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Research review paper

## Impact and application of electron shuttles on the redox (bio)transformation of contaminants: A review

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### ABSTRACT

During the last two decades, extensive research has explored the catalytic effects of different organic molecules with redox mediating properties on the anaerobic (bio)transformation of a wide variety of organic and inorganic compounds. The accumulated evidence points at a major role of electron shuttles in the redox conversion of several distinct contaminants, both by chemical and biological mechanisms. Many microorganisms are capable of reducing redox mediators linked to the anaerobic oxidation of organic and inorganic substrates. Electron shuttles can also be chemically reduced by electron donors commonly found in anaerobic environments (e.g. sulfide and ferrous iron). Reduced electron shuttles can transfer electrons to several distinct electron-withdrawing compounds, such as azo dyes, polyhalogenated compounds, nitroaromatics and oxidized metalloids, among others. Moreover, reduced molecules with redox properties can support the microbial reduction of electron acceptors, such as nitrate, arsenate and perchlorate. The aim of this review paper is to summarize the results of reductive (bio)transformation processes catalyzed by electron shuttles and to indicate which aspects should be further investigated to enhance the applicability of redox mediators on the (bio)transformation of contaminants.

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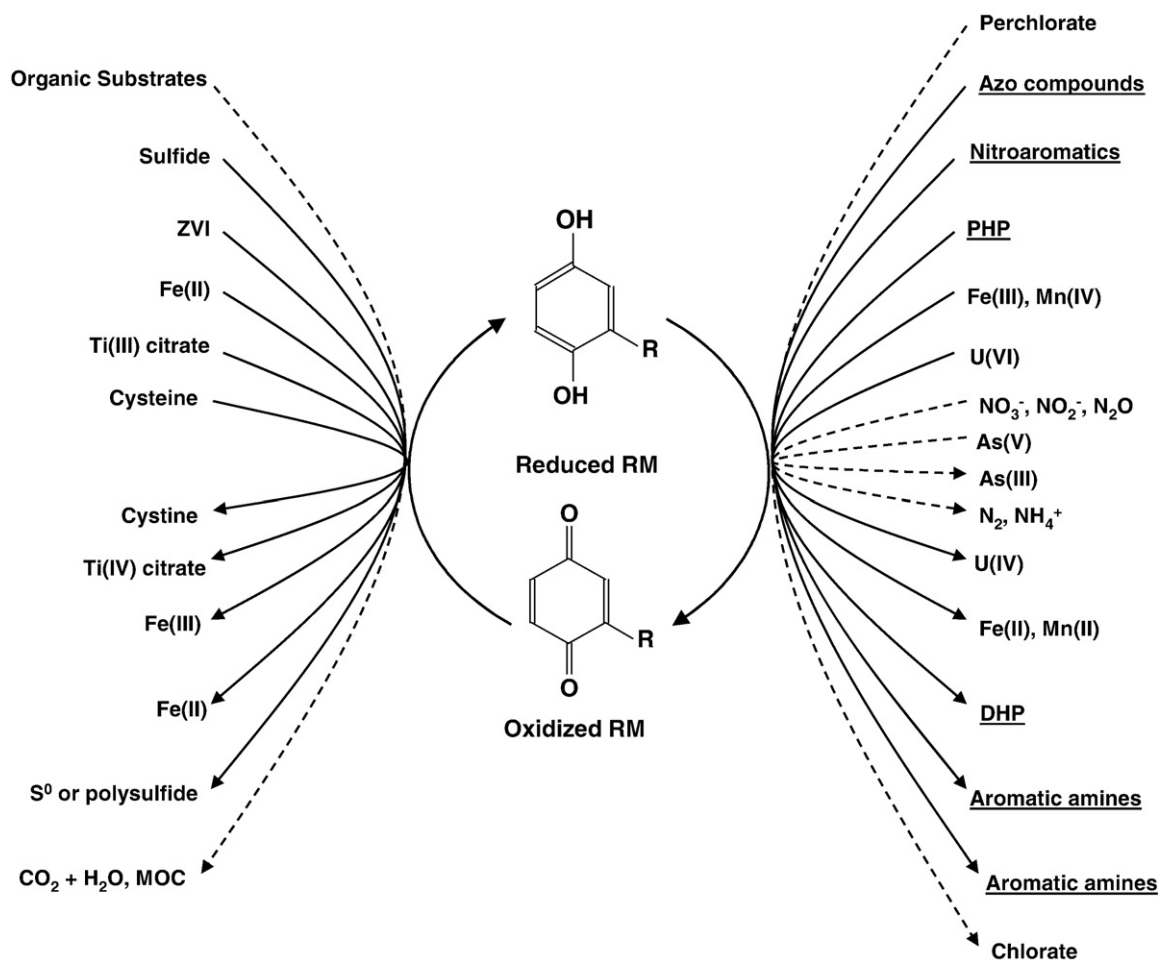
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## 1. Introduction

The chemical and petrochemical industry produces worldwide thousands of compounds each year and it is still in expansion. The global market of this sector is huge and only during the first 9 months of 2004, the sales in the USA summed up US\$110.4 billion for 26

companies of the chemical sector (Storck et al., 2005). Unfortunately, linked to the great economical benefits obtained from the chemical and petrochemical production, large volumes of wastewaters containing many different toxic and recalcitrant pollutants are being discharged (Razo-Flores et al., 2006). Among the contaminants generated are electron-withdrawing compounds, such as azo dyes,



**Fig. 1.** Abiotic (solid lines) and microbial (dashed lines) reactions involved in the reduction and oxidation of electron shuttles (e.g. quinones). ZVI, zero valent iron; MOC, more oxidized compounds; PHP, polyhalogenated pollutants; DHP, dehalogenated pollutants.

nitroaromatics, halogenated aliphatics, halogenated aromatics and metalloids, which remain unaffected during conventional aerobic wastewater treatment. In contrast, under anaerobic conditions, these contaminants can undergo reductive transformations (Field et al., 1995). During the last two decades, evidence accumulated showing the potential to convert pollutants containing electrophilic groups in their structures, in high-rate anaerobic bioreactors, such as upflow anaerobic sludge bed (UASB) and expanded granular sludge bed (EGSB) systems (Donlon et al., 1996; Christiansen et al., 1997; Razo-Flores et al., 1999; Van der Zee et al., 2001; Dos Santos et al., 2005c). However, reductive transformation of many different recalcitrant compounds proceeds very slowly due to electron transfer limitations and to toxicity effects leading to poor performance or even collapse of anaerobic bioreactors (Rodgers and Bunce, 2001; Van der Zee et al., 2001).

Since the 1990s, extensive research has been conducted to explore the catalytic effects of different electron shuttles on redox (bio) transformation processes. Electron shuttles, also referred to as redox mediators (RM), are organic molecules that can reversibly be oxidized

and reduced, thereby conferring the capacity to serve as an electron carrier in multiple redox reactions. As will be explained below, RM accelerate reactions by lowering the activation energy of the total reaction. In some cases, the presence of RM may even be a prerequisite for the reaction to take place.

RM are capable of transferring electrons in redox reactions between a wide variety of both inorganic and organic compounds. Fig. 1 illustrates most redox reactions involved in the reduction and oxidation of RM by abiotic and biological means. Reduction of RM can be promoted by chemical reaction with reductants commonly found in many anaerobic environments (e.g. sulfide, cysteine, Fe(II)) (Schwarzenbach et al., 1990; Curtis and Reinhard, 1994) or by electron donors proposed in restoration technologies (e.g. zero-valent iron (ZVI), Ti(III) citrate) (Tratnyek et al., 2001; Dror and Schlautman, 2004; Keum and Li, 2004). Furthermore, reduction of RM can be linked to the anaerobic oxidation of organic substrates by microorganisms. The most relevant RM supporting the microbial oxidation of organic compounds under anaerobic conditions are humic substances and their quinoid analogues (Field and Cervantes, 2005). Microbial

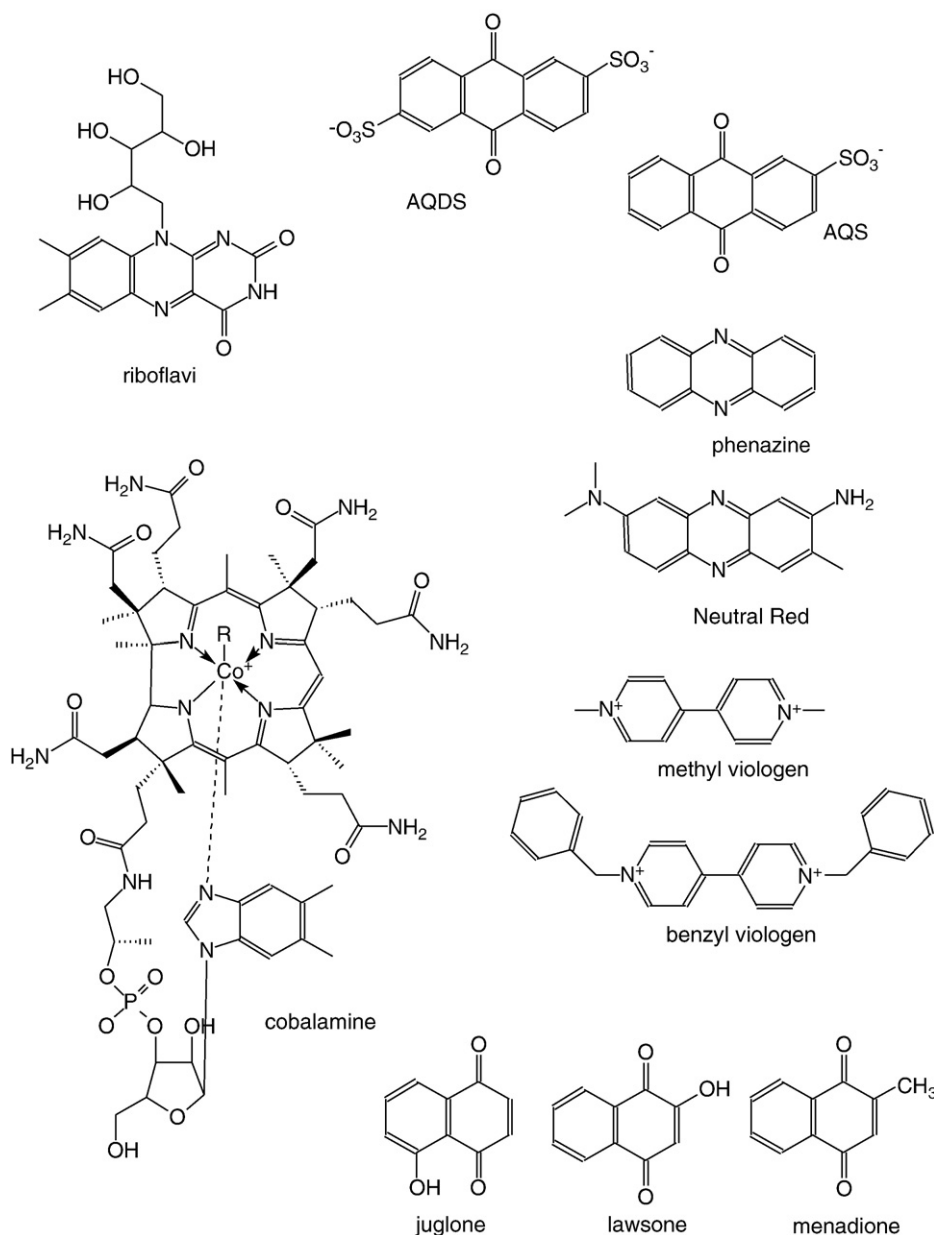


Fig. 2. Structure of the main redox mediators reported in the literature.

reduction of quinones can be linked to the anaerobic oxidation of a large variety of organic substrates including alcohols, short chain fatty acids, monosaccharides (Lovley et al., 1996; Cervantes et al., 2000a), as well as priority pollutants, such as toluene (Cervantes et al., 2001a), vinyl chloride, dichloroethene (Bradley et al., 1998) and phenolic compounds (Cervantes et al., 2000a,b). On the opposite site, several electron-withdrawing compounds accept electrons from reduced RM, thereby promoting their re-oxidation. From the environmental or public health point of view, the most relevant redox reactions involved in the re-oxidation of RM include the abiotic transfer of electrons from reduced RM towards azo dyes (Rau et al., 2002), nitroaromatics (Tratnyek and Macalady, 1989; Borch et al., 2005; Kwon and Finneran, 2006), polyhalogenated pollutants (Curtis and Reinhard, 1994; Kappler and Haderlein, 2003) and U(VI) (Fredrickson et al., 2000). In these reactions, quinones or humic materials are the most studied RM. Reduced quinones can also serve as electron donors to support the microbial reduction of perchlorate, arsenate, nitrate, nitrite and nitrous oxide (Lovley et al., 1999; Aranda-Tamaura et al., 2007).

Fig. 2 illustrates the structure of several electron shuttles reported during the redox (bio)transformation of pollutants. For a RM to be an effective electron shuttle, its standard redox potential ( $E_0'$ ) should ideally be in between those of the two eventual half reactions. However, as the RM's efficacy will also depend on the activation energy of its reduction and oxidation, in comparison to that of the non-mediated total reaction, it may occur that even compounds with  $E_0'$  lower than the primary electron donor or higher than the terminal electron acceptor may also be effective RM.

The concept of mediated electron transfer has been known for a very long time. As early as 1931, in a study on bacterial electricity generation, it was demonstrated that electromotively active organic compounds, such as benzoquinone, or inorganic compounds, such as ferricyanide, could increase the electric current of a bacterial half-cell (Cohen, 1931). To our knowledge, the first paper mentioning a role of quinoid enzyme cofactors in the reduction of azo dyes dates from the late 1960s (Roxon et al., 1967). RM-catalyzed reduction of nitroaromatics has firstly been reported by Brown (1981). Moreover, the rate of reduction of several halogenated hydrocarbons was shown to improve with increasing the amount of natural organic matter (NOM) in sediments (Peijnenburg et al., 1992), presumably due to the catalytic input of redox active groups in NOM. Nevertheless, Tratnyek and Macalady (1989) and Curtis and Reinhard (1994) were probably the first researchers to focus their research on evidencing the input of RM on the reduction of nitroaromatics and polyhalogenated pollutants, respectively. From then on, and certainly after the publication of other thorough papers on basic aspects of the role of RM (Keck et al., 1997; Kudlich et al., 1997) and the boosting attention to microbial fuel cells (Bullen et al., 2006; Logan et al., 2006) the topic became increasingly popular, both in basic and in applied research. The application of RM for wastewater treatment and aquifer bioremediation has deserved considerable attention during the last years. The first studies focused on the application of RM on reductive (bio)transformation of contaminants were carried out during the decolorization of azo dyes in high rate UASB reactors (Cervantes et al., 2001b; Van der Zee et al., 2001).

The aim of this review paper is to summarize the results of RM-catalyzed reductive (bio)transformation processes. The article describes the impact of different RM on azo dye reduction (Section 2), reductive dehalogenation (Section 3) and reduction of nitroaromatics (Section 4). Section 5 briefly exemplifies miscellaneous redox reactions catalyzed by RM. Finally, Section 6 proposes several research areas required to improve the applicability of RM on the (bio)transformation of contaminants.

## 2. Role of redox mediators on azo dye reduction

### 2.1. Introduction

Azo dyes are aromatic structures linked together by azo ( $-N=N-$ ) chromophores. They represent the largest class of dyes applied in

textile-processing industries. Due to their color and possible mutagenicity, their removal from wastewater is of major concern. For that purpose, sequential anaerobic–aerobic treatment is the most logical biological strategy for the removal of azo dyes from wastewater. The dyes' electron withdrawing azo linkages, which are very recalcitrant towards oxidative reactions, can be reduced under anaerobic conditions, yielding aromatic amines. These aromatic amines are in turn likely to be removed by aerobic treatment, through a combination of biodegradation, autooxidation and adsorption (Field et al., 1995; Van der Zee and Villaverde, 2005). Anaerobic azo dye reduction has been thoroughly investigated and several review papers summarize the results, e.g. Stolz (2001) and Pandey et al. (2007). It is clear that most, if not all, azo compounds are susceptible to reductive fission and that the capacity to reduce azo dyes under anaerobic conditions is almost ubiquitous among a wide range of bacterial species. In addition, azo dyes can be chemically reduced by bulk reducing agents, such as sulfide. Consequently, anaerobic bioreactor treatment of azo dye containing wastewaters will normally lead to a certain extent of reductive decolorization. In a recent review of a large number of bioreactor studies it was concluded that the extent of anaerobic color removal in these studies was generally high (70–100%) but that decolorization often proceeds very slowly, requiring long hydraulic residence times (Van der Zee and Villaverde, 2005). It is obvious, therefore, that accelerating azo dye reduction by electron shuttling RM will be highly relevant for the applicability of biological techniques to remove azo dyes from wastewater.

Tables 1–5 give a summary of the studies reporting the impact of RM on the rates of azo dye reduction in different biological and chemical systems. In the 'Results' columns of the tables, it can be seen that RM accelerated the reaction in most of the systems studied, often doubling to multiplying the reaction rates. In some cases, the presence of a RM even appeared as a prerequisite for the reaction to take place. However, the impact of RM was not always huge: sometimes the accelerating effect was small, sometimes absent, and in rare cases even adverse. This wide range of RM impacts reflects the differences between the investigated systems, their conditions, the dye/mediator/biomass-combinations used, etc. In the following sections, the most important factors determining the impact of RM on azo dye reduction will be discussed.

### 2.2. Redox mediator properties

Several of the studies listed in Tables 1–5 report different impacts of distinct RM under otherwise similar circumstances. Part of these differences may be directly related to the  $E_0'$  of the shuttling compounds investigated. In addition, other physico-chemical properties of the shuttling compound may play a role in determining its feasibility and strength as a mediator for azo dye reduction.

#### 2.2.1. Oxidation–reduction potential

By definition, an electron shuttling compound will only be effective as a RM for azo dye reduction if it lowers the reaction's activation energy. For that purpose, its  $E_0'$  should ideally be in between those of the two eventual half reactions, the reduction of an azo dye and the oxidation of a primary electron donor:

- A mediator's  $E_0'$  should not be much lower (ideally higher or less negative) than that of the biological reducing system (typically  $-320$  mV, representing the  $E_0'$  of NAD(P)H, the cofactor with the lowest oxidation–reduction potential) or the bulk reductant (e.g.  $-270$  mV in case of sulfide) – otherwise it will not be reduced sufficiently. Hence the observed failure of cyanocobalamin (Dos Santos et al., 2004a) and ethyl viologen (Kudlich et al., 1997) to act as a mediator is most probably due to their too low  $E_0'$  values:  $-530$  and  $-480$  mV, respectively. Methyl viologen ( $E_0' = -440$  mV) was the most reduced shuttling compound with clear ability to act as a RM

for azo dye reduction. It modestly increased the rate of Acid Red 27 reduction by *Sphingomonas* (Kudlich et al., 1997) but its impact on the reduction of Acid Yellow 23 by *Bacteroides* was huge, a 4.5-fold increase of the reaction rate, similar or even better than that of mediators with a higher  $E_0'$  (Chung et al., 1978). Especially the latter result is remarkable, since methyl viologen's  $E_0'$  is considerably lower than the indicated boundary level of  $-320$  mV.

– Likewise, a mediator's  $E_0'$  should not be much higher (ideally lower or more negative) than that of the azo dye – otherwise it will not reduce the azo linkage at sufficient rates. Since  $E_0'$ -values for azo dyes, which will be unique for each specific compound, are not available, it is less easy to estimate top boundary values for a mediator's  $E_0'$ . Polarographically determined half-wave standard potentials ( $E_{1/2}$ ) give an indication. However, reported  $E_{1/2}$ -values

**Table 1**  
Impact of redox mediators on the reductive decolorization of azo dyes by pure cultures of different microbial strains

Azo Dye <sup>a</sup>	Microorganism(s) <sup>b</sup>	Electron donor	Mediator <sup>c</sup>	RM/AD-ratio <sup>d</sup>	Results <sup>e</sup>	Reference
Acid Yellow 23	<i>Proteus vulgaris</i> (0–74 h starved cells)	Glucose	RF	0.0025–0.0175	Max. 10 ×; no additional effect RM at RM/AD >0.012	Roxon et al. (1967)
Acid Yellow 23	<i>Bacteroides thetaiotaomicron</i>	Glucose	MV, BV, CV, NR, PS FAD, MQ, FMN, JGB	0.0125	MV, BV, CV, NR: 4.5 ×; PS, FAD: 3.5 ×; MQ, FMN: 3 ×; JGB: 2.5 ×	Chung et al. (1978)
Poly-T Poly-S	18 pure cultures of anaerobic (10) and facultative (8) bacteria <sup>f</sup>	Broth	FMN	0.0119 <sup>g</sup>	Stimulatory effect depends on the dye-organism combination: max. 25 × (avg. 4.6 ×) and max. 2.3 × (avg. 1.8 ×) for Poly-T and Poly-S, resp.	Brown (1981)
Acid Red 27	<i>Sphingomonas xenophaga</i> BN6	Glucose	2-NS-metab.	(1) <sup>h</sup>	Increase (not quantified)	Keck et al. (1997, 2002)
Acid Red 27	<i>Sphingomonas paucimobilis</i> ; <i>E. coli</i> JM109	Glucose	2-NS-metab.	(1) <sup>h</sup>	Increase (not quantified)	Keck et al. (1997)
Acid Red 27	<i>Sphingomonas</i> sp. strain BN6	Glucose	AQS, LAW, AQDS, BV, MV, FAD, (EV)	0.2	AQS: 10.5 ×; LAW: 10 ×; AQDS: 6.5 ×; BV: 2 ×; MV, FAD: 1.5 ×; EV: no effect	Kudlich et al. (1997)
Acid Red 27	<i>Sphingomonas</i> sp. strain BN6	Glucose	AQS	Max. 3	Without RM: negligible reduction; AQS: increase to 2.3 $\mu\text{mol min}^{-1} \text{g}^{-1}$ protein; no additional effect RM at RM/AD >~1.4	Kudlich et al. (1997)
Acid Red 27 Acid Red 1 Food Yellow 3 Acid Black 1 DHAB	<i>Sphingomonas</i> sp. strain BN6 <i>Bacteroides fragilis</i>	Glucose Broth	AQS BV	Max. 0.6 0.17	Without RM: negligible reduction; increase to max. 4.5, 2.2, 6.6 and 3.6 $\mu\text{mol min}^{-1} \text{g}^{-1}$ protein (AR27, AR1, FY3 and AB1, resp.) Increase (not quantified)	Kudlich et al. (1997) Bragger et al. (1997)
Acid Red 27	<i>Clostridium perfringens</i>	Glucose	RF, BV	4.2	RF: ~2.5 ×, BV: ~1.6 ×	Semlé et al. (1998)
Acid Red 27	10 pure cultures from distinct taxonomic groups <sup>i</sup>	Glucose	AQS, AQDS, LAW, LAP PLUM	2.5	Stimulatory effect varies largely between different organism-RM combinations: average effect from 0.1–0.5 to 2–10 $\mu\text{mol dye min}^{-1} \text{g}^{-1}$ protein; One species, <i>H. salinarum</i> , does not reduce the dye at all.	Rau et al. (2002)
Acid Red 27 Acid Orange 20 Acid Orange 7 Food Yellow 3 Acid Red 18 Food Red 17 Acid Red 14 Acid Red 1 Acid Yellow 23 Acid Black 1 Food Black 1	<i>Sphingomonas xenophaga</i> BN6; <i>Escherichia coli</i> K12	Glucose	AQS, LAW	2.5	Stimulatory effect varies largely between different dye-organism-RM combinations: Average effect 26 ×; <i>S. xenophaga</i> : AQS about 4x more effective than LAW; <i>E. coli</i> : LAW about 37 × more effective than AQDS	Rau et al. (2002)
Reactive Black 5	<i>Shewanella</i> strain J18 143	Formate	AQDS	4	3 ×	Pearce et al. (2006)
Acid Red 27	<i>Shewanella decolorationis</i> S12	Formate	AQS, AQDS	1–5 1–12	AQS: 1.75 × at RM/AD=1–2; relative impact of RM decreases and becomes even adverse at higher RM/AD. AQDS: 1.33 × at RM/AD=1; decreasing impact, to none, at higher RM/AD.	Hong et al. (2007)
Acid Red 73 Acid Yellow 36 Acid Orange 10 Acid Orange 7 Acid Red 26	<i>Shewanella decolorationis</i> S12	Formate	AQS, AQDS	1–5 1–12	Without RM, CRE(12 h) ~19%; AQS: at RM/AD=1, CRE(12 h) ~96%; no additional effect at RM/AD=3; adverse effect at RM/AD=5; AQDS: at RM/AD=3 and 6, CRE(12 h) ~95%; no additional effect at higher RM/AD	Hong et al. (2007)
Reactive Red 2	<i>Methanothermobacter thermoautotrophicus</i> ΔH (65 °C); <i>Methanothermobacter</i> related strain NJ1 (55 °C); <i>Methanosarcina barkeri</i> (37 °C)	H <sub>2</sub>	RF	0.12	Thermophilic strains: only dye reduction in the presence of RF mesophilic strain: RF accelerates dye reduction	Dos Santos et al. (2006)
Pigment Orange 5 <sup>j</sup>	<i>Shewanella</i> strain J18 143	Formate	AQDS	2	~4 × (30 °C)	Pearce et al. (2008)
Acid Orange 52 'Dye I' Acid Orange 7 'Dye III'	<i>Issatchenkia occidentalis</i> (yeast) <sup>k</sup>	Glucose	AQS	Max. 9.5	AO52: max. 3 ×; no additional effect RM at RM/AD >2.1; 'Dye I': similar results as with AO52; AO7 and 'Dye III': adverse effect of RM (not quantified)	Ramalho et al. (2004)

**Table 2**  
Impact of redox mediators on the reductive decolorization of azo dyes by cell-free enzymatic extracts

Azo dye <sup>a</sup>	System	Mediator <sup>b</sup>	RM/AD-ratio <sup>c</sup>	Results <sup>d</sup>	Reference
Acid Yellow 23	Crude enzyme preparations (supernatant fraction) of <i>Proteus vulgaris</i> + NADPH-generating system	RF, FMN, FAD	Max. 0.536	RF: max. 14 × ; FMN: max. 16 × ; FAD: max. 3 ×	Roxon et al. (1967)
Acid Yellow 9	Cell extracts (supernatant fraction) of <i>Streptococcus faecalis</i> + NADH-generating system	RF, FMN, FAD	0.0033	RF: 1.8–3.6 × ; FMN: 1.5–3.1 × ; FAD: 1.4–2.4 ×	Scheline and Nygaard (1970)
Acid Red 1	Cell-free extracts of <i>Streptococcus faecalis</i> + NADH-generating system	RF, FMN, FAD	Max. 1.34	Without RM negligible reduction; with RM increase to 2.7, 2.3 and 2.0 μmol h <sup>-1</sup> mg <sup>-1</sup> protein (RF, FMN, FAD, respectively); very limited additional effect RM at RM/AD > 0.4	Walker et al. (1971)
Food Yellow 3 Acid Yellow 23 Poly-T, Poly-S	Cell free extracts of rat cecal bacteria + NADPH-generating system	BV	2.1–2.3 <sup>e</sup>	50 × (FY3), 164 × (AY236), 123 × (Poly-T), 118 × (Poly-S)	Brown (1981)

<sup>a</sup> Colour Index generic names dyes are given whenever available; popular trivial names are Tartrazine (Acid Yellow 23), Sunset Yellow (Food Yellow 3); Poly-S and Poly-T are polymeric azo dyes based on Food Yellow 3 and Acid Yellow 23, respectively.

<sup>b</sup> BV = Benzyl viologen, FAD = Flavin adenine dinucleotide, FMN = Flavin mononucleotide, RF = Riboflavin.

<sup>c</sup> RM/AD-ratio = molar ratio redox mediator: azo dye (unless otherwise stated).

<sup>d</sup> '×' expresses the x-fold increase of zero-order reduction rates as compared to the RM-free controls; avg. = average.

<sup>e</sup> RM/AD-ratio as moles mediator per mol (eq) dye monomer.

have a broad range (from –530 to –180 mV) and the differences between values reported for the same azo dye can vary as much as 200 mV (Dubin and Wright, 1975; Bragger et al., 1997; Semd e et al., 1998). Another and probably better indication was the result of an experiment in which the reduction of the azo dye Acid Red 27 by a large number of chemically reduced quinoid shuttling compounds with different  $E_0'$  values was investigated. This experiment demonstrated that this particular dye, which has an  $E_{1/2}$  of around –250 mV (Dubin and Wright, 1975; Semd e et al., 1998) or around –350 mV (Bragger et al., 1997), could only be reduced by quinones with  $E_0'$  values below –50 mV (Rau et al., 2002). For other azo dyes, this threshold value, which reflects the dye's  $E_0'$  in combination with the reaction's activation energy, may be different, but it is reasonable to assume that the observed failure of 1,4-Benzoquinone to act as a mediator (Dos Santos et al., 2005c) and the poor impact reported for Menadione (Chung et al., 1978; Albuquerque et al., 2005) are most probably due to their too high  $E_0'$ -values: +263 and –19 mV, respectively.

– In the  $E_0'$  range between these boundaries, there will be two opposing trends with respect to a mediator's  $E_0'$  and its impact on the rate of azo dye reduction. In the second step of mediated azo dye reduction, i.e. the chemical reaction between the reduced

mediator and the azo dye, the reaction rate will increase with decreasing  $E_0'$  of the mediator, as has been demonstrated with the reduction of Acid Red 27 by a series of chemically reduced quinones with different  $E_0'$  values (Rau et al., 2002). In the first step of mediated azo dye reduction, i.e. reduction of the mediator, an opposite trend, an increase of the reaction rate with increasing  $E_0'$  of the mediator, can be expected. The rate-limiting step of mediated azo dye reduction will thus be influenced by the mediator's  $E_0'$ , with either the reduction of the dye or that of the mediator being rate-limiting, at higher and lower  $E_0'$  values, respectively. At midrange, combination of both trends may reveal the optimum mediator's  $E_0'$  (unique for each dye/reducing system combination), where both reaction steps proceed at equal rate. However, in biological systems (Tables 1–4), such an optimum  $E_0'$  could not be confirmed: studies with multiple redox mediators under otherwise similar conditions did not show any relationship between a mediator's  $E_0'$  and the rate of azo dye reduction (Chung et al., 1978; Kudlich et al., 1997; Rau et al., 2002), and mediators with identical  $E_0'$  (FAD, FMN and AQS) often had largely different impacts. For instance, Kudlich et al. (1997) observed that Acid Red 27 reduction by *Sphingomonas* BN6 was stimulated to a decreasing extent by the following mediators (values between brackets show  $E_0'$

#### Notes to Table 1:

<sup>a</sup> Colour Index generic names dyes are given whenever available; popular trivial names are Naphthol Blue Black (Acid Black 1), Amaranth (Acid Red 27), Tartrazine (Acid Yellow 23), Methyl Orange (Acid Orange 52), Sunset Yellow (Food Yellow 3); DHAB = 4,4'-Dihydroxyazobenzene; Poly-S and Poly-T are polymeric azo dyes based on Food Yellow 3 and Acid Yellow 23, respectively; 'Dye I' = *m*-[(4-dimethylamino)phenylazo]benzenesulfonic acid, 'Dye III' = *m*-[(2-hydroxy-1-naphthyl)azo]benzenesulfonic acid.

<sup>b</sup> Although culture conditions vary between studies, anaerobic conditions prevailed when reducing azo dyes (unless otherwise stated).

<sup>c</sup> AQS = 9,10-Anthraquinone-2,6-disulfonate, AQS = 9,10-Anthraquinone-2-sulfonate, BV = Benzyl viologen, CV = Crystal Violet, EV = Ethyl viologen, FAD = Flavin adenine dinucleotide, FMN = Flavin mononucleotide, JGB = Janus Green B, LAP = Lapachol (2-Hydroxy-3-(3-methyl-2-butenyl)-1,4-naphthoquinone), LAW = Lawsone (2-Hydroxy-1,4-naphthoquinone), NR = Neutral Red, 2-NS-metab. = metabolites of aerobic biodegradation of 2-Naphthosulfonate by *Sphingomonas* strain BN6, MQ = Menadione, MV = Methyl viologen, PLUM = Plumbagin (5-Hydroxy-2-methyl-1,4-naphthoquinone), PS = Phenosafranin, RF = Riboflavin.

<sup>d</sup> RM/AD-ratio = molar ratio redox mediator: azo dye (unless otherwise stated).

<sup>e</sup> '×' expresses the x-fold increase of zero-order reduction rates as compared to the RM-free controls; avg. = average.; resp. = respectively; CRE = color removal efficiency; dye names are logically abbreviated.

<sup>f</sup> Anaerobes: *Clostridium ramosum*; *Clostridium paraputrificum*; *Clostridium sporogenes*; *Lactobacillus catenaforme*; *Acidaminococcus fermentans*; *Bacteroides fragilis*; *Ruminococcus bromii*; *Veillonella parvula*; *Peptococcus prevotii*; *Bifidobacterium adolescentis*; *Eubacterium aerofaciens*; facultatives: *Salmonella typhimurium* LT-2; *Salmonella paratyphi*; *Proteus vulgaris*; *Klebsiella pneumoniae*; *Pseudomonas aeruginosa*; *Salmonella typhimurium* TA1537; *Streptococcus faecium*.

<sup>g</sup> RM/AD-ratio as moles mediator per mol (eq) dye monomer.

<sup>h</sup> The original 2-NS-concentration was 0.5 mM, equal to the dye concentration subsequently applied.

<sup>i</sup> α-Proteobacteria: *Sphingomonas xenophaga* BN6 and *Agrobacterium tumefaciens*; β-Proteobacteria: *Ralstonia eutropha* 335 and *Hydrogenophaga palleronii*; γ-Proteobacteria: *Escherichia coli* K12; 'FCB-Pylum': *Flexibacter filiformis*; low GC: *Bacillus subtilis*; high GC: *Rhodococcus erythropolis*; Lactic acid bacteria: *Lactobacillus plantarum*; Halobacteria: *Halobacterium salinarum*.

<sup>j</sup> As commercial dispersion GemSpere Orange EX5.

<sup>k</sup> Microaerobic conditions (shaking flasks exposed to air), albeit with soluble oxygen concentrations below detection level when azo dye reduction took place.

**Table 3**  
Impact of redox mediators on the reductive decolorization of azo dyes by mixed microbial cultures under batch, anaerobic conditions

Azo dye <sup>a</sup>	System <sup>b</sup>	Electron donor <sup>c</sup>	Mediator <sup>d</sup>	RM/AD-ratio <sup>e</sup>	Results <sup>f</sup>	Reference
Acid Red 27	AAS	Glucose	AQS	Max. 3	0 → 3 μmol/(min. g protein); no additional effect at RM/AD >~1.2	Kudlich et al. (1997)
Acid Red 27	AAS	Glucose	AQS, LAW, Henna leaves <sup>g</sup>	Max. 2 max. 6 g mmol <sup>-1</sup>	AQS: max. 10–15 ×; LAW: max. 7–12 ×; Henna leaves: max. 9 × (z.o.)	Rau et al. (2002)
Reactive Orange 14	AGS	Glucose	RF	0.13	1.5–2 ×	Cervantes et al. (2006)
Reactive Orange 14 Direct Blue 53 Direct Blue 71 Acid Orange 52	AGS	Glucose	AQDS, LAW, RF	0.05	Stimulatory effect varies largely between different dye-RM combinations: avg. 2 ×; max. 3.8 ×	Encinas-Yocupicio et al. (2006)
Acid Orange 52	AGS	Glucose	AD-metab.	0.04 <sup>h</sup>	~2 ×	Encinas-Yocupicio et al. (2006)
Reactive Red 2	AGS	VFA	AQDS	Max. 1.56	Max. 7 ×	Van der Zee et al. (2001)
Mordant Yellow 10	AGS	VFA	RF	Max. 0.11	Max. 2 × (z.o.)	Field and Brady (2003)
Hydrolyzed Reactive Red 2	AGS at 55 °C	VFA, Glucose/ VFA, Glucose, H <sub>2</sub>	AQDS	0.04	VFA: 1.4 ×; Glucose/VFA: 1.6 ×, Glucose: 2.3 ×; H <sub>2</sub> : 2.4x	Dos Santos et al. (2003)
Hydrolyzed Reactive Red 2	AGS at different T	VFA, Glucose/ VFA	AQDS	0.4	VFA: 1.7 ×, 1.9 ×, 1.9 ×, 1.6 ×, 1.7 ×, at 45, 55, 60, 65 and 75 °C, resp.; Glucose/VFA: 2.0 ×, 2.6 ×, 2.4 ×, 2.1 × and 1.5 ×, at 45, 55, 60, 65 and 75 °C, resp.	Dos Santos et al. (2003)
Reactive Red 2 Acid Orange 7 Mordant Yellow 10 Hydrolyzed Reactive Red2	AGS at 55 °C AGS at different T	Glucose/VFA Glucose/VFA	AQDS, AQS, RF, (CNB12)	0.04	Stimulatory effect varies largely between different dye-RM combinations: avg. 1.7 × (RR2 and AO7) or 1.5 × (z.o., MY10); CNB12 not effective	Dos Santos et al. (2004a)
Hydrolyzed Reactive Red2	AGS at 55 °C	Glucose/VFA	AQDS	0.04	30 °C: 5 ×; 55 °C: 1.5 ×; k of RM-free control is 6 × higher at 55 °C as compared to 30 °C	Dos Santos et al. (2004b)
Hydrolyzed Reactive Red2	AGS at 55 °C	Glucose/VFA	AQDS	0.04	1.4–1.7-×	Dos Santos et al. (2004b)
Hydrolyzed Reactive Red2	AGS at 55 °C	Glucose/VFA	AQDS	Max. 33	Max. 5.9 ×; hardly any additional effect at RM/AD >4	Dos Santos et al. (2004b)
Hydrolyzed Reactive Red 2	AGS at different T	Glucose	AQS, (BQ), (AQS+BQ)	0.17	AQS: 3.8 × and 2.3 ×, at 30 and 55 °C, resp.; BQ: slight stimulation at 30 °C, slight adversary effect at 55 °C; AQS+BQ: no effect; k of RM-free control is 5.6 × higher at 55 °C as compared to 30 °C	Dos Santos et al. (2005b)
Reactive Red 2 Reactive Orange 14	AGS at different T, w or w/o BES or vancomycin	Glucose	RF	0.04	30 °C: 2.4 × and 2.2x; 55 °C: 1.7 × and 2.9 ×, RR2 and RO14, resp.; k of RM-free control is 3.7 and 4.4 × higher at 55 °C than at 30 °C, RR2 and RO14, resp.; BES hardly affects dye reduction (w or w/o RM); vancomycin strongly affects dye reduction (w or w/o RM)	Dos Santos et al. (2005a)
Reactive Red 2	AGS at different T, w or w/o BES or vancomycin	Acetate, H <sub>2</sub> , formate, methanol	RF	0.04	Acetate: 3.7 × and 1.5 ×; H <sub>2</sub> : 4.6 × and 3.8 ×; formate: 2.1 × and 2.0 ×; methanol: 2.8 × and 2.4 × (30 and 55 °C, respectively); w or w/o RM, BES stimulates azo dye reduction with H <sub>2</sub> and formate but has no effect with acetate or methanol	Dos Santos et al. (2005a)
Reactive Red 2	Crushed AGS at 30 °C, w or w/o BES	H <sub>2</sub>	RF	0.04	5.3 ×; BES stimulates azo dye reduction w or w/o RM	Dos Santos et al. (2005a)
Reactive Red 2	AGS at different T	Glucose	RF	Max. 0.67	Max. 23 × and 6 × at 30 and 55 °C, resp.; At RM/Dye >0.17: k (30 °C)=k (55 °C)	Dos Santos et al. (2005a)
Reactive Red 2 Reactive Red 4 Reactive Orange 14 Reactive Orange 14	AGS at 55 °C AGS at 55 °C	Glucose Glucose at different conc.	RF	0.04	RR2: 1.7 ×; RR4: no stimulation; RO14: 2.9 ×; BES hardly affects dye reduction (w or w/o RM); vancomycin strongly affects dye reduction (w or w/o RM)	Dos Santos et al. (2006)
Reactive Red 2 Reactive Orange 14	AGS at 55 °C	Acetate, H <sub>2</sub> , Formate, Methanol	RF	0.04	1.4–4.0 ×: impact RM increases with increasing glucose concentrations	Dos Santos et al. (2006)
Reactive Red 2 Reactive Orange 14	AGS at 55 °C	Acetate, H <sub>2</sub> , Formate, Methanol	RF	0.04	Acetate: 1.5 × and 4.6 ×, H <sub>2</sub> : 3.8 ×–7.1 × (RR2 and RO14, resp.); formate: 2.0 ×, methanol: 2.4 × (RR2) w or w/o RM, BES stimulates azo dye reduction with H <sub>2</sub> and formate but has no effect with acetate and methanol	Dos Santos et al. (2006)
Acid Orange 6	AGS at 30 °C	Acetate	RF	Max. 0.06	Big impact at RM/AD=0.02; small additional accelerating effect at RM/AD=0.04 and 0.06	Yemashova and Kalyuzhnyi (2006)
Reactive Red 24	CAS	YE/Peptone	Alginate beads with AQ	NA	Faster decolorization in comparison to controls with anthraquinone-free alginate beads	Guo et al. (2007)
Reactive Red 24 Reactive Red 2 Acid Red 1 Acid Red 14 Acid Red 72 Acid Black 1 Reactive Red 24	CAS CAS	YE/Peptone	BA	0–4.33	Max. 1.9 ×, 1.7 ×, 1.5 ×, 1.3 ×, 2.3 ×, 1.8x (z.o., RR24, RR2, AR1, AR14, AR72, AB1, respectively);no additional effect at RM/AD >~2.6	Guo et al. (2008)
Reactive Red 24	CAS	YE+Peptone	Different AQ-compounds <sup>i</sup>	NA	Increase with all RM tested (not quantified)	Guo et al. (2008)
Acid Orange 7	Partially digested AAS	Acetate	Graphite	NA	88% decolorization in 20 h, as compared to no decolorization in 24 h in the absence of graphite	Mezohegyi et al. (2007)

**Table 4**  
Impact of redox mediators on the reductive decolorization of azo dyes by mixed microbial cultures in anaerobic continuous reactors

Azo dye (AD) <sup>a</sup>	System <sup>b</sup>	HRT <sup>c</sup> (h)	Electron donor <sup>d</sup>	Mediator (RM) <sup>e</sup>	RM/AD-ratio <sup>f</sup>	Results <sup>g</sup>	Reference
Acid Orange 7	UASB with AGS	2–6	VFA	AQDS	Max. 0.105	At HRT=6 h, CRE increases from 86% (w/o RM) to max. 97%; effect RM increases with decreasing HRT	Cervantes et al. (2001a,b)
Reactive Red 2	UASB with AGS	5.9	VFA	AQDS	Max. 0.97	CRE increases with increasing RM conc., from ~40% (no RM) to 85% (RM/AD=0.12) to >98% (RM/AD=0.97)	Van der Zee et al. (2001); Dos Santos et al. (2003)
Hydrolyzed Reactive Red 2	EGSB with AGS at 55 °C	10	Glucose/VFA	AQDS	Max. 0.171	Average CRE 95%, only slightly lower than CRE of RM-free control reactor (91%); CRE of RM-free reactor more sensitive to changes in ED/AD-ratio	Dos Santos et al. (2003)
Hydrolyzed Reactive Red2, textile wastewater	EGSB with AGS at different T	10–2.5	VFA	AQDS	0.023–0.06	Huge impact RM at 30 °C; relatively small effect of AQDS at 55 °C; CRE (30 °C) < CRE (55 °C) CRE decreases with decreasing HRT	Dos Santos et al. (2005b,c)
Acid Orange 7	SBR with AS (anaerobic phase)	40 <sup>h</sup>	SA	MQ, AQDS	0.7	CRE increases from ~5% (w/o RM) to max. 25% (MQ) and ~70% and ~90% (AQDS; RM/AD-ratios 0.7 and 1.4, resp.)	Albuquerque et al. (2005)
Hydrolyzed Reactive Red 2	UASB	5.3	VFA	PAC	NA	CRE of PAC-free control reactor ~35%, CRE of PAC-amended reactors ~45% and ~95% with 0.4 and 10 g PAC L <sup>-1</sup> , respectively	Van der Zee et al. (2003)
Acid Orange 7	UPBR with partially digested AAS	Max. 0.26 <sup>i</sup>	Acetate	GAC (dye-saturated), Graphite, Aluminium	NA	AC (67 g L <sup>-1</sup> ): 99% CRE at $\tau$ =2 min. Graphite (67 g L <sup>-1</sup> ): 77% CRE at $\tau$ =9.7 min. Aluminium (68 g L <sup>-1</sup> ): 33% CRE at $\tau$ =15.8 min.	Mezohegyi et al. (2007)

<sup>a</sup> Colour Index generic names; a popular trivial name for Acid Orange 7 is Orange II.

<sup>b</sup> UASB=upflow anaerobic sludge bed reactor; EGSB=expanded granular sludge blanket reactor; SBR=sequential batch reactor; UPBR=upflow packed bed reactor; AAS=aerobic activated sludge (under anaerobic conditions); AGS=anaerobic granular sludge.

<sup>c</sup> HRT=hydraulic residence time.

<sup>d</sup> VFA=volatile fatty acids; SA=hydrolyzed sizing agent (starch derivative).

<sup>e</sup> AQDS=9,10-Anthraquinone-2,6-disulfonate, GAC=granular activated carbon, MQ=Menadione, PAC=powdered activated carbon.

<sup>f</sup> RM/AD-ratio=molar ratio redox mediator: azo dye.

<sup>g</sup> AC=activated carbon, CRE=color removal efficiency; ED=electron donor;  $\tau$ =space time.

<sup>h</sup> 24 h-cycles with an anaerobic phase of 10.5 h.

<sup>i</sup> Space time ( $\tau$ ) rather than hydraulic residence time.

in mV): AQS(-218)>Lawson(-139)>AQDS(-184)>Benzyl viologen (-360)>Methyl viologen(-440)>, FAD (-219). Moreover, the impact of five quinoid mediators on the reduction of Acid Red 27 by ten pure cultures of distinct classes of bacteria showed large differences between cultures, with different sequences of these five compounds when ranged according to their effect (Rau et al., 2002). These results reflect the diverging and apparently  $E_0'$ -independent preferences of biological mediator reduction. In contrast, in chemical systems, the mediator's  $E_0'$  may very well be more important in determining its reduction rate. However, none of the studies listed in Table 5 allows for either confirming or rejecting such a relationship.

From the above it is clear that mediated azo dye reduction, at least in biological systems, is largely independent of the mediator's  $E_0'$  and that this independence is probably mostly due to reduction of the mediator rather than to that of the dye. Therefore, it is likely that the mechanism of mediator reduction is responsible for the observed different impacts of different mediators. However, since diverging mediator preferences of different microorganisms suggest they use

distinct enzyme systems to reduce mediators, any possible correlation between the mediators' physico-chemical properties (e.g. size, charge, functional groups, etc.) and their impact on azo dye reduction is inevitably vague. The clearest trend, one that seems almost general, is that AQS is a more powerful mediator than AQDS (Rau et al., 2002; Dos Santos et al., 2004a; Hong et al., 2007). Although it cannot be excluded that this difference is solely a result of  $E_0'$  differences, it is possible that the presence of an additional sulfonate group in AQDS diminishes the ease at which the compound approaches the enzyme.

### 2.2.2. Ability to cross the cell membrane

A property of electron shuttling compounds that deserves attention is the ability to cross cell membranes. It has been demonstrated that the azoreductase activity of cell extracts can be much higher than that of intact cells and that the cell membrane forms a barrier for dyes and mediators (Kudlich et al., 1997; Russ et al., 2000). Hence, mediated azo dye reduction by *Sphingomonas xenophaga*, with the sulfonated quinone AQS as the optimum mediator, is presumably associated with the membrane-bound respiratory chain enzyme NADH:ubiquinone oxidoreductase (Kudlich et al., 1997; Rau et al., 2002), and several other species are likely to use

#### Notes to Table 3:

<sup>a</sup> Colour Index generic names are given whenever available; popular trivial names are Naphthol Blue Black (Acid Black 1), Methyl Orange (Acid Orange 52), Amaranth (Acid Red 27), Sunset Yellow (Food Yellow 3).

<sup>b</sup> AAS=aerobic activated sludge (under anaerobic conditions), AGS=anaerobic granular sludge; CAS=mixture of salt-tolerant bacteria.

<sup>c</sup> VFA=volatile fatty acids (in these cases mixtures of acetate, propionate and butyrate), YE=yeast extract.

<sup>d</sup> AD-metab.=metabolites of anaerobic reduction of the azo dyes Direct Blue 53 or Direct Blue 71 (autocatalysis); AQ=9,10-Anthraquinone; AQDS=9,10-Anthraquinone-2,6-disulfonate; AQS=9,10-Anthraquinone-2-sulfonate; BA=Bromoamine acid (1-Amino-4-bromoanthraquinone-2-sulfonic acid); BQ=1,4-Benzoquinone; CNB12=Cyanocobalamin (Vitamin B12); LAW=Lawson (2-Hydroxy-1,4-naphthoquinone); RF=Riboflavin.

<sup>e</sup> RM/AD-ratio=molar ratio redox mediator: azo dye (unless otherwise stated).

<sup>f</sup> 'x' expresses the x-fold increase of the pseudo first-order reduction rate constants (not marked) or zero-order reduction rate constants (marked with 'z.o') as compared to the RM-free controls; avg.=average; resp.=respectively; dye names are logically abbreviated.

<sup>g</sup> Henna leaves are a source of Lawson.

<sup>h</sup> assuming 2 mol catalytic aromatic amine per mol reduced azo dye.

<sup>i</sup> 1-Aminoanthraquinone; 1-Amino-2-bromo-4-hydroxyanthraquinone; 1,4-Diaminoanthraquinone; 2-Aminoanthraquinone; 1,4-Dihydroxyanthraquinone.



**Table 5**  
Impact of redox mediators on the reductive decolorization of azo dyes by different electron donors under abiotic conditions

Azo dye <sup>a</sup>	Electron donor <sup>b</sup>	Mediator <sup>c</sup>	RM/AD-ratio <sup>d</sup>	Results <sup>e</sup>	Reference
Acid Red 27	NADPH	FAD	3.1	Without FAD: less than 5% reduction	Russ et al. (2000)
Acid Red 27 (Acid Orange 7) (Acid Orange 52) (Acid Yellow 23) (4-AB) (4-HAB)	NADPH (generating system)	BV	13.3	Acid Red 27: increase (not quantified); other azo compounds: no reaction	Semdé et al. (1998)
Reactive Orange 14 0.15 mM	Sulfide	RF	0.13	31–45 ×	Cervantes et al. (2006)
Acid Orange 7 0.25–0.3 mM	Sulfide	AN AQDS	Max. 4	Max. 13 ×	Van der Zee et al. (2000)
Acid Orange 7 0.25–0.3 mM	Sulfide	Autoclaved AGS	Max. 2 Max 36 gVSS mmol <sup>-1</sup>	Max. 105 × Max. 10 ×	Van der Zee et al. (2000)
Reactive Red 2 0.3 mM	Sulfide at different T	AQS	0.04	1.2-fold decrease of activation energy due to AQS	Dos Santos et al. (2004a)
Hydrolyzed Reactive Red 2 0.3 mM	Sulfide 55 °C	AQS	0.04	1.3–1.5 ×	Dos Santos et al. (2004a)
4-AAB bound to epoxy-activated Sepapharose beads	ZVI	JUG HEM, Chlorophyll, HA	10 100 g mmol <sup>-1</sup> 2.5 gC mmol <sup>-1</sup>	Without RM: no dye reduction; JUG: complete reduction <sup>f</sup> ; other RM: some reduction	Weber (1996)
4-AAB bound to epoxy-activated Sepapharose beads	ZVI	Pond sediment; pond sediment-associated water; (water-free sediment)	NA	Pond sediment: very slow but steady reduction; pond sediment-associated water: some initial reduction after which the reaction stops; water-free sediment: no reduction	Weber (1996)
Pond sediment-adsorbed Disperse Blue 79	ZVI	Pond sediment w or w/o JUG	NA	Some reduction; small effect of JUG	Weber (1996)
Acid Yellow 9	Cathodic reduction	AQS	Max. 0.15	Without RM: no dye reduction	Bechtold et al. (1997)

<sup>a</sup> Colour Index generic names dyes are given whenever available; popular trivial names are Amarant (Acid Red 27), Tartrazine (Acid Yellow 23); 4-AAB=4-Aminoazobenzene, 4-HAB=4-Hydroxyazobenzene.

<sup>b</sup> ZVI=Zero-valent iron.

<sup>c</sup> AGS=anaerobic granular sludge, AN=1-Amino-2-naphthol, AQ=9,10-Anthraquinone, AQDS=9,10-Anthraquinone-2,6-disulfonate, 9,10-AQS=Anthraquinone-2-sulfonate, BV=Benzyl viologen, CBN12=5'-deoxyadenosylcobalamin (vitamin B12), FAD=Flavin adenine dinucleotide, HEM=Hematin, HG=Haemoglobin, JUG=Juglone (...), Poly-R478=a purple polyanthraquinone dye, RA=Resazurin, RF=Riboflavin.

<sup>d</sup> RM/AD-ratio=molar ratio redox mediator: azo dye (unless otherwise stated).

<sup>e</sup> '×' expresses the ×-fold increase of the pseudo first-order reduction rate constants (not marked) or zero-order reduction rate constants (marked with 'z.o') as compared to the RM-free controls; avg.=average; resp.=respectively.

<sup>f</sup> Aniline recovery 80%, equal to the aniline recovery with free 4-AAB.

similar membrane-associated enzyme systems. However, as has recently been shown for *E. coli*, the mechanism can also be based on intracellular enzymes in combination with a mediator that diffuses through the cell membrane. *E. coli*'s mediator-dependent 'azoreductase' was identified as two cytosolic oxygen-insensitive nitroreductases, acting as quinone reductases. The mediator was Lawsons, the only quinone with a mediator-feasible  $E_0'$  that could be reduced by the enzymes (Rau and Stolz, 2003). Lawsons's apparent ability to cross the cell membrane is probably related to its small size and its relative lipophilicity (no charged substituents). Depending on the preferences of intracellular reductases in other species, there may be more shuttling compounds with membrane-crossing properties that could act as powerful mediators for azo dye reduction.

### 2.2.3. Solubility vs. non-solubility

While the large majority of the studies listed in Tables 1–5 dealt with water-soluble compounds as redox mediators for azo dye reduction, a few investigations demonstrated redox-mediating properties of non-soluble materials. Especially activated carbon (Van der Zee et al., 2003; Mezohegyi et al., 2007), to a lesser extent graphite (Mezohegyi et al., 2007) and to some extent alginate beads with immobilized anthraquinone (Guo et al., 2007) have been shown to accelerate azo dye reduction in biological systems. As graphite, which was shown to act as mediator in contrast to the non-conductive material aluminium (Mezohegyi et al., 2007), does not contain specific groups on its surface, it is clear that catalysis of biological azo dye reduction does not necessarily depend on redox-active surface groups. Nevertheless, the considerably higher dye reduction kinetics obtained with activated carbon as compared to

graphite (Mezohegyi et al., 2007) indicates that surface groups play an important role. Therefore, activated carbon or other insoluble materials with surface-associated or entrapped redox-active functional groups can be considered the most logical candidates as insoluble redox mediators. The catalytic properties of such insoluble materials enable their use as biologically regenerable RM in anaerobic bioreactors. As these materials can be retained in the reactor for prolonged time, they are attractive alternatives to soluble RM that need to be dosed continuously.

### 2.3. Mediator/dye-ratio

Tables 1–5 show that a wide range of molar mediator/dye-ratios has been applied. In several studies, two or more ratios have investigated, usually by changing the mediator concentration (Roxon et al., 1967; Walker et al., 1971; Kudlich et al., 1997; Van der Zee et al., 2000; Cervantes et al., 2001b; Van der Zee et al., 2001; Rau et al., 2002; Dos Santos et al., 2003; Field and Brady, 2003; Dos Santos et al., 2004b; Albuquerque et al., 2005; Dos Santos et al., 2005b; Dos Santos et al., 2005c; Dos Santos et al., 2005d; Yemashova and Kalyuzhnyi, 2006; Hong et al., 2007). Dye reduction rates increase with increasing mediator concentrations. Mediator gradients typically show that the effect is especially strong at low mediator/dye ratios and levels off at higher ratios. The mediator/dye-ratios beyond which there is no additional effect of raising the mediator concentration are mostly rather high: it is not unusual that maximum azo dye reduction rates have not yet been reached at molar ratios as high as 1 (Kudlich et al., 1997; Van der Zee et al., 2001; Dos Santos et al., 2004b).

## 2.4. Temperature

Reaction rates will increase with increasing temperatures because a higher fraction of the molecules will have a kinetic energy higher than the activation energy. This effect, as described by the Arrhenius equation, will be more pronounced if the reaction's activation energy is higher. Raising the temperature will thus result in a relatively higher increase of the rate of non-mediated azo dye reduction (high activation energy) in comparison to that of mediated azo dye reduction (lower activation energy). It can be expected that chemical azo dye reducing systems behave accordingly, as appears to be confirmed by the results of [Dos Santos et al. \(2004a\)](#), the only study in [Table 5](#) that investigated chemical azo dye reduction at different temperatures. In this study it was observed that the first-order reaction rate constant of non-mediated reduction of Reactive Red 2 (0.3 mM) by sulfide (4.5 mM) was 2.2-fold higher at 55 °C than at 30 °C, and AQS (12 μM) increased these rate constants 1.9-fold and 1.7-fold at 30 °C and 55 °C, respectively ([Dos Santos et al., 2004a](#)). In biological systems, there will be limits to the temperature range of individual cultures and enzyme systems, as has been demonstrated for non-mediated azo dye reduction by several pure cultures ([Chang and Kuo, 2000](#); [Chang et al., 2001](#); [Yu et al., 2001](#); [Pearce et al., 2006](#); [Angelova et al., 2008](#)) but not for mediated azo dye reduction. Mixed cultures such as sludge will undergo population shifts when the temperature is changed. It must be taken into account, therefore, that the results of the studies by [Dos Santos et al. \(2005c\)](#) on azo dye reduction by mesophilic and thermophilic sludge may at least partly reflect changes of biomass properties.

Most of the results from these studies show the Arrhenius trends, i.e. higher basic azo dye reduction rates and a relatively smaller impact of mediators with thermophilic sludge as compared to with mesophilic sludge. For example, the first-order reaction rate constant of non-mediated reduction of hydrolyzed Reactive Red 2 (0.3 mM) was 5.6-fold higher with thermophilic sludge (55 °C) than with mesophilic sludge (30 °C), and AQS (12 μM) increased these rate constants 3.8-fold and 2.3-fold at 30 °C and 55 °C, respectively ([Dos Santos et al., 2005c](#)). Comparison of these results with the ones cited above for the reaction with sulfide, shows a general feature of sludge vs. sulfide, i.e. a much stronger effect of temperature on non-mediated azo dye reduction by sludge than by sulfide. This phenomenon may be the result of differences in the microbial composition between mesophilic and thermophilic sludge. Alternatively, it may also reflect a temperature-dependent change of the activation energy, similar to that observed for non-mediated azo dye reduction by two distinct pure cultures ([Angelova et al., 2008](#)). In contrast, explanations based on the release of intracellular mediators as a result of higher cell decay at higher temperatures are probably not valid: it would imply that, due to the higher pool of mediators, the impact of additional mediators is lower with sludge than with sulfide, something which is obviously not the case. Some of the results from the studies by [Dos Santos](#) and co-workers are not in agreement with the Arrhenius trends. The anomalous decrease of the rate of both mediated and non-mediated azo dye reduction observed at temperatures beyond the optimum temperature of 60 °C ([Dos Santos et al., 2003](#)) probably reflects the temperature optimum of the sludge inoculum. In contrast, there is no clear explanation for the lower relative impact of AQDS on the reduction of hydrolyzed Reactive Red 2 at 45 °C than at 55 and 60 °C ([Dos Santos et al., 2003](#)), and for the higher relative impact of riboflavin on the reduction of Reactive Orange 14 at 55 °C than at 30 °C ([Dos Santos et al., 2005b](#)).

## 2.5. Electron donors

RM have been shown to accelerate or even enable azo dye reduction by microorganisms from many distinct taxonomic and trophic groups. The primary electron donor of biological azo dye

reduction may therefore play a role in determining azo dye reduction rates: in individual cultures by changing enzyme activities, and in mixed cultures by stimulating specific (trophic groups of) microorganisms. The substrate type may thus affect both the rates of direct (non-mediated) azo dye reduction and those of mediator reduction, albeit not necessarily to the same extent.

Four of the studies listed in [Table 4](#), all of them using anaerobic granular sludge, compared the effects of different substrates on mediated and non-mediated azo dye reduction ([Van der Zee et al., 2001](#); [Dos Santos et al., 2003](#); [Dos Santos et al., 2005b](#); [Dos Santos et al., 2006](#)). In one of these studies, evidence was provided for a substrate-selective effect on the reduction of a mediator: It was observed that acetate stimulated the AQDS-mediated reduction of Reactive Red 2 by AQDS-adapted sludge much more than propionate, butyrate or hydrogen. In contrast, acetate neither stimulated non-mediated dye reduction, nor did it have a pronounced effect on mediated azo dye reduction by non-adapted adapted sludge. Therefore, these results suggest that enrichment of bacteria or enzyme systems that coupled AQDS-reduction to oxidation of acetate had taken place ([Van der Zee et al., 2001](#)). The other substrate effect studies were all performed with consortia not previously adapted to the mediator. Azo dye reduction rates were different with each particular substrate, and mediators always increased these rates, albeit to different extents. The values mentioned in the 'Results' column in [Table 3](#) express, for each particular substrate investigated, the impact of the mediator (riboflavin or AQDS) on the rate of azo dye reduction, and should therefore mostly be interpreted as the effect of this substrate on mediator reduction. In some cases, a positive correlation between these values and the basic non-mediated rates has been observed. For example, both the non-mediated rates of azo dye reduction by thermophilic sludge and the relative impact of AQDS increased along the substrate range volatile fatty acids (VFA)–Glucose+VFA–Glucose–H<sub>2</sub> ([Dos Santos et al., 2003](#)), and a similar correlation was found for azo dye reduction by thermophilic sludge and the relative impact of riboflavin with Endogenous substrate–Acetate–Formate–H<sub>2</sub> ([Dos Santos et al., 2006](#)). In other cases, however, the observed correlation was negative. For example, along the substrate range acetate–methanol–formate, the non-mediated azo dye reduction rates by mesophilic sludge increased but the relative impact of riboflavin decreased ([Dos Santos et al., 2006](#)). In addition, contradicting results have been reported for the effect of hydrogen on mediated vs. non-mediated Reactive Red 2 reduction by unadapted mesophilic sludge: whereas both studies reported that hydrogen was the substrate with the highest dye reduction rates, the high relative impact of riboflavin observed by [Dos Santos et al. \(2005a\)](#) contrasts sharply to the rather small relative impact of AQDS observed by [Van der Zee et al. \(2001\)](#). From these conflicting trends it is impossible to draw conclusions with respect to substrate preferences for mediator reduction. It is obvious, however, that the overall azo dye reduction process (as well as non-mediated azo dye reduction) is much more associated with hydrogen and glucose oxidation than with the oxidation of VFA.

## 2.6. Trophic groups and selective inhibitors

The higher azo dye reduction rates with glucose as compared to with VFA (as discussed in the previous sub-section) suggest that fermentative bacteria contribute more to azo dye reduction than acetogens and methanogens. More information about the involvement of fermentative and methanogenic bacteria was obtained in experiments to the effect of selective inhibitors on azo dye reduction by anaerobic granular sludge in the presence of different electron donors ([Dos Santos et al., 2005b](#); [Dos Santos et al., 2006](#)). Vancomycin, a selective inhibitor of Gram-positive bacteria, was shown to suppress both non-mediated and mediated azo dye reduction: it decreased the reduction rates of Reactive Red 2 and Reactive Orange 14 by both mesophilic and thermophilic anaerobic sludge with 50–90% and 30–55%, with glucose and methanol as the electron donors, respectively, regardless of the presence or absence of riboflavin. As

most fermentatives are Gram-positive bacteria, these observations suggest that their involvement in azo dye reduction is considerable.

In contrast to vancomycin, 2-Bromoethanesulfonate (BES), the selective inhibitor of methanogens, usually did not have much effect on azo dye reduction by granular sludge. The only exception was a considerable increase of the non-mediated reduction rates of Reactive Red 2 and Reactive Orange 14 by thermophilic sludge with hydrogen as the electron donor. In addition, BES also stimulated, albeit weakly, the non-mediated reduction of Reactive Red 2 in some other situations, i.e. with thermophilic sludge and glucose or formate, as well with mesophilic sludge and hydrogen. These observations point at substrate competition between methanogenesis and dye reduction, indicating that non-mediated azo dye reduction was to some extent linked to oxidation of hydrogen and formate (directly), or to fermentation of glucose (indirectly, by production of methanogenic substrates). Interestingly, BES did not raise any of the azo dye reduction rates in the presence of the mediator riboflavin. On the contrary, its effect, although insignificant, was always negative (Dos Santos et al., 2005b; Dos Santos et al., 2006). This suggestion of a small contribution of methanogens to riboflavin reduction was confirmed by the results of pure culture assays with hydrogenotrophic methanogens. All of the three strains used in these assays, the thermophiles *Methanothermobacter thermoautotrophicus* ΔH and a *Methanothermobacter*-related strain NJ1 and the mesophile *Methanosarcina barkeri*, were found capable of riboflavin-mediated azo dye reduction. In contrast, only the mesophilic strain could reduce the azo dye Reactive Red 2 in the absence of riboflavin (Dos Santos et al., 2006). The combined results of these sludge and pure culture studies indicate that the contribution of methanogens to azo dye reduction by anaerobic sludge is ambiguous but generally limited.

### 3. Role of redox mediators on reductive dechlorinations

#### 3.1. Introduction

Polychlorinated compounds (PCC) constitute an important group of contaminants widely spread in wastewaters, groundwater, soils and off-gasses, due to improper handling, leakage, etc. Due to their potential hazard for the environment and public health, many PCC are listed as priority pollutants by the USA environmental protection agency (EPA). PCC are commonly used as degreasing agents and solvents in the metal-industry, as cooling fluids in refrigerators, as pesticides, fungicides or insecticides, or as dielectric fluids or flame retardants (Van Eckert, 1999).

The role of RM, with different structures and properties, on the reductive dechlorination of different PCC has been tested during the last two decades. Tables 6 and 7 summarize the impacts of these RM on reductive dechlorination processes. The impact of RM on dechlorinating reactions varies among the different reaction systems evaluated. Some RM increase the rate and extent of dechlorination of distinct PCC by several orders of magnitude, under different experimental conditions. However, several dechlorinating reactions were hardly affected or were even inhibited when different RM were incorporated. A number of variables are at stake determining the impact of RM in different dechlorinating reactions, which will be discussed in the following sections.

#### 3.2. Main factors determining the input of RM in reductive dechlorinations

##### 3.2.1. pH

A few studies report on the effect of pH on reductive dechlorination processes in the presence of different RM. For instance, abiotic incubations for the reductive dechlorination of hexachloroethane (HCA) by sulfide (0.25 mM), in the presence of soil humic acids (HA, 25 mg C/L), showed a direct relationship between the pseudo-first-order rate constant ( $k$ ) for the reduction of HCA and the pH (Curtis and Reinhard, 1994). The  $k$  value increased up to ~5-fold by varying the pH

from 7.2 to 8.0 in the assays. The first-order dependence of the reaction on pH was attributed to deprotonation of the redox active groups in HA (e.g. quinones), which was previously proposed during the reduction of methyl parathion by reduced quinones at different pH values (Tratnyek and Macalady, 1989, see below).

The last hypothesis was corroborated by further experiments indicating that the  $k$  value for HCA reduction by anthrahydroquinone-2,6-disulfonate (AH<sub>2</sub>QDS) strongly depends on the pH. Curtis and Reinhard (1994) postulated that the overall rate expression for HCA reduction was considered to be the sum of three independent, parallel, first-order reactions:

$$k'_{\text{HCA}} = k_0[\text{A}(\text{OH})_2] + k_1[\text{A}(\text{OH})\text{O}^-] + k_2[\text{A}(\text{O})_2^{2-}]$$

Where  $[\text{A}(\text{OH})_2]$ ,  $[\text{A}(\text{OH})\text{O}^-]$ , and  $[\text{A}(\text{O})_2^{2-}]$  represent the concentrations of the fully protonated, the monophenolate, and the diphenolate forms of AH<sub>2</sub>QDS and  $k_0$ ,  $k_1$ , and  $k_2$  are the corresponding rate constants. It was observed that  $k_2$  was ~8-fold higher 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.

Similar results occurred during the reductive dechlorination of HCA and carbon tetrachloride (CT) by cysteine (5 mM) in the presence of iron porphyrin (30 μM) at different pH values. The second-order rate constants for the reductive dechlorination of HCA and CT increased up to 4- and 5-fold, respectively, by raising the pH from 6.0 to 9.3 (Perlinger et al., 1998).

Recent experiments suggest that different reversible functional groups are involved in redox reactions catalyzed by humic substances depending on the pH prevailing on the reaction system. Evidence was provided that non-quinoid (NQ) structures in humus have electron-carrying capacity (ECC) at pH 6.5; whereas different quinoid (Q<sub>1</sub> and Q<sub>2</sub>) structures showed ECC at pH 8 (Ratasuk and Nanny, 2007). Q<sub>1</sub> was defined as quinone moieties with electron-withdrawing groups adjacent to Q<sub>1</sub>, while Q<sub>2</sub> contains either no substituents near the quinone or have nearby electron-donating groups with additional substituents hindering hydrogenolysis through steric interactions. NQ sites accounted for 21–56% of ECC in different humic materials; whereas Q<sub>1</sub> and Q<sub>2</sub> accounted for 13–58% and 8–50% of the total ECC, respectively (Ratasuk and Nanny, 2007).

##### 3.2.2. Temperature

As described above (azo dyes section), reaction rates will enhance with increasing temperatures because a higher fraction of the molecules will have a kinetic energy higher than the activation energy. This was evident during the reductive dechlorination of HCA by sulfide (1 mM), in the presence and in the absence of juglone, at different incubation temperatures (Perlinger et al., 1996). The reduction rate of HCA by sulfide increased ~15-fold by raising the temperature from 25 °C to 65 °C in non-mediated reactions; whereas the rate of juglone-mediated reduction increased ~10-fold. The inferior effect of temperature on the reduction rate of HCA observed in juglone-mediated systems, compared to controls lacking juglone, could be attributed to differences in the apparent activation energy ( $E_a$ ) required in the reactions. In agreement with this observation, a decrease in the  $E_a$  was determined when dechlorinating reactions were conducted with different concentrations of juglone. Certainly, the  $E_a$  obtained in non-mediated reactions was 75 ± 5 kJ/mol; whereas in the presence of 20 μM juglone,  $E_a$  decreased to 52 ± 5 kJ/mol, and in the presence of 200 μM juglone,  $E_a$  was 46 ± 7 kJ/mol (Perlinger et al., 1996). A lower  $E_a$  in mediated reactions implies that the presence of a RM facilitate the reactants to achieve their reactive state, thus accelerating the transfer of electrons.

Comparison of the reduction rates of HCA by AH<sub>2</sub>QDS, achieved at 25 °C (Kappler and Haderlein, 2003) and at 50 °C (Curtis and Reinhard, 1994), in both cases conducted at pH 8.2 and a AH<sub>2</sub>QDS/HCA-ratio of 200, also agrees with the Arrhenius equation. Indeed, the  $k$  value

**Table 6**  
Impact of redox mediators on reductive dechlorination reactions under abiotic conditions

Reaction	Electron donor/system	Mediator	Ratio pollutant/mediator	Results	References
C <sub>2</sub> Cl <sub>6</sub> → C <sub>2</sub> Cl <sub>4</sub>	Sulphide or Fe (II)	HEM, BOM or HA	Molar ratio C <sub>2</sub> Cl <sub>6</sub> /HEM of 0.025; 0.1 μmol C <sub>2</sub> Cl <sub>6</sub> /mg C-HA; 0.125 μmol C <sub>2</sub> Cl <sub>6</sub> /mg BOM	7- to 45-fold higher first-order reduction rates in RM amended incubations compared to controls	Curtis and Reinhard (1994)
CCl <sub>4</sub> → CHCl <sub>3</sub>	Sulphide or Fe (II)	HEM or HA	Molar ratio CCl <sub>4</sub> /HEM of 0.025; 0.1 μmol CCl <sub>4</sub> /mg C-HA	2- to 7-fold and 6- to 18-fold higher first-order reduction rates in RM amended incubations compared to controls in Fe(II) and sulphide supplemented incubations, respectively	Curtis and Reinhard (1994)
CHBr <sub>3</sub> → CH <sub>2</sub> Br <sub>2</sub>	Sulphide or Fe (II)	HEM or HA	Molar ratio CHBr <sub>3</sub> /HEM of 0.025; 0.1 μmol CHBr <sub>3</sub> /mg C-HA	No significant impact of RM with Fe(II) as electron donor. Hematin stimulated the reduction of CHBr <sub>3</sub> by sulphide, which was not reduced in the absence of RM	Curtis and Reinhard (1994)
C <sub>2</sub> Cl <sub>6</sub> → C <sub>2</sub> Cl <sub>4</sub>	Sulphide	JUG	Molar ratio of 4 × 10 <sup>-3</sup>	10-fold higher first-order reduction rates in JUG-amended incubations compared to controls	Perlinger et al. (1996)
TCE → VC, 1,1-DCE + 1,2-DCE, acetylene, ethylene, ethane	ZVI	NOM	0.625 to 1.25 μmol TCE/mg NOM	Decrease by 21 to 39% on the first-order reduction rates by increasing the amount of NOM	Tratnyek et al. (2001)
CCl <sub>4</sub> → UP	ZVI	AQDS, JUG, FA or HA	Molar ratio of 0.021 to 2.1 and 0.017 to 1.7 for AQDS and JUG, respectively; 4.25 to 17 μmol CCl <sub>4</sub> /mg FA and 17 μmol CCl <sub>4</sub> /mg HA	2- and 3-fold higher first-order reduction rates in JUG and AQDS amended incubations, respectively, compared to controls. Decrease by 35% and 50% on the first-order reduction rates by increasing the amount of FA and HA, respectively.	Tratnyek et al. (2001)
C <sub>2</sub> Cl <sub>4</sub> → ethylene and ethane	Titanium (III) citrate	Solubilized MPP (with cosolvent)	Molar ratio of 30	Complete C <sub>2</sub> Cl <sub>4</sub> conversion in 24 h for RM-amended cultures. No conversion of C <sub>2</sub> Cl <sub>4</sub> in the absence of RM	Dror and Schlautman (2004)
C <sub>2</sub> Cl <sub>4</sub> → ethylene and ethane	Pd/ZVI <sup>a</sup>	HA <sup>b</sup> , AQDS, LAW or HQ	0.075 to 0.2 μmol C <sub>2</sub> Cl <sub>4</sub> /mg HA. For the model RM the molar ratio was 0.012	2- to 3-fold higher first-order reduction rates in model RM amended incubations compared to controls. HA decreased dechlorination rates compared to controls	Doong and Lai (2005)
CCl <sub>4</sub> → CHCl <sub>3</sub> + UP	Sulphide or cysteine	AQDS, BQ, JUG, NQ, LAW or MQ	Molar ratio of 0.02	4- to 5-fold higher first-order reduction rates in LAW and AQDS amended incubations, compared to controls. For the rest of the RM the enhancement on the first-order reduction rates varied from 20- up to 71-fold	Doong and Chiang (2005)
TCE → ethane + ethane + VC + cis-DCE	Solid-state electrode polarized at -500 mV	MV	Molar ratio of 1	Electrochemical reduction of MV promoted the dechlorination of TCE, which did not occur when MV was omitted on the experiments	Aulenta et al. (2007)

HEM, hematin; BOM, boreal organic matter; HA, humic acids; JUG, juglone, NOM, natural organic matter; AQDS, anthraquinone-2,6-disulfonate; FA, fulvic acids; MPP, metalloporphyrins; LAW, lawson; HQ, hydroquinone; BQ, benzoquinone; NQ, naphthoquinone; MQ, menadione; MV, methyl viologen; TCE, trichloroethylene; VC, vinyl chloride; DCE, dichloroethylene; UP, unidentified products; RM, redox mediator.

<sup>a</sup> Palladized Zero Valent Iron.

<sup>b</sup> C<sub>2</sub>Cl<sub>4</sub> added after 24-h equilibrium between pd/ZVI and HA.

obtained for the reduction of HCA was ~3-fold higher at 50 °C (Curtis and Reinhard, 1994) compared to the reduction rate obtained at 25 °C (Kappler and Haderlein, 2003).

### 3.2.3. Redox potential

As discussed above (azo dyes section), the  $E_0'$  of a RM should ideally be in between those of the two eventual half reactions at stake,

**Table 7**  
Impact of redox mediators on the microbial reduction of polychlorinated pollutants

Reaction	Electron donor/system	Mediator	Ratio pollutant/mediator	Results	References
CCl <sub>4</sub> → CHCl <sub>3</sub> + CO	Lactate <i>Shewanella alga</i> strain BrY	CNB12	Molar ratio of 0.13	No conversion of CT by <i>Shewanella alga</i> strain BrY in the absence CNB12; whereas 92% of CT conversion to CO within 3 weeks of incubation	Workman et al. (1997)
Octa-CDD → Tetra-CDD	Contaminated sediment without EED	CAT, RES or 3,4-DHBA	Molar ratio of 0.11	4- to 6-fold higher conversion in RM amended cultures compared to control	Barkovskii and Adriaens (1998)
CCl <sub>4</sub> → CHCl <sub>3</sub> + UP	Lactate <i>Shewanella putrefaciens</i> 200	NOM in soil	~ 10 × 10 <sup>-3</sup> μmol CCl <sub>4</sub> /mg NOM	29% of added CT (19.5 μM) converted after 33 h in the absence of NOM; whereas 64% of CCl <sub>4</sub> converted after 18 h in the presence of NOM	Collins and Picardal (1999)
CCl <sub>4</sub> → CHCl <sub>3</sub> + CO <sub>2</sub> + CS <sub>2</sub>	Fructose <i>Acetobacterium woodii</i>	HOB12	Molar ratio of 47	30-fold faster conversion rate in HOB12-amended cultures compared to non-mediated incubations. Addition of HOB12 also increased the extent of mineralization (up to 31% measured as <sup>14</sup> CO <sub>2</sub> )	Hashsham and Freedman (1999)
CCl <sub>4</sub> → CHCl <sub>3</sub> + CH <sub>2</sub> Cl <sub>2</sub> + PCE + UP	Anaerobic sludge or <i>Geobacter</i> sp. provided with different electron donors	AQDS or HA	Molar ratio CCl <sub>4</sub> /AQDS of 2 to 5; 0.05 μmol CCl <sub>4</sub> /mg HA	6-fold higher first-order reduction rates in AQDS amended sludge incubations compared to controls. AQDS and HA stimulated the reduction of CCl <sub>4</sub> by <i>Geobacter</i> sp., which did not convert CCl <sub>4</sub> in the absence of RM	Cervantes et al. (2004)
CCl <sub>4</sub> → CHCl <sub>3</sub> + Cl <sup>-</sup> + UP	Anaerobic sludge provided with VFA	AQDS, RF, CNB12, HOB12	Molar ratio of 10	3.8-, 4.0, 13.3- and 13.6-fold higher first-order reduction rates in AQDS, RF, CNB12 and HOB12 amended cultures, respectively, compared to controls	Guerrero-Barajas and Field (2005)

HA, humic acids; AQDS, anthraquinone-2,6-disulfonate; CAT, catechol; RES, resorcinol; 3,4-DHBA, 3,4-dihydroxybenzoic acid; RF, riboflavin; CNB12, cyanocobalamin; HOB12, hydroxycobalamin; UP, unidentified products; CDD, chlorinated dibenzo-*p*-dioxins, RM, redox mediator; VFA, volatile fatty acids; EED, external electron donor.

the reduction of an electron-withdrawing pollutant and the oxidation of a primary electron donor.

There are several reports indicating the relationship between the  $E_0'$  of the RM and the dechlorinating rate observed for different PCC. During the reductive dechlorination of CT by ZVI, for instance, AQDS increased the reduction rate of CT at a higher extent (up to 3-fold higher than non-mediated control) compared to the catalytic effects of juglone (up to 2.2-fold higher than non-mediated control) (Tratnyek et al., 2001). The last correlation is expected as AQDS has a more negative  $E_0'$  compared to juglone. Comparable results were reported during the reductive dechlorination of perchloroethylene (PCE) by ZVI in the presence of different quinoid RM (Doong and Lai, 2005). Hydroquinone, with the lowest  $E_0'$ , promoted the maximum reduction rates of PCE by ZVI; whereas *p*-benzoquinone, with the highest  $E_0'$ , caused the lowest reduction rates for PCE.

Biological dechlorination rates catalyzed by distinct RM also showed a high correlation between the  $E_0'$  of the RM and the reductive dechlorinating rates. The last scenario occurred when AQDS, riboflavin (RF), and cyanocobalamin (CNB12) were tested as RM during the anaerobic reduction of CT by anaerobic sludge (Guerrero-Barajas and Field, 2005). The observed development of reduction rates of CT was consistent with a decreasing trend of  $E_0'$  of the RM tested. CNB12, which has the most negative  $E_0'$  (–530 mV) enhanced 13.3-fold the reduction rate of CT, compared to the non-mediated control; whereas RF ( $E_0'$  of –208 mV) and AQDS ( $E_0'$  of –184 mV) increased the reduction rate of CT by 4- and 3.7-fold, respectively (Guerrero-Barajas and Field, 2005).

Despite all these lines of evidence, it is important to emphasize that a change in  $E_0'$  of the RM does not always reflect a direct correlation with dechlorinating kinetics. For example, the reduction rate of CT by sulfide, in the presence of different quinoid RM, was evaluated as a function of the  $E_0'$  of the quinones tested (Doong and Chiang, 2005). Juglone (JQ), naphthoquinone (NQ) and benzoquinone (BQ) were found to be more effective electron-transfer mediator than AQDS during the reductive transformation of CT by sulfide. The reduction rates observed in the presence of JQ and NQ were 10–14 higher than those obtained with AQDS, despite that the driving force for CT transformation could be expected to be greater for AQDS than that for NQ based on the  $E_0'$  values. Moreover, JQ, NQ and BQ increased the reduction rate of CT a greater extent compared to the catalytic effects of AQDS and menadione (MQ), which also contrasts with the previously reported correlation between the  $E_0'$  values and reductive transformation rates (Tratnyek et al., 2001; Rau et al., 2002; Doong and Lai, 2005; Guerrero-Barajas and Field, 2005). The last results mean that the  $E_0'$  of quinoid RM may not be the only factor that controls the reduction rate of PCC, and that the chemical structures may play an important role in determining the reactivity of quinoid compounds (Doong and Chiang, 2005).

The reduction rate of an electron-withdrawing pollutant by a reduced RM would theoretically increase with decreasing  $E_0'$  of the RM; but the reduction rate of the RM by sulfide will decrease with decreasing  $E_0'$ . The last observation may explain the lower catalytic input of AQDS on CT dechlorination, compared to the catalysis observed with BQ and JQ (Doong and Chiang, 2005). Nevertheless, according to the same hypothesis, dechlorination of CT would proceed faster by previously reduced AQDS than by reduced BQ or JQ.

### 3.2.4. Reactive species involved in reductive dechlorination reactions

Different reactive species including semiquinone, hydroquinone, and mercaptoquinone have been shown to successfully transfer electrons from bulk reductants to electron-withdrawing organic pollutants (Schwarzenbach et al., 1990; Curtis and Reinhard, 1994; Perlinger et al., 1996). The mechanism of formation of reactive species during reductive transformation processes has been linked to the characteristic redox cycling properties of the quinone compounds (Doong and Chiang, 2005).

Experimental evidence suggests that the concentration of semiquinone radicals, produced during reductive transformations, controls the rate of conversion of organic pollutants (Scott et al., 1998; Rau et al., 2002). Further research indicates that the amounts of deprotonated hydroquinone and semiquinone radicals are correlated to the degradation efficiency of priority pollutants in AQDS- and HA-amended incubations under reducing conditions (Tratnyek and Macalady, 1989; Curtis and Reinhard, 1994; Scott et al., 1998). Monophenolate (Schwarzenbach et al., 1990) and mercaptojuglone (Perlinger et al., 1996) have also been identified as the dominant reactive species during reductive transformations mediated by JQ in the presence of sulfide as electron donor. However, the rate of degradation of HCA was more specifically linked to the production of mercaptojuglone in its hydroquinone or semiquinone form at different pH values (Perlinger et al., 2002). A preliminary report elucidated the mechanism by which JQ might decrease the  $E_a$  in dechlorination reaction with sulfide as electron donor. Mercaptojuglone is the reactive species readily formed in incubation assays including both sulfide and JQ (Perlinger et al., 1998). Mercaptojuglone enhanced the reductive dechlorination of different PCC at a higher extent than that expected by the input of reduced JQ alone (Perlinger et al., 1998).

More recently, results derived from electron paramagnetic resonance, UV–vis spectroscopy and electrospray ionization mass spectroscopy of different quinoid RM, clearly showed that three groups of quinones can be categorized based on the formation of the active electron-transfer mediators involved in the reductive transformation of CT (Doong and Chiang, 2005). JQ, BQ and NQ produced mercaptoquinones as reactive species, which enhanced up to 71-fold the reduction rate of CT, compared to the control lacking quinones. In contrast, MQ and LQ, which were reduced to their corresponding hydroquinone by sulfide, promoted a much lower enhancement on the reduction rate of CT. Moreover, reduction of AQDS by sulfide produced semiquinone radicals, which moderately increased the reduction rate of CT. Therefore, the catalytic effects of different quinoid RM strongly depends on the type of reactive species produced during reductive transformation processes. Mercaptoquinone is a more efficient reactive species than either semiquinone radicals or hydroquinone for transferring electrons in a reducing environment containing thiol reductants (Doong and Chiang, 2005). The last observations could explain the remarkably higher dechlorination rate of CT promoted by LQ, compared to reactions mediated by AQDS, despite the lower  $E_0'$  of the former RM (Doong and Chiang, 2005).

### 3.2.5. Variety of electron donors and natural sources of RM

Two half-reactions are at stake during reductive dechlorinations mediated by RM: 1) reduction of redox active groups by a proper electron donor; and 2) subsequent reductive dechlorination of PCC by reduced RM (Fig. 1). Therefore, the overall reaction rate depends on the capacity of the external electron donor to transfer electrons to the RM and on the ability of the reduced RM to channel the electrons to PCC. According to the experimental data available in the literature, the reduction of different RM, by chemical and biological processes, proceeds generally faster than the subsequent transfer of electrons from the reduced RM to PCC. For instance, the reduction of AQDS and HA by sulfide occurs within a few hours (Dunnivant et al., 1992; Cervantes, unpublished results), whereas reductive dechlorinations of different PCC by reduced AQDS or HA demand several days of incubation (Curtis and Reinhard, 1994; Kappler and Haderlein, 2003; Cervantes et al., 2004). A wide variety of microorganisms, including iron-reducing, sulfate-reducing, halorespiring, fermentative and methanogenic microorganisms, are also capable of rapidly reducing HA or quinones, which could then be available to promote reductive dechlorinations in anaerobic environments (Field and Cervantes, 2005). Ferrous iron is another reductant commonly found in anaerobic environments, but its reducing capacity is minor compared to sulfide,

which is reflected by the lower dechlorination rates achieved with the former (Curtis and Reinhard, 1994).

For biologically mediated dechlorinations, glucose, formate and hydrogen generally promote higher dechlorination rates, compared to those obtained with methanol and acetate, which agrees with the faster quinone-reducing activities observed with the former substrates (Cervantes et al., 2000a,b; 2004; Guerrero-Barajas and Field, 2005).

Many different model RM, such as quinoid- and flavin-based compounds, have a higher solubility compared to natural sources of RM (e.g. humic materials), which may also imply different catalytic inputs during reductive dechlorinations. It is conceivable that RM with a higher solubility could be more easily reduced, either chemically or biologically, compared to RM remaining suspended on the reaction system, which in turn would promote faster dechlorination rates. The last observation explains the higher dechlorination rates generally achieved with soluble model RM than with suspended NOM (Schwarzenbach et al., 1990; Dunnivant et al., 1992; Tratnyek et al., 2001). Furthermore, suspended NOM could even inhibit dechlorination reactions when insoluble electron donors participate in dechlorination reactions. For example, the presence of suspended NOM in dechlorination assays, with ZVI as an electron donor, decreased the rate and extent of dechlorination of trichloroethylene (TCE) and PCE by competitive sorption onto reactive sites of ZVI surface (Tratnyek et al., 2001; Doong and Lai, 2005). In contrast, different soluble RM, such as AQDS and LQ, increased the rate and extent of dechlorination of different PCC by ZVI (Tratnyek et al., 2001; Doong and Lai, 2005).

The content of redox active groups in NOM also plays a role on determining the catalytic input of RM in dechlorination reactions. For instance, the reductive transformation of CT by *Shewanella putrefaciens* 200 was enhanced to a greater extent by HA compared to CT dechlorination mediated by fulvic acids (FA) (Collins and Picardal, 1999). A larger content of quinones has been reported in HA compared to FA (Stevenson, 1994), which may explain the last phenomenon. Nevertheless, despite the inherent variations in concentration,

accessibility, and reactivity of redox-active groups in HA originated from a variety of environments, the capacity of electrochemically reduced HA to achieve the abiotic dechlorination of PCC was fairly constant (Kappler and Haderlein, 2003).

#### 4. Role of redox mediators on the reduction of nitroaromatic pollutants

##### 4.1. Introduction

Mono- and polynitroaromatic compounds (PNACs) are widely used as agrochemicals, explosives, textile dyes, and as chemical intermediates for different manufacturing sectors. Due to their widespread use and improper disposal, contamination of soils, sediments and groundwater by PNACs is a ubiquitous problem. Several reports have confirmed the toxic and adverse effects of PNACs on biological ecosystems and on human health. Consequently, the restoration of PNACs-contaminated sites is a matter of concern among environmental protection groups (Hofstetter et al., 1999).

Tables 8 and 9 recapitulate the experimental data obtained during the reductive (bio)transformation of PNACs mediated by different RM. Most reports deal with the abiotic transformation of PNACs illustrating the catalytic effects of model RM or NOM from different origins (Table 8). Recently, reductive biotransformation of PNACs by distinct microorganisms has also been reported (Table 9). In this section the input of different RM on the reductive (bio)transformation of PNACs will be discussed, as well as the different aspects governing the catalytic reactions.

##### 4.2. Main factors determining the input of RM in the reduction of nitroaromatic pollutants

###### 4.2.1. pH

Several studies have documented the effect of pH during reductive transformation of PNACs mediated by different redox reactive groups.

**Table 8**  
Impact of redox mediators on reduction of nitroaromatic compounds under abiotic conditions

Reaction	Electron donor/system	Mediator	Ratio pollutant/mediator	Results	References
Various nitroaromatics <sup>a</sup> → Anilines	Sulfide or cysteine	JUG, LAW or IPP	Molar ratio of 0.4 to 50	Second-order rate constants for the reduction of nitroaromatics between $3.0 \times 10^{-3}$ and $3.3 \times 10^2 \text{ M}^{-1} \text{ s}^{-1}$ in the presence of RM, whereas no significant reduction occurred in the absence of RM	Schwarzenbach et al. (1990)
Various nitroaromatics <sup>b</sup> → Anilines	Sulfide	NOM from different origins	1.21 $\mu\text{mol NA/mg C-NOM}$	Second-order rate constants for the reduction of nitroaromatics between $3.0 \times 10^{-6}$ and $3.3 \times 10^{-2} \text{ h}^{-1} (\text{mg C-NOM/L})^{-1}$ in the presence of NOM, whereas no significant reduction occurred in the absence of NOM at pH of 7.2	Dunnivant et al. (1992)
Various 2,6-DNAH <sup>c</sup> → corresponding anilines	ZVI	HA or dichlone	0.5 $\mu\text{mol 2,6-DNAH/mg HA}$	HA amendments decreased the first-order rate constant of reduction of 2,6-DNAH for 1.2–10 fold compared to controls lacking HA. Dichlone amendments counteracted the negative effects of HA achieving similar reduction rates to the control lacking RM	Keum and Li (2004)
PCNB → PCA	Fe(II)	DOM from different origins	0.06 to 0.1 $\mu\text{mol PCNB/mg C-DOC}$	First-order rate constants of reduction of PCNB up to ~8-fold higher in DOC-amended assays compared to controls without DOC. Unfiltered NOM inhibited the reductive process	Hakala et al. (2007)
Various nitroaromatics <sup>d</sup> → Anilines	Sulfide	JUG	NA	Second-order rate constants for the reduction of nitroaromatics between $2.3 \times 10^{-3}$ and $6.9 \times 10^3 \text{ M}^{-1} \text{ s}^{-1}$ in the presence of JUG, whereas no significant reduction occurred in the absence of JUG	Hofstetter et al. (1999)
NB → Aniline	ZVI	FA, AQDS or LAW	12.5 to 50 $\mu\text{mol NB/mg FA}$ ; molar ratio of 2.5 and 4.4 for AQDS and LAW amended incubations, respectively	Up to 20% decrease on first-order rate constants of reduction of NB in FA-amended assays; whereas a 1.2- and 1.52-fold increase was observed in LAW- and AQDS-amended assays	Tratnyek et al. (2001)

<sup>a</sup> Including NB, 2-, 3- and 4-methyl-NB, 2-, 3- and 4-chloro-NB, 2-, 3- and 4-acetyl-NB.

<sup>b</sup> Including the same nitroaromatics considered by Schwarzenbach et al. (1990).

<sup>c</sup> Including benfluralin, ethalfuralin, nitratin, oryzalin, pendimethalin and trifluralin.

<sup>d</sup> Including TNT, 2-amino-4,6-DNT, 4-amino-2,6-DNT, 2,4-diamino-6-NB, 2,6-diamino-4-NB, 4-NT, 4-chloro-NB, 4-acetyl-NB, 1,3-DNB, 1,4-DNB, 2-NA, and 3-NA. NB, nitrobenzene; TNT, tri-nitro-toluene; DNT, di-nitro-toluene; NT, nitro-toluene; DNB, di-nitro-benzene; NA, nitro-aniline; DNAH, di-nitro-aniline; PCNB, penta-chloro-nitro-benzene; PCA, penta-chloro-aniline; HA, humic acids; JUG, juglone; NOM, natural organic matter; DOM, dissolved organic matter; DOC, dissolved organic carbon; NA, not available; AQDS, anthraquinone-2,6-sulphonate; FA, fulvic acids; IPP, ironporphyrin; LAW, lawsone; RM, redox mediator.

**Table 9**  
Impact of redox mediators on the microbial reduction of nitroaromatic compounds

Reaction	Electron donor/system	Mediator	Ratio pollutant/mediator	Results	References
TNT → 2,4-DHANT and UP	Sucrose/ <i>Cellulomonas</i> sp. strain ES6	AQDS	Molar ratio TNT/AQDS between 0.06 and 6	Up to 3.7-fold increase in first-order rate constant of reduction of TNT in AQDS-amended assays compared to controls lacking AQDS	Borch et al. (2005)
RDX or HMX → nitrite, MDNA, nitrous oxide, ammonium, formaldehyde, formic acid and CO <sub>2</sub>	Glucose/ <i>Clostridium</i> sp. EDB2	HA or AQDS	Molar ratio RDX/AQDS of 0.075 to 1.5; molar ratio HMX/AQDS of 0.0275 to 0.55. For HA-amended assays the ratio RDX/HA and HMX/HA were 0.075 to 1.5 and 0.0275 to 0.55 μmol/g, respectively	Complete removal of RDX in 5 and 8 days in the presence of HA and AQDS, respectively, compared to 10 days required in unamended controls; 28–60% higher mineralization of RDX in HA- and AQDS-amended assays compared to controls. Similar results obtained with HMX.	Bhushan et al. (2006)
RDX → TNX	Acetate-PCIO/ <i>Geobacter metallireducens</i> strain GS-15 or <i>Geobacter sulfurreducens</i> strain PCA	HA or AQDS	Molar ratio RDX/AQDS of $8 \times 10^{-3}$ . For HA-amended assays the ratio RDX/HA was 160 μmol/g	Addition of HA or AQDS to Fe(III)-containing microbial cultures increased 5- to 66-fold the reduction rate of RDX compared to unamended controls	Kwon and Finneran (2006)
RDX → nitrite, MDNA, nitrous oxide, ammonium, formaldehyde, and CO <sub>2</sub>	Acetate/ <i>Geobacter metallireducens</i>	AQDS	NA	Addition of AQDS increased the rate and extent of mineralization of RDX by <i>Geobacter metallireducens</i>	Kwon and Finneran (2008)

TNT, tri-nitro-toluene; RDX, hexahydro-1,3,5-trinitro-1,3,5-triazine; HMX, octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine; MDNA, methylene-dinitramine; TNX, hexahydro-1,3,5-trinitroso-1,3,5-triazine; DHANT, dihydroxylamine-6-nitrotoluene; PCIO, poorly crystalline iron oxide; HA, humic acids; NA, not available; AQDS, anthraquinone-2,6-sulphonate; UP, unidentified products.

The first detailed description came after the study of [Tratnyek and Macalady \(1989\)](#), who evaluated the capacity of different reduced quinone moieties to reduce methyl parathion over the pH range between 5.5 and 9.0. Reduction of this nitroaromatic pesticide by reduced indigodisulfonate proceeded at the fastest rate at pH around 7.1, but declined rapidly on both sides of this maximum. Similar results were obtained during the reduction of methyl parathion by AH<sub>2</sub>QDS, but the maximum reduction rate occurred at a pH around 6.0. Further inspection of the reaction systems, based on  $E'_0$ -pH diagrams for both RM, revealed that the concentration of the hydroquinone with one of the hydroxyl groups dissociated (monophenolate form) is substantial at  $E'_0$ /pH conditions at which the highest reduction rate of methyl parathion occurred. Based on these observations, the authors developed a connection model between deprotonation of -OH groups of the RM and the reduction rate of methyl parathion. A high correlation between the reduction rate of methyl parathion and the calculated concentration of monophenolate species of both RM was obtained ([Tratnyek and Macalady, 1989](#)).

The same deprotonation model was used by [Schwarzenbach et al. \(1990\)](#) to describe the effect of pH on the reduction of different PNACs by sulfide or cysteine, mediated by quinoid RM. The authors proposed the following equation to calculate the reduction rate of PNACs as a function of the concentration of the monophenolate ([HQ<sup>-</sup>]) and biphenolate ([Q<sup>-2</sup>]) species of the reduced RM:

$$k_{\text{obs}} = k_{\text{HQ}^-}[\text{HQ}^-] + k_{\text{Q}^{-2}}[\text{Q}^{-2}]$$

where  $k_{\text{HQ}^-}$  and  $k_{\text{Q}^{-2}}$  are the second-order rate constant for the reaction of the nitroaromatic compound with the hydroquinone monophenolate and biphenolate, respectively. Non-dissociated hydroquinone species were negligibly reactive, as compared to the dissociated species, and therefore were not considered in the equation. Experimental data revealed that for the 2 quinoid RM evaluated (JQ and LQ), the biphenolate species was ~500-fold more reactive than the monophenolate species. Thus, pH changes, particularly at pH values above 7, have a dramatic impact on the reduction rate of PNACs.

Furthermore, a somewhat more complex pH dependence of the reaction rates was found for the reduction of nitrophenols, as these compounds are weak acids, and may thus, be present in both the non-dissociated and the dissociated forms. Evidence indicated that the non-dissociated species of several nitrophenols studied reacted ~3-fold faster with the monophenolate form of reduced LQ than their dissociated species ([Schwarzenbach et al., 1990](#)).

Similar pH dependence was also observed during the reductive transformation of 3-chloro-nitrobenzene by sulfide, mediated by NOM from different origins ([Dunnivant et al., 1992](#)), corroborating the higher reactivity of dissociated quinoid-redox groups in NOM, compared to that of non-dissociated species.

In a more recent study, the effect of pH on the rate of reduction of hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) by AH<sub>2</sub>QDS was investigated ([Kwon and Finneran, 2008](#)). As the pH increased from 6.2 to 9.2 at constant AH<sub>2</sub>QDS concentration (100 μM), the reduction rate of RDX increased 61-fold. Furthermore, the authors found that the reduction rate of RDX was very closely related to the concentration of the monophenolate hydroquinone. In addition, results obtained with cell suspensions of *Geobacter metallireducens* degrading RDX, showed a decrease on the reduction rate of this contaminant by raising the pH in the microbial incubations in the absence of AQDS. Nevertheless, in AQDS-amended cell incubations of *G. metallireducens*, the reduction rate of RDX was significantly increased, compared to the reaction rates observed without AQDS, but remained similar within the whole pH range tested (6.8, 7.9 and 9.2) ([Kwon and Finneran, 2008](#)).

#### 4.2.2. Redox potential

The reduction of several nitroaromatic pollutants under different redox conditions has been investigated. The effect of the  $E'_0$  of a model system, including the reduced form of either indigodisulfonate or AQDS, on the rate of reduction of methyl parathion was studied ([Tratnyek and Macalady, 1989](#)). Experiments were conducted at a  $E'_0$  range between -37 to -208 mV, and -90 to -140 mV, for indigodisulfonate- and AQDS-supplied incubations, respectively. The results showed that the reduction rate of the nitroaromatic pesticide increased as  $E'_0$  became more negative with both quinones evaluated. This trend observed is reasonable insofar as a low  $E'_0$  reflects a high concentration of reduced species in the reaction system, which are then available to promote the reduction of methyl parathion.

Further studies also revealed a high correlation between the  $E'_0$  of the reaction system and the rate of reduction of 3-chloro-nitrobenzene by different concentrations of sulfide in the presence of different sources of NOM ([Dunnivant et al., 1992](#)). As expected, a more negative value of  $E'_0$ , achieved by a higher concentration of sulfide, promoted a higher reduction rate of the nitroaromatic pollutant. Moreover, the authors compared the capacity of reduced NOM, obtained by bubbling hydrogen into platinum-amended solutions, with the incubations described above. The results indicated slightly lower reduction rates than those obtained with sulfide, but revealed that reduced functional

groups of NOM, independent of the reduction method employed, are capable of mediating the reduction of nitroaromatic compounds. The increased reduction rate of 3-chloro-nitrobenzene observed by increasing the  $E_0$  was attributed to the additional reduction of reactive functional groups in NOM exhibiting a more negative reduction potential.

The reduction of nitrobenzene by ZVI was explored in the presence of different RM (Tratnyek et al., 2001). The obtained results also indicate the influence of the  $E_0$  on the reaction rate. Indeed, AQDS, which has a more negative  $E_0$  (–184 mV) than LQ (–137 mV), promoted a higher reduction rate of nitrobenzene than the latter RM.

#### 4.2.3. Biodegradation pathway

Although limited studies have been conducted to elucidate the impact of RM on the reduction of PNACs by microorganisms, the literature reveals that RM not only accelerate the reduction of PNACs, but also have a remarkable effect on the biodegradation pathway (Borch et al., 2005; Bhushan et al., 2006; Kwon and Finneran, 2006; Kwon and Finneran, 2008).

The reduction rate of 2,4,6-trinitrotoluene (TNT) by *Cellulomonas* sp. strain ES6 was increased up to 3.6-fold by the addition of different concentrations of AQDS (10–1000  $\mu$ M, (Borch et al., 2005). Hydroxylaminodinitrotoluenes, 2,4-dihydroxylamino-6-nitrotoluene (2,4-DHANT), and tetranitroazoxytoluenes were the major metabolites observed in pure cultures of strain ES6 in the absence of AQDS. The presence of AQDS promoted the rapid initial formation of arylhydroxylamines, such as 2,4-DHANT, which were further transformed into several unidentified polar products. Moreover, results obtained in the presence of strain ES6, ferrihydrite and 100  $\mu$ M AQDS showed further alterations on the TNT biodegradation pathway. Increased reduction rates of 4-hydroxyaminodinitrotoluene (2-HADNT) and 4-aminodinitrotoluene (4-ADNT) to 2,4-diaminonitrotoluene (2,4-DANT) were observed (Steps 1.1, 6 and 8, Fig. 3). Only traces of 2-HADNT and 2,4-DHANT were observed, whereas 2,4-DANT accounted for up to 79% of the mass balance at the end of the incubation period (70 h). The input of AQDS on ferrihydrite-amended cultures was explained by an increased concentration of surface-associated redox active Fe(II), promoted by AQDS, which then enhanced the Fe(II)-mediated TNT reduction (Borch et al., 2005).

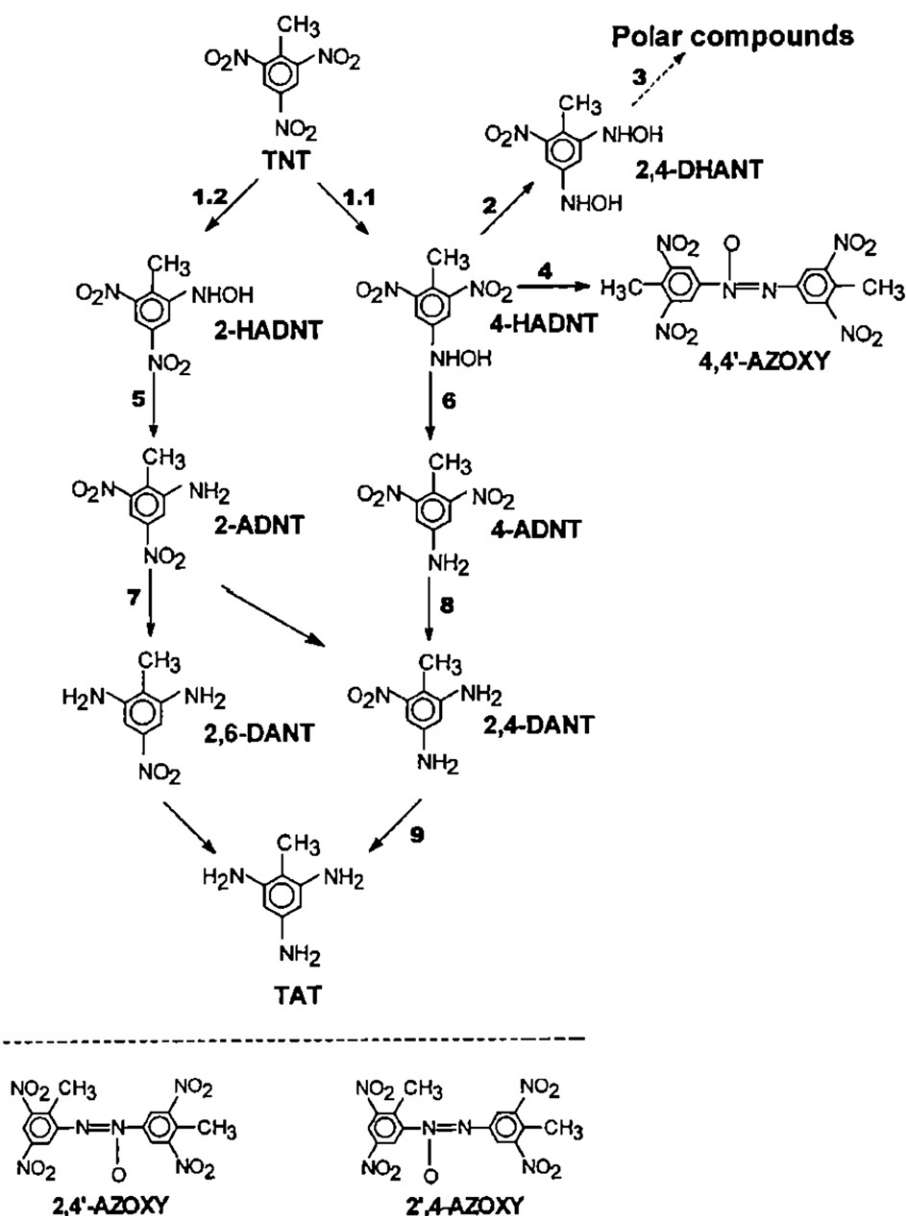


Fig. 3. Pathways and metabolites observed during the AQDS-mediated degradation of TNT by *Cellulomonas* sp. strain ES6 (after Borch et al., 2005).



The catalytic effects of HA and AQDS on the biodegradation of hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) and octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX) by *Clostridium* sp. strain EDB2 was studied (Bhushan et al., 2006). The experimental data revealed that the presence of either HA or AQDS, in cell suspensions of strain EDB2 degrading these cyclic nitramines, not only increased the biotransformation rates, but also the extent of mineralization of both pollutants. Up to 40% of uniformly labeled  $^{14}\text{C}$ -RDX (150  $\mu\text{M}$ ) was mineralized to  $^{14}\text{CO}_2$  when microbial incubations were performed in the presence of HA or AQDS; whereas only 28% of mineralization occurred in the absence of RM after 45 days of incubation. Moreover, of the initial amount of uniformly labeled  $^{14}\text{C}$ -HMX (55  $\mu\text{M}$ ) added to microbial incubations of strain EDB2, only 5% was mineralized to  $^{14}\text{CO}_2$  in the absence of RM, while 22% of mineralization was detected in both HA- and AQDS-amended cultures (Bhushan et al., 2006).

In a recent study, the impact of AQDS on the biodegradation of RDX by *G. metallireducens* was evaluated (Kwon and Finneran, 2008). In abiotic incubations including  $\text{AH}_2\text{QDS}$  as a sole electron donor, for the reduction of uniformly labeled  $^{14}\text{C}$ -RDX, the main products obtained were methylenedinitramine (MEDINA), formaldehyde, nitrous oxide, nitrite and ammonium, but no  $^{14}\text{CO}_2$  was detected. During the incubation of *G. metallireducens* in the absence of AQDS, formaldehyde was the primary carbon metabolite produced and hexahydro-1-nitroso-3,5-dinitro-1,3,5-triazine (MNX) did not accumulate. However, MEDINA was less significant compared to the level detected in abiotic conditions; whereas ammonium appeared as the main nitrogenous product. The reduction of RDX by *G. metallireducens* was significantly increased in the presence of AQDS and formaldehyde and MEDINA were concurrently produced; however, MEDINA was further metabolized leading to a greater extent of mineralization evidenced by a higher production of  $^{14}\text{CO}_2$  (Kwon and Finneran, 2008).

## 5. Role of redox mediators in miscellaneous processes

### 5.1. Introduction

The reactions dealt with so far were all reductive (bio)transformations of organic pollutants, and RM-catalysis of these reactions was based on a mostly extracellular reaction between a chemically or biologically reduced mediator with the organic electron-accepting pollutant. A similar mechanism is responsible for RM-catalysis of the reduction of vat and anthraquinone dyes (Section 5.2). RM may also play a role in the biological reduction of (bulk) inorganic electron acceptors such as nitrate, perchlorate and arsenate, through a mechanism that involves biological oxidation instead of reduction of the external RM (Section 5.2). In addition, there are other types of both biological and chemical oxidation–reduction processes that depend on or are accelerated by redox mediators. Important examples of such processes are the (dissimilatory) reduction of oxidized metal minerals (e.g. ferric iron) and anode reduction in microbial fuel cells (MFC), where RM serve to facilitate the transport of electrons respectively from and to solid materials (Section 5.3). Moreover, inversely analogous to anode reduction, also cathode oxidation can be catalyzed by RM (Section 5.4). Another type of processes that can be accelerated by RM is the chemical or biological oxidation of bulk reductants such as sulfide (Section 5.5). Furthermore, RM are known to catalyze enzymatic oxidation of organic compounds (Section 5.6). Most of these RM-catalyzed processes are beyond the scope of this paper and have already received ample attention in other review articles. In the sections below we will only briefly discuss these topics, wherever possible with reference to relevant review papers.

### 5.2. Reduction of vat and anthraquinone dyes

Redox mediators have been shown to accelerate the reduction of two classes of non-azo dyes, vat dyes and anthraquinone dyes. The

principle of vat dyeing is sequential reduction and oxidation. In the reduction step, vat dyes such as indigo are solubilized (and often decolorized), and in the consecutive oxidation step, the dyes regain their color and precipitate on the fabric. It has been demonstrated that quinones can speed up the chemical reduction of vat dyes by dithionite (Roessler et al., 2003), as well as electrochemical vat dye reduction (Roessler and Jin, 2003). Moreover, it has been shown that the reductive decolorization of an anthraquinone dye by mesophilic anaerobic sludge can be accelerated by quinones (Bisschops et al., 2005; Dos Santos et al., 2005a). Both types of dyes are structures with quinone moieties, and their decolorization is presumably a result of the reduction of quinone to hydroquinone. Although this reaction can be assumed reversible once the dyes are again exposed to oxygen (Seigneur et al., 1996), the results of studies to anaerobic treatment of anthraquinone dyes normally point at irreversible reduction (Fontenot et al., 2002; Dos Santos et al., 2005a).

### 5.3. Reduction of soluble inorganic electron acceptors

A small number of papers mention a role of RM in the reduction of soluble inorganic electron acceptors, i.e. ‘normal’ electron acceptors such as dissolved oxygen and nitrate, as well as less usual electron acceptors such as perchlorate and selenite. The reduction of some of these electron acceptors can be a purely chemical reaction (e.g. chemical sulfide oxidation by oxygen) but most electron acceptors exclusively require biotransformation for their reduction, coupled to the oxidation of primary electron donors. A crucial difference with the reductive biotransformation reactions dealt with so far is that the biological reduction of these soluble inorganic electron acceptors typically requires their uptake by the cells. As most external RM do not cross cell membranes, they are not likely to play a role in shuttling electrons directly to these electron acceptors. RM may only play a role in accelerating these reductions by transporting electrons to the cells. It has been established that many facultative anaerobes can capture electrons from chemically reduced AQDS ( $\text{AH}_2\text{QDS}$ ) to respire oxygen and nitrate, but not Fe(III), Mn(IV) and elemental sulfur (Coates et al., 2002). In this study,  $\text{AH}_2\text{QDS}$  was not added as a mediator, but as a bulk electron donor in the absence of an intrinsic mechanism of AQDS reduction. In other studies, an (electro)chemical source of electrons was available, enabling RM to shuttle electron from this source to the cell. AQDS has been successfully applied in bioelectric perchlorate reduction to shuttle electrons from a cathode to the dissimilatory perchlorate reducing bacteria *Dechloromonas* and *Azospira* (Thrash et al., 2007). Likewise, it has been observed that 1,2-naphthoquinone-4-sulfonate stimulates the simultaneous removal of sulfide and nitrate, possibly by facilitating the transfer electrons from sulfide to denitrifying bacteria (Aranda-Tamaura et al., 2007). In the absence of either an electrochemical source of electrons or a bulk reductant that reacts directly with the mediator, evidence for a role of RM is very limited. The observation that arsenate ( $\text{As(V)}$ ) reduction by lactate-fed anaerobic granular sludge was somewhat accelerated by AQDS (Field et al., 2004) may have been related to AQDS reduction coupled to lactate oxidation followed by the use of  $\text{AH}_2\text{QDS}$  as electron donor for arsenate-reducing bacteria. These results contrast the observation that AQDS had no effect on the reduction of arsenate by VFA-fed anaerobic sludge in continuous packed-column reactors (Sierra-Alvarez et al., 2005). Another study reported an accelerating effect of AQDS in combination with yeast extract on the reduction of selenite ( $\text{Se(IV)}$ ) to insoluble selenium ( $\text{Se}^0$ ) by glucose-fed *Enterobacter taylorae*. In contrast, AQDS did not affect the rate of the reduction of selenate ( $\text{Se(VI)}$ ) to  $\text{Se(IV)}$ . The different results regarding the effect of AQDS on the two steps of  $\text{Se(IV)}$  reduction were attributed to redox potential differences between the couples involved (Zhang et al., 2007) but it may also have been due to the location of the reaction, with AQDS only able to interfere with  $\text{Se(IV)}$  reduction. It has been pointed out that, in order to avoid intracellular precipitation of

insoluble products (in this case  $\text{Se}^0$ ), bacteria are more likely to use an exocellular respiratory mechanism (Gralnick and Newman, 2007).

#### 5.4. Reduction of insoluble materials (minerals and anodes)

Microbial processes depending on the transfer of electrons to solid electron acceptors present a highly relevant category of oxidation–reduction reactions with a significant role of RM. These involve the reduction of insoluble metal oxides, e.g. dissimilatory Fe(III)- and Mn(IV)-reduction, and anode reduction for electricity generation in microbial fuel cells (MFC). These processes have been the subject of a large number of research papers, and the mechanism of extracellular electron transfer, which is largely similar in both processes, has received ample attention and several review articles have been published, some of them mainly focused on respiration of minerals (Newman, 2001), others exclusively on MFC (Rabaey and Verstraete, 2005; Bullen et al., 2006; Logan et al., 2006; Lovley, 2006; Davis and Higson, 2007; Du et al., 2007; Schröder, 2007), others on both (Stams et al., 2006; Gralnick and Newman, 2007). For the transfer of electrons from the oxidation of primary substrates by bacteria to either oxidized metal minerals or the anode surface, one can distinguish between direct and mediated electron transfer (DET and MET, respectively). DET is based on membrane-bound enzymes and direct physical contact of the cell membrane or a membrane organelle (electropili) with the solid electron acceptor. In contrast, MET depends on RM, either externally added compounds such as AQS or shuttling compounds produced by the bacteria themselves, such as phenazines. In addition, in MFC an in-between category of electron transfer that has been alternately ranged under DET or MET, uses non-diffusive mediators immobilized on the anode surface. It is interesting to note the entanglement that may arise from the concepts: whereas the presence of RM is not an exclusive prerequisite for electricity generation, it may very well be that the mechanism of electron transfer in so-called ‘mediator-less’ MFCs depends (partly) on biogenic diffusive mediators. Moreover, stimulation of electricity production by non-diffusive mediators on the electrode surface still requires a DET or MET reduction mechanism.

Several metal-reducing bacteria, such as species of *Geobacter*, *Shewanella* and *Rhodospirillum rubrum*, are capable of DET via c-type cytochromes, and it has recently been demonstrated that at least some of them (e.g. *Geobacter sulfurreducens* and *S. putrefaciens*) have the additional quality of forming electrically conductive pili or nanowires that enable them to reach electron-accepting surfaces at a certain distance from the cell (Reguera et al., 2005; Gorby et al., 2006). The other mechanism of electron transfer, ‘self-mediated’ MET via biogenic RM, is also receiving growing attention. It has been shown that *Pseudomonas aeruginosa* produces pycocyanine and phenazine-1-carboxamide as RM (Rabaey et al., 2005). Moreover, recent evidence showed that several *Shewanella* species excrete the flavins riboflavin and FMN that serve as RM in electron transfer reaction (Marsili et al., 2008; Von Canstein et al., 2008). As several RM have lower redox potentials than the cytochromes responsible for DET, it has been pointed out that the amount of extractable electric energy can be higher with MET than with DET (Schröder, 2007). An important drawback of MET, however, is that diffusive mediators (either externally added or endogenously produced) may easily be lost from continuous flow systems such as MFC.

#### 5.5. Electron transfer from cathodes

Whereas RM can be involved in transferring electrons to fuel cell anodes, they also can facilitate the transfer of electrons from fuel cell cathodes to terminal electron acceptors. Especially mediated electrochemical cathodic reduction of oxygen to superoxide and peroxide has been thoroughly investigated, as summarized in the review article by Šljukić et al. (2005). In addition, RM have been used to shuttle electrons from cathodes to bacteria, either for the reduction of

electron accepting components such as TCE (Aulenta et al., 2007) perchlorate (Thrash et al., 2007) and sulfate (Kim et al., 1990), or for manipulating product formation of fermentative conversions such as glucose fermentation to propionate by a *Propionibacterium* species (Emde and Schink, 1990) or to butanol by *Clostridium acetobutylicum* (Peguín and Soucaille, 1996). In analogy to anodic reduction, cathodic oxidation can be based on MET as well as on DET. A recent review article on Bioelectrical Reactors (BER) provides an adequate summary of electrically stimulated biotransformation reactions and the mechanisms of electron transfer (Thrash and Coates, 2008).

#### 5.6. Sulfide oxidation

While the redox couple sulfide/sulfur(polysulfide) is a potential electron shuttle in biotransformation reactions, e.g. reductive dehalogenation (De Wever et al., 2000), the oxidation of sulfide itself may be affected by external RM. It has been demonstrated that dissolved organic matter (DOM, peat humic acid) plays a redox-active role in sulfide cycling (Heitmann and Blodau, 2006; Blodau et al., 2007; Heitmann et al., 2007). It was also observed that the presence of DOM affects the way sulfide is oxidized, with thiosulfate rather than elemental sulfur or polysulfide as the main reaction products (Heitmann and Blodau, 2006; Heitmann et al., 2007). Furthermore, some chemical sulfide oxidation processes in practice, so-called ‘liquid redox processes’, use catalysts (Dalrymple et al., 1989). For example, in the Stretfort process, sulfide is oxidized by oxidized transition metals that are regenerated by organic RM that eventually shuttle electrons to oxygen (Steudel, 1996). Moreover, activated carbon, which is known to act as RM in azo dye reduction (Van der Zee et al., 2003; Mezohegyi et al., 2007) is also known to catalyze the reaction between sulfide and oxygen (Choi et al., 1991; Dalai et al., 1999). In this case, however, it is unknown whether an electron shuttling mechanism is involved or whether it is merely surface-catalysis.

#### 5.7. Enzymatic oxidation of organic compounds

Several oxidative enzymes, especially laccases and peroxidases, show higher activity or depend on RM for the oxidation of wide array of organic substrates. Typical RM of these kind of enzymes are e.g. veratryl alcohol (3,4-dimethoxy benzyl alcohol) and 1-hydroxybenzotriazole, logically compounds with a higher  $E_0'$  than the RM in reductive biotransformation reactions. A recent review paper on this topic provides a comprehensive summary (Husain and Husain, 2008).

### 6. Future perspectives on the application of redox mediators on the reductive (bio)transformation of contaminants

#### 6.1. Introduction

There certainly are great potentials for the application of RM on the reductive (bio)transformation of several priority pollutants both in contaminated groundwater and in wastewaters originated from different industrial sectors. Nonetheless, to warrant a successful output of the application of RM, several important topics should be considered in future research. The purpose of this section is to underline the main research areas demanded to enhance the applicability of RM in aquifer restoration and wastewater treatment.

#### 6.2. Cost-effective sources of redox active groups

Several redox active groups have been reported to play a significant role in the reductive (bio)transformation of priority pollutants. The large list of redox active molecules includes cytochromes (Picardal et al., 1993), flavines (Guerrero-Barajas and Field, 2005), cobalamins (Zou et al., 2000), porphyrins (Koons et al., 2001), pyridines (Lee et al., 1999), phenazines (Hernandez et al., 2004) and quinones (Field and Cervantes, 2005).

Quinones, active functional groups in humus, have been proposed as the most appropriate RM for the reductive (bio)transformation of a number of priority pollutants (Field and Cervantes, 2005). The main reason for considering quinones for redox (bio)transformation processes is that they are very abundant in humus, the most plentiful and cheaply available organic source in the biosphere. Moreover, the remarkable stability of HS in the environment, evidenced by their large residence time (>500 years) (Stevenson, 1994), prevents the deterioration of this material during bioremediation processes. Furthermore, humus is generally considered as a non-hazardous material and does not lead to the production of toxic byproducts (Perminova et al., 2005).

However, to take advantage of this abundant source of RM, careful selection of the origin of the humic material should be undertaken. Certainly, the ECC of several HS from a variety of origins was quantified (Ratasuk and Nanny, 2007). Highly aliphatic FA and HA extracted from different landfill leachates did not have any ECC and therefore are not recommended as a suitable source for RM. In contrast, FA and HA originated from peat, river sediments or soils showed an ECC ranging from 25 up to 538 micro-equivalents per g HS at different pH values.

### 6.3. Engineered HS for their application as RM in redox reactions

As underlined by Perminova et al. (2005), the principal factor limiting the application of HS in remediation technologies is the intrinsic variability in redox properties observed among humic materials and their fractions. Therefore, engineered humic materials are demanded in order to accomplish the redox mediating properties required during the conversion of a large number of priority pollutants through redox reactions.

Quinoid-enriched humic materials with enhanced redox properties were synthesized by two different strategies (Perminova et al., 2005). The first approach was related to the oxidation of phenolic fractions associated with the humic aromatic core. In a second strategy, polycondensation of these phenolic fragments was carried out with hydroquinone and catechol. Redox characterization of the copolymers obtained revealed that the reducing capacity of this synthesized humic material ranged between 1 and 4 mmol/g, which was much higher than the parent materials and the oxidized derivatives. Therefore, preferential application of the copolycondensation approach was advised.

Water soluble sulfonated humic materials were produced from leonardite and peat in order to evaluate their metal-binding capacity for Fe(III) and Hg(II) (Yudov et al., 2005). Enrichment of HA with sulfonate groups was postulated as an approach for remediation techniques applied during heavy metals removal from contaminated sites. The solubility of metal–HA complexes was estimated as an amount of strong metal-binding sites (SBS) in the humic materials. The sulfonated materials were characterized by a 2-fold increase in the amount of SBS compared to the parent materials. The enhanced solubility achieved in HA by this strategy may also be useful to distribute redox active groups of HA in contaminated aquifers for restoration purposes.

Different humic derivatives have also been synthesized and immobilized in silica gel to enhance their applicability in remediation technologies based on reactive barriers (Perminova et al., 2007). Nevertheless, considering the wide variety of redox reactions in which HS could be demanded, further research is required in order to obtain engineered HS, which fulfill different redox characteristics.

### 6.4. Immobilization of RM

Quinoid RM not necessarily have to be supplied abundantly in bioremediation processes to accelerate the redox transformation of different contaminants, as they are being recycled during the transfer

of electron from an electron donor to the terminal electron acceptor (Fig. 1). However, continuous addition of RM should be supplied in anaerobic bioreactors in order to achieve increased conversion rates, which raise the costs of treatment.

An approach to eliminate the prerequisite of continuous supply of RM is to create a niche for their immobilization in anaerobic bioreactors. However, scarce attempts to apply immobilized quinoid RM for the anaerobic conversion of priority pollutants have been reported. One of the alternatives available considers the application of activated carbon (AC) as a natural source of quinoid RM and its potential for reducing azo dyes have been explored in anaerobic bioreactors (Van der Zee et al., 2003). An AC-amended bioreactor performed a higher decolorizing capacity (>90% of decolorization) compared to a bioreactor control lacking AC (<40% of decolorization) during the reductive decolorization of RR2. Evidence indicated that the redox mediating capacity of AC was the main cause of the enhanced decolorization observed as the improved RR2 removal observed was 121-fold higher than the adsorption capacity of the AC. However, the catalytic effects of AC gradually decrease attributed to its continuing wash-out from the reactor. More recently, an upflow packed-bed reactor (UPBR) containing biological AC was tested for the continuous decolorization of AO7 (Mezohegyi et al., 2007). The treatment system achieved high decolorization efficiency of AO7 at very short space times: up to 99% decolorization in 2 min. Thus, the UPBR seems to be a promising system for anaerobic redox conversions, but needs to be proved with more recalcitrant pollutants.

Another immobilizing approach reported is to insert quinoid RM within different materials through polymerization procedures (Guo et al., 2007). Through this immobilizing technique, quinoid RM remain entrapped within the synthesized polymer keeping their redox mediating capacity. In this study, anthraquinone (AQ) entrapped in calcium alginate promoted up to 2-fold increase on the rate of decolorization of different azo dyes by a salt-tolerant bacterial culture, compared to controls lacking AQ. Disadvantages of this strategy are: 1) mass transfer limitations since a major part of the RM is embedded within the polymeric material making its accessibility dependent on diffusion; 2) gradual lost of redox catalysts due to disruption of the polymeric material owing to weak mechanical strength of the materials explored.

Lately, functionalized polypyrrole (PPy) composites were enriched with AQDS during the electropolymerization of pyrrole monomer on activated carbon felt (ACF) electrode. The resulting composite (ACF/PPy/AQDS) was tested as a RM for the reductive biotransformation of nitroaromatic compounds (Li et al., 2008). This study showed that ACF/PPy/AQDS exhibited good catalytic activity and stability and its addition effectively accelerated the reductive transformation of different nitroaromatic pollutants by an anaerobic consortium. Thus, this strategy represents a promising tool to immobilize RM for remediation technologies.

### 6.5. Overcoming limitations during catalysis involving RM

As discussed in the present review, the catalytic effects of RM greatly fluctuate among the different redox reactions studied so far. On one hand, the literature provides plentiful information documenting the enhancement of redox reactions involved during the (bio) transformation of several organic and inorganic pollutants in which the rate of conversion increased several orders of magnitude. On the other hand, several studies evidenced inhibitory effects of HS during the reductive (bio)transformation of electron-withdrawing contaminants. For instance, abiotic incubations amended with different concentrations of HA showed a decrease of up to 50% on the reduction rate of NB, TCE and CT by ZVI, compared to controls incubated in the absence of HA (Tratnyek et al., 2001). The inhibitory effects of HA were attributed to competitive sorption of HA onto reactive sites of ZVI limiting the transfer of electrons between this electron donor and the

electron-withdrawing pollutants. The last scenario was also observed during the reductive transformation of PCE (Doong and Lai, 2005) and 2,6-DNAH (Keum and Li, 2004) by ZVI in which HA amendments caused a decrease of up to 10-fold on the rate of reduction of these contaminants compared to controls lacking HA.

Not only HA, but also model quinoid RM, such as AQDS, have also inhibited the reductive conversion of electron-withdrawing contaminants. Indeed, the microbial dechlorination of 2,3,5,6-tetrachlorobiphenyl by a bacterial enrichment culture was completely inhibited by the addition of HA or AQDS by non-clarified mechanisms (Cutter et al., 1998). Furthermore, AQDS addition to a methanogenic enrichment culture promoted a higher reduction rate of FeOOH, but decreased both the rate of dechlorination of pentachloroaniline and methanogenesis probably due to competition among these processes for the reducing equivalents available in the incubations (Okutman Tas and Pavlostathis, 2007).

The information available in the literature provides a broad perspective of the different experimental conditions (pH, temperature, type and concentration of RM, type and concentration of electron donor, and redox potential), which affect the catalytic impact of RM. However, scarce information has been reported to elucidate the mechanisms by which RM are involved in different redox reactions and the bottlenecks to consider during redox catalysis. In some cases, the reduction of RM by different microorganisms has been pointed out as the rate-limiting step during redox reactions (Rau et al., 2002). In contrast, other studies evidenced that the reduction of oxidized contaminants by reduced RM is the rate-limiting step (Rau et al., 2002; Jiang and Kappler, 2008). Therefore, further research is demanded in order to elucidate the mechanisms limiting the catalytic effects of RM during the anaerobic (bio)transformation of priority pollutants.

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