Technical Note: Uncovering the influence of methodological variations on the extractability of iron bound organic carbon~~.~~

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Abstract~~.~~

Association of organic carbon (OC) with reactive iron (Fe$_R$) represents an important mechanism by which OC is protected against remineralisation in soils and marine sediments. Recent studies indicate that the molecular structure of organic compounds and/or the identity of associated Fe$_R$ phases exerts a control on the ability of an OC-Fe$_R$ complex to be extracted by the citrate-bicarbonate-dithionite (CBD) method. ~~While~~However, many variations of ~~this method exist in~~ the ~~literature,~~CBD extraction are used, and these are often uncalibrated to each other, rendering comparisons of OC-Fe$_R$ values extracted ~~by~~via the different ~~method iterations~~methods impossible. Here, we created ~~a~~ synthetic ferrihydrite ~~sample~~samples coprecipitated with simple organic structures and subjected these to modifications of the most common CBD method. ~~Method~~We altered some of the method parameters (reagent concentration, time of the extraction and sample preparation methods) ~~were altered~~ and measured Fe$_R$ recovery ~~measured~~ to determine which (if any) modifications ~~resulted in~~affected the ~~greatest~~ release of Fe$_R$ from the ~~sediment~~synthetic sample. We provide an assessment of the reducing capacity of Na dithionite in the CBD method (the amount of Fe reduced by a fixed amount of dithionite) and find that the concentration of dithionite deployed can limit OC-Fe$_R$ extractability for sediments with a high Fe$_R$ content. Additionally, we show that extending the length of any CBD extraction offers no benefit in removing Fe$_R$. ~~Finally~~Moreover, we demonstrate that for synthetic OC-Fe$_R$ samples~~, the almost universal technique of~~ dominated by ferrihydrite, freeze~~-~~-drying samples can significantly reduce OC-Fe$_R$ extractability~~ and we offer insight into how,~~ this ~~may translate~~appears to ~~environmental samples using Arctic Ocean~~be less of an issue for natural marine sediments~~. These results~~ where natural aging mechanisms may mimic the freeze-drying process for more stable Fe phases. While our study is not an all-inclusive method comparison and is not aimed at delivering the "perfect" extraction setup, our findings provide a ~~valuable perspective on how~~collected summary of critical factors which influence the efficiency of ~~this extraction could be improved to provide a more accurate assessment~~the CBD extraction for OC-Fe$_R$. As such, we provide a valuable platform from which OC-Fe$_R$ values obtained under different methods can be interpreted and future studies of sediment ~~OC-Fe$_R$ content. Accurate determinations of OC-Fe$_R$ in sediments and soils represents an important step in improving our understanding of, and ability to model, the global~~ carbon ~~cycle~~cycling can build upon.

1. Introduction

~~Constraining parameters of biogeochemical cycles remains one of the largest challenges in the development of Earth system and climate models (Achterberg, 2014). Understanding in which environments organic carbon (OC) persists, the mechanisms which facilitate its preservation and the extent of such mechanisms is crucial for our understanding of the global carbon cycle. Marine sediments represent the largest sink for OC~~Marine sediments represent the largest sink for organic carbon (OC) on Earth (Hedges and Keil, 1995), and as such the preservation of OC here is crucial in controlling atmospheric carbon dioxide (CO$_2$) levels ~~and maintaining an oxygenated Earth~~over geological timescales (Canfield, 1993). Preservation of OC has been linked to different mechanistic and environmental factors, e.g. intrinsic recalcitrance of biomacromolecules, physical protection of OC by organic/inorganic matrices and redox conditions (Burdige, 2007 and references therein). ~~Physical~~The physical protection of OC by association with reactive iron (Fe$_R$) minerals, via mono or multi-layer adsorption and/or coprecipitation, is thought to represent a significant mechanism by which OC is preserved in marine sediments, accounting for 10-20% of the ~~preserved sediment~~sedimentary OC pool (Lalonde et al., 2012; Salvadó et al., 2015; ~~Faust~~Ma et al., ~~2020~~2018; Zhao et al., 2018; Wang et al., 2019; ~~Ma~~Faust et al., ~~2018~~2020, 2021). Additionally, the OC-Fe$_R$ interaction is equally important in extending the residence time of OC in soils,~~ important~~ for water retention (Rawls et al., 2003), resilience to erosion, and overall soil fertility via nutrient bioavailability (Milne et al., 2015). The prevalence of OC-Fe$_R$ is generally greater in soils than in sediments, accounting for approximately 40% of soil total organic carbon (TOC) ~~as per ~~(Wagai and Mayer ~~(~~2007~~) and~~,, Zhao et al. ~~(~~2016~~).~~)

~~Extraction of reactive Fe bound OC (OC-Fe$_R$) has been conducted by various iterations of the citrate-bicarbonate-dithionite (CBD) method, originally in soils (Deb, 1950; Mehra and Jackson, 1958) before being applied to marine sediments by Lalonde et al. (2012). The OC-Fe$_R$ extraction operates on the principle that reductive dissolution of reactive Fe phases with sodium (Na) dithionite liberates Fe$_R$-bound OC from the sediment matrix. The dissolution is conducted at circumneutral pH buffered with sodium bicarbonate and trisodium citrate to prevent hydrolysis of OC (Mehra and Jackson, 1958; Lalonde et al., 2012). Despite the longevity of this method, recent studies have identified inefficiencies. For example, Adhikari and Yang (2015) reported that only 5-44% of OC was released from hematite-humic acid complexes upon Fe dissolution. Fisher et al. (2020) also document incomplete (<60%) reduction of OC-ferrihydrite complexes by the same method and show that the molecular composition of associated OC has a large influence on Fe reactivity, with carboxyl rich compounds being most resistant towards extraction. As the extraction is operationally defined (i.e. extracts based upon susceptibility of an individual compound/mineral to chemical treatment, not upon the identity of that compound/mineral), organic matter (OM) composition and Fe phase crystallinity both have the ability to alter the reactivity of an Fe-OC compound to a point where a compound is not extracted any more by the CDB method. These findings contrast the previous understanding of the CBD method performed in an experimental context which states that this extraction will "fully reduce all solid reactive iron phases and associated organic carbon" (Lalonde et al., 2012). So far, our developed knowledge has allowed for a cautious understanding and~~

~~interpretation of OC-Fe$_R$ values, but it has yet to be seen whether this extraction can be improved in order to provide a more accurate assessment of the extent to which OC-Fe$_R$ represents an important mechanism for OC preservation in marine sediments.~~

~~Here, we synthesised a ferrihydrite sample coprecipitated with simple organic compounds spiked into marine sediment, mimicking a natural marine sediment matrix containing OC-Fe$_R$. This method allows for the creation of a sample with a known concentration of reducible Fe, therefore an accurate determination of ferrihydrite extraction efficiency can be obtained by subtracting end state Fe from initial concentrations while accounting for mass loss. Here we aim to maximise Fe extractability in order to increase the proportion of the OC-Fe$_R$ pool extracted by CBD.~~

~~Systematic improvements to the CBD method have not yet been attempted, most likely due to the multiple constraints associated with trying to quantitatively extract both OC and Fe. One such constraint is the pH at which the extraction is conducted; lowering the pH below circumneutral values will result in hydrolysis of OC, releasing OC not associated with Fe$_R$ from the sediment matrix. It should be noted, however, that lower pH extractions are known to be more efficient at extracting the targeted reactive Fe phases if the co-extracted organic compounds are not of interest (Thompson et al., 2019).~~

Extraction of OC bound to reactive iron (OC-Fe$_R$) has been conducted by various iterations of the citrate-bicarbonate-dithionite (CBD) method, originally in soils (Deb, 1950; Mehra and Jackson, 1958) before being applied to marine sediments by Lalonde et al. (2012). The OC-Fe$_R$ extraction operates on the principle that reductive dissolution of reactive Fe phases with sodium (Na) dithionite exclusively and quantitatively liberates Fe$_R$-bound OC from the sediment matrix. The dissolution is conducted at circumneutral pH buffered with sodium bicarbonate and trisodium citrate to prevent partial hydrolysis of OC (Mehra and Jackson, 1958; Lalonde et al., 2012). The circumneutral pH CBD extraction has also been used as part of the original SEDEX protocol for the extraction of Fe bound phosphate (Fe$_P$) (Ruttenberg, 1992;Kraal et al., 2012). Although thermodynamically different from the CBD extraction for OC-Fe$_R$ (8 hours at 25 °C vs. 15 minutes at 80 °C), Slomp et al. (1996) found no difference between the efficiency of this phosphate extraction and the shortened high temperature extraction of Mehra and Jackson (1958). While Ruttenberg (1992) and Thompson et al. (2019) report 90-100% of synthetic ferrihdyrite is extracted by the CBD method for Fe$_P$, the dithionite-to-sample ratio in their studies was more than double the ratio used in the OC-Fe$_R$ extraction by Lalonde et al. (2012) (1.125g dithionite for 0.5g sediment vs. 0.25g dithionite for 0.25g sediment). However, new findings indicate that CBD is less efficient at extracting crystalline hematite than previously thought, with 18.4 ± 0.7% of Fe in a synthetic hematite sample recovered by Thompson et al. (2019). Similarly, this inefficiency has been shown in the context of OC-Fe$_R$ extractions conducted at the lower dithionite strength where Adhikari and Yang (2015) report 5-44% of OC was released from hematite-humic acid complexes upon Fe dissolution. Fisher et al. (2020) also document incomplete (<60%) reduction of OC-ferrihydrite complexes by the same method (0.25g of dithionite) and show that the molecular composition of associated OC has a large influence on Fe reactivity towards dithionite, with carboxyl rich compounds being most resistant towards extraction. As the extraction is operationally defined based upon the susceptibility of an individual compound/mineral to chemical treatment and not upon the identity of that compound/mineral, OC composition and Fe phase crystallinity both have the ability to alter the reactivity, and therefore susceptibility, of an OC-Fe$_R$ compound to extraction by CBD treatment. These findings contrast with previous understanding of the CBD method performed in an experimental context, which states that this extraction will reduce “all solid reactive iron phases and the organic carbon associated with these phases” (Lalonde et al., 2012). Given the incomplete extraction of OC-Fe$_R$ in synthetic samples and the apparent sensitivity of the method to changes in OC composition and Fe phase, we investigated whether differences in method parameters can alter the amount of OC-Fe$_R$ extracted from a given matrix, in order to better understand the robustness of the method and to determine how methodological variation in the CBD extraction can affect the comparability of OC-Fe$_R$ results. This is an important consideration to validate OC-Fe$_R$ results from different studies, and to build a global understanding of the extent to which OC-Fe$_R$ interactions contribute to the carbon cycle.

In previous studies using the ~~CDB~~CBD method, concentrations of Na dithionite, and the ratio of Na dithionite to sample mass in the reaction, were not uniform~~, and a summary of the most substantive differences is shown in Table 1. This demonstrates how~~ (Table 1). Thus, the same extraction has been conducted with different “chemical strengths” which, for an operationally defined extraction, ~~makes~~could make comparison of results from such experiments impossible. Despite these wide variations in Na dithionite concentration, ~~previous studies have not conducted~~a systematic ~~assessments~~assessment of the reductive strength of dithionite for soils or sediments has not been conducted. Additionally, earlier studies make reference to repeating the extraction multiple times for Fe rich samples (Mehra and Jackson, 1958; Aguilera and Jackson, 1953), or to altering sample mass to account for variability in Fe contents (Wagai and Mayer, 2007). Such considerations have been lost in more recent iterations of the ~~CDB~~CBD method applied to sediments (Lalonde et al., 2012~~).~~

~~Our approach is targeted towards testing variations in~~), and the ~~physical parameters~~effect of these method alterations on OC-Fe$_R$ remains largely unknown due to the ~~CBD method without changing the chemical “recipe” (including pH conditions)~~lack of ~~the reductive dissolution method. Stages of the CBD method were individually tested for different extraction times, Na dithionite concentrations and sample preparation methods.~~

~~Reaction time was investigated as~~cross-calibration. Similarly, some studies have extended the run time of the CBD extraction from the ~~most common time of~~original 15 minutes (~~Lalonde et al., 2012;~~ Aguilera and Jackson, 1953; Mehra and Jackson, 1958;Lalonde et al., 2012)~~.~~. Patzner et al. (2020) performed the CBD extraction of Lalonde et al. (2012), adjusted to room temperature, over 16 hours and Wagai and Mayer (2007) ~~performed a 16 hour extraction (substituting citrate with weak HCL acid rinses to avoid use of organic compounds), and Patzner et al. (2020) extended to 6 hours. The usefulness of extending a dithionite based extraction is questionable due to the known rapid decomposition of dithionite in aqueous form suggesting, a quick loss of reduction potential~~also performed a 16 hour extraction, adjusted to be citrate free. In this application citrate, used to complex Fe, was substituted with a weak HCl rinse to redissolve precipitated Fe in an attempt to avoid the interference of citrate in OC quantification. As the effect of extending reaction times is unknown, but appears unlikely to be of significant benefit in improving the amount of Fe liberated due to the rapid degradation of Na dithionite in aqueous form (Lister and Garvie, 1959; Lem and Wayman, 1970)~~. Finally, sample preparation methods were examined since the commonly applied method of freeze drying is thought to cause particle aggregation, which may artificially shield Fe phases from reduction. The alternative approach of using a sediment slurry has been applied to soils (Chen et al., 2020) but has not been investigated in the context of marine sediments. ~~, we also included reaction time as a variable.

~~After conducting extractions under a wide range of parameter variations, we aimed to establish whether there was one clear set of optimum conditions for all CBD extractions or whether the extraction approach should be dynamic, i.e. adjusted to chemical and physical characteristics of any individual sample set.~~

To address the question of how methodological variation affects OC-Fe$_R$ extraction, we synthesised OC-Fe$_R$ ferrihydrite samples coprecipitated with simple organic compounds and mixed them at various ratios with marine sediment, mimicking a natural marine sediment matrix containing OC-Fe$_R$, as deployed in Fisher et al. (2020). This method allows for the creation of a synthetic marine sediment sample with a known content of reducible Fe, therefore an accurate determination of ferrihydrite extraction efficiency can be obtained within methodological error. To prepare the OC-Fe$_R$ samples, we used three different organic structures with increasing carboxyl content (1 COOH, 2 COOH or 3 COOH groups) to produce three different OC-Fe$_R$ samples. Our approach is targeted towards testing variations in the physical parameters and chemical concentrations of the CBD method without changing the reagents used for the reductive dissolution. As such, stages of the CBD method were individually tested for different sample preparation methods (freeze-dried vs. untreated slurries), Na dithionite concentrations and extraction times. Since freeze-drying is thought to induce particle aggregation, which may artificially shield Fe phases from reduction (Chen et al., 2020), the freeze-drying method is compared with sediment slurries to determine the effect of sample preparation method on Fe$_R$ extraction.

2. Methods

2.1 Synthesis of ferrihydrite coprecipitates~~.~~

~~A coprecipitation of ferrihydrite with an organic carbon compound (hexanedioic acid) was conducted as~~To produce synthetic OC-Fe$_R$ samples 2-line ferrihydrite was chosen to represent Fe$_R$ as it is readily precipitated in low temperature, oxic, circumneutral pH conditions and is therefore ubiquitous in soils and sediments (Zhang et al., 2018). In addition, it has an established ability to be experimentally coprecipitated with organic matter (e.g. Eusterhues et al., 2008;Eusterhues et al., 2011;Eusterhues et al., 2014).

To produce an OC-Fe$_R$ sample using an organic structure containing 2 COOH groups, we coprecipitated 2-line ferrihydrite with hexanedioic acid, via the method described in Fisher et al. (2020). Briefly, 3 g of hexanedioic acid ($C_6H_{10}O_4$) was dissolved in 250 mL of deionised (DI) water with 20 g of Fe (III) nitrate nonahydrate [Fe(NO3)3.9H2O]. 1 M potassium hydroxide (KOH) solution was ~~then~~ added by titration to achieve a pH of 7.0 ± 0.3 to precipitate 2-line ferrihydrite ~~according to the original method of (~~Schwertmann and Cornell ~~(~~2000). The resultant slurry was rinsed 5 times in 5 L of DI water over 4 days until gravitationally settled~~, the~~. The pH was ~~then~~ raised to 7 through the dropwise addition of 0.1 M NaOH solution, centrifuged (2750 g, 20 mins), and the precipitate retained. The precipitate was then either immediately frozen and freeze~~-~~-dried, or ~~retained in slurry form and~~ stored at 4 °C~~, as dictated by~~ for use in the ~~experimental conditions detailed in section 2.4~~sample preparation method experiment.

~~Two~~To produce OC-Fe$_R$ samples using an organic structure containing 1 or 3 COOH groups, two additional precipitates were prepared according to the method described above, but substituting hexanedioic acid with pentanoic ($C_5H_{10}O_2$) or 1,2,4-~~Butanetricarboxylic~~butanetricarboxylic acid ($C_7H_{10}O_6$~~) for use in the sample preparation experiment. These~~), respectively. The acids used therefore differ in their carboxyl group content (pentanoic- 1 COOH, hexanedioic- 2 COOH, 1,2,4-Butanetricarboxylic- 3 COOH), ~~and factor thought to influence their binding to Fe$_R$ (Mikutta, 2011; Karlsson and Persson, 2012, 2010). This step was taken to investigate whether there was a relationship between the binding association and the sample state (i.e, to check if compounds were more easily removed in the slurry state because they were initially weakly bound or due to a true difference in physical behaviour between slurry and dry samples).~~a factor thought to influence their binding to Fe$_R$ via bonding between carboxyl groups and mineral hydroxyls (Karlsson and Persson, 2010;Mikutta, 2011;Karlsson and Persson, 2012). The coprecipitations produced three OC-Fe$_R$ samples with an increasing number of carboxyl groups, resulting in increasing molar C/Fe ratios of 0.04 (pentanoic), 0.25 (hexanedioic) and 0.70 (1,2,4-Butanetricarboxylic). The mass of organic acids used was determined through batch coprecipitations with varying organic contents, and the masses used here represent the saturation point, where a greater addition of organic molecules did not result in an increased OC association with ferrihydrite.

2.2 Spiking of marine sediments~~.~~

~~The~~ To spike marine sediment with the experimentally produced OC-Fe$_R$ ~~content of synthetic samples was varied~~coprecipitates, different amounts of OC-Fe$_R$ were added to ~~explore whether mechanistic trends persisted at environmentally relevant concentrations. To achieve this, we mixed the precipitate with~~ aliquots of a marine sediment ~~‘carrier’ material as described by Fisher et al. (2020), using the same original carrier~~ sample ~~and similarly treated~~from the Barents Sea (sediment core depth 33.5 cm; station B6, E40; cruise JR16006; see Hopkins (2017)). This sediment was freeze-dried, ashed

(650 °C, 12 hrs) to ~~liberate~~remove OC, and fumigated with HCl vapour to remove inorganic carbon. The resulting material was predominantly siliciclastic in nature with a Fe content of 16.33 mg/g. The ratio of ~~ferrihydrite-organic~~OC-Fe$_R$ coprecipitate relative to marine sediment was differed to create a ~~concentration gradient, and these ratios are~~content matrix, e.g. the sample refered to as "20% OC-Fe$_R$" with a total mass of 0.25g contains 0.200g (80 wt%) of marine sediment and 0.50g (20 wt%) of the OC-Fe$_R$ coprecipitate, the full composition of all samples is detailed in Table 2. ~~Spiking~~Mixing was achieved by agitation of either the freeze~~-~~-dried coprecipitate with the sediment~~ carrier~~ or the dry weight equivalent of untreated slurry samples with the sediment~~ carrier~~. Dry weight of slurry samples was determined by drying 10 x 1 mL aliquots of coprecipitate slurry at 40 °C to calculate mg/ml of coprecipitate and taking the mean value.

2.3 Citrate-bicarbonate-dithionite reduction of Fe$_R$

~~Reductive~~To reduce Fe$_R$ in the synthetic freeze-dried and untreated slurry OC-Fe$_R$ coprecipitates that were spiked into the marine sediment, reductive dissolution of reactive Fe phases was conducted according to an established CBD protocol ~~(Lalonde et al., 2012; Salvadó et al., 2015). A~~(Lalonde et al., 2012;Salvadó et al., 2015). The synthetic sediment sample (0.25 g, or dry weight equivalent for slurry samples) was added to 13 mL of 0.11 M sodium bicarbonate ($NaHCO_3$) and 0.27 M trisodium citrate ($Na_3C_6H_5O_7$) solution in a 50 ml centrifuge tube, then placed in a water bath at 80~~–~~°C to pre-heat. Subsequently, 0.25 g of sodium dithionite was dissolved in 2 ml of 0.11 M $NaHCO_3$ and 0.27 M $Na_3C_6H_5O_7$ solution and added to the pre-heated mixture before ~~agitation and further heating at 80 °C for 15 mins. Despite known degradation of sodium dithionite in aqueous solutions, no difference in Fe extractability was observed when compared to addition of dithionite in solid phase for repeat samples. Prior dissolution of Na dithionite also allowed for a more rapid and less labour-intensive addition when performing parallel sample analysis, compared to individual additions of 0.25 g dry Na dithionite as detailed in earlier methods (Lalonde et al., 2012; Salvadó et al., 2015). A control extraction was conducted alongside~~vortexing and further heating at 80 °C for 15 minutes. A parallel control extraction was conducted, replacing Na dithionite and trisodium citrate with Na chloride at an equivalent ionic strength; 13 mL of 1.6 M NaCl and 0.11 M $NaHCO_3$, followed by 0.22 g NaCl dissolved in 2 mL of the 1.6 M NaCl and 0.11 M $NaHCO_3$ solution. Following the extraction, samples were centrifuged (3000 g, 10 mins) and the supernatant was retained. A three times rinse cycle using artificial seawater was then conducted on the precipitate to remove any residual dissolved Fe, a 15 ml aliquot from each of these rinses was retained and combined per sample. All supernatants were acidified to pH <2 with 12 N HCl to prevent Fe precipitation.

2.4 ~~Alteration of method conditions.~~

~~In order to investigate potential improvements to~~Testing the ~~method, it was necessary to alter individual parameters~~impact of methodological variations

To test the impact of ~~the extraction protocol. Each parameter was changed in isolation in order to allow any subsequent change in Fe extractability to be associated with this variable. Sample~~different sample preparation methods~~ were compared by~~, the results from the extraction of the synthetic freeze~~-~~-dried samples ~~compared to~~and the untreated slurry samples ~~as previously~~

~~described. The~~from the spiked marine sediment aliquots were compared. To test the impact of different extraction conditions, the amount of Na dithionite added to a reaction was ~~also changed, with~~varied, using amounts both lower (0.125 g) and higher (0.375 g, 0.500 g, 0.625 g) than the standard addition of 0.25 g Na dithionite per 0.25 g of dried sample~~ performed~~. Where the dithionite addition was changed in the reduction reaction, an equivalent change was made for the control experiment to maintain the equivalence of ionic strengths. ~~Finally,~~To test the impact of different reaction ~~time~~times, this was increased ~~at 3 time points beyond the usual 15 minutes reaction (~~to 30, 45 and 60 ~~mins).~~

minutes with manual shaking of each sample every 15 minutes. For the sample preparation and Na dithionite experiments, repeats were conducted ~~over a concentration gradient~~overl the content matrix dependent on the amount of OC-Fe$_R$ ~~spiked into~~added to the sediment. While performing these extractions on pure synthetic OC-Fe$_R$ is useful for uncovering a mechanistic trend, dilution with ~~OC~~ carbon-free sediment to lower OC-Fe$_R$ contents in the sample ensured any trends uncovered are noticeable at environmentally relevant conditions (OC-Fe$_R$ <50 wt%). Repeats of samples across this ~~concentration~~content gradient are in lieu of direct replicates for each unique sample condition. These were not possible due to yield limitations imposed by ferrihydrite coprecipitate synthesis (net ~5g per 5 L rinse solution). ~~It was essential that all~~All samples within any one experiment originated from the same batch of ferrihydrite~~ as OC adsorption and Fe content are not consistent across batches. Outliers can still be identified by comparison to the trends present in replicates at differing concentrations of OC-Fe$_R$~~.

2.5 Environmental sample treatment~~.~~

To allow comparison between sample preparation methods applied to samples containing synthetic OC-Fe$_R$, natural samples were ~~evaluated and subjected~~subject to the same methods of CBD extraction and Fe elemental analysis. Arctic Ocean seafloor sediment was collected (Cruise: JR17007, ~~Latitude (N): 80.1167, Longitude (E): 30.06827, water depth 283 m,~~ station B16, sediment depth 22-23 cm; Faust et al 2021), of which half was ~~freeze~~ thawed following freezing at the point of sampling, and half was freeze~~-~~-dried.

2.6 Elemental analysis for iron~~.~~

Initial ~~concentrations of~~ Fe contents in synthetic samples were obtained by digesting ~2 mg of dried sample in 1 mL 12N HCl at room temperature followed by a 10-fold dilution of the extract with 1% HCl solution. ~~Further dilutions~~Dilutions of initial samples, in addition to the extraction supernatants, were ~~made as necessary, dependent on Fe content,~~conducted using MilliQ water to produce a subsample within the ~~detectable window (~~1–10 ppm Fe~~). Fe concentrations~~ range. Iron contents for both the initial samples and supernatants from the extraction were determined by atomic absorption spectroscopy (Thermo Fisher iCE3300 AAS). Calibration was performed using matrix matched standards and quality control was confirmed following every 10 samples by repeat sampling of calibration standards to check for drift. Supernatants from control experiments were also measured for Fe content and these were diluted 20-fold to prevent salt blockages~~,~~; the supernatant from seawater rinses remained undiluted except for where the Fe concentration in solution was >10 ppm, whereby these were diluted 10-fold.

~~Extraction~~The recovery of Fe following the extractions was calculated by subtracting the ~~amount of Fe lost in the~~ control ~~experiment from~~ corrected loss of Fe from the initial sample Fe ~~lost following~~ content. Maximal extraction~~, then subtracting this~~ of Fe is defined as the point from ~~the initial Fe of each sample.~~which further addition of Na dithionite does not further increase the extraction of Fe.

2.7 Elemental analysis for carbon~~.~~

Carbon content was determined for all OC-Fe$_R$ contents ~~of synthetic samples~~ (20-50%) both before and after Fe extraction to determine whether OC-Fe$_R$ was completely recovered (given that the Fe this OC is bound to was incompletely reduced across the series). Carbon contents were measured using a LECO-SC144DR C&S analyser. ~~Carbon content was not measured for all samples, but was used during the experiment where Na dithionite concentrations varied (see section 3.1). This was performed to ensure that at the end point samples with incomplete Fe recovery also experienced incomplete OC recovery, as expected due to the <1 OC:Fe molar ratio of our coprecipitates. This measurement can therefore be used to confirm the choice of %Fe loss as a proxy for %OC-Fe$_R$ recovery across the entire dithionite concentration gradient.~~ The LECO analyser was calibrated with, and quality control checked against, a known standard (LECO 502-694). All carbon samples were analysed in an oven~~-~~-dried state (40 °C, 12 hours). Carbon ~~loss~~recovery was calculated ~~according~~by subtracting the remaining carbon content from the solid phase of the extracted sample from the initial carbon content. Correction by normalisation to sample mass was made to ~~Supplementary equation 1 of Fisher et al. (2020), adapted from Peter and Sobek (2018) and Salvadó et al. (2015), to correct~~account for mass loss during the dissolution. Instrument error for the LECO analyser was low (≤1% RSD) due to drift calibration throughout the analytical run.

3. Results

3.1 ~~Addition~~The effect of varying ~~quantities~~addition of Na dithionite on Fe extractability

~~While the concentration of Na dithionite in the CDB extraction was varied considerably over method iterations, the contemporary~~The CBD method of Lalonde et al. (2012) requires a 0.25 g addition ~~relative to~~ of Na dithionite per 0.25 g of dried sediment sample. ~~Here, the~~The mass of dithionite added to our reaction was adjusted (0.125 g, 0.375 g, 0.500 g, 0.625 g) while the ~~sediment mass remained at 0.25 g. The~~ mass of the OC-Fe$_R$ spiked sediment sample remained at 0.25 g. These adjusted Na dithionite masses were applied to OC-Fe$_R$ spiked sediment samples varying in their OC-Fe$_R$ contents (20-50% of total sample mass), and the %Fe extracted from these ~~adjusted extractions is shown in Fig. 1, and this figure~~ samples was measured (Fig. 1). This can be interpreted as ~~a visualisation of~~ the reduction capacity of Na dithionite relative to initial Fe content. ~~All samples~~Our results show that all samples have incomplete reduction of Fe, regardless of Na dithionite addition, with ~~those samples containing the least Fe proving extractable for the greatest proportion of Fe.~~the highest recovery of Fe in samples with low OC-Fe$_R$ contents. The 30 and 40 wt% OC-Fe$_R$ containing samples ~~tracked~~show almost identical ~~paths~~trends for their ~~extractable %~~Fe extractability, while the 20 wt% OC-Fe$_R$ sample is more readily extracted and the 50~~%~~ wt% OC-Fe$_R$ sample is ~~the~~ least ~~extractable~~extracted.

For ~~the~~ sediment ~~sample~~samples containing 20 wt% OC-Fe$_R$, maximal Fe extraction occurs at the ~~baseline~~original 0.25 g dithionite addition (89%), while ~~for sediments~~ maximal Fe extraction occurs with ~~a~~ greater dithionite additions for samples with a greater initial ~~%~~OC-Fe$_R$ content. For the 30 and 40 wt% OC-Fe$_R$ samples, maximal Fe extraction ~~occurs past the baseline. For the 30 and 40% OC-Fe mix, maximal Fe extraction~~(~88%) occurs at 0.5 g Na dithionite addition ~~where ~88% of Fe is extracted. At~~. For the 50 wt% OC-Fe$_R$ sample, 60% of total Fe is extracted at both 0.5 g and 0.625 g Na dithionite additions. ~~From this, we can deduce~~Thus, the maximal %Fe in sediment extractable by 0.25 g Na dithionite lies between a 20 and 30 wt% OC-Fe$_R$ ~~mix~~composition, equivalent to 7-10 wt% ~~Fe~~Fe$_R$ content in the sediment.

Therefore, assuming a 0.25 g sample size, the absolute amount of Fe which could be extracted for any sample would be between 17.5 and 25 mg. OC-Fe$_R$ extracted (i.e. OC recovery) was ~~measured for~~determined at the point at which maximal Fe extraction was achieved by addition of excess Na dithionite. The OC-Fe$_R$ ~~values are shown in blue and~~results (Fig. 1) indicate OC-Fe$_R$ extraction ~~is~~to be incomplete (<100%) ~~across all concentrations. Extraction of OC-Fe$_R$ was roughly similar to Fe, variable within 10%.~~in all our experiments.

3.2 The effect of sample preparation methods on Fe extractability

~~Two forms of each synthetic sample were prepared, one freeze dried and one as a slurry (referred to as 'wet'). Recovery of Fe following extraction is shown as %Fe extracted in Fig. 2. A total of 3 different coprecipitates (with varying C content) at 5 different OC-Fe:sediment ratios are shown with solid colours representing the freeze dried form and patterns representing the slurry samples. Overall, a~~A greater proportion of Fe is extracted from the marine sediment spiked with slurry samples (referred to as 'wet') than from the same sediments spiked with freeze~~-~~-dried samples (referred to as 'dry') for all coprecipitates at all OC-Fe$_R$ contents (Fig. 2). ~~concentrations. Dry~~ The dry spiked sediment samples achieve a maximum Fe extractability of 71% (for the sediment spiked with 3 COOH~~,~~ OC-Fe$_R$, at 60~~%),~~ wt% OC-Fe$_R$ content), while ~~in slurries~~for the wet spiked sediment samples up to 87% is recovered (with 3 COOH~~,~~ OC-Fe$_R$, at 100~~%). No~~ wt% OC-Fe$_R$ content). A 100% recovery of added Fe was not achieved in any of the experiments.

~~Typically, Fe recovery from samples~~Iron extractability increases with the number of carboxyl groups in the OC-Fe$_R$ coprecipitate for both ~~dried~~dry and wet spiked sediment samples; this trend is clearly shown ~~at~~for sediments with 100, 60 and 40~~% concentrations of initial~~ wt% OC-Fe$_R$ content ~~(shown by different shapes). The~~. However, the 1 COOH OC-Fe$_R$ spiked sample at the 80~~% concentration~~ wt% OC-Fe$_R$ ratio is ~~an outlier to this trend, proving~~ extractable for a greater mean amount of Fe compared to the 2 COOH ~~precipitate~~ OC-Fe$_R$ spiked sample for both the ~~dried~~dry and ~~slurried sample.~~wet preparation methods. Additionally, no trend in Fe extractability with the number of carboxyl groups is present for the 20 wt% OC-~~Fe~~

~~concentration~~Fe$_R$ spiked sediment samples, however, Fe ~~concentrations~~contents at this level are comparatively low which may obscure trends within this data series.

Following the experiments ~~on~~with marine sediment spiked with synthetic OC-Fe$_R$ compounds, a similar investigation was conducted on ~~environmental~~marine sediment samples ~~to observe whether the trend observed for synthetic samples could be replicated.~~where no OC-Fe$_R$ had been added. This experiment only ~~differed from the previous one in comparing~~compared freeze-dried vs. freeze-thawed (not slurry) samples. ~~Freeze dried samples were extractable for 22.34% (± 4.05 (1 S.D)) Fe compared to 22.68% (± 6.67) for freeze thawed samples.~~ There ~~was~~is no notable difference in the amount of Fe extracted for ~~environmental~~sediment samples which had been freeze-dried compared to those which were freeze-thawed.

~~3.3 Variability in exposure time~~ Recovery of ~~a sample to CBD treatment.~~

~~Following the method of Lalonde et al. (2012) the CBD extraction~~Fe from freeze-dried samples is ~~performed over a period of 15 minutes.~~ 22.34% Fe (± 4.05 (1 S.D.)) compared to 22.68% (± 6.67) for freeze-thawed samples.

3.3 The effect of CBD extraction time on Fe extractability

Here we examined ~~whether extending this time period would increase~~ the ~~amount~~effect of longer (>15 min) extraction time periods on the Fe ~~extracted~~extraction. All other parameters of the extraction remained the same~~, a~~ as in Lalonde et al., (2012). A 2 COOH ~~coprecipitate~~OC-Fe$_R$ spiked sediment sample at a 60~~% concentration relative to labile sediment~~ wt% OC-Fe$_R$ content was used in ~~freeze dried~~dry and wet forms. ~~Times were advanced~~Extraction time was extended in 15 minute increments from 15 minutes to 60 minutes, and results ~~from the subsequent extractions~~ are shown in Fig. 3. The percentage of Fe extracted remains ~~consistent~~very similar across the time series for both wet and ~~dried~~dry samples, and there is no evidence that increasing the extraction duration systematically increases Fe liberation.

4.0 Discussion

~~Chemical extraction of OC-Fe$_R$ from sediment samples remains an important and widely used method for determining the fate of marine organic compounds. Like all chemical extraction techniques, the method used here is operationally limited in its ability to remove OC-Fe$_R$, with evidence for incomplete extraction of some OC-Fe$_R$ complexes (Adhikari and Yang, 2015; Fisher et al., 2020). Despite these apparent method inefficiencies, it is difficult to propose any substantive changes to the CBD method due to the neutral pH constraints required to prevent OC hydrolysis. Therefore, we targeted physical, as opposed to chemical, aspects of the CBD method, with an aim to increase Fe liberation from synthetic marine sediments containing OC-Fe$_R$ complexes. Out of the varied experimental parameters, increasing the amount of Na dithionite added to the reaction was most successful in increasing Fe liberation for samples containing >20% OC-Fe$_R$ (~7 wt% Fe). The process of sample preparation was found to have a significant effect on Fe loss, with non-freeze dried samples proving extractable for a much~~

~~greater proportion of Fe compared to freeze-dried samples, likely due to particle aggregation. However, we were unable to replicate this phenomenon in natural sediment samples, potentially due to freeze-thaw induced aggregation of the non-freeze dried samples. Finally, an increase in reaction time was found to have no effect on increasing Fe extractability. Here we consider the implications of these findings and discuss the practicality of applying these changes to the CBD method.~~

4.1 Concentration of Na dithionite as a primary control on OC-Fe$_R$ extraction~~.~~

~~Sodium dithionite as a reducing agent, buffered by bicarbonate and citrate, has been used to extract reactive Fe phases from a range of media. One of the most important variables across these methods is the ratio of Na dithionite relative to the sample size and its iron oxide (Fe_2O_3) content. Here, we altered the method of~~Here, we altered the CBD method (Lalonde et al. (2012) by changing the mass of Na dithionite added to the CBD extraction for four synthetic sediments, each differing in Fe content, to determine whether an increased concentration of Na dithionite ~~would liberate more~~affects Fe ~~than the standard method~~liberation.

~~For the~~The four synthetic samples ~~we~~ subjected to dithionite reduction~~, these~~ (20-50 wt% initial OC-Fe$_R$ content) differed in ~~composition~~Fe content (7-24 wt% Fe~~, 20-50% initial OC-Fe$_R$ content). The concentration of Fe in these samples results in an effective dithionite to (wt) Fe reduction reaction~~ ,) resulting in a dithionite to Fe mass ratio of 1:0.07-0.24. ~~This is multiple times~~These ratios represent a stronger reductive power than the concentration of dithionite previously used in incomplete Fe extractions. For example, Adhikari and Yang (2015) report <50~~ wt~~% Fe was extracted with a dithionite to Fe ratio of 1:0.8 for a humic-hematite complex. While reactive Fe content in ~~bulk natural~~many marine sediments is ~~usually~~ below 7 wt% Fe ~~(Raiswell and Canfield, 1998; Canfield, 1989), inherent heterogeneity and clustering of Fe~~(e.g. Canfield, 1989;Raiswell and Canfield, 1998), spatial and temporal variation in Fe fluxes to the seafloor can ~~be seen in the Iron L$_3$-edge XAS spectra of~~result in unusually Fe-rich sediments, e.g. near hydrothermal vents (Poulton and Canfield, 2006) or in Fe-Mn nodules ~~(Barber et al., 2017). This has the potential to drive wt% Fe higher in small samples of sediment such as those treated by the method (0.25 g).~~(Hein et al., 1997). Additionally, OC-~~Fe~~Fe$_R$ has been observed at ~~concentrations~~contents exceeding 40% (of total OC) in terrestrial environments (Zhao et al., 2016;~~-~~Patzner et al., 2020) and 50% in sandy beach sediments of subterranean estuaries (e.g. 56.31% ± 5.56 Martinique Beach, Canada (Sirois et al., 2018)~~), explaining the choice to include samples with high OC-Fe compositions in the matrix.~~). We find that the sample containing 20 wt% OC-~~Fe~~Fe$_R$ (~7 wt% Fe) is maximally extracted for its reactive Fe component under the 0.25 g (0.1 M) treatment ~~as described by Lalonde et al. (2012) (Fig. 1).~~(Fig. 1). Maximal extraction ~~here~~ is defined as the point ~~from~~at which further ~~addition~~additions of ~~Na~~ dithionite ~~does~~do not increase Fe liberation. Here, the ~~extraction of Fe beyond the amount of Fe extracted under the previous dithionite addition mass ± error. For example, the~~sample containing 20 wt% OC-Fe$_R$ ~~sample subject to 0.25 g dithionite is removable for~~liberates 88.79% ± 3.55 of Fe$_{Total}$ ~~while 0.~~with a 0.25g dithionite addition, and increasing this to 0.375 g ~~addition extracts~~of dithionite only marginally increases Fe liberation to 90.94% ± 3.64; as these values are within error~~, it can be said~~ of each other, we conclude that maximal extraction is achieved with ~~0.25 g dithionite addition per the original method of Lalonde et al. (2012).~~the lower 0.25g dithionite content.

At increased ~~concentrations of~~ OC-Fe$_R$ contents, 0.25 g of Na dithionite ~~is~~seems to be no longer sufficient to achieve maximal extraction. The samples with 30 and 40 wt% OC-Fe$_R$ content, which follow almost identical trajectories~~,~~ (Fig. 1), reach maximal extraction at 0.5 g/0.2 M with 88.65% ± 3.54 and 88.22% ± 3.53 of Fe$_{Total}$ recovered, respectively. These values are within the error of maximal extraction for 20 wt% OC-Fe$_R$ and are significantly higher than the amount of Fe liberated under the standard 0.25 g/0.1 M extraction (63.03% ± 2.52 and 67.21% ±~~2.69, respectively). This finding demonstrates that the OC-Fe$_R$ composition would not be correctly determined following the method of Lalonde et al. (2012) for these OC-Fe$_R$-rich sediments, and the overall extent of OC-Fe$_R$ in the marine sediment pool would be underestimated. While 30-40% OC-Fe$_R$ content is above the average for marine sediments, many samples exist in the 20-30% range. Indeed, the average value for marine sediment OC-Fe$_R$ composition given by Lalonde et al. (2012) is greater than 20% with individual marine sediments recorded as exceeding 30% OC-Fe$_R$ (e.g.~~ 2.69, respectively). This finding demonstrates that in OC-Fe$_R$ rich sediments, the 0.1 M or weaker Na dithionite methods could underestimate the true OC-Fe$_R$ content of these samples. In any case, comparisons of OC-Fe$_R$ extracted by different dithionite contents <0.2 M for these sediments would not be comparable due to underestimations in weaker treatments. While these high OC-Fe$_R$ content sediments of 30-40% OC-Fe$_R$ (of total OC) are above the average for natural marine sediments, they do exist in areas with high OC accumulation rates (e.g. shallower waters on coastal shelves) where ~45% of global OC burial is thought to occur (Hedges and Keil, 1995), and sediments have been documented as exceeding 30% OC-Fe$_R$ (e.g. Equatorial Pacific 0°N, 34.79% (Barber et al., 2017)).

The indication that Na dithionite at the 0.25 g/0.1 M addition is increasingly inefficient with increasing OC-Fe$_R$ content is ~~confounded~~reinforced at the 50 wt% OC-~~Fe~~Fe$_R$ (24 wt% Fe) composition. ~~Here, %~~The amount of Fe extracted is increased from 39.96% ± 1.60 with 0.1 M (0.25g) Na dithionite to 59.58% ± 2.38 at double strength (0.2 M). ~~Note, however, that this~~This differs from the previous compositions in reaching a maximum at ~60% Fe, as opposed to the ~90% achieved for 20-40 wt% OC-Fe$_R$. Given that %Fe removed does not increase with further addition of Na dithionite (0.625 g), the amount of Na dithionite is no longer the limiting factor in extracting Fe from such very OC-Fe$_R$ rich samples~~.~~ (Fig 1). It ~~is likely~~might be that another reagent, potentially trisodium citrate, ~~may become~~becomes limiting. In the extraction reaction, citrate acts as a complexing agent to keep Fe dissolved in solution ~~(Lalonde et al., 2012; Sirois et al., 2018)~~(Lalonde et al., 2012;Sirois et al., 2018). If the increased strength dithionite treatment increases dissolved Fe beyond the complexing capacity of citrate, then excess Fe likely precipitates out of solution before measurement. However, Henkel et al. (2016) found that a reduced concentration of citrate is sufficient to fully complex the reduced Fe pool when the extraction is performed under anoxic conditions, which may remove the need to further increase the addition of citrate as an organic reagent.

~~Measurement of OC-Fe$_R$ extracted for the concentration of Na dithionite at which maximum Fe is extracted showed incomplete OC-Fe$_R$ loss (Fig. 1). The similarity of OC-Fe$_R$ and raw Fe extraction values indicates that OC and Fe are reductively released from the sediment in comparable proportions, as is expected due to the low molar OC:Fe$_R$ ratio of the coprecipitate (~0.7:1). From these results, it is apparent that Na dithionite concentration can limit the extractability of reducible Fe and associated OC in Fe rich sediments, and that current approaches could benefit from using increased strength Na dithionite compared to the 0.1 M treatment currently used. Based on the set of experiments we conducted, an increase to 0.2 M would be sufficient. However, if increasing the amount of Na dithionite beyond its current level, other considerations need to be made, such as the decomposition of Na dithionite in AAS standards which may skew quantifications (Taylor and Crowder, 1983). Additionally, the reduction in maximal Fe extraction seen for the 50% OC-Fe$_R$ sample, thought to be due to rapid precipitation of reduced Fe, suggests that the concentration of sodium bicarbonate and trisodium citrate may need to be changed to maintain the buffering and complexation capacity of the extraction. It is important to note that by increasing the concentration of these organic reagents, the background DOC of the experiment will also increase, which has the potential to interfere in quantification of OC released from Fe in the reduction. It would be useful to include background DOC detection for samples (as per Patzner et al., (2020)) to avoid the accidental inclusion of organic reagents in OC-Fe$_R$ determination.~~

Excess Fe precipitation out of solution may further explain the observation in OC extraction for the 50 wt% OC-Fe$_R$ sample whereby this sample appears to experience a greater loss of OC compared to Fe during the extraction, despite a molar C/Fe ratio of <1. In reality, it is likely that the actual Fe$_R$ loss is greater than detected for this sample, due to Fe precipitation before measurement, and greater than the measured OC loss, following the expected trend based on the C/Fe ratio. This is observed for all other samples with a lower initial OC-Fe$_R$ content where Fe$_R$ loss was not thought to be influenced by excess precipitation and Fe$_R$ loss was greater than that for OC (Fig.1). Nevertheless, our results show that out of the varied experimental parameters, increasing the amount of Na dithionite in the reaction had the greatest effect in increasing Fe liberation for samples containing >20 wt% OC-Fe$_R$ (~7 wt% Fe). Therefore, this indicates that for OC-Fe$_R$ rich sediments (≥ 30 wt %), OC-Fe$_R$ extraction values determined by methods which differ in their concentrations of dithionite below 0.2 M are not comparable, since for these samples OC-Fe$_R$ continues to be extracted with increasing additions of Na dithionite.

4.2 ~~Freeze~~ Sediment freeze-drying ~~of samples~~ as a limiting factor on Fe reduction~~.~~

~~Typically, chemical extractions have been performed on freeze dried samples, but how this process affects the physical properties of samples and their subsequent behaviour towards chemical reagents has not been defined. We found that the Fe extraction efficiency from freeze dried sediment samples was much less than that measured for chemically identical samples retained in slurry form (i.e. not freeze dried) (Fig. 2). The scope of extractions has expanded over time and this wet-chemical treatment is now performed on a diverse range of solid phases, including soils (e.g. Deb, 1950; Mackenzie, 1954; Zhao et al., 2016; Wagai and Mayer, 2007; Schulten and Leinweber, 1995), clays (Aguilera and Jackson, 1953; Deb, 1950; Mehra and Jackson, 1958; Mitchell and Mackenzie, 1954), plant roots (Taylor and Crowder, 1983), cryoconite accumulated on glaciers~~

~~(Cong et al., 2018), estuarine sediments (e.g. Jokinen et al., 2020; Zhao et al., 2018) and marine sediments (e.g. Barber et al., 2017; Lalonde et al., 2012; Salvado et al., 2015), with each material possessing unique physical and chemical characteristics. To aid the retrieval of samples from often remote locations, freeze drying has become established as an almost universal preservation/preparation method for solid samples. Removal of the aqueous phase decreases sample mass and prevents the need for frozen storage. Freeze drying inhibits processes of microbial degradation from occurring in the sediment sample, preserving the biochemical profile. Alternative treatments such as air drying are considered to be more aggressive as they can alter the chemical composition of samples and may inflict significant changes on sediment chemistry, including losses of biomarkers (McClymont et al., 2007).~~

~~We postulate that freeze drying-induced aggregation of sediment particles could result in reduced Fe extractability compared to non-dried samples since grain size is a known key factor in limiting determination of bioavailable Fe~~Chemical extractions of Fe are typically performed on freeze-dried sediment samples. Removal of the aqueous phase decreases sample mass and prevents the need for frozen storage, and the biochemical profile is preserved through inhibiting microbial degradation. Alternative treatments such as air drying are considered to be more aggressive as they can alter the chemical composition of samples and may inflict significant changes on sediment chemistry, including losses of biomarkers (McClymont et al., 2007) and changes in speciation of heavy metals (Zhang et al., 2001). However, how this process affects the physical properties of samples and their subsequent behaviour towards chemical reagents has not been widely considered. We found that the Fe extraction efficiency from freeze-dried sediment samples containing freeze-dried freshly precipitated ferrihydrite was much lower than that measured for chemically identical samples containing freshly precipitated ferrihydrite present in slurry form, i.e. not freeze-dried (Fig. 2).

In line with previous studies, we suggest that freeze-drying may result in reduced Fe extractability compared to not freeze-dried samples due to particle aggregation or transformation of ferrihydrite to a more stable phase. Aggregation can produce ‘shielded’ sediment particles (Chen et al., 2020), and this may inhibit Fe reduction by reducing the reactive mineral surface area exposed to dithionite. This could be overcome, e.g. by crushing, but would introduce further variability, e.g. in grain size (Raiswell et al., 1994)~~. Aggregation could reduce surface contact with dithionite, preventing reduction of ‘shielded’ sediment particles, while this could be overcome (e.g. by crushing), and this in itself would introduce further variability in grain size (Raiswell et al., 1994).The influence of freeze drying on grain~~ and would be unlikely to be effective against nanoparticles. The influence of freeze-drying on particle size has been previously noted, particularly for sediment with a high clay content (>39%) (Keiser et al., 2014). McKeague and Day (1966) similarly report that finer grinding of sediment resulted in an increased extraction of Fe. These findings indicate that particle size is a critical parameter in determining the amount of Fe extracted, however, ~~all~~current methods ~~fail to~~do not define ~~what is meant by~~ the particle size of “finely ground~~”. This lack of definition introduces an error of reproducibility as particle size is certain to vary with different sample preparation methods and therefore two identical chemical treatments may vary in strength because of physical differences in the sediment sample.~~

~~The alternate tested method of using wet samples has largely been avoided, with only a few studies (e.g. van Bodegom et al., 2003;Chen et al., 2020) reporting the use of a wet slurry sample in soils and none for~~” sediments. ~~Chen et al. (2020) justify their use of slurries as being to “minimise the physical-protection mechanisms of aggregation”, acknowledging~~An alternate hypothesis, that mineralogical transformation of ferrihdyrite during freeze-drying ~~methods are likely to result in superficial particle protection.~~

~~Despite the benefits of conducting analyses on slurried samples in being able to extract a greater amount of OC-Fe$_R$, and therefore gain a better understanding of sediment C content, there are several considerations to be made. Firstly, determining the dry weight equivalent of a slurried sample is difficult as each subsample is inherently heterogeneous. Density tests for the tested synthetic samples indicated this contributes up to ±5% error which, while significant, is less than the difference seen between~~may lead to reduced Fe ~~extraction from slurries compared to dried samples (up to 53%, 2 COOH, 100% OC-Fe, Fig.~~ recovery, was ruled out by X-ray diffraction (XRD) characterisation of a representative~~2). The use of fresh ‘wet’ samples appears to be the only method by which aggregation can be avoided with drying, freezing and thawing all producing aggregates (IAEA, 2003). However, the use of wet sediments is likely to be inappropriate for some analyses or sample sites.~~

~~Fresh sediments retain their microbiological components which can result in biological degradation of pollutants, release of ammonia and chemical degradation via hydrolysis and oxidation (Schwab, 1980). It has also been show that freeze drying can result in elevation of DOC in sediment samples (Geffard et al., 2004), further Barbanti and Bothner (1993) report an increased amount of OC (20-44 % greater) and some metals (Zn, Cu) in the coarse fraction of~~ freeze~~-dried sediment compared to slurry samples, suggesting freeze drying can similarly alter sediment chemistry. Therefore, it should be acknowledged that any storage method of water-containing samples is likely to cause some level of chemical change, and samples cannot truly be regarded as pristine. While this cannot easily be avoided, we suggest that rigorous documentation is key to making resulting data sets comparable.~~

~~Although extraction efficiency of Fe from~~-dried sample, which confirmed the identity to still be 2-line ferrihydrite~~ is improved through the use of wet samples, this only achieves a maximal extraction efficiency of 87% (3 COOH, 100% OC-Fe$_R$) (Fig. 2). For Fe$_R$ phases associated with less complex OC, the extraction efficiency is even lower, e.g. for the 1 COOH sample at the same concentration (100% OC-Fe$_R$) only 30% of Fe is liberated. Trends between Fe extraction and carboxyl content have been discussed in~~ (Fisher et al., 2020)~~ and are mirrored here in Fig. 2, besides an inflation in 1 and 2 COOH values at the 20% concentration, likely due to errors in small numbers as a result of the dilution. The 1 COOH complex at 80% OC-Fe also appears inflated and out of step with the trends set by other concentrations with no obvious explanation. While application of the CBD method to slurried samples has the ability to increase the proportion of Fe associated with OC extracted, this protocol~~

~~may not always be practicable. Additionally, the inability to fully extract Fe even when sediments are in a slurry form indicates that other limiting factors persist which prevent complete extraction of Fe_R phases by the circumneutral CBD method.~~.

To reduce the effect of aggregation during freeze-drying, a few studies on soils used fresh slurry samples (e.g. van Bodegom et al., 2003;Chen et al., 2020). Wet thawed samples have been used more widely in the sequential extraction of Fe (e.g. Wehrmann et al., 2014;Riedinger et al., 2017;Laufer et al., 2020) and the Arctic marine sediment sample used in our analysis was similarly thawed following freezing on collection. We find that our thawed sample shows no difference in its recovery for Fe compared to the dried variant of this sample. Natural aging processes within the sediment could explain this lack of freeze-drying effect in older sediments due to both the physical effects of aging on Fe minerals, and the transformation of poorly crystalline reactive ferrihydrite phases to more crystalline phases at 22cm depth (Faust et al., 2021), which are unlikely to be extracted by the neutral pH CBD extraction.

Although our results show that extraction efficiency of Fe from ferrihydrite is better in wet sediment samples, it is still only possible to achieve a maximal extraction efficiency of 87% (with 3 COOH OC-Fe_R, at 100 wt% OC-Fe_R content) (Fig. 2). The extraction efficiency for Fe_R phases associated with less complex OC is even lower, e.g. only 30% of Fe is liberated for the 1 COOH OC-Fe_R spiked sediment sample at the same OC-Fe_R content (100 wt%). Even though such samples consisting exclusively of ferrihydrite-associated OC are unlikely to occur in nature, on the whole our results confirm previously identified relationships between Fe extraction and carboxyl content, where Fe extraction using the CDB method is more efficient for OC-Fe_R that contains more carboxyl rich OC (Fisher et al., 2020). This is attributed to the greater amorphicity of ferrihydrite coprecipitated with carboxyl rich OC, i.e. the resultant mineral phase is less crystalline than ferrihydrite coprecipitated with less carboxyl rich OC, and is therefore easier to reductively dissolve. An inflation of the Fe extractability for the 1 and 2 COOH OC-Fe_R spiked sediment samples at the 20 wt% OC-Fe_R content is likely due to uncertainty as a result of the dilution. The 1 COOH OC-Fe_R spiked sediment sample at 80 wt% OC-Fe_R also appears inflated and out of step with the trends set by the samples at other OC-Fe_R contents, with no obvious explanation.

While application of the CBD method to slurried samples could increase the extracted proportion of Fe associated with OC, such an approach may not always be practical; either due to practical considerations, such as the difficulty in transporting heavy wet sediments, or when there is a need to preserve the sediment profile, for example, protecting anoxic sediments from oxic biological transformations. Additionally, the inability to fully extract Fe even when sediments are in a slurried state indicates that other limiting factors to Fe extractability persist, which prevent complete extraction of Fe_R phases by the circumneutral CBD method. Nevertheless, our results confirm that the process of sample preparation has a large effect on Fe recovery, with non-freeze-dried synthetic OC-Fe_R samples that contain ferrihydrite and carboxyl OC being extractable for a much greater proportion of Fe compared to their freeze-dried equivalents. However, this finding was not replicated for naturally aged Arctic Ocean samples, where Fe$_R$ content was dominated by more stable phases such as hematite and goethite, indicating that older natural sediments are less influenced by freeze-drying compared to freshly precipitated ferrihydrite.

4.3 Rapid reduction of Fe$_R$ by Na dithionite~~.~~

~~One parameter of the extraction method which has remained largely consistent across all iterations of CBD treatment is an extraction length of 15 minutes (Mehra and Jackson, 1958; Wagai and Mayer, 2007; Lalonde et al., 2012). As we observed incomplete Fe extraction (Fig. 1) for all our samples, a range of CBD extraction times were trialled to understand whether increasing the length of a reaction would increase Fe liberated, as seen for other chemical Fe extractions; oxalate, for example, is known to continue to extract Fe beyond a standard 1 hour treatment (McKeague and Day, 1966). Additionally, as previously mentioned, some iterations of the CBD method have been repeated multiple times in succession to extract the full Fe$_{CBD}$ pool, but it is unclear whether time or reagent concentration limit full extraction of this pool on the first treatment.~~

One parameter of the extraction method which has remained largely consistent across all iterations of CBD treatment is the extraction length of 15 minutes (Mehra and Jackson, 1958;Wagai and Mayer, 2007;Lalonde et al., 2012). As we observed incomplete Fe extraction (Fig. 1) for all our synthetic samples, a range of CBD extraction times were trialled to understand if reaction time and Fe extraction have a positive correlation, as seen in some iterations of the CBD method for OC-Fe$_R$ (e.g. Wagai and Mayer, 2007). In some applications of the CBD method, the extraction stage is repeated multiple times for the same sample in order to fully extract Fe$_{CBD}$ (e.g. Aguilera and Jackson, 1953;Mehra and Jackson, 1958), but it is still very difficult to attribute full CBD extraction to this multiple extraction protocol because the parameter that prevents full extraction of CBD extractable Fe in the first place is unknown.

Exposure time to CBD of ~~wet and~~freeze dried and slurried OC-Fe$_R$ synthetic samples ~~(~~spiked into marine sediment (using 2-COOH, OC-Fe$_R$ at 40~~%) to CBD~~ wt% OC-Fe$_R$ content) was increased from ~~the standard~~a 15~~-~~ minute treatment in 15~~-~~ minute intervals up to 60 minutes total exposure time (Fig. 3). No difference was observed for the amount of extractable Fe across the time series~~, concluding~~ and we therefore propose that an increase in chemical exposure time has no ~~difference~~effect on Fe extractability~~. This shows that time is not a limiting factor~~ for OC-Fe$_R$ phases in ~~the CBD extraction~~natural samples, and~~ that~~ reductive dissolution of the susceptible Fe phases occurs ~~rapidly. We would perhaps not expect any benefit from increasing the length of CBD treatment as~~relatively fast. This finding is in agreement with the fact that dithionite, the reductive component, is known to undergo degradation to form sodium thiosulfate and bisulfite in aqueous solutions with a rapid second order rate constant (K_2) of 3.0 ~~(g-molecule/~~mol L~~)~~$^{-1}$ min^{-1} at 79.4 °C, ~~indicating~~hence reducing conditions are unlikely to be sustained for long (Lister and Garvie, 1959).

~~While increasing extraction time has no benefit for extracting Fe with the purpose of determining the OC-Fe$_R$ pool, a recent adaptation of the CBD method has extended the time of the extraction in order to compensate for a reduction in the temperature~~

~~of the reaction. Patzner et al. (2020) performed a 16 hour CBD extraction at room temperature on permafrost samples to, in the first instance, determine %OC-Fe$_R$, then subsequently apply scanning electron microscopy (SEM) and nanoscale secondary ion mass spectrometry (nanoSIMS) to analyse the extracted organominerals. Here the authors had to alter the CBD method as they were concerned that exposure of organic compounds to high temperature may alter OC structure and fate, which they wished to analyse. This raises an interesting question as to whether temperature and length of the extraction can compensate for each other to achieve the overall same %Fe extraction.~~

~~In our series of experiments, temperature was not altered as we saw no benefit to decreasing temperature, and therefore energy, of the reaction as we were focused on maximising %Fe extraction. While we saw no benefit from increasing extraction time, likely due to rapid decomposition of Na dithionite, the decomposition process may occur much slower at room temperature due to the decreased reaction energy. The authors of this pre-print (Patzner et al., 2020) have yet to calibrate their method against the standard 80 °C treatment, however, the values they obtain for %OC-Fe in permafrost soils appear agreeable, if not a little higher than much of the literature for terrestrial samples (e.g. Zhao et al., 2016). This could potentially be a benefit to the CBD method in preserving the structural component of OC, which would subsequently allow for much wider analysis on the extracted OC, such as biomarkers, which has previously been limited by both transformation of C in extraction and by sample size. This may allow us to better understand the origins and molecular makeup of OC involved in mineral preservation processes~~However, Patzner et al. (2020) adapted the CBD method whereby time was extended to compensate for a reduction in the temperature of the reaction. A low temperature approach was not tested in our study as we focused on thermodynamically increasing the efficiency of the reaction; however, this adaptation may prove useful should non-destructive analysis be required. For example, subsequent analysis of biomarkers in extracted organics is currently not possible due to temperature induced transformation and degradation of OC when heated to 80 °C. These types of analyses may allow us to better understand the origins and molecular composition of OM involved in mineral-based preservation processes, and offers promising scope for future experimentation with the CBD method.

5. Conclusion

~~Reductive dissolution of OC-Fe$_R$ by CBD is an important and widely used method for quantifying mineral associated OC in sediments and soils, but has been shown to be misunderstood in its efficiency. Varying, often uncalibrated iterations of the method have made comparison of these extraction values impossible, compromising our ability to gain an understanding of the true extent of OC-Fe$_R$ in the global carbon cycle. In this study we aimed to address these uncertainties in the CBD method to understand which, if any, parameters of this method could be changed to improve extraction of the targeted Fe$_R$ phases and associated OC.~~

~~We found that the mass of dithionite added to a sample appears to be limiting in extracting the total easily reducible Fe pool for Fe rich sediments and a doubling of Na dithionite addition for these sediments can increase Fe extracted from ~60% to ~90%, representing a much more complete removal of the OC-Fe~~$_R$ ~~pool. We suggest that if future studies were to increase Na dithionite addition in the CBD method this should be followed by a similar increase in trisodium citrate to ensure the entire reduced Fe pool is complexed, preventing precipitation of Fe before quantification. This additional input of an organic reagent may be offset by subtraction of background DOC values from the final C content of the sediment post extraction as per the method described by Patzner et al. (2020).~~

~~Freeze drying induced aggregation appears to reduce Fe liberation in synthetic coprecipitates that were freeze dried relative to slurried, however, we were unable to replicate this increased extraction for natural samples. While we speculate this may be due to the use of freeze thawed samples, which can introduce aggregation in itself, it is hard to see a practical implementation of this adjustment for marine sediments due to the difficulty in transport of pristine samples. Consideration should also be given to the error introduced in determining dry weight if slurried samples are to be used.~~

~~Finally, increase of reaction time (up to 1 hr, 4x standard) showed no benefit for Fe extraction Given that alteration of sample preparation methods would be impractical and extraction time offered no benefit, we suggest future work should focus on increasing the concentration of Na dithionite used in extracting a range of environmental samples known to differ in their OC-Fe~~$_R$ ~~content. However, in highlighting these methodological inefficiencies, even if they cannot be entirely offset we have established an empirical basis from which future studies can understand the likely errors associated with CBD extractions and can interpret their results accordingly.~~

Reductive dissolution of OC-Fe$_R$ using the CBD method is an important and widely used protocol for quantifying mineral-associated OC in sediments and soils. In this study we aimed to address the uncertainty around variations in the preparation method for samples subject to CBD and some of the method parameters used during the extraction, to understand if these factors have an effect on the extraction efficiency of OC-Fe$_R$ and therefore the interpretation of OC-Fe$_R$ data. Our results show that the mass of dithionite added to a sample has a strong control on the extractability of the easily reducible Fe pool, and that this is particularly acute for reactive Fe-rich sediments, where a doubling of Na dithionite addition for these sediments can increase Fe$_R$ recovery from ~60% to ~90%. While a 0.1 M Na dithionite concentration appears to be sufficient for most marine sediments with average reactive Fe contents, data produced with lower dithionite to solid ratios should not be compared to those extracted by a greater concentration of dithionite. Earlier studies e.g. Mehra and Jackson (1958), Wagai and Mayer (2007) accounted for this issue by varying the dithionite to solid ratio based on Fe contents. We suggest that this approach should be redeployed for the extraction of Fe$_R$ from Fe rich environments (>30% OC-Fe$_R$), whereby an increase in dithionite concentration was shown to extract a greater amount of OC-Fe$_R$ than the standard 0.1 M approach. We also show that freeze-drying reduces Fe liberation from synthetic coprecipitates containing ferrihydrite when compared to the equivalent sample in slurried form, likely due to particle aggregation. However, the effects of freeze-drying are negligible for aged sediments where the reactive Fe mineralogy is dominated by more stable phases. While we recognise that the use of fresh sediment slurries is rarely practical, we suggest that for all samples, particularly the uppermost surface sediments, grain size or the grinding method should be reported alongside OC-Fe$_R$ extractions to aid comparability of samples beyond current descriptions of “finely ground”. Where sediment slurries are used, 10 aliquots should be oven-dried to determine the dry mass equivalent of the sample to ensure the dithionite to solid ratio is maintained; for our samples we find that variability in the dry mass contained in a slurry sample introduces an error of up to ±5%. Finally we show that an increase of reaction time (up to 1 hr) results in no additional Fe extractability over the typical 15 minutes reaction time. Given the variability in often uncalibrated extraction protocols within the literature, we show that comparison of the results from different studies are problematic when attempting to elucidate the true extent of OC-Fe$_R$ in the global carbon cycle. Future work to quantify the global importance of the OC-Fe$_R$ sink requires a uniform methodological approach to be deployed across a range of environments, with modification for Fe$_R$ rich environments. These extractions should be conducted with the knowledge that this represents the operationally defined, not absolute, OC-Fe$_R$ content of sediments.

Author Contributions

BJF, JCF, OWM, CLP and CM designed the study, ~~BF~~BJF conducted the experimental work with support from JCF and OWM. JCF supplied the Arctic sample material. BJF, JCF, OWM, CLP and CM all contributed towards analysis of the data sets. BJF prepared the first draft of the manuscript and all authors contributed towards review and editing of the final version.

Competing Interests

The authors declare no competing interests.

Data Availability

The equation used to compute OC-Fe$_R$ is available in Fisher et al., (2020), examples of implementation can be found in the associated electronic annex of that paper (https://data.mendeley.com/datasets/gpt8f8kpcs/1). Raw figure data for this manuscript is available ~~upon~~from the lead author on request.

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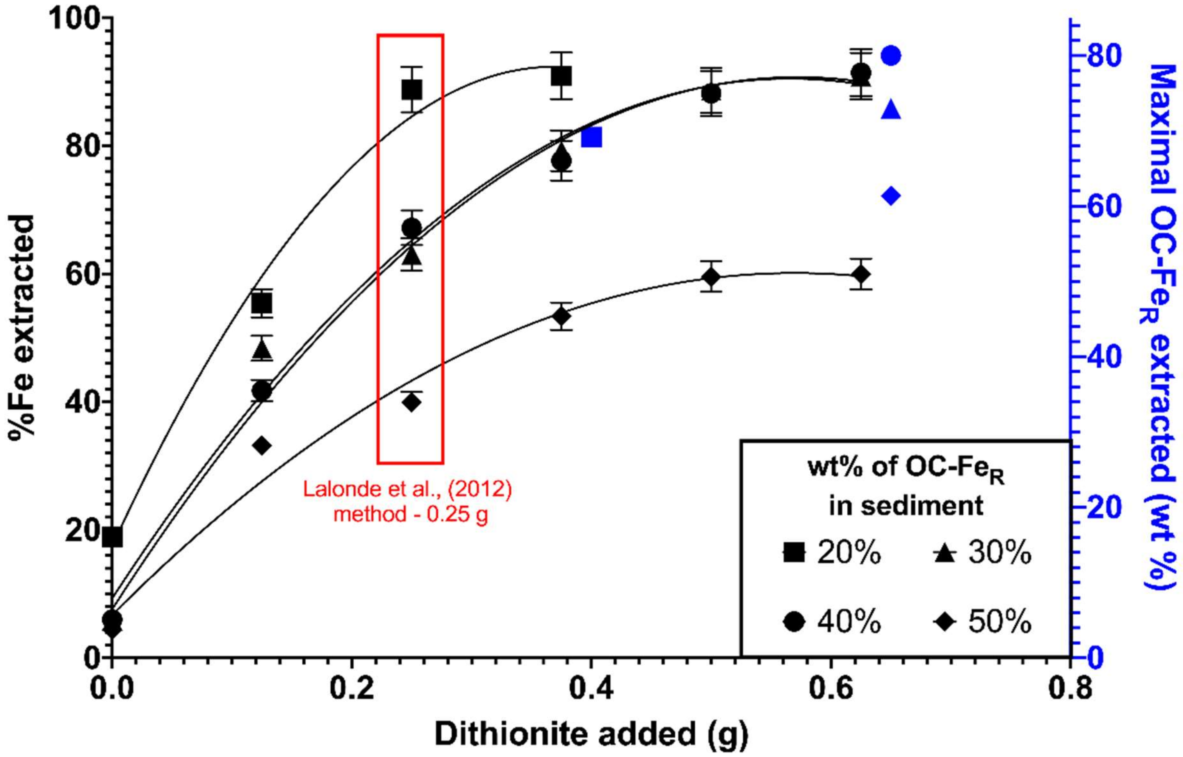

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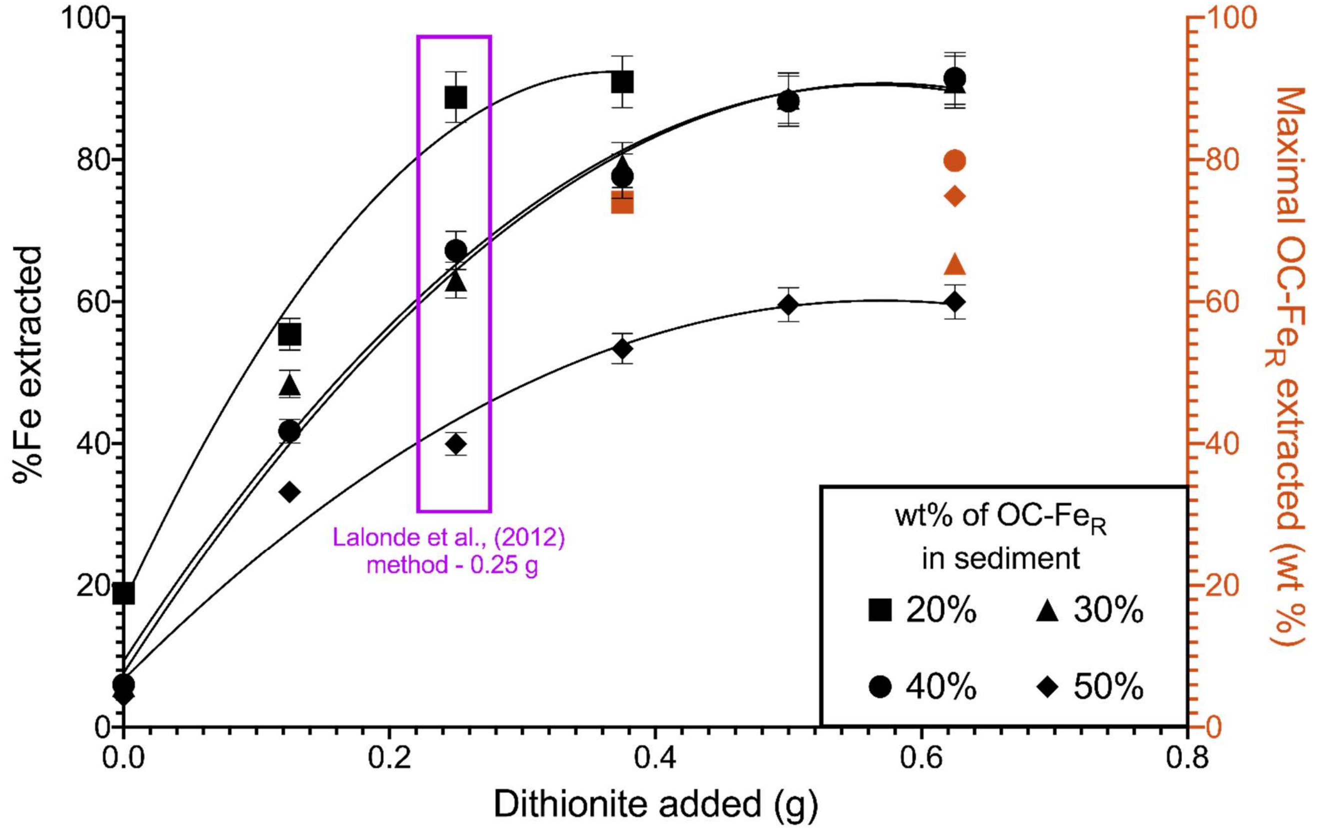
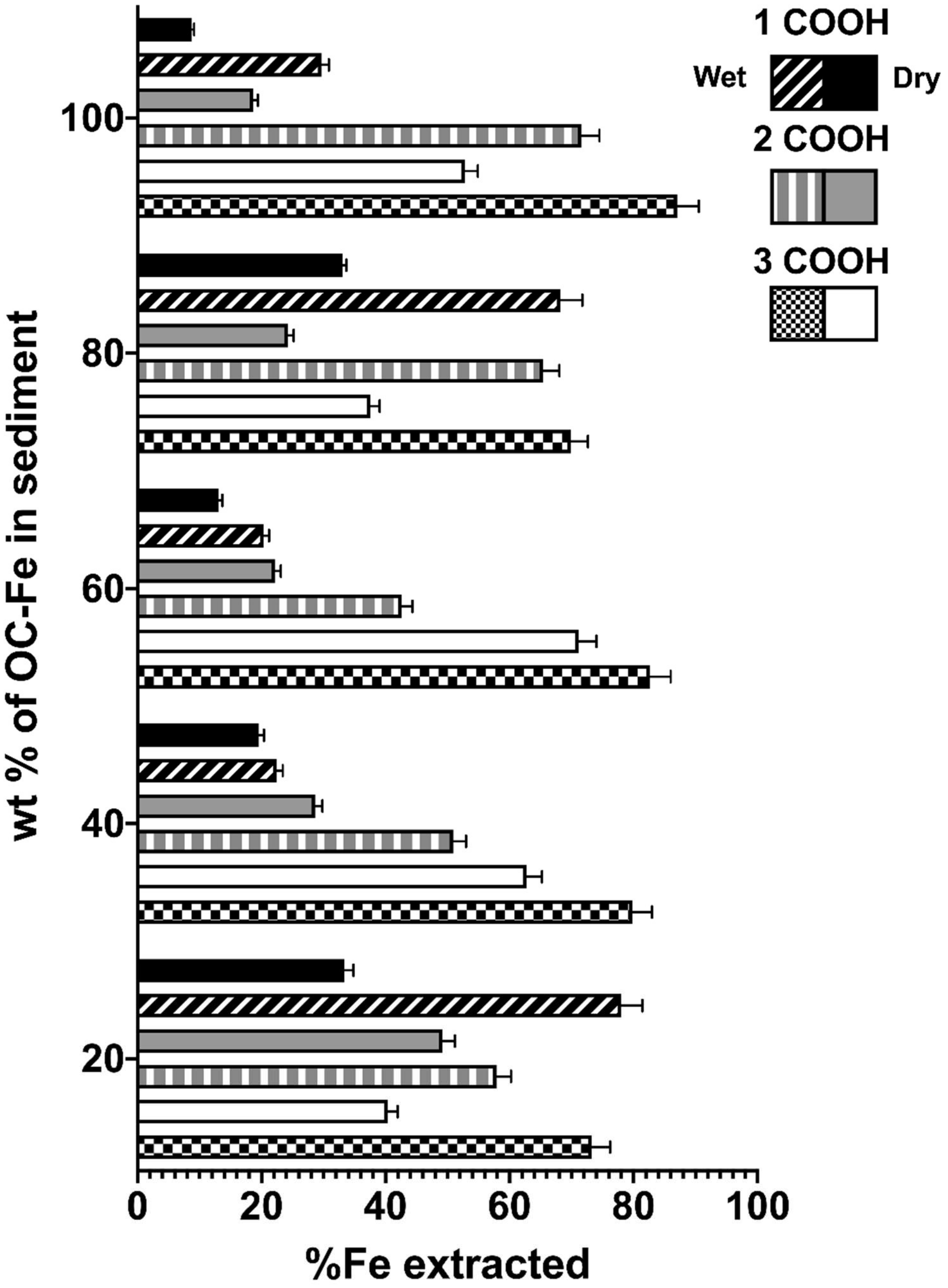


Figure 1: Reduction capacity of Na dithionite in the extraction estimated from %Fe extracted with varying Na dithionite additions across an OC-Fe$_R$ concentration gradient. Error bars show maximal compound instrument error~~. Blue~~, lack of error bars indicates error is too small to be visualised. Red shapes indicate the amount of OC-Fe$_R$ extracted for the concentration of Na dithionite at which Fe is maximally extracted for that sample (black).


1 COOH
Wet
Dry
2 COOH
3 COOH
100
80
60
40
20
wt % of OC-Fe in sediment
0
20
40
60
80
100
%Fe extracted

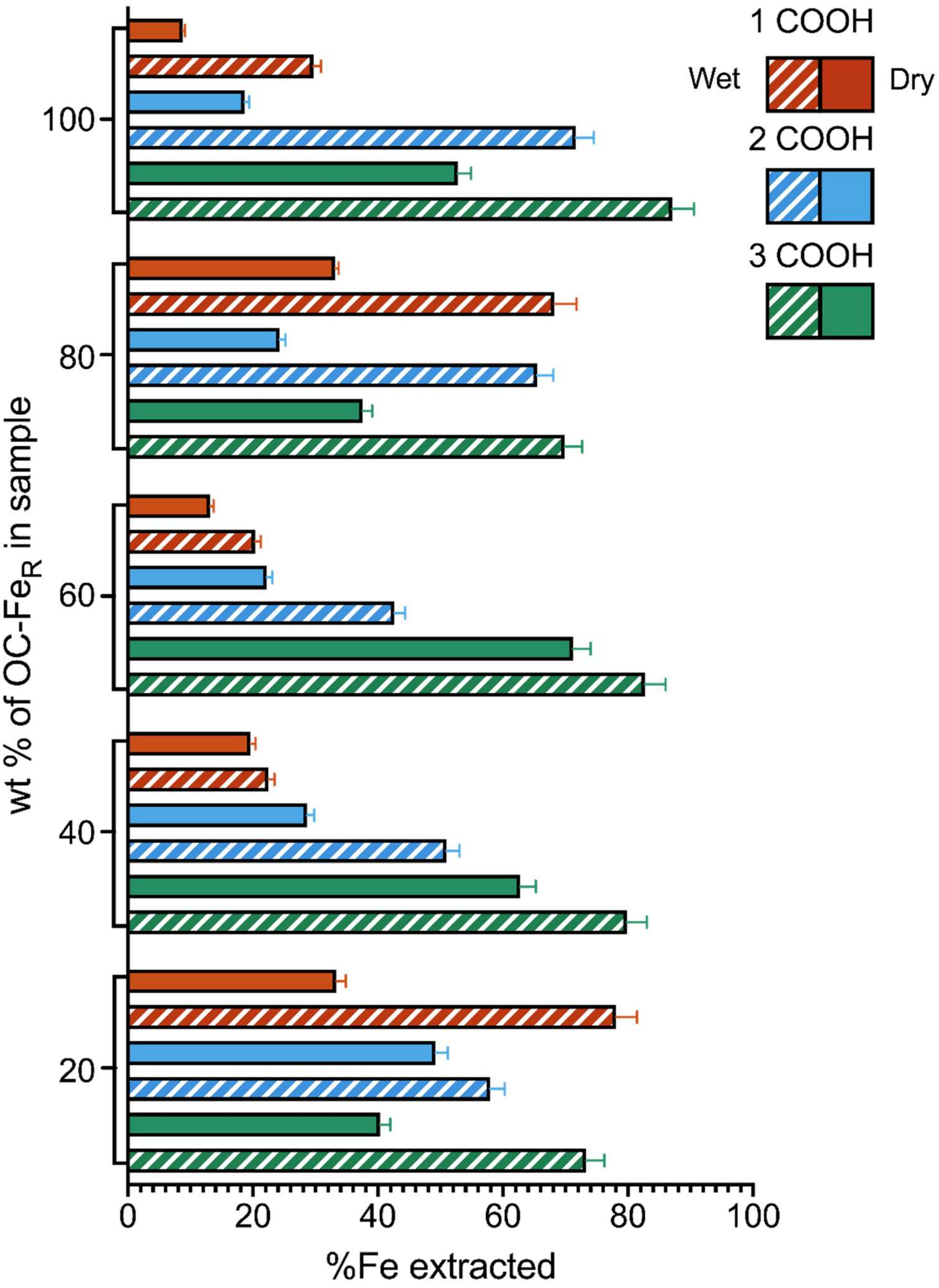
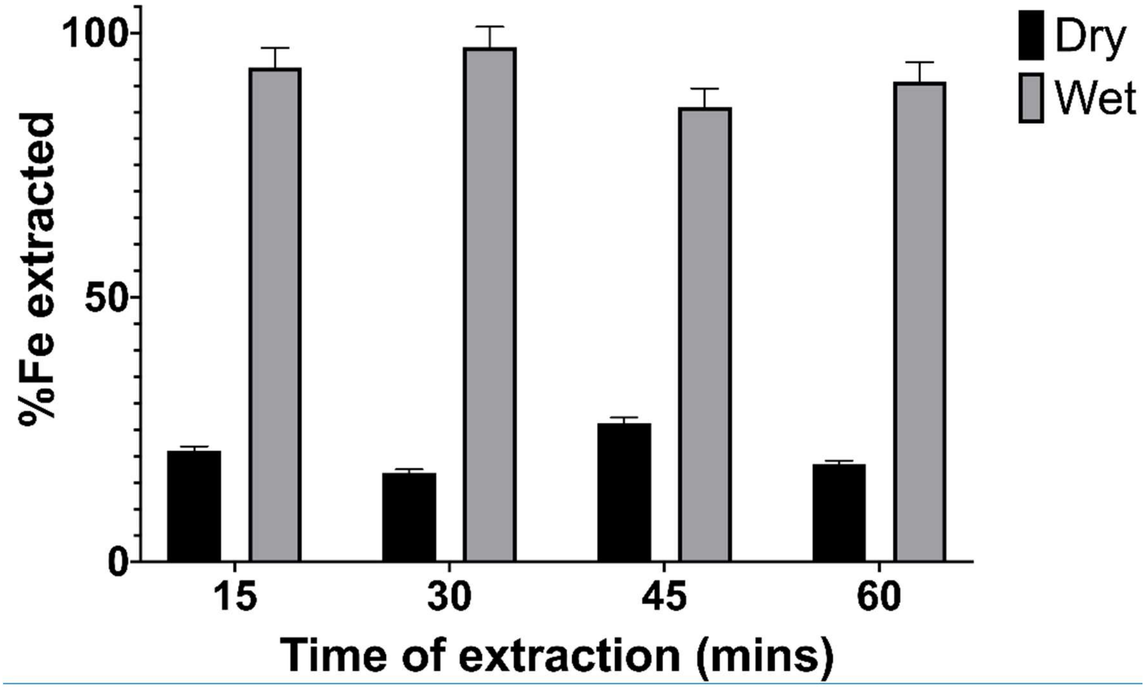


Figure 2~~-~~: Fe recovery from freeze~~-~~-dried vs slurry coprecipitates. Solid bars show dried samples while patterns show the wet (slurry) samples. 1/2/3 COOH refers to the number of carboxyl groups present in the coprecipitated organic acids. Each error bar shows maximum compound error.


100
50
0
%Fe extracted
15
30
45
60
Time of extraction (mins)
Dry
Wet

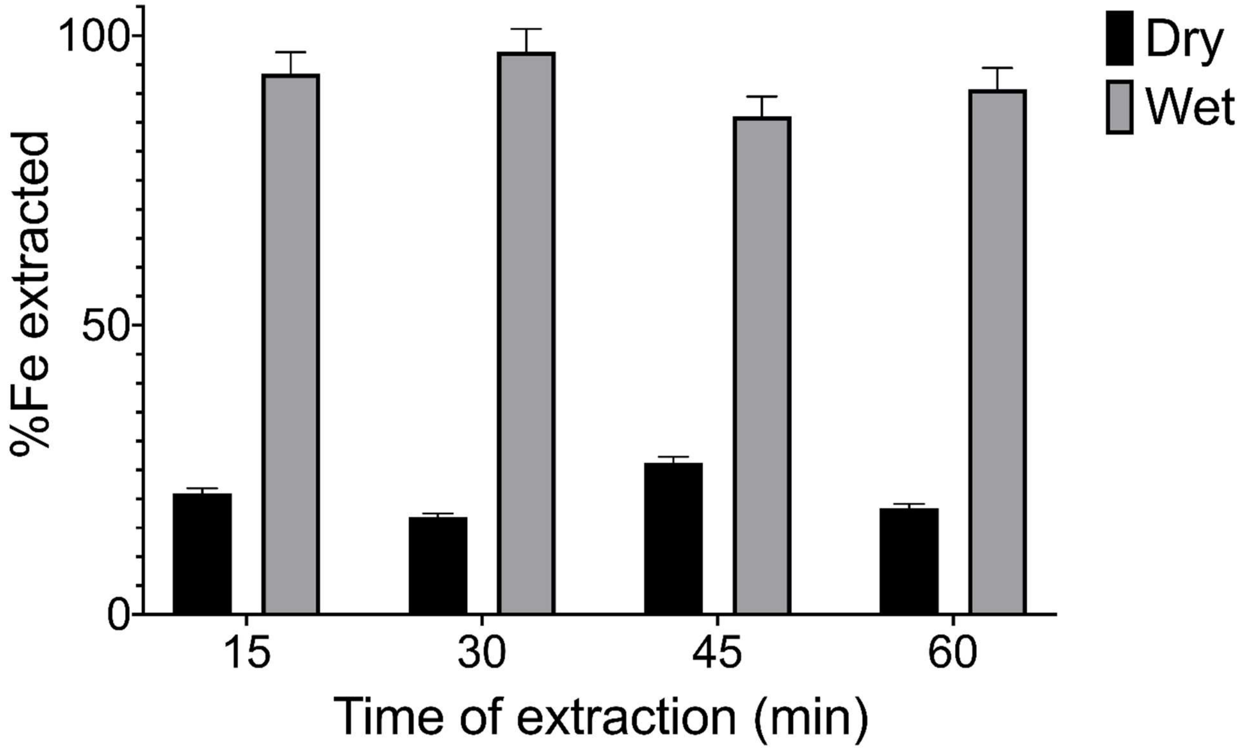


Figure 3~~-~~: %Fe extracted across a time series for CBD extraction. Error bars show compound maximal instrument error. The sample used in this experiment was a ~~synthetic~~spiked sediment ~~spiked with~~comprised of 60 wt% sediment and 40 wt% of a 2 COOH OC-Fe$_R$ coprecipitate~~ at 40% OC-Fe$_R$.~~

Reference	Dithionite concentration	Sample to solution addition (mg mL $^{-1}$)	Dithionite to sample mass ratio
Aguilera and Jackson (1953)	0.144 M	12.5[a,b]	1:0.5[a]
(Mehra and Jackson, 1958)	0.128 M	Soils: 88.89[b] Clays: 22.22	1:4 1:1
Wagai and Mayer (2007)	0.049 M	Fe rich: 4.3 Fe poor: 7.1	1:0.5 1:1.2
Lalonde et al. (2012) **Zhao et al. (2016)**	0.1 M	Sediments (Lalonde) and Soils (Zhao): 16.67	1:1

Table 1: Comparison of dithionite strength to sample mass in iterations of the CBD method applied to soils and sediments.

[a] Sample size in this method is variable due to variable Fe_2O_3 contents; samples should not exceed 0.5 g Fe_2O_3 so may be a 10 g sample with 5% Fe_2O_3 content or a 1 g sample with 50% Fe_2O_3 content. The ratio given is calculated on the basis of a 0.5 g Fe_2O_3 sample, so represents a minimal rather than absolute ratio. Dithionite concentration is based on a 40 ml reaction, while Aguilera and Jackson (1953) refer to the addition of a dithionite solution without reporting the exact volume.

[b] If any sample exceeds 5% Fe_2O_3, the extraction should be repeated an additional 1-2 times.

~~%~~wt% OC-~~Fe:sediment~~Fe$_R$ in sample	20	30	40	50	60	80	100
OC-Fe$_R$ Coprecipitate (mg)	50	75	100	125	150	200	250
Sediment (mg)	200	175	150	125	100	50	0

Table 2: Concentration matrix of spiked samples.