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A Revised Iron Extraction Protocol for Environmental Samples Rich in Nitrite and Carbonate

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ABSTRACT

Wet-chemical iron extraction is widely applied to quantify the mineral-bound ferriferous fraction of sediments and soils. As previously shown, this method is strongly affected by the composition of the soil/sediment. Samples enriched in mostly microbially produced nitrite require the removal of the nitrite-containing aqueous phase or the replacement of HCl with sulfamic acid (SA) as the extractant. In this study, we show that sedimentary carbonate buffers SA, inhibiting the stabilization of Fe(II) and effective extraction of iron. We therefore provide a revised extraction protocol which allows the preservation of low pH conditions, leading to efficient iron extraction in nitrite- and carbonate-enriched samples.

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Iron quantification; sediments; soils

Introduction

Iron (Fe) is the most abundant redox active transition metal in the earth crust. Naturally it occurs mainly as reduced ferrous (Fe(II)) and oxidized ferric Fe (Fe(III)) (Cornell & Schwertmann 2003; Ehrlich et al. 2016). Natural soils and sediments contain around 4%wt total Fe on average, ranging from as less as 0.2% up to 55% (Murad & Fischer 1987; Essington 2015). Fe biogeochemical cycling is affected by a number of abiotic and microbial redox processes (Schmidt et al. 2010; Melton et al. 2014), which strongly impact carbon (C) (Lalonde et al. 2012), oxygen (O) (Kanzaki & Murakami 2013), nitrogen (N) (Benz et al. 1998) and sulfur (S) cycles (Canfield et al. 1992) as well as nutrient and contaminant mobility (Borch et al. 2010; Vaughan & Lloyd 2011). The availability of Fe, including solubility and crystallinity, as well as the redox speciation of Fe are, hence, of interest for the characterization of environmental soil and sediment samples and lab experiments.

To identify and quantify Fe species and to characterize Fe mineralogy, various methods can be used, e.g., X-ray diffraction, Mössbauer spectroscopy or synchrotron-based X-ray absorption spectroscopy (Posth et al. 2014). As these methods are costly, time consuming and require sophisticated analytical equipment, wet-chemical Fe extraction and subsequent quantification—and in some cases speciation analysis—of dissolved Fe represent valid alternatives that are commonly used in the fields of geochemistry and geomicrobiology. A variety of protocols for wet-chemical Fe extraction have been developed, differing in the type of extracting agent, incubation time and conditions such as temperature, light or shaking (Heron et al. 1994; Kostka & Luther 1994; Poulton & Canfield 2005; Wallmann et al. 1993). Different protocols allow the extraction of different operationally defined Fe mineralogical and redox fractions and consequently to acquire information about Fe crystallinity, e.g., extraction of "adsorbed or carbonate bound Fe" by shaking with 1 M Na-acetate at room temperature for 1–5 h (Tessier et al. 1979), extraction of "poorly crystalline" Fe minerals by shaking with 0.5–1 M HCl at room temperature (Heron et al. 1994; Porsch & Kappler 2011) or extraction of "total Fe" by concentrated HCl at 70°C for up to 24 h (Heron et al. 1994; Porsch & Kappler 2011). The sequential application of extraction protocols using increasing concentrations of acids allows the extraction of several Fe fractions from the very same sample, providing detailed information about the Fe mineral composition (Heron et al. 1994; Poulton & Canfield 2005; Tessier et al. 1979). The quantification of extracted Fe is commonly done using spectrophotometric assays (Braunschweig et al. 2012; Verschoor & Molot 2013) based on agents such as ferrozine (Stookey 1970) or phenanthroline (Clark 1962). Ferrozine as well as phenanthroline form stable colored complexes with Fe^{II}_{diss}. The concentration of these complexes, that is proportional to the concentration of Fe^{II}_{diss}, can be quantified via their absorption at 562 or 533 nm, respectively. Extracted Fe^{III}_{diss} is reduced to Fe^{II}_{diss} by hydroxylamine hydrochloride or ascorbic acid (Verschoor & Molot 2013) and the so-obtained total Fe is subsequently analyzed in the same way. Fe^{III}_{diss} can then be calculated as the difference of total Fe and Fe^{II}_{diss}. Both the extraction and the quantification of iron can be affected by interfering ions in samples that have to be analyzed. The ferrozine assay is strongly affected by interference with heavy metals such as cobalt and copper (Stookey 1970; Kundra et al. 1974) or high concentrations of Fe^{III}_{diss} (Im et al. 2013). The interfering agents compete with the complexation of Fe^{II}_{diss} and ferrozine. For known sample compositions, these

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interferences can be handled by including the respective agents to standard solutions used for the calibration.

A major interfering compound for classical Fe extraction is nitrite (Klueglein & Kappler 2013), which is formed as an intermediate of denitrification processes (Klueglein et al. 2014). Under acidic conditions, which are required for Fe extraction, nitrite is protonated instantaneously to nitrous acid which decomposes into NO_2 and NO, both rapidly oxidizing Fe(II) abiotically according to the following equations:

$$NO_2^- + H^+ \rightarrow HNO_2 \quad (1)$$

$$2\ HNO_2 \rightarrow NO_2 + NO + H_2O \quad (2)$$

$$NO_2 + 2\ Fe^{2+} + 2\ H^+ \rightarrow 2\ Fe^{3+} + NO + H_2O \quad (3)$$

$$NO + Fe^{2+} + H^+ \rightarrow Fe^{3+} + HNO \quad (4)$$

$$2\ HNO \rightarrow N_2O\uparrow + H_2O \quad (5)$$

This effect is massively increased under oxic conditions as NO_2 is regenerated by the reaction of NO with O_2 (Klueglein & Kappler 2013). Fe^{II}_{diss} is subsequently not stabilized by HCl; on the contrary, the oxidation of Fe^{II}_{diss} is even enhanced. The oxidation of Fe^{II}_{diss} by nitrite is often prevented by centrifugation or filtering to separate the liquid phase containing nitrite from the solid phase of which iron needs to be extracted by acidification. However, adsorbed nitrite as well as nitrite remaining in the porewater of the sample may react with Fe(II) during the subsequent acidic extraction of iron. This reaction can be inhibited by using 40 mM sulfamic acid (SA; HSO_3NH_2) as the extractant instead of 1 M HCl. SA reacts with nitrous acid forming nitrogen gas and sulfuric acid (Marouf-Khelifa et al. 2006).

$$HNO_2 + HSO_3NH_2 \rightarrow H_2SO_4 + N_2 + H_2O \quad (6)$$

It was therefore suggested by Klueglein & Kappler (2013) to replace HCl with SA for Fe extraction in samples potentially containing nitrite. Fe extraction using SA has proven to be successful in lab systems such as culture media as well as for environmental samples (Laufer et al. 2016a; Li et al. 2015; Robertson et al. 2016; Xiu et al. 2016). In this study, we show that when analyzing Fe-spiked fresh water sediments we discovered that high carbonate contents of the sediments strongly interfered with the extraction of Fe using SA as the extractant. We, therefore, revised the existing Fe extraction protocols with the aim (i) to develop a Fe extraction protocol suitable for sediment and soil samples that contain carbonate as well as nitrite and (ii) to determine the range of carbonate and nitrite concentrations that require the application of different available protocols.

Materials and methods

Medium preparation

All experiments were performed using freshwater medium (FWM) containing the following salts (per liter): 0.6 g KH_2PO_4, 0.3 g NH_4Cl, 0.025 g $MgSO_4 * 7\ H_2O$, 0.4 g $MgCl_2 * 6\ H_2O$ and 0.1 g $CaCl_2 * 2\ H_2O$. The medium was prepared anoxically in a Widdel flask with a headspace of N_2/CO_2 (90:10) and was buffered with 22 mM bicarbonate buffer (Hegler et al. 2008). Additionally, 1 ml of a vitamin solution, 1 ml of a trace element solution and 1 ml of a selenite–tungstate solution were added to one liter medium (Ehrenreich & Widdel 1994). The pH was adjusted to 7.1 using either anoxic 1 M HCl or anoxic 0.5 M Na_2CO_3.

Sediment samples

Sediment samples were taken in September 2015 from the littoral zone of Lake Constance (47°41′42.63″N, 9°11′40.29″E) at 0.4 m water depth (Melton et al. 2012). Samples were transported to the laboratory at 4°C and stored at 10°C until processing. Sediment mineralogy and composition were determined via micro-X-ray diffraction (μXRD) (Bruker D8 Discover X-ray diffraction instrument; Bruker AXS GmbH, Germany) and X-ray fluorescence (XRF) (Bruker AXS S4 Pioneer X-ray fluorescence spectrometer; Bruker AXS GmbH, Germany). The carbonate content was quantified by a gravimetric method for loss of carbon dioxide (Loeppert & Suarez 1982) using 1 g of dried sediment and 10 ml of 3 M HCl. Loss of CO_2 was determined by the difference between initial and final weights of flask + acid + sediment. The carbonate (CO_3^{2-} %) content of the sediment was then calculated as described by Loeppert & Suarez (1982) according to the following equation:

$$CO_3^{2-}\ \% = \left(\frac{\text{g } CO_2 \text{ lost}}{\text{g soil}}\right) * \left(\frac{\text{g } CO_3^{2-}\ \text{mol}^{-1}}{\text{g } CO_2 \text{mol}^{-1}}\right) * 100 \quad (7)$$

with $(\frac{\text{g } CO_2 \text{ lost}}{\text{g soil}})$ describing the weight portion of the original soil sample that was lost as CO_2 and $(\frac{\text{g } CO_3^{2-}\ \text{mol}^{-1}}{\text{g } CO_2\ \text{mol}^{-1}})$ used to calculate the amount of CO_3^{2-} of which this CO_2 was produced.

Experimental setup

For each setup 5 g natural freshwater sediment was mixed with 50 ml anoxic FWM and spiked either with 5 mM ferrihydrite prepared according to Raven et al. (1998) and Amstaetter et al. (2012) and 2 mM $NaNO_2$ or with 5 mM $FeCl_2$ and 2 mM $NaNO_2$. All setups were prepared in triplicates. All extraction experiments were performed right after mixing medium and sediment to prevent biotic or abiotic reactions in the setups.

For extraction of poorly crystalline iron and stabilization of ferrous iron, one of the following three acids was used: 1 M HCl (further on referred to as HCl) or 40 mM SA prepared in Millipore (MQ) water (further on referred to as SA/MQ) or 40 mM SA prepared in 1 M HCl (further on referred to as SA/HCl).

1 ml subsamples of sediment/medium slurry were taken using syringes (needle diameter 1.8 mm) and transferred into 1.5 ml Eppendorf tubes. For separation of dissolved and solid Fe fractions, additional 1 ml subsamples were centrifuged for 5 min at 14,000 g. The supernatant was mixed 1:10 with one of the acids defined above in order to stabilize ferrous iron. The sediment pellet was mixed with 900 μl acid (Figure 1a). For quantification of overall Fe concentrations, the slurry was acidified in 1:10 (100 μl slurry + 900 μl acid) or 1:2 (500 μl slurry + 500 μl acid) mixtures (Figure 1b). All extraction samples containing sediment

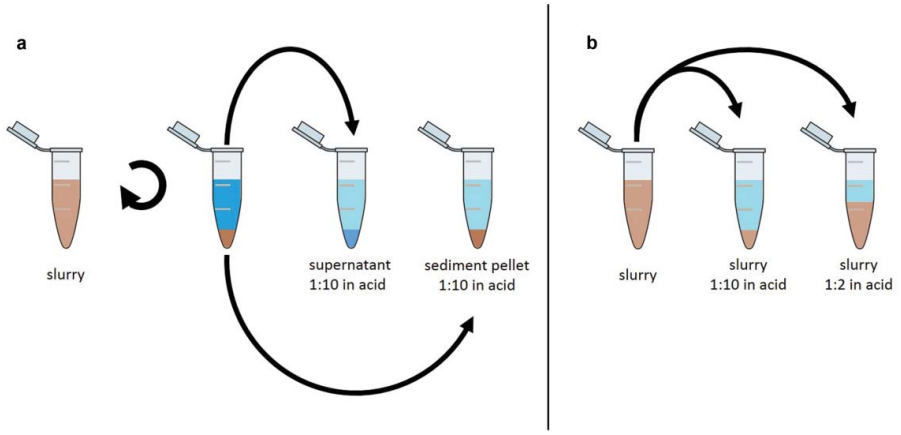

Figure 1. Microcosm subsample processing for iron extraction and stabilization. (a) Quantification of dissolved iron from the supernatant and solid phase iron from the sediment pellet sum up to total iron. (b) Sediment slurry measurements allow direct quantification of total iron.

were incubated at room temperature on a shaker for 1 h at 150 rpm to dissolve poorly crystalline iron.

All sample preparations were performed under anoxic N_2 atmosphere.

Analytical methods

Total iron and Fe(II) were measured spectrophotometrically using the ferrozine assay according to Stookey (1970) but adapted for microtiter plates. 20 μl of a sample (medium or extracted Fe from sediment or slurry, each stabilized in acid as described in the paragraph above) was mixed with 80 μl of 1 M HCl to quantify Fe^{II}_{diss} or with 80 μl hydroxylamine hydrochloride (HAHCl, 10% w/v in 1 M HCl) to quantify total Fe, i.e., $Fe^{II}_{diss} + Fe^{III}_{diss}$. 100 μl of ferrozine solution (0.1% w/v in 50% ammonium acetate) was added after 30 min incubation time for HAHCl. The ferrozine was given 5 min to react with Fe^{II}_{diss} before absorbance at 562 nm was measured in a plate reader (Multiskan^{TM} GO Microplate Spectrophotometer, Thermo Scientific). Three technical replicates of each sample have been measured. Fe^{III}_{diss} concentrations were calculated as the difference of total iron and Fe^{II}_{diss}. Standard Fe(II) solutions were prepared as dilutions of $Fe(II)(NH_4)_2(SO_4)_2$ in either 1 M HCl, 40 mM SA/MQ or 40 mM SA/HCl.

For nitrite quantification, subsamples of batch microcosms were diluted in the same way as for Fe quantification. In case of HCl, this should lead to oxidation of Fe(II) by nitrite while both mixtures containing SA should remove nitrite and, hence, stabilize Fe(II). In contrast to Fe extraction, nitrite samples were measured immediately after dilution using a continuous flow analysis (CFA) system (AutoAnalyzer 3 HR, SEAL Analytical) (Laufer et al. 2016b). To account for possible matrix effects, the respective acids without sample were measured and background levels were subtracted.

pH was measured in the supernatant of Fe extractions using pH indicator strips (Merck).

Results and discussion

Properties of natural lake constance sediment

μXRD analysis showed that the Lake Constance littoral sediment consisted mainly of quartz, calcite and dolomite. This was supported by XRF data showing 56.35% SiO, 16.23% CaO and 2.27% MgO with CaO and MgO accounting for calcite and dolomite. The presence of these carbonate minerals was confirmed by the loss of CO_2 after acidification which revealed a carbonate content of 19.6 $\pm$ 2.8%.

The total Fe content of dry weight sediment according to XRF ranges from 1.31% to 1.58%. In batch microcosm setups without amendment of Fe this resulted in 0.61 $\pm$ 0.19 mM ferrous and 0.04 $\pm$ 0.02 mM ferric iron. Nitrite could not be detected in non-amended setups.

Influence of sediment on extractant pH

Table 1 shows the pH in all extraction setups. When using 1 M HCl or SA/HCl, the pH was less than 1. The pH of the extraction setups using SA/MQ increased with increasing sediment content. In 1:10 (v/v) mixtures of the slurry with SA/MQ the pH stayed below 2, but increased to pH 5.5 in 1:2 (v/v) mixtures of slurry with SA/MQ and even up to pH 6 when pure pelleted sediment was acidified with SA/MQ. This buffering effect is caused by sedimentary carbonate as confirmed by titration of SA/MQ with Na_2CO_3 solution (Figure 2).

Effect of aqueous nitrite on Fe speciation in supernatant samples

Figure 3 shows the results of the quantification of dissolved ferrous and dissolved ferric iron as well as nitrite from the supernatant that was collected after centrifugation of the sediment slurry (Figure 1a) that was spiked with 2 mM nitrite and either 5 mM $FeCl_2$ or 5 mM ferrihydrite.

Acidification of the supernatant of the microcosms spiked with Fe(II) and nitrite (Figure 3a) with 40 mM

Table 1. pH values of acidified samples from batch microcosm using different acids.

	Supernatant 1:10	Slurry 1:10	Slurry 1:2	Pellet 1:10
1 M HCl	0–0.5	0–0.5	0.5–1	0–0.5
40 mM SA in 1 M HCl	0–0.5	0–0.5	0.5–1	0–0.5
40 mM SA	0.5–1	1.5–2	5–5.5	5.5–6

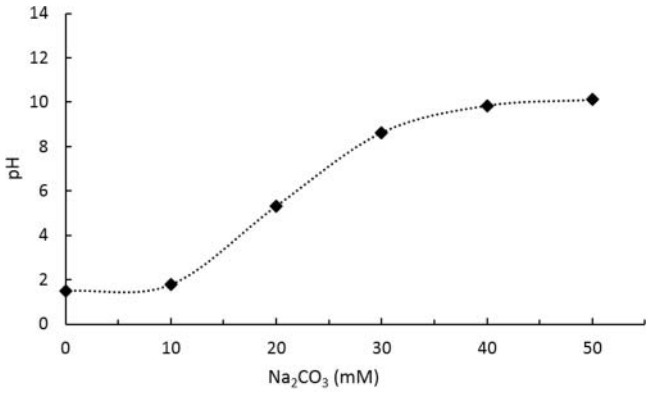

Figure 2. Stepwise addition of Na_2CO_3 solution to 40 mM sulfamic acid shows the strong buffering effect of carbonate at concentrations higher than 10 mM.

SA/MQ in order to stabilize the iron speciation that resulted in a complete removal of nitrite by reaction with SA, thus stabilizing a total of 4.18 ± 0.05 mM Fe(II) with no Fe(III) detectable. Using 1 M HCl to acidify the supernatant resulted in the stabilization of 2.27 ± 0.04 mM Fe (II) and the oxidation of 1.73 ± 0.16 mM Fe(II) to Fe(III) coupled to a removal of 1.34 mM nitrite. This corresponds to the oxidation of 43.2 ± 2.6% of the Fe(II) initially present in the supernatant by reaction with nitrite. This data confirms the abiotic oxidation of Fe(II) by nitrite and the prevention of this reaction by the use of SA as shown by Klueglein & Kappler 2013. The acidification of the supernatant with 40 mM SA/HCl provided very similar results as SA/MQ, also leading to a complete removal of nitrite by reaction with SA while stabilizing 4.02 ± 0.08 mM Fe(II), also with no detectable Fe(III).

In microcosms that were spiked with the Fe(III) mineral ferrihydrite and nitrite (Figure 3b), we detected neither Fe (II) nor Fe(III) after acidification of the supernatant with 1 M HCl. However, we detected 1.75 ± 0.06 mM nitrite in the supernatant. The fact that nitrite is preserved in samples in which Fe(II) is absent in the supernatant again confirm that the oxidation of Fe(II) in the samples shown in Figure 3a was caused by the reaction with nitrite. The use of SA/HCl or SA/MQ both provided complete removal of nitrite. Using SA/HCl we detected 0.03 ± 0.01 mM Fe(II) and no Fe(III) whereas acidification of the supernatant with SA/MQ resulted in 0.02 ± 0.02 mM Fe(II) and 0.16 ± 0.01 mM Fe(III).

Effect of sediment carbonate on Fe extraction from pelleted sediment

Figure 4 shows solid-phase ferrous and ferric iron extracted from sediment pellets after centrifugation of sediment slurry (Figure 1a) spiked with 2 mM nitrite and either 5 mM $FeCl_2$ or 5 mM ferrihydrite.

Extracting Fe from the pelleted sediment of setups that were spiked with Fe(II) and nitrite (Figure 4a), acidification with 1 M HCl or SA/HCl resulted in similar outcomes. With 1 M HCl, 2.58 ± 0.26 mM Fe(II) and 0.40 ± 0.09 mM Fe(III) were extracted while using SA/HCl 2.53 ± 0.54 mM Fe(II) and 0.19 ± 0.04 mM Fe(III) were extracted. Nitrite was not detected in any of the pellet extractions, indicating that it remained mainly in the aqueous phase discussed in the above section. If nitrite was also present in the sediment porewater, its concentration was too low to be detected. In extracts using SA/MQ the pH was increased up to pH 6, consequently the solubility of Fe was impaired and only 0.79 ± 0.04 mM Fe(II) and 0.09 ± 0.06 mM Fe(III) were extracted.

Extractions from the pelleted sediment of setups that were spiked with Fe(III) and nitrite (Figure 4b) using 1 M HCl resulted in 1.05 ± 0.38 mM Fe(II) and 3.69 ± 0.37 mM Fe(III). Similar results were obtained for extraction using SA/HCl; 1.30 ± 0.16 mM Fe(II) and 4.18 ± 0.51 mM Fe(III) were detected. When using SA/MQ, the pH reached 6 and prevented again effective Fe extraction; only 0.12 ± 0.02 mM Fe(II) and 0.04 ± 0.01 mM Fe(III) were extracted.

Combined effects of nitrite and sediment carbonate on Fe extraction and speciation

We could show that the separation of the carbonate-rich sediment and the nitrite-rich supernatant of microcosm subsamples would qualitatively be a sufficient mean to prevent interactions of Fe(II) and nitrite during acidification. This would require the subsequent stabilization of the aqueous Fe(II) in the supernatant using SA and the extraction of solid-phase Fe using HCl. However, our results also showed that summing up the quantified dissolved and solid Fe fractions resulted in overestimations of the actual sedimentary Fe content rather than allowing an accurate quantification. After spiking the microcosms with 5 mM $FeCl_2$ or ferrihydrite a total Fe concentration of 5–6 mM should be expected, considering the 0.65 ± 0.21 mM total residual sediment Fe (0.61 mM Fe(II) and 0.4 mM Fe (III)). Apart from the microcosms that were amended with 5 mM ferrihydrite and the

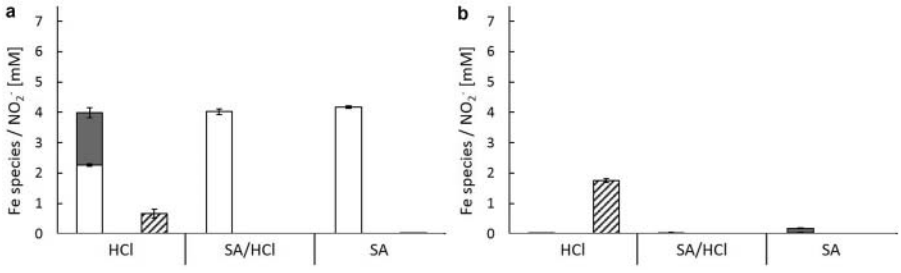

Figure 3. Quantification of ferrous iron (open bars), ferric iron (filled bars) and nitrite (hatched bars) in the supernatant of batch microcosm setups with freshwater Lake Constance sediment using different extractants. Ferrous and ferric iron are shown as stacked bars. Standard deviations are derived from biological triplicates. (a) Setups spiked with 5 mM Fe(II) and 2 mM nitrite. (b) Setups spiked with 5 mM ferrihydrite and 2 mM nitrite.

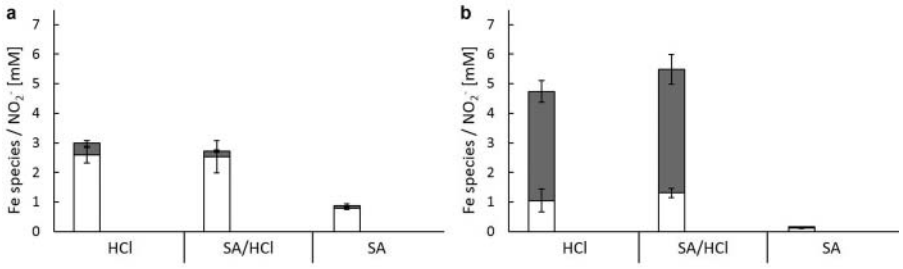

Figure 4. Quantification of ferrous iron (open bars), ferric iron (filled bars) and nitrite (hatched bars) in the pellet of batch microcosm setups with freshwater Lake Constance sediment using different extractants. Ferrous and ferric iron are shown as stacked bars. Standard deviations are derived from biological triplicates. (a) Setups spiked with 5 mM Fe(II) and 2 mM nitrite. (b) Setups spiked with 5 mM ferrihydrite and 2 mM nitrite.

extraction performed with SA/HCl (total Fe = 5.84 ± 0.67 mM), all other samples showed inaccurate total Fe concentrations, ranging from 4.74 ± 0.75 mM up to 6.98 ± 0.55 mM (Figures 3 and 4). Such variations might be caused by the heterogeneity of the sandy sediments. Withdrawing slurry subsamples from the microcosms using syringes or pipets cause variations between the replicates with respect to grain size and grain content in each subsample. Due to this variable sediment content, the Fe concentrations determined after separation of sediment and medium are slightly error-prone. The subsequent calculation of the concentrations per fraction leads to an error propagation and consequently amplifies varieties of the calculated Fe concentrations. Variations in the sediment content also occur when Fe is extracted from the slurry as a whole. However, as the calculation of total Fe concentrations is in that case obsolete, the error propagation is omitted and the varieties are less pronounced. Apart from this expected higher accuracy, Fe extractions and nitrite quantifications from the sediment slurry as a whole were performed to prove the applicability of SA/MQ on samples concurrently enriched in both nitrite and carbonate.

To assess the influence of the sediment content on the Fe extraction efficiency, two different slurry:extractant ratios were chosen. In extractions with a 1:10 (v/v) ratio, 100 μl of the slurry was acidified with 900 μl of the extractant. In extractions with a 1:2 (v/v) ratio, 500 μl of the slurry was acidified with 500 μl of the extractant (Figure 1). Ferrous and ferric iron, as well as the nitrite concentrations, detected in slurry subsamples of microcosms that were spiked with 2 mM nitrite and either 5 mM Fe(II) or 5 mM ferrihydrite are shown in Figure 5.

In Fe(II)- and nitrite-spiked sediment slurries mixed in a ratio of 1:10 (v/v) (Figure 5a) with 1 M HCl, 3.87 ± 1.08 mM Fe(II) was stabilized while 2.10 ± 0.12 mM Fe(III) was detected. This sums up to 5.97 ± 1.20 mM total Fe of which 35.9 ± 6.9% were oxidized during the extraction while only 0.05 ± 0.02 mM nitrite were left. Extracting Fe in a 1:10 (v/v) mixture with SA/HCl, a total amount of 5.48 ± 1.14 mM Fe(II) was stabilized. The 1:10 (v/v) mixture of sediment slurry and SA/MQ contained 5.30 ± 0.77 mM Fe(II). Nitrite was removed completely by reaction with SA in mixtures with SA/HCl as well as in mixtures with SA/MQ. Although all measured total Fe concentrations were within the expected range of 5–6 mM, HCl as the only extractant was insufficient to stabilize Fe(II). Oxidation by nitrite revealed the formation of Fe(III). Both setups containing SA could prevent this reaction and thereby stabilize Fe(II).

When extracting Fe from Fe(III)- and nitrite-spiked sediment slurries (Figure 5b) mixed 1:10 (v/v) with 1 M HCl, 0.23 ± 0.24 mM Fe(II) and 5.55 ± 0.37 mM Fe(III) were

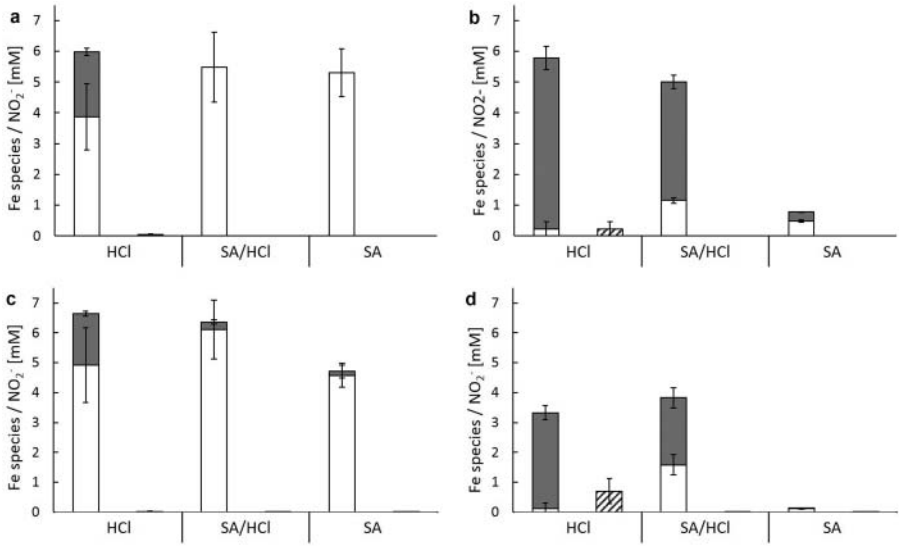

Figure 5. Quantification of ferrous iron (open bars) and ferric iron (filled bars) and nitrite (hatched bars) in slurry samples of batch microcosm setups with freshwater Lake Constance sediment using different extractants. Ferrous and ferric iron are shown as stacked bars. Standard deviations are derived from biological triplicates. (a) 1:10 (v/v) mixtures of slurry spiked with 5 mM Fe(II) and 2 mM nitrite; (b) 1:10 (v/v) mixtures of slurry spiked with 5 mM ferrihydrite and 2 mM nitrite; (c) 1:2 (v/v) mixtures of slurry spiked with 5 mM Fe(II) and 2 mM nitrite; and (d) 1:2 (v/v) mixtures of slurry spiked with 5 mM ferrihydrite and 2 mM nitrite.

detected while 0.23 ± 0.23 mM nitrite was left. Compared to that, the extraction in a 1:10 (v/v) mixture with SA/HCl revealed concentrations of 1.16 ± 0.09 mM Fe(II) and 3.85 ± 0.21 mM Fe(III) and a complete removal of nitrite. Mixing the slurry 1:10 with SA/MQ also resulted in a complete removal of nitrite, yet only 0.49 ± 0.04 mM Fe(II) and 0.28 ± 0.01 mM Fe(III) were detected. While both HCl and SA/HCl efficiently extracted Fe from the slurries, only SA/HCl was able to also stabilize Fe(II). The slight pH increase in the SA/MQ extract to 1.5–2 (see Table 1) affected the extraction efficiency significantly.

Upon mixing Fe(II)- and nitrite-spiked sediment slurries 1:2 (v/v) (Figure 5c) with 1 M HCl, 4.92 ± 1.26 mM Fe(II) were stabilized and 1.72 ± 0.09 mM Fe(III) were detected. This corresponds to 26.5 ± 5.6% of the total Fe which was oxidized while only 0.02 ± 0.01 mM nitrite was left. In 1:2 (v/v) mixtures of slurries with SA/HCl, 6.12 ± 0.99 mM Fe(II) and 0.24 ± 0.08 mM Fe(III) were detected. Upon mixing the sediment slurry 1:2 (v/v) with SA/MQ, 4.58 ± 0.41 mM Fe(II) and 0.13 ± 0.22 mM Fe(III) could be detected. In summary, again, 1 M HCl could not stabilize all Fe(II) in contrast to SA/HCl.

In 1:2 (v/v) mixtures of Fe(III)- and nitrite-spiked slurries (Figure 5d) and 1 M HCl, 0.11 ± 0.19 mM Fe(II) and 3.22 ± 0.24 mM Fe(III) were extracted and 0.70 ± 0.41 mM nitrite were detected. In mixtures with SA/HCl, 1.58 ± 0.34 mM Fe(II) and 2.24 ± 0.34 mM Fe(III) could be detected, whereas nitrite was completely removed. Using SA/MQ, only 0.11 ± 0.01 mM Fe(II) and 0.01 ± 0.00 mM Fe(III) were detected. Nitrite was completely removed. Again, in SA/MQ extracts the pH increased up to 6, causing almost complete inhibition of the extraction of Fe. In extractions using HCl or SA/HCl, on the other hand, only a slight increase to pH 1 was measured in the 1:2 (v/v) mixtures with slurries. Yet even this minor pH change resulted in significantly lowered detected total Fe concentrations of 3.33 ± 0.43 mM and 3.82 ± 0.68 mM, respectively. By mixing acid and sample in a 1:2 ratio, the actual acid concentration within the extraction setup is reduced by half, resulting in such pH increases. The acid concentration then needs to be adapted to retain a concentration of approximately 1 M in the final extraction setup.

Implications and recommendation for protocol application

Biogeochemical Fe conversion processes are often investigated in batch microcosm experiments in which environmental samples are spiked with dissolved or solid-phase Fe species and other electron donors and acceptors such as nitrate or organic carbon (Sobolev & Roden 2002; McBeth et al. 2011; Mortimer et al. 2011; Laufer et al. 2016a). The changes in Fe speciation are then followed over time by wet-chemical extractions followed by spectrophotometric analyses of the Fe redox species (Stookey 1970; Braunschweig et al. 2012; Verschoor & Molot 2013). It is known that high concentrations of other sample components (e.g., silicates or metals other than Fe) can interfere with the Fe analysis (Kundra et al. 1974; Anastácio et al. 2008; Im et al. 2013) yet also Fe extraction can be affected as shown for intermediate reaction products of the denitrification process (Klueglein & Kappler 2013; Yan et al. 2015). Apart from the commonly used separation of solid and liquid phase prior to Fe extraction (Weber et al. 2006; Chakraborty et al. 2011), the protocol suggested by Klueglein & Kappler (2013), using 40 mM SA instead of 1 M HCl as extracting agent has been proven to be highly efficient and quantitatively accurate for the extraction of poorly crystalline Fe from bacterial batch cultures containing nitrite (Klueglein & Kappler 2013; Klueglein et al. 2015; Li et al. 2015; Xiu et al. 2016) and was successfully applied to sediment and slurry samples (Laufer et al. 2016a; Robertson et al. 2016). However, in the present study we found that the extraction of Fe from a carbonate-rich freshwater sediment using 40 mM SA was insufficient. Our findings demonstrate that in addition to nitrite, sedimentary carbonate significantly affects the extraction efficiency. In carbonate-enriched samples, the low pH caused by SA will be buffered by the natural bicarbonate content and the extraction of Fe decreases in efficiency due to the increased pH in the sample. A combination of 40 mM SA with 1 M HCl was efficient in both (i) removing nitrite from the sample by reaction with SA and (ii) retaining the required pH at which Fe can be extracted efficiently. When working with samples high in carbonate and nitrite we, therefore, recommend to combine the extractants SA and HCl in order to obtain correct Fe data. We have identified the following threshold conditions that require the application of accordingly adapted Fe extraction protocols: The threshold concentration of nitrite for the application of SA as extractant depends on the Fe concentrations of the sample. As under anoxic low pH conditions each nitrite molecule can oxidize up to 2 Fe^{II}_{diss} molecules (see Equations 1–4), the alterations in Fe^{II}_{diss} and Fe^{III}_{diss} concentrations lie within the order of magnitude of the nitrite concentrations. In this study we measured oxidation of 1.29 and 1.08 Fe^{II}_{diss} molecules per nitrite molecule in the supernatant and slurry samples, respectively. This is lower than potentially possible yet the produced NO_2 and NO might react not only with Fe^{II}_{diss} but also with, e.g., organic carbon (Schmidt & Matzner 2009). Considering an error of the ferrozine assay in the low percent range (≤5%) for the described protocol (Im et al. 2013), a nitrite concentration that is three orders of magnitude lower than the Fe^{II}_{diss} concentration would cause errors that are within the range or even smaller than the technical errors of the analysis. In environmental samples the highly reactive nitrite is found only in low μM concentrations (Melton et al. 2012; Lin & Taillefert 2014; Laufer et al. 2016a), yet it can accumulate up to mM concentrations during denitrification, in particular after spiking samples with nitrate (Glass & Silverstein 1998; Weber et al. 2006; Robertson et al. 2016). The threshold concentration for carbonate, requiring the combination of SA with HCl is 100 mM within the measured sample for a 1:10 dilution in acid, or 10 mM in the final mixture, according to the buffering capacity of carbonate for SA as shown in Figure 2. Carbonate contents of soils and sediments range from a low percent range (Mortimer et al. 2011; Haverkamp et al. 2014; Harter et al. 2014) up to over 80% (Loeppert & Suarez 1982). For less strongly diluted samples, i.e., 1:2 mixtures with acid as shown in this study, the subsequent dilution of the acid has to be considered as well. Although samples containing 1 M HCl or SA/HCl could maintain a pH of 1 (Table 1), the extraction efficiency of this setups was affected and the extracted total Fe concentrations were significantly lower than in other setups (Figure 5d).

Thresholds and potential interferences are summarized in Figure 6 to provide a clear and easy guideline to choose the appropriate extractant to obtain quantitatively accurate results. In order to choose the appropriate extraction protocol it is

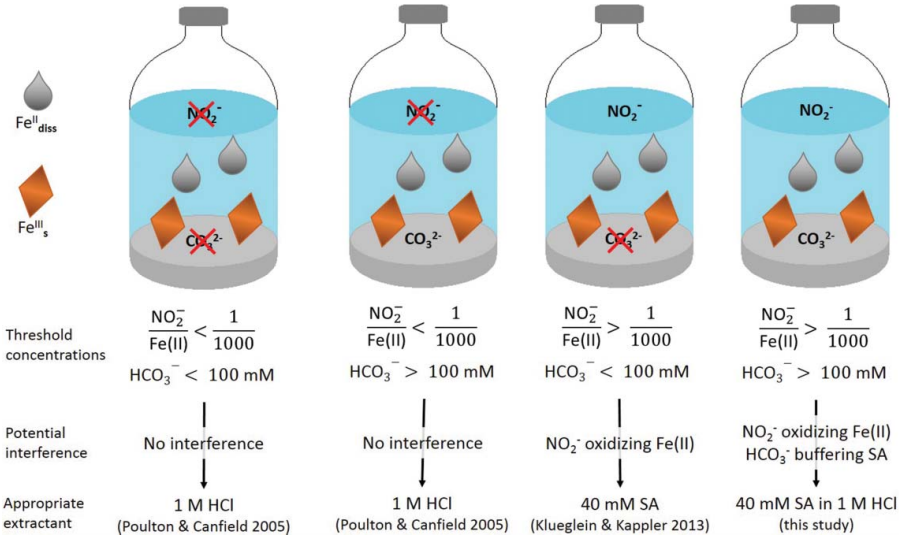

Figure 6. Overview and guideline to choose appropriate Fe extraction protocols for wet-chemical analysis based on nitrite and carbonate threshold concentrations in natural lab sediment and soil samples.

crucial to determine the sample properties prior to the extraction. For exact sample analysis nitrite can be determined spectrophotochemically (Kamphake et al. 1967; Sawicki & Scaringelli 1971) and carbonate can be quantified by loss-of-CO_2 measurements or titrations (Loeppert & Suarez 1982). However, rough estimations of nitrite and carbonate levels of the sample can be achieved by semi-quantitative quick tests such as nitrate/nitrite test strips (e.g., Merck MQuant Nitrite Test), proof of CO_2 gas loss by adding some HCl to the sample or by measuring the pH of acid sediment mixtures as they would be used for Fe extraction (Loeppert & Suarez 1982). Such quick tests allow to estimate threshold conditions and to choose the correct extraction protocol (Figure 6) for exact Fe quantification. In case of doubt or if, e.g., low sample quantities do not allow additional tests, we strongly recommend to follow the presented protocol in order to prevent possible nitrite and carbonate interference.

The protocol we present here, using a combination of 40 mM SA in 1 M HCl effectively extracts poorly crystalline Fe minerals and prevents the reaction of Fe(II) with reactive N species from sediment, soil and laboratory samples that are enriched in nitrite and carbonate. As nitrite is an obligate intermediate in denitrification, it should also be considered to use SA alone or in combination with HCl on samples that are spiked with or naturally enriched in nitrate, e.g., by the influence of wastewaters or agriculture (Constantin & Fick 1997; Glass & Silverstein 1998; Oenema et al. 1998; Smolders et al. 2010). Due to the prevalence of microbial processes in the production of nitrite, the use of SA is of special relevance for the study of microbial Fe conversion processes in environmental samples.

An equivalent protocol for extraction of higher crystalline or total Fe has not been tested, yet a subsequent sequential extraction with higher concentrated HCl (e.g., 6 M HCl) would not be affected once nitrite has been successfully removed by SA. For the use of higher acid concentrations, however, further precautions such as anoxic storage of the initial sample and anoxic processing of the extraction should be considered (Porsch & Kappler 2011).

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