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GUIDELINES FOR FRESH & MARINE WATER QUALITY

Optimisation of the US EPA (1991) pH-2 extraction method for measuring potentially bioavailable iron (iron III)

Report

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Water Quality Guidelines is a joint initiative of the Australian and New Zealand governments, in partnership with the Australian states and territories.

Cataloguing data

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Executive summary

Public submissions on the draft default guideline values technical briefs for iron in freshwater and marine water identified that the pH-2 extraction method (US EPA 1991, Method 200.1) that had been recommended for the analysis of iron in water was not validated for iron. Advice provided to the Project Coordination Group from analytical environmental chemists recommended the refinement of the US EPA (1991) method and validation of its application for the measurement of potentially bioavailable iron. Included in those recommendations was the need for a clear method that ensured consistent interpretation by analytical laboratories and provided a standardised approach to enable accreditation by the National Association of Testing Authorities.

This document describes the validation study used to optimise the US EPA (1991) method for the measurement of potentially bioavailable iron using extractions at pH 2 from both freshwater and seawater samples. The recommended method results in reasonable recovery of potentially bioavailable iron from all diluent types tested, particularly if samples are processed within 3 days and a 16-hour extraction period at pH 2 is used. It should be noted that the method provides a measurement of the fraction of iron that may potentially become available to a biological receptor and that in an aquatic environment, some of this iron may ultimately not be available to an aquatic organism.

1 Introduction

A number of metals, including iron (Fe^{3+} or Fe (III)), have low solubility in freshwater and marine water but are toxic at concentrations above their solubility limit. Research has shown that certain particulate and colloidal (precipitated) forms of Fe^{3+} can contribute to toxicity (ECCC 2024; Balsamo Crespo et al. 2023). The draft Australian and New Zealand default guideline values (DGVs) for aquatic ecosystem protection for Fe^{3+} in freshwater and marine water were released for public comment in June 2020. The draft DGVs recognised that particulate forms of Fe^{3+} could contribute to toxicity and, to account for this, recommended a pH-2 extraction method (US EPA 1991) for the analysis of potentially bioavailable Fe^{3+} in environmental water samples. However, several public submissions on the draft DGVs identified that the recommended analytical method had not been validated for Fe^{3+}.

Advice provided to the ANZG (2018) Guidelines Project Coordination Group from a number of analytical environmental chemists recommended the refinement of the US EPA (1991) method and validation of its application for the measurement of potentially bioavailable Fe^{3+} in environmental water samples. Included in those recommendations was the need for a clear method that ensured consistent interpretation by analytical laboratories and provided a standardised approach to enable accreditation by the National Association of Testing Authorities. Consequently, the current study aimed to validate the US EPA (1991) method for Fe^{3+} and to document the method so that it can be readily adopted by commercial laboratories.

Issues that were addressed through experimental validation in the current study included the optimal acidification period, optimal pH-adjustment process, maximum holding time prior to extraction, and validation of the method for Fe^{3+} (Table 1).

Issues that required only minor adjustments to the text of the method or more detailed explanatory text (Table 2) were (i) sample volume required and (ii) acceptability of sample acidification in the laboratory.

Issues not requiring further consideration (Table 3) have been addressed, and text has been included in the method to minimise ambiguity concerning the type of acid that should be used, the recommended pH and requirements for cooling samples. Table 1, Table 2 and Table 3 list the issues that were identified and provide a summary of the corresponding results and conclusions drawn from the validation study. The results are discussed in more detail in Section 3.

Table 1 Summary of issues that should be further addressed via additional research and development or validation

Aspect of method	Description of issue
Acidification period	Currently, the method prescribes 'a minimum of 16 hours' for the acidification period. It is suggested that laboratory validation be done on the method to give clarity to laboratories performing this analysis. For example, a timeline of '16–24 hours' would be clearer. Perhaps a much shorter time is sufficient. A Certified Reference Material could be used to validate the time. It is recommended that the time range be specified.
Study results	The validation study found a 16-hour extraction period to be optimal, especially when combined with samples being filtered within a 3-day holding time. This combination provided 100% recovery of potentially bioavailable Fe from both synthetic freshwater and mine-impacted freshwater, and 74% and 86% recovery from lake water and seawater, respectively. For synthetic freshwater samples that had a 3-day holding time, an 8-hour extraction period provided 91% recovery, and a 4-hour extraction period provided 82% recovery. Given that an 8-hour extraction period is likely to be less convenient for analytical laboratories to apply than a 16-hour period, the 16-hour extraction period is recommended, but a range of 8–16 hours is considered acceptable. In the interest of achieving consistency in the way the method is applied, a 16-hour extraction period rather than a range is recommended.
pH adjustment	The process of checking and adjusting pH multiple times carries a risk of sample contamination (e.g. dipping of electrodes, washing electrodes, adding nitric acid or ammonium hydroxide). The pH adjustment process could be rationalised.
Study results	Sub-sampling for pH measurement and the subsequent pH adjustment of samples have been included in the method as options, provided that the pH of the sample is confirmed following adjustment through measurement of a subsequent sub-sample. In some cases, this may not possible if sample volume does not allow for multiple sub-samples to be taken. The method used for pH adjustment in this validation study involved using a separate clean probe for blanks and clean samples and using a different probe for samples that had elevated iron. In addition to this, the probe used for samples that had elevated iron was rinsed and dried between measurements of different samples. This process allowed for direct measurement from the sample bottle and very fine adjustment of pH. There were no instances of contamination of samples or blanks using this method.
Metals listed under analysis	The US EPA (1991) method does not reference iron. It is likely the method could also be applied to iron (and laboratories would validate the method regardless). However, the National Association of Testing Authorities could choose not to accredit the use of the method in this way, given that iron is not specified in the list of analytes for which the method can be used.
Study results	The validation study has demonstrated that the method is suitable for both freshwater and seawater samples.

Table 2 Summary of issues requiring minor procedural adjustments

Aspect of method	Description of issue
Sample volume	The sampling requirements in US EPA 200.1 state to take an 800-mL sample. This volume can be significantly reduced.
Study results	The validation study used 200-mL samples. Balsamo Crespo et al. (2022) used 250-mL samples. This 200–250-mL volume range was considered acceptable and provided sufficient volume for taking duplicate samples from each sample bottle at multiple time points. Given that a laboratory using this method would only be taking a set of filtered samples for a single time point, 100 mL would be sufficient volume and, as such, this was the final recommended volume.
Field addition of acid	This is not practical, especially for remote sites, and needs amending. It is difficult to transport acids without Dangerous Goods permits, and acids cannot be sent via air transport. Also, environmental consultants often have occupational health and safety rules that they cannot touch acids. It would be best for the client to send an unpreserved sample to the laboratory and for the laboratory to add the acid in a controlled environment.
Study results	Addition of acid in the laboratory is acceptable and recommended over acidification in the field. This has been incorporated in the method.
3-day holding time	This will be difficult for remote northern Australia sites.
Study results	The validation study found that if samples cannot be processed within 3 days of collection, processing within 7 days is acceptable, provided a 16-hour extraction period at pH 2 is used. This process still provided 96% recovery of bioavailable iron from synthetic freshwater, 64% recovery from lake water and 86% recovery from seawater.

Table 3 Summary of issues not requiring further consideration

Aspect of method	Description of issue
Type of acids to use	US EPA 200.1 (US EPA 1991) uses only nitric acid. In contrast, APHA 3030C (APHA 2018) and US EPA 3010A (US EPA 1992a) and US EPA 3005A (US EPA 1992b) also use hydrochloric acid. When undertaking multi-element analyses, both hydrochloric acid and nitric acid are needed to completely digest and solubilise the different metals and metalloids. Is using two acids more efficient than one?
Study results	This validation study found that nitric acid alone (50% v/v) is appropriate for extraction of the bioavailable fraction of iron.
Recommended pH	It is not clear why the sample pH should be between 1.65 and 1.85. A validation study could resolve this.
Study results	Both the study by Balsamo Crespo et al. (2022) and this validation study found that pH-2 extraction was sufficient for suitable recovery of potentially bioavailable iron. Lower pH does not appear necessary for successful recovery and was likely to have been included in the original method as a matter of convenience/simplification of the process of addition of nitric acid. Based on observations from this study, a 200-mL sample of synthetic freshwater that had an original pH of 7.7 requires ~350 µL of 50% v/v nitric acid to adjust to pH 2. However, the volume of acid required for samples of natural water of varied pH and physicochemical composition will likely vary, so it is recommended that pH is measured during the acidification process and over the extraction period to ensure it remains at 2 ± 0.1.
Sample cooling	Reference to a 4 °C cooler is out of date and contradicts National Environment Protection Measures. Storing samples at 4 °C would be difficult in remote areas. Water samples for metal analyses are generally considered acceptable when stored at ambient temperature, so this aspect appears to be outdated. A validation study would resolve this.
Study results	For the validation study, samples were kept at room temperature (~22 °C) over the holding period and extraction period. Based on this, keeping samples at 4 °C in the field prior to acidification does not seem necessary. Following pH-2 extraction and filtration, samples were refrigerated until being sent for analyses and kept under ice overnight during freight to the external laboratory. However, commercial laboratories have indicated that, for metal samples, it is not necessary to keep them cool once they have been acidified (US EPA 2018).

2 Methods

The design of the validation study closely followed that of Balsamo Crespo et al. (2023), and details of the method used are described in Appendix A. Samples were analysed for dissolved and total recoverable Fe using inductively coupled plasma mass spectrometry (ICP-MS) by Envirolab Services (Sydney).

The first trial was conducted in synthetic freshwater (supplemented ultra-pure water, i.e. > 18 MΩ/cm) spiked with Fe in 3 forms: (i) freshly precipitated iron oxyhydroxide (prepared immediately prior to spiking), (ii) well-mineralised suspended particulate Fe and (iii) a combination of both. Before spiking with Fe, the diluent was modified to align with the synthetic diluent used by Balsamo Crespo et al. (2023). Physicochemical parameters of the synthetic diluent were pH 7.7, electrical conductivity (EC) 150 µS/cm, alkalinity 80 mg/L (measured as calcium carbonate [$CaCO_3$]) and dissolved organic carbon (DOC) 3.7 mg/L (through the addition of a fulvic-acid standard). Acid extractions were carried out with the addition of 50% v/v nitric acid (HNO_3) to pH 2.0 ± 0.1. HEPES buffer (2.5 mM strength) was used to minimise shifts in pH throughout the extraction period.

The same design was used with unfiltered and unmodified lake water whose physicochemical parameters were pH 7.5 (no buffer added), EC 44 µS/cm, alkalinity 21 mg/L $CaCO_3$ and DOC 4.6 mg/L. Mine-impacted water elevated in Fe (sourced from Frances Creek mine site, NT, 13°36'23"S 131°51'04"E) was also compared to the Fe-spiked lake water to determine if this source of Fe had different potential bioavailability.

The design was repeated using unfiltered and unmodified seawater of the following physicochemical parameters: pH 8.1, EC 53 µS/cm, DOC 0.7 mg/L and alkalinity 120 mg/L $CaCO_3$.

3 Results

3.1 pH control

Water-quality parameters and pH control are detailed in Appendix B. While pH shift was minimised by using HEPES buffer in the synthetic freshwater (pH shifts of ≤ 0.1 over 14 days), pH control within 0.03 units was still possible for lake water and seawater without the use of buffer through minor manual adjustments with 2-M or 10-M sodium hydroxide (NaOH) or 50% v/v HNO_3 at day 3, 7 or 14, as necessary.

3.2 Recovery of potentially bioavailable iron from synthetic freshwater

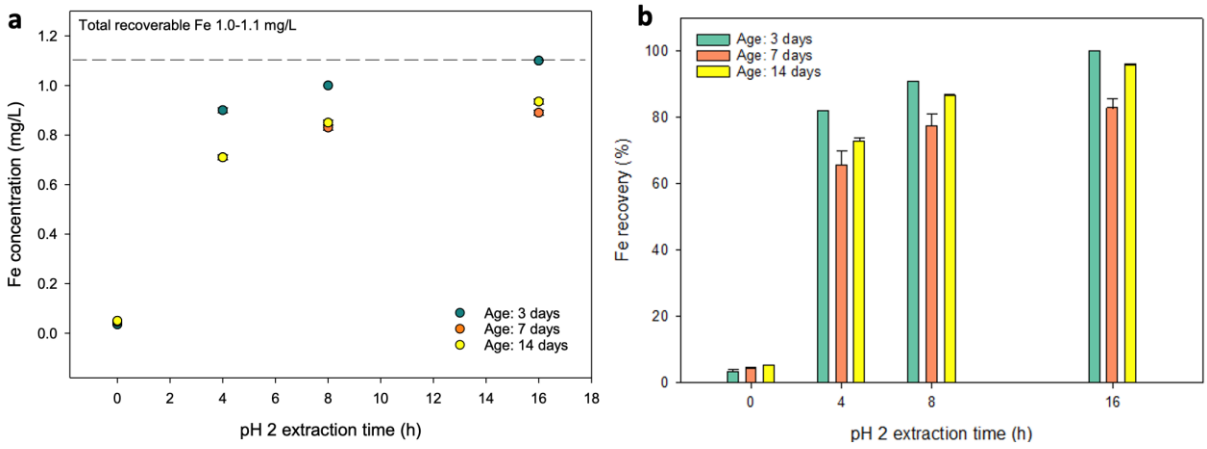
Data generated from the validation study regarding Fe dissolution and percent recovery from synthetic freshwater are detailed in Appendix C (raw data tables) and Figure 1. Optimal conditions for recovery of potentially bioavailable Fe were a 3-day holding time with a 16-hour extraction period at pH 2. This resulted in 100% recovery of bioavailable Fe in synthetic freshwater samples containing 1.1 mg/L total recoverable Fe (Figure 1a, b). Recovery decreased for samples that had been stored for 7 or 14 days (83% and 96% recovery, respectively; Figure 1b). There was insufficient replication to detect if these differences in recovery were statistically significant. In comparison, Balsamo Crespo et al. (2023) recovered 97%, 96% and 94% for samples aged 3 days, 7 days and 14 days, respectively, following a 16-hour pH-2 extraction period. Shorter extraction periods of 4 hours and 8 hours were less effective at recovering Fe than a 16-hour extraction period, regardless of sample age (Figure 1a, b). Recovery of suspended particulate Fe in turbid synthetic water remained low for all extraction periods (≤ 0.16 mg/L or ≤ 0.7%; Figure 1c, d), indicating that this method does not extract non-bioavailable suspended particulate Fe and, hence, would not materially overestimate the fraction of bioavailable Fe. This was also the case when synthetic freshwater was spiked with a combination of fresh iron oxyhydroxides and suspended particulate Fe. Recovery generally reflected what was added in the form of fresh iron oxyhydroxides (1.2 mg/L Fe; Figure 1e), and recovery was optimal for samples that had a 3-day holding time and 16-hour extraction. Overall, percent recovery was low (≤ 3%; Figure 1f) when accounting for the concentration of suspended particulate Fe, indicating that this fraction was not influencing the measurement of potentially bioavailable Fe.

Figure 1 Iron dissolution and percent recovery for each age (holding time in days) and pH-2 extraction period for synthetic freshwater

Bars = standard deviation, *n* = 4.

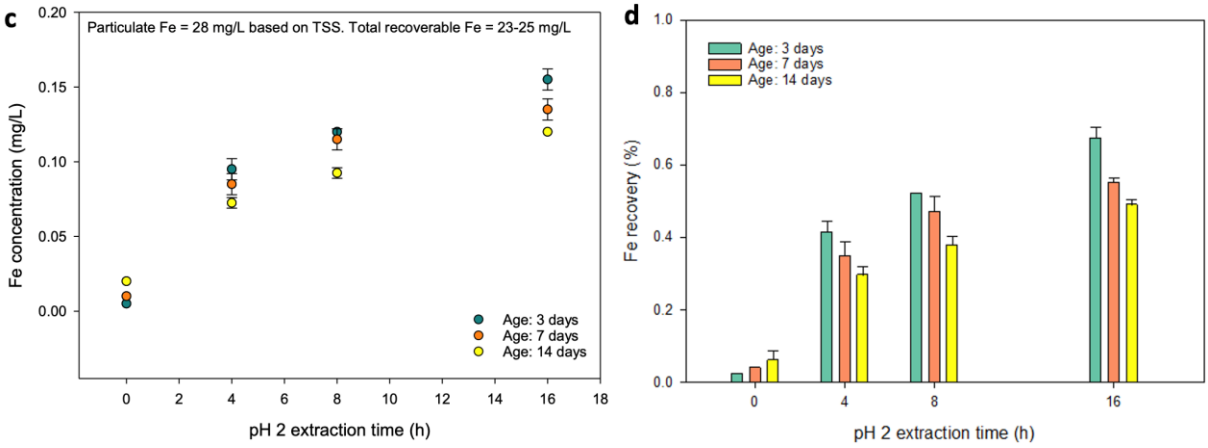
Fresh iron oxyhydroxide spiked solution ($FeCl_3.6H_20$)

(a) Iron dissolution, (b) iron recovery



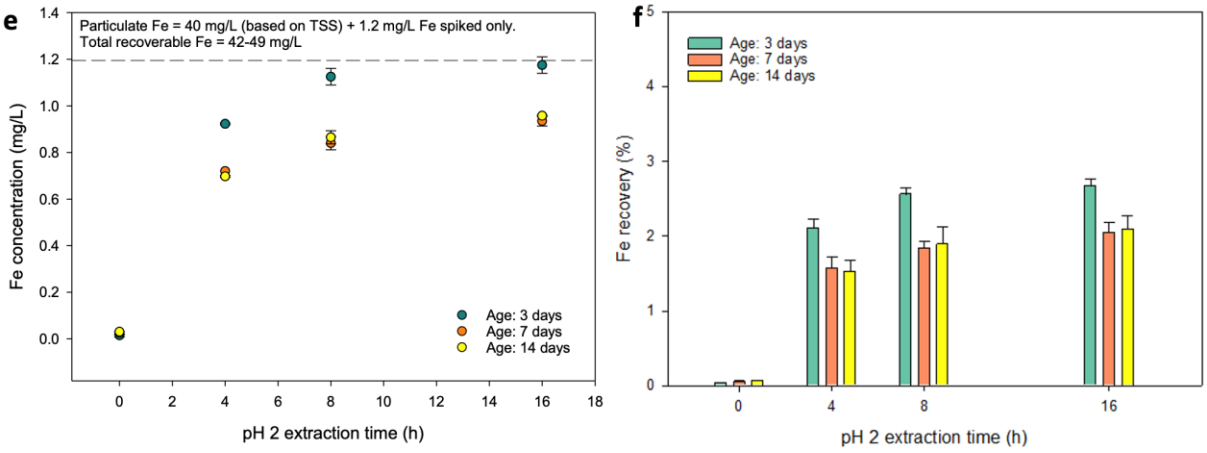
Suspended particulate iron oxyhydroxide (simulation of turbid water)

(c) Iron dissolution, (d) iron recovery



Combined suspended particulate and fresh iron oxyhydroxides

(e) Iron dissolution, (f) iron recovery



3.3 Recovery of potentially bioavailable iron from lake water

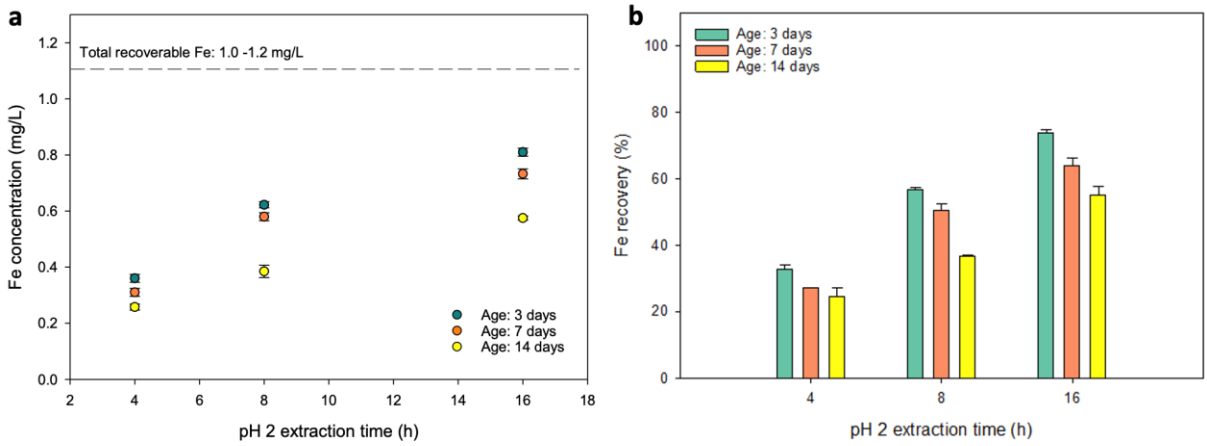
A similar pattern of recovery of potentially bioavailable Fe was observed for natural lake water samples (Appendix C and Figure 2). Recovery of potentially bioavailable Fe was greatest for 3-day-old samples, compared to 7-day-old and 14-day-old (Figure 2a, b) – 0.82 mg/L Fe was recovered from samples containing a total of 1.1 mg/L Fe. A 16-hour extraction period also produced the greatest recovery of Fe, although this only reached a maximum of 75%, compared to the 100% recovery in synthetic freshwater. This lower recovery is likely due to components in the natural water that could complex Fe (greater DOC concentrations and alkalinity of the lake water) and reduce the fraction of bioavailable Fe. Recovery of suspended particulate Fe in lake water was comparable to that in synthetic water (≤ 0.6%; Figure 2d), indicating it would not contribute to an overestimate of bioavailable Fe. Lake water spiked with a combination of fresh iron oxyhydroxides and suspended particulate Fe generally reflected what was added in the form of fresh iron oxyhydroxides (1 mg/L of 1.2 mg/L Fe recovered, or 83%; Figure 2e), and recovery was optimal for samples that had a 3-day holding time and 16-hour extraction (Figure 2e).

Figure 2 Iron dissolution and percent recovery for each age (holding time in days) and pH-2 extraction period for lake water (Lake Bennett, NT)

Bars = standard deviation, *n* = 4.

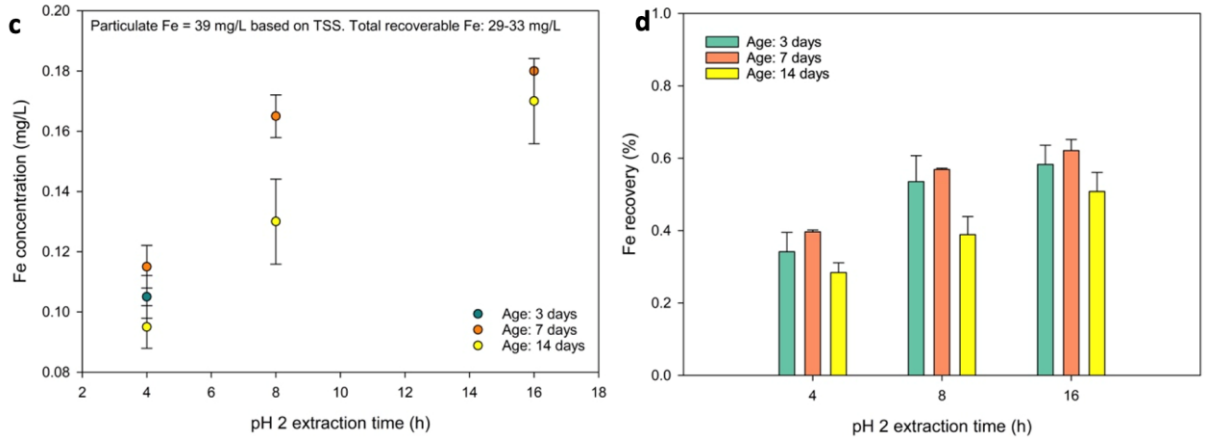
Fresh iron oxyhydroxide spiked solution ($FeCl_3.6H_20$)

(a) Iron dissolution, (b) iron recovery



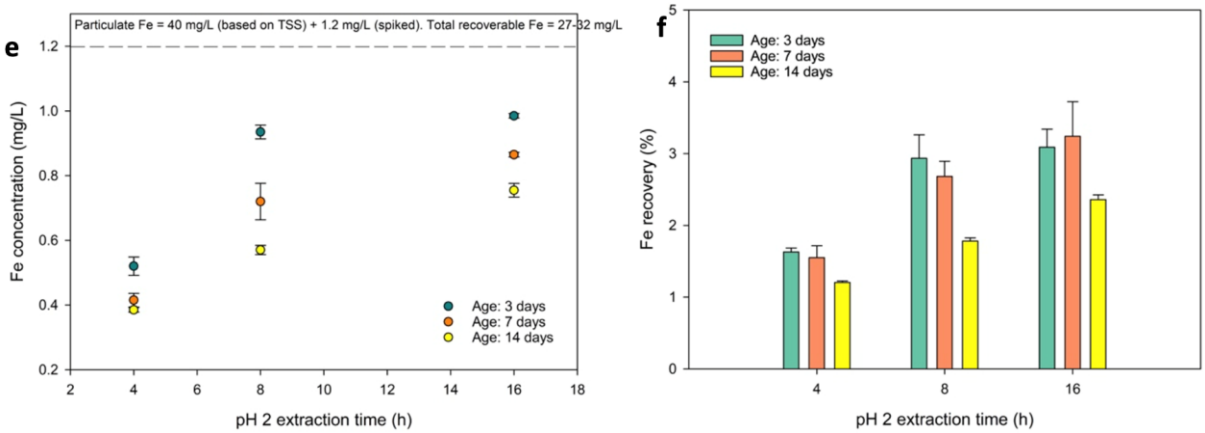
Suspended particulate iron oxyhydroxide (simulation of turbid water)

(c) Iron dissolution (note: 3-d values for 8-h and 16-h = 7-d values), (d) iron recovery



Combined suspended particulate and fresh iron oxyhydroxides

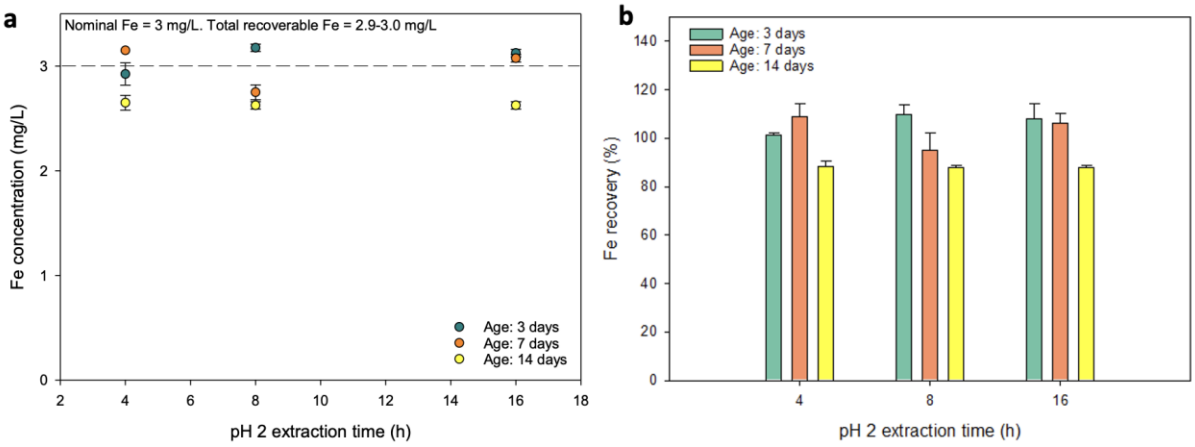
(e) Iron dissolution, (f) iron recovery



3.4 Recovery of potentially bioavailable iron from mine-impacted freshwater

Optimal conditions for recovery of Fe in the mine-impacted water elevated in Fe (3 mg/L) showed equally efficient recovery for 3-day-old (100% to 109%) and 7-day-old samples (95% to 109%), and 14-day old samples had slightly less recovery (87% to 88%) (Appendix C and Figure 3a, b). However, this water was not highly turbid (total suspended solids = 64 mg/L), and water more elevated in Fe could be targeted from this site for further investigation if deemed necessary. In the interests of providing a standard method for all water types, the recommendation of samples being processed within 3 days and using a 16-hour extraction period could also apply for these sample types.

Figure 3 a) Iron dissolution and b) percent recovery for each age (holding time in days) and pH-2 extraction period for mine-impacted water elevated in iron (Frances Creek, NT)
Bars = standard deviation, *n* = 4.



3.5 Recovery of potentially bioavailable iron from seawater

A 3-day holding time and 16-hour extraction period at pH 2 was also suitable for recovery of potentially bioavailable Fe from seawater – 0.87 mg/L Fe were recovered from samples containing a total of 1.0 mg/L Fe (86.5%; Appendix C and Figure 4a, b). Percent recovery of potentially bioavailable Fe from seawater spiked with fresh iron oxyhydroxides was similar across all holding times and extraction periods (80% to 87%; Figure 4b), and the concentration recovered from samples aged 3 days was similar to the recovery from lake water (0.81 mg/L Fe from samples containing 1.1 mg/L total Fe, or 74%; Figure 2a, b). While higher concentrations of potentially bioavailable Fe were recovered from 14-day-old samples (0.96 mg/L Fe; Figure 4a) compared to samples aged 3 days and 7 days (0.87 mg/L Fe), the total recoverable Fe measured for the 14-day-old samples was also higher (1.2 mg/L), resulting in similar percent recovery across treatments. There is insufficient information to determine the cause of the slightly higher recovery of Fe from 14-day-old samples compared to samples aged 3 days and 7 days. Percent recovery of suspended particulate Fe in seawater (~2% to 4%; Figure 4d) was slightly higher than that in lake water (~0.6%; Figure 2d) but still minimal compared to the proportion of potentially bioavailable Fe recovered (86.5%). Lower concentrations of suspended particulate Fe recovered from 3-day-old samples (2.3%; Figure 4d) compared to 7-day-old (3.2%) and 14-day-old (4.4%) samples, suggests that processing seawater samples within 3 days may be optimal for minimising interference from the suspended particulate fraction when measuring potentially bioavailable Fe. Figure 4d and Figure 4e show that the

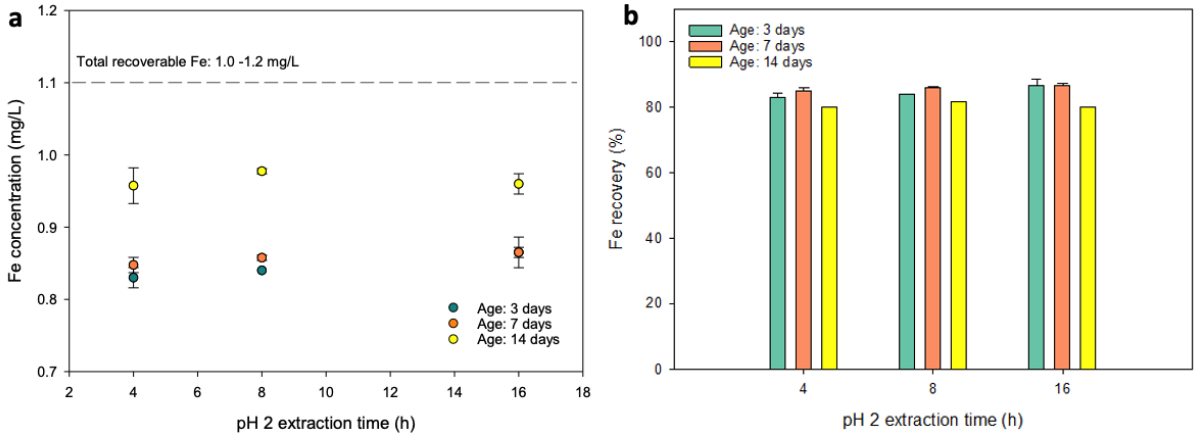
concentration of Fe recovered from the combined treatment in 3-day-old samples (~1.5 mg/L Fe or < 5%) was close to the concentration of freshly spiked oxyhydroxides (1.2 mg/L Fe), indicating minimal interference from suspended particulate Fe.

Figure 4 Iron dissolution and percent recovery for each age (holding time in days) and pH-2 extraction period for seawater (Nightcliff, NT)

Bars = standard deviation, *n* = 4.

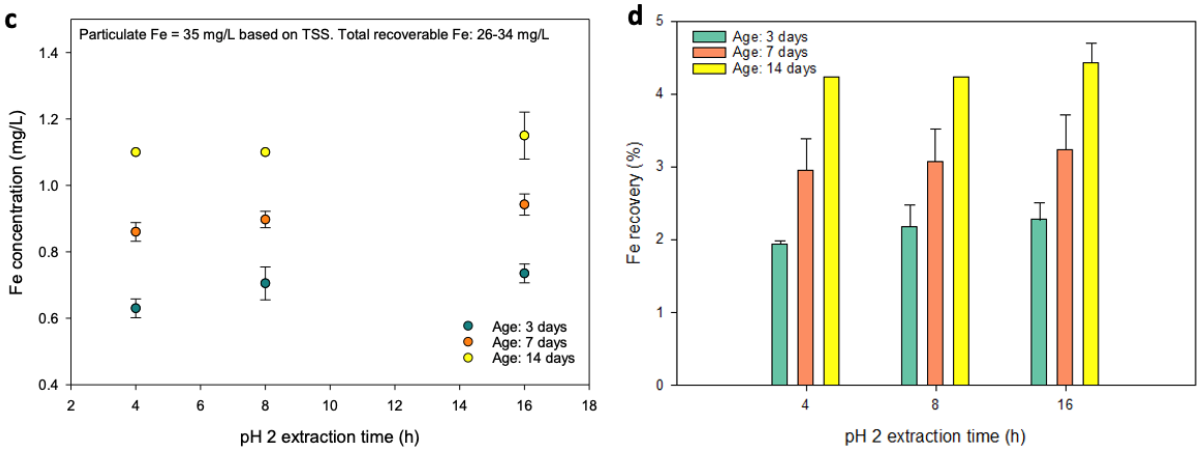
Fresh iron oxyhydroxide spiked solution ($FeCl_3.6H_20$)

(a) Iron dissolution, (b) iron recovery



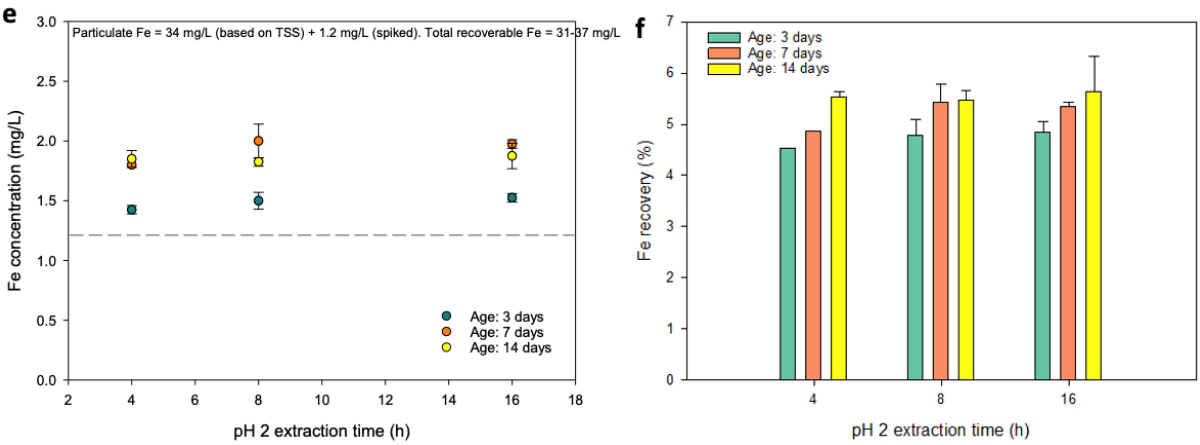
Suspended particulate iron oxyhydroxide (simulation of turbid water)

(c) Iron dissolution, (d) iron recovery



Combined suspended particulate and fresh iron oxyhydroxides

(e) Iron dissolution, (f) iron recovery



4 Conclusion

The study validated the acceptability of the US EPA (1991) method for measuring potentially bioavailable Fe. The recommended method is outlined in Appendix D.

This method provides reasonable recovery of potentially bioavailable Fe from all diluent types tested, particularly if samples are processed within 3 days and a 16-hour extraction period at pH 2 is used. The method is preferred over more aggressive methods, as it excludes unwanted solubilisation of mineralised, non-bioavailable forms of iron. It should be noted that the analysis extracts the fraction of iron (Fe^{3+}) that may potentially become available to a biological receptor. In an aquatic environment, some of this Fe^{3+} may ultimately not be available to an aquatic organism.

Glossary and acronyms

Term	Definition
$CaCO_3$	Calcium carbonate, a measure of alkalinity.
Default guideline value (DGV)	A guideline value recommended for generic application in the absence of a more specific guideline value (e.g. a site-specific value), in the *Australian and New Zealand Guidelines for Fresh and Marine Water Quality*. Formerly known as 'trigger values'.
Diluent	A diluting agent.
DO	Dissolved oxygen.
DOC	Dissolved organic carbon.
EC	Electrical conductivity.
Fe	Iron.
HCl	Hydrochloric acid.
HNO_3	Nitric acid.
ICP-MS	Inductively coupled plasma mass spectrometry.
NaOH	Sodium hydroxide.
RO	Reverse osmosis.
TOC	Total organic carbon.
Toxicity	The inherent potential or capacity of a material to cause adverse effects in a living organism.
TSS	Total suspended solids.

Appendix A. Internal laboratory procedure for validation of method

Day prior to test start (to allow diluent equilibration)

Preparing synthetic diluent

1) Soak a polyethylene water container overnight in 10% HNO_3 and triple rinse in reverse-osmosis (RO) water followed by a triple rinse in ultra-pure water (> 18 MΩ/cm).
2) Prepare 10 L of synthetic diluent using the following steps.
3) Add ultra-pure water (> 18 MΩ/cm) to 5-L volumetric flask, then add items 4–6.
4) Add 80 mg/L $CaCO_3$ (800 mg for 10 L).
5) Add 3.9 mg/L DOC (7.623 mg/L of Suwannee River Fulvic Acid standard = 76.2 mg for 10 L).
6) Add 50 mL of 0.5 M HEPES (for a 2.5 mM HEPES strength).
7) Top up to 5 L with ultra-pure water and pour into a 10 L water container.
8) Add another 5 L of ultra-pure water to the water container, close the cap and mix well.
9) Measure and record temperature (this was done at air-conditioned room temperature, ~22 °C), EC, pH and dissolved oxygen (DO). EC will be ~150 µS/cm, pH should be ~7.7, and DO should be close to 100%.
10) Measure total and DOC using high-temperature combustion (APHA 2018 Method 5310B, TOC-5000A; Shimadzu).

Collecting natural diluent

1) Prepare a high-density polyethylene container (acid washed in 10% HNO_3, triple rinsed in RO water and triple rinsed with ultra-pure water) to use for water collection.
2) Prior to collection, check container is clean by measuring EC after rinsing with ultra-pure water (EC should be < 1 µS/cm).
3) Once at the site for water collection, triple rinse the container with the water to be collected before filling the container.
4) On arrival at the lab, measure and record water parameters (temperature, EC, pH, DO) of the diluent as soon as possible.
5) Measure total organic carbon (TOC) and DOC using high-temperature combustion (APHA 2018 Method 5310B, TOC-5000A; Shimadzu).

Day of test start

Stock solutions

1) Freshly precipitated iron oxyhydroxides: 500 mg/L Fe stock solution.
   a) Make up 100 mL stock solution.

b) Weigh out 0.24203 g of iron (III) chloride hexahydrate (analytical grade, Sigma-Aldrich) and dissolve into ultra-pure water with 0.05-M HNO_3 to keep iron in solution (by adding 2.5 mL of 2-M HNO_3 solution).

c) Check pH. Aim for pH of 1.34 so Fe will remain as Fe^{3+}.

d) Store in 250-mL high-density polyethylene bottle and use on the same day of preparation.

2) Mineralised suspended particulate fraction: 3 g/L oxyhydroxide stock solution

a) Weigh 600 mg of iron (III) hydroxide, alpha-phase (analytical grade, Thermo Fisher Scientific) into 200 mL of ultra-pure water.

b) Vigorously mix the suspension.

c) Store in 250-mL bottle and use on the same day as preparation.

Test design

A = control or blank (ultra-pure water, no Fe added).

B = freshly spiked oxyhydroxides.

C = mineralised suspended particulate.

D = combination of B and C.

Example for blank treatment: Aa = 3-day samples, Ab = 7-day samples, Ac = 14-day samples.

Volumes required

- 2 L of each treatment (A, B, C, D) is required.
- 1.2 L will be needed for the extractions (6 × 200 mL), allowing for 2 replicates for each of the 3-day, 7-day and 14-day holding times.
- Remaining 800 mL can be used for chemistry samples (metals, alkalinity, total suspended solids [TSS] and TOC/DOC and for checking water parameters).

Spiking treatments with iron

1) B: for final concentration of 1.2 mg/L Fe, add 4.8 mL of stock solution #1 to 2-L volumetric flask and make up to volume with the diluent.

2) C: for 80 mg/L TSS, add 53.33 mL of stock solution #2 into 2-L volumetric flask. Vigorously mix stock solution before sub-sampling. Reflux the pipette tip a few times, drawing solution in and out before taking final aliquot.

3) D: add 4.8 mL of stock solution #1 and 53.33 mL of stock solution #2 to 2-L flask.

4) Use same 2-L flask for A, B and D treatments. Use separate flask for C (particulate-only treatment).

5) Transfer solution from the flask to the 250-mL sample bottles as quickly as possible to avoid loss of Fe to the volumetric flasks.

6) Aim for treatments to be prepared and in bottles by 1 pm so that sampling for the 4-hour, 8-hour and 16-hour extractions can be carried out at 5 pm, 9 pm and 5 am, respectively.

7) Position all bottles in holders on benchtop shaker (150 RPM) and leave to shake over the 3-day, 7-day and 14-day periods.

Chemistry sampling at test start

1) Check water parameters on the base diluent using clean pH probe (pH should be around 7.7 for the synthetic water).
2) A–D: collect a 0.45-µm filtered sample and an unfiltered sample to be analysed for standard metal suite and alkalinity.
3) C and D only: sample at least 200 mL for measurement of total suspended solids.
4) Measure TOC/DOC for treatments A–D.

Day 3

12–1 pm

1) Take an acid blank (pipette 10 ml of Suprapure HNO_3 directly into sample tube).
2) Take 0.45-µm filtered samples of all Day 3 bottles (Aa1, Aa2, Ba1, Ba2, Ca1, Ca2, Da1, Da2), and acidify to 1% (with 100 µL Suprapur HNO_3).
3) Acidify the above bottles to pH 2 ± 0.03 using 50% v/v HNO_3 for extraction (bottles should be acidified by 1 pm).
4) Record the time and pH of each bottle after acidification and the volume of acid added. Use clean pH probe for all A (or diluent-only) samples.
5) Put all bottles back on the shaker.

5 pm (4-hour extraction)

1) Check and record pH of all bottles and adjust if necessary using Suprapur 50% v/v HNO_3 drop-wise with micropipette.
2) Take the 4-hour samples: 8 × 10-mL, 0.45-µm filtered samples from Day 3 bottles (Aa1, Aa2, Ba1, Ba2, Ca1, Ca2, Da1, Da2), and acidify to 1% (with 100 µL Suprapur HNO_3).

9 pm (8-hour extraction)

1) Check and record pH of all bottles and adjust if necessary using Suprapur 50% v/v HNO_3 drop-wise with micropipette.
2) Take the 8-hour samples: 10-mL ,0.45-µm filtered samples from all Day 3 bottles (Aa1, Aa2, Ba1, Ba2, Ca1, Ca2, Da1, Da2), and acidify to 1% (with 100 µL Suprapur HNO_3).

5 am following day (16-hour extraction)

1) Check and record pH of all bottles.
2) Take the 16-hour samples: 10-mL, 0.45-µm filtered samples from all Day 3 bottles (Aa1, Aa2, Ba1, Ba2, Ca1, Ca2, Da1, Da2), and acidify to 1% (with 100 µL Suprapur HNO_3).
3) Mix remaining sample in bottle well, and transfer to 2 x 50-mL sample tubes for the total-recoverable fraction. Acidify to 1% (with 50 µL Suprapur HNO_3).

Day 7

Repeat steps above for Day 7 samples: Ab1, Ab2, Bb1, Bb2, Cb1, Cb2, Db1, Db2.

Day 14

Repeat steps above for Day 14 samples: Ac1, Ac2, Bc1, Bc2, Cc1, Cc2, Dc1, Dc2.

Sample storage and submission for analyses

Store samples in a refrigerator at ≤ 4 °C prior to analyses of dissolved Fe and total recoverable Fe by ICP-MS. Total recoverable samples with suspended particulate Fe added (C and D samples) should be subjected to the additional extraction steps listed below to ensure the particulate fraction is not under-measured. This does not affect the final method reported in Appendix D for the measurement of bioavailable Fe.

US EPA 200.8 (1994) Section 11.3 for turbid samples

1) Evaporate the 50-mL sample down to 5 mL.
2) Digest sample for 30 minutes with addition of 2 mL HNO_3 (diluted 1:2) and 5 mL of hydrochloric acid (HCl) (diluted 1:5).
3) Increase volume to 50 mL by adding 5 mL of HCl 20% and the rest Milli-Q water.
4) Transfer sample to clean 10-mL polypropylene tube and filter through 0.45-µm syringe filter if required.

Appendix B. pH control

Table B.1 pH values for synthetic freshwater prepared as per Appendix A

Test code: 1899 Fe synthetic freshwater (diluent for treatments B–D) 3 July 2023. Measurements on Day 0:

- pH: 7.64
- conductivity: 152 µS/cm
- dissolved oxygen: 98.4%
- temperature: 21°C.

	Initial replicate 1	Initial replicate 2	4-hour replicate 1	4-hour replicate 2	8-hour replicate 1	8-hour replicate 2	16-hour replicate 1	16-hour replicate 2
Day 3 (Aa)	1.995	1.993	1.992	2.001	2.020	1.980	2.015	1.974
Day 7 (Ab)	1.990	2.005	1.995	1.984	1.980	1.970	1.980	2.015
Day 14 (Ac)	2.002	2.010	1.998	1.992	2.014	2.024	2.025	2.025
Average	**1.996**	**2.003**	**1.995**	**1.992**	**2.005**	**1.991**	**2.007**	**2.005**
Std error	**0.000**	**0.010**	**0.000**	**0.000**	**0.010**	**0.020**	**0.010**	**0.020**
Day 3 (Ba)	1.990	2.001	1.993	1.970	1.971	2.004	2.006	2.020
Day 7 (Bb)	1.991	2.009	1.998	2.007	1.995	2.007	2.014	1.984
Day 14 (Bc)	2.001	1.983	1.995	1.989	2.013	2.005	2.025	2.019
Average	**1.994**	**1.998**	**1.995**	**1.989**	**1.993**	**2.005**	**2.015**	**2.008**
Std error	**0.000**	**0.010**	**0.000**	**0.010**	**0.010**	**0.000**	**0.010**	**0.010**
Day 3 (Ca)	2.000	1.980	1.994	1.985	1.980	1.985	2.009	2.008
Day 7 (Cb)	1.986	1.996	2.010	1.992	2.004	2.000	2.024	2.016
Day 14 (Cc)	2.009	2.012	2.027	2.000	2.009	2.018	2.024	2.005
Average	**2.000**	**2.000**	**2.010**	**1.990**	**2.000**	**2.000**	**2.020**	**2.010**
Std error	**0.010**	**0.010**	**0.010**	**0.000**	**0.010**	**0.010**	**0.010**	**0.000**
Day 3 (Da)	1.998	2.001	1.980	1.974	1.982	1.989	2.010	2.016
Day 7 (Db)	1.983	1.999	2.003	2.003	2.020	2.010	2.028	2.008
Day 14 (Dc)	2.010	2.015	2.000	2.005	2.007	2.009	2.007	2.013
Average	**1.997**	**2.005**	**1.994**	**1.994**	**2.003**	**2.003**	**2.015**	**2.012**
Std error	**0.010**	**0.010**	**0.010**	**0.010**	**0.010**	**0.010**	**0.010**	**0.000**

Table B.2 pH values for lake water from Lake Bennett, NT, prepared as per Appendix A

Test code: 1900 Fe lake water (for treatments B–D), collected 29 September 2023. Measurements on day of collection:

- pH: 7.52
- conductivity: 44.3 µS/cm
- dissolved oxygen: 99%
- temperature: 25.5°C.

Measurements on Day 0 (2 October 2023):

- pH: 7.36
- conductivity: 44.9 µS/cm.

	Initial replicate 1	Initial replicate 2	4-hour replicate 1	4-hour replicate 2	8-hour replicate 1	8-hour replicate 2	16-hour replicate 1	16-hour replicate 2
Day 3 (Aa)	1.998	—	—	—	—	—	2.008	—
Day 7 (Ab)	2.011	—	—	—	—	—	2.013	—
Day 14 (Ac)	2.030	—	1.986	—	1.986	—	2.024	—
Average	**2.013**	—	**1.986**	—	**1.986**	—	**2.015**	—
Std error	**0.010**	—	—	—	—	—	**0.000**	—
Day 3 (Ba)	1.990	2.006	2.006	1.997	1.992	2.010	2.018	1.993
Day 7 (Bb)	2.004	2.006	1.994	1.993	1.980	2.028	2.018	2.011
Day 14 (Bc)	2.006	2.006	2.008	1.982	2.004	1.990	1.989	1.985
Average	**2.000**	**2.006**	**2.003**	**1.991**	**1.992**	**2.009**	**2.008**	**1.996**
Std error	**0.005**	**0.000**	**0.004**	**0.004**	**0.007**	**0.011**	**0.010**	**0.008**
Day 3 (Ca)	2.000	1.987	2.016	1.998	2.011	2.004	1.994	1.986
Day 7 (Cb)	2.011	1.995	2.011	1.998	2.012	1.998	2.020	1.990
Day 14 (Cc)	1.998	2.008	2.006	2.019	2.016	2.023	1.980	2.002
Average	**1.998**	**2.008**	**2.006**	**2.019**	**2.016**	**2.023**	**1.980**	**2.002**
Std error	**0.000**	**0.010**	**0.000**	**0.010**	**0.000**	**0.010**	**0.010**	**0.000**
Day 3 (Da)	1.993	2.011	2.002	2.006	2.009	2.010	1.994	1.986
Day 7 (Db)	2.018	2.016	2.004	2.006	2.004	2.006	1.980	1.980
Day 14 (Dc)	1.973	1.988	1.995	2.020	2.008	2.018	2.010	1.989
Average	**1.995**	**2.005**	**2.000**	**2.011**	**2.007**	**2.011**	**1.995**	**1.985**
Std error	**0.010**	**0.010**	**0.000**	**0.000**	**0.000**	**0.000**	**0.010**	**0.000**

Table B.3 pH values for mine water from Frances Creek, NT, prepared as per Appendix A

Test code: 1900 Fe mine water collected 29 September 2023. Measurements on day of collection:

- pH: 2.97
- conductivity: 3,195 µS/cm
- dissolved oxygen: 98.5%
- temperature: 25.7°C.

Measurements on Day 0 (2 October 2023):

- pH: 2.98
- conductivity: 3,200 µS/cm.

	Initial replicate 1	Initial replicate 2	4-hour replicate 1	4-hour replicate 2	8-hour replicate 1	8-hour replicate 2	16-hour replicate 1	16-hour replicate 2
Day 3 (Ea)	2.020	2.020	2.016	2.008	2.018	2.016	2.001	1.999
Day 7 (Eb)	2.021	2.019	2.007	1.999	2.005	1.987	1.988	1.978
Day 14 (Ec)	2.012	2.004	2.015	2.016	2.006	2.004	2.020	2.013
Average	**2.018**	**2.014**	**2.013**	**2.008**	**2.010**	**2.002**	**2.003**	**1.997**
Std error	**0.000**	**0.010**	**0.000**	**0.000**	**0.000**	**0.010**	**0.010**	**0.010**

Table B.4 pH values for seawater from Nightcliff Jetty, NT, prepared as per Appendix A

Test code: 1901 Fe seawater (for treatments B–D), collected 6 November 2023. Measurements at time of collection:

- pH: 8.13
- conductivity: 53.3 mS/cm
- dissolved oxygen: 100.1%
- temperature: 24.8°C.

Measurements at test start:

- pH: 8.14
- conductivity: 53.5 mS/cm
- dissolved oxygen: 103%
- temperature: 18.5°C.

	Initial replicate 1	Initial replicate 2	4-hour replicate 1	4-hour replicate 2	8-hour replicate 1	8-hour replicate 2	16-hour replicate 1	16-hour replicate 2
Day 3 (Aa)	2.023	—	2.002	—	—	—	1.998	—
Day 7 (Ab)	2.000	—	—	—	1.983	—	1.993	—
Day 14 (Ac)	1.990	—	1.984	—	2.025	—	1.983	—
Average	**2.004**	—	**1.993**	—	**2.004**	—	**1.991**	—
Std error	**0.010**	—	**0.010**	—	**0.020**	—	**0.000**	—
Day 3 (Ba)	1.998	1.989	1.974	1.983	2.000	1.989	2.010	1.994
Day 7 (Bb)	2.026	2.013	1.994	1.996	2.000	1.992	1.996	1.987
Day 14 (Bc)	1.993	2.018	1.994	2.019	1.987	1.999	2.000	2.010
Average	**2.006**	**2.007**	**1.987**	**1.999**	**1.996**	**1.993**	**2.002**	**1.997**
Std error	**0.010**	**0.010**	**0.010**	**0.010**	**0.000**	**0.000**	**0.000**	**0.010**
Day 3 (Ca)	2.002	2.001	1.980	1.993	1.989	1.996	1.993	1.990
Day 7 (Cb)	2.015	2.008	1.998	2.001	1.990	1.993	2.009	2.008
Day 14 (Cc)	1.987	1.976	1.991	1.977	1.995	1.986	2.005	1.992
Average	**1.987**	**1.976**	**1.991**	**1.977**	**1.995**	**1.986**	**2.005**	**1.992**
Std error	**0.010**	**0.010**	**0.010**	**0.010**	**0.000**	**0.000**	**0.000**	**0.010**
Day 3 (Da)	1.982	1.985	1.985	1.983	2.000	1.996	1.996	1.997
Day 7 (Db)	2.006	2.006	2.000	1.997	1.992	1.992	1.994	1.986
Day 14 (Dc)	1.987	2.005	1.984	2.002	1.999	2.018	2.002	2.020
Average	**1.992**	**1.999**	**1.990**	**1.994**	**1.997**	**2.002**	**1.997**	**2.001**
Std error	**0.010**	**0.010**	**0.010**	**0.010**	**0.000**	**0.010**	**0.000**	**0.010**

Appendix C. Iron recovery data

Table C.1 Synthetic freshwater: blanks

Replicate	Fraction[a]	Age (days)	Time (hours)	Fe (mg/L)[b]	Avg of reps 1 and 2 Fe (mg/L)
Aa1	Pre-extraction	3	0	< 0.001	< 0.001
Aa1	Acidified pH 2 and filtered	3	4	< 0.001	< 0.001
Aa1	Acidified pH 2 and filtered	3	8	< 0.001	< 0.001
Aa1	Acidified pH 2 and filtered	3	16	< 0.001	< 0.001
Aa1	**Total recoverable**	**3**	**16**	**< 0.001**	**< 0.001**
Aa2	Pre-extraction	3	0	< 0.001	
Aa2	Acidified pH 2 and filtered	3	4	< 0.001	
Aa2	Acidified pH 2 and filtered	3	8	< 0.001	
Aa2	Acidified pH 2 and filtered	3	16	< 0.001	
Aa2	**Total recoverable**	**3**	**16**	**< 0.001**	
Ab1	Pre-extraction	7	0	< 0.001	< 0.001
Ab1	Acidified pH 2 and filtered	7	4	< 0.001	< 0.001
Ab1	Acidified pH 2 and filtered	7	8	< 0.001	< 0.001
Ab1	Acidified pH 2 and filtered	7	16	< 0.001	< 0.001
Ab1	**Total recoverable**	**7**	**16**	**< 0.001**	**< 0.001**
Ab2	Pre-extraction	7	0	< 0.001	
Ab2	Acidified pH 2 and filtered	7	4	< 0.001	
Ab2	Acidified pH 2 and filtered	7	8	< 0.001	
Ab2	Acidified pH 2 and filtered	7	16	< 0.001	
Ab2	**Total recoverable**	**7**	**16**	**< 0.001**	
Ac1	Pre-extraction	14	0	< 0.001	< 0.001
Ac1	Acidified pH 2 and filtered	14	4	< 0.001	< 0.001
Ac1	Acidified pH 2 and filtered	14	8	< 0.001	< 0.001
Ac1	Acidified pH 2 and filtered	14	16	< 0.001	< 0.001
Ac1	**Total recoverable**	**14**	**16**	**< 0.001**	**< 0.001**
Ac2	Pre-extraction	14	0	< 0.001	
Ac2	Acidified pH 2 and filtered	14	4	< 0.001	
Ac2	Acidified pH 2 and filtered	14	8	< 0.001	
Ac2	Acidified pH 2 and filtered	14	16	< 0.001	
Ac2	**Total recoverable**	**14**	**16**	**< 0.001**	

[a] Pre-extraction samples were 0.45-µm filtered only. Acid-extracted samples were acidified to pH 2 and then 0.45-µm filtered following the specified duration.

[b] Value is the average of 2 duplicate sub-samples from the same bottle.

Table C.2 Synthetic freshwater: freshly spiked oxyhydroxides

Replicate	Fraction[a]	Age (days)	Time (hours)	Fe (mg/L)[b]	Recovery %	Avg of reps 1 and 2 Fe (mg/L)	Avg recovery %
Ba1	Pre-extraction	3	0	0.04	3.6	0.04	3.2
Ba1	Acidified pH 2 and filtered	3	4	0.90	81.8	0.90	81.8
Ba1	Acidified pH 2 and filtered	3	8	1.00	90.9	1.00	90.9
Ba1	Acidified pH 2 and filtered	3	16	1.10	100.0	1.10	100.0
Ba1	**Total recoverable**	**3**	**16**	**1.10**	**100.0**	**1.10**	**100.0**
Ba2	Pre-extraction	3	0	0.03	2.7		
Ba2	Acidified pH 2 and filtered	3	4	0.90	81.8		
Ba2	Acidified pH 2 and filtered	3	8	1.00	90.9		
Ba2	Acidified pH 2 and filtered	3	16	1.10	100.0		
Ba2	**Total recoverable**	**3**	**16**	**1.10**	**100.0**		
Bb1	Pre-extraction	7	0	0.04	3.8	0.05	4.2
Bb1	Acidified pH 2 and filtered	7	4	0.72	68.6	0.71	65.6
Bb1	Acidified pH 2 and filtered	7	8	0.84	80.0	0.83	77.3
Bb1	Acidified pH 2 and filtered	7	16	0.89	84.8	0.89	82.8
Bb1	**Total recoverable**	**7**	**16**	**1.05**	**100.0**	**1.10**	**100.0**
Bb2	Pre-extraction	7	0	0.05	4.5		
Bb2	Acidified pH 2 and filtered	7	4	0.69	62.7		
Bb2	Acidified pH 2 and filtered	7	8	0.82	74.5		
Bb2	Acidified pH 2 and filtered	7	16	0.89	80.9		
Bb2	**Total recoverable**	**7**	**16**	**1.10**	**100.0**		
Bc1	Pre-extraction	14	0	0.05	5.1	0.05	5.1
Bc1	Acidified pH 2 and filtered	14	4	0.72	73.5	0.71	72.8
Bc1	Acidified pH 2 and filtered	14	8	0.85	86.7	0.85	86.7
Bc1	Acidified pH 2 and filtered	14	16	0.94	95.9	0.94	95.9
Bc1	**Total recoverable**	**14**	**16**	**0.98**	**100.0**	**0.98**	**100.0**

Replicate	Fraction[a]	Age (days)	Time (hours)	Fe (mg/L)[b]	Recovery %	Avg of reps 1 and 2 Fe (mg/L)	Avg recovery %
Bc2	Pre-extraction	14	0	0.05	5.2		
Bc2	Acidified pH 2 and filtered	14	4	0.70	72.2		
Bc2	Acidified pH 2 and filtered	14	8	0.84	86.6		
Bc2	Acidified pH 2 and filtered	14	16	0.93	95.9		
Bc2	**Total recoverable**	**14**	**16**	**0.97**	**100.0**		

[a] Acid-extracted samples were acidified to pH 2 and then 0.45-µm filtered following the specified duration.

[b] Value is the average of 2 duplicate sub-samples from the same bottle.

Table C.3 Synthetic freshwater: particulate

Replicate	Fraction[a]	Age (days)	Time (hours)	Avg Fe (mg/L)[b]	Recovery (%)	Avg of reps 1 and 2 Fe (mg/L)	Avg % recovery
Ca1	Pre-extraction	3	0	0.01	0.02	0.01	0.02
Ca1	Acidified pH 2 and filtered	3	4	0.10	0.43	0.10	0.41
Ca1	Acidified pH 2 and filtered	3	8	0.12	0.52	0.12	0.52
Ca1	Acidified pH 2 and filtered	3	16	0.16	0.70	0.16	0.67
Ca1	**Total recoverable[c]**	**3**	**16**	**23.00**	**100.0**	**23.00**	**100.00**
Ca2	Pre-extraction	3	0	0.01	0.02		
Ca2	Acidified pH 2 and filtered	3	4	0.09	0.39		
Ca2	Acidified pH 2 and filtered	3	8	0.12	0.52		
Ca2	Acidified pH 2 and filtered	3	16	0.15	0.65		
Ca2	**Total recoverable**	**3**	**16**	**23.00**	**100.0**		
Cb1	Pre-extraction	7	0	0.01	0.04	0.01	0.04
Cb1	Acidified pH 2 and filtered	7	4	0.08	0.32	0.09	0.35
Cb1	Acidified pH 2 and filtered	7	8	0.11	0.44	0.12	0.47
Cb1	Acidified pH 2 and filtered	7	16	0.14	0.56	0.14	0.55
Cb1	**Total recoverable**	**7**	**16**	**25.00**	**100.00**	**24.50**	**100.00**
Cb2	Pre-extraction	7	0	< 0.01	0.04		
Cb2	Acidified pH 2 and filtered	7	4	0.09	0.40		
Cb2	Acidified pH 2 and filtered	7	8	0.12	0.50		
Cb2	Acidified pH 2 and filtered	7	16	0.13	0.50		
Cb2	**Total recoverable**	**7**	**16**	**24.00**	**100.00**		

Replicate	Fraction[a]	Age (days)	Time (hours)	Avg Fe (mg/L)[b]	Recovery (%)	Avg of reps 1 and 2 Fe (mg/L)	Avg % recovery
Cc1	Pre-extraction	14	0	0.02	0.10	0.02	0.06
Cc1	Acidified pH 2 and filtered	14	4	0.07	0.30	0.07	0.30
Cc1	Acidified pH 2 and filtered	14	8	0.09	0.40	0.09	0.38
Cc1	Acidified pH 2 and filtered	14	16	0.12	0.50	0.12	0.49
Cc1	**Total recoverable**	**14**	**16**	**25.00**	**100.00**	**24.50**	**100.00**
Cc2	Pre-extraction	14	0	0.01	0.04		
Cc2	Acidified pH 2 and filtered	14	4	0.08	0.30		
Cc2	Acidified pH 2 and filtered	14	8	0.10	0.40		
Cc2	Acidified pH 2 and filtered	14	16	0.12	0.50		
Cc2	**Total recoverable**	**14**	**16**	**24.00**	**100.00**		

[a] Acid-extracted samples were acidified to pH 2 and then 0.45-µm filtered following the specified duration.

[b] Value is the average of 2 duplicate sub-samples from the same bottle.

[c] A stronger digestion method was used for the total recoverable fraction to improve recovery (Section 11.3 in US EPA 200.1). Nominal iron based on total suspended solids was 28 mg/L. Recovery using standard US EPA method was 0.4–6.4 mg/L Fe.

Table C.4 Synthetic freshwater: combination of freshly spiked oxyhydroxides and particulates (B and C)

Replicate	Fraction[a]	Age (days)	Time (hours)	Avg Fe (mg/L)[b]	Recovery (%)	Avg of reps 1 and 2 Fe (mg/L)	Avg % recovery
Da1	Pre-extraction	3	0	0.01	0.02	0.02	0.03
Da1	Acidified pH 2 and filtered	3	4	0.92	2.19	0.92	2.10
Da1	Acidified pH 2 and filtered	3	8	1.10	2.62	1.13	2.56
Da1	Acidified pH 2 and filtered	3	16	1.15	2.74	1.18	2.67
Da1	**Total recoverable[c]**	**3**	**16**	**42.00**	**100.00**	**44.00**	**100.00**
Da2	Pre-extraction	3	0	0.02	0.04		
Da2	Acidified pH 2 and filtered	3	4	0.93	2.01		
Da2	Acidified pH 2 and filtered	3	8	1.15	2.50		
Da2	Acidified pH 2 and filtered	3	16	1.20	2.61		
Da2	**Total recoverable**	**3**	**16**	**46.00**	**100.00**		
Db1	Pre-extraction	7	0	0.02	0.05	0.03	0.05
Db1	Acidified pH 2 and filtered	7	4	0.72	1.68	0.72	1.57
Db1	Acidified pH 2 and filtered	7	8	0.82	1.91	0.84	1.83
Db1	Acidified pH 2 and filtered	7	16	0.92	2.14	0.94	2.04
Db1	**Total recoverable**	**7**	**16**	**43.00**	**100.00**	**46.00**	**100.00**
Db2	Pre-extraction	7	0	0.03	0.06		
Db2	Acidified pH 2 and filtered	7	4	0.72	1.47		
Db2	Acidified pH 2 and filtered	7	8	0.86	1.76		
Db2	Acidified pH 2 and filtered	7	16	0.95	1.94		
Db2	**Total recoverable**	**7**	**16**	**49.00**	**100.00**		

Replicate	Fraction[a]	Age (days)	Time (hours)	Avg Fe (mg/L)[b]	Recovery (%)	Avg of reps 1 and 2 Fe (mg/L)	Avg % recovery
Dc1	Pre-extraction	14	0	0.03	0.07	0.03	0.07
Dc1	Acidified pH 2 and filtered	14	4	0.70	1.63	0.70	1.52
Dc1	Acidified pH 2 and filtered	14	8	0.89	2.06	0.87	1.89
Dc1	Acidified pH 2 and filtered	14	16	0.96	2.22	0.96	2.09
Dc1	**Total recoverable**	**14**	**16**	**43.00**	**100.00**	**46.00**	**100.00**
Dc2	Pre-extraction	14	0	0.03	0.06		
Dc2	Acidified pH 2 and filtered	14	4	0.70	1.42		
Dc2	Acidified pH 2 and filtered	14	8	0.85	1.72		
Dc2	Acidified pH 2 and filtered	14	16	0.96	1.96		
Dc2	**Total recoverable**	**14**	**16**	**49.00**	**100.00**		

[a] Acid-extracted samples were acidified to pH 2 and then 0.45-µm filtered following the specified duration.

[b] Value is the average of 2 duplicate sub-samples from the same bottle.

[c] A stronger digestion method was used for the total recoverable fraction to improve recovery (Section 11.3 in US EPA 200.1). Nominal iron based on total suspended solids was 40 mg/L. Recovery using standard US EPA method was 1.5–8 mg/L Fe.

Table C.5 Natural freshwater (Lake Bennett): blanks

Number of blanks minimised to reduce chemistry costs.

Replicate	Fraction[a]	Age (days)	Time (hours)	Fe (mg/L)
Aa	Acidified pH 2	3	16	< 0.001
Aa	**Total recoverable**	**3**	**16**	**< 0.001**
Ab	Acidified pH 2	7	16	< 0.001
Ab	**Total recoverable**	**7**	**16**	**< 0.001**
Ac	Acidified pH 2	14	16	< 0.001
Ac	**Total recoverable**	**14**	**16**	**< 0.001**

[a] Acid-extracted samples were acidified to pH 2 and then 0.45-µm filtered following the specified duration.

Table C.6 Natural freshwater (Lake Bennett): freshly spiked oxyhydroxides

Replicate	Fraction[a]	Age (days)	Time (hours)	Fe (mg/L)[b]	Recovery %	Avg of reps 1 and 2 Fe (mg/L)	Avg % recovery
Ba1	Acidified pH 2 and filtered	3	4	0.37	33.6	0.36	32.73
Ba1	Acidified pH 2 and filtered	3	8	0.62	55.9	0.62	56.59
Ba1	Acidified pH 2 and filtered	3	16	0.82	75.0	0.81	73.64
Ba1	**Total recoverable**	**3**	**16**	**1.10**	**100.0**	**1.10**	**100.00**
Ba2	Acidified pH 2 and filtered	3	4	0.35	31.8		
Ba2	Acidified pH 2 and filtered	3	8	0.63	57.3		
Ba2	Acidified pH 2 and filtered	3	16	0.80	72.7		
Ba2	**Total recoverable**	**3**	**16**	**1.10**	**100.0**		
Bb1	Acidified pH 2 and filtered	7	4	0.32	26.7	0.31	26.97
Bb1	Acidified pH 2 and filtered	7	8	0.59	49.2	0.58	50.49
Bb1	Acidified pH 2 and filtered	7	16	0.75	62.1	0.73	63.77
Bb1	**Total recoverable**	**7**	**16**	**1.20**	**100.0**	**1.15**	**100.00**
Bb2	Acidified pH 2 and filtered	7	4	0.30	27.3		
Bb2	Acidified pH 2 and filtered	7	8	0.57	51.8		
Bb2	Acidified pH 2 and filtered	7	16	0.72	65.5		
Bb2	**Total recoverable**	**7**	**16**	**1.10**	**100.0**		
Bc1	Acidified pH 2 and filtered	14	4	0.27	26.5	0.26	24.61
Bc1	Acidified pH 2 and filtered	14	8	0.37	36.9	0.38	36.63
Bc1	Acidified pH 2 and filtered	14	16	0.57	57.0	0.58	54.86
Bc1	**Total recoverable**	**14**	**16**	**1.00**	**100.0**	**1.05**	**100.00**

Replicate	Fraction[a]	Age (days)	Time (hours)	Fe (mg/L)[b]	Recovery %	Avg of reps 1 and 2 Fe (mg/L)	Avg % recovery
Bc2	Acidified pH 2 and filtered	14	4	0.25	22.7		
Bc2	Acidified pH 2 and filtered	14	8	0.40	36.4		
Bc2	Acidified pH 2 and filtered	14	16	0.58	52.7		
Bc2	**Total recoverable**	**14**	**16**	**1.10**	**100.0**		

[a] Acid-extracted samples were acidified to pH 2 and then 0.45-µm filtered following the specified duration.

[b] Value is the average of 2 duplicate sub-samples from the same bottle.

Table C.7 Natural freshwater (Lake Bennett): particulate

Replicate	Fraction[a]	Age (days)	Time (hours)	Fe (mg/L)[b]	Recovery %	Avg of reps 1 and 2 Fe (mg/L)	Avg % recovery
Ca1	Acidified pH 2 and filtered	3	4	0.11	0.4	0.11	0.34
Ca1	Acidified pH 2 and filtered	3	8	0.17	0.6	0.17	0.54
Ca1	Acidified pH 2 and filtered	3	16	0.18	1.0	0.18	0.58
Ca1	**Total recoverable[c]**	**3**	**16**	**29.00**	**100.0**	**31.00**	**100.00**
Ca2	Acidified pH 2 and filtered	3	4	0.10	0.3		
Ca2	Acidified pH 2 and filtered	3	8	0.16	0.5		
Ca2	Acidified pH 2 and filtered	3	16	0.18	0.5		
Ca2	**Total recoverable**	**3**	**16**	**33.00**	**100.0**		
Cb1	Acidified pH 2 and filtered	7	4	0.12	0.4	0.12	0.40
Cb1	Acidified pH 2 and filtered	7	8	0.17	0.6	0.17	0.57
Cb1	Acidified pH 2 and filtered	7	16	0.18	0.6	0.18	0.62
Cb1	**Total recoverable**	**7**	**16**	**30.00**	**100.0**	**29.00**	**100.00**
Cb2	Acidified pH 2 and filtered	7	4	0.11	0.4		
Cb2	Acidified pH 2 and filtered	7	8	0.16	0.6		
Cb2	Acidified pH 2 and filtered	7	16	0.18	0.6		
Cb2	**Total recoverable**	**7**	**16**	**28.00**	**100.0**		
Cc1	Acidified pH 2 and filtered	14	4	0.10	0.3	0.10	0.28
Cc1	Acidified pH 2 and filtered	14	8	0.14	0.4	0.13	0.39
Cc1	Acidified pH 2 and filtered	14	16	0.18	0.5	0.17	0.51
Cc1	**Total recoverable**	**14**	**16**	**33.00**	**100.0**	**33.50**	**100.00**

Replicate	Fraction[a]	Age (days)	Time (hours)	Fe (mg/L)[b]	Recovery %	Avg of reps 1 and 2 Fe (mg/L)	Avg % recovery
Cc2	Acidified pH 2 and filtered	14	4	0.09	0.3		
Cc2	Acidified pH 2 and filtered	14	8	0.12	0.4		
Cc2	Acidified pH 2 and filtered	14	16	0.16	0.5		
Cc2	**Total recoverable**	**14**	**16**	**34.00**	**100.0**		

[a] Acid-extracted samples were acidified to pH 2 and then 0.45 µm filtered following the specified duration.

[b] Value is the average of 2 duplicate sub-samples from the same bottle.

[c] The total recoverable fraction was measured using a stronger digestion method for turbid waters to improve recovery (Section 11.3 in US EPA 200.1). Nominal iron based on total suspended solids was 39 mg/L.

Table C.8 Natural freshwater (Lake Bennett): combination of freshly spiked oxyhydroxides and particulate (B and C)

Replicate	Fraction[a]	Age (days)	Time (hours)	Fe (mg/L)[b]	Recovery %	Avg of reps 1 and 2 Fe (mg/L)	Avg % recovery
Da1	Acidified pH 2 and filtered	3	4	0.54	1.6	0.52	1.63
Da1	Acidified pH 2 and filtered	3	8	0.92	2.7	0.94	2.94
Da1	Acidified pH 2 and filtered	3	16	0.99	3.0	0.99	3.09
Da1	**Total recoverable[c]**	**3**	**16**	**34.00**	**100.0**	**32.00**	**100.00**
Da2	Acidified pH 2 and filtered	3	4	0.50	1.7		
Da2	Acidified pH 2 and filtered	3	8	0.95	3.2		
Da2	Acidified pH 2 and filtered	3	16	0.98	3.3		
Da2	**Total recoverable**	**3**	**16**	**30.00**	**100.0**		
Db1	Acidified pH 2 and filtered	7	4	0.43	1.4	0.42	1.55
Db1	Acidified pH 2 and filtered	7	8	0.76	2.5	0.72	2.68
Db1	Acidified pH 2 and filtered	7	16	0.87	2.9	0.87	3.24
Db1	**Total recoverable**	**7**	**16**	**30.00**	**100.0**	**27.00**	**100.00**
Db2	Acidified pH 2 and filtered	7	4	0.40	1.7		
Db2	Acidified pH 2 and filtered	7	8	0.68	2.8		
Db2	Acidified pH 2 and filtered	7	16	0.86	3.6		
Db2	**Total recoverable**	**7**	**16**	**24.00**	**100.0**		
Dc1	Acidified pH 2 and filtered	14	4	0.39	1.2	0.39	1.20
Dc1	Acidified pH 2 and filtered	14	8	0.58	1.8	0.57	1.78
Dc1	Acidified pH 2 and filtered	14	16	0.74	2.3	0.76	2.36
Dc1	**Total recoverable**	**14**	**16**	**32.00**	**100.0**	**32.00**	**100.00**

Replicate	Fraction[a]	Age (days)	Time (hours)	Fe (mg/L)[b]	Recovery %	Avg of reps 1 and 2 Fe (mg/L)	Avg % recovery
Dc2	Acidified pH 2 and filtered	14	4	0.38	1.2		
Dc2	Acidified pH 2 and filtered	14	8	0.56	1.8		
Dc2	Acidified pH 2 and filtered	14	16	0.77	2.4		
Dc2	**Total recoverable**	**14**	**16**	**32.00**	**100.0**		

[a] Acid-extracted samples were acidified to pH 2 and then 0.45 µm filtered following the specified duration.

[b] Value is the average of 2 duplicate sub-samples from the same bottle.

[c] The total recoverable fraction was measured using a stronger digestion method for turbid waters to improve recovery (Section 11.3 in US EPA 200.1). Nominal iron based on total suspended solids was 46 mg/L.

Table C.9 Mine water: Frances Creek

Replicate	Fraction[a]	Age (days)	Time (hours)	Fe (mg/L)[b]	Recovery %	Avg of reps 1 and 2 Fe (mg/L)	Avg % recovery
Ea1	Acidified pH 2 and filtered	3	4	2.85	101.8	2.93	100.89
Ea1	Acidified pH 2 and filtered	3	8	3.15	112.5	3.18	109.58
Ea1	Acidified pH 2 and filtered	3	16	3.15	113.0	3.13	107.92
Ea1	**Total recoverable[c]**	**3**	**16**	**2.80**	**100.0**	**2.90**	**100.00**
Ea2	Acidified pH 2 and filtered	3	4	3.00	100.0		
Ea2	Acidified pH 2 and filtered	3	8	3.20	106.7		
Ea2	Acidified pH 2 and filtered	3	16	3.10	103.3		
Ea2	**Total recoverable**	**3**	**16**	**3.00**	**100.0**		
Eb1	Acidified pH 2 and filtered	7	4	3.15	112.5	3.15	108.75
Eb1	Acidified pH 2 and filtered	7	8	2.80	100.0	2.75	95.00
Eb1	Acidified pH 2 and filtered	7	16	3.05	108.9	3.08	106.13
Eb1	**Total recoverable**	**7**	**16**	**2.80**	**100.0**	**2.90**	**100.00**
Eb2	Acidified pH 2 and filtered	7	4	3.15	105.0		
Eb2	Acidified pH 2 and filtered	7	8	2.70	90.0		
Eb2	Acidified pH 2 and filtered	7	16	3.10	103.3		
Eb2	**Total recoverable**	**7**	**16**	**3.00**	**100.0**		
Ec1	Acidified pH 2 and filtered	14	4	2.70	90.0	2.65	88.33
Ec1	Acidified pH 2 and filtered	14	8	2.65	88.3	2.63	87.50
Ec1	Acidified pH 2 and filtered	14	16	2.60	86.7	2.63	87.50
Ec1	**Total recoverable**	**14**	**16**	**3.00**	**100.0**	**3.00**	**100.00**

Replicate	Fraction[a]	Age (days)	Time (hours)	Fe (mg/L)[b]	Recovery %	Avg of reps 1 and 2 Fe (mg/L)	Avg % recovery
Ec2	Acidified pH 2 and filtered	14	4	2.60	86.7		
Ec2	Acidified pH 2 and filtered	14	8	2.60	86.7		
Ec2	Acidified pH 2 and filtered	14	16	2.65	88.3		
Ec2	**Total recoverable**	**14**	**16**	**3.00**	**100.0**		

[a] Acid-extracted samples were acidified to pH 2 and then 0.45 µm filtered following the specified duration.

[b] Value is the average of 2 duplicate sub-samples from the same bottle.

[c] The total recoverable fraction was measured using a stronger digestion method for turbid waters to improve recovery (Section 11.3 in US EPA 200.1). Nominal iron based on information from the mine was 3 mg/L.

Table C.10 Natural seawater (Nightcliff): blanks

Number of blanks minimised to reduce chemistry costs.

Replicate	Fraction[a]	Age (days)	Time (hours)	Fe (mg/L)
Aa	Acidified pH 2	3	16	< 0.001
Aa	**Total recoverable**	**3**	**16**	**< 0.001**
Ab	Acidified pH 2	7	16	< 0.001
Ab	**Total recoverable**	**7**	**16**	**< 0.001**
Ac	Acidified pH 2	14	16	< 0.001
Ac	**Total recoverable**	**14**	**16**	**< 0.001**

[a] Acid-extracted samples were acidified to pH 2 and then 0.45-µm filtered following the specified duration.

Table C.11 Natural seawater (Nightcliff): freshly spiked oxyhydroxides

Replicate	Fraction[a]	Age (days)	Time (hours)	Fe (mg/L)[b]	Recovery %	Avg of reps 1 and 2 Fe (mg/L)	Avg % recovery
Ba1	Acidified pH 2 and filtered	3	4	0.82	82.0	0.83	83.00
Ba1	Acidified pH 2 and filtered	3	8	0.84	84.0	0.84	84.00
Ba1	Acidified pH 2 and filtered	3	16	0.88	88.0	0.87	86.50
Ba1	**Total recoverable**	**3**	**16**	**1.00**	**100.0**	**1.00**	**100.00**
Ba2	Acidified pH 2 and filtered	3	4	0.84	84.0		
Ba2	Acidified pH 2 and filtered	3	8	0.84	84.0		
Ba2	Acidified pH 2 and filtered	3	16	0.85	85.0		
Ba2	**Total recoverable**	**3**	**16**	**1.00**	**100.0**		
Bb1	Acidified pH 2 and filtered	7	4	0.84	84.0	0.85	84.75
Bb1	Acidified pH 2 and filtered	7	8	0.86	85.5	0.86	85.75
Bb1	Acidified pH 2 and filtered	7	16	0.86	86.0	0.87	86.50
Bb1	**Total recoverable**	**7**	**16**	**1.00**	**100.0**	**1.00**	**100.00**
Bb2	Acidified pH 2 and filtered	7	4	0.86	85.5		
Bb2	Acidified pH 2 and filtered	7	8	0.86	86.0		
Bb2	Acidified pH 2 and filtered	7	16	0.87	87.0		
Bb2	**Total recoverable**	**7**	**16**	**1.00**	**100.0**		
Bc1	Acidified pH 2 and filtered	14	4	0.98	81.3	0.96	79.79
Bc1	Acidified pH 2 and filtered	14	8	0.98	81.3	0.98	81.46
Bc1	Acidified pH 2 and filtered	14	16	0.97	80.8	0.96	80.00
Bc1	**Total recoverable**	**14**	**16**	**1.20**	**100.0**	**1.20**	**100.00**

Replicate	Fraction[a]	Age (days)	Time (hours)	Fe (mg/L)[b]	Recovery %	Avg of reps 1 and 2 Fe (mg/L)	Avg % recovery
Bc2	Acidified pH 2 and filtered	14	4	0.94	78.3		
Bc2	Acidified pH 2 and filtered	14	8	0.98	81.7		
Bc2	Acidified pH 2 and filtered	14	16	0.95	79.2		
Bc2	**Total recoverable**	**14**	**16**	**1.20**	**100.0**		

[a] Acid-extracted samples were acidified to pH 2 and then 0.45-µm filtered following the specified duration.

[b] Value is the average of 2 duplicate sub-samples from the same bottle.

Table C.12 Natural seawater (Nightcliff): particulate

Replicate	Fraction[a]	Age (days)	Time (hours)	Fe (mg/L)[b]	Recovery %	Avg of reps 1 and 2 Fe (mg/L)	Avg % recovery
Ca1	Acidified pH 2 and filtered	3	4	0.65	1.9	0.63	1.94
Ca1	Acidified pH 2 and filtered	3	8	0.67	2.0	0.71	2.18
Ca1	Acidified pH 2 and filtered	3	16	0.72	2.0	0.74	2.27
Ca1	**Total recoverable[c]**	**3**	**16**	**34.00**	**100.0**	**32.50**	**100.00**
Ca2	Acidified pH 2 and filtered	3	4	0.61	2.0		
Ca2	Acidified pH 2 and filtered	3	8	0.74	2.4		
Ca2	Acidified pH 2 and filtered	3	16	0.76	2.4		
Ca2	**Total recoverable**	**3**	**16**	**31.00**	**100.0**		
Cb1	Acidified pH 2 and filtered	7	4	0.84	2.6	0.86	2.94
Cb1	Acidified pH 2 and filtered	7	8	0.88	2.8	0.90	3.07
Cb1	Acidified pH 2 and filtered	7	16	0.92	2.9	0.94	3.22
Cb1	**Total recoverable**	**7**	**16**	**32.00**	**100.0**	**29.50**	**100.00**
Cb2	Acidified pH 2 and filtered	7	4	0.88	3.3		
Cb2	Acidified pH 2 and filtered	7	8	0.92	3.4		
Cb2	Acidified pH 2 and filtered	7	16	0.97	3.6		
Cb2	**Total recoverable**	**7**	**16**	**27.00**	**100.0**		
Cc1	Acidified pH 2 and filtered	14	4	1.10	4.2	1.10	4.23
Cc1	Acidified pH 2 and filtered	14	8	1.10	4.2	1.10	4.23
Cc1	Acidified pH 2 and filtered	14	16	1.20	4.6	1.15	4.42
Cc1	**Total recoverable**	**14**	**16**	**26.00**	**100.0**	**26.00**	**100.00**

Replicate	Fraction[a]	Age (days)	Time (hours)	Fe (mg/L)[b]	Recovery %	Avg of reps 1 and 2 Fe (mg/L)	Avg % recovery
Cc2	Acidified pH 2 and filtered	14	4	1.10	4.2		
Cc2	Acidified pH 2 and filtered	14	8	1.10	4.2		
Cc2	Acidified pH 2 and filtered	14	16	1.10	4.2		
Cc2	**Total recoverable**	**14**	**16**	**26.00**	**100.0**		

[a] Acid-extracted samples were acidified to pH 2 and then 0.45-µm filtered following the specified duration.

[b] Value is the average of 2 duplicate sub-samples from the same bottle.

[c] The total recoverable fraction was measured using a stronger digestion method for turbid waters to improve recovery (Section 11.3 in US EPA 200.1). Nominal iron based on total suspended solids was 35 mg/L.

Table C.13 Natural seawater (Nightcliff): combination of freshly spiked oxyhydroxides and particulate (B and C)

Replicate	Fraction[a]	Age (days)	Time (hours)	Fe (mg/L)[b]	Recovery %	Avg of reps 1 and 2 Fe (mg/L)	Avg % recovery
Da1	Acidified pH 2 and filtered	3	4	1.45	4.5	1.43	4.52
Da1	Acidified pH 2 and filtered	3	8	1.45	4.5	1.50	4.77
Da1	Acidified pH 2 and filtered	3	16	1.50	5.0	1.53	4.84
Da1	**Total recoverable[c]**	**3**	**16**	**32.00**	**100.0**	**31.50**	**100.00**
Da2	Acidified pH 2 and filtered	3	4	1.40	4.5		
Da2	Acidified pH 2 and filtered	3	8	1.55	5.0		
Da2	Acidified pH 2 and filtered	3	16	1.55	5.0		
Da2	**Total recoverable**	**3**	**16**	**31.00**	**100.0**		
Db1	Acidified pH 2 and filtered	7	4	1.80	4.9	1.80	4.86
Db1	Acidified pH 2 and filtered	7	8	2.10	5.7	2.00	5.41
Db1	Acidified pH 2 and filtered	7	16	2.00	5.4	1.98	5.34
Db1	**Total recoverable**	**7**	**16**	**37.00**	**100.0**	**37.00**	**100.00**
Db2	Acidified pH 2 and filtered	7	4	1.80	4.9		
Db2	Acidified pH 2 and filtered	7	8	1.90	5.1		
Db2	Acidified pH 2 and filtered	7	16	1.95	5.3		
Db2	**Total recoverable**	**7**	**16**	**37.00**	**100.0**		
Dc1	Acidified pH 2 and filtered	14	4	1.80	5.6	1.85	5.53
Dc1	Acidified pH 2 and filtered	14	8	1.80	5.6	1.83	5.46
Dc1	Acidified pH 2 and filtered	14	16	1.95	6.1	1.88	5.62
Dc1	**Total recoverable**	**14**	**16**	**32.00**	**100.0**	**33.50**	**100.00**

Replicate	Fraction[a]	Age (days)	Time (hours)	Fe (mg/L)[b]	Recovery %	Avg of reps 1 and 2 Fe (mg/L)	Avg % recovery
Dc2	Acidified pH 2 and filtered	14	4	1.90	5.4		
Dc2	Acidified pH 2 and filtered	14	8	1.85	5.3		
Dc2	Acidified pH 2 and filtered	14	16	1.80	5.1		
Dc2	**Total recoverable**	**14**	**16**	**35.00**	**100.0**		

[a] Acid-extracted samples were acidified to pH 2 and then 0.45-µm filtered following the specified duration.

[b] Value is the average of two duplicate sub-samples from the same bottle.

[c] The total recoverable fraction was measured using a stronger digestion method for turbid waters to improve recovery (Section 11.3 in US EPA 200.8). Nominal iron based on total suspended solids was 34 mg/L.

Appendix D. Method for the determination of potentially bioavailable iron in environmental freshwater and seawater samples through extraction at pH 2

Scope

This method can be used to determine acid-soluble Fe in freshwater and seawater. Results from this method may be used to assess the potential impact of bioavailable Fe^{3+} on water quality and aquatic biota.

Summary of the method

This method describes the procedure for preparing an aqueous sample for determination of acid-soluble Fe prior to analysis by ICP-MS. This method does not detail the requirements for ICP-MS analyses, as it is expected that analytical laboratories will already have their established procedures in place to meet their quality-assurance/quality-control requirements for ICP-MS. The method has been adapted from the US EPA (1991) method for determination of metals (other than Fe) in environmental samples and has been validated for Fe through several studies that can be referred to for further details (Balsamo-Crespo et al. 2022; ANZG 2024).

Safety

This method involves the use of concentrated and dilute HNO_3. Concentrated HNO_3 should be used in a fume cupboard. Safety glasses and a laboratory coat should be used for protection of the skin and eyes when working with acids. Refer to the safety recommendations listed in the Safety Data Sheet relevant to the strength of HNO_3 being used.

Apparatus

- calibrated pH meter
- platform mixer (optional)
- glassware: Schott bottle for 50% v/v HNO_3
- calibrated micropipettes
- sample storage bottles: polyethylene, wide-mouthed to facilitate direct pH measurement and adjustment, a 250-mL bottle containing 200-mL sample volume is recommended; however, 100 mL would be sufficient volume to enable pH measurement)
- sample-collection tubes (15-mL high-density polyethylene tubes for a 10-mL sample volume)
- 30-mL or 50-mL disposable, sterilised plastic syringes

- 0.45-µm syringe filters (mixed-cellulose-ester membrane filters or polyethersulfone membrane filters are suitable).

Reagents or standards

- ultra-pure deionised water (> 18-MΩ/cm resistivity at 25 °C).
- concentrated HNO_3 of ultra-high purity grade for trace analysis (e.g. Suprapur) for sample acidification
- 50% v/v HNO_3 prepared by adding an equal volume of ultra-high-purity grade HNO_3 to ultra-high-purity deionised water
- concentrated nitric acid of American Chemical Society grade or reagent grade for preparation of 10% HNO_3 acid baths for cleaning of labware
- 3 pH buffer solutions: pH 2, 4 and 7, or pH 2, 4 and 10. The use of commercially available certified solutions is recommended.

Sample collection

For detailed information regarding the recommended methods for grab sampling, refer to the *Water monitoring and sampling manual* (DES 2018). General principles are listed below.

- Collect and handle samples in a way that minimises contamination.
- Wear disposable gloves during sample collection and replace as necessary (e.g. at each new site).
- Pre-soaking or acid washing sample bottles or tubes is not required if it can be shown through procedural blanks and laboratory quality-assurance procedures that the containers are not introducing any contamination.
- Once at the site, pre-rinse the sample bottle with water from the site prior to sample collection, if possible.
- Collect samples promptly, and recap the bottle as soon as possible to minimise airborne sources of contamination.
- Collect a minimum sample volume of 100 mL to provide sufficient volume for pH measurement.

Sample storage and preservation

- Once samples have been transported to the laboratory, process them as soon as possible.
- Acidify samples in the laboratory, rather than in the field, to avoid safety risks associated with the transportation and handling of concentrated HNO_3.
- If samples are being collected from a remote location, where there will be a delay until processing occurs, they can be held for up to 14 days at room temperature (~22 °C) prior to processing. However, optimal recovery of Fe^{3+} is achieved if samples can be processed within 3 days. Acceptable holding time may decrease if samples are exposed to temperatures > 22 °C. If those conditions are anticipated, store samples on ice in a cooler during transit.
- Once samples have undergone pH-2 extraction and have been filtered and acidified to 1% HNO_3, they do not need to be refrigerated prior to ICP-MS analysis and they have a recommended holding time of 6 months (US EPA 2018).

pH meter calibration

- Specific instructions for the calibration of the pH meter will be dependent on the pH meter being used.
- Calibrate the pH meter on the day of use.
- Include in the calibration a minimum of 2 points, one of which being pH 2. However, calibration at 3 points – pH 2, pH 4 and pH 7 or 10 – is ideal.

pH 2 extraction

1) Starting with any blanks and using a clean, calibrated pH probe, measure the pH of the sample directly in the sample bottle. If there are contamination concerns over measuring pH directly from the sample bottle, and there is sufficient sample volume to sub-sample for pH measurement, take a sub-sample to measure pH. Based on the pH reading, adjust pH directly in the sample bottle. Take a subsequent sub-sample to confirm final pH. Alternatively, if there is insufficient volume to sub-sample for this process, use a clean probe for blanks or reference samples and a separate probe for samples elevated in Fe. A procedural laboratory blank (see Quality control) can also be used to identify whether this process is resulting in any sample contamination.
2) Acidify the sample to pH 2 ± 0.1 by adding 50% v/v HNO_3 directly to the bottle using a micropipette (as a guide, a 200-mL sample with original pH of ~7.7 will require around 300 µL of 50% v/v HNO_3 to acidify to pH 2). If sample volume is limited, acidification can be done in a 50-mL sample tube, adding approximately 50 µL of 50% v/v HNO_3 to 40 mL of sample (if starting pH is close to neutral).
3) Wait for the pH reading to settle and, if pH is still within range, recap the bottle or tube and place it on a platform mixer or bottle roller for 16 hours at room temperature so that samples are continuously agitated.
4) Repeat steps 1–3 for all samples, processing them in order of lowest to highest nominal Fe concentrations (if known). The pH probe should be rinsed with ultra-pure deionised water and dried between samples.

Sample filtration

Samples are filtered to < 0.45 µm, ideally using syringe filters. A sample volume of 10–15 mL is generally considered acceptable. The use of syringe filters minimises the surface area (compared to larger-volume field filtration kits) where Fe^{3+} could potentially adsorb to the inner surface and be lost from the sample. If possible, rinse the syringe and filter in sample water prior to taking the sample. To do this, draw up a full syringe of sample, attach the filter, and push the sample through the filter to waste. When drawing up the sample, avoid allowing contact between the sample and the syringe stopper in the syringe chamber.

Sample acidification

Filtered samples are acidified to 1% HNO_3 using ultra-high purity grade HNO_3.

Sample analyses

Samples are analysed for dissolved iron by ICP-MS.

Quality control

It is recommended, if possible, that the following blanks accompany a batch of samples:

- acid blank: pipette ultra-high-purity grade HNO_3 directly into a sample tube without filtration to check for contamination introduced by the concentrated acid
- laboratory blank: collect high-purity deionised water directly into a sample tube and acidify to 1% HNO_3.
- procedural laboratory blank: collect high-purity deionised water into a sample bottle and process the blank in the same way as a field sample (pH-2 extraction, 0.45-µm syringe filtration into a sample tube, acidification to 1% HNO_3). This checks for any contamination introduced throughout the whole laboratory process.
- field blank: take high-purity deionised water into the field and, at the time of sample collection, collect it into a sample bottle of the same type as that used for the sample. This field blank is processed the same way as the field sample/s through pH-2 extraction, 0.45-µm filtration and acidification to 1% HNO_3.

With regards to ICP-MS analyses, laboratories will have their own established quality-control procedures and acceptability criteria. This will usually involve blank samples, duplicate samples, matrix spikes and running of standard reference material of known concentration.

References

ANZG (Australian and New Zealand Guidelines) (2018) *Australian and New Zealand Guidelines for Fresh and Marine Water Quality*, Australian and New Zealand governments and Australian state and territory governments.

APHA (American Public Health Association) (2018) *Standard methods for the examination of water and wastewater,* 23rd edition, APHA, American Water Works Association, Water Environment Federation, doi:10.2105/SMWW.2882.041.

Balsamo Crespo E, Reichelt-Brushett A, Smith RE, Rose AL and Batley GE (2023) 'Improving the measurement of iron(III) bioavailability in freshwater samples: methods and performance', *Environmental Toxicology and Chemistry*, 42(2):303–316, doi:10.1002/etc.5530.

DES (Department of Environment and Science) (2018) *Monitoring and sampling manual: Environmental Protection (Water) Policy 2009*, DES, Queensland Government, Brisbane, accessed 22 March 2024.

ECCC (Environment and Climate Change Canada) (2024) *Federal environmental quality guidelines – iron*, Canadian Environmental Protection Act, 1999, ECCC, Quebec.

US EPA (United States Environmental Protection Agency) (1991) 'Method 200.1 Determination of acid-soluble metals', in *Methods for the determination of metals in environmental samples*, EPA/600/4-91/010, Office of Research and Development, Cincinnati.

US EPA (1992a) *SW-846 Test Method 3010A: acid digestion of aqueous samples and extracts for total metals for analysis by flame atomic absorption spectroscopy (FLAA) or inductively coupled plasma spectroscopy (ICP)*.

US EPA (1992b) *SW-846 Test Method 3005A: acid digestion of waters for total recoverable or dissolved metals for analysis by flame atomic absorption (FLAA) or inductively coupled plasma (ICP) spectroscopy*.

US EPA (1994) 'Method 200.8 Determination of trace elements in waters and wastes by inductively coupled plasma – mass spectrometry', Revision 5.4, EPA/600/4-91/010, Cincinnati.

US EPA (2018) 'Chapter 3, Inorganic analytes', in *Test methods for evaluating solid waste: physical/chemical methods compendium, SW-846*, Version VI, accessed 7 November 2023.