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Activated Carbon Fibers as Biological Supports and Redox Mediators in the Biotic and Abiotic Anaerobic Transformation of Nitroaromatic Compounds

Presenta:

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Constancia de aprobación de la tesis

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que se desarrolló bajo la dirección de

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Dedicatoria

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Abbreviations

AAQ	2-Aminoanthraquinone
ACF	Activated carbon fiber
AQDS	Anthraquinone,2,6-disulfonate
AP or 4AP	4-Aminophenol
AW	Fabric (unmodified) ACFs
3CA	3-Chloroaniline
3CNB	3-Chloronitrobenzene
COD	Chemical oxygen demand
DNAN	2,4-Dinitroanisole
HS^-	Hydrosulfide ion
H_2S	Hydrogen sulfide
HPLC-DAD	High performance liquid chromatography - Diode array detection
MENA	2-Methoxy, 5-nitroaniline
NAC	Nitroaromatic compounds
Na_2S	Sodium sulfide
NP or 4NP	4-Nitrophenol
OX	ACFs modified with nitric acid
PZC	Point of zero charge
UHPLC-QTOF-MS	Ultra high pressure liquid chromatography - quadrupole time of flight - mass spectrometry
VFA	Volatile fatty acid

Resumen

El objetivo del presente trabajo de tesis fue demostrar que las fibras de carbón activado (FCAs) actúan como mediadores redox en la reducción química (abiótica) y biológica de compuestos nitro-aromáticos (CNA) en sistemas en lote y continuo. En la parte fisicoquímica se estudió la reducción en lote de dos nitroaromaticos modelo, 4-nitrofenol (NF) y 3-cloronitrobenceno (CNB), usando FCAs como mediadores redox y Na_2S como donador de electrones. La caracterización de las propiedades fisicoquímicas y el análisis de la literatura reveló la importancia de estructuras quinónicas en la superficie de las FCAs para oxidar el Na_2S y reducir el CNA modelo a su correspondiente amina aromática. De forma similar, también se exploró el anclaje de antraquinonas en la superficie de las FCAs con el objetivo de aumentar la tasa de reducción de NP. Los resultados de este anclaje demostraron que los materiales modificados con antraquinona,2,6-disulfonato (AQDS) mejoraron la reducción de NP en un 49% comparado con las FCAs sin modificar.

Las FCAs fueron utilizadas como soportes biológicos y mediadores redox en la biotransformación continua del nitroaromático NP usando lodo granular metanogénico y etanol como fuente de carbono. Los resultados mostraron que utilizando una concentración en exceso de fuente de carbono los bioreactores empacados con FCAs con diferentes cantidades de grupos quinónicos no mostraban diferencias significativas en la biotransformación de NP con el bioreactor control sin FCAs. Después de una serie de modificaciones a la concentración de la fuente de carbono se logró visualizar una mayor biotransformación de NP en los reactores empacados con FCAs, mientras que el reactor control mostró un menor rendimiento. Se piensa que el mayor contenido de grupos funcionales con actividad redox así como otras propiedades fisicoquímicas de los materiales carbonosos promovieron la mayor reducción de NP.

Por otro lado, en la parte biológica se estudió primero la biotransformación del compuesto explosivo 2,4-dinitroanisole (DNAN) y su derivado 2-metoxy,5-nitroanilina (MENA) en columnas de suelo con flujo ascendente y utilizando etanol como fuente de carbono exógena. Una columna que no recibió etanol y que dependió del carbono orgánico endógeno del suelo sirvió como control de un proceso de atenuación natural. Los resultados fueron contundentes al demostrar que el proceso de biotransformación no solo incluía la reducción del grupo nitro, sino procesos de O-demetilación de DNAN y MENA, junto con la N-acetilación de aminas aromáticas y la formación de dímeros y trímeros. Finalmente también se demostró que al promover las condiciones reductivas en las columnas de suelo con el uso de etanol se obtenía una mayor concentración de aminas aromáticas,

mientras que sin etanol se promovían otros procesos de biotransformación como la O-demetilación.

Finalmente, la relevancia científica de este trabajo está en la generación de conocimiento respecto a los procesos fisicoquímicos y biológicos que intervienen en la biotransformación de compuestos nitroaromáticos. Las contribuciones más representativas se basan en el uso de técnicas de caracterización de carbones activados para explicar el fenómeno de reducción de compuestos nitroaromáticos en fase acuosa. Por otro lado, este trabajo también aporta conocimiento en el área biotecnológica respecto a los procesos de biotransformación de compuestos nitroaromáticos cuando estos entran en contacto con el suelo (simulado en columnas de flujo ascendente) y cuando están en presencia/ausencia de alguna fuente de carbono exógena. Tomando en cuenta los resultados de esta tesis se puede concluir que el uso de FCAs como mediadores redox en sistemas fisicoquímicos y biológicos puede ser una tecnología viable para la eliminación de contaminantes recalcitrantes, aunque su aplicación a gran escala aún requiere futuros estudios.

Abstract

The objective of this thesis work was to demonstrate that activated carbon fibers (ACFs) functioned as redox mediators in the chemical (abiotic) and biological reduction of nitroaromatic compounds (NACs) in aqueous solutions. Physicochemical studies included the reduction of two model nitroaromatic compounds, 4-nitrophenol (NP) and 3-chloronitrobenzene (CNB) using ACFs as redox mediators and Na_2S as electron donor. Physicochemical characterization of ACFs and the literature analysis revealed the critical role of quinone structures in the oxidation of Na_2S and the reduction of NACs to aromatic amines. Also, the anthraquinone anchorage on ACF surface also exhibited a higher redox activity. The results showed that materials with anthraquinone,2,4-disulphonate (AQDS) improved in 49% the reduction of NP when compared to the unmodified materials.

ACFs were used as biological supports and redox mediators in the continuous biotransformation of NP using methanogenic granular sludge and ethanol as carbon source. The results showed that an excess of carbon source affected the biotransformation of NP since bioreactors packed with ACFs with different concentrations of redox-active groups did not exhibited significant differences with the control reactor without ACFs. After modifications in the concentration of ethanol (exogenous carbon source) the ACF-packed reactors achieved a higher biotransformation of NP while the control reactor exhibited a low efficiency. It was hypothesized that a higher concentration of redox-active groups together with other ACF physicochemical properties increased the biotransformation of NP as compared with the control reactor.

On the other hand, the biotransformation of explosive NACs 2,4-dinitroanisole (DNAN) and its reduced product 2-methoxy,5-nitroaniline (MENA) was studied in up-flow anaerobic soil columns amended with ethanol as an exogenous carbon source. The control bioreactor relied only in endogenous carbon source present in soil and simulated the natural attenuation of the explosive compounds. The results clearly showed that the biotransformation process included not only nitro group reduction but also included O-demethylation of DNAN and MENA, together with the N-acetylation of aromatic amines and the generation of dimers and trimers. Finally, it was demonstrated that the concentration of aromatic amines was higher under the reductive conditions promoted with ethanol, while the lack of it promoted other biotransformation processes like O-demethylation.

Finally, the scientific relevance of this work relies in the generation of knowledge about physicochemical and biological processes involved in the biotransformation of nitroaromatic compounds. The most representative contributions are based in

the use of characterization techniques for ACFs in order to explain the reduction of nitroaromatic compounds under anaerobic conditions. On the other hand, this work also contributes to biotechnology with the study of the biotransformation of nitroaromatic compounds when they reach the soil (simulated in up-flow glass columns) in the presence/absence of an exogenous carbon source. Taken as a whole, the results of this thesis suggest that the use of ACFs as redox mediators in chemical and biological systems is a potential technology for the elimination of recalcitrant pollutants, although their industrial application require further studies.

CHAPTER 1.- INTRODUCTION

1.1. Nitroaromatic compounds – Uses, environmental issues, and remediation technologies

Nitroaromatic compounds (NACs) are characterized by the presence of at least one nitro group in the aromatic structure and belong to the largest and most important groups of industrial chemicals in use today [1]. Nitroaromatics such as 2,4,6-trinitrotoluene (TNT) or other products with nitro groups such as hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX), and octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX) have been used extensively as the main components of explosive formulations by the US military [2]. However, they are currently being replaced by low-sensitive high-temperature detonation substitutes like 2,4-dinitroanisole (DNAN) [3, 4]. On the other hand, several pharmaceutical products such as acetaminophen [5] and anpirtoline [6] are produced from nitrophenols or chloronitrobenzenes, respectively. Moreover, nitrobenzene, nitrotoluenes, nitrophenols, and their halogenated derivatives serve as starting compounds in the production of a wide variety of pesticides [1, 7]. The extended use of NACs in industry is followed by their potential unwanted release into the environment in the form of gaseous emissions, solid residues, and wastewater.

NACs could be released to air directly from a variety of incomplete combustion process [8] such as diesel and gasoline engine exhaust [9]. Unconsumed ordnance on firing ranges and wastewaters from ammunition processing factories could accumulate solid and liquid residues which could leak into nearby surface waters and then into aquifers [4]. The hazard of NACs in the environment is their toxicity to living organisms [7] and the mutagenicity of the nitroso and hydroxyl-amine derivatives [10] produced under the redox (reductive/oxidative) conditions in air, water and soil. According to the "Wiley's Remediation Technologies Handbook" written by Jay H. Lehr [11] there are several technologies currently available in order to eliminate or reduce the toxicity of the NACs (adapted from reference 11):

Physicochemical Technologies:

- **Granular Activated Carbon (GAC) -** GAC is a commercially available ex situ contaminant-removal technology that extracts contaminants from liquid and airstreams by adsorption. GAC is generally used to collect low levels of contaminants. It is typically used to remove organics but can also be used to adsorb and concentrate inorganics for further treatment or as a polishing treatment in conjunction with other remediation technologies.
- **ECO Purification Systems USA, Inc. -** This technology uses catalytic oxidation to destroy dissolved organic contaminants in a fixed-bed reactor. Ozone and the polluted water pass through the reactor where the organic contaminants are oxidized to form carbon dioxide and water. Clean water is discharged, and residual ozone is recycled or destroyed within the treatment system.
- **The ARS Technologies, Inc.** - Ferox (^{SM}) process is an in situ remediation technology for the treatment of chlorinated hydrocarbons, leachable heavy metals, and other contaminants. The process involves the subsurface injection and dispersion of reactive zero-valence iron powder into the saturated or unsaturated zones of a contaminated area.
- **ManTech Environmental Corporation -** Electro Chemical GeoOxidation (ECGO) is an in situ technology designed to remediate organic-contaminated soil and groundwater as well as liquid and sludge waste streams. The process works by applying an electrical current to probes driven into the ground at contaminated sites.
- **Electrokinetic Remediation -** The technology employs a low-intensity direct electrical current to desorb and remove ionic and polar organic contaminants from the subsurface. The current is applied across electrode pairs that have been placed in the ground on either side of the contaminated zone. Surfactants and/or complexing agents may be introduced at the electrodes to enhance contaminant removal rates.

Biological Technologies:

- **Molasses Treatment for Bioremediation** - Molasses has been used as a carbon and energy source to encourage the anaerobic bioremediation of soil and groundwater contaminated with metals, explosives, and chlorinated solvents. The molasses can be added to excavated, screened soil, or injected directly into the subsurface via wells.
- **Grace Bioremediation Technologies (Daramend®) -** Daramend accelerates degradation of the target compounds by combining contaminated soil with solid-phase, organic, and inorganic soil amendments of specific particle size and nutrient content.
- **Idaho Research Foundation, Inc., and White Shield, Inc. -** The Simplot Anaerobic Biological Remediation (SABRE) process is a patented, ex situ technology used to treat soils contaminated with nitroaromatic compounds. Researchers isolated a selection of anaerobic bacteria based on their ability to degrade nitroaromatic compounds with the total destruction of intermediate compounds by the completion of treatment. These bacteria are the basis of the SABRE process.
- **Electrokinetically Enhanced Bioremediation -** Electrokinetically enhanced bioremediation is an in situ process for the treatment of soils and groundwater contaminated with petroleum hydrocarbons and other compounds easily biodegraded under anaerobic conditions. Bench-scale tests have shown that the application of an electric field provides electrokinetic transport of nutrients and biodegrading bacteria to areas of contamination. In addition, microbial growth is enhanced, nitrate transport can be predicted, and beneficial temperature increases can be achieved to areas of contamination.

Additional remediation technologies such as advanced oxidation processes, incineration or biological GAC-supported treatments are described in Rodgers and

Bunce 2001 [12]. The proper remediation strategy depend (mostly) on the concentration of NACs since energy intensive treatments such as incineration may be too expensive at low concentrations or the toxicity of high NAC concentrations could limit the applicability of bioremediation [12]. Among the current technologies, bioremediation is a low cost and environmentally friendly approach to treat NACs in diluted aqueous solutions.

1.2. Biotransformation of nitroaromatic compounds and the role of redox mediators

Bioremediation usually employs aerobic conditions to promote mineralization of organic compounds to CO_2 and H_2O [12]. In the case of NACs they can be biodegraded under aerobic conditions, but the possibility of oxidative degradation decreases if the number of nitro substituents increases [7] due to electrostatic repulsions between the electronegative groups (e.g. nitro moieties) and the oxygenases [13]. On the other hand, reduction of nitro group on NACs is catalyzed by a group of enzymes known as oxygen insensitive nitroreductases [7, 14]. These flavoproteins widely distributed in nature have been suggested to mediate the sequential transfer of 2 electrons from NADH or NADPH to the nitro moiety of NACs [14-16]. The reduction of an aromatic nitro group to the corresponding amino derivative is commonly assumed to occur in three steps [17] (see Figure 1.1).

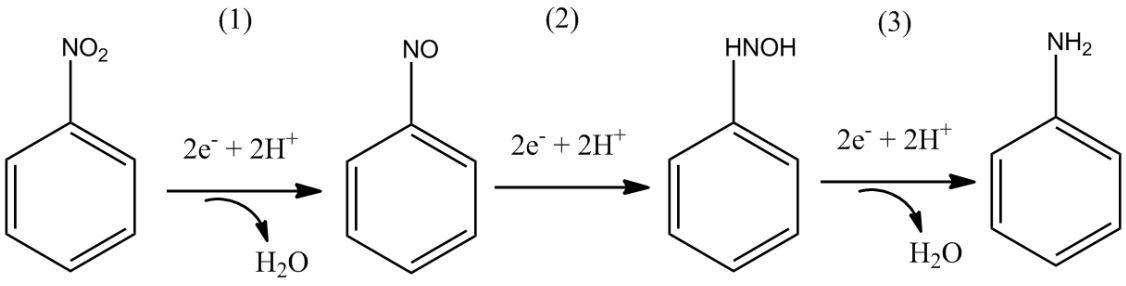

Figure 1.1. Steps in the reduction of a NAC to an aromatic amine: electron and proton transfer to 1) NAC, 2) nitroso compound, and 3) hydroxylamine compound.

Nitroso and hydroxylamine compounds are widely known intermediates from nitro group reduction [7, 17].Usually the nitroso derivatives have been reported as short-

live compounds not detected at the end of the reduction process [18], leading to hydroxylamine or amine derivatives as the possible end products [7]. The resulting amino-aromatic derivatives are troublesome for the nucleophilic attack of anaerobes [19] or any other chemical reducing agent, but they could be treated in a secondary aeration process where they can polymerize [20, 21] and/or attach to hydrophobic synthetic materials without further damage to the environment.

The anaerobic biotransformation of NACs has been studied with detail in the past decades. For more information about the biotransformation of NACs the reader should be directed to the several articles [22-24] and reviews [1, 2, 7, 25, 26] available in literature detailing the aerobic and/or anaerobic metabolic routes for the remediation of mono- and poly-nitroaromatic compounds. Nonetheless, in recent years the kinetics of biotransformation of NACs has been catalyzed by the use of soluble redox mediators. These chemical substances accept electrons from the microbial oxidation of an organic compound (e.g. glucose) and then shuttle the electrons to the nitroaromatic, azo, or polychlorinated compounds of interest [27-38]. The catalytic effect of the soluble redox mediators depend strongly on their chemical structure [35] and the experimental conditions used to reduce the electron withdrawing group [29]. The success of a redox mediator is usually correlated with the presence of quinone groups in their structure due to the property of these functional groups to accept and donate electrons reversely in the form of quinone/hydroquinone redox reactions (see Figure 1.2). However, the main disadvantage of soluble redox mediators under continuous conditions is their tendency to be lost in the bioreactors effluent solution, together with the associated expenses to maintain catalytic levels of such compounds inside the anaerobic system [39, 40]. For this reason it is highly desirable to immobilize the redox mediators on the surface of easy-handling materials capable of remain inside the anaerobic systems for long periods of time.

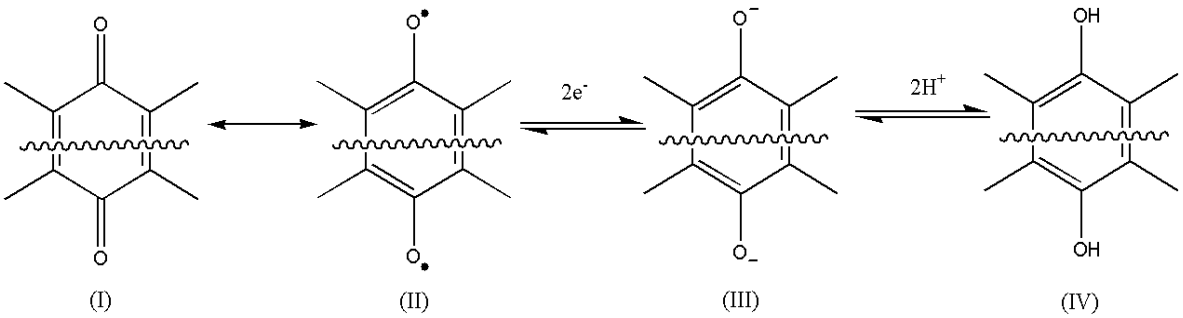

Figure 1.2. Electron transfer activity of quinone sites (adapted from Leon y Leon and Radovic [41]). From left to right: two carbonyl groups (quinone, structure I) stabilize radicals (structure II) via resonance; the radicals can reversibly accept electrons and become anions (structure III); anions could reversibly accept protons from solution and lead to phenolic hydroquinone sites (structure IV).

1.3. State of the art in the use of redox mediators in anaerobic systems

The latest reports on the use of redox mediators to speed up the abiotic and biotic anaerobic reduction of several chemical compounds have followed three different mainstreams:

1) **Use of soluble redox mediators** in the transfer of electrons from a chemical [17, 37, 42-45] or biological [28-35, 37, 38] source to the nitro, azo or other halogenated chemicals. See Table 1.1.
2) **Use of solid redox mediators** in the transfer of electrons from a chemical [39, 46-59] or biological [39, 52, 57, 60-67] source to the nitro, azo or other halogenated chemicals. See Table 1.2.
3) **Use of a solid support (usually with electro active functionalities) for the immobilization of a redox mediator** in the transfer of electrons from a chemical [68, 69] or biological [40, 70-77] source to the nitro, azo or other halogenated chemicals. See Table 1.3.

Table 1.1. Use of soluble redox mediators in the transfer of electrons from a chemical or biological source to the nitro, azo or other halogenated chemicals: Case 1.

		Abiotic electron donor	Soluble redox mediator	Biotic electron source	Inoculum	Pollutant	Batch (B) / Continuous (C)	Ref.
Case 1 - Soluble Redox Mediators	ABIOTIC	Na_2S	Quinone and Iron Porphyrin			Nitroaromatics	B	17
		Na_2S	AQDS			Acid Orange 7 and Reactive Red 2	B	37
		Perchloric acid in acetonitrile	NADH			Nitrobenzene	B	42
		Na_2S	Juglone			Hexachloroethane	B	43
		Na_2S	Iron Porphyrin and Mercaptojuglone			Polyhalogenated Methanes and Ethanes	B	44
		NaHS and cysteine	Anthraquinones			Carbon tetrachloride	B	45
	BIOTIC		AQDS	VFA mix	Anaerobic sludge	Azo dyes	C	28
			AQDS	Ethanol	Anaerobic sludge	Azo dyes	C	29
			AQDS	Formate and Glucose	Anaerobic sludge	Azo dyes	B and C	30
			AQDS, AQS and riboflavin		Anaerobic sludge	Azo dyes	B	31
			AQDS	VFA mix	Anaerobic sludge	Acid Orange 7	C	32
			Riboflavin	Glucose	Anaerobic sludge	Azo dyes	B	33
			Humic substances, AQDS, NQS	Glucose	Anaerobic sludge	Carbon tetrachloride, and Reactive Red 2	B	34
			Anthraquinones		Taxonomically different bacteria	Azo dyes	B	35
			AQDS	VFA mix	Anaerobic sludge	Acid Orange 7 and Reactive Red 2	B	37
			Humic substances and AQDS	VFA mix	Anaerobic sludge	Carbon tetrachloride	B	38

Table 1.2. Use of solid redox mediators in the transfer of electrons from a chemical or biological source to the nitro, azo or other halogenated chemicals: Case 2.

		Abiotic electron donor	Solid redox mediator and/or support	Biotic electron source	Inoculum	Pollutants	Batch (B) / Continuous (C)	Ref.
Case 2 - Solid Redox Mediators	ABIOTIC	Na_2S	GAC			Acid Orange 7	B	39
		Na_2S	NOM			Nitroaromatics	B	46
		ZVI	ZVI, humus, diclone			Nitroaromatics	B	47
		Na_2S	Black carbon			RDX	B	48
		Na_2S	Black carbon			Nitrobenzenes	B	49
		H_2O_2	Activated carbon			2-nitrophenol	B	50
		Na_2S	Graphene oxide			Nitrobenzene	B	51
		Na_2S	Graphene oxide			RR2, 3-chloronitrobenzene	B and C	52
		Dithiothreitol	Graphite- and black carbon (soot)			Nitroaromatics	B	53
		Na_2S	Graphite, activated carbon, and diesel soot			Nitroaromatics	B	54
		$NaBH_4$	N-doped graphene			4-nitrophenol	B	55
		$NaBH_4$	Graphene oxide			4-nitrophenol	B	56
		Na_2S	GAC			Azo dyes	B	57
		Na_2S	Black carbon			Nitroaromatics	B	58
		Na_2S	Black carbon			RDX	B	59
	BIOTIC		GAC	VFA mix	Anaerobic sludge	Acid Orange 7	B and C	39
			Graphene oxide	Lactate/ethanol	Anaerobic sludge	RR2, 3-chloronitrobenzene	B and C	52
			GAC	Different carbon sources	Anaerobic biomass	Azo dyes	B	57
			GAC, GAC-H2, Xerogels, Nanotubes	VFA mix	Anaerobic sludge	Azo dyes	B	60
			Carbon cloth	Glucose	Domestic wastewater	Nitrobenzene	B	61
			ACFs	Glucose	Anaerobic sludge	Azo dye methyl red	B	62
			ACFs	Glucose	Anaerobic sludge	methyl red	B	62
			GAC	Acetate	Anaerobic mixed culture	Acid Orange 7	C	63
			GAC	Acetate	Anaerobic mixed culture	Rhodamine	C	64
			GAC	Acetate	Anaerobic mixed culture	Azo dyes	C	65
			GAC	Acetate	Anaerobic mixed culture	Acid Orange 7	C	66
			Graphene	Glucose	Anaerobic sludge	Nitrobenzene	B	67

Table 1.3. Use of a solid support for the immobilization of a redox mediator in the transfer of electrons from a chemical or biological source to the nitro, azo or other halogenated chemicals: Case 3.

		Abiotic electron donor	Soluble redox mediator	Solid redox mediator and/or support	Biotic electron source	Inoculum	Pollutants	Batch (B) / Continuous (C)	Ref.
Case 3 - Solid support for the immobilization of redox mediators	ABIOTIC	Na_2S	Diethylenetriamine + anthraquinone-2-sulfonic acid	Graphene oxide			Acid Yellow 36	B	68
		Hydrazine monohydrate	AQDS, anthracene	CNT			Nitrobenzene	B	69
	BIOTIC		Humic substances	Anion exchange resin	Glucose	Anaerobic sludge	Carbon tetrachloride, and Reactive Red 2 (RR2)	B	75
			Anthraquinone-2-sulfonate	Poly(ethylene terephthalate) fiber	Peptone, yeast extract	Cells of S. alga	Azo dyes and nitroaromatics	B	73
			AQS	PET-Cloth	Glucose	*Escherichia coli* K-12	Azo dye red 18	B	72
			Anthraquinone	Polyvinyl alcohol		Salt-tolerant bacteria	Azo dyes	B	77
			AQDS	Metal-oxides nanoparticles	Glucose	Anaerobic sludge	Reactive Red 2 (RR2)	B	74
			AQDS, NQS	Anion exchange resin	Glucose	Anaerobic sludge	Azo dyes	B	40
			Polypyrrole/AQDS	Active carbon felt	Glucose	Sludge	Azo dyes	B	70
			Polypyrrole/AQDS	Active carbon felt	Glucose	Sludge	Nitroaromatics	B	71
			Four anthraquinone dyes	Ter-ethylene fiber and cotton textile	Yeast extract, peptone	*Halomona* sp. GYW	Azo dyes	B	76
			Riboflavin	Cellulose		Anaerobic sludge	Remazol Golden Yellow RNL	B	78
			AQS, AQDS	Polyurethane	Glucose	Salt-tolerant AQS-reducing bacteria	Reactive Red K-2G	B	79
			AQS	PET fiber	Sodium formate	*S.alga*	Azo dyes and nitroaromatics	B	73

From the above experimental research we could conclude that the immobilization of redox active functionalities on solid supports has brought technological improvements towards the industrial application of solid redox mediators in the bioremediation of recalcitrant compounds.

1.3.1. Immobilization of redox mediators in the biotransformation of compounds with electron withdrawing groups

Several materials have been used for the immobilization of redox mediators in the biotransformation of pollutants with electron withdrawing groups, like azo or nitro compounds. Materials such as poly(ethylene terephthalate) fiber [72, 73], polyvinyl alcohol [77], metal-oxide nanoparticles [74], anion exchange resins [40, 75], and different forms of activated carbons [70, 71, 80] were used to immobilize redox active chemical compounds with quinone functional groups on their structure. Among the materials mentioned before, activated carbons outstand because of the possibility of tailoring their physical and chemical properties, especially the surface quinone concentration.

1.3.2. Physicochemical properties of activated carbon relevant for redox processes

Activated carbons are widely known for their wide range of chemical and physical properties that vary according to the conditions used in their fabrication. One of the most important features of an activated carbon is the porosity distribution since a high content of micropores (pore diameter < 2 nm) will increase the specific surface area, the van der Waals forces (from the near proximity of carbon atoms)[81], and the capacity to adsorb NACs onto the activated carbon. On the other hand, the presence of certain functional groups (see Figure 1.3) modify the surface charge, the point of zero charge and the electrostatic interactions of the activated carbon that could led to an increased adsorption of NACs.

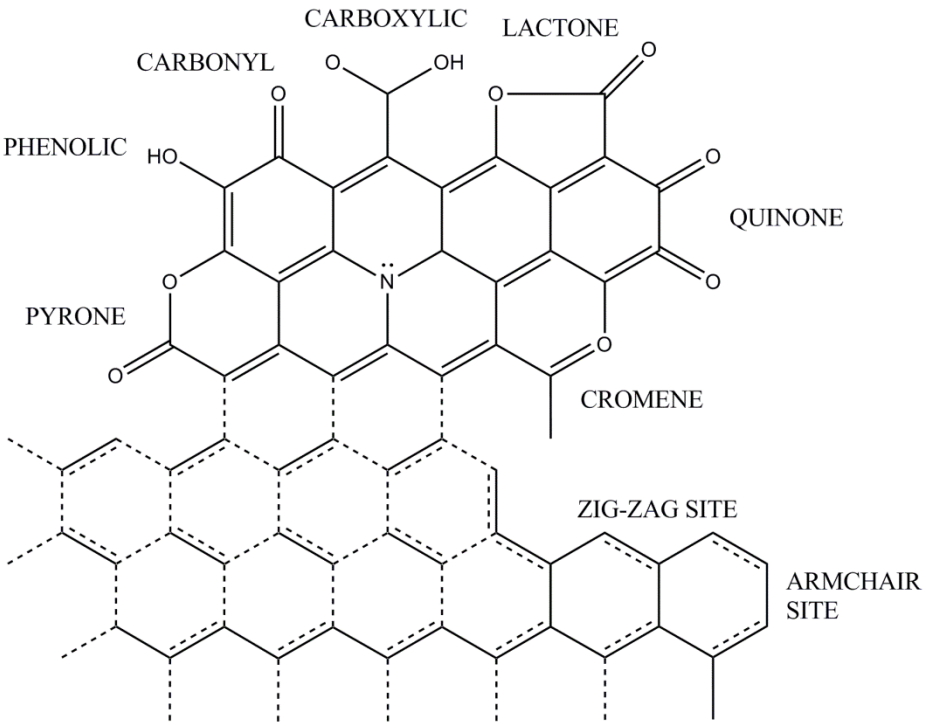

Figure 1.3. Variety of surface functional groups in activated carbons.

In the past, the main application of activated carbons in biological systems was as biological carrier that allowed the biofilm formation in the external surface. Such biofilm formation was promoted because the adsorption properties of activated carbon allow it to buffer high concentrations of toxic substrate which would otherwise exceed the tolerance concentration of anaerobic bacteria [82-89]. However, activated carbons can catalyze many reactions [41, 90-92] due to the electrochemical potentials commonly reported of 0.6-0.8 V [93], and the presence of redox active functionalities of the quinone-hydroquinone type couples suggested to contribute to this electron accepting-donating (redox) behavior [41].

As shown in Tables 1.2 and 1.3, several studies have taken advantage of the presence of quinone moieties at the surface of activated carbons and used such knowledge towards the improvement in the reaction rates of transformation of several pollutants with electron withdrawing groups. Under abiotic conditions, it has been demonstrated that activated carbons could catalyze the reduction of a wide variety of xenobiotic compounds because of the presence of quinone functionalities

[39, 62] or high content of electron rich sites on their basal planes (π electrons) [57]. However, under biotic conditions the enhancement in the biotransformation of any recalcitrant pollutant might be influenced not only by the surface chemistry of the activated carbon, but also by biofilm formation on the carbon surface.

With respect to activated carbons used as redox mediators, activated carbon fibers (ACFs) offer several advantages as compared to granular (GAC) and powder (PAC) forms. In comparison, the effective area for biofilm growth in GACs tend to decrease dramatically because of the clogging of pores resulting from this microbial growth [94], while in ACFs the decrease in the effective area is much less because these expose (on average) 100 times more external specific surface area [95, 96] than GACs. Additionally, these materials have some features that make them attractive for their potential application in bioreactors that can catalyze the transformation of recalcitrant pollutants, such as high tensile strength, high surface area, light-handed, acid and base resistance, and easy to manipulate due to their textile characteristics.

1.4. Motivation of this research

The basic understanding of the biotic and abiotic processes governing the NACs reduction with the use of redox mediators still needs further research in order to elucidate their applicability as a bioremediation technology. Despite the technical advantages of the ACFs as compared to other activated carbons, their study as redox mediator in the abiotic and biotic reduction of NACs is rather meager at present [61, 71]. Moreover, from all the research studies available in literature only some of them have been carried out under continuous conditions in the biotransformation of azo dyes [39, 63-66], and none (to the author's knowledge) in the biotransformation of nitroaromatic compounds. Finally, the studies dealing with the use of activated carbon as redox mediator in the biotransformation of environmental pollutants usually omits the role of the biofilm developed onto the carbon surfaces, as well as the interactions bacteria-surface involved. Due to these reasons, the main motivation of this study was to evaluate ACFs with different

quinone content as redox mediators in the continuous biotransformation of nitroaromatic compounds.

1.5. Hypothesis

Activated carbon fibers serve as redox mediators and as supporting material for biological growth in the biotransformation of several nitroaromatic compounds under continuous conditions.

1.6. Objectives

1.6.1. General objectives

Prove that activated carbon fibers (ACFs) with different surface chemical properties could work as redox mediators in the chemical (abiotic) reduction of nitroaromatic compounds (3-chloronitrobenzene and 4-nitrophenol) to their amino derivatives with Na_2S as electron donor.

Study the biotransformation of a new nitroaromatic explosive (2,4-dinitroanisole) in soil columns under continuous anaerobic conditions in order to elucidate the main biotransformation products.

Demonstrate that activated carbon fibers with different content of quinone functional groups enhance the anaerobic biotransformation of 4-nitrophenol under continuous conditions by using ethanol as an exogenous electron donor.

1.6.2. Specific objectives

- Measure the textural properties (e.g. pore diameter, pore volume, and specific surface area) and chemical characteristics (e.g. point of zero charge, concentration of acid and basic active sites) of ACFs in order to explain the chemical and biological reduction experiments of NACs.
- Modify the textural and chemical properties of ACFs using several methods such as chemical oxidation with nitric acid, thermal reduction under inert atmosphere (He:H, 99:1%), and anchorage of anthraquinone molecules on activated carbon fibers surface in order to improve the reduction of NACs.

- Quantify the amount of NACs that could be adsorbed on each ACF by adsorption isotherms and rule out this phenomenon as the main mechanisms behind a decrease in NAC concentration.
- Prove that hydrogen sulfide (H_2S) could donate electrons to ACFs with the concomitant reduction of NACs in aqueous solutions (batch experiments) in order to demonstrate the chemical role of ACFs as redox mediators.
- Perform continuous experiments for the reduction of explosive NACs in soil columns in order to determine and quantify the complex metabolic biotransformation products of the reduction of NACs under anaerobic conditions.
- Build up hybrid UASB reactors with fixed ACFs inside for the continuous biotransformation of NACs.
- Select the experimental conditions necessary to prove that ACFs serve not only as biological support, but also as redox mediators in the continuous biotransformation of NACs.

1.7. Scope and structure of thesis

The scope of this thesis covers the abiotic and biological reduction of nitroaromatic compounds under anoxic conditions. This thesis was organized as follows:

Chapter 1: In this chapter we reviewed the industrial uses, environmental issues and remediation technologies available for the anthropogenic pollutants known as nitroaromatic compounds (NACs). We focused our study towards the biotransformation of NACs and the use of redox mediators to speed up the reaction rates. A state of the art in the current use of soluble and solid redox mediators in the biotransformation of pollutants with electron withdrawing groups was also offered. Physicochemical properties of activated carbons were presented. These properties set the theoretical motivation to conduct the research reported in this thesis.

Chapter 2: The use of activated carbon fibers (ACFs) as redox mediator in the chemical oxidation of Na_2S and the reduction of NACs to their aminoaromatic

derivatives was explored. In order to elucidate the role of specific surface functional groups in the reduction process, several modified ACFs were prepared from the as-received material (AW). These modified materials with different concentration of functional groups include acid oxidized ACFs (OX) and thermally reduced ACFs (700): these were treated with 8 M HNO_3 and at 700°C under an inert atmosphere of hydrogen:helium (1:99%), respectively. The results showed an increased reduction of NACs to their respective aromatic amines by at least 1.38 times with the use of OX materials as compared to AW materials, while 700 materials hindered the reduction process. These results were explained by the higher presence of redox-active carbonyl (quinone) groups in the surface of OX as compared to AW and 700.

Chapter 3: In the search for improved catalytic materials, redox active anthraquinone molecules were anchored on the as-received ACFs (AW). 2-aminoanthraquinone (AAQ) and anthraquinone-2,6-disulfonate (AQDS) were anchored by chemical modification with $SOCl_2$. The anchorage of these molecules had a clear effect on the physical and chemical properties of the original materials. The ACFs modified with AQDS exhibited a better redox performance due to an improvement in the catalytic properties of the material (49%) towards the reduction of NP to 4-aminophenol, providing sodium sulfide as the electron donor. HPLC and spectrometric scans, at the end of the reduction experiments, showed that AQDS remained anchored on ACFs.

Chapter 4: For this experimental stage we integrated all the physicochemical concepts related with the use of ACFs as redox mediators (chapters 2 and 3) with the biological factors that affect the biotransformation of NACs in order to prove that ACFs could also perform as redox mediators in biological systems (chapter 4). A novel bioreactor with fixed ACF disks and anaerobic granular sludge in the bottom worked as a hybrid UASB reactor used for the 4-nitrophenol (NP) biotransformation. All the ACFs with different quinone content allowed the biofilm formation and reduced NP with high efficiencies (>95%). Modifications in the concentration of ethanol (exogenous carbon source) allowed an increased

biotransformation efficiency in the bioreactors packed with ACFs in the order OX > AQDS > AW (2.11-, 1.97-, and 1.47-fold increase in biotransformation as compared to the control reactor, respectively).

Chapter 5: In this chapter we shifted from the chemical and biological reduction experiments with ACFs to the continuous biotransformation of NACs in soil. The biotransformation of 2,4-dinitroanisole (DNAN) and 2-methoxy-5-nitroaniline (MENA) was studied in two anaerobic soil columns in aims to imitate the seepage of these contaminants through saturated soil. In this experimental stage two powerful analytical techniques (HPLC and UHPLC-tandem mass spectrometer) were used to quantify/identify the metabolites of DNAN and MENA produced under anaerobic conditions. Several biotransformation processes were identified such as nitro-group reduction, O-demethylation, N-acetylation and even secondary reactions such as the polymerization of reactive nitroso compounds with aromatic amines (condensation reactions). The results also showed that ethanol (exogenous carbon source) promoted a higher detection of aminoaromatic compounds (~66%) in the effluent of the anaerobic columns while the lack of ethanol promoted the O-demethylation of DNAN and MENA (<50%) in order to obtain energy for microbial metabolism and NACs reduction.

Chapter 6: In this chapter we presented a general overview to the main conclusion of this thesis and discuss some hypotheses that need future research in order to fully elucidate the chemical and biological mechanisms of reduction of NACs.

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CHAPTER 2.- ACTIVATED CARBON FIBERS AS REDOX MEDIATORS FOR THE INCREASED REDUCTION OF NITROAROMATICS

ABSTRACT

Activated carbon fibers (ACFs) were used as redox mediators in the anaerobic chemical reduction of two model nitroaromatic compounds: 4-nitrophenol and 3-chloronitrobenzene. The effect of ACF chemical properties on the reduction of nitroaromatic compounds was measured by chemical oxidation of ACFs with 8 M nitric acid and thermal treatment at 700°C under an inert atmosphere of hydrogen:helium (1%:99%), before their use in batch experiments. The results show that ACFs are necessary to transform the nitroaromatic compounds to their corresponding aromatic amines, indicating the function of ACFs as redox mediators. Furthermore, the oxidation and thermal modification of ACFs clearly changes the redox properties of these materials, with the former being the better option to increase the concentration of quinone groups (1.68 times), and therefore the redox mediating capacity of ACFs (up to 1.38 times).

2.1. Introduction

It has been reported that the use of chemical substances with quinone groups in their structure can catalyze the biotransformation of recalcitrant pollutants (e.g. azo dyes) to aromatic amines, which in a secondary treatment (aerobic treatment) can be biodegraded more easily [1-3]. Such chemical substances with capacity to receive and yield electrons are known as redox mediators and the catalysis in the transformation of recalcitrant pollutants is within their potential applications. Activated carbons are well known not only by their excellent adsorbent properties,

Chapter adapted from:
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but also by the presence of a diversity of surface functional groups, including quinone groups [4].

Despite the fact that it has been demonstrated that granular activated carbons can act as redox mediators (through surface quinone groups) in the anaerobic biotransformation of recalcitrant compounds such as azo dyes [1, 5-8], there is clearly a lack of information on their application for other pollutants and for distinct forms of activated carbon, as it is the case of nitroaromatic compounds and activated carbon fibers (ACFs). The ACFs have unique characteristics compared with granular or powder activated carbons that make them suitable for their use as catalyst in the transformation of recalcitrant pollutants. In comparison, the effective area for biofilm growth in granular activated carbons tend to decrease dramatically because of the clogging of pores resulting from this microbial growth [9], while in ACFs the decrease in the effective area is much less because these expose (on average) 100 times more external specific surface area [10, 11] than granular activated carbons. Additionally, these materials have some features that make them attractive to their potential application in bioreactors that can catalyze the transformation of recalcitrant pollutants, such as high tensile strength, high surface area, light-handed, acid and base resistance, and easy to manipulate due to their textile characteristics.

As known, nitroaromatic compounds are among the most used chemicals in industry today in a wide range of products. This is due to the high electronegativity of the nitro group (-NO_2) bonded to the aromatic ring that confers unique properties, attractive in chemical synthesis. The complexity of these compounds can increase with the number of aromatic rings or substituents on the chemical structure, as it is the case for pharmaceutical products, dyes and explosives. The toxicity of nitroaromatic compounds is associated to the products formed in the reduction of nitro groups (aromatic hydroxylamine derivatives) [12], which can react with biomolecules, including DNA, causing toxic and mutagenic effects [13].

The main disadvantage of the widespread use of nitroaromatic compounds is both soil and groundwater pollution. Although some nitroaromatic compounds are intentionally applied to the environment (i.e., pesticides), improper handling and/or storage practices by both producers and end users have resulted in their accidental release to the environment in nations throughout the world [14].

The aim of this work was to demonstrate that ACFs can act as effective redox mediators in the abiotic transformation of two model nitroaromatic compounds: 4-nitrophenol (4NP) and 3-chloronitrobenzene (3CNB), and to explain the reduction mechanism that takes place in batch experiments under non-oxidizing conditions. Furthermore, it is well established that activated carbons can be modified chemically by several methods in order to increase their catalytic properties [7, 8, 15-19]. Consequently, ACFs were oxidized with nitric acid and treated thermally under an inert atmosphere of hydrogen and helium in order to elucidate the role of surface functional groups on ACF in the reduction of nitroaromatics.

2.2. Experimental

2.2.1 Chemicals

All chemicals (>99% purity) were used as received. 4NP and 4-aminophenol (4AP) were purchased from Sigma-Aldrich (Mexico City, Mexico) and Riedel-de Haën (Seelze, Germany), respectively; 3CNB and 3-chloroaniline (3CA) from Aldrich (Missouri, USA); sodium sulfite (Na_2SO_3) and sodium sulfate (Na_2SO_4) from J. T. Baker (Mexico City, Mexico); sodium sulfide (used as electron donor, $Na_2S{\cdot}9H_2O$) and sodium thiosulfate ($Na_2S_2O_3{\cdot}5H_2O$) from Fisher-Scientific (New Jersey, USA); nitric acid 70% (ACS grade) from Fermont (Monterrey, Mexico). The basal medium (pH=7.6) contained several compounds in concentrations that do not absorb UV and/or visible light from 200 to 400 nm (g/L): $NaHCO_3$ (5.0), NH_4Cl (0.28), K_2HPO_4 (0.25), $MgSO_4{\cdot}7H_2O$ (0.1), $CaCl_2{\cdot}2H_2O$ (0.01), and trace elements (1 mL/L), as described elsewhere [20].

Polyacrylonitrile based ACF with commercial name AW1105 was purchased from KoTHmex (Taichung, Taiwan). The ACF was used directly as obtained from the supplier.

2.2.2 Chemical modification of ACFs

2.2.2.1 Chemical oxidation

Experiments were performed according to the method described by Rangel and Streat [16] as follows: 200 mL of HNO_3 7.68 M were placed into a reactor (three-necked round-bottomed flask of 250 mL) half-submerged in a constant temperature water bath at 85°C. Afterwards, 5 g of ACFs were added and a constant flow of nitrogen was applied at inside-bottom of reactor in order to mix the acid and ACF along 90 min of oxidation. The modified sample was removed from the acid solution and rinsed with deionized water.

2.2.2.2 Thermal modification

10 g of ACFs (previously modified with HNO_3) were placed inside a rotary reactor (Carbolite, HRT 1175 model) and heated to 700 °C at 15 °C/min. A mix of hydrogen: helium (1:99%) was passed throughout the reactor at a flow rate of 0.5-1.0 L/min during 90 min. The inert atmosphere in the reactor was kept until the temperature of the sample reached the room temperature. Finally, the sample was stored in a plastic bag.

2.2.3 Textural and surface chemistry characterization of ACFs

Surface area (m^2/g), average pore diameter (nm), and pore volume (cm^3/g) were determined by nitrogen adsorption/desorption at 77 K (BET method [21]) using a Micromeritics ASAP 2020 surface analyzer. Specific surface functional groups on ACFs were measured by potentiometric titrations as described by Boehm [22], and the point of zero charge (PZC) was obtained by the procedure reported by Rangel and Streat [16].

2.2.4 Fourier transform-infrared (FTIR) spectra of ACFs

Three samples of ACFs (fabric [AW1105], oxidized [AW1105 OX] and thermally treated [AW1105 700]) were blended with KBr at a ratio of 1:99% (w/w), and then

pressed into a disk for FTIR analysis. The spectra (32 scans) were recorded on a Thermo-Scientific FTIR (Nicolet 6700 model) spectrophotometer under ambient conditions.

2.2.5 Adsorption isotherms

These experiments were conducted to evaluate the nitroaromatic compounds adsorption capacity onto different ACFs samples. 10 mg of ACFs and 10 mL of basal medium (pH = 7.6, see section 2.2.1) with solutions of nitroaromatics at different concentrations (0-500 μM) were added into plastic tubes of 15 mL. Then the tubes were sealed and agitated for 48 h at 150 rpm and 25°C. The concentrations of nitroaromatics remaining in the solution were measured spectrophotometrically as described in section 2.2.6. All experiments were performed by triplicate.

2.2.5 Chemical reduction of nitroaromatics

The capacity of ACFs to act as redox mediators was evaluated providing a primary electron donor (Na_2S with pKa = 14.15 [23, 24], or hydrosulfide ion (HS^-) and sulfhydric acid (H_2S) at pH 7.6) and the final electron acceptor (4NP or 3CNB) into a glass serum flask. In this procedure the electrons will be transferred from Na_2S to activated carbons [1], being the latter the responsible to shuttle electrons from carbons to the chemical compounds of interest, reducing it in the process. The overall chemical reaction expected to be mediated by ACFs is: $Ar\text{-}NO_2 + Na_2S = Ar\text{-}NH_2$ + elemental sulfur (S^0), where $Ar\text{-}NO_2$ and $Ar\text{-}NH_2$ represent the nitroaromatic (4NP or 3CNB) and aminoaromatic (4AP or 3CA) compounds, respectively.

The mass of ACFs needed in these experiments was calculated from adsorption isotherms and are shown in Table 2.1.

Table 2.1. Experimental design of chemical reduction of nitroaromatics mediated by activated carbon fibers (ACFs).

# (triplicate runs)	Experiment	Species	ACF	ACF mass (mg)	Basal Media (mL)	Na_2S 26.1mM (mL)	Nitroaromatic 1.27mM (mL)
1	Nitroaromatic Stability	4NP			30		20
2	Direct Chemical Reduction	4NP + Na_2S			25	5	20
3	Adsorption	4NP + ACF	AW1105	5.00	30		20
4			AW1105 OX	7.46			
5			AW1105 700	3.91			
6	Mediated Chemical Reduction	4NP + Na_2S + ACF	AW1105	5.00	25	5	20
7			AW1105 OX	7.46			
8			AW1105 700	3.91			
9	Nitroaromatic Stability	3CNB			30		20
10	Direct Chemical Reduction	3CNB + Na_2S			25	5	20
11	Adsorption	3CNB + ACF	AW1105	2.63	30		20
12			AW1105 OX	5.26			
13			AW1105 700	0.88			
14	Mediated Chemical Reduction	3CNB + Na_2S + ACF	AW1105	2.63	25	5	20
15			AW1105 OX	5.26			
16			AW1105 700	0.88			

A 25 mL aliquot of basal medium was added into 117 mL serum flask containing ACF. The flasks were sealed with butyl rubber stoppers and the gas headspace was flushed for 3 min with a gas mixture of N_2:CO_2 (80/20%). Inside an anaerobic chamber (with an atmosphere composed of N_2:H_2 (95:5%)), 5 mL of sulfide were added with a syringe from a 26.1 mM Na_2S stock solution (HS^- is the reactive species). The bottles were incubated horizontally for 24 h at 25 °C in a rotary shaker at 110 rpm. After preincubation, 20 mL of 4NP or 3CNB were added from a 1.27 mM stock solution in order to obtain an initial nitroaromatic solution of 0.5 mM and a total sulfide concentration of 2.61 mM. Experiments were carried out during 2 and 15 days for 3CNB and 4NP, respectively. Samples of 0.3 mL were taken at

selected times and the concentration of nitroaromatic was analyzed as described in section 2.2.6. Controls without ACFs and/or Na_2S were included to measure the stability of nitroaromatics, the direct reduction of nitroaromatics by sulfide and the adsorption onto ACFs. All experimental treatments were performed by triplicate.

2.2.6 Analytical methods

The concentrations of nitroaromatics remaining were measured by diluting 0.1 mL of sample with 1.9 mL of 0.1 M phosphate buffer (pH 7.15) to an absorbance less than 1.0. The spectrophotometer used was a Thermo Scientific UV-vis (Genesis 10uv model), and the wavelengths used to quantify the 4NP and 3CNB in solution were 400 and 265 nm, respectively. Even if total reduction of 4NP occurs the wavelengths at which the 4AP absorb in the UV-vis spectra (200-300 nm, see Figure 2.1a) do not affect the final measurements of 4NP (400 nm). However, caution has to be taken in the case of 3CNB. This is because in the transformation of more than 56% of 3CNB to 3CA the signal detected by the UV-vis spectrometer corresponds to the 3CA (200-300 nm, see Figure 2.1b). For this reason, the quantification of both nitroaromatics by high-performance liquid chromatography (HPLC) is highly recommended.

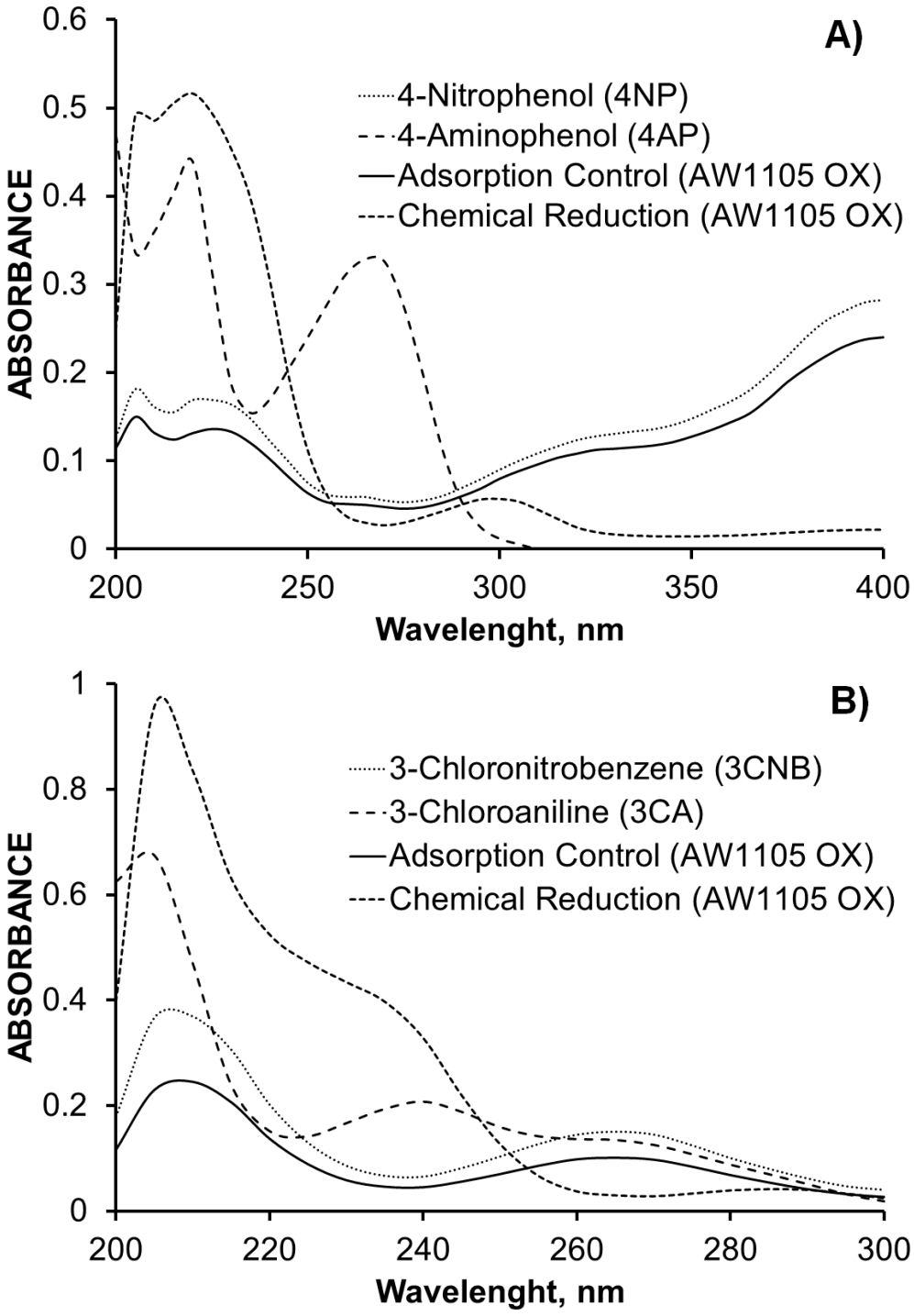

Figure 2.1. UV spectra for a) 4-nitrophenol (4NP) and b) 3-chloronitrobenzene (3CNB) chemical reduction experiments.

The concentrations of 4AP, 3CA, HS^- and S^0 were measured by HPLC. The chromatograph (Agilent Technologies, 1200 series) was equipped with a column Synergy 4U Hydro-RP 80R (250 x 4.60 mm, 4 micron) from Phenomenex. 2 µL of sample was injected with an autosampler. The carrier liquid, composed of 60% of deionized water and 40% acetonitrile, was pumped at a flow rate of 1 mL/min. 4AP, 3CA, HS^- and S^0 were detected at 25°C and wavelengths of 230-400 nm with an Agilent Technologies diode array detector.

2.3. Results and discussion

2.3.1 Characterization of ACFs

Commercial ACF AW1105 has a BET surface area of 1098 m^2/g. This value was kept almost without changes after chemical oxidation (1052 m^2/g) and thermal modification (1144 m^2/g), indicating that these treatments were not destructive under the conditions studied. Additionally, the average pore diameter and pore volume did not significantly change, obtaining values of 2.02, 1.91, 1.99 nm and 0.37, 0.37, 0.39 cm^3/g for AW1105, AW1105 OX and AW1105 700, respectively. These results indicated the microporous nature of ACFs, which can be a determining factor on the reduction of nitroaromatics due to the high probability to have both reactive groups (edge sites) over the carbon surface and exposure to nitroaromatics in solution.

The chemical and thermal modifications caused changes on the surface chemistry of ACFs as noted on Table 2.2. Firstly, the total concentration of acid groups increased from 1.9 to 3.5 milli-equivalents (meq)/g when AW1105 was oxidized with nitric acid, mainly due to the formation of carboxylic and lactones groups. Carbonyl groups (two carbonyl groups = quinone group [25]) increased in the same way 1.68 times. According to literature, only quinone and chromene groups have been proposed to act as redox mediators [19, 25], which means that the increase in the concentration of quinone groups could improve the redox properties of AW1105 OX. On the other hand, the thermal modification caused gasification of

carboxyl groups and depletion on lactonic and carbonyl groups by factors of 2.96 and 1.24, respectively. Sample AW1105 700 was heated under H_2 atmosphere to gasify loosely bounded and highly (re)active carbon atoms [18] like carboxyl, lactone, phenolic and anhydride groups [15] in order to favor the presence of quinone groups on the ACF surface. Table 2.2 show the concentration of carbonyl groups on AW1105 700 was less than AW1105 and AW1105 OX. This means that the temperature of 700 °C was high enough to reduce some part of the quinone groups to phenolic groups, indicating that AW1105 700 could probably have less redox activity than AW1105 and AW1105 OX.

Table 2.2. Surface chemical properties of activated carbon fibers (ACFs) non-modified (AW1105), modified with nitric acid (AW1105 OX), and modified thermally under inert conditions (AW1105 700).

Name of ACF	Surface functional groups, (meq/g)						Point of Zero Charge (pH)
	Acid groups					Total basic groups	
	Carboxylic	Lactonic	Phenolic	Carbonyl	Total acid groups		
AW1105	0.160	0.740	0.225	0.775	1.900	0.109	5.16
AW1105 OX	0.960	0.940	0.300	1.300	3.500	0.257	3.41
AW1105 700	0.000	0.250	1.438	0.625	2.313	0.703	9.30

The PZC shown on Table 2.2 and Figure 2.2 also reflects the changes on the surface chemistry of ACFs. The PZC of AW1105 changed to the acid range (PZC=3.41) when modified with nitric acid, but this changed to basic (PZC=9.30) when thermally modified. The adsorption and reduction experiments were carried out at an initial pH of 7.6. According to Figure 2.2, at this pH the overall surface charge on AW1105 OX was the most negative, while the AW1105 700 showed the opposite behavior (electropositive) and the AW1105 exhibited an electronegative character. Considering that nitroaromatic compounds are electronegative molecules, a low PZC of AW1105 OX and the electronegative character at pH 7.60 will probably cause electrostatic repulsions that would affect the adsorption process. However, the higher concentration of quinone groups in AW1105 OX could benefit the redox process due to the reversible transformation of quinone groups to hydroquinones [25] (electropositive groups) or the formation of anion

complexes by HS^-. The opposite applies to AW1105 700, where the affinity to adsorb nitroaromatic compounds could be an advantage to catalyze their transformation, but the minor presence of quinone groups could counteract this property.

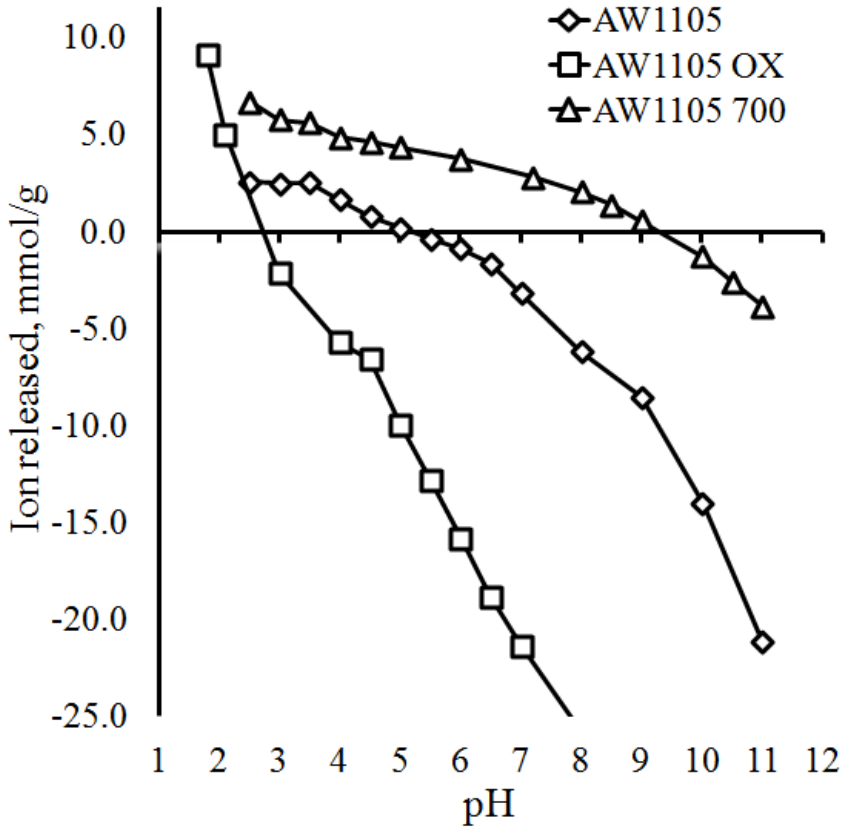

Figure 2.2. The point of zero charge (PZC) of modified activated carbon fibers (ACFs) and surface charge at different pH.

2.3.1.2 FTIR spectra of ACFs

Figure 2.3 shows the FTIR spectra of non-modified and modified samples, which were performed to support the presence of oxygenated surface functional groups, like quinone groups, on ACFs. It is to be noted that peaks at 2850-2950 cm^{-1} (C-H stretching in sp^3 hybridized carbons) and 667 cm^{-1} (C-S stretching) appear 'inverse' because these are more intense in the *s*-polarized scans than in the *p*-polarized scans [26], and a *p*:*s* ratio was used to present the FTIR spectra. This is because *p*-

polarized spectra are more sensitive to the bonded interface (affected by electron resonance in ACF) than the outer surface [27].

The FTIR spectra of acid oxidized ACF (AW1105 OX) suffered significant changes. The increase in intensity of bands ranging from 1740-1600 cm^{-1} (C=O stretching vibrations in quinones, ketones, aldehydes, lactones, anhydrides and carboxylic acids) can be attributed to the formation of carboxylic, lactonic and quinone groups, as obtained by Boehm titrations (see Table 2.2). Also, the formation of carboxylate ions and anhydride groups on the surface of AW1105 OX increased the absorption in the bands ranging from 1600-1380 and 1300-900 cm^{-1}, respectively [28]. However, in the thermally modified AW1105 700 the majority of the surface functional groups previously created in AW1105 OX was gasified. This can be supported by the decrease in intensity of the bands on the FTIR spectra, especially at 1740-1600 cm^{-1} were the carboxyl and lactone groups are identified. Additionally, FTIR spectra of ACFs contacted with Na_2S (HS^-) for 1 day (ACFs were rinsed with deionized water 3 times and dried before analysis) was obtained in order to determine changes on the chemical composition of ACFs. Results in Figure 2.3 show that all ACFs + HS^- exhibit a peak at 667 cm^{-1} corresponding to a C-S linkage. This kind of bond can be formed due to the high nucleophilicity of the reactive HS^- species and the charge deficiency on the carbon of the carbonyl compounds on all ACFs studied. The nucleophilic addition reaction may create anions (increase in the band near 1400 cm^{-1}) on the carbon surface such as carboxylate ion or quinone anions (reversible proton transfer leads to hydroquinone sites [25]), which could help the ACFs to reduce the nitroaromatic compounds.

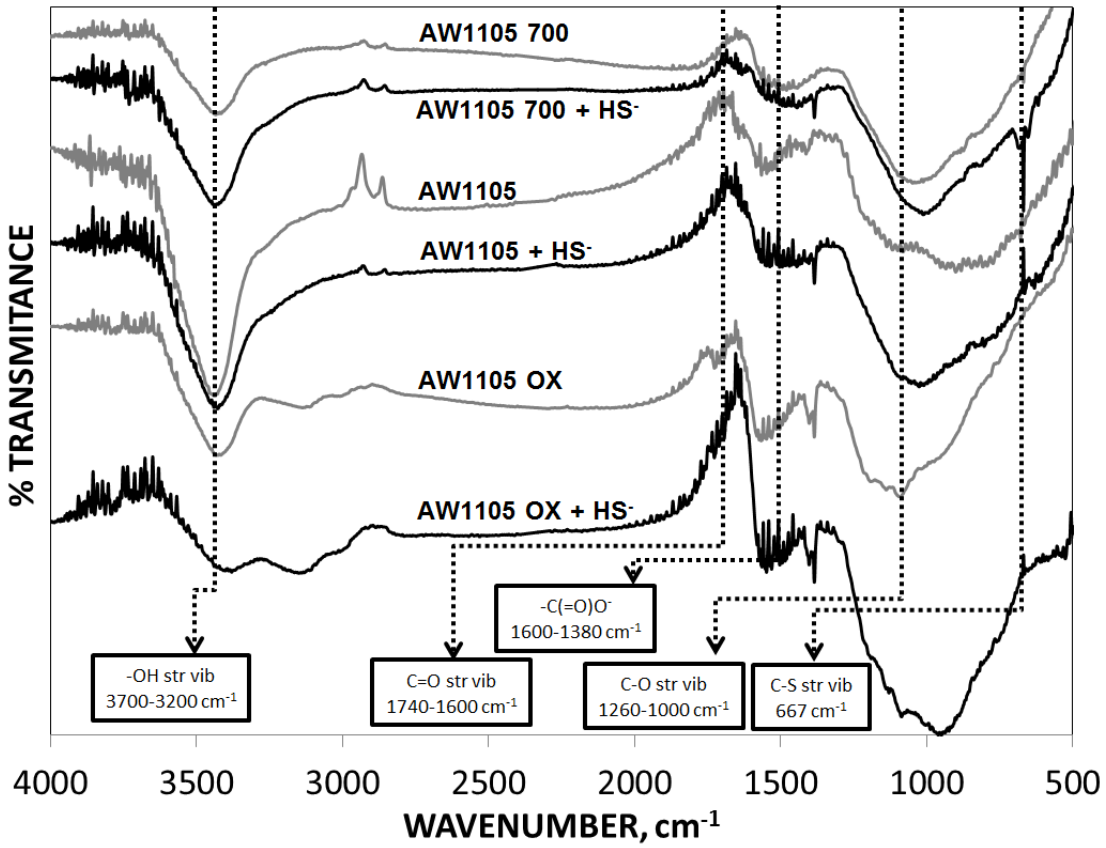

Figure 2.3. FT-IR spectra of AW1105 activated carbon fibers. Nitric acid oxidized sample (AW1105 OX) and thermally treated fiber under inert conditions (AW1105 700). FT-IR spectra of samples pre-incubated for 1 day with Na_2S ([Na_2S] = 4.33 mM) were also added in this figure.

2.3.2 Adsorption Isotherms

The adsorption capacities of modified and as-received ACFs to adsorb nitroaromatics 4NP and 3CNB are reported in Figure 2.4. The maximum adsorption capacities for the two nitroaromatic compounds (q_e) follow this order: AW1105 700 > AW1105 > AW1105 OX. This can be explained considering that both nitroaromatics are electronegative in nature and that AW1105 OX exhibits a net negative surface charge at pH 7.6 (see Figure 2.2), which causes electrostatic repulsions between adsorbent-adsorbate and hence a low adsorption by this material. The opposite applies for AW1105 700, were the net positive surface charge at pH 7.6 increase the affinity for both, 4NP and 3CNB.

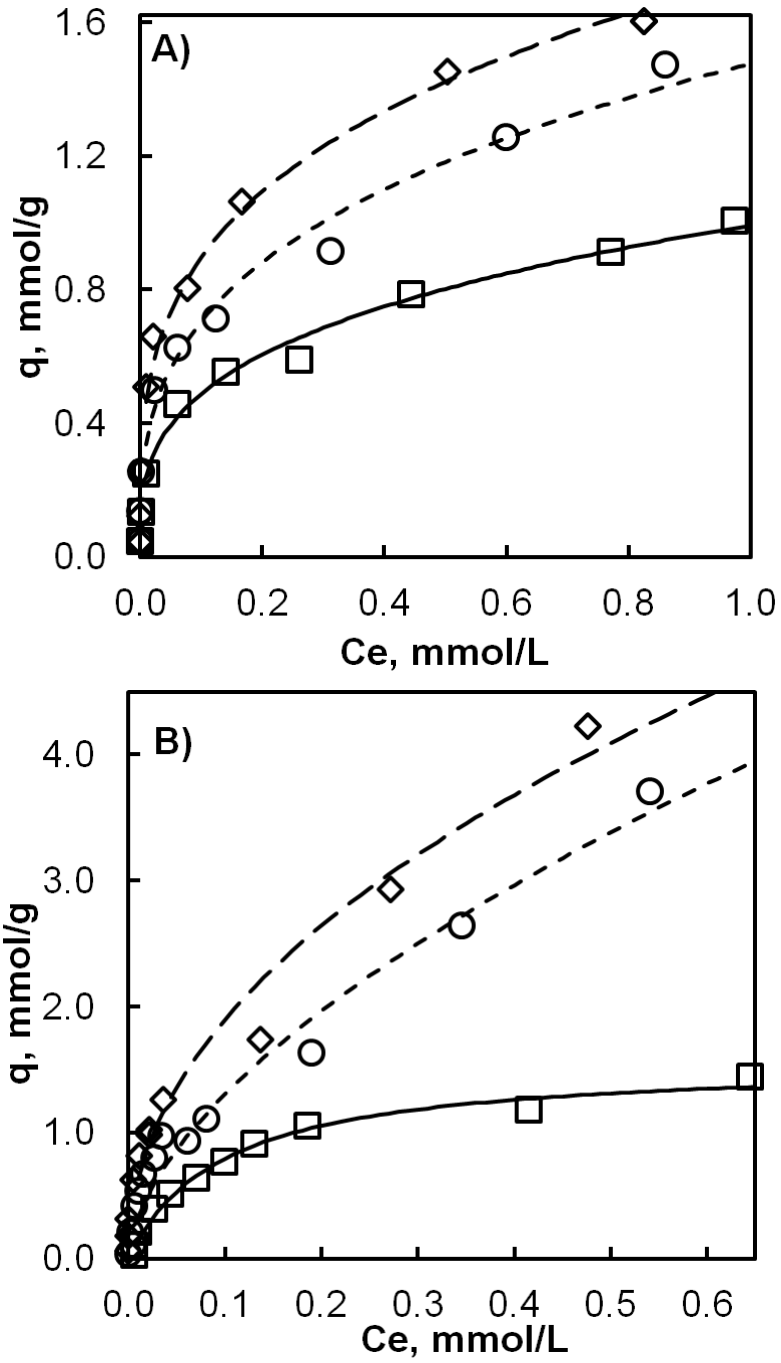

Figure 2.4. Adsorption isotherms of A) 4-nitrophenol (4NP) and B) 3-chloronitrobenzene (3CNB) on as-received activated carbon fiber (AW1105, circles), modified by chemical oxidation with nitric acid (AW1105 OX, squares) and thermally modified under inert conditions (AW1105 700, diamonds). The experiments were conducted at pH 7.6 and 25°C.

It was found that the Freundlich model fitted best to experimental data, and thus the values of q_e for AW1105 700, AW1105 and AW1105 OX at C_e = 0.30 mmol/L and pH 7.6 were 1.23, 1.00, 0.69 mmol/g for 4NP, and 3.21, 2.50, 1.18 mmol/g for 3CNB, respectively. Furthermore, higher values of q_e for 3CNB highlight the fact that the more electronegative the substituent on the aromatic ring, the highest the adsorption will be on basic ACFs. Considering the electropositive nature of hydroquinone groups, it is expected that anaerobic reduction of 3CNB proceeds faster than of 4NP, due to the higher electrostatic interactions between 3CNB and the hydroquinone groups. Furthermore, the *meta*-position of Cl makes 3CNB vulnerable to electronic attack by anions through the electron deficient aromatic ring of this molecule, improving its reduction.

2.3.3 ACFs as redox mediators and the effect of chemical properties on nitroaromatic reduction

ACF catalysis of 4NP and 3CNB by sulfide is shown in Figures 2.5 and 2.6, respectively. In Figure 2.5, two controls were performed: one measured the nitroaromatic stability along the experiment (4NP alone) and the second showed the resistance of nitroaromatic to accept electrons from the primary source (4NP + Na_2S). These controls were the same for all ACFs studied. Figure 2.5 shows that the adsorption controls (ACF + nitroaromatic) diminished the concentration of 4NP less than 20% in all cases. Additional spectrometric scans (200 – 400 nm, see Figure 2.1a) show that 4NP preserved the same spectra at the end of the experiments, but with lower concentration, which indicates that the remaining 4NP was not reduced to 4AP.

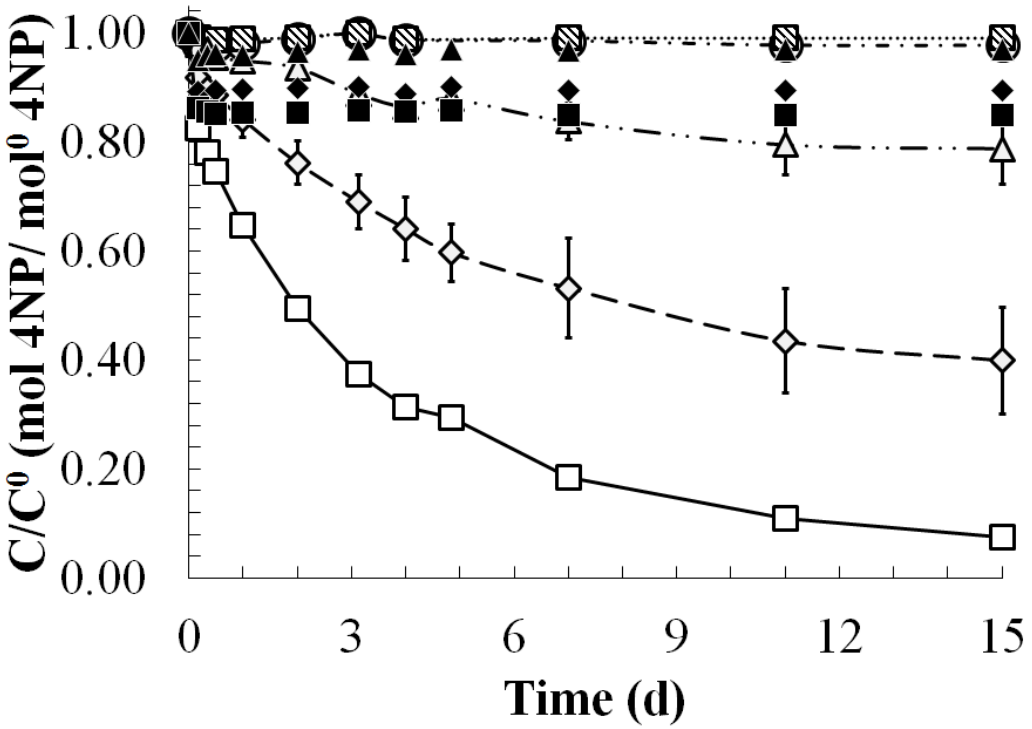

Figure 2.5. Activated carbon fiber (ACF) catalysis of 4-nitrophenol (4NP) reduction by sulfide. 4NP concentration control (circle symbols), direct chemical reduction control (striped squares, 4NP + Na_2S), adsorption controls (full symbols, 4NP + ACF) and reduction experiments (open symbols, 4NP + Na_2S + ACF) of AW1105 (diamonds), AW1105 OX (squares), AW1105 700 (triangles).

On the other hand, the chemical reduction experiments (electron donor + ACF + nitroaromatic) exhibited a greater decrease in the concentration of 4NP in all ACFs. The difference between these experiments and the adsorption controls can be attributed to the reduction of 4NP to 4AP. To confirm the presence of 4AP, samples from the chemical reduction experiments and standard solutions of Na_2S, 4NP and 4AP were analyzed by HPLC. The most representative chromatogram obtained is shown in Figure 2.7-I), and correspond to the ACF that display the best performance to transform 4NP to 4AP (AW1105 OX). Several chemical substances were identified and 4AP is between them, with a retention time of 3.045 min (peak D). These results clearly demonstrate the property of ACF to act as redox mediators in the transformation of 4NP to 4AP. Experiments with other ACF and

the replicates of this experiment exhibited the same peaks, but with different concentrations (result not shown).

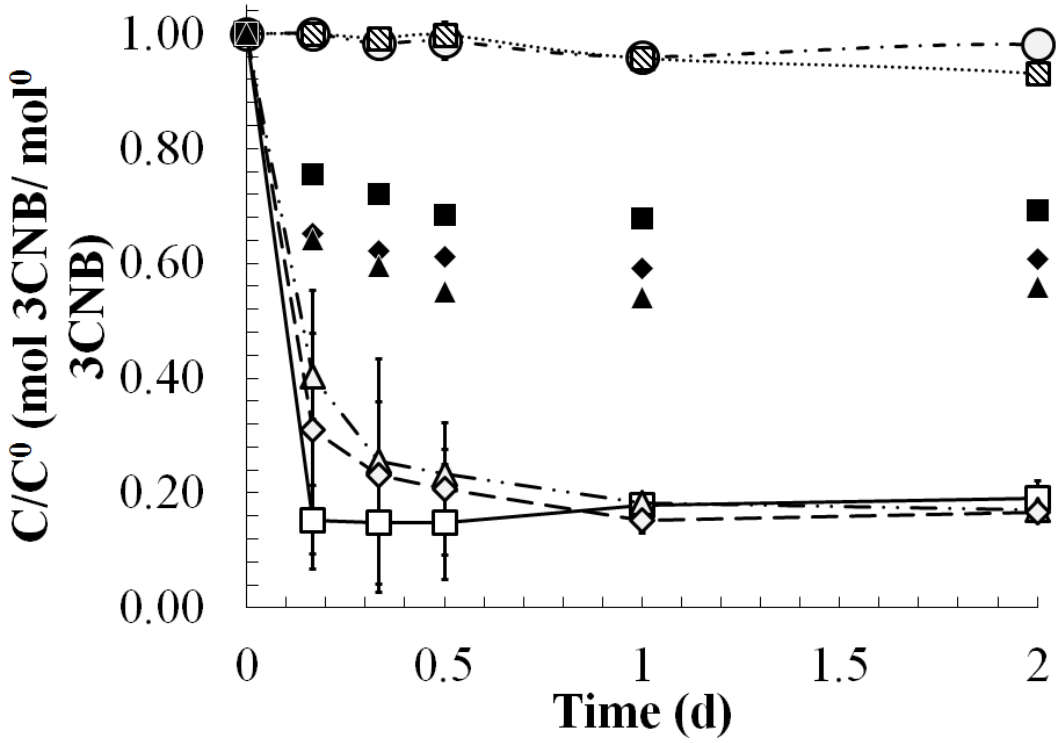

Figure 2.6. Activated carbon fiber (ACF) catalysis of 3-chloronitrobenzene (3CNB) reduction by sulfide. 3CNB concentration control (circle symbols), direct chemical reduction control (striped squares, 3CNB + Na_2S), adsorption controls (full symbols, 3CNB + ACF) and reduction experiments (open symbols, 3CNB + Na_2S + ACF) of AW1105 (diamonds), AW1105 OX (squares), and AW1105 700 (triangles).

Considering that the ACFs analyzed in this study have almost the same surface area and pore size distributions (see section 2.3.1), the distinct capacities of ACFs to catalyze the 4NP reduction by sulfide can rather be explained by their surface chemistry. ACFs with higher capacities to transform 4NP to 4AP follow the next order: AW1105 OX > AW1105 > AW1105 700. This may be related to a greater presence of carboxyl, lactonic, carbonyl (see Table 2.2) and anhydride groups (see Figure 2.3) in AW1105 OX in comparison to the other ACFs. It has been suggested that carboxyl groups could, in principle, resonate in concert with other functional groups in order to participate in the electron transfer process, however, the energy

needed to transfer electrons could actually break bonds (in simple molecules) rather than altering them as previously stated [25]. Additionally, lactones in acidic solutions could undergo one-electron transfer process, but in basic solutions (pH > 7), lactone rings would open and carboxyl groups would be formed [25]. This means that carboxyl and lactone like groups are unlikely to act as redox mediators in the transformation of nitroaromatics to aminoaromatics, leaving quinone groups (carbonyl like) as the most probable responsible of catalytic activity in AW1105 OX. From that point of view, the relative poor concentration of quinone groups in AW1105 700 might explain the lowest capacity to reduce 4NP to 4AP as shown in Figure 2.5. Other studies about the catalysis of azo dyes by activated carbon [7, 8] reported that carbons with basic properties (thermally treated) exhibit a better performance to transfer electrons (through quinonic groups or delocalized π-electrons) to the azo group, transforming dyes to their amino derivative more effectively. However, azo dyes have a more complex structure than the nitroaromatic compounds reported herein. The presence of more aromatic rings in the structure of azo dyes could help not only to interact more efficiently with electropositive groups on the ACF surface (hydroquinones, π-electrons) but also to delocalize electrons from the carbon surface groups to the azo group (-N=N-) more easily than to the benzene ring present in 4NP or 3CNB.

In the case of 3CNB (see Figure 2.6), the two performed controls showed a similar behavior than 4NP. The concentration of 3CNB alone and with Na_2S did not change noticeably within 2 days of the experiment, indicating the resistance of 3CNB to be reduced directly by Na_2S. The adsorption control showed that less than half of the 3CNB available on the solution was adsorbed on ACF surface. Despite this fact, differences between adsorption controls and reduction experiments can be attributed to transformation of 3CNB to 3CA. HPLC was employed to confirm the consumption of 3CNB and the formation of 3CA. As can be seen in Figure 2.6, AW1105 OX had the best performance to catalyze the 3CNB reduction, and thus the chromatogram of these experiment is shown in Figure 2.7. The total consumption of 3CNB (retention time = 13.76 min, peak J),

and concomitant formation of 3CA (retention time =10.59 min, peak I) was observed. These results demonstrated that ACF can act as redox mediators in the transformation of 3CNB to 3CA.

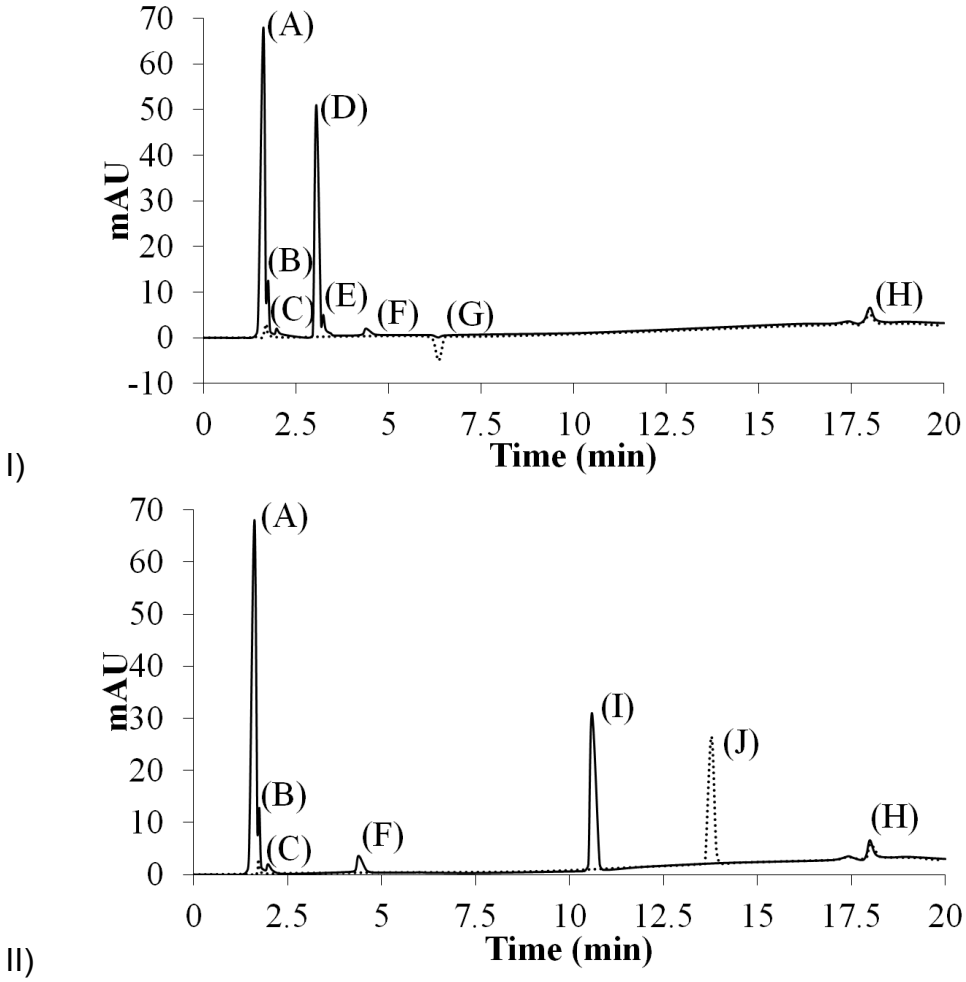

Figure 2.7. Chromatograms of AW1105 OX catalysis of I) 4NP and II) 3CNB reduction by sulfide. The peaks in the chromatograms correspond to elemental sulfur (S^0, A), thiosulfate ion (B), sulfate ion (C), 4-aminophenol (4AP, peak D), sulfite ion (E), hydrosulfide ion (HS^-, peak F), 4-nitrophenol (4NP, peak G), 3-chloroaniline (3CA, peak I), non-identified substance from basal media (H) and 3-chloronitrobenzene (3CNB, peak J). Pointed and solid lines represent the initial and final sample, respectively.

As in the case of 4NP, ACFs with higher capacities to transform 3CNB to 3CA followed the next order: AW1105 OX > AW1105 > AW1105 700. The explanation of this trend can be regarded as a result of the higher concentration of quinones on

the surface of AW1105 OX compared with AW1105 700. However, an important difference between 4NP and 3CNB reduction is the time in which each one of them reach equilibrium (15 and 1 days, respectively). It is to be noted that the mass of ACF employed in the 3CNB reduction experiments was less than that applied in experiments with 4NP (see Table 2.1), but this cannot explain why less mass of ACF reduces 3CNB faster. In this work we suggest two pathways for the faster reduction of 3CNB: 1) the chloride group in 3CNB is more electronegative than the hydroxyl substituent in 4NP, and this could allow 3CNB to interact more easily with hydroquinone groups (formed in the reduction of quinone groups) in order to transfer the electrons to the nitro group, reducing them in the process, 2) the FTIR evidence also suggests the formation of sulfur complexes (intermediates) on carbon surfaces that could help to create anions (carboxylate ions and/or quinone anions) susceptible to donate electrons to the nitroaromatic compounds through the partial positive charge of the aromatic ring in 3CNB. Previous studies have also documented the positive effects of electron-withdrawing substituents, such as Cl^-, on the reductive transformation of nitroaromatics mediated by quinones [29].

2.3.4 Proposed mechanism of nitroaromatic reduction

It was clear from the reduction experiments that the formation of reactive sites on ACF, due to the presence of HS^-, was a key step on the reduction of 4NP and 3CNB to 4AP and 3CA, respectively. Since FTIR spectra (Figure 2.3) revealed the formation of a C-S bond at 667 cm^{-1} in pre-incubated ACFs with HS^-, it is plausible to assume that mercaptoquinones might have been formed and also served as redox functional groups during the reductive transformation of 4NP and 3CNB. Previous studies have documented the involvement of mercaptoquinones during the reductive dechlorination of polychlorinated solvents mediated by different quinones provided with sulfide as electron donor [30, 31]. However, the energy required to create a C-S bond on a resonance-stabilized surface is high, and consequently, unlikely to be the dominant reduction mechanism. The tendency of sulfur compounds to act as reductant instead of nucleophiles is partly due to the weakness of the C-S bond (270 kJ/mol) [32]. In support to the latter statement,

chromatograms for both 4NP and 3CNB showed that more than 80% of the initial HS^- was detected as elemental sulfur (S^0) at the end of the chemical reduction experiments, while the rest of the HS^- could be chemically adsorbed on ACFs or transformed into several compounds (thiosulfate, sulfite or sulfate ions [29, 33], see Figure 2.7), given the reductive conditions in the medium. This points out that the oxidation of HS^- and the concomitant reduction of nitroaromatic compounds was a dynamic process that involves the participation of carbonyl sites (quinone groups) on ACFs. In Figure 2.8, a model was proposed for the hydrogen sulfur ion oxidation ($HS^- + OH^- = S^0 + H_2O + 2e^-$), the transfer of electrons to quinone groups (structure A), the formation of hydroquinones (structure B), and the 'electron shuttle' to nitroaromatic compounds and its intermediates [29, 34]. As previously discussed, without ACFs the direct chemical reduction of 4NP and/or 3CNB by HS^- (Na_2S) cannot take place, as observed in Figure 2.4 and 2.5.

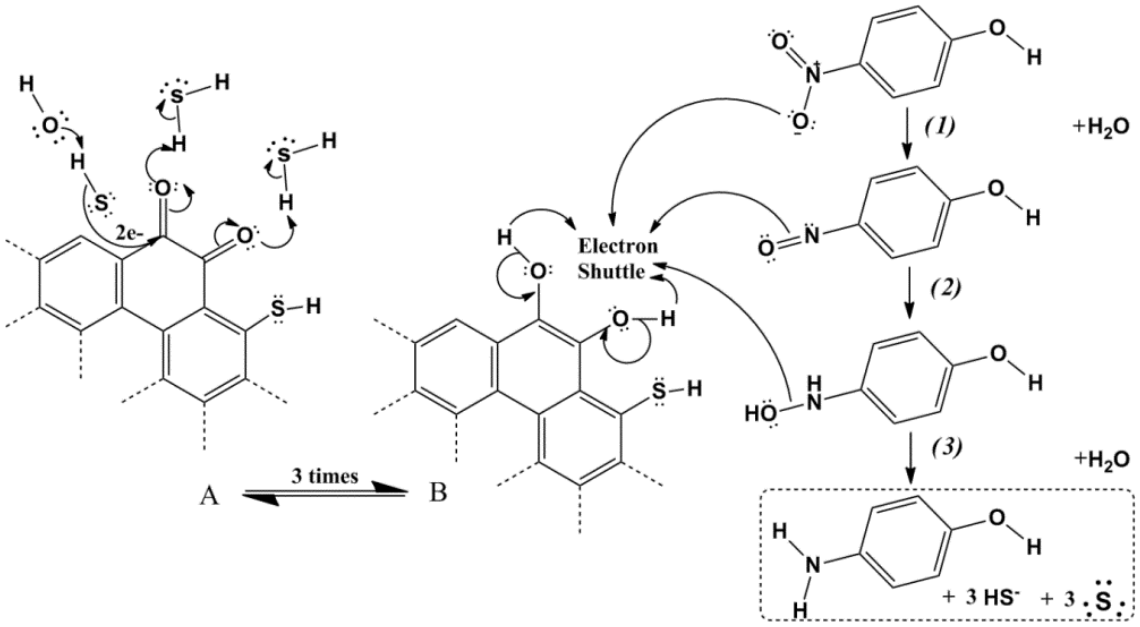

Figure 2.8. Proposed mechanism for nitroaromatic reduction (4NP in Figure). A) Hydrosulfide ion (HS^-) oxidation by OH^- and transfer of electrons to quinones trough resonance; B) Hydroquinone formation and 'Electron Shuttle' to nitroaromatics and their intermediates (reactions 1, 2 and 3); The dotted square marks the end products (aromatic amine) of these redox reactions.

Until now, the presence of surface functional groups on ACFs had not been demonstrated to act as effective redox mediators on the reduction of nitroaromatic compounds by sulfide as primary electron donor. The great external surface area that these materials expose to the solution in comparison to granular activated carbons, in conjunction to their mechanical properties, makes ACFs a potential material for aqueous and gas catalysis of hazardous materials. Moreover, combining the ability of some microorganisms to transform nitroaromatic compounds to aromatic amines with the ability of ACFs to accept electrons and donate them to the nitroaromatics, one can approach to an environmentally friendly source of primary electrons that can help not only to transform a major quantity of pollutants in aqueous solution, but also to generate low cost energy in the form of biogas. Finally, it should be mentioned that the amino aromatics produced from the reduction of nitroaromatic compounds are dangerous to human health, but in a posterior treatment they can be biodegraded [35] or transformed into less dangerous complex humic compounds [36]. On the other hand, aromatic amines are one of the largest groups of feedstocks used by the chemical industry [14], and their recovery from effluents of nitroaromatic reduction processes might be desirable. For these reasons, further studies have to be conducted in order to find out the catalytic properties of ACFs with other pollutants and biological electron donors.

2.4. Conclusions

Chemical reduction experiments demonstrate that ACFs can act as redox mediators accepting electrons from the oxidation of HS^- species and transferring them to the nitroaromatic compounds studied (4NP and 3CNB), reducing and transforming them to their respective aminoaromatic compounds (4AP and 3CA). The formation of the aromatic amines was confirmed by HPLC. The presence of surface functional groups, i.e. quinone groups (mercaptoquinones to a lesser extent), on ACFs can promote their catalytic activity to reduce nitroaromatic compounds under non-oxidizing conditions. This is supported by the increase in the concentration of quinone groups on ACFs modified with nitric acid (AW1105 OX) and the higher capacity to transform 4NP to 4AP and 3CNB to 3CA, compared

to the unmodified ACFs and those treated thermally at 700 °C under inert conditions. The catalytic performance of all ACFs was greater on 3CNB due to the high electronegativity of this molecule that permits a faster interaction with hydroquinone groups and/or anions over the carbon surface (electropositive and reactive groups), compared with 4NP.

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CHAPTER 3.- ANCHORAGE OF ANTHRAQUINONE MOLECULES ONTO ACTIVATED CARBON FIBERS TO ENHANCE THE REDUCTION OF 4-NITROPHENOL

ABSTRACT

The development of carbon materials with redox functional groups on their surface is an attractive alternative for the faster treatment of contaminants with electron withdrawing groups like 4-nitrophenol (4NP). In the search for better catalytic materials, here we report the anchorage of two anthraquinones on the surface of polyacrylonitrile Activated Carbon Fibers (ACFs). 2-aminoanthraquinone (AAQ) and anthraquinone-2,6-disulfonate (AQDS) were anchored on $SOCl_2$ treated carbon materials. The results show that fixation of AAQ and AQDS on ACFs caused changes in the surface area and the pore size distribution of ACFs, but only AQDS materials (8.3% in weight) showed an improvement in the catalytic properties of the material (49%) towards the reduction of 4NP to 4-aminophenol, providing sodium sulfide as the electron donor. HPLC and spectrometric scans, at the end of the reduction experiments, showed that AQDS remained anchored on ACFs. Immobilized AQDS on ACFs was shown to be effective as solid-phase redox mediator improving the reductive transformation of 4NP. Other applications for the method of immobilization of redox mediators on the surface of ACFs reported herein could be found in other fields of study such as energy storage and microbial fuel cells.

3.1. Introduction

The use of carbon materials as catalyst (on its own or as a support for metal active elements) is increasingly important in biotechnology and electrochemistry. This is promoted mainly because the ease of manipulation of the physical and chemical

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Amezquita-Garcia, H. J.; Razo-Flores, E.; Cervantes, F. J.; Rangel-Mendez, J. R., Anchorage of anthraquinone molecules onto activated carbon fibers to enhance the reduction of 4-nitrophenol. *Journal of Chemical Technology and Biotechnology* **2014,** *early view.* DOI: 10.1002/jctb.4478

properties of the carbon surface. The concentration and properties of the functional groups in the carbon materials can lead to their application as catalyst in the transformation and detoxification of priority pollutants. The fact that activated carbons have quinone groups in their chemical structure [1] had motivated their use as redox mediator in biological [2, 3] and abiotic processes [2, 4-6] with positive results in the reductive transformation of hazardous pollutants. The most recent studies in this area have reported the tailoring of the physicochemical properties of activated carbons [5, 7, 8] and the immobilization of model redox mediators with quinone moieties in order to increase the reduction rate of azo dyes [9] and nitroaromatic compounds [10] in solution.

Several methods have been reported to achieve a high concentration of quinone groups in carbon materials, including: oxidation with acid media (HNO_3, [11] H_2SO_4 [12]), modification with O_3, [13] and adsorption or immobilization of electroactive species such as benzoquinones, [14] anthraquinone, [15, 16] 2-aminoanthraquinone (AAQ) [17] and anthraquinone disulfonate (AQDS) [9, 10, 18]. Similar strategies have been applied in the fields of energy storage and microbial fuel cells, where activated carbons were modified with a high concentration of electroactive functionalities (e.g. quinone groups) to generate promising electrical double layer capacitors [15, 19, 20] and anode materials [21, 22], respectively. Considering the importance of developing electro-active and/or electro-catalytic materials in multiple disciplines, this study describes a method to immobilize two model quinone molecules (AAQ and AQDS) on the surface of Activated Carbon Fibers (ACFs) utilizing thionyl chloride ($SOCl_2$), which has only been used in the amidization of activated carbons [1] and carbon nanotubes [23, 24]. Furthermore, the effects of the anchored quinones on both the physicochemical properties of ACFs and on the reduction of a model hazardous pollutant (4-nitrophenol, 4NP) under anoxic conditions were studied.

3.2. Experimental

3.2.1. Chemicals

Polyacrylonitrile based ACF with commercial name AW1105 (AW) was purchased from KoTHmex (Taichung, Taiwan). The ACF was used directly as obtained from the supplier.

All chemicals (>99% purity) were used as received. 4-nitrophenol (4NP), pyridine and AAQ were purchased from Sigma-Aldrich (Mexico City, Mexico); 4-aminophenol (4AP) from Riedel-de Haën (Seelze, Germany); thionyl chloride ($SOCl_2$), sodium sulfite (Na_2SO_3) and sodium sulfate (Na_2SO_4) from J. T. Baker (Mexico City, Mexico); sodium sulfide ($Na_2S{\cdot}9H_2O$) from Fisher-Scientific (New Jersey, USA); and AQDS from TCI (Tokyo, Japan). The basal medium (pH=7.6) contained the following components (g/L): $NaHCO_3$ (5.0), NH_4Cl (0.28), K_2HPO_4 (0.25), $MgSO_4{\cdot}7H_2O$ (0.1), $CaCl_2{\cdot}2H_2O$ (0.01), and trace elements (1 mL/L), as described elsewhere [25].

3.2.2. Chemical functionalization of ACFs

The first step in the anchoring of molecules with quinone groups onto the ACF surface was the formation of chloride groups starting from the hydroxyl moieties present in the carboxylic (-COOH) and phenolic (-OH) groups. For this purpose, $SOCl_2$ and pyridine were used as dehydrating medium [26] and solvent, [27] respectively. $SOCl_2$ is a very corrosive agent that reacts vigorously with water and moisture, forming HCl and SO_2. It has to be handled carefully under a fume hood, similar to strong inorganic acids [28]. One gram of ACFs (AW) was submerged in an amber frosted glass of 125 mL with 40 mL of a mixture of $SOCl_2$ and pyridine at a ratio of 0.25 mL $SOCl_2$/mL pyridine (AW-Cl). After 24 h of shaking at 110 rpm and 25°C, the material was washed in a fume hood with ethanol until no pyridine and $SOCl_2$ were observed in the remaining solution by UV-vis spectra (190-300 nm). The samples were dried in an oven at 90°C for 12 h. A portion of 300 mg of AW-Cl were mixed with 25 mL of one of the following solutions at boiling point temperature: AQDS dissolved in water at 1 g/L (AW-AQDS) or AAQ dissolved in

ethanol at 1 g/L (AW-AAQ). The solvents are different given the solubility limitations of each anthraquinone molecule. Right after, samples were stirred at 110 rpm for 12 h, and finally rinsed with deionized water 3 times (no AQDS or AAQ were detected in the remaining solution by spectroscopic screening in the range of 190-400 nm). Samples were dried in an oven at 100°C for 12 h and stored until use in chemical reduction experiments.

3.2.3. Textural and surface chemistry characterization of ACF

Surface area (m^2/g), average pore diameter (nm), and pore volume (cm^3/g) were determined by nitrogen adsorption/desorption at 77 K (BET method) [29] using a Micromeritics ASAP 2020 surface analyzer. The pore size distribution was determined from the data obtained in the t-plot method provided by this equipment. The concentration of carboxylic, lactonic, fenolic, carbonyl, and basic groups in the ACFs was calculated by titration with 0.1 N solutions of sodium bicarbonate, sodium carbonate, sodium hydroxide, sodium ethoxide and hydrochloric acid, respectively, as described by Boehm [30]. The Point of Zero Charge (PZC) was obtained by an automatic titrator Metler Toledo (PL 70) following the described method by Arcibar-Orozco et al [31].

3.2.3.1. Fourier transform-infrared (FTIR) spectra of ACF

Four samples of ACF (as received (AW), modified with $SOCl_2$ (AW-Cl), with AAQ (AW-AAQ), and with AQDS (AW-AQDS)) were blended with KBr at a ratio of 0.1:99.9 (w/w), and then the pellets were obtained to conduct FTIR analysis. The spectra (32 scans) were recorded on a Thermo-Scientific FTIR (Nicolet 6700 model) spectrophotometer under ambient conditions.

3.2.4. Influence of anchored quinone molecules on the reduction of 4-nitrophenol

Reduction experiments as well as analytical procedures were performed as reported in a previous work [5]. A 25 mL aliquot of basal medium was added into 60 mL serum flasks containing a square of 0.63 cm^2 of as received or modified ACFs. The flasks were sealed with butyl rubber stoppers and the liquid was flushed for 3

min with a gas mixture of N_2:CO_2 (80:20%). Inside a glove box with an atmosphere composed of N_2:H_2 (95:5%), 5 mL of sodium sulfide (H_2S *(aq)* and HS^- in solution) were added with a syringe from a 26 mM stock solution (at this point [HS^-] = 4.33 mM). Previous results have indicated that the high nucleophilicity of sulfide species lead to an effective transfer of electrons to the ACFs [5] and this is expected to promote a faster reduction of 4NP in the solution. A general scheme for the expected reduction of 4NP with HS^- and the anthraquinone-modified ACFs can be observed in Figure 3.1. The bottles were pre-incubated horizontally for 24 h at 25 °C in a rotary shaker at 110 rpm. The next step was to add 20 mL of 4NP from a 1.27 mM stock solution in order to obtain an initial nitroaromatic solution of 0.5 mM and a total sulfide concentration of 2.6 mM. Experiments were carried out for 7 days and samples of 0.3 mL were taken at selected times and the concentration of 4NP and 4AP were analyzed as described in section 3.2.5. Controls without ACFs and/or sulfide were included to measure the stability of 4NP, the direct reduction of 4NP by sulfide and the adsorption of 4NP onto ACF. All experimental treatments were performed by triplicate.

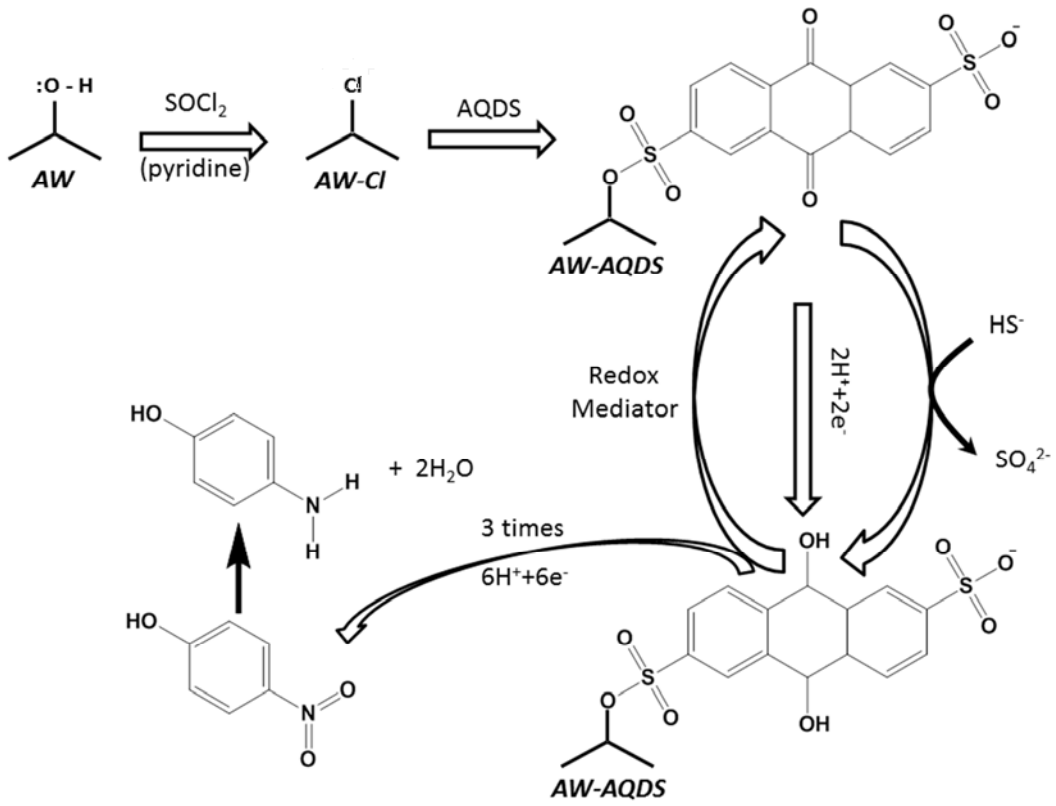

Figure 3.1. Graphic representation of the modification of ACFs (AW) surface with $SOCl_2$ (AW-Cl) and AQDS (AW-AQDS), followed by the use of anthraquinone functionalities as redox mediators in the reduction of 4NP to 4AP, using HS^- as electron donor.

3.2.5 Analytical methods

The concentration of 4NP was measured spectrophotometrically at 400 nm by diluting 0.1 mL of sample with 1.9 mL of 0.1 M phosphate buffer (pH 7.15) to an absorbance less than 1.0. The spectrophotometer used was a Thermo Scientific UV-vis (Genesis 10uv model). Detection of the remaining 4NP and the generated 4-aminophenol (4AP) was obtained by high-performance liquid chromatography (HPLC). The HPLC (Agilent Technologies, 1200 series) was equipped with a column Synergy 4U Hydro-RP 80R (250 x 4.60 mm, 4 micron) from Phenomenex. Samples of 2 µL were injected with an autosampler. The carrier liquid, composed of 60% of deionized water and 40% acetonitrile, was pumped at a flow rate of 1

mL/min. 4AP and 4NP were detected at wavelengths of 230-400 nm and 25 ^{o}C with an Agilent Technologies diode array detector.

3.3. Results and discussion

3.3.1. Characterization of ACFs

Table 1 includes the surface properties of AW after modification with $SOCl_2$, AQDS and AAQ. In the first case, the treatment of AW with $SOCl_2$ (AW-Cl) did not modify noticeably the surface area (from 1098 to 1033 m^2/g). However, surface area corresponding to micropores decreased 20% and mesopores between 2 and 10 nm rise 19%. This change in pore size distribution of AW could be attributed to the damage of micropores by the interaction with $SOCl_2$, leading to a greater concentration of mesopores between 2 and 10 nm. This could benefit the mass transfer of 4NP and its subsequent transformation to 4AP. A graphical representation of the changes in pore size distribution can be found in Figure 3.2. The properties of AW-Cl also changed when exposed to AQDS (AW-AQDS) or AAQ (AW-AAQ) solutions. In both cases a decrease in surface area, pore volume, average pore diameter and percentage of mesopores between 2 and 10 nm was observed (see Table 3.1). These results indicated that pores previously created in AW-Cl (from 2 to 10 nm) were partially clogged by the attachment of AQDS and AAQ on the pore walls of ACFs (the projected diameter of the quinone molecules is greater than 1 nm). Differences in the grade of clogging between AW-AQDS and AW-AAQ could be due to the different amounts of AQDS (1 g/L = 2.36 mM) and AAQ (1 g/L = 4.48 mM) used to prepare each material.

Table 3.1. *Physical properties of as-received (AW) and modified ACFs with $SOCl_2$ (AW-Cl), AQDS (AW-AQDS) and AAQ (AW-AAQ).*

Physical properties of ACFs	AW	AW-Cl	AW-AQDS	AW-AAQ
Surface Area (m^2/g)	1098	1033	704	581
Pore Volume (cm^3/g)	0.368	0.345	0.195	0.168
Average Pore Diameter (nm)	2.02	2.00	1.82	1.84
Micropore (μ < 2 nm) surface area (m^2/g)	583	341	310	295
Mesopore (2 < μ < 10 nm) surface area (m^2/g)	489	659	368	257
Mesopore (10 < μ < 50 nm) surface area (m^2/g)	21	27	18	21
Macropore (μ > 50 nm) surface area (m^2/g)	5	6	13	8

Moreover, it could be observed in Table 3.2 remarkable changes in surface chemistry of AW, AW-Cl, AW-AQDS and AW-AAQ. In the first place, the phenolic sites disappeared in AW-Cl due to the reaction of hydroxyl moieties with $SOCl_2$, yielding chlorine groups (C-Cl) attached on the carbonaceous surface (product A, Figure 3.3). Carbonyl groups increased with the anthraquinone modification in AW-AAQ and AW-AQDS, but only AW-AQDS showed a higher concentration of carboxylic acids (3 < pKa < 6). This could be attributed to the sulfonate groups (pKa = -6) [32] anchored on the surface of AW-AQDS, and also to the reaction of water (acting as a nucleophile) with the remaining C-Cl, C-O-SO-Cl and CO-Cl groups (products A, B and C, Figure 3.3) in the final step of sample preparation (rinsing). In fact, the increase in the concentration of acidic groups (from 1.9 to 5.55 meq/g) shifted the PZC of the material to pH 3.06. Previous work have demonstrated that materials with lower PZC hinders the adsorption of nitroaromatic compounds onto ACFs, but improve their reduction due to the greater concentration of quinone groups in low-PZC materials [5]. On the other hand, the PZC for the materials AW-Cl and AW was 5.1 and 5.16, respectively. These results indicate that the replacement of the OH- by Cl- groups in AW-Cl did not change significantly the PZC of the carbon materials. The PZC of AW-AAQ and AW-AQDS was 3.79 and 3.06, respectively, indicating the high concentration of common

acidic groups such as lactonic and carbonyl sites. However, main differences in the redox properties should be expected due to the differences in the polarity of the quinone structures AAQ and AQDS.

Table 3.2. *Chemical properties of as-received (AW) and modified ACFs with $SOCl_2$ (AW-Cl), AQDS (AW-AQDS) and AAQ (AW-AAQ).*

Chemical Properties	AW	AW-Cl	AW-AQDS	AW-AAQ
Carboxylic (meq/g)	0.16	0.75	3.74	0.63
Lactonic (meq/g)	0.74	1.25	0.81	1.13
Phenolic (meq/g)	0.23	0.09	0.00	0.00
Carbonyl (meq/g)	0.78	0.29	1.00	0.75
Total acidic groups (meq/g)	1.90	2.38	5.55	2.50
Total basic groups (meq/g)	0.11	0.48	0.20	0.38
PZC (pH)	5.16	5.10	3.06	3.79

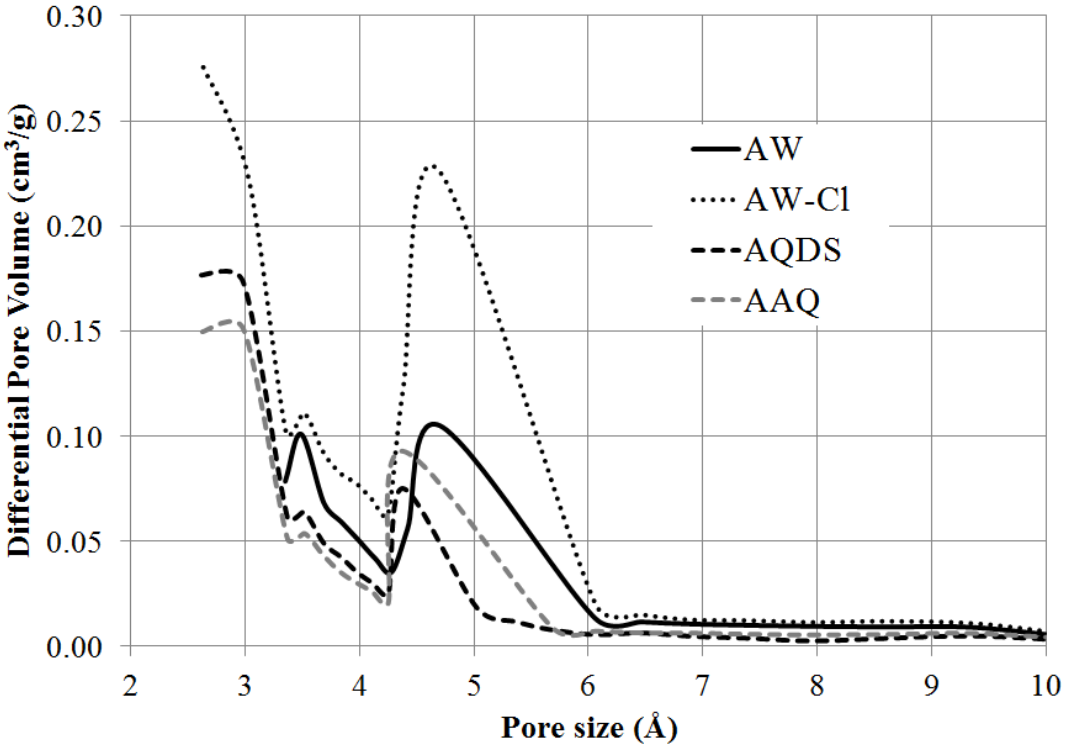

Figure 3.2. Differential pore volume using the t-plot method for chemically modified ACFs. As-received (AW) and modified ACFs with SOCl2 (AW-Cl), AQDS (AW-AQDS) and AAQ (AW-AAQ).

FT-IR absorption bands of chlorine groups (C-Cl, A in Figure 3.3) on the surface of AW can be found in Figure 3.4, where the material AW-Cl exhibits a strong band at 615 cm^{-1} due to chlorine groups attached on the surface of carbon (C-Cl stretching [33]). Additionally, it is possible that a complete reaction between $SOCl_2$ and hydroxyl groups did not take place, leading to aromatic sulphonyl chlorides (C-O-SO-Cl, B in Figure 3.3) detected as a shoulder at 1225-1210 cm^{-1} (S=O stretching) and 1107 cm^{-1} (C-O stretching). Some evidence of acyl chloride groups (CO-Cl, C in Figure 3.3) can be seen at 1740 cm^{-1} (C=O stretching) together with the peak at 615 cm^{-1} (C-Cl stretching [33]).

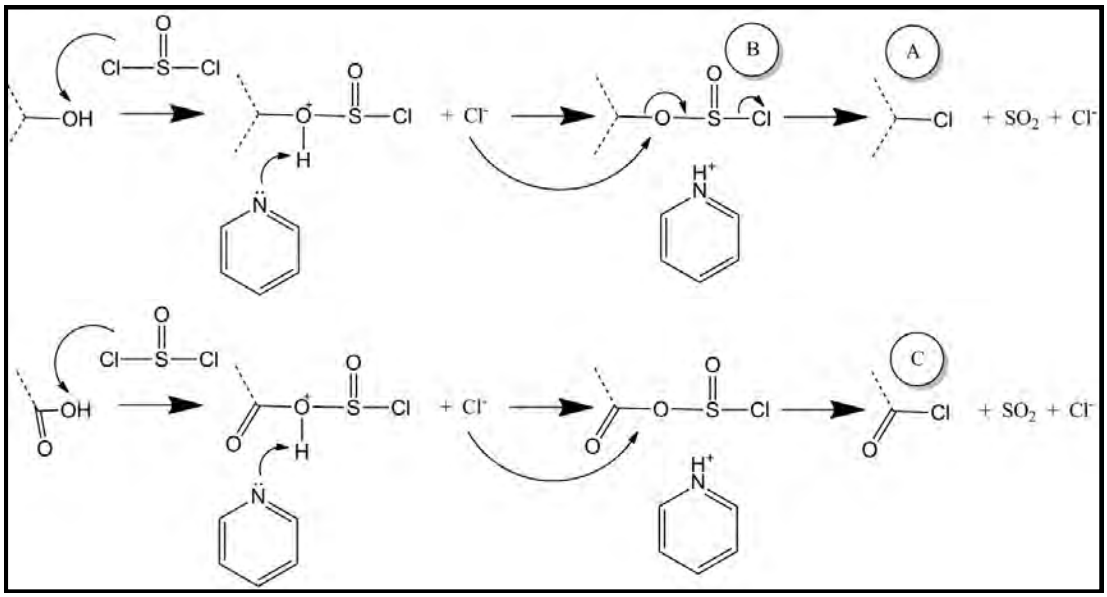

Figure 3.3. Expected substitution reactions in AW due to reaction of $SOCl_2$ with hydroxyl groups present in ACFs (pyridine was used as the solvent). For simplicity, chlorinated products A, B and C are represented as –C-Cl, -C-O-SO-Cl and –CO-Cl, respectively.

After modification of AW-Cl with AQDS and AAQ, the intensity of peaks corresponding to C-Cl, C-O and S=O stretching (C-O-SO-Cl) decreased due to the substitution by the sulfonate (AW-AQDS) or amino (AW-AAQ) groups. The anchoring of AQDS on ACFs surface is evidenced by the absorption bands found at (cm^{-1}) 1420-1330 (asymmetric SO_2 stretching), 1235-1145 (symmetric SO_2

stretching), 1020-850 (asymmetric SO stretching), 830-650 (symmetric SO stretching), 700-600 (S-C stretching) and 610-500 (SO_2 deformation vibration [33]). On the other hand, the anchoring of AAQ led to the presence of secondary aromatic amines detectable at 1360-1250 cm^{-1} (C_{ACF}-N stretching) and 1280-1080 cm^{-1}, (N-$C_{anthraquinone}$ stretching [33]). The absorption band corresponding to N-H stretch (3450-3400 cm^{-1}) is overlapped due to the adsorbed moisture (3550-3230 cm^{-1}, hydrogen bonded OH stretching vibrations). Other groups present in AW-AQDS and AW-AAQ are ketone groups, generated by the reaction of remaining chlorine groups with water of the rinse process (1770-1700 cm^{-1} [C=O stretching]), and quinones (1680-1650 cm^{-1}) in the anthracene structure (1640 and 1550 cm^{-1}) of AQDS and AAQ. The detection of quinone groups in AW-AQDS and AW-AAQ is relevant for the purpose of this study since it indicates the availability of these redox active functional groups in modified ACFs, which are expected to promote a faster reduction of 4NP during their application in the redox conversion of this pollutant.

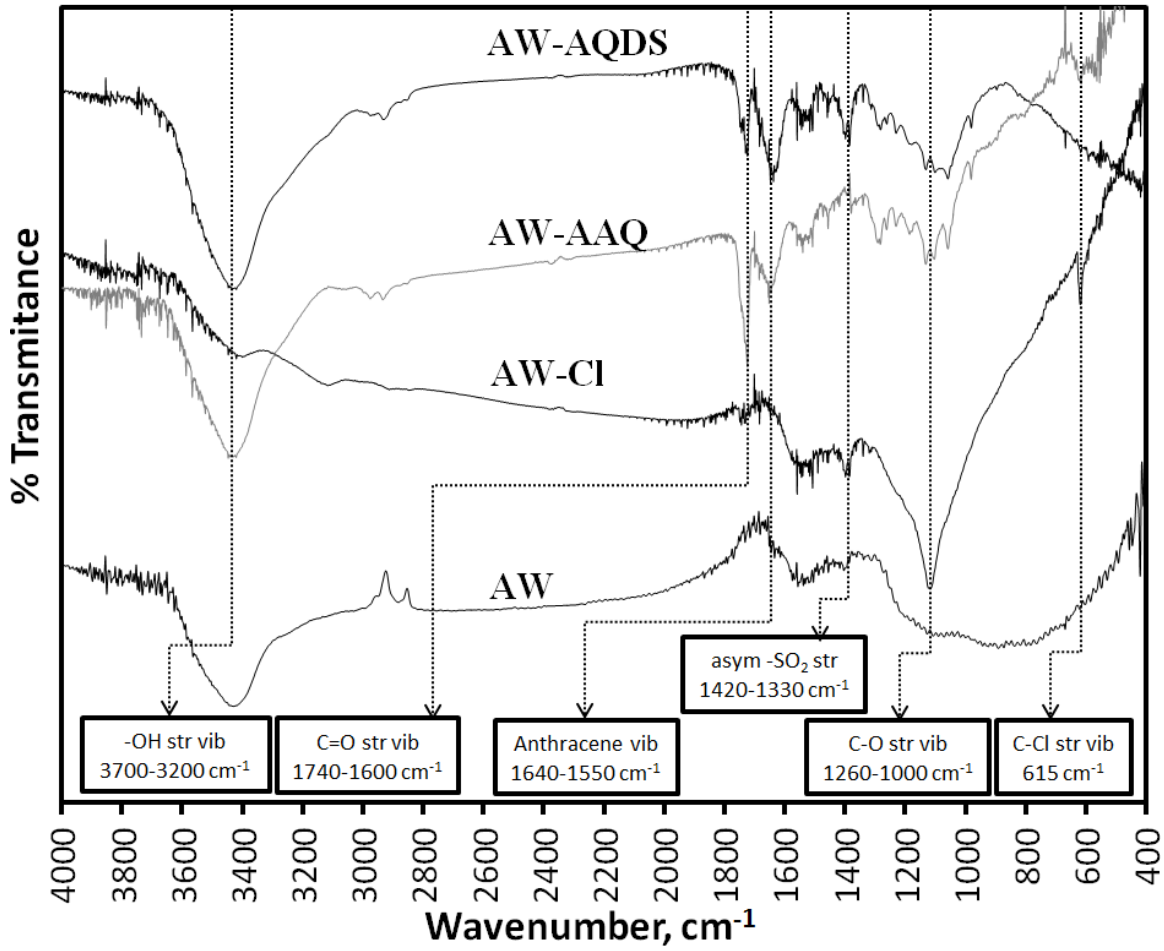

Figure 3.4. FT-IR spectra of as-received (AW) and modified ACFs with $SOCl_2$ (AW-Cl), AQDS (AW-AQDS) and AAQ (AW-AAQ).

3.3.2. Effect of chemical properties of ACFs on 4-nitrophenol reduction

Different reduction rates of 4-nitrophenol (4NP) by the use of sulfide (HS^-) as electron donor and ACFs as redox mediator are shown in Figure 3.5. This figure shows that controls lacking ACFs or HS^- did not show significant removal of 4NP along the experiment due to the resistance of 4NP to be reduced by the medium itself and by the presence of HS^-. These controls were the same for all ACFs studied. The adsorption experiments for AW, AW-Cl, AW-AQDS and AW-AAQ showed an adsorption of 4NP of 17, 30, 18 and 22%, respectively. However, incubations provided with both ACF and HS^- exhibited a greater decrease in the concentration of 4NP in all ACFs studied due to the reduction of 4NP to 4AP. At

the end of the experiments (7 days), the materials that improved the reduction of 4NP to 4AP (at the same conditions of pH = 7.6, T = 25 °C and [HS^-] = 2.6 mM) followed the next order AW-AAQ < AW-Cl = AW < AW-AQDS. The presence of 4AP was evidenced by HPLC analysis for all treatments amended with ACFs and HS^-, while no 4AP was detected in incubations lacking either ACF or HS^-. The chromatogram of the material that performed better (AW-AQDS) in the catalytic reduction of 4NP to 4AP is shown in Figure 3.6. At the end of the experiments (day 7), the HPLC results showed that around 15% of the original 4NP did not reacted and remained in the solution. Around 65% of the 4NP reduced was detected as 4AP in the samples. The lack in the detection of new compounds other than 4AP supports the hypothesis that the other 45% of the 4NP could be reduced to 4AP and then adsorbed into the surface of the materials.

In these experiments, if all AQDS anchored on AW-AQDS could be detached and pass to the solution due to the high nucleophilicity of HS^-, then the concentration of AQDS in the solution would be around 14 mg/L and a peak in the chromatogram would be observed at 2.0 min, but this was not the case. Similar to HPLC results, UV-vis scans from 200-600 nm (see Figure 3.7) confirmed that AQDS in incubations supplied with AW-AQDS remained in the carbonaceous structure since no signals was observed at 260 nm, were AQDS could be detected. These results clearly demonstrate that AW-AQDS acts as effective redox mediator in the transformation of 4NP to 4AP.

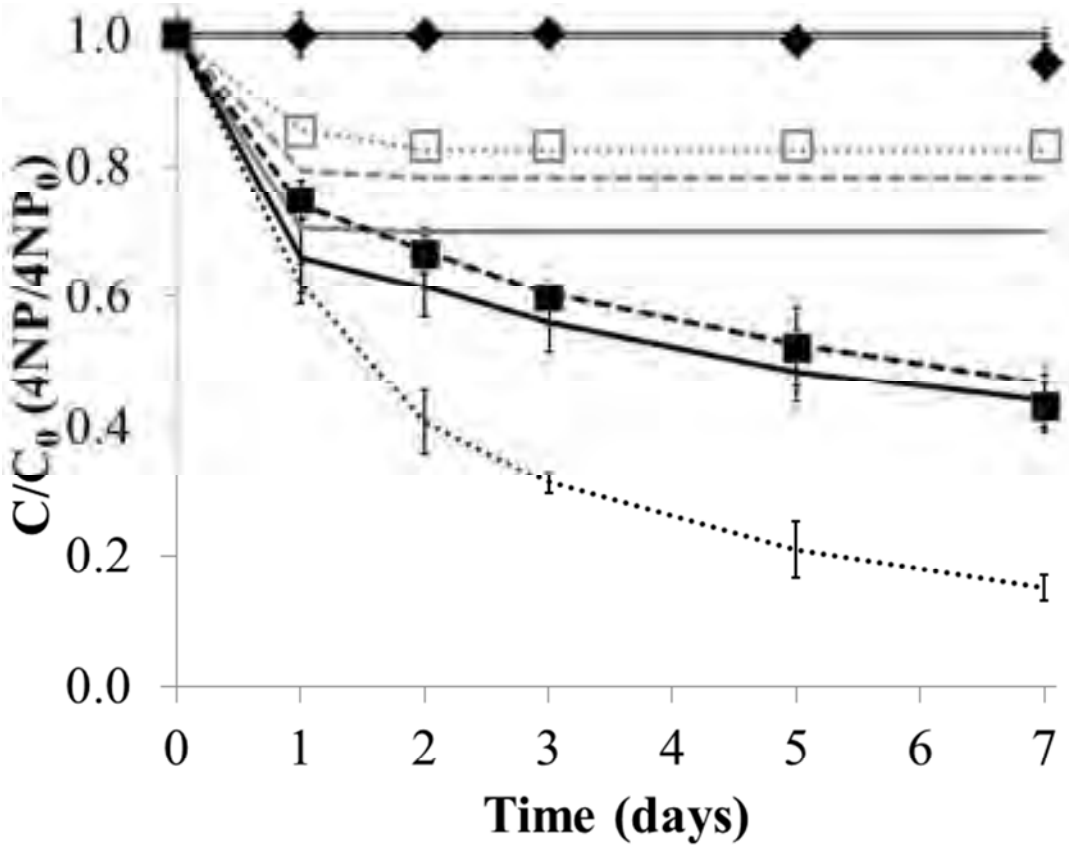

Figure 3.5. 4NP chemical reduction experiments: 4NP (double line), 4NP + HS^- (diamonds). The adsorption (4NP + ACF) and chemical reduction experiments (4NP + ACF + HS^-) are denoted in gray and black color, respectively, for AW (squares), AW-Cl (continuous line), AW-AAQ (hyphen line) and AW-AQDS (dotted line).

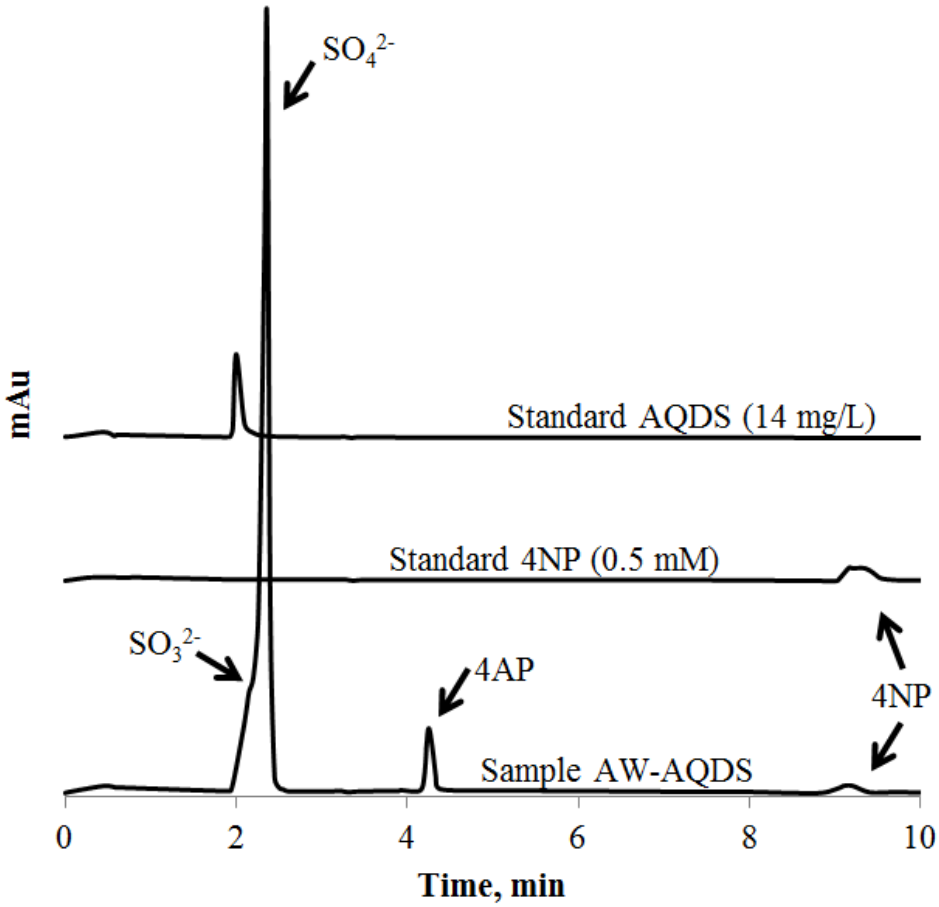

Figure 3.6. HPLC chromatograms at the end of the reduction experiments of 4NP to 4AP when using AW-AQDS as redox mediator and HS^- as electron donor.

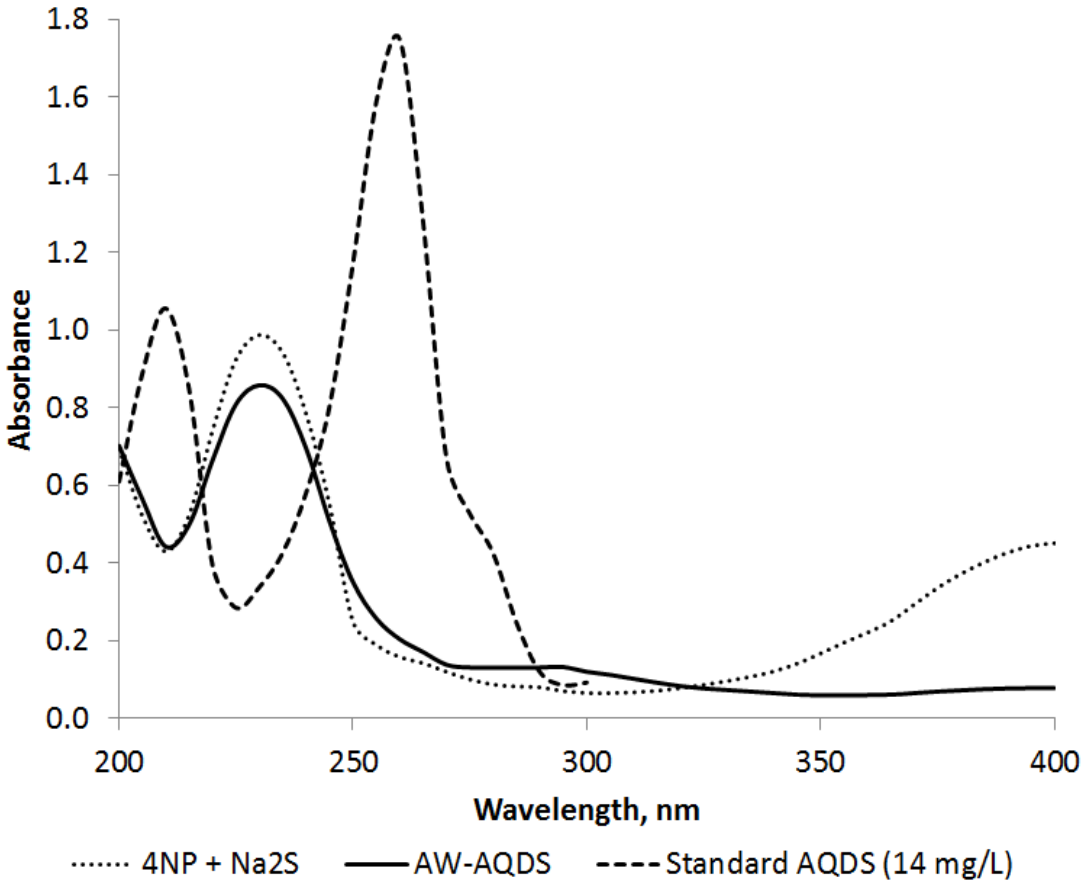

Figure 3.7. Spectrometric UV-vis scans at the end of the reduction experiments when using AW-AQDS as redox mediator.

Our results indicated that modified materials such as AW-Cl and AW-AAQ did not differ significantly in their reduction percentage of 4NP at day 7 compared to AW (53-57% of reduction). On the other hand, at day 1 the reduction of 4NP by AW-Cl (34%) was higher than AW and AW-AAQ (25%). This difference in the first day of the experiments could be due to the greater adsorption of 4NP onto AW-Cl (continuous gray line in Figure 3.5), however, similarities at the end of the experiments could be explained because of the similarities in the concentration of total acidic groups in the materials AW, AW-Cl and AW-AAQ. Even though quinone groups have been reported to catalyze the reduction of nitroaromatic compounds, [5] the quinone groups anchored in AW-AAQ were not active under the conditions studied or at least were not involved in the catalytic transformation of 4NP to 4AP.

In fact, the nitro group in 4NP tends to be solvated by water molecules in aqueous solutions while AAQ molecules are hydrophobic in nature, resulting in the lack of interaction of the quinone groups with 4NP. Previous reports have demonstrated that covalently grafted AAQ onto chemically modified graphene contributed to increase the redox capacitance of these materials [17]. These means that AAQ could present redox activity when attached to graphene (complex with amphoteric character), but might not when the (pore) surface is covered or surrounded with hydrophobic (AAQ) active sites in AW-AAQ.

On the other hand, AQDS molecules have two acidic (sulfonic) groups highly soluble in water that are capable to interact (electrostatically trough the electrical double layer) with 4NP and other promoters of the electron transfer reactions such as protons (H^+) [34] and ions [35]. These interactions can lead to a greater catalytic activity of the quinone group in AQDS that is reflected in an increased reduction (49%) of 4NP by AW-AQDS compared to the original material (AW). The introduction of sulfonic ($-SO_3H$) acidic groups on carbon materials was reported to enhance the catalytic etherification of isopentene with methanol to produce *tert*-amyl methyl ether [36]. Other studies have also reported carbon materials with AQDS anchored on the surface by electro-polymerization with positive results in the biotransformation of electron withdrawing pollutants (azo dyes [9] and nitroaromatic compounds [10]). However, the electro-polymerization method used in this case caused total blockage of pores of carbon materials, resulting in the misuse of the catalytic and adsorptive properties of the ACFs.

Much of the contribution of this work is based on the development of a new methodology to prepare materials with quinone functionalities attached on the surface of ACFs. In this case we focus the application towards the catalytic reduction of a model nitroaromatic compound (4NP), considering that mesoporous materials would be more advantageous than microporous ones, in order to minimize diffusion limitations and catalysis deactivation, [37] and that the narrow porosity of ACFs could provide regions where the surface potentials overlap and the 4NP molecules might interact with more than one surface [32]. The application of

materials such as AW-AQDS could be expanded to the catalytic reduction of chemical compounds with a more positive redox potentials than that of 4NP (-1.02 V vs Ag) [38] or even used in supercapacitors applications, given the redox activity of the quinone molecules anchored on the carbon materials. Future work will include electrochemical studies and the effect of parameters such as the pH and the concentration of electron donor (HS^-) on the catalytic activity of ACFs.

3.4. Conclusions

The modification of polyacrylonitrile based activated carbon fibers with $SOCl_2$ yields materials highly susceptible to substitution of the chlorine groups by the sulfonate ($-SO_3^-$) or the amino ($-NH_2$) group present in the anthraquinone molecules studied (AQDS and the AAQ, respectively). The anchorage of the AQDS and AAQ on the surface of ACFs was supported by FTIR, HPLC and UV-Vis analysis. The hydrophobic nature of AAQ could explain its lowest catalytic activity in the reduction of 4NP to 4AP, while the acidic nature of the sulfonate groups in AQDS and the low PZC of the AW-AQDS could help to attract key elements in the reduction of the nitro group (protons and ions) as well as 4NP. Both the chemical and physical properties of the original ACF changed noticeably, but only the material modified with AQDS (AW-AQDS) increased its reduction capacity by 49%.

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CHAPTER 4.- ACTIVATED CARBON FIBERS WITH REDOX ACTIVE FUNCTIONALITIES IMPROVES THE CONTINUOUS ANAEROBIC BIOTRANSFORMATION OF 4-NITROPHENOL

ABSTRACT

The number of studies dealing with the use of activated carbons as redox mediators (electron shuttle catalyst) in biological systems has grown rapidly in recent years. However, evidence explaining the role of the surface chemistry of activated carbons in the biotransformation of recalcitrant pollutants under continuous conditions is rather meager at present. This study offers a discussion about the role of the chemistry of activated carbon fibers (ACFs) on the increased reduction of a model nitroaromatic compound, 4-nitrophenol (NP), under continuous conditions and using novel UASB-packed reactors. ACFs with different physicochemical properties and different levels of redox-active groups were studied, including un-modified (AW), HNO_3 treated (OX) and modified with anthraquinone-2,6-disulfonate (AQDS) ACFs. The results indicate that biofilm formation could cover up the effect of the different ACFs studied during the first days of the continuous experiment. However, with modifications in the concentration of ethanol (exogenous carbon source) the interaction bacteria-ACF surface was enhanced and resulted in increased biotransformation efficiencies of 1.47-, 1.97- and 2.11-fold for the reactors packed with AW, AQDS and OX materials, respectively, as compared with the control reactor, which lacked any carbonaceous support. We correlated the observed NP biotransformation efficiencies in the order OX > AQDS > AW with the content or redox-active groups such as carbonyl groups (quinone moieties) on the ACF surface in the order 1.3 > 1.0 > 0.78 milliequivalents/g, respectively. The present work proposes a novel treatment concept to enhance the reductive biotransformation of contaminants and

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provides a deep understanding on the complex subject of using activated carbons as redox mediators in biological systems.

Keywords: Biofilm, quinone, redox mediator, activated carbon fibers, biotransformation, nitroaromatic.

4.1. Introduction

Xenobiotic compounds usually contain molecular arrangements that are not normally found in nature and therefore they are hard to metabolize by microorganisms. For some anthropogenic pollutants with strong electronegative groups, like azo dyes or nitroaromatic compounds, anaerobic conditions foster biotransformation into less harmful compounds [1] or products that could be detoxified in a secondary treatment with air [2]. Several studies have improved the kinetics of anaerobic (biotic and abiotic) transformation of recalcitrant compounds with the use of electron shuttles (redox mediators, RM) that could accept electrons (RM becomes reduced) from chemical substances or the fortuitous metabolism of a carbon source by microorganisms and donate them (RM becomes oxidized) to the pollutant of interest. Heretofore most of the research in RM as catalyst for biological applications has focused in anaerobic environments were the consumption of a carbon source (e.g. ethanol, glucose, volatile fatty acids) by pure or mixed microbial populations generate the energy necessary to reduce the soluble (e.g. anthraquinone-2,6-disulfonate (AQDS)) or immobilized RM (e.g. graphene, graphite carbon nanotubes, black carbon, granular activated carbon, and activated carbon fibers), and subsequently, shuttle that energy to the targeted electron withdrawing compounds in aqueous solutions (e.g. nitroaromatic, azo, and polychlorinated compounds) [3-6]. Among the recent advances in the use of RM, the immobilization of redox functionalities (i.e. quinone moieties) is regarded as a promising strategy to reduce costs and accelerate biotransformation of xenobiotic compounds [7].

Activated carbons are widely known to have versatile applications due to the possibility of tailoring their chemical and physical properties, including the quantity of carbonyl (quinone) groups on their surface. In biotechnology, the role of activated carbons has often been limited to support bacteria and to adsorb toxic compounds that could hinder biological degradation/transformation of xenobiotic pollutants [8, 9]. However, recent studies have taken advantage of the presence or introduction of quinone groups on the surface of activated carbons towards an improvement in the rate of anaerobic biotransformation of recalcitrant compounds such as azo dyes [3, 4, 6, 10] or nitroaromatic compounds [11]. From the above studies, only some have been carried out under continuous conditions in the biotransformation of azo dyes [3, 10], and none (to the author's knowledge) in the biotransformation of nitroaromatic compounds.

The goal of this study was to demonstrate that activated carbon fibers (ACFs) could serve not only as a support media for the growing of anaerobic microorganisms, but also as RM in the continuous biotransformation of the model nitroaromatic compound, 4-nitrophenol (NP). The amount of redox active functionalities (carbonyl structures) onto ACFs were increased by HNO_3 treatment and by anchoring AQDS molecules on the ACF surface as described in previous studies [12, 13]. The contribution of the physicochemical properties of ACFs on the reduction of NP is also discussed to fully disclose the role of ACFs as RM in continuous systems.

4.2. Experimental

4.2.1. Chemicals

4-nitrophenol (4NP) and 4-aminophenol (4AP) were purchased (>99% purity) from Sigma-Aldrich (Mexico City, Mexico) and Riedel-de-Haën (Seelze, Germany), respectively. Ethanol (absolute grade) was purchased from Fermont (Monterrey N.L., Mexico). The aqueous mineral media had the following composition (g/L): $NaHCO_3$ (5.0), NH_4Cl (0.28), KH_2PO_4 (0.25), $MgSO_4 \cdot 7H_2O$ (0.1), $CaCl_2 \cdot 2H_2O$ (0.01), and trace elements (1 mL/L).

4.2.2. Characterization of activated carbons

Polyacrylonitrile ACF (AW) were oxidized with HNO_3 8M (OX) and pretreated with $SOCl_2$ prior to AQDS anchorage on ACF surface (AQDS) as described in previous studies [29, 30]. The surface area (m^2/g) was determined by nitrogen adsorption/desorption at 77 K (BET method) using a Micromeritics ASAP 2020 surface analyzer. Specific surface functional groups on ACFs were measured by potentiometric titrations as described by Boehm [14], and the point of zero charge (PZC) was obtained by the procedure reported by Rangel and Streat [15].

4.2.3. Bioreactor design and operational conditions

Novel lab scale up-flow anaerobic sludge bed (UASB)-packed reactors were inoculated with anaerobic granular sludge (AGS) with 10.6% of volatile suspended solids (VSS) from a UASB reactor treating wastewater from a brewery factory (Cd. Obregon, Sonora, Mexico). The polyacrylate UASB reactors (5:22 cm of internal diameter:height) were packed with polyvinyl chloride (PVC) disks of 5:4:1 cm (external diameter: internal diameter: height) as showed in Figure 4.1. The ACFs of 4 cm in diameter were fixed to the PVC disks with a nylon cord. Two holes of 0.5 cm in diameter were cut in order to facilitate the flow of biogas and liquid to the top of the reactor. The holes created in the ACF disks were intercalated from disk-to-disk to promote the complete mixture of the fluid passing through the UASB reactor.

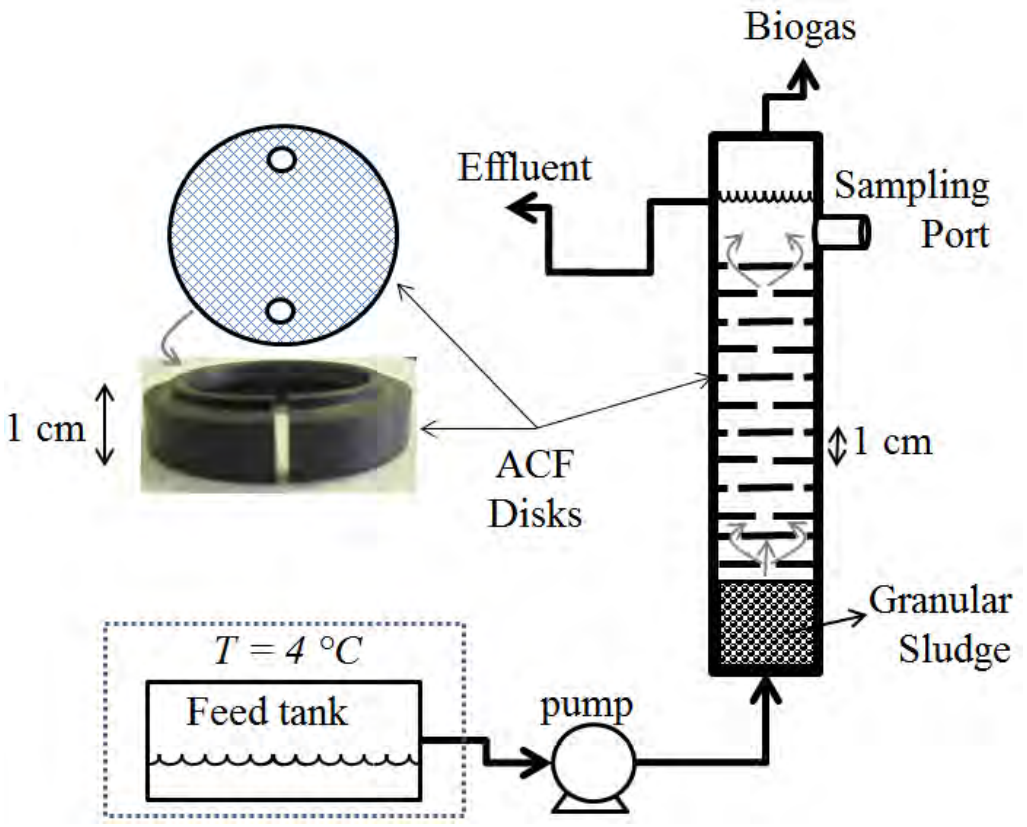

Figure 4.1. Bioreactor design for the anaerobic reduction of 4NF with and without the presence of ACFs. Room temperature 25-32 °C.

Four UASB reactors (working volume of 400 mL) were inoculated at the bottom with 25 g VSS/L (94.3 g of AGS per reactor). A total of 12 disks were inserted to different UASB reactors: 1) control reactor with only AGS (un-packed), 2) reactor packed with AGS and 2.87 g of AW, 3) reactor packed with AGS and 3.37 g of OX, and 4) reactor packed with AGS and 3.34 g of AQDS-anchored ACF (AQDS). The difference in the mass of AW, OX, and AQDS is related to the modification of the ACFs (increase on the oxygen content and/or moisture adsorbed) rather than the area exposed by each face of the ACF disk (5.7 cm^2). Selected chemical properties of the ACFs used in the study are shown in Table 4.1.

Table 4.1. Selected chemical properties of ACFs used in this study (for more details see references 12 and 13).

PACKING MATERIAL	Surface Area (m^2/g)	PZC (pH)	Total acidic groups (meq/g)	Total basic groups (meq/g)	Carboxylic (meq/g)	Lactonic (meq/g)	Phenolic (meq/g)	Carbonyl (meq/g)
AW	1098	5.16	1.90	0.11	0.16	0.74	0.23	0.78
OX	1052	3.41	3.50	0.26	0.96	0.94	0.30	1.30
AQDS	704	3.06	5.55	0.20	3.74	0.81	0.00	1.00

Ethanol was provided as an exogenous electron-donating substrate that the anaerobic microorganisms could use to reduce NP by co-metabolism according to the equations 1-4. Note that these equations consider the hydrogen produced in the fermentation process [16] as an effective electron donor and acetate is not consumed by the anaerobic microorganisms. Continuous dosing of ethanol to all bioreactors followed two stages: 1) Acclimation of AGS to ethanol consumption for 4 (retention time 12 h) and 3 (retention time 8 h) days in phase 1 and 2, respectively; 2) Biotransformation of NP (75 mg/L) with 4 different amounts of carbon source as specified in Table 4.2. The day at which NP and NP-saturated ACF disks were first added to the reactor was defined as day 0 of the reactor operation (start of Period I). Biotransformation of NP to AP, pH, biogas production and the COD removal efficiency were monitored throughout the experiment.

(1) Ethanol fermentation (ref 16) : $\frac{3}{2}\left[CH_3CH_2OH + H_2O \rightarrow CH_3COO^- + 2H_2 + H^+\right]$

(2) Hydrogen oxidation : $3\left[H_2 \rightarrow 2H^+ + 2e^-\right]$

(3) Nitro - group reduction : $R - NO_2 + 6H^+ + 6e^- \rightarrow R - NH_2 + 2H_2O$

(4) Biotransformation of NP :

$$R - NO_2 + \frac{3}{2}CH_3CH_2OH \rightarrow R - NH_2 + \frac{3}{2}CH_3COO^- + \frac{1}{2}H_2O + \frac{3}{2}H^+$$

Table 4.2. The operational conditions during continuous operation of bioreactors treating 4-nitrophenol (NP).

PERIOD	*A1*	*A2*	*I*	*II*	*III*	*IV*
days	*4*	*3*	*7*	*10*	*14*	*7*
Ethanol influent (mg/L)	1000	697	697	41	0	20.5
Ethanol influent (mg COD/L)	2087	1455	1455	86	0	43
Ethanol loading rate (mg COD/L-d)	4174	4364	4364	257	0	128
NP influent (mg/L)			75	75	75	75
NP loading rate (mg/L-d)			225	225	225	225
Hydraulic retention time (h)	12	8	8	8	8	8
Biogas-CH_4 expected (mL CH_4/d)*	648	678	678	40	0	20

A1 = acclimation phase 1

A2 = acclimation phase 2

*T=30°C, P=1 atm. Biogas-CH_4 expected considering the use of hydrogen and acetate (produced in ethanol fermentation) as electron donors for the methanogenesis and without NP reduction.

4.2.4. Saturation of ACFs with NP

Prior to insertion of ACF disks into the bioreactors, they were saturated in a separate batch system with NP and basal mineral media, until the final concentration in the aqueous solution at equilibrium was 75 mg NP/L (initial pH 8.75, final pH 6.8, without pH control). The materials AW, OX, and AQDS have an adsorption capacity (at equilibrium concentration of 75 mg NP/L) of approximately 160 [12], 110 [12], and 75 (unpublished results) mg NP/g ACF, respectively. This saturation of ACF disks with NP was performed in order to discard the adsorption process as the main mechanism behind NP removal in the bioreactors.

4.2.5. Analytical

Chemical oxygen demand (COD) of the influent and effluent of reactors and VSS were measured according to standard methods [17]. Volatile fatty acids (VFA) were quantified by capillary electrophoresis (Agilent CE G1600A) using the Agilent Basic Anion Buffer [18]. Biogas generation was quantified by the use of 500 or 100 mL inverted burettes, depending on the expected production of biogas per day (see Table 4.2). CH_4 and CO_2 concentrations were determined by gas chromatography (GC, Agilent Technologies, 6090N series) equipped with a column Alltech Hayesep (10'x1/8"x0.085" SS) and a thermal conductivity detector (TCD at 250 °C, N_2 gas,

reference flow = 30 mL/min, make up flow = 8 mL/min). N_2 was used as carrier gas at 12.9 mL/min. Oven and injector temperatures were 60 and 250 °C, respectively.

An HPLC (Agilent Technologies, 1200 series) equipped with a C18 silica based column Phenomenex, Synergy 4U Hydro-RP 80R (250 x 4.6 mm, 4 micron) was used to quantify NP and AP (influent and effluent of the reactors) at wavelengths of 400 and 195 nm, respectively. Samples of 20 µL were injected with a mobile phase of acetonitrile: water (40:60) at flow of 0.5 mL/min. Samples were diluted with ascorbic acid to a final concentration of 200 ppm in order to prevent AP auto-oxidation in air [1].

4.3. Results

4.3.1 Acclimation of the inoculum to ethanol consumption

Table 4.3 shows several variables measured during the continuous biotransformation of NP such as pH, COD, COD removal, methane production and biogas composition. The first strategic step towards the biotransformation of NP was the acclimation of the inoculum seed to ethanol consumption (fermentation) and the concomitant methanogenesis in each of the four bioreactors. The adaptation to methanogenesis from ethanol was achieved with the use of two retention times 12 h (phase 1) and 8 h (phase 2) at a similar loading rates (Table 4.2). At the end of the acclimation period all the bioreactors achieved a high COD removal efficiency (> 83.8%) and acetate was the only metabolite of ethanol fermentation detected by capillary electrophoresis at the effluent of the reactors as shown in Figure 4.2. The protons produced by acidogenic fermenting bacteria caused a slight decrease in effluent pH of all bioreactors of about 0.5 units (Table 4.3). Syntrophic interactions between hydrogenotrophic and acetoclastic methanogens took advantage of other fermentation products such as CO_2 and H_2 (H_2 not detected by the GC method used) for the production of CH_4 (average biogas composition of 80:20% CH_4:CO_2) as shown in Table 4.3. The methane produced in each bioreactor (504-673 mL CH_4/d) was close to the theoretical volume expected to be produced from 697 mg ethanol/L at 1 atm, T = 30 °C (678

mL CH_4/day, Table 4.2). These results during the acclimation period demonstrated that the inoculum used in each bioreactor presented similar methanogenic activity and stability which set the starting reference for the evaluation of different packing materials in the biotransformation of NP under continuous conditions.

Table 4.3. Variables measured during continuous operation of bioreactors treating 4-nitrophenol (NP).

SAMPLE	PERIOD	pH	COD (g/L)	COD removal*	Biogas-CH_4 (mL CH_4/d)	Ratio CH_4:CO_2 %
INFLUENT	*A1*	8.75 +/- 0.09	1.99 +/- 0.05			
	A2	8.7 +/- 0.04	1.37 +/- 0.03			
	I	8.69 +/- 0.06	1.47 +/- 0.03			
	II	8.73 +/- 0.11	0.21 +/- 0.07			
	III	8.39 +/- 0.13	0.13 +/- 0.01			
	IV	8.52 +/- 0.05	0.13 +/- 0.01			
CONTROL	*A1*	8.13 +/- 0.05	0.20 +/- 0.02	90.2 +/- 1.0	592 +/- 24	81:19 +/- 0
	A2	8.19 +/- 0.06	0.16 +/- 0.00	88.3 +/- 0.2	504 +/- 45	83:17 +/- 2
	I	8.21 +/- 0.08	0.35 +/- 0.11	76.2 +/- 7.6	498 +/- 78	83:17 +/- 1
	II	8.61 +/- 0.09	0.22 +/- 0.05	-8.3 +/- 17.2	ND	ND
	III	8.32 +/- 0.14	0.15 +/- 0.00	-20 +/- 9.5	ND	ND
	IV	8.44 +/- 0.06	0.14 +/- 0.02	-14.9 +/- 8.7	ND	ND
AW	*A1*	8.12 +/- 0.04	0.21 +/- 0.06	89.6 +/- 3.0	673 +/- 37	80:20 +/- 1
	A2	8.17 +/- 0.04	0.22 +/- 0.00	83.8 +/- 0.3	609 +/- 63	82:18 +/- 2
	I	8.17 +/- 0.05	0.39 +/- 0.21	73.3 +/- 13.7	484 +/- 99	82:18 +/- 1
	II	8.58 +/- 0.10	0.20 +/- 0.06	2.2 +/- 15.2	ND	ND
	III	8.35 +/- 0.17	0.11 +/- 0.01	10.3 +/- 11.4	ND	ND
	IV	8.45 +/- 0.06	0.11 +/- 0.01	10.8 +/- 0.5	ND	ND
OX	*A1*	8.15 +/- 0.02	0.21 +/- 0.06	89.4 +/- 3.2	624 +/- 84	79:21 +/- 2
	A2	8.18 +/- 0-02	0.16 +/- 0.04	88.4 +/- 3.0	540 +/- 59	82:18 +/- 2
	I	8.16 +/- 0.02	0.33 +/- 0.11	77.5 +/- 6.9	543 +/- 49	82:18 +/- 1
	II	8.6 +/- 0.06	0.19 +/- 0.05	6.7 +/- 9.5	ND	ND
	III	8.38 +/- 0.13	0.12 +/- 0.00	4.6 +/- 7.9	ND	ND
	IV	8.45 +/- 0.07	0.13 +/- 0.02	0.0 +/- 10.6	ND	ND
AQDS	*A1*	8.20 +/- 0.02	0.21 +/- 0.07	89.4 +/- 3.6	567 +/- 90	80:20 +/- 2
	A2	8.18 +/- 0.02	0.15 +/- 0.00	89.3 +/- 0.3	603 +/- 63	84:16 +/- 3
	I	8.18 +/- 0.02	0.19 +/- 0.05	86.9 +/- 3.8	575 +/- 65	82:18 +/- 0
	II	8.54 +/- 0.14	0.18 +/- 0.07	11.5 +/- 21.2	ND	ND
	III	8.39 +/- 0.09	0.09 +/- 0.00	29.0 +/- 4.7	ND	ND
	IV	8.46 +/- 0.52	0.09 +/- 0.00	27.4 +/- 2.6	ND	ND

* COD removal = (influent COD-final COD)/influent COD*100

ND = Not Detected

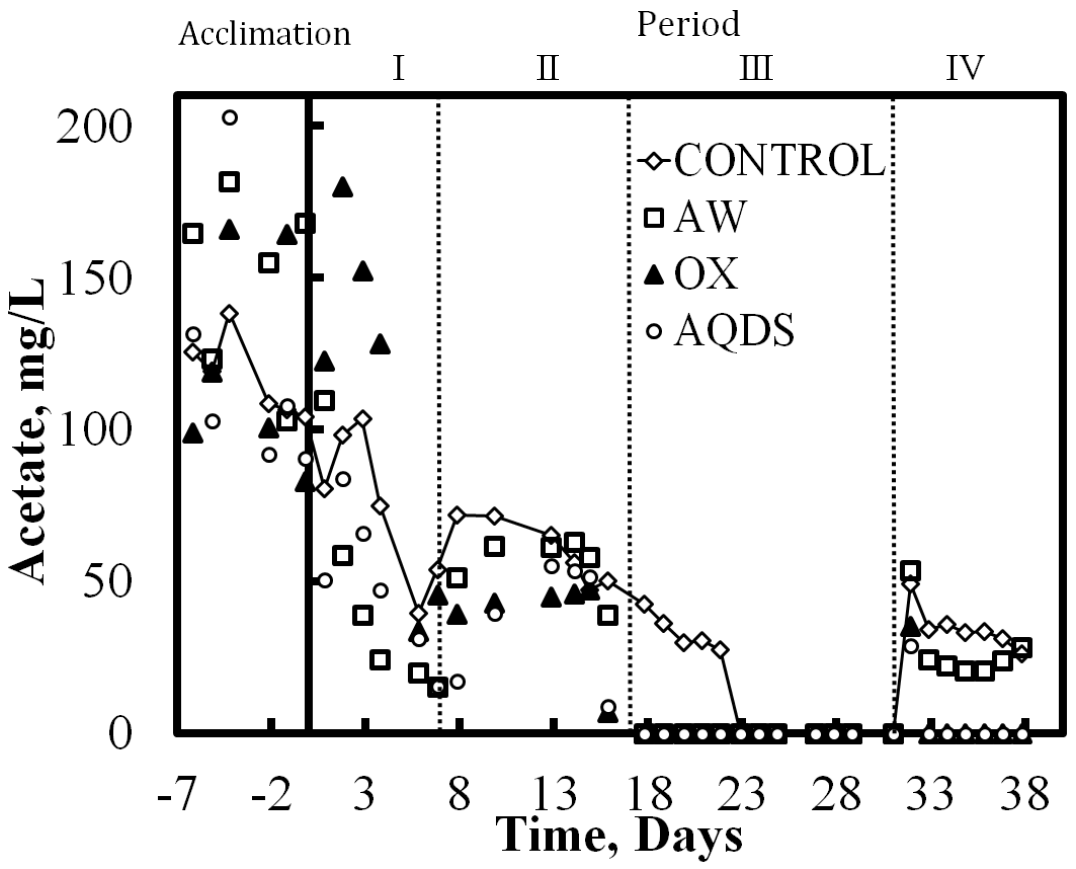

Figure 4.2. Acetate production from ethanol fermentation during the continuous biotransformation of NP to AP. Legend: CONTROL (diamonds and line), AW (squares), OX (triangles), and AQDS (circles).

4.3.2. Biotransformation of NP with excess of carbon source in Period I

At the end of acclimation period, ACF disks saturated with NP (see section 4.2.4) were inserted into the bioreactors in a process that took less than 20 min per bioreactor. Right after this process, NP (75 mg/L) and ethanol (697 mg/L) in basal mineral media were fed into the system (day 0). In the first day of Period I (Figure 4.3) we observed that the control reactor removed more NP (> 95%) than the reactors packed with AW, OX or AQDS (~ 80%). This result could be explained by desorption of NP due to the presence of other molecules competing for the same adsorption sites in ACF surface. In fact, the close up in Figure 4.4 shows that NP was biotransformed to AP from day 1. AP is an electropositive compound that

could be adsorbed more easily by electrostatic interactions with the negatively charged surfaces. According to Table 4.3 the average working pH of all bioreactors in Period I was 8.18 while the PZC (Table 4.1) of AW, OX and AQDS was below this value (5.16, 3.41 and 3.06, respectively). The PZC<pH means that ACF materials were negatively charged, especially OX and AQDS materials. In the first two days of NP biotransformation the adsorption of AP was greater in the bioreactors packed with electronegative materials in the order: OX>AW~AQDS>control (see Figure 4.4). AQDS materials were previously reported to suffer partial pore clogging and a reduction in surface area due to a chemical modification with $SOCl_2$ and the subsequent anchorage of anthraquinone molecules of projected diameter greater than 1 nm [13]. The partial pore clogging in AQDS materials might hinder the adsorption of AP (projected diameter of 6 Å) in the micropores of the material, resulting in a lower adsorption as compared with OX bioreactors. AP adsorption onto ACF surface might cause desorption of NP to the bulk solution due to surface charge modifications and/or adsorption site competitions. Around days 6 to 7 the detection of NP in the effluent of all bioreactors was negligible (> 98% removal) and the recovery of AP was >80% presumably because the bioreactors reached an equilibrium between the NP adsorbed/desorbed, the biotransformation of NP by the anaerobic consortium to AP, and the adsorption of other products such as acetate ions and AP on ACF surface.

It has to be noted that the similar efficiencies in the removal of NP between un-packed and packed bioreactors at the end of Period I (> 98% removal) are better explained by an excess in the concentration of ethanol or reducing equivalents. According to equations 1 to 4, 1.5 mol of ethanol are required to reduce 1 mol of NP. Therefore, we needed to provide only 37.2 mg/L of ethanol (0.81 mmol/L ethanol) in the feed stream in order to reduce the 75 mg/L (0.54 mmol/L) of NP entering the bioreactors. Nonetheless, the use of an excess of carbon source in Period I showed that NP was being reduced without changes in the variables of operation (Table 4.3) as compared to the acclimation period. These results

demonstrated the stability of the anaerobic bioreactors masked the effect of the chemical properties of ACFs in the biotransformation of NP.

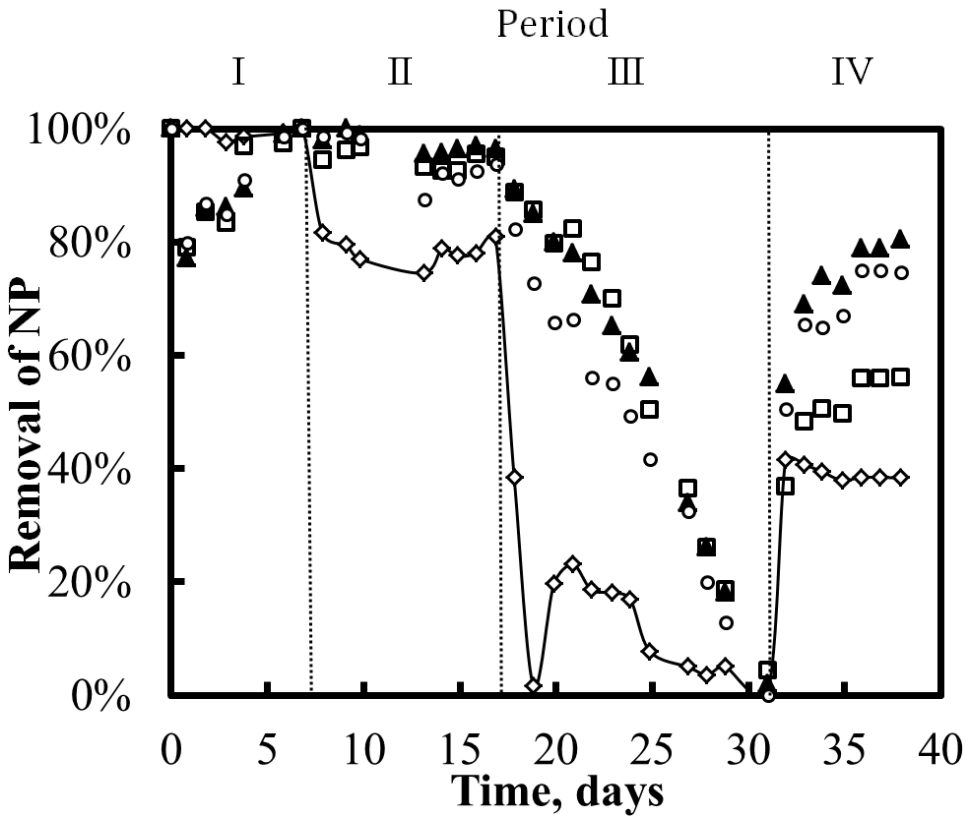

Figure 4.3. Removal efficiency of NP as calculated by (NP_{feed}-$NP_{effluent}$)/NP_{feed} × 100. Legend: CONTROL (diamonds and line), AW (squares), OX (triangles), and AQDS (circles).

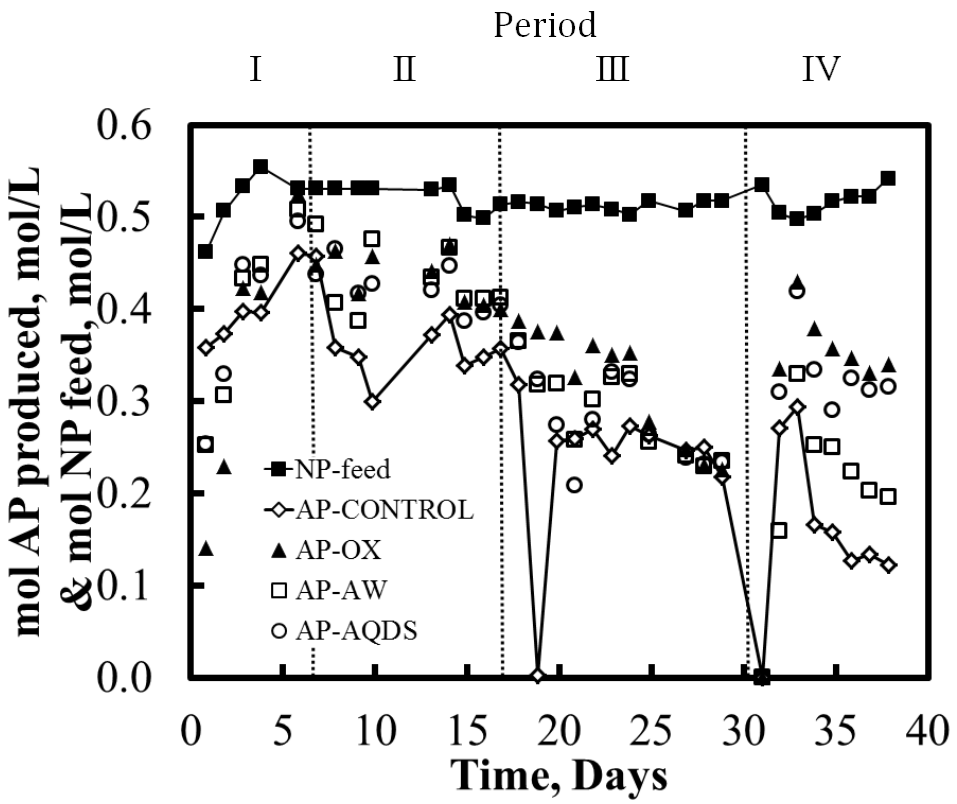

Figure 4.4. Production of AP from the reduction of NP.

4.3.3. Improved efficiency of ACF-packed bioreactors in Period II

Although a high biotransformation of NP to AP was desired, we did not observed performance differences at the end of Period I between the un-packed (control) and packed UASB reactors. The 697 mg/L of ethanol used in Period I represented a 19 times excess in the reducing equivalents needed for NP reduction and for this reason the ethanol concentration was decreased to 41 mg/L (1.1 times excess) in Period II. As consequence of these changes, the pH rose in all reactors to values closer to the feed stream pH (Table 4.3). COD values in Table 4.3 exhibited little differences between influent and effluent of all bioreactors, resulting in low COD removals. This low COD removals could be explained by the reduction of NP (1.47 g COD/g NP) to a product (AP) with higher theoretical COD (2.44 g COD/g AP) and/or by residual organic matter desorbed from AGS and ACFs in Period I. Furthermore, the biogas production dropped suddenly in Period II and remained undetectable through the rest of the experiment (Periods III and IV). In Table 4.2 it

could be observed that the expected methane production per day in Period II was 40 mL CH_4/d. The lack in the detection of CH_4 in all bioreactors means that the reducing equivalents were channeled to the nitroaromatic reduction or the acetate production instead to the methanogenesis process. The latter statement is supported not only by the lack of CH_4 detection, but also by the high NP removal efficiencies observed in all bioreactors (> 81%) and the presence of acetate in the effluent of the bioreactors (see Figure 4.2). Other authors have also found that nitrobenzene reduction by anaerobic sludge and reduced graphene oxide (RGO)/anaerobic sludge systems was independent on methanogenesis, but dependent on acetogenesis [19]. It is shown in Figure 4.2 that the acetogenesis process was still active in Period II supporting our hypothesis that NP reduction was independent on methanogenesis, but dependent on acetogenesis. Lastly, the lack of CO_2 detection in the biogas could be explained by both their low production and its solubility in the basal mineral media.

The higher removal of ACF-packed bioreactors (> 94%) in comparison to the control reactor (~ 81%) was the main result of Period II. The similar removal efficiencies of AW, OX and AQDS bioreactors strongly suggested that ACF materials only served as biological support in Period II. Literature evidence about the use of activated carbons as biological supports sustain that: 1) adsorption properties of activated carbons allow it to buffer high concentrations of toxic substrate which would otherwise exceed the tolerance concentration of anaerobic bacteria [8, 9, 20]; 2) activated carbons promote biomass growth possibly by the presence of crevices, pores, and other surface irregularities that provide microbial attachment sites and offer protection from hydrodynamic shearing forces [8, 20]; and 3) adsorption properties of activated carbons increase the concentration of substrate at the liquid-solid interface promoting biological growth [20, 21]. The common feature of these studies is that they consider activated carbons as inert materials with adsorption properties that only support bacterial attachment.

In the field of carbon materials it is widely known that activated carbons can catalyze many reactions [22] depending on the type and concentration of functional

groups on the activated carbons. Many carbon materials exhibit electrochemical potentials of ca. 0.6-0.8 V [23], and the presence of redox active functionalities of the quinone-hydroquinone type couples are suggested to contribute to this electron accepting-donating (redox) behavior [24]. Several studies have taken advantage of the presence of quinone moieties at the surface of activated carbons and used such knowledge towards the improvement in the rates of transformation of several pollutants. Under abiotic conditions, it has been demonstrated that activated carbons could catalyze the reduction of a wide variety of xenobiotic compounds because of the presence of quinone functionalities [3, 12, 13, 25] or the high content of electron rich sites on their basal planes (π electrons) [26]. However, under biotic conditions the enhancement in the biotransformation of any recalcitrant pollutant might be influenced not only by the surface chemistry of the activated carbon, but also by the biofilm formation on the carbon surface.

Literature evidence about the use of activated carbons as RM in the biotransformation of environmental pollutants usually omits the role of the biofilm formation and their interaction with carbon surfaces. van der Zee et al., [3] proved that under abiotic conditions the granular activated carbon (GAC) accepted electrons from the Na_2S oxidation and shuttle them to the azo dye (acid orange 7) reducing it in the process. However, under biotic conditions they did not discussed that the mixture of GAC with the anaerobic biomass could promote the attachment of more cells than the control reactor (which lacked of GAC).The latter would improve the kinetics of biotransformation of azo dyes by increasing the cell mass concentration rather than the presence of quinones on GAC surface. Other studies that have attributed higher reduction rates in biological systems due to specific chemical properties of the activated carbons also lacked of any discussion on the biofilm over carbon surfaces [6, 10].

The adhesion of bacteria to surfaces, and their subsequent formation of biofilms, is rapid and is remarkably unaffected by the physical or chemical nature of the surface concerned [27]. Moreover, the reaction rates can be increased not only by increased cell mass concentration (biofilm formation) in the reactor [28], but also by

faster mass transfer because of a shorter diffusion distance of the exogenous carbon sources into the biofilm [29]. Although the present study did not evaluate the biofilm formation on ACF surface, the negative influence of biofilm in the blockage of redox functionalities was observed in Figure 4.3, because the bioreactors packed with different ACFs removed NP with similar efficiencies (~ 94-97% reduction). Similar results were reported by Ríos-del Toro et al [25] were the reduction rates for the azo dye methyl red were enhanced by 8-fold when using ACFs as biological support, but the biofilm greatly reduced its redox mediating capacity mainly due to diffusion restrictions and/or blockage of redox active functionalities in ACFs [25]. In order to disclose the role of ACF functional groups as RM in the biotransformation of NP further restrictions to the amount of carbon source were implemented aiming to decrease the negative effects of the biofilm on ACF functional groups.

4.3.4. Effect of extreme oligotrophic conditions in NP biotransformation (Period III)

Restrictions in the exogenous carbon source in Period III (0 g/L) allowed the depletion of endogenous substrates like acetate (see Figure 4.2) in all bioreactors and the concomitant reduction in the efficiency of removal of NP as observed in Figure 4.3. In order to biotransform NP, the control reactor relied only in endogenous substrates in AGS while UASB-packed reactors used both the endogenous substrates in AGS and additional substrates adsorbed on ACFs (e.g. acetate ions and/or cell lysed products). These differences in endogenous substrates might explain the higher efficiencies of UASB-packed reactors in the reduction of NP in Period III as compared with the control bioreactor.

The gradual decrease in NP reduction was coupled to a gradual decrease in the AP produced as shown in Figure 4.4. This phenomenon is similar to the NP desorption explained in Period I (see section 4.3.2). Since the endogenous substrates were being depleted in Period III, the reducing equivalents and the removal of NP decreased, leading to an increase in NP concentration and the competition with AP for the adsorption sites. The AP desorption in the control

reactor (which lacked of ACF) is quite unclear, but exist the possibility of AP desorption from the extracellular polymeric substances (EPS) present in the AGS. EPS vary in their composition, but the majority are polyanionic (electronegative) due to the presence of either uronic acids or ketal-linked pyruvate [30]. This property is important because it allows association of divalent cations such as Ca^{2+} and Mg^{2+}, which have been shown to cross-link with the polymer strands and provide greater binding force in a developed biofilm [31]. This ability of EPS to interact with electropositive molecules such as cations may allow AP adsorption in the first place. The lack of NP reduction in Period III created NP concentration gradients at the interface AGS surface-bulk liquid that promoted the gradual desorption of AP to the aqueous media, which is responsible for the AP detection observed in Figure 4.4.

At the end of Period III it was concluded that endogenous substrates in all bioreactors were consumed due to the stopped biological transformation of NP, while at the same time AGS and all ACF disks inside AW, OX and AQDS were saturated again with NP.

4.3.5. Effect of the surface chemistry of ACFs in NP biotransformation (Period IV)

After the extreme conditions achieved in Period III, the reactivation of resilient bacteria was promoted by the addition of a small amount of ethanol (20.5 mg/L) during Period IV. This low ethanol concentration corresponds to only 55 % of the ethanol required to reduce 75 mg/L of NP, assuming that only H_2 acts as an effective electron donor and acetate is not consumed by the anaerobic microorganisms. In oligotrophic environments, organic nutrients tend to associate with available surfaces, and to trigger local biofilm development [27]. Under these new conditions we observed marked differences in the removal of NP between the control and the ACF-packed bioreactors (see Figure 4.3). The efficiency in the removal of NP followed the order: OX>AQDS>AW>Control corresponding to 80, 75, 56 and 38 % of NP removal, respectively. In other words, the reactors packed with AW, AQDS and OX materials enhanced the removal of NP by 1.47-, 1.97- and

2.11-fold, respectively. This enhanced removal could not be explained by the adsorption capacities of ACFs at equilibrium concentration of 75 mg NP/L (AW>OX>AQDS of 160, 110, and 75 mg NP/g ACF, respectively) neither the surface area of the materials (AW>OX>AQDS of 1098, 1052, and 704 m^2/g ACF, respectively). As other authors have suggested, when carbons are used as catalyst, as evidenced by the effectiveness of materials with both very low (< 1 m^2/g) and very high surface area (> 1000 m^2/g), the proton- and/or electron-transfer properties are more important [32]. Such proton- and/or electron-transfer properties could improve if redox active functionalities such as pyrones [33, 34], chromenes [34] and/or carbonyl (quinone) groups [24] are present in ACF surface. According to Table 4.1 the higher content of lactonic, carbonyl and total basic sites (pyrones, chromenes, and other non-specific groups [34]) in the order OX>AQDS>AW was correlated with the higher removal of NP observed in the packed bioreactors (OX>AQDS>AW). In other words, these functional groups could act as RM in the transfer of electrons from the anaerobic consumption of ethanol by bacteria to the final electron acceptor NP. In spite of the fact that lactones have been proposed to undergo one-electron transfer process in acidic solutions, in basic solutions (pH>7) lactone rings would open and carboxyl groups would be formed making these functional groups unlikely to act as RM [12, 24].

The microorganisms capable of using quinone moieties as terminal electron acceptors for the oxidation of several ecologically significant substrates are called quinone-respiring bacteria [35]. The evidence in Period IV showed that bioreactors packed with materials with high concentration of electroactive sites on their surface (OX>AQDS>AW) contributed to a greater extent to enhance the removal of NP to AP, suggesting the presence of quinone-respiring microorganisms. It is also interesting to note that the NP removal in OX and AQDS bioreactors (80 and 75 %, respectively) was higher than the expected NP biotransformation by 20.5 mg/L of ethanol (55 % reduction) in Period IV. These results indicated that H_2 is not the only electron donor utilized for the nitro group reduction (see equations 1-4), but also acetate is being metabolized and providing reducing equivalents for NP

reduction. The fermentation of 20.5 mg/L of ethanol in equation 4 will generate 26.3 mg/L of acetate at the effluent of all bioreactors in Period IV. However, in Figure 4.2 only the control and AW bioreactors showed an effluent concentration of 33.6 and 20.5 mg/L of acetate, respectively, while in OX and AQDS the acetate was not detected, indicating their consumption and use as an additional electron donor. The exact mechanism of electron transfer from the bacteria to the ACF surface, and from ACF surface to NP is not clear. However, several mechanisms for bacteria-surface respiration in microbial fuel cells (MFCs) have been proposed by Lovley [36]: a) direct electron transfer via outer-surface c-type cytochromes, b) long-range electron transfer via microbial nanowires, c) electron flow through a conductive biofilm matrix containing cytochromes, and d) soluble electron shuttles. Taken as a whole, the results from Period IV offer evidence of an improved removal of NP with the use of ACFs with electroactive-rich functionalities and low concentrations of carbon source. The biofilm developed in those ACFs probably changed their morphology and activity with each carbon source restriction from Period I to IV. For all these reasons, further microbiological studies are needed in order to provide evidence of biofilm formation, morphology and/or enrichment with quinone-respiring respiring bacteria.

Conclusive results about the role of specific functional groups are (as in any carbonaceous system) open to question [22]. The surface groups over any activated carbon do not behave as classical functions of organic chemistry because the reactivity of the molecule (e.g. quinone groups) could change depending on the molecular environment and the individual reaction. Quoting the opinion of D.L. Trimm about surface complexes over carbon “it seemed better to consider them as combined structures presenting numerous mesomeric forms largely favored by their location on the polyaromatic frame” [22]. Considering this information, in this study one could conclude that there is a correlation between the observed effect (enhanced removal of NP) and the presence of specific redox active functional groups (i.e. carbonyl or quinone groups) on the surface of ACFs, only if the biofilm formed onto ACFs is modified by carbon source restrictions. There are more

factors involved in the biotransformation of recalcitrant pollutants using activated carbons as RMs, but their study in such complex systems will be subject of future research.

4.4. Conclusions

Ethanol (exogenous carbon source) degradation by the anaerobic granular sludge provided the electrons and protons necessary for the methanogenesis process in the acclimation period. In the first experimental stage (Period I) the reducing conditions in all bioreactors allowed the biotransformation of NP to AP with high efficiencies (> 98%). The first carbon source restriction in Period II resulted in enhanced NP removal in all ACF-packed reactors (> 94%) in comparison to the control reactor (~ 81%), but hindered the redox mediating capacity of the ACFs with different quinone concentrations. Period II also showed that NP removal occurred at expenses of the methanogenesis process, meaning that the electrons equivalents were channeled to the nitro-group reduction. The total privation of exogenous carbon source in Period III ended up with the lack of NP removal due to the total consumption of endogenous substrates, while at the same time all bioreactors were saturated with NP. Finally, the small amount of ethanol fed in Period IV demonstrated that the surface chemistry of the ACF materials improved the removal of NP under continuous conditions. The materials with higher concentration of electroactive functionalities exhibited a correlation with the higher removal of NP in the order OX > AQDS > AW. The latter results showed that the anaerobic NP biotransformation was enhanced by 1.47-, 1.97- and 2.11-fold when the bioreactors were packed with OX, AQDS and AW materials, respectively. The results and discussion provided in this work strongly suggest the study and discussion of the biofilm formation over the carbon (or any other redox active materials) surfaces when they are used as redox mediators.

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CHAPTER 5.- PATHWAYS OF 2,4-DINITROANISOLE (DNAN) AND 2-METHOXY-5-NITROANILINE (MENA) BIOTRANSFORMATION IN CONTINUOUS FLOW-THROUGH ANAEROBIC SOIL COLUMNS

ABSTRACT

Insensitive munitions (IM's) like 2,4-dinitroanisole (DNAN) are being tested as replacement for 2,4,6-trinitrotoluene (TNT) due to its better resistance to unintended detonations due to shock impacts and elevated temperature. Little is known about the environmental fate and impact of IM's. The objective of this work was to study the biotransformation of DNAN and one of its biotransformation products, 2-methoxy-5-nitroaniline (MENA), in two flow-through anaerobic soil columns receiving different levels of organic carbon. The influent of "column 1" (C1) was amended with ethanol (100 mg/L) as exogenous electron donor, whereas "column 2" (C2) did not receive ethanol and therefore relied on endogenous substrates in soil organic matter as electron donor. The columns were operated initially with MENA (first 62 days) and subsequently DNAN (80 remaining days). Based on high performance liquid chromatography diode array detector (HPLC-DAD) detection MENA (150 μM) was converted in both columns. MENA was removed and 2,4-diaminoanisole (DAAN) was observed as an important intermediate in C1 with ethanol as an exogenous electron donor. In the C2 column with only endogenous substrates 2-amino-4-nitrophenol (ANP) was detected alongside some DAAN. The use of ultra-high pressure liquid chromatography-quadrupole time of flight mass spectrometry (UHPLC-QTOF-MS) confirmed the detection of ANP in C2, and also revealed a wide range of additional metabolites in both columns. The metabolites detected indicated nitro group reduction, O-demethylation and N-acetylation reactions as well as dimerization reactions, suggesting a tendency of the unstable intermediates, such as nitroso and aromatic

amino, to couple even in the absence of elemental oxygen. Ecotoxicity analyses using the Microtox™ bioassay evidenced that anaerobic treatment increased the inhibitory potential of the aqueous medium. The results taken as a whole indicate DNAN and MENA are readily reduced yielding reactive intermediates that undergo coupling reactions to yield toxic intermediates. Under conditions in which electron-donor is limiting there is a greater tendency for the microbial community to O-demethylate the methoxy group yielding phenols such as ANP.

5.1. Introduction

Defense industries worldwide are interested in the replacement of 2,4,6-trinitrotoluene as a munitions compound (TNT) because of its suspected toxicity and susceptibility to unintended detonations [1]. Insensitive munitions (IM's) are a new class of energetic materials that are less likely to explode if dropped, shot at or hit by an explosive during transport. The U.S. Army recently approved the use of IM's such as 2,4-dinitroanisole (DNAN) as replacement of TNT [2]. The increased production and use of compounds like DNAN also bring concerns about the environmental impact of wastewaters, accidental spills or residual from the incomplete detonation of explosives.

The electron withdrawing nature of the nitro groups [3] makes oxidation of polynitroaromatic compounds problematic. For this reason, the initial biotransformation usually involves the reduction of the nitro group. The biotransformation products of DNAN under both aerobic [4-8] and anaerobic [5, 8, 9] conditions typically leads to the formation of metabolites with reduced nitro groups. However, evidence of O-demethylation of DNAN followed by oxidative biodegradation of 2,4-dinitrophenol has also recently been reported by an aerobic bacterium isolated from a munitions wastewater treatment plant [6]. Also, aerobic batch experiments of the biotransformation of DNAN in artificially contaminated soil microcosms demonstrated that the reduction of at least one nitro group occur, leading to the major end-product 2-amino-4-nitroanisole (or 2-methoxy-5-nitroaniline, MENA) [4]. Batch experiments with anaerobic sludge on the other hand have demonstrated a rapid biotransformation of DNAN to MENA and 2,4-

diaminoanisole (DAAN) using H_2 as an electron donating substrate [5]. Hawari et al. [8] also reported that reduction of DNAN with either zero valent iron or bacteria regioselectively produced MENA which, under strict anaerobic conditions formed DAAN. Platten et al. [9, 10] reported the effective use of anaerobic fluidized bed bioreactors (AFBB) seeded with anaerobic digester biomass in the biotransformation of DNAN to DAAN, using ethanol as carbon source. The results indicate that anaerobic mixed cultures readily reduce DNAN to its aromatic amine counterparts. If released into the environment, DNAN will be in contact with soil and subjected to aerobic and anaerobic biotransformation processes. The anaerobic biotransformation of DNAN in soil under continuous flow (similar to the conditions found in environment) has not been reported and their study is important to understand to what extent biotransformation of DNAN will take place in the field as dissolved DNAN leaches through the soil.

The present study evaluates the anaerobic biotransformation of DNAN and its main biotransformation product, MENA, in soil under continuous flow through conditions. MENA or DNAN aqueous solutions were fed to soil columns to imitate the seepage of these contaminants through saturated soil. One soil column was continuously supplied with ethanol as an electron donating substrate (column 1, C1); whereas a second column relied solely on endogenous substrates (column 2, C2). High performance liquid chromatography diode array detector (HPLC-DAD) and ultra-high pressure liquid chromatography - quadrupole time of flight - mass spectrometry (UHPLC-QTOF-MS) was used to provide evidence of the main metabolites produced inside the soil columns.

5.2 Methods

5.2.1 Chemicals

DAAN (99.4%) and MENA (98%) were purchased from Sigma-Aldrich (St. Louis, MO, USA). DNAN (98%) was obtained from Alfa Aesar (Ward Hill, MA, USA). Chemical structures are shown in Figure 5.1.

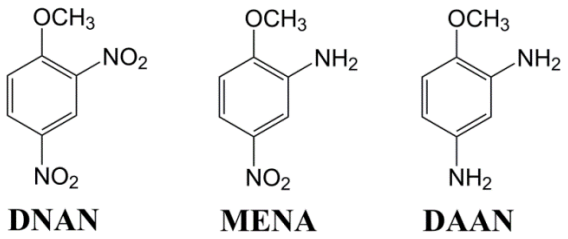

Figure 5.1. Molecular structure of the compounds DNAN, and two common reductive biotransformation products, MENA and DAAN, used in this study.

5.2.2 Anaerobic soil-columns for the biotransformation of DNAN and MENA

The anaerobic soil column experiments (see Figure 5.2) were carried out in 400 mL columns packed with a mixture of 150 mL (157 g) of North Carolina Camp Butner Soil and 150 mL of Colorado Sand (260 g), in order to facilitate permeability.

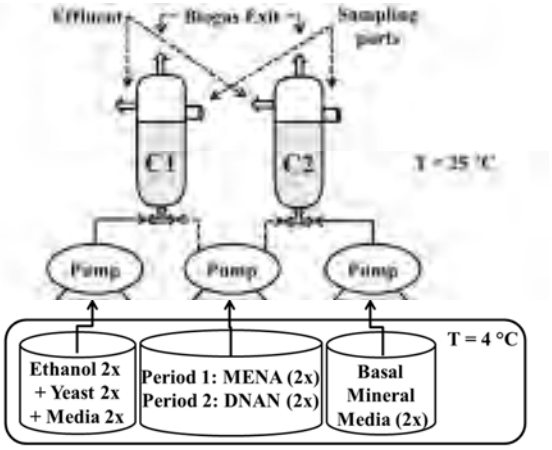

Figure 5.2. Graphic representation of the anaerobic columns 1 (C1) and 2 (C2) used in this study. The fill in columns represents the soil from Camp Butner (North Carolina, USA) (soil composition = sandy loam (68.7:19.8:11.5 sand:silt:clay) with 20.7 g organic carbon/kg; composed 2.6% of secondary minerals and contains 0.7 g oxalic acid extractable Fe/kg).

The columns were continuously fed with basal media containing 0.15 mM of MENA for the first period (62 days) and 0.075 mM of DNAN during a second period (following 80 days of operation). The empty bed hydraulic retention time of the

columns was 28 h. C1 feeding solution contained ethanol as an exogenous electron-donating substrate that the microorganisms in soil could use according to the reaction: $CH_3CH_2OH + H_2O \rightarrow CH_3COO^- + 2H_2 + H^+$ [11]. The H_2 produced was expected to provide the necessary electron equivalents ($H_2 \rightarrow 2H^+ + 2e^-$) required to reduce the nitro groups in the MENA and DNAN following the reaction:

$R\text{-}NO_2 + 6e^- + 6H^+ \rightarrow R\text{-}NH_2 + 2H_2O$ [12, 13]

where "R" represents the rest of the molecule. C2 did not receive ethanol and consequently relied on endogenous organic matter in the soil to supply electron donating equivalents.

The basal mineral media and the nitroaromatic compound solution (concentrated 2×) were placed in different containers and mixed before they entered the columns as shown in Figure 5.2. The 1× concentration of the basal mineral medium is as follows (mg/L): Na_2HPO_4 (10), $CaCl_2{\cdot}2H_2O$ (15), $MgSO_4{\cdot}7H_2O$ (15), NH_4Cl (15), $NaHCO_3$ (400) and 1 mL/L of trace elements. The composition of the trace elements was (mg/L): H_3BO_3 (50), $FeCl_2{\cdot}4H_2O$ (2000), $ZnCl_2$ (50), $MnCl_2{\cdot}4H_2O$ (50), $(NH_4)Mo_7O_{24}{\cdot}4H_2O$ (50), $AlCl_3{\cdot}6H_2O$ (90), $CoCl_2{\cdot}6H_2O$ (2000), $NiCl_2{\cdot}6H_2O$ (50), $CuCl_2{\cdot}2H_2O$ (30), $NaSeO_3{\cdot}5H_2O$ (100), EDTA (1000), resazurin (2000), 36% HCl (1 mL). The final pH of the basal media was adjusted to 7.2 with HCl. The concentrated nitroaromatic solutions (2×) containing 0.3 mM MENA and 0.15 mM DNAN were prepared using ultrapure water (NANOpure Infinity™, Barnstead International, Dubuque, IA). In the case of the C1, 200 mg/L of ethanol and 10 mg/L of yeast extract (YE) were added to the concentrated basal media (2×), so that the concentration after mixture with the nitroaromatic solution would be 100 and 5 mg/L of ethanol and YE, respectively. The ethanol added was supplied in about a 10-fold excess of the amount needed to fully reduce MENA or DNAN to their corresponding aromatic amines (10.3 mg/L). In the case of C2, the mineral medium fed to the column was similar to C1 but it lacked both ethanol and YE in the feeding solution.

5.2.3 Analytical

5.2.3.1 Gas Chromatography for ethanol and volatile fatty acid analysis

The presence of ethanol and several volatile fatty acids (VFA) such as acetate, propionate and butyrate were analyzed using an Agilent Technologies 7890 GC system (Santa Clara, CA, USA), with a fused silica Stabilwax®-DA column (30 m × 530 µm × 0.25 µm, Restek, State College, PA, USA). The carrier gas was helium, 5.2 mL/min, and samples were measured in a flame-ionization detector (FID). Injector and detector temperatures were both at 280 °C. To achieve a better separation of the VFAs the following temperature program was used: 105 °C (2 min), gradient 105-150 °C (8 °C/min), followed by another gradient 150-180 °C (15 °C/min). An autosampler was used for injecting samples of 1 µL. The retention times for ethanol, acetate, propionate, and butyrate were 1.4, 3.6, 4.16, and 5.36 min, respectively.

5.2.3.2 Metabolite detection by High Performance Liquid Chromatography-Diode Array Detector (HPLC-DAD)

The products of the reduction of DNAN and MENA in liquid phase were routinely analyzed using an Agilent 1290 series (Santa Clara, CA) HPLC-DAD. 20 µL samples were separated using an E1 Acclaim Explosives column (4.6 mm × 240 mm, 5 µm; Dionex, Salt Lake City, UT) and a mobile phase of water:acetonitrile (55:45 v/v) at 40 °C. The separation was run isocratically at a flow rate of 1 mL/min for 12 min. The detector was set to scan for wavelengths of 210, 230, 254, 260, and 400 nm. Detection of DNAN (retention time (rt) = 10.7 min), and MENA (rt = 7 min) was performed at 254 nm, while DAAN (rt = 16 min) was detected at 210 nm. Ascorbic acid (AA) was added to the samples in the second half of period 2 as an antioxidant agent. An aliquot of 1 mL of ascorbic acid 1000 mg/L was added inside a 5 mL syringe (free of air), and then 4 mL of sample were pulled out the column and mixed with the ascorbic acid (final concentration = 200 mg/L). The time expended between the preparation of the sample (1 mL) and the injection of 20 µL into the HPLC column did not exceeded 2 minutes in all cases.

5.2.3.3 Metabolite detection by Ultra High Pressure Liquid Chromatography-Quadrupole Time of Flight-Mass Spectrometry (UHPLC-QTOF-MS)

High resolution full scan mass spectra were obtained in two steps: 1) separation of 20 µL sample in an Ultimate 3000 UHPLC from Dionex-Thermo, using a E1 Acclaim Explosives column (4.6 mm × 240 mm, 5 µm; Dionex, Salt Lake City, UT) with a mobile phase of water:acetonitrile (55:45 v/v) at a flow of 1 mL/min and 40 °C, and 2) infusion into a TripleTOF^{TM} 5600 quadrupole TOFMS (AB Sciex, Framingham, MA) equipped with Electro Spray Ionization (ESI) source kept at 700 °C. Spectra were obtained in ESI positive ion mode with a capillarity setting of 5.5 kV and declustering potential of 50 V. Curtain gas, desolvation gas, and nebulizer gas levels were kept at 30, 35, and 35 psi, respectively, with nitrogen. The spectral data was processed using the Analyst TF 1.5.1 software application and the MultiQuant tool, while the molecular formulae was identified using the Formula Finder 2.0.2.0.

5.2.4. Microbial inhibition bioassays

Aqueous samples were taken in both the influent and effluent of the soil columns and subjected to the luminescent bacterium *Aliivibrio fischeri* (Lot No. 13F4067A, Modern Water Inc., New Castle, DE, USA) to determine their ecotoxicological potential. The procedure was conducted in a Microtox ® model 500 analyzer (Strategic Diagnostics, Inc. SDIX, Newark, DE, USA), and the experimental details can be found elsewhere [14]. Additional sample solutions tested included MENA 0.05 mM.

5.3 Results and Discussion

5.3.1 Removal of ethanol and effluent VFA concentration

Ethanol was metabolized via VFA to generate electron donor for the nitro-group reduction. The ethanol consumption (A), total VFA (B) and each individual VFA species (acetate (C), propionate (D) and butyrate (E)) in the effluent of C1 are

shown in Figure 5.3. Over the first 20 days, microorganisms enriched to the anaerobic conversion of ethanol to acetate which became a stoichiometric conversion. At that time acetate was the main VFA detected. Between days 27-30 there was an inadvertent 10-fold increase in the supplied ethanol concentration. As a consequence, the concentration of acetate, butyrate and ethanol in the effluent of C1 increased with a parallel decrease in the effluent pH (Figure 5.4). Immediately after correcting this problem, acetate, butyrate and ethanol in the effluent progressively declined. Ethanol and butyrate declined below 0.5 mM each by day 45. Acetate levels dropped below 0.5 mM by day 80. Soon after the organic overloading, the pH recovered to the normal value (pH ~ 7). After day 80, the concentration of ethanol and butyrate reached values below the detection limit, and acetate was only detected on a few events at relatively low concentrations, indicating that the VFAs after day 80 were largely consumed. There is evidence in the literature that anaerobic mixed communities use ethanol as a carbon source and produced CH_4 as the final metabolite [9]. However, anaerobic production of CH_4 is a complex process involving many classes of bacteria and several intermediate steps [15]. Methanogens required to produce CH_4 are not expected to be naturally enriched in upland soils, therefore several months were probably required to achieve an enrichment of methanogens needed to remove VFA in the C1. Methane production was not measured in C1 or C2 probably due to the low concentration of substrate involved yielding in C1 maximally (< 30 cm^3/day). Inhibition of methanogenic archeas by nitroaromatic compounds is known [16, 17]; however, as will be discussed, the nitroaromatics were readily reduced to less toxic aromatic amines.

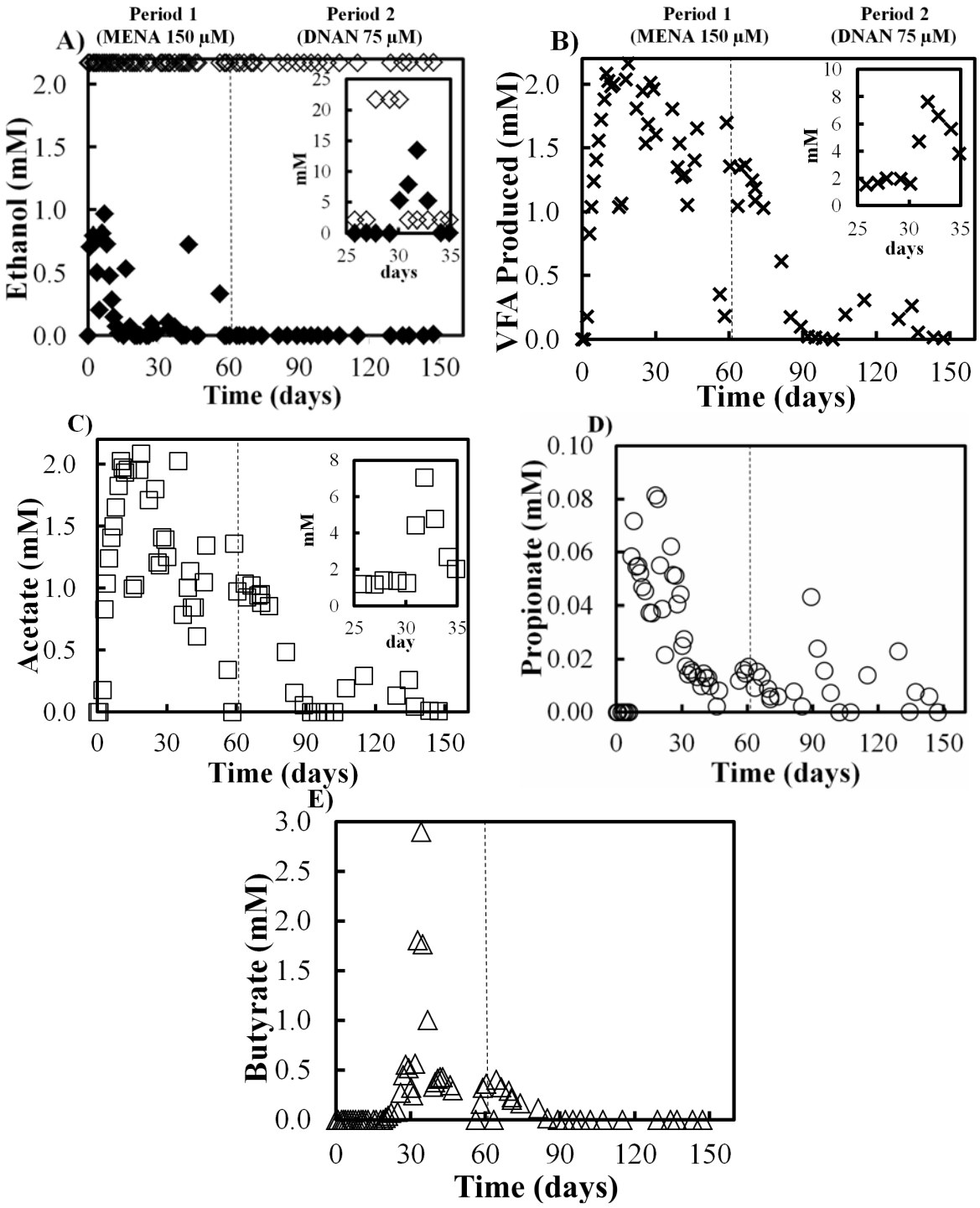

Figure 5.3. Ethanol (A), total VFA (B) and individual VFA (C,D and E) concentrations in Column 1. Legend in A: ethanol concentrations in the influent (open diamond) and effluent (filled diamond); Note during days 27-30, the influent ethanol concentration was 10-fold higher than normal.

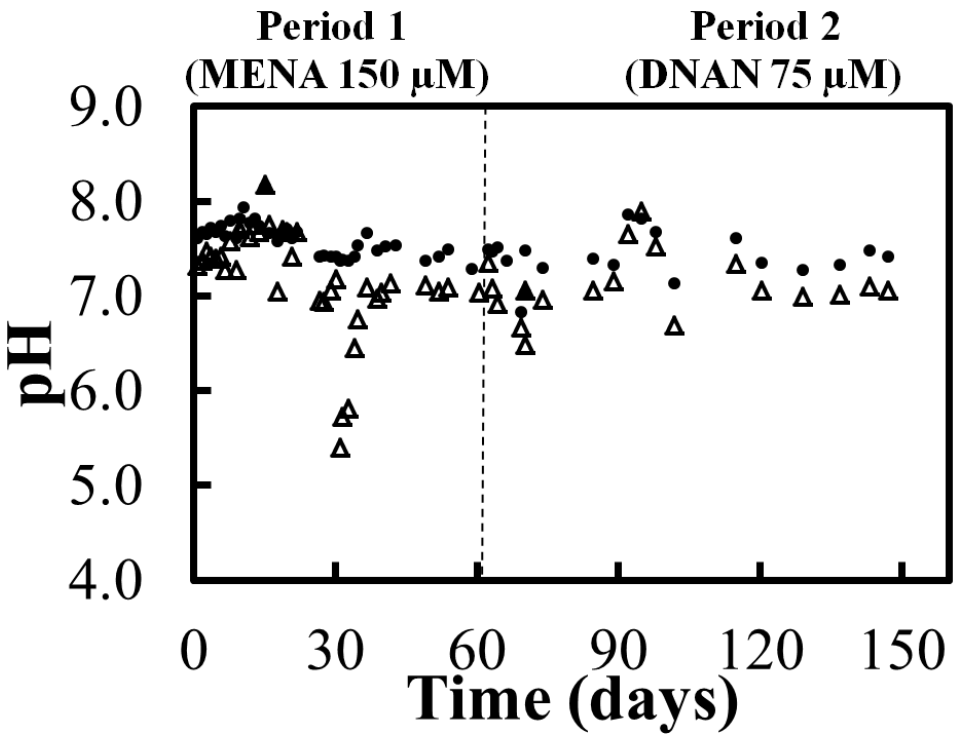

Figure 5.4. Variation of the effluent pH in Columns 1 (circles) and 2 (triangles).

In the case of C2, the VFA production was very low compared to C1 due to the lack of ethanol and YE in the feed (Figure 5.5). Nonetheless during the first 60 days, acetate and to a lesser extent propionate was detectable in the range of 0.02 to 0.12 mM, indicating fermentation of endogenous substrates in soil. Recent reports indicate that propionate and acetate are important products of organic matter (OM) fermentation in anoxic environments [18]. Degradation is initiated by hydrolysis of polymers (e.g. OM) to oligomers and then to monomers, which become fermentatively degraded to CO_2, H_2 and acetate, as well as other simple carbon compounds such as fatty acids (e.g. propionate) and alcohols [18]. Such metabolites could be used as an energy source in C2 for the biotransformation of MENA and DNAN (natural attenuation).

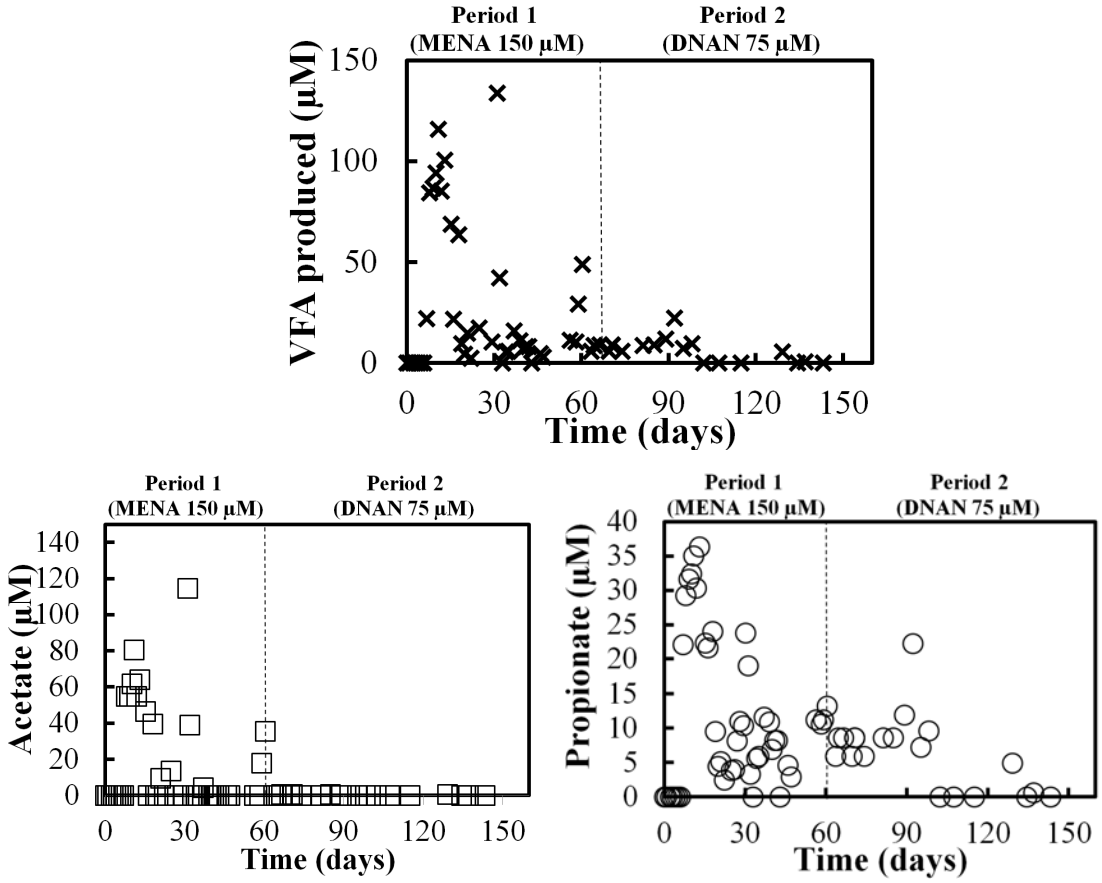

Figure 5.5. Total VFA produced (top graph) and individual VFA concentration (acetate and propionate) in the effluent of Column 2.

5.3.2 Soil column biotransformation of MENA and DNAN

The results in the section 5.3.1 evidenced that the anaerobic columns we studied exhibit microbial activity because the consumption of ethanol in C1 and the production of VFAs in both C1 and C2. The addition of a carbon source to C1 showed noticeably differences in the pathway of biotransformation of MENA and DNAN, mainly in the type and amount of reduced metabolites detected by HPLC-DAD. The results in Figure 5.6A and 5.6B show the biotransformation of nitroaromatic compounds in the soil columns with and without added ethanol, C1 and C2, respectively. At the start of the experiment, MENA was fed to the columns and it was initially detectable in the effluent, peaking around day 5 to 10 and thereafter declining rapidly or slowly in columns C1 and C2, respectively. MENA was clearly transformed since 150 µM of this compound in the influent was

decreased to 30 to 45 μM at the peak effluent MENA concentrations. The effluent MENA concentrations decreased further to 0 and 5 μM by the end of period 1, corresponding to MENA removal efficiencies of 100 and 97% in C1 and C2, respectively. The better removal of MENA in C1 results can be attributed to the conversion of ethanol, generating electron donating intermediates like H_2 that could foster nitro-group reduction of MENA. Similarly, it has been proven that anaerobic biotransformation of DNAN to MENA and/or DAAN readily occur using ethanol fermentation to H_2 [9], or H_2 alone as electron donor [5]. The detection of the reduced compound DAAN was better under the best reducing conditions achieved in C1. These results are in agreement with other studies where the detection of DAAN was achieved under strictly anaerobic conditions [5, 8, 9].

In the case of C2 (Figure 5.6B), reduction of MENA occurred albeit at a slower rate compared to C1. This suggested the participation of endogenous substrates generated in the breakdown of OM by native microorganisms present in soil [18]. These endogenous substrates could serve as a source of energy for microorganisms that helped the biotransformation of MENA to DAAN (detected on a few occasions in C2 during period 1). However, the HPLC-DAD results indicated that MENA was biotransformed to other products. The use of the UHPLC-QTOF-MS confirmed the presence of the new metabolite 2-amino-4-nitrophenol (ANP, *m/z* = 155.0451, see Table 5.1 and compound 6 in Figure 5.7) after day 25 (period 1). The ANP was a product of the O-demethylation of MENA, and was amendable to analysis with the HPLC-DAD by using the calibration curve of MENA, due to the strong similarity of their UV-vis spectra (Figure 5.8). The retention time of this compound was 4.7 min and the maximum absorption peak was at 254 nm.

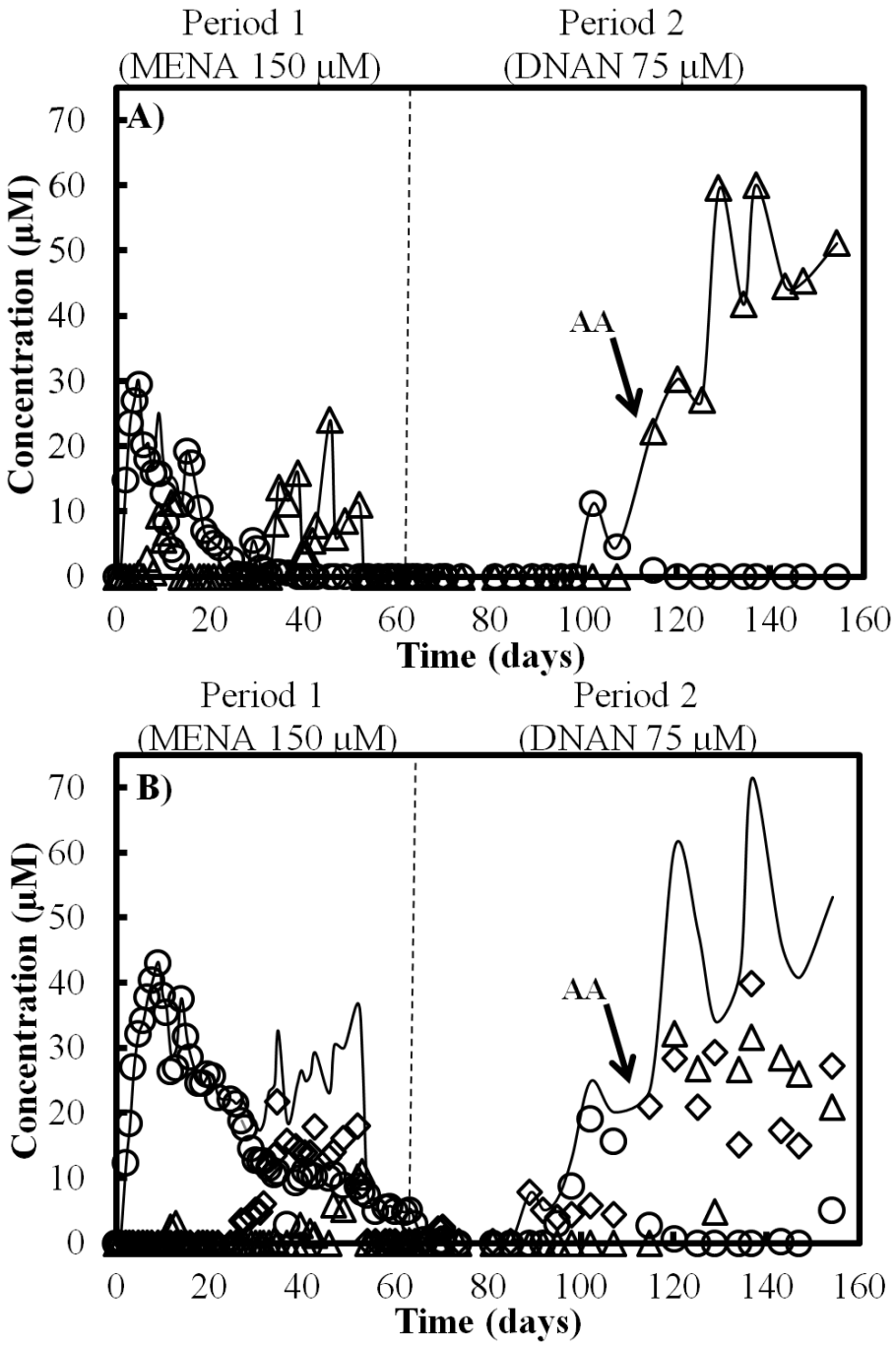

Figure 5.6. MENA, DNAN and biotransformation products detected in the effluent of Columns 1 (panel A) and 2 (panel B) after reduction of the nitroaromatic compounds MENA (open circles) and DNAN (not ever detected in effluent). Additional biotransformation products in the effluent detected by HPLC-DAD were DAAN (open triangles) and ANP (open diamonds). The mass balance is represented as a continuous line and stands for the sum of DAAN, ANP, and MENA in the effluent. AA indicated the start of the use of ascorbic acid in the effluent samples.

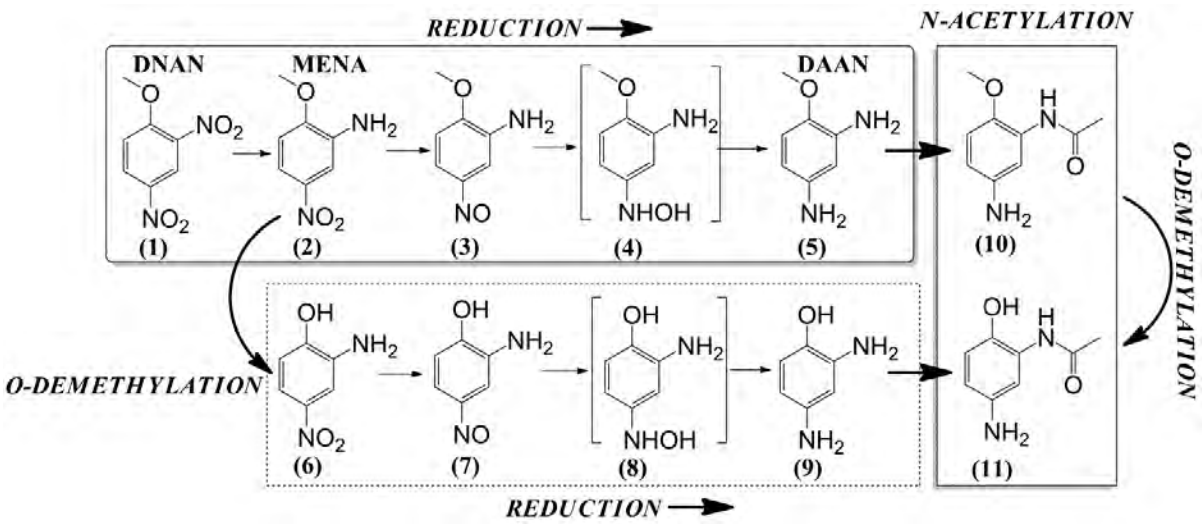

Figure 5.7. Monomers detected in the effluent of Columns 1 and 2 by UHPLC-QTOF-MS, and the main metabolic routes proposed. The compounds in parenthesis were not detected.

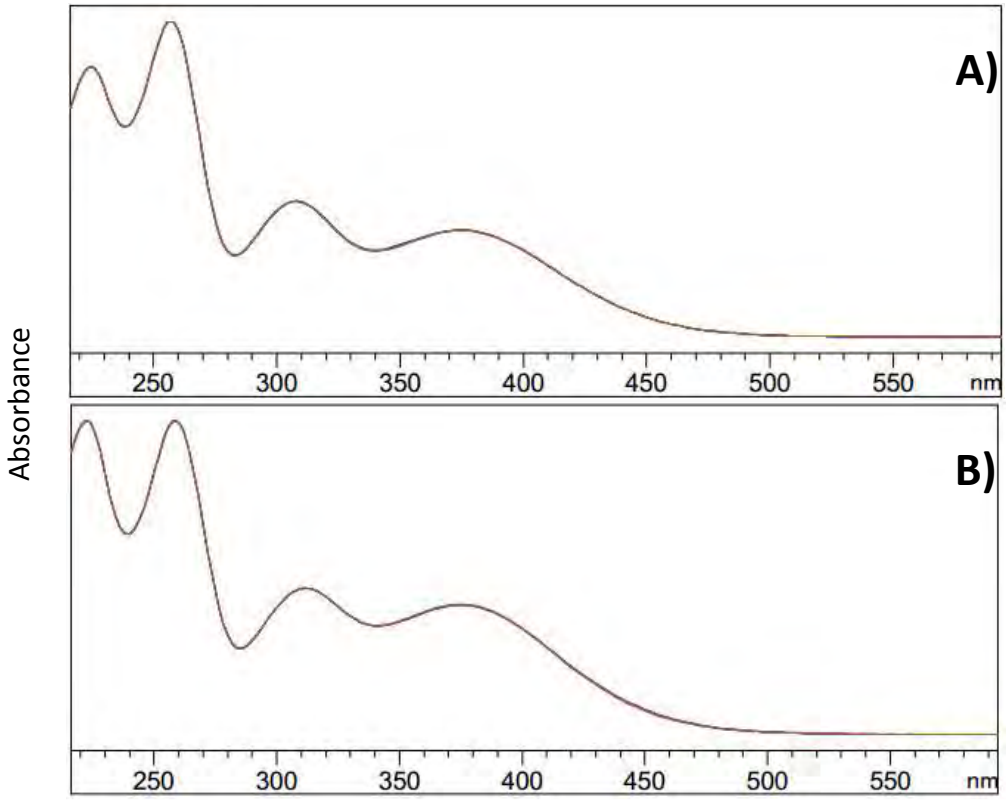

Figure 5.8. Similarities in the UV-vis spectra of A) MENA and B) ANP.

At around the time the nitroaromatic compound in the feeding solution shifted from MENA to DNAN (at the start of period 2), DNAN was readily reduced by the microbial community in the columns, since DNAN was not detected in all effluent samples (Figures 5.6A and 5.6B). Likewise, in this time period there was seldom any detection of reduced metabolites such as MENA or DAAN. Likewise in this time period there was seldom any detection of reduced metabolites such as MENA or DAAN. The difference between the DNAN fed (75 μM) and the sum of metabolites recovered in the effluent solution (DAAN, MENA, ANP) resulted in very poor mass balances as indicated by the continuous lines in Figure 5.6. The difficulty to detect aromatic amine compounds was suspected to be related to their autoxidation with air as several authors have reported with a variety of aromatic amines [3, 9, 19, 20]. Likewise the recovery of aromatic amines was problematic with an older HPLC column. To improve the measurement of aromatic amines, starting on day 115, a new HPLC column was installed and we started to routinely recover effluent samples from both columns directly from the anaerobic zone with an aliquot of ascorbic acid used as antioxidant to prevent autoxidation of samples (labeled “AA” in Figure 5.6) [3, 17, 20]. The recovery of DAAN in the samples of C1 (from day 115 onwards) improved after the use of ascorbic acid by 57 (±18 %). DAAN and ANP recoveries from C2 were 33 % (±12 %) and 32 % (±12 %), respectively. However, a complete mass balance was never achieved probably due to the formation of other metabolites not amendable to detection by the HPLC-DAD, or possible polymerization or irreversible binding of the metabolites such as DAAN to humic substances. Reduced metabolites of TNT, were shown to be adsorbed [21], or become incorporated into different soil fractions (humic acids, fulvic acids, and humin) [22], or form azo compounds [23] even under anaerobic conditions. This suggests that the reduced products of DNAN could also be retained or subjected to other unwanted transformations inside the anaerobic soil columns, which resulted in the incomplete mass balances observed in C1 (57 ±18 %) and C2 (66 ±16 %).

5.3.3 Metabolite detection in the biotransformation of MENA and DNAN by UHPLC-QTOF-MS

In addition to the biotransformation products of MENA and DNAN detected by HPLC-DAD, additional products were detected by UHPLC-QTOF-MS. Figure 5.7 show the suggested structures for monomeric metabolites detected by UHPLC-QTOF-MS in both soil columns (C1 and C2), whereas Figure 5.9 show the dimer and trimer structures. In Table 5.1 provides the measured and calculated molecular masses $[M+H]^+$ of the suggested structures in Figures 5.7 and 5.9. The absolute mass of the suggested structures differed from the measured mass by only -5.49 to 2.99 ppm. The fragmentation spectra and fragment losses for each compound in Table 5.1 were performed, but Figure 5.10 only showed DAAN as a representative example. At the beginning of period 1 (day 27) only some of the metabolites in Table 5.1 were detected. For instance, structures **2**, **5** (monomers), **12**, **13, 14** (dimers), and **19** (trimer) were detected at the effluent of C1, but only structure **6** ($[M+H]^+$ = 155.0451) was detected in C2 together with the metabolites found in C1. At the end of the experimental run (end of period 2, day 154) the number of metabolites detected increased to 15 and 16 in C1 and C2, respectively (structure **6** only in C2). Additionally, aromatic amines are susceptible to autoxidation upon exposure to air [9, 20, 24-26]. Thus there was concern that O_2 exposure during sampling and handling may influence the products detected. Previously the detection of a dimer compound during anaerobic treatment of DNAN was attributed to autoxidation [9]. To counteract possible polymerization during sampling and handling, we minimized the time between sample analysis (< 1 h) and added ascorbic acid to the samples. By taking all these cautions the samples at the end of period 2 with AA achieved a better signal-to-noise ratio in the detection of all the 16 compounds in both columns (25 and 20% improvement in C1 and C2, respectively), as observed in Figure 5.11. These results give some insights about the complexity in the biotransformation of MENA and DNAN when entering to the environment through soil seepage. An explanation about how the metabolites in Figures 5.7 and 5.9 were produced inside C1 and C2 was offered.

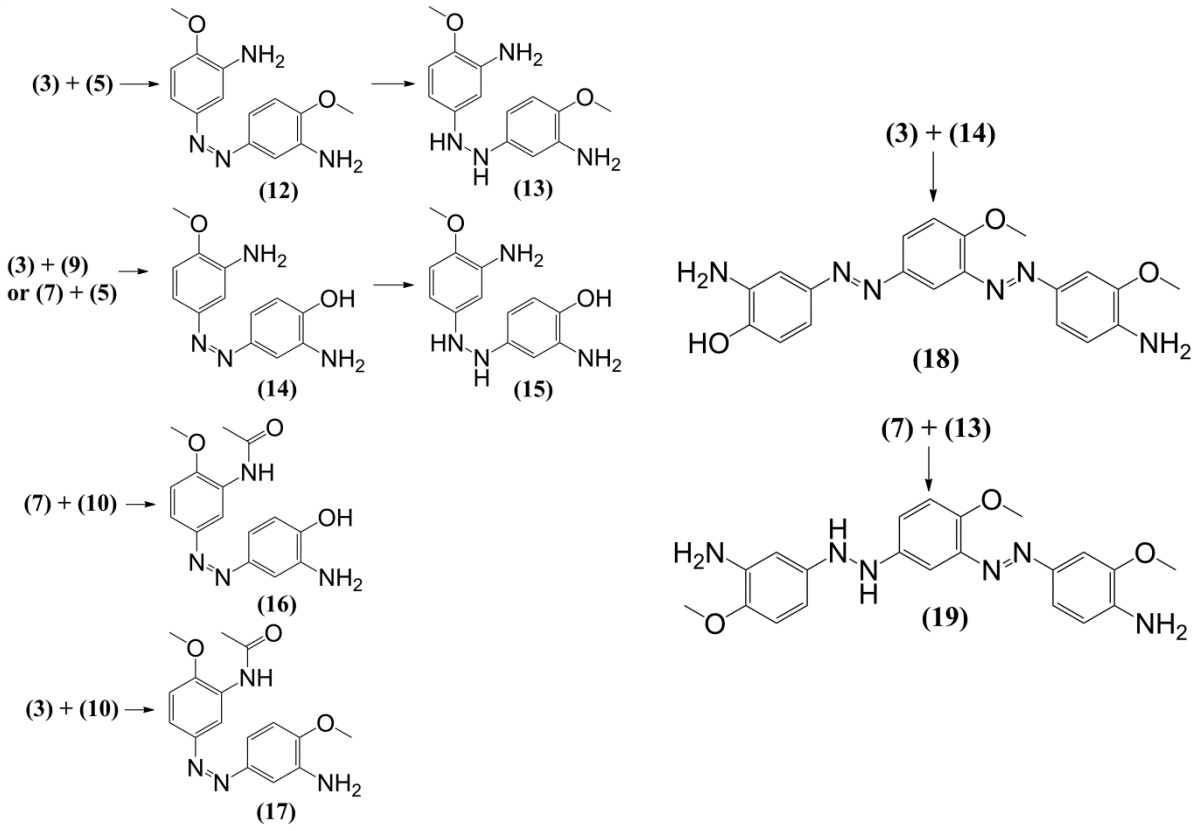

Figure 5.9. Dimers (left) and trimers (right) detected in the effluent of Columns 1 and 2 by UHPLC-QTOF-MS, and the proposed condensation reactions.

Table 5.1. Molecular weight, retention times and m/z values of the compounds detected by UHPLC-QTOF-MS in the effluent of Columns 1 and 2.

Structure in Figures 5.7 and 5.9	Molecular formula [M]	Retention time (min)	Calculated[a] [M+H]+	Measured [M+H]+	Difference (ppm)
2	$C_7H_8N_2O_3$	7.6	169.0608	169.0611	-1.77
3	$C_7H_8N_2O_2$	12.1	153.0659	153.0663	-2.61
5	$C_7H_{10}N_2O$	12.0	139.0866	139.0870	-2.88
6	$C_6H_6N_2O_3$	4.7	155.0451	155.0451	0.00
7	$C_6H_6N_2O_2$	12.1	139.0502	139.0501	0.72
9	$C_6H_8N_2O_1$	17.7	125.0709	125.0712	-2.40
10	$C_9H_{12}N_2O_2$	4.0	181.0972	181.0971	0.55
11	$C_8H_{10}N_2O_2$	3.8	167.0815	167.0810	2.99
12	$C_{14}H_{16}N_4O_2$	13.7	273.1346	273.1361	-5.49
13	$C_{14}H_{18}N_4O_2$	18.7	275.1503	275.1499	1.45
14	$C_{13}H_{14}N_4O_2$	2.0	259.1190	259.1196	-2.32
15	$C_{13}H_{16}N_4O_2$	3.1	261.1346	261.1349	-1.15
16	$C_{15}H_{16}N_4O_3$	7.6	301.1295	301.1308	-4.32
17	$C_{16}H_{18}N_4O_3$	9.2	315.1452	315.1452	0.00
18	$C_{20}H_{20}N_6O_3$	12.0	393.1670	393.1670	0.00
19	$C_{21}H_{24}N_6O_3$	12.1	409.1983	409.1983	0.00

[a]ChemBioDraw Ultra 12.0.2.1076

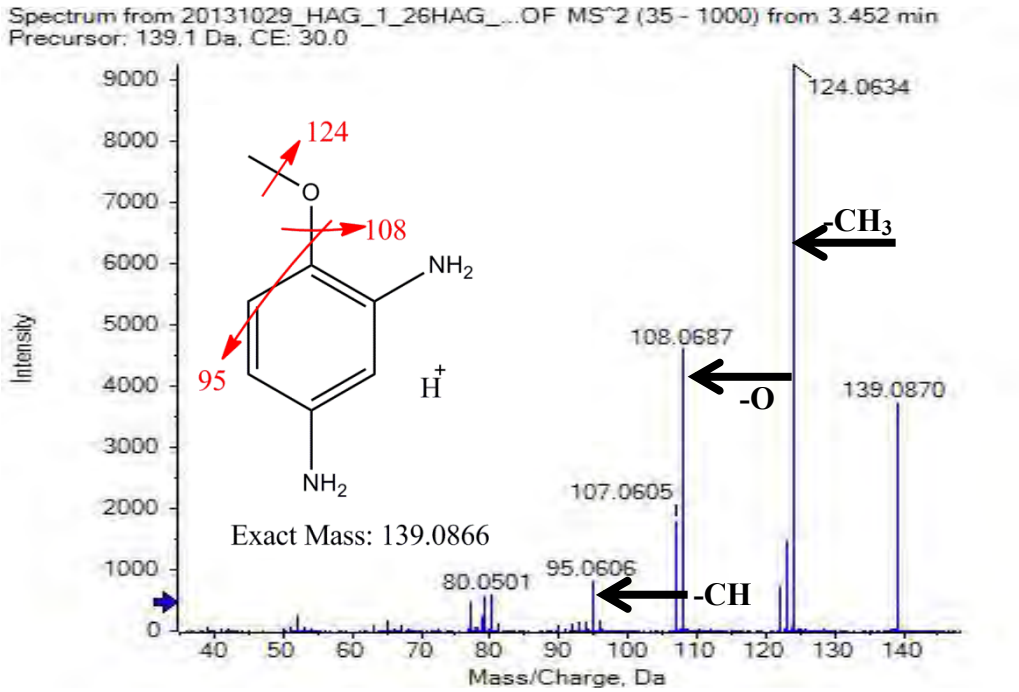

Figure 5.10. MS/MS Fragmentation spectra for DAAN (compound 5, *m/z* = 139.0870).

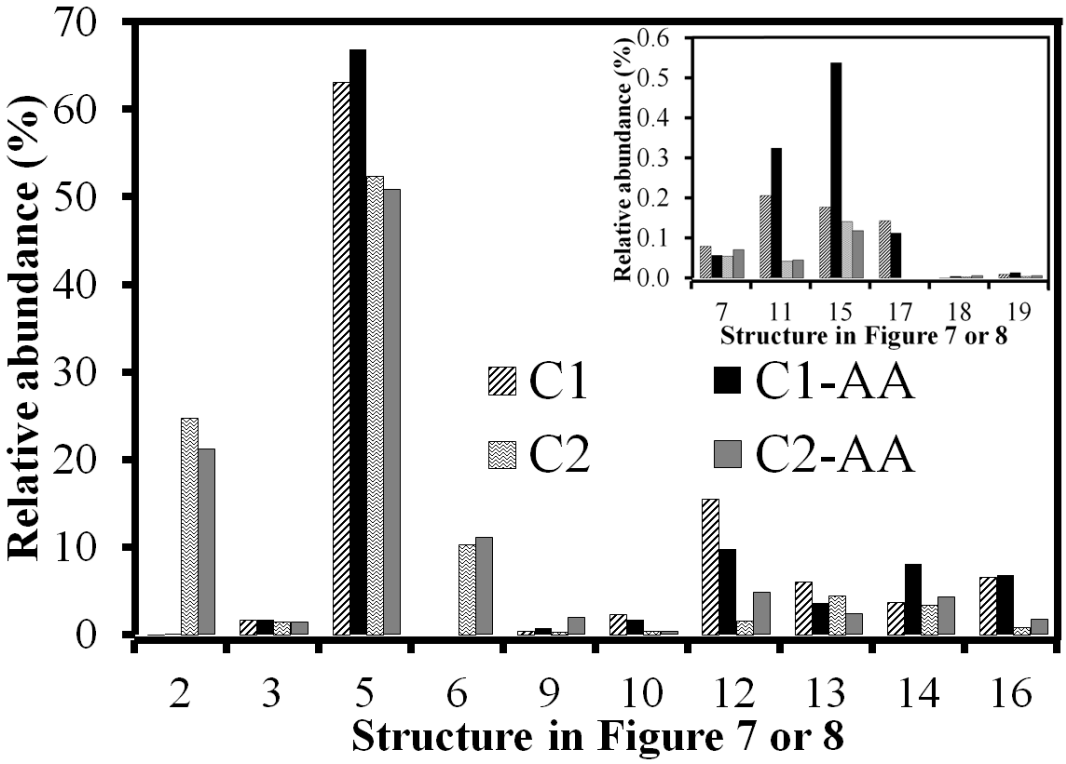

Figure 5.11. Relative abundance of the different chemical compounds detected by UHPLC-QTOF-MS in the effluent of the anaerobic columns with (C1-AA and C2-AA) and without ascorbic acid (C1 and C2).

5.3.3.1 Nitro-group reduction

The reduction of the nitro-group to an amino group was identified as the dominant process in both columns driving the biotransformation of MENA and DNAN. This was corroborated by the high relative abundance of DAAN (Figure 5.11) at the end of period 2, with 63 and 52 % for C1 and C2, respectively. The reduction of nitro groups is catalyzed by a group of enzymes known as oxygen insensitive nitroreductases [13, 27]. These are widely distributed in nature and are flavoproteins that have been suggested to mediate the sequential transfer of 2 electrons from NADH or NADPH to the nitro moiety of nitrosubstituted compounds [27-29]. The initial 6 e^- and 6 H^+ transfer by cometabolism of the anaerobic community in soil to DNAN (structure 1, Figure 5.7) favored the reduction of the nitro group in the *ortho* position as evidenced by the detection of the metabolite MENA (structure 2, Figure

5.7). The anaerobic reduction of the *ortho* nitro group in dinitro-aromatic compounds like 2,4-dinitrotoluene (DNT) [19, 30], 4,4-dinitrophenol (DNP) [31] and DNAN [5, 6, 9] also has been observed to be favored due to the strong electron deficiency created in the aromatic ring by the nitro groups [13]. On the other hand, the reduction of the *para* nitro group in MENA has been shown to act as a bottleneck in the fully biotransformation of DNAN to DAAN [5]. Nevertheless, period 1 helped to overcome such complications by adaptation of the soil microbial community to biotransform MENA, which was sequentially reduced first to nitroso-intermediates (**3** and **7**) and subsequently to amine groups (**5** (DAAN) and **9),** putatively via hydroxyl amine intermediates. Nitroso and hydroxylamine compounds are widely known intermediates from nitro group reduction [12, 13]. Usually the nitroso derivatives have been reported as short-lived compounds not detected at the end of the reduction process [32], leading to hydroxylamino derivatives as the possible end products [13]. The fact that we detected nitroso intermediates instead of hydroxylamine derivatives by UHPLC-QTOF-MS remains unclear, although the relative abundance of the compounds **3** and **7** in C1 and C2 was 1.6 and 1.4% and 0.06 and 0.07% (with AA), respectively.

5.3.3.2 O-demethylation of MENA and reduced compounds

Evidence of O-demethylation was also evident in both columns. O-demethylation can first be witnessed by comparing monomeric metabolites **2**, **3**, **5** and **10** with those of their O-demethylated counterparts, **6**, **7**, **9** and **11** (Figure 5.7). The comparison indicates that MENA (**2**), **3**, **5** (DAAN) and **10** were O-demethylated. The only O-demethylated (monomeric) metabolites detected in C1 were **7**, **9** and **11**, while in C2 an additional compound **6** (ANP) was detected. ANP was the most abundant O-demethylated product as shown in Figure 5.11, which points out to the greater pressure to utilize O-methyl substituents as a one-carbon energy-source in C2. Other studies have demonstrated that acetogen microorganisms like *Acetobacterium woodii* could be capable of grow methylotrophically on the O-methyl substituents of aromatic compounds [33]. Acetogens of this kind are present in many anaerobic environments and their activity can convert methoxylated one-

carbon substrates to acetate [34]. Bache and Pfennig [33] proposed that in the case of the strict anaerobic *Acetobacterium woodii* either a transmethoxylation or hydrolytic cleavage of the phenylether bond to methanol and the corresponding hydroxyl derivative of the aromatic compound is involved in the O-demethylation process. Theoretically, one mole of methanol could be oxidized to CO_2, 6 e^- and 6 H^+ [35], which is just enough energy to reduce a nitro group. In Figure 5.5 we observed a very low production of acetate in the early stages of the experiment which could be attributed not only to the OM fermentation (see section 5.3.1) but also to the presence of acetogenic microorganisms. Other microorganisms such as *Nocardioides* sp. JS1661 (isolated from a DNAN manufacturing plant) also have the ability to remove the methyl group (O-demethylation) from DNAN (without requirement of cofactors or oxygen), releasing methanol and forming DNP in the process [6]. Perreault et al. [4] also observed the O-demethylation of some DNAN-reduced metabolites under aerobic conditions with the associated release of formaldehyde, while Richard and Weidhaas [7] only observed the O-demethyaltion of DNAN to DNP. The reduction of DNAN, followed by O-demethylation of MENA could be used as a source of energy by the microbial population present in both C1 and (mainly) C2 and consequently, improving the biological reduction DAAN (C1) or DNAN > MENA > ANP (C2). Additional evidence of O-demethylation for C1 and C2 is apparent from the detection of compounds **14**, **15**, **16** and **18** in Figure 5.9 and 4.11 since each have a free hydroxyl group.

5.3.3.3 N-acetylation of reduced compounds

The metabolite structures also provide evidence of N-acetylation reactions during MENA and DNAN biotransformation. The most reduced metabolites DAAN (**5**) and 2,4-diaminophenol (DAP) (**9**) were converted to their N-acetylated counterparts **10** and **11**, respectively (Figure 5.7). Also the presence of N-acetyl groups in compounds **16** and **17** (Figure 5.9) provide evidence of N-acetylation having taken place. Some N-acetylated derivatives in the biotransformation of TNT were observed by Hawari et al. [36] and Knicker et al. [37]. Moreover, N-acetyled biotransformation products of DNAN in soil were also reported in Olivares et al.

(anaerobic) [5], Platten et al (anaerobic) [9] and Perreault et al. (aerobic) [4]. This kind of biotransformations has been reported to be catalyzed by arylamine N-acetyltransferases (NATs) which transfer an acetyl group from acetyl-coenzyme A to a xenobiotic acceptor substrate [38]. NATs can be present in both prokaryotes and eukaryotes, and play an important role in the detoxification of environmental carcinogens with amine groups [39], like DAAN and DAP. Other studies have demonstrated that aromatic amines used as precursors in the hair dye industry, have dramatically reduced their genotoxic potential after the N-acetylation processes [40]. N-acetylation reactions occurring in both C1 and C2 might be the first step towards a protection for the mixed cultures in soil against the toxicity or mutagenicity of aromatic amines [41] such as DAAN, DAP, hydroxylamine and/or nitroso compounds.

5.3.3.4 Coupling of unstable compounds

Evidence of coupling of biotransformation intermediates to dimers and trimers was also evident in this study. The biotransformation products, compounds **12**–**19**, corresponding to azo and hydrazine linked dimers and trimers (Figure 5.9) provide conclusive proof that coupling of intermediates had taken place in the absence of O_2. There are two main mechanisms by which the coupling reactions can occur. The first mechanism involves coupling between nitroso- and hydroxyl-amine intermediates and is the most widely accepted mechanism [42-45]. The coupling reaction is initiated by a nucleophilic attack from the hydroxylamine to the nitroso-group which produces an intermediate compound, N,N'-dihydroxy-hydrazine [46]. This intermediate loses a water to form the stable dimer, azoxybenzene. The azoxybenzene can be further reduced to azobenzene [43, 45]. The second mechanism involves coupling between nitroso-intermediates and aromatic amines. The coupling reaction is initiated by a nucleophilic attack of the amine to the nitroso-group which produces an intermediate compound, N-hydroxy-hydrazine [47, 48]. Upon loss of a water, this intermediate is directly converted to an azobenzene. Since our results indicate the presence of nitroso (**3** or **7**) and aminoaromatic compounds (**2**, **6**, **5**, **9**, **10** and **11**) in the anaerobic effluent of C1 and C2, the

second mechanism might be more plausible to generate the dimer and trimer structures found in Figure 5.9. Several publications have reported the presence of dimeric or trimeric compounds after exposure of diamino- or triamino-aromatic compounds to air. Yang et al. [19] reported that the reduction of DNT produced 2,4-diaminotoluene (DAT) under anoxic conditions, but after incubation of DAT in sediment under aerobic conditions the metabolites detected by LC-MS shifted to azo- and hydrazine-dimers of DAT, and even a hydrazine-trimer was detected in sediment acetone extracts. Platten et al. [9] found that DAAN formed the azo 2,4-diaminoanisole dimer (compound **12** in Figure 5.9) upon exposure of the samples with air. Perreault et al. [4] showed that the biotransformation of DNAN under aerobic conditions also promoted the coupling reactions between nitroso and hydroxylamino compounds with the concomitant generation of azo compounds. Nonetheless, these polymeric compounds also have been shown to occur under anoxic conditions. Hawari et al. [22] showed that 2,4,6-triaminotoluene (TAT) reacted under strictly anaerobic conditions and produced azo derivatives, suggesting the biotic nature of their formation. Even with the use of ascorbic acid as antioxidant agent in the analysis of anaerobic samples Olivares et al. [5] found several dimeric structures formed by anaerobic coupling of DNAN reduction products. Taken as a whole, the evidence indicates the formation of polymeric material inside anaerobic systems, including soil.

Finally, the gap between the concentration of DNAN and MENA fed and the amount of metabolites recovered at the effluents of C1 and C2 (Figures 5.6A and 5.6B, respectively) could be partially explained by sorption or irreversible binding of hydrophobic compounds into soil OM. Thorn and Kennedy [49] demonstrated that several reduced compounds of TNT underwent nucleophilic addition reactions (under aerobic conditions) with quinone and other carbonyl groups in the soil humic acid to form both heterocyclic and nonheterocyclic condensation products. On the other hand, in the absence of oxygen, catechol and hydroquinone groups are prevented from oxidizing to quinones and therefore from undergoing nucleophilic addition by the aniline (aromatic amine) [50]. Under anoxic conditions the

nucleophilic addition reactions could take place due to the presence of unreacted quinone moieties in soil [50], which are the major substrate for the nucleophilic addition of aromatic amines to humic substances [51, 52]. Elovitz and Weber [53] reported that covalent binding of diaminonitrotoluenes (DANTs) was lower under anoxic conditions than aerobic, and the potential for covalent binding through nucleophilic addition is expected to increase with N-electron density. Hawari et al [8] also found that an increase in nucleophilicity of DNAN reduced metabolites supported an increase in the binding capacity towards electronegative sites (C=O, COOH). One can speculate that part of the electropositive metabolites detected in this study (monomers **5** and **9** in Figure 5.7, and dimer and trimers in Figure 5.9) underwent irreversible binding with the electronegative surface functional groups in soil (e.g. unreacted quinones), leading to differences between the concentration of DNAN and MENA fed and the amount of metabolites detected at the effluents of C1 and C2 by HPLC-DAD and UHPLC-QTOF-MS.

5.3.3.5 Azo bond reduction and hydrazine derivatives

Lastly, the spectrum of products detected in Figure 5.9 indicates that azo bonds were subjected to reduction forming the hydrazine bonds. Azo dimers, compounds **12** and **14**, were transformed to hydrazine dimers, compounds **13** and **15**, respectively. Other authors have reported the detection of hydrazine-intermediates in the biotransformation of azo dyes [54-56], and DNAN [5]. The reducing conditions prevailing inside C1 and C2, together with the presence of cytoplasmic non-specific enzymes (azo reductases [57]) in the mixed soil microbial community might account for the cleavage of azo bonds previously formed by coupling reactions (see section 5.3.3.4 and proposed coupling reactions in Figure 5.9) to hydrazine-metabolites, and if the reaction conditions the azo bond would be cleaved yielding their original constituents DAAN and DAP.

5.3.4 Microbial inhibition bioassays

The microbial toxicity impact of metabolites formed during anaerobic treatment compared to DNAN was evaluated using the Microtox test. The results in Figure 5.12 show that the toxicity of the influent and effluent of both soil columns with a

dilution factor of 2 (thus DNAN in the influent was tested at 37.5 µM, and compared to 2× diluted effluent). The results indicate that the feed stream of C1 was 6.9 times more toxic to *A. fischeri* than the influent of C2, probably due to the presence of ethanol which acted as an antiseptic agent with the sensitive marine bacterium. The effluent of C1 was 46 and 1.7 times more toxic than the influent DNAN (without ethanol or C2 influent) and the effluent of C2, respectively. Usually, the aromatic amines have been reported to be less toxic than the parent nitroaromatic compounds [14, 17, 58], but some of the reduced metabolites can behave exactly the opposite. It has been demonstrated that the reduction of 2,4-dinitrotoluene produced the more toxic intermediates 4-amino,2-nitrotoluene and 2-amino,4-nitrotoluene [59]. MENA is the metabolite of reduction of DNAN and also has been proved to be about 1.18 times more toxic than the parent compound [14]. Another explanation for the observed toxicity could be attributed to the presence of nitroso compounds (structures **3** and **7**, Figure 5.7) which have been correlated to be highly reactive and toxic to living microorganisms because they can modify proteins and DNA [41]. However, the relative abundance of nitroso compounds in both C1 and C2 seemed to be very similar (see Figure 5.11). For these reason, an alternative hypothesis for the enhanced toxicity of the effluent of C1 could be related to the presence of coupling products such as the dimers and trimers showed in Figure 5.9. Similar results were found by Carpenter et al. [60] which concluded that the aerobic biotransformation products of ^{14}C-labeled TNT reacted with lipids, fatty acids, and protein constituents of the microbial flora, forming macromolecular structures of the polyamide type. From literature we could visualize that the biotransformation products of TNT under aerobic conditions also include dimer and trimer structures [19] which are very similar to the structures found in this work. In Figure 5.11 we observed that for samples without AA there was a greater presence (relative abundance) of dimers **12**, **13**, **15** in C1 compared to C2 samples. These biotransformation products of DNAN could react in the presence of air (provided in the Microtox test) with the constituents of *A. fischeri* (i.e. lipids, fatty acids, and protein constituents) increasing the toxicity of C1 samples compared

with C2. However, a more detailed study about the toxicity of those intermediates is necessary in the future work.

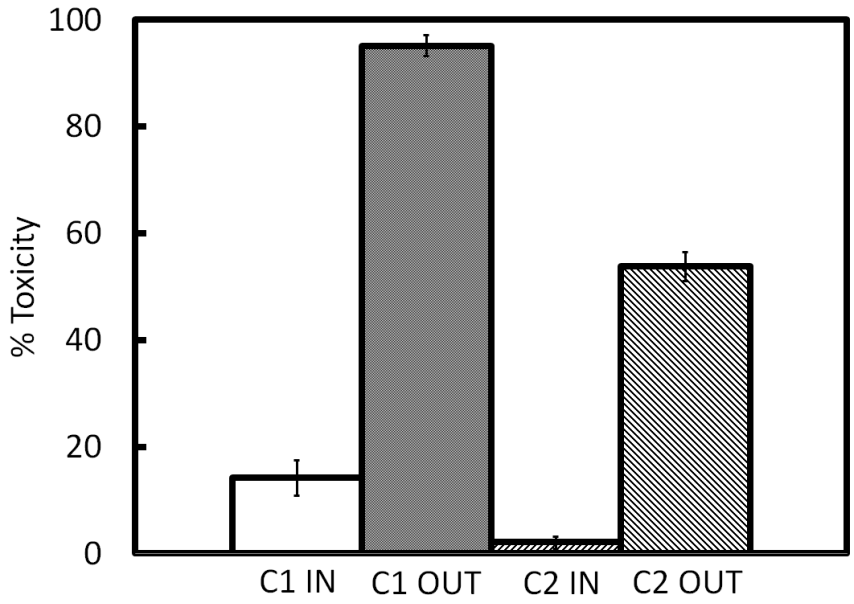

Figure 5.12. Toxicity at minute 30 of the influent and effluent of Columns 1 and 2 when DNAN was used in the feed stream (final concentration in assay = 37.5 µM).

5.4 Conclusions

The environmental fate of DNAN in soil was complex and the results presented herein provide evidence that the addition of a carbon source can increase the rate of biotransformation of MENA and DNAN. The lack of an exogenous electron donor in C2 promoted the adaptation of the microorganism to grow methylotrophically by O-demethylation of MENA and DNAN. The autoxidation of the most common aromatic amines produced inside the anaerobic columns (DAAN and ANP) was stopped by the addition of freshly prepared ascorbic acid, but the detection of dimers and trimers reflected that some coupling reactions were still taking place inside the columns. The detection of new metabolites of the reduction of intensive munitions was achieved by the use of the UHPL-QTOF-MS. The detection of N-acetylated compounds suggested that the microorganisms were starting the detoxification of the toxic compounds inside the columns. The global

effect of the biotransformation of DNAN was an increased toxicity in the effluent of the anaerobic columns due to the production of toxic intermediates such as nitroso and/or polymeric compounds (dimers and trimers). Future research will focus in the aerobic treatment of the anaerobic toxic waste for the complete bioremediation and detoxification of the nitroaromatic solutions.

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CHAPTER 6. GENERAL DISCUSSION AND FINAL REMARKS

Study of the anaerobic reduction of nitroaromatic compounds (NACs) is a complex subject that has attracted the attention of two different fields of research: biotechnology and catalysis. Both areas have merged together with the use of redox mediators that catalyze the reduction of recalcitrant compounds such as NACs, azo dyes or other chemical compounds prone to reduction processes. In this thesis work it was explored the reduction of NACs from the chemical (chapters 2 and 3) and the biological perspectives (chapter 5), including the merge of both concepts (chapter 4) with the use of activated carbon fibers (ACFs) as biological support in the biotransformation of 4-nitrophenol under continuous anaerobic conditions. In this final chapter the main conclusions of this thesis work will be reviewed and discuss the new scientific evidence in literature in aims to highlight the contribution of this study in the biotechnological application of ACFs as redox mediators.

6.1. Activated carbon fibers as redox mediators for the increased reduction of nitroaromatics

The first strategic step was to demonstrate that ACFs acted as redox mediators between the chemical oxidation of $Na_2S{\cdot}9H_2O$ and the reduction of 4-nitrophenol (NP) or 3-chloronitrobenzene (CNB). To prove this hypothesis it was demonstrated that both NACs were stable during the 15 days of the batch experiment. Figures 2.5 and 2.6 displayed that concentrations of control experiments with only NP or CNB decreased 0 and 5%, respectively. In second place, it was showed that the direct chemical reduction of both NACs with the electron donor Na_2S (HS^- as the proposed reactive species) was less than 5%. In the third place, adsorption isotherms were determined for each NAC onto three ACFs with different content of quinone functional groups (fabric polyacrylonitrile ACF “AW”, acid oxidized ACF “OX” and thermally reduced ACF “700”) as shown in Figure 2.4. The results of such isotherms helped to calculate the mass of ACF necessary to adsorb 20% of the NAC in the solution. The adsorption controls in Figures 2.5 and 2.6 showed

that adsorption of NP and CNB was less than 20 and 50% in all ACFs, respectively. Finally, chemical reduction experiments combined the three elements of the reaction: electron donor (Na_2S), redox mediator (AW, OX or 700), and electron acceptor (NACs). In all cases it was demonstrated that the presence of the three elements reduced more the concentration of both NP and CNB than the controls experiments mentioned previously (see Figures 2.5 and 2.6). These results together with the HPLC evidence of reduced compounds were indication that the ACFs acted as redox mediators in the oxidation of Na_2S and the reduction of NP to 4-aminophenol (AP) and CNB to 3-chloroaniline (CA), respectively. A more detailed study of the physicochemical characteristics of all ACFs revealed that the higher reduction of NACs was correlated to the higher presence of quinone functionalities on acid treated "OX" ACFs. OX materials presented the most negative surface charge at the experimental pH of 7.6 because of its lower point of zero charge (PZC = 3.41) than AW (PZC = 5.16) and 700 (PZC = 9.3) ACFs. Nonetheless, the NACs with a partial negative charge on the nitro group overcome the negative surface charge of OX materials being reduced to their respective aminoaromatic compounds in the process. As part of the mechanism of NAC reduction it was proposed: 1) the oxidation of the hydrosulfide ion (HS^-) on ACF surface, 2) followed by the electron and proton transfer to the quinone groups, 3) the formation of hydroquinones and 4) the final electron and proton shuttle to the NAC and its intermediates (see Figure 2.8). However, this proposed mechanism needs further explanation of the factors involved in the redox reaction.

6.1.1. Adsorption mechanisms

The choice of activated carbon as adsorbent for a specific application is usually based on its surface characteristics such as specific surface area, pore volume, pore sizes, and, not too often, surface chemistry [1]. However, in redox processes, both chemical and physical characteristics of the materials are connected and play an important role in the reactivity of the activated carbons. For example, the affinity of active carbons to retain water (chemical characteristic) and a higher presence of

micropores (physical characteristic) were reported as important for H_2S (pKa = 7.6) adsorption [1] and oxidation [1-3], respectively.

On the other hand, adsorption of electronegative NACs is governed mainly by electrostatic interactions with the ACF surface [4]. In this work, CNB remains with two electronegative groups (i.e. –Cl and $–NO_2$) at pH 7.6 in the chemical reduction experiments of Figures 2.5 and 2.6. Under the same conditions, the pH of the solution promoted the partial deprotonation of NP (pKa = 7.15) to 4-nitrophenoxide ion [5] (NPi) which led to the presence of two electronegative groups on the molecule (i.e. $–O^-$ and $–NO_2$). Moreover, at pH=7.6 Na_2S is dissociated to H_2S and HS^- species with even distribution in the aqueous solution. From a practical perspective, electronegative materials such as OX probably hindered the adsorption of the electronegative reactants (CNB or NPi, and HS^- species) by electrostatic repulsions. However, all ACFs have an amphoteric character because they have separate, not coinciding, acid and basic surface sites [6] as seen in Table 2.2. Functional sites such as γ pyrone-type, chromene, diketone or quinone groups, on one hand, and delocalized π-electrons of the basal planes, on the other, are assumed to have a basic nature [7]. Considering that basic sites are electropositive in nature, it is highly probable that the adsorption of electronegative reactants (electron donor and NACs) took place onto basic sites where they can react.

6.1.2. Redox active groups

Quinones have been proposed to undergo reversible electron and proton transfer processes [6] as showed in Figures 1.2 and 2.8. However, other basic sites (e.g. γ pyrone-type, chromene, and diketone groups) could also (in theory) explain the redox behavior of carbon materials [7]. According to theoretical calculations performed by Montes-Moran et al. [7], several pyrone-like structures can effectively accept two H^+ and two e^- from anthrahydroquinone molecules (E^0 = 0.13 V), which were considered as representative of quinonoid structures on carbon surfaces [8]. The computed electrochemical potentials exhibited values of -0.58 to 1.1 V depending on the positions of the pyrone groups on the polyaromatic frame.

Products of pyrone transformation could be considered as semi-hydroquinones (hydroxyl groups neighboring carbonyl/semi-quinone structures) ready to donate two H^+ and two e^-. In theory, chromene and diketones could also participate in redox reactions since they are carbonyl/semi-quinone structures that could change their electrochemical potentials as a function of their physicochemical environment. In this work, the oxidation of Na_2S could occur in basic sites (i.e. quinones, γ pyrone-type, chromene, and diketone structures) with the formation of electron-rich hydroquinones and/or semi-hydroquinones. Such redox active functional groups could donate protons and electrons to the NACs adsorbed on the same or neighboring basic sites, transforming them to their aminoaromatic derivatives. The exact nature of the surface complexes involved in the reduction reaction is, as in any carbonaceous system, open to question [9]. Moreover, the transfer of six H^+ and six e^- to the nitro group remains unclear, but it is hypothesized that microporosity of the ACFs might play an important role due to the overlap of several electrochemical potentials at pore openings [6] that could led to successful nitroaromatic reduction.

6.1.3. Presence of inorganic impurities

There are several reports in literature where catalytic amounts of impurities on the carbon surface had a significant effect on the kinetic experiments. For example, Cariaso and Walker [2] studied the gas phase oxidation of H_2S over carbon surfaces and found that impurities in the carbon had a strong influence on the catalytic reaction. Regular activated carbons from wood, coal, nutshells and fruit stones, all contain up to several percentage of indigenous impurities [10]. Indeed, it is very difficult to completely purify carbon materials, and carbons that are useful on an industrial scale because they usually have a large ash content [9]. In order to consider carbon materials as catalyst (or redox mediators) one have to ensure an absolute minimum of inorganic impurities which, if present, would invalidate kinetic data [10]. Recent progress has been made on the use of 'metal free' carbon nanotubes (CNT) as redox mediators (catalyst) in the reduction of nitrobenzene by using hydrazine monohydrate as electron donor [11]. Wu et al. reported that Fe

impurities had no or marginal effect on the reactions, because it was embedded in the CNTs and could not interact with the reactants (CNTs and nitrobenzene). The authors also concluded that the activity of the CNT was mainly derived from carbonyl groups.

In order to fully validate the reduction models proposed in this thesis future research must be performed on reduction systems lacking inorganic impurities in both the activated carbon surface and in the aqueous solution.

6.2. Anchorage of anthraquinone molecules onto activated carbon fibers to enhance the reduction of 4-nitrophenol

In the search for better catalytic materials, the second strategic step of this thesis work focused on the anchorage of redox active functionalities on the surface of ACFs. Two anthraquinone molecules previously reported to have redox properties 2-aminoanthraquinone (AAQ) [12] and anthraquinone disulfonate (AQDS) [13-15] were anchored on ACF surface by chemical modification with $SOCl_2$. FT-IR analysis showed the presence of absorption bands related with the anthraquinone structures anchored on the ACF surface. Moreover, the specific surface area and the pore size distribution of the ACFs changed due to the attachment of AAQ and AQDS with a projected diameter greater than 1 nm. In spite of those changes, ACFs with AQDS molecules anchored on the surface (AW-AQDS) improved the reduction of NP by 49% as compared to the as-received materials (AW). Materials with AAQ molecules anchored on ACF surface (AW-AAQ) exhibited almost the same performance as compared to AW probably due to the hydrophobicity of the AAQ molecule that hindered the adsorption of the reactants (HS^- and NP) on the active sites (i.e. quinone groups).

The study on the anchorage of anthraquinone molecules on ACF resulted in three materials with different content of quinone sites on their surface (see Table 4.1). Each of the materials AW, OX and AQDS could potentially increase the biological reduction of NP under continuous conditions, but more insights were needed in the biotransformation of NACs in order to explain better the biological experiments. For

these reasons, the biotransformation NACs under continuous conditions was studied in chapter 4 with the use of ACFs as redox mediators and biological supports.

6.3. Activated carbon fibers with redox active functionalities improves the continuous anaerobic biotransformation of 4-nitrophenol

In this experimental stage all the physicochemical concepts related with the use of ACFs as redox mediators (chapters 2 and 3) were fused with the biological factors that affect the biotransformation of NACs in order to prove that ACFs could also perform as redox mediators in biological systems (chapter 4).

To start with the biotransformation experiments we designed a novel concept bioreactor where the ACFs were supported on disks inside the anaerobic column (see Figure 4.1). After this procedure, the anaerobic granular sludge (AGS, inoculum) was acclimated to ethanol (carbon source) consumption as described in section 4.2.3. After the acclimation period, the NP was fed and the ACFs disks (previously saturated with NP) were inserted into the column just above the inoculum. These strategies allowed the planktonic bacteria to travel in the up-flow stream and attach to the ACFs where the biofilm could build up over the time. The control reactor lacked of any carbonaceous material inside the column, but worked at the same conditions as the other columns packed with as-received (AW), acid treated (OX), and anthraquinone modified (AQDS) bioreactors. Generally speaking, all four bioreactors exhibited a high biotransformation of NP (>98%) in the first 6 days of the continuous experiment. This high performance was attributed to the high concentration of ethanol used in Period I (56 times the quantity of ethanol necessary to reduce 75 ppm of NP). During Period II the ethanol concentration was reduced to 3.3 times the ethanol required to reduce 75 mg/L of NP. Such carbon source limitation showed that ACFs improved the reduction of NP as compared with the control reactor, but the biofilm developed over carbon surfaces masked the effect of the active functional groups on the transformation of NP. The total restriction of exogenous carbon source to the bioreactors for 14 days (Period III) promoted a decrease in the NP removal and the saturation of ACFs

with NP. Finally in Period IV (ethanol fed at 1.6 times the amount required to reduce 75 mg/L of NP) the microbial activity was promoted by feeding ethanol at low concentrations. The biotransformation of NP was enhanced by the higher presence of redox active groups (e.g. carbonyl) in the surface of ACFs in the order: OX > AQDS > AW. The main conclusion of this experimental stage might be that biofilm development over ACF surface biotransformed NP in a very short period of time when the exogenous carbon and electron donating source are fed in large excess into the bioreactors. On the other hand, it was observed that the biofilm could modify its NP biotransformation efficacy influenced by the surface chemistry of the ACF materials when the carbon and electron donor sources (exogenous and endogenous) are extremely limited. From the biotechnological perspective, the application of activated carbon as redox mediator in the biotransformation of recalcitrant pollutants could take advantage of the results presented herein. The reader is encouraged to analyze the biofilm development and morphology changes with the proper analytical tools commercially available and consider those results in the discussion of any carbon used in biological systems. A short academic stay in the University of Arizona helped to acquire more insights about the biotransformation of nitroaromatic compounds as explained in the following section

6.4. Pathways of 2,4-dinitroanisole (DNAN) and 2-methoxy-5-nitroaniline (MENA) biotransformation in continuous flow-through anaerobic soil columns

In this stage of the thesis the anaerobic biotransformation process of several NACs showed an increased level of complexity that led not only to the production of the corresponding aromatic amines, but also to a plethora of metabolites. Such diversity of metabolic pathways was promoted by carbon source limitations and/or the use of soil as the attachment media for microorganisms to grow. In any case, the biotransformation of 2,4-dinitroanisole (DNAN) and 2-methoxy-5-nitroaniline (MENA) was studied in two soil columns receiving different amounts of exogenous carbon source: 100 mg/L of ethanol in column 1 (C1) and column 2 (C2) relied only on endogenous organic matter present in soil. The presence of an extra source or

reducing equivalents in C1 promoted the reduction of MENA and DNAN to 2,4-diaminoanisole (DAAN) as the main reduction product, while in C2 there was a metabolite not observed in C1, 2-amino-4-nitrophenol (ANP). The production of ANP in C2 was related to the selective carbon source pressure in this column that led to the O-demethylation of MENA and DNAN that served as a source of energy for microorganisms to grow. More monomers (Figure 5.7), dimers and trimmers (Figure 5.9) from the reduction of MENA and DNAN were detected in the effluent with the use of UHPLC-QTOF-MS (Ultra High Pressure Liquid Chromatography-Quadrupole Time of Flight-Mass Spectrometry). Such metabolites suggested the existence of microorganisms in the mixed soil community capable not only of nitro group reduction, but also of O-demethylation and N-acetylation reactions. Finally, the detection of dimer and trimers revealed a complex system where reactive nitroso-, hydroxylamino- and aminoaromatic compounds probably reacted in the presence of air or other oxidizing agents found in soil.

With the experience acquired in this experimental stage in the biotransformation of NACs it was set up the experimental system necessary for the continuous biotransformation of NP in hybrid UASB-ACF biofilm reactors.

6.5. PERSPECTIVES

The study of the biological transformation of NACs with the use of ACFs as biological support and redox mediators is not an easy subject. Many areas of knowledge have to be considered when explaining the combined physical and biological results such as: organic and inorganic catalysis, organic and inorganic chemistry, physical chemistry, analytical chemistry, solid-state chemistry, electrochemistry, soil chemistry, microbial toxicity, biotransformation processes (reduction, O-demethylation, N-acetylation, polymerization, and more), microbial groups in anaerobic ecosystems, biofilm development concepts, among others. However, most of the subjects were just mentioned in this thesis work and will require the further research in order to fully evaluate the application of ACFs as redox mediators in the biotransformation of recalcitrant pollutants.

Some of the perspectives generated in this thesis include:

1) Effect of the inorganic impurities in the chemical and biological transformation of NACs. In previous sections it was stated that inorganic impurities (trace redox-active metal ions, acid and basic Lewis salts) could have a large effect in the performance of activated carbons as catalyst (redox mediator). The same applies for biological systems were the biotransformation of recalcitrant pollutants could be enhanced by the presence in the solution of soluble redox active compounds such as Fe or Mn salts. The future design of chemical and biological experiments where the inorganic impurities have no or marginal effect on the results (reduction of NACs) is an important future perspective.
2) The role of specific functional groups of ACFs on the chemical and biological transformation of recalcitrant pollutants is open to many questions, especially because the method of fabrication and chemical modification of active carbons are not specific about the graphitization (sp^2 and sp^3 carbon content) or the distribution of surface chemical groups on the graphitic layer (the reactivity depends on it). The use of more analytical techniques and/or more “simple” structures (fully characterized and studied activated carbons or graphene materials) will help us to understand better the carbon use in redox systems and the role of specific functional groups in oxidation or reduction process.
3) The use of pure microbial cultures might facilitate the results interpretation and mechanism discussion in the use of ACFs as redox mediators for the biotransformation of pollutants.
4) The biofilm formation over ACF surface and their interactions towards the reduction/removal of nitroaromatic compounds is an interesting subject that requires further studies such as: biofilm development over time, diffusion of substrates and nitroaromatics in the biofilm, specific contributions of bacterial co-metabolism, surface functional groups in ACFs and other

factors (e.g. biomolecules, ions) in the reduction/removal of nitroaromatic compounds.

5) All this thesis work was focused on the chemical and/or biological reduction of NACs, however, there is a great window of opportunity for the oxidation of recalcitrant compounds. The aerobic degradation of several mono-nitroaromatic compounds has been reviewed by Ju and Parales [16]. On the other hand, activated carbons are widely known to perform oxidation reactions and their use in the oxidation of recalcitrant pollutants is a promising technology [6, 9, 17, 18].

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Scientific products

List of publications

Amezquita-Garcia, H. J.; Razo-Flores, E.; Cervantes, F. J.; Rangel-Mendez, J. R., Activated carbon fibers as redox mediators for the increased reduction of nitroaromatics. *Carbon* **2013,** *55*, 276-284. DOI: 10.1016/j.carbon.2012.12.062

Amezquita-Garcia, H. J.; Razo-Flores, E.; Cervantes, F. J.; Rangel-Mendez, J. R., Anchorage of anthraquinone molecules onto activated carbon fibers to enhance the reduction of 4-nitrophenol. *Journal of Chemical Technology and Biotechnology* **2014,** *In press*. DOI: 10.1002/jctb.4478

Amezquita-Garcia, H. J.; Olivares, C.; Abrell, L.; Sierra-Alvarez, R.; Rangel-Mendez, J.R.; Razo-Flores, E.; Field, J. A., Pathways of 2,4-dinitroanisole (DNAN) and 2-methoxy, 5-nitroaniline (MENA) biotransformation in continuous flow-through anaerobic soil columns. **In preparation**.

Amezquita-Garcia, H. J.; Rangel-Mendez, J. R.; Cervantes, F. J.; Razo-Flores, E., Activated carbon fibers with redox active functionalities improves the continuous anaerobicbiotransformation of 4-nitrophenol. **In preparation**.