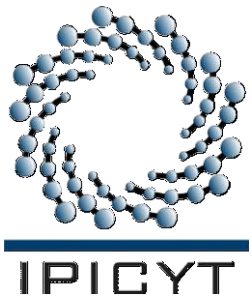

INSTITUTO POTOSINO DE INVESTIGACIÓN CIENTÍFICA Y TECNOLÓGICA, A.C.

POSGRADO EN CIENCIAS APLICADAS

Strategies to immobilize humic substances for their application on the reductive biotransformation of recalcitrant pollutants

Tesis que presenta

Claudia Margarita Martínez Rodríguez

Para obtener el grado de

Doctora en Ciencias Aplicadas

En la opción de

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Directores de la Tesis:

Dr. Francisco Javier Cervantes Carrillo

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San Luis Potosí, S.L.P. 17 de Diciembre de 2013

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Constancia de aprobación de la tesis

La tesis **"Strategies to immobilize humic substances for their application on the reductive biotransformation of recalcitrant pollutants"** presentada para obtener el Grado de Doctora en Ciencias Aplicadas en la opción de Ciencias Ambientales fue elaborada por **Claudia Margarita Martínez Rodríguez** y aprobada el **diecisiete de diciembre de dos mil trece** por los suscritos, designados por el Colegio de Profesores de la División de Ciencias Ambientales del Instituto Potosino de Investigación Científica y Tecnológica, A.C.

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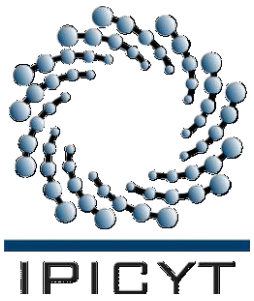

Institutional Credits

This Dissertation was developed in the laboratories of Environmental Sciences Division of Instituto Potosino de Investigación Científica y Tecnológica, A.C., under the supervision of Dr. Francisco J. Cervantes Carrillo and Dr. María de Lourdes Berenice Celis García

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DOCTORA EN CIENCIAS APLICADAS
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sobre la Tesis intitulada:

Strategies to immobilize humic substances for their application on the reductive biotransformation of recalcitrant pollutants

que se desarrolló bajo la dirección de

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A Diego,

el tesoro de mi vida

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Abbreviations

AER	anion exchange resin
i-Leonardite	immobilized leonardite on resin
a-Leonardite	immobilized leonardite on γ-Al_2O_3 NPs
AH_2QDS	anthrahydroquinone-2,6-disulfonate
AQDS	anthraquinone-2,6-disulfonate
CS	factory of candies
CT	carbon tetrachloride
COD	chemical oxygen demand
CF	chloroform
4-CP	4-chlorophenol
2,4-DCP	2,4-dichlorophenol
DCM	dichloromethane
GWC	compost produced with gardening wastes
EAC	electron accepting capacity
EGSB	expanded granular sludge bed
ETC	electron transferring capacity
HRM	humus reducing microorganisms
HRT	hydraulic residence time
HA	humic acids
HS	humic substances
K_{d1}	first order rate constants for reduction of CT
K_{d2}	second-order rate constants for reduction of CT

SRR	specific reduction rate
NPs	nanoparticles
PS	sludge originated from a wastewater treatment plant treating effluents from a paper-mill factory
pHpzc	point of zero charge
RR2	reactive red 2
RM	redox mediator
S	sediment
SCP	soil of cocoa plantation
SDF	soil of deciduous forest
TEA	terminal electron acceptor
TOC	total organic carbon
UASB	upflow anaerobic sludge blanket
VSS	volatile suspended solids

Resumen

Martínez, C.M. Estrategias de inmovilización de sustancias húmicas y su aplicación en la biotransformación reductiva de contaminantes recalcitrantes. Tesis Doctoral, IPICYT

Las sustancias húmicas (SH) y sus compuestos análogos, como las quinonas, han sido empleados como mediadores redox (MR) para acelerar la conversión de contaminantes prioritarios susceptibles a reacciones de óxido-reducción (redox), como colorantes azo, compuestos polihalogenados alifáticos y aromáticos, así como nitroaromáticos, los cuales son persistentes o escasamente reducidos en los sistemas de tratamiento anaerobios de aguas residuales. La aplicación de SH o sus análogas quinonas han mejorado significativamente la conversión redox de dichos contaminantes. Sin embargo, la principal limitación en la aplicación de las SH como MR en los sistemas de tratamiento en continuo, es que demanda su adición continua para incrementar las tasas de conversión, lo cual no es viable desde el punto de vista económico y ambiental. Por lo tanto, recientemente se han explorado varias estrategias para inmovilizar SH y quinonas modelo, con el objetivo de desarrollar sistemas que integren la capacidad redox de MR inmovilizados y promover incrementos en las tasas de conversión. Esta tesis se enfocó en evaluar dos diferentes estrategias de inmovilización de SH (SH adsorbidas sobre resinas de intercambio iónico y sobre nanopartículas de γ-Al_2O_3) para aplicarlas en la biotransformación redox de contaminantes recalcitrantes en experimentos en lote y en bioreactores operados en flujo continuo. Adicionalmente, este trabajo evaluó la capacidad redox de SH extraídas de ambientes y residuos ricos en materia orgánica, con el fin de promover la aplicabilidad de SH inmovilizadas extraídas de fuentes naturales en el tratamiento de contaminantes recalcitrantes.

Los resultados presentados en este trabajo demostraron por primera vez, que las SH inmovilizadas pudieron servir como MR de fase sólida efectivos para promover la remoción simultánea de fenol y rojo reactivo 2 (RR2) en un reactor UASB, durante periodos de operación prolongados sin la necesidad de la constante adición del MR. El reactor UASB suplementado con SH inmovilizadas, logró eficiencias de eliminación de fenol y RR2 superiores al 70 % y 85 % respectivamente (Capítulo 3). Los resultados presentados en el capítulo 4 demostraron que fue posible lograr la co-inmovilización de microorganismos reductores de humus (MRH) con SH soportadas sobre nanopartículas (NP) de γ-Al_2O_3, como una novedosa estrategia de inmovilización de SH. Gránulos grandes con aglomeración apropiada de biomasa y de NPs de Al_2O_3 fueron obtenidos en la presencia de SH, en comparación con aquellos producidos en la ausencia de SH. Sin embargo, al evaluar el efecto catalítico de estos gránulos en la reducción del RR2 y del 2,4-diclorofenol (DCF), la cantidad de SH contenida en dichos gránulos y el bloqueo de los grupos redox en el humus, fueron posibles factores que limitaron la capacidad redox de las SH inmovilizadas durante la biodegradación simultánea de ambos contaminantes. Finalmente, en el Capítulo 5 se demostró que SH extraídas de fuentes naturales actuaron como MR durante la biodegradación simultánea de fenol y tetracloruro de carbono (TCC). Los

resultados también indicaron que la capacidad aceptora de electrones (CAE) de las fuentes de SH derivadas de ambientes ricos en materia orgánica, dependió de la fuente y de las características de las SH evaluadas. Así mismo, la CAE no estuvo relacionada con su capacidad para lograr la reducción del TCC. Por lo tanto, estos últimos resultados sugieren la importancia de seleccionar la fuente de SH apropiada para degradar el contaminante de interés, con el objetivo de promover la aplicación de SH inmovilizadas, extraídas de fuentes naturales.

Abstract

Martínez, C.M. Strategies to immobilize humic substances for their application on the reductive biotransformation of recalcitrant pollutants PhD Thesis. IPICYT

Humic substances (HS) and quinone analogues, have lately been explored as redox mediators (RM) for accelerating the redox conversion of priority pollutants, such as azo dyes, aliphatic and aromatic poly-halogenated contaminants, as well as nitroaromatics, which are persistent or poorly reduced in anaerobic wastewater treatment systems. The application of HS or quinone analogues has significantly enhanced the redox conversion of these pollutants. However, the main limitation for the application of HS as RM in continuous wastewater treatment sytems is that their continuous addition must be provided to increase conversion rates, which is economically and environmentally non-viable. Therefore, several attempts to immobilize HS and quinone model compounds have recently been explored to develop engineered systems integrating the redox mediating capacity of immobilized RM in order to promote enhanced conversion rates. This thesis was focused on evaluating two different strategies of immobilization HS (HS adsorbed on an anion exchange resin and on γ-Al_2O_3 nanoparticles) to achieve the redox biotransformation of recalcitrant pollutants in batch cultures and in bioreactors operated in continuous flow experiments. Additionally, this work evaluates the redox mediating capacity of HS derived from organic rich environments and wastes, in order to promote the applicability of immobilized HS derived from natural sources in the removal of recalcitrant pollutants.

The results presented in this work demonstrated for first time that immobilized HS could serve as effective solid-phase RM promoting the simultaneous removal of phenol and reactive red 2 (RR2) in long-term operation of a UASB reactor without the need of continuous supply of the RM. The UASB reactor supplied with immobilized HS, achieved phenol and RR2 removal efficiencies higher than 70% and 85 % respectively, throughout the long-term experiments (Chapter 3). The results presented in Chapter 4 demonstrated that it was possible to achieve the co-immobilization of humus reducing microorganism (HRM) with HS supported on γ-Al_2O_3 nanoparticles (NPs), as a novel strategy of immobilization of HS. Larger granules with appropriate agglomeration of biomass and γ-Al_2O_3 NPs were obtained in the presence of HS as compared to those produced in the absence of HS. However, during the evaluation of the catalytic effect of these granules on the reduction of RR2 and 2,4-dichlorophenol (2,4-DCP), the amount of HS contained in the produced granules and the obstruction of redox active functional groups in HS, could be factors that limited the catalytic effect of these granules during the reduction of RR2 and DCP.Finally, in Chapter 5, it was demonstrated that HS derived from a natural source acted as effective RM during the simultaneous biodegradation of phenol and carbon tetrachloride (CT). The results also indicated that the electron-accepting capacity (EAC) of HS derived from different organic-rich environments depended on the source and characteristics of HS and that the EAC was not related to their reducing capacity to achieve CT dechlorination. Therefore,

the last results obtained suggest the importance of selecting the appropriate source of HS to remove the contaminant of interest in order to promote the applicability of immobilized HS derived from natural sources in the removal of recalcitrant pollutants.

1

Introduction

Due to the increased discharge of wastewaters containing priority pollutants, which have a negative effect on the environment and on public health, new strategies to decrease treatment costs and time required for removal of these contaminants have been proposed.

Conventional and innovative biological treatment processes have received great attention due to their competitive cost, effectiveness, ability to produce less waste and environmental benignity (Chen et al 2003; Volesky 2007). However, several organic industrial and agricultural pollutants, containing electron-accepting functional groups in their structure, are recalcitrant and remain unaffected during conventional aerobic wastewater treatment processes. In contrast, these pollutants are susceptible to reductive biotransformation under anaerobic conditions using high-rate anaerobic bioreactors such as upflow anaerobic sludge blanket (UASB) and expanded granular sludge bed (EGSB) systems, although long hydraulic residence times (HRT) are required in these treatment processes to achieve efficient removal of the contaminants (Van der Zee and Villaverde 2005).

During the last two decades, evidence has demonstrated that addition of redox mediators (RM), such as humic substances (HS) and their quinone model compounds, (*e.g* anthraquinone-2,6-disulfonate, 1,4-benzoquinone, 1,4-naphthoquinone, juglone, menaquinone, lawsone) can enhance the reductive conversion of contaminants in anaerobic treatment systems (Van der Zee and Cervantes 2009).

Initially, humic compounds were studied mostly as source of carbon, micronutrients or for their general impact on the growth of microorganisms (Müller-Wegener, 1988). Subsequently, it was discovered that natural organic matter, particularly HS, and quinone model compounds, were able to accelerate the microbial and abiotic reductive transformation of different electron accepting pollutants (*e.g* azo dyes, nitroaromatics and polychlorinated pollutants, among others) by increasing the rate of redox reactions by several orders of magnitude (Curtis and Reinhard 1994; Rau et al. 2002; Cervantes et al. 2004; Doong and Chiang 2005; Li et al. 2009; Liu et al. 2009) and in some cases, they were indispensable for reactions to take place. However, strategies to immobilize and maintain RM in anaerobic bioreactors are demanded in order to extrapolate their catalytic properties in full-scale applications. In this sense, several attempts to immobilize HS and quinone model compounds have lately been explored in order to promote enhanced conversion rates. However, the vast majority of immobilizing techniques has been proposed and tested with expensive quinone model compounds, and their applicability has only been demonstrated in batch experiments. Nowadays, there are scarce reports showing the impact of immobilized HS on the redox biotransformation of contaminants (Alvarez et al. 2012; Cervantes et al. 2011b; Cervantes et al. 2013). Although these investigations have demonstrated that immobilized HS had good catalytic capacity during the redox processes evaluated, further studies are demanded to optimize the immobilizing techniques before application in full-scale wastewater treatment systems.

This thesis is focused on evaluating two different strategies to co-immobilize HS and humic-reducing microorganisms (HRM) to achieve the redox biotransformation

of recalcitrant pollutants in batch and continuous flow experiments. The immobilizing techniques tested include HS adsorbed on an anion exchange resin and on γ-Al_2O_3 nanoparticles, which have previously been reported to serve as effective solid-phase RM during the redox conversion of contaminants by anaerobic consortia (Alvarez et al. 2012; Cervantes et al. 2010). Additionally, this work evaluates the redox mediating capacity of HS derived from organic rich environments and wastes during the biodegradation of contaminants.

Thesis rationalization

Several techniques to immobilize RM have been reported in recent years (Alvarez et al. 2010; Cervantes et al. 2010; Guo et al. 2007; Guo et al. 2010; Lu et al. 2010; Wang et al. 2009b; Yuan et al. 2012). All these studies reported that the immobilization procedure applied did not limit the catalytic properties of RM tested, thus demonstrating the effective role of immobilized RM on the redox biotransformation of organic pollutants. However, in all these studies electron-shuttling model compounds were used. Scarce reports have shown the impact of immobilized HS on the redox biotransformation of contaminants (Alvarez et al. 2012; Cervantes et al. 2011b). Although these investigations demonstrated that immobilized HS had good catalytic capacity during the redox processes evaluated, the application of immobilized HS as RM is an issue that had not been studied and requires further studies to optimize the redox conversion of recalcitrant pollutants during for their application in full-scale wastewater treatment systems. This dissertation will contribute to develop two strategies to co-immobilize HS and HRM in anaerobic bioreactors. Immobilized HS will then be tested for their effectiveness

to serve as solid-phase RM during the biodegradation of recalcitrant pollutants, commonly found in industrial effluents, such as phenol compounds, azo dyes and polihalogenated solvents. The thesis will also contribute on the understading of the mechanisms involved during the biodegradation of the studied contaminants, mediated by immobilized HS, based on the characterization of HS evaluated and monitoring of the products derived from the reductive biotransformation of the pollutants. Moreover, this work will contribute to identify suitable natural sources of HS for their application in the biodegradation of contaminants.

Hypotheses

The principal hypotheses of this thesis are:

1) Immobilized HS on an anion exchange resin will serve as effective RM to achieve the biodegradation of phenol and reactive red 2 in long-term operation of a UASB reactor.
2) Granulation will be an efficient strategy to co-immobilized HS and HRM in anaerobic bioreactors. Immobilized HS by this technique will maintain their catalytic properties to enhance the redox conversion of reactive red 2 and 2,4-dichlorophenol.
3) The capacity of HS derived from organic rich enviroments and wastes and wastes to achieve the reductive dechlorination of carbon tetrachloride will depend on the electron accepting capacity of these HS. HS derived from one of these organic sources will act as effective RM during the simultaneous biodegradation of phenol and carbon tetrachloride.

General objective

Evaluate the redox-mediating capacity of immobilized HS during the simultaneous biodegradation of recalcitrant pollutants in batch and continuous flow experiments.

Particular objectives

1. Evaluate the redox-mediating capacity of immobilized HS on an anion exchange resin, to achieve the simultaneous removal of phenol and Reactive Red 2, in a continuous high-rate anaerobic reactor.
2. Evaluate the capacity of a granulation process to co-immobilize humus reducing microorganisms and HS supported on γ-Al_2O_3 nanoparticles and assess their redox mediating capacity during the reductive biotransformation of Reactive Red 2 and 2,4-dichlorophenol.
3. Evaluate the redox mediating capacity of HS derived from organic rich environments and wastes, during the simultaneous biodegradation of phenol and carbon tetrachloride by anaerobic consortia.

Thesis outline

Chapter 2 of this thesis presents a literature review reporting the contribution of HS in different environmental processes, emphasizing its role on the redox conversion of recalcitrant pollutants in anaerobic wastewater treatment systems. Chapter 3 presents the application of immobilized HS on an anion exchange resin, to serve as RM to achieve the removal of two representative pollutants commonly found in textile effluents (phenol and Reactive Red 2) in a UASB reactor. Chapter 4 reports the co-immobilization of humus reducing microorganism (HRM) with HS coated γ-

Al_2O_3 nanoparticles by granulation. Likewise, this chapter presents the application of these granules on the reductive biotransformation of two recalcitrant pollutants, Reactive red 2 (RR2) and 2,4-dichlorophenol (2,4-DCP). Chapter 5 describes the electron transferring capacity of three different sources of HS derived from organic rich environments and wastes. Furthermore, selected source of HS was applied to achieve the simultaneous removal of phenol and carbon tetrachloride (CT) in batch tests. Finally, chapter 6 presents the final remarks and future prospects of this thesis.

2

Contribution of HS in different environmental process

Abstract

Humic substances (HS) constitute a very abundant class of organic compounds that are chemically heterogeneous and widely distributed in terrestrial and aquatic environments. Evidence accumulated during the last decades indicates that HS play relevant roles on the transport, fate and redox conversion of organic and inorganic compounds both in chemically and microbially driven reactions. The present review underlines the contribution of humus reducing microorganisms in relevant environmental processes, such as biodegradation of recalcitrant pollutants; redox conversion of industrial contaminants in anaerobic wastewater treatment systems; and on the microbial production of nano-catalysts and alternative energy sources.

A modified version of this chapter has been published as: Martinez CM, Alvarez LH, Celis LB, Cervantes FJ (2013) Humus reducing microorganisms and their valuable contribution in environmental processes. Appl Microbiol Biotechnol 97: 9897-9905

2.1 Introduction

Humic substances (HS) are the stable organic matter accumulating in soil from the decomposition of plant litter. These are composed of high molecular weight dark colored substances. The composition of HS is largely heterogeneous, but the core structure is best represented as a condensed polyphenol with quinone moieties (Figure 2.1). The core polyphenolic structure is occasionally substituted with amines and aliphatics (Stevenson 1994). HS are considered to be a product of phenol polymerization, involving both phenols derived from the fungal breakdown of lignin and tannins, as well as phenols formed as secondary metabolites of plants and microorganisms. The polymerization is caused by oxidative coupling catalyzed by oxidative enzymes (peroxidases, laccases, phenol oxidases, among others) and metal oxides (*e.g.* Mn^{4+}), as well as by autoxidation. During the coupling reactions, amino acids are copolymerized into the polymers (Field 2001).

HS are very resistant to biodegradation as evidenced by its millenary residence time in soil ranging up to a few thousand years (Stevenson 1994). Thus, it is evident that the carbonaceous structure of HS is hardly metabolized by microorganisms. Hundreds to thousands of years or even geological time scales are required before assimilation of carbon through biological processes (Field 2001). Nevertheless, HS play important roles in metals transport and soil fertility (Macalady and Walton-Day 2011). Furthermore, the presence of redox mediating functional groups in HS promote three important roles, which significantly impact the redox conversion and fate of a large variety of inorganic as well as organic contaminants: 1) as terminal electron acceptor for microbial respiration; 2) as redox mediator (RM) (electron shuttle) between an electron donor and an electron-accepting compound; and 3) as

electron donor for the microbial reduction of more oxidized electron acceptors. Different literature reviews have already reported on the microbial utilization and transformation of HS (Filip et al. 2011); on the microbial interactions with HS (Van Trump et al. 2006); and on their application as redox RM for bioremediation purposes (Van der Zee and Cervantes 2009). The present review provides comprehensive and updated information regarding the biodiversity of humus reducing microorganisms (HRM) and the redox mediating functional groups in HS. The review also underlines the role of HS on (1) anaerobic microbial oxidation of recalcitrant pollutants, (2) mitigation of greenhouse gases emission from anoxic environments, (3) redox conversion of recalcitrant pollutants in anaerobic wastewater treatment systems, and (4) new developments such as microbial production of nanocatalysts and alternative energy sources.

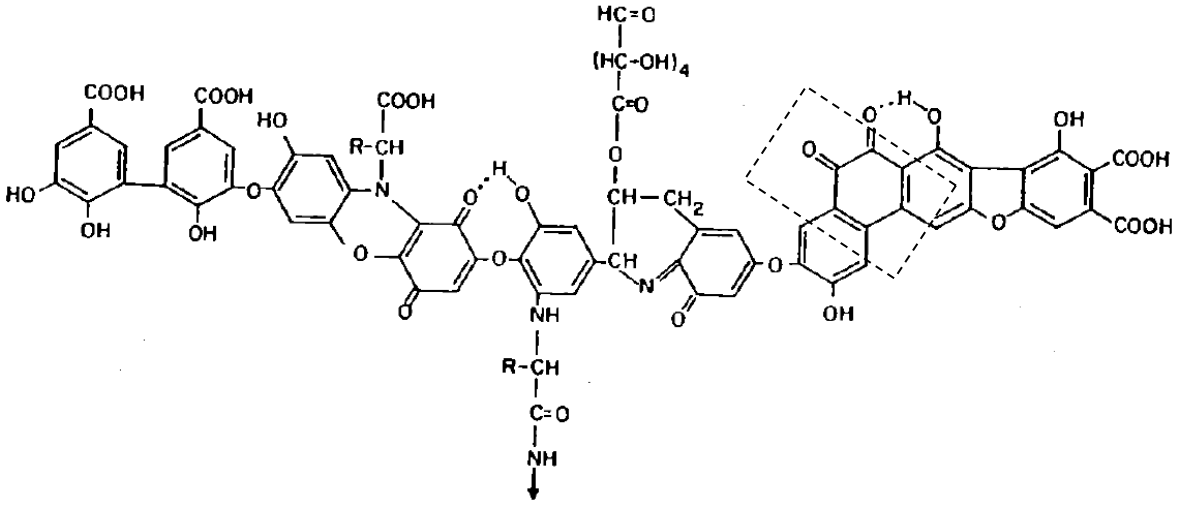

Figure 2.1 Model structure of humic substances (Stevenson, 1994). Dashed squares indicate quinone group

2.2 Biodiversity of humus reducing microorganisms

The pioneer study reporting the capacity of microorganisms to utilize HS as terminal electron acceptor (TEA) was reported by Lovley et al. (1996a). This research group firstly observed that the application of HS, as a chelating agent to increase the availability of Fe(III) oxides, promoted a greater biodegradation of hydrocarbons by Fe(III)-reducing microorganisms as compared to synthetic chelating compounds (Lovley et al. 1996b), even though HS has an inferior chelating capacity. Based on the findings reported by Tratnyek and Macalady (1989), Lovley et al. (1996a) hypothesized that microorganisms could be capable of transferring electrons derived from anaerobic microbial oxidation of these contaminants to redox active functional groups (*e.g.* quinones) in HS. Therefore, it was demonstrated that the iron-reducing bacteria *Geobacter metallireducens* and *Shewanella alga* could use humic acids (HA) and the humic model compound, anthraquinone-2,6-disulfonate (AQDS), as TEA coupled to the anaerobic oxidation of acetate and hydrogen (Lovley et al. 1996a). Following reports suggested the ubiquity of HRM based on a wide diversity of environments in which microbial HS or quinone reducing activities were observed (Coates et al. 1998; Cervantes et al. 2000a). Since then, the topic has extensively been studied and the reported works indicate a widespread variety of microorganisms that have the capacity of reducing humus or quinoid model compounds. Most HRM are Fe(III)-reducing bacteria and archaea, but the vast phylogenetic miscellany also includes denitrifying, sulfate-reducing, halorespiring, fermentative and even methanogenic microorganisms (Table 2.1). Nevertheless, microbial reduction of humus is not universal and there are reports documenting the

lack of some microorganisms, including Fe(III)-reducers like *Georgfuchsia toluolica* (Weelink et al. 2009), to use HS or quinones as TEA.

Most microorganisms with the capacity to transfer electrons to HS or quinoid analogues have coupled this reducing process to microbial growth, which is recognized as humus respiration. Nevertheless, anoxic substrates oxidation linked to humus reduction by several microorganisms, including all archaea tested, did not yield detectable microbial growth (Table 2.1), suggesting unspecific biochemical activity in these cases. The term humus-reducing microorganisms is generally used for those with the capacity to couple anoxic substrates oxidation to extracellular electron transfer to HS regardless if the microbial process is linked to microbial growth.

The biochemical pathway involved in the microbial reduction of HS has a number of similarities with the extracellular electron transfer to insoluble electron acceptors, such as metal oxides and electrodes. For instance, the c-type cytochrome Omcs is a common electron carrier involved in the microbial respiration of insoluble Fe(III) and HS by *Geobacter sulfurreducens* (Qian et al. 2011). Moreover, menaquinone plays a key role transferring electrons during the microbial reduction of both metal oxides and HS in *Shewanella* species (Newman and Kolter 2000; Bird et al. 2011). A recent review presented the updated understanding related to biochemical mechanisms involved in the microbial reduction of insoluble Fe(III), which are expected to be related with those involved in the microbial reduction of HS (Bird et al. 2011). However, considering the extensive diversity of humus-reducing microorganisms, much remains to be elucidated to establish the biochemical basis

supporting the microbial reduction of HS, for instance by fermentative bacteria and methanogens.

Table 2.1 Phylogenetic Diversity of Quinone- or Humus-Reducing Microorganisms Reported in the Literature

Phylogeny	Strain	TEA*	Growth*	Reference
Archaea				
Methanococcales	*Methanococcus thermolithitrophicus*	AQDS	ND	Lovley et al. 2000
	Methanococcus voltaei	AQDS	-	Bond and Lovley 2002
Methanobacteriales	*Methanobacterium thermoautotrophicum*	AQDS	ND	Lovley et al. 2000
	Methanobacterium palustre	AQDS, HA	-	Bond and Lovley 2002
Methanosarcinales	*Methanosarcina barkeri*	AQDS, HA	-	Bond and Lovley 2002
	Methanolobus vulcani	AQDS	-	Bond and Lovley 2002
	Methanosphaera cuniculi	AQDS, HA	-	Bond and Lovley 2002
Methanomicrobiales	*Methanospirillum hungatei*	AQDS	-	Cervantes et al. 2002
Methanopyrales	*Methanopyrus kandleri*	AQDS	ND	Lovley et al. 2000
Thermoproteales	*Pyrobaculum islandicum*	AQDS, HA	ND	Lovley et al. 2000
	Pyrodictium abyssi	AQDS	ND	Lovley et al. 2000
	Thermococcus celer	AQDS	ND	Lovley et al. 2000
Thermococcales	*Pyrococcus furiosus*	AQDS	ND	Lovley et al. 2000
Archaeoglobales	*Archaeoglobus fulgidus*	AQDS	ND	Lovley et al. 2000
Bacteria				
Gram Negative				
γ-Proteobacteria	*Pantoea agglomerans*	AQDS	+	Francis et al. 2000
	Shewanella alga	AQDS, HA	+	Fredrickson et al. 2000b
	Shewanella putrefaciens	AQDS	+	Lee et al. 2000

Table 2.1 Continued

	Shewanella sacchrophila	AQDS	ND	Lovley et al. 1998
	Shewanella cinica	HA	ND	Huang et al. 2010
	Aeromonas hydrophila	AQDS	ND	Lovley et al. 1998
	Geospirillum barnseii	AQDS	ND	Lovley et al. 1998
	Wolinella succinogenes	AQDS, HA	ND	Lovley et al. 1998
	Escherichia coli K12	AQS, L	ND	Rau et al. 2002
α-Proteobacteria	*Sphingomonas xenophaga* BN6	AQS	ND	Rau et al. 2002
	Paracoccus versutus GW1	AQDS	+	Li et al. 2013
	Azospirillum humicireducens	AQDS	+	Zhou et al. 2012
β-Proteobacteria	*Ralstonia eutropha335*	AQS	ND	Rau et al. 2002
	Thauera humireducens	AQDS	+	Yang et al. 2013a
	Comamonas koreensis strain *CY01*	AQDS	+	Wang et al. 2009a
δ-Proteobacteria	*Geobacter metallireducens*	AQDS, HA	+	Lovley et al. 1996a
	Geobacter sulfurreducens	AQDS, HA	ND	Lovley et al. 1998
	Geobacter humireducens	AQDS, HA	ND	Lovley et al. 1998
	Geobacter JW-3	AQDS, HA	+	Coates et al. 1998
	Geobacter TC-4	AQDS, HA	+	Coates et al. 1998
	Geobacter grbiciae	AQDS	ND	Coates et al. 2001
	Desulfovibrio G11	AQDS	-	Cervantes et al. 2002
	Desulfovibrio idahonensis	AQDS	ND	Sass et al. 2009
	Desulfovibrio vulgaris	DMBQ, NQ	ND	Tatsumi et al. 2000
	Desulfuromonas acetexigens	AQDS	ND	Lovley et al. 1998
	Desulfuromonas SDB-1	AQDS	+	Coates et al. 1998
	Desulfuromonas FD-1	AQDS	+	Coates et al. 1998
	Desulfobulbus propionicus	AQDS	+	Holmes et al. 2004

Table 2.1 Continued

Deinococci	*Deinococcus radiodurans*	AQDS	-	Fredrickson et al. 2000a
Halanaerobiales	*Fuchsiella alkaliacetigena*	AQDS	+	Zhilina et al. 2012
Thermotogales	*Thermotoga maritima*	AQDS	ND	Lovley et al. 2000
Thermoanaerobacteriales	*Calderihabitans maritimus*	AQDS	+	Yoneda et al. 2013
Gram positives				
Thermoanaerobacteriales	*Thermoanaerobacter siderophilus*	AQDS	+	Slobodkin et al. 1999
	Carboxydothermus pertinax	AQDS	+	Yoneda et al. 2012
	Carboxydothermus ferrireducens	AQDS	+	Gavrilov et al. 2012
	Carboxydothermus siderophilus	AQDS	+	Slepova et al. 2009
	Carboxydothermus hydrogenoformans	AQDS	+	Henstra and Stams 2004
	Thermoterrabacterium ferrireducens	AQDS	+	Henstra and Stams 2004
	Moorella humiferrea	AQDS, HA	+	Nepomnyashchayaa et al. 2012
Bacillales	*Bacillus subtilis*	AQS, AQDS, L	ND	Rau et al. 2002
	Bacillus pseudofirmus MC02	AQDS, HA	+	Ma et al. 2012
	Planococcus sp. MC01	AQDS	ND	Ma et al. 2013
	Bacillus thermotolerans	AQDS	+	Yang et al. 2013b
	Anaerobacillus alkalilacustre	AQDS	+	Zavarzina et al. 2009
Actinomycetales	*Propionibacterium freudenreichii*	HA	-	Benz et al. 1998
	Kocuria rosea HN01	AQDS	ND	Chen et al. 2013a
	Corynebacterium humireducens MFC-5	HA, FA	ND	Wu et al. 2012
Lactobacillales	*Enterococcus cecorum*	HA	-	Benz et al. 1998
	Lactococcus lactis	HA, ACNQ	ND	Benz et al. 1998

Table 2.1 Continued

	Lactobacillus plantarum	ACNQ	ND	Yamazaki et al. 2002
Clostridiales	*Desulfitobacterium hafniense DP7*	AQDS	+	Luijten et al. 2004
	Thermincola ferriacetica	AQDS	+	Zavarzina et al. 2007
	Thermincola potens	AQDS	ND	Carlson et al. 2012
	Desulfitobacterium dehalogenans	AQDS, HA	+	Cervantes et al. 2002
	Desulfitobacterium PCE1	AQDS	+	Cervantes et al. 2002
Campylobacteriales	*Sulfurospirillum barnesii*	AQDS	+	Luijten et al. 2004
	Sulfurospirillum deleyianum	AQDS	+	Luijten et al. 2004
	Sulfurospirillum arsenophilum	AQDS	+	Luijten et al. 2004

*AQDS, anthraquinone-2,6-disulfonate; AQS, anthraquinone-2-sulfonate; ACNQ, 2-amino-3-carboxy-1,4-naphthoquinone; NQ, 1,4-naphthoquinone; DMBQ, 2,6-dimethyl-1,4-benzoquinone; L, lawsone (2-hydroxy-1,4-naphthoquinon); HA, humic acids; FA, fulvic acids; TEA, terminal electron acceptor; ND, not determined. Positive symbol indicates that microbial reduction of quinones or humus was linked to growth; negative symbol indicates no correlation between reduction of quinones or humus to growth.

2.3 Redox mediating functional groups in HS

The pioneer studies documenting the role of HS on the reduction of nitroaromatic compounds pointed out at quinones as the RM functional groups in HS, stimulating the transfer of electrons during the redox reactions evaluated (Tratnyek and Macalady 1989; Dunnivant et al. 1992). Several lines of evidence subsequently collected, support the hypothesis that quinones are the main electron transferring functional groups in humus. Electron spin resonance measurements revealed direct evidence that quinone moieties are the actual functional groups accepting electrons during the microbial reduction of HS (Scott et al. 1998). Moreover, genetic evidence provided a common biochemical basis for quinone and humus reduction in *Shewanella putrefaciens MR*. The study showed that menaquinone was involved in the electron transport chain of *S. putrefaciens MR* during the reduction of HS and the quinone model compound AQDS. Mutants of this organism, lacking the ability to

synthesize menaquinone, were unable to reduce both AQDS and HS (Newman and Kolter 2000). Further experiments showed a high agreement between the quinone content of HS samples, determined by the method developed by Schnitzer and Riffaldi (1972), with their electron transferring capacities (ETC) (Sposito 2011). A high correlation was also reported between the ETC and the infrared spectra intensity corresponding to quinone moieties (~1600-1700 cm^{-1}) in different HA extracted from a variety of organic rich environments (Hernández-Montoya et al. 2012). Additional studies have also reported a linear correlation of the electron accepting capacity of a dozen of commercial HS samples, determined by an electrochemical method, with their C/H molar ratio and aromaticity (Aeschbacher et al. 2010). The authors concluded that quinone moieties likely dominated these redox reactions. Likewise, the values of electron accepting capacity obtained in this investigation were strongly and positively correlated with those reported by Ratasuk and Nanny (2007) for commercial humic samples. All this information suggests that quinones are important redox functional moieties in humus.

Further studies have also suggested that non-quinone functional groups in HS samples significantly contribute to their ETC. Firstly, Ratasuk and Nanny (2007) showed that HA reduced at pH 6.5 in a H_2/Pd reaction system apparently had lost their quinone moieties; however, these HA did not lose their ETC (determined by reaction with ferric citrate). By selectively reducing HS samples at pH 6.5 in a H_2/Pd reaction system, quinone groups are no longer available because they are protonated forming phenolic groups (pKa=9.9), thus blocking the ETC attributable to quinone moieties. Further evidence of this blocking mechanism avoiding quinone reduction was revealed by infrared spectra (Hernández-Montoya et al. 2012).

Indeed, HS samples previously reduced with H_2-Pd at pH 8 showed the typical spectral signal associated with hydroquinone groups at ~1360 cm^{-1}. In contrast, HS samples previously reduced with H_2-Pd at pH 6.5 did not show the hydroquinone spectral signal, but aromatic ketone groups remained intact. These studies also revealed that non-quinone functional groups accounted for up to 44-58% of the total ETC quantified in a variety of HS samples derived from organic rich environments (Ratasuk and Nanny 2007; Hernández-Montoya et al. 2012). Furthermore, it was verified that the blocking mechanism imposed in the H_2/Pd reaction system at pH 6.5 was reversible and that *Geobacter sulfurreducens* was capable of reducing both quinones and non-quinone functional groups in different HS samples (Hernández-Montoya et al. 2012).

The significant electron transferring capacity detected in these humic samples linked to non-quinone functional groups might be associated with nitrogenous and sulfurous redox mediating functional groups, such as dimethyl sulfone, 3-(methylthio)-propanoic acid, N-methyl aniline, and 1-methyl-2,5-pyrrolidinedione, as reported by Fimmen et al. (2007).

There are ongoing efforts to coherently understand the chemical structure and the redox chemistry involved in abiotic and microbial electron transfer in HS. An extensive variety of spectroscopic and chromatographic techniques have been employed to characterize the structural features of HS. Prominent among the non-destructive methods are infrared, ultraviolet-visible, fluorescence, and nuclear magnetic resonance spectroscopies, mass spectroscopy and all its variations; X-ray techniques, and chromatographic methods, such as gel electrophoresis. Regarding analytical tools employed to understand the redox chemistry of HS, the most useful

reported so far are Fourier-Transformed Infrared Spectroscopy, electron paramagnetic resonance, and fluorescence spectroscopy. These techniques have contributed to elucidate the redox state and redox activity of HS (for an updated overview see Macalady & Walton-Day 2011). Redox state refers to the relative extent that the redox active functional groups are oxidized or reduced; whereas redox activity is described in terms of the capacity of HS to accept and donate electrons. Despite the significant advances to reveal the chemical aspects determining the redox state and redox activity of HS, greater efforts are required to reach a more complete understanding of these issues in order to predict the behavior of HS in biogeochemical processes occurring in natural ecosystems and in HS-mediated redox reaction taking place in engineered systems.

2.4 Humic substances as terminal electron acceptors for bioremediation purposes

After recognition of HS reduction as a microbial respiratory process (Lovley et al. 1996a), several studies have been reported on the capacity of microbial consortia to achieve the anaerobic oxidation of recalcitrant pollutants linked to the reduction of HS or humic model compounds (Table 2.2). Bradley et al. (1998) firstly reported the mineralization of vinyl chloride and dichloroethene coupled to the reduction of HA or AQDS by organic rich sediment. The anaerobic oxidation of phenolic compounds was also achieved by AQDS-reducing consortia (Cervantes et al. 2000b). Further studies documented the important role of AQDS-reducing consortia on the anaerobic oxidation of phenolic compounds even in the presence of alternative electron acceptors, such as nitrate and sulfate (Cervantes et al. 2008). Hydrocarbons have also been degraded by humus-reducing consortia. The

mineralization of toluene was firstly documented in humus-reducing sediments. Mineralization of [^{13}C] toluene to $^{13}CO_2$ was linked to the microbial reduction of HA or AQDS by enriched consortia (Cervantes et al. 2001a). Furthermore, the anaerobic oxidation of benzene coupled to HA or AQDS reduction was evidenced in an enrichment culture derived from a contaminated lagoon (Cervantes et al. 2011a). Moreover, it has been reported the anaerobic oxidation of methyl-*tert*-butyl-ether under AQDS-reducing conditions by an enrichment culture originated from a contaminated sediment (Wei and Finneran 2009). More recently, the anaerobic oxidation of benzene (Zhang et al. 2012) and phenanthrene (Ma et al. 2011) by pure cultures was documented under AQDS-reducing conditions. Thus, HS may be a more important TEA promoting the bioremediation of contaminated environments than previously considered. Organic rich environments are of particular interest because HS could support the anaerobic oxidation of organic pollutants by serving as TEA, thus contributing to the intrinsic bioremediation of these ecosystems. HS may also greatly stimulate the anoxic biodegradation of organic contaminants in oligotrophic environments linking the anaerobic oxidation of these pollutants to the reduction of other TEA. Particularly, HS may channel electrons from anoxic pollutant oxidation to metal oxides reduction by serving as electron shuttles; a scenario which was shown to occur during the anaerobic oxidation of hydrocarbons (Lovley et al. 1996b; Anderson and Lovley 1999; Cervantes et al. 2001a). Furthermore, recycling of redox mediating functional groups in HS has also been promoted during the simultaneous biodegradation of phenol and carbon tetrachloride by anaerobic consortia (Martinez et al. 2012).

Table 2.2 Organic priority pollutants biodegraded under anaerobic conditions with humic substances serving as terminal electron acceptor

Pollutant	TEA	Inocula	Reference
***cis*-dichloroethene**	AQDS	Organic rich stream sediments	Bradley et al. 1998
Vinyl chloride	AQDS, HA	Organic rich stream sediments	Bradley et al. 1998
***p*-cresol**	AQDS	Anaerobic sludge	Cervantes et al. 2000b
Phenol	AQDS	Anaerobic sludge	Cervantes et al. 2000b
Toluene	AQDS, HA	Enrichment culture from sediments	Cervantes et al. 2001a
MTBE	AQDS	Contaminated aquifer sediment	Wei and Finneran 2009
Benzene	AQDS, HA	Sediment from contaminated lagoon	Cervantes et al. 2011b
Phenanthrene	AQDS	Pure culture of *Pseudomonas aeruginosa*	Ma et al. 2011
Benzene	AQDS	Pure culture of *Geobacter sp.*	Zhang et al. 2012

AQDS, anthraquinone-2,6-disulfonate; HA, humic acids; MTBE, methyl-*tert*-butyl-ether; TEA, terminal electron acceptor

Even though the evidence collected during the last decades clearly indicate the great potential to apply HS for accelerating the redox (bio)transformation of contaminants, scarce patents or technological developments have been reported, which take advantage of the redox properties of HS for cleaning up contaminated sites (Perminova et al. 2007b; Cervantes et al. 2012). Therefore, further efforts should be directed to develop engineered treatment processes integrating suitable sources of HS for these applications.

2.5 Impact of humic substances on mitigation of greenhouse gases emissions from anaerobic ecosystems

Several investigations have suggested that HS may play a significant role on decreasing the emission of greenhouse gases in different environments. For instance, Bradley et al. (1998) reported that methane biosynthesis (in an organic

rich sediment) linked to the mineralization of vinyl chloride and dichloroethene, shifted toward CO_2 production when the sediment was supplemented with HA as terminal electron acceptor. An increase in the CO_2:CH_4 ratio during the anaerobic oxidation of organic substrates is ecologically relevant because CH_4 is significantly more powerful as a greenhouse gas than is CO_2. Later, Cervantes et al. (2000a, 2008) reported the inhibition of methanogenesis in sludge and sediment incubations provided with AQDS as terminal electron acceptor. Reduction of AQDS became the preferred pathway over methanogenesis when volatile fatty acids, hydrogen or phenolic compounds were supplied as substrates. From the thermodynamic point of view, AQDS reduction is more favorable than methanogenesis. Thus, thermodynamics might play an important role for the prevalence of quinone respiration over methanogenesis (Cervantes et al. 2000a).

However, competition and thermodynamics are not the only reasons to explain the occurrence of quinone reduction instead of methanogenesis. Indeed, several methanogenic archaea have been shown to shift their physiology from CH_4 production towards the reduction of AQDS (Lovley et al. 2000; Cervantes et al. 2002). Thus, a decrease of CH_4 production in organic rich environments may also be explained by this metabolic versatility of methanogens.

Under methanogenic conditions after inorganic terminal electron acceptor (NO_3^-, Fe (III), Mn (IV) and SO_4^{2-}) have been depleted, CO_2 and CH_4 are produced in equal amounts resulting in a 1:1 ratio of CO_2:CH_4 (Conrad 1999). However, some studies have reported that the ratio of CO_2:CH_4 in many freshwater peatland soils is frequently >1, suggesting the reduction of non-methanogenic terminal electron acceptor in these freshwater environments (Blodau 2002; Yavit and Seidman-Zager

2006). In this sense, a number of researchers have attributed the high CO_2:CH_4 ratio to utilization of HS as organic terminal electron acceptor (Blodau and Deppe 2012; Heitman et al. 2007; Keller and Bridgham 2007; Keller et al. 2009; Neubauer et al. 2005).

Recently, Heitmann et al. (2007) demonstrated that dissolved organic matter acted as an important electron acceptor in a Canadian peatland, contributing indirectly (by regenerating oxidized sulfur species for sulfate reduction) to high CO_2:CH_4 ratios. Likewise, Keller et al. (2009), who evaluated the effect of different sources of HS in the production of CO_2 and CH_4, reported that HS could strongly limit the production of CH_4 by serving as terminal electron acceptor in wetlands. More recently, results obtained by Blodau and Deppe (2012) also demonstrate that HS served as terminal electron acceptor and contributed to decrease CH_4 release from the Mer Bleue bog, Canada.

Although not yet experimentally proven, but proposed by Blodau and Deppe (2012), there is another mechanism by which humus could also diminish methane emissions from organic rich environments: by serving as terminal electron acceptor to promote anaerobic methane oxidation in these ecosystems. This is thermodynamically feasible as HS have similar or even more positive standard redox potential (Straub et al. 2001) than terminal electron acceptor previously reported to support anaerobic methane oxidation (*e.g.* sulfate).

Recent reports have also documented the role of reduced HS (*e.g.* hydroquinones) on mitigating emission of greenhouse gases. The reduced form of AQDS (anthrahydroquinone-2,6-disulfonate, AH_2QDS) could promote the reduction of nitrous oxide by a denitrifying consortium by serving as a sole electron donor

(Aranda-Tamaura et al. 2007). Thus, reduced HS could significantly contribute to attenuate emissions of this powerful greenhouse gas in soils and sediments.

Most HS present in soils and sediments are in particulate form, rather than dissolved, and the capacity of humus reducing microorganisms to reduce solid-phase HS has recently been demonstrated (Roden et al. 2010). Keller & Takagi (2013) reported that microbial reduction of solid-phase HS is responsible of up to 61% of the total carbon mineralization in a bog soil, thus suppressing methane emissions in this ecosystem. However, further *in situ* measurements are required in order to decipher more precisely the role of solid-phase HS on mitigating the emission of greenhouse gases in organic rich environments. New studies should also be focused on challenging issues, such as documenting this role of HS in Arctic peat soil (Lipson et al 2010) and other organic rich ecosystems, which are dramatically changing due to the global climate change currently in course.

2.6 Application of quinones and humic substances for the redox conversion of recalcitrant pollutants in anaerobic wastewater treatment systems

During the last two decades evidence has demonstrated the effective role of HS and quinone analogues as RM for accelerating the redox conversion of priority pollutants, such as azo dyes, aliphatic and aromatic poly-halogenated compounds, nitroaromatics and metalloids (Figure 2.2). Some reviews have already underlined the potential of applying these RM in wastewater treatment systems (Van der Zee and Cervantes 2009; Watanabe et al. 2009). Since then, research has expanded their role to a broader spectrum of contaminants, including 2,4-dichlorophenoxyacetic acid (Wang et al. 2009a), 4-chlorobiphenyl (Wang et al. 2011a), pentachlorophenol (Zhang and Katayama 2012), 1,1,1-trichloro-2,2-bis(4-

chlorophenyl) ethane (DDT, Cao et al. 2012) and tetrabromobisphenol-A (Wang et al. 2013a).

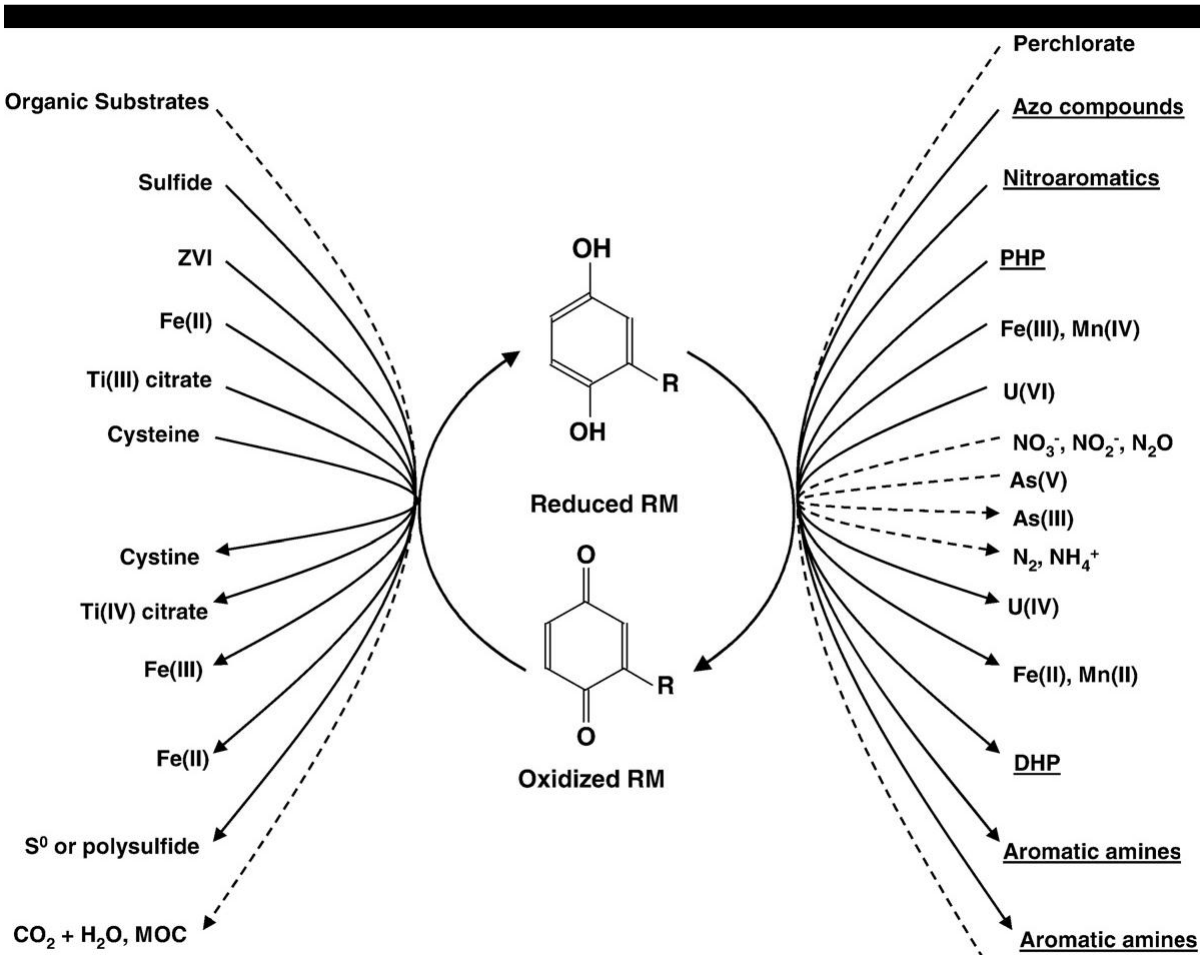

Figure 2.2 Abiotic (solid lines) and microbial reactions (dashed lines) in the reduction and oxidation of electron shuttles (e.g. quinones). ZVI, zero valent iron; MOC, more oxidized compounds; PHP, polyhalogenated pollutants; DHP, dehalogenated pollutants (Van der Zee and Cervantes 2009)

Most of these contaminants are persistent or poorly reduced in anaerobic wastewater treatment systems and the application of HS or quinone analogues have significantly enhanced their redox conversion (Cervantes et al. 2001b; Van der Zee et al. 2001). Nevertheless, continuous addition of RM should be provided to

maintain the increased conversion rates, which is economically and environmentally non-viable.

Therefore, several attempts to immobilize HS and quinone model compounds have lately been explored to develop engineered systems integrating the redox mediating capacity of immobilized RM in order to promote enhanced conversion rates. Table 2.3 provides a compilation of studies reporting the impact of immobilized RM to accelerate the redox conversion of contaminants. RM have been immobilized by entrapment in calcium alginate, polyvinyl alcohol-H_3BO_3 and agar (Guo et al. 2007), by covalent binding on ceramsites (Yuan et al. 2012) and polyurethane foam (Lu et al. 2010; Wang et al. 2013b), by adsorption on metal oxides nanoparticles (Alvarez et al. 2010), by electrostatic attraction on the surface of anion exchange resins (Cervantes et al. 2010), and by electro-polymerization on activated carbon felt (Wang et al. 2009b).

Table 2.3 Impact of immobilized redox mediators on the reductive biotransformation of priority pollutants

Redox mediator[a]	Supporting material	Immobilization mechanism	Pollutants[b]	Results[c]	References
Quinone model compounds					
Anthraquinone	Calcium alginate	Entrapment	AB, RBR, AS, ARB, ARG, RBRK	1.5-2 + for all dyes, with CRE >95% at different pH (6-10), with a good reusability after four cycles	Guo et al. 2007
Anthraquinone	Calcium alginate	Entrapment	Nitrate	Denitrification rate: 2.0 +	Guo et al. 2010

Table 2.3 Continued

AQDS	Calcium alginate	Entrapment	AR3R	CRE was ~100% and 34% for treatment with and without AQDS respectively, after 12 h of incubation	Su et al. 2009
AQDS	Activated carbon felt	Electro-polymerization	NB, DNT	NB: 5.1 +; 2,4-DNT: 5.7 +; 2,6-DNT: 4.8 +. RE >90% after six cycles for 2,4-DNT	Li et al. 2008
AQDS	Activated carbon felt	Electro-polymerization	RR120	3.2 + and 80% of CRE after six repeated experiments	Li et al. 2009
AQDS	Activated carbon felt	Electro-polymerization	5 reactive dyes, 4 acid dyes and 2 direct dyes	CRE ranging between 65-92%, with 1-3 + for all dyes tested	Wang et al. 2009b
AQDS	$Al(OH)_3$ (15 nm)	Adsorption	RR2	7.5 +, with a CRE of 43% after 12 h with AQDS	Alvarez et al. 2010
AQDS, NQS	Anion exchange resin	Anion exchange	RR2, MO, MR	with AQDS RR2: 1.9 +; MO: 3.4 +; MR: 2.5 + with NQS RR2: 3.8 +; MO: 8.8 +; MR: 3.5 +	Cervantes et al. 2010
AQS	Polyurethane foam	Covalent	Amaranth	5.0 +, with CRE of 98.7% after 10 repeated experiments	Lu et al. 2010
AQS	Ceramsites	Adsorption/ Covalent	AY36, RR2, AR27, AO7	CRE over 98% for all azo dyes	Yuan et al. 2012

Table 2.3 Continued

AQDS	Polyurethane foam	Covalent	Nitrobenzene	Nitrobenzene bio-reduction 95.6% +, aniline formation 94.3%+	Wang et al. 2013b
Humic substances					
HA	Ion exchange resin	Anion exchange	CT, RR2	CT: 4.0 +, with RE of ~74% to CF RR2: 2.0 +, with CRE of ~70%	Cervantes et al. 2011b
FA	γ-Al_2O_3 (63 µm)	Adsorption	CT	Adsorption and reduction of CT was observed. 10.4 +, with a RE of 90%	Alvarez et al. 2012
HA	Ion exchange resin	Anion exchange	Phenol and RR2	UASB reactor supplied with immobilized HA showed color and phenol removal efficiencies higher than control UASB reactor without HA	Martinez et al. (2013)

[a] AQS, anthraquinone-2-sulfonate; AQDS, anthraquinone-2,6-disulfonate; NQS, 1,2-Naphthoquinone-4-sulfonate; HA, humic acids; FA, fulvic acids

[b] AB, Acid Black 10B; RBR, Reactive Brilliant Red X-3B; AS, Acid Scarlet GR; ARB, Acid Red B; ARG, Acid Red G; RBRK, Reactive Brilliant Red K-2BP; NB, Nitrobenzene; DNT, 2,4- and 2,6-dinitrotoluene; RR2, Reactive Red 2; MO, Methyl Orange; MR, Methyl Red; RR120, Reactive Red 120; AR3R, Acid Red 3R; AY36, Acid Yellow; AR27, Acid red 27; AO7, Acid Orange 7; CT, Carbon tetrachloride

[c] + refers to the increase in conversion rate compared to the controls without redox mediator; CRE, color removal efficiency; RE, reduction efficiency; CF, Chlroform

Among the immobilizing techniques developed, several limitations have been identified during their application, such as mass transfer limitations due to RM entrapment within the immobilizing material (Guo et al. 2007; Lu et al. 2010),

disruption of supporting material due to weak mechanical properties (Guo et al. 2007), desorption of immobilized RM under high anions concentrations (Cervantes et al. 2010), the possibility of washout of RM from bioreactors due to small size of immobilizing material (Alvarez et al. 2010) and poor immobilizing capacity (Yuan et al. 2012). Furthermore, the vast majority of immobilizing techniques have been proposed and tested with expensive quinone model compounds, and their applicability have only been demonstrated in batch experiments (Table 2.3). Since HS constitutes the largest fraction of organic matter on earth, which is economically available in organic rich environments (*e.g.* soil and sediments) and wastes (*e.g.* composts), it would be convenient to immobilize HS (derived from those sources) using the available techniques and verify if after immobilization, HS maintain their redox mediating capacity. A few reports have documented that immobilized HS can serve as effective RM accelerating the reductive biotransformation of contaminants in batch incubations (Cervantes et al. 2011b; Alvarez et al. 2012, Cervantes et al. 2013).

More recently, Martinez et al. (2013) evaluated the catalytic effect of immobilized HS on an anion exchange resin, to achieve the simultaneous removal of two representative pollutants commonly found in textile effluents (*e.g*. phenol and Reactive Red 2 (RR2)) in a continuous high-rate anaerobic reactor. Efficient removal of these recalcitrant pollutants could be achieved in long-term operation of a continuous bioreactor supplemented with immobilized HS without the need of their continuous addition. A control bioreactor lacking immobilized HS collapsed after 120 days of operation due to the toxicity imposed by RR2.

Further efforts have been done to develop new strategies of immobilization of HS in alternative materials (Table 2.4). Although these materials have not been applied in redox reactions yet, they seem to fulfill the requirements to serve as a solid-phase RM for the reductive biotransformation of recalcitrant pollutants, have low cost and good adsorption capacities, as well as hydrolytical stability.

Immobilizing techniques should be combined with engineered HS, which can be synthetized in order to increase their redox activity for the (bio)transformation of contaminants. HS have been modified to enhance their redox properties, for instance, by enriching their structure with quinones (Perminova et al. 2005) or with sulfur-containing functional groups (Schmeide et al. 2012). Thus, multidisciplinary research should be conducted in order to develop engineered HS, which can be integrated by immobilizing techniques to bioremediation technologies. The challenge to accomplish these developments is to identify HS derived from cheap resources, such as organic rich environments and wastes, with the appropriate redox activity for specific catalytic applications. For instance, it has recently been demonstrated that HS having the highest electron accepting capacity do not necessarily show the greater reactivity towards a specific pollutant (Martinez et al. 2012). Thus, further studies are required to elucidate the relationship between the structural and chemical characteristics of HS and their redox activity. Furthermore, immobilizing techniques should be designed in order to allow the redox mediating functional groups present in HS remain available for catalysis after immobilization in the supporting material. These issues should be the subject in future research.

Table 2.4 Immobilization techniques for humic substances with potential for their application as solid-phase redox mediator

Redox mediator[a]	Material	Immobilization mechanism	Results	References
HA **FA**	Epoxypropylsilica Epoxypropylcellulose Chloromethylated styrene-divinylbenzene Polyacrilamide	Covalent and entrapment	Good adsorption properties and hydrolytical stability	Klavins and Apsite 1997
PAHA	Aminopropyl silica Glutaraldehyde-activated aminopropyl silica	Physical and chemical bonds	Good adsorption properties and stability in a wide range of pH	Koopal et al. 1998
HA	Silica gel	Covalent	Good adsorption properties and thermal stability	Prado et al. 2004
HA	Silica matrices	Covalent	Good adsorption properties and stability	Luo et al. 2007
HA	Silica gel	Covalent	Good adsorption properties and stability	Perminova et al. 2007
HA	γ-Al_2O_3 (20-50 μm)	Adsorption	Good adsorption properties and stability	Alvarez and Cervantes 2012
HA	Sodium alginate and hydroxyl ethyl cellulose blending	Covalent	Good adsorption properties and stability	Chen et al. 2012
HA	Chitosan/zeolite composite Surfactant modified	Electrostatic interaction and hydrophobic interactions	Thermal stability and HA adsorption capacities decreased with increasing solution pH between 4 to 12	Lin and Zhan 2012

[a] HA, humic acids; FA, fulvic acids; PAHA, purified Aldrich humic acid

2.7 Potential role of quinones in the biosynthesis of nano-catalysts

Some microorganisms are capable to change the oxidation state of metals and concomitantly deposit them outside or inside their cell membrane. These nano-

materials, usually called biogenic nano-catalysts, have the capacity to catalyze redox reactions, which have been applied for the treatment of different organic and inorganic pollutants such as, heavy metals, drugs, biocides, chlorinated solvents and pesticides (Forrez et al. 2011; Hennebel et al. 2009). Emerging research has revealed the potential role of quinones on the biosynthesis of nano-catalysts. For instance, Wang et al. (2011b) reported enhanced reduction of selenite (Se(IV)) and tellurite (Te(IV)) by *Escherichia coli* in presence of several quinone model compounds. The presence of quinones as RM not only accelerated the microbial reduction of Se(IV) and Te(IV), but also promoted the extracellular accumulation of Se(0) nano-spheres and Te(0) nano-rods catalysts.

More recently, Pat-Espadas et al. (2012) reported the use of AQDS as RM to accelerate the reduction of Pd(II) by *Geobacter sulfurreducens.* The incorporation of AQDS promoted a faster extracellular production of Pd(0) nano-catalyst than a control incubated in the absence of AQDS. The production of extracellular nano-catalysts represents an attractive alternative to facilitate their recovery from microbial incubations because a significant fraction could easily be separated from microbial cells as compared to conventional microbial incubations, which typically report produced nano-catalysts associated to biomass (Forrez et al. 2011; Hennebel et al. 2009). Lately, Tuo et al. (2013) demonstrated that biogenic Pd(0) nano-catalysts produced by *Geobacter sulfurreducens* in the presence of AQDS showed effective catalytic reduction of Cr(VI).

The results obtained in these investigations suggest that microbial production of nano-catalysts in the presence of HS could be exploited for the bioremediation of industrial effluents contaminated by precious metals, which could be recovered as

nano-catalysts for many different industrial applications. However, this is an emerging area, which demands substantial research activity in order to understand the environmental factors determining the structural and catalytic properties of biogenic nano-catalysts synthetized in the presence of quinones. The challenge is to develop microbial processes, which could become competitive against physical-chemical synthetizing processes, producing nano-catalysts with appropriate size, shape and catalytic capacity.

2.8 Role of humic substances in the production of bioenergy

It has been demonstrated that different microorganisms can generate electricity and hydrogen using several organic compounds, including organic matter present in wastewater as energy source (Watanabe 2008). Electrons released from the microbial oxidation of organic matter are transferred to intracellular electron acceptors (*e.g*. NAD^+), and then to the respiratory chain. Nevertheless, if electrons flow is directed to an extracellular electron acceptor or electrode (*i.e*. anode), microbial oxidation of organic matter can be coupled to electricity production. Some studies have shown that electron transfer to the anode can be accelerated by using different RM, including HS and quinone analogues, which enhanced bioelectricity production (Watanabe et al. 2009). Similarly, RM have also been proven to promote an increment on biohydrogen production. Table 2.5 shows recent studies related with bioelectricity and biohydrogen production by quinone- and humus-reducing microorganisms.

Table 2.5 Electricity and hydrogen production by quinone- or humus-reducing microorganisms

Electron shuttle[a]	Microorganisms or enzymes	Results*	Reference
Production of bioelectricity			
PQQ	Flavo-oxidoreductases and NAD^+-dependent enzymes	The maximum electrical power was 8 μW, at an external load of 3 kΩ.	Willner et al. 1998
HNQ	*Klebsiella pneumonia* (anodic compartment) *Leptothrix discophora* (cathodic compartment)	Production of peak power density of 126.7±31.5 mW/m^2 with a 50 Ω resistor. HNQ facilitated electron transfer to anode. Manganese was microbiologically oxidized and deposited in cathode as MnO_2.	Rhoads et al. 2005
AQDS	*Shewanella oneidensis*	Addition of AQDS increased the current and power in a range of 30% to 100%.	Ringeisein et al. 2006
AQDS, HA	*Clostridium cellulolyticum*	Resarzurin (non humic redox mediator) greatly enhanced current production as compared with MFC with AQDS or HA.	Sund et al. 2007
HA	Domestic wastewater was used as inoculum	HA increased the power density up to 67% respect to the control without HA.	Huang and Angelidaki 2008
ACNQ	*Lactococcus lactis*	External addition of ACNQ generated a catalytic current of −100 mV SHE, which is similar to that developed by *L. lactis*.	Freguia et al. 2009

Table 2.5 Continued

HS	Domestic wastewater was used as inoculum	Presence of HS increased the maximum power density up to 84% and 30%, for glucose and xylose, respectively.	Thygesen et al. 2009
DTBBQ	*Klebsiella pneumoniae*	Anode coated and uncoated with microfiltration membrane achieved a maximum voltage (mV) outputs of 316 and 426, respectively.	Deng et al. 2010
AQDS	*Geobacter sulfurreducens*	The power density was 1.0 mW/cm^2 of anode surface area (27-fold higher respect to the control without AQDS).	Adachi et al. 2010
HAQ	*Geobacter metallireducens*	Addition of HAQ increased the voltage from 170 mV to 290 mV.	Tang et al. 2010
HA	*Escherichia coli*	With HA, the maximum power generation was 6.75 µW, while the current was boosted to the highest value of 34.4 µA.	Nasirahmadi and Safekordi 2012
AQDS, HA	Anaerobic sludge	MFC with AQDS and HA showed an increment of power density of 36% and 15%, respectively, as compared to MFC lacking redox mediators.	Sun et al. 2013
Quinoid intermediaries of phenolic compounds	*Aeromonas sp., Aeromonas hydrophila, Acinetobacter johnsonii, P. hauseri, Enterobacter cancerogenus*	The results obtained from a reductive decolorization indicate a possible application for bioelectricity production.	Chen et al. 2013b

Table 2.5 Continued

Production of biohydrogen			
AQDS, HS	*Clostridium beijerinckii*	Reduced HS produced less H_2 than reduced AQDS after 4 days. Fe(III) inhibited H_2 production.	Hatch and Finneran 2008
AQDS	*Clostridium beijerinckii*	H_2 molar yield with 250 µM of reduced AQDS was 1.4-fold higher respect to the control without AQDS.	Ye et al. 2011
AQDS	*Clostridium beijerinckii*	Reduced AQDS (100- 500 mM) increased the H_2 production rates (mmol/L-hr) from 0.8-1.35 to 1.2-2.7, using different substrates.	Ye et al. 2012
AQDS	*Clostridium beijerinckii*	H_2 production was 1.94-fold higher using 2000 µM of reduced AQDS respect to the control without AQDS.	Ye et al. 2013
AQDS	*Clostridium beijerinckii and Geobacter metallireducens*	There was an increment (%) in the maximum cumulative H_2 production (52), specific H_2 production rate (38), and H_2 molar yield (34), using reduced AQDS in relation to the control lacking AQDS.	Zhang et al. 2013a
HS, FA, indigo dye, juglone, lawsone, AH_2QDS	*Clostridium beijerinckii and Geobacter metallireducens*	All RM increase the H_2 production (%) (61-98), specific H_2 production rate (%) (157-368), and H_2 molar yield (%) (14-45)	Zhang et al. 2013b

[a] HNQ, 2-hydroxy-1,4-naphthoquinone; PQQ, pyrroloquinoline quinone; AQDS, anthraquinone-2,6-disulfonate; HA, humic acids; ACNQ, 2-amino-3-dicarboxy-1,4-naphthoquinone; HS, humic substances; DTBBQ, 2,6-di-tert-butyl-p-benzoquinon; HAQ, 1-hydroxy-4-aminoanthraquinone; MFC, microbial fuel cell; SHE, standard hydrogen electrode; AH_2QDS, anthrahydroquinone-2,6-disulfonate; RM, redox mediator

Addition of quinones greatly enhanced the production of bioelectricity. For instance, respect to controls lacking RM, incubations amended with AQDS increased the power density up to 27-times using *Geobacter sulfurreducens* (Adachi et al. 2010), and up to 100% using *Shewanella oneidensis* (Ringeisein et al. 2006). Likewise, using domestic wastewater as inoculum, HS also promoted an increment on power density of 67% and 87% with xylose and glucose, respectively (Huang and Angelidaki 2008; Thygesen et al. 2009). It has also been documented that bioelectricity generation can also be enhanced by endogenous RM such as 2-amino-3-dicarboxy-1,4-naphthoquinone (Freguia et al. 2009), and 2,6-di-*tert*-butyl-*p*-benzoquinone (Deng et al. 2010), using the strains *Lactococcus lactis* and *Klebsiella pneumoniae*, respectively. Bioelectricity production has also been coupled to biotransformation of electron accepting contaminants. Sun et al. (2013) used AQDS and HS in the anodic compartment of a microbial fuel cell to promote the reductive decolorization of an azo dye coupled to bioelectricity generation. Addition of AQDS and HS increased up to 3.5- and 5.6-fold the rate of decolorization, along with an increment on maximum power density of 26% and 44%, respectively, as compared to the control lacking RM. Bioelectricity production is not exclusively dependent of organic matter oxidation. Certainly, other energy sources (*i.e.* sun light) can be coupled to the process. For instance, Yagishita et al. (1997) documented that *Synechococcus* sp. (a photosynthetic microorganism) improved its capacity to convert solar energy to electric energy in the presence of 2-hydroxy-1,4-naphthoquinone.

In the case of biohydrogen production, there are some studies reporting the use of RM (mainly AQDS). The two main impediments for the industrial production of

biohydrogen are: the low yield and limited production rate (Levin et al. 2004). Nevertheless, addition of RM has improved both the yield and production rate of the process. Certainly, AH_2QDS increased the production rate of biohydrogen between 1.2 to 2.7-fold (Ye et al. 2012; Ye et al. 2013); and also, the hydrogen molar yield was increased up to 1.4-fold; in all cases, incubations were compared to the control lacking AH_2QDS. The microorganism involved in these studies was *Clostridium beijerinckii*.

More recently, Zhang et al. (2013b) reported the positive effect of HS in biohydrogen production in a co-culture of *Clostridium beijerinckii* and *Geobacter metallireducens*. The results obtained in this investigation also confirm the applicability of HS for enhancing the production of this alternative energy source. Nevertheless, further research is needed in order to optimize and scale up hydrogen producing processes. Studies that show the potential to produce biohydrogen from organic wastes in quinone-mediated continuous flow bioreactors are encouraged. Particularly, biohydrogen producing processes in which immobilized HS are applied will be attractive.

2.9 Future perspectives

This review highlights the contribution of humus reducing microorganisms in relevant environmental processes, such as biodegradation of recalcitrant pollutants in contaminated sites, mitigation of greenhouse gases emission in organic rich ecosystems, redox conversion of contaminants in engineered treatment systems, and microbial production of nano-catalysts and alternative energy sources. Further research is demanded to fully characterize and understand the structural and catalytic properties of HS to enhance their contribution on transport, fate and

biodegradation of contaminants, as well as on mitigating emission of greenhouse gases, in natural environments. Moreover, multidisciplinary work is also required to develop engineered HS with enhanced redox properties for their application in bioremediation processes and to create wastewater treatment systems in which immobilized HS have been integrated for the removal of recalcitrant pollutants from industrial effluents. The enhanced production of nano-catalysts and bioenergy promoted by HS and quinone analogues, underlined in the present review, suggest the great potential for exploring and optimizing these processes for industrial applications.

3

Immobilized humic substances as redox mediator for the simultaneous removal of phenol and Reactive Red 2 in a UASB reactor

Abstract

The present study reports a novel treatment concept combining the redox-mediating capacity of immobilized humic substances (HS) with the biodegrading activity of anaerobic sludge for the simultaneous removal of two representative pollutants of textile wastewaters (*e.g*. phenol and Reactive Red 2 (RR2)) in a high-rate anaerobic reactor. The use of immobilized HS (1 g TOC L^{-1}), supported on an anion exchange resin) in an UASB reactor, increased the decolorization efficiency of RR2 (~ 90%), extent of phenol oxidation (~ 75%) and stability as compared to a control UASB reactor operated without immobilized HS, which collapsed after 120 days of dye introduction (50-100 mg L^{-1}). Increase in the concentration of immobilized HS (2 g TOC L^{-1}) further enhanced the stability and efficiency of the UASB reactor. Detection of aniline in the effluent as RR2 reduction product confirmed that reduction of RR2 was the major mechanism of dye removal. This is the first demonstration of immobilized HS serving as effective RM for the removal of recalcitrant pollutants from wastewater in a high-rate anaerobic bioreactor.

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3.1 Introduction

Environmental problems generated by the textile industry have received increased attention for several decades because this industrial sector is one of the largest generators of contaminated effluents, which mainly arise from dyeing and finishing processes, and it is associated with water pollution caused by discharges of untreated or poorly treated effluents. In terms of total volume, the global dyestuff production is 3.4 X 10^{10} kg per year, which accounts for annual global sales of nearly US$ 6 billion (Cervantes and Dos Santos 2011). Release of these contaminated effluents into the environment is undesirable, not only due to aesthetic aspects, but also because many dyes and their breakdown products are toxic and/or mutagenic to life (Dos Santos et al. 2004). While different costly physical and chemical techniques have been used for achieving removal of dyes, the treatment of textile wastewaters by biological treatment systems represents a great challenge, due to the great variability in composition and to toxicological effects causing inhibition or collapse of bioreactors (Sponza and Isik 2004; Van der Zee et al. 2001). Under anaerobic conditions most azo dyes are susceptible to reductive decolorization (Stolz 2001), although the reduction rate may be rather low, especially for dyes with high polarity or complicated structure, such as some sulfonated reactive azo dyes. This represents a serious problem for the application of high-rate anaerobic bioreactors for the treatment of textile wastewater, because long hydraulic retention time (HRT) (typically ranging from 24 up to 72 h) are demanded to achieve acceptable color removal efficiencies (Van der Zee and Villaverde 2005).

During the last two decades, it has been reported that addition of redox mediators

(RM), such as humic substances (HS) and their quinone model compounds could significantly enhance the reduction rate of azo dyes by accelerating the transfer of electron equivalents derived from microbial substrate oxidation to the electron-accepting azo dyes (Stolz 2001; Van der Zee and Cervantes 2009). Although evidence collected clearly indicated the great potential for applying these RM to increase the reductive decolorization of several azo dyes in continuous flow experiments (Cervantes et al. 2001b; Costa et al. 2010; Dos Santos et al. 2003; 2005; Rodrigues et al. 2012; Van der Zee et al. 2001), the main limitation is that continuous addition of the RM should be provided to increase conversion rates, which is economically and environmentally non-viable.

To overcome this limitation, several attempts to immobilize RM have been explored during the last years (Table 2.3). However, all previous studies have been developed using costly synthetic RM and their catalytic capacity has only been demonstrated in batch experiments. Moreover, a few reports have showed the impact of immobilized HS on the redox biotransformation of contaminants also in batch experiments. (Alvarez et al. 2012; Cervantes et al. 2011b; Cervantes et al. 2013).

Recently, Lian et al. (2011) reported for the first time the effect of functional bio-carrier modified by disperse turquoise blue S-GL as RM on the decolorization of disperse scarlet S-BWFL in bioreactors. Although this investigation demonstrates that it is possible to use immobilized RM in bioreactors, no previous reports have documented long-term impact of immobilized HS for achieving removal of recalcitrant pollutants in continuous bioreactors.

The aim of the present investigation was to evaluate the catalytic effect of

immobilized HS, on an anion exchange resin (AER), to serve as RM to achieve effective removal of two representative pollutants commonly found in textile effluents (*e.g.* phenol and RR2) in a continuous high-rate anaerobic reactor (Figure 3.1).

Phenol was selected as a model contaminant because it is a common constituent of many different industrial effluents, including those derived from textile factories. RR2 was selected as an azo model compound to challenge the treatment concept as it has been reported to be very recalcitrant and toxic for anaerobic consortia causing even the collapse of high-rate anaerobic bioreactors (Beydilli and Pavlostathis 2005; Van der Zee et al. 2001). The concurrence of phenol and azo dyes is expected not only in textile wastewaters, but also on several industrial discharges such as those generated from the pharmaceutical and chemical sectors. To our knowledge, the present study constitutes the first demonstration of immobilized HS serving as effective RM for the removal of recalcitrant pollutants from wastewater in a high-rate anaerobic bioreactor.

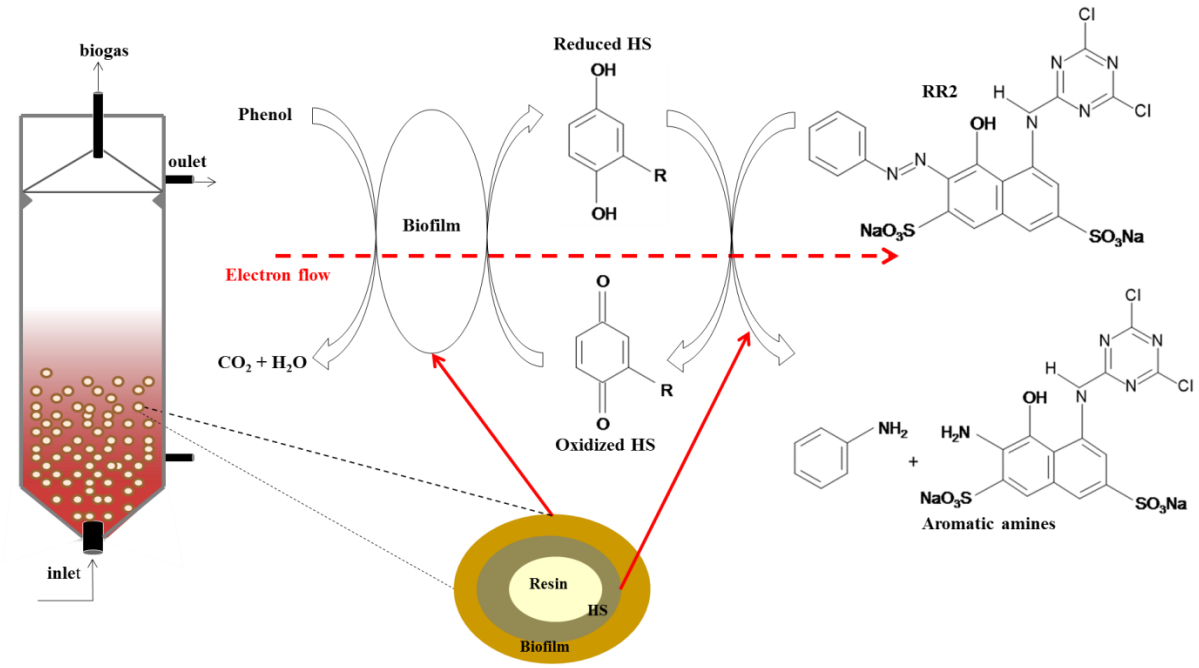

Figure 3.1 Redox reactions involved in the simultaneous biodegradation of phenol and reactive red 2 mediated by immobilized humic substances on anion exchange resin in an UASB rector

3.2 Materials and methods

3.2.1 Chemicals

RR2 (Procion Red MX-5B, ~50% of purity) was purchased from Sigma-Aldrich Company and used without additional purification. The source of HS was Leonardite obtained from the International Humic Substances Society (IHSS, Catalogue No. 1BS104L). HS were sulfonated to increase solubility and achieve immobilization on an AER (Rohm and Haas, AMBERJET 4600 CL, Philadelphia, PA) as previously described (Cervantes et al. 2011b). The main characteristics of this AER are presented in Table 3.1, and previously reported by Cervantes et al (2010).

In order to achieve the sulfonation of HS, 100 g of Leonardite were dried at 50°C for one day and placed into 1000 mL beaker, cooled in an ice bath, thereafter 200 ml of cooled chlorosulphonic acid ($HClSO_3$) were added with in small portions (50 ml)

with permanent stirring during approximately 6 hours. When the reaction was completed, the mixture was poured into 4 L of iced distilled water to hydrolyze the rest of $HClSO_3$, the obtained solution was left for 12 hours at room temperature. Then, the supernant was discarded, and the precipitate was washed out with distelled water until the supernant solution gave negative reaction to $AgNO_3$. The obtained precipitate of sulphonated Leonardite was dried at 50°C. The capacity of the AER AMBERJET 4600 CL to adsorb sulfonated leonardite was determined by adsorption isotherms with initial leonardite concentrations ranging 100–6500 mg Total Organic Carbon (TOC) L^{-1}. Adsorption isotherms were carried out using a weight/volume ratio of 0.020 g mL^{-1}, in a volume of 15 mL and at pH 7.0 with constant temperature of 25 °C and with continuous shaking (180 rpm). The initial and equilibrium concentration of TOC was measured after 3 days was measured to determine the quantity of TOC adsorbed per gram of AER by a mass balance relationship using Eq. (3.1):

$$qe = V (C_0 - Ce) / W \qquad (3.1)$$

Where qe is the adsorption capacity (mg g^{-1}), V is the volume of sulfonated Leonardite solution (L), C_0 and Ce are the initial and equilibrium concentrations of sulfonated Leonardite (mg TOC L^{-1}) respectively, and W the weight (g) of AER. For simplicity the term i-Leonardite will be used to refer to immobilized HS on the AER. The mechanism of immobilization of HS on AER is presented in Figure 3.1.

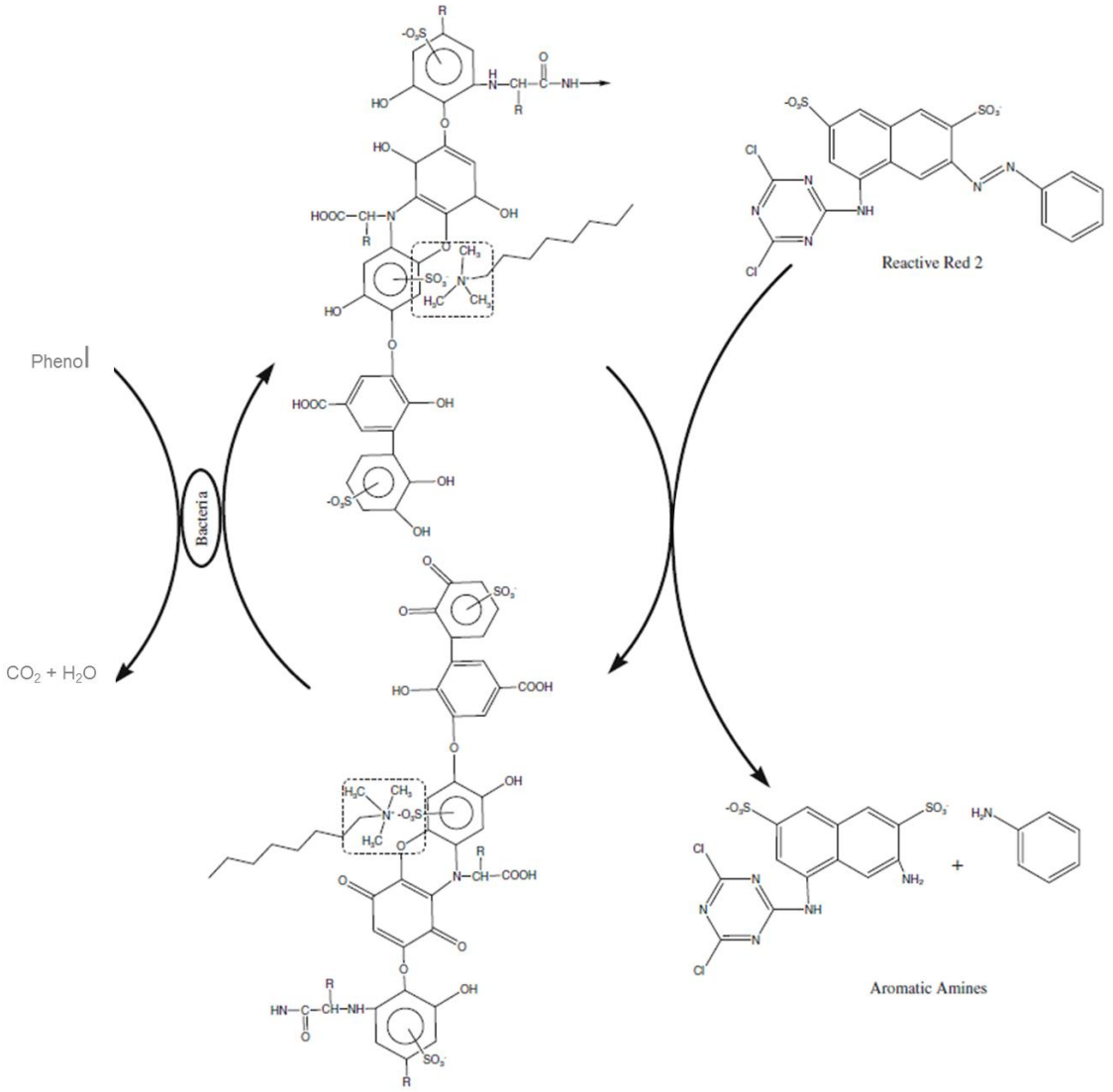

Figure 3.2 Proposed mechanisms involved in the reductive biotransformation of Reactive Red 2 (RR2) by humus-reducing microorganisms mediated by immobilized sulfonated HS on an anion exchange resin (AER). Immobilized HS served as terminal electro acceptor for humus-reducing microorganism supporting the anaerobic oxidation of phenol during the firss step. Reduced HS then transfer the reducing equivalents to the electron-accepting azo bond of RR2 in the second step. Dashed squares indicate the interaction between sulfonated groups inserted in HS and the quaternary amine present at the AER

Table 3.1 Characteristics of AMBERJET resin 4600 CL (Cervantes et al. 2010)

Functtional group	Quaternary amine
Morphology	Spherical, non-porous
Diameter	2 mm
Specific weight	1.25 g cm^3
Specific surface	0.40 m^2 g^{-1}
pH	4 - 12

3.2.2 Adsorption of RR2 and aniline

Experiments were conducted to estimate the extent of adsorption of RR2 and aniline (one of the products derived from azo reduction of RR2) onto untreated resin and i-Leonardite. To determine the amount of adsorbed RR2, different quantities of RR2 (10 – 800 mg L^{-1}) were added to serum vials containing 225 mg of untreated resin or i-Leonardite, equivalent to 1000 mg TOC L^{-1}. Batch vials were sealed with Teflon stoppers and aluminum caps and incubated at 28 °C in the dark and agitated (80 rpm) during 48 h before sampling.

The adsorbed amount of aniline was determined on untreated resin and i-Leonardite, which were previously exposed to RR2 (i.e. 225 mg of untreated resin or i-Leonardite). After 48 h of incubation (28 ºC, 80 rpm), 2 mL of sample were taken for further analysis.

3.2.3 Bioreactors operation

Two upflow anaerobic sludge blanket (UASB) reactors were operated with a HRT of 12 h, in a controlled temperature room (28 ± 1 °C). The reactors were named R1 (control reactor to which untreated resin was added) and R2 (reactor supplemented

with i-Leonardite). Both reactors were made of glass and were 32 cm height and 5 cm in diameter, for a total volume of 0.33 L and were inoculated with 5 g volatile suspended solids (VSS) L^{-1} composed of a mixture of an anaerobic granular sludge and a sediment at a ratio of 4:1 (in terms of VSS). The anaerobic sludge was incorporated to enrich the bioreactors with a methanogenic consortium and was obtained from a full-scale UASB reactor treating brewery effluents (Sonora, Mexico). To achieve homogeneous distribution in both reactors, the anaerobic granular sludge was disintegrated in an anaerobic chamber and mixed with the sediment. Reactors were operated for five periods distinguished by the concentration of RR2 and i-Leonardite added to reactor 2. During periods I, II and III, 17.6 g of untreated resin and i-Leonardite were added to R1 and R2, respectively; the amount of i-Leonardite supplied to R2 was equivalent to 1 g TOC L^{-1}. In periods IV and V, the amount of i-Leonardite added to R2 was doubled to 2 g TOC L^{-1}. The reactors were fed with synthetic wastewater comprising phenol as the sole substrate, RR2, balanced nutrients and trace elements. The basal medium contained (g L^{-1}): NH_4Cl (0.28), K_2HPO_4 (0.25), $MgSO_4 \cdot 7H_2O$ (0.1), $CaCl_2 \cdot 2H_2O$ (0.01), $NaHCO_3$ (5) and 1 ml L^{-1} of trace elements (Martínez et al. 2012).

Before addition of RR2, both bioreactors were progressively acclimated to phenol as a sole energy source during 8 months (Figure 3.2). During the acclimation period, acetate was initially added as co-substrate. Initial influent phenol and acetate concentrations were 300 and 200 mg chemical oxygen demand (COD) L^{-1}, respectively. During the next period, the concentration of phenol was increased to 400 mg COD L^{-1} and to 500 mg COD L^{-1} at the end. Acetate concentration was decreased accordingly in order to have a total COD concentration of 500 mg L^{-1}.

After the acclimation period in which steady state removal of phenol was observed, the reactors were fed with 50 mg L^{-1} of RR2 (period I), and, subsequently, the dye concentration was increased to 75 mg L^{-1} (period II) and to 100 mg L^{-1} (period III). In order to further evaluate the catalytic effects of i-Leonardite on the simultaneous removal of phenol and RR2, the concentration of i-Leonardite in R2 was increased to 2 g TOC L^{-1}, the concentrations of RR2 evaluated in the reactors were 50 mg L^{-1} (period IV) and, finally, 100 mg L^{-1} (period V).

3.2.4 Analyses

Phenol was analyzed by gas chromatography (GC, Agilent technologies 7890 series) coupled to a flame ionization detector. Separation was achieved with a 5% phenyl-95% arylene-siloxane (30 m × 0.250 mm ID, 0.25 lm (df)) DB-5MS fused-silica capillary column from Agilent Technologies. Helium was used as carrier gas at 2.5 mL min^{-1} column flow-rate and nitrogen was the makeup gas at 30 mL min^{-1}. The temperatures of injector and detector were maintained at 250 and 300°C, respectively. The oven temperature was programmed from 35°C (1 min hold) to 280°C (8°C min^{-1}). Color removal was determined photometrically by UV/visible spectrophotometry (Thermo-cientific Genesys 10 UV) at 539 nm. Aniline was measured by HPLC. The HPLC was equipped with two reverse phase columns Synergi 4uHydro-RP80A (250 · 4.60 mm, 4 micron) from Phenomenex. The carrier liquid, composed of 60% of deionized water and 40% acetonitrile, was pumped at a flow rate of 1 mL min^{-1}. Aniline was detected using a diode array at 230 nm. The amount of biogas produced was recorded using the water displacement method. The content of methane and carbon dioxide in the biogas were analyzed by gas chromatograph (GC, Agilent Technologies 6890 N series) equipped with a thermal

conductivity detector and a column Hayesep D (Alltech, Deerfield, Illinois, USA) with the following dimensions: 3.048 m × 3.18 mm × 2.16 mm. Nitrogen was used as carrier gas with a flow-rate of 12 mL min^{-1}. Temperatures of the injection port, oven and the detector were 250, 60 and 250°C, respectively. Nitrogen was used as carrier gas with a flow-rate of 12 mL min^{-1}. COD and VSS were analyzed according to Standard Methods.

3.3 Results

3.3.1 Performance of UASB reactors

In order to evaluate the capacity of the anaerobic consortia for achieving the simultaneous removal of phenol and RR2, two UASB reactors were operated: a control reactor to which untreated resin was added (R1) and a reactor supplemented with immobilized HS (R2) on the same resin (i-Leonardite). The experimental set up was divided into 8 experimental periods, including reactors start-up (acclimation period). During the acclimation period (210 days), acetate was added as co-substrate and its concentration was progressively decreased in order to have a total COD concentration of 500 mg L^{-1} with phenol as a sole electron donor at the end of this period. After the acclimation period, the reactors were operated for five periods distinguished by the concentrations of RR2 and i-Leonardite applied (Figures 3.3 and 3.4).

3.3.2 Phenol removal

Phenol removal efficiencies higher than 60% were achieved in both reactors during the initial acclimation period, and no statistical difference was observed in their phenol removal efficiency ($p = 0.05$) (Figure 3.3). However, it was evident that during the third period (when phenol was supplied as the sole energy source),

phenol removal efficiencies for R1 and R2 were higher than those obtained in the first two periods, probably due to a gradual adaptation of the consortia to phenol, and phenol removal efficiencies around 80% were achieved in both reactors at the end of the acclimation period.

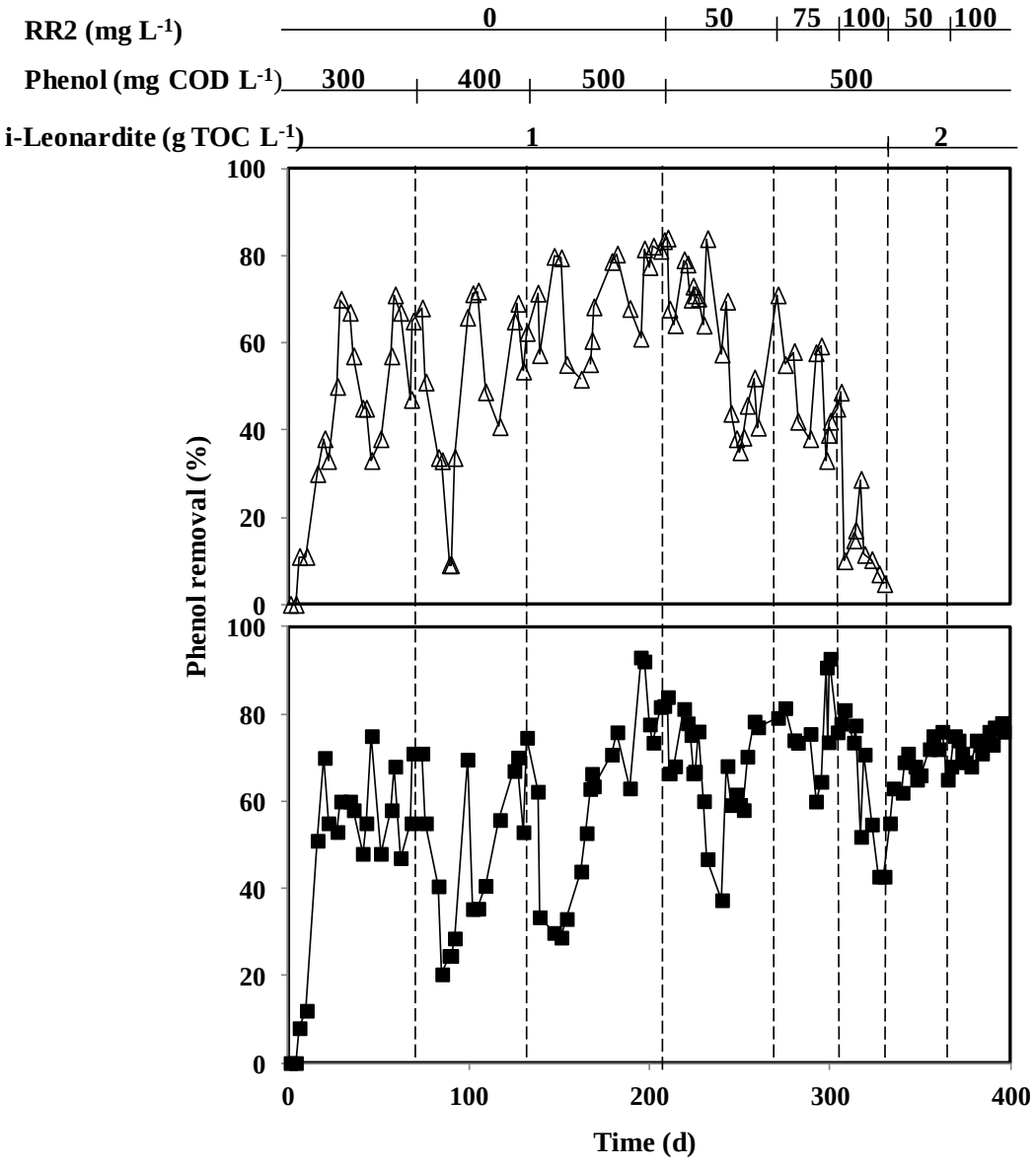

Figure 3.3 Phenol removal efficiencies achieved in the UASB reactor supplemented with untreated resin (open triangles) and in the UASB reactor supplemented with immobilized humic substances (i-Leonardite) on the resin (closed squares)

Addition of RR2 exerted inhibitory effects in both reactors as reflected in lower phenol removal efficiency (Figure 3.3) as compared to the performance observed during the acclimation period in the absence of this recalcitrant azo dye. Respect to the last acclimation period, phenol removal efficiency decreased 24% in R1 and 15% in R2. The next increment of RR2 to 75 mg L^{-1}, did not affect phenol oxidation in R2 (Table 3.2). The average phenol removal efficiency in R2 was 76.6%. In contrast, phenol removal in R1 decreased to 47.1% under the same conditions (Figure 3.3). During the subsequent increase of RR2 concentration to 100 mg L^{-1}, the average phenol removal efficiencies in both reactors decreased 60% in R1 and 16% in R2. R1 collapsed after 120 days of operation probably due to RR2 toxicity and its activity could not be recovered, whereas R2 maintained a reasonable phenol removal capacity (64.9%). The increase of i-Leonardite concentration in R2 further improved its performance during the last two periods, reaching similar phenol removal efficiency as that observed with 75 mg RR2 L^{-1}. COD analysis confirmed a higher biodegradation of phenol in R2 than in R1 in all experimental periods (Table 3.2).

3.3.3 Color removal

After the acclimation period with phenol as substrate (8 months), RR2 was then introduced at a concentration of 50 mg RR2 L^{-1} (80 µM) in both reactors (Table 3.2). At the beginning of this period, decolorization efficiencies around 80% were observed in both reactors presumably due to initial RR2 adsorption onto untreated resin (R1) and onto i-Leonardite (R2) (Figure 3.4).

Table 3.2 Performance of the UASB reactors during the simultaneous removal of phenol and RR2

Period	I		II		III		IV	V
Parameter	**R1**	**R2**	**R1**	**R2**	**R1**	**R2**	**R2**	**R2**
RR2 influent (μM)	70 ± 10		110 ± 10	130 ± 10	200± 10	170±20	100±30	180±30
Color removal (%)	56.5±20.6	78.5± 9.7	37.2±10.9	92.04±2.6	16.3±12.4	76.6±9.5	91.4±4.5	88.9 ± 6
Phenol removal (%)	60.1±11.2	66.1±15.7	49.5±12.3	76.5 ± 9.7	19.8±15.7	64.9±15.1	67.7±5.9	72.6 ±3.8
COD removal (%)	67.5±11.6	73.8 ± 7.3	37.2 ± 3.5	70.5 ± 5.6	25.8±17.8	52.3 ± 8.7	67.8±8.9	81.7 ±4.9
Aniline effluent (μM)	20 ± 10	30 ± 10	10 ± 2	70 ± 10	4 ± 0.0	50 ± 10	70 ± 0.5	130 ± 20

R1 control reactor to which untreated resin was added, R2 reactor supplemented with i-Leonardite

Experimental data derived from adsorption isotherms indicate that the maximum adsorption capacities of RR2 on untreated resin and on i-Leonardite were 54.2±1.6 and 63.6±4.2 mg g^{-1}, respectively. Experimental data were best described by Langmuir model based on the determination coefficient (R^2 = 0.98). Figures 3.5 and 3.6 show the adsorption data with untreated resin and with i-Leonardite, respectively, together with their fit to the adsorption isotherm equations of Langmuir (Qo and *b* are constants of the Langmuir equation, with Qo = maximum adsorption capacity [mg adsorbed RR2 per g material] and *b* = affinity constant [mg. L^{-1}]). The low values for parameter *b* (*b* < 1) indicated a high RR2 affinity for both materials evaluated. According to adsorption isotherms data, saturation of both untreated resin and i-Leonardite occurred during the first few days of operation at all concentrations of RR2 tested. As the adsorption capacity of these materials became exhausted, decolorization efficiencies gradually decreased to 13% and 67% in R1 and R2, respectively (Figure 3.4). During the next experimental period, the concentration of RR2 was increased to 75 mg L^{-1}. R1 showed a considerable

operational instability and poor decolorization efficiency was obtained at the end of this period.

In contrast, R2 responded with a significant increase in the average decolorization efficiency (Table 3.2). Likewise, the average decolorization efficiency of R2 during this period was 1.32-fold higher than with 50 mg RR2 L^{-1} probably due to adaptation of the consortium to RR2. When RR2 concentration was increased to 100 mg L^{-1}, the decolorization efficiencies of both reactors gradually decreased. During this period, the average decolorization efficiency for R2 dropped down to 76%. In contrast, R1 collapsed after 120 days of dye addition, presumably due to RR2 toxicity (Figure 3.4), and the capacity of R1 to achieve the simultaneous removal of phenol and RR2 could not be recovered. In the following periods, the concentration of i-Leonardite was increased to 2 g TOC L^{-1} in R2, to further increase the catalytic impact of i-Leonardite for the simultaneous removal of phenol and RR2. During both periods, the average decolorization efficiencies of R2 increased considerably (Figure 3.4). With 50 mg RR2 L^{-1}, R2 reached the same decolorization efficiencies observed previously (~ 92%, Table 3.1), and no statistical difference concerning decolorization was observed ($p = 0.05$). In the last period (with 100 mg RR2 L^{-1}), average decolorization efficiency was higher than 80 %.

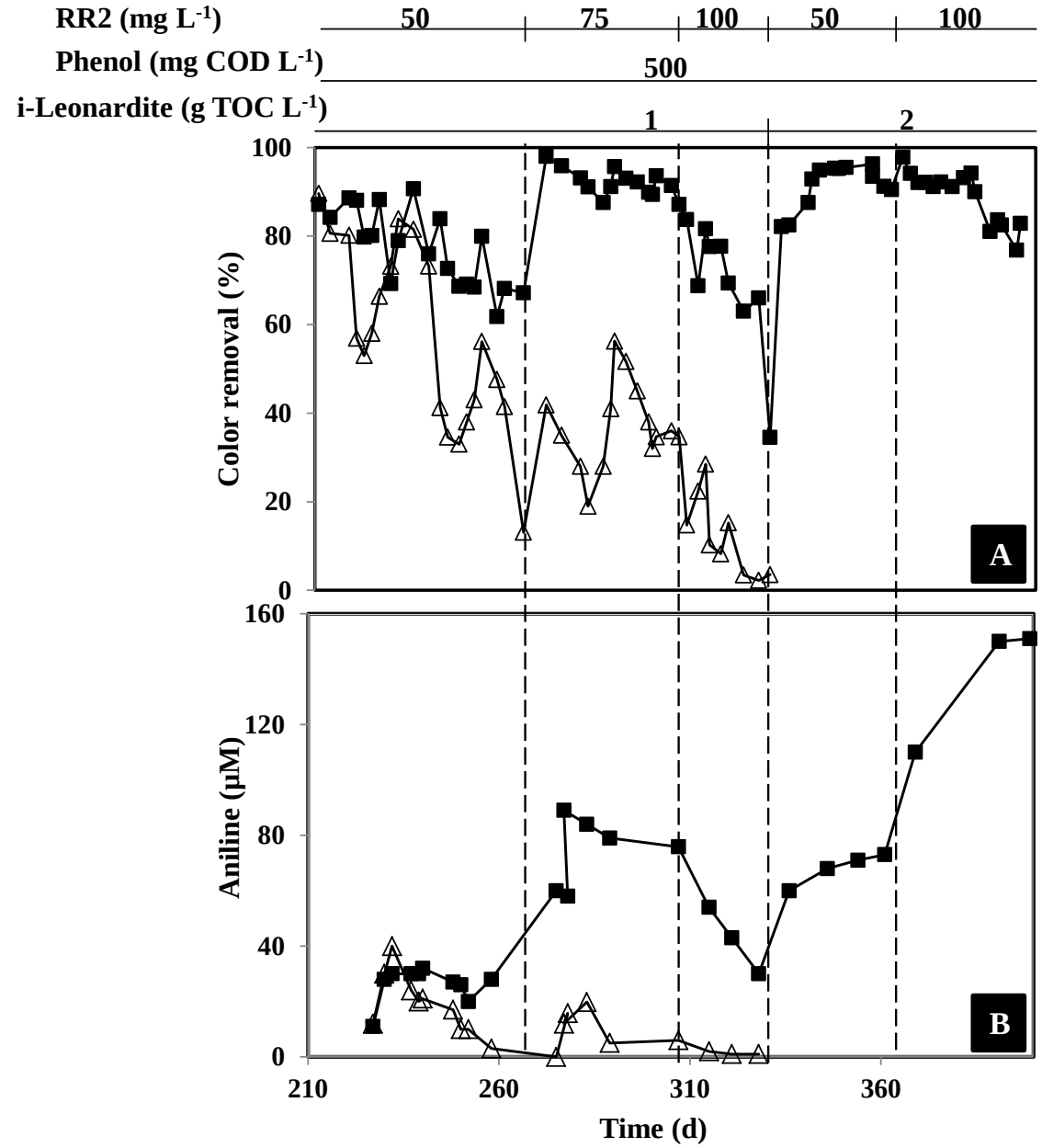

Figure 3.4 Decolorization of reactive red 2 in the control reactor supplemented with untreated resin (open triangles) and in the reactor supplemented with immobilized humic substances (i-Leonardite) on the resin (closed squares). (A) Color removal efficiencies of RR2 and (B) Concentration of aniline in the effluent

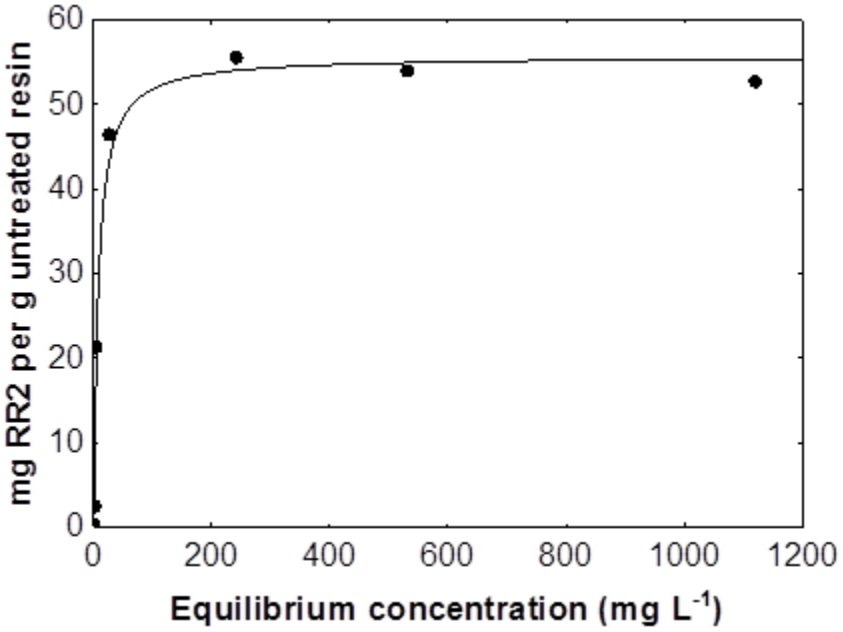

Figure 3.5 Adsorption isotherm of reactive red 2 on untreated resin. Experimental data (circles). Langmuir fit: Q = (Qo·Ce)/(*b*+Ce) = (54.8·Ce)/(0.03+Ce) (continuous line)

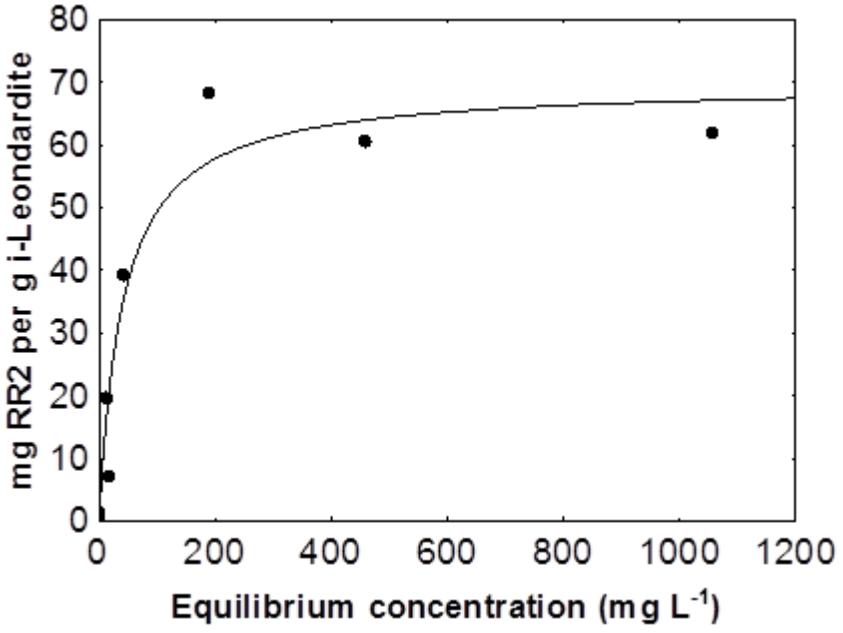

Figure 3.6 Adsorption isotherm of reactive red 2 on immobilized humic substances on the resin (i- Leonardite). Experimental data (circles). Langmuir fit: Q = (Qo·Ce)/(*b*+Ce) = (65.7·C)/(0.02+Ce) (continuous line)

3.3.4 Aniline as reduction product from RR2 decolorization

Under anaerobic conditions, the complete reduction of the RR2 involves the transfer of four-electrons to produce two aromatic amines as final products of this reductive decolorization; one of the produced aromatic amines is aniline and its detection is a clear evidence of the azo bond cleavage. Accordingly, in this investigation quantification of aniline was the measured parameter to confirm RR2 reduction (Figure 3.4). Data summarized in Table 3.2 revealed that higher decolorization efficiency of RR2 corresponded to higher aniline concentrations in R2 effluent (Table 3.2). With 75 mg L^{-1}, aniline recovery represented 63% of the amount of RR2 reduced, while that observed during the last two periods (at 50 and 100 mg RR2 L^{-1}) in which i-Leonardite concentration was increased two-fold, aniline recovery increased to 75 and 72%, respectively. Likewise, according to adsorption isotherms (data not showed), the amount of adsorbed aniline onto i-Leonardite previously exposed to RR2, did not represent more than 1% of aniline to be quantified. Considering the recovery of aniline obtained in the different experimental periods of this investigation, it can be observed that aniline recoveries are significantly higher than those obtained in other studies in which other redox mediators have been evaluated (Van der Zee et al. 2003). In fact, analysis of amines derived from azo dyes cleavage is challenging due to their instability during sampling and analysis; thus, careful handling is recommended to prevent their (auto)oxidation, such as using an reducing agent (*e.g.* ascorbic acid) in the diluting buffer (Van der Zee et al. 2001).

3.4 Discussion

The present investigation demonstrates the feasibility of removing two representative pollutants commonly found in textile effluents, one being oxidized (phenol), the other being reduced (RR2), in a high-rate anaerobic reactor supplemented with i-Leonardite as RM without the need of continuous addition of the RM.

The UASB reactor supplied with i-Leonardite showed high efficiency to achieve the simultaneous removal of phenol and RR2. In the present study, it was demonstrated that i-Leonardite could act as effective solid-phase RM promoting the simultaneous removal of phenol and RR2 in long-term operation of a UASB reactor without the need of continuous supply of the RM.

The robustness of the treatment concept developed can be compared with decolorizing treatment processes using other redox mediators for the reductive decolorization of RR2 in continuous bioreactors. However, all previously reported processes were supplied with readily biodegradable co-substrates, such as volatile fatty acids, glucose or ethanol (Table 3.3), whereas in the present study, the UASB reactor supplied with i-Leonardite was operated with phenol as the sole energy source achieving phenol removal efficiencies higher than 70% throughout the long-term experiments. Furthermore, the UASB reactor supplied with i-Leonardite was operated with the highest specific RR2 loading reported so far in the literature (40 mg RR2 g VSS.d^{-1}) achieving color removal ranging 80-90%, just as effective as those bioreactors previously supplied with RM which demanded their continuous addition (Table 3.3). In contrast, the UASB reactor lacking i-Leonardite collapsed after 120 days of exposure to RR2 and could not be recovered, thus demonstrating

the effectiveness of i-Leonardite as solid-phase RM promoting the efficient removal of the two model priority pollutants evaluated (phenol and RR2). Moreover, the present study was conducted with non-hydrolyzed RR2, which has been reported to be more toxic than previously hydrolyzed RR2 (Dos Santos et al. 2003; 2005; Van der Zee et al. 2001). The color removal efficiency obtained in the UASB reactor amended with i-Leonardite was also comparable with that obtained in an UASB reactor supplied with granular activated carbon as RM (Van der Zee et al. 2003).

Although limited, i-Leonardite showed some capacity to absorb both RR2 and aniline, which could represent a mechanism to mitigate the inhibitory effects of these contaminants towards anaerobic bacteria responsible for achieving the decolorizing process studied. Both RR2 and aniline have previously been reported to be extremely toxic to methanogens at concentrations lower than those prevailing in the present experimentation (Dos Santos et al. 2003; Van der Zee et al. 2001, Donlon et al. 1995). Thus, i-Leonardite could mitigate the inhibitory effects caused by compounds contained in textile effluents, by adsorbing a fraction of these compounds and accelerating their reduction, which is relevant considering the great complexity and variability in composition of textile wastewaters. Immobilized HS have previously been reported to decrease the inhibitory effects of contaminants by adsorbing a fraction during their redox conversion in batch experiments (Alvarez et al. 2012).

Immobilized HS as a natural RM present some other advantages underlined by Cervantes et al. (2011b): (1) humus is the most plentiful and cheaply available organic fraction in the biosphere; (2) humus is considered as a non-hazardous material and does not lead to the production of toxic byproducts and; (3) its stability

in the environment prevents its deterioration during bioremediation processes. In this study, the remarkable stability of i-Leonardite and the robust immobilizing technique applied contributed to the retention of this natural RM in the UASB reactor amended with i-Leonardite, without losing its redox mediating properties to catalyze the continuous removal of phenol and RR2 (Figures 3.3 and 3.4). The robustness of the immobilizing technique was verified during the whole acclimation period (first 200 days of operation of the bioreactor) since spectrophotometric screening did not detect any detachment of i-Leonardite from R2 when monitoring the effluent of the treatment system. This monitoring could not be maintained after introduction of RR2 in the bioreactor due to interference of the dye during the spectrophotometric screening. Nevertheless, the robustness and stability showed by the bioreactor supplied with immobilized HS is strong evidence that they remained in the treatment system promoting the simultaneous removal of phenol and RR2.

It has been documented that the microbial reduction of immobilized HS by anaerobic sludge is the rate-limiting step during the reductive decolorization of RR2 mediated by i-Leonardite (Cervantes et al. 2013). Thus, the high biomass concentration usually applied in UASB reactors and the fact that enrichment and immobilization of HRM is feasible in this type of bioreactors (Cervantes et al. 2003), could overcome these kinetic limitations during the application of i-Leonardite for the removal of recalcitrant pollutants from industrial wastewaters.

Table 3.3 Reductive decolorization of reactive red 2 (RR2) achieved with different redox mediators and different substrates

Substrate	Azo dye	Specific dye load (mg RR2 (gVSS.d)$^{-1}$	Color removal (%)	RM	Reference
VFA	RR2	27	98	AQDS	Van der Zee et al. 2001
Glucose-VFA	Hydrolyzed RR2	10	92	AQDS	Dos Santos et al. 2003
VFA	Hydrolyzed RR2	6.5	90	Activated carbon	Van der Zee et al. 2003
Glucose-VFA	Textile wastewater – hydrolyzed RR2	40	88	AQDS	Dos Santos et al. 2005
Ethanol	RR2	6.5	84	AQDS	Braúna et al. 2009
Ethanol	RR2	13	85	AQDS	Rodriguez et al. 2012
Phenol	RR2	20 - 40	80 - 90	i-Leonardite	This study

VFA, volatile fatty acids; RR2, red reactive 2; AQDS, anthraquinone 2,6 disulfonate; i-Leonardite, humic substances derived from leonardite immobilized onto the anion exchange resin

3.5 Conclusions

The present investigation demonstrates the feasibility of removing two representative pollutants in a high-rate anaerobic reactor supplemented with i-Leonardite as RM without the need of continuous addition of the RM.

The application of this novel treatment concept is an attractive strategy that: 1) will prevent the continuous dosing of these natural redox active immobilized compounds to the treatment system; 2) will mitigate the inhibitory effects caused by extreme fluctuations of contaminants from industrial effluents; 3) will increase the rate and

extent of removal of pollutants and, 4) will ensure that the redox catalytic properties of i-Leonardite can withstand long-term operation.

The development of this new treatment concept will contribute to the efficient removal of recalcitrant pollutants, commonly found in the textile and other industrial sectors. The treatment concept is conceived to treat high loadings of pollutants in a limited space due to the increased conversion rate expected by the redox properties of immobilized HS. By applying this treatment concept, the industrial sector will benefit by the fact that treated water could be recycled to achieve more sustainable and economically feasible productive processes.

4

Co-immobilization of humus and humus-reducing microorganisms by granulation for the anaerobic degradation of recalcitrant pollutants

Abstract

The present study evaluated the possibility of co-immobilization of humus reducing microorganism (HRM) and humic substances (HS), supported on γ-Al_2O_3 nanoparticles (NPs), by granulation. The results obtained revealed granules with appropriated agglomeration of biomass and γ-Al_2O_3 NPs in the presence of HS. However, the amount of HS contained in the produced granules and the obstruction of redox active functional groups in HS could be factors that limited the catalytic effect of these granules during the reduction of reactive red 2 (RR2) and 2,4-dichlorophenol (DCP). Both aspects were studied by performing further incubations supplied with an external amount of HS and with disintegrated sludge. In this sense, the presence of HS significantly increased the rate of RR2 and 2,4-DCP reduction, compared to controls lacking HS. Likewise, the detection of aniline and 4-chlorophenol (4-CP) as principal reduction products of RR2 and 2,4-DCP respectively, confirmed the reduction of both contaminants.

A modified version of this chapter has been published as:, Alvarez LH, Martinez CM, Cervantes FJ (2013). Anaerobic granulation using humus-reducing microorganisms and γ-Al_2O_3 nanoparticles coated with humic acids. Proceedings of the XII World Congress on Anaerobic Digestion. Oral Presentation. Santiago de Compostela.

4.1 Introduction

This thesis is focused on evaluating two different strategies to co-immobilize HS and humic-reducing microorganisms (HRM) to achieve the redox biotransformation of recalcitrant pollutants in batch and continuous flow experiments. The previous chapter demonstrated that with the first immobilization strategy evaluated in this project (immobilization of HS on anion exchange resin), it was possible to mantain the HS in the supplemented UASB reactor achieving the effective removal of phenol and Reactive Red 2 (RR2) in long-term operation. In the present chapter the results obtained during the evaluation of the other immobilization strategy, HS adsorbed on γ-Al_2O_3 NPs are presented.

Due to the great advantages of nan-materials, applied in different industrial sectors, such as electronic, materials engineering, food, transportation, cosmetic, energy, pharmaceutical, biomedical, automotive, agriculture, fishing, manufacturing, security and consumers goods, are demanding these engineered materials. However, little research has been focused on their application in bioremediation purposes, specially in the development of wastewater treatment systems effective to remove recalcitrant pollutants. Some studies have been focused on elucidating the interaction of natural organic matter (eg. HS) and NPs once released into the environment (Lin et al. 2010; Saleh et al. 2010; Wang et al. 2008), and on mitigation of their toxicity in the presence of HS (Alvarez and Cervantes 2012; Dasari and Hwang 2010; Lin et al. 2012; Van Hoecke et al. 2011; Zhao et al 2013). In the last case, a possible explication for the toxicity attenuation is that HS can coat on NPs and serve as a physical barrier preventing the attachment of NPs to microorganisms (Lin et al. 2012). Lin et al. (2012) evaluating the effect of NPs coated with HS,

reported that the presence of HS limited agglomeration and co-precipitation of TiO_2 NPs on *Chlorella sp* due to increased electrostatic repulsion between TiO_2 NPs and negatively charged algal cell. Furthermore, HS acted as an antioxidant agent by reacting with reactive oxygen species, which reduced the oxidative stress and toxicity of TiO_2 NPs. More recently, Zhao et al. (2013) also suggested electrostatic repulsion as the principal mechanism for mitigation of toxicity of CuO NPs on *E. coli.* The capacity of metal oxides to adsorb HS has also been used for bioremediation purposes. For instance, HS immobilized on alumina particles were shown to effectively serve as solid-phase redox mediator (RM) for accelerating the reductive dechlorination of carbon tetrachloride by an anaerobic consortium (Alvarez et al. 2012). Previously, the humic model compound anthraquinone 2,6-disulfonate (AQDS), adsorbed on $Al(OH)_3$ NPs, was also demonstrated as effective solid-phase RM during the reductive decolorization of the azo dye RR2 (Alvarez et al. 2010). Furthermore, Alvarez and Cervantes (2011) proposed that co-immobilization of humus reducing microorganisms and NPs covered with HS (as RM) during granular sludge formation could be possible. This granular sludge with enhanced redox properties could be used in bioreactors in order to eliminate the prerequisite of continuous addition of HS and prevent their wash-out from bioreactors. Nevertheless, co-immobilization of HS and HRM by granulation has not been reported in the literature.

The two main goals of this study were: 1) to develop and characterize granular sludge produced for the co-immobilization of HRM with HS coated γ-Al_2O_3 NPs and 2) to assess the effects of these granules on the removal of two recalcitrant pollutants. In this work, RR2 and 2,4-dichlorophenol (DCP) were used as model

pollutants. Likewise, γ-Al_2O_3 NPs were chosen since they have shown high capacity to adsorb HS and good settling capacity in preliminary studies (Alvarez et al. 2010; 2012).

4.2 Materials and methods

4.2.1 Chemicals

NPs of γ-Al_2O_3 (20-50 nm and >150 m^2/g) were purchased from Inframat Advanced Materials (Manchester, CT USA). The source of HS was Leonardite obtained from the International Humic Substances Society (IHSS, Catalogue No. 1BS104L). HS were sulfonated and adsorbed on γ-Al_2O_3 NPs as previously described (Cervantes et al. 2011). RR2 (Procion Red MX-5B, ~50% of purity) and 2,4-DCP were purchased from Sigma-Aldrich Company and used without additional purification. All other chemicals used in this study were of analytical grade.

4.2.2 Adsorption of HS on γ-Al_2O_3 NP

Both the capacity of γ-Al_2O_3 NPs to adsorb Leonardite and their surface charge distribution were carried out as described by Alvarez and Cervantes (2012). Leonardite was previously sulfonated in order to enhance their solubility and affinity to γ-Al_2O_3 NPs, and also to allow that quinone groups present in Leonardite remain available after adsorption (see Chapter 3 for details on sulfonation process). The capacity of NP of γ-Al_2O_3 to adsorb Leonardite was determined by adsorption isotherms using the batch equilibrium technique. Different concentrations of Leonardite (100 to 5800 mg Total Organic Carbon (TOC) L-1) were prepared using deionized water, and then adjusting the pH to 4 with 0.1 M HCl. Then, 0.2 g of γ-Al_2O_3 and 10 mL of Leonardite solution (previously filtered with 0.22 μm) were mixed in polypropylene vials. The vials were placed on a shaker (160 rpm at 27°C)

during three days, until the equilibrium was accomplished. After centrifugation (3200 rpm, 10 min) the supernatant was analyzed in order to determine the equilibrium concentration of Leonardite in terms of TOC; and the adsorption capacity determined by a mass balance, (according to Eq. 3.1 in Chapter 3). For simplicity, the term a-Leonardite will be used to refer to the adsorbed Leonardite on γ-Al_2O_3 NPs.

4.2.3 Surface Charge Distribution of γ-Al_2O_3 NPs

Batch experiments were conducted to determinate the surface charge distribution of γ-Al_2O_3 NPs at different pH values under a CO_2-free atmosphere. N_2 was bubbled for 15 min into the solutions and also in the headspace of vials before sealing. First, 0.4 g of γ-Al_2O_3 NPs was dispensed in vials of 50 mL, and portions of 0.1 M NaCl were used to maintain a constant ionic strength. Initial pH values (2 to 12) were obtained by adding NaOH or HCl 0.1 M, for a total volume of 25 ml. After 4 days in stirring (150 rpm at 25°C), the final pH was measured and the surface charge (expressed as the amount of ions released) was obtained with a mass balance based on pH change.

4.2.4 Procedure for granulation

Two upflow anaerobic sludge blanket (UASB) reactors were operated with a hydraulic retention time (HRT) of 36 h in a controlled temperature room (28 ± 1 °C). The reactors were named R1 (control reactor to which untreated γ-Al_2O_3 NPs were added) and R2 (reactor supplemented with a-Leonardite). Both reactors were made of glass, for a total volume of 1.1 L. R2 was supplemented with 30 g of a-Leonardite, which represents ~2.4 g of Total Organic Carbon (TOC) of Leonardite L^{-1}, while R1 was amended with the same amount of NPs, but using untreated γ-

Al_2O_3 NPs. Both reactors were inoculated with 25 g volatile suspended solids (VSS) L^{-1} of a methanogenic consortium obtained from a full-scale UASB reactor treating brewery effluents (Sonora, Mexico). The granular sludge was previously reported to reduce both suspended (Alvarez and Cervantes 2012) and immobilized Leonardite (Martinez et al. 2013). To achieve homogeneous distribution in both reactors, anaerobic granular sludge was disintegrated using a sieve of 250 µm in an anaerobic chamber prior to be used. The reactors were fed with glucose (2 g chemical oxygen demand (COD) L^{-1}), peptone (1 g COD L^{-1}), a mixture of propionate, butyrate, and acetate (1 g COD L^{-1}) as substrate, balanced nutrients and trace elements. The basal medium contained (g L^{-1}): NH_4Cl (0.28), K_2HPO_4 (0.25), $MgSO_4 \cdot 7H_2O$ (0.1), $CaCl_2 \cdot 2H_2O$ (0.2), $FeSO_4$ (0.032), $NaHCO_3$ (5) and 1 ml L^{-1} of trace elements (Martinez et al. 2013). The reactors were firstly operated in batch mode with recirculation during one week in order to prevent an important wash-out of either γ-Al_2O_3 NPs or a-Leonardite. Then, operation in continuous mode followed during the remaining experimental period.

4.2.5 Characterization of produced granules

In both reactors granules were visible after 60 days of continuous operation and the reactors were mantained and operated under the same conditions for approximately one year. After 380 days, aproximately 200 mL of already formed granules were withdrawn from both reactors and characterized based on their size and content of γ-Al_2O_3 NPs. The size distribution of granules was carried out by using different sieves (0.25 to ≥ 2.0 mm). Samples for determination of content of γ-Al_2O_3 NPs were digested with a mixture of HCl:HNO_3:HF (2:2:1) as follows: 1) in the effluent: 5 mL of water were mixed with 5 mL of acids solution; 2) in the granules: a defined

portion of sample (~ 0.5 g) was suspended in 10 mL of acids solution. Both water and granules samples were maintained in a shaker until the digestion was completed. Finally, dissolved Al^{3+} was measured by Inductively Coupled Plasma (ICP-AES) (Varian 730 series, Palo Alto, CA, USA).

4.2.6 Biodegradation tests

Assays were conducted in batch mode by duplicate in glass serum bottles with a liquid volume of 80 mL. Anaerobic basal medium previously described in chapter 3 (Martinez et al. 2012), was directly transferred to the vials, which were then inoculated with 0.5 g VSS L^{-1} of granules extracted from R1 and R2. All assays were supplied with glucose as substrate (1 g COD L^{-1}). Reduction assays were performed with disintegrated granules and with untreated granules. This assays were supplemented with immobilized HS to achieve a final concentration of HS of 2 g COT L^{-1}. Before addition of RR2 or 2,4-DCP, methanogenic activity was verified in all incubations. Once the production of methane in all cultures was similar, RR2 and 2,4 DCP were added to a final concentration of 0.6 mM and 0.12 mM, respectively. Sterile controls were also included in the experimental protocol in order to identify potential physicochemical processes (e.g. adsorption) involved during RR2 and 2,4-DCP removal. All experimental treatments were incubated at (28 ± 1 °C) in the dark at 150 rpm.

4.2.7 Analyses

Methane concentration in biogas was quantified in 100 µL headspace samples in a gas chromatograph (GC, Agilent Technologies 6890N series) equipped with a thermal conductivity detector and a column Hayesep D (Alltech, Deerfield, Illinois, USA) with the following dimensions: 3.048m × 3.18mm × 2.16 mm. Nitrogen was

used as carrier gas with a flow-rate of 12 mL min^{-1}. Temperatures of the injection port, oven and the detector were 250, 60 and 250 °C, respectively. Nitrogen was used as carrier gas with a flow-rate of 12 mL min^{-1}. Color removal (RR2) was determined photometrically by UV/visible spectrophotometry (Thermo-cientific Genesys 10 UV) at 539 nm. Aniline, 2,4-DCP and 4-chlorophenol were measured by high performance liquid chromatography (HPLC). The HPLC was equipped with two reverse phase columns Synergi 4uHydro-RP80A (250 • 4.60 mm, 4 micron) from Phenomenex. The carrier liquid composed of 60% of deionized water and 40% acetonitrile, was pumped at a flow rate of 1 mL min^{-1}. All contaminants were detected using a diode array at 230 nm. The detection time for aniline, 2,4-DCP and 4-chlorophenol were 7.8, 13.46 and 10.83 min. respectively. COD and VSS were analyzed according to Standard Methods (APHA, 1998).

4.3 Results

4.3.1 Surface charge distribution and adsorption of HS on γ-Al_2O_3 NPs

The capacity of γ-Al_2O_3 NPs to adsorb Leonardite and the surface charge distribution of this material were determined. Surface charge distribution data of γ-Al_2O_3 NPs at different pH values are given in Figure 4.1. Surface charge of γ-Al_2O_3 NPs decreased with the increase of pH. The pH point of zero charge (pHpzc) of γ-Al_2O_3 was 8.1, which implies that positive charges prevail on their surface below this pH value, whereas negative ones above this value. Thus, according to surface charge distribution, an appropriate pH value for the adsorption of Leonardite on γ-Al_2O_3 NPs was 4, since sulfonated Leonardite is negatively charged at this pH value due to the presence of sulfonated group (SO_3^- pKa_1 = 1.76 and pKa_2 =7.2).

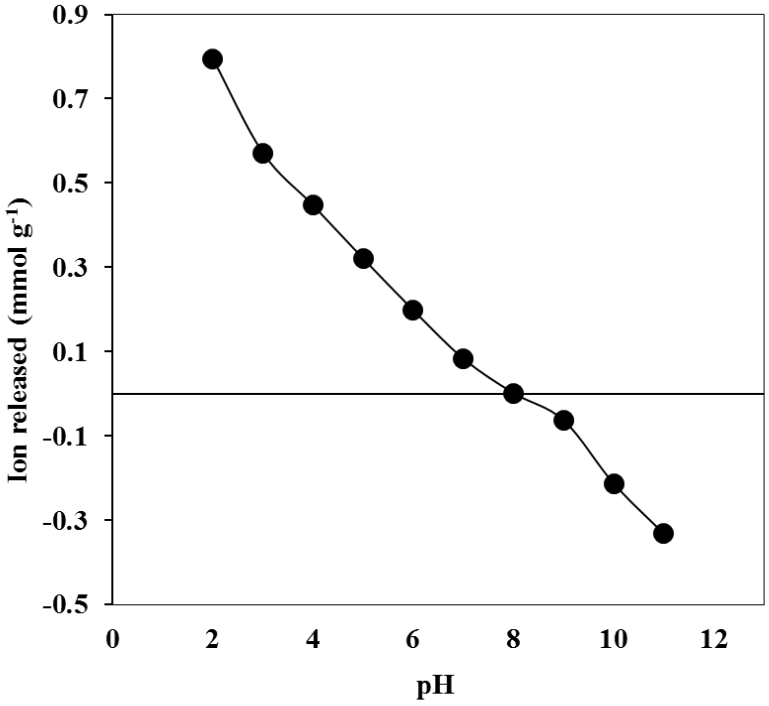

Figure 4.1 Surface charge distribution of γ-Al_2O_3 NP at different pH values

Adsorption assays revealed an adsorption capacity of Leonardite on γ-Al_2O_3 NPs of ~165 mg/g. After six desorption cycles, the adsorption capacity became stable at 89.5 mg TOC g^{-1} (Figure 4.2). This is an adsorption value higher than that obtained by Alvarez et al. (2012), who immobilized fulvic acids on γ-Al_2O_3 (12 mg TOC g^{-1}), but in that study alumina particles with an average size > 63 μm were used, whereas in this study the size of the alumina NPs was between 20-50 nm. The proposed immobilization mechanism of sulfonated leonardite on γ-Al_2O_3 NPs is presented in Figure 4.3. Some studies have reported different mechanism of natural organic matter on mineral surfaces: anion sorption, ligand exchange, protonation, hydrogen bonding, cation bridging, and physical adsorption. In this study, the immobilization was conducted at pH 4 at which γ-Al_2O_3 NPs are positively charged,

while sulfonated groups in Leonardite are negatively charged. Thus, the obtained results suggest that immobilization of HS on γ-Al_2O_3 is principaly due to electrostatic interaction. However, it is possible that other mechanisms are also involved.

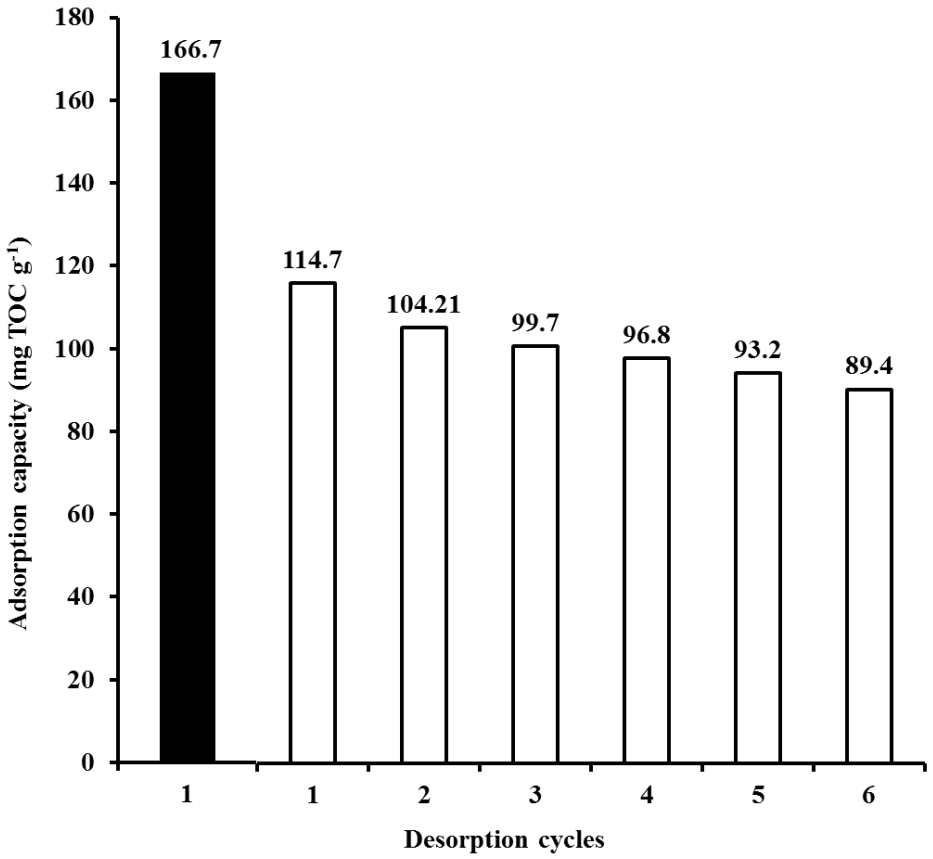

Figure 4.2 Adsorption capacity of Leonardite on γ-Al_2O_3 NPs after six desorption cycles

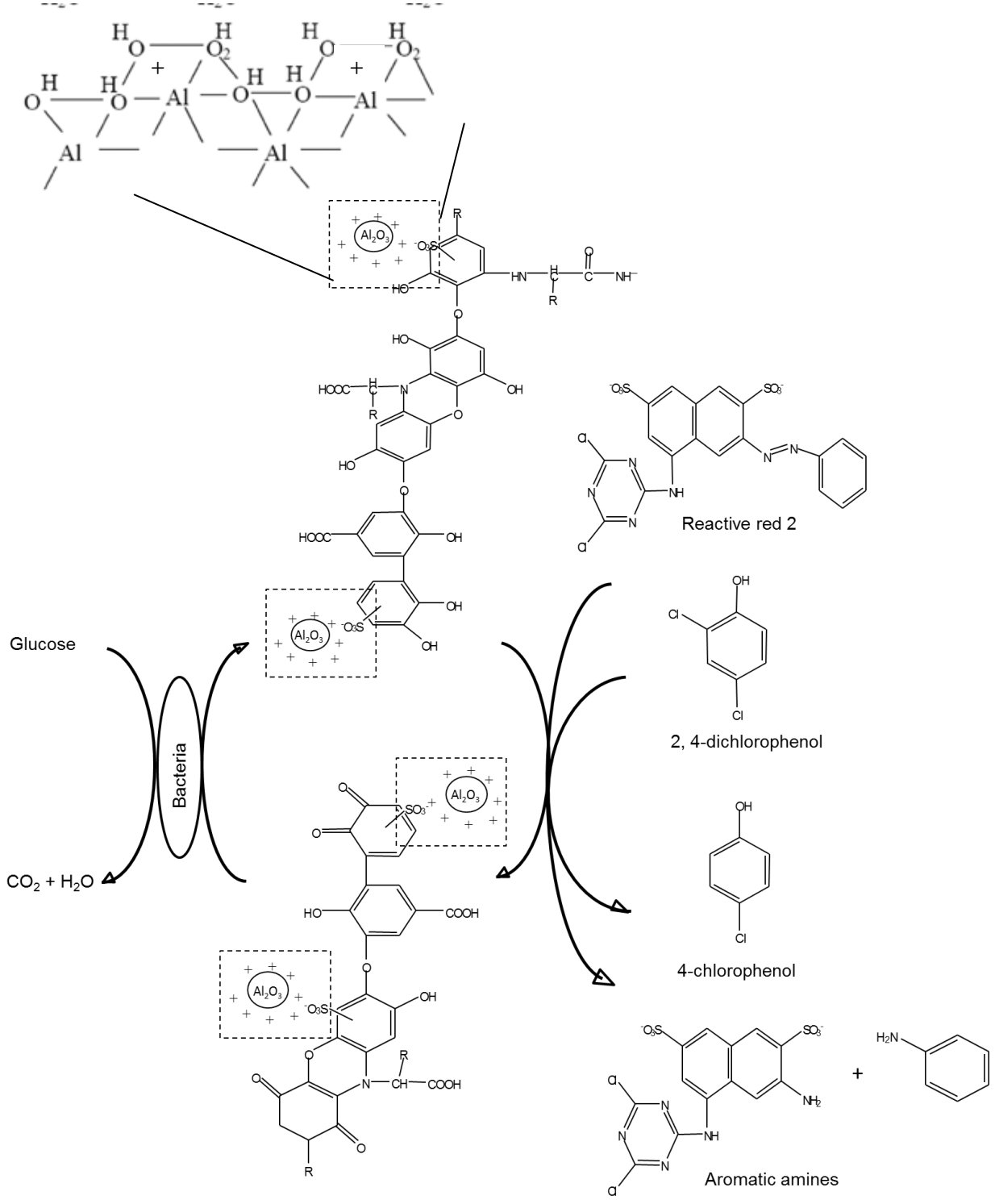

Figure 4.3 Proposed mechanisms involved in the reductive biotransformation of Reactive Red 2 (RR2) and 2,4 dichlorophenol (DCP) by humus-reducing microorganism mediated by immobilized sulfonated HS on γ-Al_2O_3 NPs. Immobilized HS served as terminal electron acceptor for humus-reducing microorganisms supporting the anaerobic oxidation of glucose in the first step. Reduced HS then transfer the reducing equivalents to the electron-accepting group of RR2 and DCP in the second step. Dashed squares indicate the interaction between sulfonated groups inserted in HS and the positive charges on γ-Al_2O_3 NPs.

4.3.2 Performance of UASB reactors during granulation

COD removal efficiencies at steady state were ~95.5% ± 2.2 and ~96.7% ± 2.0 for R1 and R2, respectively (Figure 4.4).

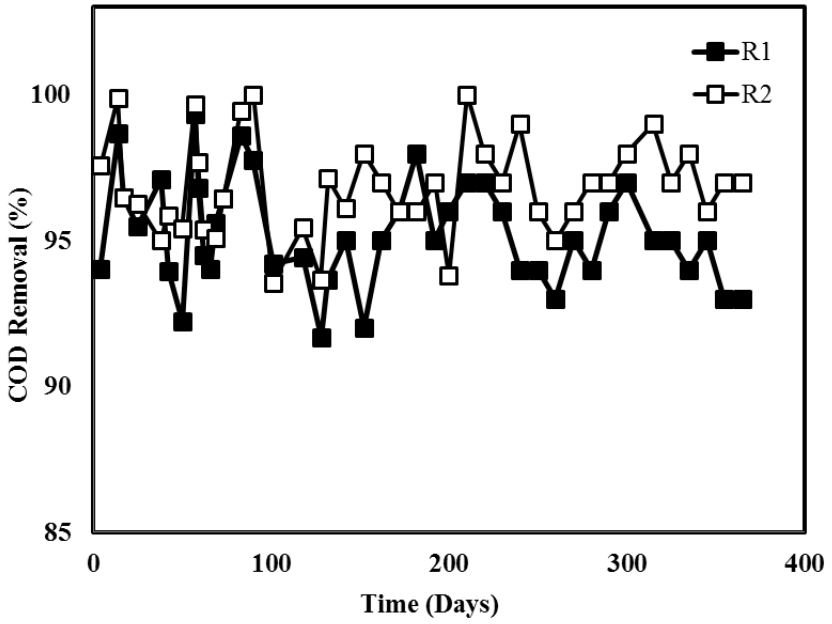

Figure 4.4 COD removal efficiencies during the granulation process

Figure 4.5 shows that the effluent concentration of VSS and Al^{3+} decreased over time when continuous mode was established. During the first 25 days of operation, wash-out of VSS decreased from ~275 to ~100 mg L^{-1}. Later, the effluent concentration of VSS was stabilized at around 50 mg L^{-1} in both reactors (Figure 4.5a). On the other hand, the initial content of Al^{3+} in the effluent for R1 and R2 were 39 and 35 mg L^{-1}, respectively. The concentration of Al^{3+} gradually decreased up to ≤1.0 mg L^{-1} and was stabilized after 60 days of continuous operation (Figure 4.5b). In order to confirm that Al_3^+ was not coming out from the reactor after one

year of operation, its concentration was determined again. The results confirmed that aluminium was not present in the effluent. Thus, the quantified amount of Al^{3+} washed out was 5.1 g of Al^{3+} (corresponding to ~20% of Al_2O_3 NPs initially added in R2). Speciation analysis revealed that alumina NPs are in solid state at the pH of operation in both reactors (Figure 4.6). Thus, γ-Al_2O_3 NPs are expected to be stable once immobilized by the granulation process.

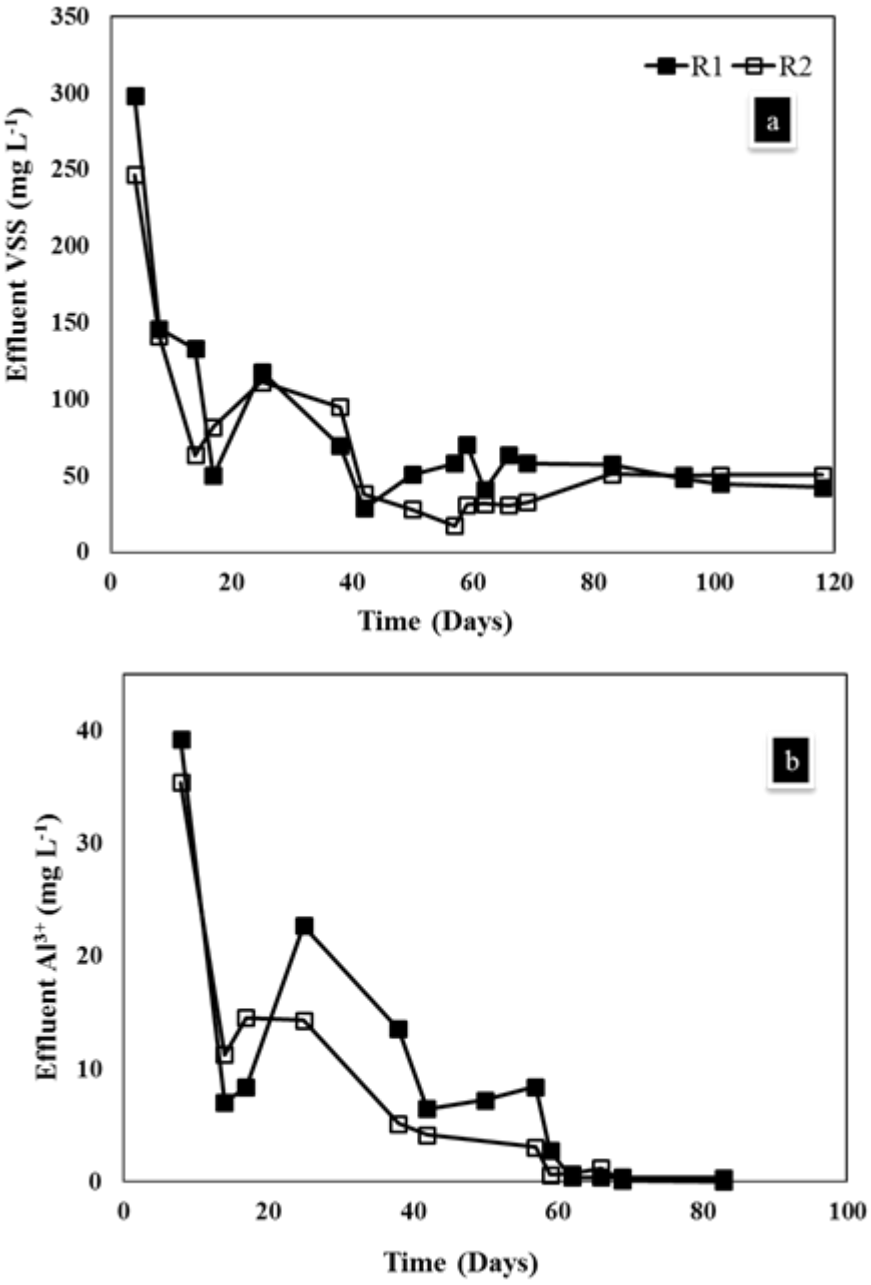

Figure 4.5 Effluent concentrations of a) VSS and b) Al^{3+} during the starting stage in both reactors

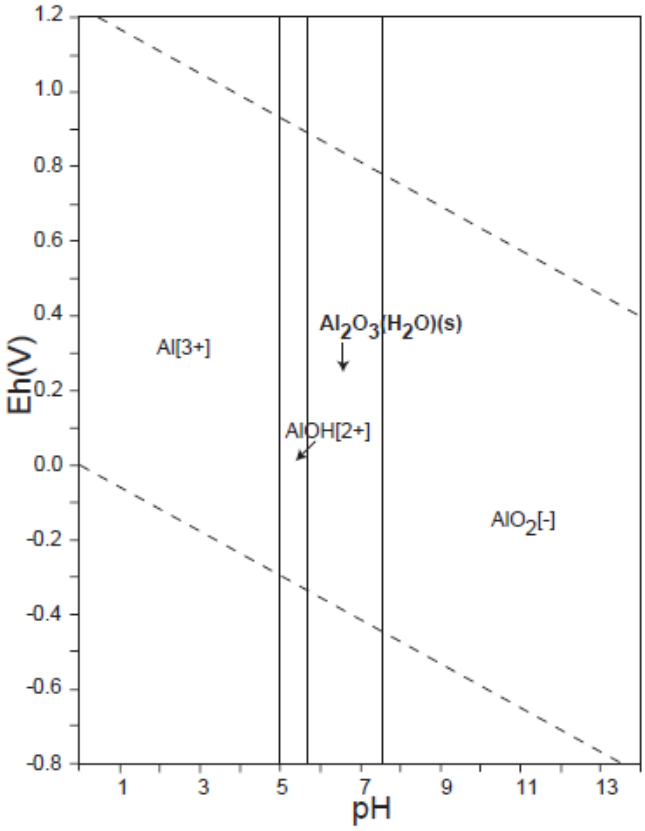

Figure 4.6 Eh-pH diagram of the system Al_2O_3

4.3.3 Characterization of granules

Once operational parameters were stable after approximately one year of continuous operation, granular biomass samples were extracted from each reactor for characterization. Figure 4.7a shows size distribution (by weight) of produced granules from both reactors. In fact, granules were well established as soon as 40 days of bioreactors operation. Size distribution of granules was very different in both reactors. While 73% of granules from R2 were in average of ≥0.5 to ≤2.0 mm, only 45% of granules from R1 were found in the same range of size. Furthermore, 45% of granules from R1 were in the range of 0.25 to 0.5 mm. Thus, these results indicated that granules formed in the presence of Leonardite were, on average,

larger than granules formed in the absence of Leonardite.

As indicated in Figure 4.7b, the Al^{3+} content in granules of R1 and R2 was similar regardless of size or if NPs were coated with HS or not, indicating agglomeration of biomass and γ-Al_2O_3 NPs in both reactors. The average aluminum content was 5.07 and 5.69 mg Al^{3+} g^{-1} in sludge samples from R1 and R2, respectively. According to this value and considering the adsorption capacity of a-Leonardite (89.5 mg TOC g^{-1}), the average content of HS in granules formed in R2 was 0.96 mg TOC g granule $^{-1}$ or 13.74 mg TOC g VSS^{-1}, which corresponds to a concentration of 343 mg TOC L^{-1} in R2.

Reduction of RR2 and 2,4-DCP by granular sludge

Biodegradation experiments were firstly conducted with granular sludge produced from both reactors without providing external HS. Thus, the actual concentration of HS prevailing in the incubations inoculated with granules derived from R2 was 0.96 mg TOC L^{-1}, while no HS were present in experiments performed with granules from R1. Results obtained from these experiments could suggest that the amount of HS contained in the granules from R2 (0.96 mg TOC g $^{-1}$) was insufficient to promote an increased reduction of the studied contaminants. Under these conditions, the specific reduction rates (SRR) obtained were similar with granules from R1 and R2. Thus, the immobilized amount of HS in granules from R2 might have been too low to enhance the reductive biotransformation of RR2 and 2,4-DCP. In order to exclude this effect, the concentration of immobilized HS was increased to 2 g TOC in incubations with granules derived from R2. Moreover, reduction assays were also conducted with disintegrated granules in order determine if the catalytic effect of HS might be inhibited due to integration of HS at the inner part of the

granules.

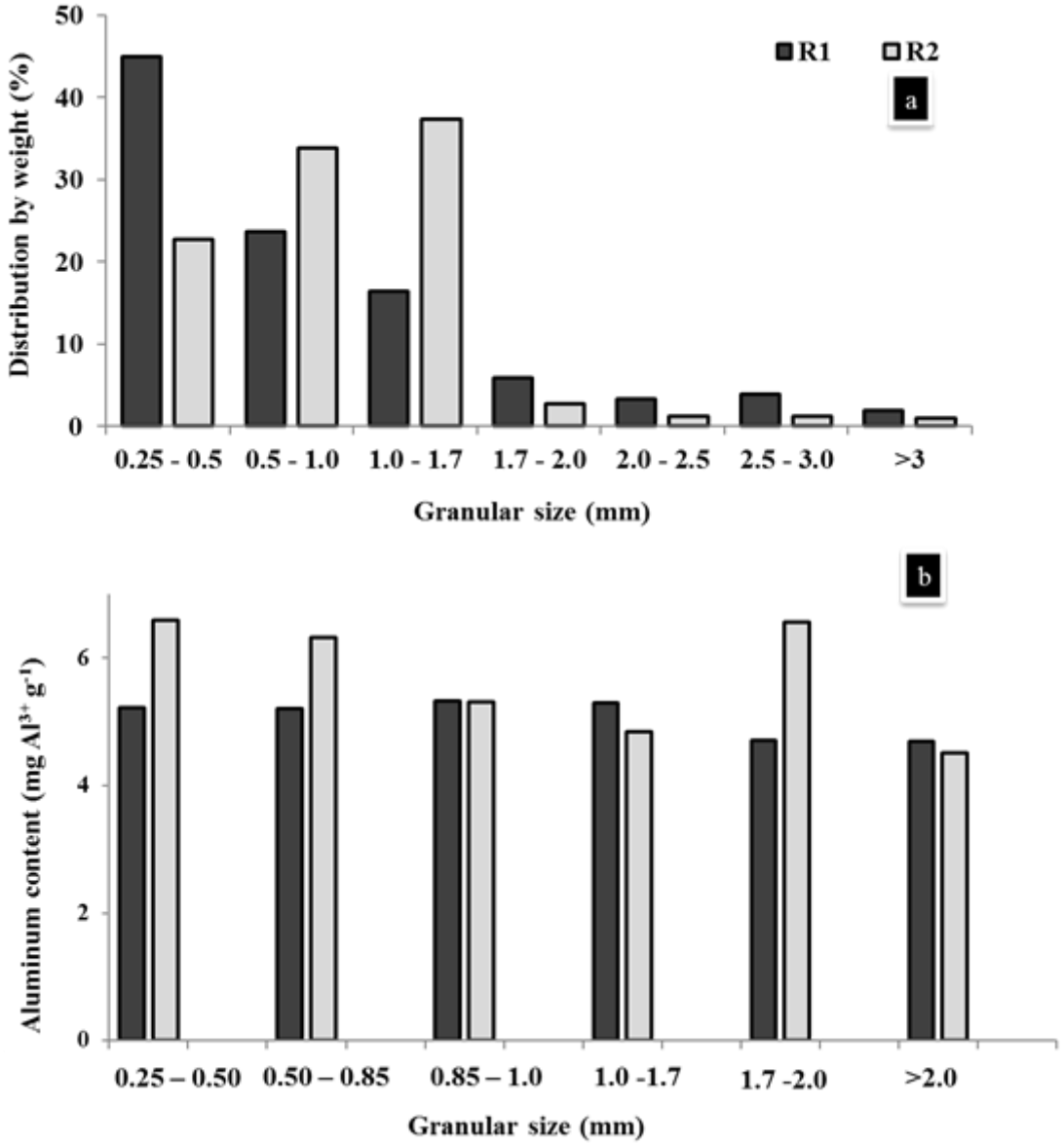

Figure 4.7 a) Size distribution (by weight) of granules b) content of Al^{3+} after 1 year of continuous operation

Table 4.1 shows SRR and mass balances obtained in the different treatments evaluated after one week of incubations. The results indicated that addition of external HS significantly increased the SRR of RR2. In the presence of HS with disintegrated granules and granular sludge around 50% of RR2 was reduced after

one week of incubation. In contrast, in controls lacking HS only 25% of RR2 was reduced. The calculated SRR in the presence of HS with disintegrated granules and granular sludge were 7.28 µmol (gSSV.d) $^{-1}$ and 7.04 µmol (gSSV.d) $^{-1}$, respectively. For controls lacking HS, the SRR values were 3.24 µmol (gSSV.d)$^{-1}$ and 3.88 µmol (gSSV.d) $^{-1}$, respectively. Thus, the presence of HS increased 2.2 and 1.9-fold the SRR value compared to the control incubated without HS.

Results presented in Table 4.1 also indicated that disintegration of granules did not affect the reduction of RR2 since no significance differences were found in the reduction rates obtained

Table 4.1. Specific reduction rate and mass balance for the reduction of RR2 in the different culture conditions evaluated after one week of incubation

Culture conditions	SRR (µmol gSSV.d^{-1})	$RR2_i$ µM	$RR2_f$ µM	Aniline µM	Reduction (%)	Recovery (%)
Disintegrated sludge from R2	7.28	780 ± 2.46	460 ± 4.2	288 ± 1.5	45.5	96.5
Granular sludge from R2	7.04	730 ± 3.57	451 ± 2	257 ± 3.4	44.8	96.7
Disintegrated sludge from R1	3.24	800 ± 0.49	670 ± 0.4	111 ± 3.5	22.5	97.7
Granular sludge from R1	3.88	840 ± 2.21	627 ± 2	178 ± 4.1	22.1	95.1

Initial concentration: RR2 600 µM, HS 2g/L. Results represent average from duplicate incubations
$RR2_i$ initial concentration of RR2, $RR2_f$ final concentration of RR2, R1 control reactor to which untreated γ-Al_2O_3 NPs were added, R2 reactor supplemented with a-Leonardite
Reduction = (Aniline / $RR2_i$) X 100; % Recovery = ($RR2_f$ + Aniline)/ $RR2_i$ x 100

Reduction of RR2 was also reflected on the production of aniline as its reduction product. Data summarized in Table 4.1 revealed that higher decolorization efficiency of RR2 corresponded to higher aniline production. In the presence of HS,

aniline recovery represented more than 90% of the amount of RR2 reduced and around 80% in controls lacking HS. Considering the recovery of aniline obtained in these assays, it can be observed that aniline recoveries are significantly higher than those obtained in other studies in which the same RM was used (Martinez et al. 2013). Meanwhile, neither reduction of RR2 nor detection of aniline occurred in sterile controls.

Regarding assays for 2,4-DCP reduction, results indicated that addition of external immobilized HS significantly increased the reduction of this contaminant (Table 4.2). In the presence of immobilized HS with disintegrated sludge and with granular sludge, 70% and 55 % of 2,4-DCP was reduced, respectively, after 11 days of incubation. In contrast, controls lacking HS showed less than 23% of 2,4-DCP reduction. The calculated SRR in the presence of HS with disintegrated and granular sludge was 11.12 µmol (gSSV.d) $^{-1}$ and 7.86 µmol (gSSV.d) $^{-1}$, respectively. For controls lacking HS, the k_{DCP} values were 3.77 µmol (gSSV.d) $^{-1}$ and 1.03 µmol (gSSV.d) $^{-1}$ respectively. Thus, the presence of HS increased 3 and 7 fold the SRR value compared to the controls incubated without HS.

Certainly, assays conducted with disintegrated sludge showed faster and higher extent of reduction of 2,4-DCP than that obtained with granular sludge in the presence of HS. Reduction of 2,4-DCP was also reflected on the detection of 4-chlorophenol (4-CP) as its reduction product. Data summarized in Table 4.2 revealed that higher reduction efficiency of 2,4-DCP corresponded to higher 4-CP concentration. In the presence of HS, 4-CP recovery represented more than 90% of the amount of 2,4-DCP reduced and less than 80% in controls lacking HS.

Table 4.2 Specific reduction rate and mass balance for the reduction of 2,4-DCP by the different culture conditions evaluated after eleven days of incubation

Culture conditions	SRR (μmol gSSV.d^{-1})	2,4-DCP$_i$ μM	2,4-DCP$_f$ μM	4-CP μM	Reduction (%)	Recovery (%)
Disintegrated sludge from R2	11.12	75 ± 3.2	16.1 ± 4.2	56.5 ± 2.6	74.5	95.7
Granular sludge from R2	7.86	78 ± 1.5	32.2 ± 3.4	42.9 ± 5.2	54.4	95.2
Disintegrated sludge from R1	3.77	78 ± 3.7	60.7 ± 2.8	14.9 ± 1.3	19.0	96.4
Granular sludge from R1	1.03	73 ± 2.6	67.4 ± 3.1	2.4 ± 2.6	22.9	94.7

Initial concentration: 2,4-DCP 120 μM, HS 2g/L. Results represent average from duplicated incubations
2,4-DCP$_i$ initial concentration of 2,4-DCP, 2,4-DCP$_f$ final concentration of 2,4-DCP, 4-CP 4-chlorophenol, R1 control reactor to which untreated γ-Al_2O_3 NPs were added, R2 reactor supplemented with a-Leonardite
Reduction = (4-CP / 2,4-DCP$_i$) X 100; % Recovery = (2,4-DCP$_f$ + 4-CP)/ 2,4-DCP$_i$ x 100

4.4 Discussion

The main objective of this study was to explore the possibility to co-immobilize HRM with HS supported on γ-Al_2O_3 NPs, as another strategy to maintain HS within a continuous reactor. The obtained results demonstrated for the first time that it was possible to achieve this co-immobilization previously proposed by Alvarez and Cervantes (2011). We consider that during this co-immobilization process, the majority of the microorganisms present in the granules are HMR, since it was previously determined that this sludge was able to reduce the humic model compound anthraquinone 2,6-disulfonate (AQDS). Furthermore, the large diversity of microorganisms capable of reducing HS (see Table 2.1 in Chapter 2) also supports this hypothesis.

The present results showed that larger granules with appropriate agglomeration of biomass and γ-Al_2O_3 NPs were obtained in the presence of HS (R2) as compared to those produced in the absence of HS (R1). These results are in agreement with previous studies (Alvarez and Cervantes 2012; Zhao et al. 2013), which have documented that γ-Al_2O_3 NPs coated with HS interact better with anaerobic microorganisms and decrease the toxicity of NPs. Thus, this could explain why NPs modified with HS promoted a better granulation process. The absence of toxicological effects of γ-Al_2O_3 NPs was also evidenced by the high COD removal efficiency achieved in both reactors during the granulation process (Figure 4.4). However, during the evaluation of the catalytic effect of these granules on the reduction of RR2 and 2,4-DCP, some limitations were observed.

According to the collected evidence, the amount of HS contained in the produced granules (0.96 mg TOC g^{-1}) in R2, could have been was insufficient to promote an increased SRR of RR2 and 2,4-DCP.

Cervantes et al. (2011b) who evaluated the reduction of RR2 (0.3 mM) in the presence of immobilized HS (same source used in the present study and equivalent to 100 mg TOC L^{-1}), supported on an anion exchange resin, reported that immobilized HS increased 2-fold the rate of decolorization of RR2 as compared with sludge incubations lacking HS. In the present study, no significant catalytic effect of HS supported on γ-Al_2O_3 NPs was observed even though a higher amount of immobilized HS (343 mg TOC L^{-1}) were supplied. This inconsistency might have been due to the different inocula used.

Furthermore, interaction of redox-mediating functional groups of immobilized HS with biomass components or exopolymeric substances might also have interferred

with the capacity of immobilize HS to promote the reductive biotransformation of the studied pollutants.

Recently, Rios-del Toro et al. (2013) evaluated if the formation of biofilm on activated carbon fiber (ACF), affected its redox-mediating capacity. The authors reported that biofilm formation drastically decreased the redox capacity of ACF during the reduction of methyl red. Likewise, the rate-limiting step during the reduction of this azo dye, was the electron transfer from the microbially reduced ACF to the contaminant. In other words, the biofilm hindered functional groups on ACF reducing the catalytic capacity due to the obstruction of redox functional groups by biomass components.

Although in this investigation more experiments are needed to confirm the obstruction of redox active functional groups in HS, we can suggest that in the present work, the redox mediating capacity of HS was affected during their co-immobilization with HRM in the granulation process promoted in R2. This could be due to integration of HS coated γ-Al_2O_3 NPs in the inner part of the produced granules, which could have limited their accessibility for catalyzing the redox conversions evaluated. Furthermore, redox active functional groups in HS, such as quinones, might have interacted with biomass components, thus blocking their redox mediating capacity.

Finally, another limitation encountered during the co-immobilization of HS and HRM during the present study was the loss of an important fraction (~20%) of the a-Leonardite initially supplied during the early stages of granulation (days 1 to 80), which significantly decreased the amount of a-Leonardite available after the granulation process was completed. Previously, Alvarez et al. (2012) reported that

γ-Al_2O_3 NPs showed better settling capacity as compared to other NPs studied ($Al(OH)_3$, and TiO2.), fact which in this study, was observed during the granulation process. However, further studies are required in order to verify that redox active functional groups (e.g. quinones) in immobilized HS remain available for catalysis after the granulation process.

4.5 Conclusions

The present study demonstrated for the first time the possibility to co-immobilize HRM with HS supported on γ-Al_2O_3 NPs, as another strategy to maintain HS within a continuous reactor. In the presence of HS, larger granules with appropriate agglomeration of biomass and γ-Al_2O_3 NPs were obtained. However, the amount of HS contained in the produced granules and obstruction of redox active functional groups in HS, could be factors that limited the catalytic effect of these granules during the reduction of RR2 and 2,4-DCP. Thus, more studies are needed in order to verify that redox active functional groups (e.g. quinones) in immobilized HS remain available after the granulation process. Likewise, the amount of a-Leonardite initially supplied during the early stages of granulation, is a parameter that must be considered in order to ensure a proper amount of a-Leonardite available after the granulation process.

5

Simultaneous biodegradation of phenol and carbon tetrachloride mediated by humic substances

Abstract

The capacity of an anaerobic sediment to achieve the simultaneous biodegradation of phenol and carbon tetrachloride (CT) was evaluated, using humic substances (HS) as redox mediator (RM). The presence of HS in sediment incubations increased the rate of biodegradation of phenol and the rate of dehalogenation (2.5-fold) of CT compared to controls lacking HS. Further experiments revealed that the electron-accepting capacity (EAC) of HS derived from different organic-rich environments was not associated with their reducing capacity to achieve CT dechlorination. The collected kinetic data suggest that the reduction of CT by reduced HS was the rate-limiting step during the simultaneous biodegradation of phenol and CT. To our knowledge, the present study constitutes the first demonstration of the simultaneous biodegradation of two priority pollutants mediated by HS.

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5.1 Introduction

During the last two decades, evidence have reported that addition of redox mediators (RM), such as humic substances (HS) and their quinone model compounds could significantly enhance the reduction rate of several recalcitrant pollutants by accelerating the transfer of electron equivalents derived from microbial substrate oxidation to the electron-accepting contaminants (Van der Zee and Cervantes 2009). However, as pointed out before, the main limitation in the application of HS as RM in continuous systems is that their continuous addition must be provided to increase conversion rates, which is economically and environmentally non-viable. In this sense, some strategies to immobilize HS have previously been reported (Chapter 2, Table 2.3 and 2.4). However, only a few reports have documented that immobilized HS can serve as effective RM during the reductive biotransformation of recalcitrant pollutants in batch incubations (Alvarez et al. 2012, Cervantes et al. 2011b, Cervantes et al. 2013), and several of these strategies of immobilization have not been applied in redox reactions yet (Chapter 2, Table 2.4).

The results presented in previous chapters demonstrated the effectiveness of immobilized HS to serve as solid-phase RM to accelerate the biodegradation of representative pollutants in a high-rate anaerobic reactor without the need of continuous addition. Although these results show the potential for the use of immobilized HS in the treatment of recalcitrant pollutants, studies to identify suitable sources of HS, such as organic rich environments and wastes are needed. Further information is also required in order to elucidate the main mechanisms involved in

the redox reactions mediated by HS and to promote their applicability. For instance, Perminova et al. (2005) suggested that the principal factor limiting the application of HS in remediation technologies is the intrinsic variability in redox properties observed among humic materials and their fractions. Indeed, the electron transferring capacity (ETC) greatly varies among HS extracted from different organic rich environments (Ratasuk and Nanny 2007) and scarce information is available in the literature clarifying the appropriate HS source demanded in redox reactions for remediation purposes. Moreover, very limited information has been reported to elucidate the rate-limiting step during the redox biotransformation of contaminants. In some cases, the reduction of RM by different microorganisms has been pointed out as the rate-limiting step during redox reactions (Rau et al. 2002). In contrast, other studies evidenced that the reduction of electron-accepting contaminants by reduced HS or hydroquinones is the rate-limiting step (Rau et al. 2002). Therefore, further research is demanded in order to elucidate the mechanisms limiting the catalytic effects of HS during the anaerobic (bio)transformation of priority pollutants, in order to evaluate their catalytic properties and to promote the applicability of immobilized HS derived from natural sources in wastewater treatment systems.

The aim of the present work was to study the kinetic aspects involved during the simultaneous biodegradation of phenol and carbon tetrachloride (CT) mediated by HS derived from different natural sources (Figure 5.1). Phenol and CT were selected as model pollutants because they frequently contaminate soils and water bodies due to their widespread industrial use and high incidence due to improper disposal, leaking storage tanks, and spills (Cervantes et al. 2000b; 2004). The study

assesses the capacity of three anaerobic consortia to oxidize phenol with the humic model compound, anthraquinone 2.6-disulfonate (AQDS), as terminal electron acceptor (TEA); evaluates the capacity of three sources of HS as reducing agent of CT; and selects the proper inoculum and source of HS to achieve the simultaneous removal of phenol and CT.

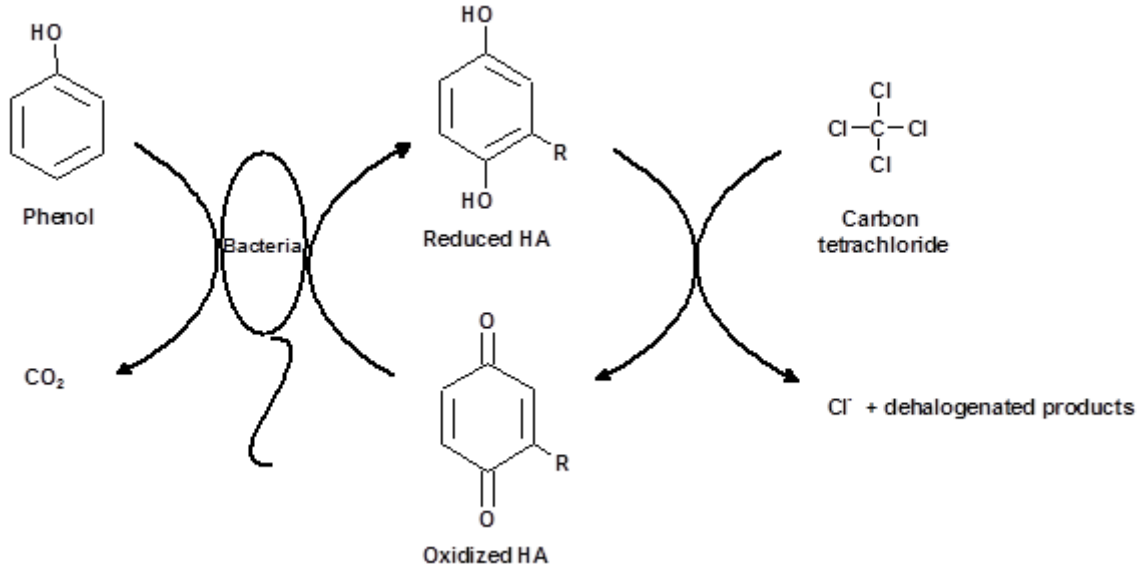

Figure 5.1 Mechanism proposed for the simultaneous biodegradation of phenol and CT mediated by humic substances

5.1 Materials and methods

5.1.1 Inocula and basal medium

Anaerobic granular sludge samples were collected from two full-scale UASB reactors treating effluents from a factory of candies (CS) (San Luis Potosi, Mexico) and from a paper-mill factory (PS) (Eerbeek, The Netherlands). Additionally, a sediment (S) was collected from a lagoon (Marland) contaminated with hydrocarbons located in Ebano (San Luis Potosí, Mexico). The content of volatile suspended solids (VSS) in these anaerobic consortia were (in % wt/wt): 4.98, 9.94 and 3.75 respectively, for CS, PS and S. The basal medium used in all batch

experiments was prepared as previously described (Cervantes et al. 2000). The basal medium was flushed with N_2/CO_2 (80%/20%) by passing this gas mixture through the liquid bulk for 10 minutes and was used without sterilization in all the experiments.

5.1.2 Biodegradation of phenol with AQDS as TEA

Incubations were conducted in batch mode by duplicate in glass serum bottles with a liquid volume of 50 mL. Anaerobic basal medium supplemented with AQDS (5 mM) was transferred directly to the glass serum bottles, which were inoculated by adding 2 g VSS L^{-1} of each consortium and sealed with Teflon stoppers and aluminum caps. Vials were then flushed with N_2/CO_2 (80%/20%) for 10 minutes. Finally, phenol was added as substrate at the initial concentration of 1.35 mM. Controls without phenol were also incubated to correct for endogenous AQDS reduction. All bioassays were incubated at 28 °C. The pH of the medium was controlled at 7.2 by a bicarbonate/CO_2 buffer (60 mM).

To determine if the evaluated consortia were also able to degrade phenol under methanogenic conditions, incubations lacking AQDS were included. Controls without phenol were also included to correct for the endogenous methanogenic activity. Samples from every experimental unit were periodically taken, centrifuged and filtered (0.2 µm), for determining the concentration of volatile fatty acids, such as acetate, propionate and butyrate, as well as to measure the reduction of AQDS [as anthrahydroquinone-2,6-disulfonate (AH_2QDS)]. Production of methane was determined by monitoring the composition of the produced biogas. All bioassays were incubated at 28 °C in the dark with mild shaking.

AQDS reduction and CH_4 production rates were determined using the maximum slope observed on linear regressions considering at least three sampling points. The coefficient of determination (R^2) was higher than 0.9 for all microbial activities calculated.

5.1.3 Reduction of CT by HS

Three different sources of HS were tested, which originated from a soil of cocoa plantation (SCP), soil of deciduous forest in San Luis Potosí, Mexico (SDF) and compost produced with gardening wastes (GWC). HS were extracted with NaOH 0.1 M (Sigma-Aldrich, solution titrated to 0.0987 M) under a nitrogen atmosphere, according to the standardized procedure of the International Humic Substance Society (IHSS). Table 5.1 shows the general characteristics of the HS evaluated. Chemical reduction experiments were performed in batch mode by triplicate in glass serum bottles with a liquid volume of 50 mL. Serum flasks were filled with basal medium containing HS (4g L^{-1}), Pd as catalyst (8 pellets) and sealed with Teflon stoppers and aluminum caps. The vials were flushed with H_2 for 1 hour to saturate the vials with this reducing agent and were incubated for one week to achieve complete reduction of HS. Table 5.1 shows the electron accepting capacity (EAC) of the tested HS determined by the ferrozine technique (Lovley et al. 1996b). After complete reduction of HS, Pd pellets were removed and solutions were diluted to establish an initial reducing capacity of HS at 150 micro-electron equivalents (μEq) gHS^{-1}. Reduced HS solutions were then transferred (in an anaerobic chamber with a N_2/H_2 atmosphere) to serum flasks sealed with Teflon stoppers and aluminum crimps and previously flushed with N_2/CO_2 (80%/20%) for 20 minutes. Finally, CT (100 μM referred to the liquid volume) was added to the serum flasks (from a

concentrated anaerobic stock solution). The assays were incubated at 28 °C in the dark with a mild shaking. The concentration of CT and products derived from its reductive dechlorination, such as chloroform (CF) and dichloromethane (DCM), was determined over time to assess the rate of dechlorination of CT.

Table 5.1 General characteristics and electron accepting capacity of each HS evaluated

Code	Description	Localization (state) and geographic coordinate	Typical Vegetation		Soil	EAC (µEq gHS^{-1})
			Specie	Genus	pH [a]	
SDF	Soil of deciduous forest of San Luis Potosí	San Luis Potosí: 22° 5′ 0″ N, 100° 38′ 0″ W	*Quercus rugosa Neé*	*Quercus*	5.65	174.9 ± 66.9
SCP	Soil from cocoa plantation	Pichucalco, Chiapas: 17º 30' N, 93º 07' W	*Theobroma cacao* L.	*Theobroma*	5.23	163.2 ± 21.3
GWC	Compost of gardening wastes	Pilot station at University of Guadalajara			6.69	347.1 ± 10.2

[a] Determined directly on the sample with a soil pH electrode
Reduction chemistry with: H_2/Pd and 4 g L^{-1} of HS. Electron accepting capacity (EAC) after 1 week of incubation. Results represent average from triplicate incubations
micro-electron equivalents, µEq; HS, humic substances

5.1.4 Simultaneous biodegradation of phenol and CT mediated by HS

The assays were conducted in batch mode by triplicate in glass serum bottles with a liquid volume of 30 mL. Anaerobic basal medium was directly transferred to the vials, which were then inoculated with 0.01 g VSS L^{-1} of sediment from Marland lagoon (inoculum S). HS derived from SCP (4 g L^{-1}) were used as RM during these experiments. All vials were sealed with Teflon stoppers and aluminum caps and flushed with N_2/CO_2 (80%/20%) for 10 minutes to establish anaerobic conditions. After that, phenol was added as external electron donor at the initial concentration of 1.35 mM**.** Before addition of CT, phenol oxidation and reduction of HS were monitored. Once phenol oxidation linked to the reduction of HS was observed, CT

was added at the initial concentration of 30 μM. Endogenous controls lacking phenol were included in order to document the coupling between phenol oxidation and CT dechlorination. Moreover, controls lacking HS were also included in order to assess their catalytic effects. Finally, sterile controls without inoculum were also included in the experimental protocol in order to identify potential physicochemical processes (e.g. adsorption) involved during phenol and CT removal. All experimental treatments were incubated at 28 °C in the dark with a mild shaking.

5.1.5 Analytical Methods

The concentration of AH_2QDS was determined on anaerobically collected samples in an anaerobic chamber by UV/visible spectrophotometry (Thermo-Scientific Genesys 10 UV) at 450 nm using a calibrating curve of AQDS chemically reduced by dithionite as previously described (Cervantes et al. 2000b).

The production of methane was quantified in 100 μL headspace samples in a gas chromatograph (GC, Agilent Technologies 6890N series) equipped with a thermal conductivity detector and a column Hayesep D (Alltech, Deerfield, Illinois, USA) with the following dimensions: 3.048m × 3.18mm × 2.16mm. Nitrogen was used as carrier gas with a flow-rate of 12 mL min^{-1}. Temperatures of the injection port, oven and the detector were 250, 60 and 250 °C, respectively. Nitrogen was used as carrier gas with a flow-rate of 12 mL min^{-1}.

The removal of CT and the production of volatile chlorinated hydrocarbons such as, CF and DCM were determined in 50 μL headspace samples by gas chromatography (GC, Agilent technologies 7890 series) coupled to a micro cell electron capture detector. Separation was achieved with a 5% phenyl-95% dimethyl-polysiloxane (30 m × 32 mm ID, 0.25μm (d_f)) HP-5 fused-silica capillary

column from Agilent Technologies (Little Falls, DE). Helium was used as carrier gas at 1 mL min^{-1} column flow-rate and nitrogen was the makeup gas at 59 mL min^{-1}. The temperatures of injector and detector were maintained at 200 ºC and 260 ºC, respectively. The oven temperature was programmed from 45 ºC (1 min hold) to 63 ºC (5 ºC min^{-1}).

Phenol was analyzed by gas chromatography (GC, Agilent technologies 7890 series) coupled to a flame ionization detector. Separation was achieved with a 5% phenyl-95% arilen-siloxane (30 m × 0.250 mm ID, 0.25µm (d_f)) DB-5MS fused-silica capillary column from Agilent Technologies. Helium was used as carrier gas at 33 cm sec^{-1} column flow-rate and nitrogen was the makeup gas at 30 mL min^{-1}. The temperatures of injector and detector were maintained at 250 ºC and 300 ºC, respectively. The oven temperature was programmed from 35 ºC (1 min hold) to 280 ºC (8 ºC min^{-1}).

The concentration of acetate, propionate and butyrate were measured using a capillary electrophoresis ion analyzer (Agilent G1600A, Waldbronn, Germany) equipped with a capillary column (50 lm ID_ 72 cm). The reduction of SCP was evaluated by the ferrozine method (Lovley et al. 1996).

5.2 Results

5.2.1 Anaerobic biodegradation of phenol

Among the different consortia studied, sediment S was the only consortium capable of anaerobically oxidizing phenol with the AQDS as TEA. The maximum respiratory rate achieved by this inoculum during the anaerobic oxidation of phenol was 7.04±0.37 µEq $gVS.h^{-1}$; in contrast, no coupling between phenol degradation and

AQDS reduction was detected with consortia CS and PS after 40 days of incubation.

On the other hand, complete conversion of phenol to methane was accomplished by both anaerobic granular sludges tested (inocula CS and PS). The maximum methane production rates calculated were 5.9±7.1 μEq $gVS.h^{-1}$ and 3.46±2.9 μEq $gVS.h^{-1}$, respectively, for CS and PS consortia. In both cases, complete phenol conversion was based on stoichiometric recovery of methane (>95%) and no detection of phenol after 50 days of incubation. Meanwhile, sediment S did not show any methanogenic activity with phenol as electron donor.

5.2.2 Reduction of CT by HS derived from different environments

Three different sources of HS were chemically reduced in a H_2/Pd reaction system and evaluated for their capacity to dechlorinate CT under abiotic conditions with reduced HS as a sole electron donor. Figure 5.2 shows the reduction of CT by the reduced HS evaluated. In all cases, the reduction of CT occurred without any lag phase. HS extracted from SCP showed the highest reducing capacity for the reductive dehalogenation of CT. After 16 days of incubation, 47.3 % of the CT initially added (100 μM) was reduced by SCP, whereas only 25 % reduction occurred with HS derived from SDF and GWC. These results contrast with the EAC quantified (Table 5.1), in which, GWC showed the highest value, indicating that EAC was not related to the capacity to reduce CT.

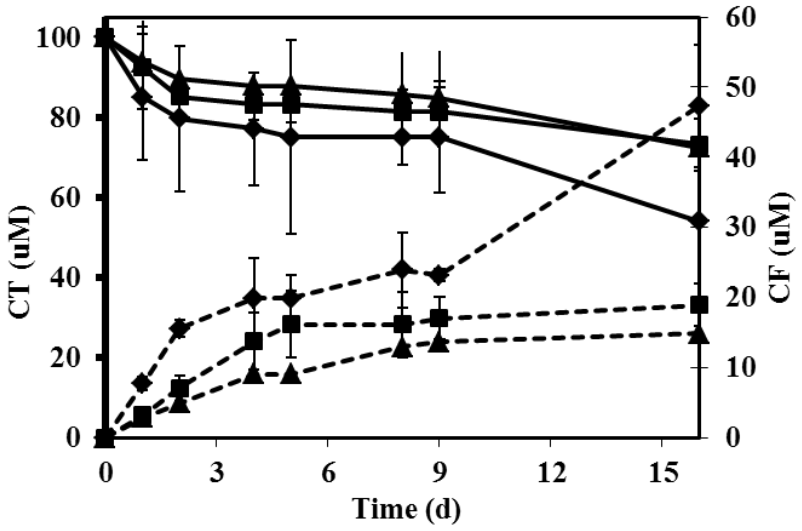

Figure 5.1 Time course of dechlorination of CT linked to CF formation by three HS (4 g $L^{-1)}$ samples previously reduced with H_2/Pd. ♦ SCP ■ SDF ▲ GWC. — CT reduced, ---- CF produced. Results represent average from triplicate incubations

CF was the only dehalogenation product detected from CT dechlorination by reduced HA in all cases. The high level of recovery obtained at the end of the incubation period suggests that unidentified dechlorination products accounted for a minor fraction of reduced CT (Table 5.2). Dehalogenation of CT by reduced HS was most accurately described by second-order kinetics (Figure 5.3). The second-order rate constants (k_{d2}) were 1.8×10^{-5}, 8.2×10^{-6} and 8×10^{-6} μM h^{-1} when reduced HS derived from SCP, SDF and GWC were used as reducing agents, respectively. Therefore, the k_{d2} value observed with HS from SCP corresponded to a 2–fold increase respect to the dechlorination rate observed with the other Hs sources. There was no reduction of CT in chemical controls including non-reduced HS.

Table 5.2 Second order rates constants (K_{d2}) and mass balance for the dechlorination of CT by the three sources of HA evaluated after 16 days of incubation

HS	k_{d2} $\mu M.h^{-1}$	CT_i μM	CT_f μM	CF_p μM	Reduction (%)	Recovery (%)
SCP	1.8×10^{-5}	102.3 ± 3	53.9 ± 20	47.3 ± 8.7	45.8	98.9
SDF	8.2×10^{-6}	98.5 ± 1.1	73.2 ± 6.4	19 ± 2.9	19.28	93.6
GWC	8×10^{-6}	98.9 ± 2.2	72.3 ± 9.3	14.8 ± 0.8	14.96	88

Initial concentration: CT 100 μM, HS 4g L^{-1}. Results represent average from triplicate incubations.
Soil of cocoa plantation (SCP), soil of deciduous forest in San Luis Potosí, Mexico (SDF) and compost produced with gardening wastes (GWC).
CT_i initial concentration of CT, CT_f final concentration of CT, CF_p produced concentration of CF.
Reduction = (CF_p / CT_i) X 100; % Recovery = (CT_f + CF_p)/ CT_i x 100

5.2.3 Simultaneous biodegradation of phenol and CT mediated by HS

HS derived from SCP showed the highest capacity to transfer electrons to CT. Likewise, sediment S was the only consortium able to oxide phenol using AQDS as TEA. Thus, these sources of HS and inoculum were used to study the simultaneous biodegradation of phenol and CT mediated by HS. Figure 5.4 shows the reduction of CT and oxidation of phenol under the different treatment conditions evaluated. In the presence of HS (Figure 5.4A), complete reduction of CT was observed after 3 days of incubation and significant reduction of CT occurred even in endogenous controls lacking phenol. In the absence of HS (Figure 5.4B), the reduction of CT was completed after 10 days of incubation. However, in this case, endogenous activity did not promote CT dechlorination. In both cases, phenol was not completely consumed. In the presence of HS, only 46±1.58 mg L^{-1} (0.20 μM) was oxidized, whereas in controls lacking HA 88±3.2 mg L^{-1} (0.40 μM) of phenol was removed. Therefore, it is evident in both cases that the coupling between phenol degradation to CT reduction occurred in sediment incubations.

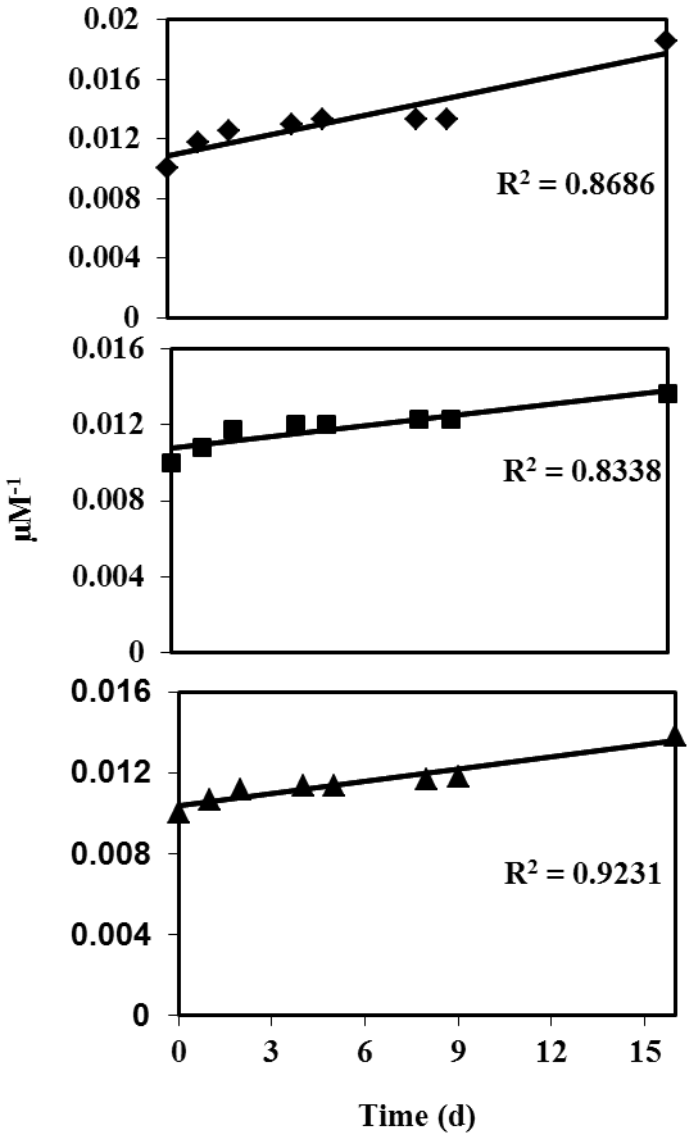

Figure 5.3 Second-order kinetics during the dechlorination of CT by different HS samples previously reduced with H_2/Pd. ♦ SCP ■ SDF ▲GWC. Results represent average from triplicate incubations

Table 5.3 shows dehalogenation rates and mass balances obtained in the different treatments evaluated after 3 days of incubation. Dehalogenation of CT followed first-order kinetics and the first-order rate constants (k_{d1}) calculated in the presence of HS derived from SCP, with and without of phenol, were 0.025 h^{-1} and 0.015 h^{-1}, respectively. For biological controls lacking HS, the k_{d1} value was 0.010 h^{-1}. Thus, the presence of HS increased 2.5–fold the k_{d1} value compared to the control

incubated in the absence of HS. HS also promoted a significant dechlorination of CT even in endogenous controls lacking phenol.

The impact of HS was also reflected in an increase in the efficiency of dechlorination by this consortium. Certainly, 77% of CT reduction occurred in sediment incubations including phenol and HS, 50% in endogenous control supplied with HS and 44% in incubations provided with phenol but lacking HS. CF and DCM were the dehalogenation products detected in all cases. Meanwhile, not significant reduction of CT and oxidation of phenol occurred in sterile controls (without consortium).

Further experiments to elucidate the role of HS on CT dechlorination pathway revealed that chemically reduced HS derived from SCP did not have the capacity to reduce CF further under abiotic conditions. Furthermore, CF could not be dechlorinated in biologically active sediment incubations when reduced HS were supplied as a unique electron donor. Therefore, these results suggest that the redox mediating capacity of HS were only involved in the first step of CT dechlorination (*e.g*. CT to CF). Nevertheless, the presence of HS in sediment incubations supplied with phenol as electron donor promoted a larger extent of CT reduction, including higher production of DCM probably due to kinetic enhancement. Namely, by promoting a faster reduction of CT to CF by the presence of HS, a higher concentration of CF would have then been available for further dechlorination in sediment incubations, thus achieving an overall more efficient dechlorination process.

Table 5.3 First order rate constants (K_{d1}) and mass balance for the dechlorination of CT by the different culture conditions evaluated after 3 days of incubation.

Culture conditions	k_{d1} (h^{-1})	CT_i µM	CT_f µM	CF_p µM	DCM_p µM	Reduction (%)	Recovery (%)
A	0.025	33.5 ± 1.18	5 ± 4.5	6.24 ± 0.7	19.8 ± 1.6	77.7	92.6
B	0.015	31.0 ± 1.16	13.3 ± 1.9	7.5 ± 5.1	8 ± 4.1	50	92.9
C	0.010	29.7 ± 4.2	14.9 ± 1.4	2.8 ± 1.5	10.5 ± 2.2	44	94.9
D	0.000	31.1 ± 1.18	30.9±1.25	0	0	0	99
E	0.000	33.3 ± 0.18	31 ± 1.6	0	0	0	93

Initial concentration: CT 30 µM, HS 4g L^{-1}. Results represent average from triplicate incubations
(A) S + HS + Phenol + CT, (B) S + HS + CT, (C) S + Phenol + CT, (D) S + CT, (E) HS + Phenol + CT
CT_i initial concentration of CT, CT_f final concentration of CT, CF_p produced concentration of CF, DCM_p produced concentration of DCM
Reduction = (CFp + DCMp / CT_i) X 100; % Recovery = (CT_f + CF_p + DCMp)/CT_i x 100

5.3 Discussion

The present study indicates that HS were able to mediate the simultaneous biodegradation of phenol and CT by increasing the rate and extent of oxidation of phenol and dechlorination of CT by the studied consortium. Certainly, addition of HS in sediment incubations increased the rate of oxidation of phenol as compared to controls lacking HS in which methanogenesis was the prevailing process. Moreover, the enhanced phenol oxidation was coupled to CT dechlorination, which was also increased both in terms of rate and extent of dechlorination (Table 5.3). The role of HS during the coupling between phenol oxidation and CT dechlorination was further emphasized by the higher rate and extent of CT reduction observed in HS-phenol-amended incubations compared to the results obtained in HS-amended endogenous controls lacking phenol.

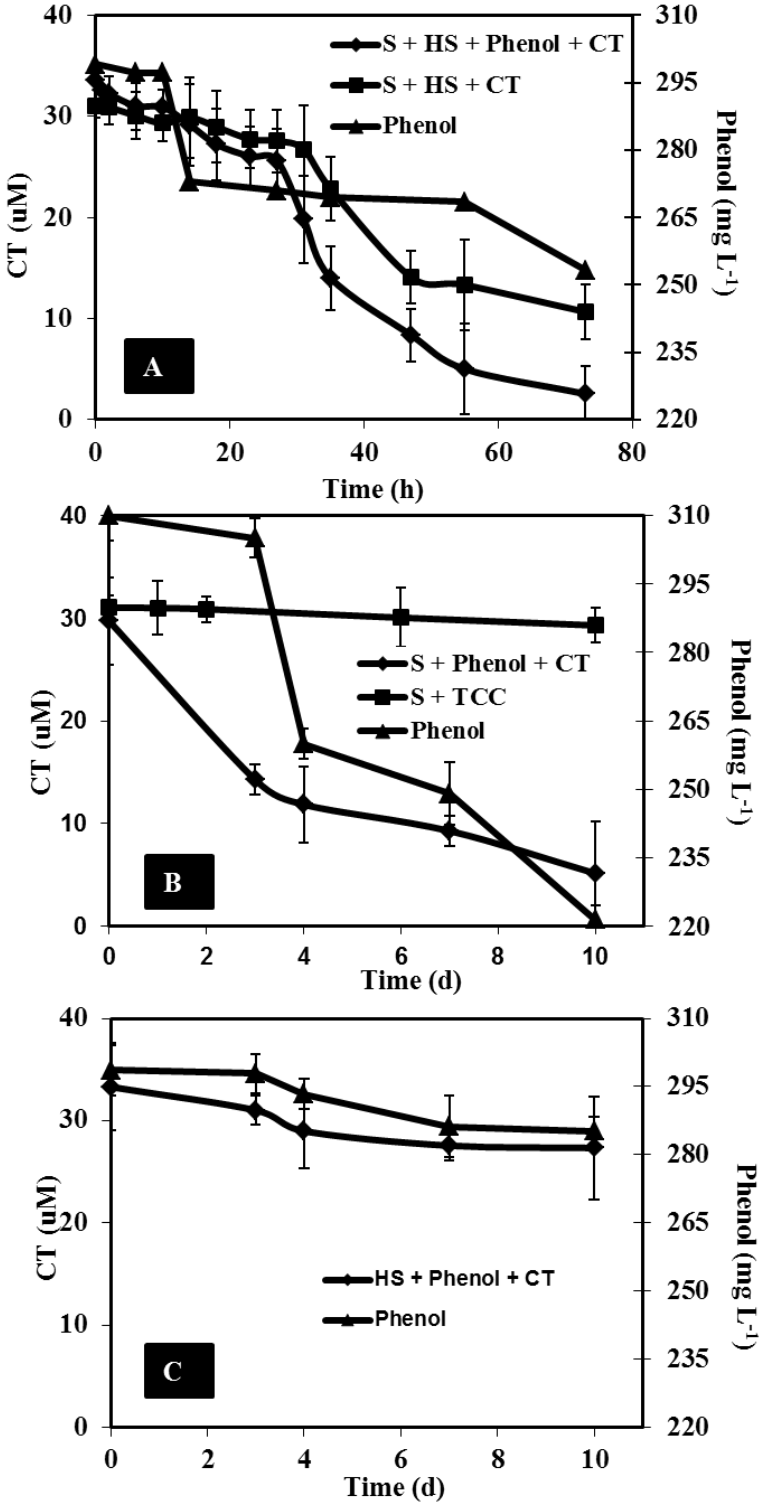

Figure 5.4 Impact of HS during the simultaneous biodegradation of CT and phenol (A) in presence of HS from SCP as RM (B) biological control lacking HS (C) chemical control without sediment. Results represent average from triplicate incubations

Although several studies have previously been documented the impact of HS by increasing the rate and extent of biodegradation of a wide variety of priority pollutants (Van der Zee and Cervantes 2009), the present work reports for the first time the simultaneous biodegradation of two contaminants mediated by HS derived from a natural source acting as effective RM (Figure 5.1).

The results from the present study also indicate that the EAC depended on the source and characteristics of HS and that the EAC was not related to its ETC to achieve CT dechlorination. Indeed, HS derived from SCP showed the lowest EAC among the different HS studied, but accomplished the reduction of CT to CF at a rate 2.2–fold faster as compared to the dechlorination rate observed with HS derived from SDF and GWC. Thus, the results suggest that HS derived from SCP have electron-transferring functional groups with a greater reactivity towards CT dechlorination than that expected from redox functional groups present in the other two HS sources. Several studies have reported that the properties of HS such as size, molecular weight, elemental composition, structure and the number and position of functional groups vary depending on the origin and age of the precursor material, and affect directly the ETC of these materials (Ratasuk and Nanny 2007).

The anaerobic biodegradation of CT in the presence of quinones or HS has been reported in previous studies. For instance, Cervantes et al. (2004) who evaluated the dechlorination of CT by anaerobic sludge in the presence of AQDS and HS, reported that the addition of these redox mediators significantly increased the k_{d1} value during CT dechlorination. However, the type of the substrate provided during this study significantly affected the rate of CT dechlorination. In the presence of AQDS (20 μM) and with glucose, acetate and methanol as electron donors, the k_{d1}

values after 27 days of incubation were, 0.174, 0.141 and 0.105 day^{-1} respectively. In the present study, the k_{d1} value obtained after 73 hours of incubation was 0.6 day^{-1}, which is higher than previously reported with redox mediators including AQDS, riboflavin and HS (Cervantes et al. 2004; Guerrero-Barajas and Field 2005). Thus, the use of phenol as an electron donor did not limit the CT dechlorination process neither in the presence nor in the absence of HS.

Considering the kinetic data collected during the biological reduction of AQDS linked to phenol oxidation and from the chemical reduction of CT by reduced HS, the results suggest that the first step in Figure 5.1 (biological reduction of quinones with phenol as electron donor) was 17.22-fold faster than the second step (transfer of reducing equivalents from reduced HS towards CT dechlorination) and therefore, this last step is considered the rate-limiting step in the whole process. Indeed, the reduction rate of AQDS was 14.09 μEq $L.h^{-1}$ during the oxidation of phenol in sediment incubations, whereas the transfer of electrons from reduced HS reduced to CT occurred at 0.818 μEq $L.h^{-1}$. Although these calculations were made with the humic model compound, AQDS, previous studies have documented that the reduction rate of AQDS and HS proceed at similar rates by different humus-reducing microorganisms (Lovley et al. 1996). Thus, the kinetic data obtained with AQDS could be extrapolated with those expected with HS.

The fact that the reduction of CT by previously reduced HS under abiotic conditions was most accurately described by second-order kinetics (Table 5.2) implies that the reaction rate depended on the concentration of both reduced redox functional groups in HS and CT. Previous studies have also reported second-order kinetics describing the reduction of other polyhalogenated pollutants, such as

hexachloroethane, by reduced HS (Kappler and Haderlein 2003). However, dechlorination of CT coupled to phenol oxidation in sediment incubations followed first-order kinetics, probably because redox functional groups in HS could be regenerated through the microbial oxidation of phenol after transferring the electrons for CT dechlorination.

5.4 Conclusions

The present work reports for the first time the simultaneous biodegradation of two contaminants, phenol and CT mediated by HS derived from a natural source acting as effective RM. Further experiments revealed that the electron-accepting capacity of HS derived from different organic-rich environments was not associated with their reducing capacity to achieve CT dechlorination. Therefore, the results suggest the importance of selecting the appropriate source of HS to remove the contaminant of interest, in order to promote the applicability of immobilized HS derived from natural sources in the treatment of recalcitrant pollutants.

6

Final remarks and perspectives

During the last two decades evidence demonstrated the effective role of humic substances (HS) and quinone analogues as redox mediators (RM) for accelerating the redox conversion of priority pollutants, such as azo dyes, aliphatic and aromatic poly-halogenated compounds, nitroaromatics and metalloids. Recently, research has expanded the role of HS to a broader spectrum of contaminants, including 2,4-dichlorophenoxyacetic acid (Wang et al. 2009a), 4-chlorobiphenyl (Wang et al. 2011a), pentachlorophenol (Zhang and Katayama 2012) and tetrabromobisphenol-A (Wang et al. 2013a). Some literature reviews have already underlined the potential of applying these RM in wastewater treatment systems (Van der Zee and Cervantes 2009; Watanabe et al. 2009). The addition of RM can promote a good performance in bioreactors (*e.g*. UASB and EGSB) by accelerating the reduction of the contaminants and can also attenuate their toxicological effects on anaerobic consortia (Van der Zee et al. 2001). However, the main limitation in the application of HS as RM in continuous systems is that their continuous addition must be provided to increase conversion rates, which is economically and environmentally non-viable. Therefore, several attempts to immobilize HS and quinone model compounds have lately been explored to develop engineered systems integrating the redox mediating capacity of immobilized RM in order to promote enhanced conversion rates (Chapter 2,Table 2.3). However, only a few reports have

documented that immobilized HS can serve as effective RM during the reductive biotransformation of recalcitrant pollutants in batch incubations (Alvarez et al. 2012, Cervantes et al. 2011b, Cervantes et al. 2013). In this sense, this thesis was focused on evaluating two different strategies of immobilization of HS (HS adsorbed on an anion exchange resin (AER) and on γ-Al_2O_3 nanoparticles (NPs)) to achieve the redox biotransformation of recalcitrant pollutants in batch and continuous flow experiments. Additionally, this work evaluates the redox mediating capacity of HS derived from organic rich environments and wastes, in order to promote the applicability of immobilized HS derived from natural sources in the treatment of recalcitrant pollutants.

Chapter 3 shows evidence fulfilling the first hypothesis presented in this dissertation (see in Chapter 1 all hypotheses). Indeed, immobilized HS, on an AER, served as effective solid-phase RM for achiving the simultaneous removal of phenol and reactive red 2 (RR2) in long-term operation of a UASB reactor. Moreover, the first part of hypothesis 2 was also demonstrated in Chapter 4. Granules integrating both HS, supported on γ-Al_2O_3 NPs, and humus-reducing microorganisms were produced and the content of HS obtained in enriched granules was 0.96 mg TOC per gram of granules after one year of operation. Nevertheless, results presented in Chapter 4 also indicated that immobilized HS by this granulation process did not maintain their redox-mediating capacity to enhance the reductive biotransformation of RR2 and 2,4-dichloro-phenol (2,4-DCP). Increased conversion rates of RR2 and 2,4-DCP were only observed by the produced granules when external HS, supported on γ-Al_2O_3 NPs, were supplied. These results suggest that redox active functional groups of immobilized HS were not available after the granulation

process probably due to interaction with biomass components or with extracellular polymeric substances. Thus, further research is demanded in order to understand the mechanisms of immobilization of HS by the granulation process in order to create strategies to preserve their redox-mediating capacity for the removal of recalcitrant pollutants.

Regarding hypothesis 3, results summarized in Chapter 5 clearly show that the capacity of HS derived from organic rich environments and wastes to achieve the reductive dechlorination of carbon tetrachloride (CT) did not depend on the electron accepting capacity of the extracted HS. However, it was demonstrated that HS originated from one of the organic sources evaluated served as effective RM enhancing the simultaneous removal of phenol and CT

Table 6.1 summarizes the advantages and disadvantages of the two immobilizing techniques evaluated in the present dissertation. The AER tested in Chapter 3 showed lower adsorption capacity (37 mg TOC g^{-1}, Cervantes et al. 2011b) as compared to γ-Al_2O_3 NPs (89 mg TOC g^{-1}, Chapter 4), which may partly be explained by the larger surface area available in the later material. Both materials have good settling capacity. However, immobilization of HS, supported on γ-Al_2O_3 NPs, depends on the granulation process, whereas HS immobilized on the AER can easily be maintained in UASB reactors due to its larger size and high specific weight. HS immobilized on the AER maintained their redox-mediating capacity after long-term operation of a UASB reactor effectively removing phenol and RR2. However, HS supported on γ-Al_2O_3 NPs apparently lost their redox-mediating capacity after the granulation process probably due to blocking of these functional groups by interaction with biomass components.

Table 6.1 Comparison between the two immobilization strategies evaluated in this investigation

Immobilization on anion exchange resin	Immobilization on γ-Al_2O_3 nanoparticles
Advantages	
Good settling capacity	High adsorption capacity compared to other immobilizing materials
Good mechanical properties	Good settling capacity
Stability	Cheap material
Disadvantages	
Expensive	Wash-out during the start-up period
Poor adsorption capacity compared to other immobilizing materials	Potential desorption with changes in pH
	Eventual obstruction of redox-mediating groups by the granulation process
Desorption of HS at high anions concentration	

6.1 Perspectives

Although our results revealed the feasibility of maintaining the immobilized HS in a high-rate anaerobic reactor and catalyze the redox conversion of recalcitrant pollutants for prolonged periods, several challenges need to be addressed to apply this immobilized RM in full scale wastewater treatment systems:

- Further studies are required to elucidate the relationship between the structural and chemical characteristics of HS and their redox activity, in order

to identify suitable HS extracted from natural sources.

- More studies are required in order to verify if the redox mediating functional groups in HS (e.g. quinones) remain available for catalysis after immobilization in the supporting material (in the case of co-immobilization of humus-reducing microorganism with HS immobilized on γ-Al_2O_3 nanoparticles)
- More studies are also demanded to develop strategies that allow overcoming the main limitations encountered during the application of immobilized RM: obstruction of redox active functional groups, disruption of supporting material, desorption of immobilized RM, washout of RM from bioreactors and poor immobilizing capacity.
- Engineered HS, which could be enriched with redox mediating functional groups to increase their catalytic properties, are also demanded for their use in real wastewater treatment systems.

All these issues should be the subject in future research.

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