DMP can be related with the lack of 3,5-DMP degradation.

Phenol degradation components such p-benzoquinone and o-benzoquinone have been described to be intermediate metabolites of catechol degradation forming yellow and brownish coloration respectively due to the chromophore groups substituted in the aromatic ring (Mijangos et al., 2006). Medium colors obtained in this study may indicate the degradation via catechol pathway. From the color, it can be inferred that different metabolic pathways were used to degrade DMPs. This could be due to different microbial communities between PCW and PFBR able to degrade DMPs isomers by different metabolic pathways.

In the 3,4-DMP culture from PCW was observed an increase in the absorbance at 375 nm while DMPs concentration was decreasing. This may indicate the possible presence of the enzyme 2,3-dioxygenase, which activity leads to the accumulation of a methyl derivate of the 2-hydroxymuconic acid semialdehyde, that is visible at this wavelength (Dorn and Knackmuss, 1978). It is suggested to continue the studies measuring the catechol enzymes activity and the intermediate metabolites of the metabolic pathways.

6.3.2 Isolates identification

From M9 single DMP isomers cultures, 50 µL were streaked in a M9 agar plate with the DMP mix as sole carbon source for 2 cycles in order to obtain pure isolates. Single bacterial

colonies proceed to DNA extraction, PCR of 16S 27f amplification and sequencing. 21 isolates were sequenced from which 8 showed purity and identity standards. Sequences were analyzed by BLAST software were the evaluation criteria were covered by more than 95% of identity, percentage of similarity of sequence in nucleotides; the identification of the isolates is listed on in table 11. A complete table with bacteria pictures (colonies and single cells) is presented in appendix 5.

Table 11: Bacteria isolated from PCW and PFBR from water samples. Identity percentages are given by blast software.

ID	Microorganism	Ident %	Isolated from
Wp1	*Achromobacter xylosoxidans A8*	98	PCW and PFBR
Wp4	*Pseudomonas monteilii SB 3101*	97	PCW
Wp7	*Pseudomonas synxantha BG33R*	96	PCW
Wp12	*Ochrobactrum anthropi ATCC 49188*	99	PCW
Rp1	*Microbacterium testaceum StLB037*	96	PFBR
Rp5	*Alicycliphilus denitrificans BC*	99	PFBR
Rp6	*Stenotrophomonas maltophilia K279a*	99	PFBR
Rp7	*Pseudomonas putida KT2440*	99	PFBR

The identified strains were used to predict gens that encode for phenol-like cleavage enzymes as following:

Achromobacter xylosoxidans A8 from Burkhorderiales order and Alcaligenacea family has being predicted to have a plasmid which encodes for the meta pathway phenol degradation-like protein, enzyme chlorocatechol-1,2dioxygenase (Jencova et al., 2004)

Pseudomonas monteilii from the Pseudomonadales family and specie group of Pseudomonas has being found to have a protein homology expressing catechol 2,3-dioxygenase enzymes in the degradation of PAHs. This enzyme is dependent on Fe^{2+} as cofactor and interferes from homology with the pseudomonas group (Isaac et al., 2013). Protocatechuate 3,4-dioxygenase enzyme has being described for this specie, therefore has being implemented

for bioaugmentation of petrochemical effluents wastewater treatment (Dueholm et al., 2014).

Pseudomonas synxantha, from pseudomonas fluorescence group was predicted as carrier of several gens related to phenol degrading-like enzymes (catechol 1,2-dioxigenase, catechol oxidase, gentisate 1,2-dioxygenase and protocatechuate 3,4-dioxigenase) (Loper et al., 2012).

Ochrobactrum anthropi from Brucellaceae family is recognized as an opportunistic human pathogen, predicted for the gen codifying for protocatechuate 3,4-dioxygenase enzyme, so far non catechol or gentisate related enzymes have been found in these species (Chain et al., 2011). *Microbacterium testaceum StLB037* from Microbacteriaceae family was reported by its complete genome by Morohoshi et al (2011) where the genes coded for gentisate 1,2-dioxygenase and protocatechuate 3,4-dioxygenase can be predicted.

For *Alicycliphilus denitrificans BC,* family Comamonadaceae the complete genome has being reported, where the gene coded for catechol 1,2-dioxygenase is predicted (Mechichi, 2003); although the close relation with *Alicycliphilus denitrificans* K601 indicate presence of the protein catechol 2,3-dioxygenase (cofactor Fe^{2+}).

Stenotrophomonas maltophilia K279a has being predicted for the gen xylE which encodes the enzyme activity of catechol 1,2-dioxygenase on catechol and methyl derivate forms. Inhibited competence form phenol substitute in ortho position (Guzik et al., 2013)

Pseudomonas putida KT2440, from pseudomonas putida group was predicted as carrier of several gens related to phenol degrading-like enzymes (catechol 1,2-dioxigenase and protocatechuate 3,4-dioxigenase) (Nelson et al., 2002).

The predictions of gens that encode phenol degradation-like bacteria do not ensure the activity of those enzymes in the obtained strains. In order to show the DMP degradation activity of each strain batch cultures were inoculated with axenic strains.

6.4 Degradation of dimethylphenols in batch cultures

The identified bacteria strains (8 isolations) were cultivated in M9 medium with the DMP mix (2,6-, 3,4- and 3,5-DMP) as sole carbon source measuring optical density and DMPs concentration by HPLC. The initial concentration for each isomer was about 70 mg/L.

Figure 22 shows DMPs residual concentration and optical density at 620 nm for all cultures. Microbial adaptation phase (lag phase) was not evident due to previous cultivations with DMPs. Exponential growth phase (log phase) occurred from 24 to 72 hours depending on the strain.

Degradation of 2,6-, 3,4- and 3,5-DMP in the given conditions was not evidenced; DMPs concentration did not decrease more than 48 mg/L. The highest DMPs concentration decrease was 16% for 3,5-DMP in *Achromobacter xylosoxidans* (Wp1) followed by 12% for 3,4-DMP in the *Pseudomonas synxantha* (Wp7) culture.

Color change was not perceived during the culture incubation time. This could be related to low or null DMPs degradation. Abiotic degradation was observed in the control with a rate of 10% f or 3,4-DMP, 11% for 2,6-DMP and 11% for 3,5- DMP after an incubation time of 144 hours.

It can be deduced that single strains were not able to degrade DMPs isomers under the experimental conditions given in this study, in contrast with the consortium cultures (PCW and PFBR) where degradation rates were higher than 70%. This suggests the better performance of the consortium as in the study of Prpich and Daugulis (2005) where the consortium outperformed single strains isolations at phenol biodegradation.

Single strains DMPs degradation underperformed in this study could be caused by uncultured microorganism presented in consortium, co-metabolism, and accumulation of metabolites or lack of inducers. Although it cannot be exclude the degradation potential of the single stains; it needs further studies under diverse culture conditions.

Another possibility of consortium outperformance is the lack of enzymes activity. Gens that encode some of the DMP degrading enzymes can be located in plasmids (DNA molecules distinct to chromosome DNA), therefore plasmid lost during replication can lead to the loss of degradation ability as demonstrated by (Jain et al., 1984) for different *Pseudomonas*

putida strains or lost by growth on non-selective media (Hopper and Kemp, 1980). It is suggested to study degradation activity with further additional substrates that enhance DMPs degradation rates such phenol or glucose as indicated by Hinteregger et al (1992).

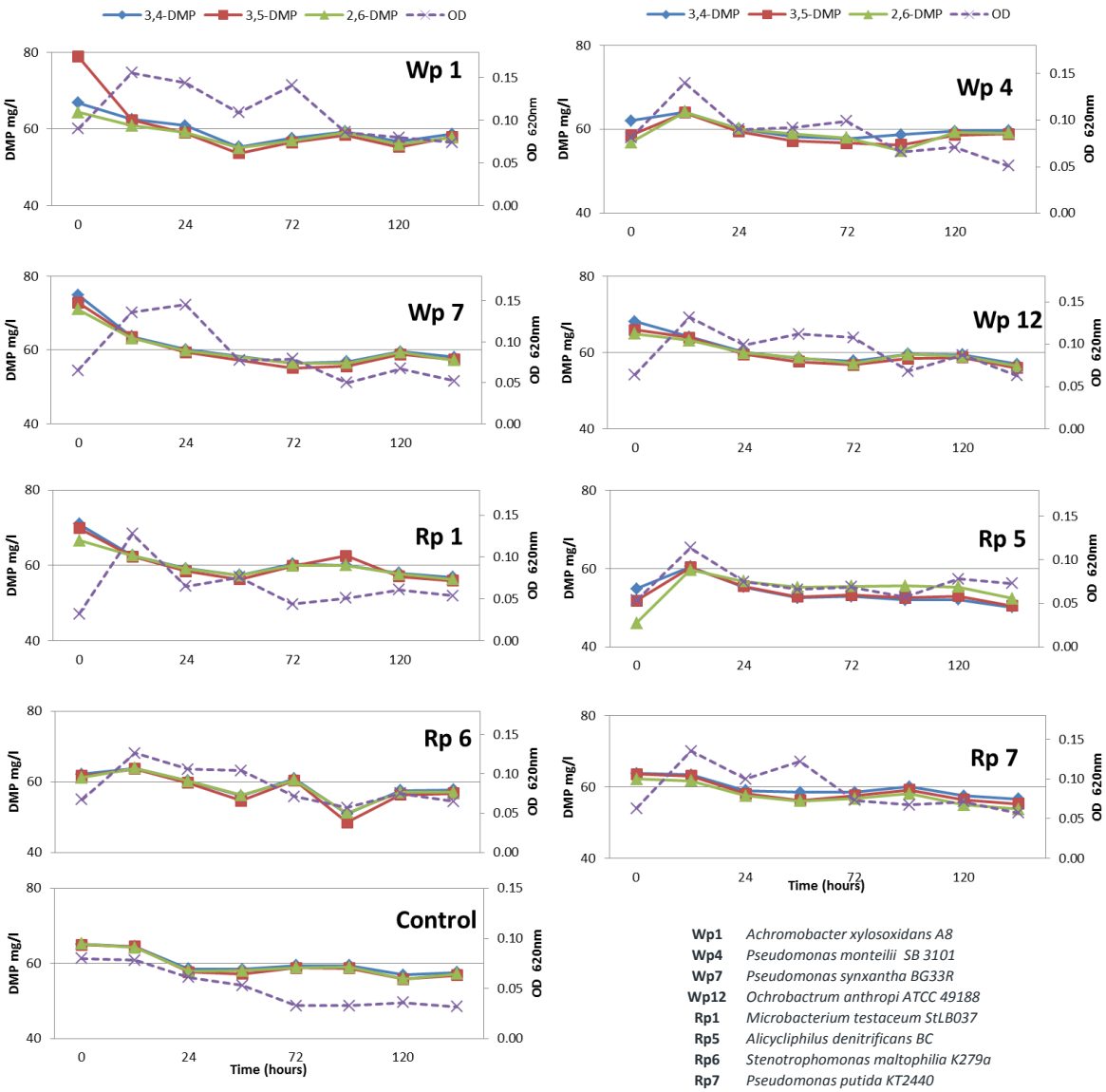

Figure 22: Optical density to 620 nm for all identified isolations for a total of 144 hours

6.4.1 Isolation from gravel samples

An additional batch culture was cultivated from gravel from the PCW, where bacteria grew poorly. This may be caused for the limited availability of cofactors of the medium implemented (minimum medium) in comparison with M9 medium. All isomers were studied

in this test (figure 23). A major removal rate was observe in 2,5-DMP with 55% removal. In this same culture after 14 days also fungal growth was detected.

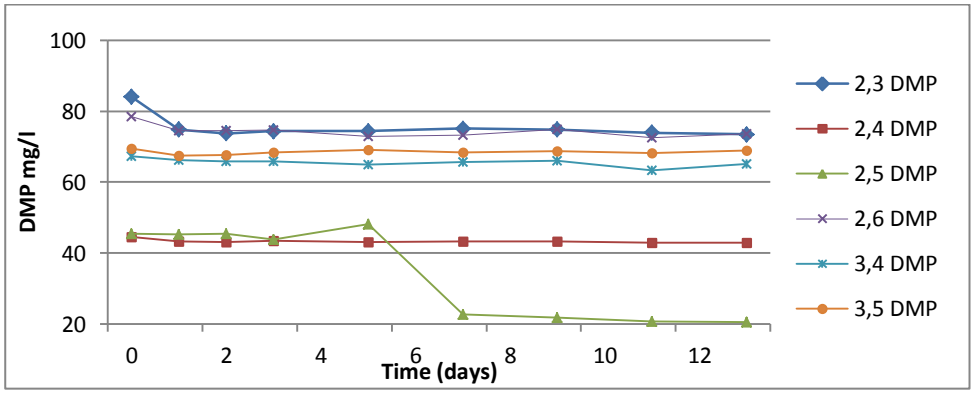

Figure 23: Degradation of separated DMP isomers from gravel PCW samples.

The fungus presented septet hyaline hyphae, un-branched phialides (narrow at the apex) hyaline fusiform conidia grouped and in strings; as it is shown in figure 24. It can be hypotetize that the fungus isolated from PCW is able to grow in a minimum medium with 2,5-DMP as sole carbon source and it can be related to *Gliomastix* spp. (*Acremonium spp.).* This specie has being isolated from coke waste water (Kwon et al., 2002), reported with polyphenol oxidase activity related to copper ions (Gouka et al., 2001)

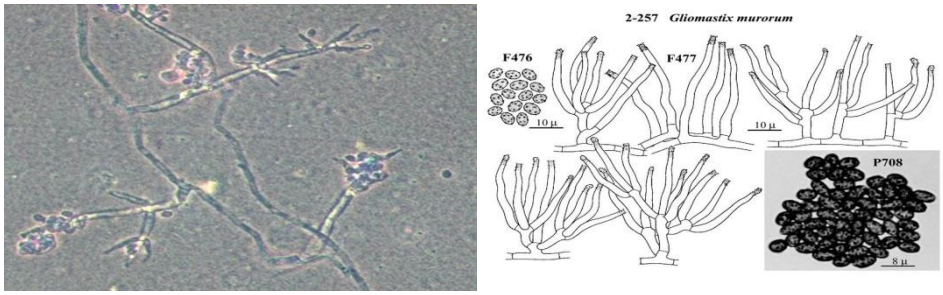

Figure 24: Left. Fungus microscopy image 40x (grow in 2,5-DMP). Right, Gliomastix murorum (Matsushima, 1975)

7. Conclusions

The experimental findings from these laboratory scale CWs suggest that CWs are a suitable treatment method for degrading/removing DMPs. DMPs removal efficiency in this study reached 96% and 100% for the planted horizontal subsurface flow CW and the Planted Fixed Bed Reactor under aerobic conditions.

The planted horizontal subsurface flow CW demonstrated better performance than the unplanted horizontal subsurface flow CW (control) due positive impact of plants as part of the matrix for biofilm formation, supporting bacterial and fungal population able to degrade DMPs.

Oxygen availability plays an important role in the degradation of 2,6-DMP. In the Planted Fixed Bed Reactor was obtained 100% removal efficiency for all isomers probably due the system design of continuously mixing which provides better dissolved oxygen transfer rates in the system. In contrast in the planted horizontal subsurface flow CW 2,6-DMP was not so well removed indicating anoxic zones with oxygen limitation.

The isolated strains were not able to degrade the single DMPs under the studied conditions. In contrast to this the complex microbial consortia were able to degrade them. At present it cannot be excluded that the degradation potential of the single stains exists. So, further studies are needed under diverse culture conditions and the consideration of possible co-metabolism.

In batch cultures and in the experimental CW stable organic carbon (metabolites) from DMPs removal/degradation was formed. This phenomenon should be further studied in order to determine DMPs metabolic degradation pathways. The knowledge of the responsible DMPs degradation enzymes represents a potential tool to evaluate biodegradation activity of carbochemical wastewater treatment by rapid in-situ method monitoring the abundance and behavior of degrading bacteria community.

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9. Appendix

Appendix 1: Composition of trace mineral solution TMS3

Compound	concentration [g/L]
Disodium salt (Titriplex III)	1.0
$FeSO_4 . 7 H_2O$	1.0
$MnCl_2 . 2 H_2O$	0.8
$CoCl_2 . 6 H_2O$	1.7
$CaCl_2 . 2 H_2O$	0.7
$ZnCl_2$	1.0
$CuCl_2 . 2 H_2O$	1.5
$NiCl_2 . 6 H_20$	0.3
H_3BO_3	0.1
$Na_2MoO_4 . 2 H_2O$	0.1
$Na_2SeO_3 . 5 H_2O$	0.02
HCl (concentrated solution)	3 (mL/L)

Appendix 2: M9 medium components (Juretschko et al., 1998)

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

Solution A (50 mL)	
$MgCl_2 \cdot 6H_2O$	0.5375 g
$CaCO_3$	0.1000 g
$FeSO_4 \cdot 7H_2O$	0.2250 g
$ZnSO_4 \cdot 7H_2O$	0.0720 g
$MnSO_4 \cdot 4H_2O$	0.0424 g
$CuSO_4 \cdot 5H_2O$	0.0125 g
$CoCL_2 \cdot 6H_2O$	0.0140 g

H_3BO_3	0.0030 g
HCl (37%)	2.565 mL

Solution B	
$MgSO_4.7H_2O$	1 M

Solution C	
$FeSO_4.7H_2O$	36 Mm

Appendix 3: Mineral salts

Compound	concentration [g/L]
Na_2HPO_4	70
KH_2PO_4	28
NaCl	5
NH_4Cl	10

Appendix 4: Trace elements

Compound	concentration [g/L]
MgSO4 . 7 H_2O	50
$FeSO_4$. 7 H_2O	5
$MnSO_4$. H_2O	2,5
$ZnCl_2$	3,2
$CaCl_2$. 6 H_2O	0.5
$BaCl_2$	0,3
$CoSO_4$. 7 H_2O	0,18
CuSO . $5H_2O$	0.18
H_3BO_3	3.25
EDTA	5
HCl (37%)	73 mL (2M)

Appendix 5: PCW and PFBR bacteria isolations

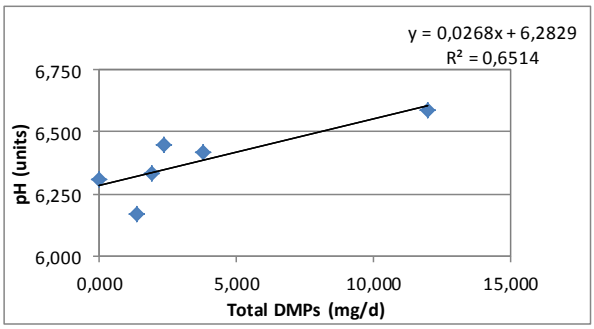
Name seq	ISOLATES SEQUENCING FROM PCW	Max score		Total score	Query cover	Ident	Colony picture	Microscope 40X
Wp1	Achromobacter xylosoxidans A8 chromosome, complete genome	1742	5227	99%	0.0	98%		
	Bordetella avium 197N chromosome, complete genome	1653	4961	99%	0.0	97%		
	Bordetella bronchiseptica 253, complete genome	1626	4878	99%	0.0	96%		
	Bordetella parapertussis Bpp5, complete genome	1626	4878	99%	0.0	96%		
Wp4	Pseudomonas monteilii SB3101, complete genome	1820	10920	99%	0.0	97%		
	Pseudomonas stutzeri A1501 chromosome, complete genome	1820	7263	99%	0.0	97%		
	Pseudomonas entomophila L48 chromosome, complete genome	1808	12657	99%	0.0	97%		
	Pseudomonas putida KT2440 chromosome, complete genome	1803	12601	99%	0.0	97%		
Wp7	Pseudomonas synxantha BG33R chromosome, whole genome shotgun sequence	1886	11278	98%	0.0	96%		
	Pseudomonas mendocina ymp chromosome, complete genome	1886	7529	98%	0.0	96%		
	Pseudomonas monteilii SB3101, complete genome	1882	11297	98%	0.0	96%		
	Pseudomonas stutzeri A1501 chromosome, complete genome	1877	7503	98%	0.0	96%		
Wp12	Ochrobactrum anthropi ATCC 49188 chromosome 2, complete sequence	1182	2365	99%	0.0	99%		
	Ochrobactrum anthropi ATCC 49188 chromosome 1, complete sequence	1182	2365	99%	0.0	99%		
	Brucella ceti TE10759-12 chromosome 2, complete sequence	1166	1166	99%	0.0	99%		
	Brucella ceti TE10759-12 chromosome 1, complete sequence	1166	2332	99%	0.0	99%		

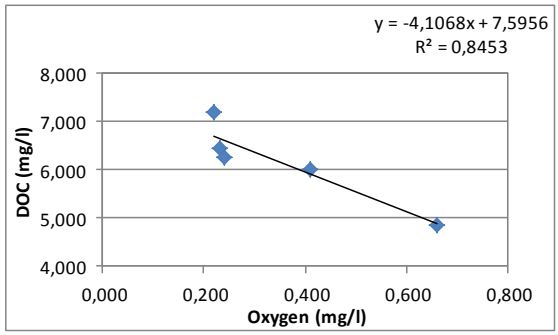
Name seq	ISOLATES SEQUENCING FROM PFBR	Max score		Total score	Query cover	Ident	Colony picture	Microscope 40X
Rp1	Microbacterium testaceum StLB037, complete genome	1820	3634	100%	0.0	96%		
	Clavibacter michiganensis subsp. michiganensis NCPPB 382 chromosome, co	1565	3130	100%	0.0	92%		
	Leifsonia xyli subsp. xyli str. CTCB07 chromosome, complete genome	1539	1539	96%	0.0	93%		
	Micrococcus luteus NCTC 2665 chromosome, complete genome	1533	3067	100%	0.0	92%		
Rp5	Alicycliphilus denitrificans BC chromosome, complete genome	1951	5853	98%	0.0	99%		
	Acidovorax ebreus TPSY chromosome, complete genome	1784	5354	98%	0.0	96%		
	Acidovorax sp. JS42 chromosome, complete genome	1784	5354	98%	0.0	96%		
	Acidovorax avenae subsp. avenae ATCC 19860 chromosome	1724	5172	98%	0.0	95%		
Rp6	Stenotrophomonas maltophilia K279a chromosome, complete genome	1977	7874	99%	0.0	99%		
	Xanthomonas hortorum pv. carotae str. M081 chromosome, whole genome s	1829	1829	99%	0.0	96%		
	Xanthomonas campestris pv. campestris str. ATCC 33913 chromosome, comp	1829	3658	99%	0.0	96%		
	Xanthomonas oryzae pv. oryzae KACC 10331 chromosome, complete genome	1823	3647	99%	0.0	96%		
Rp7	Pseudomonas putida KT2440 chromosome, complete genome	1938	13562	99%	0.0	99%		
	Pseudomonas monteilii SB3101, complete genome	1921	11529	99%	0.0	99%		
	Pseudomonas entomophila L48 chromosome, complete genome	1921	13445	99%	0.0	99%		
	Pseudomonas fulva 12-X chromosome, complete genome	1882	7520	99%	0.0	98%		

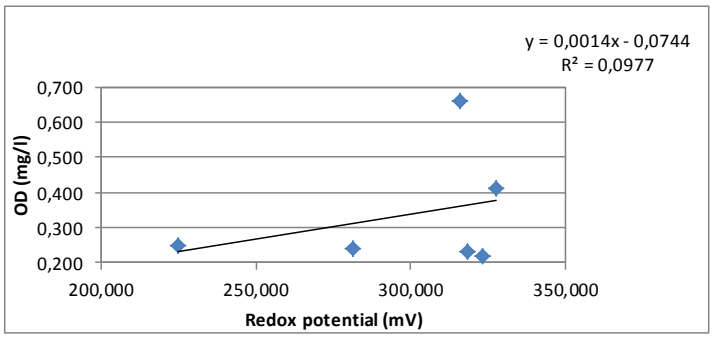
Appendix 6: PCW correlation analysis

	PCW Outflow							
	3,4 DMP	3,5 DMP	2,6 DMP	Total DMPs	pH	Redox	Oxygen	DOC
12/03/2014	0,593	0,000	1,740	2,332	6,450	323,320	0,220	7,190
19/03/2014	0,077	0,168	1,132	1,376	6,170	318,220	0,230	6,440
26/03/2014	0,000	0,000	0,000	0,000	6,310	224,880	0,250	
02/04/2014	0,000	0,000	1,899	1,899	6,330	327,880	0,410	6,020
17/04/2014	0,386	1,105	2,299	3,790	6,420	315,880	0,660	4,860
07/05/2014	0,843	2,962	8,174	11,979	6,590	281,420	0,240	6,240

	3,4 DMP	*3,5 DMP*	*2,6 DMP*	*Total DMPs*	*pH*	*Redox*	*Oxygen*	*DOC*
3,4 DMP	1,000							
3,5 DMP	0,767	1,000						
2,6 DMP	0,808	0,950	1,000					
Total DMPs	0,832	0,972	0,995	1,000				
pH	0,881	0,753	0,791	0,807	1,000			
Redox	0,120	-0,121	0,035	0,000	-0,057	1,000		
Oxygen	-0,101	0,058	-0,082	-0,047	0,074	0,312	1,000	
DOC	0,182	-0,259	-0,035	-0,083	-0,027	0,060	-0,919	1,000



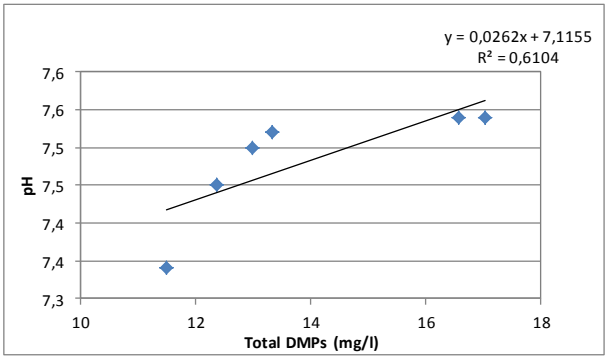


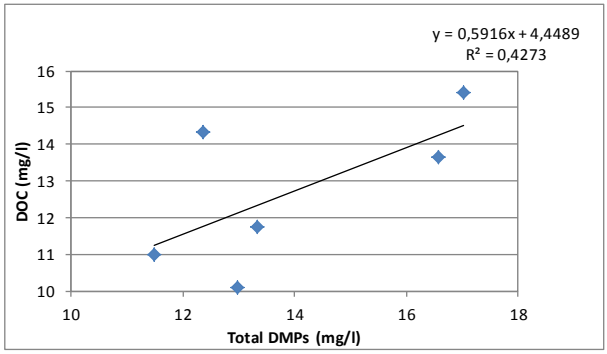


Appendix 7: UPCW correlation analysis

	UPCW Outflow							
	3,4 DMP	3,5 DMP	2,6 DMP	Total DMPs	pH	Redox	Oxygen	DOC
12/03/2014	2	5	5	12	7,5	254	0,2	14
19/03/2014	2	5	5	13	7,5	236	0,2	10
26/03/2014	2	5	5	11	7,3	249	0,2	11
02/04/2014	3	7	7	17	7,5	285	0,2	15
17/04/2014	2	6	5	13	7,5	301	0,8	12
07/05/2014	3	6	7	17	7,5	90	0,2	14

	3,4 DMP	*3,5 DMP*	*2,6 DMP*	*Total DMPs*	*pH*	*Redox*	*Oxygen*	*DOC*
3,4 DMP	1,000							
3,5 DMP	0,854	1,000						
2,6 DMP	0,945	0,939	1,000					
Total DMPs	0,950	0,967	0,992	1,000				
pH	0,798	0,760	0,741	0,781	1,000			
Redox	-0,593	-0,178	-0,429	-0,387	-0,191	1,000		
Oxygen	-0,148	0,053	-0,242	-0,119	0,208	0,495	1,000	
DOC	0,629	0,601	0,670	0,654	0,401	-0,081	-0,182	1,000





Appendix 8: PFBR correlation analysis

Concentration of components in PFBR outflow

Date	Sampling day	pH	OD	Redox	T°
19/05/2014	1	6,53	0,3405	198	28,1
20/05/2014	2	6,33	0,1008	193	27,4
21/05/2014	3	6,13	0,0422	176	27,1
22/05/2014	4	6,22	0,1113	195	31,4
23/05/2014	5	6,33	0,3716	221	24,4
	Average	6,31	0,193	196,6	27,68
	standard deviation:	0,150	0,151	16,10279	2,509

Correlation analysis

	pH	*OD*	*Eh*	*T*
pH	1			
OD	0,757864	1		
Redox	0,474468	0,834578	1	
T	-0,13919	-0,47047	-0,44818	1