Flaws in the methodologies for organic carbon analysis in seagrass blue carbon soils

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Abstract

The ability to accurately measure organic carbon (OC) in marine sediments or soils is overall taken for granted in scientific communities, yet this seemingly mundane task remains a methodological challenge when the soil matrix contains calcium carbonate ($CaCO_3$), creating inaccuracies in Blue Carbon estimates. Here, we compared five common methods combining acidification, combustion, and wet oxidation pre-treatments for determination of OC in sediments and soils containing $CaCO_3$ based on the analyses of artificial soil mixtures made of different OC and $CaCO_3$ contents, and multiple soils from Australian seagrass cores. The results obtained showed that methods involving acidification pre-treatment entailed $-17 \pm 0.2\%$ (mean ± SE) underestimation of OC content (ranging from -8% to -26%), whereas the combustion-based method was accurate for samples with high $CaCO_3$ content but entailed 32–47% overestimation in samples with low $CaCO_3$ content. The Heanes method (wet oxidation method) showed <5% deviation from the known OC content, but this method is not suitable for soil samples containing reduced iron, sulfur and potentially manganese compounds. The differences observed among methods have significant impacts on local, regional, and global Blue Carbon storage calculations. We provide key methodological guidelines for the analysis of OC in soils with high and low $CaCO_3$ contents, aiming at improving accuracy in current Blue Carbon science.

Accurate and precise measurement of organic carbon (OC) content in biomass, soils and sediments is crucial to determine the role of ecosystems as carbon sinks and in particular, to assess their contribution to climate change mitigation (i.e., Green and Blue Carbon) under the current global change emergency (Wotherspoon et al. 2015; Macreadie et al. 2021). Whereas OC in biomass and soils where calcium carbonate ($CaCO_3$) is absent can be accurately measured with methodologies developed by edaphologists since the last century (Nelson and Sommers 1983), the presence of inorganic carbon (IC) in the form of $CaCO_3$ in coastal and terrestrial soils adds difficulty and complexity to OC analyses (Van Iperen and Helder 1985). This is of special concern in Blue Carbon science (i.e., the role of coastal vegetated ecosystems in climate change mitigation), an emerging discipline where the analysis of OC is crucial and the presence of $CaCO_3$ is common, in particular for seagrass ecosystems (Mazarrasa et al. 2015; Saderne et al. 2019). As the potential of conservation and restoration of tidal marshes, mangroves, seagrasses and macroalgae (i.e., Blue Carbon) for climate change mitigation ramps up in scientific, environmental and policy circles (e.g., National Determined Contributions), accurate and precise measurements of OC in Blue Carbon soils where $CaCO_3$ is present is a pressing issue.

Direct OC determination using an elemental analyzer (EA) and indirect determination based on Loss-On-Ignition (LOI) are the most common analytical methods to estimate OC in coastal sediments and soils, and are considered to be robust techniques in Blue Carbon science (Fourqurean et al. 2014). However, the presence of $CaCO_3$ in marine settings can result in inaccurate estimates of OC using these methods, due to the effects of analytical procedures on either OC and/or IC (Byers et al. 1978; Verardo et al. 1990; Wai Ting Tung and Tanner 2003; Brodie et al. 2011). Using an EA to determine OC requires removing $CaCO_3$ prior to analysis and this is commonly achieved through the acidification of the sample (i.e., acid digestion of IC). The acid digestion pretreatment varies across studies, with differences in the acid used (e.g., HCl or H_3PO_4), the acid concentration applied (ranging from 0.5 to 10%), the time period of digestion (from few hours to more than 24 h), or whether acidification of the sample occurs in vessels followed by centrifugation and removal of excess acid, or directly within silver capsules without rinsing prior to analysis (Verardo et al. 1990; Kennedy et al. 2005; Schlacher and Connolly 2014; Wotherspoon et al. 2015; Fujisaki et al. 2022). These methodological differences can significantly bias OC estimates. For instance, the rinsing of acidified samples to remove excess acid can lead to the loss of inorganic acid-soluble components (e.g., heavy metals), leading to an overestimation of OC. On the other hand, it can also cause the loss of acid-soluble or easily-hydrolysed organic compounds, leading to an underestimation of OC (Pocklington and Hagell 1975; Byers et al. 1978). Indeed, losses of specific OC compounds during acidification pretreatment can result in OC isotopic fractionation ($\delta^{13}C$), which is a common proxy used to decipher the provenance of OC in Blue Carbon science (Kennedy et al. 2005, 2010; Komada et al. 2008). On the contrary, not rinsing the excess acid can cause losses of volatile acids and inaccuracies in weigh measurements due to the formation of hygroscopic salts and thereby, result in either an underestimation or overestimation of OC content (Byers et al. 1978). Direct acid treatment in silver capsules has been recommended to avoid losses due to acid hydrolysis (Verardo et al. 1990), but this method is not suitable when large amounts of $CaCO_3$ are present and requires more operator skill and EA and IRMS maintenance (Kennedy et al. 2005).

Other approaches to estimate OC in Blue Carbon soils include the LOI technique, which indirectly estimate OC from organic matter (OM) content determined as the loss in dry weight (DW) after combusting the sample at 400–550°C for 4–6 h (Heiri et al. 2001). OC is estimated from OM values using a linear equation resultant from plotting OM and OC contents in samples where both LOI and EA have been conducted (Fourqurean et al. 2012, 2014). Yet, LOI can lead to an overestimation of OM content due to the loss of water from clay minerals and loss of carbonate species (i.e., magnesium carbonate, manganese carbonate, and iron carbonate) at 100–550°C combustion temperatures (Byers et al. 1978; Froelich 1980; Sutherland 1998; Leong and Tanner 1999). Additionally, OM continues to be oxidized at temperatures >550°C, which can also lead to up to 10% underestimate of soil OC content (Howard et al. 2021). The LOI measurement is influenced not only by temperature and length of combustion, but also by the characteristics of the muffle furnace itself and even by the placement of crucibles within the oven (Heiri et al. 2001), leading to high variability between laboratories. Thus, LOI accuracy can vary across samples with different OC and/or IC contents, and it has been considered unreliable for substrates with low OM content (Tuffour et al. 2014).

LOI can also be combined with EA to determine OC content in marine sediments and soils, as the difference between total carbon (TC) content in the bulk sample and IC content in a combusted sample (Fourqurean et al. 2012; Wotherspoon et al. 2015; Howard et al. 2016). The wet oxidation technique is another common method for measuring OC in marine sediments (e.g., the classic Walkley-black method and the Heanes method) (Byers et al. 1978), but with little uptake within the Blue Carbon research community (Cammen 1975; Broome et al. 1986). It involves using potassium dichromate and sulfuric acid as the oxidants, with the option to accelerate the oxidation process through external heat. However, the wet oxidation method has uncertainties regarding oxidation of other constituents in the soil and the proportion of total OC oxidized (Gaudette et al. 1974; Byers et al. 1978; Leong and Tanner 1999).

The difficultly in robustly measuring OC in marine sediments or soils and, particularly, when the mineral matrix is primarily composed of calcareous materials, has been discussed at length (Byers et al. 1978; Bisutti et al. 2004; Wotherspoon et al. 2015). Despite the large amount of existing peer-reviewed literature on OC content analyses in soils and sediments with presence of $CaCO_3$, and the known issues with some methods, there is little current agreement on methods for measuring OC in Blue Carbon ecosystems. The differences in OC estimates across methods can be of one order of magnitude (Byers et al. 1978; Leong and Tanner 1999), which are highly relevant considering that OC content in Blue Carbon soils typically ranges from <1% to 20% (Fourqurean et al. 2012; Ouyang and Lee 2020). In addition, the high variability in OC and $CaCO_3$ contents within and across Blue Carbon soils, ranging from <1% to >30% OC and 0% to >99% $CaCO_3$ (Fourqurean et al. 2012; Gorham et al. 2021), adds further error across methods and complexity on choosing the best methodology for OC analyses. As a result, differences in the methodologies used across research groups and disciplines can result in large uncertainties in regional and global estimates of carbon inventories, or even large errors in the estimates when inaccurate methods are being used (Ouyang and Lee 2020).

As the number of studies providing new local, regional, and national estimates of soil OC sinks in Blue Carbon

ecosystems is increasing exponentially (de Paula Costa and Macreadie 2022), the use of standardized and accurate methodologies is urgent. Here, we tested five distinct methods for OC analyses on a set of artificially prepared soils with known contents of silicate sand, carbonate, and seagrass plant debris mixtures, and on a set of 62 seagrass soil samples with broad ranges of OC and $CaCO_3$ contents to decipher the best methodology for OC analyses in seagrass soils. The five methodologies tested rank among the most used by the Blue Carbon scientific community, and are relatively quick, easy, cheap, and standardized approaches that could be used widely by Blue Carbon research groups worldwide.

Materials and procedures

Artificial soil and seagrass soil samples

A set of four artificial soils with combined high and low OM and $CaCO_3$ contents (high $CaCO_3$–low OM, high $CaCO_3$–high OM, low $CaCO_3$–low OM, and low $CaCO_3$–high OM) were created to assess the reliability of five distinct analytical methods to estimate OC in soils where $CaCO_3$ is present (Table 1). The artificial soil samples consisted of different proportions of living seagrass *Posidonia australis* (i.e., leaves, sheaths, rhizomes, and roots containing $37.1 \pm 0.03\%$ OC), reagent grade $CaCO_3$ (12.0% IC), and acid-washed and combusted siliciclastic sand (10% HCl; 450°C for 5 h) prepared as standards of known OC and IC contents. Artificial soil mixtures totaling 100 g of DW material each were homogenized for 24 h on a roller mill before fine grinding using a hardened steel ring and puck mill. In addition, a set of 62 soil samples from multiple Australian seagrass cores (from top to 100 cm soil-depth) (Serrano et al. 2019) encompassing broad ranges of OC content and mineral properties (e.g., $CaCO_3$ content and within siliciclastic and karstic catchments) were selected. Prior to laboratory analyses, all seagrass soil samples were dried at 70°C until constant weight and finely ground to powder on a Retsch mill.

Analytical procedures

OC content was determined in bulk living seagrass *P. australis* using an EA, after scrapping the epiphytes with a razor blade, rinsing the plants with milli-Q water and drying at 70°C until constant weight. OC content was determined in five replicate subsamples of each of the artificial soil samples

and in the 62 seagrass soil samples using 4 out of 5 of the methods (see sections "Slow weak acidification and elemental analysis (SA and EA)", "Slow weak acidification and elemental analysis coupled with an isotope ratio mass spectrometer (SA and EA-IRMS)", "Loss-on-ignition and elemental analysis (LOI and EA)", and "Heanes wet oxidation"; Table 2). The remaining method (section "Quick strong acidification and elemental analysis (QA and EA)") was only applied in three replicate subsamples of each of the artificial soil samples and in 25 seagrass soil samples.

Slow weak acidification and elemental analysis (SA and EA)

About 2 g DW of powder sample was acidified with 30 mL of 1 N HCl inside 50 mL centrifuge tubes. The HCl was gradually added in 5 mL aliquots over 4 h to avoid excessive bubbling and subsequent loss of sample. The tubes were then capped and placed on a shaker table for 18 h at ~20°C. Samples were then centrifuged at 3000 rpm for 8 min and the supernatant was carefully decanted to avoid the loss of particulate OM. The samples were then rinsed with 30 mL of milli-Q water, vortexed for 2 min, centrifuged (3000 rpm for 8 min), decanted, dried at 70°C for 48 h until constant weight, and homogenized to powder. Mass loss during the acidification process was recorded by measuring the DW of sample before and after acidification. OC in the acidified samples, and TC in bulk samples were analyzed using a LECO Trumac CN analyzer (LECO Corp.). The mean precision (Standard Deviation; SD) of replicate analyses of standards was 0.02% for TC. TC measured on the bulk sample contained both OC and IC, whereas TC measured on the post-acidification sample only contained OC. IC content was estimated as the difference between TC and OC.

Slow weak acidification and elemental analysis coupled with an isotope ratio mass spectrometer (SA and EA-IRMS)

The procedures used in this method were the same as for method SA and EA (section "Slow weak acidification and elemental analysis (SA and EA)"), with the exception of the time of HCl digestion to remove IC (1 h instead of 4 h), and the manual agitation of samples instead of mechanical shacking. Both bulk and acidified samples were analyzed for TC, OC, and stable C isotopes using a Micro Cube elemental analyzer (Elementar Analysensysteme GmbH, Hanau, Germany) interfaced to a PDZ

Table 1. Weight percentages of carbonate (reagent grade $CaCO_3$), OM (*P. australis* tissue), and combusted and acid-washed siliciclastic sand used to create the four artificial soil samples.

Artificial mixtures	Proportion (%) $CaCO_3$/OM/sand	Known OC (mg g^{-1})	Known IC (mg g^{-1})
High $CaCO_3$–low OM	75/5/20	19	90
High $CaCO_3$–high OM	75/15/10	56	90
Low $CaCO_3$–high OM	20/15/65	56	24
Low $CaCO_3$–low OM	20/5/75	19	24

Table 2. Summary of methods used for organic carbon (OC) analysis.

Method	Description	Indirect/direct OC estimates	Main source(s) of potential error	N
SA and EA	Elemental analysis in preacidified samples (slow weak acidification)	Direct	Hydrolysis/dissolution/volatile of organics and/or inorganics; loss of particulate OC.	62
SA and EA-IRMS	Elemental analysis coupled with mass spectrometry in preacidified samples (slow weak acidification)	Direct	Loss of volatile acids and inaccurate weighing.	62
QA and EA	Elemental analysis in preacidified samples (quick strong acidification)	Direct	Hydrolysis/dissolution/volatile of organics and/or inorganics; loss of particulate OC.	25
LOI and EA	Elemental analysis in bulk samples for TC and in combusted samples (500°C) for TIC. Then, TOC = TC − IC	Indirect	LOI can remove IC and water from clay minerals; OC is the small residual estimated.	62
Heanes	Heanes wet oxidation	Direct	Other reduced species are co-oxidized.	62

Description of the method, indication of whether estimates of OC are direct or indirect, and main sources of potential error in OC estimates.
N = number of seagrass soil samples analyzed per method.

Europa 20–20 isotope ratio mass spectrometer (Sercon Ltd., Cheshire, UK). The mean precision (SD) of replicate analyses was 0.02% for both TC and OC, and 0.2‰ for $\delta^{13}C$. Carbon isotope ratios are expressed as δ values in parts per thousand (‰) relative to Vienna Pee Dee Belemnite.

Quick strong acidification and elemental analysis (QA and EA)

The procedures used in this method were similar to those in methods SA and EA and SA and EA-IRMS (sections "Slow weak acidification and elemental analysis (SA and EA)" and "Slow weak acidification and elemental analysis coupled with an isotope ratio mass spectrometer (SA and EA-IRMS)"), with the exception of the strength of HCl used for the digestion to remove IC, the amount of HCl used, and the duration of the digestion. Here, 10 mL of 4 N HCl was gradually added in 1 mL aliquots to 2 g DW of soil. Samples were vortexed and immediately centrifuged and rinsed following the same procedures as outlined above. Total contact time between sample and acid was kept to < 15 min. Samples were then analyzed for TC and OC as in method SA and EA (section "Slow weak acidification and elemental analysis (SA and EA)").

Loss-on-ignition and elemental analysis (LOI and EA)

Dry samples were analyzed for TC content using a CHN EA (Fisons NA1500). About 4 g of each sample was combusted at 500°C for 5 h. IC content of the combusted samples was determined with CHN EA and scaled to mass of the samples before combustion, then OC was calculated as the difference between TC and IC. TC and IC were determined in duplicate aliquots from each sample and the average of the duplicates was used as the value for each sample.

Heanes wet oxidation

The Heanes method (Heanes 1984) is a variant on the classic Walkely-Black wet oxidation method (Walkley and Black 1934) for determination of OC where heat is applied to overcome the incomplete oxidation often observed when using the Walkely-Black method. The Australian Soils and Plant Analysis Council method 6B1 was followed in this study (see Rayment and Lyons 2011 for full methodology). Briefly, 0.5 g of soil was added to 100 mL reaction tubes, and 10 mL of potassium dichromate (0.167 M $K_2Cr_2O_7$) and 20 mL of concentrated sulfuric acid (H_2SO_4) were added. The reaction tubes were then heated to 135°C for 30 min and then cooled by filling the reaction tubes to the 100 mL mark with milli-Q water. After centrifuging, the final color was read by absorption at 600 nm and compared to a calibration curve generated using a sucrose solution. Dichromate can oxidize other reduced compounds that can be present in marine settings; as such, the potential bias in results due to the oxidation by dichromate of other reduced species (e.g., pyrite, iron[II], and nitrite) was diminished by excluding from the dataset the 12 of the 62 samples analyzed that developed unexpected color and rapid reaction (Lewis et al. 2011).

Statistical analyses

Differences among OC concentration (mg OC g^{-1}) estimates between the known OC contents from the artificial soil samples and those obtained using the five analytical methods described in sections "Slow weak acidification and elemental analysis (SA and EA)", "Slow weak acidification and elemental analysis coupled with an isotope ratio mass spectrometer (SA and EA-IRMS)", "Quick strong acidification and elemental analysis (QA and EA)", "Loss-on-ignition and elemental analysis (LOI and EA)", and "Heanes wet oxidation" were assessed through Generalized Linear Models (GLM) and post-hoc Fisher least significant difference (LSD) analyses. OC concentration data were square root transformed prior to analyses to meet normality and homogeneity of variance.

Acknowledging that it is not possible to exactly estimate the real OC in seagrass soil samples, and considering that the OC estimates in the artificial soils obtained using the Heanes method were not significantly different to the known OC values across all four artificial soils ($p > 0.05$; see section "Results"), we used the OC content estimates in seagrass soils samples by the Heanes method as a reference to assess the potential bias produced by the other methods tested. To assess differences in OC content between the Heanes method and the other methods tested we used two indexes that consider differences across paired samples: the Bias and the root mean square error (RMSE). The bias was calculated as the sum of the differences in OC content between each method and the Heanes method for each seagrass soil sample divided by the number of samples analyzed ($n = 62$ in all cases except in the QA and EA method where $n = 25$). The RMSE was calculated as the sum of the squared differences in OC content between each method and the Heanes method for each seagrass sample divided by the number of samples analyzed -1.

The loss of OC in artificial soil mixtures following the SA and EA-IRMS method was calculated as the difference between the known OC contents and the OC content obtained with the SA and EA-IRMS method. The effect of OC loss during acidification on δ^{13}C fractionation was calculated as the difference between the δ^{13}C of artificial soil mixtures obtained with the SA and EA-IRMS method and the δ^{13}C of seagrass OM. Significant effects were tested through GLM and post-hoc Fisher LSD analyses.

In order to assess the influence of soil IC and OC contents on the dispersion of soil OC estimates obtained with the methods tested, we fitted exponential functions between IC contents and the dispersion of OC estimates across methods (i.e., calculated as the difference between the maximum and minimum OC values obtained across the five methods tested). Similarly, an exponential function was fitted between OC contents and the dispersion of OC estimates across methods. The significance of the relationship was tested by applying a linear regression analysis between the log transformed dispersion of OC estimates across methods and the log transformed OC and IC contents. All statistical analyses were conducted with Statistics© and RStudio.

Results

Artificial soil samples

Significantly different OC content estimates were obtained based on five different analytical methods, and the trends varied across the four artificial soil mixtures analyzed (Fig. 1 and Table 3; $p < 0.0001$). For the high CaCO$_3$–low OM artificial soil samples, the OC contents estimated by the LOI and EA and the Heanes methods (mean ± Standard Error; 18.5 ± 1.5 and 19.6 ± 0.1 mg OC g^{-1}, respectively) were similar to the known value (18.5 mg OC g^{-1}; $p > 0.05$). In contrast, the OC contents

estimated with the SA and EA, SA and EA-IRMS, and OA and EA methods (ranging from 14.5 to 16.1 mg OC g^{-1}) were significantly lower (13–22%) than the known value (Fig. 1; $p < 0.0001$). Similarly, for the high CaCO$_3$-high OM artificial soils, the OC contents estimated by the LOI and EA and the Heanes methods (58.8 ± 1.4 and 58.1 ± 0.4 mg OC g^{-1}, respectively) were similar to the known content (55.7 mg OC g^{-1}), whereas all other methods provided significantly lower OC estimates (12–26%), in particular the SA and EA-IRMS method (41.1 ± 1.2 mg OC g^{-1}; Fig. 1; $p < 0.0001$).

For the low CaCO$_3$–high OM artificial soils, the OC content estimates measured by the Heanes, SA and EA and QA and EA methods (58.0 ± 0.9, 51.1 ± 1.8, and 47.2 ± 1.1 mg OC g^{-1}, respectively) were not statistically different to the known value (55.7 mg OC g^{-1}; $p > 0.05$). On the other hand, significantly lower values (22% ± 2.8%) were obtained with the SA and EA-IRMS method (43.2 ± 1.6 mg OC g^{-1}); whereas significantly higher values (32% ± 10%) were obtained with the LOI and EA method (73.3 ± 12.3 mg OC g^{-1}; Fig. 1; $p < 0.0001$). For the low CaCO$_3$–low OM artificial soils, the OC content estimates measured by the LOI and EA method (27.2 ± 7.7 mg OC g^{-1}) were significantly higher (47% ± 19%) than the known value (18.6 mg OC g^{-1}; $p < 0.0001$), whereas the OC content estimated for all other methods examined (ranging from 15.6 to 19.2 mg OC g^{-1}) were similar to the known OC content (Fig. 1 and Table 3). Overall, the Heanes method showed the lowest difference in OC content between the known and estimated values for the four artificial soils studied, and also the LOI and EA method for the high CaCO$_3$–low OM and the high CaCO$_3$-high OM artificial soil samples (Fig. 1). δ^{13}C fractionation and loss of OC content following the SA and EA-IRMS method were significantly higher in artificial soil mixtures with a high content of OM compared to mixtures with low OM content ($p < 0.05$; Fig. 2).

Seagrass soil samples

The OC content estimates in the seagrass soil samples differed across the five analytical methods examined (Fig. 3 and Table 4). OC content estimated by the LOI and EA method tended to be higher than estimates by the Heanes method (used as a reference; bias of 4.5 mg OC g^{-1}) across the broad spectrum of samples analyzed, whereas all other methods tended to result in lower OC estimates (bias ranged from -2.2 to -3.0 mg OC g^{-1}) compared to the Heanes method. The method that resulted in highest differences in OC estimates compared to the Heanes method was the LOI and EA method (RMSE = 7.1 mg OC g^{-1}); whereas RMSE ranged from 2.7 to 5.4 mg OC g^{-1} in the other methods tested. The differences between the maximum and minimum OC values obtained across all methods tended to increase with increasing IC content ($R^2 = 0.51$, $p < 0.0001$) and OC content ($R^2 = 0.37$, $p < 0.0001$; Fig. 4). Supplementary Fig. S1 showed that the OC content was independent of IC content across the range of seagrass soil samples studied ($p > 0.05$).
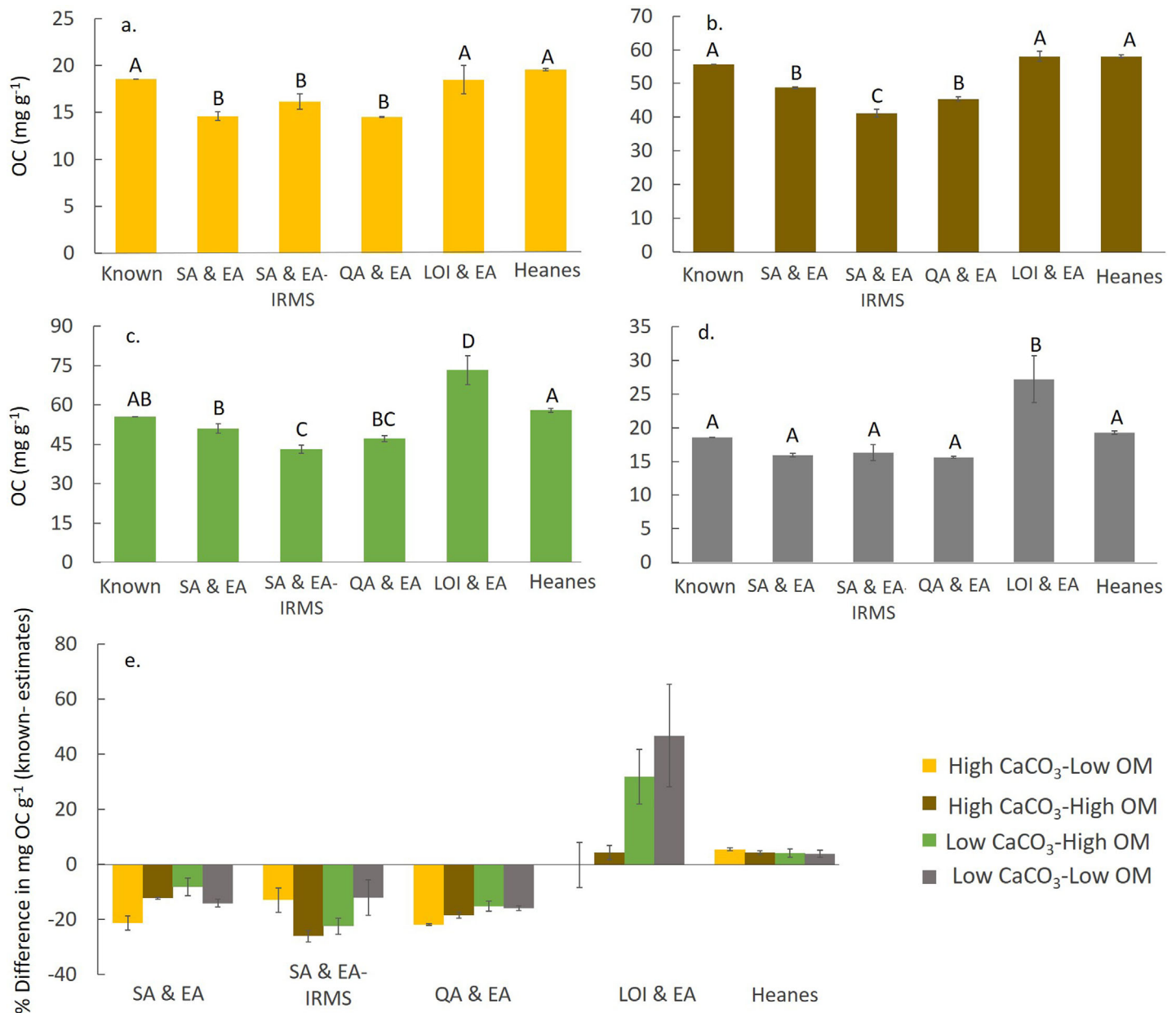
Fig. 1. Estimates of organic carbon content (mg OC g^{-1}) obtained with the five methods tested in four artificial soil samples with known concentrations of CaCO$_3$ and OM. (**a**) High CaCO$_3$–low OM; (**b**) high CaCO$_3$–high OM; (**c**) low CaCO$_3$–high OM; and (**d**) low CaCO$_3$–low OM. (**e**) Difference in % between the known and estimated OC contents (mg OC g^{-1}) across methods in artificial soil samples. Different letters indicate significant differences ($p < 0.05$).

Discussion

This study demonstrates that the common methods used to estimate OC content in Blue Carbon science can result in either over- or under-estimates of real OC content. The best tested method for obtaining accurate OC estimates in artificial soil mixtures across a wide range of OC and CaCO$_3$ contents was the Heanes method, with the important caveat that it was not applicable to all samples. On the other hand, the accuracy of the other methods tested varied according to OM and

CaCO$_3$ contents within the soil matrix (Figs. 1 and 4), pointing to the need to determine approximate OC and IC contents ad-hoc to aid the decision of which OC analytical method to use. Overall, those methods based on acid-digestion resulted in underestimates of OC content in artificial soil samples ($-17 \pm 0.2\%$; ranging from -8 to -26), whereas that based on combustion (LOI and EA) overestimated OC content by $21 \pm 1.5\%$ (ranging from 0 to 47%). This pattern was also consistent in the seagrass soil samples analyzed, with

Table 3. Results of the GLM to assess differences in organic carbon contents estimated through five different methods and the known values for each of the four types of artificial soil samples made of different OM and $CaCO_3$ contents.

Artificial soil mixtures		SS	Df	MS	F	p
High CaCO₃–low OM	Intercept	455.96	1	455.97	11,294.50	0.0000
	Treatment	1.57	5	0.31	7.80	0.0002
	Error	0.88	22	0.04		
High CaCO₃–high OM	Intercept	1375.89	1	1375.89	82,370.30	0.0000
	Treatment	6.04	5	1.21	72.39	0.0000
	Error	0.36	22	0.02		
Low CaCO₃–high OM	Intercept	1301.94	1	1301.95	9618.28	0.0000
	Treatment	11.06	5	2.21	16.35	0.0000
	Error	2.84	21	0.13		
Low CaCO₃–low OM	Intercept	501.03	1	501.03	4596.11	0.0000
	Treatment	5.091	5	1.02	9.34	0.0001
	Error	2.40	22	0.11		

SS, sum-of-squares; DF, degrees of freedom; MS, mean squares; *F*, *F* ratio; *p*: *p* values.
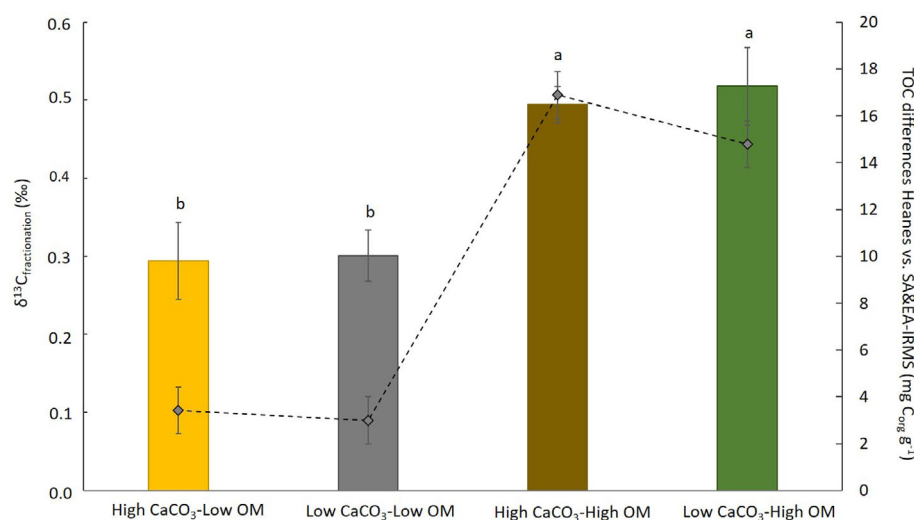
Fig. 2. Estimates of $\delta^{13}C$ fractionation (‰; left y-axis) and loss of OC content (mg OC g^{-1}; right y-axis) following the SA and EA-IRMS method in artificial soil mixtures. Different letters indicate significant differences ($p < 0.05$).

a negative bias of OC content with acid-digestion based methods ranging from -2.2 to -3.0 mg OC g^{-1}, and a positive bias of 4.5 mg OC g^{-1} for the combustion-based method relative to the Heanes method, which reinforces the hypothesis of uncertainties in current global seagrass Blue Carbon estimates. The decrease in accuracy of OC estimates across methods with increasing OC and IC contents in the soils examined showcases the importance of OM and $CaCO_3$ contents in the reliability of OC estimates (Fig. 4), adding further complexity to assessing OC in seagrass, mangrove and tidal marsh soil samples that require assessing IC content to determine OC content.

The underestimation of OC content with acidification pretreatment to remove IC has been linked to multiple chemical reactions, including the loss of easily hydrolysed organic compounds due to the heat generated during the reaction of $CaCO_3$ with acid, the loss of acid-soluble organic compounds and particulate OM when rinsing excess acid from samples, the loss of volatile acids, or inaccuracies in weigh due to the formation of hygroscopic salts ($CaCl_2$ and/or $MgCl_2$) if excess acid is not removed (Pocklington and Hagell 1975; Byers et al. 1978). Hydrolysis of organic compounds is clearly an issue with acidification-based methods where the supernatant is discarded (and washing is required to avoid the precipitation of the hygroscopic salt). In addition, there is also a strong risk of losing some particulate OM during removal of the supernatant by decanting or pipetting. The coloration of the supernatant is a clear indication that organic material is
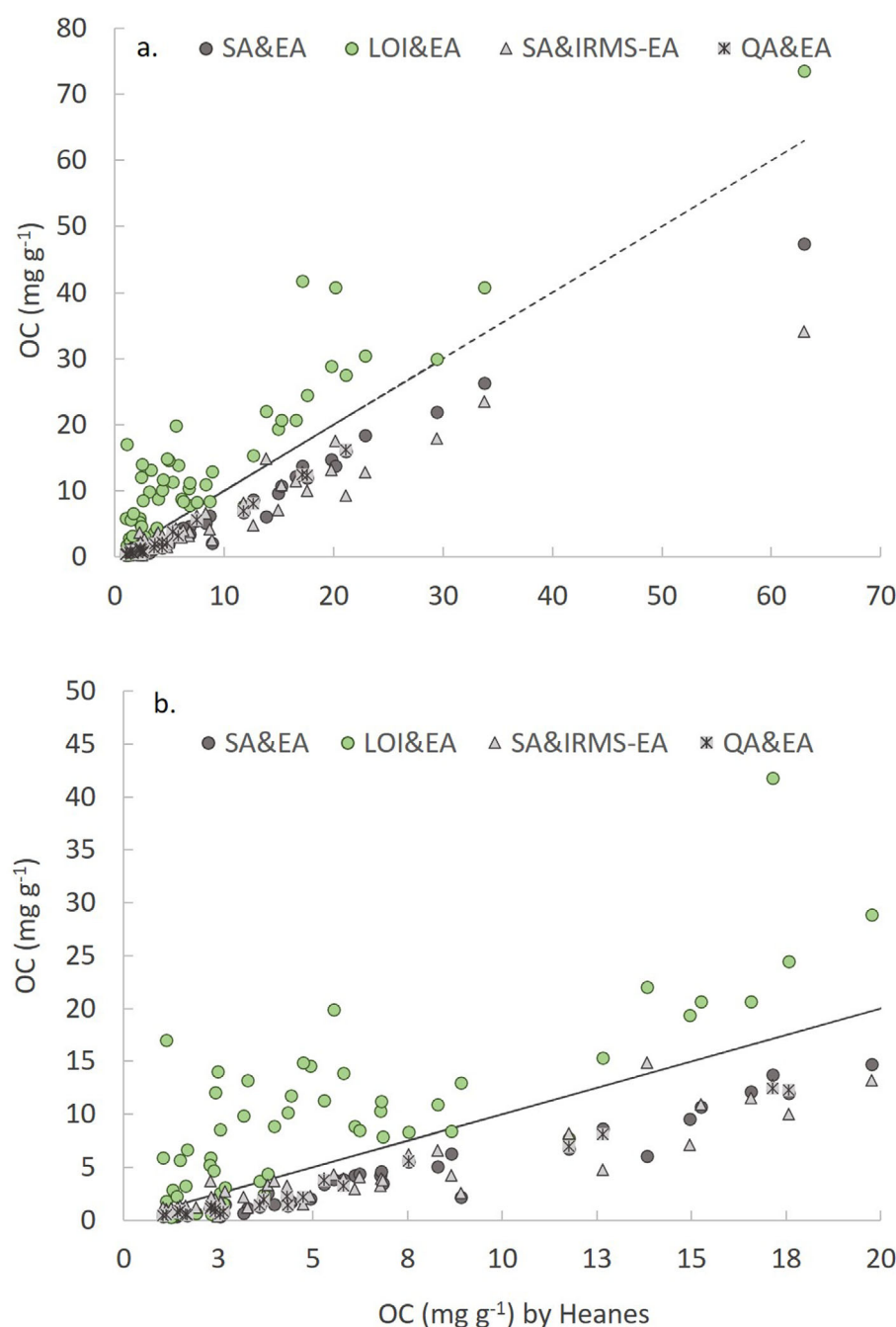
Fig. 3. (**a**) OC content (mg OC g^{-1}) estimated by the different methods plotted against the OC content estimated by the Heanes method (used as a reference). (**b**) Close up of samples with < 10 mg OC g^{-1}. The dotted line in panels (a) and (b) represents the 1 : 1 line.
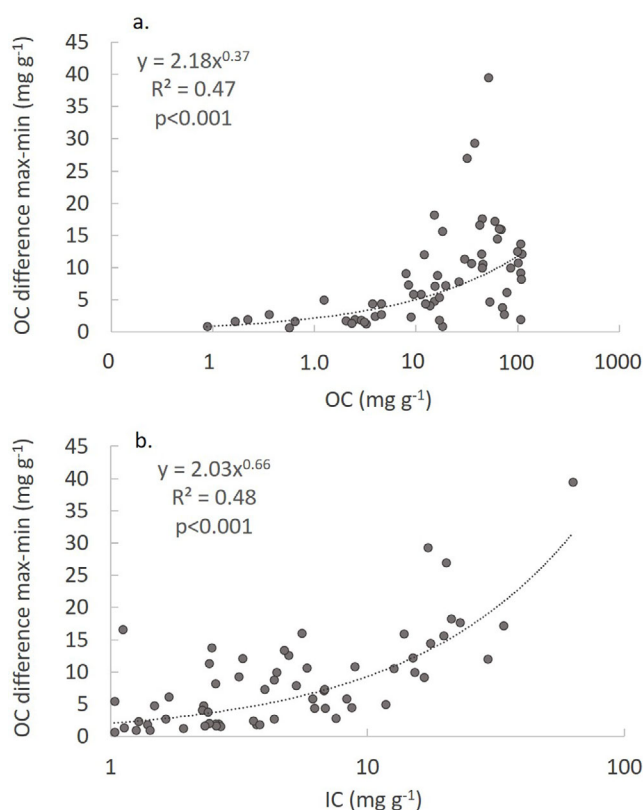
being washed from the sample during this step, which could be in part due to the extraction of metals ions (e.g., Fe^{2+}, Fe^{3+}, Ni^{2+}, Cu^{2+}, Mn^{2+}, and Cr^{3+}). Also, when carbonates are dominant, excessive reaction is possible leading to bubbling over and additional loss of material. Indeed, 5% to 10% of the samples run for methods SA and EA and SA and EA-IRMS had to be repeated using larger vessels because of bubbling over.

Another limitation of the acid-digestion methods is linked to the risk of incomplete digestion of IC due to the presence of HCl-resistant carbonate minerals (e.g., siderite; Moss et al., 1961) or when relying on the lack of effervescence as an indicator for the absence of IC (Lukasewycz and Burkhard 2005). Although the use of IRMS to estimate both OC and $\delta^{13}C$ rather than OC estimates with EA alone can reduce the precision of OC estimates, it can contribute to determine

TABLE 4. Mean $\pm$ SD estimates of organic carbon (mg OC g^{-1}) content measured by each method in Australian seagrass soils.

	SA and EA	SA and EA-IRMS	QA and EA	LOI and EA	Heanes ($n = 62$)	Heanes ($n = 25$)
Mean	5.23	5.11	3.48	12.55	8.11	5.70
SD	7.93	6.24	4.38	13.02	10.2	5.72
n	62	62	25	62	62	25
SE	1.86	2.04	1.66	3.48	2.54	2.38
RMSE	3.83	5.43	2.71	7.07		
Bias	-2.88	-3.00	-2.22	4.45		
R^2	0.99	0.93	0.98	0.84		
Slope	0.77	0.59	0.76	1.17		
Intercept	-1.04	0.33	0.85	3.06		

Summary statistics RMSE and Bias (mg OC g^{-1}) of comparisons between each method and the Heanes method. The comparisons for the SA and EA, SA and EA-IRMS, and LOI and EA methods were run based on 62 samples, whereas comparisons for the QA and EA method were run for 25 samples. The R^2, slope and intercept of the linear regressions fitted are also indicated.
N = number of samples analyzed per method.

Fig. 4. The difference between the maximum and minimum OC values for each sample across all five methods plotted as a function of (**a**) IC content; and (**b**) OC content.

indicates the loss of more labile organic compounds (e.g., proteins) that are enriched in ^{13}C and the selective preservation of less soluble compounds such as lignin that are depleted in ^{13}C relative to bulk plant OM (Kaal et al. 2022). This effect of acidification on δ^{13}C values of bulk sedimentary OC has implications for using δ^{13}C as a proxy for deciphering the provenance of sedimentary OC in Blue Carbon ecosystems (Kennedy et al. 2010; Geraldi et al. 2019) and requires further attention. The consistent underestimation of OC across acidification-based methods and sample types with either low and/or high OM and CaCO$_3$ contents ($-17\% \pm 0.2\%$) points to a systematic error in past estimates, but also offers possibilities to correct for this bias. However, the source of OM used in our artificial soil mixtures (i.e., living seagrass matter) is likely more prone to be lost during rinsing the excess of acid compared to real soils where a portion of the OM is adsorbed to mineral particles and thereby, corrections of current Blue Carbon estimates were not conducted. The accuracy of different methods for estimating OC content in Blue Carbon soils can be influenced by the origin, composition, and degree of decomposition of OC accumulated n Blue Carbon soils. For example, the soil OC accumulated in Blue Carbon ecosystems originates from multiple sources (e.g., marine macrophytes, seston, and terrestrial run-off) and experiences complex diagenetic processes upon burial and aging (Kaal et al., 2016; Liu et al. 2017). The nature of soil OC matrices found across Blue Carbon ecosystems highly varies across habitats and along the sedimentary soil sequences within the same habitat and therefore, it was not possible to constraint all these parameters in the artificial soils created. The use of whole seagrass plants consisting of leaves, leaves, sheaths, rhizomes, and roots in this study, which contain labile and recalcitrant components, aimed at embracing some of the potential variability linked to the nature of soil OC. However, further studies exploring the accuracy of methodologies for estimating OC in soil matrices

the incomplete digestion of IC. The presence of IC following acidification would result in δ^{13}C values higher than $-9‰$, owing to the δ^{13}C values of CaCO$_3$ close to zero and the δ^{13}C of seagrass and other potential OM sources $> -9‰$ (Kennedy et al. 2005, 2010). Acidification also resulted in δ^{13}C fractionation, in particular in OM-rich samples, which

with multiple OM sources and diagenesis stages are required to constrain the current bias in Blue Carbon soils.

The overestimated OC contents obtained through the method that required pre-combustion (i.e., LOI and EA) was likely due to the loss of water from clay minerals and the loss of some carbonate species (e.g., magnesium carbonate, manganese carbonate, and iron carbonate) at 400–550°C combustion temperatures (Byers et al. 1978; Froelich 1980; Sutherland 1998; Leong and Tanner 1999). Although the LOI method is an easy, rapid and inexpensive method, previous studies showed either >90% predictive power (Craft et al. 1991; Fourqurean et al. 2012) or up to 30% uncertainty in OC estimation from LOI data (Santisteban et al. 2004; Mazarrasa et al. 2017; Ouyan and Lee 2020; Gorham et al. 2021), which could be in part related to the different methodologies used. The loss of carbon from $CaCO_3$ in soils was stated to be negligible below 450°C (Davies 1974), but loss from, for example, siderite and magnesium carbonates begins at 400°C (Froelich, 1980; Gallagher and Warne, 1981). Therefore, the presence of carbonates containing Mg, Fe, or Mn in Blue Carbon soils (Koretsky et al. 2008) can entail the overlap of the thermal degradation temperatures of IC and OC and thereby, fail to separate IC from TC by dry combustion (Gibbs 1977; Mook and Hoskin 1982). Although previous studies showed that OM continues to be oxidized at temperatures >550°C (Howard et al. 2021), the loss of mass with no loss of OC during combustion at 500°C in our study could explain the overestimated OC values obtained. The large variability in the overestimation of OC content across seagrass soil samples with low-$CaCO_3$ content using the LOI and EA method (i.e., from 30% to 45% deviation from the known values) precludes using this method. However, the LOI and EA method was highly reliable for $CaCO_3$-rich samples (75%) based on the negligible bias compared to the Heanes method.

The Heanes method showed the highest similarity to the known OC contents in all artificial soil sample types and therefore, it could be the most reliable method for assessing OC content in Blue Carbon samples containing $CaCO_3$ (Fig. 1). The Heanes method overcomes the limitation of underestimating OC with classical dichromate digestion (Walkly-Black)—primarily underestimating highly resistant and charred material—by heating to 135°C (Leong and Tanner 1999). However, this method is time consuming and requires the use of significant amounts of reagents that can be harmful to humans and the environment (Kimble et al. 2001; da Silva Dias et al. 2013). In addition, the Heanes method is not suitable for seagrass soil samples containing reduced iron, sulfur and potentially manganese, which are common in marine settings and likely more abundant in Blue Carbon habitats receiving terrestrial minerals and clay (Lewis et al. 2011). Dichromate oxidizes these species not only biasing resultant OC values but often leading to a violent reaction in the test tube presenting a potential hazard. In the experience in this study, it was relatively easy to determine when material other

than OM was being oxidized because of resultant color development and speed of reaction, which occurred in 18% of the samples analyzed. However, the Heanes method presents major limitations for broad uptake in Blue Carbon science also owing to the subjectiveness of this assessment of the degree of skill required.

Overall, the difference in OC estimates between methods and the known values for the different artificial soil types was significant for acid-digestion methods ($-17 \pm 0.16\%$) in the soil artificial samples with low $CaCO_3$ contents analyzed with the LOI and EA method (32% and 47%), but negligible for the Heanes method (4–5%) and for the LOI and EA method in soil artificial samples with high $CaCO_3$ content (Fig. 1). A plausible explanation to such high error could be linked to the larger effect of weight loss from water absorbed to clays and carbonate species during combustion in samples with lower $CaCO_3$ content (Leong and Tanner 1999), and from the lack of precision when subtracting one large number (IC) from another (TC) to obtain a small, uncertain residual (OC).

These findings have significant implications for Blue Carbon accounting. For example, assuming that ~75% of the seagrass soil OC stock data used to produce global estimates (1732 Tg OC in the top meter of soil; Macreadie et al. 2021) was obtained following acidification pre-treatment methods and the remaining 25% using combustion methods (LOI), then global seagrass OC stock in the top-meter soil would be 8.4% higher than previously estimated. Thus, the predominance of acidification pre-treatment methods will result in an underestimate of OC content. In addition, a Blue Carbon project based on an existing baseline value calculated using an acidification method but using the LOI-EA method for validation and verification of carbon credits will show a large apparent increase in OC due to the method mismatch. This methodological-linked bias will also apply to other Blue Carbon ecosystems thriving in substrates where $CaCO_3$ are present, including mangroves and tidal marshes.

Recommendations

A guideline for determining the best analytical method for OC content estimates, based on the OC and $CaCO_3$ contents in soils is provided in Fig. 5, whereas Table 5 summarizes the strengths and limitations of each method tested together with recommendations for their use. The results from this study highlight the importance of determining approximate OC and $CaCO_3$ contents of target samples to aid the decision of which method to use. The range in OC and $CaCO_3$ contents across a set of samples can be rapidly and economically estimated with LOI (Heiri et al. 2001) or soil color (Rossel et al. 2006), or using a calcimeter for $CaCO_3$ content (Sherrod et al. 2002). Then after, the Heanes or LOI and EA methods should be used for estimating OC in samples with high $CaCO_3$ content (~ 75%), whereas all other soil samples could be analyzed with the Heanes method. However, despite
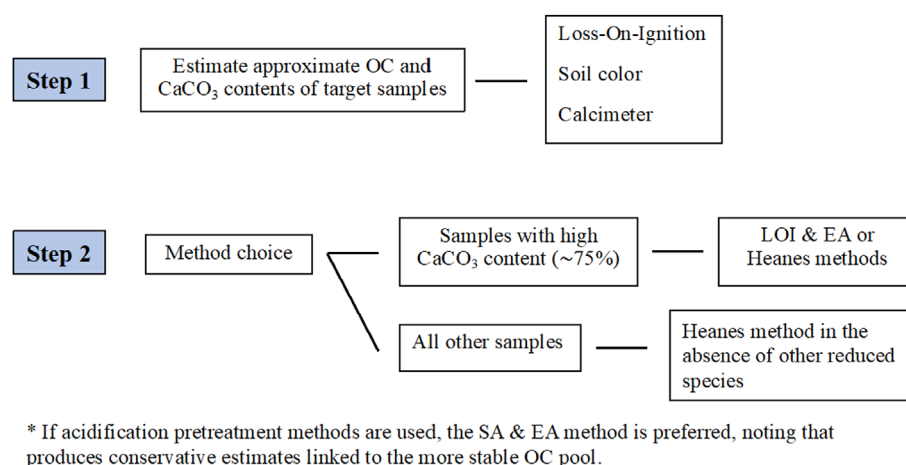
Fig. 5. Diagram showing the summary of the findings obtained in this study, including decision steps toward the best method to use for OC analyses based on sample characteristics.

Table 5. Strengths and limitations of the methods tested for organic carbon (OC) analyses, and recommendations for their use under different scenarios.

Method	Strengths	Limitations	Recommendations
SA and EA; QA and EA; SA and EA-IRMS	High precision in $CaCO_3$-poor soils. Rapid and relatively cheap.	Underestimates OC content.	Avoid acid-digestion methods if the supernatant has color. Avoid when acid-resistant carbonate minerals are present and use SA and EA-IRMS to assess incomplete digestion.
LOI and EA	High precision in $CaCO_3$-rich soils.	Overestimates OC content.	Avoid when carbonates containing Mg, Fe, or Mn are present.
Heanes	High precision.	Not applicable to all samples. Time consuming.	Do not use when reduced Fe, S, and Mn are present.

the Heanes method producing the most accurate results, chemical oxidation is not recommended as a routine method in marine sediments or soils due to the possibility of co-oxidation of other reduced species. Alternatively, if acidification pre-treatment methods are used, the SA and EA method is preferred, but acknowledging that produce conservative estimates linked to the more stable OC pool. In addition, the use of vessels >50 mL for acid pretreatment is recommended to avoid excessive bubbling and subsequent loss of sample, together with accounting for the OC content in the supernatant removed to reduce underestimation. We recommend against LOI practices in samples with low $CaCO_3$ content due to the potentially large uncertainties in OC estimates. The guidelines provided are applicable to seagrass Blue Carbon research but may not be suitable for tidal marsh and mangrove ecosystems. Indeed, the bias detected in OC

content estimates in artificial soil samples across methods was tested only in soils with 19 and 56 mg OC g^{-1}, and 24 and 90 mg IC g^{-1} and therefore, the trends observed may not be representative of samples with higher or lower OC and IC contents. Further studies of artificial samples with known organic and inorganic compositions are required to assess the accuracy of the methods tested. Thus, testing the same methodologies plus others (e.g., color, variations of the Walkley-black method, Rock-Eval method, stepwise oxidation techniques, using silver capsules for acid pretreatment to avoid rinsing the excess of acid, or the difference between TC obtained by dry combustion and $CaCO_3$ by calcimeter) across soil samples from multiple Blue Carbon ecosystems with different mineral and OM compositions (i.e., quality and quantity) are required to further enhance accuracy in OC analyses in soils with carbonates.

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Conflict of Interest

None declared.