

Evaluation of immobilized redox indicators as reversible, in situ redox sensors for determining Fe(III)-reducing conditions in environmental samples

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Abstract

An in situ methodology based on immobilized redox indicators has been developed to determine when Fe(III)-reducing conditions exist in environmental systems. The redox indicators thionine (Thi, formal potential at pH 7 (E_7^0) equals 66 mV), toluidine blue O (TB, $E_7^0 = 31$ mV), and cresyl violet (CV, $E_7^0 = -75$ mV) have been immobilized to 40–60 μm agarose beads via an amine–aldehyde coupling reaction. These beads were packed into a flow cell to allow spectrophotometric monitoring of the redox state of simple solutions and wastewater slurries pumped from in a bioreactor. Fe(II), a product of microbial activity, at levels observed in real systems reduces both the free (non-immobilized) and immobilized redox indicator to different degrees for samples with pH 6.5 or higher. At pH 7, immobilized Thi and TB are significantly reduced at Fe(II) concentrations greater than 0.1 and 0.3 mM, respectively. CV, with the lowest formal potential, requires Fe(II) levels in excess of 10 mM. The degree of reduction of the indicators (i.e. the fraction of indicator oxidized) observed during titrations can be qualitatively modeled with a simple equilibrium model based on ferrihydrite or lepidocrocite as the Fe(III)-solid phase. The reversibility of Fe(II)-indicator reactions was also demonstrated by showing that the reduced indicator becomes re-oxidized when Fe(II) levels decrease. © 2001 Elsevier Science B.V. All rights reserved.

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1. Introduction

Redox measurements in environmental systems (e.g. groundwater, soil) are fraught with difficulty due to the complexity of natural systems and the

limitations of current methods for evaluating redox status. Most commonly, the Pt electrode has been used as an in situ sensor for measurement of ‘redox level’ [1,2]. Ideally, the measured potential of the Pt electrode (vs. the standard hydrogen electrode, SHE), E_{Pt} , in a system would be equivalent to the system E_{H} . However, most redox-active species in the environment do not couple to, or poison, the Pt electrode or are at such low

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concentrations that they have little or no effect on E_{Pt} [3]. Therefore, redox measurements based on E_{Pt} can be misleading.

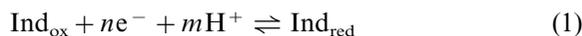
Many microbiologists, soil scientists and environmental engineers have adopted a more practical, non-equilibrium scale for redox level based on microbial activity and the dominant *terminal electron acceptor* (TEA) process. Microbes utilize redox reactions to produce adenosine triphosphate (ATP) for energy [4,5]. Through oxidation of a substrate (usually an organic acid, alcohol, sugar or H_2), electrons can be ‘shuttled’ through an electron transport mechanism, creating ATP, ADP and other important biochemical species [4,5]. The electrons are eventually transferred to another available chemical species, termed the TEA. Important TEAs include O_2 , NO_3^- , Mn(IV) (from Mn(IV) -oxides), Fe(III) (from Fe(III) -oxides), SO_4^{2-} and CO_2 . It is this dominant TEA process which defines the microbial redox level.

The Fe(III) -reducing redox level is considered one of the most important microbial redox levels in terms of its effects on the biogeochemistry of the environment. High Fe(II) concentrations in groundwater cause major problems in drinking water (e.g. discoloration) and are an important concern of water treatment facilities [6]. Nearly all facultative anaerobes are capable of using Fe(III) compounds as terminal electron acceptors (TEAs) [3] and iron (in various forms) is a major component of freshwater and oceanic sediments, constituting between 1 and 9% of the overall bulk [7].

The colorimetric determination of Fe(II) is commonly based on the Fe(II) -chelating agent 1,10-orthophenanthroline (OP or Ferrouin) [8] and can be adapted for field measurements. However, this colorimetric method requires that a sample must be taken from the system being investigated. In situ sensors based on OP have been proposed (sol-gels) but not proven for field use. The reaction of OP with Fe(II) is essentially irreversible which makes it difficult to develop a convenient sensor for Fe(II) that can monitor increases and decreases of $[\text{Fe(II)}]$ over time. Furthermore, the $\text{Fe(II)}-(\text{OP})_3$ method does not directly measure the redox characteristics of the system. There have been some attempts to correlate Pt electrode potentials to the onset of Fe(III) -reduction and pro-

duction of soluble Fe(II) [1,2]. However, Fe(III) -reducing conditions have been observed over a wide range of E_{Pt} values by different researchers (generally +100 to –100 mV [1], although as high as +600 mV and as low as –200 mV [2]). Hence, E_{Pt} does not provide an unambiguous measure of the onset of Fe(III) -reducing conditions or the transition to another microbial level.

Redox indicators which react with Fe(II) present a possible means of determining when Fe(III) -reducing conditions exist. Normally colored in their oxidized form and colorless when reduced, the absorbance of the indicator can be monitored with a spectrophotometer. As the oxidized species reacts with a reductant (e.g. Fe(II)), the absorbance decreases and a relative measure of the ‘reducing power’ of the system can be estimated. The redox half-couple of a redox indicator can be described by



where n , the number of electrons transferred, is typically 1 or 2, and m , the number of protons transferred, is typically 0, 1 or 2 and dependent on the pH [9]. Many redox indicators are reversible and couple to the Pt electrode [10–12].

The redox potential for an indicator (E_{ind}) is determined by the relative concentrations

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

(activities) of the oxidized and reduced species and the Nernst equation, where $E_{\text{ind},m}^{\circ}$ is the formal potential of the indicator and m indicates the pH. This formal potential is different for different indicators and is often a complex function of pH because many indicators have groups such as amines which can be protonated or unprotonated. Experimentally, the concentration ratio in Eq. (2) is evaluated by measuring the absorbance of one of the species, normally the colored oxidized form. In this case, the concentration ratio equals $(1 - f_{\text{ox}})/f_{\text{ox}}$ where f_{ox} is the *fraction of indicator oxidized*, determined from the absorbance of the indicator. Eq. (2) can be rewritten as

$$E_{\text{ind}} = E_{\text{ind},m}^{\circ} - \frac{RT}{nF} \ln \left(\frac{1 - f_{\text{ox}}}{f_{\text{ox}}} \right) \quad (3)$$

An indicator with a formal potential very near that of the dominant Fe(II)/Fe(III)-solid couple could be reduced by Fe(II) at an environmentally-relevant pH if the Fe(II) levels are high enough. The speciation of the indicator (fraction oxidized) could be monitored spectrophotometrically. Furthermore, if an indicator/Fe(II) equilibrium existed and a known model could be applied, the absorbance (and f_{ox}) might be useful for estimating Fe(II) levels in a system.

Previously in our laboratory, Fe(II) was shown to reversibly reduce the oxidized form of various redox indicators (e.g. thionine, methylene blue) near neutral pH [12]. Furthermore, Lemmon et al. [10,11] developed an immobilization scheme for several redox indicators including thionine. Immobilization is necessary to isolate the indicator molecules from the negatively-charged soil particles to which they strongly adsorb. In bioreactor experiments with anaerobic Bashaw soil slurries at pH 6.0, significant reduction of immobilized thionine occurred when Fe(II) concentrations increased to about 0.6 mM [10]. The direct coupling of Fe(II) to thionine (and other indicators) might present a basis for the development of a sensor which is better suited than the Pt electrode to determining when Fe(III)-reducing conditions exist in sub-surface water or soil.

In this paper, the relationship of the redox speciation of the redox indicators thionine, toluidine blue O, and cresyl violet to Fe(II) concentration and pH, in both simple solutions and complex environmental systems, is examined. All experiments were performed in a 2-l, deaerated, bioreactor system. Free (non-immobilized) and immobilized indicators were titrated with Fe(II) at various environmentally-relevant pH values in simple electrolyte solutions in the bioreactor system. The absorbance of the indicator, Fe(II) concentration, and Pt electrode potential (E_{Pt}) were measured during the titrations. The dependence of redox indicator speciation on Fe(II) concentration was compared to that predicted by thermodynamic models. In addition, soil and wastewater slurries were used to confirm that Fe(II), at naturally occurring levels, reduces the redox indicators

at about the same levels as in simple titration experiments. In these experiments, the pH was either controlled at 7.0 or allowed to assume its natural value.

2. Experimental

2.1. Instrumentation

Studies of the effects of Fe(II) concentration on redox indicator speciation were conducted in an air-tight bioreactor system, illustrated in Fig. 1 [11]. The bioreactor was used to achieve and maintain anoxic conditions for both simple electrolyte solutions and soil or wastewater slurries. A Pt-button electrode (Orion) was used for E_{Pt} measurements and a glass pH electrode (Orion) was used to measure the pH. One reference electrode (Ag/AgCl double junction, Orion) was used for all indicator electrodes. Dispensing pumps (Micro Π-Petter, FMI) were used to add acid or base to control the pH. Readings of E_{Pt} and pH were taken at operator-chosen time intervals with a PC using a QuickBASIC program developed to take electrode readings automatically and control the pH. To maintain anoxic conditions, the bioreactor was purged with N₂ gas (pre-purified grade), at a rate ranging between 10 and 50 ml min⁻¹. Residual O₂ in the tank was removed with an oxygen trap (Oxy-Trap, Alltech).

A sophisticated cross-flow filter system provided the continuous separation of liquid from solids in the reactor slurry and direct interaction of soluble redox species with the immobilized redox indicator [10,11]. The primary loop (A) included the cross-flow filter and a peristaltic pump (Masterflex, Cole-Parmer). Reactor solution flowed at 50–100 ml min⁻¹ in and out of the cavity on one side of a Durapore membrane filter (0.65 μm, Millipore). In the secondary loop, a peristaltic pump (model P-3, Pharmacia) pumped a small fraction of total flow (1–2 ml min⁻¹) to the filter, to a spectrophotometric flow cell and then back to the reactor. For off-line analysis, filtered bioreactor samples were taken with the valve in the external loop. To minimize residual O₂ leakage in the external loops, both pumps were

encased in two large, plastic containers which were purged continuously with N_2 . Except for the Tygon tubing used with the two peristaltic pumps, PEEK tubing (1/16" o.d.) with low O_2 permeability was used throughout both primary and secondary loops.

All absorbance measurements were made with a Hewlett Packard 8452A diode array spectrophotometer. The affinity beads with immobilized redox indicators were packed in a flow cell (1-mm

pathlength, Hellma 170.700-QS) and allowed to interact with filtered bioreactor solution pumped through the secondary loop. A schematic of the flow cell is shown in the inset of Fig. 1. During titrations of free dissolved indicators with Fe(II), the indicator solution in the reactor was pumped through a 1-cm pathlength flow cell (Hellma 176.730-QS) in the external loop for absorbance measurements. A spectrophotometer cuvette with a screw cap was used (10-mm pathlength, 3.7 ml

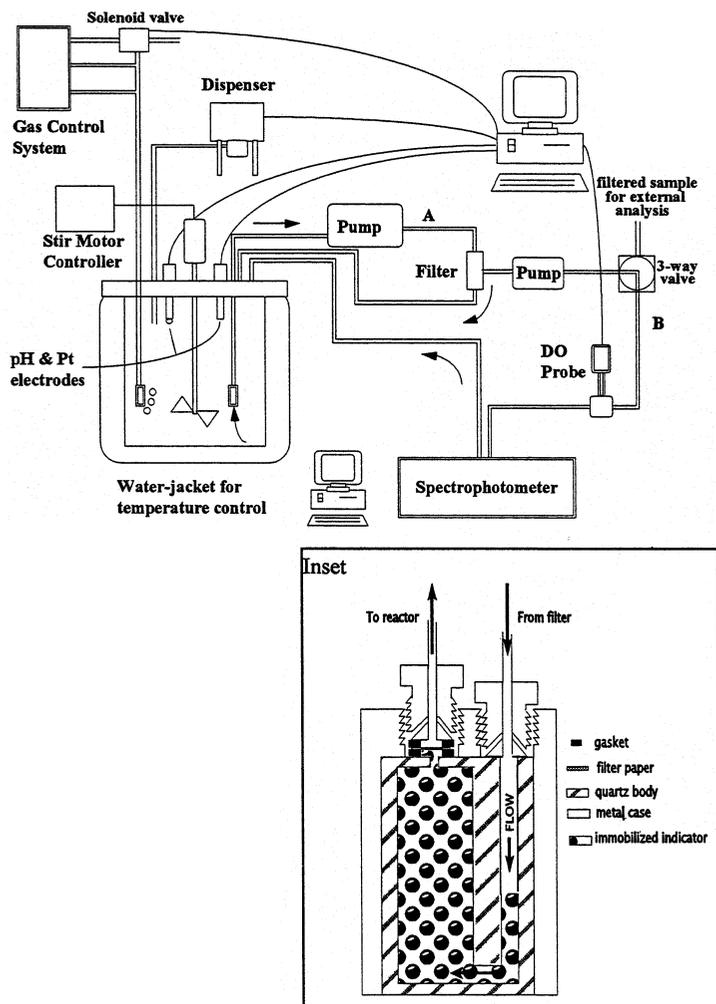


Fig. 1. Bioreactor system for controlling and maintaining redox conditions, pH and anaerobic integrity. Loop A refers to the primary external loop and loop B refers to the secondary external loop, from which samples are taken for analysis. The bioreactor has a 2-l volume and normally contained 1 liter of solution or slurry. The DO probe in loop B was not used for these experiments. The flow cell (inset) is packed with beads containing immobilized indicator, plumbed into loop B, and placed in the spectrophotometer sample compartment.

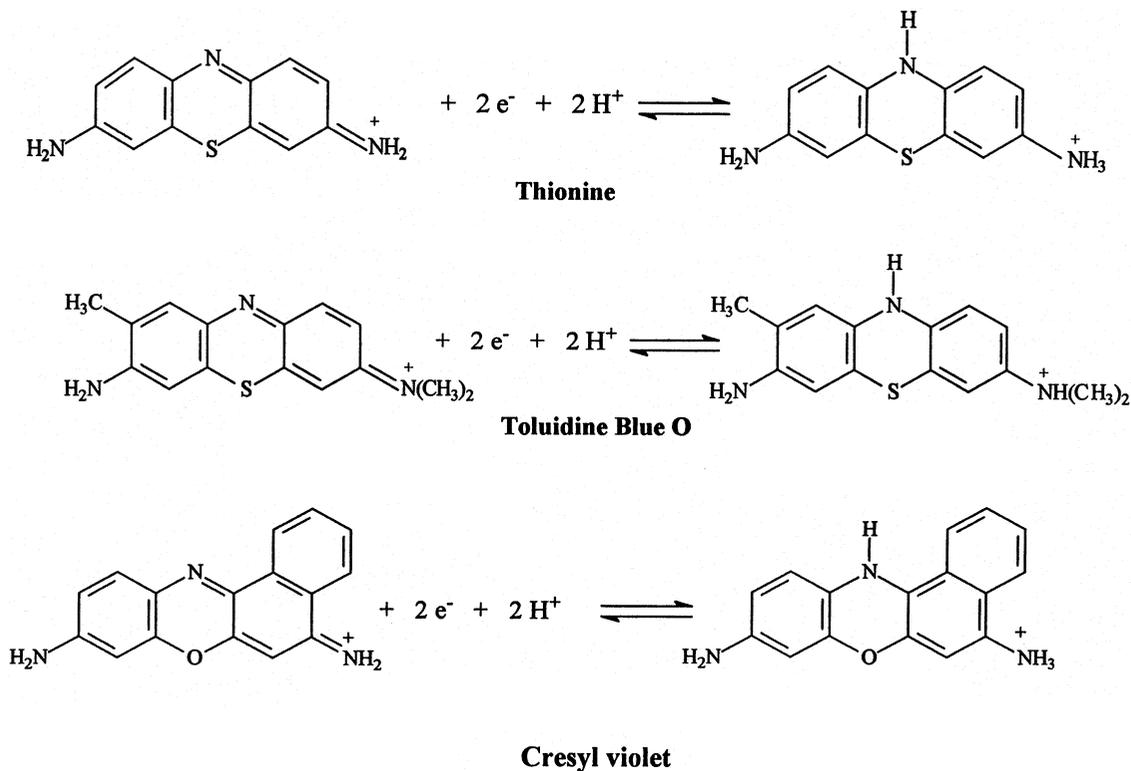


Fig. 2. Structures of thionine (Thi), toluidine blue O (TB) and cresyl violet (CV).

volume, Spectrocell) to quantify soluble Fe(II) with the OP method.

2.2. Immobilization of redox indicators

The redox indicators thionine (Thi, 3,7-diaminophenothiazin-5-ium chloride) and cresyl violet acetate (CV, 5,9-diaminobenzo[*a*]-phenoxazonium acetate) were obtained from Aldrich. Toluidine blue O (TB, tolonium chloride) was obtained from Sigma. The structures of the indicators are shown in Fig. 2. Redox indicators were immobilized on 60- μ m, cross-linked agarose (4%), affinity beads (Sterogene ALD beads) by a procedure developed by Lemmon et al. [10,11] to immobilize thionine. The immobilization scheme involved a coupling of the amine groups on the redox indicators to aldehyde groups on the affinity beads at pH 5–7 (Thi and TB) or pH 3–4

(CV), forming an imine. A NaCNBH₃ (Aldrich) solution was used to reduce the imine bond to a more stable secondary amine. Formal potentials were measured by the method developed by Lemmon et al. [10,11].

2.3. Solution preparation

All solutions and bioreactor slurries were prepared with deionized water obtained from a Millipore Milli-Q water system. Two Fe(II) solutions, 0.5 and 0.05 M in 0.1 M HClO₄, were prepared with Fe(ClO₄)₂·2H₂O (G. Frederick Smith Chemical Co.) and 70% HClO₄ (Mallinkrodt). Hydroxylamine hydrochloride (EM Science) solution (10% (w/v) in water), a 0.5% (w/v) OP (Aldrich) solution in water, and a 50 mg/l Fe(III) (Mallinkrodt) solution in 6 mM HCl (Fisher) were used to prepare Fe(II) standards. pH buffers were prepared from Na₂HPO₄·7H₂O and NaH₂PO₄ (both

from Mallinkrodt) or from TRIZMA hydrochloride (Sigma). When the computerized pH-stat was used, 1 M HCl and 1 M NaOH were added to adjust the pH. A ~240 mM solution of Ti(III) citrate was prepared with 15 ml of TiCl_3 (13% w/w in ~20% HCl, Fluka) and 7.4 g of sodium citrate dihydrate (Mallinkrodt) in 50 ml of ~0.5 M TRIZMA HCl (Sigma) made to pH 7 by addition of NaOH. The solution was then purged with N_2 and stored in a freezer for subsequent use.

2.4. *Bashaw soil and wastewater slurry samples*

Soil samples were taken from a location near Corvallis, OR in the Jackson Frazier Wetlands. The physical and chemical structure of the soil have been described in more detail [10]. The air-dried soil was first crushed, sifted through a 0.5-mm mesh twice, then sifted through a 0.25-mm mesh once until a very fine consistency was obtained. For soil bioreactor experiments, 50 g of this soil sample were added to the bioreactor along with 10 mmol of $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (Baker) and 2 mmol of NH_4Cl (EM Science). DI water was added up to the 1-liter mark and the slurry was then purged with N_2 . The stirring motor speed was maintained in a range between 50 and 100 rev. min^{-1} .

Wastewater sludge samples were obtained in 1-liter plastic bottles from the City of Corvallis Wastewater Reclamation Plant and contained significant numbers of anaerobic, reducing microbes (Fe(III)-reducers, sulfate-reducers, and methanogens). For reactor experiments, ~300 ml of this material was placed in the reactor along with ~700 ml of deaerated DI water and 10 mmol of CaCl_2 . The slurry was immediately purged with N_2 to maintain the anaerobic integrity of the system and prevent unnecessary exposure of the microbes to O_2 . Clogging in the cross-flow filter in the primary loop due to debris in the slurry was offset by reducing the stirrer rotation rate to 5–10 rev. min^{-1} , allowing larger debris to settle at the bottom of the reactor. The solution pumped through the primary loop was taken from 1 to 2" from the top of the wastewater solution.

2.5. *Iron(II) as an important reductant in soil slurries*

The effect of OP on the reduction of free Thi by Fe(II) and Ti(III) was tested. Absorption spectra were taken of 10 μM Thi at pH 7 before and after successive additions of a 50:1 molar excess of Fe(II), a 2000:1 excess of OP, and a 240:1 excess of Ti(III). For all measurements, the sample cell was capped and purged with N_2 before additions.

The effect of OP on the reduction of free Thi by Fe(II) in a Bashaw soil slurry was evaluated as follows. The Bashaw soil slurry, under anaerobic conditions in the bioreactor for several weeks, had a measured soil E_{Pt} of about -250 mV (all E_{Pt} values reported vs. SHE) and pH 7.6 (no pH stat was used). Soluble Fe(II) levels were measured to be ~130 μM and immobilized Thi in the packed flow cell was about 80% reduced ($f_{\text{ox}} = 0.2$). In the capped spectrophotometer cuvette, 1 ml of 20 μM Thi was purged and 1 ml of filtered reactor solution was added directly to the spectrophotometer cuvette via a needle attached directly to PEEK tubing in the external loop. An absorption spectrum was acquired after mixing and a 5-min equilibration time. This procedure was again repeated with 80 μl of the 10% (w/v) OP solution added as the Fe(II)-complexing agent.

2.6. *Titrations of free and immobilized indicators with Fe(II)*

In several experiments, various free and immobilized redox indicators were titrated with Fe(II) at several different pH values. TB and CV, unlike Thi, had not been previously characterized by Lemmon et al. [10] or Mobley [12]. Free (i.e. non-immobilized) indicator solutions of Thi, TB ($\lambda_{\text{max}} = 632$ nm) and CV ($\lambda_{\text{max}} = 586$ nm) were titrated with Fe(II) at pH values of 6.3, 7.0 and 7.5. The pH was maintained constant with the computer-based pH stat system.

The reactor solution was continually pumped through a 1-cm path length flow cell in the secondary loop so that the absorbance of the indicator and samples for Fe(II) determination could be acquired at any time. For these experiments, 1 liter of a deaerated 20 μM solution of the indica-

tor in 0.05–0.1 M KCl (Mallinkrodt) was placed in the reactor and 10–100 μl (0.5–5 μmol) increments of Fe(II) were injected with a syringe. Absorbance measurements were taken before the first injection and typically 0.5–1 h after each injection to allow sufficient time for the system to come to steady-state. Only one sample was taken per addition to determine [Fe(II)]. The wavelength maximum of the OP–Fe(II) complex was sufficiently separated from the wavelength maxima of the redox indicators to allow accurate measurements of Fe(II). Pt electrode measurements were taken throughout the experiment, normally with a 15-min interval.

Titration of immobilized redox indicators with Fe(II) were performed at pH 7 and 8 with 0.05 M TRIZMA buffer to maintain constant pH. No free redox indicator or additional electrolyte was used. Immobilized indicator beads were packed into the 1-mm path length cell (inset of Fig. 1) and an initial absorbance spectrum was taken. Fe(II) was added in the manner described for the free indicator. Because of the slower mass transfer rates of Fe(II) to the immobilized indicator and overall slower reaction times, 2–4 h was allowed for equilibration between additions.

To test the reversibility of the Fe(II)/TB equilibrium, portions of the Fe(II)-buffer reactor solution were removed and then deaerated buffer solution with no Fe(II) was added to bring the volume back to the initial value (to decrease the Fe(II) concentration by 25–100%). When Fe(II) levels dropped below about 100 μM , Fe(II) additions were again made to the reactor to reverse the effect.

2.7. Reactor experiments with wastewater slurries under Fe(III)-reducing conditions

Wastewater sludge was added to the bioreactor and the pH was maintained at 7.0 by automatic addition of acid. To stimulate Fe(III)-reducing conditions, an Fe(III) source, a variety of nutrients, and sodium acetate (a substrate) were added, as described by Chapelle [6]. To the wastewater slurry, 13.5 g (50 mmol) of $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ (G. Frederick Smith Chemical Co.) were added along with 6.8 g of sodium acetate (Mallinkrodt), 1.5 g of

NH_4Cl , 0.6 g of NaH_2PO_4 , 0.1 g of KCl, 2.5 g of NaHCO_3 (Mallinkrodt), and 1.0 g of NaCl (EM Science). Immobilized Thi and CV were packed into separate flow cells and placed in the external loop of the bioreactor system. Throughout the course of the experiment, absorbance measurements of the two indicators were taken and samples were withdrawn to determine Fe(II) and total sulfide (quantified with CHEMetrics methods using disposable solution vials).

3. Results and discussion

3.1. Iron(II) as a reductant of thionine in soil slurries

The effect of orthophenanthroline (OP) on the reduction of Thi by Fe(II) was initially tested. OP rapidly chelates Fe(II), forming an orange-red complex with a λ_{max} of 510 nm [8]. Without OP, the reduction of Thi by Fe(II) was rapid as indicated by the eradication of the Thi absorbance band at 600 nm. When OP was present in the reaction mixture, Thi was not reduced until Ti(III) was added. These results support the hypothesis that OP in the solution impedes the reduction of Thi by complexing the Fe(II) before it can react with Thi rather than by chemically interacting with the Thi directly so that it cannot be reduced.

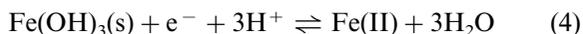
When a filtered aliquot of anaerobic soil slurry from the bioreactor with $\sim 130 \mu\text{M}$ Fe(II) was mixed with Thi, the Thi was reduced rapidly as indicated by the disappearance of the absorption band (Fig. 3A). With OP present to complex the Fe(II), the Thi band remained (Fig. 3B). The decrease in the absorbance of the Thi band is attributed to the 50% dilution of the Thi solution with 1 ml of reactor solution, rather than the partial reduction of the indicator.

The results of this study strongly support the hypothesis that the soluble Fe(II) in the soil slurry is a major reductant of the redox indicator Thi and perhaps the *primary* reductant of Thi in soil slurries under Fe(III)-reducing conditions. However, Fe(II) may not be the *only* reductant of redox indicators under Fe(III)-reducing condi-

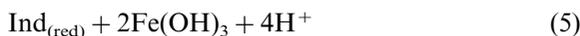
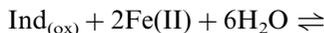
tions. Other significant reductants may be present at lower concentrations or may have slower reaction kinetics with the redox indicator.

3.2. Titrations of free indicators with Fe(II) at pH 7

The reduction of the redox indicators Thi, TB, or CV requires the transfer of two electrons and two protons (Eq. (1) with $n=2$ and $m=2$). Fe(II), acting as the reductant, transfers one electron for every Fe(II) atom. This half-reaction is



where, at relevant environmental pH values (5–8), the Fe(III) form is an insoluble solid [13]. The coupling of the two half reactions (Eqs. (1) and (4)) yields



assuming that an Fe(III)-solid phase forms. The potential of the Fe(II)/Fe(OH)₃ couple can be calculated with the Nernst equation

$$E_{\text{Fe(OH)}_3/\text{Fe(II)}} = E_m^{\circ} - \frac{RT}{nF} \ln [\text{Fe(II)}] \quad (6)$$

where E_m° is the formal potential of the Fe couple at a given pH.

For an Fe(II)/indicator system at equilibrium, the redox potentials of the indicator and Fe(OH)₃/Fe(II) couples (Eqs. (3) and (6), respectively) would be equal. From this equality, the fraction of indicator oxidized can be calculated as shown in Eq. (7).

$$f_{\text{ox}} = \frac{1}{\left(10^{\left[\frac{2}{0.059} (E_{\text{ind},m}^{\circ} - E_{\text{Fe(OH)}_3/\text{Fe(II),m}^{\circ}}) + \log [\text{Fe(II)}]^2 \right]} + 1 \right)} \quad (7)$$

The likely Fe(III) products, ferrihydrite or lepidocrocite, have E_7° (formal potential at pH 7) values of -0.182 and -0.274 V, respectively [3].

Titration data for the three free indicators with Fe(II) at pH 7.0 are shown in Fig. 4. In Fig. 4A it is clear that the order of reduction is Thi, TB and CV. For CV, reduction occurred at a considerably lower potential and required a much greater amount (and equilibrium concentration) of Fe(II) (Fig. 4B).

Formal potentials at pH 7 are compared in Table 1 and the values measured for Thi and TB in this work correspond well with literature values (except for one literature value for TB [14]) and values previously measured in this laboratory. However, the formal potential measured for CV differs significantly from the single literature value found [14].

In Table 2, measured Fe(II) levels for $f_{\text{ox}} = 0.5$ for Thi, TB and CV are compared to Fe(II) levels expected based on Fe(II)/ferrihydrite and Fe(II)/lepidocrocite models, based on mass balance, and based on total Fe(II) added. For the free indicator titrations, the measured Fe(II) levels lie between those predicted from the ferrihydrite and lepidocrocite models (although the lepidocrocite

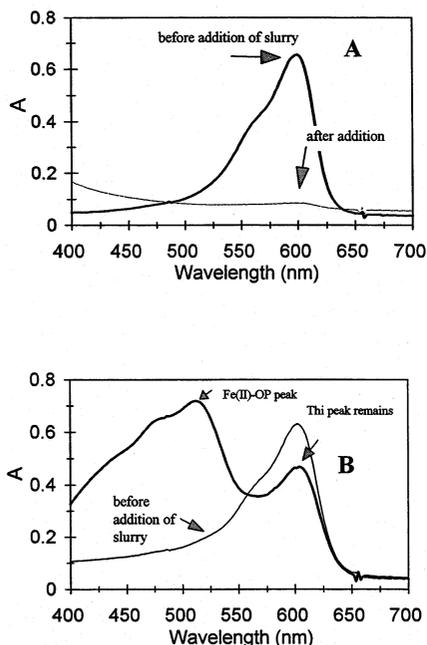


Fig. 3. Effect of orthophenanthroline (OP) on the reduction of thionine (Thi) by a soil slurry containing Fe(II). Spectrum of 1 ml of 20 μM Thi before and after addition of 1 ml of bioreactor solution, with a measured Fe(II) concentration of 130 μM , without (A) and with (B) OP. The absorbance band at 600 nm is due to oxidized Thi and the absorbance band at 510 nm is from the Fe(II)–OP complex.

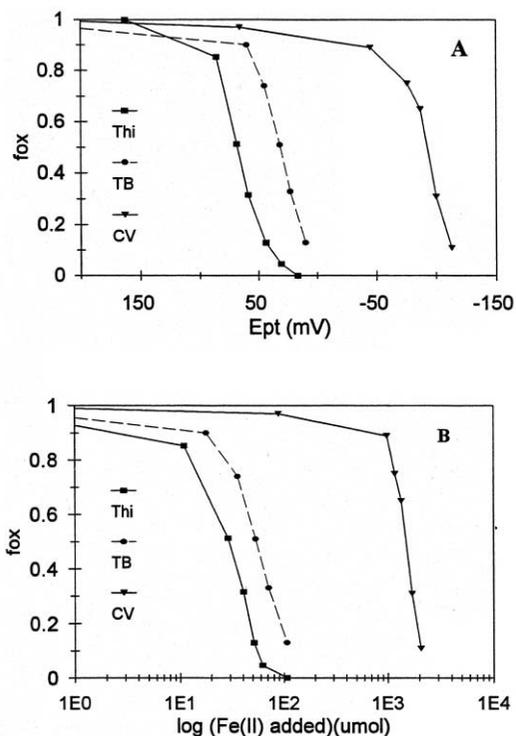


Fig. 4. Comparison of results for titrations of 20 μM Thi, TB and CV with Fe(II) at pH 7 in terms of E_{Pt} (A) and total Fe(II) added (B). The indicators poison the Pt electrode as they are reduced. CV, with the much lower formal potential, requires far more Fe(II) for reduction than the other two indicators.

model more closely predicts the levels). For Thi, the lepidocrocite model predicts that Fe(II) levels would be nearly unmeasurable at half-reduction (the detection limit with the OP method is 2–5

μM [15]) which is consistent with the results obtained.

3.3. pH dependence of reduction of free thionine with Fe(II)

The equilibrium reaction between Fe(II) and an indicator to form an iron hydroxide solid phase (Eq. (5)), such as ferrihydrite or lepidocrocite, is very pH-dependent, and the concentration of Fe(II) required to reduce a given fraction of indicator decreases with increasing pH. The E_{H}/pH dependence of the half-reaction for the redox indicator is not obvious in Eq. (2) because it is incorporated in the formal potential.

For thiazine type indicators (e.g. thionine), E_m^0 , the formal potential at a specified pH, is given by Bishop [9]. For pH values from 6 to 8 (relevant environmental pH values) this equation simplifies to

$$E_m^0 \approx E^0 + \frac{RT}{nF} \ln (K_{r1}[\text{H}^+]^2 + K_{r1}K_{r2}[\text{H}^+]) \quad (8)$$

where K_{r1} and K_{r2} are acid dissociation constants of the reduced form of the indicator. For Thi, K_{r1} and K_{r2} are $10^{-4.38}$ and $10^{-5.3}$, respectively [9].

In Fig. 5, the theoretical E_{H}/pH dependence of the free indicator Thi is compared to that for a ferrihydrite Fe(III)-solid phase at three different Fe(II) concentrations. The Thi potential is not nearly as dependent on pH (about 29 mV/pH unit in region of pH 6–8) as the Fe(II)/ferrihydrite potential, which exhibits a slope of 177 mV per unit pH. At pH about 6.5 and below, the Fe(II)/ferrihydrite equilibrium lines are above (at a

Table 1
Formal potentials of free indicators at pH 7

Indicator	$E_7^0(\text{mV})^a$	$E_7^0(\text{mV})^b$	$E_7^0(\text{mV})^c$	$E_7^0(\text{mV})^d$	$E_7^0(\text{mV})^e$ (this work)
Thionine	+64	+62	+66	NT ^f	+66
Toluidine blue	+34	-11	NT	+31	+31
Cresyl violet	Not found	-167	NT	-75	-92

^a From Bishop [9].

^b From Jacob [14].

^c From Ti(III) citrate titration [10,11].

^d Interpolated from Ti(III) citrate titration [3].

^e Interpolated from Fe(II) titration of single indicators (data from Fig. 4).

^f NT, not tested.

Table 2

Comparison of measured and calculated Fe(II) levels for $f_{\text{ox}} = 0.5$ for the free indicators Thi, TB and CV at pH 7

Indicator	E_7° (mV) ^a	[Fe(II)] (μM)			[Fe(II)] (μM) ^d calculated with Fe(II)/ferrihydrite model	[Fe(II)] (μM) ^d calculated with Fe(II)/lepidocrocite model
		Measured	Total Fe(II) added ^b	Based on mass balance ^c		
Thi	+66	NM ^c	30	10	60	2
TB	+31	28	55	30	250	7
CV	-75	1100	1500	1480	15 000	420

^a From Ti(III) citrate titration measurements [3,11].

^b Calculated as total mol of Fe(II) added to achieve half reduction ($f_{\text{ox}} = 0.5$) of a given indicator (interpolated from data in Ref. [3]).

^c Based on total Fe(II) added and assumed stoichiometric reaction of Fe(II) with redox indicator $[\text{Fe(II)}]_{\text{mb}} = [(\text{mol Fe(II) added}) - 2 \times (\text{mol of indicator reduced})] / \{\text{volume of reactor solution}\}$.

^d Based on solving Eq. (7) (in text) for [Fe(II)] with $f_{\text{ox}} = 0.5$ $[\text{Fe(II)}](M) = \text{antilog}((-1/0.059) \times (E_{\text{ind},m}^{\circ} - E_{\text{Fe},m}^{\circ}))$ where $E_{\text{Fe},m}^{\circ}$ is the formal potential for the Fe(II)/Fe(III)-solid couple at pH m , and at pH 7 is -0.182 V for ferrihydrite and -0.274 V for the lepidocrocite solid phases.

^e NM, not measured.

higher potential) that of the Thi line (Fe(II) is stable relative to Thi and Thi remains oxidized). Significantly below pH 7, only very high levels of Fe(II) ($\gg 1$ mM) would reduce Thi. Near pH 7, Fe(II) (> 100 μM) has the ability to reduce Thi, and significantly above pH 7, ferrihydrite would be the predominant form of the Fe couple ($[\text{Fe(II)}] > 10$ μM) [3]. Significantly above pH 7, even sub-micromolar levels of Fe(II) should completely reduce Thi. Clearly, pH is a more dominant factor than Fe(II) concentration in the Thi/Fe(II) equilibrium.

The results of titrations of 20 μM free Thi at pH values of 6.3 and 7.5 are shown in Fig. 6. In the figure, the Thi redox potential ($E(\text{thi})$), calculated from the indicator speciation, corresponds closely to the measured E_{Pt} during the titration. The corresponding least-squares fits based on equilibrium models for two Fe(III) oxides and the measured Fe(II) concentration are also shown. At pH 6.3, the calculated Fe(II) potentials (in the figure, $E(\text{ferr})$ and $E(\text{lep})$, respectively) lie significantly closer to the lepidocrocite equilibrium line than the ferrihydrite equilibrium line. However, at pH 7.5, the Fe(II) data appear closer to the ferrihydrite line. Because the formal potential of the Fe(II)/ferrihydrite couple is less negative than

that of the Fe(II)/lepidocrocite couple by nearly 100 mV (-0.182 vs. -0.274 V, respectively), Fe(II) would be a weaker reductant if ferrihydrite were the Fe(III)-product formed at pH 7.5.

Calculated and experimental Fe(II) levels necessary to achieve a given level of reduction of free Thi at pH 6.3 and 7.5 are summarized in Table 3. At pH 6.3, much more Fe(II) was required to reduce the Thi than at pH 7.5, following the expected pattern. The nature of the Fe(III)-hy-

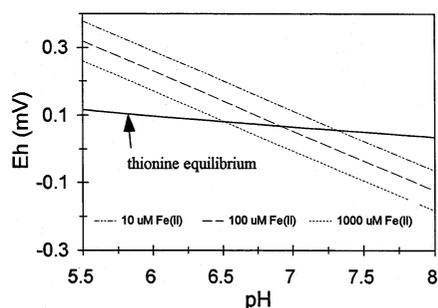


Fig. 5. Dependence of E_{H} of the free Thi couple (E_{ind}) and the Fe(II)/ferrihydrite couple on pH. The Thi line is based on Eqs. (3) and (8) with $f_{\text{ox}} = 0.5$ (half-reduced). The three Fe lines are based on Fe(II) concentrations of 10, 100 and 1000 μM and Eq. (6). For pH 6, 7 and 8, the theoretical Fe(II) concentration (based on the ferrihydrite model) to reduce half the Thi is 18 mM, 63 μM and 0.2 μM, respectively.

Table 3
Comparison of Fe(II) titration data of 20 μM free Thi at pH 6.3 and 7.5

pH	[Fe(II)] (μM) (for observable reduction)	[Fe(II)] (μM) $f_{\text{ox}} = 0.5$ measured (added) ^a	[Fe(II)] (μM) $f_{\text{ox}} < 0.1$ measured (added)	Stoichiometry ^b	[Fe(II)] (μM) ^c $f_{\text{ox}} = 0.5$ or (0.1) calculated ferrihydrite model	[Fe(II)] (μM) ^c $f_{\text{ox}} = 0.5$ or (0.1) calculated lepidocrocite model
6.3	> 100	200 (270)	500 (600)	~ 20:1	3300 (27 000)	92 (760)
7.5	NM ^d	NM (30)	35 (75)	~ 3.8:1	4 (8)	0.1 (0.2)

^a μmoles added to 1 liter.

^b Based on total Fe(II) added to completely reduce 20 μM Thi; 2:1 is expected by balanced reaction equation.

^c Based on Eq. (7) with $f_{\text{ox}} = 0.1$ and $E_{\text{ind},m}^0$ given by Eq. (8).

^d NM, not measurable.

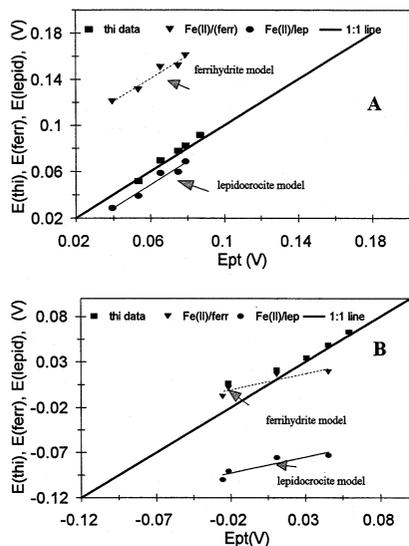


Fig. 6. Comparison of redox potentials for the titration of 20 μM free Thi with Fe(II) at pH 6.3 (A) and 7.5 (B). The redox potential of the indicator ($E(\text{thi})$) is calculated from Eqs. (3) and (8) and the measured speciation. The redox potentials based on the Fe(II)/lepidocrocite and Fe(II)/ferrihydrite equilibrium models and the measured Fe(II) concentration are calculated with Eq. (6) and the appropriate formal potential.

droxide phase formed and the extent of surface adsorption of Fe(II) may change with pH as supported by the data in Fig. 6.

3.4. Titrations of immobilized indicators with Fe(II)

Data for the titrations of immobilized Thi, TB and CV with Fe(II) at pH 7 are shown in Fig. 7 and the results are summarized in Table 4. As with the corresponding free indicators, Fe(II) concentrations about two orders of magnitude greater were required to reduce a given fraction of CV relative to Thi. Upon immobilization, the formal potentials (relative to the free indicators) of Thi and CV decreased by 12 and 6 mV, respectively, and of TB increased by 5 mV.

Much more Fe(II) (by an order of magnitude) is required to achieve a given level of reduction (Table 4) of all immobilized indicators relative to the free indicators. This behavior is not explained by the small shifts in formal potential. From Eq. (7), the concentration of Fe(II) at pH 7 required to reduce half the indicator would increase by a factor of ~ 2

due to a negative shift in the formal potential of 10 mV. The more dramatic change observed suggests that the environment (e.g. ionic strength) of the indicator immobilized within the agarose beads is different than for the free indicator or the nature of the Fe(III) oxide formed within the beads (e.g. the formal potential) may be different. Theoretically, the difference in the formal potentials of the indicator and Fe couples would have to increase by 56 mV to increase by a factor of 10 the concentration of Fe(II) required to reduce half the indicator. Also, the effective concentration of the immobilized indicator within the beads is considerably higher than that for experiments with free indicator (estimated to be over 100 μM [10,11]).

The experimental titration data shown in Fig. 7 agree more closely with the equilibrium line predicted by the Fe(II)/ferrihydrite model than that of the Fe(II)/lepidocrocite model. The Fe(II) concentration required to reduce Thi or TB is a factor of about two greater than expected from the equilibrium model based on ferrihydrite. Similar S-shaped curves calculated with lepidocrocite as the Fe(III)-solid phase (not shown) were shifted about two orders of magnitude toward lower [Fe(II)] compared to the curves shown. The significant scatter in the experimental data for all three indica-

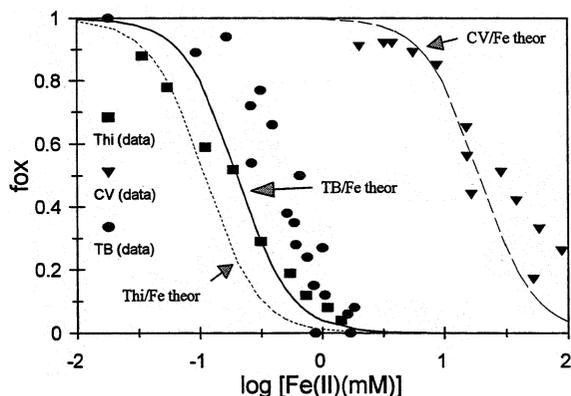


Fig. 7. Comparison of titration data of immobilized Thi, TB and CV with Fe(II) to results predicted from equilibrium models based on ferrihydrite as the Fe(III)-solid phase at pH 7. The theoretical sigmoidal-shaped curves are based on calculations for the equilibrium between a given indicator couple and the ferrihydrite/Fe(II) couple (Eq. (7)) and the measured Fe(II) concentration.

Table 4
Summary of results of titrations of immobilized indicators with Fe(II) at pH 7

Redox indicator	E_7^0 (mV) free	E_7^0 (mV) immobilized	[Fe(II)] (μM) $f_{\text{ox}} = 0.5$ immobilized (free)	[Fe(II)] (μM) $f_{\text{ox}} = 0.5$ immobilized based on lepidocrocite model ^b	[Fe(II)] (μM) $f_{\text{ox}} = 0.5$ immobilized based on ferrihydrite model ^b
Thi	+66	+52	200 (NM ^a)	3	100
TB	+31	+36	400 (28)	6	200
CV	–75	–81	16 000 (1100)	540	19 000

^a ND, not determined.

^b Based on Eq. (7).

tors may be due to small pH variations or uncertainties in absorbance values due to light scattering from the buildup of Fe(III)-solids in the flow cell over time. However, the trend of the data indicates that the model developed does work qualitatively.

Titration data at pH 8 are summarized in Table 5. As expected, substantially less Fe(II) was required to reduce the immobilized indicators relative to pH 7 as with the free indicators. With either indicator, more Fe(II) was added to achieve half reduction than predicted by equilibrium models with ferrihydrite or lepidocrocite as the solid phase.

The data in Fig. 8 illustrate the effects of raising and lowering (by dilution) the Fe(II) concentration on the speciation of immobilized TB at pH 7. Note that the redox speciation of the TB tracks Fe(II) concentration during both addition and dilution stages. These data provide strong evidence of the reversibility of the indicator/Fe(II) redox reaction. Fe(II) concentrations are a factor of 2–3 higher than predicted by the equilibrium model with ferrihydrite as the solid phase.

In many experiments involving titrations of the immobilized indicators with Fe(II), a buildup of a brownish layer (believed to be Fe(III)-oxides) on the agarose beads in the flow cell coincided with the initial reduction of the indicators. It is hypothesized that as Fe(II) concentrations decrease in the reactor solution, the Fe(III)-oxides coated on the surfaces within the beads are responsible for the re-oxidation of the indicator. The Fe(III)-solids may also provide a catalytic surface or provide a high localized Fe(II) concentration that enhances the rate of reduction of the indicator by Fe(II).

During titrations of free indicators, Fe(III)-oxide

buildup was not observed. Oxide buildup is more obvious in the packed flow cell because it is indicated spectrophotometrically by an increasing baseline absorbance and the confinement of the indicator and Fe(III) oxides to a localized small volume.

3.5. Evaluation of immobilized thionine and cresyl violet in wastewater slurries

Fig. 9A shows the time dependence of Fe(II) levels and the speciation of immobilized Thi and CV in contact with a wastewater slurry under Fe(III)-reducing conditions at pH 7. The Fe(II) levels in the wastewater slurry rose to just over 0.4 mM (~ 90 h into the experiment), which was sufficient to reduce $\sim 75\%$ of the Thi ($f_{\text{ox}} = 0.25$). The absorbance of CV decreased slightly (to $f_{\text{ox}} = 0.8$), but changes this small over a week or more can be due to changes in the packing density of the beads. The Fe(II) that was required to achieve the observed level of reduction of Thi was a factor of two or three higher than predicted by the equilibrium model based on ferrihydrite as the solid. Similar results were previously obtained for titrations of immobilized Thi with Fe(II) in a simple buffer solution (Fig. 7). In both wastewater and simple solutions at pH 7, more than 0.4 mM Fe(II) was necessary for significant reduction of CV ($f_{\text{ox}} < 0.8$).

The Pt electrode remained poised at about -260 mV for the duration of the experiment. Often a value of E_{Pt} this negative at pH 7 is indicative of the sulfate-reducers and methanogens in the slurry. However, no sulfide was detected (with CHEMet-

Table 5
Comparison of fraction of immobilized Thi and CV reduced at pH 7 and 8

Indicator	[Fe(II)] (μM) pH 7 ^a for			[Fe(II)] (μM) pH 8 for			[Fe(II)] (μM) pH 8 for	[Fe(II)] (μM) pH 8 for
	$f_{ox} \approx 0.8$	$f_{ox} = 0.5$	$f_{ox} < 0.1$	$f_{ox} \approx 0.8$	$f_{ox} = 0.5$	$f_{ox} < 0.1$	$f_{ox} = 0.5$ based on lepidocrocite model ^b	$f_{ox} = 0.5$ based on ferrihydrite model ^b
Thi	60	200	650	NM ^c	40	150	0.01	0.3
CV	10 000	16 000	ND ^d	600	ND	ND	2	60

^a Data from Table 4 ($f_{ox} = 0.5$) and estimated from Fig. 7.

^b Based on Eq. (7) and $E_{ind,m}^{0'}$ given by Eq. (8) with the assumption that the pH dependence of the formal potential of immobilized Thi and CV is similar to that of the free Thi.

^c NM, not measured.

^d ND, not determined.

rics vials, the detection limit is about 0.5 μM total sulfide). No attempt was made to measure methane in the reactor headspace.

4. Conclusions

In the soil and wastewater slurries studied, Fe(II) appears to be a major reductant of the redox indicator Thi under Fe(III)-reducing conditions. Experimental results with OP suggest that Fe(II) is indeed an *important* reductant, if not the *only* reductant, of Thi when microbially controlled Fe(III)-reducing conditions are dominant.

Thionine appears to be well suited as an indicator for Fe(III)-reducing conditions for samples with pH 6.5 or higher because the levels of Fe(II) produced under these conditions will reduce Thi to different degrees. Fe(II) levels in subsurface water can typically range from non-detectable up to the order of 1 mM [16]. Above pH ~ 6.5, Thi is reduced by Fe(II) levels greater than about 0.1 mM. Lower levels of Fe(II) (~ 10 μM or less) can substantially reduce Thi at pH values greater than ~ 7.5. Significantly below pH 6.5, [Fe(II)] must be greater than 1 mM to reduce a significant fraction of Thi which is unlikely in most field situations. TB, with a formal potential ~ 15 mV below that of Thi, requires Fe(II) levels a factor of two or three higher at a given pH than those for Thi to achieve the same level of reduction (f_{ox}).

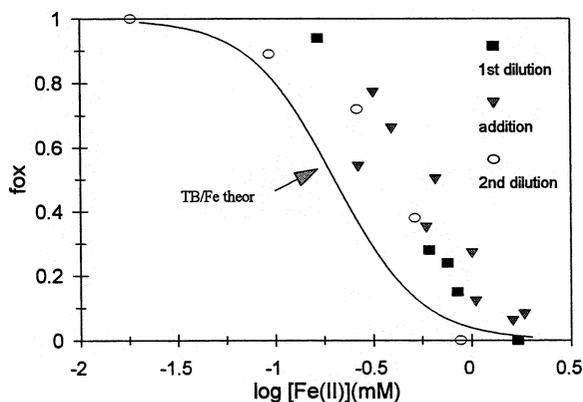


Fig. 8. Dependence of speciation of TB on Fe(II) concentration at pH 7, showing reversibility of Fe(II)–TB equilibrium. Fe(II) was first added to the reactor to completely reduce TB. The solution was then diluted, more Fe(II) was added, and the solution was diluted again.

Development of reversible field sensors to detect Fe(II) and Fe(III)-reducing conditions based on Thi and TB appears to be feasible because the Fe(II)/indicator equilibrium is reversible (i.e. reduced indicator is re-oxidized when the Fe(II) level is lowered). It is hypothesized that a buildup of Fe(III)-hydroxides in the flow cell (observed as a brownish discoloration and increase in background absorbance) is a critical aspect of this reversibility. Interestingly, initial reduction of the indicator did not occur until some discoloration was observed.

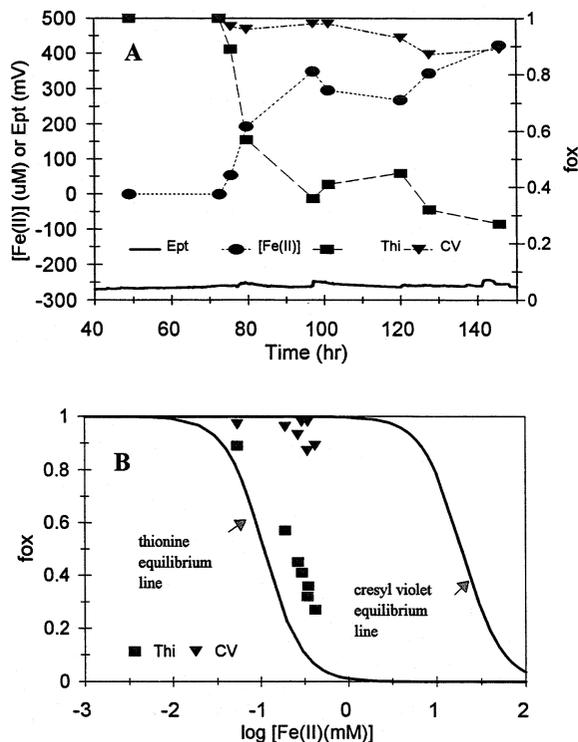


Fig. 9. Correlation of the redox speciation of immobilized Thi and CV to Fe(II) levels in a wastewater slurry (A) and comparison to equilibrium models (B) at pH 7. The equilibrium model is based on ferrihydrite as the Fe(III)-solid phase (Eq. (7)).

The degree of reduction of the indicators observed during titrations can be qualitatively predicted with a simple equilibrium model. A quantitative description is not possible because the nature of the Fe(III)-solid phase (and hence the formal redox potential) is not known, appears to vary with pH, and is affected by the immobilization. With immobilized indicators at pH 7–8, the fraction of indicator oxidized can be better predicted with a model based on the Fe(III)-solid ferrihydrite. For free Thi, experimental results are better predicted at pH 6.3 with a Fe(II)/lepidocrocite model and at pH 7.5 by the Fe(II)/ferrihydrite model.

It is difficult to determine if the equilibrium model used is appropriate or if correct parameters (e.g. formal potentials) are being applied. A more

sophisticated model would have to consider both soluble Fe(II) and Fe(II) adsorbed on the Fe(III)-hydroxides as reductants and the possibility of mixed Fe(III)-solid phases. There is also evidence that the amount of Fe(II) necessary to achieve half-reduction of an indicator increases (but not the formal potential) with indicator concentration. This uncertainty in the nature of the Fe(III)-solid phase (e.g. formal potential) and effect of adsorbed Fe(II) leads to difficulties in accurately estimating Fe(II) levels with immobilized redox indicators. Redox indicators respond not just to the Fe(II) level but to the *actual reducing ability of Fe(II)* in a particular situation which is affected by the nature of the Fe(III)-oxide or Fe(III) complexes formed and the pH.

Immobilized and free redox indicators couple to Fe(II) in much the same manner and their redox characteristics are affected similarly by pH. However, there are important differences, especially in terms of their applicability to environmental field analysis. An immobilized indicator is fixed to a sensor surface, will not be adsorbed to particles in ground water or soils, and can be retracted from the sample (allowing for multiple analyses). The free indicators studied, once mixed with the sample, cannot be extracted easily for later use because they readily adsorb to the surfaces of minerals. Nevertheless, free indicator tends to react and come to equilibrium more quickly with Fe(II) (15–30 min) than does indicator immobilized on beads (1–2 h). The slower response for immobilized indicators is due in part to the need for filtering and pumping ($0.5\text{--}1.0\text{ ml min}^{-1}$) the sample to a flow cell and the low rates of mass transfer through the beads in the flow cell. For environmental measurements of sub-surface samples, redox conditions change slowly so that fast response is not always critical. Preliminary studies indicate that thionine can be immobilized on thin membrane films with the same coupling chemistry and that the response time can be 5–10 min with high concentrations of Fe(II). Clogging problems are also reduced by replacing beads with a film format.

We are currently exploring several aspects of sensors based on redox indicators to use in the field to evaluate redox conditions in groundwater. We

propose that determination of Fe(III)-reducing conditions with immobilized indicators will be more accurate than with Pt electrode measurements and be simpler, more rapid, and possibly more accurate than off-line, spectrophotometric determination of [Fe(II)].

Immobilized redox indicators may be useful to determine when redox transformations of priority contaminants (e.g. As, TCE) are occurring. Recent bioreactor studies with soil slurries indicate that As(V) is reduced to As(III) when reduction of Thi occurs [3,17]. Reduction of thionine is not proof that Fe(II) is the reductant or of Fe(III)-reducing conditions. Thionine is reduced by other reductants than Fe(II) such as sulfide. However, reduction of Thi clearly indicates the presence of a reductant at a sufficient concentration to generate a 'redox potential' low enough to reduce thionine. This lower 'redox potential' is indicative of fairly reducing and anoxic conditions under which Fe(III) reduction could occur. Additional information is required to reach a more definitive conclusion. For example, other redox indicators such as CV, which are reduced at significantly lower redox potentials, might be used in combination with Thi to distinguish between different microbial redox levels.

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