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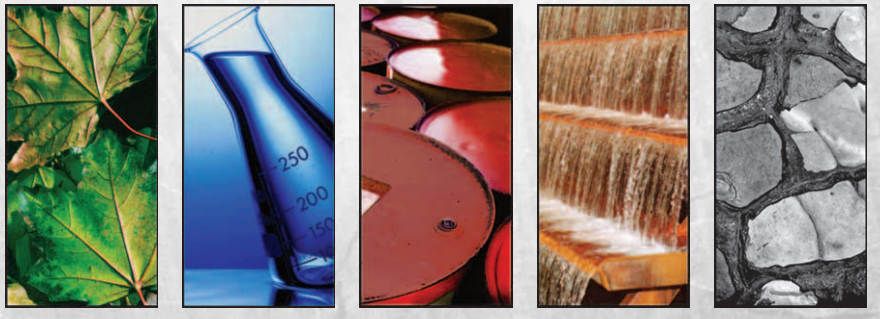

# Bioenergetics of transient and steady-state anaerobic redox processes relevant to groundwater contamination

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Institute of Environment & Resources
Technical University of Denmark

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# Preface

This thesis summarizes the findings from a Ph.D. project conducted at the Institute of Environment & Resources DTU, from February 2004 until April 2007. The study was funded through a scholarship from the Danish Research Agency (Internationalization grant for research training in Denmark).

This work is based on six journal papers:

i) Heimann AC, Friis AK & Jakobsen R (2005) Effects of sulfate on anaerobic chloroethene degradation by an enriched culture under transient and steady-state hydrogen supply. Water Res. 39(15): 3579-3586.

ii) Heimann AC, Batstone DJ & Jakobsen R (2006) *Methanosarcina* spp. drive vinyl chloride dechlorination via interspecies hydrogen transfer. Appl. Environ. Microbiol. 72: 2942-2949.

iii) Heimann AC & Jakobsen R (2006) Experimental evidence for a lack of thermodynamic control on hydrogen concentrations during anaerobic degradation of chlorinated ethenes. Environ. Sci. Technol. 40: 3501-3507.

iv) Heimann AC, Friis AK, Scheutz C & Jakobsen R (2007) Dynamics of reductive TCE dechlorination in two distinct $H_2$ supply scenarios and at various temperatures. Biodegradation 18: 167–179.

v) Heimann AC, Jakobsen R (2007) Filtration through nylon membranes negatively affects analysis of arsenic and phosphate by the molybdenum blue method. Talanta DOI 10.1016/j.talanta.2006.11.012, in press.

vi) Heimann AC, Blodau C, Postma D, Larsen F, Viet PH, Nhan PQ, Jessen S, Duc MT, Hue NTM, Jakobsen R (2007) Hydrogen thresholds and steady-state concentrations associated with microbial arsenate respiration. Environ. Sci. Technol. 41: 2311-2317.

The papers are not included in this www-version but may be obtained from the Library at the Institute of Environment & Resources, Bygningstorvet, Building 115, Technical University of Denmark, DK-2800 Kgs. Lyngby (library@er.dtu.dk).

I would like to thank my main supervisor, Associate Professor **Rasmus Jakobsen** from the Institute of Environment & Resources DTU, who guided me through this Ph.D. in a highly competent, responsive, and extremely friendly way. My acknowledgement also

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And finally, I thank my wife **Nha-Yong Au**, simply for being who she is.

Copenhagen, April 2007 Axel Heimann

# Abstract

This thesis investigates anaerobic microbial respiration of two different classes of groundwater contaminants, chlorinated ethenes and arsenic. A combination of pure and mixed culture experiments, sediment microcosm studies, and thermodynamic calculations was used to elucidate energetic constraints on a key parameter in anoxic systems, the concentration of dissolved hydrogen.

Degradation of trichloroethene (TCE) by the mixed dechlorinating culture KB-1 was studied over a wide temperature range (4–60 °C) using propionate and lactate as a slowly and rapidly fermenting substrate, respectively. While the overall rate of dechlorination was temperature-dependent, the choice of substrate and the resulting difference in $H_2$ levels strongly influenced end points of dechlorination and the occurrence of methanogenesis. Slow $H_2$ supply during propionate fermentation resulted in a lag phase prior to further dechlorination of cis-dichloroethene (*c*DCE), accompanied by a characteristic increase of $H_2$ and methane.

The same culture was used to study the impact of parameters determining the reactions' Gibbs free energy yield on $H_2$ levels during degradation of *c*DCE and vinyl chloride (VC). Changes in temperature (10-30°C) as well as variation of chloride levels (10-110 mmol/L) did not influence $H_2$ levels in a way that suggested thermodynamic control. Hence, it seems that partial equilibrium modeling is not directly applicable to dechlorinating systems.

Aceticlastic methanogens of the genus *Methanosarcina* drive dechlorination of VC by supplying *Dehalococcoides* spp. with $H_2$ derived from anaerobic acetate oxidation. This process, which was found during growth of KB-1 on acetate and VC, was studied using a combination of fluorescence in situ hybridization (FISH), radiotracer experiments, $H_2$ measurements, and thermodynamic calculations. Intriguingly, the transfer of $H_2$ to *Dehalococcoides* spp. is rendered thermodynamically favorable only by the very low $H_2$ levels maintained during dechlorination of VC.

Hydrogen thresholds for microbial respiration of arsenate (As(V)) were studied in a pure culture of *Sulfurospirillum arsenophilum*, a microbe growing by reducing As(V) to As(III). The $H_2$ thresholds (0.03-0.09 nmol/L) for this culture are among the lowest values measured so far compared with other terminal electron-accepting processes (TEAPs).

Similarly, sediment microcosm from an arsenic-contaminated aquifer in Vietnam showed rapid reduction of As(V) to As(III) accompanied by comparatively low steady

state $H_2$ levels. Collectively, these data suggest that microbial As(V)-reduction, a suspected culprit in arsenic contamination of groundwater, is a highly competitive TEAP in terms of substrate utilization and $H_2$ uptake.

# Dansk resumé

I afhandlingen undersøges anaerob mikrobiel respiration for to forskellige typer af kontaminanter i grundvandet, klorerede ethener og arsen. En kombination af ren- og blandkultur eksperimenter, sedimentmikrokosmos studier og termodynamiske beregninger blev benyttet, for at belyse energetiske begrænsninger af en nøgle parameter i anoxiske systemer, koncentrationen af opløst brint.

Triklorethen (TCE) nedbrydning af den deklorerende blandkultur KB-1 blev undersøgt ved forskellige temperaturer (4-60 °C) med hjælp af henholdsvis propionat og laktat som langsomt og hurtigt gærende substrater. Mens den overordnede dekloreringsrate var afhængig af temperaturen, havde valget af substrat og den afledte forskel i brint niveauet en stor indflydelse på de endelige produkter af dekloreringsprocessen og forekomsten af metandannelse. Langsom brintdannelse i løbet af propionat gæring resulterede i en nølefase inden videregående deklorering af cis-dikloroethene (*c*DCE), ledsaget af en karakteristisk stigning i koncentrationen af brint og metan.

Den samme kultur blev benyttet til at studere effekten af parametre, som bestemmer Gibbs fri energi for nedbrydning af *c*DCE og vinyl klorid (VC) på brint niveauet i systemet. Ændringer i temperaturen (10-30°C) og variationer af klorid niveauet (10-100 mmol/L) havde ikke en indflydelse på brint niveauet på en måde som kunne antyde termodynamisk kontrol. Dermed er partiel ligevægts modellering ikke direkte anvendelig på deklorerende systemer.

Acetiklastiske metanogener af genus *Methanosarcina* driver deklorering af VC ved at levere brint, dannet ved anaerob acetat oxidation, til *Dehalococcoides* spp. Denne proces, som blev opdaget under dyrkning af KB-1 på acetat og VC, blev undersøgt med en kombination af "fluorescence in situ hybridization" (FISH), radioaktive sporstoffer, brintmålinger og termodynamiske beregninger. Disse viste at overførselen af brint til *Dehalococcoides* spp. først bliver termodynamisk gunstig ved de lave brint niveauer, som er typisk for anaerob deklorering af VC.

Tærskelværdier for brint under mikrobiel respiration af arsenat (As(V)) blev studeret i en renkultur af *Sulfurospirillum arsenophilum*, en mikroorganisme, som vokser ved at reducere As(V) til As(III). Tærskelværdierne for brint i denne kultur (0.03-0.09 nmol/L) er blandt de laveste værdier som hidtil er målt, hvis man sammenligner med andre terminale elektron-accepterende processer (TEAPs).

Ligeledes viste sediment mikrokosmer fra en arsen-forurenet aquifer i Vietnam hurtig omsætning af As(V) til As(III) ledsaget af forholdsvis lave steady state brint niveauer.

Sammen, tyder data på, at As(V)-reduktion, mistænkt for at spille en rolle i arsen kontaminering i grundvandsystemer, er en meget konkurrencedygtig TEAP med hensyn til substrat udnyttelse og brint optage.

# 1. Introduction

Oxygen-depleted sediments are fascinating environments. The absence of aerobic respiration as the most favorable energy-yielding process allows for the development of a complex biogeochemistry featuring astounding strategies of anaerobic life. In these settings, microbial communities often get by on minute amounts of energy while displaying various interdependencies in scavenging growth-supporting substrates.

The overall pattern of electron and carbon flow in anoxic environments is a succession of several degradation reactions involving different microbes (*42*). Organic macro-molecules (e.g. carbohydrates, proteins, nucleic acids, or lipids) are degraded by the successive co-action of several microbial groups such as primary and secondary fermenters (obligate proton reducers) and methanogens (*128*). The final step in this pattern is the terminal electron-accepting process (TEAP) in which inorganic electron acceptors are reduced with low molecular weight intermediates such as acetate or hydrogen ($H_2$) (*8*). TEAPs in pristine environments may include denitrification, iron and manganese oxide reduction, sulfate reduction, or methanogenesis.

A key intermediate in anaerobic degradation is dissolved hydrogen ($H_2$) which is not only a common product of many fermentation reactions, but also the TEAP-driving electron donor with the highest turnover and therefore shortest residence time in anoxic sediments (*24*). Consequently, aqueous hydrogen concentrations in aquifers tend to be extremely low, often at nanomolar or sub-nanomolar levels. This makes dissolved hydrogen an excellent parameter to evaluate the redox state of a given system in terms of dominant and/or thermodynamically feasible TEAPs (*93*). Since the energy yield of a given TEAP decreases with the redox potential (Eh) of the electron acceptor (e.g. $Eh_{NO3-/N2} > Eh_{CO2/CH4}$) bacteria reducing nitrate are able to outcompete methanogens for $H_2$ at standard conditions. Following this line of thought, Lovley and Goodwin (*96*) empirically identified $H_2$ levels associated with different TEAPs (manganese or nitrate reduction, < 0.05 nM; iron reduction, 0.2 nM; sulfate reduction, 1-1.5 nM; methanogenesis, 7-10 nM), the $H_2$ level increasing with decreasing redox potential of the electron acceptor.

Lovley and Goodwin (*96*) also derived the same qualitative order of TEAPs from Michaelis-Menten kinetics for hydrogen uptake at steady-state conditions (no net growth). This is due to decreasing Michaelis-Menten constants ($K_m$) and increasing yield coefficients (Y) with increasing energy yields of a given reaction. Accordingly, the most straightforward approach for using $H_2$ as a descriptive redox parameter is to compare the concentration in a given system with these empirical ranges. The implicit

assumption of this approach is that $H_2$ levels characteristic of e.g. Fe(III) reduction are too low to support $H_2$-dependent sulfate reduction or methanogenesis, and so forth. Several studies on redox conditions in pristine sediments have followed this line of reasoning (*21,22,98*).

A refinement of this approach is the partial equilibrium concept (*76,77,123*). Since production of $H_2$ (i.e. fermentation) is limiting the overall rate of organic matter degradation, the TEAP is a comparatively fast process which renders it conducive to thermodynamic equilibrium calculations. Apart from the fact that steady-state conditions are not a prerequisite for this approach, it has the big advantage of accounting for the *in situ* conditions in a given system (e.g. activities of reactants, temperature). Thus, combining the information on product/educt ratios with $H_2$ levels allows for calculations of *in situ* Gibbs free energies in the system of interest.

Hoehler et al. (*67*) could nicely demonstrate the validity of this concept in a series of laboratory experiments. $H_2$ concentrations in methanogenic and sulfate reducing sediment microcosms, respectively, responded to changes in electron acceptor concentrations, pH, and temperature, maintaining a nearly constant energy yield (close to thermodynamic equilibrium; $\Delta G$ = -15 to -20 kJ per mol reaction). For obvious reasons the energy yield of a given TEAP does not reach true thermodynamic equilibrium ($\Delta G = 0$), but is restricted to a minimum metabolically convertible energy yield. This corresponds to the energy required for synthesis of 1/5 to 1/3 of an ATP unit translating into approximately -10 to -20 kJ per mol reaction (*68,128*).

The aim of this study was to elucidate the validity and applicability of these concepts in contaminated environments, in which the natural range of TEAPs is supplemented by microbial types of anaerobic respiration that utilize groundwater contaminants as electron acceptors. The focus was put on two different classes of contaminants which, while physico-chemically very different, both pose severe threats to groundwater quality worldwide. These are (1) chlorinated ethenes and (2) inorganic arsenic.

# 2. Dehalorespiration of chlorinated ethenes

## 2.1. Chlorinated ethenes in groundwater: a health hazard

Halogenated aliphatic compounds constitute a serious threat to groundwater quality in industrialized countries (*54*). Chlorinated ethenes, such as perchloroethene (PCE), trichloroethene (TCE), and their metabolites dichloroethene (DCE) and the more toxic vinyl chloride (VC), are among the most commonly encountered contaminants within this group (*1,54*). Owing to their beneficial physico-chemical properties (nearly noncorrosive and nonflammable), PCE and TCE have been heavily used as dry cleaning and metal degreasing agents since the 1940s (*147,148*). Intake of chlorinated ethenes may trigger several adverse health effects such as central nervous system depression, neurotoxicity, liver and kidney damage, liver cancers, angiosarcomas and hepatocellular carcinoma (*81,147*). Consequently, the World Health Organization has set the chlorinated ethenes guideline values for drinking water quality to 0.3-70 µg/L (depending on the chloroethene species (*147*)).

## 2.2. Anaerobic biodegradation of chlorinated ethenes

Anaerobic dechlorination of chlorinated ethenes is achievable through several biological processes, of which dehalorespiration is the most important one in terms of plume attenuation at contaminated sites (*148*). In dehalorespiration, chlorinated ethenes are used as electron acceptors, yielding energy and allowing for microbial growth (*70,71*). As chlorine atoms are successively replaced by hydrogen atoms PCE is reduced to TCE, cis-DCE (the most common of the three DCE isomers in microbial degradation), VC, and eventually ethene (*146*). This process is outlined in Figure 1.

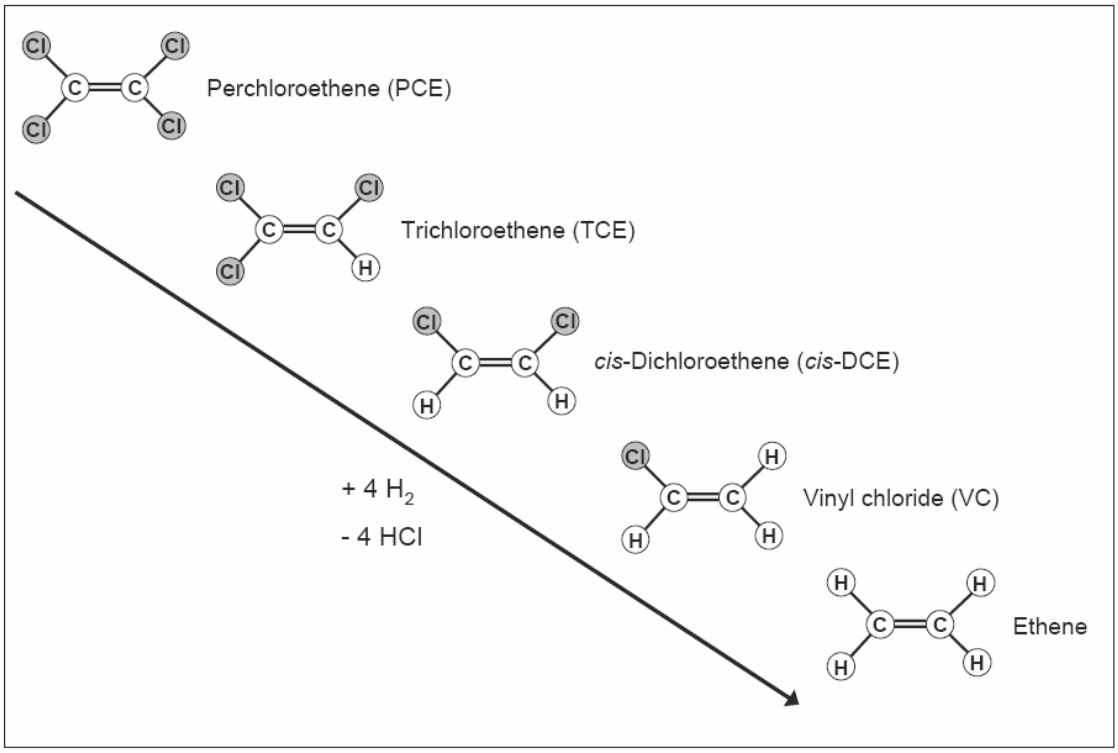


**Figure 1**. Reductive dechlorination sequence as mediated by dehalorespiratory bacteria.

In addition to dehalorespiration, reductive dechlorination can also occur cometabolically (fortuitously), catalyzed by the bacterial transition-metal coenzymes vitamin B12, coenzyme F430, and hematin (*51*) or by the CO dehydrogenase enzyme complex from *Methanosarcina thermophila* (*75*). However, cometabolic degradation is often slow and incomplete (*148*). Another degradation pathway under anaerobic conditions is the complete mineralization to $CO_2$ (*15*).

Reductive dechlorination can be stimulated by the addition of various electron donors such as acetate, lactate, formate, toluene, methanol, or yeast extract (*32,40,101,129,130,132,141,153*). These are either used directly or through $H_2$ production by fermentation (*9*). Dehalorespiring bacteria capable of growing with every chlorinated ethene species as electron acceptor are to date restricted to the genus *Dehalococcoides* (*28,29,38,58,107*), whereas the capability to reduce PCE and TCE seems to be phylogenetically widespread (*48*). Completely dechlorinating cultures enriched from contaminated sites, such as the commercially available mixed culture KB-1$^{TM}$, therefore generally contain *Dehalococcoides* species as vital contributors to site cleanup (*37,38,50*). All known *Dehalococcoides* spp. isolates require $H_2$ as ultimate electron donor (*3,58,106*). Consequently, $H_2$ is considered the most important electron donor for dehalorespiration, thus playing a key role in anaerobic dechlorination

(*46,148*). A fair amount of work done in this thesis was therefore dedicated to investigating the significance of $H_2$ levels in reductively dechlorinating environments, both in terms of competition with alternative TEAPs and the influence of bioenergetics (i.e. thermodynamic constraints) on this parameter.

## 2.3. Competitiveness of dehalorespirers for $H_2$ consumption

In general, dehalorespiring bacteria are highly competitive with respect to utilization of $H_2$ as electron donor. Both $H_2$ thresholds and steady-state concentrations during dehalorespiration are among the lowest when compared to competing TEAP (*60,92,99,152*). Accordingly, the relatively low half-velocity coefficients for $H_2$ utilization by dechlorinators (roughly an order of magnitude lower than values for methanogens) suggest that dechlorinators can outcompete methanogens at low $H_2$ concentrations (*10*).

However, even under $H_2$-limited conditions the rate of dehalorespiration can be negatively affected by the presence of alternative electron acceptors like sulfate as was demonstrated in this thesis (*59*). This effect decreases in more transient systems with a high buildup of $H_2$ concentrations, e.g. due to rapid fermentation of lactate. Other findings on the effect of sulfate on dechlorination are contradictory, ranging from no inhibition (*33,39,69*), partial inhibition (*17*), varying degrees of inhibition during different phases of microbial growth (*143*), inhibition even with ample hydrogen supply (*112*), to dechlorinators outcompeting sulfate reducers (*108*).

A rigorous comparison of dechlorination patterns under two extreme scenarios of a low-rate $H_2$ releasing substrate (propionate) versus a high-rate $H_2$ releasing substrate (lactate) over a wide temperature range was done as part of this thesis (*64*). While lactate amendment of the mixed dechlorinating culture KB-1 resulted in a rapid buildup of hydrogen to levels beyond 1000 nM, propionate served as a more moderate substrate in terms of $H_2$ production (the concentrations being several orders of magnitude lower when compared to lactate-fed cultures; Figure 2). However, especially at colder temperatures low $H_2$-release rates (propionate setup) led to slow and incomplete TCE dechlorination by KB-1 (*64*).

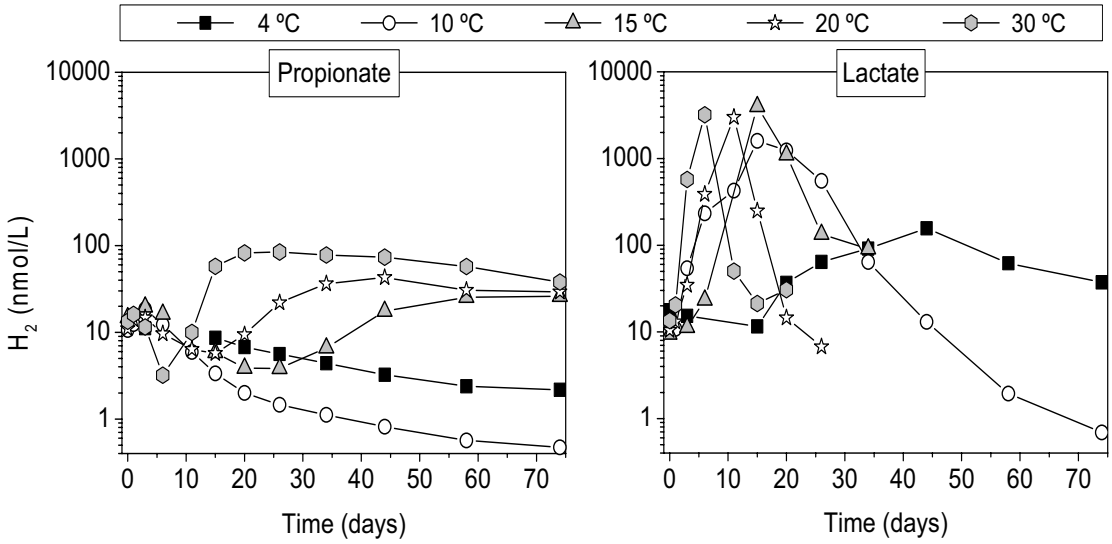


**Figure 2**. Hydrogen concentrations at different temperatures and with two different fermentable substrates

Consequently, choosing an appropriate $H_2$-donating substrate to stimulate dehalorespiration is a trade-off between rapid dechlorination and negative side effects of adding surplus donor (bioclogging, excess methane production (*45,90*)). Some studies advise to use slowly fermentable substrates such as propionate to exploit the competitive advantage of dehalorespiring bacteria at low $H_2$ levels (*44,133*). However, this point of view is challenged by others arguing that rapid and complete degradation of chlorinated ethenes is the primary goal in engineered dehalorespiring systems (*20,100*).

## 2.4. Thermodynamics and hydrogen levels in dechlorinating systems

In pristine, anoxic settings, the energy gain from TEAPs is often very close to thermodynamic equilibrium (*25*), not exceeding 10-20 kJ per mol of reaction, e.g. for methanogenesis (*68,128*). This allows for an evaluation of redox conditions in a given environment by calculating *in situ* Gibbs free energy yields and comparing them with this metabolic energy threshold (*76*).

However, for reductive dechlorination of organic hydrocarbons such as TCE or *cis*-DCE the Gibbs free energies are generally much more negative (i.e. yielding more energy) when assuming a range of typical field conditions. An example of this is shown for the reduction of cis-DCE to VC:

$$\Delta G_r = \Delta G^{0,T} + RT \ln\left(\frac{[VC][Cl^-][H^+]}{[cis-DCE][H_2]}\right) \quad (1)$$

where $\Delta G^{0,T}$ is the standard state Gibbs free energy at in-situ temperatures, R is the gas constant, and activities of the reactants are indicated by square brackets. When calculating the energy yield of this reaction assuming a 1:1 ratio of *cis*-DCE and VC, a chloride activity of 0.01, pH = 7, and a temperature of 25 ºC, the resulting energy yields are generally more negative than -100 kJ per mol of reaction (varying the aqueous hydrogen concentration between 0.1 and 10 nM; (*61*)). Similar tendencies are found for dechlorination steps involving other chlorinated ethenes.

With reductive dechlorination reactions generally proceeding far from thermodynamic equilibrium it seems likely that $H_2$ levels in these systems are controlled by kinetics rather than by bioenergetic conditions (*35*). However, since the simple respiratory chains involved in halorespiration tend to use the available energy rather inefficiently (*71*) it is not inconceivable that a more negative threshold Gibbs energy exists that controls hydrogen levels in dechlorinating environments in accordance with the partial equilibrium concept. Neither of these views has been verified experimentally.

In an effort to improve our understanding of what hydrogen concentrations in dechlorinating systems relate to, a series of laboratory experiments was conducted as part of this thesis (*61*). The experimental setup closely followed the approach taken by Hoehler et al. (*67*) in their study of sulfate reduction and methanogenesis in anaerobic sediments. While most of the parameters entering the Gibbs free energy expression for the dechlorination of *cis*-DCE or VC were kept constant, only one parameter (either the temperature or the chloride level) was varied at a time.

Results from these experiments suggested that hydrogen levels in dechlorinating systems are not controlled by thermodynamics (*61*). Varying the temperature (between 10 and 30 °C), or the chloride level (between 10 and 110 mmol chloride) did not change hydrogen concentrations in a fashion that would allow for application of the partial equilibrium concept.

## 2.5. The influence of aceticlastic methanogens on dechlorination

One aim of the present thesis was to take a closer look at the potential interaction between (i) dechlorinating bacteria that reduce toxic compounds such as VC and (ii) methanogens that convert acetate directly to methane. The motivation for this came largely from earlier experiments with the mixed culture KB-1 that repeatedly (and quite unexpectedly) showed a strong positive correlation between the rates of vinyl chloride dechlorination (an obligately hydrogenotrophic process) and the rates of aceticlastic methanogenesis when acetate was the only available electron donor (*64*).

While VC-respiring bacteria are not able to use acetate directly as electron donor, mixed dechlorinating cultures can make use of reducing equivalents of acetate by oxidizing it anaerobically to $CO_2$ plus $H_2$ (syntrophic bacteria) and subsequently oxidizing the evolved hydrogen with VC (dechlorinators) (*57*). Consequently, addition of acetate alone may be sufficient to stimulate dechlorination to ethene, regardless of the presence of methanogens.

A curious feature of some aceticlastic methanogens is their capability of producing small amounts of $H_2$ from acetate while the major part of the acetate is converted to methane (*85,95*). The pathway of hydrogen formation is identical to the reaction carried out by syntrophic bacteria (formation of $CO_2$ plus $H_2$). Hydrogenotrophic sulfate reducers or Fe(III)-reducers have been shown to utilize the evolved $H_2$ and even increase the amount of $H_2$ produced in these methanogenic systems (*2,14,122*).

Using a combination of different methods such as radiotracer experiments with [2-$^{14}$C]acetate, fluorescence in situ hybridization (FISH), rate measurements and hydrogen sampling we could show that this process is also relevant in acetate-rich dechlorinating systems (*60*). *Methanosarcina*, one of the two genera capable of aceticlastic methanogenesis, may drive dechlorination of VC by transferring $H_2$ from acetate to dehalorespiring *Dehalococcoides* spp., resulting in up to 7-fold increased dechlorination rates (as compared to non-methanogenic controls). The amount of $H_2$ produced by *Methanosarcina* greatly increases in the presence of dehalorespirers, due to the low $H_2$ levels typically maintained in VC-dechlorinating cultures (0.3-0.5 nmol/L; (*60*)). *Methanosaeta*, the other genus capable of aceticlastic methanogenesis, did not show this effect.

# 3. Microbial reduction of arsenic

## 3.1. Arsenic in drinking water: a global health disaster

Arsenic is a naturally occurring constituent of sulfidic ores and metal arsenates or arsenides, making it the 20$^{th}$ most abundant element in the Earth's crust (*27,147*). Arsenic concentrations in soils range from 0.1 to more than 1000 mg/kg (*111*). Release of arsenic into groundwater may occur upon dissolution of these arsenic-bearing minerals (*147*). Natural waters may contain arsenic levels up to more than 5000 µg/L, while typical concentrations in freshwater are below the WHO limit of 10 µg/L (*134*). There are numerous areas with high levels of arsenic in groundwater. These include Argentina, Bangladesh, Chile, China, Hungary, India, Mexico, Romania, Taiwan, USA, and Vietnam (*134*).

Probably the worst and most infamous case is Bangladesh with over 50 million people consuming water that exceeds the arsenic limit set by the WHO (*13,109*). Millions of small tube wells were installed here in the 1970s and 80s tapping drinking water from shallow aquifers. Later it turned out that water from an estimated 50% of these wells was contaminated with arsenic of natural origin, derived from sediments that had been transported from the Himalaya and deposited millions of years ago (*13,120*). The exact processes leading to the release of sediment-associated arsenic into the groundwater are still a matter of debate.

Consumption of drinking water with elevated arsenic levels may lead to a wide range of adverse health effects, most importantly various types of cancer, particularly skin, bladder and lung cancer, keratosis, cardiovascular diseases and diabetes (*116,147*). The toxicological mechanisms depend on the oxidation state of arsenic. Pentavalent arsenate (As(V)) is a structural analog of phosphate that uncouples oxidative phosphorylation inhibiting ATP synthesis, while trivalent arsenite (As(III)) is considered even more toxic showing a high reactivity towards sulfhydryl groups of proteins (*119*).

## 3.2. The biogeochemistry of arsenic

In natural waters arsenic mostly occurs as inorganic oxyanions of pentavalent arsenate (As(V)) or trivalent arsenite (As(III)) (*134*). The ratio of As(V) to As(III) can vary considerably in groundwaters, depending on the redox conditions. However, in anaerobic sediments, As(III) is generally the predominant arsenic species (*16*). In fact,

the ratio of dissolved As(V) and As(III) has been proposed as an indicator of the redox conditions in groundwater (*23*).

Dissolved arsenic may become re-associated or re-precipitated with other sedimentary mineral phases. Most importantly, arsenic in sediments is often associated with iron or aluminum (oxyhydr)oxides (*12,16,43*). In water treatment, sorption to iron oxide coatings on sand filters may therefore play an important role in arsenic removal (*78*). Consequently, release and mobilization of arsenic is often linked to the reductive dissolution of iron oxides (*5,12,104,109,121*). In a sedimentary system with a vertical redox gradient this may lead to reductive dissolution in the anoxic zone, upward diffusion and re-precipitation near the oxic sediment-water interface (*12*). Laboratory incubations of arsenic-impacted aquifer sediments from West Bengal amended with acetate and a culture enriched in Fe(III)-reducing bacteria demonstrated that metal-reducing microbes play an important role in arsenic mobilization in these environments (*73*).

As for abiotic processes, sediment-associated arsenic may be released into solution by competition for sorption sites with phosphate, carbonate or dissolved organic matter (*7,11,31*). In semi-arid or arid regions, development of high pH conditions due to mineral weathering and excessive evaporation may act as an additional mechanism for arsenic mobilization, leading to desorption of anionic As(V) from iron oxide surfaces (*134*).

Another factor influencing arsenic mobility is the transformation and re-crystallization of iron oxide minerals upon their reductive dissolution (*121*), both impacting the affinity of the mineral phase for arsenic and the specific surface area (*34*). To complicate things even further, ferrous iron ($Fe^{2+}$) released by reductive dissolution of iron oxides may re-precipitate in more reduced mineral phases (siderite) that then again may re-capture some of the arsenic from the aqueous phase (*5*). In the presence of sulfide (S(-II)) and under acidic conditions, As(III) is also known to precipitate as the arsenic sulfides orpiment ($As_2S_3$) and realgar (AsS) (*86,113,115*). In addition, dissolved sulfide chemically reduces As(V) to As(III) (*136*).

The presence of sedimentary sulfide minerals, such as pyrite ($FeS_2$) can also affect the mobility of arsenic (*12,27,82,110*). Pyrite may effectively capture arsenic at Fe/As ratios of around 1000 (*12*). In mine tailing sediments from Coeur d'Alene Lake, ID, around 70% of all arsenic is associated with sulfides (*56*). Upon oxygenation of these sediments (e.g. in response to excessive ground water abstraction) pyritic minerals may become oxidized with arsenic being released into solution (*8*).

Changes in the oxidation state of arsenic may have considerable effects on its mobility in contaminated sediments (*104*). In oxidized environments, concentrations of dissolved arsenic tend to be low while the reduction of As(V) to As(III) may release substantial amounts of arsenic into the aqueous phase (*73,104*). In fact, mobilization of arsenic by reduction to the As(III) species has been suggested as a bioremediation approach for treating contaminated soils (*36,149*).

However, the relationship between the oxidation state and the mobility of arsenic species is far from simple. In fact, adsorption studies done by Manning et al. (*103*) suggest that As(III) will be more strongly bound to iron oxide surfaces than As(V) at neutral to alkaline pH. Accordingly, in sediments of a freshwater reservoir (Olancha, CA) treated with ferric chloride As(III) remained associated with iron oxides (*47*). Similarly, Dixit and Hering (*34*) showed that in the circumneutral pH range of 6-9 As(III) was sorbed equally or even more strongly to amorphous iron oxide or goethite than As(V). Only at low pH values (below pH 5-6) was As(V) sorption more favorable than sorption of As(III). The implications are that in order to mobilize iron oxide-associated arsenic, reductive dissolution of the actual sorbent (i.e. the iron oxide) is required. This again conflicts with findings from others (as discussed below), and demonstrates the complexity of arsenic biogeochemistry which has led to a lively debate in the scientific community.

## 3.3. Microbial reduction of arsenate

Microbial reduction of As(V) to As(III) has been long known to occur in various environments, mediated by aquatic bacteria, activated sludge, or wine yeast (*27,47,115*). The formation and subsequent expulsion of As(III) by a specific transporter is a widespread microbial detoxification strategy for lowering the intracellular concentration of arsenic (*111*). Some bacteria may also reduce As(V) or As(III) to arsine ($AsH_3$, As(-III)), although only small amounts of arsine are produced by unenriched cultures (*27*). For instance, anoxic salt marsh sediment amended with millimolar levels of As(V) showed no quantitatively significant reduction beyond As(III) (*36*).

Ahmann et al. (*4*) were the first to show the existence of microbes that gain energy (i.e. grow) through the reduction of As(V) to As(III), a process termed dissimilatory arsenate respiration. An isolate obtained from arsenic-contaminated sediments could completely reduce millimolar levels of As(V) within a few days, stoichiometrically producing As(III) while using lactate as electron donor. This process takes place in the absence of oxygen. In addition, it was found that the same isolate (*Sulfurospirillum arsenophilum*) could mobilize arsenic from a solid iron arsenate phase (*5*).

To date, numerous other, phylogenetically diverse strains of arsenate-respiring microbes have been discovered. Apparently, arsenate respiration occurs in a large variety of environments ranging from freshwater sediments, hypersaline lake waters, hot springs, deep-sea hydrothermal vents, gold mines, and arsenic-treated wood to bovine rumen fluid, hamster feces, and the termite hindgut (*52,65,87,114,117,124-126,135,139,140*). In extreme environments such as arsenic-rich alkaline soda lakes (e.g. Mono Lake, California; containing ~200 µmol/L inorganic arsenic (*66*)) arsenate respiration may actually account for a significant fraction of the total carbon mineralization (*118,139*). As(V)-respiring microbes can use a variety of electron donors including lactate, acetate, formate, hydrogen and sulfide (*120*).

An important difference between microbial As(V) reduction as (i) a detoxifying process and as (ii) a respiratory process is the cellular location of the enzymes catalyzing the respective processes (*120*). For the detoxifying reduction, As(V) has to enter the cell since the responsible protein is located in the cytoplasm. In contrast, the respiratory As(V) reductase is located in the periplasm making it possible for As(V) respirers to attack sediment-bound arsenic.

All known microbes capable of dissimilatory arsenate reduction also use other electron acceptors such as nitrate, sulfate, or Fe(III) for growth (*119*), implying a low degree of specialization. Considering this together with the apparent ubiquity of arsenate-respiring microbes, it seems reasonable to assume an involvement of these bacteria in mobilization of sediment-associated arsenic in aquifers. A recent molecular approach seems promising for the detection of dissimilatory As(V) reduction in spite of the apparent phylogenetic broadness of As(V)-respiring microbes. The functional gene encoding for the common denominator of arsenate respiring microbes, the respiratory As(V) reductase, was targeted by a PCR based approach introduced by Malasarn et al. (*102*).

## 3.4. Energetics of arsenate respiration

In the redox sequence of sediments turning anaerobic (i.e. the depletion of oxygen followed by the depletion of nitrate, iron oxides, sulfate etc.) reduction of As(V) to As(III) is expected to occur between sulfate reduction and iron oxide reduction under standard conditions (Table 1). The exact energetic position of arsenate respiring microbes depends, however, on the in-situ geochemical conditions of a given site (e.g. pH, $H_2$ level, etc.).

Whether reductive dissolution of iron oxides occurs before, after, or simultaneously with the reduction of sorbed As(V) is not clear. Masscheleyn et al. (*104*) found that As-contaminated soil incubated under reducing conditions first released some As(III) into solution before dissolved Fe increased. Similarly, Zobrist et al. (*154*) showed that reductive dissolution of ferrihydrite was not required for sorbed As to be reduced and released into solution. This was nicely demonstrated in experiments using As(V) coprecipitated with either ferrihydrite or aluminum hydroxide, the latter not undergoing microbial reductive dissolution. As(V) adsorbed to the aluminum hydroxide was reduced to As(III) in spite of the unreactive mineral phase sorbent (*154*).

In contrast to this, Islam et al. (*73*) found that anaerobic incubations of West Bengal aquifer sediment microcosms first released sediment-associated arsenic after a considerable time of ongoing iron oxide reduction. This decoupling of Fe(III) and As(V) reduction was attributed to the energetic situation at in situ conditions (i.e. the initially higher redox potentials of the $Fe(OH)_3/Fe^{2+}$ couple as compared to the As(V)/As(III) couple).

In a later study, it was found that the iron oxide reducing enrichment culture derived from these sediments was dominated by a close relative of the Fe(III)-reducer *Geothrix fermentans* which is not capable of As(V) reduction (*74*). When a pure culture of *G. fermentans* was inoculated into heat-sterilized sediments from the same aquifer it reduced Fe(III), but was unable to mobilize arsenic associated with the solid phase (*74*). This suggests that reduction of iron oxides in these sediments is not sufficient to release sediment-bound arsenic and that As(V)-reducing microbes are likely involved in its mobilization.

Whether the occurrence and/or sequence of iron oxide reduction and As(V) reduction are controlled by energetic (i.e. thermodynamic) considerations is not known. While low energy yielding TEAPs (e.g. methanogenesis, sulfate reduction; see Table 1) generally follow a sequence that is largely dependent on their in situ energy yield, high energy yielding TEAPs (e.g. dechlorination, nitrate reduction) and their respective steady state hydrogen levels and thresholds tend to be limited by kinetics (e.g. enzymatic uptake of hydrogen (*61,67*)). The reduction of As(V) to As(III) yields a comparatively high amount of energy under standard conditions ($\Delta G^0$ = -162.4 kJ/mol; Table 1) as well as under in situ conditions ($\Delta G^0$ ~ -50 kJ/mol; (*63*)). This suggests that hydrogen levels in As(V)-reducing systems are probably not controlled by thermodynamics. However, the range of theses values and how they compare to values occurring in iron oxide reducing systems were previously unknown.

**Table 1**. Standard Gibbs free energies ($\Delta G^0$) for various TEAPs

| Reactions | $\Delta G^0$ (kJ/mol)[1] |
|---|---|
| $1/2\ O_2 + H_2 \rightarrow H_2O$ | -237.2 |
| $2/5\ NO_3^- + H_2 + 2/5\ H^+ \rightarrow 1/5\ N_2 + 6/5\ H_2O$ | -240.1 |
| $2\ FeOOH(a) + H_2 + 4\ H^+ \rightarrow 2\ Fe^{2+} + 4\ H_2O$ | -182.5 |
| $HAsO_4^{2-} + H_2 + 2\ H^+ \rightarrow H_3AsO_3 + H_2O$ | -162.4 |
| $VC + H_2 \rightarrow Ethene + H^+ + Cl^-$ | -100.9 |
| $1/4\ SO_4^{2-} + H_2 + 1/4\ H^+ \rightarrow 1/4\ HS^- + H_2O$ | -48.0 |
| $1/4\ HCO_3^- + H_2 + 1/4\ H^+ \rightarrow 1/4\ CH_4 + 3/4\ H_2O$ | -43.9 |
| $1/2\ HCO_3^- + H_2 + 1/4\ H^+ \rightarrow 1/4\ Acetate^- + H_2O$ | -36.1 |

[1] Calculated from the Gibbs free energies of formation from the elements (*61,84,137,142*); The following are treated as gaseous species: $O_2$, $H_2$, $N_2$, and $CH_4$

Thus, both the threshold for hydrogen uptake by As(V)-respiring microbes and steady state hydrogen levels in As(V)-reducing sediment microcosms were determined as part of this thesis (*63*). Pure cultures of *Sulfurospirillum arsenophilum*, an As(V)-respiring microorganism (*135*), consumed hydrogen to thresholds of around 0.03 to 0.09 nmol/L when reducing As(V) to As(III). Compared to hydrogen thresholds of other TEAPs these values are among the lowest measured (Figure 3) suggesting that As(V)-reducing bacteria are highly competitive at e-donor-limiting conditions.

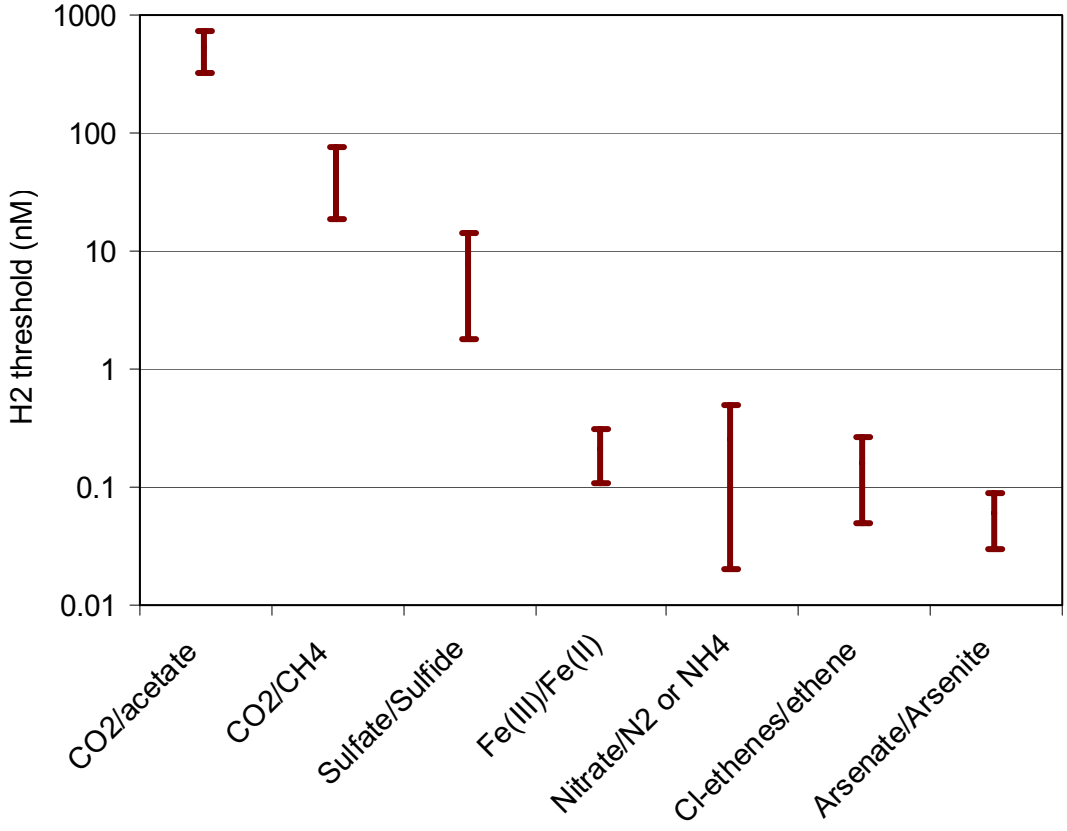


**Figure 3**. Hydrogen thresholds for various TEAPs occurring in pristine or contaminated anaerobic environments (bars indicate the range given by the following references; $CO_2$/acetate (*26*), $CO_2$/$CH_4$ (*26,94*), sulfate/sulfide (*26,99*), Fe(III)/Fe(II) (*19,97,138*), nitrate/$N_2$ or $NH_4$ (*26,55,138*), chlorinated ethenes/ethene (*92,99,138*), arsenate/arsenite (*63*)).

A quite unexpected result from this study was the high rate of As(V)-reduction in microcosms derived from an arsenic-contaminated aquifer in Vietnam (*63*). The fact that As(V)-reduction rates were high and remained largely unchanged when *S. arsenophilum* was added to the microcosms suggests a vital population of As(V)-reducing microbes in these environments.

## 3.5. Analytical detection of trace aqueous arsenic species

Increased awareness of human health problems associated with elevated arsenic levels in drinking water have resulted in the demand for analytical tools for quantifying trace

amounts of arsenic in aqueous solutions. Consequently, the last decade has seen the emergence of various sophisticated analytical methods for the precise determination of aqueous arsenic species down to nanomolar levels. The most advanced technologies combine the virtues of chromatographic separation techniques such as ion chromatography (IC) or high-performance liquid chromatography (HPLC) with atomic absorption spectrometry (AAS) (*18,91*), atomic fluorescence spectrometry (AFS) (*88,89,151*), or inductively coupled plasma mass spectrometry (ICP-MS) (*41,83,105,145,150*). In addition, electrochemical approaches (anodic stripping voltametry, ASV) (*30,131*), chemiluminescence-based methods (*72,127*), and microbial reporter technologies (*144*) are available.

A more traditional low-tech alternative is the colorimetric molybdenum blue method (*79*) which was the method of choice in the present thesis. Since the method also allows for the determination of phosphate and silicate it is often part of flow injection analysis systems (*49,53,80*). It has also been frequently employed in studies on microbial As(V)-reduction (*4,6,86*).

When using the molybdenum blue method for the determination of arsenic species during microbial As(V)-reduction we found a curious result. Samples that were filtered with nylon syringe filters showed significantly lower absorbances (6-74%) as compared to unfiltered samples (*62*). This effect was observed with arsenic- or phosphate-containing synthetic solutions as well as with pure filtered water that was subsequently spiked with either arsenite of phosphate. This indicates that one (or more) compounds eluting from the filter membranes interfere with the color formation of the method. Consequently, cautioning against the use of nylon filters in combination with this widely used assay is an important collateral result of the present thesis (*62*).

# 4. Conclusions

Chlorinated hydrocarbons and arsenic represent two distinct classes of groundwater contaminants. They differ greatly both in their physico-chemical properties and introduction pathways into drinking water resources. While chlorinated hydrocarbons are a good example of a man-made group of toxic contaminants, elevated levels of arsenic in groundwater often stem from natural sources. Despite their differences, chlorinated ethenes and arsenic both act as microbial electron acceptors in anaerobic respiration processes.

The concentration of dissolved $H_2$ is an excellent tool for studying the competitiveness and bioenergetics of both respiratory processes. $H_2$ levels during dehalorespiration are among the lowest steady state concentrations observed in anaerobic systems. Consequently, microbes that respire chlorinated ethenes can easily outcompete other TEAPs such as methanogenesis during slow $H_2$ release. This is accomplished in systems with low reactivity of organic matter and/or thermodynamic constraints on rapid fermentation (e.g. syntrophic acetate or propionate turnover).

Unlike the TEAPs acetogenesis, methanogenesis, and sulfate reduction, $H_2$ levels during chloroethene dehalorespiration are not controlled by thermodynamics. This renders dehalorespiring environments unfavorable for partial equilibrium modeling.

Aceticlastic methanogens of the genus *Methanosarcina* may greatly increase rates of chloroethene degradation by transferring $H_2$ to dehalorespiring microbes. As a response to low $H_2$ levels that are maintained during chloroethene reduction *Methanosarcina* spp. oxidize an increased fraction of acetate to $CO_2$ plus $H_2$, a process with potential relevance to acetate-rich bioengineered systems.

When respiring arsenate, *S. arsenophilum* reduces $H_2$ to thresholds that are among the lowest reported so far. This apparent competitiveness of As(V)-respiring microbes in terms of $H_2$ uptake was also observed during As(V) reduction in sediment microcosms from an arsenic-contaminated aquifer. These findings may lead to a better understanding of biogeochemical arsenic-mobilization processes that appear to be linked to the sequential microbial reduction of Fe(III) and As(V) in aquifer sediments.

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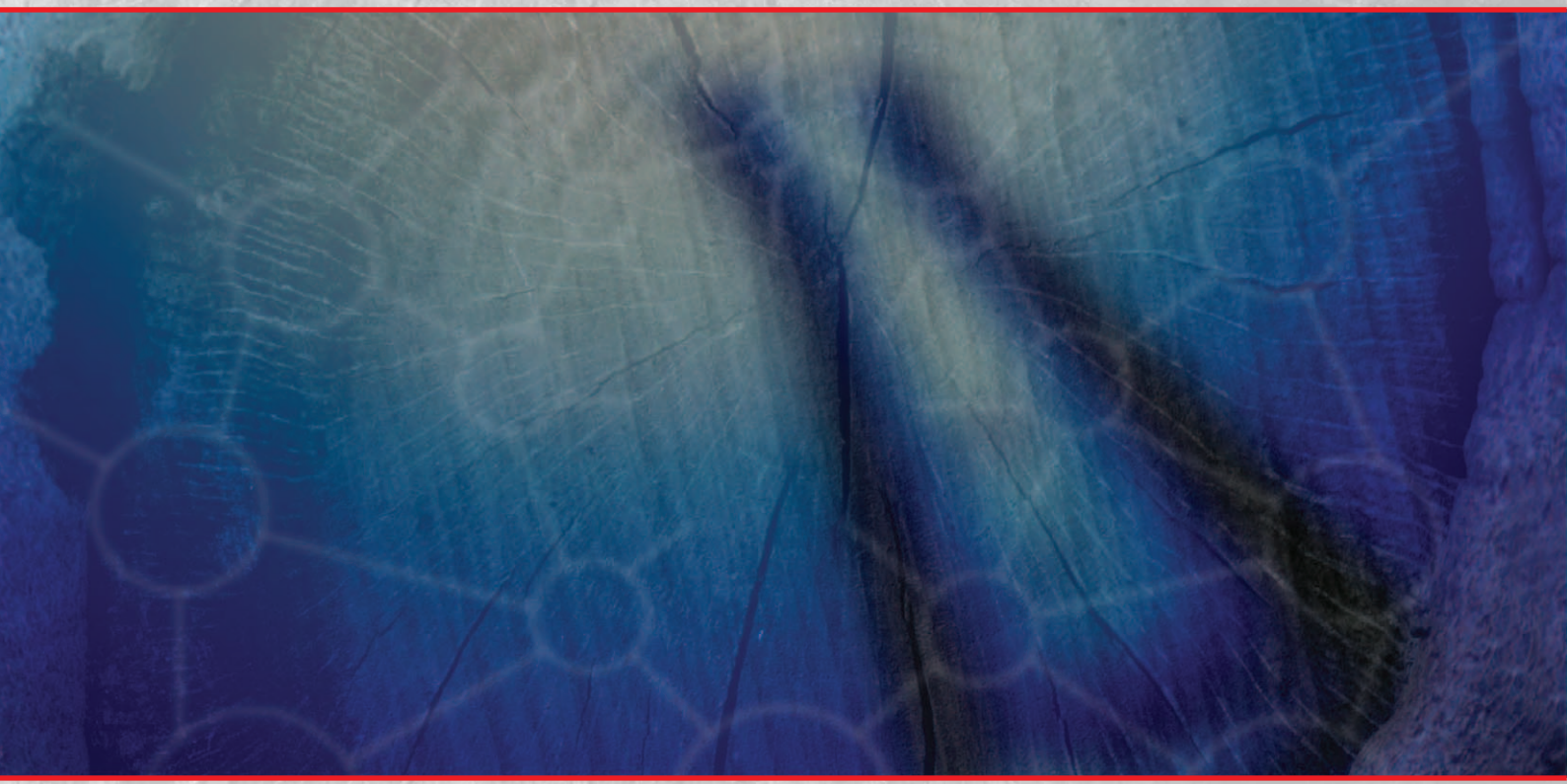

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