Propositions

1. Humus may significantly contribute to the intrinsic bioremediation of anaerobic environments contaminated with priority pollutants by serving as a terminal electron acceptor.
   This dissertation
   Bradley, P. M ., F. H. Chapelle and D. R. Lovley. (1998). Humic acids as electron acceptor for anaerobic microbial oxidation of vinyl chloride and dichloroethene. Appl. Environ. Microbiol. **64**, 3102-3105.

2. The application of catalytic concentrations of quinones, as redox mediators, to anaerobic wastewater treatment systems enhances the conversion of priority pollutants susceptible to reductive biotransformation. The accelerated conversion of this type of contaminants would result in more compact wastewater treatment systems.
   This dissertation

3. The injection of nitrate and sulfate to contaminated aquifers to stimulate the anaerobic biodegradation of aromatic hydrocarbons has more environmental risks than the benefits claimed by Hutchins *et al.* (1991) and Lovley (2000).
   Hutchins, S. R., Sewell, G. W., Kovacs, D. A. and Smith, G. A. (1991). Biodegradation of aromatic hydrocarbons by aquifer microorganisms under denitrifying conditions. Environ. Sci. Technol. **25**, 68-76.
   Lovley, D. R. (2000). Anaerobic benzene degradation. Biodegradation **11**, 107-116.

4. A humble recognition of the other's rights will help much more than millions of missiles to bring peace in the Middle East.
5. "Globalization" is a new form of Colonialism, which is contributing to increase the opulence of the rich, as well as the misery of the poor.
6. Legislation on abortion, euthanasia, drugs and sex trade, as well as gay marriages, makes The Netherlands one of the most controversial countries.
7. Those who place much effort on criticizing others are generally blind to look at their own faults.

8. If the "civilized" countries really want to eradicate the evil of terrorism, they should first come up with a correct definition of "terrorist". Otherwise they will loose all credibility.

9. Vitamin T (tacos, tortillas, tortas, tamales, tostadas, tinga, etc.) and vitamin CH (chile, chorizo, chilorio, churros, chuleta, champurrado, etc.) are essential nutrients to keep Mexicans running.

10. Dutch mood and Dutch weather are highly correlated: both are continuously changing.

Propositions belonging to the thesis entitled "Quinones as electron acceptors and redox mediators for the anaerobic biotransformation of priority pollutants"

Francisco J. Cervantes-Carrillo
Wageningen, 14 January, 2002

Quinones as Electron Acceptors and Redox Mediators for the Anaerobic Biotransformation of Priority Pollutants

Francisco J. Cervantes-Carrillo

Promotor

Prof. dr. ir. G. Lettinga

hoogleraar in de anaërobe zuiveringstechnologie en hergebruik van afvalstoffen

Co-promotor

Dr. ir. J. A. Field

associate professor, University of Arizona, USA

Samenstelling promotiecommissie

Prof. Dr. O. Monroy	Universidad Autónoma Metropolitana-Iztapalapa, Mexico
Prof. Dr. ir. A. J. M. Stams	Wageningen Universiteit
Dr. ir. P. Buurman	Wageningen Universiteit
Prof. Dr. Ae. de Groot	Wageningen Universiteit

Quinones as Electron Acceptors and Redox Mediators for the Anaerobic Biotransformation of Priority Pollutants

Francisco J. Cervantes-Carrillo

Proefschrift

Ter verkrijging van de graad van doctor
Op gezag van de rector magnificus
van Wageningen Universiteit,
Prof. dr. ir. L. Speelman,
in het openbaar te verdedigen
op maandag 14 januari 2002
des namiddags te vier uur in de Aula

CIP-DATA KONINKLIJKE BIBLIOTHEEK, DEN HAAG

Author: Cervantes-Carrillo, F. J.
Tittle: Quinones as electron acceptors and redox mediators for the anaerobic biotransformation of priority pollutants
ISBN: 90-5808-567-8
Publication year: 2002
Subject headings: Quinones, humus, redox mediator, electron acceptor, priority pollutants
Thesis Wageningen University, Wageningen, The Netherlands –with references- with general discussion and conclusions in Dutch- with abstract in Spanish.162 p.

Dedicated to my wife, Liz, and to that little person who will soon call me "Papá".

Abstract

Cervantes-Carrillo, F. J. 2002. **Quinones as Electron Acceptors and Redox Mediators for the Anaerobic Biotransformation of Priority Pollutants.** Doctoral Thesis, Wageningen University, Wageningen, The Netherlands.

Humus is the most abundant organic fraction in the biosphere. It is composed of a complex structure in which recalcitrant polymers prevail with a residence time lasting decades or even centuries. Despite the recalcitrance of humic substances, they have recently been recognized to play an important role on the anaerobic conversion of organic matter by serving as an electron acceptor for microbial respiration. Quinone moieties are the responsible electron-accepting groups accounting for the microbial reduction of humus. Quinones and humus not only serve as terminal electron acceptors for microbial respiration, but they also function as redox mediators during the transfer of electrons in microbial and chemical reactions. In this dissertation the impact of humus and quinone analogues on the anaerobic biotransformation of ecologically important substrates, as well as priority pollutants, was evaluated.

Consortia obtained from many different environments including sandy, organic rich, and contaminated sediments, as well as anaerobic and aerobic sludges, showed the capacity for oxidizing a wide variety of ecologically significant substrates, such as lactate and acetate, when the humic model compound, anthraquinone-2,6-disulfonate (AQDS), was provided as a final electron acceptor. AQDS-reducing microorganisms out-competed methanogens for most of the substrates supplied indicating that quinone reduction is a widespread physiological process, which may contribute to important carbon cycling process in many different environments. Quinone and humus reduction was also found in pure cultures of different microorganisms, such as *Desulfitobacterium* spp. and *Methanospirillum hungatei,* indicating that the ubiquity of quinone reduction may be due to the wide diversity of microorganisms with the capacity for reducing humic substances. The results also illustrate that phylogenetically distinct microorganisms can channel electrons from anaerobic substrate oxidation via quinone reduction towards the reduction of metal oxides. Quinone respiring microorganisms could also be enriched and immobilized in the microbial community of an anaerobic granular sludge of a upflow anaerobic sludge blanket (UASB) reactor. The feasibility to immobilize quinone-reducing microorganisms can be applied to accelerate the conversion of xenobiotics susceptible to reductive biotransformations such as azo dyes and polychlorinated compounds in continuous bioreactors.

The long-term goal of this research was to explore the capacity of humus respiring consortia for oxidizing priority pollutants through the reduction of humic substances. Anaerobic granular sludge originated from different wastewater treatment plants were shown to oxidize phenol and *p*-cresol coupled to the reduction of AQDS. Both phenolic contaminants were converted to methane in the absence of the humic analogue, but addition of AQDS as an alternative electron acceptor diverted the flow of electrons from methanogenesis towards quinone reduction. Priority pollutants, which were not degraded under methanogenic conditions, could also be mineralized by humus-respiring consortia when humic substances were provided as an electron acceptor. Enriched sediments from different origins readily mineralized uniformly labeled [^{13}C]toluene to $^{13}CO_2$ when humic acids or AQDS were provided as terminal electron acceptors. Negligible recovery of $^{13}CO_2$ occurred in the

absence of humic substances. Additionally, the electrons in the toluene mineralized were recovered stoichiometrically as reduced humus or AH_2QDS (reduced form of AQDS).

Humic substances were also shown to accelerate the transfer of reducing equivalents required for the anaerobic conversion of different pollutants containing electron-withdrawing groups. AQDS supplemented at sub-stoichiometric levels in granular sludge incubations enhanced the rate of conversion of carbon tetrachloride (CT) leading to an increased production of inorganic chloride. Negligible dechlorination occurred in sterile controls with autoclaved sludge and considerably less dechlorination was achieved in active controls lacking AQDS. A humus respiring enrichment culture, composed primarily of a *Geobacter* sp., derived from the same granular sludge was also shown to dechlorinate CT, yielding similar products as the AQDS-supplemented sludge consortium. Addition of catalytic levels of AQDS to a UASB reactor continuously treating the azo dye, acid orange 7 (AO7), also enhanced the biotransformation of this pollutant to the corresponding aromatic amines. High efficiency (>90 %) of decolorization of AO7 occurred even at a hydraulic residence time of 2 hours with a molar ratio of AQDS/AO7 as low as 1/100, whereas 70 % of color removal occurred in the absence of AQDS under the same hydraulic conditions.

The evidences provided in this study indicate that humic substances may play an important role on the stabilization of organic matter, as well as on the intrinsic bioremediation of contaminated environments, by serving as a terminal electron acceptor. The application of humic substances for achieving the bioremediation of contaminated aquifers can be considered. Humus and quinones can also be applied in anaerobic reactors to enhance the conversion of priority pollutants containing electron-withdrawing groups.

Resumen

Cervantes-Carrillo, F. J. 2002. **Quinonas como Aceptores de Electrones y Mediadores Redox en la Biotransformación Anaerobia de Contaminantes Prioritarios.** Tesis Doctoral, Universidad de Wageningen, Wageningen, Países Bajos.

El humus es la fracción orgánica más abundante en la biosfera. Esta compuesto de una estructura compleja en la que polímeros recalcitrantes prevalecen por décadas o incluso siglos. A pesar de ser recalcitrantes, las substancias húmicas pueden jugar un papel importante en la conversión anaerobia de substratos orgánicos mediante su participación en la respiración microbiana como aceptores de electrones. Las quinonas son los grupos funcionales que aceptan los electrones directamente durante la reducción microbiana del humus. Las quinonas presentes en el humus no solamente sirven como aceptores de electrones en la respiración microbiana, sino también, como mediadores de oxido-reducción (redox) durante la transferencia de electrones en reacciones químicas y microbianas. En esta disertación, el impacto del humus y sus análogos (quinonas) en la biotransformación anaerobia de substratos ecológicamente importantes, así como de contaminantes prioritarios, fue evaluado.

Los consorcios obtenidos de ambientes muy variados, incluyendo sedimentos arenosos, contaminados y ricos en materia orgánica, así como lodos aerobios y anaerobios, pudieron oxidar una gran diversidad de substratos ecológicamente importantes, como el acetato y el lactato, cuando el compuesto modelo, 2,6-disulfonato de antraquinona (AQDS), fue adicionado como aceptor final de electrones. En estos consorcios, los microorganismos reductores de AQDS superaron a los metanogénicos en la degradación de la mayoría de los substratos estudiados, lo cual sugiere que la reducción microbiana del humus podría contribuir significativamente en el ciclo del carbono en diferentes ambientes. La reducción de humus y AQDS también fue evidente en cultivos axénicos de diferentes microorganismos, como *Desulfitobacterium* spp. y *Methanospirillum hungatei*, indicando que la ubicuidad de la reducción microbiana del humus podria ser debido a la gran diversidad de microorganismos que son capaces de reducir substancias húmicas. Los organismos capaces de reducir quinonas también pudieron canalizar electrones hacia óxidos metálicos que fueron a su vez reducidos durante la reducción microbiana de quinonas. Este tipo de microorganismos pudieron ser enriquecidos e inmovilizados en la población microbiana del lodo granular de un reactor anaerobio de lecho de lodos de flujo ascendente (UASB). La inmovilización de microorganismos reductores del humus podría ser aplicado para acelerar la conversión de contaminantes recalcitrantes susceptibles a una reducción, como los colorantes azo o compuestos policlorinados.

La principal meta del presente estudio fue explorar la capacidad de consorcios reductores del humus para oxidar contaminantes prioritarios mediante la reducción de substancias húmicas. Los lodos granulares originados de diferentes plantas de tratamiento de aguas residuales mostraron la capacidad de oxidar fenol y *p*-cresol acoplado a la reducción de AQDS. Ambos contaminantes fenólicos fueron convertidos a metano en la ausencia de quinonas, pero la adición de AQDS como un aceptor de electrones alternativo, desvió el flujo de electrones de la metanogénesis hacia la reducción de quinonas. Contaminantes prioritarios que no fueron degradados bajo condiciones metanogénicas pudieron también ser mineralizados por consorcios respiradores del humus. Sedimentos enriquecidos de diferentes ambientes mineralizaron rápidamente [^{13}C]tolueno marcado

uniformemente a $^{13}CO_2$ cuando AQDS o ácidos húmicos fueron incluidos como aceptores de electrones. La producción de $^{13}CO_2$ en la ausencia de substancias húmicas fue despreciable. Además, los equivalentes de electrones del tolueno oxidado fueron recuperados estequiométricamente en forma de humus reducido o como AH_2QDS (forma reducida de AQDS).

Las substancias húmicas aceleraron también la transferencia de electrones requeridos para la conversión anaerobia de diferentes contaminantes conteniendo grupos electrofílicos. El suplemento de AQDS por debajo del nivel estequiométrico a incubaciones de lodo granular acelero la velocidad de conversión de tetracloruro de carbono (CT) propiciando un incremento en la producción de cloro inorgánico en el medio. La decloración ocurrida en los controles estériles fue despreciable, mientras que una decloración mucho menor ocurrió en la ausencia de AQDS. Un cultivo enriquecido capaz de reducir el humus, compuesto principalmente de una especie del genero *Geobacter* y obtenido del mismo lodo granular, mostró la misma capacidad de decloración que el consorcio original. La aplicación de niveles catalíticos de AQDS a un reactor UASB incremento la velocidad de conversión de un colorante azo, Naranja Acido 7 (AO7). Se obtuvieron altas (>90 %) eficiencias de decoloración de AO7 aun al aplicar 2 horas como tiempo de residencia hidráulico con una relación molar de AQDS/AO7 tan baja como 1/100, mientras que solo el 70 % del color fue eliminado en la ausencia de AQDS bajo las mismas condiciones hidráulicas.

Las evidencias dadas en el presente estudio indican que las substancias húmicas, al actuar como aceptores de electrones, podrían jugar un papel importante en la estabilización de la materia orgánica y en la bioremediación intrínseca de ambientes contaminados. La aplicación de substancias húmicas para lograr la bioremediación de acuíferos contaminados puede ser considerada. El humus o quinonas también pueden ser aplicados en reactores anaerobios para acelerar la conversión de contaminantes prioritarios conteniendo grupos electrofílicos.

Contents

1. General introduction 1

2. Competition between methanogenesis and quinone respiration for ecologically important substrates in anaerobic consortia 17

3. Reduction of humic substances by halorespiring, sulfate-reducing and methanogenic microorganisms 35

4. Quinones as terminal electron acceptors in anaerobic granular sludge 49

5. Quinones as terminal electron acceptors for anaerobic microbial oxidation of phenolic compounds 63

6. Anaerobic mineralization of toluene by enriched sediments with quinones and humus as terminal electron acceptors 77

7. Quinone-respiration improves dechlorination of carbon tetrachloride by anaerobic sludge 95

8. Enhanced decolourisation of acid orange 7 in a continuous UASB reactor with quinones as redox mediators 113

9. General discussion and conclusions (English and Dutch) 123

Acknowledgements

Curriculum vitae

List of publications

1

General Introduction

Role of quinones in the biodegradation of priority pollutants: a review*

*Published in part as: Field, J. A., F. J. Cervantes, F. P. Van der Zee and G. Lettinga. (2000). *Water Sci. Technol.* **42**, 215-222.

Introduction

Humic substances constitute a very abundant class of organic compounds that are chemically heterogeneous and widely distributed in terrestrial and aquatic environments. These ubiquitous substances are generally considered recalcitrant because of their remarkable stability in the environment. For instance, high molecular weight humic polymers have a residence time longer than 500 years (*34*). However, recent reports have suggested that humus may play different roles in the carbon and electron flow in anaerobic environments. Although the chemical properties of humic substances depend on the chemical characteristics of the organic matter from which they were derived, and the properties of their environment (*34*), evidences indicated that quinone moieties might be important electron acceptors in humic substances for microbial respiration (*31*). Not only do humic substances serve as terminal electron acceptor for anaerobic substrate oxidation (*22*), but they also act as redox mediators to support the reduction of metals (*13, 22, 35*), as well as polychlorinated (*1, 8*), nitro- (*10, 15, 30*) or azo- pollutants (*16, 18*). Moreover, humic substances can also serve as electron donors for anaerobic respiration (*5, 23*). Therefore, humic substances can be involved in different abiotic and microbial processes of electron transfer in anaerobic environments.

Humic substances as terminal electron acceptor

Anaerobic respiration. Initially, it was observed that a rapid anaerobic oxidation of benzene could be achieved by chelating Fe(III) in soil with different chelators, including humic acids (*24*). This phenomenon was attributed to the solubilization of Fe(III) oxides by chelators and thus making Fe(III) more available to benzene-oxidizing Fe(III)-reducing bacteria. However, it was observed that humus-Fe(III) complexes stimulated benzene degradation better than any of the chelators evaluated, such as NTA and EDTA, which had higher chelation capacity as compared to humic acids. Therefore, it was hypothesized that the stimulated benzene oxidation by humus-Fe(III) complexes was due to the coupling of two processes, with microorganisms first donating electrons to humus and the humic substances then reducing the terminal electron acceptor (Fe(III), see Figure 1). Further experiments elucidated this theory, *Geobacter metallireducens*, an Fe(III)-reducing bacterium, was capable for mineralizing [^{14}C]-acetate to $^{14}CO_2$ when highly purified soil humic acids were provided as sole terminal electron acceptors, whereas no mineralization of acetate occurred in the absence of humus (*22*). Reduction of humic substances agreed with the concomitant microbial growth of the Fe(III)-reducing organism indicating that humic substances, aside from serving as electron acceptors, provide energy for anaerobic respiration.

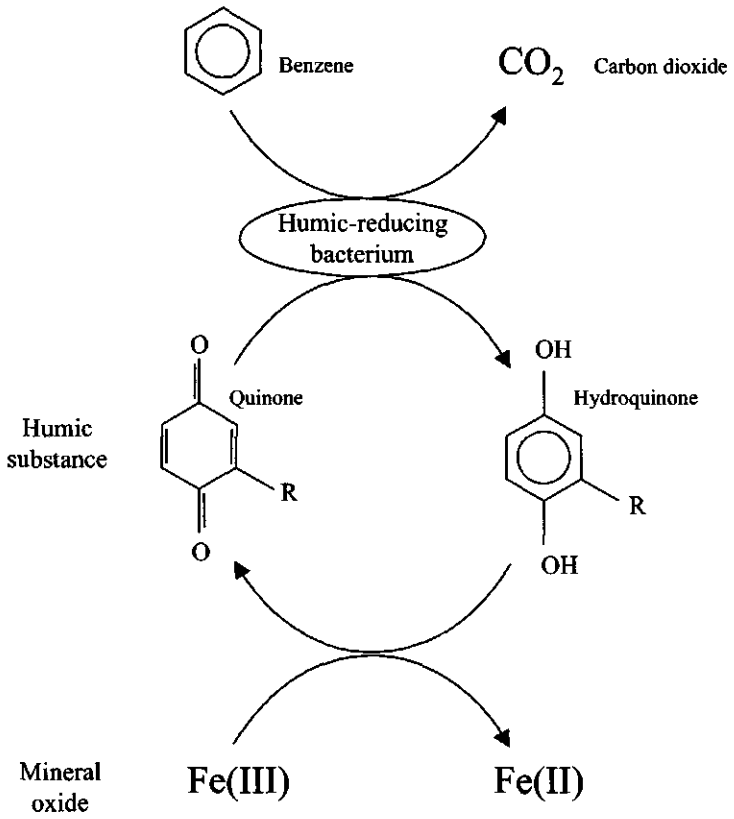


Figure 1. Recycling of quinone moieties in humus by a mineral oxide to achieve the biodegradation of benzene (*22, 24*).

Quinones. Quinones are important structural units in humus (*34*), which are the most likely candidates for the redox reactions observed. The quinone model moiety, anthraquinone-2,6-disulfonate (AQDS), was also shown to be used as a sole terminal electron acceptor by *G. metallireducens* to support cell growth on acetate. The reduced form of AQDS, anthrahydroquinone-2,6-disulfonate (AH_2QDS), was recovered in stoichiometric yields of 4 mol AH_2QDS per mol acetate oxidized (*22*). Moreover, all other microorganisms tested that have been found to have the ability to reduce humus can also reduce AQDS (*7, 20-22*). In further support of the hypothesis that quinones moieties are the responsible functional groups in humus for the electron-accepting properties, experiments were conducted correlating humus quinone content with its electron accepting capacities. The best quinone indicator is the electron spin resonance (ESR) of the reduced humus, due to the unpaired electron in the semiquinone radical. This parameter was highly correlated with the microbial electron-accepting capacity of a wide variety of humic substances collected from sediments, soil and aquatic environments (*31*). Additionally, the biochemical basis of AQDS and humus reduction in *Shewanella*

putrefaciens MR1 was investigated and found to be related to a respiratory chain utilizing menaquinone. The results from this investigation provided genetic evidence of a common biochemical basis for humus and AQDS reduction. Mutants of *Shewanella putrefaciens* MR1, lacking the ability to synthesize menaquinone, which were unable to reduce AQDS, were also unable to reduce humus (*26*). Therefore, quinone model compounds should be able to replace the function of humus as terminal electron acceptor.

Substrate and microbial diversity related to humus respiration. Microbial reduction of both humus and AQDS can support the anaerobic oxidation of a wide variety of substrates. It was suggested that members of the family *Geobacteraceae* might be the most important group of humus-reducers since *Geobacter* and *Desulfuromonas* species were isolated from different freshwater and marine sediments with acetate and AQDS as electron donor and electron acceptor, respectively (*7*). Some of these isolates were also capable of oxidizing other substrates, such as ethanol and hydrogen, through AQDS reduction. *Pantoea agglomerans* SP1, an Fe(III)-reducing facultative anaerobe member of the *Enterobacteriaceae*, was able to grow when AQDS was provided as a terminal electron acceptor for the anaerobic oxidation of different substrates (*12*). The novel thermophilic Fe(III)-reducing bacterium, *Thermoanaerobacter siderophilus* sp. nov., could also grow under AQDS-reducing conditions with peptone as a sole electron donor (*33*). Furthermore, there is a number of microorganisms that gratuitously reduce humic substances. For instance, the uranium-reducing bacterium, *Deinococcus radiodurans*, could reduce AQDS, but was not able to link AQDS reduction to growth (*14*). Lovley *et al.* (1998) conducted a qualitative screening to determine the phylogenetic diversity of quinone respiration. They observed AQDS or humus reduction by *Shewanella* species, *Desulfitobacterium dehalogenans*, *Desulfuromonas acetexigens*, *Geospirillum barnseii*, *Wolinella succinogenes*, and *Geothrix fermentens* (*21*). The authors, however, did not investigate whether the reduction of humus or AQDS by these microorganisms was linked to growth during this screening. More recently, humus and quinone reduction has also been found in a wide variety of thermophilic and hyperthermophilic microorganisms including Fe(III)-reducers (e.g. *Pyrobaculum islandicum, Pyrodictium abyssi, Thermococcus celer*) and methanogenic archaea (e.g. *Methanopyrus kandleri, Methanobacterium thermoautotrophicum*), which exhibited hydrogen-dependent AQDS reduction, but quinone reduction was not observed to be growth-linked with any of these microorganisms (*20*).

Besides the simple substrates that can be oxidized via humus reduction, it was recently reported that the microbial oxidation of vinyl chloride (VC) and dichloroethene (DCE) was stimulated by addition of humic acids or AQDS as a terminal electron acceptor by an organic rich sediment (*4*). There was not significant mineralization of the chlorinated compounds in the absence of AQDS and humus. Moreover, the biodegradation of [1,2-^{14}C]VC was coupled to the reduction of AQDS in the bioassays resulting in a net oxidation of the chlorinated pollutant to $^{14}CO_2$ whereas previous works indicated a net reduction of this compound to ethene or ethane under anaerobic conditions. These facts suggest

that humic compounds can play a potential role during the bioremediation of contaminated anaerobic sites by acting as final electron acceptors.

Table 1. Microbial reduction of humic acids (HA) or the humic analogue, AQDS, as terminal electron acceptors

Electron donor	Electron acceptor	Microorganism	Reference
Anaerobic Respiration			
Acetate	AQDS and HA	*Geobacter* sp. TC-4	7
Acetate	AQDS and HA	*Geobacter metallireducens*	22
Acetate and hydrogen	AQDS	*Pantoea agglomerans* SP1	12
Acetate, formate, lactate, ethanol, pyruvate and hydrogen	AQDS and HA	*Geobacter* sp. JW-3	7
Acetate, propionate, ethanol and succinate	AQDS	*Desulfuromonas* sp. SDB-1	7
Acetate, propionate, ethanol and succinate	AQDS	*Desulfuromonas* sp. FD-1	7
Dichloroethene and vinyl chloride	AQDS and HA	Fresh organic-rich sediment	4
Lactate and hydrogen	AQDS and HA	*Shewanella alga*	22
Peptone	AQDS	*Thermoanaerobacter siderophilus*	33
Qualitative reduction of humic substances[a]			
Not clearly specified	AQDS	*Shewanella putrefaciens*	21
Not clearly specified	AQDS	*Shewanella sacchrophila*	21
Not clearly specified	AQDS	*Aeromonas hydrophila*	21
Not clearly specified	AQDS	*Desulfitobacterium dehalogenans*	21
Not clearly specified	AQDS	*Geospirillum barnseii*	21
Not clearly specified	AQDS and HA	*Wolinella succinogenes*	21
Not clearly specified	AQDS	*Geothrix fermentens*	21
Not clearly specified	AQDS	*Desulfuromonas acetexigens*	21
Additional electron sink for fermentation[b]			
Glucose	HA	*Enterococcus cecorum*	3
Glucose	HA	*Lactococcus lactis*	3
Lactate and propionate	HA	*Propionibacterium freudenreichii*	3
Gratuitous Reduction			
Hydrogen	AQDS and HA	*Pyrobaculum islandicum*	20
Hydrogen	AQDS	*Pyrodictium abyssi*	20
Hydrogen	AQDS	*Pyrococcus furiosus*	20
Hydrogen	AQDS	*Archaeoglobus fulgidus*	20
Hydrogen	AQDS	*Thermococcus celer*	20
Hydrogen	AQDS	*Methanococcus thermolithitrophicus*	20
Hydrogen	AQDS	*Methanobacterium thermoautotrophicum*	20
Hydrogen	AQDS	*Methanopyrus kandleri*	20
Lactate	AQDS	*Deinococcus radiodurans*	14

[a]Reduction of AQDS or humus not quantified but only reported as a qualitative test. Microbial growth was not confirmed to be linked to AQDS or humus reduction.
[b]Microbial growth was not confirmed to be linked to AQDS or humus reduction.

Fermentation. Humic acid reduction was also observed by *Propionibacterium freudenreichii* and other fermentative bacteria (*3*). The addition of humic acids to a culture of *Propionibacterium freudenreichii* actively fermenting lactate resulted in the formation of more oxidized products such as acetate instead of propionate. Similar results were also observed during the lactic acid fermentation of glucose by *Enterococcus cecorum* and *Lactococcus lactis*.

Therefore, the presence of humic substances in anaerobic environments can enhance the oxidation of organic substrates by serving as a terminal electron acceptor or by serving as an additional electron sink. Table 1 summarizes the literature data concerning the oxidation of various substrates coupled to humic reducing processes.

Recycling of humic substances by metal oxides

Reduced humic substances can be oxidized directly by metal oxides in soils and sediments. Direct reduction of Fe(III) to Fe(II) by reduced humic substances in abiotic systems was reported (*17, 22*). Also hydroquinones (the reduced form of quinones) are among the best known reductants of $Mn(IV)O_2$ precipitates (*35, 36*). Table 2 illustrates the reduction of metal oxides by reduced humic substances or hydroquinones. Therefore, it is expected that quinone moieties in humus can be recycled by the reaction of their reduced form with oxidized minerals naturally present in aquifers and soils. This could allow for the recycling of humic substances as electron acceptor enabling their use at sub-stoichiometric concentrations for bioremediation of contaminated sites (Figure 1).

Table 2. Abiotic reduction of metals by different reduced humus and humic model compounds

Metal reduction	Humus or humic model compound	Reference
Fe (III) → Fe (II)	Reduced humus and AH_2QDS	17, 22
Mn (IV) → Mn (II)	3-methoxycatechol, catechol, 3,4-dihydroxybenzoic acid and hydroquinone*	35, 36

*A number of other organic compounds caused the reduction of Mn(IV) to Mn(II) at slower rates.

Humic substances as redox mediators for reductive biotransformations

Humic substances do not only stimulate the oxidation of a wide variety of organic compounds by acting as electron acceptor, but also support the reductive biotransformation of environmental pollutants such as azo dyes, substituted nitrobenzenes, polyhalogenated compounds and radionuclides by shuttling electrons between an external electron donor and those pollutants.

Azo dyes cleavage. Azo dyes are readily reductively cleaved to colorless aromatic amines by a wide variety of inocula and microorganisms under anaerobic conditions (*11*). However, recent experiments revealed that long reaction times are required for many azo dyes to achieve satisfying extent (>90%) of decolorization (*37*) and therefore, the use of redox mediators is required in these cases to speed up the reductive decolorizing processes. It was found that an accelerated decolorization capacity for

different azo dyes by anaerobically incubated cells suspensions of the bacterium, *Sphingomonas sp.* BN6, could be obtained if this strain was previously grown with 2-naphthalenesulfonic acid (2NS) aerobically (*16*). The enhancement of the azo dye reduction rate was clarified by an unidentified metabolite of 2NS acting as redox mediator. In order to confirm this theory, several quinone substances were tested, such as AQDS, anthraquinone-2-sulfonate (AQS), and 2-hydroxy-1,4-naphthoquinone, which were found to be effective as redox mediators by shuttling reduction equivalents from cell suspensions of strain BN6 incubated anaerobically to amaranth, with glucose as electron donor. Further experiments indicated that a NADH:ubiquinone oxidoreductase situated in the membrane of *Sphingomonas sp.* BN6 was responsible for the dye reduction (*18*). The quinone moieties thus, transport the electrons from the periplasm to the azo dye at a distance from the cell (Figure 2). Several other dyes were reduced by the same system (Table 3). The use of AQDS as redox mediator at catalytic concentrations also enabled the continuous treatment of a recalcitrant reactive azo dye (Reactive red 2) in anaerobic bioreactors, which otherwise was only marginally biotransformed (*39*).

The extracellular role of quinones in the reductive decolorization of azo dyes was corroborated by the regeneration of an azo-dye-saturated cellulosic anion exchange resin by *Burkholderia cepacia*. By physically separating a cell suspension of *Burkholderia cepacia* using a dialysis tube, it was demonstrated that AQS mediated the transfer of electrons from bacteria to the adsorbed dye (*19*). The results are also consistent with the use of AQDS and AQS to mediate the chemical reduction of acid orange 7 by sulfide (*38*) and the electrochemical cathodic reduction of dispersed dyes (*2*).

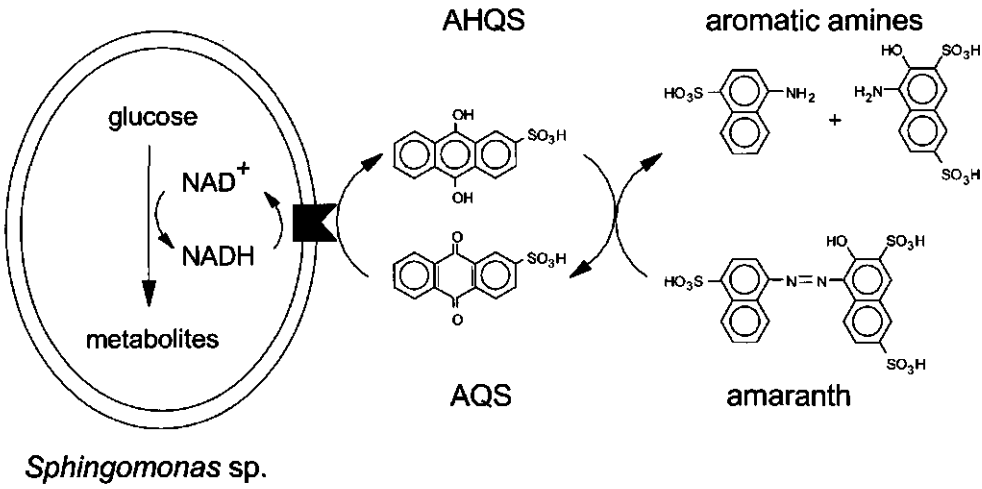


Figure 2. Proposed mechanism of anthraquinone-2-sulfonate (AQS) mediation of amaranth dye decolorization by *Sphingomonas* sp. BN6. AQS is reduced by the membrane bound respiratory chain enzyme, NADH:ubiquinone oxidoreductase (filled box) to anthrahydroquinone-2-sulfonate (AHQS), which in turn transfers the electrons to the dye, causing azo cleavage (*18*).

Reduction of nitroaromatics. Quinones and natural organic matter (NOM) from different sources were also shown to mediate the abiotic reduction of nitrobenzenes to the corresponding anilines, with sulfide as an electron donor (*10, 15, 30*). The observed pseudo-first-order rate constant for the nitro group reduction in the presence of NOM was approximately 500 times faster compared to that obtained in the absence of NOM. Furthermore, pseudo-first-order kinetics were observed in solutions containing a very low concentration of NOM. This suggested that the reductive NOM constituents act as redox mediators at catalytic concentrations. The pH-dependency of the quinone mediated reactions indicated that the dissociated species (phenolate and diphenolate) of the reduced quinones were responsible for the electron transfer (*10*). Table 3 summarizes the reductive transformation of several nitroaromatic pollutants by humic or model humic compounds reported in literature.

Reductive dehalogenation. The use of humus and quinones to mediate the reductive dehalogenation of polychlorinated pollutants is summarized in Table 3. Most studies concern abiotic processes where the reduction by bulk reducing agents is mediated by humic substances. For instance, the addition of humic model compounds, such as resorcinol and catechol, to abiotic assays including polychlorinated dibenzo-*p*-dioxins (PCDDs) was shown to stimulate the dechlorination of these contaminants (*1*). Addition of the humic analogues to reaction systems containing octa-CDDs led to the formation of the tetra-CDD group of congeners, whereas no reductive dechlorination of octa-CDDs was observed in the absence of humic model compounds. Similar dechlorination yields (4-20 %) were observed in the presence of humic acids, when the results were compared to those obtained with the humic model compounds in terms of phenolic acidity (*32*).

Both quinones and humic acids also enhanced the abiotic reductive dehalogenation of hexachloroethane (HCA), carbon tetrachloride (CT) and bromoform. The addition of these redox mediators to solution containing sulfide, elemental sulfur or Fe(II) as electron donors increased the pseudo-first-order rate constant by factors of up to 10 (*9, 28*). In the case of humic substances, quinones are implicated since AH_2QDS could mediate the direct reduction of HCA as a sole electron donor (*9*). The pseudo-first-order rate constant for HCA disappearance by AH_2QDS was observed to depend on the pH. Thus, the overall rate expression for HCA reduction was considered to be the sum of three independent, parallel, first-order reactions:

$$K'_{HCA} = K_0[A(OH)_2] + K_1[A(OH)O^-] + K_2[A(O)_2^{2-}]$$

Where $[A(OH)_2]$, $[A(OH)O^-]$, and $[A(O)_2^{2-}]$ represent the concentrations of the fully protonated, the monophenolate, and the diphenolate forms of AH_2QDS and K_0, K_1, and K_2 are the corresponding rate constants. It was observed that K_2 was about 8 times faster than K_1 while K_0 was not different from zero indicating that the dechlorination process mediated by quinones proceeds faster at high pH levels. Rate results of CT and HCA under different conditions agreed qualitatively with predictions based on

a one-electron reduction mechanism (*9*). A one-electron reduction mechanism would imply that the abiotic reduction proceeds via radical intermediates. However, the reduction of polyhalogenated alkanes and methanes by sulfide in the presence of juglone (5-hydroxy-1,4-naphthoquinone) is distinct. The reduced form of the quinone, juglone hydroquinone (1,4,5-trihydroxynaphthalene) did not cause direct dechlorination (*28*). Instead, sulfide reacted with juglone to form mercaptojuglone, which is implicated in the mediation process (*29*). The observed reaction rates are higher than those that would be expected from one-electron reduction alone because the thiol group of mercaptojuglone can transfer two electrons by acting as a nucleophile at a halogen of the polyhalogenated alkane.

In addition to the abiotic dehalogenating processes mediated by humus or quinones, the anaerobic biotransformation of CT by *Shewanella putrefaciens* 200 was also reported to be accelerated by the presence of soil organic matter (*8*). Only 29 % of the chlorinated pollutant initially added (~20 μM) was converted after 33 hours in cell suspensions without soil, whereas 64 % was transformed after only 18 hours when soil was present. Further experiments revealed that the humic acid (HA) fraction catalyzed the dechlorination reaction to a greater extent (270-442 μg of CT per gram of HA) than did the fulvic acid (FA) fraction (149-234 μg of CT per gram of FA), and the fraction containing humin and inorganic minerals (19-26 μg of CT per gram).

Humic substances can also chelate metals improving their catalytic properties for reductive dehalogenation. Humic-metal complexes were shown to mediate the abiotic reductive dechlorination of TCE with Ti(III) citrate as electron donor (*27*). Rapid TCE reduction was obtained by Ni-humic complexes, with total removal of TCE in less than 23 hours. Cu-humic complexes were less effective as catalyst, only 60 % of TCE was reduced after 150 hours. The reductive dechlorination rate of TCE in the absence of humic-metal complexes occurred five times slower than in the presence of humic acids for the experiments with copper. Only negligible reduction of TCE occurred in the experiments with nickel when the humic acids were excluded. In all cases ethene and ethane were the main end products of TCE reduction. Further experiments revealed a strong pH dependence of TCE reduction by Ni-humic complexes may be due to the variation of both Ni-humic concentration and redox potential with pH (*25*).

Reduction of radionuclides. Quinone moieties in humus can also mediate the reduction of radionuclides. Biogenic AH_2QDS caused the direct reduction of U(VI) in the absence of cells (*13*). Moreover, *Deinococcus radiodurans* R1, a radiation-resistant microorganism, could almost completely reduce U(VI) at concentrations raging from 5 to 200 μM in the presence of 100 μM AQDS. The same microorganism was unable to directly reduce U(VI) in the absence of AQDS indicating that biogenic hydroquinones were responsible for the reduction of U(VI) in the bioassays, which is consistent with the AQDS-reducing capacity of this microorganism (*14*). The same microorganism was able to reduce the soluble β-particle emitter, Tc(VII), to insoluble Tc(IV) via AQDS reduction.

Table 3. Reductive transformations of priority pollutants by humic or model humic compounds as redox mediators

Electron donor	System	Redox mediator[a]	Reductive reaction	Reference
Azo dyes				
Cathode	Abiotic	AQS, AQDS or DHAQ	Vat yellow 1 → decolorized dye	2
Glucose	Bacteria[b]	UI-2NS-M or 1,2-naphthoquinone	Amaranth → aromatic amines	16
Glucose	Bacteria[b]	AQS, AQDS or lawsone	Various azo dyes[c] → aromatic amines	18
Glucose	*Burkholderia cepacia*	AQS	Various azo dyes[d] → aromatic amines	19
Sulfide	Abiotic	AQDS or 1A2N	Acid orange 7 → aromatic amines	38
Volatile fatty acids	Anaerobic granular sludge	AQDS	Reactive red 2 → aromatic amines	39
Polyhalogenated pollutants				
Lactate	*Shewanella putrefaciens* 200	Soil organic matter	$CCl_4 \rightarrow CHCl_3$ + unidentified products	8
Unidentified donor in sediment	Abiotic	Catechol, resorcinol or humic acids	Octachlorodioxin → tetrachlorodioxin	1, 32
Sulfide or Fe(II)	Abiotic	Humic acids	$C_2Cl_6 \rightarrow C_2Cl_4$	9
Sulfide or Fe(II)	Abiotic	Humic acids	$CCl_4 \rightarrow CHCl_3$	9
Sulfide or Fe(II)	Abiotic	Humic acids	$CHBr_3$ → unidentified	9
Sulfide or sulfur	Abiotic	Juglone	$C_2Cl_6 \rightarrow C_2Cl_4$	28
Nitroaromatics				
Sulfide	Abiotic	Natural organic matter	Various Nitroaromatics[e] → anilines	10, 15
Sulfide	Abiotic	Juglone or lawsone	Various Nitroaromatics[f] → anilines	30
Radionuclides				
Lactate	*Deinococcus radiodurans*	AQDS	U(VI) → U(IV) Tc(VII) → Tc(IV)	14

[a]AQS, anthraquinone-2-sulfonate; AQDS, anthraquinone-2,6-disulfonate; DHAQ, 1,4-Dihydroxyanthraquinone; UI-2NS-M, unidentified metabolites from aerobic degradation of 2-Naphthalenesulfonate; lawsone, 2-hydroxy-1,4-naphthoquinone; 1A2N, 1-amino-2-naphthol; juglone, 5-hydroxy-1,4-naphthoquinone.

[b]Anaerobic bacteria incubated anaerobically (*Pseudomonas putida, Sphingomonas* sp., *Escherichia coli* and activated sludge).

[c]Amaranth, Acid red 1, Sunset yellow, Naphthol blue black.

[d]Acid orange 7, Reactive red 180.

[e]Nitrobenzene, 2-nitrotoluene, 3-nitrotoluene, 4-nitrotoluene, various amino-, alkyl- and chloro-nitroaromatics, and trinitrotoluene.

[f]Nitrobenzene, nitrophenols, various alkyl- and chloro-nitroaromatics.

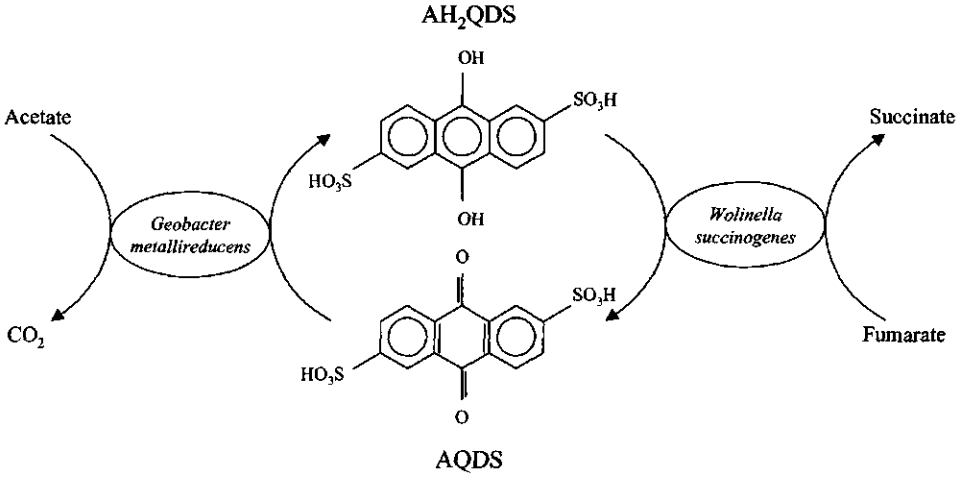


Figure 4. Quinone moieties acting as interspecies electron transfer. AQDS is firstly used as electron acceptor by *G. metallireducens* to oxidize acetate and then, AH_2QDS (reduced form of AQDS) is employed as electron donor by *W. succinogenes* for fumarate respiration (*23*).

Humic substances as electron donors to support microbial growth

The role of humic substances as an electron donor for anaerobic respiration has been recently reported indicating that hydroquinones in humus may serve as electron donors for denitrification, dissimilatory nitrate reduction to ammonium (DNRA), as well as the reduction of fumarate, and the toxic metalloids selenate and arsenate (*23*). AH_2QDS oxidation could yield energy to support growth for three fumarate-reducing microorganisms, *Shewanella alga, Geobacter sulfurreducens* and *Wolinella succinogenes*. The fact that these microorganisms did not grow when AH_2QDS or fumarate was omitted indicates that they obtain energy for growth from AH_2QDS oxidation. Further experiments indicated that quinones could act as interspecies electron transfer between different microorganisms, such as *G. metallireducens* and *W. succinogenes*. The former can oxidize acetate with quinones as electron acceptor, but cannot use fumarate as electron acceptor. *W. succinogenes* cannot oxidize acetate, but can use fumarate as electron acceptor. There was a negligible acetate oxidation when washed cell suspensions of *G. metallireducens* and *W. succinogenes* were incubated in bicarbonate buffer containing acetate and fumarate. When AH_2QDS was included in the culture, acetate was oxidized rapidly over time. The finding that there was a negligible acetate oxidation in AH_2QDS-containing cell suspensions with only *G. metallireducens* or *W. succinogenes* indicated that quinone moieties enhanced the electron transfer between the acetate-oxidizing AQDS-reducing, *G. metallireducens*, and the AH_2QDS-oxidizing fumarate-reducing, *W. succinogenes* (Figure 4). These results suggest that reduced humic substances can donate electrons to microorganisms. Hydroquinones have also been reported as a potential electron donor for the microbial reduction of (per)chlorate.

Strain CKB, a protobacterium closely related to *Rhodocyclus tenuis*, was able to oxidize AH_2QDS coupled to the reduction of (per)chlorate and the respiratory process was shown to support growth (*5*).

Significance

Previously humus was considered as inert organic matter. However, new insights from the literature indicate that humus can have active roles in anaerobic biodegradation processes including that of priority pollutants. The recent evidences reported on the role of humic compounds in electron transfer under anaerobic conditions shows that these ubiquitous compounds are mainly involved in 4 different phenomena:

1) Serving as terminal electron acceptors for the anaerobic oxidation of both readily biodegradable compounds and priority pollutants. Metal oxides can recycle the reduced humus.
2) Serving as redox mediators in the reductive (bio)transformation of priority pollutants such as polychlorinated, nitroaromatics, azo dyes and radionuclides.
3) Serving as electron donors for the anaerobic respiration of more oxidized compounds, such as nitrate, fumarate and (per)chlorate.
4) Chelating metals to improve their catalytic properties such as the reduction of polychlorinated compounds by humic-metal complexes.

Therefore, humic compounds or analogues can play different roles in the bioremediation of contaminated anaerobic sites as well as on the treatment of wastewaters containing priority pollutants.

Scope and outline of thesis

The objective of this dissertation is to evaluate the impact of humic substances or humic analogues on the anaerobic biotransformation of priority pollutants. The study is divided in two phases:

Phase 1. Evaluate the capacity of humus-respiring consortia and isolates for oxidizing ecologically important substrates and selected priority pollutants with humus or quinones as terminal electron acceptors.

Phase 2. Evaluate the role of humic substances as redox mediators in reductive processes to achieve the anaerobic biotransformation of selected priority pollutants.

Anaerobic consortia obtained from a broad diversity of environments are described in Chapter 2 for their capacity to oxidize ecologically important simple substrates, such as acetate, lactate, and methanol, with the humic analogue, AQDS, as a final electron acceptor. In Chapter 3, pure cultures of different microorganisms, previously not known to have the ability to reduce humic substances, are evaluated for their capacity to use humic substances as a terminal electron acceptor for the anaerobic oxidation of simple substrates. An AQDS-respiring enrichment derived from an anaerobic granular sludge is physiologically and phylogenetically characterized in Chapter 4. AQDS-respiring

microorganisms were also immobilized in the granular sludge of a continuous upflow anaerobic sludge blanket (UASB) reactor and the application of these microorganisms in anaerobic reactors treating wastewaters containing priority contaminants is also discussed in chapter 4. Consortia that showed the capacity to oxidize simple substrates with AQDS as a terminal electron acceptor (Chapter 2) are also tested for their ability to oxidize priority pollutants, such as *p*-cresol (Chapter 5) and toluene (Chapter 6) coupled to AQDS or humus reduction.

In Chapter 7, the role of quinone-respiring consortia on the anaerobic dechlorination of a polychlorinated pollutant (carbon tetrachloride), with quinones as redox mediators, is described. Chapter 8 illustrates the application of quinones as redox mediators to accelerate the anaerobic biotransformation of an azo dye (Acid orange 7) by anaerobic granular sludge in a continuous reactor.

Finally, the results obtained from this dissertation are discussed in chapter 9 in relation to their relevance for the bioremediation of aquifers and sediments, as well as, for the anaerobic treatment of wastewaters containing priority pollutants.

References

1. Barkovskii, A. L. and P. Adriaens. (1998). Impact of humic constituents on microbial dechlorination of polychlorinated dioxins. *Environ. Toxicol. Chem.* **17**, 1013-1020.
2. Bechtold, T., E. Burtscher and A. Turcanu. (1999). Anthraquinones as mediators for the indirect cathodic reduction of dispersed organic dyestuffs. *J. Electroanal. Chem.* **465**, 80-87.
3. Benz, M., B. Schink and A. Brune. (1998). Humic acid reduction by *Propionibacterium freudenreichii* and other fermenting bacteria. *Appl. Environ. Microbiol.* **64**, 4507-4512.
4. Bradley, P., F. Chapelle and D. R. Lovley. (1998). Humic acids as electron acceptor for anaerobic microbial oxidation of vinyl chloride and dichloroethene. Appl. Environ. Microbiol. **64**, 3102-3105.
5. Bruce, R. A., L. A. Achenbach and J. D. Coates. (1999). Reduction of (per)chlorate by a novel organism isolated from paper mill waste. *Environ. Microbiol.* **1**, 319-329.
6. Coates, J. D., V. K.. Bhupathiraju, L. A. Achenbach, M. J. Mclnerney, and D. R. Lovley. (2001). *Geobacter hydrogenophilus, Geobacter chapellei* and *Geobacter grbicie,* three new, strictly anaerobic, dissimilatory Fe(III)-reducers. *Int. J. Syst. Evol. Microbiol.* **51**, 581-588.
7. Coates, J., D. Ellis, E. L. Blunt-Harris, C. Gaw, E. Roden and D. R. Lovley. (1998). Recovery of humic-reducing bacteria from a diversity of environments. *Appl. Environ. Microbiol.* **64**, 1504-1509.
8. Collins, R. and F. Picardal. (1999). Enhanced anaerobic transformation of carbon tetrachloride by soil organic matter. *Environ. Toxicol. Chem.* **18**, 2703-2710.
9. Curtis, C. and M. Reinhard. (1994). Reductive dehalogenation of hexachloroethane, carbon tetrachloride, and bromoform by anthrahydroquinone disulphonate and humic acid. *Environ. Sci. Technol.* **28**, 2393-2401.

10. Dunnivant, F., R. Schwarzenbach and D. Macalady. (1992). Reduction of substituted nitrobenzenes in aqueous solutions containing natural organic matter. *Environ. Sci. Technol.* **26**, 2133-2142.

11. Field J. A., A. J. M. Stams, M. Kato, and G. Schraa. (1995). Enhanced biodegradation of aromatic pollutants in cocultures of anaerobic and aerobic bacterial consortia. *Antonie van Leeuwenhoek* **67**, 47-77.

12. Francis, C. A., A. Y. Obraztsova and B. M. Tebo. (2000). Dissimilatory metal reduction by the facultative anaerobe *Pantoea agglomerans* SP1. *Appl. Environ. Microbiol.* **66**, 543-548.

13. Fredrickson, J. K., J. M. Zachara, D. W. Kennedy, M. C. Duff, Y. A. Gorby, S. W. Li and K. M. Krupka. (2000). Reduction of U(VI) in goethite (α-FeOOH) suspensions by a dissimilatory metal-reducing bacterium. *Geochim. Cosmochim. Acta* **64**, 3085-3098.

14. Fredrickson, J. K., H. M. Kostandarithes, S. W. Li, A. E. Plymale and M. J. Daly. (2000) Reduction of Fe(III), Cr(VI), U(VI), and Tc(VII) by *Deinococcus radiodurans* R1. *Appl. Environ. Microbiol.* **66**, 2006-2011.

15. Hofstetter, T. B., C. G. Heijman, S. B. Haderlein, C. Holliger and R. P. Schwarzenbach. (1999). Complete reduction of TNT and other (poly)nitroaromatic compounds under iron-reducing subsurface conditions. *Environ. Sci. Technol.* **33**, 1479-1487.

16. Keck, A., J. Klein, M. Kudlich, A. Stolz, H. Knackmuss, and R. Mattes. (1997). Reduction of azo dyes by redox meditors originating in the naphthalenesulfonic acid degradation pathway of *Sphingomonas sp.* strain BN6. *Appl. Environ. Microbiol.* **63**, 3684-3690.

17. Kieft, T., J. Fredrickson, T. Onstott, Y. Gorby, H. Kostandarithes, T. Bailey, D. Kennedy, S. Li, A. Plymale, C. Spadoni, and M. Gray. (1999). Dissimilatory reduction of Fe (III) and other electron acceptors by a *Thermus* isolate. *Appl. Environ. Microbiol.* **65**, 1214-1221.

18. Kudlich, M., A. Keck, J. Klein and A. Stolz. (1997). Localization of the enzyme system involved in anaerobic reduction of azo dyes by *Sphingomonas sp.* Strain BN6 and effect of artificial redox mediators on the rate of azo dye reduction. *Appl. Environ. Microbiol.* **63**, 3691-3694.

19. Laszlo, J. A. (2000). Regeneration of azo-dye-saturated cellulosic anion exchange resin by *Burkholderia cepacia* anaerobic dye reduction. *Environ. Sci. Technol.* **34**, 167-172.

20. Lovley D. R., K. Kashefi, M. Vargas, J. M. Tor and E. L. Blunt-Harris. (2000) Reduction of humic substances and Fe(III) by hyperthermophilic microorganisms. *Chem. Geol.* **169**, 289-298.

21. Lovley, D. R., J. L. Fraga, E. L. Blunt-Harris, L. A. Hayes, E. J. P. Phillips and J. D. Coates. (1998). Humic substances as a mediator for microbially catalyzed metal reduction. *Acta Hydrochim. Hydrobiol.* **26**, 152-157.

22. Lovley, D. R., J. D., Coates, E. L. Blunt-Harris, E. J. P. Phillips and J. Woodward. (1996). Humic substances as electron acceptors for microbial respiration. *Nature* **382**, 445-448.

23. Lovley, D. R., J. L. Fraga, J. D. Coates and E. L. Blunt-Harris. (1999). Humics as an electron donor for anaerobic respiration. *Environ. Microbiol.* **1**, 89-98.

24. Lovley, D. R., J. Woodward and F. Chapelle. (1996). Rapid anaerobic benzene oxidation with a variety of chelated Fe (III) forms. *Appl. Environ. Microbiol.* **62**, 288-291.

25. Ma, H., E. J. O'Loughlin and D. R. Burris. (2001). Factors affecting humic-nickel complex mediated reduction of trichloroethene in homogeneous aqueous solution. *Environ. Sci. Technol.* **35**, 717-724.

26. Newman, D. K., and R. Kolter. (2000). A role for excreted quinones in extracellular electron transfer. *Nature* **405**, 94-97.

27. O'loughlin, E., D. Burris, and C. Delcomyn. (1999). Reductive dechlorination of trichloroethene mediated by humic-metal complexes. *Environ. Sci. Technol.* **33**, 1145-1147.

28. Perlinger J. A., W. Angst and R. P. Schwarzenbach. (1996). Kinetics of the reduction of hexachloroethane by juglone in solutions containing hydrogen sulfide. *Environ. Sci. Technol.* **30**, 3408-3417.

29. Perlinger J. A., J. Buschmann, W. Angst and R. P. Schwarzenbach. (1998). Iron porphyrin and mercaptojuglone mediated reduction of polyhalogenated methanes and ethanes in homogeneous aqueous solution. *Environ. Sci. Technol.* **32**, 2431-2437.

30. Schwarzenbach, R., R. Stierli, K. Lanz and J. Zeyer (1990). Quinone and iron porphyrin mediated reduction of nitroaromatic compounds in homogeneous aqueous solution. *Environs. Sci. Technol.* **24**, 1566-1574.

31. Scott, D., D. Mcknight, E. L. Blunt-Harris, S. Kolesar and D. R. Lovley. (1998). Quinone moieties as electron acceptors in the reduction of humic substances by humic-reducing microorganisms. *Environ. Sci. Technol.* **32**, 2984-2989.

32. Shiang-Fu, Q., A. L. Barkovskii and P. Adriaens. (1999). Reductive transformation of dioxins: an assessment of the contribution of dissolved organic matter to dechlorination reactions. *Environ. Sci. Technol.* **33**, 3837-3842.

33. Slobodkin, A. I., T. P. Tourova, B. B. Kuznetsov, N. A. Kostrikina, N. A. Chernyh, and E. A. Bonch-Osmolovskaya. (1999). *Thermoanaerobacter siderophilus* sp. nov., a novel dissimilatory Fe(III)-reducing, anaerobic, thermophilic bacterium. *Int. J. Syst. Bacteriol.* **49**, 1471-1478.

34. Stevenson, F. J. (1994). Humus chemistry: genesis, composition, reactions. John Wiley & Sons. New York, N.Y.

35. Stone, A. and J. Morgan. (1984). Reduction and dissolution of manganese (III) and manganese (IV) oxides by organics: 2. Survey of the reactivity of organics. *Environ. Sci. Technol.* **18**, 617-624.

36. Stone, A. and J. Morgan. (1984). Reduction and dissolution of manganese (III) and manganese (IV) oxides by organics: 1. Reaction with hydroquinone. *Environ. Sci. Technol.* **18**, 450-456.

37. Van der Zee, F. P., G. Lettinga and J. A. Field. (2000). Azo dye decolourisation by anaerobic granular sludge. *Chemosphere* **44**, 1169-1176.

38. Van der Zee, F. P., G. Lettinga and J. A. Field. (2000). The role of (auto)catalysis in the mechanism of an anaerobic azo reduction. *Water Sci. Technol.* **42**, 301-308.

39. Van der Zee, F. P., R. H. M., Bouwman, D. P. B. T. B., Strik, G., Lettinga and J. A. Field. (2001). Application of redox mediators to accelerate the transformation of reactive azo dyes in anaerobic bioreactors. Biotechnol. Bioeng. **75**, 691-701.

2

Competition between methanogenesis and quinone respiration for ecologically important substrates in anaerobic consortia*

*A modified version has been published as: Cervantes F. J., S. van der Velde, G. Lettinga and J. A. Field. (2000) *FEMS Microbiol. Ecol.* **34**, 161-171.

(Bennekom, The Netherlands) was also used as inoculum in this study. Additionally, three different anaerobic sediments were used in the experiments. Anaerobic Rhine sediment was collected alongside the banks of the river near Lexkesveer in Wageningen, The Netherlands. Anaerobic Petroleum Harbor sediment was collected from a contaminated site with polycyclic aromatic hydrocarbons (Amsterdam, The Netherlands). Anaerobic sediment rich in organic matter (about 50 % dry weight) was collected from a small canal ("Voorwetering", Nieuwkoop, The Netherlands). All biomass sources were stored at 4 °C before use.

The basal medium used in all the experiments contained (g l^{-1}): $NaHCO_3$, (5); NH_4Cl, (0.03); K_2HPO_4, (0.02); $MgCl_2*6H_2O$, (0.012); $CaCl_2*2H_2O$, (0.005); Na_2S, (0.013); and 1 ml l^{-1} of both trace elements and vitamins. The basal medium was flushed with N_2/CO_2 (70/30) for 10 minutes before use.

Bioassays. The assays were conducted in batch mode culture by triplicate in 117-ml glass serum bottles with a liquid volume of 50 ml (67 ml as headspace). Two sets of assays were run. In the first set, basal medium was transferred directly to the vials after flushing it and then, inoculation took place by adding 1 g of volatile suspended solids (VSS) per liter for activated and granular sludge or 10 g (dry weight) of sediment per liter for the anaerobic sediments. The vials were sealed with butyl rubber stoppers and aluminum crimps and then flushed with N_2/CO_2 (70/30) for 10 minutes. Finally, the corresponding substrate was added at the final concentration of 300 mg of chemical oxygen demand (COD) per liter. The substrates evaluated include hydrogen, acetate, propionate, methanol and lactate. Another set was amended with AQDS (20 mM) and run under the same experimental conditions. Furthermore, two more sets of assays (one with and another without AQDS) were conducted in the presence of the methanogenic inhibitor, 2-bromoethanesulphonic acid (BES) at the final concentration of 50 mM. Sterilized controls were also included for the reduction of AQDS with hydrogen as electron donor. Endogenous methane production and AQDS reduction refer to the occurrence of these phenomena by the inocula evaluated without any addition of substrate in the experiments. The pH was corroborated and remained at 7.3±0.1 in all the assays. All incubations were carried out at 30 °C in the dark. Refilling the corresponding bottles and following all the parameters at least one more time confirmed all the batch experiments.

Analyses. Analysis of AH_2QDS was carried out on anaerobically collected samples in an anaerobic chamber under N_2/H_2 (96:4) atmosphere. Samples were centrifuged (10000 g, 5 min) and then diluted in 1 cm disposable plastic cuvettes containing anaerobic bicarbonate buffer (60 mM, pH 6.7±0.1). Concentrations of AH_2QDS were determined by monitoring absorbance at 450 nm and using an extinction coefficient of 2.25 AU per mM obtained from a calibration curve of AQDS chemically reduced by dithionite. Figure 1 illustrates the spectrum of AH_2QDS obtained from the microbial reduction of AQDS by Nedalco sludge with acetate as electron donor and also by chemical reduction with dithionite. There was no difference observed in the spectrum obtained by both mechanisms of

reduction even at early steps during microbial reduction of AQDS, indicating that reduction of AQDS proceeded directly to AH_2QDS without any accumulation of partially reduced quinone intermediates.

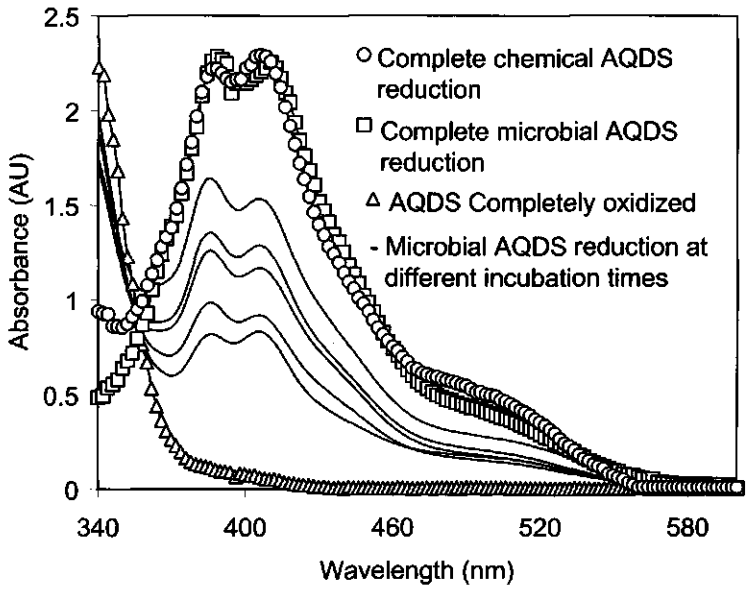


Figure 1. Spectrum of anthraquinone-2,6-disulfonate in its oxidized (AQDS) and reduced (AH_2QDS) forms. Chemical reduction of AQDS refers to the formation of AH_2QDS with dithionite as reductant and microbial AQDS reduction was achieved by "Nedalco" sludge with acetate as electron donor. Measurements were conducted in anaerobically collected samples diluted in anaerobic bicarbonate buffer (60 mM). Cuvvetes containing 0.25 mM of AQDS in all cases.

Methane production was determined by using a flame ionization gas chromatograph model 438/S (Packard-Becker, Delft, The Netherlands). The gas chromatograph was equipped with a steel column (2 m x 2 mm) packed with Porapak Q (80/100 mesh, Millipore Corp., Bedford, Mass.). The temperatures of the column, injector port, and the flame ionization detector were 60, 200 and 220 °C, respectively. Nitrogen was used, as carrier, at a flow rate of 20 ml per min and the sample injection volume was 100 µl. Hydrogen concentration was followed by gas chromatography with a thermal conductivity detector. The chromatograph was equipped with a 1.5 m x 2 mm steel column packed with Mol sieve 5A, 60-80 mesh. The temperatures for the column, injection port and detector were 40, 110, 125 °C, respectively. The carrier gas was argon at a flow rate of 20 ml per min and the sample injection volume was 100 µl.

Samples for methanol and volatile fatty acids (VFA) were analyzed after centrifugation at 10000 g for 5 min. VFA were determined by gas chromatography using a Hewlett Packard 5890 equipped with a 2 m x 2 mm glass column packed with Supelcoport (100-120 mesh) coated with 10 % Fluorad FC 431.

The temperatures of the column, injection port and flame ionization detector were 130, 200, 280 °C, respectively. The carrier gas was nitrogen saturated with formic acid (40 ml per min). Methanol was analyzed in the same way as VFA except that the temperatures of the column, the injection port and the flame ionization detector were 70, 200 and 280 °C, respectively. Lactate was analyzed by high performance liquid chromatography (HPLC) in previously centrifuged samples (10000 g, 5 min). The HPLC was equipped with an Ion 300 "organic acids" column (30 cm x 7.8 mm), which was kept at 20 °C, and with a refractive index (RI) detector. H_2SO_4 (1.25 mM) was used as mobile phase at 0.5 ml per min. The sample injection volume was 20 µl.

Redox measurements were conducted anaerobically using a WTW redox electrode with an Ag/AgCl (3 M) reference electrolyte at 25 °C. The readings were corrected for the redox potential of the reference electrode (+ 207 mV at 25 °C). The electrode was calibrated at – 480 mV using an anaerobic redox standard solution of titanium(III) citrate (*13*).

Results

Reduction of AQDS by different anaerobic consortia with acetate and hydrogen as electron donors. All the consortia evaluated were capable of using AQDS as terminal electron acceptor for oxidizing hydrogen as substrate after 5 weeks of incubation (Table 1). There was neither AQDS reduction nor hydrogen consumption in sterilized controls with autoclaved inocula (data not shown). Most of the consortia studied were also able to reduce AQDS with acetate as electron donor, but reduction of AQDS by "Shell" granular sludge and "Rhine" sediment occurred rather slowly with this substrate (Table 1). Further incubation (8 weeks in total) of these bioassays resulted in only minor reduction of AQDS (data not shown). Both substrates were partially or completely converted to methane by all the consortia in the absence of AQDS (data not shown).

Characterization of the AQDS-respiring consortia. The capacity of the methanogenic granular sludge obtained from a full-scale UASB reactor (alcohol distillery of Nedalco; Bergen op Zoom, The Netherlands) and the anaerobic sediment rich in organic matter obtained from "Voorwetering" (Nieuwkoop, The Netherlands) for oxidizing different substrates coupled to the reduction of AQDS was explored. All the substrates evaluated were completely converted to methane by "Nedalco" sludge when bicarbonate was the only electron acceptor available after two weeks of incubation. There was negligible methane production when the methanogenic inhibitor, BES, was included in the cultures. Acetate was not consumed under these inhibitory conditions whereas propionate, methanol and hydrogen were only partially converted to acetate. Lactate was also converted to acetate and propionate under these conditions (Table 2).

Table 1. Acetate and hydrogen oxidation coupled to quinone respiration by different consortia after 5 weeks of incubation[a]

Inoculum	Characteristics	Quinone respiration with acetate		Quinone respiration with hydrogen	
		% substrate[b]	% substrate consumed[c]	% substrate	% substrate consumed
Nedalco	Methanogenic granular sludge	86	128	84	84
Shell	Methanogenic granular sludge	7	101	98	98
Bennekom	Aerobic activated sludge	100	102	100	100
APH	Amsterdam petroleum harbor sediment	88	102	100	100
Voorwetering	Anaerobic sediment rich in NOM	102	109	104	104
Rhine	Anaerobic sediment	21	104	98	98

AQDS, anthraquinone-2,6-disulfonate; AH_2QDS, anthrahydroquinone-2,6-disulfonate; COD, chemical oxygen demand; NOM, natural organic matter.

[a]Initial concentration for both substrates 300 mg COD l^{-1}. AQDS concentration of 20 mM in all cases. All calculations based on mg COD per liter of liquid fluid. COD in AH_2QDS refers only to the hydrogen linked to the structure. Results are means of triplicate incubations. In all the cases the standard deviation was less than 10 %.

[b]% of substrate consumed via AQDS reduction related to the input COD = (AH_2QDS produced – endogenous AH_2QDS production)/(Input COD).

[c]% of substrate converted via AQDS reduction related to the substrate consumption = (AH_2QDS produced – endogenous AH_2QDS production)/(Input COD – substrate not consumed).

Table 2. COD balance (in mg COD l^{-1}) for the oxidation of different substrates by Nedalco sludge in the absence of AQDS after 5 weeks of incubation[a]

Culture	Endogenous	Acetate	Propionate	Methane	Recovery (%)[b]
Acetate	76	-	-	444	121
Acetate-BES	76	360	27	45	117
Propionate	76	8	7	430	117
Propionate-BES	76	150	200	66	108
Methanol	76	-	-	392	104
Methanol-BES	76	188	-	43	86[c]
Hydrogen	76	2	-	373	101[d]
Hydrogen-BES	76	252	17	72	93[d]
Lactate	59[f]	15	-	365	107[e]
Lactate-BES	59[f]	161	194.5	30	109[e]

All abbreviations refer to the same terms given in Table 1. BES: 2-bromoethanesulphonic acid.

[a]All substrates at the initial concentration of 300 mg COD l^{-1}. Results are means of triplicate incubations. In all cases the standard deviation was less than 10 %.

[b]Recovery = (identified products - endogenous COD)/(input COD).

[c]Including 98 mg COD l^{-1} remaining as methanol.

[d]No hydrogen detected at the end of the experiment.

[e]No lactate detected at the end of the experiment

[f]Conducted with another endogenous control at a different time compared to the other batch cultures.

In general, both consortia evaluated did not produce methane when AQDS (20 mM) was included in the cultures. Instead of this, there was reduction of AQDS with hydrogen, acetate and lactate as electron donors by both inocula (Figures 2a and 2b). The role of AQDS as terminal electron acceptor was evidenced by the AQDS reduction achieved beyond endogenous controls. Moreover the COD recovered as AH_2QDS (referring only to the hydrogen linked to the structure and corrected for the

endogenous AH_2QDS production) agreed with the amount of substrate consumed in all the cases (Table 1). The corrected AH_2QDS accounted for 56 to 65 % of the lactate consumed by both inocula and the remaining was recovered as propionate (Tables 4 and 5). Addition of BES to the AQDS-containing cultures did not affect the reduction of AQDS with these substrates (Figures 2c and 2d). Complete conversion of substrates in BES amended cultures, where methanogenesis was knocked out , was only made feasible if AQDS was added. This observation confirmed the role of AQDS as a terminal electron acceptor.

Table 3. COD balance (in mg COD l^{-1}) for the oxidation of different substrates by "Voorwetering" sediment in the absence of AQDS after 5 weeks of incubation[a]

Culture	Endogenous	Acetate	Propionate	Methane	Recovery (%)[b]
Acetate	14	98	-	215	100
Acetate-BES	14	195	78	10	92
Propionate	14	-	255	59	100
Propionate-BES	14	35	248	5	95
Methanol	14	-	-	303	98
Methanol-BES	14	115	-	32	97[c]
Hydrogen	14	-	-	249	83[d]
Hydrogen-BES	14	95	-	7	87[e]
Lactate	14	8	-	295	98[f]
Lactate-BES	14	157	115	12	93[f]

All abbreviations refer to the same terms given in Table 1. BES: 2-bromoethanesulphonic acid.
[a]All substrates at the initial concentration of 300 mg COD l^{-1}. Results are means of triplicate incubations. In all cases the standard deviation was less than 10 %.
[b]Recovery = (identified products - endogenous COD)/(input COD).
[c]Including 148 mg COD l^{-1} remaining as methanol.
[d]No hydrogen detected at the end of the experiment.
[e]Including 167 mg COD l^{-1} remaining as hydrogen.
[f]No lactate detected at the end of the experiment.

Negligible AQDS reduction (compared to the endogenous control) occurred with propionate by "Nedalco" sludge (Figures 2a and 2c). There was no methane production detected in the propionate-AQDS culture, which was found in the absence of AQDS (Table 2), and a small fraction of propionate was converted to higher VFA (25-50 mg COD l^{-1} mainly as butyrate and valerate) by "Nedalco" sludge. Furthermore, only partial reduction of AQDS with propionate by "Voorwetering" sediment was achieved (Figures 2b and 2d). The reduction of AQDS proceeded through the conversion of propionate to acetate. Acetate was then oxidized leading to further reduction of AQDS, but after the propionate concentration reached about 1.5 mM, there was no further conversion of this substrate (Figure 3). Nevertheless, the reaction was still thermodynamically favorable under the experimental conditions ($\Delta G'$ about – 130 kJ per mol at the end of the experiment). No methane production was detected in the propionate/AQDS culture by this sediment. Addition of BES in the propionate/AQDS experiments did not effect the pattern observed in the absence of this methanogenic inhibitor by both inocula (Tables 4 and 5).

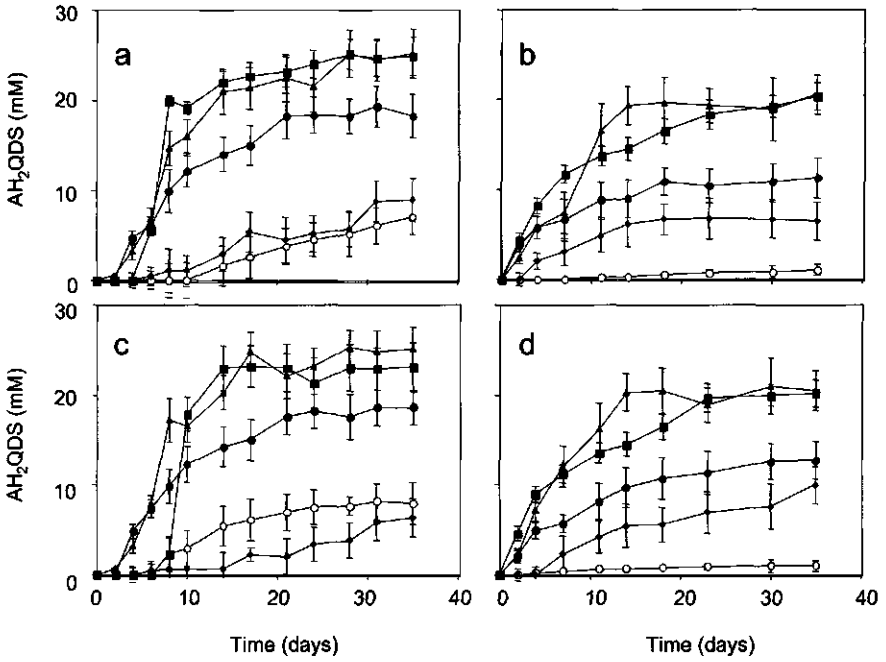


Figure 2. Reduction of anthraquinone-2,6-disulfonate (AQDS) by different consortia with different substrates. (a) Reduction of AQDS (20 mM) by "Nedalco" sludge. (b) Reduction of AQDS (20 mM) by "Voorwetering" sediment. (c) Reduction of AQDS (20 mM) by "Nedalco" sludge in the presence of BES (50 mM). (d) Reduction of AQDS (20 mM) by "Voorwetering" sediment in the presence of BES (50 mM). Measurements were conducted spectrophotometrically (450 nm) in anaerobically collected samples diluted in anaerobic bicarbonate buffer (60 mM). The results are the means of triplicate incubations and the bars indicate the standard deviation. Substrates: ■, acetate; ▲, hydrogen; ●, lactate, ◆, propionate; A, endogenous. All the substrates supplied at 300 mg COD l^{-1}. Results from repeated experiments in refilled bottles. BES: 2-bromoethanesulphonic acid.

Methanol was the only substrate that was converted to methane in the presence of the alternative electron acceptor, AQDS. In fact, after a lag phase of three weeks, methanogenesis was the preferred pathway over AQDS reduction by "Nedalco" sludge with methanol (Figure 4a). On the other hand, there was a competition between methanogenesis and acetogenesis for methanol by anaerobic "Voorwetering" sediment. Once methanol was completely depleted methanogenic activity ceased. After that time point, the consumption of the accumulated acetate led to AQDS reduction by this sediment (Figure 4b). Important fractions of COD were recovered both as methane and AH_2QDS (Table 5). On the other hand, both consortia only oxidized methanol via AQDS reduction when BES was included in the medium (Figures 4c and 4d). Methanol oxidation proceeded through the formation of acetate as an intermediate in both cases and AQDS reduction coincided with acetate accumulation (about 0.6 mM) in the cultures. These observations indicate that the reduction of AQDS was most likely caused directly by acetate. The COD recovered as AH_2QDS in the BES amended methanol-

AQDS cultures agreed with the amount of methanol consumed by both AQDS-respiring consortia (80-90 %). The remaining COD was recovered as acetate.

Table 4. COD balance (in mg COD l^{-1}) for the oxidation of different substrates by Nedalco sludge in the presence of AQDS (20 mM) after 5 weeks of incubation[a]

Culture	Endogenous	Acetate	Propionate	AH_2QDS[c]	Recovery (%)[b]
Acetate	151	109	-	402	118
Acetate-BES	151	126	-	369	111
Propionate	151	31	199	141	86[d]
Propionate-BES	151	9	213	101	78[e]
Methanol	151	42	-	71	72[f]
Methanol-BES	151	63	-	363	102[g]
Hydrogen	151	44	-	403	99[h]
Hydrogen-BES	151	50	-	404	101[h]
Lactate	98[j]	5	75	292	96[i]
Lactate-BES	98[j]	6	73	281	90[i]

All abbreviations refer to the same terms given in Table 1. BES: 2-bromoethanesulphonic acid.
[a]All substrates at the initial concentration of 300 mg COD l^{-1}. Results are means of triplicate incubations. In all cases the standard deviation was less than 10 %.
[b]Recovery = (identified products - endogenous COD)/(input COD).
[c]COD calculated only refers to the hydrogen linked to the structure.
[d]Including 24 mg COD l^{-1} as higher volatile fatty acids formed (butyrate and valerate).
[e]Including 50 mg COD l^{-1} as higher volatile fatty acids formed (butyrate and valerate).
[f]Including 240 mg COD l^{-1} as methane produced.
[g]Including 25 mg COD l^{-1} remaining as methanol.
[h]No hydrogen detected at the end of the experiment.
[i]No lactate detected at the end of the experiment.
[j]Conducted with another endogenous control at a different time compared to the other batch cultures.

Toxicity of AQDS over methanogenic activity by anaerobic granular sludge. The toxicity of AQDS over the acetoclastic metanogenic activity of "Shell" granular sludge (obtained from Moerdijk, The Netherlands) was studied. This consortium was characterized by a high methanogenic activity (0.4 g COD-CH_4 per g of VSS per day), but negligible capacity of reducing AQDS with acetate as electron donor (Table 1). The methanogenic activity of this consortium was diminished or completely abolished during the first 75 hours of incubation by the presence of AQDS in the medium (Figure 5). However, recovery of the methanogenic activity was achieved by further incubation (20 to 70 hours) in the cultures with 5, 10 and 15 mM of AQDS. Recovery of the methanogenic activity of the cultures containing 20 and 25 mM of AQDS was also achieved after 120 hours of incubation.

A toxicity effect of AQDS was also observed over methanogenic activity by "Nedalco" sludge with methanol as substrate. Methanol was completely converted to methane in the absence of AQDS after 2 weeks of incubation; whereas methane production started only after 3 weeks of incubation in the AQDS-amended (20 mM) culture with a 25 % lower methane production rate (Figure 6).

Discussion

The results presented here indicated that quinones, analogues of redox active groups in humus, could be important electron acceptors for the anaerobic biotransformation of a wide variety of substrates. Quinone respiring capacity was observed both in natural habitats, such as anaerobic sediments and soils, and in artificial habitats such as sludges from wastewater treatment plants, including aerobic treatment. These results agreed with the recovery of humic-reducing bacteria from different environments (*5*) and indicate that AQDS-respiring microorganisms are widespread in nature. This study also suggested that humic substances may play an important role in the biotransformation of organic matter during anaerobic treatment of waste streams if they are present in the system.

Table 5. COD balance (in mg COD l^{-1}) for the oxidation of different substrates by "Voorwetering" sediment in the presence of AQDS (20 mM) after 5 weeks of incubation[a]

Culture	Endogenous	Acetate	Propionate	AH_2QDS[c]	Recovery (%)[b]
Acetate	16	21	-	325	109
Acetate-BES	16	53	-	323	110
Propionate	16	153	9	119	91
Propionate-BES	16	10	167	160	109
Methanol	16	8	-	149	118[d]
Methanol-BES	16	49	-	278	105
Hydrogen	16	-	-	328	104[e]
Hydrogen-BES	16	5	-	328	105[e]
Lactate	16	6	71	181	84[f]
Lactate-BES	16	8	82	203	93[f]

All abbreviations refer to the same terms given in Table 1. BES: 2-bromoethanesulphonic acid.
[a]All substrates at the initial concentration of 300 mg COD l^{-1}. Results are means of triplicate incubations. In all cases the standard deviation was less than 10 %.
[b]Recovery=(identified products - endogenous COD)/(input COD).
[c]COD calculated only refers to the hydrogen linked to the structure.
[d]Including 210 mg COD l^{-1} as methane produced (in all other cases methane not detectable or at trace levels).
[e]No hydrogen detected at the end of the experiment.
[f]No lactate detected at the end of the experiment.

Our results suggested that hydrogen-oxidizing rather than acetate-oxidizing bacteria are the most widespread AQDS-reducing microorganisms in the consortia studied. This was evidenced by the fact that all the consortia evaluated were capable of oxidizing hydrogen coupled to complete AQDS reduction (20 mM), whereas only three of the five consortia studied achieved complete AQDS reduction when acetate was provided as electron donor after 5 weeks of incubation (Table 1). The fact that the methanogenic inhibitor, BES, did not affect the reduction of AQDS with different substrates by "Nedalco" sludge and "Voorwetering" sediment suggested that methanogenic bacteria may not be involved in the quinone respiring processes observed. Although this does not exclude the possibility that methanogens might have reduced AQDS by a pathway in which Coenzyme M reduction (BES inhibited) is not involved (*14*). Furthermore, "Shell" sludge, which showed the highest specific acetoclastic methanogenic activity of all the consortia studied, could not reduce AQDS with acetate as an electron donor. This strongly suggests that acetoclastic methanogens were not involved in quinone

respiration. Also the long lag phases observed indicate that methanogens were not reducing AQDS in the consortia evaluated. This lag phase suggested enrichment of new quinone-respiring bacteria rather than activity of the existing cells. Methanogenic archaea are expected to be very abundant in the anaerobic consortia studied based on the high methanogenic activity observed in most of them. The shorter lag phase observed in refilled bottles (Figure 2) confirmed that new populations were being enriched during these studies.

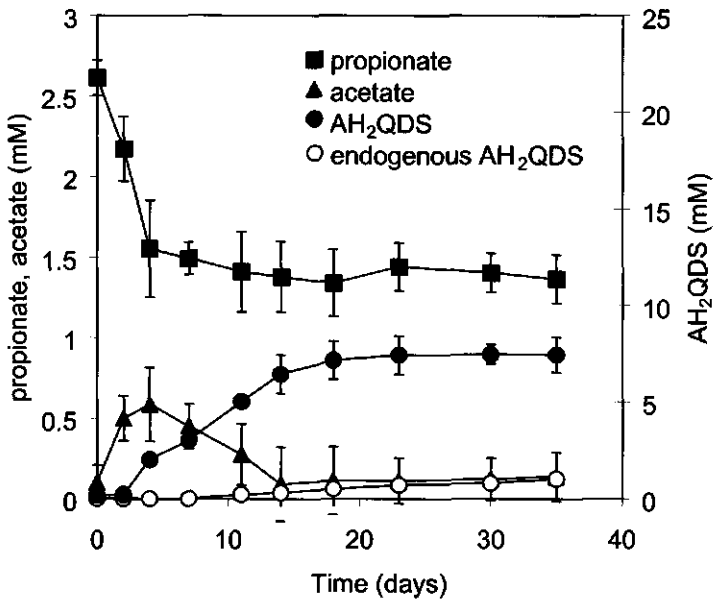


Figure 3. Reduction of anthraquinone-2,6-disulfonate (AQDS, 20 mM) coupled to the conversion of propionate to acetate by "Voorwetering" sediment. AH_2QDS measurements were conducted spectrophotometrically (450 nm) in anaerobically collected samples diluted in anaerobic bicarbonate buffer (60 mM). The results are the means of triplicate incubations and the bars indicate the standard deviation. Results from repeated experiments in refilled bottles.

Addition of AQDS (20 mM) to most cultures prevented methanogenesis. In general, quinone respiration predominated when AQDS was added. This is consistent with the fact that AQDS was found to be toxic to acetoclastic methanogenic activity in "Shell" granular sludge (Figure 5) and toxic to methylotrophic methanogenic activity in "Nedalco" sludge (Figure 6). Methanogenesis generally occurred more rapidly and with less lag time when AQDS was absent. Moreover, complete conversion of acetate to methane by anaerobic sediment obtained from Rhine river, was only possible in the absence of AQDS. There was negligible acetate conversion and methane production in the AQDS amended culture by this consortium (data not shown). Propionate/AQDS cultures also illustrate the toxic effect of AQDS, since no methane production occurred in these experiments even though there

was only minor conversion of propionate linked to AQDS reduction. Otherwise, it would have been expected that methanogens could have outcompeted the quinone-respiring bacteria for this substrate. Thus, toxicity of AQDS is an important factor for determining outcome of competition between methanogenesis and quinone respiration.

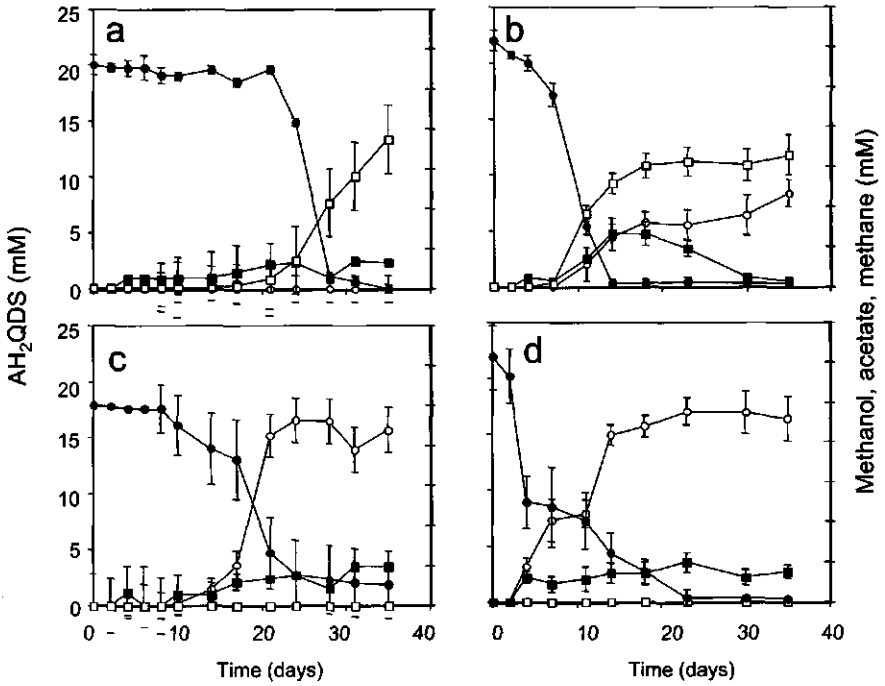


Figure 4. Conversion of methanol in the presence of anthraquinone-2,6-disulfonate (AQDS) by different consortia. (a) By "Nedalco" sludge with 20 mM of AQDS. (b) By "Voorwetering" sediment with 20 mM of AQDS. (C) By "Nedalco" sludge with 20 mM of AQDS in the presence of BES (50 mM). (d) By "Voorwetering" sediment with 20 mM of AQDS in the presence of BES (50 mM). Methane and AH_2QDS corrected for the endogenous production. AH_2QDS measurements were conducted spectrophotometrically (450 nm) in anaerobically collected samples diluted in anaerobic bicarbonate buffer (60 mM). The results are the means of triplicate incubations and the bars indicate the standard deviation. Results from repeated experiments in refilled bottles. Methanol (●); methane ( ); acetate (■); AH_2QDS (A). BES: 2-bromoethanesulphonic acid.

Table 6. Thermodynamic comparison of AQDS reduction with other microbial processes with acetate as electron donor (values calculated with data from Sober (1970) (*19*) and Thauer *et al.* (1977) (*20*))

Reaction	$\Delta G^{o'}$ (kJ per reaction)
$CH_3COO^- + 8\ Fe^{3+} + 4\ H_2O \rightarrow 8\ Fe^{2+} + 9\ H^+ + 2\ HCO_3^-$	- 808.6
$CH_3COO^- + 8/5\ NO_3^- + 3/5\ H^+ \rightarrow 2\ HCO_3^- + 4/5\ N_2 + 4/5\ H_2O$	- 791.9
$CH_3COO^- + 4\ AQDS + 4\ H_2O \rightarrow 4\ AH_2QDS + 2\ HCO_3^- + H^+$	- 73.0
$CH_3COO^- + SO_4^{2-} \rightarrow HS^- + 2\ HCO_3^-$	- 47.6
$CH_3COO^- + H_2O \rightarrow CH_4 + HCO_3^-$	- 31.0

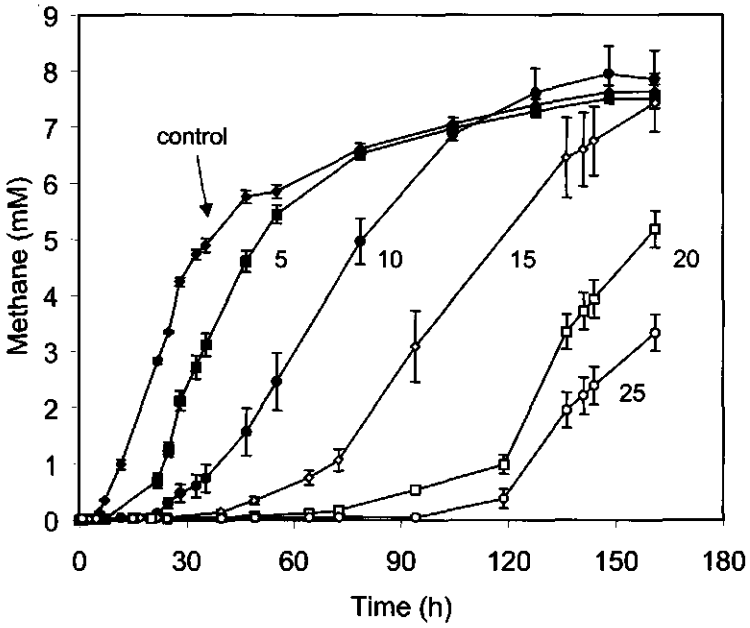


Figure 5. Toxicity effect of anthraquinone-2,6-disulfonate (AQDS) over acetoclastic methanogenic activity by "Shell" sludge. All batch experiments containing 300 mg COD-acetate l^{-1} as substrate. The numbers next to the lines indicate the concentration of AQDS in mM. The results are the means of triplicate incubations for each treatment. Bars indicate the standard deviation.

The fact that methanogenic activity was recovered by further incubation of the cultures of granular sludges exposed to AQDS suggested that the toxicity effect by this compound was reversible. AQDS (20 mM) increased the redox potential of the culture fluid to about + 130 mV, but it was decreased to about – 175 to – 250 mV depending on the extent of reduction of AQDS in the cultures. The high redox potential that prevailed at the starting time point probably interfered with biochemical processes required for methanogenesis as it is generally assumed that methane production is only possible if the redox potential is lower than – 200 to – 400 mV in the medium (*15*). Besides AQDS-respiring bacteria, methanogens in granular sludges might have mechanisms to decrease the redox potential of the medium, which allow for the methane production observed. In fact, previous experiments revealed that methanogenic bacteria, such as *Methanosarcina barkeri*, were capable of generating by themselves the redox environment, which suited the production of methane (*15*). It was shown that the capacity for decreasing the redox potential by *M. barkeri* was dependent on the amount of methanol added. This is in agreement with the fact that methanol was the unique substrate that could be converted to methane by "Nedalco" granular sludge in the presence of AQDS. The lag phase (3 weeks) observed in the methanol/AQDS culture by "Nedalco" sludge (Figure 4a) suggested that this

consortium might have also needed this period to decrease the redox potential of the medium before starting any methanogenic activity. The fact that methanol was the only substrate that could be converted to methane by "Nedalco" sludge in the presence of AQDS might be explained by biochemical aspects. Methanol bypasses most of the important biochemical steps towards formation of methyl-coenzyme M during methanogenesis compared to acetate (*16*). This hypothesis is also supported by the competition observed between methanogenesis and acetogenesis by "Voorwetering" sediment in the methanol/AQDS culture. Methanol was the only substrate that was converted to methane in the presence of AQDS, whereas the accumulated intermediate acetate, was probably consumed via quinone respiration by this consortium (Figure 4b).

The fact that reduction of AQDS by "Nedalco" and "Voorwetering" consortia in the methanol/AQDS/BES cultures, was only possible when the acetate concentration reached about 0.6 mM (Figures 4c and 4d) suggested that the reduction of the quinone moieties was not related to the direct methanol oxidation, but to the consumption of the acetate intermediate. Moreover, none of the AQDS-reducing bacteria recovered from different anaerobic sediments could use methanol as electron donor either (*5*). Thus, methanol seems not to be a suitable direct substrate for AQDS-respiring or humus-respiring microorganisms; whereas it can be directly converted to methane by methylotrophic microorganisms (*17,18*). This might have also played an important role for determining the preferred pathway during methanol depletion in our experiments because methanogenesis occurred when BES was not included in the methanol/AQDS cultures by both consortia studied even though AQDS reduction is thermodynamically more favorable than methanogenesis.

Table 7. Stoichiometry and standard free energy change ($\Delta G^{o'}$) for the reduction of AQDS with different electron donors (values calculated with data from Sober (1970) (*19*) and Thauer *et al.* (1977) (*20*))

Reaction	$\Delta G^{o'}$ (kJ per reaction)
methanol	
$CH_3OH + 3\ AQDS + 2\ H_2O \rightarrow 3\ AH_2QDS + HCO_3^- + H^+$	- 109.7
lactate	
$CH_3CHOHCOO^- + 2\ AQDS + 2\ H_2O \rightarrow CH_3COO^- + 2\ AH_2QDS + HCO_3^- + H^+$	- 93.0
acetate	
$CH_3COO^- + 4\ AQDS + 4\ H_2O \rightarrow 4\ AH_2QDS + 2\ HCO_3^- + H^+$	- 73.0
propionate	
$CH_3CH_2COO^- + 3\ AQDS + 3\ H_2O \rightarrow CH_3COO^- + 3\ AH_2QDS + HCO_3^- + H^+$	- 57.1
hydrogen	
$H_2 + AQDS \rightarrow AH_2QDS$	- 44.4

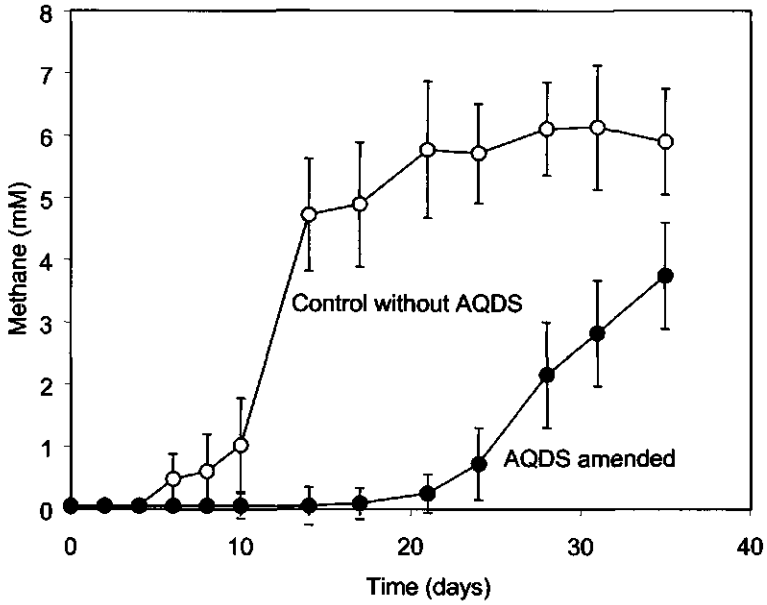


Figure 6. Toxicity effect of anthraquinone-2,6-disulfonate (AQDS, 20 mM) over methanogenic activity by "Nedalco" sludge with methanol as substrate (300 mg COD l^{-1}). The results are the means of triplicate incubations for each treatment. Bars indicate the standard deviation.

AQDS reduction is a very favorable reaction from the thermodynamic point of view. This reaction is more favorable than sulfate reduction and methanogenesis (Table 6). The thermodynamic favourability might have played an important role for the occurrence of AQDS reduction instead of methanogenesis in most of the experiments showed. However, Table 6 illustrates that iron reduction and denitrification are expected to be more favorable processes than AQDS respiration. The conversions of all the substrates tested in this study are thermodynamically feasible when AQDS is provided as terminal electron acceptor (Table 7). Conversion of propionate to acetate coupled to the reduction of AQDS was one of the most unfavorable reactions studied (Table 7). However, according to the experimental conditions applied, this reaction was thermodynamically favorable during the whole period tested by "Nedalco" sludge and "Voorwetering" sediment ($\Delta G'$ between – 155 and – 70 kJ per mol of propionate in both cases). Nevertheless, there was no reduction of AQDS after the concentration of propionate decreased to 1.5 mM in the cultures, which agreed with the point in which no further conversion of propionate to acetate was observed (Figure 3). The accumulation of higher VFA in the propionate/AQDS cultures by "Nedalco" sludge (Table 4) suggested that the transfer of electrons was truncated during these experiments due to unknown reasons.

As humus is the most abundant organic fraction in the biosphere, it may contribute to important carbon cycling process by serving as a terminal electron acceptor for the anaerobic microbial oxidation of a wide variety of ecologically important substrates. This is also supported by the fact that reduction

of AQDS by "Nedalco" and "Shell" sludges could be coupled to the oxidation of *p*-cresol (data not shown). Moreover, the fact that quinone-respiring capacity was found in a wide variety of environments, including contaminated anaerobic sites, suggested that there is an enormous potential for using humic substances to clean up anaerobic polluted sediments and aquifers. Humic substances do not necessarily have to be present in abundant supply as they can be recycled by chemical reoxidation of hydroquinones in humus with metal oxides naturally present in many anaerobic sites (*2,8*). Moreover, humic substances can also be recycled microbially, as hydroquinones can be potential electron donors for anaerobic reduction of nitrate (*21*).

Acknowledgements

This research was financially supported by The Council of Science and Technology of Mexico (CONACyT).

References

1. Stevenson, F.J. (1994) Humus chemistry: Genesis, Composition, Reactions, 2nd Edn., 496 pp. John Wiley and Sons, Inc. New York.
2. Lovley, D.R., Coates, J.D., Blunt-Harris, E.L., Phillips E.J.P. and Woodward, J.C. (1996) Humic substances as electron acceptors for microbial respiration. *Nature* **382**, 445-448.
3. Bradley, P.M., Chapelle, F.H. and Lovley, D.R. (1998) Humic acids as electron acceptors for anaerobic microbial oxidation of vinyl chloride and dichloroethene. *Appl. Environ. Microbiol.* **64**, 3102-3105.
4. Scott, D.T., McKnight, D.M., Blunt-Harris, E.L., Kolesar, S.E. and Lovley, D.R. (1998) Quinone moieties act as electron acceptors in the reduction of humic substances by humics-reducing microorganisms. *Environ. Sci. Technol.* **32**, 2984-2989.
5. Coates, J.D., Ellis, D.J., Roden, E., Gaw, K., Blunt-Harris, E.L. and Lovley, D.R. (1998) Recovery of humics-reducing bacteria from a diversity of sedimentary environments. *Appl. Environ. Microbiol.* **64**, 1504-1509.
6. Francis, C.A., Obraztsova, A.Y. and Tebo, B.M. (2000) Dissimilatory metal reduction by the facultative anaerobe *Pantoea agglomerans* SP1. *Appl. Environ. Microbiol.* **66**, 543-548.
7. Fredrickson, J.K., Kostandarithes, H.M., Li, S.W., Plymale, A.E. and Daly, M.J. (2000) Reduction of Fe(III), Cr(VI), U(VI), and Tc(VII) by *Deinococcus radiodurans* R1. *Appl. Environ. Microbiol.* **66**, 2006-2011.
8. Stone, A.T. and Morgan J.J. (1984) reduction of manganese(III) and manganese(IV) oxides by organics. 1. Reaction with hydroquinone. *Environ. Sci. Technol.* **18**, 450-456.

9. Zachara, J.M., Fredrickson, J.K., Li S.M., Kennedy, D.W., Smith, S.C. and Gassman, P.L. (1998). Bacterial reduction of crystalline Fe^{3+} oxides in single phase suspensions and subsurface materials. *Am. Mineralogist.* **83**, 1426-1443.

10. Lovley, D.R., Woodward, J.C. and Chapelle, F.H. (1996) Rapid anaerobic benzene oxidation with a variety of chelated Fe(III) forms. *Appl. Environ. Microbiol.* **62**, 288-291.

11. Anderson, R.T. and Lovley, D.R. (1999) Naphthalene and benzene degradation under Fe(III)-reducing conditions in petroleum-contaminated aquifers. *Bioremediation J.* **3**, 121-135.

12. Benz, M., Schink, B. and Brune, A. (1998) Humic acid reduction by *Propionibacterium freudenreichii* and other fermenting bacteria. *Appl. Environ. Microbiol.* **64**, 4507-4512.

13. Zehnder, A.J.B. and Wuhrmann, K. (1976) Titanium(III) citrate as a nontoxic oxidation-reduction buffering system for the culture of obligate anaerobes. *Science* **194**, 1165-1166.

14. Oremland, R.S. and Capone, D.G. (1988) Use of "specific" inhibitors in biogeochemistry and microbial ecology. In: Advances in microbial ecology (Marshall, K.C. Ed.), pp. 285-383. Plenum Press, New York.

15. Fetzer, S. and Conrad R. (1993) Effect of redox potential on methanogenesis by *Methanosarcina barkeri*. *Arch. Microbiol.* **160**, 108-113.

16. Thauer, R.K. (1998) Biochemistry of methanogenesis: a tribute to Marjory Stephenson. *Microbiology* **144**, 2377-2406.

17. Stadtman, T.C. and Barker, H.A. (1951) Studies on the methane fermentation.IX. The origin of methane in the acetate and methanol fermentations by *Methanosarcina*. *J. Bacteriol.* **16**, 81-86.

18. Vogels, G.D., Keltjens, J.T. and van der Drift, C. (1988) Biochemistry of methane production. P. 707-770. In: A.J.B. Zehnder (ed.). Biology of anaerobic bacteria. John Wiley, New York, USA.

19. Sober, H.A. (1970) Handbook of biochemistry: selected data for molecular biology. The Chemical Rubber Co., Cleveland, Ohio.

20. Thauer, R.K., Jungermann, K. and Decker, K. (1977) Energy conservation in chemotrophic anaerobic bacteria. *Bacteriol. Rev.* **41**, 100-180.

21. Lovley, D.R., Fraga, J.L., Coates, J.D. and Blunt-Harris, E.L. (1999) Humics as an electron donor for anaerobic respiration. *Environ. Microbiol.* **1**, 89-98.

3

Reduction of humic substances by halorespiring, sulfate-reducing and methanogenic microorganisms*

*Cervantes F. J., F. A. M. de Bok, T. Duong-Dac, A. J. M. Stams, G. Lettinga and J. A. Field. *Environ. Microbiol. Accepted for publication.*

Abstract

Physiologically distinct anaerobic microorganisms were explored for their ability to oxidize different substrates with humic acids or the humic analogue, anthraquinone-2,6-disulfonate (AQDS), as a terminal electron acceptor. Most of the microorganisms evaluated including e.g. the halorespiring-bacterium, *Desulfitobacterium* PCE1; the sulfate-reducing bacterium, *Desulfovibrio* G11; and the methanogenic archaeon, *Methanospirillum hungatei* JF1, could oxidize hydrogen linked to the reduction of humic acids or AQDS. *Desulfitobacterium dehalogenans* and *Desulfitobacterium* PCE1 could also convert lactate to acetate linked to the reduction of humic substances. Humus served as a terminal electron acceptor supporting growth of *Desulfitobacterium* species, which may explain the recovery of these microorganisms from organic rich environments where the presence of chlorinated pollutants or sulfite is not expected. The results suggest that the ubiquity of humus reduction found in many different environments may be due to the increasing number of anaerobic microorganisms, which are known to be able to reduce humic substances.

Introduction

Humus is the most abundant organic fraction accumulating in terrestrial and aquatic environments. Although humus itself is inert as a substrate, it has been reported as a potential electron acceptor supporting the anaerobic microbial oxidation of other substrates, such as acetate, and hydrogen (*3, 5, 15*). The microbial reduction of humus is ecologically relevant because it may serve as an important mechanism for organic substrate oxidation in many anaerobic environments, due to its abundance, and because of the high reactivity of humus with metal oxides. Microbially reduced humus can abiotically transfer electrons to Fe(III) and Mn(IV) oxides (*15, 22*) allowing for its regeneration to the oxidized form. Thus, even sub-stoichiometric concentrations of humus could mediate anaerobic substrate oxidation and reduction of metal oxides in anaerobic environments.

Quinones are structural units, which are very abundant in humus (*21*). Electron spin resonance measurements revealed direct evidence that quinone moieties are the actual functional groups accepting electrons during the microbial reduction of humus (*19*). 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 humus and the quinone model compound, anthraquinone-2,6-disulfonate (AQDS). Mutants of this organism, lacking the ability to synthesize menaquinone, were unable to reduce AQDS and humus (*17*). Thus, quinones are good analogues for the function of humus as a terminal electron acceptor. Humic acid is the fraction of humus generally containing the highest concentration of quinones (*21*), and it has also been used in different studies for exploring the microbial reduction of humus (*1, 2, 15*).

Most known quinone-reducing microorganisms are Fe(III)-reducing bacteria of the family *Geobacteraceae* capable of coupling the respiratory process to growth (*4, 5, 15*). Other Fe(III)-reducers, such as *Pantoea agglomerans* SP1(*6*) and *Thermoanaerobacter siderophilus* sp. nov. (*20*), also coupled the reduction of AQDS to growth. Besides, there are many quinone-reducing microorganisms in which microbial growth has not been confirmed. The studies include other Fe(III)-reducing bacteria from the genera *Shewanella, Desulfitobacterium, Desulfuromonas, Geospirillum, Wolinella,* and *Geothrix* (*16*); the uranium-reducing bacterium, *Deinococcus radiodurans* (*7*); and fermentative bacteria, such as *Propionibacterium freudenreichii* (*1*). Quinone reduction has also been found in thermophilic and hyperthermophilic microorganisms including Fe(III)-reducers (e.g. *Pyrobaculum islandicum, Pyrodictium abyssi, Thermococcus celer*) and methanogenic archaea (e.g. *Methanopyrus kandleri, Methanobacterium thermoautotrophicum*) in which no growth was reported (*13*). In the present study, phylogenetically distinct microorganisms, which were previously not known to have the capacity to reduce quinones, were explored for their ability to oxidize simple substrates with humic acids or AQDS as a terminal electron acceptor.

Materials and methods

Source of microorganisms. *Syntrophobacter fumaroxidans* (DSM 10017), *Desulfovibrio* G11 (DSM 7057), *Desulfitobacterium dehalogenans* (DSM 9161), *Desulfitobacterium* PCE1 (DSM 10344), *Desulforhabdus amnigenus* (DSM 10338), and *Methanospirillum hungatei* JF1 (DSM 864) were obtained from the stock culture collection of the laboratory of microbiology of the Wageningen University.

Media preparation. Bicarbonate buffered mineral basal medium (pH 7.2) was prepared as previously described (*3*). For the present study, the concentrations of NH_4Cl and K_2HPO_4 were modified to 0.1 and 0.05 g per liter, respectively, and yeast extract (0.2 g per liter) was also included. Amorphous ferric oxide was prepared as previously described (*10*). The metal oxide suspensions were washed 3 times by centrifugation and resuspended in distilled water. Finally, the metal oxides were suspended in basal medium to obtain a final concentration of 10 mM of Fe(III). Bicarbonate concentration was set at 2.5 g per liter when ferric oxide was provided as a terminal electron acceptor and HEPES (*N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid, 50 mM, pH 7.2) was included as a buffer. When humus was studied as a potential electron acceptor, humic acids (Janssen Chimica Belgium, 20 g per liter) were suspended in bicarbonate buffered mineral basal medium. All the media were flushed with N_2/CO_2 (80:20) before use.

Microbial incubations. Incubations were performed in 117-ml bottles sealed with butyl rubber stoppers and aluminum caps under a N_2/CO_2 (80/20) atmosphere at 37 °C in the dark. The basal medium was supplied with AQDS (5 mM), humic acids (Janssen Chimica Belgium, 20 g per liter) or amorphous ferric oxyhydroxide (FeOOH, 10 mM) as a terminal electron acceptor. Acetate (2 mM) or

lactate (5 mM) was provided as an electron donor from stock solutions. When hydrogen was included as an electron donor, a headspace of H_2/CO_2 (80/20, final pressure 1.7 bars) was used. Depletion of the substrate, reduction of the corresponding electron acceptor, and cell numbers were followed in time as described below. Protein concentration was also determined at the end of the incubations as described below. Controls in which no external electron donor was provided were also included to correct for the endogenous reduction of the electron acceptors. Sterile controls in which no inoculation took place were also included. All the experiments were applied in triplicate incubations for all the conditions studied.

Analytical techniques. Fe(II) production was determined by the ferrozine technique (*14*). Electrons transferred to humic substances were quantified by reducing Fe(III)-citrate with filtered culture supernatants as described previously (*15*). AQDS reduction was determined by following the formation of AH_2QDS spectrophotometrically at 450 nm as described before (*3*). All samples (1 ml) were collected under axenic conditions by conventional sterile handling techniques, and immediately transferred into anaerobic glass reaction vials (10 ml). All measurements were carried out in an anaerobic chamber containing N_2/H_2 (95/5).

The concentration of formate, acetate, lactate, hydrogen, and methane was determined by gas chromatographic methods previously described (*3*). Cell numbers were determined by phase-contrast microscopy using a Bürker-Türk counting chamber at 400 x magnification. To determine the protein content 30 ml of each culture was centrifuged at 17,500 rpm for 10 min at 4 °C. Pellets were washed with 2 ml Tris-HCl pH 8 and then centrifuged for 10 min at 14,000 rpm. Pellets were then dissolved in 200 µl of NaOH (1 M). Samples were then boiled for 10 min in closed vials and after that samples were taken for protein determination by a Lowry-based Bio-Rad DC assay.

Results

Reduction of humic substances by different anaerobic microorganisms. The capacity for different microbial groups generally found in anaerobic environments to use humic substances as a terminal electron acceptor was first evaluated with *Desulfitobacterium dehalogenans*, a previously reported halorespiring and sulfite-reducing microorganism isolated from a methanogenic lake sediment (*25*). *D. dehalogenans* reduced humic acids when either lactate or hydrogen was provided as an electron donor (Figure 1). Endogenous substrates (introduced together with inoculum) also led to a partial reduction of humus by *D. dehalogenans*. No reduction of humic acids was observed in the sterile controls lacking cells. The reduction of humic acids in the active cultures coincided with growth of *D. dehalogenans*, which was verified by increased cell numbers and protein content after incubation (data not shown). *D. dehalogenans* was also able to reduce AQDS with lactate as an electron donor (Figure 2A) and the process was also linked to an increase in cell numbers (Figure 2B). The reduction of AQDS paralleled the conversion of lactate to acetate by this organism with a ratio of reduced AQDS

to lactate converted of 1.35±0.05, which is 33 % lower compared to the stoichiometric value (2:1) according to the following reaction:

Lactate + 2 AQDS + 2 H_2O → Acetate + 2 AH_2QDS + HCO_3^- + H^+

The lower than expected ratio may be due to the capacity of *D. dehalogenans* to produce acetate from the reduction of CO_2 (*27*). Formate and other fatty acids were not detected in the cultures. No conversion of lactate was observed in cell suspensions without AQDS (data not shown).

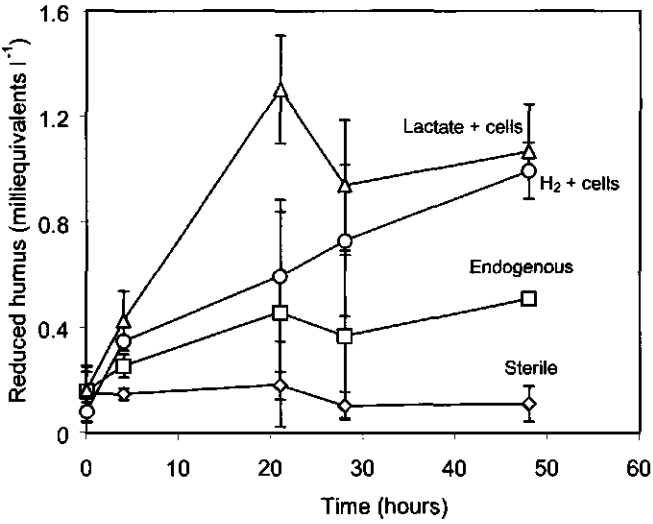


Figure 1. Reduction of humic acids (20 g per liter) by *Desulfitobacterium dehalogenans* with different substrates. The results are the means of triplicate incubations and the error bars indicate the standard deviation.

As several evidences have documented the implication of quinones during the microbial reduction of humic substances (*17, 19*) and that microorganisms that had been recovered as AQDS-reducers showed also the ability to reduce humus (*5*), AQDS was used as a model compound in further experiments with different microorganisms. Another halorespiring bacterium, *Desulfitobacterium* PCE1, isolated from a contaminated soil (*8*), readily reduced AQDS when lactate or hydrogen was provided as an electron donor and in both cases growth was observed (data not shown).

AQDS reduction was also found in the sulfate-reducing bacterium *Desulfovibrio* G11 when hydrogen was added as an electron donor (Figure 3), but growth could not reproducibly be established in this microorganism. No reduction of AQDS occurred in the absence of hydrogen or in the sterile controls lacking cells. In the active cultures, AQDS reduction approximately agreed with the stoichiometric consumption of hydrogen (ratio AQDS reduced to hydrogen consumed of 0.8±0.1). *Desulforabdus amnigenus*, another sulfate-reducer, did not reduce AQDS under the same conditions. Furthermore, *Syntrophobacter fumaroxidans*, a sulfate-reducing bacterium, which also has the capacity of

converting propionate to hydrogen and acetate in co-culture with hydrogen-oxidizing organisms (*9*), could not reduce AQDS when propionate or hydrogen was added as an electron donor.

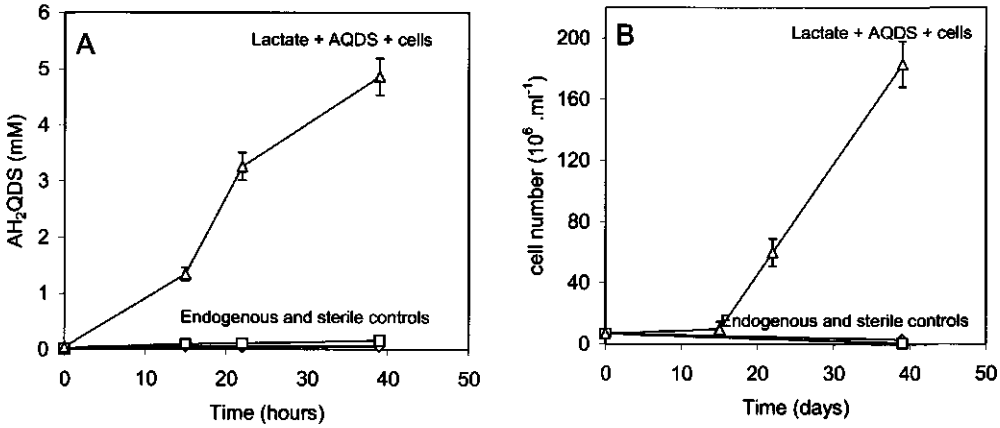


Figure 2. Reduction of AQDS (A) coupled to growth (B) by *Desulfitobacterium dehalogenans* with lactate as an electron donor. The results are the means of triplicate incubations and the error bars indicate the standard deviation.

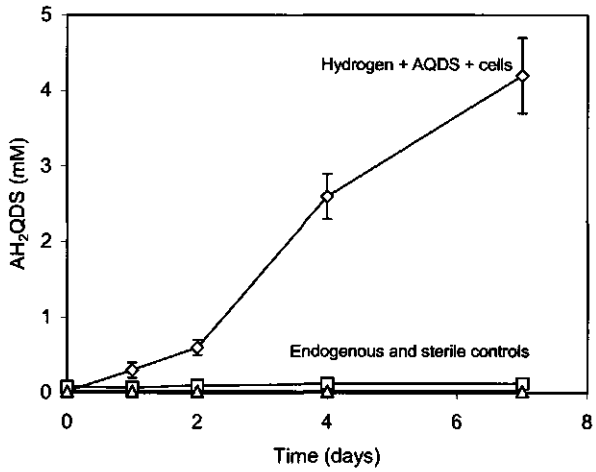


Figure 3. Reduction of AQDS by *Desulfovibrio* G11 with hydrogen as an electron donor. The results are means of triplicate incubations and the bars indicate the standard deviation.

Methanospirillum hungatei JF1, a hydrogenotrophic methanogen, could also stoichiometrically oxidize hydrogen linked to the reduction of AQDS (Figure 4), but microbial growth could not be confirmed by direct microscopic counting or protein determinations. No reduction of AQDS occurred in the absence of hydrogen or in the sterile controls. No methanogenic activity was detected by *M. hungatei* JF1 in the presence of AQDS.

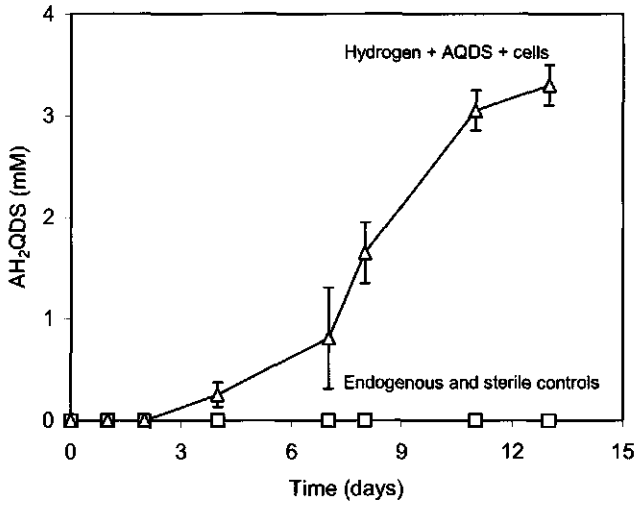


Figure 4. Reduction of AQDS by *Methanospirillum hungatei* JF1 with hydrogen as an electron donor. The results are means of triplicate incubations and the bars indicate the standard deviation.

Reduction of amorphous ferric oxide via quinone reduction by *Desulfitobacterium dehalogenans* and *Desulfovibrio* G11. The capacity of some of the microorganisms that were able to reduce humic substances to channel electrons from anaerobic oxidations to amorphous ferric oxyhydroxide via quinone reduction was also explored. Cell suspensions of *D. dehalogenans* readily transferred electrons to goethite when AQDS (500 µM) was included in the medium, provided with lactate as an electron donor (Figure 5). There was no reduction of the metal oxide in the absence of AQDS or in sterile controls containing both goethite and AQDS. Negligible production of Fe(II) was observed when no external electron donor was included. *D. dehalogenans* was also able to transfer electrons to goethite, via AQDS, when hydrogen was added as an electron donor (data not shown).

Addition of AQDS at the same level also enhanced the reduction of goethite by cell suspensions of *Desulfovibrio* G11 when hydrogen was provided as an electron donor, which reached about 5 mM of Fe(II) produced after 7 days of incubation. Meanwhile, only about 1.5 mM of Fe(II) was produced in the absence of AQDS during the same incubation period. No reduction of goethite occurred in the endogenous and sterile controls.

Discussion

In the present study physiologically different anaerobic microorganisms were explored for their capacity to oxidize different substrates with AQDS or humic acids as a terminal electron acceptor. The results give further evidence that reduction of humic substances is a physiological property that can be found in a wide variety of phylogenetically distinct microorganisms including halorespiring bacteria,

e.g. *Desulfitobacterium* species, the sulfate-reducing bacterium, *Desulfovibrio* G11, and the methanogenic archaeon, *Methanospirillum hungatei* JF1. The present study constitutes the first report indicating that halorespiring-microorganisms can couple the oxidation of different substrates to the reduction of humic acids and AQDS, and the respiratory process was shown to support microbial growth in the two *Desulfitobacterium* species evaluated. Our results also report for the first time the reduction of quinones by mesophilic methanogenic archaea. Most humus- or quinone-reducing microorganisms previously reported are Fe(III)-reducing bacteria of the family *Geobacteraceae* (*4, 5, 15*). Thermophilic and hyperthermophilic methanogenic archaea (*13*), as well as mesophilic fermentative bacteria (*1*), were also previously reported as humus-reducing organisms. Moreover, a qualitative study indicated that *Desulfitobacterium dehalogenans* could also reduce AQDS (*16*), but the authors did not investigate whether cell growth by this organism was linked to AQDS reduction. More recently, cell suspensions of *Desulfovibrio vulgaris* were shown to completely oxidized hydrogen linked to the reduction of 2-methyl-1,4-naphthoquinone (vitamin K3), which was coupled to an electrode to generate current (*23*). A periplasmic hydrogenase originated from *Desulfovibrio vulgaris* was shown to reduce vitamin K3, 2,6-dimethyl-1,4-benzoquinone, 1,4-naphthoquinone and anthraquinone-2-sulfonate. Thus, it is plausible that this microorganism is also able to reduce quinones in humus coupled to the oxidation of hydrogen.

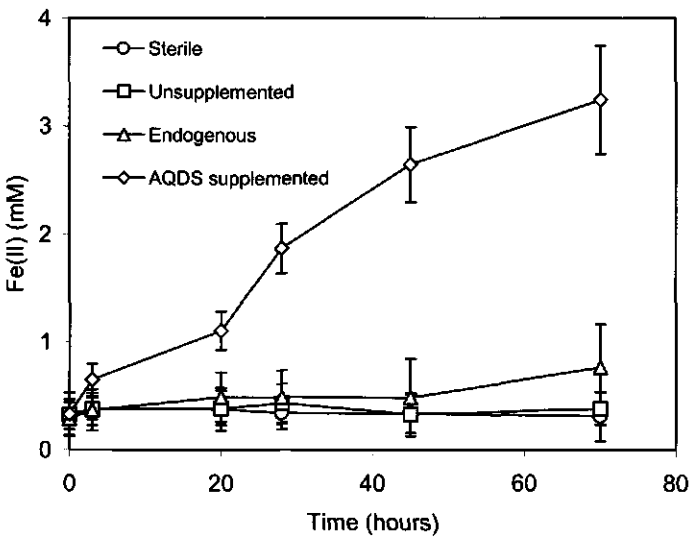


Figure 5. Reduction of ferric oxyhydroxide (10 mM) by *Desulfitobacterium dehalogenans* in AQDS (500 µM) supplemented medium with lactate (5 mM) as electron donor. The unsupplemented control conducted in the absence of AQDS. The results are means of triplicate incubations and the bars indicate the standard deviation.

The finding that *Desulfitobacterium* species were able to grow with either lactate or hydrogen when AQDS or humic acids were provided as a sole terminal electron acceptor, demonstrates that these halorespiring microorganisms coupled the electron transfer from the anaerobic oxidation of lactate and hydrogen to humus-respiration with ATP synthesis. The role of humic substances as a terminal electron acceptor was corroborated by the absence of substrate oxidation when AQDS and humus were omitted in the bioassays. The fact that humus can function as a growth supporting terminal electron acceptor for halorespiring microorganisms indicates that such organisms can be expected in organic rich pristine environments never exposed to halogenated pollutants. In fact, several *Desulfitobacterium* species have been isolated or detected by polymerase chain reaction (PCR) amplifications in sites rich in organic matter, such as forest soil, swamps, and compost, in which the presence of chlorinated pollutants and sulfite is not expected (*11, 18*).

All the microorganisms, which showed the capacity for reducing AQDS or humus in the present study, could use hydrogen as an electron donor and none of them was able to oxidize acetate via quinone reduction. The results agree with previous experiments indicating that hydrogen-oxidizing rather than acetate-oxidizing are the most widespread quinone-reducing microorganisms in nature (*3*). The results also suggest that acetate-linked humus reduction is rather associated with Fe(III)-reducing microorganisms (*4, 5, 15*). Since hydrogen is an important intermediate in the anaerobic biodegradation of organic matter in natural environments, hydrogen-oxidizing humus-reducing microorganisms may significantly contribute to anaerobic bioconversions particularly in organic rich sites where humic substances could serve as a potential electron acceptor.

The reduction of AQDS by *Desulfovibrio* G11and *Methanospirillum hungatei* JF1 could not accurately be linked to growth. Nevertheless, the lack of coupling between quinone reduction and microbial growth does not dismiss the ecological impact that these microorganisms may have in different environments because they could oxidize hydrogen, an important interspecies substrate, by co-metabolically reducing quinones in humus. Different levels of inoculation enhanced the reduction AQDS by *Desulfovibrio* G11(data not shown) suggesting that this reducing process may be related to a fortuitous enzymatic reaction developed by these microorganisms. Further experiments revealed the capacity of a cell extract obtained from *Syntrophobacter fumaroxidans* to reduce AQDS with different electron donors including hydrogen, formate and carbon monoxide (data not shown). This microorganism was unable to reduce AQDS when cells suspensions were incubated with hydrogen or propionate as an electron donor, suggesting that this strain possesses an electron carrier capable to reduce AQDS that was not excreted in the culture in the experiments conducted with entire cells. Therefore, there may be many different microorganisms capable of producing reductants of the proper redox potential to reduce quinones in humus, but not all may have the proper carrier to transfer the electrons to the final electron acceptor.

Recent biochemical experiments indicated that menaquinone, a common quinone structure found in the respiratory chain of many anaerobic bacteria, was involved in the reduction of AQDS and humus by *Shewanella putrefaciens* (*17*). Menaquinone is also present in the respiratory chain of some of the microorganisms (e.g. *Desulfitobacterium dehalogenans* (*26*)), which showed humus reduction in the present study. However, given the broad diversity of quinone-reducing microorganisms, which include methanogenic organisms lacking menaquinone, it is expected that the electron transport observed in humus reduction may include different electron carriers depending on the microorganism involved.

In the present study, most experiments were conducted with AQDS as a model compound at 5 mM to allow microbial growth to reach quantitative levels. The relatively high quinone concentration employed may not represent real concentrations found in aquatic environments. The concentration of humic substances in water bodies rarely exceeds 5 mg per litter as dissolved organic carbon (DOC) and the quinone content of the different humic fractions is generally within the range of 100-400 mmol of DOC per gram of humus (*12, 21, 24*). Thus, the quinone concentration for most aquatic environments may be within the range between 0.5 and 2 mmol of DOC (as C=O) per litter. Nonetheless, microbial growth linked to the reduction of humic acids was also observed in humic acid suspensions (20 g per litter). The microbial reduction of suspended humic substances suggests that quinones do not necessarily have to be dissolved to serve as a potential electron acceptor in humus. Other studies have also documented the microbial reduction of suspended humic acids coupled to the anaerobic oxidation of different organic compounds, including the priority pollutant, toluene (*1, 2*).

The fact that hydroquinones in humus are readily oxidized by metal oxides (e.g. Fe(III) and Mn(IV) oxides), which are very abundant in many sedimentary environments, implies that the reduction of these electron acceptors is not exclusively related to metal-reducing microorganisms, but also to all other microorganisms capable to transfer electrons to humic substances. Fermentative bacteria, e.g. *Propionibacterium freudenreichii*, were previously reported to channel electrons from anaerobic oxidations via humic acids towards Fe(III) reduction (*1*). Although it is still uncertain whether the reduction of Fe(III) in sedimentary sites proceeds directly by Fe(III)-reducers, or indirectly via quinone reduction by humus-reducers (*5*), the present study and previous results obtained with different microorganisms illustrates the possibility that many phylogenetically distinct types of organisms may contribute to the reduction of metal oxides via humus reduction. The estimated concentration of quinones that prevail in most aquatic environments (see above) may be sufficient to support the anaerobic oxidation of different substrates in oligotrophic sites where Fe(III) and Mn(IV) are very abundant. Indeed, AQDS supplied at 500 μM supported the anaerobic oxidation of lactate and hydrogen linked to the reduction of goethite by *Desulfitobacterium dehalogenans*. Moreover, there are some other examples in which the reduction of more crystalline ferric oxides was stimulated via quinone reduction when AQDS was added even at lower concentrations (*13, 28*).

Acknowledgements

This research was financially supported by The Council of Science and Technology of Mexico (CONACyT).

References

1. Benz, M., Schink, B., and Brune, A. (1998) Humic acid reduction by *Propionibacterium freudenreichii* and other fermentative bacteria. Appl. Environ. Microbiol. **64**:4507-4512.
2. Cervantes, F. J., Dijksma, W., Duong-Dac, T., Ivanova, A., Lettinga, G. and Field, J. A. (2001) Anaerobic mineralization of toluene by enriched sediments with quinones and humus as terminal electron acceptors. Appl. Environ. Microbiol. **67**:4471-4478.
3. Cervantes, F. J., van der Velde, S., Lettinga, G., and Field, J. A. (2000) Competition between methanogenesis and quinone respiration for ecologically important substrates in anaerobic consortia. FEMS Microbiol. Ecol. **34**:161-171.
4. Coates, J. D., Bhupathiraju, V. K., Achenbach, L. A., Mclnerney, M. J., and Lovley, D. R. (2001) *Geobacter hydrogenophilus, Geobacter chapellei* and *Geobacter grbicie*, three new, strictly anaerobic, dissimilatory Fe(III)-reducers. Int. J. Syst. Evol. Microbiol. **51**: 581-588.
5. Coates, J. D., Ellis, D. J., Roden, E., Gaw, K., Blunt-Harris, E. L., and Lovley, D. R. (1998) Recovery of humics-reducing bacteria from a diversity of sedimentary environments. Appl. Environ. Microbiol. **64**:1504-1509.
6. Francis, C. A., Obraztsova, A. Y., and Tebo, B. M. (2000) Dissimilatory metal reduction by the facultative anaerobe *Pantoea agglomerans* SP1. Appl. Environ. Microbiol. **66**:543-548.
7. Fredrickson, J. K., Kostandarithes, H. M., Li, S. W., Plymale, A. E., and Daly, M. J. (2000) Reduction of Fe(III), Cr(VI), U(VI), and Tc(VII) by *Deinococcus radiodurans* R1. Appl. Environ. Microbiol. **66**:2006-2011.
8. Gerritse, J., Renard, V., Pedro-Gomes, T. M., Lawson, P. A., Collins, M. D., and Gottschal, J. C. (1996) *Desulfitobacterium* sp. strain PCE1, an anaerobic bacterium that can grow by reductive dechlorination of tetrachloroethene or ortho-chlorinated phenols. Arch. Microbiol. **165**:132-140.

9. Harmsen, H. J. M., Kengen, K. M. P., Akkermans, A. D. L., and Stams, A. J. M. (1995) Phylogenetic analysis of two syntrophic propionate-oxidizing bacteria in enrichments cultures. Syst. Appl. Microbiol. **18**:67-73.

10. Kostka, J. and Nealson, K. H. (1998) Isolation, cultivation and characterization of iron- and manganese-reducing bacteria, In *Techniques in microbial ecology*. Burlage, R. S., Atlas, R., Stahl, D., Geesey, G., and Sayler, G. (eds). New York: Oxford University Press, pp. 58-78.

11. Lanthier, M., Villemur, R., Lépine, F., Bisaillon, J., and Beaudet, R. (2001) Geographic distribution of *Desulfitobacterium frappieri* PCP-1 and *Desulfitobacterium* spp. in soils from the province of Quebec, Canada. FEMS Microbiol. Ecol. **36**:185-191.

12. Leenheer, J. A. (1981) Comprehensive approach to preparative isolation and fractionation of dissolved organic carbon from natural waters and wastewaters. Environ. Sci. Technol. **15**:578-587.

13. Lovley D. R., Kashefi, K., Vargas, M., Tor, J. M., and Blunt-Harris, E. L. (2000) Reduction of humic substances and Fe(III) by hyperthermophilic microorganisms. Chem. Geol. **169**:289-298.

14. Lovley, D. R., and Phillips, E. J. P. (1986) Availability of ferric iron for microbial reduction in bottom sediments of the fresh-water tidal Potomac River. Appl. Environ. Microbiol. **52**:751-757.

15. Lovley, D. R., Coates, J. D., Blunt-Harris, E. L., Phillips, E. J. P., and Woodward, J. C. (1996) Humic substances as electron acceptors for microbial respiration. Nature **382**:445-448.

16. Lovley, D. R., Fraga, J. L., Blunt-Harris, E. L., Hayes, L. A., Phillips, E. J. P., and Coates, J. D. (1998) Humic substances as a mediator for microbially catalyzed metal reduction. Acta Hydrochim. Hydrobiol. **26**:152-157.

17. Newman, D. K., and Kolter, R. (2000) A role for excreted quinones in extracellular electron transfer. Nature **405**:94-97.

18. Sanford, R. A., Cole, J. R., Löffler, F. E., and Tiedje, J. M. (1996) Characterization of *Desulfitobacterium chlororespirans* sp. nov., which grows by coupling the oxidation of lactate to the reductive dechlorination of 3-chloro-4-hydroxybenzoate. Appl. Environ. Microbiol. **62**:3800-3808.

19. Scott, D. T., McKnight, D. M., Blunt-Harris, E. L., Kolesar, S. E., and Lovley, D. R. (1998) Quinone moieties act as electron acceptors in the reduction of humic substances by humics-reducing microorganisms. Environ. Sci. Technol. **32**:2984-2989.

20. Slobodkin, A. I., Tourova, T. P., Kuznetsov, B. B., Kostrikina, N. A., Chernyh, N. A., and Bonch-Osmolovskaya, E. A. (1999) *Thermoanaerobacter siderophilus* sp. nov., a novel dissimilatory Fe(III)-reducing, anaerobic, thermophilic bacterium. Int. J. Syst. Bacteriol. **49**:1471-1478.

21. Stevenson, F. J. (1994) Humus chemistry: genesis, composition, reactions. New York: Wiley.

22. Stone, A. T., and Morgan, J. J. (1984) Reduction of manganese(III) and manganese(IV) oxides by organics. 1. Reaction with hydroquinone. Environ. Sci. Technol. **18**:450-456.

23. Tatsumi, H., Kano, K., and Ikeda, T. (2000) Kinetic analysis of fast hydrogenase reaction of *Desulfovibrio vulgaris* cells in the presence of exogenous electron acceptors. J. Phys. Chem. **104**:12079-12083.

24. Thurman, E. M. and Malcolm, R. L. (1981) Preparative isolation of aquatic humic substances. Environ. Sci. Technol. **15**:463-466.

25. Utkin, I., Woese, C., and Wiegel, J. (1994) Isolation and characterization of *Desulfitobacterium dehalogenans* gen. Nov., sp. nov., an anaerobic bacterium which reductively dechlorinates chlorophenolic compounds. Int. J. Syst. Bacteriol. **44**:612-619.

26. Van de Pas, B. A. (2000) Biochemistry and physiology of halorespiration by *Desulfitobacterium dehalogenans*. PhD thesis. Wageningen University, Wageningen, The Netherlands.

27. Van de Pas, B. A., Jansen, S., Dijkema, C., Schraa, G., de Vos, W. M., and Stams, A. J. M. (2001) Energy yield of respiration on chloroaromatic compounds in *Desulfitobacterium dehalogenans*. Appl. Environ. Microbiol. **67**:3958-3963.

28. Zachara, J.M., Fredrickson, J. K., Li, S. M., Kennedy, D. W., Smith, S. C., and Gassman, P. L. (1998) Bacterial reduction of crystalline Fe^{3+} oxides in single phase suspensions and subsurface materials. American Mineralogist **83**:1426-1443.

4

Quinones as terminal electron acceptors in anaerobic granular sludge*

*Cervantes F. J., T. Duong-Dac, A. E. Ivanova, K. Roest, A. D. L. Akkermans, G. Lettinga and J. A. Field. *Submitted*

corresponding medium was continuously pumped from a container kept at 4 °C in a fridge. The reactors with a volume of 160 ml were placed in a 30 °C room and operated with a hydraulic residence time of 6 hours throughout the study. The consumption of substrates was monitored over time as described below. AQDS reduction was followed in the AQDS-supplemented reactor as described below. The produced off-gases were collected from both reactors by a liquid displacement device, from which samples were taken to determine the methane concentration as described below. After about one year of continuos operation sludge samples were obtained from both reactors to determine changes in the microbial population by DGGE.

Microbial incubations. Incubations were performed in 117-ml bottles sealed with butyl rubber stoppers and aluminum caps under a N_2/CO_2 (80/20) atmosphere at 37 °C in the dark. The basal medium was supplied with one of the following electron acceptors: AQDS (5 or 20 mM), humic acids (Janssen Chimica Belgium, 20 g per liter), nitrate (2 mM), sulfate (1.25 mM), elemental sulfur (10 mM), fumarate (50 mM), ferric iron chelated with nitrilotriacetic (Fe(III)-NTA, 10 mM) or amorphous ferric oxyhydroxide (FeOOH, 10 mM). One of the following substrates was provided as electron donor: acetate (2 mM), formate (5 mM), ethanol (5 mM), lactate (5 mM), benzoate (0.5 mM) and phenol (0.5 mM). When hydrogen was included as an electron donor, a headspace of H_2/CO_2 (80/20, final pressure 1.7 bars) was used. For evaluating the impact of humus on the reduction of amorphous ferric oxyhydroxide, humic acids (Janssen Chimica Belgium, 2 g per liter) were included in the medium. Depletion of the substrate, reduction of the corresponding electron acceptor, and cell numbers were followed in time as described below. Controls in which no external electron donor was provided were also included to correct for the endogenous reduction of the electron acceptors. Sterile controls in which no inoculation took place were also included. All the experiments were applied in triplicate incubations for all the conditions studied.

Analytical techniques. Fe(II) production was determined by the ferrozine technique (*20*). Samples (0.5 ml) were collected under axenic conditions (nearby a flame) and were immediately transferred into 2-ml polypropylene reaction vessels containing 0.5 ml of 1 N HCl to prevent autoxidation. Electrons transferred to humic acids were quantified by reducing Fe(III)-citrate with liquid samples as described before (*21*). AQDS reduction was determined spectrophotometrically at 450 nm in an anaerobic chamber as described before (*3*). The concentration of VFA, formate, lactate, ethanol, hydrogen, and methane were determined by previously described gas chromatographic methods (*3*). Benzoate and phenol concentrations were determined by high performance liquid chromatography (HPLC) as described previously (*4*). Cell numbers were determined by phase-contrast microscopy using a Bürker-Türk counting chamber at 400 x magnification.

Sulfate concentrations were determined by injecting 30 µl samples by an auto-sampler (Marathon) in a HPLC equipped with a VYDAC ion chromatography column (302 IC, 250 x 4.6 mm). The temperature of the column and detector (Waters 431 conductivity detector) were 20 and 35 °C,

respectively. As eluent 0.018 M potassium biphthalate, at a rate of 1.2 ml per min, was used. Samples for sulfate analysis were fixed by 2- to 4-fold dilution with a 0.1 M zinc acetate solution, centrifuged (10000 g, 3 min) and diluted with demineralized water. Nitrate and nitrite concentrations were also determined by HPLC equipped with the same column used for sulfate analysis and at the same temperature. 30 µl samples were also injected by an auto-sampler (Marathon). Potassium dihydrogen phosphate (10 g per liter, pH 3) adjusted by phosphoric acid was used as eluent at a flow rate of 1.5 ml per min. Nitrate and nitrite were detected by ultra violet detector (783 UV Detector-Kratos Analytical USA) at a wavelength of 205 nm. All samples were centrifuged (10000 g, 3 min) before analysis.

DNA isolation. To determine the phylogenetic diversity of the microbial population of the studied granular sludge from laboratory reactors and enrichment cultures, DGGE, cloning and sequencing methods were used. Total DNA was extracted from 1-ml homogenized sludge samples and from 10-ml enrichment cultures as previously described (*24*).

PCR. The 16S rRNA-genes were amplified from the genomic DNA by Polymerase Chain Reaction (PCR) using a *Taq* DNA polymerase kit from Life Technologies (Gaithersburg, Md.) with primers for conserved domains. Complete eubacteria 16S rDNA was selectively amplified using 7-f (5'-AGAGTTTGAT(C/T)(A/C)TGGCTCAG-3') and 1510-r (5'-ACGG(C/T)TACCTTGTTACGACTT-3') primers [15] with the following thermocycling program: 94 °C for 5 min; 25 cycles of 94 °C for 30 s, 52 °C for 20 s, and 68 °C for 40 s; and 68 °C for 7 min. The reactions were subsequently cooled to 4 °C. For DGGE use a specific region of eubacteria 16S rDNA (V6-V8 region) was amplified using 968-GC-f (5'-CGCCCGGGGCGCGCCCCGGGCGGGGCGGGGGCACGGGGGGAACGCGAAGAACC TTAC-3') and 1401-r (5'-CGGTGTGTACAAGACCC-3') primers (*23*) using the same thermocycle program but with 35 cycles and an annealing temperature of 56 °C. All primers were purchased from MWG-Biotech (Ebersberg, Germany). Size and amount of PCR products were estimated by 1% agarose gel (w/v) electrophoresis and ethidium bromide staining.

DGGE. DGGE analysis of the amplicons was performed on 8% (w/v) polyacrylamide gels containing denaturant gradients of 40 to 55%. A 100% denaturant corresponds to 7M urea (GIBCO BRL) and 40% (v/v) formamide (Merck). Electrophoresis was performed in 0.5 x Tris-Acetic acid-EDTA buffer (20 mM Tris, 10 mM acetic acid and 0.5 mM EDTA pH 8) at 85 V and 60 °C for 16 hours using a DGENE™ System apparatus (BioRad, Hercules, CA). Previously a voltage of 200 V was applied for 5 min. Silver-staining and development of the gels were performed according to Sanguinetti et al. (*25*) with minor modifications. Colour fixation solution (8x) was composed by 200 ml of 96 % (v:v) ethanol including 10 ml of acetic acid and 40 ml of demineralized water. The silver staining solution was prepared by adding 0.4 g $AgNO_3$ to 200 ml 1x colour fixing solution. The developer agent was composed by 10 mg of $NaBH_4$ included in a mixture of 200 ml of 1.5 % NaOH solution and 600 ml of formaldehyde. The colour preservation solution contained 50 ml 96 % (v:v) ethanol, 20 ml of glycerol and 130 ml of demineralized water.

The negative image of DGGE was obtained after drying the gel overnight at 60 °C. The DGGE technique was also applied to assess the biodiversity of archaea present in the consortia studied, but only the results obtained from the eubacterial screening is shown since no archaea were found in the enrichment culture, but only in the anaerobic granular sludge.

Cloning and sequencing. The amplified 16S rDNA products were purified by a QIAquick Kit (Qiagen GmbH, Hilden, Germany) and cloned in E. coli JM109 by using the pGEM®-T Easy Vector System (Promega, Leiden, The Netherlands) with ampicillin selection and blue/white screening, according to the manufacture's manual. The inserts were screened by Restriction Fragment Length Polymorphism (RFLP) analysis with the enzyme *MspI* (fragments were compared in a 2 % Boehringer (Boehringer Mannheim GmbH, Mannheim, Germany) agarose gel (w/v) electrophoresis and ethidium bromide staining) and by mobility comparison on DGGE. Plasmids of selected transformant were purified using the QIAprep spin miniprep kit (Qiagen GmbH, Hilden, Germany). Sequencing analysis was carried out with the Sequenase sequencing kit (Amersham, Slough, United Kingdom) using the sequencing primers Sp6 (5'-GATTTAGGTGACACTATAG-3'), complementary to one adjacent sequence of the pGEM®-T cloning site, T7 (5'-TAATACGACTCACTATAGGG-3'), complementary to the other adjacent sequence of the pGEM®-T cloning site, and internal primer 533 (5'-GTGCCAGC(A/C)GCCGCGGTAA-3') labeled with IRD8000 (MWG-Biotech, Ebersberg, Germany). The sequences were automatically analyzed on a LI-COR (Lincoln, NE, USA) DNA sequencer 4000L and corrected manually. A similarity search of the 16S rDNA sequence, derived from the enrichment clone, was performed by using the NCBI sequence search service, available on the internet (http//www.ncbi.nlm.nih.gov/blast/blast.cgi?Jform=0). A 1520 base pairs size cloned fragment was sequenced. The sequence was submitted in the GenBank database (Accession number AF404348).

Results

Characterization of an AQDS-reducing culture obtained from an anaerobic granular sludge. An AQDS-reducing enrichment culture was obtained from an anaerobic granular sludge originated from a full-scale UASB reactor (Nedalco, Bergen op Zoom, The Netherlands), that previously showed the capacity to degrade a wide variety of organic substrates coupled to AQDS reduction (*3, 4*). The enrichment was recovered from the highest positive dilutions of granular sludge in liquid AQDS-supplemented medium and was transferred to media containing AQDS (5 mM) several times using acetate as electron donor. DGGE analysis indicated that one microorganism was predominant in the enrichment culture. Sequence analysis revealed that this microorganism was 97 % related in 1520 base pairs to *Geobacter sulfurreducens*, a previously reported iron-reducing microorganism (*9*).

The enrichment culture could reduce AQDS with acetate, hydrogen or formate as an electron donor (Figure 1), and the molar ratio substrate consumption to AQDS reduction was 1.2:4, 1.3:1, and 1.1:1

when acetate, hydrogen or formate was provided, respectively, which approximately agrees with the expected stoichiometry of the reactions (1:4, 1:1, and 1:1, respectively). The same enrichment could not reduce AQDS with other substrates such as ethanol, lactate, methanol, benzoate or phenol. Acetate oxidation was also observed in basal medium containing humic acids (Janssen Chimica Belgium, 20 g per liter) as a terminal electron acceptor by the AQDS-reducing enrichment and the acetate consumption paralleled the increase in cell numbers in the culture (Figure 2). Furthermore, the electrons transferred to the humic acids during acetate oxidation were quantified at the end of the experiment by reducing Fe(III)-citrate with liquid samples obtained from this culture. The amount of Fe(II) recovered was 6.12 milliequivalents per liter (corrected for the endogenous control), which fits with the amount of acetate converted (6.23 milliequivalents electron per liter). This inoculum could also oxidize acetate with Fe(III)-NTA as a terminal electron acceptor, but not with nitrate, sulfate, elemental sulfur or fumarate. Insoluble ferric oxyhydroxide was not a suitable electron acceptor to support acetate oxidation by the *Geobacter* enrichment, but addition of humic acids (Janssen Chimica Belgium, 2 g per liter) allowed the complete reduction of this electron acceptor (Figure 3) and the electron equivalents recovered as Fe(II) accounted for 74 % of the acetate consumed.

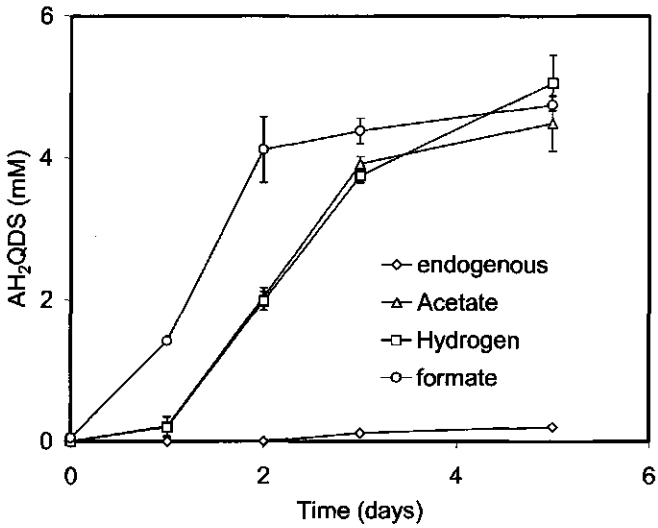


Figure 1. Reduction of anthraquinone-2,6-disulfonate (AQDS, 5 mM) by *Geobacter* enrichment with different substrates. The results are means of triplicate incubations and the bars indicate the standard deviation.

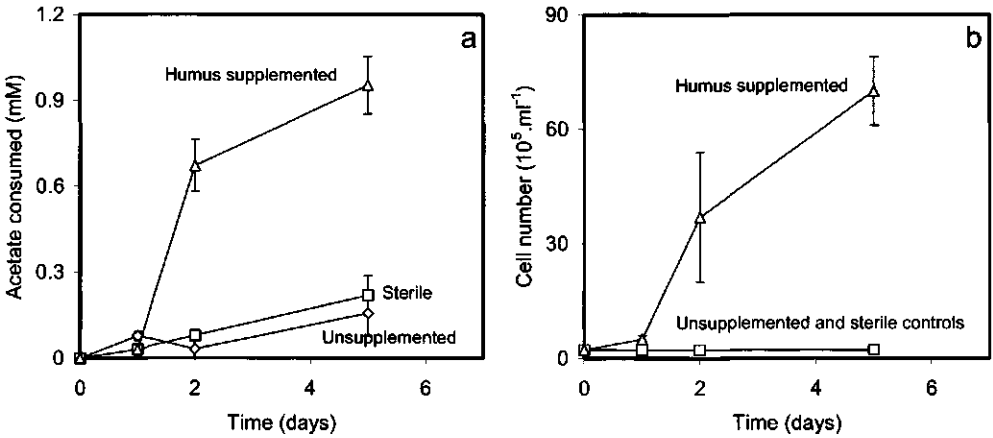


Figure 2. Acetate oxidation (a) coupled to growth (b) by *Geobacter* enrichment in humic acids (20 g per liter) supplemented medium. Unsupplemented control conducted in the absence of humic acids. The results are means of triplicate incubations and the bars indicate the standard deviation.

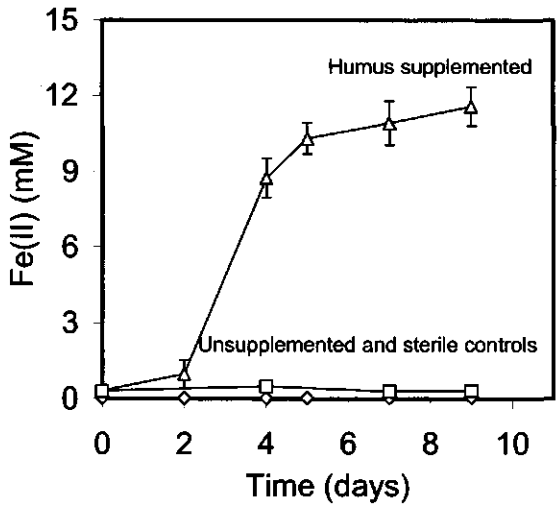


Figure 3. Reduction of ferric oxyhydroxide (10 mM) by *Geobacter* enrichment in humus (2 g per liter) supplemented medium with acetate (2 mM) as electron donor. The unsupplemented control conducted in the absence of humus. The results are means of triplicate incubations and the bars indicate the standard deviation.

Competition between methanogenesis and AQDS reduction in continuous reactors. The original anaerobic granular sludge from which the enrichment culture was obtained was continuously fed with an AQDS (12.5 mM) supplemented medium in a laboratory-scale UASB reactor. Total AQDS reduction was achieved in the reactor and sustained even after 11 months of operation, which accounted for 71 % of the VFA removal in the UASB reactor. Methane production eventually occurred in the AQDS-supplemented reactor accounting for up to 30 % of the VFA removal (Figure

4a). A control reactor was operated under methanogenic conditions in the absence of AQDS. Methanogenic activity was verified by monitoring the off-gas collected by a liquid displacement device. However, accurate quantification of the methane production was only made feasible after solving leakage problems in both systems. Figure 4b shows that the VFA removal in the methanogenic reactor was highly (92 %) recovered as methane once quantification problems were solved. After 11 months of continuous operation, sludge samples were obtained from both reactors to compare the microbial population of these consortia with the phylogenetic pattern observed in the enrichment culture. Figure 5 shows the DNA-based profiles from the bacterial community of the methanogenic reactor and from the AQDS-supplemented reactor. A significant change in the DNA-based profiles was observed in the reactors after this period. The DGGE gel also shows that the *Geobacter sulfurreducens*-related species clone, which prevailed in the enrichment culture, had the same dominant band found in the DNA-based profile of the enrichment. The same DNA fraction, however, did not appear as a dominant band in any of DNA-profiles of the reactors. Nevertheless, granular sludge obtained from both reactors showed the capacity to reduce AQDS with acetate as an electron donor in batch experiments. Remarkably, even the sludge operated under defined methanogenic conditions for 11 months was also able to reduce AQDS under the same conditions (Figure 6). The granular sludge from the AQDS-supplemented reactor could completely reduce AQDS (20 mM) after 3 days of incubation, whereas the sludge from the methanogenic reactor reduced AQDS at the same rate after a lag phase of several days.

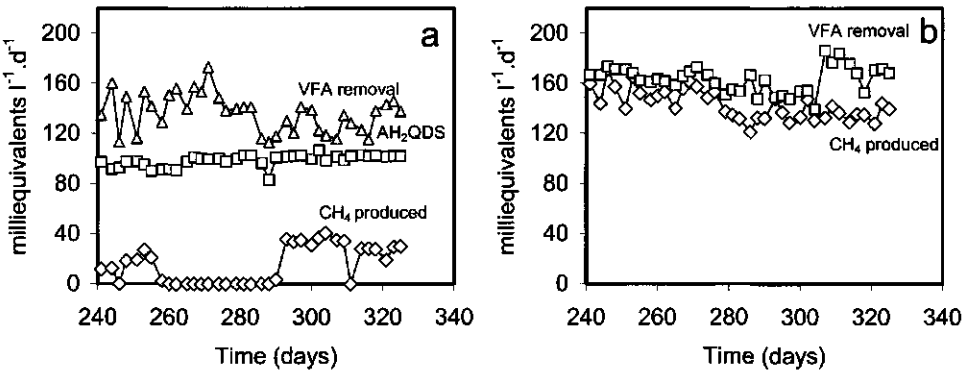


Figure 4. Methane production, AQDS reduction and VFA removal from the (a) AQDS-supplemented and (b) methanogenic UASB reactors operated at a hydraulic residence time of 6 hours.

Discussion

In the present study an AQDS-reducing enrichment culture derived from anaerobic granular sludge was characterized. The enrichment culture was predominated by a microorganism closely related to

Geobacter sulfurreducens and could grow with acetate, formate or hydrogen at the expense of AQDS, humic acids or chelated Fe(III) reduction. The DGGE method did not verify that the same type of microorganism was dominant in the anaerobic bioreactor continuously operated for a prolonged period with AQDS as a terminal electron acceptor. Nonetheless, the DNA-profile showed new bands (a, b, c and d in lane 3 of Figure 6) that appeared dominant in the microbial community of the AQDS-supplemented reactor compared to the consortium of the methanogenic reactor. The new dominant DNA-fractions may represent quinone-reducing microorganisms, yet to be identified, which were enriched and immobilized in the microbial community of the granular sludge of the AQDS-supplemented reactor, permitting the extensive reduction of AQDS and oxidation of acetate with a hydraulic residence time of 6 hours. The results indicate that it is feasible to apply quinone-reducing microorganisms in continuous bioreactors. The quinone-reducing ability can potentially be important in wastewaters rich in humic substances. Since acetate is an important intermediate in anaerobic digestion, acetate-oxidizing quinone-reducing microorganisms may significantly contribute to anaerobic substrate oxidation in wastewater treatment systems. The wide variety of organic substrates, including priority pollutants, such as *p*-cresol (*4*) and toluene (*2*) that can be oxidized through the microbial reduction of quinone moieties in humus further emphasizes the significance of humic substances serving as a terminal electron acceptor.

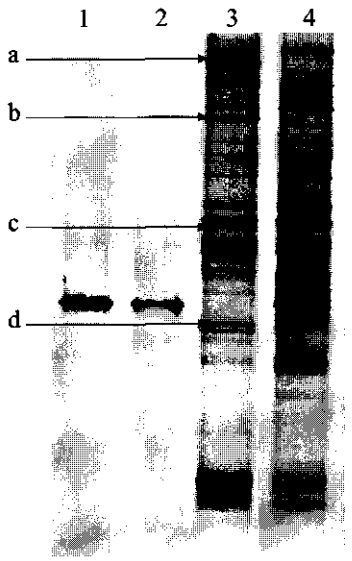


Figure 5. DGGE gel for 16S rDNA fragments obtained from granular sludge, *Geobacter* enrichment and a clone obtained from the dominant DNA fraction from the *Geobacter* enrichment. (1) Clone from *Geobacter* related microorganism in enrichment, (2) *Geobacter* enrichment, (3) granular sludge from AQDS-supplemented UASB reactor, and (4) granular sludge from the methanogenic UASB reactor. a, b, c, and d refer to new dominant bands found in the DNA profile of the sludge obtained from the AQDS-supplemented reactor.

The quinone reducing activity can also be applied to accelerate the reduction of xenobiotics susceptible to reductive biotransformations such as azo dyes (*14, 16, 27*); carbon tetrachloride (*8*); as well as metals and radionuclides (*12, 18*). In fact, the enrichment culture in which a *Geobacter* sp. prevailed was shown to generate reducing equivalents via humus-respiration, which allowed for the reductive dechlorination of carbon tetrachloride (data not shown). Moreover, addition of sub-stoichiometric concentrations of AQDS to a laboratory-scale UASB reactor inoculated with the original granular sludge enhanced the reductive biotransformation of an azo dye pollutant (*5*). Thus, quinone-reducing microorganisms may also play an important role on the reductive biotransformation of different priority pollutants in wastewater streams.

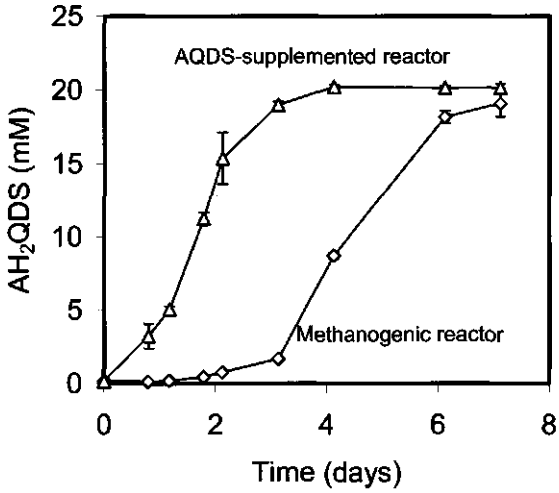


Figure 6. Reduction of anthraquinone-2,6-disulfonate (AQDS, 20 mM) by anaerobic granular sludge obtained from the methanogenic and AQDS-supplemented UASB reactors with acetate (1 g COD per liter) as electron donor. The results are means of triplicate incubations and the bars indicate the standard deviation.

In this study, quinone respiring activity was observed to rapidly develop in methanogenic granular sludges, including reactor sludge obtained after long-term operation under defined methanogenic conditions. Quinone reduction has also been observed in other methanogenic consortia with these characteristics (*3*). The activity could be due to gratuitous reduction of AQDS by methanogens or associated syntrophic bacteria (*1, 19*). Selective inhibition of methanogenic activity in the same granular sludge suggested that acetoclastic methanogens were not involved in the AQDS-reducing activity observed in this consortium, because AQDS reduction still occurred in the presence of bromoethanesulfonic acid (*3*). Furthermore, all the acetate-oxidizing quinone-reducing microorganisms reported in the literature belong to the *Geobacteraceae* family (*6, 7, 21, 22*). Thus, the AQDS-reducing capacity of the methanogenic consortium may rather be associated to the presence of

the *Geobacter* related species, which prevailed in minor proportion in the sludge community for a prolonged period by an unknown electron acceptor. This factor may significantly contribute to the ubiquity of quinone-reducing microorganisms in nature because the seasonal fluctuations in terminal electron acceptors eventually occurring in soil and sediments may not strongly affect the survival of humus-respiring community in these habitats.

Acknowledgements

This research was financially supported by The Council of Science and Technology of Mexico (CONACyT).

References

1. Benz, M., Schink, B. and Brune, A. (1998) Humic acid reduction by *Propionibacterium freudenreichii* and other fermentative bacteria. Appl. Environ. Microbiol. 64,4507-4512.
2. Cervantes, F. J., Dijksma, W., Duong-Dac, T., Ivanova, A., Lettinga, G. and Field, J. A. (2001) Anaerobic mineralization of toluene by enriched sediments with quinones and humus as terminal electron acceptors. Appl. Environ. Microbiol. 67, 4471-4478.
3. Cervantes, F. J., van der Velde, S., Lettinga, G. and Field, J. A. (2000) Competition between methanogenesis and quinone respiration for ecologically important substrates in anaerobic consortia. FEMS Microbiol. Ecol. 34,161-171.
4. Cervantes, F. J., van der Velde, S., Lettinga, G. and Field, J. A. (2000b) Quinones as terminal electron acceptors for anaerobic microbial oxidation of phenolic compounds. Biodegradation 11, 313-321.
5. Cervantes, F. J., van der Zee, F. P., Lettinga, G. and Field, J. A. (2001) Enhanced decolourisation of acid orange 7 in a continuous UASB reactor with quinones as redox mediators. Water Sci. Technol. 44, 123-128.
6. Coates, J. D., Bhupathiraju, V. K., Achenbach, L. A., Mclnerney, M. J. and Lovley, D. R. (2001) *Geobacter hydrogenophilus, Geobacter chapellei* and *Geobacter grbiciae*, three new, strict anaerobic, dissimilatory Fe(III)-reducers. Int. J. Syst. Evol. Microbiol. 51, 581-588.
7. Coates, J. D., Ellis, D. J., Roden, E., Gaw, K., Blunt-Harris, E. L. and Lovley, D. R. (1998) Recovery of humics-reducing bacteria from a diversity of sedimentary environments. Appl. Environ. Microbiol. 64,1504-1509.
8. Collins, R. and Picardal, F. (1999) Enhanced anaerobic transformation of carbon tetrachloride by soil organic matter. Environ. Toxicol. Chem. 18, 2703-2710.
9. Cord-Ruwisch, R., Lovley, D. R. and Schink, B. (1998) Growth of *Geobacter sulfurreducens* with acetate in syntrophic cooperation with hydrogen-oxidizing anaerobic partners. Appl. Environ. Microbiol. 64,2232-2236.

10. Field, J. A., Cervantes, F. J., van der Zee, F. P. and Lettinga, G. (2000) Role of quinones in the biodegradation of priority pollutants: a review. Water Sci. Technol. 42, 215-222.

11. Francis, C. A., Obraztsova, A. Y. and Tebo, B. M. (2000) Dissimilatory metal reduction by the facultative anaerobe *Pantoea agglomerans* SP1. Appl. Environ. Microbiol. 66,543-548.

12. Fredrickson, J. K., Kostandarithes, H. M., Li, S. W., Plymale, A. E. and Daly, M. J. (2000) Reduction of Fe(III), Cr(VI), U(VI), and Tc(VII) by *Deinococcus radiodurans* R1. Appl. Environ. Microbiol. 66,2006-2011.

13. Kostka, J. and Nealson, K. H. (1998) Isolation, cultivation and characterization of iron- and manganese-reducing bacteria. In: Techniques in microbial ecology (Burlage, R. S., Atlas, R., Stahl, D., Geesey, G. and Sayler, G., Eds.), pp. 58-78, Oxford University Press, New York.

14. Kudlich, M., Keck, A., Klein, J. and Stolz, A. (1997) Localization of the enzyme system involved in anaerobic reduction of azo dyes by *Sphingomonas* sp. Strain BN6 and effect of artificial redox mediators on the rate of azo dye reduction. Appl. Environ. Microbiol. 63, 3691-3694.

15. Lane, D. J. (1991) 16S/23S rRNA sequencing. In: Nucleic acid techniques in bacterial systematics (Stackebrandt, E. and Goodgellow, M., Eds.), pp. 115-147. Wiley & Sons, Chichester, United Kingdom.

16. Laszlo, J.A. (2000) Regeneration of azo-dye-saturated cellulosic anion exchange resin by *Burkholderia cepacia*. Environ. Sci. Technol. 34, 167-172.

17. Lettinga, G., van Velsen, A. F. M., Hobma, S. W., de Zeeuw, W. and Klapwijk, A. (1980) Use of the upflow sludge blanket (USB) reactor concept for biological wastewater treatment, especially for anaerobic treatment. Biotechnol. Bioeng. 22, 699-734.

18. Lloyd, J. R., Sole, V. A., van Praagh, C. V. G. and Lovley, D. R. (2000) Direct and Fe(II)-mediated reduction of technetium by Fe(III)-reducing bacteria. Appl. Environ. Microbiol. 66, 3743-3749.

19. Lovley D. R., Kashefi, K., Vargas, M., Tor, J. M. and Blunt-Harris, E. L. (2000) Reduction of humic substances and Fe(III) by hyperthermophilic microorganisms. Chem. Geol. 169,289-298.

20. Lovley, D. R. and Phillips, E. J. P. (1986) Availability of ferric iron for microbial reduction in bottom sediments of the fresh-water tidal Potomac River. Appl. Environ. Microbiol. 52,751-757.

21. Lovley, D. R., Coates, J. D., Blunt-Harris, E. L., Phillips, E. J. P. and Woodward, J. C. (1996) Humic substances as electron acceptors for microbial respiration. Nature 382,445-448.

22. Lovley, D. R., Fraga, J. L., Blunt-Harris, E. L., Hayes, L. A., Phillips, E. J. P., and Coates, J. D. (1998) Humic substances as a mediator for microbially catalyzed metal reduction. Acta Hydrochim. Hydrobiol. 26, 152-157.

23. Nübel, U., Engelen, B., Felske, A., Snaidr, J., Wieshuber, A., Amann, R. I., Ludwig, W. and Backhaus, H. (1996) Sequence heterogeneities of genes encoding 16S rRNAs in *Paenibacillus polymyxa* detected by temperature gradient gel electrophoresis. J. Bacteriol. 178, 5636-5643.

24. Oude Elferink, S. J. W. H., Rinia, H. A., Bruins, M. E., de Vos, W. M. and Stams, A. J. M. (1997) Detection and quantification of *Desulforabdus amnigenus* in anaerobic granular sludge by dot blot hybridization and PCR amplification. J. Appl. Microbiol. 83, 102-110.

25. Sanguinetti, C. J. E., Dias, N. and Simpson, A. J. (1994) Rapid silver staining and recovery of PCR products separated on polyacrylamide gels. BioTechniques 17, 914-921.

26. Scott, D. T., McKnight, D. M., Blunt-Harris, E. L., Kolesar, S. E. and Lovley, D. R. (1998) Quinone moieties act as electron acceptors in the reduction of humic substances by humics-reducing microorganisms. Environ. Sci. Technol. 32,2984-2989.

27. van der Zee, F. P., Bouwman, R. H. M., Strik, D. P. B. T. B., Lettinga, G., and Field, J. A. (2001) Application of redox mediators to accelerate the transformation of reactive azo dyes in anaerobic bioreactors. Biotechnol. Bioeng. **75**, 691-701.

28. Zachara, J.M., Fredrickson, J. K., Li, S. M., Kennedy, D. W., Smith, S. C. and Gassman, P. L. (1998) Bacterial reduction of crystalline Fe^{3+} oxides in single phase suspensions and subsurface materials. Am. Mineralogist 83,1426-1443.

5

Quinones as terminal electron acceptors for anaerobic microbial oxidation of phenolic compounds*

*A modified version has been published as: Cervantes F. J., S. van der Velde, G. Lettinga and J. A. Field. (2000) *Biodegradation* **11**, 313-321.

Abstract

The capacity of anaerobic granular sludge for oxidizing phenol and *p*-cresol under anaerobic conditions was studied. Phenol and *p*-cresol were completely converted to methane when bicarbonate was the only terminal electron acceptor available. When the humic model compound, anthraquinone-2,6-disulfonate, was included as an alternative electron acceptor in the cultures, the oxidation of the phenolic compounds was coupled to the reduction of the model humic compound to its corresponding hydroquinone, anthrahydroquinone-2,6-disulfonate. These results demonstrate for the first time that the anaerobic degradation of phenolic compounds can be coupled to the reduction of quinones as terminal electron acceptor.

Introduction

Phenols are common constituents of industrial aqueous effluents from processes such as polymeric resin production, oil refining and coking plants. Phenol is both toxic and lethal to fish at relatively low concentrations (e.g. 5-25 mg l^{-1}) and imparts objectionable tastes to drinking water at much lower concentrations (*13*). Due to their widespread use, phenolic compounds are common contaminants of water bodies, which receive untreated streams containing these compounds. It has been shown that phenol can be degraded by microorganisms participating in methanogenic consortia (*24*). Additionally, phenol can be degraded anaerobically by pure cultures using alternative electron acceptors such as sulfate (*1*), nitrate (*23*) and ferric iron (*18*).

In this study humus is considered as a terminal electron acceptor for phenolic compounds degradation. Humus is the stable organic matter accumulating in sediments and soils. It has been recently reported to play an active role in the anaerobic oxidation of various organic compounds, such as functioning as a terminal electron acceptor for the microbial oxidation of acetate (*17*). A microbial humus-respiring consortium obtained from an organic rich streambed sediment was also shown to mineralize vinyl chloride and dichloroethene under anaerobic conditions (*3*). The fact that humus was serving as the electron acceptor for the anaerobic oxidation of these contaminants was demonstrated by stimulating the oxidation through the addition of humic acids or the humic model compound, anthraquinone-2,6-disulfonate (AQDS).

Quinone moieties are the most likely candidates for the redox reactions observed in humus. This is supported by recent experiments, which correlated the humic substance quinone content with their electron accepting capacity (*19*). Therefore, quinone model compounds should be able to replace the function of humus as terminal electron acceptor. Most known humus-reducing microorganisms are capable of transferring electrons to AQDS, reducing it to anthrahydroquinone-2,6-disulfonate (AH_2QDS) (*4, 10, 17*). Furthermore, isolation of AQDS-reducing microorganisms from a variety of sediments consistently resulted in the recovery of microorganisms that could also reduce humic acids (*4*).

The fact that there is a wide variety of organic compounds which can be utilized by a humus-respiring consortia (*7*) leads to the question of whether humus or humic model compounds can also achieve the oxidation of phenolic compounds by acting as terminal electron acceptors. In this study, the capacity of two different anaerobic granular sludges for oxidizing phenolic compounds with AQDS as a terminal electron acceptor was explored.

Materials and methods

Inocula and basal medium. Methanogenic granular sludge from a full-scale upflow anaerobic sludge blanket (UASB) reactor treating effluent from an alcohol distillery of Nedalco (Bergen op Zoom, The Netherlands) and from a full-scale UASB reactor treating wet oxidized industrial effluent of Shell Nederland Chemie (Moerdijk, the Netherlands) were used for the present study. These consortia were chosen based on their capacity for both degrading phenolic compounds under methanogenic conditions and for reducing AQDS with readily biodegradable substrates such as hydrogen and acetate. Both granular sludge sources were washed and sieved to remove the fine particles before use in the batch tests. Both biomass sources were stored at 4 °C before use.

The basal medium used in all batch experiments contained (g l^{-1}): $NaHCO_3$, (5); NH_4Cl, (0.03); K_2HPO_4, (0.02); $MgCl_2.6H_2O$, (0.012); $CaCl_2.2H_2O$, (0.005); Na_2S, (0.013); and 1 ml l^{-1} of both trace elements and vitamins solutions. The trace elements solution contained (mg l^{-1}): $FeCl_2.4H_2O$, (2000); H_3BO_3, (50); $ZnCl_2$, (50); $CuCl_2.2H_2O$, (38); $MnCl_2.4H_2O$ (500); $(NH_4)_6Mo_7O_{24}.4H_2O$, (50); $AlCl_3.6H_2O$, (90); $CoCl_2.6H_2O$, (2000); $NiCl_2.6H_2O$, (92); $Na_2SeO.5H_2O$, (162); EDTA, (1000); and 1 ml l^{-1} of HCl (36 %). The vitamins solution contained (mg l^{-1}): biotin, (20); *p*-aminobenzoate, (50); pantothenate, (50); folic acid dihydrate, (20); lipoic acid, (50); pyridoxine, (100); Nicotinamide, (50). Thiamine, riboflavin and cyanocobalamine were prepared separately in individual flasks and were added to the basal medium at the final concentrations of 100, 50 and 10 μg l^{-1}, respectively. The basal medium was flushed with N_2/CO_2 (70/30) by passing this gas mixture through the liquid bulk for 10 minutes and was used without sterilization in the experiments.

Bioassays for the biodegradation of phenolic compounds under anaerobic conditions. The assays were conducted in batch mode by triplicate cultures in 117-ml glass serum bottles with a liquid volume of 50 ml (67 ml as headspace). Two sets of assays were run. In the first set, basal medium was transferred directly to the vials and then, inoculation took place by adding 1 g of volatile suspended solids (VSS) per liter in the cultures. The vials were sealed with butyl rubber stoppers and aluminum crimps and then flushed with N_2/CO_2 (70/30) for 10 minutes. Finally, either phenol or *p*-cresol was added as substrate at the final concentration of 300 mg of theoretic chemical oxygen demand (COD) per liter; namely, 1.35 mM for phenol and 1.1 mM for *p*-cresol. Another set was amended with AQDS (25 mM) and run under the same experimental conditions. Furthermore, two more sets of assays were conducted (one with and the other without AQDS) in the presence of the methanogenic inhibitor, 2-

bromoethanesulphonic acid (BES) at a final concentration of 50 mM. Controls without phenolic compounds to correct for endogenous methane production and AQDS reduction were also conducted. Sterilized controls were also included to discard chemical transformations. The pH under these conditions was monitored and remained at 7.3±0.1 in all the assays.

Analyses. Analysis of AH_2QDS was carried out on anaerobically collected samples in an anaerobic chamber under N_2/H_2 (96:4) atmosphere. The anaerobic chamber was a Type B Coy chamber (Coy Laboratory Products Inc.) made of pressed polished clear vinyl with a manual airlock installed. Samples (0.5 ml) were collected by using 1 ml disposable syringes and centrifuged (10000 g, 5 min) under these conditions and then diluted in 1 cm disposable plastic cuvettes containing anaerobic bicarbonate buffer (60 mM, pH 6.7±0.1). Concentrations of AH_2QDS were determined by monitoring absorbance at 450 nm and using an extinction coefficient of 2.25 absorbance units per mM obtained from a calibration curve of AQDS chemically reduced by dithionite.

Phenol and *p*-cresol were analyzed on previously centrifuged samples (10000 g, 5 min) by gas chromatography using a Hewlett Packard 5890 gas chromatograph equipped with 2 m x 6 mm x 2 mm glass column packed with Supelcoport (100-120 mesh) coated with 10 % Fluorad FC 431. The temperatures of the column, the injector port and the flame ionization detector were 130, 200 and 280 °C, respectively. The carrier gas was nitrogen saturated with formic acid (40 ml/min). The retention times were 9.3 and 13.7 min for phenol and *p*-cresol, respectively. The sample injection volume was 10 µl.

Using a flame ionization gas chromatograph model 438/S (Packard-Becker, Delft, The Netherlands), methane production was determined. The gas chromatograph was equipped with a steel column (2 m x 2 mm) packed with Porapak Q (80/100 mesh, Millipore Corp., Bedford, Mass.). The temperatures of the column, injector port, and the flame ionization detector were 60, 200 and 220 °C, respectively. Nitrogen was used, as carrier, at a flow rate of 20 ml/min and the sample injection volume was 100 µl. Volatile fatty acids (VFA) were analyzed as previously described (*16*).

The intermediates benzoate and *p*-hydroxybenzoate were analyzed with high performance liquid chromatography (HPLC) at the end of the experiments. Samples from the batch experiments were centrifuged (10000 g, 5 min) and diluted in demineralized water, and 10-µl samples were injected with a Marathon autosampler (Separations, Hendrik Ido Ambacht, The Netherlands). These compounds were detected spectrophotometrically with a Spectroflow 783 UV detector (Kratos Analytical, Hendrik Ido Ambacht, The Netherlands) at their maximum absorbance (218 nm). Methanol with 2 % demineralized water (A) and triethylamine (5 mM) in acetate buffer (10 mM) (B) were used as liquid phase and were pumped (Separations High Precision Pump Model 104, Separations, Hendrik Ido Ambacht, The Netherlands) at a flow rate of 500 µl min^{-1} first through a Separations GT-103 degaser (Hendrik Ido Ambacht, The Netherlands) and afterwards through a reverse-phase C18 column (Chromosphere C18, Chrompack, Bergen op Zoom, The Netherlands). The liquid phase composition

was 15 % of solution A and 85 % of solution B; namely, the final composition was 4.25 mM of triethylamine and 3.7 mM of methanol in acetate buffer (8.5 mM). The retention times were 2.45 min and 4.38 min for *p*-hydroxybenzoate and benzoate, respectively.

The VSS content of the granular sludges was determined by subtracting the ash content from the dry weight after the sludge was incubated overnight at 105 °C. The ash content was determined after the dry sludge was heated at 550 °C for 2 hours. The sample size used in the analyses was 10 g of wet sludge.

Results

Complete conversion of both phenol and *p*-cresol to methane was observed when batch experiments were conducted in the absence of AQDS and BES by the anaerobic granular sludge obtained from a full-scale UASB reactor treating effluent from an alcohol distillery of Nedalco (Figure 1). There was a lag phase of about 30 days before this granular sludge started to consume the phenolic compounds, but after this period, degradation took place in both bioassays. Evidence of complete degradation is based on the elimination of the phenolic compounds and recovery of the stoichiometric amounts of methane in excess of that produced in the endogenous substrate control cultures (Tables 1 and 2).

Phenol was completely consumed by "Nedalco" granular sludge also in the presence of BES in which negligible methane production was observed (Figure 2A). Further analyses revealed that phenol was completely converted to benzoate under these conditions (Table 1). On the other hand, *p*-cresol was partially converted after 105 days of incubation by "Nedalco" sludge, only 56 % of *p*-cresol was consumed (Figure 2B) and benzoate was only detected at low levels under these conditions (Table 1). The very low recovery observed in the balance for the *p*-cresol-BES culture (Table 1) suggests that this phenolic compound was converted to another unidentified intermediate by this consortium. The missing COD could not be attributed to either VFA or to *p*-hydroxybenzoate, which were only present at trace levels at the end of the experiment (data not shown).

Phenol degradation could also be coupled to AQDS reduction by "Nedalco" granular sludge (Figure 3A), but at a 3-fold lower extent of degradation as compared to methanogenic conditions (Table 2). The coupling between phenol degradation and AQDS reduction is evidenced by the amount of phenol degraded, which fits with the COD recovered as hydrogen in AH_2QDS corrected for the endogenous AQDS reduction (Table 2). No benzoate was detected at the end of the experiment under these conditions. When BES was included in the phenol-AQDS culture, the extent of phenol degradation was slightly lower (Table 2) and it was partially converted to benzoate (Table 1). In the presence of BES no significant AQDS reduction was observed as compared to the endogenous AQDS reduction (Figure 3B). Further experiments confirmed that benzoate oxidation can be coupled to AQDS reduction by this granular sludge (about 100 mg COD l^{-1} of benzoate recovered as AH_2QDS, corrected for endogenous AQDS reduction, after 5 months of incubation). Neither phenol conversion nor AQDS

reduction was observed in sterilized controls with autoclaved "Nedalco" sludge. Moreover, there was no methanogenic activity detected in the presence of AQDS.

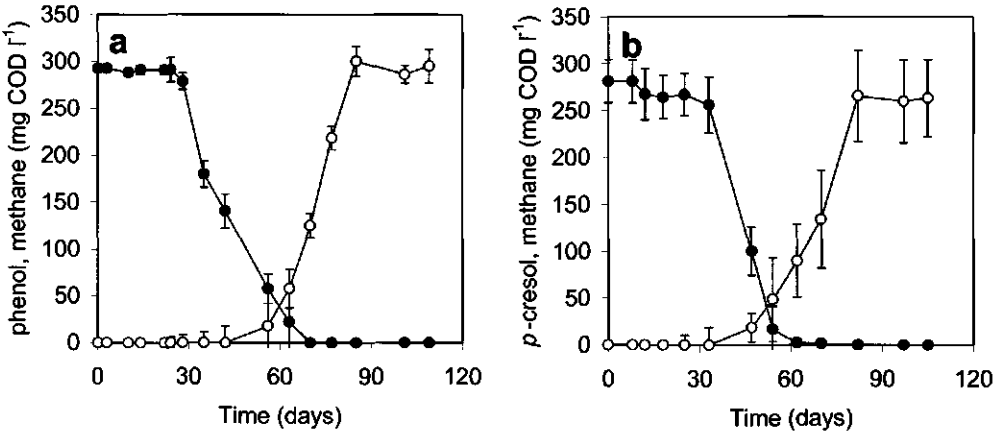


Figure 1. Conversion of phenolic compounds (Δ) to methane (A) by "Nedalco" sludge. (a) Phenol. (b) *p*-cresol. Methane production reported as mg COD per liter of culture fluid and corrected for endogenous methanogenesis.

Table 1. COD balance (in mg COD l^{-1}) in different cultures for phenol and *p*-cresol biodegradation by "Nedalco" sludge after 15 weeks of incubation

Culture (added substrate)	Endogenous[1]	Phenol	p-Cresol	Benzoate	CH_4[3]	AH_2QDS[4]	Recovery[5] (%)
Phenol (293)	406±18	ND[2]	ND	ND	701±57	-	100.7
Phenol-BES (293)	12±2	ND	ND	312±4	15±6	-	107.5
Phenol-AQDS (292)	161±14	205±7	ND	ND	ND	262±54	104.8
Phenol-AQDS-BES (283)	157±12	202±7	ND	159±0.7	ND	137±19	120.5
p-cresol (281)	214±41	ND	ND	ND	477±29	-	93.6
p-cresol-BES (299)	10±4	ND	132±35	8±3	10±6	-	46.8
p-cresol-AQDS (265)	117±7	ND	84±45	ND	ND	297±101	99.6
p-cresol-AQDS-BES (263)	113±10	ND	149±4	ND	ND	224±21	98.9

[1]Endogenous production of methane (AQDS not present) or AH_2QDS (AQDS present) in sludge controls.
[2]ND: Not detected.
[3]Total production of methane not corrected for endogenous methane production. Methane concentration expressed as mg COD per liter of culture fluid.
[4]COD calculated only refers to the hydrogen linked to the structure, total concentration of AH_2QDS measured not corrected for endogenous AH_2QDS production.
[5]Recovery=(identified products - endogenous COD)/(initial COD), no VFA were detected in all the samples at the end of the experiment.

Table 2. Extent of degradation and ratio reduced products:substrate consumed (RRPSC) for phenol and *p*-cresol by "Nedalco" and "Shell" sludge under different anaerobic conditions after 15 weeks of incubation[1]

	Extent of degradation (%)		RRPSC (%)[2]	
Culture Conditions	"Nedalco" sludge	"Shell" sludge	"Nedalco" sludge	"Shell" sludge
Phenol	100±0	100±0	101±8	113±5
Phenol-BES	100±0	100±0	Not applicable[3]	Not applicable
Phenol-AQDS	30±3	38±12	116±21	47±27[4]
Phenol-AQDS-BES	28±3	41±3	Not applicable	Not applicable
p-cresol	100±0	100±0	94±6	102±3
p-cresol-BES	56±12	70±5	Not applicable	Not applicable
p-cresol-AQDS	69±16	96±1	99±34	79±14
p-cresol-AQDS-BES	44±2	100±0	98±8	106±8

[1]Data from experiments with "Shell" sludge after 20 weeks of incubation.
[2]RRPSC=(total CH_4-endogenous CH_4)/(phenol or *p*-cresol consumed) for methanogenic culture, RRPSC=(total AH_2QDS-endogenous AH_2QDS)/(phenol or *p*-cresol consumed) for the AQDS containing cultures. Ratio based on mg COD per liter of culture fluid. COD calculated as AH_2QDS only refers to the hydrogen linked to the structure.
[3]Not applicable refers to the lack of coupling between degradation and methanogenesis or AQDS reduction.
[4]*Total recovery 75 % including 28 mg COD* l^{-1} as benzoate.

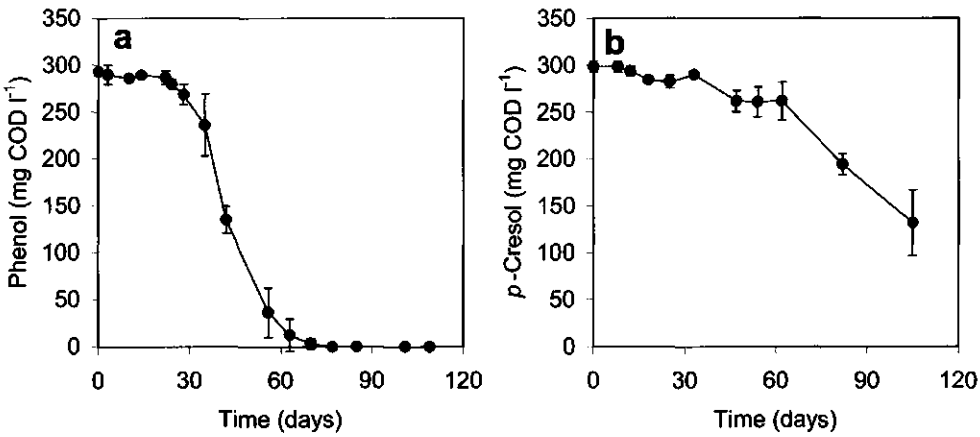


Figure 2. Phenol (a) and *p*-cresol (b) degradation by "Nedalco" sludge in the presence of BES (50 mM).

Oxidation of *p*-cresol could also support AQDS reduction by "Nedalco" granular sludge both in the presence and in the absence of BES (Figure 4). Evidence is based on decrease in *p*-cresol and concomitant increase in AH_2QDS production beyond the level observed in the endogenous substrate control. Furthermore, the COD recovered as hydrogen in AH_2QDS (corrected for endogenous AH_2QDS production) agrees with the amount of *p*-cresol consumed in both experiments (Table 2). There was no benzoate nor VFA detected at the end of the experiment under these conditions (Table 1). There was no methanogenic activity in the presence of AQDS in the experiments for *p*-cresol degradation. In sterilized controls neither *p*-cresol conversion nor AQDS reduction was observed. The extent of degradation of *p*-cresol observed in the presence of AQDS was about the same level

compared to that observed in the *p*-cresol-BES culture, but about 1.5-2.0-fold lower than under methanogenic conditions (Table 2).

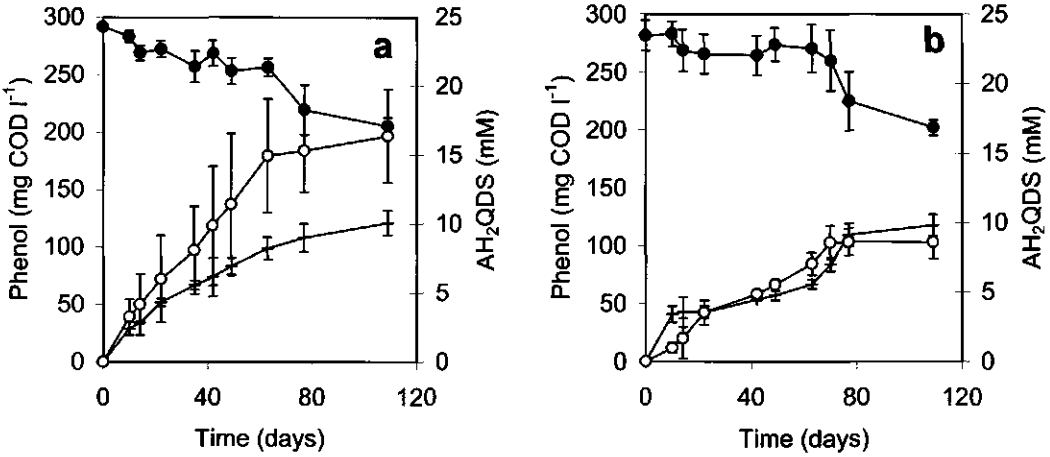


Figure 3. Degradation of phenol (Δ) by "Nedalco" sludge. (a) In the presence of AQDS (25 mM). (b) In the presence of AQDS (25 mM) and BES (50 mM). (A), AH_2QDS; (+), endogenous AH_2QDS.

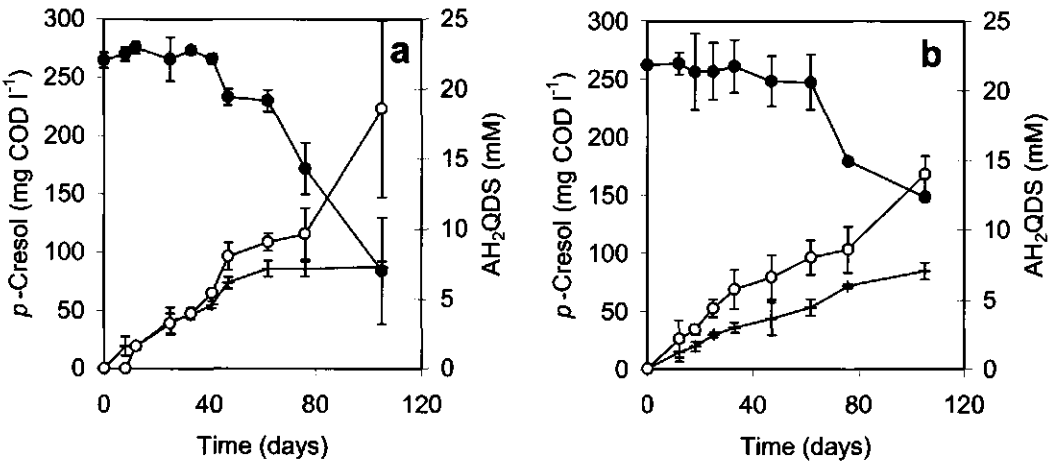


Figure 4. Degradation of *p*-cresol (Δ) by "Nedalco" sludge. (a) In the presence of AQDS (25 mM). (b) In the presence of AQDS (25 mM) and BES (50 mM). (A), AH_2QDS; (+), endogenous AH_2QDS.

Additional experiments were carried out with anaerobic granular sludge obtained from a full-scale UASB reactor treating wet oxidized industrial effluent of Shell Nederland Chemie. This sludge referred to as "Shell" sludge was able to completely degrade *p*-cresol coupled to AQDS reduction both in the absence and in the presence of BES (Figure 5). This is evidenced by the consumption of this phenolic compound, which fits with the COD recovered as hydrogen in AH_2QDS corrected for the

endogenous AQDS reduction (Table 2). However, there was only minor phenol degradation linked to AQDS respiration by "Shell" sludge after 5 months of incubation. The COD recovered as AH_2QDS accounted only for 47 % (Table 2) of the phenol degraded (about 100 mg COD-phenol l^{-1}) and benzoate was detected as an intermediate (28 % of the phenol degraded). When BES was included in the phenol-AQDS culture, no coupling between phenol degradation and AQDS reduction was observed by "Shell" sludge. There was no methane production by "Shell" sludge in the AQDS amended media. Moreover, neither conversion of the phenolic compounds nor reduction of AQDS was observed in the sterilized controls with autoclaved "Shell" sludge.

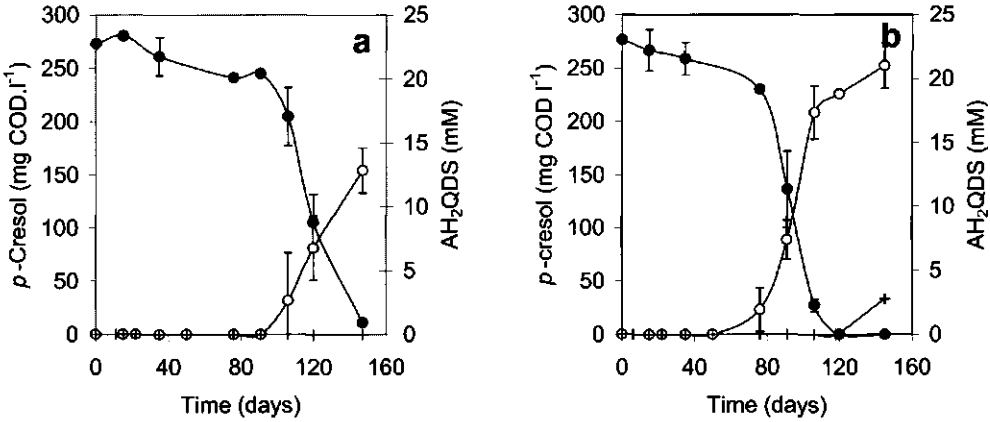


Figure 5. Degradation of *p*-cresol (Δ) by "Shell" sludge. (a) In the presence of AQDS (25 mM). (b) In the presence of AQDS (25 mM) and BES (50 mM). (A), AH_2QDS; (+), endogenous AH_2QDS.

Both phenolic compounds were completely converted to methane when bicarbonate was the only electron acceptor available by "Shell" granular sludge after 3 months of incubation (data not shown). The COD recovered as methane (corrected for the endogenous methane production) agreed with the amount of phenol and *p*-cresol consumed (Table 2). Phenol was completely converted to benzoate (98 % of recovery) when the methanogenic inhibitor, BES, was included in the culture; whereas about 70 % of *p*-cresol was consumed under the same conditions. *p*-cresol was mainly converted to VFA, but this COD only accounted for about 60 % of the consumed *p*-cresol indicating that this phenolic compound was transformed to another unidentified intermediate also by "Shell" sludge when BES was included in the medium.

Discussion

The observation that phenol and *p*-cresol degradation occurred under methanogenic conditions is consistent with numerous, previous reports, which indicate that these pollutants can be utilized by methanogenic consortia (*2, 6, 21, 24*). This also agrees with thermodynamics, which indicates that

conversion of these phenolic compounds is favorable under methanogenic conditions (see Table 3). The lag phase observed during these experiments was due to the inocula used, which were not previously exposed to the phenolic contaminants. Time was required for the growth of the responsible degrading bacteria and the development of the enzymatic systems involved in the degradation pathway.

The complete conversion of phenol to benzoate in the presence of the methanogenic inhibitor, BES, agrees with previous reports, which showed the same pattern by inhibiting the culture. Field and Lettinga (*8*) observed complete conversion of phenol to benzoate in methanogenic cultures that were inhibited by an excess concentration of phenol. Knoll and Winter (*15*) used an atmosphere of 80 % H_2 and 20 % CO_2 to stimulate feedback inhibition and this also led to the accumulation of benzoate during degradation of phenol under methanogenic conditions. Conversion of phenol to benzoate is feasible according to thermodynamics if hydrogen is available from endogenous substrates ($\Delta G^{\circ\prime}$ = -64.9 kJ mol^{-1}). Considering the proposed pathway of phenol degradation, which proceeds through benzoyl-CoA (*12*), it seems that equilibrium is reached between benzoyl-CoA and benzoate when inhibitory conditions predominate in the culture and no further transformation occurs towards saturating the aromatic ring, which are the next steps in the pathway (Figure 6). This may be explained by thermodynamics which indicates that conversion of benzoate to acetate is an unfavorable reaction, whereas the global conversion of benzoate to methane is thermodynamically favorable (Table 3), but this last reaction did not occur under these conditions due to the presence of BES.

The limited conversion of *p*-cresol achieved when BES was included in the medium suggests that this methanogenic inhibitor has a stronger effect on the degradation of *p*-cresol compared to the effect observed during phenol degradation by both inocula tested. Since there was no major accumulation of benzoate or *p*-hydroxybenzoate during these experiments, other intermediates such as *p*-hydroxybenzyl alcohol and *p*-hydroxybenzaldehyde, which are formed during the conversion of *p*-cresol to *p*-hydroxybenzoate (*5*), might have accumulated.

In this study we observed that quinones can be used as alternative electron acceptors for supporting the anaerobic oxidation of phenols. Upon addition of the model compound, AQDS, the flow of electrons was diverted away from methanogenesis and was directed towards quinone reduction. The coupling of phenol and *p*-cresol degradation to quinone reduction was supported by the stoichiometric recovery of electrons in the reduced quinone, AH_2QDS, as compared to the amount of phenols degraded (see Table 2).

AQDS reduction was most likely related to the oxidation of intermediates (e.g. benzoate) and not to the direct oxidation of phenol by "Nedalco" sludge. This is suggested by the negligible AQDS reduction observed (compared to the endogenous control) when BES was included in the phenol-AQDS culture in which partial conversion of phenol to benzoate was observed by "Nedalco" sludge.

This theory is also supported by the fact that benzoate degradation led to AQDS reduction by this anaerobic granular sludge.

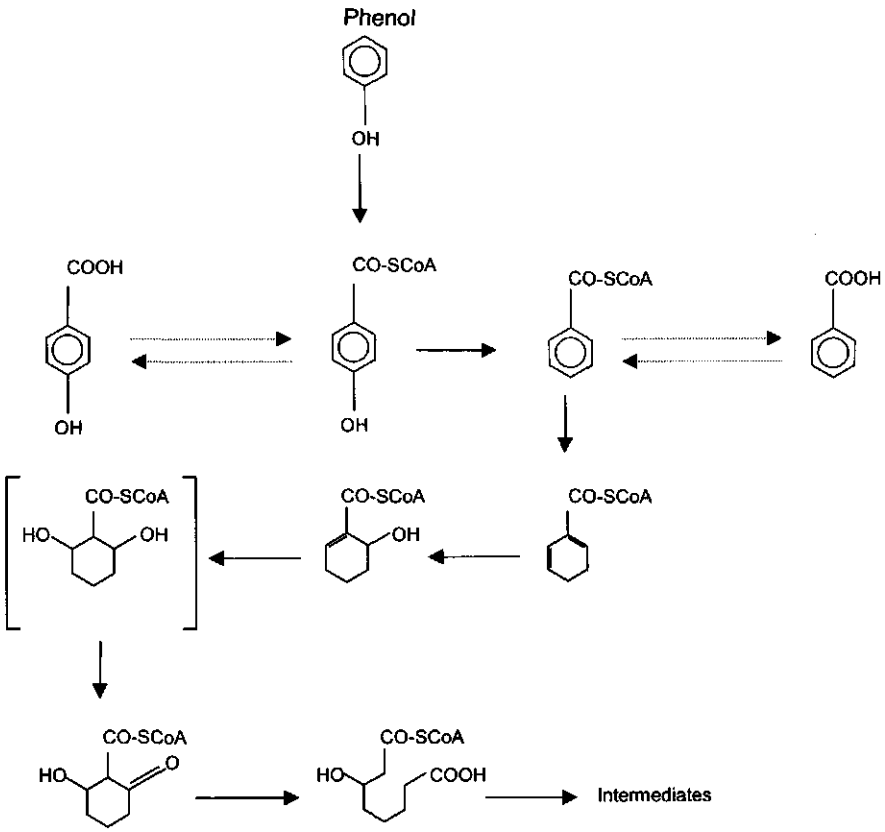


Figure 6. Proposed pathway involved in the anaerobic degradation of phenol (*9, 12*).

On the other hand, the coupling between *p*-cresol degradation and AQDS reduction was observed both in the presence and in the absence of BES, without any accumulation of intermediates by both consortia evaluated. This may indicate that *p*-cresol degradation was carried out directly through quinone respiration by both sources of anaerobic granular sludge.

Addition of AQDS to the consortia prevented methanogenesis. This may be due to the fact that AQDS was inhibitory to methanogens or that AQDS was the preferred electron acceptor over bicarbonate. AQDS increased the redox potential of the culture fluid (data not shown). This high redox potential probably interferes with biochemical processes required for methanogenesis.

Thermodynamically, AQDS reduction is more favorable than methanogenesis (Table 3) and therefore, it can be expected that AQDS reduction would proceed instead of methanogenesis according to this

point of view. However, this does not agree with the slower degradation for both phenolic compounds observed with AQDS as alternative electron acceptor compared to that obtained under methanogenic conditions by both sources of granular sludge. The slower degradation rates may be attributed to the type of inocula used in these experiments, which are characterized by a high methanogenic activity and thus, may only contain few quinone respiring microorganisms. This may also explain the longer lag phase observed during biodegradation of *p*-cresol via AQDS reduction by "Shell" sludge (Figure 5) compared to that observed under methanogenic conditions (only one month as lag phase).

Table 3. Reactions involved in the degradation of phenolic compounds under anaerobic conditions (all $\Delta G^{\circ\prime}$ values are calculated with data from references *14* and *22*)[1]

Reaction	$\Delta G^{\circ\prime}$ (kJ reaction^{-1})
phenol:	
$C_6H_6O + 6.5\ H_2O \rightarrow 3.5\ CH_4 + 2.5\ HCO_3^- + 2.5\ H^+$	- 155.3
$C_6H_6O + 17\ H_2O + 14\ AQDS \rightarrow 14\ AH_2QDS + 6\ HCO_3^- + 6\ H^+$	- 302.0
p-cresol:	
$C_7H_8O + 7.5\ H_2O \rightarrow 4.25\ CH_4 + 2.75\ HCO_3^- + 2.75\ H^+$	- 187.5
$C_7H_8O + 20\ H_2O + 17\ AQDS \rightarrow 17\ AH_2QDS + 7\ HCO_3^- + 7\ H^+$	-365.7
benzoate:	
$C_7H_5O_2^- + 7\ H_2O \rightarrow 3\ CH_3COO^- + HCO_3^- + 3\ H^+ + 3\ H_2$	+ 70.4
$C_7H_5O_2^- + 7.75\ H_2O \rightarrow 3.75\ CH_4 + 3.25\ HCO_3^- + 2.25\ H^+$	- 124.1
$C_7H_5O_2^- + 19\ H_2O + 15\ AQDS \rightarrow 15\ AH_2QDS + 7\ HCO_3^- + 6\ H^+$	- 281.5

[1] $\Delta G^{\circ\prime}$ for reactions with AQDS include reduction of AQDS by hydrogen according to Nernst equation with data from reference *20*. $\Delta G^{\circ\prime} = -44.4$ kJ mol^{-1}.

To our knowledge, this investigation is the first report of quinones serving as a terminal electron acceptor to support the oxidation of phenolic compounds under anaerobic conditions. Thus, the results have important implications for bioremediation of anaerobic sites contaminated with phenolic compounds. In fact, the results suggest that humus, which is very abundant in many anaerobic sites and rich in quinone moieties, may contribute to the bioremediation capacity of sites contaminated with aromatic compounds by serving as a terminal electron acceptor. These results also suggest that quinones may play an active role in the biodegradation of plant material, which contains a variety of simple and complex phenolic substances (*11*). Therefore, quinones in humus may contribute to important carbon cycling process in the biosphere.

Conclusions

The results presented in this study indicate that quinones can contribute in the oxidation of phenolic compounds by serving as terminal electron acceptors. The results also suggest that humus may be a potential electron acceptor for the biodegradation of aromatic compounds in anaerobic sites. This information needs to be considered in future studies of electron and carbon flow in soils and sediments as it may have important implications for the biotransformation of organic matter.

Acknowledgements

This study was financially supported by Council of Science and Technology of Mexico (CONACyT). We thank Alfons Stams for providing advice during thermodynamics calculations.

References

1. Bak, F. and Widdel, F. (1986) Anaerobic degradation of phenol derivatives by *Desulfobacterium phenolicum* sp nov. *Arch. Microbiol.* **146**, 177-180.
2. Boyd, S. A., Shelton, D. R., Berry, D. and Tiedje, J. M. (1993) Anaerobic degradation of phenolic compounds in digested sludges. *Appl. Environ. Microbiol.* **46**, 50-54.
3. Bradley, P. M., Chapelle, F. H. and Lovley, D. R. (1998) Humic acids as electron acceptors for anaerobic microbial oxidation of vinyl chloride and dichloroethene. *Appl. Environ. Microbiol.* **64**, 3102-3105.
4. Coates, J. D., Ellis, D. J., Roden, E., Gaw, K., Blunt-Harris, E. L. and Lovley, D. R. (1998) Recovery of humics-reducing bacteria from a diversity of sedimentary environments. *Appl. Environ. Microbiol.* **64**, 1504-1509.
5. Cunane, L. M., Chen, Z. W., Shamala, N., Mathews, F. S., Cronin, C. N. and McIntire, W. S. (2000) Structures of the flavocytochrome *p*-cresol methylhydroxylase and its enzyme-substrate complex: gated substrate entry and proton relays support the proposed catalytic mechanism. *J. Mol. Biol.* **295**, 357-374.
6. Dwyer, D. F., Krumme, M. L., Boyd, S. A. and Tiedje, J. M. (1986) Kinetics of phenol degradation by an immobilized methanogenic consortium. *Appl. Environ. Microbiol.* **52**, 345-351.
7. Field, J. A., Cervantes, F. J., Van der Zee, F. P. and Lettinga, G. (2000) Role of quinones in the biodegradation of priority pollutants: A review. *Water Sci. Technol.* **42**, 215-222.
8. Field, J. A. and Lettinga, G. (1989) The effect of oxidative coloration on the methanogenic toxicity and anaerobic biodegradability of phenols. *Biol. Wastes.* **29**, 161-179.
9. Field, J. A., Stams, A. J. M., Kato, M. and Schraa, G. (1995) Enhanced biodegradation of aromatic pollutants in cocultures of anaerobic and aerobic bacterial consortia. *Antonie van Leeuwenhoek.* **67**, 47-77.
10. Francis, C. A., Obraztsova, A. Y. and Tebo, B. M. (2000) Dissimilatory metal reduction by the facultative anaerobe *Pantoea agglomerans* SP1. *Appl. Environ. Microbiol.* **66**, 543-548.
11. Harborne, J. B. (1980) Plant phenolics. In: Bell EA & Charlwood BV (Eds) Secondary plants products (pp 329-402). Springer-Verlag, New York
12. Heider, J. and Fuchs, G. (1997) Review: Anaerobic metabolism of aromatic compounds. *Eur. J. Biochem.* **243**, 577-596.
13. Hill, G. and Robinson, C. W. (1975) Substrate inhibition kinetics: phenol degradation by *Pseudomonas putida*. *Biotechnol. Bioeng.* **17**, 1599-1615.

14. Holliger, C., Stams, A. J. M. and Zehnder, A. J. B. (1988) Anaerobic degradation of recalcitrant compounds. In: Hall, E. R. and Hobson, P. N. (Eds) Anaerobic digestion, Fifth international symposium on anaerobic digestion (pp 211-224). Bologna.

15. Knoll, G. and Winter, J. (1989) Degradation of phenol via carboxylation to benzoate by a defined, obligate syntrophic consortium of anaerobic bacteria. *Appl. Microbiol. Biotechnol.* **30**, 318-324.

16. Kortekaas, S., Vidal, G., Yan-Ling, H., Lettinga, G. and Field, J. A. (1998) Anaerobic-aerobic treatment of toxic pulping black liquor with upfront effluent recirculation. *J. Ferment. Bioeng.* **86**, 97-110.

17. Lovley, D. R., Coates, J. D., Blunt-Harris, E. L., Phillips, E. J. P. and Woodward, J. C. (1996) Humic substances as electron acceptors for microbial respiration. *Nature.* **382**, 445-448.

18. Lovley, D. R. and Lonergan, D. J. (1990) Anaerobic oxidation of toluene, phenol and *p*-cresol by the dissimilatory iron-reducing organism, GS-15. *Appl. Environ. Microbiol.* **56**, 1858-1864.

19. Scott, D. T., McKnight, D. M., Blunt-Harris, E. L., Kolesar, S. E. and Lovley, D. R. (1998) Quinone moieties act as electron acceptors in the reduction of humic substances by humics-reducing microorganisms. *Environ. Sci. Technol.* **32**, 2984-2989.

20. Sober, H. A. (1970) Handbook of biochemistry: selected data for molecular biology. The Chemical Rubber Co., Cleveland, Ohio.

21. Tawfiki, K. H., Lépine, F., Bisaillon, J. G., Beaudet, R., Hawari, J. and Guiot, S. R. (2000) Effects of bioaugmentation strategies in UASB reactors with a methanogenic consortium for removal of phenolic compounds. *Biotechnol. Bioeng.* **67**, 417-423.

22. Thauer, R. K., Jungermann, K. and Decker, K. (1977) Energy conservation in chemotrophic anaerobic bacteria. *Bacteriol. Rev.* **41**, 100-180.

23. Tschech, A. and Fuchs, G. (1987) Anaerobic degradation of phenol by pure cultures of newly isolated denitrifying pseudomonads. *Arch. Microbiol.* **148**, 213-217.

24. Wang YT, Suidan MT & Ritman BE (1986) Anaerobic treatment of phenol by an expanded-bed reactor. *J. WPCF.* **58**, 227-233.

6

Anaerobic mineralization of toluene by enriched sediments with quinones and humus as terminal electron acceptors *

*A modified version has been published as: Cervantes F. J., W. Dijksma, T. Duong-Dac, A. Ivanova, G. Lettinga and J. A. Field (2001). *Appl. Environ. Microbiol.* **67**, 4471-4478.

Abstract

The anaerobic microbial oxidation of toluene to CO_2 coupled to humus respiration was demonstrated in enriched anaerobic sediments from Amsterdam Petroleum harbor (APH) and Rhine river. Both highly purified soil humic acids (HPSHA) and the humic quinone moiety model compound, anthraquinone-2,6-disulfonate (AQDS) were utilized as terminal electron acceptors. After two weeks of incubation, 50 and 85 % of added uniformly labeled [^{13}C]toluene was recovered as $^{13}CO_2$ in HPSHA and AQDS supplemented APH sediment enrichment cultures, respectively; whereas negligible recovery occurred in unsupplemented cultures. The conversion of [^{13}C]toluene agreed with the high recovery of electrons as reduced humus or as anthrahydroquinone-2,6-disulfonate. APH sediment was also able to use nitrate, and amorphous manganese dioxide as a terminal electron acceptor to support the anaerobic biodegradation of toluene. Addition of substoichiometric amounts of humic acids in bioassays containing amorphous ferric oxyhydroxide as a terminal electron acceptor led to more than 65 % conversion of toluene (1 mM) after 11 weeks of incubation, which paralleled the partial recovery of electron equivalents as acid extractable Fe(II). Negligible conversion of toluene and reduction of Fe(III) occurred in these bioassays when humic acid was omitted. The present study provides clear quantitative evidence for the mineralization of an aromatic hydrocarbon by humus respiring microorganisms. The results indicate that humic substances may significantly contribute to the intrinsic bioremediation of anaerobic sites contaminated with priority pollutants by serving as a terminal electron acceptor.

Introduction

Toluene is an important constituent of gasoline accounting for 5-7 % (wt/wt) of its composition (39). Due to leaks in underground fuel storage tanks, improper disposal techniques and spills of all types of petroleum products, widespread contamination of toluene has occurred in soil, sediment and groundwater. The relatively high aqueous solubility of toluene of 515 mg/liter at 20 °C (39) accounts for its mobility in the environment. Due to its toxicity, toluene is considered as a priority pollutant by the Environmental Protection Agency (39). Toluene is a depressant of the central nervous system (39), and an enhancing agent in skin carcinogenesis (12).

Microbial degradation of toluene readily occurs under aerobic conditions (32, 33) by a wide variety of aerobic bacteria utilizing several monooxygenases and a dioxygenase to initiate the attack. However, many polluted sites are often depleted of oxygen. Consequently, alternative degradation pathways under anaerobic conditions are important in determining the fate of toluene. In the absence of oxygen, various investigators have shown that toluene degradation is linked to methanogenesis, sulfate-, nitrate-, and iron reduction (16). Recently, toluene degradation was also shown to occur linked to the reduction of manganese oxides (21, 22) and to a fermentative oxidation process with fumarate as a terminal electron acceptor (29). These alternative electron acceptors either occur naturally in

groundwater and sediments (e.g., iron) or are possible additives to stimulate *in situ* biodegradation processes.

In the present study, humus is evaluated as a potential electron acceptor for toluene biodegradation. Humus is the stable organic matter accumulating in sediments and soils (35). Although humus is generally considered to be inert for microbial catabolism, it has recently been reported to play an active role in the anaerobic oxidation of a wide variety of ecologically relevant organic substrates (e.g., acetate, lactate) as well as hydrogen, by serving as a terminal electron acceptor (4, 7, 9, 28). These studies demonstrate that reduction of humic substances may be an important mechanism for organic substrate oxidation in many anaerobic environments. Quinone moieties of humus are implicated as the redox active groups (31) accepting the electrons. Anthraquinone-2,6-disulfonate (AQDS) has been used as a defined model for such moieties (7, 9, 17, 28). Most humus-respiring microorganisms are also capable of transferring electrons to AQDS, reducing it to anthrahydroquinone-2,6-disulfonate (AH_2QDS) and therefore, quinone model compounds imitate the function of humus as terminal electron acceptor. Since reduced humus and hydroquinones are readily oxidized by Fe(III) and Mn(IV) (28, 36), humus only needs to be present at substoichiometric concentrations to be an effective electron acceptor as long as these metal oxides are abundant in the sediment. Thus, humus can link the degradation of substrates to dissimilatory metal reduction.

Aside from the simple substrates initially tested, evidence is accumulating that more complex substrates are degraded by quinone respiration. The anaerobic microbial oxidation of phenol and *p*-cresol in granular sludge was recently found to be coupled to the reduction of AQDS (8). Addition of humic acids or AQDS was also shown to stimulate the mineralization of the priority pollutants, vinyl chloride and dichloroethene, by a humus-respiring consortium under anaerobic conditions (5).

The fact that there are a wide variety of organic compounds, which can be utilized by humus-respiring consortia, leads to the question whether humus can also support the anaerobic oxidation of toluene by serving as a terminal electron acceptor. In this study, the capacity of two different sediments for oxidizing toluene with humic acids or AQDS as terminal electron acceptor was explored. The results constitute a clear quantitative demonstration for the mineralization of an aromatic hydrocarbon priority pollutant by humus respiring microorganisms.

Materials and methods

Sediments. Two different sediments were used for the present study. Petroleum Harbor sediment was dredged from the Amsterdam Petroleum Harbor, which was constructed for storage and transshipment of petroleum and coal. Around the Petroleum Harbor (APH), industrial activities developed and oil tanks were built. At the beginning of World War II, oil storage tanks were destroyed and large quantities of oil leaked into the harbor, causing major oil contamination of the sediment. Diverse other sources, such as industrial discharges, shipping, and tanker cleaning have also contributed to

contamination of the sediment. As a consequence, Amsterdam Petroleum Harbor sediment, referred to as "APH sediment" in this study, is contaminated with oil and polycyclic aromatic hydrocarbons (11). Anaerobic Rhine sediment was collected alongside the banks of the river near Lexkesveer in Wageningen, The Netherlands. This sediment was chosen because toluene, benzene and naphthalene have been detected as contaminants in Rhine water (20). This sediment has been previously shown to degrade aromatic compounds, such as toluene and sulfanilic acid, under different redox conditions (21, 37). Both sources of inoculum were able to oxidize hydrogen and acetate with AQDS as terminal electron acceptor (7).

Sediment incubations. Bicarbonate buffered basal medium (pH 7.2) was prepared as previously described (7). For the present study, the concentrations of NH_4Cl and K_2HPO_4 were modified to 0.1 and 0.05 g per liter, respectively. The basal medium was supplied with one of the following electron acceptors: AQDS (25 mM), nitrate (10 mM), sulfate (6.25 mM). AQDS was previously dissolved in boiled water and then all the components of the basal medium were included. The medium was cooled in a stream of N_2/CO_2 (80:20). All the media were dispensed in 117-ml glass serum bottles after being flushed with N_2/CO_2 (80:20) at the final volume of 50 ml (67 ml as headspace) and then inoculation took place by adding 10 g (dry weight) per liter of previously homogenized sediment. The vials were sealed with Viton stoppers (Maag Technic AG, Dübendorf, Switzerland) and aluminum crimps and were flushed with N_2/CO_2 (80:20). Sulfate and nitrate were added from anaerobic and sterilized stock solutions in distilled water. Toluene (1 mM final concentration) was added from a stock solution in hexadecane. Hexadecane did not exceed 0.2 % (v/v) of the liquid volume in the bioassays. Biodegradation of toluene was also confirmed in the absence of hexadecane, but the results presented in this study came from experiments in which toluene was added in hexadecane to facilitate minimal handling error during its addition. All the bioassays were statically incubated in a 30 °C room and were manually shaken before sampling to ensure homogenous distribution of toluene. Sterile controls were prepared under the same conditions and autoclaved for 20 minutes at 120 °C two times prior to addition of toluene. Controls without toluene addition, but with the same amount of hexadecane added, were also included to correct for the endogenous reduction of the different electron acceptors provided and to verify the absence of hexadecane metabolism. All the experiments were applied in triplicate incubations for all the conditions studied. Toluene degradation and reduction of the corresponding electron acceptor was followed in time as described below.

Metal oxides as terminal electron acceptors for the anaerobic toluene degradation. The capacity of APH sediment for degrading toluene with insoluble metal oxides as terminal electron acceptors was also explored. Vernadite (amorphous MnO_2) and Goethite (amorphous FeOOH) were prepared as previously described (2, 19). The metal oxide suspensions were washed 3 times by centrifugation and resuspended in distilled water. Finally, the metal oxides were suspended in basal medium to obtain a final concentration of 25 mM and 50 mM of Mn(IV) and Fe(III), respectively. Bicarbonate

concentration was set at 2.5 g per liter in these experiments and HEPES (50 mM, pH 7.2) was included as a buffer. The metal suspensions were flushed with N_2/CO_2 (80:20) and homogeneously distributed in 117-ml glass serum bottles at the final volume of 50 ml (67 ml as headspace). The vials were inoculated with 10 g (dry weight) per liter of APH sediment and sealed with Viton stoppers and aluminum crimps. All the bioassays were conducted in a N_2/CO_2 (80:20) atmosphere. When the impact of humic substances on the biodegradation of toluene with metal oxides was studied, humic acids (Janssen Chimica Belgium, 2 g per liter) were added to the medium and distributed in the same form as described above. Toluene was added to the cultures from a stock solution in hexadecane. Sterile and endogenous controls were prepared in the same manner as described for the bottles with alternative electron acceptors evaluated and all bioassays were incubated under the same conditions as described above. Toluene degradation was followed in time as described below and the reduction of the metal oxides was also measured at the end of the experiment as described below.

Mineralization of [^{13}C]toluene with AQDS and humic substances as terminal electron acceptor. Bioassays in which anaerobic degradation of toluene was observed coupled to the reduction of AQDS were decanted and refilled with anaerobic fresh medium (containing 25 mM of AQDS) in a N_2/H_2 (95:5) atmosphere. The vials were sealed again with Viton stoppers and aluminum crimps and flushed with N_2/CO_2 (80:20) before adding more toluene (1 mM). The bioassays were refilled 3 times (when all toluene had been depleted) in the same way before transferring the sediment to the vials for the studies with uniformly labeled [^{13}C]toluene. The basal medium was prepared without bicarbonate addition for the studies with [^{13}C]toluene and amended with AQDS (5 mM) or with highly purified soil humic acids (HPSHA, 12 g per liter) obtained from the International Humic Substances Society (IHSS). The media were neutralized by adding sodium hydroxide or hydrochloric acid and buffered with sodium phosphate (10 mM, pH 7.2). The media were homogeneously dispensed into 57-ml glass serum bottles (final volume 25 ml with a headspace of 32 ml) and the enriched sediment was added at 10 g (dry weight) per liter in the bioassays under anaerobic conditions. The vials containing the enriched sediment were flushed with pure nitrogen gas and then uniformly labeled [^{13}C]toluene was added from a stock solution in anaerobic and sterile distilled water. All the experiments were applied in triplicate incubations for all the conditions studied. All the bioassays were statically incubated in a 30 °C room and were manually shaken before sampling to ensure homogenous distribution of toluene. The production of $^{13}CO_2$ from [^{13}C]toluene and the depletion of [^{13}C]toluene was monitored in time as described below. The electrons transferred to AQDS and to HPSHA during [^{13}C]toluene degradation was also followed as described below. Sterile controls were prepared under the same conditions and autoclaved for 20 minutes at 120 °C two times prior to addition of [^{13}C]toluene. Controls without [^{13}C]toluene addition were also included to correct for the background level of $^{13}CO_2$ and reduction of AQDS and humus by endogenous substrates in the enrichment culture.

Analytical techniques. The toluene concentrations in 100 µl headspace samples were determined by gas chromatography (Hewlett Packard Series II 5890) and a flame ionization detector. The chromatograph was equipped with a CP-sil 8CB column and helium (4.3 ml per min) was used as a carrier gas. The temperature of the injection port, oven and detector, were 225, 120 and 225 °C, respectively. Standards were prepared in basal medium containing the same amount of sediment (10 g dry weight per liter) used for the experiments and therefore, reflect the equilibrium in toluene concentrations between the headspace and the sediment. Toluene was added to the standard bottles from a stock solution in hexadecane. The standard bottles were previously autoclaved for 20 minutes at 120 °C two times and incubated at 30 °C overnight before adding toluene (4 hrs before analysis).

Concentrations of AH_2QDS were determined spectrophotometrically by monitoring absorbance at 450 nm in an anaerobic chamber as previously described (7). Mn(II) production was estimated by measuring the accumulation of soluble manganese in 0.5 N hydrochloric acid at the end of the experiment as previously described (25). Samples were collected in an anaerobic chamber with a N_2/H_2 (96:4) atmosphere. After 30 min, acidified culture medium (1 ml) was filtered through a 0.2 µm filter and properly diluted before determining the concentration of Mn(II) by atomic adsorption spectroscopy (SpectrAA-300, Varian Nederland B. V.). An air-acetylene flame was used and the wavelength was at 403.1 nm with a lamp current of 5 mA. Fe(II) production was determined by measuring the accumulation of HCl-soluble Fe(II) at the end of the experiment. As previously described (24), the amount of Fe(II) that was soluble after a 30-min extraction in 0.5 N hydrochloric acid was determined with ferrozine. Samples for Fe(II) determinations were also collected in an anaerobic chamber with a N_2/H_2 (96:4) atmosphere. Methane production was determined as previously described (7).

Electrons transferred to humic substances were quantified as previously described (28). Samples were collected in an anaerobic chamber with a N_2/H_2 (96:4) atmosphere and filtrated through a 0.2 µm-pore-diameter filter. Anaerobic Fe(III)-citrate solution (10 mM final concentration) was added to filtrates and after 30 min of reaction, sub-samples were taken for Fe(II) determination. When no Fe(III)-citrate was added to liquid samples and Fe(II) determinations were carried out, negligible recovery of electrons was achieved beyond the endogenous control indicating the lack of iron bound in the sources of humus applied.

Sulfate concentrations were determined by injecting 30 µl samples by an auto-sampler (Marathon) in a HPLC (high performance liquid chromatography) equipped with a VYDAC ion chromatography column (302 IC, 250 x 4.6 mm). The temperature of the column and detector (Waters 431 conductivity detector) were 20 and 35 °C, respectively. As eluent 0.018 M potassium biphthalate, at a rate of 1.2 ml per min, was used. Samples for sulfate analysis were fixed by 2- to 4-fold dilution with a 0.1 M zinc acetate solution, centrifuged (10000 g, 3 min) and diluted with demineralized water. Nitrate and nitrite concentrations were also determined by HPLC equipped with the same column used for sulfate

analysis and at the same temperature. 30 µl samples were also injected by an auto-sampler (Marathon). Potassium dihydrogen phosphate (10 g per liter, pH 3) adjusted by phosphoric acid was used as eluent at a flow rate of 1.5 ml per min. Nitrate and nitrite were detected by ultra violet detector (783 UV Detector-Kratos Analytical USA) at a wavelength of 205 nm. All samples were centrifuged (10000 g, 3 min) before analysis.

Production of $^{13}CO_2$ from [^{13}C]toluene was quantified based on the ratio of $^{13}CO_2$ to $^{12}CO_2$ in 100 µl headspace samples. Carbon has two stable isotopes, with ^{12}C comprising 98.89 % and ^{13}C comprising 1.11 % of the total abundance (14). Samples were injected in a gas chromatograph (Hewlett Packard 5890 Series II) equipped with a fused silica capillary column (PoraplotQ, Chrompack, the Netherlands), which was connected to a mass spectrometer selective detector (Hewlett Packard 5971 Series). Helium was used as a carrier gas at a flow rate of 1.5 ml per min. The temperature of the injector port and detector were 100 and 280 °C, respectively. The oven temperature was maintained at 40 °C during the first 3 min and then gradually (20 °C per min) increased to 240 °C for achieving [^{13}C]toluene quantification in the same samples. The extent of mineralization of [^{13}C]toluene was calculated according to the concentrations of $^{13}CO_2$ measured in the headspace, which were corrected for the theoretical amount of $^{13}CO_2$ dissolved in the liquid phase based on Henry's law. This was corroborated by taking representative bioassays at the end of the experiments from which total recovery of $^{13}CO_2$ was achieved by acidification with concentrated hydrochloric acid. The data obtained from these representative cultures were very closely related (more than 90 % of similarity) to those theoretically calculated.

Chemicals. AQDS was purchased from Aldrich Chemical (Milwaukee, Wis.). Toluene (99.5 %) and humic acid sodium salt were purchased from Janssen Chimica (Geel, Belgium). Hexadecane (99 %) was purchased from Acros Organics (Geel, Belgium). Uniformly labeled [^{13}C]toluene (99 % ^{13}C] was purchased from Campro Scientific (Veenendaal, The Netherlands). Highly purified soil humic acids were purchased from the IHSS. The elemental composition of this soil humic acids was as follows (in % of dry weight): carbon, 58.1; hydrogen, 3.7; oxygen, 34.1; nitrogen, 4.1; sulfur, 0.4; and it had a phenolic-OH content of 1.73 mol per Kg of dry humus. Further information can be obtained at the website of the IHSS (http://www.ihss.gatech.edu). All other chemicals were obtained from Merck (Damstadt, Germany).

Results

Biodegradation of toluene with alternative electron acceptors. APH sediment degraded toluene in the absence of oxygen when AQDS was included in the medium. During the initial exposure, toluene (1 mM) was completely eliminated after 2 months of incubation (with a lag phase of 40 days) and there was a concomitant reduction of AQDS to AH_2QDS. When these bioassays were decanted and refilled with fresh medium containing AQDS (25 mM) and toluene (1 mM), the lag phase was

significantly decreased and the same rate of toluene degradation was observed (Fig. 1A). There was no significant toluene disappearance when bicarbonate was provided as a sole electron acceptor nor was methane production detectable. If toluene was incubated with AQDS in autoclaved sediment, no significant loss of toluene was observed. In the biologically active sediment, the toluene consumption agreed with the reduction of AQDS (Fig. 1B). The ratio of AQDS reduction (corrected for the endogenous control) to toluene degradation was 20.2±5.2 (mean ± standard error; n=3), which is very close to the stoichiometric value (Table 1) suggesting that toluene was probably completely converted to carbon dioxide under these conditions. Only negligible endogenous AQDS reduction occurred when toluene was omitted in the cultures, but including the same amount of hexadecane (0.2 % v/v). No reduction of AQDS was detected in the sterilized control.

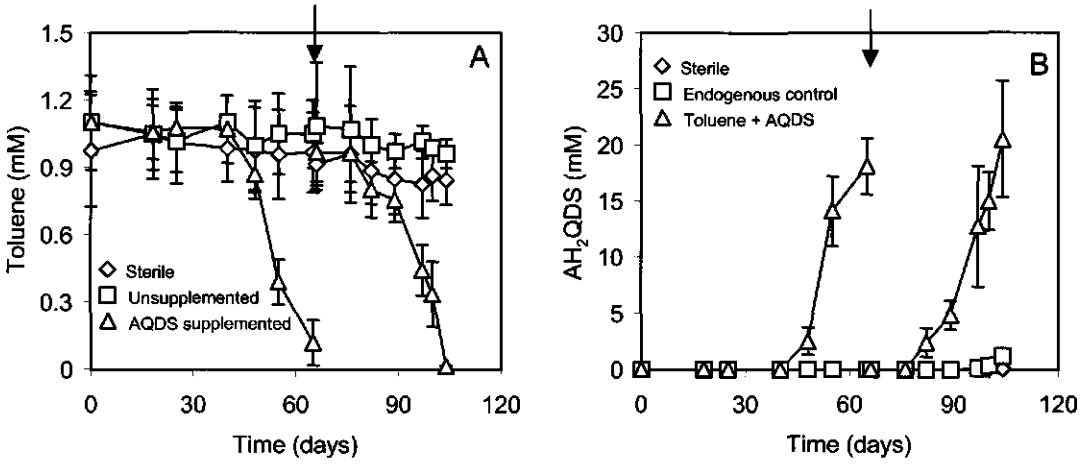


Figure 1. Simultaneous toluene conversion (A) and AQDS reduction (B) by APH sediment in anaerobic culture bottles containing bicarbonate-buffered basal medium supplemented with 25 mM of AQDS. The unsupplemented control was prepared in the same manner without AQDS. Endogenous control (without toluene addition) containing the same amount of hexadecane (0.2 % v/v) used for toluene addition. AQDS reduction was quantified spectrophotometrically as the increase in absorbance at 450 nm. Data are means ± standard deviation for triplicate incubations in each treatment. Arrows indicate addition of fresh medium containing AQDS and toluene in depleted bioassays.

The possibility that toluene degradation in APH sediment could also be linked to the reduction of other anoxic electron acceptors was explored. Of all the alternative electron acceptors tested, only nitrate, Mn(IV) and AQDS supported toluene degradation. No toluene degradation was detected under sulfate reducing or methanogenic conditions after 4 months of incubation. Also no degradation of toluene was observed when Fe(III) in the form of goethite was used as a direct electron acceptor during the same incubation time. These results coincided with the absence of methane production and Fe(II) production as well as the lack of sulfate elimination during the experiments.

Toluene conversion agreed with the reduction of nitrate by APH sediment and the ratio of nitrate reduction (corrected for the endogenous control) to toluene degradation was 5.9±0.7 (mean ± standard error; n=3), which is very closely related to the stoichiometric value (Table 1). Toluene conversion by APH sediment was also evident with the addition of amorphous MnO_2 in the medium. Parallel with toluene conversion in the MnO_2-supplemented cultures was the partial recovery of acid-extractable Mn(II) accounting for 40 % of electron equivalents in toluene consumed.

Table 1. Thermodynamic comparison for the biodegradation of toluene with alternative electron acceptors (values calculated with data from references 20, 34, 38)

Reaction	$\Delta G^{o'}$ (kJ/mol)
$C_7H_8 + 36\ Fe^{3+} + 21\ H_2O \rightarrow 36\ Fe^{2+} + 43\ H^+ + 7\ HCO_3^-$	- 3629.6
$C_7H_8 + 7.2\ NO_3^- + 0.2\ H^+ \rightarrow 3.6\ N_2 + 0.6\ H_2O + 7\ HCO_3^-$	- 3554.8
$C_7H_8 + 18\ MnO_2 + 18\ H_2CO_3 \rightarrow 7\ CO_2 + 18\ MnCO_3 + 22\ H_2O$	- 3358.8[a]
$C_7H_8 + 36\ FeO(OH) + 36\ H^+ \rightarrow 7\ CO_2 + 36\ Fe(OH)^+ + 22\ H_2O$	- 1443.6[a]
$C_7H_8 + 18\ AQDS + 21\ H_2O \rightarrow 18\ AH_2QDS + 7\ H^+ + 7\ HCO_3^-$	- 319.7
$C_7H_8 + 4.5\ SO_4^{2-} + 3\ H_2O \rightarrow 4.5\ HS^- + 2.5\ H^+ + 7\ HCO_3^-$	- 205.2
$C_7H_8 + 7.5\ H_2O \rightarrow 4.5\ CH_4 + 2.5\ H^+ + 2.5\ HCO_3^-$	- 130.7

[a]Solid-phase free energies were used

Humic acid stimulation of toluene biodegradation linked to metal oxides reduction. To explore the potential link between biodegradation of toluene and dissimilatory reduction of metal oxides by channeling the electrons via humus respiration, APH sediment incubations were supplemented either with goethite (FeOOH, 50 mM) or with vernadite (MnO_2, 25 mM) together with a substoichiometric amount of humic acids (Janssen Chimica Belgium, 2 g per liter). The electron accepting capacity of Janssen humic acids was determined as previously described (28) with an acetate-oxidizing humus-respiring enrichment culture indicating an average electron uptake of 0.306 milliequivalents per g of humic acids (Janssen). Namely, addition of Janssen humic acids at this level could only account for the biodegradation of 1.7 % of the toluene added in the cultures (1 mM). Nevertheless if these low levels of humic acid were added, more than 65 % of the toluene was depleted in the goethite-humus supplemented bioassays by APH sediment after 11 weeks of incubation (Fig.2A). The consumption of toluene in these cultures paralleled the partial recovery of electron equivalents as acid-extractable Fe(II), accounting for 30 % of the electron equivalents in the toluene consumed. Negligible conversion of toluene and release of acid-extractable Fe(II) occurred in the goethite-supplemented cultures when humic acids were omitted from the medium. Likewise, none of these phenomena appeared in sterilized incubations with autoclaved sediment supplemented with goethite and humic acids (Fig. 2A).

When the same source of humic acids was applied in vernadite-supplemented cultures at the same level, toluene conversion proceeded with a shorter lag phase time compared to that observed in the absence of humic acids (Fig. 2B). Toluene was completely depleted in both cases after 5 weeks of

incubation and this coincided with the partial recovery of acid-extractable Mn(II), accounting for 34 % of electron equivalents in toluene consumed.

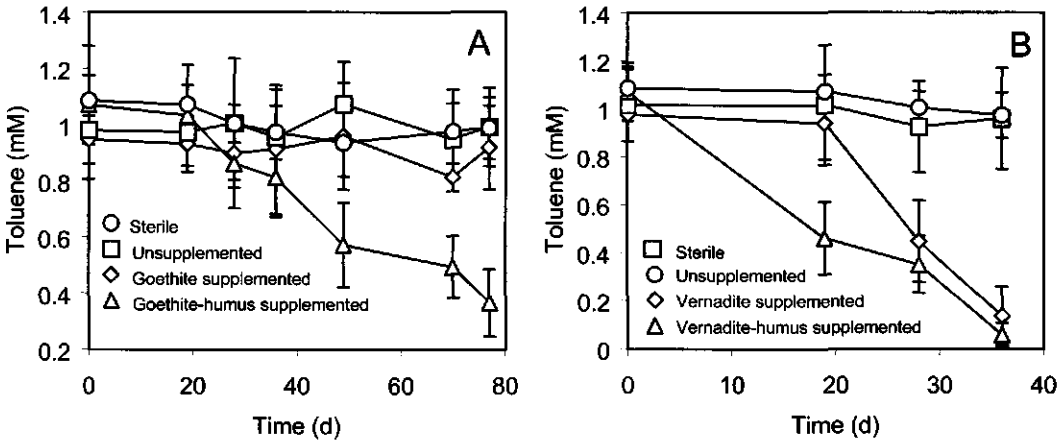


Figure 2. Conversion of toluene by APH sediment in anaerobic culture bottles containing HEPES-buffered basal medium supplemented with (A) amorphous ferric oxyhydroxide (goethite, 50 mM) or (B) amorphous manganese dioxide (vernadite, 25 mM). Goethite-humus and vernadite-humus supplemented cultures also containing 2 g humic acids (Janssen Chimica Belgium) per liter. Unsupplemented controls prepared in the same manner without metal oxide and humus. Sterile controls containing both metal oxide and humus with autoclaved sediment. Data are means ± standard deviation for triplicate incubations in each treatment.

[^{13}C]toluene conversion to $^{13}CO_2$ with AQDS and humic substances as terminal electron acceptor. To confirm mineralization of toluene to CO_2 under anoxic quinone and humus respiring conditions, enrichment cultures from sediment samples were incubated with uniformly labeled [^{13}C]toluene. Enriched APH sediment was able to convert [^{13}C]toluene to $^{13}CO_2$ in medium supplemented with AQDS (5 mM) or with HPSHA (12 g per liter) without any lag phase (Fig. 3A). There was negligible recovery of $^{13}CO_2$ in the endogenous control in the absence of [^{13}C]toluene and in the presence of [^{13}C]toluene and HPSHA incubated with autoclaved sediment. In the absence of AQDS and HPSHA, less than 7 % of the added [^{13}C]toluene was recovered as $^{13}CO_2$ probably due to the presence of small amounts of AQDS remaining in the sediment from previous enrichment. This was confirmed by the slight orange color developed in these controls and by the slight reduction of Fe(III)-citrate by the culture fluid from these controls (Table 2). The conversion of [^{13}C]toluene to $^{13}CO_2$ by APH sediment was concomitantly coupled to an increase in electrons recovered as AH_2QDS or as reduced humus in the cultures (Fig. 3B). In fact, there was a high recovery of both [^{13}C]carbon and electrons in the AQDS and HPSHA cultures (Table 2). Enriched sediment obtain from the Rhine river was also able to convert [^{13}C]toluene to $^{13}CO_2$ with AQDS or HPSHA as terminal electron acceptor, but the rate of toluene mineralization was slower compared to that observed with enriched

APH sediment (Fig. 3C). Controls showed no significant recovery of $^{13}CO_2$ and [^{13}C]toluene conversion. The extent of [^{13}C]toluene mineralization observed (about 1.8±0.1 milliequivalents per liter in both cases) paralleled the stoichiometric recovery of electrons as AH_2QDS or as reduced humus (Fig. 3D). There was negligible recovery of electrons in the sterilized and endogenous (without [^{13}C]toluene addition) controls.

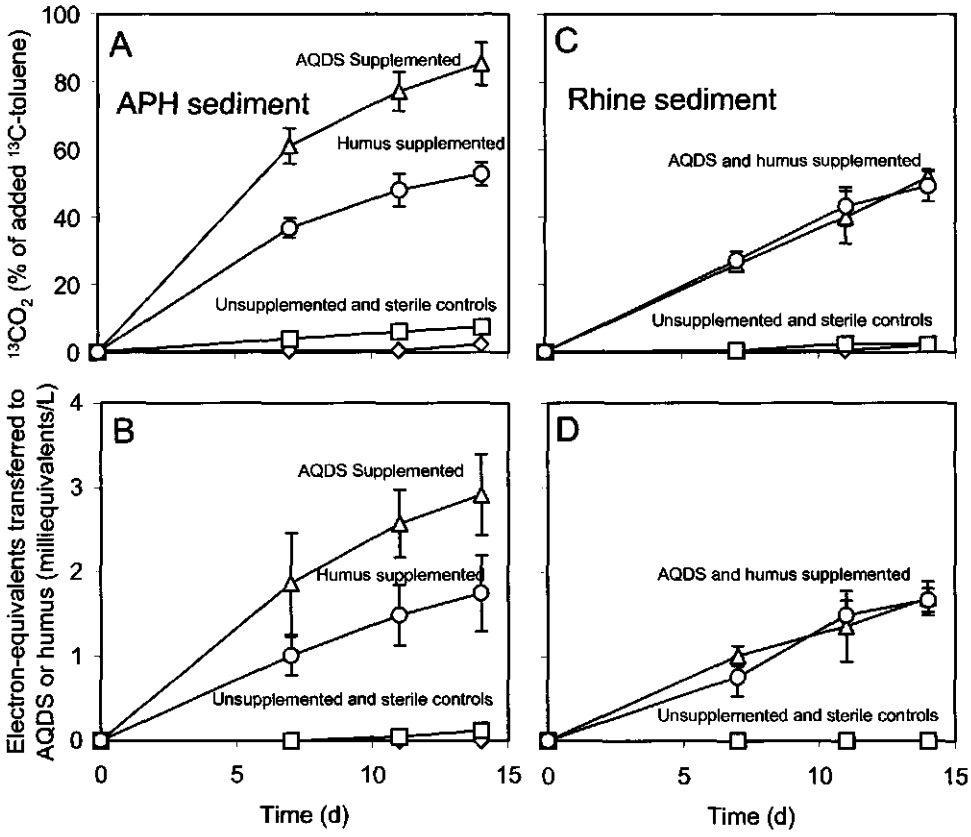


Figure 3. Mineralization of [^{13}C]toluene to $^{13}CO_2$ (A and C) coupled to the reduction of AQDS or humus (B and D) by enriched APH (A and B) or Rhine (C and D) sediments in anaerobic culture bottles containing phosphate-buffered basal medium supplemented with AQDS (5 mM) or with highly purified soil humic acids (12 g per liter). Uniformly labeled [^{13}C]toluene added at the initial concentration of 100 µM referred to the liquid volume. Unsupplemented control prepared in the same manner without AQDS and humus. All data corrected for the endogenous control (without [^{13}C]toluene addition). Data are means ± standard deviation for triplicate incubations in each treatment.

Discussion

Humic substances as a terminal electron acceptor for the anoxic microbial oxidation of toluene. In the present study humic acids and the humic model compound, AQDS, were explored as potential

electron acceptors to achieve anoxic microbial oxidation of toluene by different inocula. Toluene biodegradation was coupled to the reduction of humic acids and AQDS by APH and Rhine sediments. The results from this study demonstrate multiple evidences that the humic compounds are implicated in the anoxic biodegradation of toluene. Firstly, toluene biodegradation became feasible when the anaerobic sediments were supplied with HPSHA and AQDS. Secondly, the electron equivalents from the consumed toluene were highly recovered as AH_2QDS (85 %) and reduced humic acids (65 %), respectively when AQDS and HPSHA served as the terminal electron acceptors. Thirdly, uniformly labeled [^{13}C]toluene was mineralized to $^{13}CO_2$ and the recovery of ^{13}C-labeled carbon as $^{13}CO_2$ accounted for 74-91 % of the [^{13}C]toluene consumed. The results constitute a clear quantitative demonstration of anoxic aromatic hydrocarbon biodegradation linked to the reduction of quinones and humic acids.

Previously, Lovley *et al.* (28) hypothesized that humus had served as a direct electron acceptor during benzene biodegradation when humic acids were added as chelators to increase Fe(III) oxide bioavailability for a benzene-degrading Fe(III)-reducing consortium in contaminated sediment. This hypothesis was based on the observation that humic acids stimulated benzene biodegradation better than synthetic chelators (e.g. EDTA and NTA) even though humus had inferior chelating properties (27). The mechanism proposed implies that benzene had been degraded with humic substances acting as the direct electron acceptor and the obtained reduced humus had been recycled back to the oxidized form by chemical reaction with Fe(III) oxides. The impact of AQDS on the anaerobic benzene oxidation was also studied in three different sites of Fe(III)-reducing sediments (1). Stimulation of benzene oxidation was observed at one site when 600 μM AQDS was applied, which may have been due to the use of AQDS as an electron acceptor, but the reduction of AQDS was not demonstrated. The same sediment sample did not oxidize benzene when 300 μM AQDS was applied, yet the amount of benzene added (12 μM) would have only required 180 μM AQDS for complete oxidation. Strains of *Geobacter* have been isolated that can oxidize toluene with Fe(III) as an electron acceptor (10). The same strains can also reduce AQDS with acetate; thus, it is conceivable that they could couple toluene oxidation to AQDS reduction.

The Gibbs free energy of toluene degradation linked to AQDS reduction is more favorable than degradation linked to sulfate reduction and methanogenesis (Table 1). Thermodynamic differences might partly explain why toluene degradation in this study readily occurred with AQDS, but not with sulfate or bicarbonate as electron acceptors. Biodegradation of toluene coupled to sulfate reduction (3, 30) and methanogenesis (13, 18, 40) has previously been reported to occur but these processes usually require long lag periods before rates become appreciable.

The anoxic biodegradation of toluene with humic substances as terminal electron acceptors was not evident at all sites but only found in historically polluted sites indicating long-term enrichment of hydrocarbon-degrading microorganisms after prolonged exposure to aromatic hydrocarbon pollutants.

Sludge, soil and sediment material from pristine sites previously reported to degrade readily biodegradable compounds with AQDS as terminal electron acceptor (7) were not able to degrade toluene under AQDS-reducing conditions (data not shown).

Table 2. Balances of electrons and [^{13}C]carbon for the anaerobic conversion of uniformly labeled [^{13}C]toluene with anthraquinone-2,6-disulfonate (AQDS) and highly purified soil humic acids (HPSHA) as terminal electron acceptors by enriched APH sediment after 2 weeks of incubation[a]

Culture	[^{13}C]toluene added	Products and remaining toluene		Total recovery (%)[b]
Electron Equivalent Balance (in milliequivalents per liter)[c]				
Unsupplemented	3.7	[^{13}C]toluene remaining	3.4	
		Fe(III)-citrate reduced by liquid fluid	0.1	95
		Total	3.5	
AQDS-supplemented	3.4	[^{13}C]toluene remaining	ND[d]	
		AH_2QDS	2.9	85
		Total	2.9	
HPSHA-supplemented	3.5	[^{13}C]toluene remaining	0.9	
		Fe(III)-citrate reduced by liquid fluid	1.7	74
		Total	2.6	
[^{13}C]Carbon Balance (in mmol ^{13}C per liter)[e]				
Unsupplemented	0.75	[^{13}C]toluene remaining	0.69	
		$^{13}CO_2$	0.05	99
		Total	0.74	
AQDS-supplemented	0.69	[^{13}C]toluene remaining	ND	
		$^{13}CO_2$	0.63	91
		Total	0.63	
HPSHA-supplemented	0.71	[^{13}C]toluene remaining	0.18	
		$^{13}CO_2$	0.39	80
		Total	0.57	

[a]Data represent mean values obtained from triplicate incubations for the different conditions applied and standard deviations were in general within 10 % of the mean value. Negligible conversion of [^{13}C]toluene and reduction of the corresponding electron acceptor occurred in sterilized incubations with autoclaved sediment.
[b]Total recovery of electrons = (electrons recovered in electron acceptor + [^{13}C]toluene not consumed)/([^{13}C]toluene added). Total recovery of [^{13}C]carbon = (carbon recovered as $^{13}CO_2$ + [^{13}C]toluene not consumed)/([^{13}C]toluene added).
[c]Corrected for endogenous controls. Less than 2 % of endogenous reduction occurred in all cases.
[d]ND, not detected.
[e]Corrected for the background level of $^{13}CO_2$ found in the absence of [^{13}C]toluene.

APH sediment showed the capacity to utilize other more favorable electron acceptors (nitrate and Mn(IV)) to support biodegradation of toluene reflecting that this consortium may contain a wide variety of microorganisms with different capacities to degrade aromatic hydrocarbons or that

microorganisms involved in toluene biodegradation may achieve this anoxic process with different electron acceptors. Other consortia have previously showed the capacity to degrade toluene with both nitrate and Mn(IV) as a terminal electron acceptor (23).

Humic acid stimulation of toluene biodegradation linked to metal oxides reduction. Goethite was not utilized directly as an electron acceptor by APH sediment to achieve anoxic biodegradation of toluene. Conversion of toluene was only made feasible by supplementing the goethite-containing cultures with substoichiometric levels of humic acid in terms of electron accepting equivalents. The electron accepting capacity of Janssen humic acids could only account for 1.7 % of the potentially degradable toluene and yet 65 % of the toluene was degraded in these experiments. The stimulation can thus only be accounted for by a chelating effect of humic acids with Fe(III) (27) or a redox mediating effect (28). Based on previous observations in the literature that demonstrate the involvement of humic substances as redox mediators linking the oxidation of simple substrates (e.g. acetate) to goethite reduction (17, 26, 28), it is plausible that goethite reduction by toluene degraders in APH sediment was a result of reduced humic acids acting as electron shuttles in the goethite-humus bioassays. Non-iron reducing bacteria, e.g. *Propionibacterium freudenreichii*, were recently reported to channel electrons from anaerobic oxidations via humic acids towards Fe(III) reduction, suggesting that dissimilatory iron reduction in soil and sediments may not be exclusively related to iron-reducing microorganisms (4). Hydroquinones in humus can reach micropores that remain inaccessible to Fe(III)-reducing microorganisms (41) and may eliminate the need of direct contact between humus-reducing microorganisms and metal oxides as a pre-requisite for achieving anoxic organic matter oxidation.

The partial recovery of electron equivalents from converted toluene either as Mn(II) or Fe(II) in the metal oxides-humus-supplemented cultures may be explained by a series of post-reduction biogeochemical reactions. Biogenic Fe(II) and Mn(II) might have undergone sorption to bacteria or to the residual metal oxide surface, as well as precipitation with sulfide (6, 42), which may have accounted for a decreased recovery during the acid extraction technique applied.

Ecological implications. The results presented in this study for toluene and previous results with vinyl chloride and dichloroethene (5) suggest that humus, the most abundant organic fraction in nature, may be a more important electron acceptor for bioremediation of contaminated environments than previously thought. Biodegradation of recalcitrant contaminants may take place in organic rich sediments, wetlands, eutrophic lakes, and in microniches in compost, where humic substances could serve as a potential electron acceptor for the anoxic microbial oxidation of a wide variety of organic pollutants. Moreover, quinone or humus reducing bacteria and activities have previously been found in many organic matter rich environments (7, 9). Therefore, intrinsic bioremediation may be much larger than previously considered in these habitats. Humic substances may also greatly stimulate the anoxic biodegradation of organic contaminants in oligotrophic environments as well by linking the

biodegradation of these pollutants to the reduction of other electron acceptors. Particularly, quinones in humus may channel electrons from anoxic pollutant oxidation to metal oxide reduction by serving as redox mediators, which was shown to be the case for the anoxic oxidation of methyl *tert*-butyl ether (15).

Acknowledgments

This research was financially supported by Council of Science and Technology of Mexico (CONACyT). We thank Wim Roelofsen for technical assistance during measurements of labeled toluene and Alfons Stams for critical review.

References

1. Anderson, R. T. and D. R. Lovley. (1999) Naphthalene and benzene degradation under Fe(III)-reducing conditions in petroleum-contaminated aquifers. *Bioremediat. J.* **3,** 121-135.
2. Atkinson, R. J., A. M. Posner and J. P. Quirk. (1967). Adsorption of potential-determining ions at the ferric oxide-aqueous electrolyte interface. *J. Phys. Chem.* **71,** 550-558.
3. Beller, H. R., A. M. Spormann, P. K. Sharma, J. R. Cole and M. Reinhard. (1996) Isolation and characterization of a novel toluene-degrading sulfate-reducing bacterium. *Appl. Environ. Microbiol.* **62,** 1188-1196.
4. Benz, M., B. Schink and A. Brune. (1998) Humic acid reduction by *Propionibacterium freudenreichii* and other fermentative bacteria. *Appl. Environ. Microbiol.* **64,** 4507-4512.
5. Bradley, P. M., F. H. Chapelle and D. R. Lovley. (1998) Humic acids as electron acceptors for anaerobic microbial oxidation of vinyl chloride and dichloroethene. *Appl. Environ. Microbiol.* **64,** 3102-3105.
6. Burdige, D. J. and K. H. Nealson. (1986) Chemical and microbiological studies of sulfide-mediated manganese reduction. *Geomicrobiol. J.* **4,** 361-387.
7. Cervantes, F. J., S. van der Velde, G. Lettinga and J. A. Field. (2000) Competition between methanogenesis and quinone respiration for ecologically important substrates in anaerobic consortia. *FEMS Microbiol. Ecol.* **34,** 161-171.
8. Cervantes, F. J., S. van der Velde, G. Lettinga and J. A. Field. (2000). Quinones as terminal electron acceptors for anaerobic microbial oxidation of phenolic compounds. *Biodegradation* **11,** 313-321.
9. Coates, J. D., D. J. Ellis, E. Roden, K. Gaw, E. L. Blunt-Harris and D. R. Lovley. (1998) Recovery of humics-reducing bacteria from a diversity of sedimentary environments. *Appl. Environ. Microbiol.* **64,** 1504-1509.

10. Coates, J. D., V. K. Bhupathiraju, L. A. Achenbach, M. J. Mclnerney and D. R. Lovley. (2001) *Geobacter hydrogenophilus, Geobacter chapellei* and *Geobacter grbiciae*, three new, strict anaerobic, dissimilatory Fe(III)-reducers. *Int. J. Syst. Evol. Microbiol.* **51,** 581-588.

11. Cuypers, M. P., J. T. C. Grotenhuis and W. H. Rulkens. (1998) Characterisation of PAH-contaminated sediments in a remediation perspective. Wat. Sci. Technol. **37:**157-164.

12. Dean, B. J. (1978) Genetic toxicology of benzene, toluene, xylenes and phenols. *Mutat. Res.* **47,** 75-97.

13. Edwards, E. A. and D. Grbiæ- Grbiæ. (1994) Anaerobic degradation of toluene and *o*-xylene by a methanogenic consortium. *Appl. Environ. Microbiol.* **60,** 313-322.

14. Faure, G. (1986) Principles of isotope geology. John Wiley and Sons. New York.

15. Finneran, K. T. and D. R. Lovley. (2001) Anaerobic degradation of methyl *tert*-butyl ether (MTBE) and *tert*-butyl alcohol (TBA). *Environ. Sci. Technol.* **35,** 1785-1790.

16. Frazer, A. C., P. W. Coschigano and L. Y. Young. (1995) Toluene metabolism under anaerobic conditions: A review. *Anaerobe* **1,** 293-303.

17. Fredrickson, J. K., H. M. Kostandarithes, S. W. Li, A. E. Plymale and M. J. Daly. (2000) Reduction of Fe(III), Cr(VI), U(VI), and Tc(VII) by *Deinococcus radiodurans* R1. *Appl. Environ. Microbiol.* **66,** 2006-2011.

18. Grbiæ- Grbiæ, D. and T. M. Vogel. (1987) Transformation of toluene and benzene by mixed methanogenic cultures. *Appl. Environ. Microbiol.* **53,** 254-260.

19. Kostka, J. and K. H. Nealson. (1998). Isolation, cultivation and characterization of iron- and manganese-reducing bacteria, p. 58-78. *In* R. S. Burlage, R. Atlas, D. Stahl, G. Geesey and G. Sayler (ed.), Techniques in microbial ecology, Oxford University Press, New York.

20. Langenhoff, A. A. M. (1997) Ph.D. thesis. Biodegradation of toluene, benzene and naphthalene under anaerobic conditions. Wageningen University, Wageningen, The Netherlands.

21. Langenhoff, A. A. M., A. J. B. Zehnder and G. Schraa. (1996) Behavior of toluene, benzene and naphthalene under anaerobic conditions in sediment columns. *Biodegradation* **7,** 267-274.

22. Langenhoff, A. A. M., D. L. Brouwers-Ceiler, J. H. L. Engelberting, J. J. Quist, J. G. P. N. Wolkenfelt, A. J. B. Zehnder and G. Schraa. (1997) Microbial reduction of manganese coupled to toluene oxidation. *FEMS Microbiol. Ecol.* **22,** 119-127.

23. Langenhoff, A. A. M., M. Briglia, I. Nijenhuis N. C. G. Tan, A. J. B. Zehnder and G. Schraa. (1997) Characterization of a manganese-reducing, toluene-degrading enrichment culture. *FEMS Microbiol. Ecol.* **24,** 113-125.

24. Lovley, D. R. and E. J. P. Phillips. (1986). Availability of ferric iron for microbial reduction in bottom sediments of the fresh-water tidal Potomac River. *Appl. Environ. Microbiol.* **52,** 751-757.

25. Lovley, D. R. and E. J. P. Phillips. (1988) Novel mode of microbial energy metabolism: organic carbon oxidation coupled to dissimilatory reduction of iron and manganese. *Appl. Environ. Microbiol.* **54,** 1472-1480.

26. Lovley, D. R., J. L. Fraga, E. L. Blunt-Harris, L. A. Hayes, E. J. P. Phillips and J. D. Coates. (1998) Humic substances as a mediator for microbially catalyzed metal reduction. *Acta hydrochim. Hydrobiol.* **26,** 152-157.

27. Lovley, D. R., J. C. Woodward and F. H. Chapelle. (1996) Rapid anaerobic benzene oxidation with a variety of chelated Fe(III) forms. *Appl. Environ. Microbiol.* **62,** 288-291.

28. Lovley, D. R., J. D. Coates, E. L. Blunt-Harris, E. J. P. Phillips and J. C. Woodward. (1996) Humic substances as electron acceptors for microbial respiration. *Nature* **382,** 445-448.

29. Meckenstock, R. U. (1999) Fermentative toluene degradation in anaerobic defined syntrophic cocultures. *FEMS Microbiol. Lett.* **177,** 67-73.

30. Rabus, R., R. Nordhaus, W. Ludwig and F. Widdel. (1993) Complete oxidation of toluene under strictly anoxic conditions by a new sulfate-reducing bacterium. *Appl. Environ. Microbiol.* **59,** 1444-1451.

31. Scott, D. T., D. M. McKnight, E. L. Blunt-Harris, S. E. Kolesar and D. R. Lovley. (1998) Quinone moieties act as electron acceptors in the reduction of humic substances by humics-reducing microorganisms. *Environ. Sci. Technol.* **32,** 2984-2989.

32. Smith, M. R. (1990) The biodegradation of aromatic hydrocarbons by bacteria. *Biodegradation* **1,** 191-206.

33. Smith, M. R. (1994) The physiology of aromatic hydrocarbon degrading bacteria, p. 347-378. *In* C. Ratledge (ed.), Biochemistry of microbial degradation. Kluwer Academic Publishers, Dordrecht.

34. Sober, H. A. (1970) Handbook of biochemistry: selected data for molecular biology. The Chemical Rubber Co., Cleveland, Ohio.

35. Stevenson, F. J. (1994) Humus chemistry: genesis, Composition, Reactions, 2nd ed. Wiley, New York.

36. Stone, A. T. and J. J. Morgan. (1984) Reduction of manganese(III) and manganese(IV) oxides by organics. 1. Reaction with hydroquinone. *Environ. Sci. Technol.* **18,** 450-456.

37. Tan, N. C. G., F. X. Prenafeta-Boldú, J. L. Opsteeg, G. Lettinga and J. A. Field. (1999) Biodegradation of azo dyes in cocultures of anaerobic granular sludge with aerobic aromatic amine degrading enrichment cultures. *Appl. Microbiol. Biotechnol.* **51,** 865-871.

38. Thauer, R. K., K. Jungermann and K. Decker. (1977) Energy conservation in chemotrophic anaerobic bacteria. *Bacteriol. Rev.* **41,** 100-180.

39. U.S. Public Health Service. (1989) Toxicological profile for toluene. Publication ATSDR/TP-89/23. Agency for Toxic Substances and Disease Registry, U.S. Public Health Service, Atlanta.

Chapter 7

Abstract

The impact of humic acids and the humic model compound, anthraquinone-2,6-disulfonate (AQDS), on the biodegradation of carbon tetrachloride (CT) by anaerobic granular sludge was studied. Addition of both humic acids and AQDS at sub-stoichiometric levels enhanced the rate of conversion of CT leading to an increased production of inorganic chloride, which accounted for 40-50 % of the CT initially added. Negligible dechlorination occurred in sterile controls with autoclaved sludge or in active controls lacking humic substances. Accumulation of chloroform (1-10 %) and dichloromethane (traces) also accounted for the CT converted. The accumulation of a chlorinated ethene, tetra(per)chloroethylene (PCE, up to 9 % of added CT), is also reported for the first time as an intermediate of CT degradation. The enhanced CT conversion observed could be attributed to humus-respiring bacteria in the sludge as evidence by the selective inhibition of quinone respiration with the antibiotic, neomycin. Also, a humus-respiring enrichment culture (composed primarily of a *Geobacter* sp.) derived from the granular sludge was shown to dechlorinate CT, yielding similar products as the AQDS-supplemented granular sludge consortium.

Introduction

Carbon tetrachloride (CT) is a toxic, carcinogenic compound listed as a priority pollutant by the U. S. Environmental Protection Agency. CT can produce liver and kidney damage in mammals by accidental acute exposure incidents. Chronic exposure of humans to CT has resulted in neurological effects and it also has lethal effects on humans and animals at high doses (*1, 2*). CT was the favorite chlorinated hydrocarbon dry-cleaning agent used until its utilization was banned in the 1950s at which time it was gradually replaced by trichloroethylene (TCE) and tetra(per)chloroethylene (PCE) (*3*). Other industrial activities, such as degreasing processes, usage as a grain fumigant, as well as production of chlorinated paraffin wax and chlorofluorocarbons have also demanded a large amount of this solvent. Improper disposal, leaking storage tanks, and spills during all these activities have led to a widespread contamination of soils, wastewaters, groundwater, sediments, and off-gases by CT.

CT generally resists aerobic biotransformation because it is an oxidized species with its carbon atom having the same oxidation state as CO_2. However, there are a few reports indicating that oxygen substitution of the carbon atom of CT may lead to the formation of CO_2 via carbonyl-containing intermediates (*4*). As a polyhalogenated hydrocarbon, CT readily undergoes chemical and microbial reductive transformations (*5, 6*). Anaerobic biological conversions include different cometabolic reductive pathways in which reactions are catalyzed by reduced cofactors present in microorganisms, such as cytochromes (*7*), cobalamins (*8, 9*), porphyrins (*10, 11*), pyridines (*12*) and factor F_{430} (*13*), or by biogenic inorganic reducing agents, such as pyrite (*14*), Fe(II) and sulfide (*5*). Microorganisms with different physiological characteristics, including methanogens (e.g. *Methanobacterium thermoautotrophicum*) (*15*), acetogens (e.g. *Acetobacterium woodii*) (*8*), nitrate-reducers (e.g.

Pseudomonas stutzeri) (*12*), and iron-reducers (e.g. *Shewanella putrefaciens*) (*7*) were shown to convert CT under axenic conditions. Biodegradation of CT also occurred in mixed cultures containing different consortia under methanogenic- (*16, 17*) and sulfate-reducing (*18*) conditions.

Mechanisms of microbially catalyzed reductive dehalogenation of CT are not well understood and may be species and compound dependent. Most inocula evaluated are capable to reductively convert CT to chloroform (CF). As the number of chlorine substituents decreases, removal of additional chlorine substituents becomes energetically and kinetically more difficult (*6*). However, rapid biological dechlorination of CT to CO_2 and inorganic chloride, with minor accumulation of lower chlorinated methanes, may occur when appropriate conditions prevail. It has been reported that the mechanism of mineralization of CT may proceed through the formation of a trichloromethyl radical followed by its reduction to dichlorocarbene, which subsequently can react with water leading ultimately to the formation of CO_2 via either formate or CO (*19*). Dichlorocarbene may also react with sulfide resulting in CS_2 formation (*17*) and the trichloromethyl radical can be converted to hexachloroethane (HCA) via dimerization reactions (*20, 21*).

Humic substances and quinones, representative of structural moieties in humus, were also shown to mediate the abiotic reductive dehalogenation of polyhalogenated pollutants by inorganic electron donors (*22*). The conversion of HCA to PCE by Fe(II), sulfide or elemental sulfur was significantly stimulated up to 10-fold by the presence of humus or quinones (*5, 23*). The reduced humic model compound, anthrahydroquinone-2,6-disulfonate (AH_2QDS), could also directly cause dechlorination of HCA (*5*). In a similar fashion, quinones and natural organic matter from different sources could also catalyze the abiotic transformation of nitrobenzenes and azo dyes to the corresponding aromatic amines by sulfide (*24, 25*). Although many abiotic experiments have shown that quinones and humic substances can catalyze reductive dehalogenation reactions, there is much less evidence that such compounds are involved in biological dehalogenation. The anaerobic biotransformation of CT by *Shewanella putrefaciens* 200 was recently reported to be accelerated by the presence of high-organic-carbon soil, but the actual functional groups responsible for the enhanced CT transformation rates were not elucidated (*26*). The role of quinones as redox mediators to stimulate the microbial reduction of azo dyes has on the other hand been demonstrated (*27-30*).

In the present study, the impact of humic acids and the humic model compound, anthraquinone-2,6-disulfonate (AQDS), on the dechlorination of CT by anaerobic granular sludge was explored. The results indicate that CT conversion rates and dechlorination efficiency by anaerobic granular sludge were increased by the addition of sub-stoichiometric quantities of AQDS. The enhancement could be attributed to humus respiring bacteria in the sludge.

Materials and methods

Inocula and basal medium. Methanogenic granular sludge from a full-scale upflow anaerobic sludge blanket (UASB) reactor treating effluent from an alcohol distillery of Nedalco (Bergen op Zoom, The Netherlands, referred to as "Nedalco" sludge) and from a full-scale UASB reactor treating wet oxidized industrial effluent of Shell Nederland Chemie (Moerdijk, The Netherlands, referred to as "Shell" sludge) were used for the present study. Both sources of granular sludge were continuously fed in lab-scale UASB reactors with basal medium containing a mixture of acetate (4.45 mM), propionate (0.07 mM), and butyrate (0.05 mM) to obtain active biomass for the batch experiments. The influent was set at the initial concentration of 1.5 g chemical oxygen demand (COD) l^{-1} with the described mixture of volatile fatty acids. The reactors were operated in a 30 °C room with a hydraulic residence time of 6 hours. The COD removal efficiency was higher than 95 % in both reactors under steady state conditions. The inocula were washed and sieved to remove fine particles before use in the batch tests. A humus-respiring enrichment culture obtained from "Nedalco" granular sludge was also used in this study. The enrichment, composed almost exclusively by a *Geobacter sulfurreducens* related species (referred to as *Geobacter* NS1), was shown to degrade acetate, hydrogen and formate using AQDS as terminal electron acceptor. Moreover, the oxidation of acetate was also shown to be linked to humus respiration by the same enrichment (data not shown).

The basal medium used in all batch experiments contained (g l^{-1}): $NaHCO_3$, (5); NH_4HCO_3, (0.04); K_2HPO_4, (0.02); $MgSO_4.7H_2O$, (0.015); $Ca(OH)_2$, (0.003); Na_2S, (0.013); and 1 ml l^{-1} of trace elements. The experiments conducted with the enrichment culture were carried out in basal medium also containing 1 ml l^{-1} of vitamins solution with a composition previously described (*31*).

Bioassays for the reductive biotransformation of CT with quinones and humic acids as redox mediators. The experiments were conducted in batch mode by triplicate in 117-ml serum bottles with a liquid volume of 50 ml (67 ml as headspace). Anaerobic basal medium supplemented with AQDS (5, 20 or 50 μM) or with highly purified soil humic acids (0.5 g l^{-1}) obtained from the International Humic Substances Society (IHSS) was transferred directly to the vials and then, inoculation took place by adding 0.25 g of volatile suspended solids (VSS) l^{-1} in the cultures. The vials were sealed with Viton stoppers (Maag Technic AG, Dubendorf, Switzerland) and aluminum crimps and then flushed with N_2/CO_2 (80/20) for 10 minutes. The pressure was set at 1.5 bars in all the bottles with this gas mixture. Acetate, glucose, or methanol was provided as co-substrate at the final concentration of 1 g COD l^{-1} from stock solutions prepared in sterile anaerobic distilled water. When hydrogen was provided as a co-substrate a headspace of N_2/H_2 (80/20) was established in the bioassays at the final pressure of 1.5 bars. When the effect of different levels of acetate (20, 100 and 500 μM) on the conversion of CT was evaluated, inoculation was provided at 0.1 g VSS l^{-1} to decrease any interference provided by endogenous substrates. Bioassays performed with the enrichment culture obtained from "Nedalco" sludge were inoculated with a washed cells suspension propagated in basal medium amended with

AQDS (5 mM) and acetate (2 mM). Cells were harvested after complete reduction of AQDS under anaerobic conditions and resuspended in basal medium without AQDS. The cells suspension contained $7 \cdot 10^6$ cells ml^{-1} and was added at 5 % (v/v) to the bioassays. All bioassays were pre-incubated overnight prior to addition of CT and co-substrate. CT was added to the vials from a stock solution at the final concentration of 100 µM referred to the liquid volume. The stock solution of CT was prepared in sterile anaerobic distilled water. All batches were incubated in a 30 °C room and softly shaken. When the impact of different specific antibiotics over the dechlorination of CT was studied, vancomycin (100 mg l^{-1}), gentamycin (100 mg l^{-1}), neomycin (100 mg l^{-1}) and 2-bromoethanesulphonic acid (BES, 30 mM) were included in the cultures from stock solutions. Batch controls to which no inoculum was added were included to check any leakage of the chlorinated compounds through the stoppers. Sterile controls including autoclaved sludge were prepared by autoclaving the vials for 15 min at 121 °C two times before starting the experiments. Batch controls, to which no addition of CT was provided, were also included to correct for the background level of chloride. When the effect of different co-substrates on the conversion of CT was studied, an endogenous control lacking any external electron donor was also included. Conversion of CT, accumulation of intermediates and release of inorganic chloride were determined in the bioassays in time as described below. Methane production and reduction of AQDS and humus were also monitored in time as described below.

Analytical techniques. The removal of CT, and the production of volatile chlorinated hydrocarbons, such as CF, dichloromethane (DCM), methyl chloride (MC), HCA, TCE and PCE were determined in 100 µl headspace samples by gas chromatography (GC, Hewlett Packard 6890 series) equipped with an electron capture detector. The gas chromatograph was equipped with a CP-Poraplot Q column and operated under the following conditions: injector temperature, 200 °C; detector temperature, 250 °C; oven temperature, 70 °C. Helium was the carrier gas, and nitrogen was the makeup gas. Total gas flow was 60 ml min^{-1}. The retention times of MC, DCM, CF, CT, TCE, PCE and HCA were 2.5, 3.1, 3.8, 4.5, 8.8, 9.2, and 9.4 min, respectively. Identification of volatile chlorinated intermediates was confirmed in 100 µl headspace samples, which were injected in a gas chromatograph (Hewlett Packard 5890 Series II) equipped with a fused silica capillary column (PoraplotQ, Chrompack, The Netherlands) and connected to a mass spectrometer (MS) selective detector (Hewlett Packard 5971 Series). Helium was used as a carrier gas at a flow rate of 1.5 ml per min. The temperature of the injector port, detector and oven were 200, 280 and 70 °C, respectively.

Chloride concentrations were determined by an absorbable organic halogen (AOX) analyzer (ECS 1600, Euroglas Analytical Instruments, Delft, The Netherlands). Samples (50-100 µl) were directly injected into the micro-colorimeter that contained 75 % (v/v) of acetic acid in water as electrolyte. The concentration of volatile fatty acids, hydrogen and methane was determined by previously described chromatographic methods (*32*). Reduction of AQDS was determined qualitatively by observing a

development of orange color in the culture fluid or quantitatively by following the formation of AH_2QDS at 450 nm in an anaerobic chamber as previously described (*31*). Reduction of humic substances was also followed under anaerobic conditions as described before (*33*).

Results

Biodegradation of CT by anaerobic granular sludge. Two sludges were chosen for evaluating the effect of AQDS on CT removal. The first sludge, "Nedalco", has robust quinone-respiring activity with acetate as substrate; while the second sludge, "Shell", has insignificant quinone respiring activity with acetate (*32*). Reductive biotransformation of CT was achieved by "Nedalco" granular sludge without any lag phase. About 60 % of the CT initially added (100 μM) was converted after 12 days of incubation by this inoculum (Fig. 1). The conversion of CT paralleled the partial recovery of inorganic chloride from these cultures at the end of the experiment (Table 1). There was no CT conversion in basal medium controls lacking sludge and only minor conversion occurred in the cultures containing autoclaved "Nedalco" sludge. Addition of AQDS (20 μM) enhanced both the biodegradation of CT (Fig. 1) and the efficiency of dechlorination by this consortium (Table 1). The reduction of AQDS was qualitatively evident in the bioassays by the development of orange color in the culture fluid due to the formation of AH_2QDS.

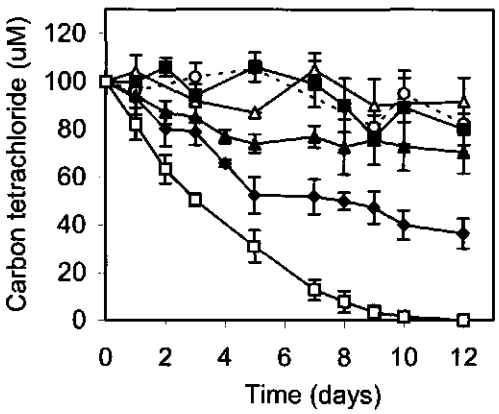


Figure 1. Conversion of CT by "Nedalco" sludge (0.25 g VSS l^{-1}) with acetate as electron donor (1 g COD l^{-1}) and AQDS (20 μM) as redox mediator. Conditions: △, chemical control; ○, chemical control with AQDS; ▲, autoclaved sludge without AQDS; ■, autoclaved sludge with AQDS; ◆, living sludge without AQDS; , living sludge with AQDS. Results obtained from triplicate incubations and error bars indicate the standard deviation.

The reductive biotransformation of CT by "Shell" sludge proceeded slower compared to the rate of conversion observed in "Nedalco" sludge. Furthermore, AQDS addition had a negligible effect. After 12 days only, 37 and 51 % of the CT was removed, respectively, in batches unsupplemented and

supplemented with AQDS. No orange color development was evident indicating negligible AQDS reduction by "Shell" sludge.

Lower chlorinated methanes – CF, DCM, but not MC – were detected as intermediates during CT degradation by both sources of granular sludge regardless the presence of AQDS accounting for less than 10 % of the initial amount of CT in all cases after 12 days of incubation (Table 1). No methanogenic activity was detected in any source of granular sludge during the conversion of CT.

Table 1. Chlorine balance for the biodegradation of CT (100 μM)[a] by "Nedalco" granular sludge (0.25 g VSS l^{-1}) with acetate (1 g COD l^{-1}) as an electron donor after 12 days of incubation

Culture conditions	Chlorine concentration (μM)				Recovery (%)	
	CT	CF	DCM	Cl^{-}[b]	Cl^{-}[c]	Total[d]
Unsupplemented control	144	33	BDL[e]	64	16±1	60±9
AQDS (20 μM) supplemented	BDL	21	BDL	172	43±10	48±11
Autoclaved Sludge Control	216	3	BDL	59	15±3	70±17
Sterilized Medium Control	368	BDL	BDL	ND[f]	ND	92±8

[a]400 μM in terms of chlorine linked to CT.
[b]Values corrected for the background level of chloride in the absence of CT.
[c]Efficiency of chloride release compared to the initial amount of CT. Values represent means of triplicate incubations ± standard deviation.
[d]Total amount of products compared to CT at time zero. Values represent means of triplicate incubations ± standard deviation.
[e]Below detection limit.
[f]Not detected beyond the background level of chloride observed in the absence of CT.

Addition of different concentrations of AQDS was shown to accelerate the reductive dechlorination of CT by "Nedalco" sludge. This was evidenced by the quicker CT conversion observed by increasing the AQDS concentration in the medium (Fig. 2A). Moreover, increasing the AQDS concentration in the cultures led to an enhanced production of inorganic chloride in the medium (Fig. 2B). CF and DCM were also detected as intermediates during these experiments and they accounted for less than 10 % of the CT initially added after 12 days of incubation.

Effect of different antibiotics on the conversion of CT by granular sludge. The impact of four different antibiotics (BES; vancomycin; gentamycin and neomycin) on the bioconversion of CT by "Nedalco" sludge was studied. None of the inhibitors had any effect on the conversion of CT in the absence of AQDS. In all cases about 60 % of CT was converted after 12 days of incubation regardless of the presence or absence of antibiotics (data not shown). On the other hand, when the experiments were performed with AQDS (20 μM) in the culture medium, protein synthesis inhibitors (gentamycin and neomycin) cancelled the enhanced activity due to AQDS supplementation so that the rates were similar to the AQDS-unsupplemented control (Fig. 3). The methanogenic inhibitor, BES, had no effect except for a delaying in achieving full activity for 3 days in the bioassays. The broad eubacterial cell-wall synthesis inhibitor, vancomycin, had an intermediate effect on the conversion of CT with AQDS as redox mediator (Fig 3). The negative impact of neomycin and gentamycin was also evident on the efficiency of dechlorination of CT by "Nedalco" sludge (Table 2) and the same inhibitors were shown

to inhibit the reduction of AQDS as indicated by the negligible orange color developed in bioassays including these antibiotics. In contrast, inclusion of BES and vancomycin to CT-degrading cultures did not affect the reduction orange color development by "Nedalco" sludge.

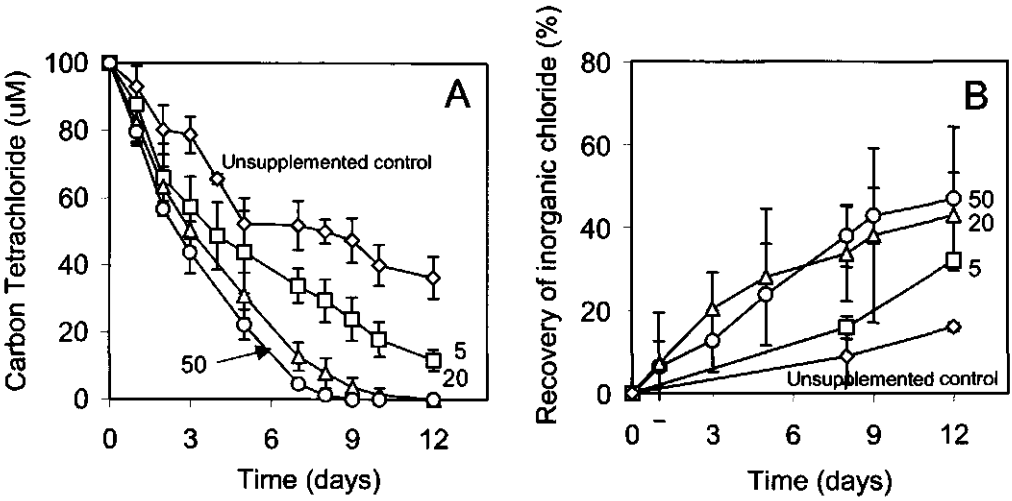


Figure 2. (A) Conversion of CT; and (B) recovery of inorganic chloride from CT biodegradation by "Nedalco" granular sludge (0.25 g VSS l^{-1}) at different AQDS concentrations with acetate (1 g COD l^{-1}) as an electron donor. Unsupplemented control refers to bioassays conducted in the absence of AQDS. The number next to the lines indicates the concentration of AQDS (in μM) in supplemented cultures. Recovery of chloride referred to the amount added of chlorine as CT and corrected for the chloride present in basal medium in the absence of CT. Results obtained from triplicate incubations and error bars indicate the standard deviation.

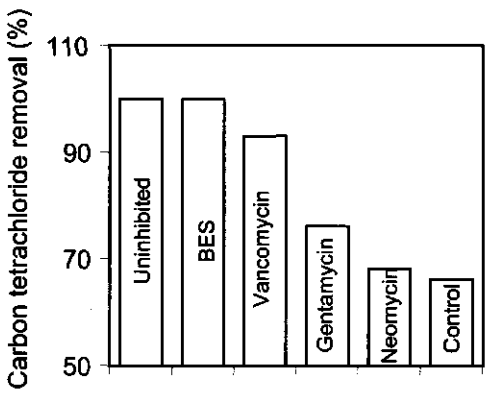


Figure 3. CT removal by "Nedalco" granular sludge (0.25 g VSS l^{-1}) after 12 days of incubation with acetate as electron donor (1 g COD l^{-1}) and AQDS (20 μM) as redox mediator in the presence of different antibiotics. Uninhibited refers to bioassays conducted in the absence of inhibitors including AQDS. Control refers to bioassays conducted in the absence of AQDS and inhibitors. All the antibiotics, except for BES (30 mM), applied at 100 mg l^{-1}. Data represent average from triplicate incubations and the standard deviation was within 5 % of the mean value in all cases.

Table 2. Chlorine balance for the biodegradation of CT (100 μM)[a] by "Nedalco" sludge (0.25 g VSS l^{-1}) with acetate as an electron donor (1 g COD l^{-1}) in the presence of AQDS (20 μM) and different antibiotics after 12 days of incubation

Culture conditions	Chlorine concentration (μM)				Recovery (%)	
	CT	CF	DCM	Cl^{-b}	Cl^{-c}	Total[d]
Unsupplemented control	136	33	BDL[e]	58	15±12	57±14
AQDS-Neomycin	128	33	2	72	18±2	59±8
AQDS-Gentamycin	96	42	2	96	24±15	59±9
AQDS-Vancomycin	28	30	BDL	157	39±14	54±14
AQDS-BES	BDL	30	BDL	169	42±8	50±8
AQDS-Uninhibited	BDL	24	2	162	40±4	47±10

[a]400 μM in terms of chlorine linked to CT.
[b]Values corrected for the background level of chloride in the absence of CT.
[c]Efficiency of chloride release compared to the initial amount of CT. Values represent means of triplicate incubations ± standard deviation.
[d]Total amount of products compared to CT at time zero. Values represent means of triplicate incubations ± standard deviation.
[e]Below detection limit.

Effect of different substrates on the conversion of CT by granular sludge. Different substrates were compared as a primary electron donor for the reductive biodegradation of CT by "Nedalco" sludge. In medium lacking AQDS, methanol and acetate were poor electron donors, only negligibly improving the rate of CT conversion compared to the endogenous control (not supplied with any external electron donor). In contrast, hydrogen and glucose significantly contributed to enhance the reductive process (Fig. 4A). When the same substrates were provided as an electron donor in medium containing AQDS (20 μM), the rate of conversion proceeded faster, regardless of the type of electron-donor included (Fig. 4B). Most noteworthy, is the fact that acetate and methanol became effective electron-donors only when AQDS was provided to the medium. CT conversion in the endogenous substrate control in the presence of AQDS was slow and similar to the rate observed for the endogenous control in the absence of AQDS. Addition of any of the electron donors studied together with AQDS also improved the efficiency of dechlorination by the granular sludge (Table 3).

In this experiment (and onwards), monitoring of chloroethanes and chloroethenes became part of the analytical protocol. In addition to the accumulation of lower chlorinated methanes (CF and DCM) from the anaerobic bioconversion of CT, PCE was also identified as an important intermediate which was consistently prevalent in many of the treatments. Its identification was confirmed by MS analysis. The PCE concentrations accounted for up to 9 % of the CT converted in terms of chlorine when H_2 was used as electron-donor with AQDS (Table 3). PCE was not detected in endogenous controls nor when glucose was used as the electron donor, otherwise its concentration was higher for any given electron donor with AQDS supplemented compared to unsupplemented cultures. None of the other chlorinated two-carbon intermediates monitored were detected during this and other experiments.

Further experiments revealed no significant difference in the rate of conversion of CT by "Nedalco" sludge when different levels of acetate (20 100 and 500 μM) were provided as an external electron

donor in the presence of AQDS. Almost complete conversion of CT was achieved for all the concentrations of acetate provided, including sub-stoichiometric levels of the co-substrate, after 4 weeks of incubation. Only about 40 % of the CT initially added disappeared in the endogenous control during the same incubation period. The addition of different levels of acetate, however, showed an enhanced efficiency of dechlorination by increasing the concentration of the co-substrate leading to 31, 48 and 51 % of dechlorination at 20, 100 and 500 μM of acetate, respectively, after 4 weeks of incubation. Only 15 % of the CT included in the endogenous control (without any external electron donor) was dechlorinated during the same incubation period. The accumulation of CF, DCM and PCE during this experiment occurred at the same level compared to that observed in previous assays.

The conversion of CT by "Nedalco" sludge in the absence of AQDS was only evident when acetate was added at 500 μM (70 % of conversion after 4 weeks of incubation). No significant difference in the extent of conversion of CT was observed at 20 and 100 μM of acetate compared to the endogenous control and in all these cases the conversion of CT did not exceed 40 % of the amount initially added during the same incubation period.

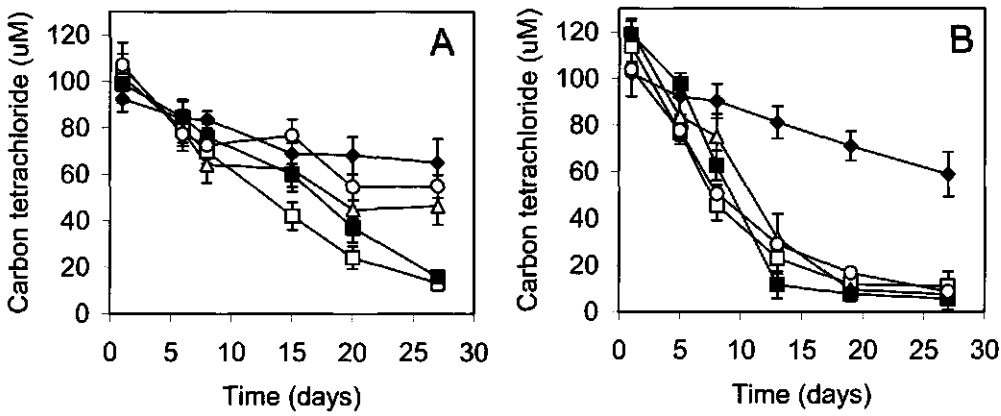


Figure 4. Conversion of CT by "Nedalco" granular sludge (0.1 g VSS l^{-1}) in the absence (A) and in the presence (B) of AQDS (20 μM) with different substrates as electron donors. All substrates provided at 1 g COD l^{-1}. When hydrogen was included as an electron donor, a headspace of H_2/CO_2 (80/20) was established with a total pressure of 1.5 bars. Substrates: ◆, endogenous control (No external substrate provided); △, acetate; ○, methanol; ■, Glucose; , hydrogen. Results obtained from triplicate incubations and error bars indicate the standard deviation.

Biodegradation of CT by a humus-respiring enrichment culture. A humus-respiring enrichment culture originated from "Nedalco" granular sludge was also studied for its capacity to degrade CT under anaerobic conditions. The enrichment, predominated by a *Geobacter* sp., was previously shown to oxidize acetate, formate and hydrogen when AQDS, humic acids and Fe(III)-NTA were provided as terminal electron acceptors (data not shown). The oxidation of these substrates agreed with the

recovery of AH_2QDS, Fe(II), and reduced humus from these cultures. The humus-respiring inoculum was unable to convert CT when AQDS and humus were omitted in the medium. However, addition of AQDS (5 μM) or highly purified soil humic acids (0.5 g l^{-1}) to cell suspensions of this enrichment allowed for the anaerobic biodegradation of CT (Fig. 5), which agreed with the recovery of AH_2QDS and reduced humus from these cultures (data not shown). Neither conversion of CT nor reduction of AQDS or humus was observed in the sterile controls lacking cells or in bioassays inhibited by the addition of neomycin (data not shown). The impact of AQDS and humic acids in the active controls was also favorable reflected on the partial recovery of inorganic chloride from these incubations, which was not observed in the unsupplemented and sterile controls. The recovery of inorganic chloride in the AQDS- and humus-supplemented bioassays accounted for 43 and 52 %, respectively, of the chlorine initially linked to CT after 22 days of incubation. Additionally, CF, DCM and PCE also accumulated during the conversion of CT by the enrichment culture accounting for 5, 2, and 3 %, respectively, of the CT-chlorine initially added in the AQDS-supplemented bioassays during the same incubation period. Likewise, 5, 2, and 2 % of the chlorine initially linked to CT was recovered as CF, DCM, and PCE, respectively, in the humus-supplemented cultures.

Table 3. Chlorine balance for the biodegradation of CT (100 μM)[a] by "Nedalco" sludge (0.1 g VSS l^{-1}) with different electron donors after 27 days of incubation

Culture conditions	Chlorine concentration (μM)					Recovery (%)	
	CT	CF	DCM	PCE	Cl^{-}[b]	Cl^{-}[c]	Total[d]
Endogenous[e] control	260	2	BDL[f]	BDL	48	13±5	84±18
Hydrogen	53	4	1	12.4	135	33±12	50±12
Acetate	185	3	BDL	2	99	23±8	68±9
Glucose	64	9	BDL	BDL	112	28±10	47±6
Methanol	220	3	BDL	BDL	85	20±5	72±8
AQDS-Endogenous control	237	4	BDL	BDL	67	16±5	75±14
AQDS-Hydrogen	44	4	BDL	35	223	49±12	67±11
AQDS-Acetate	30	2	BDL	17	181	38±9	48±7
AQDS-Glucose	23	5	1	BDL	184	39±7	44±9
AQDS-Methanol	35	4	BDL	8	158	38±7	49±10

[a]400 μM in terms of chlorine linked to CT. All substrates added at 1 g COD l^{-1}. Hydrogen supplied with a headspace of H_2/CO_2 (80/20) at 1.5 bars.
[b]Values corrected for the background level of chloride in the absence of CT.
[c]Efficiency of chloride release compared to the initial amount of CT. Values represent means of triplicate incubations ± standard deviation.
[d]Total amount of products compared to CT at time zero. Values represent means of triplicate incubations ± standard deviation.
[e]No external electron donor provided
[f]Below detection limit.

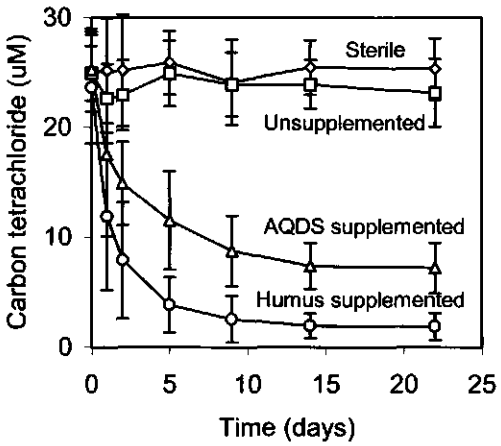


Figure 5. Conversion of CT (25 μM) by a humus-respiring enrichment culture originated from "Nedalco" sludge in the presence of AQDS (5 μM) or highly purified soil humic acids (0.5 g l^{-1}) with acetate (1 g COD l^{-1}) as an electron donor. Sterile control containing both AQDS and humus in the absence of cells. Unsupplemented control conducted in the absence of AQDS and humus. Results obtained from triplicate incubations and error bars indicate the standard deviation.

Discussion

The present study indicates that quinone groups in humus may play an important role in the conversion of polychlorinated pollutants by increasing their rate and extent of dechlorination in anaerobic consortia. CT dechlorination by an anaerobic granular sludge was enhanced by addition of the humic model compound, AQDS, at sub-stoichiometric concentrations. The role of quinone moieties was further emphasized by the addition of AQDS or highly purified humus to cell suspensions of a humus-respiring enrichment culture derived from the sludge. Dechlorination of CT by the enrichment culture was only made feasible by supplementing the cultures with these quinone-containing compounds. The lack of any conversion in sterile medium and autoclaved sludge (or cell) controls indicates that biological mechanisms were important in the transformation of CT.

The present study provides multiple evidences that quinone-respiring microorganisms were responsible for the enhanced conversion of CT observed. First, enhancement of CT-conversion was only observed upon addition of AQDS or humus into the media. Second, the enhanced CT-conversion was only significant in bioassays with measurable quinone respiring activity. Third, antibiotics targeting protein translation (neomycin and gentamycin) cancelled the stimulating effect of AQDS on CT-dechlorination in anaerobic granular sludge and the same antibiotics completely inhibited CT-dechlorination by the quinone-respiring enrichment culture. Presumably the antibiotics prevent the synthesis of new proteins required for quinone respiration. The partial inhibition caused by vancomycin coincides with the effect of the inhibitor on eubacterial growth (by blocking cell wall synthesis), but not necessarily inhibiting the activity of existing cells. BES, a specific inhibitor of

methanogens, did not significantly inhibit CT-dechlorination by the granular sludge, suggesting that methanogens were not directly involved.

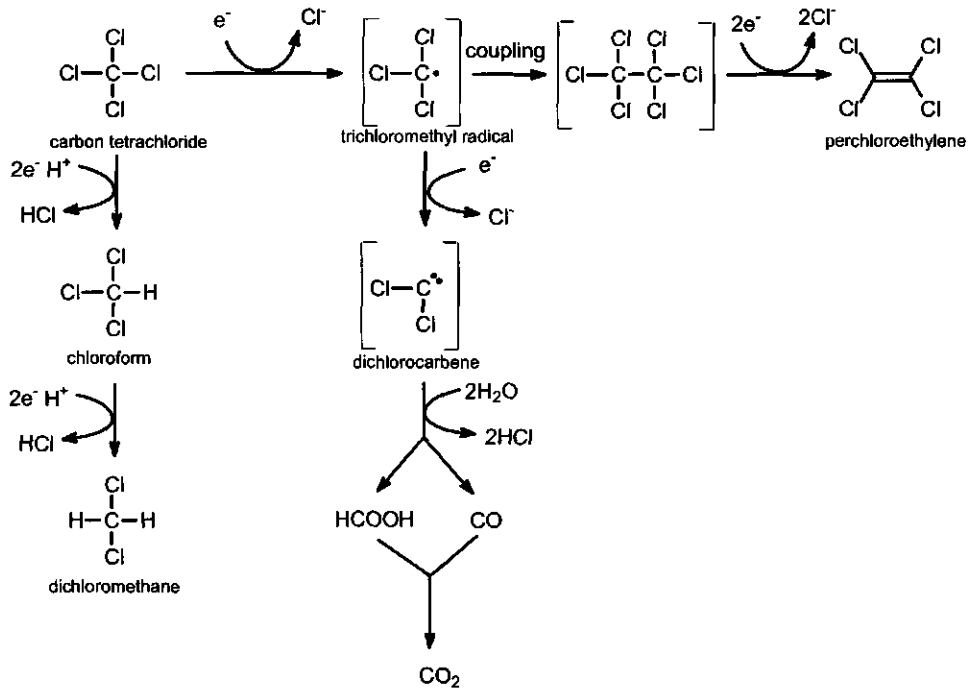


Figure 6. Proposed formation of degradation products observed in this study. Compounds in brackets were not identified, and $^{14}CO2$ as a major product of [^{14}C]-CT was confirmed in a previous study with anaerobic granular sludge (*17*). Formate and CO were also previously observed in an electrolytic model system (*19*).

Anaerobic biodegradation of CT was previously described under different conditions including methanogenic (*15-17*), sulfate-reducing (*18*), nitrate-reducing (*12*), and Fe(III)-reducing (*7, 34*) conditions by mixed or pure cultures. To our knowledge, the present study constitutes the first report for the anaerobic biodegradation of CT under humus-reducing conditions. Addition of high-organic-carbon soil to cell suspensions of *Shewanella putrefaciens* 200 was previously shown to accelerate the anaerobic conversion of CT to CF and unidentified products (*26*), but the actual functional groups and mechanisms involved were not elucidated during this process. More recently, Fe(III)-reducing bacteria were shown to stimulate the conversion of CT to CF (*34*). The mechanism proposed includes the biological reduction of Fe(III) to Fe(II), followed by the chemical conversion of CT to CF by the biogenic Fe(II). Most quinone-respiring bacteria reported in the literature are Fe(III)-reducers of the family *Geobacteraceae* (*35*) and the quinone-respiring enrichment culture utilized here was also predominated by a *Geobacter* sp. The present study indicates that these Fe(III)-reducing bacteria may

have a more important role in the conversion of polychlorinated pollutants than previously considered. Fe(III)-reducing microorganisms may significantly contribute to the conversion of polychlorinated pollutants by generating reducing equivalents via quinone- and humus-respiration in organic rich sediments and soils, as well as in wetlands, eutrophic lakes, and in microniches in composts. Quinones in humus, once being microbially reduced may transfer the reducing equivalents to the chlorinated compounds. Indeed the chemical dechlorination of HCA by AH_2QDS was demonstrated (*5*). This regenerating mechanism allows for the application of humic substances at sub-stoichiometric concentrations for the bioremediation of contaminated sites. The enhanced conversion of CT observed in the present study occurred in all cases at sub-stoichiometric concentrations of AQDS. In the humus-respiring enrichment culture, 5 μM AQDS supported up to 43 % dechlorination of 25 μM CT (equivalent to 43 μM AH_2QDS in electron equivalents). Our results also indicate that addition of an external electron donor is necessary to maintain the reductive biodegradation of CT. A wide variety of substrates could be used as an electron donor to enhance the reductive process. This agrees with the fact that a wide variety of organic substrates can be oxidized via quinone or humus respiration (*31-33, 35, 36*). Quinones were also able to extend the possible electron donors utilized by anaerobic granular sludge consortium to acetate and methanol that otherwise were not utilized to support CT dechlorination. Very low concentrations of electron donor had a large impact on dechlorination. For example, 20 μM acetate (160 microelectron equivalents l^{-1}) removed an additional 40 to 50 μM of CT beyond that observed in the endogenous control in granular sludge.

The main product of CT conversion was inorganic chloride accounting for 40 to 50% of the chlorine initially linked to CT in various experiments. Additionally, the accumulation of lower chlorinated methanes, CF (from 1 up to 10 % of added CT) and to a lesser extent DCM (traces), was observed. These intermediates suggest a sequence of two-electron reductive hydrogenolysis as a dechlorinating pathway in this study. Lower chlorinated methanes do not account for the bulk of the chlorine observed. Thus, alternative pathways are likely. Previously, CO_2 was found as a major end product of CT degradation by anaerobic granular sludge, which could be explained by the hydrolysis of a dichlorocarbene intermediate (*17*). Also in the present study the accumulation of a chlorinated ethene, PCE (from 0.5 up to 9 % of added CT) is reported for the first time as an intermediate of CT degradation. The formation of PCE, suggests a coupling reaction occurred between C_1 radicals, such as trichloromethyl radical resulting from a one-electron-reduction of CT. The most likely explanation for the formation of PCE during the biodegradation of CT, is the coupling of two trichloromethyl radicals to form HCA (*20, 21*). HCA is then readily reduced to PCE preventing HCA accumulation. The rapid reduction of HCA to PCE is reported for anaerobic granular sludge (*37*). Also the chemical conversion of HCA to PCE by hydroquinones is reported (*5*). Figure 6 illustrates a scheme accounting for the reactions observed in this study. The products identified could not account for approximately 40 to 50% of the chlorine of the CT-converted. Identifying the missing chlorine-containing products

should be the subject of future research. However, since coupling was shown to occur as evidenced by PCE formation, it is reasonable to assume that at least some of the missing products may be complex polymerized products or adducts with biomass components (*38, 39*).

The accumulation of PCE is certainly undesirable because it is also an environmental pollutant. However PCE is also amendable to anaerobic bioremediation by halorespiring bacteria (*40*). Depending on the halorespiring microorganisms present at a given site, either partial or complete dechlorination occurs. Partial reduction of PCE leads to the potential accumulation of carcinogenic chlorinated ethenes, such as dichloroethene and vinyl chloride, which are more recalcitrant under anaerobic conditions. The presence of humic substances, however, may contribute to attenuate the accumulation of these pollutants, since both AQDS and humic acids were previously shown to serve as a terminal electron acceptors supporting the anaerobic oxidation of dichloroethene and vinyl chloride to mineralized products (*36*).

Acknowledgements

This research was financially supported by the Council of Science and Technology of Mexico (CONACyT).

References

1. Recknagel, R. O.; Glende, E. A. (1973) Carbon tetrachloride hepatotoxicity: an example of lethal cleavage. *Crit. Rev. Toxicol.* **2**, 263-297.
2. Van Agteren; M. H.; Keuning, S. (1998) Handbook on biodegradation and biological treatment of hazardous organic compounds. Kluwer Academic Publishers: Dordrecht, The Netherlands.
3. Wentz, M. The evolution of environmentally responsible fabricare technologies. (1995) *Am. Drycleaner* **62**, 52-62.
4. Lewis, T. A.; Crawford, R. L. (1993) Physiological factors affecting carbon tetrachloride dehalogenation by the denitrifying bacterium *Pseudomonas* sp. strain KC. *Appl. Environ. Microbiol.* **59**, 1635-1641.
5. Curtis, G. P.; Reinhard M. (1994) Reductive dehalogenation of hexachloroethane, carbon tetrachloride, and bromoform by anthrahydroquinone disulfonate and humic acid. *Environ. Sci. Technol.* **28**, 2393-2401.
6. Vogel, T. M.; Criddle, C. S.; McCarty, P. L. (1987) Transformations of halogenated aliphatic compounds. *Environ. Sci. Technol.* **21**, 722-736.
7. Picardal, F. W.; Arnold, R. G.; Couch, H.; Little, A. M.; Smith, M. A. (1993) Involvement of cytochromes in the anaerobic biotransformation of tetrachloromethane by *Shewanella putrefaciens* 200. *Appl. Environ. Microbiol.* **59**, 3763-3770.

8. Hashsham, S. A.; Freedman, D. L. (1999) Enhanced biotransformation of carbon tetrachloride by *Acetobacterium woodii* upon addition of hydroxocobalamin and fructose. *Appl. Environ. Microbiol.* **65**, 4537-4542.

9. Zou, S.; Stensel, H. D.; Ferguson, J. F. (2000) Carbon tetrachloride degradation: effect of microbial growth substrate and vitamin B_{12} content. *Environ. Sci. Technol.* **34**, 1751-1757.

10. Gantzer, C. J.; Wackett, L. P. (1991) Reductive dechlorination catalyzed by bacterial transition-metal coenzymes. *Environ. Sci. Technol.* **25**, 715-722.

11. Koons, B. W.; Baeseman, J. L.; Novak, P. J. (2001) Investigation of cell exudates active in carbon tetrachloride and chloroform degradation. *Biotechnol. Bioeng.* **74**, 12-17.

12. Lee, C.-H.; Lewis, T. A.; Paszczynski, A.; Crawford, R. L. (1999) Identification of an extracellular catalyst of carbon tetrachloride dehalogenation from *Pseudomonas stutzeri* strain KC as pyridine-2,6-bis(thiocarboxylate). *Biochem. Biophys. Res. Commun.* **261**, 562-566.

13. Krone, U. E.; Laufer, K.; Thauer, R. K. (1989) Coenzyme F_{430} as a possible catalyst for the reductive dehalogenation of chlorinated C_1 hydrocarbons in methanogenic bacteria. *Biochemistry* **28**, 10061-10065.

14. Kriegman-King, M. R.; Reinhard, M. (1994) Transformation of carbon tetrachloride by pyrite in aqueous solution. *Environ. Sci. Technol.* **28**, 692-700.

15. Egli, C. R.; Scholtz, R.; Cook, A. M.; Leisinger, T. Anaerobic dechlorination of tetrachloromethane and 1,2-dichloroethane to degradable products by pure cultures of *Desulfitobacterium* sp. and *Methanobacterium* sp. *FEMS Microbiol. Lett.* **1987**, *43*, 257-261.

16. De Best, J. H.; Hunneman, P.; Doddema, H. J.; Janssen, D. B.; Harder, W. Transformation of carbon tetrachloride in an anaerobic packed-bed reactor without addition of another electron donor. *Biodegradation* **1999**, *10*, 287-295.

17. Van Eekert, M. H. A.; Schröder, T. J.; Stams, A. J. M.; Schraa, G.; Field, J. A. (1998) Degradation and fate of carbon tetrachloride in unadapted methanogenic granular sludge. *Appl. Environ. Microbiol.* **64**, 2350-2356.

18. De Best, J. H.; Salminen, E.; Doddema, H. J.; Janssen, D. B.; Harder, W. (1998) Transformation of carbon tetrachloride under sulfate reducing conditions. *Biodegradation* **8**, 429-436.

19. Criddle, C. S.; McCarty, P. L. (1991) Electrolytic model system for reductive dehalogenation in aqueous environments. *Environ. Sci. Technol.* **25**, 973-978.

20. Fowler, S. J. L. (1969) *Br. J. Pharmacol.* **37**, 733-737.

21. Anders, M. W. (1982) In *Metabolic Basis of Detoxification*; Jakoby, W. B., Bend, J. R., Caldwell, J., Eds.; Academic Press: New York; pp 29-49.

22. Field, J. A.; Cervantes, F. J.; Van der Zee, F. P.; Lettinga, G. (2000) Role of quinones in the biodegradation of priority pollutants: a review. *Water Sci. Technol.* **42**, 215-222.

23. Curtis, G. P. Ph. D. (1991) Thesis, Stanford University, Stanford, CA.

24. Schwarzenbach, R. P.; Stierli, R.; Lanz, K.; Zeyer, J. (1990) Quinone and iron porphyrin mediated reduction of nitroaromatic compounds in homogeneous aqueous solution. *Environ. Sci. Technol.* **24**, 1566-1574.

25. Van der Zee, F. P.; Lettinga, G.; Field, J. A. (2000) The role of (auto)catalysis in the mechanism of an azo dye reduction. *Water Sci. Technol.* **42**, 301-308.

26. Collins, R.; Picardal, F. (1999) Enhanced anaerobic transformation of carbon tetrachloride by soil organic matter. *Environ. Toxicol. Chem.* **18**, 2703-2710.

27. Cervantes, F. J.; Van der Zee, F. P.; Lettinga, G.; Field, J. A. (2001) Enhanced decolourisation of acid orange 7 in a continuous UASB reactor with quinones as redox mediators. *Water Sci. Technol.* **44**, 123-128.

28. Kudlich, M., A. Keck, J. Klein and A. Stolz. (1997) Localization of the enzyme system involved in anaerobic reduction of azo dyes by *Sphingomonas* sp. strain BN6 and effect of artificial redox mediators on the rate of azo dye reduction. *Appl. Environ. Microbiol.* **63**, 3691-3694.

29. Laszlo, J.A. (2000) Regeneration of azo-dye-saturated cellulosic anion exchange resin by *Burkholderia cepacia* anaerobic dye reduction. *Environ. Sci. Technol.* **34**, 167-172.

30. Van der Zee, F. P.; Bouwman, R. H. M.; Strik, D. P. B. T. B.; Lettinga, G.; and Field, J. A. (2001) Application of redox mediators to accelerate the transformation of reactive azo dyes in anaerobic bioreactors. *Biotechnol. Bioeng.* **75**, 691-701.

31. Cervantes, F. J.; van der Velde, S.; Lettinga, G.; Field, J. A. (2000) Quinones as terminal electron acceptors for anaerobic microbial oxidation of phenolic compounds. *Biodegradation* **11**, 313-321.

32. Cervantes, F. J.; van der Velde, S.; Lettinga, G.; Field, J. A. (2000) Competition between methanogenesis and quinone respiration for ecologically important substrates in anaerobic consortia. *FEMS Microbiol. Ecol.* **34**, 161-171.

33. Cervantes, F. J.; Dijksma, W.; Duong-Dac, T.; Ivanova, A.; Lettinga, G.; Field, J. A. (2001) Anaerobic mineralization of toluene by enriched sediments with quinones and humus as terminal electron acceptors. *Appl. Environ. Microbiol.* **67**, 4471-4478.

34. Gerlach, R.; Cunningham, A. B.; Caccavo Jr., F. (2000) Dissimilatory iron-reducing bacteria can influence the reduction of carbon tetrachloride by iron metal. *Environ. Sci. Technol.* **34**, 2461-2464.

35. Coates, J. D.; Ellis, D. J.; Roden, E.; Gaw, K.; Blunt-Harris, E. L.; Lovley D. R. (1998) Recovery of humics-reducing bacteria from a diversity of sedimentary environments. *Appl. Environ. Microbiol.* **64**, 1504-1509.

36. Bradley, P. M., F. H. Chapelle and D. R. Lovley. (1998) Humic acids as electron acceptors for anaerobic microbial oxidation of vinyl chloride and dichloroethene. *Appl. Environ. Microbiol.* **64**, 3102-3105.

37. Van Eekert, M. H. A.; Stams, A. J. M.; Field, J. A.; Schraa, G. (1999) Gratuitous dechlorination of chloroethanes by methanogenic granular sludge. *Appl. Microbiol. Biotechnol.* **51**, 46-52.

38. DeGroot, H.; Haas, W. (1981) *Biochem. Pharmacol.* **30**, 2343-2347.

39. Sipes, I. G.; Krishna, G.; Gillette, J. R. (1977) *Life Sci.* **20**, 1541-1548.

40. Middeldorp, P. J. M.; Luijten, M. L. G. C.; Van der Pas, B. A.; Van Eekert, M. H. A.; Kengen, S. W. M.; Schraa, G.; Stams, A. J. M. (1999) Anaerobic microbial reductive dehalogenation of chlorinated ethenes. *Bioremediation J.* **3**, 151-169.

8

Enhanced decolourisation of acid orange 7 in a continuous UASB reactor with quinones as redox mediators*

*A modified version has been published as: Cervantes F. J., F. P. van der Zee, G. Lettinga and J. A. Field (2001). *Water Sci. Technol.* **44**(4), 123-128.

Abstract

The reductive biotransformation of acid orange 7 (AO7) was explored in a lab-scale upflow anaerobic sludge blanket (UASB) reactor at low hydraulic residence times (HRT). A colour removal of 85 % was achieved when the reactor was operated at a HRT of 6 hours, but decreased up to 70 % when the HRT was lowered to 2 hours. Addition of the quinone model compound, anthraquinone 2,6-disulfonate (AQDS), as redox mediator, allowed for a considerably higher decolourising efficiency (≥90 % at all the HRT evaluated). The results indicate that the use of catalytic concentrations of AQDS (AQDS/AO7 molar ratio about 0.01) can accelerate decolourising processes achieving satisfying extent of decolourisation.

Introduction

Synthetic azo dyes are extensively used as dyes for textiles, food, and cosmetics. More than 7 x 10^5 tons of these dyes are produced annually world-wide (Zollinger, 1987). Most of the azo dyes, which are released into the environment, originate from the textile industry and the dyestuff manufacturing industry (Meyer, 1981). Their discharge is undesirable, not only for aesthetic reasons, but also because many azo dyes and their breakdown products have been proven to be toxic to aquatic life (Chung and Stevens, 1993) and mutagenic to humans (Chung and Cerniglia, 1992). They are frequently found chemically unchanged under aerobic conditions (Levine, 1991). However, under anaerobic conditions, many bacteria gratuitously reduce azo dyes by reductive cleavage of the azo bound to colourless aromatic amines (Field *et al.*, 1995). The reductive ring fission of the azo linkage is generally presumed to be an unspecific extracellular process in which reducing equivalents from an external electron donor are transferred to the dye (Laszlo, 2000).

Preliminary experiments showed that long reaction times are required for many azo dyes to achieve satisfying extent (>90%) of decolourisation, which may represent a serious problem for applying high-rate anaerobic treatment as the first stage in the biological degradation of azo dyes (Van der Zee *et al.*, 2001). However, quinone based redox mediating compounds have been recently reported to accelerate azo reduction rates by shuttling reducing equivalents from bacteria to azo dyes (Kudlich *et al.*, 1997, Laszlo, 2000). Therefore, the use of these redox mediators may eliminate the transfer of reduction equivalents as a rate-limiting step in decolourising processes. These observations are consistent with the use of the quinone model compounds, anthraquinone disulfonate (AQDS) and anthraquinone sulfonate (AQS), to mediate the electrochemical cathodic reduction of dispersed dyes (Bechtold *et al.*, 1999). Furthermore, other reports indicate that quinone moieties can also mediate abiotic reductive dehalogenation and nitroaromatic reduction, by ferrous iron or sulfide (Field *et al.*, 2000). Quinones increased the reduction rate of these reductive processes by one to several orders of magnitude.

In the present study, the role of the quinone model compound, AQDS, in accelerating the reductive biotransformation of Acid Orange 7 (AO7) was explored in a continuos upflow anaerobic sludge blanket (UASB) reactor.

Materials and methods

A 1.3-L UASB reactor (Figure 1) was placed in a 30 °C room and seeded with methanogenic granular sludge (30 g of volatile suspended solids (VSS) per litre) obtained from a full-scale UASB reactor treating effluents of an alcohol distillery of Nedalco (Nergen op Zoom, The Netherlands). The lab-scale UASB reactor was fed with a volatile fatty acids (VFA) mixture (acetate:propionate:butirate=1:1:1 based on chemical oxygen demand (COD) ratio) at a final concentration of 1.5 g COD/L prepared in basal medium. The concentration of the azo dye AO7 was kept at 100 mg AO7/L throughout the study. Eventually, different concentrations of AQDS were added in the medium. The influent was kept at 4 °C and continuously pumped to the reactor with a peristaltic pump.

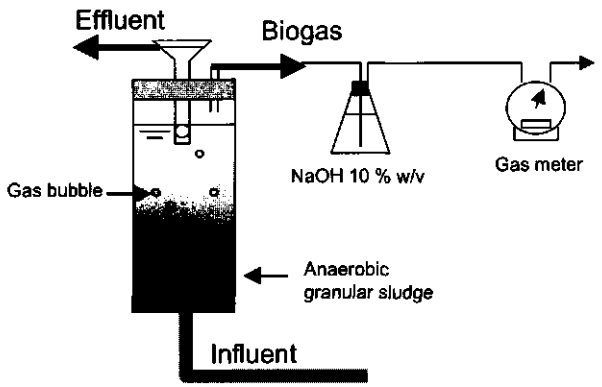


Figure 1. Schematic diagram of the UASB system used for the present study.

The basal medium contained (mg/L): NH_4Cl (280), K_2HPO_4 (250), $MgSO_4 \cdot 7H_2O$ (100), $CaCl_2 \cdot 2H_2O$ (10) and 1 ml/L of trace elements and the pH was adjusted to 7 in the influent. Methane production was measured with a wet test gas meter after washing the biogas in a 10 % NaOH solution to remove carbon dioxide.

Colour was measured spectrophotometrically with a Spectronics 60 spectrophotometer (Milton Ray Analytical Products Division, Belgium) at the AO7's wavelength of maximum absorbance (484 nm). Liquid phase samples (0.75 ml) were centrifuged (10000 g, 3 min) and diluted up to an absorbance of less than 1 in a phosphate buffer (10.86 g l^{-1} $NaH_2PO_4 \cdot 2H_2O$; 5.38 g l^{-1} $Na_2HPO_4 \cdot H_2O$). The buffer contained freshly added ascorbic acid (~200 mg l^{-1}) to prevent autooxidation reactions. Without dye,

absorbance of the medium and buffer was less than 0.5 % of the absorbance right after dye addition. Soluble COD and VSS were analysed according to standard methods (APHA, 1985). Sulfanilic acid was measured by high performance liquid chromatography (HPLC) according to the method described by Tan *et al.* (1999).

Acid orange 7 (Orange II, dye content 98 %) was purchased from Aldrich Chemical Company Ltd., Gillingham, England. Sulfanilic acid (99 %, A. C. S. reagent) was purchased from Sigma-Aldrich Chemie GmbH, Steinheim, Germany. Anthraquinone-2, 6-disuphonic acid, disodium salt and 1-amino-2-naphthol hydrochloride (technical grade, 90 %) were purchased from Aldrich Chemical Company Inc., Milwaukee, USA.

Results and discussion

During the start-up period, the UASB reactor was operated for 75 days at a VFA-COD loading rate of 5.3 g COD/L.d, including 100 mg AO7/L in the medium, with a hydraulic residence time (HRT) of 6 hours. The total removal of soluble COD achieved during this period was about 80 % and removal of colour was about 85 % when steady state conditions were reached.

Table 1. Performance of the decolourising UASB reactor at different AQDS concentrations in steady state with a HRT of 6 hours

AQDS (μM)	0*	3	10	30
COD Removal (%)	80.1±0.2	79.1±0.9	81.1±0.1	86.7±0.2
Decolourisation (%)	85.7±1.1	97.3±0.7	98.8±0.1	98.9±0.2
CH_4 production (g COD-CH_4/L-d)	4.24±0.1	4.18±0.1	4.19±0.2	4.21±0.1

*Initial period prior to any exposition to AQDS

After this period, the UASB reactor was supplied with different AQDS concentrations while the COD loading rate was kept constant. A significant improvement in the colour removal occurred in the UASB reactor directly after amending the medium with AQDS when the colour removal increased up to 97-99 % (Table 1). This represented an increase in the AO7 reduction rate from 39.9 ± 0.5 μmol/L.hr (without AQDS) to 45.8 ± 0.2 μmol/L.hr (for all different concentrations of AQDS applied). When AQDS was intermittently omitted in the medium, colour removal decreased as shown in Figure 2. However, it was surprisingly observed that in these intermittent periods colour removal did not drop back to 85 %, but only decreased to 94 %. This suggests that some quinones might have been retained in the reactor stimulating the azo dye reduction at catalytic concentrations. Moreover, the complete decolourisation achieved with the lowest AQDS concentration tested (3 μM) also suggests that quinone moieties can act at catalytic concentrations as redox mediators. Namely, a molar ratio of AQDS/AO7 of about 0.01 was sufficient to achieve an efficient decolourising process. These

results are consistent with previous experiments (Kudlich *et al.*, 1997), which revealed that addition of AQDS and AQS at different levels accelerated the anaerobic microbial transformation of different azo dyes (e.g. amaranth, acid red 1, sunset yellow, naphthol blue black).

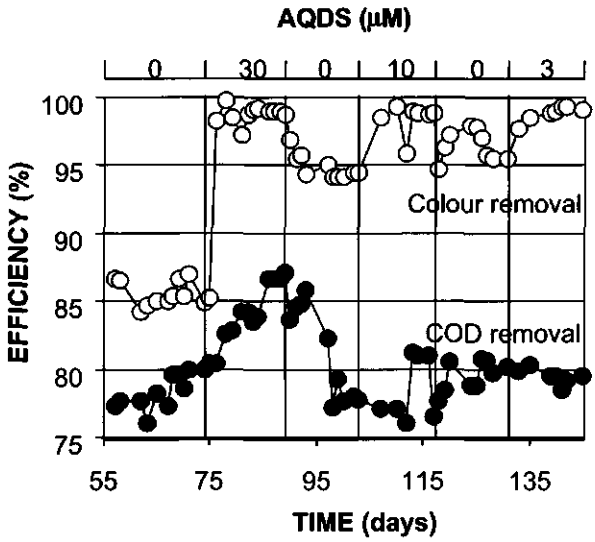


Figure 2. Performance of the decolourising UASB reactor at different AQDS concentrations with a HRT of 6 hours.

Figure 3 illustrates the role of AQDS during the decolourisation of AO7 with a mixture of VFA as potential electron donors. These substrates are converted to methane and carbon dioxide by the anaerobic consortium during the first step of the process. The biologically generated electrons reduce AQDS to the corresponding hydroquinone (AH_2QDS), which subsequently transfers the electrons to the azo dye. The reaction of AH_2QDS with the azo dye regenerates the quinone moieties to their oxidised form. Recycling the AQDS/AH_2QDS couple in this form enables its use at catalytic concentrations as a redox mediator to accelerate the reduction of the azo dye. This may explain the same extent of stimulation achieved in the decolourisation of AO7 by adding different concentrations of AQDS in the reactor. Moreover, similar rates of reduction of AO7 (20-90 μmol/L.hr) were obtained in pure cultures by *Burkholderia cepacia* when different concentrations of AQS were added, even with an AQS/AO7 ratio as high as 6.25 (Laszlo, 2000). Therefore, quinone moieties do not necessarily have to be present in abundant supply to accelerate the decolourisation of azo dyes, as they can be easily recycled in the decolourising process.

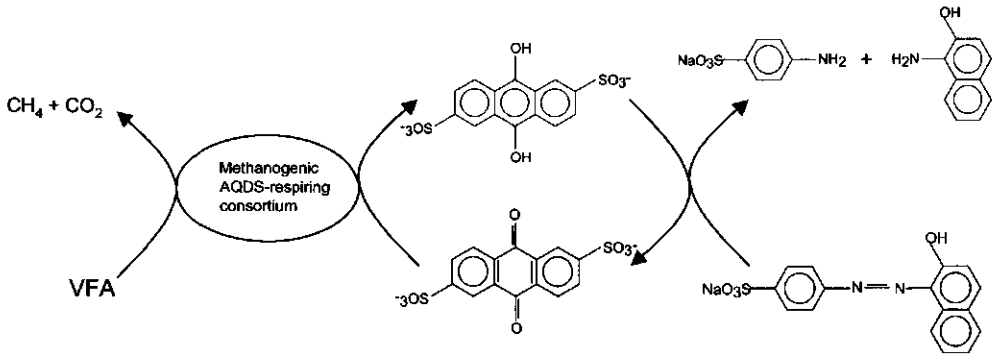


Figure 3. Proposed mechanism for the anaerobic microbial reduction of acid orange 7 by a methanogenic AQDS-respiring consortium with AQDS as redox mediator.

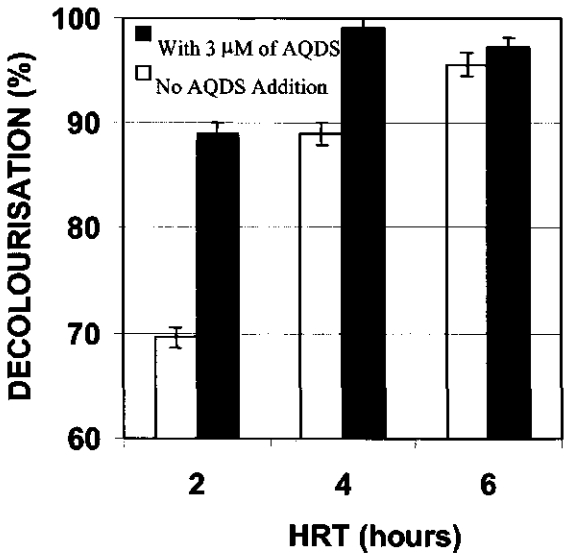


Figure 4. Colour removal from the UASB reactor at different HRT in steady state. Data obtained after exposing the reactor with different AQDS concentrations at 6 hours as HRT.

Reduction of AO7 leads to the formation of the aromatic amines, sulfanilic acid and 1-amino-2-naphthol. The latter is an unstable aromatic amine that can undergo autooxidation reactions, but sulfanilic acid is rather stable and was followed during the conversion of AO7 in the decolourising reactor. The results showed no further conversion of sulfanilic acid in the continuous reactor or in batch experiments both in the absence and in the presence (25 mM) of AQDS (data not shown). However, the capacity of "Nedalco" sludge to oxidise aromatic compounds (e.g. phenol, *p*-cresol) both under methanogenic and AQDS reducing conditions (Cervantes *et al.*, 2000) suggests that

aromatic amines characterised by the presence of hydroxyl or carboxyl groups may be degraded by this inoculum during the anaerobic reduction of azo dyes containing these functional groups. The anaerobic biodegradation of this type of aromatic amines has been previously reported (Kuhn and Suflita, 1989; Razo-Flores *et al.*, 1996).

After the lowest concentration of AQDS was tested, the HRT was gradually decreased in the UASB reactor keeping the same VFA and AO7 concentrations in the influent. This resulted in decreasing efficiency of decolourisation in the reactor. However, when AQDS (3 μM) was added in the medium, a significant improvement in decolourisation was obtained at all the HRT tested (Figure 4), whereas the COD removal efficiency did not differ significantly in all cases (data not shown). This indicates that AQDS can mediate a satisfying extent of decolourisation of AO7 at catalytic concentrations, even at a HRT of 2 hours. The results also support preliminary studies, which indicated that, the transfer, rather than the production of reducing equivalents was the rate-limiting factor for determining the rate of reduction of azo dyes (Van der Zee *et al.*, 2001).

The application of anaerobic digestion as the first step for the treatment of textile wastewaters may constitute a sustainable technology considering the following aspects. Azo dyes cleavage may be carried out at low HRT, which would demand very compact anaerobic digesters. Redox mediators do not necessarily have to be present in abundant supply, as they can be easily recycled during the decolourising process (see Figure 2). Model redox mediators can be replaced by humic substances, which are very abundant in nature and have inert properties. Humus is characterised by a high content in quinone groups in its structure (Stevenson, 1994) and therefore, represents the most abundant source of quinone moieties. Moreover, addition of AQDS to UASB decolourising reactors has also enhanced the reductive biotransformation of other azo dyes, e.g. reactive red 2, which were decolourised at a much lower rate (data not shown).

Conclusions

The results presented in this study demonstrate that the quinone model compound, AQDS, can accelerate the rate of azo dye (AO7) reduction by mediating the transfer of reducing equivalents to the dye. Results also suggest that AQDS can mediate a satisfying extent of decolourisation at catalytic concentrations (AQDS/AO7 molar ratio about 0.01), even with a HRT of 2 hours. Therefore, the use of redox mediators, such as AQDS, eliminates the transfer of reducing equivalents as the rate-limiting step in anaerobic decolourising processes.

Acknowledgements

This research was financially supported by The Council of Science and Technology of Mexico (CONACyT).

References

1. APHA (1985). *Standard Methods for Examination of Water and Wastewater.* 16th edn, American Public Health Association, Washington DC.
2. Bechtold, T., Burtscher, E. and Turcanu, A. (1999). Anthraquinones as mediators for the indirect cathodic reduction of dispersed organic dyestuffs. *Journal of Electroanalytical Chemistry.* **465**, 80-87.
3. Cervantes, F.J., Van der Velde, S., Lettinga, G. and Field, J.A. (2000). Quinones as terminal electron acceptors for anaerobic microbial oxidation of phenolic compounds. *Biodegradation* **11**, 313-321.
4. Chung, K.T. and Cerniglia, C.E. (1992). Mutagenicity of azo dyes: Structure-activity relationships. *Mutat. Res.* **277**, 201-220.
5. Chung, K.T. and Stevens, S.E.J. (1993). Degradation of azo dyes by environmental microorganisms and helminths. *Environ. Toxicol. Chem.***12**(11), 2121-2132.
6. Field, J. A., Stams, A. J. M., Kato, M. and Schraa G. (1995). Enhanced biodegradation of aromatic pollutants in cocultures of anaerobic and aerobic bacterial consortia. *Antonie van Leeuwenhoek.* **67**, 47-77.
7. Field, J.A., Cervantes, F.J., Van der Zee, F. and Lettinga, G. (2000). Role of quinones in the biodegradation of priority pollutants: A review. *Water Sci. Technol.* **42**(5-6), 215-222.
8. Kudlich, M., Keck, A., Klein, J. and Stolz, A. (1997). Localization of the enzyme system involved in anaerobic reduction of azo dyes by *Sphingomonas sp.* Strain BN6 and effect of artificial redox mediators on the rate of azo dye reduction. *Appl. Environ. Microbiol.* **63**, 3691-3694.
9. Kuhn, E.P. and Suflita, J.M. (1989). Anaerobic biodegradation of nitrogen-substituted and sulfonated benzene aquifer contaminants. *Hazardous Waste Hazardous Mater.* **6**, 121-134.
10. Laszlo, J.A. (2000). Regeneration of azo-dye-saturated cellulosic anion exchange resin by *Burkholderia cepacia. Environ. Sci. Technol.* **34**, 167-172.
11. Levine, W. G. (1991). Metabolism of azo dyes: Implication for detoxification and activation. *Drug Metab. Rev.* **23**, 253-309.
12. Meyer, U. (1981). Biodegradation of synthetic organic colorants. *FEMS Symp.* **12**,371-385.
13. Razo-Flores, E., Donlon, B.A., Field, J.A. and Lettinga, G. (1996). Biodegradability of N-substituted aromatics and alkylphenols under methanogenic conditions using granular sludge. *Water Sci. Technol.* **33**(3), 47-57.
14. Stevenson, F.J. (1994). Humus chemistry: Genesis, Composition, Reactions. 2nd Edn., Wiley, New York. 496 pp.

15. Tan, N.C.G., Prenafeta-Boldu, F.X., Opsteeg, J.L., Lettinga, G. and Field, J.A. (1999). Biodegradation of azo dyes in cocultures of anaerobic granular sludge with aerobic aromatic amine degrading enrichment cultures. *Appl. Microbiol. Biotechnol.* **51**, 865-871.

16. Van der Zee, F.P., Field, J.A. and Lettinga, G. (2001). Azo dye decolourisation by anaerobic granular sludge. *Chemosphere* **44**, 1169-1176.

17. Zollinger, H. (1987). Colour chemistry-synthesis, properties and applications for organic dyes and pigments, p.92-102. VCH Publishers, Inc., New York, N.Y.

9

General Discussion and Conclusions

Introduction

Humus is the most abundant organic fraction in the biosphere. Humic substances are poorly biodegradable polymers formed during the decomposition of plant material in soil and sediments. The remarkable recalcitrance of humus is reflected by its long residence times in the environment, which exceeds 500 years (*44*). However, it has recently been recognized that humic substances may play an important role in the anaerobic biodegradation and biotransformation of organic as well as inorganic compounds. Humus can serve as a terminal electron acceptor supporting the anaerobic microbial oxidation of a wide variety of organic substrates. Microbially reduced humus can transfer electrons to metal oxides, such as Fe(III) and Mn(IV), allowing for the regeneration of humus to the oxidized form. Thus, even sub-stoichiometric concentrations of humic substances can mediate both anaerobic substrate oxidation and metal oxide reduction. Humic substances can also serve as electron shuttles abiotically transferring electrons from an external electron donor to priority pollutants, which are susceptible to reductive transformations (e.g., polyhalogenated compounds, nitroaromatics, azo dyes and radionuclides). Moreover, reduced humic substances can also serve as an electron donor to achieve the microbial reduction of more oxidized electron acceptors, such as nitrate, fumarate and (per)chlorate. Figure 1 summarizes the different mechanisms that contribute to the recycling of humic substances in nature. In this closing chapter, the results obtained from the studies included in this dissertation will be discussed. The discussion focuses on the ubiquity and diversity of humus-respiring microorganisms and on the competition of quinone-respiring microorganisms over methanogens for different ecologically important substrates. Microbial humus reduction is also discussed from the ecological point of view. Likewise, the role of humic substances on the bioremediation of aquifers and sediments, as well as, on the anaerobic treatment of wastewaters containing priority pollutants will be discussed here.

Ubiquity and diversity of humus-respiring microorganisms

Humus reduction has recently been recognized as a novel respiratory pathway, which may contribute to the anaerobic microbial oxidation of different ecologically important substrates (*33*). Various evidences have documented the implication of quinone sub-structural units of humus during the microbial reduction of humic substances (*33, 37, 41*) and thus, the terms "humus respiration" and "quinone respiration" will be used alike in the present chapter.

In chapter 2 anaerobic consortia obtained from different environments showed quinone-respiring capacities (*8*). In all the consortia evaluated, including sandy and organic rich sediments, contaminated soils, and anaerobic as well as aerobic sludges originating from wastewater treatment plants, quinone-respiring bacteria out-competed methanogens for a number of distinct simple organic substrates, such as acetate and lactate, as well as hydrogen. The results are consistent with the recovery of humus-

reducing bacteria from many different habitats (*15*) and indicate that quinone-respiring microorganisms are widespread in nature.

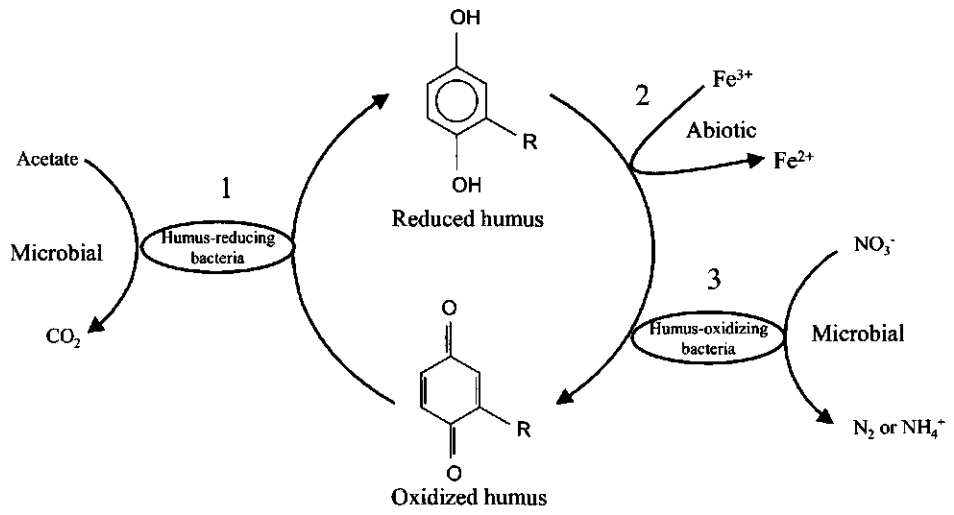


Figure 1. Abiotic and microbial reactions involved in the redox cycling of quinone moieties in humus: 1) microbial reduction of humus coupled to the anaerobic oxidation of an organic substrate (acetate); 2) abiotic oxidation of reduced humus by a metal oxide (Fe^{3+}); 3) reduced humus as an electron donor for the microbial reduction of a more oxidized electron acceptor (NO_3^-).

Figure 2 shows the phylogenetic tree including all the humus-reducing microorganisms reported in the literature. It can be observed that a broad diversity of physiologically distinct microbial groups can reduce humic substances when they are supplied as a terminal electron acceptor for the anaerobic oxidation of different substrates. Most of the humus-reducing microorganisms can oxidize hydrogen linked to the reduction of humus or the humic analogue, anthraquinone-2,6-disulfonate (AQDS); whereas acetate-linked humus reduction is rather associated with Fe(III)-reducing microorganisms members of the family *Geobacteraceae* (*12, 14, 15, 33*). The wide diversity of microorganisms, including Fe(III)-reducers (e.g., *Geobacter* spp. (*14, 15, 33*)), nitrate-reducers (e.g., *Shewanella putrefaciens* (*34, 37*)), sulfur-reducers (e.g., *Desulfuromonas* spp. (*15, 34*)), fermentative bacteria (e.g., *Propionibacterium freudenreichii* (*4*), and methanogenic archaea (e.g., *Methanococcus thermolithotrophicus* (*30*)), that can reduce humic substances may be an important factor for determining the ubiquity of humus-reduction in many anaerobic environments. Moreover, the ability to reduce humic substances have also been reported in hyperthermophilic microorganisms, such as

Pyrobaculum islandicum, which are the organisms most closely related to the last common ancestor of extant organisms suggesting that the last common ancestor had the ability to reduce humus (*30*). In chapter 3 of this dissertation different microorganisms generally found in anaerobic environments, which were previously not known to have the ability to reduce humic substances, are reported as quinone-reducers for the first time. All the microorganisms, including the sulfate-reducing bacterium, *Desulfovibrio* G11, the halorespiring microorganisms, *Desulfitobacterium* PCE1 and *Desulfitobacterium dehalogenans*, and the hydrogenotrophic methanogen, *Methanospirillum hungatei*, oxidized hydrogen linked to the stoichiometric reduction of AQDS. The *Desulfitobacterium* spp. were also able to oxidize lactate coupled to humus and AQDS reduction and the respiratory process was shown to support growth (*11*). The capacity of halorespiring microorganisms to grow under humus-reducing conditions suggests that humus may be the natural occurring electron acceptor for these microorganisms explaining their occurrence in pristine sites, where the presence of chlorinated pollutants and sulfite is not expected (*27, 39*).

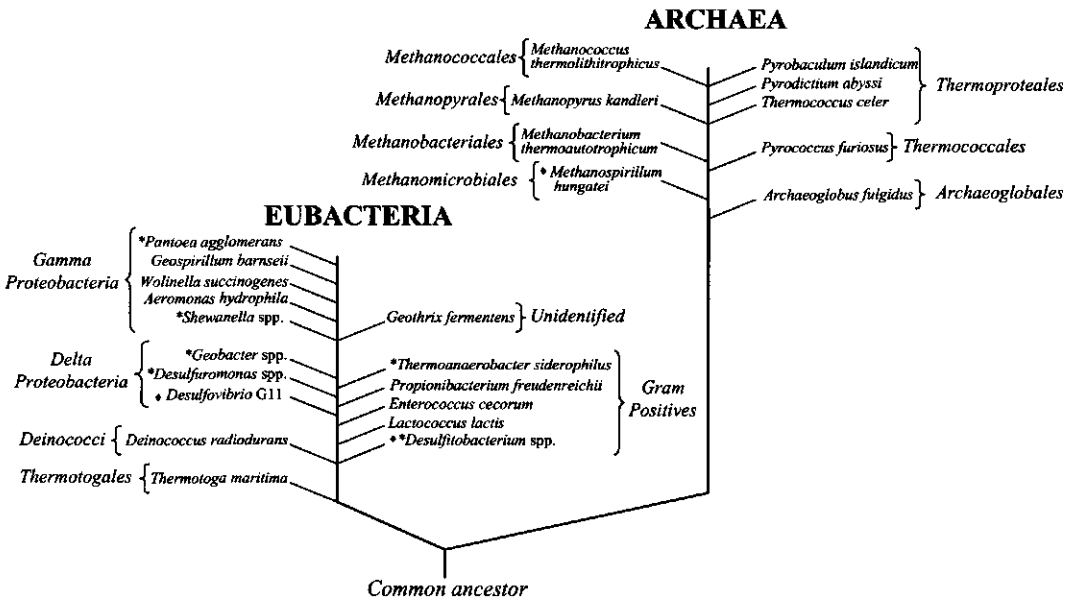


Figure 2. Phylogenetic diversity of humus-reducing microorganisms reported in the literature. The scheme does not show any similarity among the different phylogenetic groups illustrated (data obtained from references *4, 11, 14, 15, 22, 23, 30, 33, 34, 42*).
*, Microorganisms in which quinone respiration has been confirmed to support microbial growth. ♦, Contribution of this dissertation to the phylogenetic tree.

Given the versatility of several humus-reducing microorganisms, which can utilize a number of different electron acceptors, it is expected that the seasonal fluctuations in terminal electron acceptors

occurring in soil and sediments may not strongly affect the survival of humus-reducing microorganisms in these habitats. The versatility of humus-respiring microorganisms is also reflected in the wide diversity of substrates that can be oxidized via quinone respiration (*8*, *20*). Table 1 summarizes the substrates reported in the literature supporting quinone or humus respiration in different pure and mixed cultures. Furthermore, the reduction of humic substances has also been linked to the anaerobic oxidation of recalcitrant compounds by different consortia (see below). The metabolic versatility of humus-respiring bacteria may also be an important factor, which accounts for the ubiquity of these microorganisms.

Table 1. Anaerobic microbial oxidation of ecologically important substrates and priority pollutants linked to the reduction of humus or the humic model compound AQDS

Substrate	Electron acceptor	Inoculum	Reference
Ecologically important substrates			
Hydrogen	Humus or AQDS	Most humus reducing microorganisms in mixed or pure culture	8, 11, 15, 22, 30, 33
Acetate	Humus or AQDS	Pure cultures of *Geobacter* spp. and *Desulfuromonas* spp. and several consortia from different environments	8, 12, 14, 15, 22, 33, 34
Formate	AQDS	Pure cultures of *Geobacter* spp.	12, 15
Lactate	Humus or AQDS	Pure cultures of *Geobacter* spp., *Desulfitobacterium* spp., *Shewanella alga*, *Propionibacterium freudenreichii* and *Deinococcus radiodurans*, and several consortia from different environments	4, 8, 11, 15, 23, 33
Propionate	Humus or AQDS	Pure cultures of *Desulfuromonas* spp. and *Propionibacterium freudenreichii*, and several consortia from different environments	4, 8, 15
Ethanol	AQDS	Pure cultures of *Geobacter* spp. and *Desulfuromonas* spp.	15
Pyruvate	AQDS	Pure cultures of *Geobacter* spp.	15
Succinate	AQDS	Pure cultures of *Desulfuromonas* spp.	15
Glucose	Humus	Pure cultures of *Enterococcus cecorum* and *Lactococcus lactis*	4
Priority pollutant substrates			
Dichloroethene and vinyl chloride	Humus or AQDS	Fresh organic rich sediment	6
♦Phenol and *p*-cresol	AQDS	Anaerobic granular sludge from different origins	9
♦Toluene	Humus or AQDS	Anaerobic sediments from different origins	7

♦Contribution of this dissertation to the diversity of substrates that can be oxidized through the reduction of humic substances

Considering all these observations, it is not surprising that reduction of humic substances and recovery of quinone-respiring bacteria had been found in many different natural environmental, such as organic rich and oligotrophic sediments and soils (*8, 15*), as well as artificial habitats, such as sludges from wastewater treatment plants (*12*). The presence of humus-reducing microorganisms in wastewater treatment systems may have important implications for the anaerobic biotransformation of priority pollutants via quinone respiration (see below).

Competition of quinone respiration over methanogenesis for ecologically important substrates

Chapter 2 describes the addition of the humic analogue, AQDS, as an electron acceptor for sediment, soil, or sludge incubations. The AQDS-additions prevented methanogenesis and AQDS reduction became the preferred pathway for the conversion of most of the simple substrates provided (*8*). In the absence of AQDS, all the substrates, including hydrogen, acetate, lactate, propionate and methanol were partially or completely converted to methane.

There are multiple reasons that can explain why quinone-respiration out-competes methanogenesis. As shown in Figure 3, the reduction potential of the AQDS/AH_2QDS electron acceptor couple is superior to that of methanogenesis and sulfate-reduction. AQDS reduction is also thermodynamically superior than the reduction of the main Fe(III) forms found in nature, such as goethite and magnetite, which are poorly bioavailable. There is more free energy available for utilizing AQDS as an electron acceptor compared to these final electron acceptors. Humic substances from different origins have also showed redox potential within the same superior range (Figure 3). Microorganisms that reduce electron acceptors with a higher redox potential generally out-compete methanogens for hydrogen and acetate because they gain more energy from the oxidation of these substrates (*5, 31, 32, 52*). Therefore, competitive effects due to thermodynamic differences may contribute for the prevalence of quinone-respiring organisms over methanogens. However, other alternative electron acceptors, such as some metal oxides, as well as nitrate, are higher up in the reduction potential tower compared to quinone respiration (Figure 3). Consequently, denitrification and dissimilatory reduction of metal oxides, which are more bioavailable, such as vernadite and ferrihydrite, would be expected to out-compete quinone respiration.

In many of the consortia tested, initially there was a relatively high concentration of methanogens compared to the initial quinone-respiring population, thus thermodynamics can not account completely for the sudden predominance of quinone respiration over methanogenesis. Thus the fact that AQDS increased the redox potential (up to + 130 mV) of the culture fluid when supplied as an electron acceptor (*8*) may be important. The elevated redox potential may interfere with biochemical processes required for methanogenesis as it is generally assumed that methane production is only feasible at redox potentials below – 200 mV (*19*). AQDS supplied at high concentrations was even shown to be

toxic for acetoclastic and methylotrophic methanogenesis in anaerobic granular sludges (*8*). Toxicity may also play an important role for enabling quinone-reducing microorganisms to out-compete methanogens.

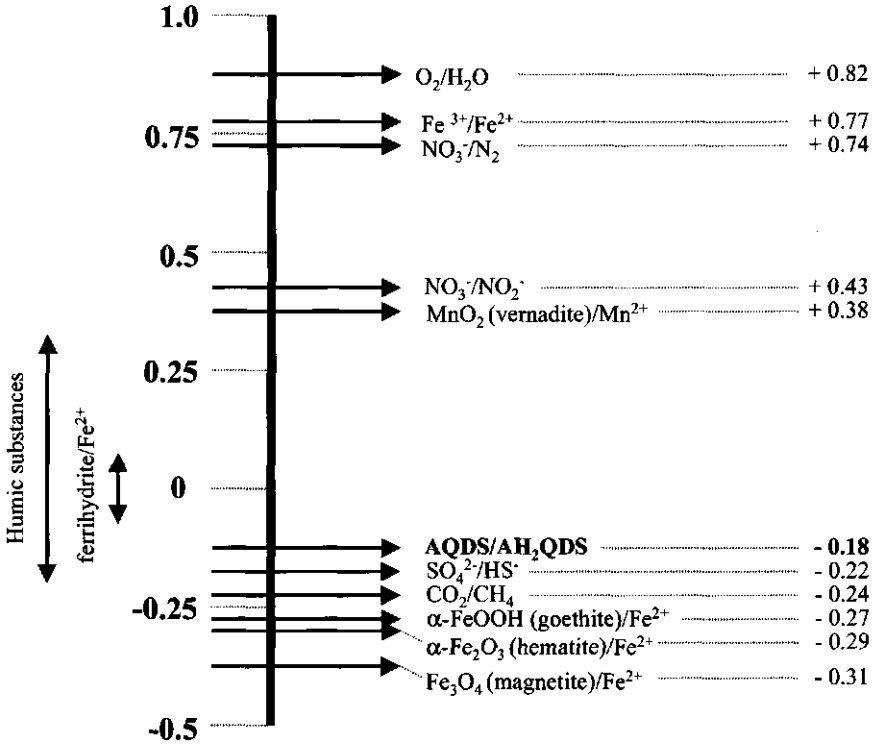


Figure 3. Reduction potentials (in V) under standard conditions ($E^{'0}$) of the main redox couples in nature. Data obtained from references *43* and *46*.

Ecological impact of microbial humus reduction

The broad spectrum of ecologically important substrates (Table 1), that can be oxidized through quinone-reducing processes indicates that quinones in humus may significantly contribute to carbon cycling process by serving as a terminal electron acceptor. The ecological relevance of microbial reduction of humic substances is further emphasized by the abundance of this organic fraction in many anaerobic sites, such as soil, sediments, swamps, eutrophic lakes, and compost.

Moreover, the high reactivity of hydroquinones in humus with metal oxides, such as Fe(III) (*33*) and Mn(IV) (*45*), allows for their regeneration to the oxidized. The recycling mechanism implies that even sub-stoichiometric concentrations of humic substances may lead to both anaerobic substrate oxidation and reduction of metal oxides (Figure 1), which are very abundant in many sedimentary environments. Oxidation of organic substrates via humus-respiration occurs more rapid than oxidation of substrates

linked directly to the reduction of metal oxides (*33*) probably because soluble humic substances are more readily accessible for microbial reduction than are insoluble metal oxides, which is also reflected on the higher redox potential observed in humus compared to the main Fe(III) forms in nature. AH_2QDS has been shown to access micropores inside geological oxides that cannot be reached by bacteria (*53*); thus, it is still questionable whether the reduction of Fe(III) and Mn(IV) proceeds directly by metal-reducers, or indirectly via quinone reduction by humus-reducers in nature. Humus-reducing microorganisms, such as *Propionibacterium freudenreichii*, which do not have the ability to reduce Fe(III), were shown to channel electrons from anaerobic oxidations, via humus reduction, towards the reduction of amorphous Fe(III) oxides (*4*). In chapter 3, the capacity of an halorespiring microorganism, *Desulfitobacterium dehalogenans*, and a sulfate-reducing bacterium, *Desulfovibrio* G11, for reducing amorphous Fe(III) oxides via quinone reduction is reported for the first time. *Desulfitobacterium dehalogenans* was unable to reduce the metal oxide in the absence of humic substances, but addition of sub-stoichiometric concentrations of AQDS (500 μM) allowed for the reduction of Fe(III), which was coupled to the oxidation of lactate or hydrogen. AQDS added at the same level enhanced the reduction of Fe(III) by *Desulfovibrio* G11 with hydrogen as an electron donor (*11*). The results suggest that many phylogenetically distinct types of organisms may contribute to the reduction of metal oxides via humus reduction in sedimentary habitats.

Humus as an electron acceptor for the bioremediation of aquifers and sediments

After humus reduction was recognized as a respiratory pathway in 1996 (*33*), a number or investigations have reported the potential of humic substances to serve as a terminal electron acceptor for achieving the microbial oxidation of recalcitrant compounds. The first finding that humic substances could act as electron acceptors for the anaerobic oxidation of priority pollutants was reported in an organic rich streambed sediment capable of mineralizing vinyl chloride and dichloroethene under humus-reducing conditions (*6*). These chlorinated contaminants were negligibly degraded in the absence of humic substances under anaerobic conditions, whereas addition of humic acids greatly stimulated the recovery of $^{14}CO_2$ from [1,2-^{14}C]vinyl chloride and [1,2-^{14}C]dichloroethene. The role of humic substances as an electron acceptor was corroborated in AQDS-supplemented bioassays in which the mineralization of [1,2-^{14}C]vinyl chloride was concomitant to the reduction of AQDS. In the present dissertation multiple priority pollutants were shown to be vulnerable to anaerobic biodegradation under quinone-reducing conditions. In chapter 5, anaerobic sludges originated from different wastewater treatment plants were shown to convert phenol and *p*-cresol under AQDS-reducing conditions and the conversion of the phenolic contaminants agreed with the stoichiometric recovery of the corresponding hydroquinone (anthrahydroquinone-2,6-disulfonate, AH_2QDS) in the bioassays (*9*). The phenolic compounds were completely converted to methane in the absence of humic substances, but addition of AQDS as an alternative electron acceptor diverted the

flow of electrons from methanogenesis towards quinone respiration. These results demonstrate for the first time that quinones in humus may contribute to the anaerobic microbial oxidation of phenolic compounds by serving as a terminal electron acceptor. Most importantly is the fact that priority pollutants (e.g., toluene), which were not degraded under methanogenic conditions, could be mineralized by humus-respiring consortia when humic substances were provided as an electron acceptor. In chapter 6, enriched anaerobic sediments originated from the Rhine river and from the Amsterdam Petroleum Harbor (APH) readily mineralized uniformly labeled [^{13}C]toluene to $^{13}CO_2$ when humic acids or AQDS were provided as terminal electron acceptors (*7*). After two weeks of incubation, 50 and 85 % of added [^{13}C]toluene was recovered as $^{13}CO_2$ in humus- and AQDS-supplemented enrichment cultures of APH sediment, respectively, whereas negligible recovery occurred in the absence of humic substances. Additionally, the electron equivalents in the toluene mineralized were recovered stoichiometrically as reduced humus or AH_2QDS. These evidences constitute the first direct and quantitative demonstration for the mineralization of an aromatic hydrocarbon by humus-respiring microorganisms.

Therefore, humus may be a more important electron acceptor for the bioremediation of contaminated environments than previously considered. Organic rich environments are particularly concerned because humic substances could support the anaerobic oxidation of organic pollutants by serving as a terminal electron acceptor and thus, contributing to the intrinsic bioremediation of these habitats.

Addition of humic acids (*35*) or AQDS (*1*) to petroleum-contaminated aquifer sediments rich in Fe(III) oxides, but deficient in humus, showed promising stimulation of the anaerobic biodegradation of benzene at some sites. Humic substances are also claimed to stimulate the anaerobic degradation of the gasoline additive, methyl-*tert*-butyl ether, in aquifer sediments provided with Fe(III) as a final electron acceptor (*21*). Moreover, in chapter 6 of this dissertation it was shown that APH sediment did not use amorphous Fe(III) oxide as an electron acceptor for toluene degradation, but when sub-stoichiometric amounts of humic acids were included in sediment incubations, toluene biodegradation could be coupled to the reduction of Fe(III). Presumably humus-respiring microorganisms transferred electrons from anaerobic toluene oxidation to the metal oxide via humus reduction (*7*). Figure 4 summarizes the mechanisms of anaerobic toluene biodegradation in the humus-respiring consortium. Therefore, an eventual technology based on pumping humic substances or quinones into oligotrophic aquifers and sediments to enhance the anaerobic oxidation of priority pollutants can also be considered. All the Fe(III)-reducing microorganisms evaluated are also able to utilize humus or AQDS as an electron acceptor (*12, 14, 15, 30, 33, 34*) and quinones have much higher aqueous solubility than alternatives, such O_2, Fe(III) and Mn(IV), which may warrant good transport into the aquifer. The regeneration of hydroquinones in humus by chemical reaction with metal oxides may allow for the application of humic substances in contaminated sites at sub-stoichiometric concentrations as long as the mechanisms for achieving the recycling process are also available. For engineered bioremediation

systems with groundwater recirculation, quinone recycling can also be achieved by direct chemical reaction of pumped up hydroquinones with oxygen and the resulting recycled quinones can be reinjected into the aquifer.

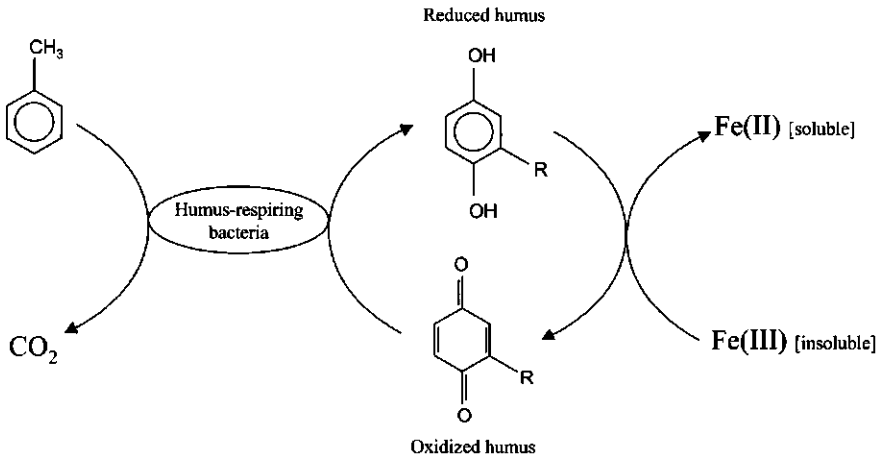


Figure 4. Anaerobic oxidation of toluene in the quinone-respiring Amsterdam Petroleum Harbor sediment (*7*).

The application of humic substances for achieving bioremediation of contaminated aquifers and sediments have some other advantages. Humus does not represent any risk when applied in aquifers and sediments deficient in organic matter given its inert properties. Although humic substances may enhance the movement of a contaminant plume in ground waters due to their ability to bind hydrocarbons and heavy metals (*44*), the increased mobilization may also improve hydrocarbon bioavailability, which could also improve its biodegradation. Another important implication is the possible solubilization of metals, such as Fe(II) and Mn(II), during the regeneration of hydroquinones in humus. However, Fe(II) and Mn(II) may undergo sorption to bacteria or to the residual metal oxide surface. The results presented in chapter 6 illustrate this possibility. Only 30-34 % of the reducing equivalents as Fe(II) or Mn(II), which were expected during the anaerobic oxidation of toluene, could be recovered when goethite (α-FeOOH) and vernadite (MnO_2) were provided as a terminal electron acceptor in the presence of humic acids (*7*). Furthermore, aerobic, phototrophic and nitrate-reducing microorganisms can oxidize substantial amounts (up to 8-10 mM) of soluble Fe(II) leading to the formation of insoluble ferrihydrite, which is excreted outside the cells (*46*). Biogenic ferrihydrite has been shown to be a suitable electron acceptor for iron-reducing bacteria (*47*) indicating that biological Fe(II) oxidation indeed contributes to the recycling of iron in anaerobic environments. Another advantage of the application of humic substances for the bioremediation of anaerobic sites is represented by the lack of undesirable intermediates during the microbial reduction of humic

substances. The injection of nitrate and sulfate for the bioremediation of contaminated environments has limitations due to the accumulation of intermediates (e.g., nitrite) or end products (e.g., sulfide), which represent pollutants themselves (*2, 25*). Therefore, the utilization of humic substances to stimulate the bioremediation of contaminated anaerobic sites containing recalcitrant pollutants represents an attractive alternative.

Humus as a redox mediator for the reductive (bio)transformations of priority pollutants

Humic substances do not only serve as final electron acceptors for the anaerobic oxidation of pollutants. Humus and quinone analogues can also stimulate the reductive (bio)transformation of azo dyes, nitroaromatics, polyhalogenated contaminants and radionuclides by shuttling electrons between an external electron donor and those pollutants. Mechanisms mediated by humic substances include abiotic as well as biological processes in which quinones or humus accelerate the reductive transformations by one to several orders of magnitude.

The most well studied abiotic mediation of humic substances in reductive reactions is the conversion of hexachloroethane (HCA) to tetrachloroethylene, also known as perchloroethylene (PCE), by ferrous iron, sulfide or elemental sulfur (*17*). Addition of quinones accelerated up to 10-fold the rate of these chemical reactions and the reduced quinone moiety (AH_2QDS) could also cause the direct dechlorination of HCA (*17*). Addition of humic model compounds, such as resorcinol and catechol, to abiotic assays including chlorinated dibenzo-*p*-dioxins (CDDs) led to the dechlorination of these contaminants (*3*). Reactions systems containing octa-CDDs and any of the humic analogues achieved the formation of the tetra-CDDs group of congeners, whereas no reductive dechlorination of octa-CDDs occurred in the absence of humic model compounds. Quinones and humus also enabled the abiotic reduction of nitroaromatics (*18, 40*) and azo dyes (*50*) to the corresponding aromatic amines with bulk reducing agents commonly found in anaerobic environments, such as sulfide and ferrous iron.

Humus and quinones have also been implicated in the reductive biotransformations of priority pollutants. *Deinococcus radiodurans*, a radiation-resistant microorganism, could reduce the soluble radionuclides, U(VI) and Tc(VII), to their insoluble species, U(IV) and Tc(IV), when AQDS was provided as a redox mediator at sub-stoichiometric concentrations, whereas no reduction of the radionuclides occurred in the absence of AQDS (*23*). The precipitation of these ionization radiation emitters through quinone reduction may have important implications for the bioremediation of metal- and radionuclide-contaminated sites.

Cell suspensions of *Shewanella putrefaciens* including soil organic matter converted carbon tetrachloride (CT) to chloroform and unidentified products at a higher rate compared to bioassays lacking organic matter (*16*). The humic acid fraction was shown to catalyze the conversion of CT at a greater extent than did the fulvic acid and humin constituents, but the actual functional groups and

mechanisms involved in the redox reactions were not elucidated. The evidences presented in chapter 7 indicate that quinone-respiring microorganisms may play a more important role on the conversion of polyhalogenated pollutants than previously considered. Addition of AQDS at sub-stoichiometric levels enhanced the rate and extent of dechlorination of CT by anaerobic granular sludge leading to an increased production of inorganic chloride, which accounted for 40-50 % of the chlorine initially linked to the chlorinated contaminant (*10*). A wide variety of substrates could be used as an electron donor to enhance the dechlorinating process, which agrees with the fact that a broad spectrum of compounds can be oxidized through quinone respiration (see above). AQDS was also able to extend the possible electron donors utilized by the anaerobic sludge to acetate and methanol, which otherwise were not used to support CT dechlorination in the absence of quinones. The enhanced CT dechlorination observed was attributed to humus-respiring bacteria in the sludge as evidenced by the selective inhibition of quinone respiration with the antibiotic, neomycin. Neomycin inhibited both AQDS reduction and the enhanced CT conversion by the anaerobic granular sludge. The methanogenic inhibitor, 2-bromoethanesulfonic acid, which showed no effect on AQDS reduction, did not inhibit the conversion of CT by the same inoculum. Furthermore, a humus-respiring enrichment culture, composed primarily of a *Geobacter sulfurreducens* related species, derived from the granular sludge was also shown to dechlorinate CT in the presence of either AQDS or humic acids at low concentrations, yielding similar products as the AQDS-supplemented sludge. The conversion of CT was paralleled by the reduction of AQDS and humus in the bioassays constituting the first demonstration that quinone-respiration can contribute as a mechanism generating electron equivalents for dechlorinating processes. No conversion of CT was observed in the absence of humic substances by the enrichment culture. Chloroform and dichloromethane accounted for a minor proportion (1-10 %) of the CT converted in the bioassays supplemented with humic substances. The accumulation of a chlorinated ethene, PCE, is also reported for the first time as an intermediate of CT biodegradation in this study. The most likely explanation for the accumulation of PCE (up to 9 % of the CT initially added) during the conversion of CT, is the coupling of two trichloromethyl radicals to form HCA. HCA, in turn, could be readily converted to PCE either by abiotic reduction with hydroquinones (*17*) or by further microbial dechlorination (*51*). The accumulation of PCE is undesirable because it could lead to the formation of lower chlorinated pollutants, such as dichloroethene and vinyl chloride, which are more recalcitrant than PCE under anaerobic conditions. The presence of humic substances, however, may contribute to attenuate the accumulation of these contaminants, since both AQDS and humus, as previously shown, can serve as a terminal electron acceptor for the anaerobic microbial oxidation of dichloroethene and vinyl chloride to mineralization products (*6*).

Impact of humus on the anaerobic treatment of wastewaters containing priority pollutants

Azo dyes, nitroaromatics and polyhalogenated pollutants are common constituents of wastewaters originated from the chemical and petrochemical industry, which have been shown to be toxic for the biological systems continuously treating these discharges. As electron-withdrawing pollutants, however, they can be converted in anaerobic reactors allowing for the detoxification of the systems. A number of reports have indicated that quinone moieties could accelerate the reductive biotransformation of these pollutants. For instance, different quinone structures have been shown to accelerate the reduction of azo dyes to the corresponding colorless aromatic amines (*26, 28*).

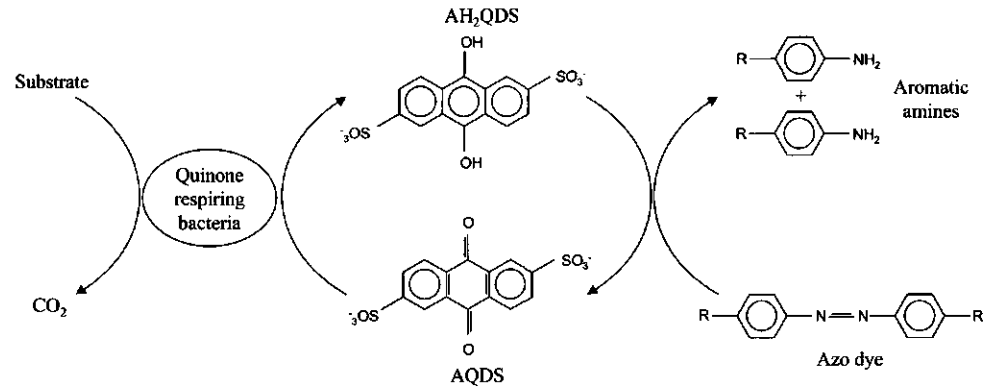


Figure 5. Mechanism of AQDS mediated reduction of azo dyes. Quinone-respiring bacteria reduce AQDS to the corresponding hydroquinone, AH_2QDS, which in turn directly transfers the electrons to the dye resulting in azo cleavage.

In chapter 8 of this dissertation, the application of different levels of AQDS to accelerate the reductive decolorization of Acid Orange 7 (AO7) in a continuous upflow anaerobic sludge blanket (UASB) reactor is reported (*13*). High efficiency (>90 %) of decolorization of AO7 occurred even at a hydraulic residence time of 2 hours with a molar ratio of AQDS/AO7 as low as 1/100, whereas 70 % of color removal occurred in the absence of AQDS under the same hydraulic conditions. Moreover, recent experiments have revealed that addition of AQDS at very low concentrations also enhanced the reductive biotransformation of other azo dyes (e.g. Reactive Red 2) which are decolorized at a much lower rate in the absence of quinones (*49*). The enhancement in the decolorizing process not only lead to detoxification (*49*), but it also accelerated the reductive process, which in turn, could decrease the hydraulic residence time required (*13*) enabling more compact anaerobic reactors. Since microbially reduced quinones are responsible for the terminal reaction with the azo dyes, the quinone-reducing microorganisms do not need to be in direct contact with the dye to carry out the decolorizing process (*28*). Figure 5 shows the mechanism of azo dye reduction mediated by quinones. Thus, quinones, as

redox mediators, do not necessarily have to be present in abundant supply, as they can be easily recycled during the reductive mechanisms. According to these evidences, the application of humus or quinones to wastewater treatment reactors could enhance the biotransformation of different contaminants susceptible to reductive conversion.

High rate wastewater treatment systems, such as UASB and expanded granular sludge blanket (EGSB) reactors, in which anaerobic granular sludge is developed, represent attractive technologies for achieving the anaerobic conversion of priority pollutants. Anaerobic granular sludge maintains superior settling characteristics and the washout of biomass is decreased to a minimum by creating a quiescent zone within the reactors, enabling the sludge particles to flocculate, to settle, and to be entrapped in a secondary sludge blanket (*29*). Therefore, quinone-respiring bacteria, which have been shown to play an important role on the reductive biotransformation of priority pollutants (*10*), may be retained in the reactors facilitating their involvement in reductive conversions. In fact, in chapter 4, an AQDS-respiring enrichment culture that was developed in anaerobic granular sludge is described. The enriched quinone-respiring granular sludge of a UASB reactor was capable of continuously reducing AQDS for a prolonged period in steady state indicating that quinone-respiring microorganisms can be immobilized in the microbial community of the anaerobic granular sludge (*12*). Furthermore, immobilization of other types of microorganisms with the capacity to degrade different priority pollutants has successfully been achieved in UASB reactors allowing for the continuous conversion of the contaminants (*24*, *48*). Therefore, granular sludge constitutes a suitable form of immobilization of quinone-respiring microorganisms in continuous anaerobic reactors. Further advantages of the UASB and EGSB reactors is the hydraulic and gas mixing conditions that prevail in the systems, which allow for minimal biological dead-space and prevent localized high concentrations of the toxic pollutants present in the wastewater. Moreover, the granular structure provides protection to microorganisms inside the granules attenuating the toxic impact of the contaminants (*38*).

Concluding remarks

Humus was generally considered as an inert material in the past due to its remarkable stability in the environment. However, the evidences discussed here indicate that quinone moieties in humus can play different roles contributing to the anaerobic biodegradation and biotransformation of ecologically important substrates, as well as priority pollutants. Humic acids and quinone model compounds supported the anaerobic microbial oxidation of several important substrates by serving as a terminal electron acceptor in many different environments. Quinone-respiring activity was ubiquitously observed both in natural environments, such as sandy and organic rich sediments, and in artificial habitats, such as sludges originated from wastewater treatment plants. The capacity to reduce humic substances was also found in axenic cultures of phylogenetically distinct microorganisms, which oxidized different substrates via humus reduction, indicating that many different microorganisms may

be involved in the reduction of humic substances in nature. Humus-respiring consortia also showed the capacity of mineralizing priority pollutants, such as toluene, when humus and quinones were provided as a final electron acceptor. Thus, a technology based on injecting humic substances into aquifers and sediments to stimulate the bioremediation of contaminated sites can be considered. Humic substances do not necessarily have to be supplied abundantly to stimulate the bioremediation of these sites. Microbially reduced quinones in humus can be recycled by chemical reaction with metal oxides, which are abundant in many anaerobic environments, allowing for the application of humic substances at sub-stoichiometric levels.

Quinones and humus also served as redox mediators accelerating the reductive biotransformation of different contaminants with electron-withdrawing groups. Catalytic concentrations of AQDS and humic acids enhanced both the rate and extent of dechlorination of carbon tetrachloride by quinone-respiring consortia. Addition of micromolar concentrations of AQDS to a UASB reactor continuously treating an azo dye also allowed for a decrement in the hydraulic residence time required for an efficient decolorizing process. Therefore, the application of catalytic levels of quinones or humus to anaerobic reactors may enhance the reductive biotransformation of priority pollutants in wastewaters. The enhanced biotransformation of these contaminants may have important implications because it may lead to detoxification of the microbial community in wastewater treatment systems. Likewise, the application of humic substances may demand more compact wastewater treatment systems due to the accelerated conversion of priority pollutants.

References

1. Anderson, R. T. and D. R. Lovley. (1999) Naphthalene and benzene degradation under Fe(III)-reducing conditions in petroleum-contaminated aquifers. *Bioremediat. J.* **3**, 121-135.
2. Anderson, R. T. and D. R. Lovley. (2000). Anaerobic bioremediation of benzene under sulfate-reducing conditions in a petroleum-contaminated aquifer. *Environ. Sci. Technol.* **34**, 2261-2266.
3. Barkovskii, A. L. and P. Adriaens. (1998). Impact of humic constituents on microbial dechlorination of polychlorinated dioxins. *Environ. Toxicol. Chem.* **17**, 1013-1020.
4. Benz, M., Schink, B. and Brune, A. (1998) Humic acid reduction by *Propionibacterium freudenreichii* and other fermentative bacteria. *Appl. Environ. Microbiol.* **64**,4507-4512.
5. Bodegom, P. M. and Stams, A. J. M. (1999) Effects of alternative electron acceptors and temperature on methanogenesis in rice paddy soils. *Chemosphere* **39**, 167-182.
6. Bradley, P. M., F. H. Chapelle and D. R. Lovley. (1998) Humic acids as electron acceptors for anaerobic microbial oxidation of vinyl chloride and dichloroethene. *Appl. Environ. Microbiol.* **64**, 3102-3105.

7. Cervantes, F. J., Dijksma, W., Duong-Dac, T., Ivanova, A., Lettinga, G. and Field, J. A. (2001) Anaerobic mineralization of toluene by enriched sediments with quinones and humus as terminal electron acceptors. *Appl. Environ. Microbiol.* **67**, 4471-4478.

8. Cervantes, F. J., van der Velde, S., Lettinga, G. and Field, J. A. (2000) Competition between methanogenesis and quinone respiration for ecologically important substrates in anaerobic consortia. *FEMS Microbiol. Ecol.* **34**,161-171.

9. Cervantes, F. J., van der Velde, S., Lettinga, G. and Field, J. A. (2000) Quinones as terminal electron acceptors for anaerobic microbial oxidation of phenolic compounds. Biodegradation 11, 313-321.

10. Cervantes, F. J., Vu-Thi-Thuy, L., Lettinga, G. and Field, J. A. Quinone-respiration improves dechlorination of carbon tetrachloride by anaerobic sludge. *Submitted.*

11. Cervantes, F.J., De Bok F. A. M., Duong-Dac T., Stams, A. J. M., Lettinga G. and Field J. A. Reduction of humic substances by halorespiring, sulfate-reducing and methanogenic microorganisms. *Submitted.*

12. Cervantes, F.J., Duong-Dac T., Ivanova, A.E., Roest K., Akkermans A. D. L., Lettinga G. and Field J. A. Quinones as terminal electron acceptors in anaerobic granular sludge. *Submitted.*

13. Cervantes, F.J., van der Zee, F.P., Lettinga, G. and Field, J.A. (2001). Enhanced decolourisation of acid orange 7 in a continuous UASB reactor with quinones as redox mediators. *Water Sci. Technol.* **44**, 123-128.

14. Coates, J. D., Bhupathiraju, V. K., Achenbach, L. A., McInerney, M. J., and Lovley, D. R. (2001) *Geobacter hydrogenophilus, Geobacter chapellei* and *Geobacter grbicie*, three new, strictly anaerobic, dissimilatory Fe(III)-reducers. *Int. J. Syst. Evol. Microbiol.* **51**, 581-588.

15. Coates, J. D., Ellis, D. J., Roden, E., Gaw, K., Blunt-Harris, E. L. and Lovley, D. R. (1998) Recovery of humics-reducing bacteria from a diversity of sedimentary environments. *Appl. Environ. Microbiol.* **64**,1504-1509.

16. Collins, R. and Picardal, F. (1999) Enhanced anaerobic transformation of carbon tetrachloride by soil organic matter. *Environ. Toxicol. Chem.* **18**, 2703-2710.

17. Curtis, C. and Reinhard M. (1994) Reductive dehalogenation of hexachloroethane, carbon tetrachloride, and bromoform by anthrahydroquinone disulphonate and humic acid. *Environ. Sci. Technol.* **28**, 2393-2401.

18. Dunnivant, F., Schwarzenbach R. and Macalady D. (1992) Reduction of substituted nitrobenzenes in aqueous solutions containing natural organic matter. *Environ. Sci. Technol.* **26**, 2133-2142.

19. Fetzer, S. and Conrad R. (1993) Effect of redox potential on methanogenesis by *Methanosarcina barkeri. Arch. Microbiol.* **160**, 108-113.

20. Field, J. A., Cervantes, F. J., van der Zee, F. P. and Lettinga, G. (2000) Role of quinones in the biodegradation of priority pollutants: a review. *Water Sci. Technol.* **42**, 215-222.

21. Finneran, K. T. and Lovley D. R. (2001) Anaerobic degradation of methyl *tert*-butyl ether (MTBE) and *tert*-butyl alcohol (TBA). *Environ. Sci. Technol.* **35**, 1785-1790.

22. Francis, C. A., Obraztsova, A. Y. and Tebo, B. M. (2000) Dissimilatory metal reduction by the facultative anaerobe *Pantoea agglomerans* SP1. *Appl. Environ. Microbiol.* **66**,543-548.

23. Fredrickson, J. K., Kostandarithes, H. M., Li, S. W., Plymale, A. E. and Daly, M. J. (2000) Reduction of Fe(III), Cr(VI), U(VI), and Tc(VII) by *Deinococcus radiodurans* R1. *Appl. Environ. Microbiol.* **66**,2006-2011.

24. Hörber, C., Christensen, N., Arvin, E. and Ahring, B. K. (1999) Tetrachloroethene dechlorination kinetics by *Dehalospirillum multivorans* immobilized in upflow anaerobic sludge blanket reactors. *Appl. Microbiol. Biotechnol.* **51**, 694-699.

25. Hutchins, S. R., Sewell G. W., Kovacs D. A. and Smith G. A. (1991) Biodegradation of aromatic hydrocarbons by aquifer microorganisms under denitrifying conditions. *Environ. Sci. Technol.* **25**, 68-76.

26. Kudlich, M., Keck, A., Klein, J. and Stolz, A. (1997) Localization of the enzyme system involved in anaerobic reduction of azo dyes by *Sphingomonas* sp. Strain BN6 and effect of artificial redox mediators on the rate of azo dye reduction. *Appl. Environ. Microbiol.* **63**, 3691-3694.

27. Lanthier, M., Villemur, R., Lépine, F., Bisaillon, J., and Beaudet, R. (2001) Geographic distribution of *Desulfitobacterium frappieri* PCP-1 and *Desulfitobacterium* spp. in soils from the province of Quebec, Canada. *FEMS Microbiol. Ecol.* **36**, 185-191.

28. Laszlo, J. A. (2000) Regeneration of azo-dye-saturated cellulosic anion exchange resin by *Burkholderia cepacia*. *Environ. Sci. Technol.* **34**, 167-172.

29. Lettinga, G., van Velsen, A. F. M., Hobma, S. W., de Zeeuw, W. and Klapwijk, A. (1980) Use of the upflow sludge blanket (USB) reactor concept for biological wastewater treatment, especially for anaerobic treatment. *Biotechnol. Bioeng.* **22**, 699-734.

30. Lovley D. R., Kashefi, K., Vargas, M., Tor, J. M. and Blunt-Harris, E. L. (2000) Reduction of humic substances and Fe(III) by hyperthermophilic microorganisms. *Chem. Geol.* **169**,289-298.

31. Lovley, D. R. (1985) Minimum threshold for hydrogen metabolism in methanogenic bacteria. *Appl. Environ. Microbiol.* **49**, 1530-1531.

32. Lovley, D. R. and Phillips, E. J. P. (1987) Competitive mechanisms for inhibition of sulfate reduction and methane production in the zone of ferric iron reduction in sediments. *Appl. Environ. Microbiol.* **53**, 2636-2641.

33. Lovley, D. R., Coates, J. D., Blunt-Harris, E. L., Phillips, E. J. P. and Woodward, J. C. (1996) Humic substances as electron acceptors for microbial respiration. *Nature* **382**,445-448.

34. Lovley, D. R., Fraga, J. L., Blunt-Harris, E. L., Hayes, L. A., Phillips, E. J. P., and Coates, J. D. (1998) Humic substances as a mediator for microbially catalyzed metal reduction. *Acta Hydrochim. Hydrobiol.* **26**, 152-157.

35. Lovley, D. R., Woodward J. C. and Chapelle F. H. (1996) Rapid anaerobic benzene oxidation with a variety of chelated Fe(III) forms. *Appl. Environ. Microbiol.* **62**, 288-291.

36. Lovley, D. R., Fraga J. L., Coates J. D. and Blunt-Harris E. L. (1999) Humics as an electron donor for anaerobic respiration. *Environ. Microbiol.* **1**, 89-98.

37. Newman, D. K., and Kolter, R. (2000) A role for excreted quinones in extracellular electron transfer. *Nature* **405**, 94-97.

38. Razo-Flores, E., Donlon, B, Lettinga, G. and Field, J. A. (1997) Biotransformation and biodegradation of N-substituted aromatics in methanogenic granular sludge. *FEMS Microbiol. Rev.* **20**, 525-538.

39. Sanford, R. A., Cole, J. R., Löffler, F. E., and Tiedje, J. M. (1996) Characterization of *Desulfitobacterium chlororespirans* sp. nov., which grows by coupling the oxidation of lactate to the reductive dechlorination of 3-chloro-4-hydroxybenzoate. *Appl. Environ. Microbiol.* **62**, 3800-3808.

40. Schwarzenbach, R., Stierli, R., Lanz, K. and Zeyer, J. (1990) Quinone and iron porphyrin mediated reduction of nitroaromatic compounds in homogeneous aqueous solution. *Environs. Sci. Technol.* **24**, 1566-1574.

41. Scott, D. T., McKnight, D. M., Blunt-Harris, E. L., Kolesar, S. E. and Lovley, D. R. (1998) Quinone moieties act as electron acceptors in the reduction of humic substances by humics-reducing microorganisms. *Environ. Sci. Technol.* **32**,2984-2989.

42. Slobodkin, A. I., Tourova, T. P., Kuznetsov, B. B., Kostrikina, N. A., Chernyh, N. A., and Bonch-Osmolovskaya, E. A. (1999) *Thermoanaerobacter siderophilus* sp. nov., a novel dissimilatory Fe(III)-reducing, anaerobic, thermophilic bacterium. *Int. J. Syst. Bacteriol.* **49**, 1471-1478.

43. Sober, H. A. (1970) Handbook of biochemistry: selected data for molecular biology. The Chemical Rubber Co., Cleveland, Ohio.

44. Stevenson, F. J. (1994) Humus chemistry: genesis, Composition, Reactions, 2nd ed. Wiley, New York.

45. Stone, A. T. and Morgan J. J. (1984) Reduction of manganese(III) and manganese(IV) oxides by organics. 1. Reaction with hydroquinone. *Environ. Sci. Technol.* **18**, 450-456.

46. Straub, K. L., Benz, M. and Schink, B. (2001) Iron metabolism in anoxic environments at near neutral pH. *FEMS Microbiol. Ecol.* **34**, 181-186.

47. Straub, K. L., Hanzlik, M. and Buchholz-Cleven, B. E. E. (1998) The use of biologically produced ferrihydrite for the isolation of novel iron-reducing bacteria. *Syst. Appl. Microbiol.* **21**, 442-449.

48. Tawfiki-Hajji, K., Lepine, F., Bisaillon, J. G., Beaudet, R., Hawari, J. and Guiot, S. R. (2000) Effects of bioaugmentation strategies in UASB reactors with a methanogenic consortium for removal of phenolic compounds. *Biotechnol. Bioeng.* **67**, 417-423.

49. Van der Zee, F. P., Bouwman, R. H. M., Strik, D. P. B. T. B., Lettinga, G., and Field, J. A. (2001) Application of redox mediators to accelerate the transformation of reactive azo dyes in anaerobic bioreactors. *Biotechnol. Bioeng.* **75**, 691-701.

50. Van der Zee, F. P., Lettinga, G. and Field, J. A. (2000) The role of (auto)catalysis in the mechanism of an azo dye reduction. *Water Sci. Technol.* **42**, 301-308.

51. Van Eekert, M. H. A., Stams, A. J. M., Field, J. A. and Schraa, G. (1999) Gratuitous dechlorination of chloroethanes by methanogenic granular sludge. *Appl. Microbiol. Biotechnol.* **51**, 46-52.

52. Ward, D. M. and Winfrey, M. R. (1985) Interactions between methanogenic and sulfate-reducing bacteria in sediments. *Adv. Aquat. Microbiol.* **3**, 141-179.

53. Zachara, J.M., Fredrickson, J. K., Li, S. M., Kennedy, D. W., Smith, S. C. and Gassman, P. L. (1998) Bacterial reduction of crystalline Fe^{3+} oxides in single phase suspensions and subsurface materials. *Am. Mineralogist.* **83**,1426-1443.

9

Algemene Discussie en Conclusies

Introductie

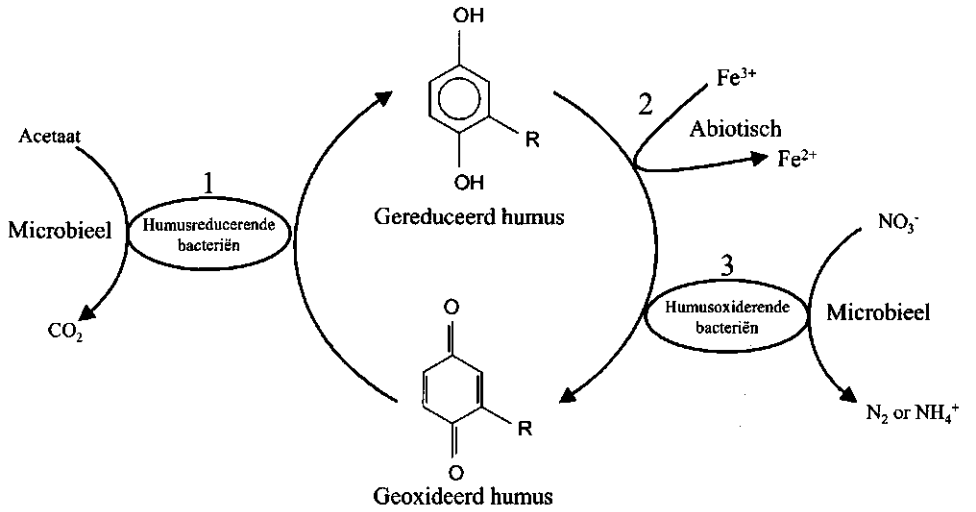
Humus is de meest voorkomende organische fractie in de biosfeer. Humusstoffen zijn biologisch slecht-afbreekbare polymeren die gevormd worden gedurende de decompositie van plantenmateriaal in bodems en sedimenten. Lange verblijftijden van meer dan 500 jaar geven blijk van de opmerkelijke persistentie van humus in het milieu (*44*). Het is recentelijk echter onderkend dat humeuze verbindingen een belangrijke rol kunnen spelen in de anaërobe biotransformatie en biodegradatie van zowel organische als anorganische stoffen. Humus kan dienen als terminale elektronenacceptor voor de anaërobe microbiële oxidatie van een gevarieerde reeks organische stoffen. Microbieel gereduceerd humus kan elektronen overdragen op metaaloxiden zoals Fe(III) en Mn(IV). Hierdoor vindt regeneratie plaats van de geoxideerde vorm van humus. Humus kan daarom, ook wanneer het slechts aanwezig is in substoichiometrische hoeveelheden, als mediator ('bemiddelaar') optreden bij zowel de anaërobe oxidatie van organische substraten als de anaërobe reductie van metaaloxiden. Humuszuur kan eveneens als mediator dienst doen in de abiotische overdracht van elektronen van een externe elektronendonor op reduceerbare zwartelijststoffen (b.v. polyhalogeenverbindingen, nitroaromaten, azokleurstoffen en radionucliden). Bovendien kunnen gereduceerde humusstoffen dienen als elektronendonor voor de microbiële reductie van hoger-geoxideerde elektronenacceptoren zoals nitraat, fumaraat en (per)chloraat. Figuur 1 vat de verschillende mechanismen samen die bijdragen aan de kringloop van humus in de natuur. In dit afsluitende hoofdstuk zullen de resultaten van het onderzoek dat is opgenomen in dit proefschrift worden besproken. De discussie richt zich op de alomtegenwoordigheid en diversiteit van humusrespirerende micro-organismen en op de competitie tussen chinonrespirerende en methanogene micro-organismen met betrekking tot de omzetting van verschillende ecologisch belangrijke substraten. Ook zal microbiële humusreductie vanuit ecologisch gezichtspunt worden besproken en voorts zal worden ingegaan op de rol van humuszuren in de bioremediatie van waterbodems en sedimenten en in de anaërobe behandeling van zwartelijststoffen in afvalwater.

Alomtegenwoordigheid en diversiteit van humusrespirerende micro-organismen

Het is pas recentelijk onderkend dat humusreductie als respiratieroute een bijdrage kan leveren aan de anaërobe microbiële oxidatie van verschillende ecologisch belangrijke verbindingen (*33*). Volgens verscheidene aanwijzingen behelst microbiële humusreductie de reductie van chinongroepen in de humusstoffen (*33, 37, 41*). Daarom worden in dit hoofdstuk de termen "humusademhaling of –respiratie" en "chinonademhaling of –respiratie" naast elkaar gebruikt.

In hoofdstuk 2 van dit proefschrift werd vastgesteld dat anaërobe consortia uit verschillende milieus chinonrespirerende capaciteit vertoonden (*8*). De geëvalueerde consortia waren afkomstig van zandige sedimenten, van sedimenten die juist rijk waren aan organische stof, van verontreinigde bodems en van slib uit zowel anaërobe als aërobe afvalwaterzuiveringsinstallaties. De chinonrespirende micro-

organismen in al deze consortia verdrongen de methanogenen tijdens de competitie om een aantal verschillende eenvoudige substraten zoals acetaat, lactaat en waterstof. Deze resultaten zijn in lijn met de ophoping van humusreducerende micro-organismen uit vele verschillende verspreidingsgebieden (*15*) en geven aan dat humusrespirerende micro-organismen wijdverbreid zijn in de natuur.

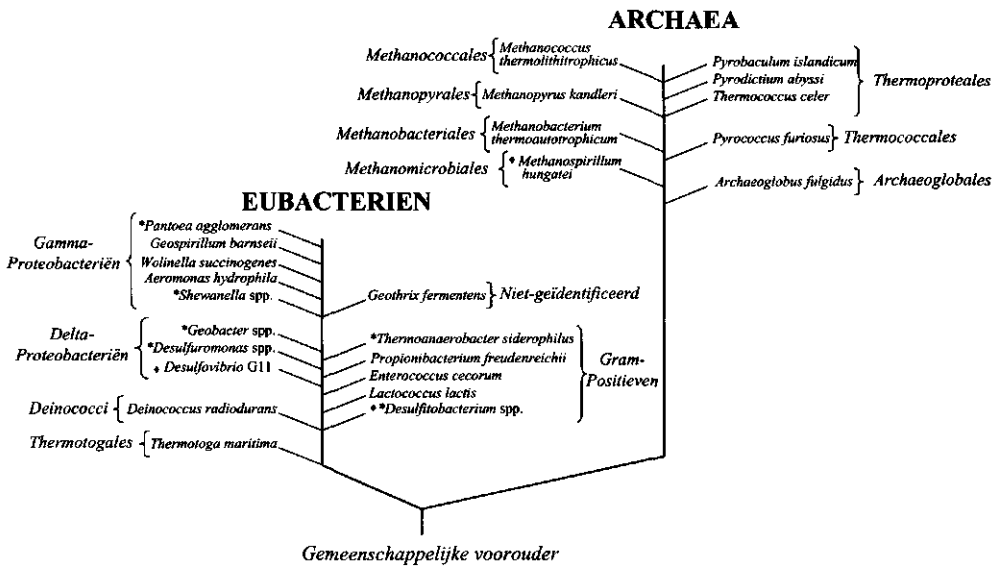


Figuur 1. Abiotische en microbiële reacties die betrokken zijn bij de redoxcyclus van chinon-structuren in humus: 1) microbiële humusreductie gekoppeld aan de anaërobe oxidatie van een organisch substraat (acetaat); 2) abiotische oxidatie van gereduceerd humus door een metaaloxide (Fe^{3+}); 3) gereduceerd humus als een elektronendonor voor de microbiële reductie van een hoger-geoxideerde elektronenacceptor (NO_3^-).

Figuur 2 toont de fylogenetische stamboom van alle in de literatuur gerapporteerde humusreducerende micro-organismen. De figuur illustreert dat bacteriën uit een brede verscheidenheid aan fysiologisch verschillende groepen humus kunnen reduceren als terminale elektronenacceptor in de anaërobe oxidatie van verschillende substraten. De meeste humusreducerende micro-organismen kunnen de oxidatie van waterstof koppelen aan de reductie van humus of van de humus-analoge verbinding antrachinon-2,6-disulfonaat (AQDS); terwijl oxidatie van acetaat door humusreducerende micro-organismen meestal is voorbehouden aan Fe(III)-reducerende leden van de familie *Geobacteraceae* (*12, 14, 15, 33*). De brede verscheidenheid aan humusreducerende micro-organismen, waaronder Fe(III)-reduceerders (b.v. *Geobacter* spp. (*14, 15, 33*)), nitraatreduceerders (b.v. *Shewanella putrefaciens* (*34, 37*)), sulfaatreduceerders (b.v. *Desulfuromonas* spp. (*15, 34*)), fermentatieve bacteriën (b.v. *Propionibacterium freudenreichii* (*4*)) en methanogene archaebacteriën (b.v. *Methanococcus thermolithitrophicus* (*30*)) is waarschijnlijk bepalend voor de alomtegenwoordigheid van humusreductie in vele anaërobe milieus. Bovendien is humusreductie eveneens gerapporteerd voor hyperthermofiele micro-organismen zoals *Pyrobaculum islandicum*. Deze hyperthermofielen zijn

de organismen die het dichtst gerelateerd zijn aan de laatste gemeenschappelijke voorouder van alle huidige levensvormen, dus wellicht heeft deze laatste gemeenschappelijke voorouder reeds het vermogen gehad om humus te reduceren (*30*).

In hoofstuk 3 van dit proefschrift werden verschillende in anaërobe milieus algemene micro-organismen, waarvan niet bekend was dat ze humus konden reduceren, voor de eerste maal als chinon-reduceerders herkend. Al deze micro-organismen, waaronder de sulfaatreducerende bacterie *Desulfovibrio* G11, de halorespirerende bacteriën *Desulfitobacterium* PCE1 en *Desulfitobacterium dehalogenans* en de hydrogenotrofe methanogeen *Methanospirillum hungatei*, oxideerden waterstof gekoppeld aan stoichiometrische reductie van AQDS. De *Desulfitobacterium* spp. waren eveneens in staat oxidatie van lactaat te koppelen aan de reductie van humus en AQDS. Dit respiratoire proces bleek de groei van de culturen te bevorderen (*11*). Het vermogen van halorespirerende micro-organismen om onder humusreducerende omstandigheden te groeien doet vermoeden dat humus de natuurlijke elektronenacceptor voor deze micro-organismen is, hetgeen zou verklaren waarom ze kunnen worden aangetroffen in ongerepte gebieden waar de aanwezigheid van gechloreerde verontreinigingen en sulfiet geenszins te verwachten is (*27, 39*).



Figuur 2. Fylogenetische diversiteit van de uit de literatuur bekende humusreducerende micro-organismen. Het schema laat zien dat er geen gelijkvormigheid is tussen de verschillende fylogentische groepen (data van referenties *4, 11, 14, 15, 22, 23, 30, 33, 34, 42*). *, micro-organismen waarvan is vastgesteld dat chinonrespiratie de groei bevordert; ♦, bijdrage van dit proefschrift aan de fylogenetische stamboom.

Aangezien verscheidene humusreducerende micro-organismen veelzijdig zijn voor wat betreft de elektronenacceptoren die ze kunnen gebruiken, valt te verwachten dat de seizoensgebonden fluctuaties

in het aanbod van terminale elektronenacceptoren in bodems en sedimenten het overleven van deze organismen in deze milieus niet sterk zal beïnvloeden. De veelzijdigheid van humusrespirerende micro-organismen uit zich ook in de brede diversiteit van elektronendonoren voor chinonademhaling (*8, 20*). Tabel 1 geeft een overzicht van de in de literatuur vermelde substraten die door verschillende rein- of mengculturen via chinon- of humusademhaling kunnen worden geoxideerd. De reductie van humuszuren blijkt verder eveneens gekoppeld te kunnen worden aan de anaërobe oxidatie van persistente verbindingen (zie hieronder). De metabole veelzijdigheid van humusrespirerende bacteriën verklaart waarschijnlijk in belangrijke mate waarom deze micro-organismen zo alomtegenwoordig zijn.

Tabel 1. Anaërobe microbiële oxidatie van ecologisch belangrijke substraten en zwartelijststoffen, gekoppeld aan de reductie van humus of van de humus-modelverbinding AQDS.

Substraat	Elektronenacceptor	Inoculum	Referentie
Ecologisch belangrijke substraten			
Watersof	Humus of AQDS	Vooral humusredurende micro-organismen in meng- of reincultuur	8, 11, 15, 22, 30, 33
Acetaat	Humus of AQDS	Reinculturen van *Geobacter* spp. en *Desulfuromonas* spp., alsmede verscheidene consortia uit diverse milieus	8, 12, 14, 15, 22, 33, 34
Formaat	AQDS	Reinculturen van *Geobacter* spp.	12, 15
Lactaat	Humus of AQDS	Reinculturen van *Geobacter* spp., *Desulfitobacterium* spp., *Shewanella alga, Propionibacterium freudenreichii* en *Deinococcus radiodurans*, alsmede verscheidene consortia uit diverse milieus	4, 8, 11, 15, 23, 33
Propionaat	Humus of AQDS	Reinculturen van *Desulfuromonas* spp. en *Propionibacterium freudenreichii*, alsmede verscheidene consortia uit diverse milieus	4, 8, 15
Ethanol	AQDS	Reinculturen van *Geobacter* spp. en *Desulfuromonas* spp.	15
Pyruvaat	AQDS	Reinculturen van *Geobacter* spp.	15
Succinaat	AQDS	Reinculturen van *Desulfuromonas* spp.	15
Glucose	Humus	Reinculturen van *Enterococcus cecorum* en *Lactococcus lactis*	4
Zwartelijststoffen			
Dichlooretheen en vinylchloride	Humus of AQDS	Vers eutroof sediment	6
♦Fenol en *p*-cresol	AQDS	Anaëroob korrelslib van verschillende oorsprong	9
♦Tolueen	Humus of AQDS	Anaëroob sediment van verschillende oorsprong	7

♦Dit proefschrift

Gezien al deze waarnemingen is het niet verrassend dat in vele verschillende habitats, in natuurlijke milieus zoals eutrofe of juist oligotrofe sedimenten en bodems (*8, 15*), maar ook in kunstmatige milieus zoals slib van afvalwaterzuiveringsinstallaties (*12*), humusreductie is geconstateerd en chinon-respirerende bacteriën werden aangetroffen. De aanwezigheid van humusreducerende micro-organismen in afvalwaterzuiveringssystemen kan belangrijk zijn voor de anaërobe biotransformatie van zwartelijststoffen via chinonademhaling (zie hieronder).

Competitie tussen chinonademhaling en methanogenese voor ecologisch belangrijke substraten

Hoofdstuk 2 beschreef wat er gebeurt als de humuszuur-analoge verbinding AQDS wordt toegevoegd als elektronenacceptor aan incubaties van sediment, bodem of slib. AQDS-additie verhinderde methanogenese, omdat AQDS-reductie meestal werd verkozen als omzettingsroute van de eenvoudige substraten die werden getest (*8*). In afwezigheid van AQDS werden alle substraten, waaronder waterstof, acetaat, lactaat, propionaat en methanol, gedeeltelijk of volledig omgezet in methaan.

Er zijn vele redenen ter verklaring van het verdringen van methanogenese door chinonademhaling. Zoals blijkt uit Figuur 3 is de redoxpotentiaal van het AQDS/AH_2QDS-koppel gunstiger dan dat van methanogenese en sulfaatreductie. AQDS-reductie is ook thermodynamisch gunstiger dan reductie van de gebruikelijkste Fe(III)-vormen in de natuur, zoals goethiet en magnetiet. Voor het gebruik van deze vormen van ijzer als terminale elektronenacceptor is minder vrije energie beschikbaar dan voor het gebruik van AQDS. Humusstoffen van verschillende oorsprong vertonen een redoxpotentiaal die vergelijkbaar is met die van AQDS (Figuur 3). Micro-organismen die elektronenacceptoren met hogere redoxpotentiaal gebruiken, winnen in het algemeen de competitie met methanogenen om waterstof en acetaat, omdat ze meer energie halen uit de oxidatie van deze substraten (*5, 31, 32, 52*). Competitieve effecten als gevolg van thermodynamische verschillen zullen daarom bijdragen aan het overwicht van chinonrespirerende organismen ten opzichte van methanogenen. Andere alternatieve elektronenacceptoren, zoals sommige metaaloxiden en nitraat, staan echter hoger dan AQDS en humus in het rijtje van redoxpotentialen (Figuur 3). Dientengevolge kan men verwachten dat denitrificatie en dissimilatorische reductie van metaaloxiden zoals vernadiet en ferrihydriet het zullen winnen van chinonrespiratie.

In veel van de geteste consortia was de populatie chinonrespirerende bacteriën in eerste instantie relatief gering ten opzichte van de populatie methanogenen. Thermodynamica kan daarom de plotselinge overhand van chinonrespiratie ten opzichte van methanogenese niet volledig verklaren. De toename van de redoxpotentiaal (tot +130 mV) van het cultuurmedium door de introductie van AQDS zou hier weleens belangrijk geweest kunnen zijn. De verhoogde redoxpotentiaal zou kunnen interfereren met de voor methanogenese vereiste biochemische processen, want het wordt algemeen aangenomen dat methaanproductie alleen mogelijk is bij redoxpotentialen die lager zijn dan –200 mV

(*19*). Het werd zelfs aangetoond dat hoge concentraties AQDS toxisch waren voor acetoclastische en methylotrofe methanogenese in anaëroob korrelslib (*8*). Toxiciteit kan er dus ook voor zorgen dat chinonreducerende micro-organismen in staat zijn om methanogenen te overheersen.

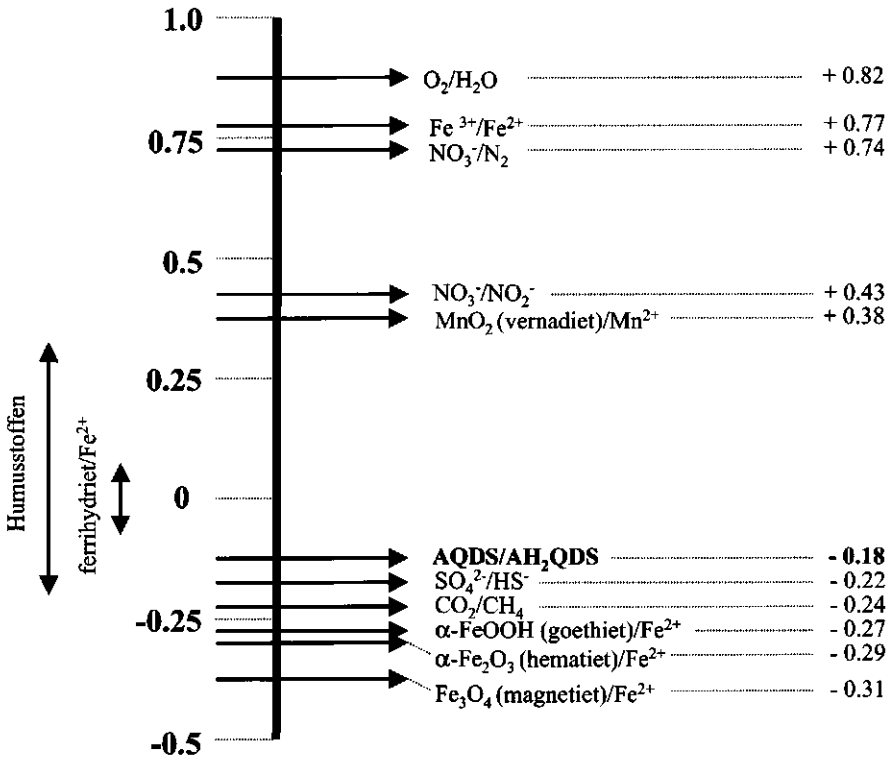


Figure 3. Redoxpotentialen (in V) onder standaardcondities ($E^{'0}$) van de belangrijkste redoxkoppels in de natuur. Data van referenties *43* en *46*.

De ecologische betekenis van microbiële humusreductie

Het brede spectrum van ecologisch belangrijke substraten dat kan worden geoxideerd door chinon-reducerende processen (Tabel 1), wijst erop dat chinonen in humus, door op te treden als terminale elektronenacceptor, weleens significant zouden kunnen bijdragen aan de koolstofkringloop. De ecologische relevantie van microbiële reductie van humusstoffen wordt verder benadrukt door de overvloedige aanwezigheid van deze organische fractie in vele anaërobe milieus, zoals bodems, sedimenten, moerassen, eutrofe meren en compost. Bovendien zorgt de hoge reactiviteit van hydro-chinonen in humus met metaaloxiden zoals Fe(III) (*33*) en Mn(IV) (*45*) voor regeneratie van de geoxideerde vorm. Dit kringloopmechanisme houdt in, dat zelfs substoichiometrische concentraties humusstoffen kunnen leiden tot zowel anaërobe oxidatie van substraten als reductie van de -in vele

sedimentaire milieus overvloedig voorkomende- metaaloxiden (Figuur1). Oxidatie van organische substraten via humusademhaling geschiedt sneller dan oxidatie van substraten die direct gekoppeld zijn aan de reductie van metaaloxiden (*33*). Dit wordt waarschijnlijk veroorzaakt doordat opgeloste humuszuren beter beschikbaar zijn dan onopgeloste metaaloxiden, hetgeen ook blijkt uit de hogere redoxpotentiaal van humus vergeleken met die van de meest voorkomende Fe(III)-vormen in de natuur. Van AH_2QDS is aangetoond dat het binnendringt in microporiën binnenin geologische oxiden die niet door bacteriën kunnen worden bereikt (*53*). Het valt daarom te betwijfelen of de reductie van Fe(III) en Mn(IV) in de natuur direct wordt veroorzaakt door metaalreduceerders, ofwel dat het indirect geschiedt, via chinonreductie door humusreduceerders. Humusreducerende micro-organismen die, zoals bijvoorbeeld *Propionibacterium freudenreichi*, niet het vermogen hebben om Fe(III) te reduceren, blijken elektronen van anaërobe oxidatiereacties via humusreductie naar amorfe Fe(III)-oxiden te kunnen leiden (*4*). In hoofdstuk 3 werd voor de eerste maal verslag gedaan van het vermogen van twee micro-organismen (de halorespirerende bacterie *Desulfitobacterium dehalogenans* en de sulfaatreducerende bacterie *Desulfovibrio* G11) om amorfe Fe(III)-oxiden te reduceren via chinonreductie. *Desulfitobacterium dehalogenans* was niet in staat om het metaaloxide te reduceren in afwezigheid van humusstoffen, maar het toevoegen van substoichiometrische concentraties AQDS (500 µM) leidde ertoe dat Fe(III) werd gereduceerd, gekoppeld aan de oxidatie van lactaat of waterstof. Toevoeging van eenzelfde hoeveelheid AQDS aan *Desulfovibrio* G11, die zonder AQDS en met waterstof als elektronendonor wèl in staat was om Fe(III) te oxideren, verbeterde de Fe(III)-reductie (*11*). Deze resultaten geven aan dat, vooral in veel sedimentaire milieus waar metaaloxiden overvloedig aanwezig zijn, vele fylogenetisch verschillende typen organismen een bijdrage kunnen leveren aan de reductie van metaaloxiden via humusreductie.

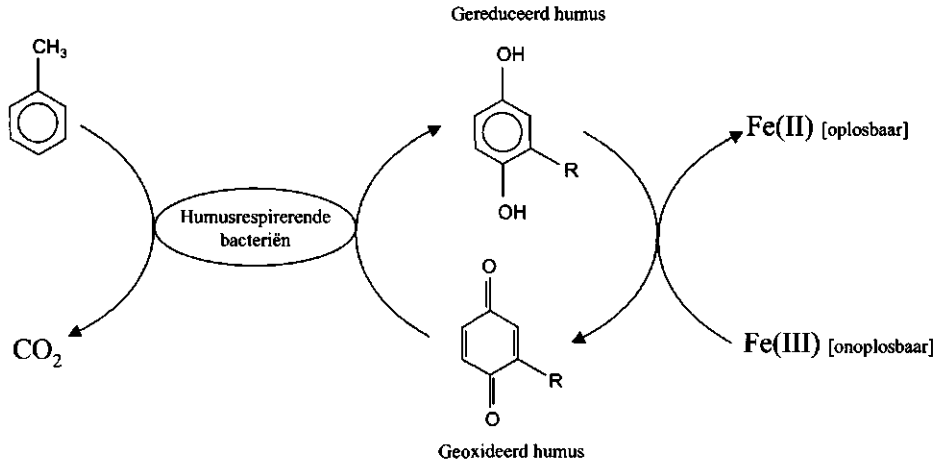
Humus als elektronenacceptor voor de bioremediatie van waterbodems en sediments

Nadat humusreductie in 1996 (*33*) was herkend als ademhalingsroute is er onderzoek verricht naar de mogelijkheden van humus als elektronenacceptor in de microbiële oxidatie van persistente verbindingen. De eerste publicatie waarin gerapporteerd werd dat humusstoffen optraden als elektronenacceptor voor de anaërobe oxidatie van zwartelijststoffen handelde over een eutroof beekbedsediment dat vinylchloride en dichlooretheen mineraliseerde onder humusreducerende condities (*6*). Deze gechloreerde verontreinigingen werden niet of nauwelijks afgebroken onder anaërobe condities in afwezigheid van humusstoffen, terwijl het toevoegen van humuszuren de vorming van $^{14}CO_2$ van [1,2-^{14}C]vinylchloride en [1,2-^{14}C]dichlooretheen sterk stimuleerde. De elektronenaccepterende rol van humusstoffen werd bevestigd in biologische tests in aanwezigheid van AQDS, waarin de mineralisering van [1,2-^{14}C]vinylchloride gelijktijdig verliep met de reductie van AQDS. In dit proefschrift werd van vele zwartelijststoffen aangetoond dat ze onder chinonreducerende omstandigheden anaërobe biodegradatie kunnen ondergaan. In hoofdstuk 5 werd aangetoond dat het

anaërobe slib van verschillende afvalwaterzuiveringsinstallaties in staat was fenol en *p*-cresol om te zetten onder AQDS-reducerende omstandigheden (*9*). De omzetting van deze fenolverontreinigingen was in overeenstemming met de stochiometrische vorming van het corresponderende hydrochinon (antrahydrochinon-2,6-disulfonaat, AH_2QDS). Fenol en *p*-cresol werden in afwezigheid van humusstoffen volledig omgezet naar methaan, maar het toevoegen van AQDS leidde ertoe dat de elektronenstroom omboog van methanogenese naar chinonrespiratie. Deze resultaten tonen voor de eerste keer dat chinonen in humus, door op te treden als alternatieve elektronenacceptor, bij kunnen dragen aan de anaërobe microbiële oxidatie van fenolverbindingen. Belangrijker is echter dat humusrespirerende consortia in staat waren om zwartelijststoffen te mineraliseren die onder methanogene omstandigheden niet werden afgebroken, b.v. tolueen. In hoofstuk 6 werd namelijk gerapporteerd dat verrijkte anaërobe sedimenten van de Rijn en van de Amsterdamse Petroleumhaven (APH) het uniform gelabelde [^{13}C]tolueen vlot naar $^{13}CO_2$ mineraliseerden indien humuszuren of AQDS werden aangeboden als terminale elektronenacceptor: na twee weken incubatie was respectievelijk 50 % en 85 % van het toegevoegde [^{13}C]tolueen door APH-sediment gemineraleerd (*7*). Bovendien werden de elektronenequivalenten van het gemineraliseerde tolueen stoichiometrisch teruggevonden als gereduceerd humus of als AH_2QDS. Deze resultaten vormen het eerste directe en kwantitatieve bewijs voor de mineralisering van een aromatische koolwaterstof door humusrespirerende micro-organismen. Humus kan dus voor de bioremediatie van verontreinigde milieus weleens een belangrijkere elektronenacceptor zijn dan oorspronkelijk werd aangenomen. Een en ander heeft vooral betrekking op eutrofe milieus, aangezien humusstoffen de anaërobe oxidatie van organische verontreinigingen kunnen helpen in hun rol als terminale elektronenacceptor. Het toevoegen van humuszuren (*35*) of AQDS (*1*) aan met petroleum verontreinigde waterbodemsedimenten die rijk waren aan Fe(III)-oxiden maar arm aan humus, leidde op sommige plaatsen tot veelbelovende stimulering van de anaërobe biodegradatie van benzeen. Ook wordt beweerd dat humusstoffen de anaërobe afbraak van methyl-*tert*-butyl ether, een loodvervanger in benzine, kunnen stimuleren in waterbodemsedimenten waaraan Fe(II) als terminale elektronenacceptor is toegevoegd (*21*). In hoofdstuk 6 van dit proefschrift werd bovendien aangetoond dat amorf Fe(III)-oxide niet door APH-sediment als elektronenacceptor voor tolueenafbraak kon worden gebruikt, tenzij wanneer substoichiometrische hoeveelheden humuszuur werden toegevoegd. Vermoedelijk zorgden de humusrespirerende micro-organismen voor de overdracht van elektronen van tolueen naar het ijzeroxide via humusreductie (*7*). Figuur 4 vat samen hoe tolueen anaëroob wordt afgebroken in het humusrespirerende consortium.

Het zou dus kunnen worden overwogen om, teneinde de anaërobe oxidatie van zwartelijststoffen te verbeteren, een technologie te ontwikkelen die gebaseerd is op het injecteren van humuszuren of chinonen in oligotrofe waterbodems en sedimenten. Het vermogen om humus of AQDS als elektronenacceptor te gebruiken blijkt een eigenschap te zijn van alle daarop geteste Fe(III)-reducerende micro-organismen (*12, 14, 15, 30, 33, 34*) en waarschijnlijk wordt, omdat chinonen een

veel hogere wateroplosbaarheid hebben dan alternatieve elektronenacceptoren zoals O_2, Fe(III) en Mn(IV), goed transport naar de waterbodem gewaarborgd. Vanwege de regeneratie van hydrochinonen in humus in chemische reacties met metaaloxiden, vergt het gebruik van humusstoffen voor het schoonmaken van verontreinigde terreinen slechts substoichiometrische concentraties, zolang de mechanismen om de kringloop in stand te houden maar beschikbaar zijn. Voor geconstrueerde bioremediatiesystemen met grondwaterrecirculatie kan de chinonkringloop ook in stand worden gehouden door de geïnjecteerde hydrochinonen in een direct-chemische reactie met zuurstof te laten reageren tot chinonen, die vervolgens weer terug worden geleid naar de waterbodem.



Figuur 4. Anaërobe oxidatie van tolueen in het chinonrespirerende Amsterdam Petroleumhaven-sediment (*7*).

De toepassing van humusstoffen om bioremediatie van verontreinigde waterbodems en sedimenten te bewerkstelligen heeft enkele andere voordelen. Er is geen risico verbonden aan het gebruik van humus in waterbodems en sedimenten met een tekort aan organische stof, want humus is verder inert. Hoewel humuzuren de verplaatsing van een verontreinigingspluim in grondwater kunnen versnellen door kool-waterstoffen en zware metalen te binden (*44*) kan de versnelde mobilisatie ook de biobeschikbaarheid -en daarmee de biologische afbraak- van koolwaterstoffen verbeteren. Een ander belangrijk gevolg is dat metalen als Fe(II) en Mn(II) oplosbaar worden ten gevolge van de regeneratie van hydrochinonen in humus. Fe(II) en Mn(II) kunnen echter sorptie ondergaan, aan bacteriën of aan het oppervlak van het resterende metaaloxide. De resultaten die in hoofstuk 6 werden gepresenteerd illustreren deze mogelijkheid. Tijdens de anaërobe oxidatie van tolueen met goethiet (α-FeOOH) en vernadiet (MnO_2) als terminale elektronenacceptor in aanwezigheid van humuszuur kon slechts 30-34% van de verwachte hoeveelheid reductie-equivalenten als Fe(II) en Mn (II) worden teruggevonden (*7*). Verder kan oplosbaar gemaakt Fe(II) niet alleen door aërobe bacteriën worden teruggeoxideerd, maar ook door fototrofe en nitraatreducerende micro-organismen die flinke hoeveelheden (8 tot 10 mM) ferreus

ijzer oxideren tot ferrihydriet dat buiten de cel wordt afgezet (*46*). Biogeen ferrihydriet blijkt een geschikte elektronenacceptor voor ijzerreducerende bacteriën te zijn (*47*), hetgeen aangeeft dat biologische Fe(II)-oxidatie inderdaad bijdraagt aan de kringloop van ijzer in anaërobe milieus.
Nog een voordeel van het gebruik van humus voor de bioremediatie van anaërobe terreinen is dat er gedurende de microbiële reductie van van humusstoffen geen ongewenste intermediairen worden gevormd. Het injecteren van nitraat of sulfaat voor de bioremediatie van verontreinigde milieus kent beperkingen in verband met de accumulatie van intermediaren (b.v. nitriet) of eindproducten (b.v. sulfide) die zelf verontreigingen zijn (*2, 25*). Het gebruik van humusstoffen is dan ook een aantrekkelijk alternatief voor de bioremediatie van anaërobe terreinen die met persistente stoffen verontreinigd zijn.

Humus als redoxmediator voor de reductieve (bio)transformatie van zwartelijststoffen

Humusstoffen en chinon-analogen doen niet alleen dienst als terminale elektronenacceptor voor anaërobe oxidatie van verontreinigingen, maar ze kunnen ook de reductieve (bio)transformatie van azokleurstoffen, nitroaromaten, polyhalogeenverbindingen en radionucliden stimuleren, middels de overdracht van elektronen van een externe elektronendonor naar deze verontreinigingen. Processen die door humusstoffen worden geholpen kunnen zowel abiotisch als biologisch zijn. Chinonen of humus kunnen deze reductieve omzettingen één tot enkele orden van grootte versnellen.
De best-bestudeerde abiotische redoxmediatie door humusstoffen is de reductieve omzetting van hexachloorethaan (HCA) naar tetrachloorethyleen (ook bekend als perchloorethyleen, PCE) door ferreus ijzer, sulfide of elementair zwavel (*17*). Toevoeging van chinonen versnelde de snelheid van deze chemische reacties tot het tienvoudige. Daarnaast bleek ook dat de gereduceerde chinonstructuur (AH_2QDS) in staat was HCA direct te dechloreren (*17*). Toevoeging van humus-modelstoffen, zoals resorcinol en catechol, in abiotische proeven met gechloreerde dibenzo-*p*-dioxines (CDDs) leidde tot dechlorering van deze verontreinigingen (*3*). In reactiesystemen met octa-CDDs en een humus-analoge verbinding werd vorming van de corresponderende tetra-CDDs bereikt, terwijl octa-CDDs niet reductief gedechloreerd werden in afwezigheid van humus-modelstoffen. Chinonen en humus maakten voorts mogelijk dat nitroaromaten (*18, 40*) en azokleurstoffen (*50*) abiotisch naar hun corresponderende aromatische aminen werden gereduceerd door gewone bulk-reductanten in anaërobe milieus, zoals sulfide en ferreus ijzer.
Humus en chinonen zijn ook betrokken bij de reductieve biotransformatie van zwartelijststoffen. *Deinococcus radiodurans*, een stralingsresistent micro-organisme, kon de opgeloste radionucliden U(VI) en Tc(VII) reduceren naar hun onopgeloste vormen U(IV) en Tc(IV) als AQDS in sub-stoichiometrische concentraties werd toegevoegd als redoxmediator, terwijl de radionucliden niet gereduceerd werden in afwezigheid van AQDS (*23*). Precipitatie van deze ionisatiestralingsbronnen

door chinonreductie kan van belang zijn voor de bioremediatie van terreinen die verontreinigd zijn met metaal- en radionucliden.

Celsuspensies van *Shewanella putrefaciens* met organisch bodemmateriaal zetten koolstoftetrachloride (CT) sneller om in chloroform en andere, niet-geïdentificeerde, producten dan celsuspensies zonder organisch bodemmateriaal (*16*). De humuszuurfractie bleek de omzetting van CT in sterkere mate te katalyseren dan de fulvozuurfractie en de huminefractie, maar de daarvoor verantwoordelijke functionele groepen en de mechanismen werden niet opgehelderd.

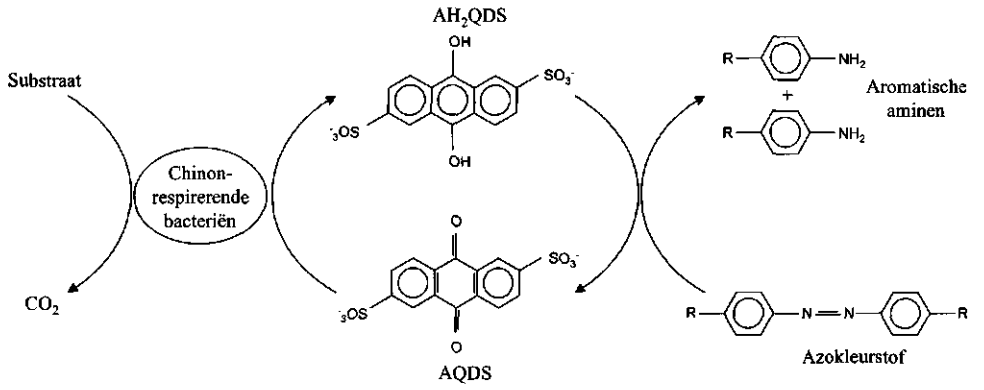
De gegevens die in hoofdstuk 7 werden gepresenteerd geven aan dat chinonrespirerende micro-organismen bij de omzetting van polyhalogeenverontreinigingen een belangrijkere rol kunnen spelen dan tot dusver werd aangenomen. Het toevoegen van substoichiometrische hoeveelheden AQDS verhoogde zowel de snelheid als de mate van CT-dechlorering door anaëroob korrelslib. Dit leidde tot toenemende productie van anorganisch chloride, tot 40-50 % van het chloorgehalte van de oorsponkelijke gechloreerde verbinding (*10*). Een grote variëteit aan substraten kon dienen als elektronendonor in het verbeterde dechloreringsproces. Dit is in overeenstemming met het brede spectrum van verbindingen dat via chinonademhaling kan worden geoxideerd (zie hierboven).

AQDS was bovendien in staat om het aantal mogelijke elektronendonoren voor de reductieve dechlorering van CT door anaëroob slib uit te breiden met acetaat en methanol, substraten die in afwezigheid van chinonen niet worden aangewend voor deze reactie. Deze verbeterde CT-dechlorering kon worden toegeschreven aan humusrespirerende bacteriën in het slib, want het antibioticum neomycine, een selectieve remmer van chinonrespiratie, onderdrukte zowel de reductie van AQDS als de verbeterde omzetting van CT. Voorts werd waargenomen dat een uit het korrelslib opgehoopte humusrespirerende cultuur, die voornamelijk bestond uit een soort die gerelateerd was aan *Geobacter sulfurreducens*, in staat was om, in aanwezigheid van lage concentraties AQDS of humuszuur, CT te dechloreren onder vorming van dezelfde reactieproducten als wanneer de reactie werd uitgevoerd door korrelslib in aanwezigheid van AQDS. In de biologische tests verliep de omzetting van CT parallel aan de reductie van AQDS en humus, terwijl in afwezigheid van humusstoffen omzetting van CT door de ophopingscultuur niet werd waargenomen. Dit resultaat demonstreert voor het eerst dat chinonrespiratie een bijdrage kan leveren aan het vrijmaken van elektronenequivalenten voor dechloreringsprocessen. Slechts een klein gedeelte (1-10%) van het omgezette CT kwam vrij als chloroform en dichloormethaan. Ook werd er een gechloreerde etheen, PCE, gevormd. Nooit eerder werd gerapporteerd dat PCE een intermediair van CT-biodegradatie kan zijn. De aannemelijkste verklaring voor de accumulatie van PCE (tot 9% van het oorspronkelijk toegevoegde CT) gaat uit van de koppelingsreactie van twee trichloormethylradicalen tot HCA. HCA zou dan vervolgens gemakkelijk kunnen worden omgezet in PCE, ofwel door abiotische reductie met hydrochinonen (*17*) ofwel door verdere microbiële dechlorering (*51*). PCE-accumulatie is onwenselijk, omdat het kan leiden tot de vorming van lager gechloreerde verontreinigingen, zoals dichlooretheen en vinylchloride,

waar onder anaërobe omstandigheden verder niets mee gebeurt. Aanwezigheid van humusstoffen zou er echter toe bij kunnen dragen dat deze verontreinigingen minder accumuleren, want AQDS en humus kunnen, zoals eerder is aangetoond, beide dienst doen als terminale elektronenacceptor voor de microbiële mineralisatie van dichloorethaan en vinylchloride (*6*).

De betekenis van humus voor de anaërobe behandeling van afvalwaters die zwartelijststoffen bevatten

Azokleurstoffen, nitroaromaten en polyhalogeenverbindingen zijn gewone verontreinigingen in afvalwater van chemische en petrochemische industrieën. Van deze stoffen is bekend dat ze toxisch zijn voor continue biologische afvalwaterbehandelingssystemen. Aangezien het verontreinigingen betreft met een elektronenzuigende werking kunnen ze echter in anaërobe reactoren worden omgezet, zodat detoxificatie optreedt. In een aantal rapportages is erop gewezen dat chinongroepen de reductieve biotransformatie van deze verontreinigingen kunnen versnellen. Zo bleek bijvoorbeeld dat verschillende chinonstructuren de reductie van azokleurstoffen tot de corresponderende kleurloze aromatische aminen versnellen (*26, 28*).



Figuur 5. Mechanisme van de door AQDS versnelde reductie van azokleurstoffen. Chinonrespirerende bacteriën reduceren AQDS naar het corresponderende hydrochinon, AH_2QDS, dat de elektronen vervolgens direct overdraagt op de kleurstof, hetgeen resulteert in splitsing van de azoband.

Hoofdstuk 8 van dit proefschrift behandelde de toepassing van verschillende concentraties AQDS om de reductieve ontkleuring van Acid Orange 7 (AO7) in een continue opwaarts doorstroomde slibbedreactor (UASB) te versnellen (*13*). AO7 werd met hoge efficiëntie ontkleurd, zelfs bij een hydraulische verblijftijd van 2 uur en een molaire AQDS/AO7-verhouding van slechts 1/100. In afwezigheid van AQDS, maar onder gelijke hydraulische condities, werd daarentegen slechts 70% kleurverwijdering bereikt. Uit recent onderzoek bleek bovendien dat AQDS bij zeer lage concentraties eveneens de reductieve biotransformatie verbetert van kleurstoffen (b.v. Reactive Red 2), die in

afwezigheid van AQDS met een veel lagere snelheid worden ontkleurd (*49*). De verbeterde ontkleuring leidde niet alleen tot detoxificatie (*49*), maar ook tot een zodanige versnelling van het reductieve proces, dat de vereiste hydraulische verblijftijd kon worden verkort (*13*). Aangezien de terminale reactie van het ontkleuringsproces een chemische reactie is tussen de azokleurstof en de microbieel gereduceerde chinonen, is het niet nodig dat er direct contact plaatsvindt tussen chinon-reducerende micro-organismen en de kleurstof (*28*). Figuur 5 toont het mechanisme van de door chinonen versnelde reductie van azokleurstoffen. Als redoxmediatoren hoeven chinonen dus niet per se overvloedig aanwezig te zijn, want ze kunnen gemakkelijk worden teruggevormd door reductie.

Gezien het voorafgaande valt het te verwachten dat, middels toepassing van chinonstructuren in anaërobe continu-reactoren, de biotransformatie van reduceerbare zwartelijststoffen verbeterd kan worden. Afvalwaterzuiveringssytemen met hoge opwaartse snelheid en korrelslib, zoals de UASB-reactor en de geexpandeerde korrelslibbedreactor (EGSB), zijn aantrekkelijke technieken om anaërobe omzetting van zwartelijststoffen te bewerkstelligen. Anaëroob slib kent uitstekende bezinkings-eigenschappen en de uitspoeling van biomassa wordt tot een minumum beperkt doordat er in de reactoren een stille zone is ingesteld die de slibdeeltjes in staat stelt te flocculeren of te bezinken (*29*). Het zou daarom kunnen dat chinonrespirerende bacteriën, waarvan gebleken is dat ze een belangrijke rol spelen in de reductieve biotransformatie van zwartelijststoffen (*10*), in het slibbed vastgehouden worden, waardoor hun betrokkenheid bij reductieve omzettingen wordt vergemakkelijkt. In feite werd in hoofdstuk 4 reeds gewag gemaakt van een chinonrespirerende cultuur die was opgehoopt uit korrel-slib. De UASB-reactor vanwaaruit dit korrelslib afkomstig was reduceerde AQDS continu gedurende een langdurige en stabiele periode. Een en ander betekent dat chinonrespirerende micro-organismen geïmmobiliseerd kunnen worden in de microbiële gemeenschap van het anaërobe korrelslib (*12*). In UASB-reactoren is bovendien met succes de immobilisatie van andere typen zwartelijststoffen-omzettende micro-organismen verwezenlijkt (*24*, *48*). Daarom bezit korrelslib een geschikte vorm voor immobilisatie van chinonrespirerende micro-organismen in continue anaërobe reactoren. Een verder voordeel van UASB- en EGSB-reactoren is de goede menging van vloeistof en gas. Hierdoor is de biologisch-dode ruimte minimaal en wordt verhinderd dat er lokaal hoge concentraties voorkomen van toxische verontreinigingen in het afvalwater. Tenslotte zorgt de korrelstructuur van het slib voor bescherming van de micro-organismen in de korrel, zodat de toxische werking van de veront-reinigingen wordt afgezwakt (*38*).

Concluderende opmerkingen

Humus werd in het verleden doorgaans beschouwd als een inert materiaal, aangezien het opmerkelijk stabiel is in het milieu. Wat in dit proefschrift werd besproken geeft echter aan dat humusstoffen op verschillende manieren een rol kunnen spelen in de anaërobe biodegradatie van ecologisch belangrijke substraten en zwartelijststoffen. Humuszuren en chinon-modelstoffen dragen bij aan de microbiële

oxidatie van verscheidene belangrijke substraten door in vele verschillende milieus dienst te doen als elektronenacceptor. Chinonrespirerende activiteit is alomtegenwoordig, zowel in natuurlijke milieus, van zandige sedimenten tot sedimenten die juist rijk zijn aan organische stof, als in kunstmatige habitats, zoals slib van afvalwaterzuiveringsinstallaties. Het vermogen om humusstoffen te reduceren wordt ook aangetroffen in axenische culturen van fylogenetisch verschillende groepen micro-organismen, die humusreductie koppelen aan de oxidatie van uiteenlopende substraten. Dit wijst erop dat vele verschillende micro-organismen betrokken kunnen zijn bij de reductie van humusstoffen in de natuur. Humusrespirerende consortia hebben bovendien het vermogen om zwartelijststoffen, waaronder tolueen, te oxideren in aanwezigheid van humusstoffen of chinonen als terminale elektronenacceptor. Een technologie die gebaseerd is op het injecteren van humusstoffen in verontreinigde waterbodems en sedimenten zal daarom de bioremediatie van de verontreinigingen kunnen stimuleren. Het is daartoe niet noodzakelijk dat humusstoffen in grote hoeveelheid worden toegevoegd, want de microbieel gereduceerde chinonen in humus kunnen worden geregenereerd doordat ze chemisch reageren met de metaaloxiden die in veel anaërobe milieus overvloedig aanwezig zijn. Toepassing van humusstof-injectie vereist daarom slechts substoichiometrische hoeveelheden. Chinonen en humus kunnen ook dienen als redoxmediatoren die de reductieve biotransformatie van verontreinigingen met elektronenzuigende groepen versnellen. Katalytische concentraties AQDS en humuszuur verbeterden namelijk zowel de snelheid als de mate van dechlorering van koolstoftetrachloride door chinonrespirerende consortia en tevens leidde het toevoegen van micromolaire concentraties AQDS tijdens de behandeling van azokleurstoffen in UASB-reactoren tot een zodanige versnelling van het ontkleuringsproces, dat de voor een efficiënte ontkleuring benodigde hydraulische verblijftijd, verlaagd kon worden. Toepassing van katalytische concentraties chinonen of humusstoffen in anaërobe reactoren kan daarom de reductieve omzetting van zwartelijststoffen in afvalwater in sterke mate verbeteren. Deze verbetering kan belangrijke gevolgen hebben: het kan leiden tot verlaging van de toxiciteit van afvalwaters voor de aanwezige microbiële gemeenschappen en het kan leiden tot verlaging van de benodigde hydraulische verblijftijd en dus tot compactere reactoren.

Referenties

1. Anderson, R. T. and D. R. Lovley. (1999) Naphthalene and benzene degradation under Fe(III)-reducing conditions in petroleum-contaminated aquifers. *Bioremediat. J.* **3**, 121-135.
2. Anderson, R. T. and D. R. Lovley. (2000). Anaerobic bioremediation of benzene under sulfate-reducing conditions in a petroleum-contaminated aquifer. *Environ. Sci. Technol.* **34**, 2261-2266.
3. Barkovskii, A. L. and P. Adriaens. (1998). Impact of humic constituents on microbial dechlorination of polychlorinated dioxins. *Environ. Toxicol. Chem.* **17**, 1013-1020.
4. Benz, M., Schink, B. and Brune, A. (1998) Humic acid reduction by *Propionibacterium freudenreichii* and other fermentative bacteria. *Appl. Environ. Microbiol.* **64**,4507-4512.

5. Bodegom, P. M. and Stams, A. J. M. (1999) Effects of alternative electron acceptors and temperature on methanogenesis in rice paddy soils. *Chemosphere* **39**, 167-182.

6. Bradley, P. M., F. H. Chapelle and D. R. Lovley. (1998) Humic acids as electron acceptors for anaerobic microbial oxidation of vinyl chloride and dichloroethene. *Appl. Environ. Microbiol.* **64**, 3102-3105.

7. Cervantes, F. J., Dijksma, W., Duong-Dac, T., Ivanova, A., Lettinga, G. and Field, J. A. (2001) Anaerobic mineralization of toluene by enriched sediments with quinones and humus as terminal electron acceptors. *Appl. Environ. Microbiol.* **67**, 4471-4478.

8. Cervantes, F. J., van der Velde, S., Lettinga, G. and Field, J. A. (2000) Competition between methanogenesis and quinone respiration for ecologically important substrates in anaerobic consortia. *FEMS Microbiol. Ecol.* **34**,161-171.

9. Cervantes, F. J., van der Velde, S., Lettinga, G. and Field, J. A. (2000) Quinones as terminal electron acceptors for anaerobic microbial oxidation of phenolic compounds. Biodegradation 11, 313-321.

10. Cervantes, F. J., Vu-Thi-Thuy, L., Lettinga, G. and Field, J. A. Quinone-respiration improves dechlorination of carbon tetrachloride by anaerobic sludge. *Submitted.*

11. Cervantes, F.J., De Bok F. A. M., Duong-Dac T., Stams, A. J. M., Lettinga G. and Field J. A. Reduction of humic substances by halorespiring, sulfate-reducing and methanogenic microorganisms. *Submitted.*

12. Cervantes, F.J., Duong-Dac T., Ivanova, A.E., Roest K., Akkermans A. D. L., Lettinga G. and Field J. A. Quinones as terminal electron acceptors in anaerobic granular sludge. *Submitted.*

13. Cervantes, F.J., van der Zee, F.P., Lettinga, G. and Field, J.A. (2001). Enhanced decolourisation of acid orange 7 in a continuous UASB reactor with quinones as redox mediators. *Water Sci. Technol.* **44**, 123-128.

14. Coates, J. D., Bhupathiraju, V. K., Achenbach, L. A., McInerney, M. J., and Lovley, D. R. (2001) *Geobacter hydrogenophilus, Geobacter chapellei* and *Geobacter grbicie*, three new, strictly anaerobic, dissimilatory Fe(III)-reducers. *Int. J. Syst. Evol. Microbiol.* **51**, 581-588.

15. Coates, J. D., Ellis, D. J., Roden, E., Gaw, K., Blunt-Harris, E. L. and Lovley, D. R. (1998) Recovery of humics-reducing bacteria from a diversity of sedimentary environments. *Appl. Environ. Microbiol.* **64**,1504-1509.

16. Collins, R. and Picardal, F. (1999) Enhanced anaerobic transformation of carbon tetrachloride by soil organic matter. *Environ. Toxicol. Chem.* **18**, 2703-2710.

17. Curtis, C. and Reinhard M. (1994) Reductive dehalogenation of hexachloroethane, carbon tetrachloride, and bromoform by anthrahydroquinone disulphonate and humic acid. *Environ. Sci. Technol.* **28**, 2393-2401.

18. Dunnivant, F., Schwarzenbach R. and Macalady D. (1992) Reduction of substituted nitrobenzenes in aqueous solutions containing natural organic matter. *Environ. Sci. Technol.* **26**, 2133-2142.

19. Fetzer, S. and Conrad R. (1993) Effect of redox potential on methanogenesis by *Methanosarcina barkeri*. *Arch. Microbiol.* **160**, 108-113.

20. Field, J. A., Cervantes, F. J., van der Zee, F. P. and Lettinga, G. (2000) Role of quinones in the biodegradation of priority pollutants: a review. *Water Sci. Technol.* **42**, 215-222.

21. Finneran, K. T. and Lovley D. R. (2001) Anaerobic degradation of methyl *tert*-butyl ether (MTBE) and *tert*-butyl alcohol (TBA). *Environ. Sci. Technol.* **35**, 1785-1790.

22. Francis, C. A., Obraztsova, A. Y. and Tebo, B. M. (2000) Dissimilatory metal reduction by the facultative anaerobe *Pantoea agglomerans* SP1. *Appl. Environ. Microbiol.* **66**,543-548.

23. Fredrickson, J. K., Kostandarithes, H. M., Li, S. W., Plymale, A. E. and Daly, M. J. (2000) Reduction of Fe(III), Cr(VI), U(VI), and Tc(VII) by *Deinococcus radiodurans* R1. *Appl. Environ. Microbiol.* **66**,2006-2011.

24. Hörber, C., Christensen, N., Arvin, E. and Ahring, B. K. (1999) Tetrachloroethene dechlorination kinetics by *Dehalospirillum multivorans* immobilized in upflow anaerobic sludge blanket reactors. *Appl. Microbiol. Biotechnol.* **51**, 694-699.

25. Hutchins, S. R., Sewell G. W., Kovacs D. A. and Smith G. A. (1991) Biodegradation of aromatic hydrocarbons by aquifer microorganisms under denitrifying conditions. *Environ. Sci. Technol.* **25**, 68-76.

26. Kudlich, M., Keck, A., Klein, J. and Stolz, A. (1997) Localization of the enzyme system involved in anaerobic reduction of azo dyes by *Sphingomonas* sp. Strain BN6 and effect of artificial redox mediators on the rate of azo dye reduction. *Appl. Environ. Microbiol.* **63**, 3691-3694.

27. Lanthier, M., Villemur, R., Lépine, F., Bisaillon, J., and Beaudet, R. (2001) Geographic distribution of *Desulfitobacterium frappieri* PCP-1 and *Desulfitobacterium* spp. in soils from the province of Quebec, Canada. *FEMS Microbiol. Ecol.* **36**, 185-191.

28. Laszlo, J. A. (2000) Regeneration of azo-dye-saturated cellulosic anion exchange resin by *Burkholderia cepacia*. *Environ. Sci. Technol.* **34**, 167-172.

29. Lettinga, G., van Velsen, A. F. M., Hobma, S. W., de Zeeuw, W. and Klapwijk, A. (1980) Use of the upflow sludge blanket (USB) reactor concept for biological wastewater treatment, especially for anaerobic treatment. *Biotechnol. Bioeng.* **22**, 699-734.

30. Lovley D. R., Kashefi, K., Vargas, M., Tor, J. M. and Blunt-Harris, E. L. (2000) Reduction of humic substances and Fe(III) by hyperthermophilic microorganisms. *Chem. Geol.* **169**,289-298.

31. Lovley, D. R. (1985) Minimum threshold for hydrogen metabolism in methanogenic bacteria. *Appl. Environ. Microbiol.* **49**, 1530-1531.

32. Lovley, D. R. and Phillips, E. J. P. (1987) Competitive mechanisms for inhibition of sulfate reduction and methane production in the zone of ferric iron reduction in sediments. *Appl. Environ. Microbiol.* **53**, 2636-2641.

33. Lovley, D. R., Coates, J. D., Blunt-Harris, E. L., Phillips, E. J. P. and Woodward, J. C. (1996) Humic substances as electron acceptors for microbial respiration. *Nature* **382**,445-448.

34. Lovley, D. R., Fraga, J. L., Blunt-Harris, E. L., Hayes, L. A., Phillips, E. J. P., and Coates, J. D. (1998) Humic substances as a mediator for microbially catalyzed metal reduction. *Acta Hydrochim. Hydrobiol.* **26**, 152-157.

35. Lovley, D. R., Woodward J. C. and Chapelle F. H. (1996) Rapid anaerobic benzene oxidation with a variety of chelated Fe(III) forms. *Appl. Environ. Microbiol.* **62**, 288-291.

36. Lovley, D. R., Fraga J. L., Coates J. D. and Blunt-Harris E. L. (1999) Humics as an electron donor for anaerobic respiration. *Environ. Microbiol.* **1**, 89-98.

37. Newman, D. K., and Kolter, R. (2000) A role for excreted quinones in extracellular electron transfer. *Nature* **405**, 94-97.

38. Razo-Flores, E., Donlon, B, Lettinga, G. and Field, J. A. (1997) Biotransformation and biodegradation of N-substituted aromatics in methanogenic granular sludge. *FEMS Microbiol. Rev.* **20**, 525-538.

39. Sanford, R. A., Cole, J. R., Löffler, F. E., and Tiedje, J. M. (1996) Characterization of *Desulfitobacterium chlororespirans* sp. nov., which grows by coupling the oxidation of lactate to the reductive dechlorination of 3-chloro-4-hydroxybenzoate. *Appl. Environ. Microbiol.* **62**, 3800-3808.

40. Schwarzenbach, R., Stierli, R., Lanz, K. and Zeyer, J. (1990) Quinone and iron porphyrin mediated reduction of nitroaromatic compounds in homogeneous aqueous solution. *Environs. Sci. Technol.* **24**, 1566-1574.

41. Scott, D. T., McKnight, D. M., Blunt-Harris, E. L., Kolesar, S. E. and Lovley, D. R. (1998) Quinone moieties act as electron acceptors in the reduction of humic substances by humics-reducing microorganisms. *Environ. Sci. Technol.* **32**,2984-2989.

42. Slobodkin, A. I., Tourova, T. P., Kuznetsov, B. B., Kostrikina, N. A., Chernyh, N. A., and Bonch-Osmolovskaya, E. A. (1999) *Thermoanaerobacter siderophilus* sp. nov., a novel dissimilatory Fe(III)-reducing, anaerobic, thermophilic bacterium. *Int. J. Syst. Bacteriol.* **49**, 1471-1478.

43. Sober, H. A. (1970) Handbook of biochemistry: selected data for molecular biology. The Chemical Rubber Co., Cleveland, Ohio.

44. Stevenson, F. J. (1994) Humus chemistry: genesis, Composition, Reactions, 2nd ed. Wiley, New York.

45. Stone, A. T. and Morgan J. J. (1984) Reduction of manganese(III) and manganese(IV) oxides by organics. 1. Reaction with hydroquinone. *Environ. Sci. Technol.* **18**, 450-456.

46. Straub, K. L., Benz, M. and Schink, B. (2001) Iron metabolism in anoxic environments at near neutral pH. *FEMS Microbiol. Ecol.* **34**, 181-186.

47. Straub, K. L., Hanzlik, M. and Buchholz-Cleven, B. E. E. (1998) The use of biologically produced ferrihydrite for the isolation of novel iron-reducing bacteria. *Syst. Appl. Microbiol.* **21**, 442-449.

48. Tawfiki-Hajji, K., Lepine, F., Bisaillon, J. G., Beaudet, R., Hawari, J. and Guiot, S. R. (2000) Effects of bioaugmentation strategies in UASB reactors with a methanogenic consortium for removal of phenolic compounds. *Biotechnol. Bioeng.* **67**, 417-423.

49. Van der Zee, F. P., Bouwman, R. H. M., Strik, D. P. B. T. B., Lettinga, G., and Field, J. A. (2001) Application of redox mediators to accelerate the transformation of reactive azo dyes in anaerobic bioreactors. *Biotechnol. Bioeng.* (In press).

50. Van der Zee, F. P., Lettinga, G. and Field, J. A. (2000) The role of (auto)catalysis in the mechanism of an azo dye reduction. *Water Sci. Technol.* **42**, 301-308.

51. Van Eekert, M. H. A., Stams, A. J. M., Field, J. A. and Schraa, G. (1999) Gratuitous dechlorination of chloroethanes by methanogenic granular sludge. *Appl. Microbiol. Biotechnol.* **51**, 46-52.

52. Ward, D. M. and Winfrey, M. R. (1985) Interactions between methanogenic and sulfate-reducing bacteria in sediments. *Adv. Aquat. Microbiol.* **3**, 141-179.

53. Zachara, J.M., Fredrickson, J. K., Li, S. M., Kennedy, D. W., Smith, S. C. and Gassman, P. L. (1998) Bacterial reduction of crystalline Fe^{3+} oxides in single phase suspensions and subsurface materials. *Am. Mineralogist.* **83**,1426-1443.

Acknowledgements

The adventure in The Netherlands has become to its end and I would like to acknowledge the contribution of those who helped me to conclude the research summarized here. First of all, I would like to express my most sincere gratitude to my supervisor, Jim Field, for introducing me to this very interesting topic. Jim, thanks for your encouragement and excellent guidance, I have indeed no words, which can express my appreciation for you. Reyes, for your hospitality and support from the very beginning, gracias!. I am also indebted to my promotor, Gatze Lettinga, for allowing me to become part of his "anaerobic community". Gatze, "bedank" for your support, advice and guidance. I extent my appreciation to Dora Lettinga for her kind reception.

Very significant was the experimental work carried out by Sijrk van der Velde and Wouter Dijksma, and special mention to the brilliant contribution of the Vietnamese students, Duong Dac Tuan and Vu Thi Thuy Linh.
A number of colleagues and staff members from the Department of Environmental Technology also helped me in one way or another. The technical assistance of Ilse, Johannes, Geert, Hillion, Sjoerd, Jo and Winnie is greatly appreciated, as well as the collaboration and constructive discussion of Frank and Nico. The cooperation of Frank de Bok, Maurice Luijten, Kees Roest, Anna Ivanova, Miriam van Eekert, Caroline Plugge, Fons Stams and Wim Roelofsen from the Department of Microbiology was indeed valuable for this research. I appreciate the valuable discussion with Spyros Pavlostatis. Spyros, for your time, much obliged!.
I would like to extent my gratefulness to the ladies from the secretary, Heleen, Liesbeth and Anita, who helped in many different ways and at several times and to my office-room-mates, Adrie and Paula, for sharing the office in a nice way.
Elias, it's funny that we have met here, and not in our home country, thanks for your suggestions. My recognition to my former supervisors at UAM-Iztapalapa, Oscar Monroy and Jorge Gómez, for introducing me to the field of Environmental Biotechnology.
Estas líneas en español las dedico a mis padres, hermanos y demás familiares que a pesar de la distancia siempre me mostraron de diferentes maneras su cariño y apoyo. Ustedes son también parte de este logro. Gracias por todo.
Liz, hemos llegado a nuestra meta. La aventura no ha sido fácil, pero nos ha permitido vivir una inolvidable experiencia que nos ha llenado de muy gratos recuerdos. Tu amor incondicional y apoyo constante representa mi mayor estimulo y satisfacción. Gracias por todo. Por si fuera poco, hemos concluido nuestra tesis con la bendición de nuestro bebe. Ustedes son lo más importante para mí. Los amo.

Francisco J. Cervantes-Carrillo
Wageningen, The Netherlands. January, 2002.

Curriculum vitae

The author of this dissertation, Francisco Javier Cervantes Carrillo, was born on February 19th, 1972 in Cd. Obregón, Sonora, Mexico. In 1995, he obtained the Bachelor of Science degree in Biotechnology Engineering from Instituto Tecnológico de Sonora (ITSON) with *Magna cum lauda*. He also became "Distinguished Graduate" from ITSON in 1995. His Master of Science degree was granted in 1998 from the Department of Biotechnology of Universidad Autónoma Metropolitana-Iztapalapa (UAM-I). The topic of the master thesis was "Physiological and biochemical aspects of the nitrogen removal from concentrated wastewaters". He obtained the prize "University Merit" from UAM-I in 1998. From 1997 till the end of 1998 he was an associated lecturer-researcher of the Department of Biotechnology of UAM-I. In January 1999, he started his Ph.D. studies at the Department of Environmental Technology of Wageningen University and the results are summarised in the present dissertation. In February 2002, he will assume a position as a lecturer-researcher in a post-graduate program in Biotechnology and Natural Resources at ITSON. His permanent address will be:

Instituto Tecnológico de Sonora
Dirección de Investigación y Estudios de Posgrado (DIEP)
5 de Febrero 818 sur
85000, Cd. Obregón, Sonora
Mexico.

List of Publications

1. **Cervantes, F.**, Monroy, O. and Gómez, J. (1998). Accumulation of intermediates in a denitrifying process at different copper and high nitrate concentrations. *Biotechnol. Lett.* **20**, 959-961.
2. **Cervantes, F.**, Monroy, O. and Gómez, J. (1999). Influence of ammonium on the performance of a denitrifying culture under heterotrophic conditions. *Appl. Biochem. Biotechnol.* **81**, 13-23.
3. **Cervantes-Carrillo, F.**, Pérez-Trevilla, J. and Gómez, J. (2000) Avances en la Eliminación Biológica del Nitrógeno de las Aguas Residuales. *Revista Latinoamericana de Microbiología.* **42**,73-82. In Spanish with abstract in English.
4. Field, J.A., **Cervantes, F. J.**, van der Zee, F. and Lettinga, G. (2000). Role of quinones in the biodegradation of priority pollutants: a review. *Water Sci. Technol.* **42** (5-6), 215-222.
5. **Cervantes, F. J.**, van der Velde, S., Lettinga, G. and Field, J.A. (2000). Competition between methanogenesis and quinone respiration for ecologically important substrates in anaerobic consortia. *FEMS Microbiol. Ecol.* **34**, 161-171.
6. **Cervantes, F. J.**, van der Velde, S., Lettinga, G. and Field, J.A. (2000). Quinones as terminal electron acceptors for anaerobic microbial oxidation of phenolic compounds. *Biodegradation.* **11**, 313-321.
7. **Cervantes, F. J.**, De La Rosa, D.A. and Gómez, J. (2001). Nitrogen removal from wastewaters at low C/N ratios with ammonium and acetate as electron donors. *Bioresource Technol.* **79**, 165-170.
8. **Cervantes, F. J.**, van der Zee, F.P., Lettinga, G. and Field, J.A. (2001). Enhanced decolourisation of acid orange 7 in a continuous UASB reactor with quinones as redox mediators. *Water Sci. Technol.* **44**(4), 123-128.
9. **Cervantes, F.J.**, Dijksma, W., Duong-Dac T., Ivanova, A., Lettinga, G. and Field, J.A. (2001). Anaerobic mineralization of toluene by enriched sediments with quinones and humus as terminal electron acceptors. *Appl. Environ. Microbiol.* **67**, 4471-4478.
10. **Cervantes, F. J.**, de Bok F. A. M., Duong-Dac T., Stams, A. J. M., Lettinga G. and Field J. A. Reduction of humic substances by halorespiring, sulfate-reducing and methanogenic microorganisms. *Environ. Microbiol. Accepted for publication.*
11. **Cervantes, F. J.**, Duong-Dac T., Ivanova, A.E., Roest K., Akkermans A. D. L., Lettinga G. and Field J. A. Quinones as terminal electron acceptors in anaerobic granular sludge. *Submitted.*
12. **Cervantes, F. J.**, Vu-Thi, T.L., Lettinga, G. and Field, J.A. Quinone-respiration improves dechlorination of carbon tetrachloride by anaerobic sludge. *Submitted.*

The research described in this thesis was carried out at the Department of Environmental Technology of the Wageningen University and financially supported by the Council of Science and Technology of Mexico (Grant No. 94665).

Cover design: Elizabeth Levario and Francisco J. Cervantes