Geoderma 430 (2023) 116327

Contents lists available at ScienceDirect

Geoderma

journal homepage: www.elsevier.com/locate/geoderma

Quantifying mineral-associated organic matter in wetlands as an indicator of the degree of soil carbon protection

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ARTICLE INFO

Handling Editor: Cornelia Rumpel

Keywords:
Soil organic matter
Mineral-organic associations
Physical protection
Physical fractionation
Wetlands

ABSTRACT

As atmospheric carbon (C) concentrations increase, so too has interest in understanding the mechanisms that preserve C within the soil organic matter (SOM). Mineral-associated organic matter (MAOM) is one pool of SOM recently shown to protect soil organic C from mineralization. However, most MAOM research has been in agriculture and forest mineral soils. Given the magnitude of soil C stored in wetlands, this study sought to determine the abundance of MAOM in wetlands. A standard method for quantifying MAOM in terrestrial soils was tested, modified, and optimized with three unique wetland soils. Using a physical fractionation method followed by a density fractionation, it was concluded that field moist soils and a dispersant were necessary for fractionating wetland soils (0–50 cm). The Bayhead Swamp soil had the greatest total C (484.07 ± 1.25 mg C g soil $^{-1}$), but the smallest MAOM pool (0.63 %); most of the C was in the light fraction as particulate organic matter (POM, >98 %). The sandy-peat Cypress Dome soil also lacked MAOM (1.09 %) and was dominated by POM (66.26 %). The silt-rich Brackish Marsh had the least total C (94.37 ± 1.67 mg C g soil $^{-1}$), but the largest fraction of protected C (24.60 % MAOM). Nitrogen (N) followed similar trends to C, except in the Brackish Marsh where nearly half the total N was MAOM. This research represents the first step in evaluating the role of MAOM in C persistence in organic-rich wetland soils; future work should consider C physical fraction, in addition to C quantity.

1. Introduction

Wetlands play a major role in the global carbon (C) cycle, storing 20–30 % of the total C (Lal, 2008) while only representing approximately 6 % of the global land surface (Mitsch and Gosselink, 2007). Most of this C is stored as soil organic matter (SOM; Donato et al., 2011), prompting significant interest in understanding the factors affecting SOM persistence in wetland soils. The turnover rate of SOM is known to be influenced by temperature and oxygen availability, as well as the physicochemical and molecular properties of the parent plant litter (Oades, 1988). Historically, biochemical protection (sometimes referred to as selective preservation) has been considered a dominant pathway for soil C preservation. This concept focuses on plant litter's degree of inherent recalcitrance, or resistance to microbial decomposition due to the high molecular weight and complexity and/or low nutrient availability of the input litter (von Lützow et al., 2006; Kleber, 2010). For example, one study observed that after a two-year incubation, 43–57 % of plant litter had decomposed, but only 5–12 % of the lignin, suggesting the more recalcitrant lignin molecules may persist longer in the soil than labile (i.e., smaller, higher nutrient) molecules (Hall et al., 2020). Along with biochemical protection, the prevalence of anaerobic conditions in wetlands, a form of environmental protection (Schmidt et al., 2011) that shifts microbial respiration to slower, less efficient metabolic pathways requiring alternative electron acceptors, limits C mineralization and promotes SOM persistence (Reddy and DeLaune, 2008). The widely accepted concepts of biochemical and environmental protection further intersect with the 'enzymatic latch' hypothesis, which asserts the suppression of the oxidative enzyme phenol oxidase in wetlands also contributes directly to the vast accumulation of soil C in these ecosystems (Freeman et al., 2001; Saraswati et al., 2016). These basic concepts have dominated the wetland scientific literature about soil C for decades (e.g., Reddy and Patrick, 1975; Cahoon and Reed, 1995; Twilley et al., 1997; Chambers et al., 2013; Chambers et al., 2016; Hodgkins et al., 2018; Steinmuller et al., 2020; Sapkota and White, 2021), with little or no attention given to other potential mechanisms for soil C preservation.

An alternative pathway for SOM preservation increasingly studied in terrestrial soils is mineral associated organic matter (MAOM). Defined as chemical bonds between SOM and mineral surfaces and occlusion within

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https://doi.org/10.1016/j.geoderma.2023.116327
Received 16 September 2022; Received in revised form 31 December 2022; Accepted 4 January 2023

micropores or small aggregates < 53 μm, MAOM formation renders the SOM inaccessible to decomposers (Six et al., 2002; Kleber et al., 2015). MAOM is considered the most stable form of SOM, able to persist for centuries to millennia (von Lützow et al., 2007) with limited susceptibility to changing environmental conditions (Song et al., 2012; Rocci et al., 2021). The physical and chemical protection afforded by MAOM formation is contrasted against particulate organic matter (POM), which is defined as light-weight fragments of partially undecomposed plant or fungal-derived compounds, unprotected SOM, which typically has a faster turnover rate and higher C-to nitrogen (N) ratio than MAOM (Lavallee et al., 2020; Cotrufo and Lavallee, 2022). Contemporary soil C research conducted in upland agroecosystems, forests, permafrost, and grasslands routinely focus on MAOM as the primary mechanism for long-term SOM stabilization and C preservation (e.g., Cambardella and Elliott, 1993a; Six et al., 1998; Cotrufo et al., 2015; Ye et al., 2019) introducing a new knowledge gap: what role does MAOM play in soil C preservation within wetland soils?

Although numerous wetland studies quantify total C stocks (e.g., Twilley et al., 1992; Chmura et al., 2003; Nahlik & Fennessy, 2016), few have performed more detailed analyses of the physical form or chemical properties of the wetland SOM, which is known to influence the turnover rate and stability of the C (Sollins et al., 1996). Of the studies that have investigated the molecular and chemical characteristics of the wetland SOM, methods have included sequential extractions, spectrophotometry, Fourier Transform Infrared Spectroscopy (FTIR) and Nuclear Magnetic Resonance (NMR) (e.g., Dodla et al., 2008; Tfaily et al., 2014; Hodgkins et al., 2018; Steinmuller and Chambers, 2019). Mineral associations have been investigated in a few tidal or seasonally inundated mineral wetland soils (LaCroix et al., 2019; Maietta et al., 2019; Kottkamp et al., 2022), but these studies have not employed the full density and physical fractionation sequence commonly applied to the study of MAOM in terrestrial soils (Six et al., 1998; Del Galdo et al., 2003; Haddix et al., 2016). In wetlands, correlations between SOM and fine minerals, such as silts and clays, have been identified (Lewis et al., 2021), which is commonly observed in terrestrial soils (Hassink, 1994; Feller and Beare, 1997; Hassink, 1997). Recent wetlands work has also demonstrated the importance of specific metal complexes, suggesting, for example, extractable aluminum may predict soil C in wetter soils (LaCroix et al., 2019) and iron-OM interactions may contribute to C storage in some marshes (Seyfferth et al., 2020). However, most wetland soils research neglects to investigate mineral associations at all, possibly because the availability of minerals is low in many types of autochthonous peat-forming wetland soils (e.g., Histosols). In organic-rich wetland soils, common in ecosystems characterized by frequent or continuous waterlogging, POM in varying states of decay may be the dominant form of SOM. Still, considering the overwhelming evidence that associations between SOM and minerals decreases the decay rate of the organic matter (Mikutta et al., 2007), quantifying its presence is critical to understanding the long-term stability of wetland soil C during climatic change. Moreover, an understanding of the prevalence and role of MAOM in diverse wetlands will improve models of C dynamics that consider different C pools and turnover rates (Robertson et al., 2019).

In addition to chemical protection of SOM through MAOM formation, terrestrial soils research also commonly investigates physical protection through aggregate formation (e.g., Cambardella and Elliott, 1993a; Jastrow, 1996; Six et al., 1998; Christensen, 1992). This too has been largely ignored in the wetland soil research as it pertains to a mechanism for soil C preservation. Aggregates protect C from mineralization by spatially separating soil fauna and enzymes from available C (Killham et al., 1993), as well as reducing oxygen availability and gas diffusion to minimize microbial activity (Sexstone et al., 1985). Aggregation is positively correlated with the presence of silt and clay minerals (Kemper and Koch, 1966, Six et al., 2002) and may incorporate MAOM in the smallest size fraction (Cotrufo and Lavallee, 2022). Aggregates formation typically involves organic binding agents categorized by their level of protection, from polysaccharides (short-term aggregates) to polyvalent cations and polymers (persistent aggregates). These organic compounds bind primary minerals, bacteria, and plant debris to create soil structure (Tisdall and Oades, 1982; Chaney and Swift, 1986). This definition implies the necessity of mineral sediments for aggregate formation. Therefore, it is unclear if the environmental conditions found in wetlands are appropriate to promote aggregation, particularly in Histosols with low or negligible mineral matter.

The few studies that have quantified aggregates in wetlands were conducted in soils with higher mineral content (not Histosols) and have consistently found macroaggregates ($>$2 mm) were the dominant size fraction (Hossler and Bouchard, 2010; Maietta et al., 2019). Macroaggregates also dominated the single study found in Histosols, though the soil studied had been drained for 100 yr for agricultural purposes (Wright and Inglett, 2009). Although the macroaggregates in these wetland studies contained the greatest C content, others have suggested aggregates > 2 mm are the least stable size fraction of aggregate, typically being of a younger age and having a faster turnover rate than microaggregates (250 μm −53 μm; Six et al., 2002; von Lützow et al., 2006, 2007). A better understanding of aggregate formation and physical protection of SOM in wetlands will further enhance the understanding of the C cycle in wetland soils and is also a key step to identifying the appropriate methodology to quantify MAOM in wetlands.

The goal of this study was twofold: 1) to optimize the published physical and density soil fractionation methods that quantify MAOM for use on wetland soils, and 2) to determine the abundance of MAOM, and its contribution to soil total C and N pools, in three distinct types of wetland soils. Within goal #1, two specific sub-questions developed, a) how does the initial moisture status of wetland soils affect the resulting particle size distribution, and b) are soil aggregates present in wetlands soils, thus requiring the use of a dispersant? These goals were achieved through a laboratory experiment that empirically compared the results of soil drying and the use (or lack of) a dispersant on particle size distribution, followed by quantification of total C and N content for each size and density fraction.

2. Methods

2.1. Soil selection

Three unique wetland soil types were chosen to exemplify a diversity of organic matter and mineral contents commonly found within wetland soils (Table 1). Soil samples down to 50 cm were collected using the push core method The Bayhead Swamp soil is from a depressional swamp located on the campus of the University of Central Florida in

Table 1
Site locations, characteristics, and physicochemical properties of the three wetland soil types used in this study. Data represents means and standard error ($n = 3$).

Wetland	Bayhead Swamp	Cypress Dome	Brackish Marsh
Coordinates	28°36′25.55″N 81°12′6.05″W	28°36′14.56″N 81°11′40.85″W	29°26′31.41″N 89°54′18.10″W
Soil Taxonomic Class	hyperthermic Histic Humaquepts	hyperthermic Aeric Alaquods	Euic, hyperthermic Typic Haplosaprists
Dominant Vegetation	*Magnolia virginiana*	*Taxodium distichum* var. *imbricarium*	*Spartina alterniflora*
Soil Moisture (%)	88.93 ± 0.67	33.22 ± 1.42	69.12 ± 1.58
Organic Matter (%)	86.80 ± 0.02	11.92 ± 0.53	20.15 ± 0.12
Total C (mg C/g)	484.07 ± 1.25	88.17 ± 11.95	94.37 ± 1.67
Total N (mg N/g)	18.23 ± 0.03	5.40 ± 0.42	4.63 ± 0.03

Orlando, Florida. This wetland was selected for its Histosol-like characteristics and high SOM content. The wetland is continuously flooded, and the dominant vegetation present is swamp bay (*Persea palustris*; Steinmuller and Chambers, 2018). The Cypress Dome soil was collected from another depressional wetland at the University of Central Florida which is intermittently flooded and is dominated by pond cypress (*Taxodium distichum* var. *imbricarium;* Steinmuller and Chambers, 2018). This wetland was chosen due to the high sand content. The third wetland soil analyzed was from a Brackish Marsh in coastal Louisiana dominated by cord grass (*Spartina alterniflora*). Soil from this location was chosen due to the high silt (>50 %) and clay (>20 %) content (Sapkota et al., 2021).

2.2. *Soil moisture status prior to particle size fractionation*

To determine the most effective soil moisture conditions for quantifying MAOM within wetland soils, 20 replicate subsamples of the bulk soil from the Bayhead Swamp were analyzed. Ten subsamples were oven dried at 70 °C until constant weight, while the other 10 were maintained at field moisture status. Once the oven-dried soils reached constant weight, a mortar and pestle were used to break apart the dried, compacted, single mass of soil that formed during oven-drying. Since performing a particle size fractionation was the goal, caution was taken not to demolish individual soil particles, but to break-apart aggregates to particle size. Field-moist soils retained the same moisture status as when they were collected and were weighed to the equivalent of 20 g of dry soil. Both the field-moist soils and the oven-dried soils were then subjected to particle size fractionation as outlined below.

2.3. *Particle size fractionation*

The first step of MAOM quantification is to separate the soils by particle size (Six et al., 1998). For each wetland soil type, samples were sequentially wet sieved through three sieves: 2 mm, 250 μm, and 53 μm. Beginning with the largest sieve (2 mm), the soil sample was placed on the sieve screen, which was submerged in a shallow tub of deionized (DI) water slightly larger than the diameter of the sieve itself. The sieve was lifted-up, then resubmerged, at a rate of 50 reps within 2 min, making sure the soil completely broke the surface of the water on each rep (Cambardella and Elliott, 1993b). After sieving through the 2 mm sieve, the soil–water mixture that passed through the sieve was transferred onto the sieve screen of the next sieve size down (250 μm) and the wet sieving was repeated. The remaining soil on top of each sieve was collected and transferred into pre-weighed plastic containers. This procedure was repeated with all 3 sieves resulting in four size fractions, >2 mm, 2 mm-250 μm, 250 μm-53 μm, and < 53 μm. Each size fraction was collected and transferred into a pre-weighed plastic containers and placed in a drying oven at 70 °C until constant weight.

2.4. *Determining need for a dispersant to break-down soil aggregates*

Following the physical fractionation, each size fraction was transferred into a 125 mL or 250 mL Nalogene plastic bottle, depending on the mass of the sample. Five randomly selected samples of each size fraction had 0.5 % sodium hexametaphosphate solution added to the bottle, and the remaining 5 samples had DI water added; both treatments created a 1:10 soil to solution ratio. The purpose of the 0.5 % sodium hexametaphosphate was to act as a dispersant (Cambardella and Elliott, 1992), which would break up any present aggregates and free any organic matter that was entrapped within the aggregates. While using sodium hexametaphosphate is commonly used has a dispersant, complete dispersion of aggregates is often difficult to achieve (see review by Christensen, 1992). The purpose of the DI water was to serve as a control to determine if changes in the distribution of soil masses among the four size fractions after a second sieving were a result of aggregate break-down/dispersion, or simply an artifact of wet-sieving the soils a second time. Soils were exposed to either treatment after initial wet sieving to ensure initial particle distribution was a result of soil moisture levels (field moist vs oven dried) and not a result of being exposed to DI or hexametaphosphate. After slurry creation, all samples were vortexed for 30 s and placed on an orbital shaker for 18 h at 120 RPM. Each sample was wet sieved again, as described above. After wet sieving, samples were again oven dried at 70 °C until constant weight.

2.5. *Density fractionation*

Soil from each wetland type was composited by size fraction following the physical fractionation, resulting in four total size fractions for each sample. For only the soil samples exposed to hexametaphosphate in section 2.4, the particle size fractionation was followed by a density fractionation to separate the light fraction (LF; also known as unassociated POM with a density < 1.85 g cm^{-3}) from the heavy fraction (HF; complexed mineral-associate OM with a density > 1.85 g cm^{-3} (Castellano et al., 2015; Cotrufo et al., 2015). Within the HF, we define MAOM as the < 53 μm HF, while all other size fractions are referred to as "coarse" HF. While it is common for MAOM to be fractionated by either physical fractionation (Cambardella and Elliott, 1992; Cotrufo et al., 2019; Ye et al.,2019) or density fractionation (Golchin et al., 1994; Sollins et al., 2009). The decision to follow the physical fractionation with a density fraction was based on the large amount of LF in the highly organic soils and the combination of physical and density fractions have been done in other studies (Six et al., 1998; Haddix et al., 2020). Sodium polytungstate made to a density of 1.85 g cm^{-3} was the solution used for the density fractionation. This solution is commonly used as it is less hazardous than alternative solutions and can be recycled and used again (Six et al., 1999). First, 2 g of soil were added to a centrifuge tube and 25 mL of 1.85 g cm^{-3} sodium polytungstate were added. Samples were placed on an orbital shaker for 18 h at 120 RPM. Once off the shaker, 10 mL of 1.85 g cm^{-3} sodium polytungstate were used to wash-off soil particles from the caps and sides of the centrifuge tube to ensure all soil was in solution. Samples rested for 30 min prior to centrifuging to promote an effective density fractionation. Samples were then centrifuged at 500 RPM for 30 min and 20 °C.

The LF floating after centrifuging was aspirated out using a pipette, placed on a 0.45 μM filter, and rinsed with DI water using a vacuum filtration system to rinse off any remaining sodium polytungstate solution from the soil. The soil sample was then backwashed onto a pre-weighed container using DI water. Additional 1.85 g cm^{-3} sodium polytungstate was added to the centrifuge tube and soil to replace the removed solution bringing the final volume around 35 mL. Centrifuging, aspirating, filtering, and backwashing were repeated with the second round of sodium polytungstate, then again with DI to rinse whatever solution may have remained. All density fractions were backwashed into pre-weighed containers and oven dried at 70 °C until constant weight.

2.6. *Total C and N*

Once fully dried, the density-fractionated soil samples were ground using ceramic balls in a SPEX 8000 M Mixer/Mill (SPEX Sample Prep, Metuchen, NJ, USA) until fully homogenized. Next, these samples were analyzed for total C and N by weighing 5 mg into tin capsules and combusting the samples in an Elementar Vario Micro Cube (Elementar Americas Inc., Mount Laurel, NJ, USA).

2.7. *Statistical analysis*

Statistical analysis was performed using R version 4.0.3 (R Foundation for Statistical Computing, Vienna, Austria) within RStudio (RStudio Team, 2020). All data wrangling was done using R packages tidyverse (Wickham et al., 2019), while all visuals were made using R package ggplot2 (Wickham, 2016). For all ANOVA's, residuals of the model were tested for normality using Shapiro-Wilk's test ($p > 0.05$), as well as visually using histograms and normal Q-Q plots, and homogeneity was determined by Levene's Test ($p > 0.05$). When the data did not meet the assumptions of an ANOVA after transformations, a generalized linear model (GLM) was used, and the best distribution was determined using the fitdistrplus R package (Delignette-Muller and Dutang, 2015). If multiple distributions fit the data, the model with the lowest AICc score was chosen, and if two models had the same score, the model was selected based on visuals of the residuals. Following either an ANOVA or GLM, the emmeans package was used to determine post hoc testing which used a Tukey adjustment (Russell, 2020).

To determine whether using field moist or oven-dried soil impacted the particle size distribution, t-tests were used to compare percent soil mass distributed among each size fraction. Normality and homogeneity of variance were tested as described above and in Fox and Weisberg (2019). If variances were equal ($p > 0.05$), then an unpaired Student *t*-test was used; if the variances were unequal ($p < 0.05$), then an unpaired Welch *t*-test was used. If the data did not meet the assumptions of a *t*-test, a Wilcoxon rank sum test was used. Data is presented in the form of mean ± standard error for percent of the initial soil mass distributed to each size fraction.

To determine the need for a dispersant, a two-way ANOVA with an interaction between treatment (dispersant vs DI water) and size fraction (parameter ∼ size fraction * treatment) was used to predict the change in percent soil mass between the initial physical fractionation and the physical fractionation after being exposed to the treatment solution. Bayhead Swamp oven-dried sample data did not meet the assumptions of ANOVA, alternatively a GLM was used using a Gaussian distribution with an identity link to predict the change in percent soil mass (parameter ∼ size fraction * treatment). Data is again presented in the form of mean ± standard error for percent of the initial soil mass distributed to each size fraction.

When analyzing total C and N data, a GLM (parameter ∼ size fraction * density), where size fraction is each size fraction and density is the LF or HF. For total C and N within the Bayhead Swamp and Cypress Dome samples, a Gamma distribution with a log link was used to predict total C and N. A Gamma distribution with an inverse link was used to predict total C and N for the Brackish Marsh.

3. Results

3.1. Soil moisture status for particle size fractionation

Air drying soils prior to physical fractionation, as is typically done with terrestrial soils (Six et al., 1998; Denef et al., 2013; Soong and Cotrufo, 2015), was determined to be unachievable for the Bayhead Swamp soil due to the high % moisture (∼89 %). Therefore, the resulting soil mass distribution among size fractions when using field-moist soil vs oven-dried soil for physical fractionation was compared (Fig. 1). Following fractionation, oven-dried soils had a greater percent mass (61.85 ± 3.54 %) within the largest size fraction (>2 mm) compared to the field-moist soils (24.20 ± 1.64 %; Wilcoxon rank sum test statistic (W) < 0.001, $p < 0.001$; Fig. 1a). There was no difference in soil mass in the 2 mm-250 μm size fraction (Fig. 1b), with the average of the two treatments being 28.91 ± 2.08 % of the total soil mass. Field-moist soils had a greater percent of soil mass (28.65 ± 1.89 %) in the 250 μm-53 μm size fraction compared to oven-dried soil (6.07 ± 0.85 %; *t*-test statistic (t) = 10.88, $p < 0.001$; Fig. 1c). The < 53 μm size fraction contained 17.91 ± 1.33 % of the field-moist soil mass and only 3.49 ± 0.35 % of the oven-dried soil mass (t = 10.80, $p < 0.001$; Fig. 1d).

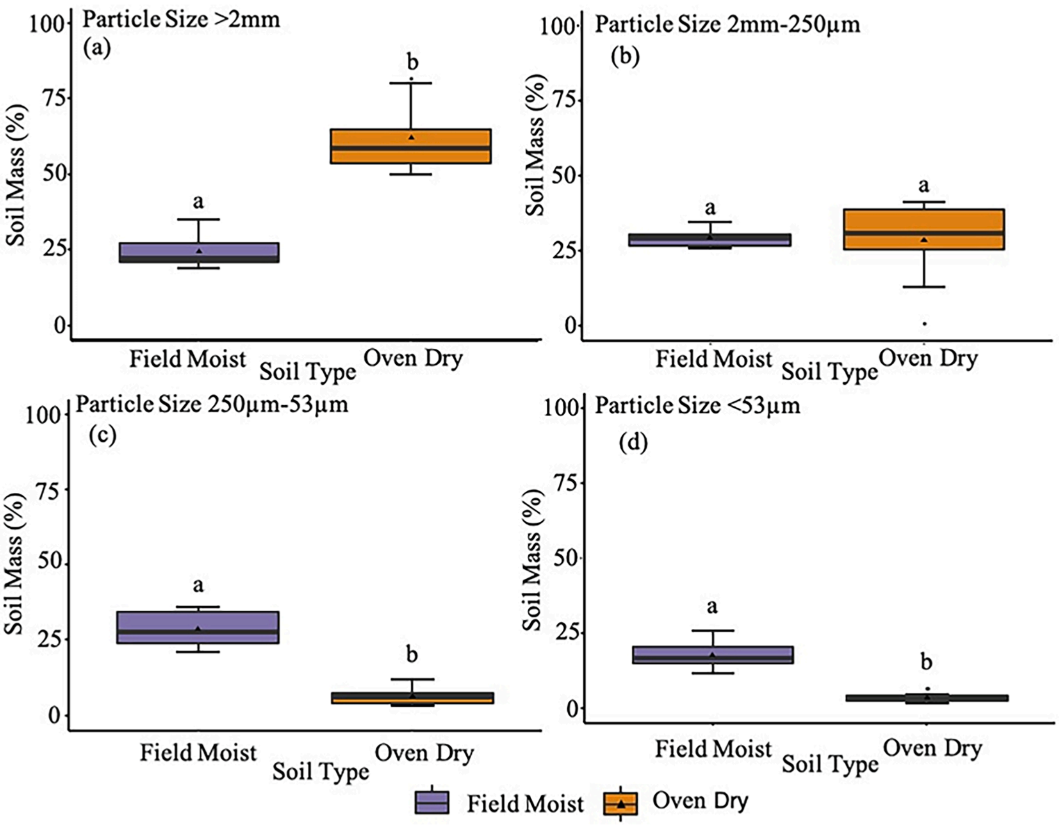

Fig. 1. Particle size distribution of Bayhead Swamp soil after wet sieving at field moisture status, as compared to after oven-drying at 70 °C until constant weight. Box plots represent median (horizontal line) and interquartile range. Means are represented by triangles and outliers by dots. Error bars represent standard error (n = 10). Different letters denote $p < 0.05$.

3.2. Need for a dispersant to break-down soil aggregates

In general, all soils (regardless of dispersant or DI water treatment) lost soil mass in the largest size fraction (>2 mm) and gained mass in the smallest fraction (<53 μm) during the second size fractionation. The effect of the second fractionation on the middle size fractions (2 mm-250 μm and 250 μm-53 μm) varied with soil type. Significant differences in the percent change of the soil mass in one or more size fractions between those soils treated with the dispersant, and those treated with the DI water control (i.e., a size fraction * treatment effect), were considered indicative of the need for a dispersant to break-apart existing aggregates.

The percent change in particle size distribution for the field-moist Bayhead Swamp soils did not differ between the dispersant and DI water control treatments (size fraction * treatment effect, F = 1.31, p = 0.289; Fig. 2a). For both treatments, the > 2 mm size fraction decreased by 10.32 ± 1.86 % and the 2 mm-250 μm size fraction percent soil mass decreased 8.68 ± 1.99 %. Within the two smallest size fractions, both treatments gained a similar mass. The 250 μm-53 μm size fraction gained an average of 2.42 ± 0.63 % and the < 53 μm fraction gained an average of 16.58 ± 1.11 %, regardless of treatment.

The interaction of size fraction * treatment for the oven-dried Bayhead Swamp soils was not significant (F = 2.19, p = 0.109; Fig. 2b), but some of the pairwise comparisons were noteworthy. The average decrease in the > 2 mm size fraction was 14.15 ± 5.51 % for both treatments. Soil in the 2 mm-250 μm size fraction generally decreased for the dispersant treatment an average of 3.35 ± 1.97 % and increased in the DI water control an average of 9.01 ± 8.91 %, but within treatment variability limited significance (p = 0.065). There was little change in the 250 μm-53 μm size fraction (+0.54 ± 1.07 %) and no difference between treatments. Oven-dried Bayhead Swamp soils treated with the dispersant generally gained more soil mass in the < 53 μm size fraction (16.79 ± 6.40 %) than soils treated with the DI water control (4.97 ± 0.92 %; p = 0.077).

The Cypress Dome soils (initially fractionated as field-moist only) demonstrated a significant size fraction * treatment effect (F = 4.52, p = 0.011; Fig. 2c). There was a greater reduction in percent soil mass in the largest (>2 mm) size fraction for samples treated with the dispersant (28.25 ± 2.72 %) than control samples (13.29 ± 3.82 %; p = 0.008). This resulted in a generally greater gain in mass in the 2 mm-250 μm fraction and < 53 μm fraction of the dispersant treated soil, compared to the DI water control treatment. Specifically, the 2 mm-250 μm dispersant soils gained 5.45 ± 1.18 % in soil mass while the control soils lost an average of 4.32 ± 7.75 % mass (p = 0.071). In the < 53 μm size fraction, the dispersant soils gained 7.43 ± 0.27 % soil mass while the DI water control soils only gained 0.70 ± 0.21 % (p = 0.205). The 250 μm-53 μm size fraction was unaffected by treatment, gaining an average percent soil mass of 16.13 ± 4.50 %.

The Brackish Marsh soils (also initially fractionated as field-moist only) showed a significant size fraction * treatment effect (F = 5.55, p = 0.004; Fig. 2d). Soils from the Brackish Marsh generally lost soil mass in all 3 larger size fractions and the magnitude of mass loss was generally greater in the dispersant treated soils than the DI water control soils. Specifically, the dispersant treatment resulted in the > 2 mm size fraction loosing 7.57 ± 1.23 % mass, compared to a 4.53 ± 1.27 % mass loss in the DI water control (p = 0.049). Within the 2 mm-250 μm size fraction, the soils exposed to the dispersant lost 0.59 ± 0.28 % soil mass,

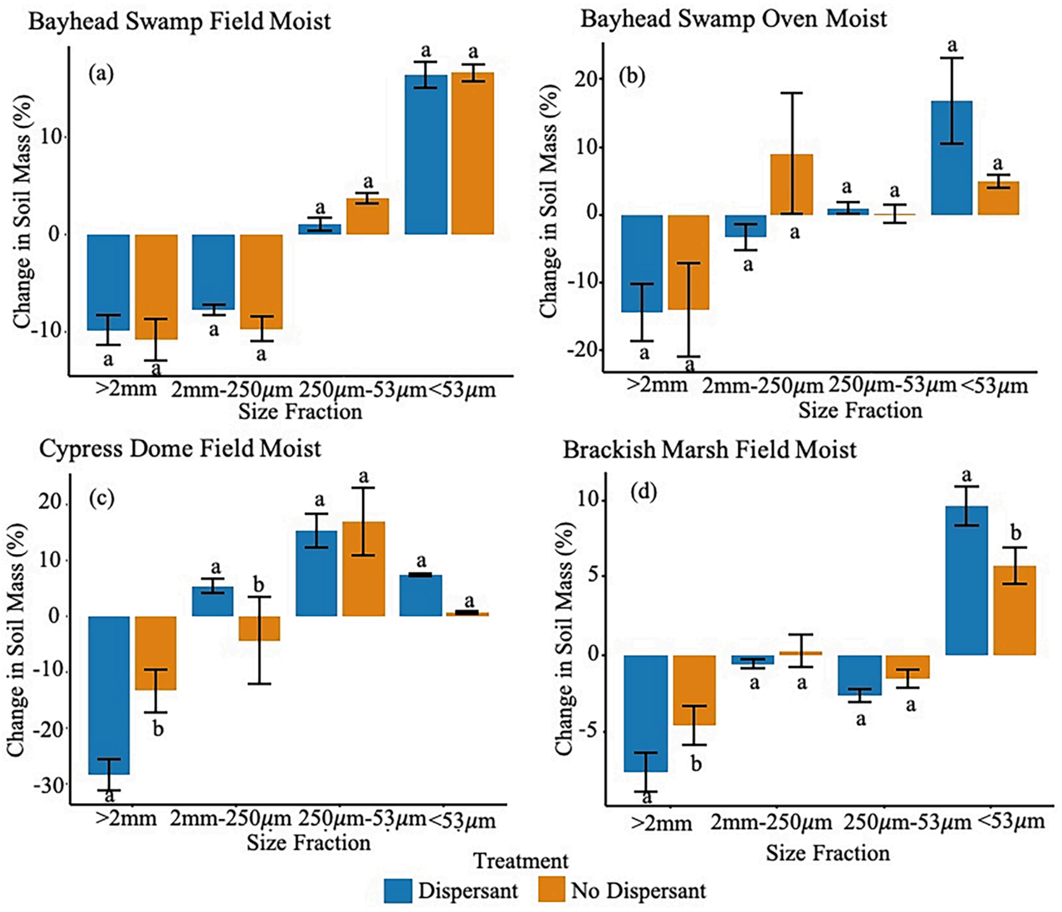

Fig. 2. Change in particle size distribution between initial particle size fraction and particle size fractionation after either a dispersant (sodium hexametaphosphate) or no dispersant (DI water control). Data are means and standard error (n = 5). Different letters denote significant differences between dispersant and no dispersant treatments in each size fraction at $p < 0.05$.

compared to the DI water control, which gained 0.26 ± 1.06 % soil mass ($p = 0.572$). In the 250 μm-53 μm size fraction, the dispersant treated soils lost 2.65 ± 0.42 % soil mass, while the DI water control treatment soils lost 1.52 ± 0.59 % ($p = 0.451$). These small treatment differences in the larger size fractions resulted in a significantly greater gain in soil mass for the dispersant treated soils in the < 53 μm size fraction (10.81 ± 1.54 %) when compared to the DI water control (5.79 ± 1.17 %, $p = 0.002$).

3.3. Total C content by wetland soil type

The majority of the C in the Bayhead Swamp soil (98.86 %) was found in the LF (Fig. 3a) with a weak interaction between size fraction * density ($F = 2.68$, $p = 0.068$). The > 2 mm and 2 mm-250 μm LFs had the largest quantity of C, with 29.99 % (215.58 ± 19.34 mg C g soil^{-1}) and 29.96 % (215.36 ± 18.28 mg C g soil^{-1}) of the total C, respectively. The size of the C pool in the LF decreased with the two smallest size fractions. In the HF, the > 2 mm size fraction contained 0.30 mg C g soil $^{-1}$ (0.04 %), which was less than the 2 mm-250 μm fraction (0.41 % of the total; 2.96 ± 1.95 mg C g soil $^{-1}$; $p = 0.006$). The < 53 μm HF contained 4.56 ± 2.43 mg C g soil $^{-1}$ (0.63 % of the total) which was greater than the > 2 mm and 250 μm-53 μm HFs ($p = 0.003$ and $p = 0.004$, respectively).

Similar to the Bayhead Swamp, the Cypress Dome soils had the majority of the C in the LF (66.24 %) with a weak interaction of size fraction * density ($F = 2.41$, $p = 0.087$; Fig. 3b). Soils in the > 2 mm LF again had the largest quantity of C (42.33 % of the total; 65.71 ± 19.73 mg C g soil^{-1}), which was more C than the LF of 250 μm-53 μm (4.81 %; 7.46 ± 1.57 mg C g soil^{-1}; $p = 0.007$). Within the HF, the > 2 mm and 2 mm-250 μm size fractions were similar, making up 15.10 % and 16.45 %, respectively. There was less C associated within the 250 μm-53 μm HF (1.11 %; 1.72 ± 1.16 mg C/gsoil), and the < 53 μm HF (1.09 %; 1.69 ± 1.17 mg C g soil^{-1}) when compared to the > 2 mm HF ($p = 0.001$ for both).

Brackish Marsh soils had the majority of the C (71.23 %) in the LF with a significant interaction of size fraction * density ($F = 38.95$, $p < 0.001$; Fig. 3c). Within the LF, the > 2 mm fraction had the largest C pool, comprising 36.42 % of the total C (39.40 ± 3.08 mg C g soil^{-1}), which was greater than the 2 mm-250 μm fraction (15.13 %; 16.36 ± 2.11 mg C g soil^{-1}, $p = 0.023$) and the 250 μm-53 μm fraction (13.63 %; 14.74 ± 3.01, $p = 0.010$). The < 53 μm LF had 6.07 %; 6.56 ± 1.74 mg C g soil^{-1} and was also less than the > 2 mm LF ($p < 0.001$). Carbon content within the three largest sized HFs were comparable with 2.04 %, 1.07 %, 1.04 % of the total C, respectively. The < 53 μm HF had the most C of all the HFs, containing 24.60 % of the total C (26.60 ± 1.07 mg C g soil^{-1}, $p < 0.001$ for all three comparisons).

3.4. Total N content by wetland soil type

Similar to the total C, total N in the Bayhead Swamp soils were predominantly within the LF (98.72 %) with a weak interaction between size fraction * density ($F = 2.65$, $p = 0.071$; Fig. 4a). Within the LF, the three largest size fractions had similar proportions of N. Nitrogen in the < 53 μm LF was generally less than in the 2 mm-250 μm LF with 16.42 % of the total N (4.05 ± 0.44 mg N g soil^{-1}, $p = 0.059$). Of the N in the HF, 0.10 % of the total (0.02 mg N g soil^{-1}) was > 2 mm, which was less than the 2 mm-250 μm fraction (0.39 %; 0.10 ± 0.05 mg N g soil^{-1}, $p = 0.031$). The 250 μm-53 μm HF was similar to the > 2 mm HF, but the < 53 μm HF had 0.66 % of the total N (0.16 ± 0.08 mg N g soil^{-1}), which was greater than N in the > 2 mm size and 250 μm-53 μm size fractions ($p = 0.006$ and $p = 0.009$, respectively).

Within the Cypress Dome soils, N followed a similar trend to C with the majority being within the LF (65.58 %) and a weak interaction between size fraction * density ($F = 2.71$, $p = 0.064$; Fig, 4b). Within the LF, the > 2 mm size fraction had the largest pool of N (3.53 ± 1.10 mg N g soil^{-1}) followed by the 2 mm-250 μm fraction. Light fraction 250 μm-

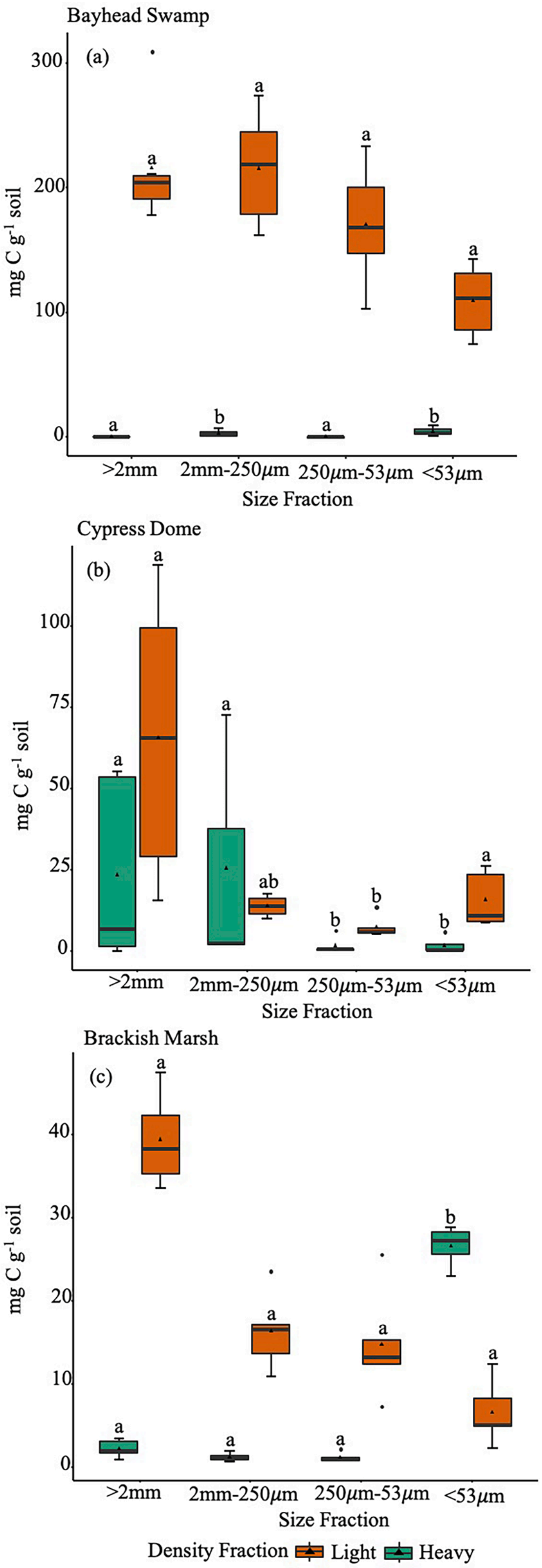

Fig. 3. Total carbon across all 8 size and density fractions after the physical and density fractionations for each wetland soil type. Box plots represent median (horizontal line) and interquartile range. Means are represented by triangles and outliers by dots. Error bars represent standard error ($n = 5$). Different letters represent significant differences ($p < 0.05$) between size fractions based on the density (light fraction (LF) or heavy fraction (HF)).

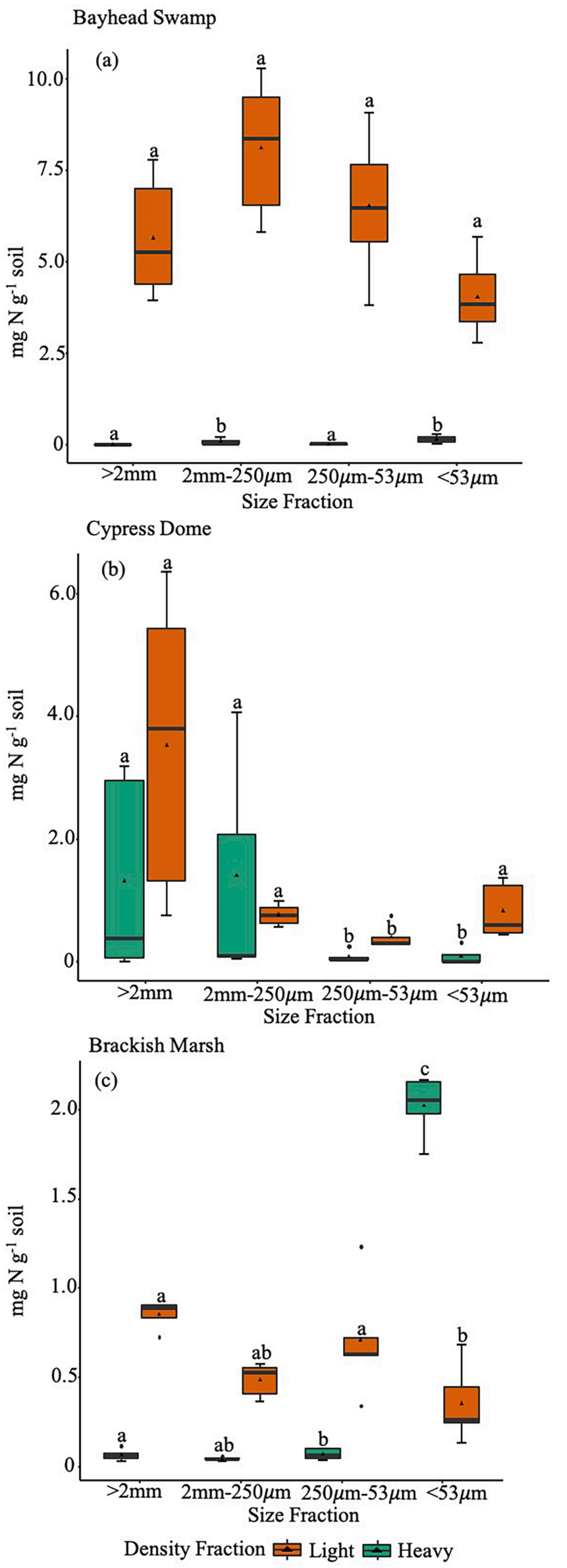

Fig. 4. Total nitrogen across all 8 size and density fractions after the physical and density fractionations for each wetland soil type. Error bars represent standard error ($n = 5$). Comparisons across particle size fractions were made only for each density fraction. Different letters represent significant differences ($p < 0.05$) between size fractions based on the density (light or heavy).

53 μm had less N (0.40 ± 0.09 mg N g soil^{-1}) than the > 2 mm size fraction ($p = 0.004$). The < 53 μm LF contained 9.83 % of the total N (0.83 ± 0.20 mg N g soil^{-1}). Within the HF, 15.65 % of the total N was > 2 mm (1.32 ± 0.72 mg N g soil^{-1}), which is greater than both 250 μm-53 μm (0.08 ± 0.04 mg N g soil^{-1}) and < 53 μm (0.09 ± 0.06 mg N g soil^{-1}) size fractions ($p < 0.001$ for both).

Within the Brackish Marsh soil, the interaction of size fraction * density predicted total N ($F = 90.87$, $p < 0.001$; Fig. 4c), and the LF contained 51.15 % of the total N. The > 2 mm LF was the largest LF N pool (18.49 %; 0.85 ± 0.04 mg N g soil^{-1}) and did not differ from the particle size fractions 2 mm-250 μm and 250 μm-53 μm. The < 53 μm LF made up a smaller proportion of the total N with only 7.67 % (0.35 ± 0.10 mg N g soil^{-1}), which was less than the LF of > 2 mm and 250 μm-53 μm ($p = 0.012$ and $p = 0.046$, respectively). Within the HF, total N was similar across the three largest size fractions being 1.43 %, 0.93 %, and 1.50 % of the total N, respectively. Total N within the < 53 μm HF was greater than all three other size fractions containing 43.96 % (2.02 mg N g soil^{-1}) of the total N ($p < 0.001$ for all three other HFs).

3.5. *C:N ratios by wetland soil type*

The C:N values of each fraction within the three different soils are highlighted in Table 2. The interaction of size fraction * density was significant for the C:N of the Bayhead Swamp soils ($F = 14.60$, $p < 0.001$). Within the LF, C:N values decreased from > 2 mm size fraction to 2 mm-250 μm ($p = 0.001$). The 250 μm-53 μm and < 53 μm LFs had lower C:N. In the HF, >2 mm particle size had a lower C:N than in 2 mm-250 μm ($p = 0.026$) and < 53 μm ($p = 0.007$) HFs. The C:N for HF 250 μm-53 μm was<2 mm-250 μm ($p < 0.001$).

The Cypress Dome soils C:N was not predicted by size fraction or density ($F = 1.50$, $p = 0.236$). Within the LF, there was no difference of C:N values across size fractions, which ranged from 17.9 to 29.5 In the HF, C:N values were similar between the first two fractions. The < 53 μm HF had the highest C:N value compared to 250 μm-53 μm ($p = 0.040$).

Size fraction nor density predicted C:N for the Brackish Marsh soils ($F = 0.641$, $p = 0.594$), but C:N generally decreased across size fractions and some pairwise comparisons were significant. Within the LF, >2 mm size fraction had a C:N value greater than both 250 μm-53 μm and < 53 μm size fractions ($p = 0.022$ and $p = 0.008$, respectively). The C:N ratio of the 2 mm-250 μm size fraction did not differ from > 2 mm fraction. Within the HF, >2 mm size fraction had a larger C:N value compared to 250 μm-53 μm and < 53 μm size fractions ($p = 0.005$ and $p < 0.001$, respectively).

Table 2
C:N values across wetland for each particle size fraction and density fractionation (LF = light fraction, HF = heavy fraction). Error bars represent standard deviation ($n = 5$). Different letters denote $p < 0.05$. Asterisks denote only 1 sample was recovered out of the 5 replicates.

Wetland	Density Fraction	>2mm	2 mm-250 μm	250 μm-53 μm	<53 μm
Bayhead Swamp	LF	39.7 ± 1.6^{a}	26.7 ± 1.9^{b}	26.2 ± 0.8^{b}	27.1 ± 1.1^{b}
	HF	12.8^{a}	26.4 ± 0.1^{b}	12.0 ± 0.0^{a}	31.1 ± 0.1^{b}
Cypress Dome	LF	19.4 ± 0.9^{a}	18.1 ± 0.2^{a}	18.8 ± 0.3^{a}	19.1 ± 0.3^{a}
	HF	19.2 ± 1.6^{a}	24.1 ± 6.1^{a}	17.9 ± 3.4^{ab}	29.5 ± 7.5^{ac}
Brackish Marsh	LF	46.4 ± 3.1^{a}	33.3 ± 2.4^{a}	20.8 ± 0.25^{a}	18.6 ± 0.6^{b}
	HF	43.7 ± 19.0^{a}	26.4 ± 2.3^{ab}	16.9 ± 2.4^{b}	13.2 ± 0.4^{b}

4. Discussion

4.1. Saturated soils should be fractionated at field moisture status

While physical fractionation methods have been well-established for terrestrial soils and typically begin with air-dried (Six et al., 1998; Del Galdo et al., 2003; Cotrufo et al., 2015) or oven dried (Cambardella and Elliott, 1993b; Denef et al., 2013; Soong and Cotrufo, 2015) samples, our results suggest field saturated wetland soils require a different approach. Specifically, soils are recommended to be maintained at field moisture status for particle size fractionation to prevent creating artificial aggregates during the drying process, as demonstrated by altered soil size distributions for the Bayhead Swamp soil. Specifically, the > 2 mm size fraction averaged 37.7 % greater when the Bayhead Swamp soil was subjected to oven-drying, as compared to when it was sieved at field moisture status. While there appeared not to be an impact on the 2 mm-250 μm particle size, percent soil mass within the 250 μm-53 μm and < 53 μm size fractions were 22.5 % and 14.4 % less, respectively, when oven dried as compared to field moist. This suggests oven drying an organic soil (i.e., 70° C until constant weight; Reddy et al., 2013) that is typically saturated or inundated under field conditions promotes the formation of *de novo* aggregates, causing the potential for an overestimation of the largest size fraction (>2 mm) and underestimation of the smaller size fractions. With low-density soils such as this (~89 % moisture), air-drying was unachievable. Therefore, sieving organic-rich wetland soils at field moisture status is believed to provide the most accurate quantification of the *in-situ* particle size distribution. Following this determination, the remaining two soil types were only sieved at field moisture status.

4.2. Minerals and intermittent hydrology may promote aggregation

The change in the distribution of size fractions for the field moist Bayhead Swamp soil did not differ when the dispersant or DI water (control) was used, with a change in soil mass of only 1.5–2.5 % across all four size fractions. This suggests soil aggregates were not present to be broken-up by the sodium hexametaphosphate solution (Gee et al., 1986) and this step was not necessary for the field moist Bayhead Swamp soil. This soil is classified as a hyperthermic Histic Humaquepts (Soil Survey Staff, 2022), but the pedon studied contained an Oa horizon > 450 cm and had a SOM content of 89–98 %, suggesting it is better classified as a Histosol (L.G. Chambers, unpublished data). Another study did observe aggregates within a Histosol (Wright and Inglett, 2009), but this difference may be due to the fact these soils were a previous wetland that had been drained in the early 1900′s and used for agriculture for more than a century. When the Bayhead Swamp soil was oven-dried, the < 53 μm size fraction gained 4-fold more soil mass when exposed to the dispersant, while the larger size fractions lost mass. Together, this suggests drying naturally water-logged Histosols, either through artificial drainage or in the lab, may promote aggregation. It remains unclear if Histosols under continuous inundation naturally form aggregates in the field, as others have also noted the wettest soils along a hydrologic transect lacked the macroaggregates observed further upslope (Kottkamp et al., 2022).

The largest size fraction (>2 mm) of the Cypress Dome soil decreased by 2 times more with the dispersant than without, indicating the presence of aggregates in this sandy-organic wetland soil and the need for a dispersant for accurate particle size fractionation. The high SOM content and low availability of fine (silt and clay) minerals in this soil had led us to hypothesize it would not contain aggregates when fractionated at field moisture, but this site differs from the Bayhead Swamp in that it is not continuously saturated. Rather, the water level in the Cypress Dome has been documented to drop>20 cm below the soil surface during the dry season (e.g., Jan.-May; L.G. Chambers, unpublished data), which may have enhanced natural aggregate formation. Others have observed that soils with long-term fluctuating water levels can promote water stable aggregates (Denef et al., 2001).

Finally, the Brackish Marsh soil had a large quantity of silt and clay minerals (Sapkota et al., 2021), which have been observed to increase soil aggregation (Franzluebbers and Arshad, 1996). The dispersant treatment reduced the soil mass in > 2 mm and 250 μm-53 μm size fractions, leading to a 5.1 % increase in the mass of the < 53 μm size fraction. This indicates the presence of soil aggregates and the potential to underestimate the size of the MAOM pool if a dispersant was not used. The Brackish Marsh not only contains fine minerals but is also intertidal. In sum, this data suggests a possible interaction between soil type and hydrology in the promotion of soil aggregation in wetlands. Utilizing a dispersant on any wetland soil that contains quantifiable mineral matter or is exposed to a variable hydropattern is recommended to ensure accurate particle size fractionation where investigators are not interested in accounting for soil aggregates.

4.3. Soil C content and its degree of protection varied by wetland

Total C mass was highest within the Bayhead Swamp soil (484.07 ± 1.25 mg g soil^{-1}), but it was almost all (98.7 %) in the LF. The LF is often classified as POM, or partially decomposed plant litter, and is considered the unprotected form of soil C (Six et al., 2002). Since unoccluded POM lacks the physical or chemical protection afforded by aggregation or mineral associations, it typically has a faster turnover rate than MAOM, despite being dominated by large complex molecules that require enzyme depolymerization (von Lützow et al., 2007; Kleber et al., 2015). Importantly, only 0.63 % of all the soil C in the Bayhead Swamp is protected as MAOM, indicating a high degree of vulnerability for this soil C pool if the environmental protection afforded by saturation was removed.

Compared to the C rich Bayhead Swamp soil with almost entirely unprotected POM, the Cypress Dome and Louisiana Marsh soils both had measurable amounts of C associated with the HF. While the Cypress Dome had very little C within the MAOM (<53 μm HF) pool (1.09 %), it did have measurable C within the > 2 mm HF (15.1 %) and the 2 mm-250 μm HF (16.5 %). This larger HF C could be considered "heavy POM" rather than highly stable MAOM (Cotrufo and Lavallee, 2022) or even "coarse-MAOM" (Samson et al., 2020). Furthermore, considering the large variability in C concentrations found within our heavy coarse fraction, more research is needed to validate its presence. For example, the carbon in the heavy coarse fraction could represent relatively stable aggregates that have persisted after being exposed to a dispersant. We utilized sodium hexametaphosphate as our dispersant (Cambardella and Elliott, 1992), but others have employed ultrasonic vibration, hand or mechanical shaking with water or various chemical dispersants, or shaking with glass or agate balls; complete dispersion is known to be difficult (see review by Christensen, 1992). Depending on the goal, others should consider additional dispersing efforts to break all aggregates and get a true particle size fractionation. Furthermore, the nature of this particular soil, being high in both fibrous SOM and sand, may have uniquely limited the ability to achieve a clear density separation. Larger POM may be weighed down by sand particles during the density fractionation without actually being part of a true aggregate or coated with minerals. Future work should investigate the efficacy of different dispersing techniques on wetland soils to confirm if heavy-POM is more common in this ecosystem type, how it is formed, and what its ecological significance is.

The Brackish Marsh soil had the least total C (94.37 ± 1.67 mg g soil^{-1}) but the largest fraction in the form of MAOM, 24.6 % of the total C. An additional 4.2 % of total C was found in the HFs of the three larger particle size fractions (i.e., heavy coarse fraction), but these pools had much lower C concentrations than observed in the Cypress Dome and are therefore more likely to be unassociated minerals resulting from a successful density separation. Similar to the Bayhead Swamp, the concentration of C in the LF tended to decrease with particle size, while the < 53 μm HF (MAOM) had dramatically (10 times) more C than the other

HFs. The distribution of C among the size and density pools of the Brackish Marsh soil most closely resembles the distribution of C seen in terrestrial soils studies (Six et al., 1998; Cotrufo et al., 2019; Haddix et al., 2020; Yu et al., 2022). This may be because this soil was the only one of the three with appreciable silt and clay content to form MAOM.

4.4. High N content and low C:N is associated with fine minerals

Soil total N was generally distributed in a similar manner to total C among the different size and density fractions of the Bayhead Swamp and Cypress Dome soils. For example, 98.7 % of the total N was in the LF of the Bayhead Swamp while the total N of the Cypress Dome soil was primarily distributed among the LF (65.6 %) and heavy coarse fraction (32.65 %). Due to the overwhelming dominance of POM (and lack of MAOM) in these soil types, C:N ratios are generally high, vary little, and reflect ratios typically observed in unprotected plant litter (Six et al., 2002). The Bayhead Swamp soil C:N averaged 25.2, with slightly higher values in the largest POM (~40) and some of the heavy coarse fraction appearing to contain low C:N (however, this assumed to be an artifact of the extremely small mass of heavy coarse fraction in this soil). The Cypress Dome soil had surprisingly consistent C:N across all size and density fractions (~18–30).

In contrast, the Brackish Marsh soil had about half (52.1 %) of the total N in the LF and most of the remaining (44.0 %) in the MAOM fraction, despite MAOM containing only 24.5 % of the total C. While C has consistently been shown to be more stable and persistent when incorporated into the MAOM fraction (e.g., Castellano et al., 2015, Lavallee et al., 2020) the implications for N persistence of MAOM are less clear. Some studies suggest elevated rates of N mineralization from the MAOM fraction (Turner et al., 2017), while others indicated repressed rates of N mineralization (Swanston et al., 2004).

The distribution of total N in the Brackish Marsh soils is reflected in the C:N, which is lowest in the MAOM pool (~13), intermediate in the heavy coarse fraction (~22), and highest in the POM (43–47). This is consistent with recent frameworks which asserts microbial byproducts (*in vivo*) and soluble organic matter (*ex vivo*) are the major precursors of MAOM (Cotrufo et al., 2013; Cotrufo et al., 2015; Cotrufo and Lavallee, 2022; Haddix et al., 2016; Liang et al., 2017). Interestingly, the mean C:N of the Brackish Marsh LF generally declined with particle size, suggesting a relationship between POM size and degree of microbial processing. Fewer studies density fractionate individual size fractions, as was done here, but doing so may enhance the functional relevance of particle size fractionations (Samson et al., 2020).

5. Conclusion

A method traditionally used for quantifying MAOM within terrestrial soils was modified and used across wetland soils with varying physicochemical properties. It was observed that using field moist soils was most effective in representing *in-situ* soil size fractions when compared to oven dried soil; air drying soils is often unattainable due to the saturated conditions of wetland soils. Despite that the dispersal technique may not have fully dispersed all present aggregates, a dispersant was needed to break-apart aggregates when the wetland soils had either a non-negligible amount of minerals present and/or an intermittent hydroperiod. Although the Bayhead Swamp soil, characterized by permanent waterlogging and virtually no minerals, showed no evidence of the existence of natural aggregates, we still recommend including the use of a dispersant unless these unique conditions have been previously confirmed.

Contrary to the conditions found in the terrestrial and mineral-based soils used for most previous MAOM studies, minerals appear to be limiting MAOM formation in these organic wetland soils, while POM abounds (representing up to 99.4 % of the soil mass and 98.9 % of the total C). In the Cypress Dome soil that contain only minerals in the sand size fraction, a sizable pool of carbon in the heavy coarse fraction was found, which has not been commonly documented in other studies. Further research is needed to better understand if this was a methodological artifact (occluded POM due to insufficient dispersion of aggregates) or a unique property of sandy organic soils that may be ecologically significant. As predicted, the silt and clay rich Brackish Marsh contained the most MAOM and supported existing theories about soil physical fractionation developed in terrestrial soil, including a low C:N in the MAOM fraction indicative of labile and microbial C sources. In this soil, a linear decrease in C:N with size was observed in both the heavy and light fractions, suggesting a direct correlation between size and source, or degree of decomposition. The low C:N of the Brackish Marsh MAOM pool also caused nearly half of all soil total N to be found in this fraction.

Overall, this study represents a critical first step toward improving the understanding of the mechanisms that promote C persistence in organic wetland soils beyond environmental controls (e.g., microbial inhibition due to waterlogging). This is particularly important in wetlands vulnerable to environmental change, where temporary or permanent shifts in hydrology could remove environmental constraints on soil microbial activity and leave unprotected POM subject to rapid mineralization. Through this lens of differentiating SOM based on vulnerability, as protected (MAOM) or unprotected (POM), our results demonstrate that the soil with the largest total C pool (Bayhead Swamp), was the least protected, while the soil with the least total C (Brackish Marsh) was the most. On a mass basis, only 4.56 ± 2.43 mg C g soil^{-1} were protected (stabilized) in the Bayhead Swamp, while 26.60 ± 1.07 mg C g soil^{-1} were protected in the Brackish Marsh. Future soil C inventories should consider not just the quantity of soil C, but also the form of that C as it relates to degree of protection.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgements

The authors would like to thank John R. White for collecting the Brackish Marsh soils for the analysis and Jason Anandappa, Paul Boudreau and Chelsea Nitsch for lab assistance. This work was partially funded by the U.S. Army Corp of Engineers, Engineering with Nature® Program through Cooperative Ecosystem Studies Unit Agreement W912HZ-21-2-0051, the Interagency Climate Change NASA program grant no. 2017-67003-26482/project accession no. 1012260 from the USDA National Institute of Food and Agriculture, and National Science Foundation Chemical Oceanography Grant (#1635837) to LGC, and the Society of Wetland Scientists Biogeochemistry Section Research Grant (to AJM).

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