Evaluation of redox indicators for determining sulfate-reducing and dechlorinating conditions

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Abstract

An in situ methodology based on covalently bonded redox indicators has been developed for determining when sulfate-reducing conditions exist in environmental samples. Three immobilized redox indicators [thionine (Thi, formal potential at pH 7 \(E^\circ_0 = 52\text{mV} \))], cresyl violet (CV, \(E^\circ_0 = -81\text{mV} \)), and phenosafranine (PSaf, \(E^\circ_0 = -267\text{mV} \)) were tested for their response to sulfide in synthetic solutions and under sulfate-reducing conditions in wastewater slurries. The byproduct of the sulfate-reducing process, sulfide, was found to couple well to CV in the concentration range of 1–100 \(\mu\text{M} \) total sulfide ([S(-II)]) and the pH range of 6–8. Thi, the indicator with the highest formal potential, reacts rapidly with sulfide at levels well below 1 \(\mu\text{M} \) while PSaf, the indicator with the lowest formal potential, does not couple to sulfide at levels in excess of 100 \(\mu\text{M} \) [S(-II)]. The degree of reduction of the indicators (i.e., the fraction of cresyl violet oxidized) in contact with a given level of sulfide can be modeled qualitatively with an equilibrium expression for [S(-II)]-indicator based on the Nernst equation assuming that rhombic sulfur is the product of sulfide oxidation. In a groundwater sample with dechlorinating microbes, reduction of Thi and partial reduction of CV correlated with dechlorination of TCE to cis-DCE.

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1. Introduction

The sulfate-reducing microbial redox level is one of the most reducing redox levels in environmental systems. In terms of formal reduction potentials \((E^\circ_1\), all potentials here are referenced to the standard hydrogen electrode (SHE)), sulfate reduction to sulfide occurs at about \(-220\text{mV} \) at pH 7 \(\text{(Brock et al., 1994)} \). This low redox level is attained in environmental systems when conditions are completely anaerobic and the terminal electron acceptors (TEAs) such as \(\text{NO}_3^-, \text{Mn(IV)} \) and \(\text{Fe(III)} \) (usually) have been depleted \(\text{(Chapelle, 1993)} \). Sulfate-reducers use sulfate as the TEA and commonly lactate, acetate, or \(\text{H}_2 \) as the electron donor in the process of creating \(\text{ATP} \), producing sulfide \(\text{(Brock et al., 1994; Chapelle, 1993)} \).

Microbial reductive dechlorination of the organic contaminants such as trichloroethylene (TCE) to \(\text{cis-dichloroethylene} \) (cis-DCE) normally occurs under highly reducing conditions including sulfate-reducing conditions \(\text{(Rifai et al., 1995)} \). In some microbial cultures, partial transformation of tetrachloroethylene (PCE) or TCE to \(\text{cis-DCE} \) occur as conditions change...
from aerobic to anaerobic, accompanied by the release of sulfide (Semprini, 1997).

The appearance of sulfide in environmental systems (e.g., groundwater aquifers) can have important consequences for the geochemistry of the local environment (Brock et al., 1994; McBride, 1994). Sulfide precipitates many valent metal species including Fe(II), Cu(II), Ni(II), Hg(II) and Cd(II). The bioavailability and toxicity of trace metals such as Pb and Cd in sediments can be controlled by the iron sulfide and are evaluated by determining the amount of acid-volatile sulfide (Di Toro et al., 1992). Sulfide is toxic to many organisms, as it combines with iron in cytochromes and other essential iron compounds in the cell (Brock et al., 1994).

Sulfate-reducing conditions in environmental systems are normally determined by monitoring decreases in sulfate concentration or increases in sulfide concentration. Common means of measuring sulfate include ion chromatography with a conductivity detector and gravimetric or turbidimetric methods based on Ba(II) as a precipitating agent (APHA, 1995). Sulfide is commonly measured with the colorimetric methylene blue method, iodometric titrimetry, or potentiometry with an Ag/Ag2S electrode (APHA, 1995).

Sulfate determination in the field is often difficult. Ion chromatographs are not particularly amenable to field use and gravimetric methods require filtering and drying of the precipitate. Turbidimetric methods, although simple to use in the field, are unselective and subject to interferences by particulate matter in the sample, with a detection limit of only about 1 mg SO4\(^{2-}\)/L (~10 \(\mu\)M) (APHA, 1995). Additionally, there is always the risk of oxidizing reduced sulfur species (e.g., HS\(^{-}\), SO3\(^{2-}\)) to sulfate during sampling, sample storage, or analysis.

Methods for field determination of sulfide are available, but oxidation before determination is always an issue. The methylene blue method is a colorimetric method selective for total sulfide (S\(^{-}\), HS\(^{-}\) and H2S) (APHA, 1995). It is used for commercial field testing kits that provide a detection limit of about 0.06 mg/L (~0.6 \(\mu\)M) total sulfide (CHEMetrics, 1997). Reducing agents such as SO3\(^{2-}\) can interfere with the color development. The use of Ag/Ag2S electrodes in the field is also common and allow direct, in situ measurements of S(II) (detection limit of ~3 mM total sulfide (Frevert, 1980)). Frevert (1980) found that the Ag/Ag2S electrode was more suited than the methylene blue method for making sulfide measurements under conditions of varying ionic strength. However, for quantitative measurements, the Ag/Ag2S electrode requires calibration (APHA, 1995) which is time consuming and very difficult for in situ analysis in the field. Furthermore, Jeroschewski et al. (1996) point out that the Ag/Ag2S electrode can respond to other species (i.e., a mixed potential is observed) and the response deviates from Nernstian below 10 \(\mu\)M total sulfide. Iodometric methods are not selective for sulfide and also respond to sulfate and other reduced sulfur species (APHA, 1995).

Some evidence suggests that the Pt electrode potential (\(E_{Pt}\)) can be used to determine when sulfate-reducing conditions exist. Patrick and Connell (1968), using a soil slurry in an anaerobic bioreactor in which \(E_{Pt}\) could be maintained constant by addition of O2, found that the onset of sulfate reduction occurred at an \(E_{Pt}\) of approximately ~150 mV. In studies of anaerobic marine sediments under sulfate-reducing conditions, Berner (1963) reports \(E_{Pt}\) values between ~150 and ~250 mV. A plot of \(E_{Pt}\) versus log of S\(^{2-}\) concentration calculated from Ag/Ag2S electrode measurements was reasonably linear from 10\(^{-8}\) to 10\(^{-11}\) M S\(^{2-}\) and experimental \(E_{Pt}\) values were close to those predicted with the Nernst equation based on rhombic sulfur as the oxidized sulfur species.

However, there are serious limitations to the use of \(E_{Pt}\) for determining sulfate-reducing conditions including fouling of the electrode surface. Under sulfate-reducing conditions, \(E_{Pt}\) values have been reported to vary between 0 and ~300 mV by different researchers (Lovley and Goodwin, 1988) and overlap \(E_{Pt}\) values observed under Fe(III)-reducing or methanogenic conditions.

In this paper, the application of three redox indicators, thionine (Thi), cresyl violet (CV), and phenosafranine (PSaF), for evaluating sulfate-reducing conditions is presented. These indicators were covalently immobilized to agarose affinity beads and packed into spectrophotometer flow cells. The immobilized indicators were titrated with sulfide at pH 6–8 to determine the effects of pH and sulfide level on indicator speciation (degree of indicator reduction) and the results are compared to predictions made using equilibrium models. Filtered solution from a wastewater slurry in a bioreactor, under sulfate-reducing conditions was pumped through the flow cells. Reduction of the indicators was monitored spectrophotometrically while relevant parameters of the system including [S(II)] and the potential at a Pt electrode (redox potential) were measured concurrently. In a related study, redox indicator speciation was monitored during the microbially mediated redox transformation of trichloroethylene (TCE) to cis-dichloroethylene (cis-DCE) in a bioreactor with a groundwater sample from a contaminated site, rich in TCE dechlorinators.

2. Theory of redox indicators

Redox indicators provide an alternative means of determining when sulfate-reducing conditions exist. Normally the oxidized form is colored and the reduced form is colorless and the absorbance of the redox indicator can be monitored with a spectrophotometer.
As the oxidized indicator reacts with a reductant (e.g., S(-II)), the absorbance decreases and the “reducing power” of the sample can be estimated. The redox half-reaction of a redox indicator is described by

\[
\text{Ind}_{\text{ox}} + n\text{e}^- + m\text{H}^+ \rightarrow \text{Ind}_{\text{red}}.
\]  
(1)

where \(\text{Ind}_{\text{ox}}\) is the oxidized form of the indicator, \(\text{Ind}_{\text{red}}\) is the reduced form, \(n\) is the number of electrons transferred (typically 1 or 2), and \(m\) is the number of protons transferred (typically 0, 1 or 2) and is dependent on the pH (Bishop, 1972).

Many redox indicators are reversible and couple to the Pt electrode (Lemmon et al., 1996; Lemmon, 1995; Mobley, 1992). The redox potential for an indicator \((E_{\text{ind}})\) is determined by the relative concentrations (activities) of the oxidized and reduced species and the Nernst equation,

\[
E_{\text{ind}} = E_{\text{ind}, \text{pH}}^\circ - \frac{RT}{nF} \ln \left( \frac{[\text{Ind}_{\text{red}}]}{[\text{Ind}_{\text{ox}}]} \right),
\]  
(2)

where \(E_{\text{ind}, \text{pH}}^\circ\) is the formal potential of the indicator at the specified pH. This formal potential is different for different indicators and is often a complex function of pH since many indicators have groups such as amines which can be protonated or deprotonated. Experimentally, the concentration ratio in Eq. (2) is normally evaluated by measuring the absorbance of the colored oxidized form and equals \((1-f_{\text{ox}})/f_{\text{ox}}\) where \(f_{\text{ox}}\) is the fraction of indicator oxidized. This fraction is determined as the ratio of the measured absorbance to the absorbance of the fully oxidized form of the indicator. Eq. (2) can then be rewritten as

\[
E_{\text{ind}} = E_{\text{ind}, \text{pH}}^\circ - \frac{RT}{nF} \ln \left( \frac{1-f_{\text{ox}}}{f_{\text{ox}}} \right).
\]  
(3)

Lemmon et al. (1995, 1996) developed an immobilization scheme for several redox indicators. Immobilization is necessary to isolate the indicator molecules from negatively charged soil particles to which they strongly adsorb. Moreover, immobilization of a given indicator allows the development of an in situ sensor that responds to certain reductants and oxidants in environmental systems.

Jones and Ingle (1999, 2001) found that the immobilized redox indicator Thi (formal potential at pH 7 \((E_\text{f}^\circ)\) of +52 mV) coupled well to the reductant Fe(II) \((E_\text{f}^\circ = +54 \text{mV} \text{ for } ~100 \mu\text{M Fe(II)} \text{ at pH 7 with ferricyrdate as the oxidized solid})\). Furthermore, evidence supports the hypothesis that under Fe(III)-reducing conditions, Fe(II), the product of this TMA process, is a primary reductant of Thi. These results suggest that a redox indicator with a formal potential close to the formal potential of a given TMA process may be useful for determining when a given redox level exists.

An indicator for sulfate-reducing conditions could be chosen so that it responds to (is reduced by) a reduced sulfur species such as SO\textsuperscript{3}\textsuperscript{2-} or S(-II) produced under sulfate-reducing conditions. Of these reduced sulfur species, sulfide (H\textsubscript{2}S, HS\textsuperscript{-}, or S\textsuperscript{2-}) is the most common and less transitory product of this TMA process. Sulfite, the initial product of sulfate reduction, is rapidly reduced to sulfide by sulfate-reducing microorganisms (Brock et al., 1994). Furthermore, oxidation of S(-II) (by a given redox indicator) would produce a solid sulfur species (e.g., rhombic sulfur, Srhmb) in a two-electron transfer process rather than produce SO\textsuperscript{3}\textsuperscript{2-} which would require a transfer of eight electrons (Brock et al., 1994; Bishop, 1972). The Srhmb/S\textsuperscript{2-} half-reaction

\[
S^0(\text{rhmb}) + 2e^- = _S^2-(aq)
\]  
(4)

at 25 °C, has a redox potential based on the Nernst equation (Berner, 1963) of

\[
E_{S/S^2-} = -0.475 - 0.0295 \log[S^2-(aq)].
\]  
(5)

The value of \([S^2-(aq)]\) is calculated from the total sulfide concentration \((\text{H}_2\text{S} + [\text{HS}^-] + [S^2-])\) and appropriate \(K_a\)'s (Jones, 1999):

\[
[S^2-(aq)] = \frac{[S(-II)]}{[S^0(\text{rhmb})]} \frac{[S(-II)]}{1 + [H^+]//10^{-14} + [H^+]^2/10^{-7.1}10^{-14}}.
\]  
(6)

At pH 7 and 25 °C, the redox potentials for 1, 10 and 100 μM total sulfide from Eq. (5) are −81, −111, and −140 mV, respectively.

In our laboratory, three redox indicators, Thi, CV, and PSaf, have been characterized in terms of how they couple to sulfide in solution and how they could be useful for determining when sulfate-reducing conditions exist (Lemmon et al., 1996; Lemmon, 1995; Mobley, 1992; Jones, 1999). Of these indicators, CV is the indicator of choice for sulfide. Immobilized CV has a formal potential \((E_\text{f}^\circ = -81 \text{mV})\) nearest the Srhmb/S\textsuperscript{2-} couple at pH 7 and for levels of sulfide found in anaerobic environmental samples (−81 mV for 1 μM total sulfide).

3. Materials and methods

3.1. Instrumentation

All experiments were conducted in an airtight bioreactor system. Shown schematically in Fig. 1, which has been described previously in detail (Lemmon et al., 1996; Jones, 1999). The system includes a sophisticated cross-flow filter for separating liquid from the solid content in the bioreactor and is configured with a Pt-button electrode for \(E_{Pt}\) measurements, a glass pH electrode for pH measurements, and a sulfide electrode to monitor sulfide levels. A microcomputer system is used to monitor electrode potentials, and in some cases,
to control dispensing pumps (FMI Micro P-Petter) to add acid or base to maintain constant pH.

To remove O₂ and promote anoxic conditions, the bioreactor was initially purged with N₂ for several hours or overnight (~50 mL/min) with a gas control system based on a N₂ tank, oxygen trap, and mass flow controller. To minimize loss of H₂S, methane, or other gases which were produced in experiments, the N₂ flow rate was decreased to very low flow values (a bubble or so every 10–20 s) or turned off completely, after anaerobic conditions were achieved (as noted by $E_{Pt} < 0$ mV).

For titration experiments of immobilized indicators with sulfide, the affinity beads with a given immobilized redox indicator were packed into a flow cell (Hellma, 170.700-QS, 1-mm pathlength) and allowed to interact with filtered bioreactor solution pumped through the external loop (Lemmon et al. 1996; Jones, 1999). Absorbance measurements of the immobilized indicators were made with a Hewlett Packard 8452A diode array spectrophotometer. Sulfide injections were made with a 100-μL syringe through a septum (Chemi-inert) in the reactor lid.
Gas headspace samples (for TCE and cis-DCE) were withdrawn through the septum in the reactor lid with a 100-μL gas-tight syringe (Hamilton, SampleLock). Methane was quantified with a HP5890 GC and a calibration method developed by Roberts (1997). TCE and cis-DCE were quantified with a HP 6890 GC with a PID/FID detector and a calibration method developed by Vancheeswaran et al. (1999).

3.2. Chemicals

Deionized water, obtained from a Millipore Milli-Q water system, was used for titration experiments of immobilized redox indicators with sulfide and for preparation of all standards. The redox indicators, Thi, CV, and PSaf, shown in Fig. 2, were obtained from Aldrich. Redox indicators were covalently bonded on 60-μm, cross-linked agarose (4%), affinity beads (Sterogene ALD beads) with a procedure developed by Lemmon (Lemmon, 1995; Lemmon et al., 1996) for Thi and PSaf and modified slightly for immobilization of CV (Jones, 1999; Jones and Ingle, 2001). The immobilization scheme involves a coupling of the amine groups on the redox indicators to aldehyde groups on the affinity beads, forming an imine, which is then reduced to a more stable secondary amine.

A solution of ~0.1 M total sulfide was prepared with Na2S·9H2O (Mallinckrodt) for sulfide titrations. Filtered bioreactor samples were taken from a three-way valve in the external loop and analyzed for total sulfide with CHEMetrics Vacu-vial® ampules which employ the colorimetric methylene blue method for sulfide analysis (APHA, 1995; CHEMetrics, 1997). A small, portable spectrophotometer (Multi-Filter Photometer, CHEMetrics) was used to measure the absorbance. A 0.1 M Cu(NO3)2·2.5H2O (Mallinckrodt) solution was used as a precipitating agent for sulfide.

For titration experiments, buffers were used to adjust the pH of electrolyte systems in the bioreactor and 0.05 M KCl (Mallinckrodt) was used as an electrolyte. Phosphate buffer was prepared with Na2HPO4·7H2O and NaH2PO4 (both from Mallinckrodt). In bioreactor experiments with wastewater slurries, sodium acetate (Mallinckrodt) and then lactic acid (85% or ~10.9 M, Mallinckrodt) was used as an electron donor for sulfate-reducers. Sodium sulfate (Baker), magnesium sulfate (anhydrous, Baker), and potassium phosphate (Mallinckrodt) were used as nutrients. CaCl2·2H2O (Baker) and NH4Cl (EM Science) were added to wastewater slurries to increase ionic strength and to provide a microbial nitrogen source, respectively. The computerized pH-stat system was used to add 1 M

![Thionine](attachment:image1.png)

![Cresyl Violet](attachment:image2.png)

![Phenosafranine](attachment:image3.png)

Fig. 2. Structures and reduction reactions for thionine (Thi), cresyl violet (CV), and phenosafranine (PSaf).
lactic acid (made to pH 1 with HCl (Mallinckrodt)) or 1 M NaOH to adjust the pH.

Solutions of 0.1 M Fe(II) solution in 0.1 M HClO₄, hydroxylamine hydrochloride (10% (w/v) in water), 0.5% (w/v) 1,10-orthophenanthroline, and 50 mg/L Fe(III) solution in 6 mM HCl were prepared as previously described (Jones and Ingle, 2001). For the TCE/groundwater experiment, trichloroethylene (TCE, Aldrich, 99.5%) was used.

3.3. Titrations of immobilized redox indicators with sulfide

The immobilized redox indicators, Thi and CV, were titrated with sulfide at pH 6–8 while immobilized PSaf was titrated only at pH 7. A 0.05 M phosphate buffer was used along with the pH stat system to control the pH.

Each of the three immobilized indicators were packed into separate flow cells (1-mm path length, Fig. 1) (Lemmon et al., 1996) and an initial absorbance spectrum of each was taken. The cells were placed in the external loop and reactor solution was pumped through them. After deaeration of the reactor solution, aliquots (10–100 µL) of ~0.1 M sulfide were injected into the reactor solution via the septum and indicator absorbance, total sulfide level, \( E_{Pt} \), and \( E_{S^2-} \) were measured periodically. In general, 2–3 h were required between additions for the absorbance of the indicators to reach steady-state.

3.4. Behavior of redox indicators in wastewater slurries under sulfate-reducing conditions

Wastewater sludge samples were obtained in 1-L plastic bottles from the City of Corvallis Wastewater Reclamation Plant and contained significant numbers of anaerobic, reducing microbes (methanogens and sulfate-reducers). The samples were stored in a refrigerator for subsequent use. Over time, the slurry separated into two phases: an aqueous upper phase with some particulate matter and a thick organic, sludge-like lower phase.

For bioreactor experiments, ~250 mL of the aqueous upper phase was placed in the reactor along with 750 mL of deaerated tap water. To the reactor slurry, 6.8 g of sodium acetate (50 mmol), 1 g each of sodium sulfate, ammonium chloride and calcium chloride (3.7 mmol, 18.7 mmol, and 6.8 mmol, respectively), 2 g (16.6 mmol) of magnesium sulfate and 0.5 g (3.7 mmol) of potassium phosphate were added. This list of nutrients was adopted from Chapelle (1993) for sulfate-reducing conditions. The slurry was initially purged with N₂ (3–4 h) to maintain the anaerobic integrity of the system and prevent unnecessary exposure of the microbes to O₂. Later (~150 h into the experiment), 4.6 mL (50 mmol) of lactic acid were added to the slurry to act as the electron donor for sulfate-reduction (in place of acetate) because no reduction of sulfate was noted (based on sulfide measurements with CHEMetrics vials).

To minimize clogging of the cross-flow filter in the primary loop due to debris in the slurry, the stirrer controller was turned down to 5–10 rpm, allowing larger debris to settle at the bottom of the reactor. The solution pumped through the primary loop was drawn 1–2 in below the surface of the wastewater solution rather than near the bottom.

The wastewater experiment was conducted in the same manner as titrations of the immobilized indicators (Thi, CV and PSaf) with sulfide including use of the pH stat, except sulfide was produced naturally by the microbes (i.e., no sulfide additions to the reactor were made) and \( E_{S^2-} \) was not measured. Near the end of the experiment, Cu(II) was added to precipitate out the sulfide in the reactor.

3.5. Degradation of TCE in a groundwater sample

A 1-L groundwater sample was obtained from the Dept. of Environmental Engineering at Oregon State University. This sample was originally acquired from a TCE-contaminated site at Lawrence Livermore National Laboratory. It is described in more detail and has been shown to be rich in microbes that dechlorinate TCE to cis-DCE (Vancheeswaran et al., 1999, 2003).

First, the groundwater sample was purged with purified N₂ to remove volatile background components (TCE, cis-DCE, H₂S). Next, the reactor was autoclaved to sterilize the system. Finally, ~970 mL of groundwater was added to the reactor in an anaerobic glove box to minimize exposure of the microbes to O₂ and 80 mL of 100% CO₂ was injected into the reactor headspace via the injection port to decrease the pH of the system (which tended to increase over time). To minimize the risk of O₂ contamination, the pH-stat system was not used.

Immobilelized Thi and CV were packed into flow cells and placed in the external loop of the reactor. Approximately 50 mg of TCE (0.38 mmol) was spiked into the reactor. To minimize O₂ leakage into the system, the pumps were turned on for 2–3 h per day rather than continuously, and absorbance data for the indicators were taken every 1–2 days. Levels of TCE and cis-DCE were monitored by GC over time (taking 50-µL gas headspace samples) and occasionally a sample was taken for sulfide determination. \( E_{Pt} \) and \( E_{S^2-} \) (the potential of the sulfide electrode) were continuously monitored.

4. Results and discussion

4.1. Titration of immobilized indicators with sulfide

The formal potentials at pH 7 of the redox indicators Thi, CV, and PSaf immobilized on agarose beads were
experimentally determined to be +52, -81, and -286 mV, respectively (Lemmon et al., 1996; Jones, 1999). For free (unbound) indicators, the formal potentials for Thi, CV, and PSaf at pH 7 are 66, -75, and -267 mV, respectively, and hence only slightly different from the immobilized indicators. The sulfide concentration necessary to reduce these indicators should increase in the order of highest to lowest formal potential (Thi, CV, PSaf). The data for titrations of immobilized Thi and CV with sulfide at pH 7 shown in Fig. 3 are consistent with this prediction. In fact, Thi is effectively completely reduced throughout the titration while sulfide levels are never large enough to reduce PSaf significantly.

The calculated curves in Fig. 3 are based on the reaction shown below:

$$\text{Ind}_{\text{ox}} + S^{2-}_{\text{aq}} = \text{Ind}_{\text{red}} + S^{0}_{\text{rhmb}} \quad (7)$$

The fraction of oxidized indicator ($f_{\text{ox}}$) is determined from below:

$$f_{\text{ox}} = \frac{1}{\{10^{\frac{2}{M}}(E'_{\text{ind, pH}} - E_{S^{2-}}) + 1\}} \quad (8)$$

This equation is derived by equating the potential of the indicator half-reaction (right side of Eq. (3)) to the potential of the sulfide half-reaction (Eq. (5)) and solving for $f_{\text{ox}}$. The value of $E'_{S^{2-}}$ is calculated from the total sulfide concentration $[\text{S(-II)}_{\text{tot}}]$ measured after steady-state was reached for a given sulfide addition and Eqs. (5) and (6) and the pH.

The data indicate that CV couples well to sulfide in the range of 1–100 μM total sulfide while Thi is useful for low level detection (<1 μM total sulfide). PSaf does not appear useful for sulfide detection at levels expected in environmental samples. The slight apparent reduction of PSaf ($f_{\text{ox}} \approx 0.8$ at $[\text{S(-II)}_{\text{tot}}] > 100 \mu$M is not theoretically predicted and probably an experimental artifact due to shifts in the baseline absorbance from the buildup of solids (e.g., $S^{0}_{\text{rhmb}}$) in the flow cell. The final absorbance of PSaf did not significantly change after contact with DI water saturated with atmospheric O$_2$ supporting the hypothesis that PSaf was not reduced.

The formal potential of CV (~81 mV at pH 7 and $f_{\text{ox}} = 0.5$) is the same as the value calculated for the $S^{0}_{\text{rhmb}}$/S$^{2-}$(aq) couple at 1 μM total sulfide (Eq. (5)). Hence, reduction of CV should be observed with total sulfide levels around 1 μM. From Fig. 3, the experimental values of total sulfide necessary to reduce a given fraction of CV are greater by about a factor of 3 (to the right of the equilibrium curve) than those predicted by the equilibrium model. This discrepancy may be due to non-equilibrium conditions (i.e., slow kinetics of the sulfide/indicator reaction or slow diffusion into the beads) or an incorrect formal potential for the sulfur half-reaction (e.g., activity coefficient effects or formation of reduced sulfur species different from $S^{0}_{\text{rhmb}}$ (e.g., polysulfides, S$^{2-}_3$, S$^{2-}_2$)).

As expected, Thi is reduced at much lower concentrations of sulfide than for CV and theoretically, all the Thi should be reduced with 0.01 μM total sulfide. The value of $f_{\text{ox}}$ for Thi is effectively zero throughout the titration, except for the first data point which is unexpectedly high (~0.2 compared to the calculated value of less than 0.01). The one unexpected value is attributed to slow reaction kinetics and diffusion into the beads or the difficulty of accurately determining low sulfide concentrations. Effectively, the titration data for Thi with sulfide cannot be determined with certainty because it requires that sulfide concentrations can be accurately measured that are below the method detection limit of about 1 μM (CHEMetrics, 1997). It has previously been demonstrated that similar titration data for Thi with Fe(II) do follow the predicted equilibrium model (Jones and Ingle, 2001).

During these titrations, the potential of the Pt electrode varied between ~0.14 and ~0.23 V and was quite scattered, while the sulfide electrode potential decreased from about ~0.25 to ~0.32 V. The experimental values of $E_{\text{Pt}}$ are about 50–100 mV more negative than those predicted by Eq. (5). For total sulfide levels ranging from about 0.3 μM to 100 μM at pH 7, the expected $E_{\text{Pt}}$ range is about ~0.07 V to ~0.14 V (Berner, 1963).

Thi and CV were also titrated with sulfide at pH 6 and 8. Thi was completely reduced at all pH values tested by the time any sulfide was detected. As shown in Fig. 4, titration curves of CV at pH 7 and 8 tended to overlap, but more sulfide was necessary to reduce CV at pH 6 (>10 μM for $f_{\text{ox}} = 0.5$).
The observed behavior is consistent with expected changes in redox potential of the two couples with pH as shown in Fig. 5. As pH decreases, the reduction potential of the sulfide couple increases more rapidly below pH 7 (~59 mV per decade) than that of the indicator (~29 mV per decade) as H₂S becomes the dominant form (rather than HS⁻). The total (equilibrium) sulfide concentration necessary to achieve half-reduction of CV (f₀ₓ = 0.5) at pH 6 is calculated to be ~6 μM.

4.2. Response of indicators in wastewater slurries under sulfate-reducing conditions

The time dependence of the fraction oxidized of immobilized Thi and CV in contact with wastewater slurry at pH 7 under sulfate-reducing conditions is shown in Fig. 6. At ~195 h, the flow cell was placed in the external flow loop and Thi was completely reduced within minutes. As time progressed, CV was over half-reduced (f₀ₓ ≈ 0.3) when total sulfide levels reached ~10 μM (expected f₀ₓ of ~0.1) and nearly completely reduced (f₀ₓ ≈ 0.1) when concentrations exceeded 100 μM (expected f₀ₓ ~ 0). These sulfide levels are greater than expected from the titration data in Fig. 3. In this experiment, the higher turbidity added a positive bias to the sulfide results obtained with the methylene blue method and made it more difficult to estimate the absorbance of the indicator due to baseline shifts because of particle buildup in the flow cell. With the complex sample, partial clogging of the cross-flow filter increased the probability of introducing O₂ into the secondary loop. The initial concentration of sulfate in the reactor solution was greater than 0.89 mM (85 mg/mL), which is above the upper limit of the calibration range of the turbidimetric method (CHEMetrics, 1997).

To evaluate the importance of sulfide as the reductant of the indicators, 0.2 mmol of Cu(II) was first added to the reactor (after 295 h, not shown in Fig. 6) when [S(-II)ₜₒₜ] was above 100 μM. The sulfide precipitated as CuS and no sulfide was detected with the spectrophotometric method. Next, the sample flow cell containing immobilized Thi was momentarily removed from the loop and O₂-saturated DI water was injected into the cell to re-oxidize the indicator. The cell was then placed back in the external loop and no significant reduction of Thi was observed after several hours. This absence of reduction of Thi after removing the soluble sulfide is consistent with the hypothesis that sulfide is the major reductant of the redox indicators under sulfate-reducing conditions.

Fig. 4. Titration data for immobilized CV thionine titrated at pH 6–8.

Fig. 5. Dependence of calculated redox potentials for the CV (E(CV)) and S(-II)/S⁰ couple (E_S²⁻) on pH. The redox potentials are calculated from Eq. (3) and Eqs. (5) and (6), respectively.

Fig. 6. Time dependence of redox indicator speciation, sulfide concentration in a waste water slurry reaching sulfate-reducing conditions at pH 7. During the first 150 h, acetate was used as the electron donor (substrate) for the sulfate-reducing bacteria, but no sulfide was detected. At 150 h, lactate was added as the substrate. Sulfide was detected (by smell) at 190 h, at which point Thi and CV were placed in the external loop.
4.3. Response of indicators during dechlorination of TCE

Results of the experiment with groundwater involving microbial reduction of TCE are shown in Fig. 7. Gradual reduction of Thi occurred from the onset of the experiment and Thi was completely reduced after 400 h (Fig. 7A) when the cis-DCE level reached ~6 μM. After ~200 h, cis-DCE was detected and its concentration increased rapidly after 400 h. A significant fraction of CV was reduced ($f_{ox} \approx 0.6$) after ~600 h. No sulfide was detected in samples taken at about 100 h and near the end of the experiment.

As shown in Fig. 7B, the value of $E_{S^{2-}}$ remained above ~250 mV (expected for $[S(-II)]_{(tot)} < 0.3 \mu$M) over most of the experiment, although it fell to ~300 mV (equivalent to ~1 μM) for a few hours (at about 100 h). $E_{Pt}$ varied significantly throughout the experiment, reaching levels as high as +100 mV and as low as ~150 mV. The potential of the Pt electrode is not particularly indicative of the start of dechlorination, although dechlorination only occurs when the potential is significantly below 0 V. What species control the measured “redox” potential are not known.

The results suggest that complete reduction of Thi and partial reduction of CV is indicative of the onset of TCE reduction to cis-DCE for the culture studied. The reductant (or reductants) that was (were) responsible for completely reducing Thi and partially reducing CV was (were) not identified. Low levels of sulfide, below that detectable by the colorimetric method, could be responsible for the reduction of Thi. Reductants of biochemical origin (e.g., ferredoxins, cytochromes, hydroquinones, cobalamins) may also be responsible for reduction of the indicators.

4.4. Concept of a ‘redox window’ with immobilized redox indicators

A redox scale based on immobilized redox indicators for environmental applications would require a series of indicators with potentials covering the range of about +400 to ~350 mV (at pH 7), which react reversibly with the relevant reductants and oxidants in groundwater and can be monitored spectrophotometrically. As an environmental system became more reducing, one of the indicators would be completely reduced, just as the next (in terms of redox potential) started reducing. To characterize fully the range of environmental redox potentials, as many as 10–12 reversible immobilized indicators (each spanning a redox range of about 60 mV) might be necessary. Any two colored, reversible redox indicators define a “redox window” based on the differences in formal potentials.

Two indicators, Thi and CV, have been identified as useful for this purpose. It takes much lower concentrations of the stronger reductant sulfide (0.3–100 μM) to reduce Thi or CV than Fe(II) (0.1–10 mM) (Lemmon et al., 1996; Jones and Ingle, 2001), and a higher concentration of either reductant to reduce CV compared to Thi. Other immobilized redox indicators could also be employed. In particular, PSaf with a low formal redox potential ($E_f^{th} = -286 $ mV) may be useful for detecting very reducing levels such as methanogenic conditions (Lemmon et al., 1996).

Fortunately, the formal potentials of Thi and CV are very near the calculated potentials for important redox couples and define a significant “redox window”. Not only do both indicators couple well to important major reductants (Fe(II) (Lemmon et al., 1996; Jones, 1999) and S(-II), respectively), but their relative “location” in the environmental redox scale overlaps redox potentials at which transformations of common contaminants and other redox-active species occur. This concept is shown schematically in Fig. 8. The Thi equilibrium curve overlaps both the Fe(III)-reducing potential region and the potential region for transformations of As(V) to As(III). In a recent study (Bos, 1996), the reduction of...
CH4(g) couple (based on the SO4 McBride, 1994). The sulfate-reducing (S) region is calculated concentration would be required for half-reduction of reduction potential were in the same potential region as detecting the onset of sulfate reduction. If the CV DCE transformation (occurring between 0.35 and 0.45 V at pH 7). Hence, CV is useful for total sulfide at pH 7 (equivalent to the formal potential CV started, while Cr (VI) would be completely reduced during the progression to more reducing conditions (at 4.5. pH effects on redox potentials

As pH increases, the redox potentials for most couples illustrated in Fig. 8 shift to a lower or more negative values but to different extents. For the region of pH 6–8, EH for Thi changes ~30 mV per pH unit, while the potential shift for the Fe(OH)3/Fe(II) couple is more pronounced, ~177 mV per pH unit. At pH 6, the center of the redox region for the Fe(OH)3/Fe(II) couple (Fe region) shifts to the left (more positive potentials) about 120 mV more than the redox region for the Thi couple. This difference makes Fe(II) a much poorer reductant for Thi at pH 6 than at pH 7 ([Fe(II)] required is ~30 mM rather than ~0.1 mM for fOx = 0.5). However, if lepidocrocite, instead of ferrihydrite, were the Fe(III)-solid phase formed at pH 6 (Jones and Ingle, 2001), the Fe(II) levels would not need to be so high ([Fe(II)] ≤ 0.9 mM for fOx = 0.5). At pH 8, the Fe redox region is shifted more to the right (negative) of the Thi region, and Fe(II) is an even better reductant than at pH 7.

For CV in the region of pH 6–8, a shift of about ~30 mV per pH unit is expected, which is the same for the S0(rhmb)/H2S couple in the pH region of 7–8. As pH decreases to 6, however, H2S becomes the dominant reduced sulfur species and a two-proton transfer occurs (~60 mV per pH unit) for the couple. Therefore, the S0(rhmb)/H2S couple shifts ~30 mV further to the left (more positive) relative to the CV reduction zone and more total sulfide is required for reduction of CV. However, CV can still be used as an effective indicator of sulfate-reducing conditions (see Fig. 5).

The S region (SO4 2-/H2S couple) has a similar pH dependence as the S0(rhmb)/H2S couple, but remains considerably more negative relative to the CV couple across this pH range. Of the other couples shown, the
redox potential over the pH region 6–8 decreases with increasing pH by 59 mV per pH unit for the CO₂/CH₄ couple (denoted C), ~120 mV per pH unit for the As couple (Bos, 1996) and ~100 mV per pH unit for the Fe couple (Bard et al., 1985). The $E_{H/H}$ dependency for TCE/cis-DCE was not given in the reference (Semprini, 1997).

5. Conclusions

As demonstrated with wastewater slurry, immobilized CV is well suited for the detection of sulfate-reducing conditions and responds to total sulfide in the range of 1–100 μM. Experimental results support the hypothesis that sulfide is the primary reductant of CV under sulfate-reducing conditions. Thi couples well to low levels (< 1 μM) of total sulfide.

Previously, Thi was found to be useful for predicting Fe(III)-reducing conditions (Lemann et al., 1996; Jones and Ingle, 2001) when Fe(II) levels reach about 0.1 mM near pH 7. However, reduction of Thi in contact with an environmental system is not necessarily proof that Fe(II) is the reductant, or that Fe(III)-reducing conditions exist, because stronger reductants such as low level sulfide (< 1 μM) can also reduce Thi. We have continued to use both Thi and CV as tools in many other studies and observed behavior consistent with that reported here.

Evaluating a sample with two or more indicators such as Thi and CV helps to delineate between different possible reduction levels in microbially active samples. For example, if Thi is reduced but CV is not reduced, O₂ is absent and either Fe(III)-reducers are quite active and dominant, or more reducing microbes such as sulfate-reducers are responsible for producing sub-micro-molar levels of stronger reductants such as sulfide. On the other hand, if both Thi and CV are reduced, O₂ is absent, Fe-reducers are likely not active, and either sulfate-reducers are quite active, producing total sulfide levels on the order of 10 μM, or even more reducing microbes (e.g., methanogens) are dominant. pH plays a strong role in the indicator-reductant equilibrium and should be monitored and taken into account with any assessment of redox status with redox indicators.

Immobilized redox indicators can indicate when “redox conditions” or the activity of select microbial populations is either not suitable or appropriate for the transformations of some priority contaminants. For the dechlorinating culture studied in this work, significant reduction of Thi was indicative of conditions sufficiently reducing for redox transformations of TCE to cis-DCE. Reduction of Thi does not necessarily prove that dechlorination is occurring as additional microbial conditions may be necessary. However, if Thi is completely oxidized, it is unlikely that dechlorination is occurring because the theoretical redox potential would be about +0.1 V or higher around pH 7. Significant reduction of CV was not a necessary condition for dechlorination with the culture studied here, but would always be an indication of a low theoretical redox potential (approximately ~0.1 V or less) that could be appropriate for dechlorination.

Determination of redox status in anaerobic samples such as Fe(III)-reducing, sulfate-reducing, or dechlorinating conditions based on redox indicators provides unique advantages compared to conventional methods. Immobilized redox indicators can be configured in flow cells for direct on-line sampling of groundwater or wastewater or deployed in the subsurface, eliminating the need for sample storage and minimizing exposure to O₂. Redox indicator measurements have the potential of providing a better assessment of redox level than Pt electrode measurements and can be simpler than off-line, spectrophotometric determination of [S(II)]. Any method for determination of sulfate-reducing conditions in groundwater based on measuring sulfide or using sulfide as a reductant including redox indicators can give misleading results. Sulfide levels recovered from a given zone can be affected by precipitation of sulfide, leaching of sulfur species from mineral sources, or movement of sulfur species between different zones of the water column or aquifer (Chapelle et al., 1995).

We are exploring new methods to immobilize redox indicators to enhance their applicability in the field for the development of in situ sensors. Procedures are being developed to immobilize redox indicators on thin membrane films instead of affinity beads, to minimize clogging and improve response time. We are also designing flow cells and miniature spectrometers which can be introduced directly into the sample of interest. Immobilized redox indicators will be further evaluated in field and laboratory studies to “calibrate” the reduction of different indicators to major types of microbial conditions and types of contaminant transformations. It would be interesting to explore the correlation between the response of redox indicators such as CV and the level of acid-volatile sulfide in sulfide rich sediments. Near neutral pH, CV would respond to sufficiently high levels of soluble S(II) in equilibrium with metal sulfides.

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