

Blue carbon dynamics in restored UK saltmarshes

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Abstract

Saltmarsh restoration by breaching the seawall surrounding an area used for agriculture is posited as a natural climate solution because these blue carbon ecosystems can accumulate organic carbon (OC) at rates exceeding those of other ecosystems. This thesis examined blue carbon dynamics in restored UK saltmarshes across a wide scale, from the stocks of OC in several sites to the chemical composition of the organic matter (OM) that comprises that stock and the microorganisms that determine its fate. In Chapter Two, it was concluded that sites between eight and 102-years old had OC stocks of a similar magnitude to their natural counterparts, but a larger share of the OM originated from an external, marine setting. Chapter Three involved a methodological assessment of the fluorometric microplate enzyme assay method, a technique that determines extracellular enzyme activity in soils. Conclusions from this chapter were applied to Chapter Four to assess the microbial community composition and functioning of neighbouring natural and restored marshes. Measuring redox conditions showed that the restored marsh was more oxygen limited than the natural. This resulted in a microbial community dominated by sulphate reducing taxa, especially in the deep sediment layer representative of a relic agricultural field. Finally, in Chapter Five, the teabag index and microbial DNA sequencing were employed to determine the rates and drivers of OM decomposition in the same neighbouring marshes. Although the restored marsh was dominated by anaerobic microbial taxa, OM decomposition and stabilisation rates did not differ between the sites. Together, these results suggest that less of the OM in restored sites is autochthonous and, even under strongly reducing conditions, microbial communities remain capable of driving OM remineralisation.

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Incorporated Works

Two of the Chapters comprising this thesis have been submitted to academic journals and are available as preprints:

- Chapter Two – Based upon Gore, C., Mueller, P., Chapman, M., dal Molin, F., Schmiedl, G., Milker, Y., Benson, L., Marca, A. and. and Nolte, S. (accepted; pending revisions) “Organic matter composition and origin differ between restored and natural UK saltmarshes up to 100 years after the breach”, *Biogeosciences*. Preprint available at: <https://doi.org/10.5194/egusphere-2026-1559>. The author contributions were as follows: CG: Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Conceptualization. PM: Writing – review & editing, Supervision, Conceptualization. MC: Writing – review & editing, Methodology, Conceptualization, Supervision. FDM: Writing – review & editing, Supervision, Formal analysis. GS: Writing – review & editing. YM: Writing – review & editing. LB: Writing – review & editing, Supervision. SN: Writing – review & editing, Supervision, Methodology, Conceptualization, Funding acquisition.
- Chapter Three – Based upon Gore, C., Nolte, S., Lehtovirta-Morley, L., Mittmann-Goetsch, J. and Mueller, P. (accepted; pending revisions) "Storage temperature and diluent cause inconsistencies in commonly used fluorometric microplate enzyme assays conducted with saltmarsh soils", *Limnology and Oceanography: Methods*. Preprint available at: <https://dx.doi.org/10.2139/ssrn.5678185>. The author contributions were as follows: CG: Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Conceptualization. SN: Conceptualization, Funding Acquisition, Supervision, Writing – Review & Editing, Methodology. LLM: Conceptualization, Supervision, Writing – Review & Editing, Methodology, Resources. JMG: Writing – Review & Editing, Methodology. PM: Conceptualization, Supervision, Writing – Review & Editing, Methodology.

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Chapter One

Introduction

1.1 Introduction

Saltmarshes are vegetated coastal ecosystems, characterised by regular tidal inundation, that mark the transition from terrestrial to marine environment (Levin *et al.*, 2001). In 2020, the global extent of these habitats was found to be 52,880 km², marking them as important parts of coastlines across 120 countries (Worthington *et al.*, 2024; Fig. 1.1). This wide spatial extent means that different areas grouped together as saltmarshes often vary greatly in terms of structure and function, with the result that many individual studies focused upon specific areas but fitted within a global context are needed (Yando *et al.*, 2023).

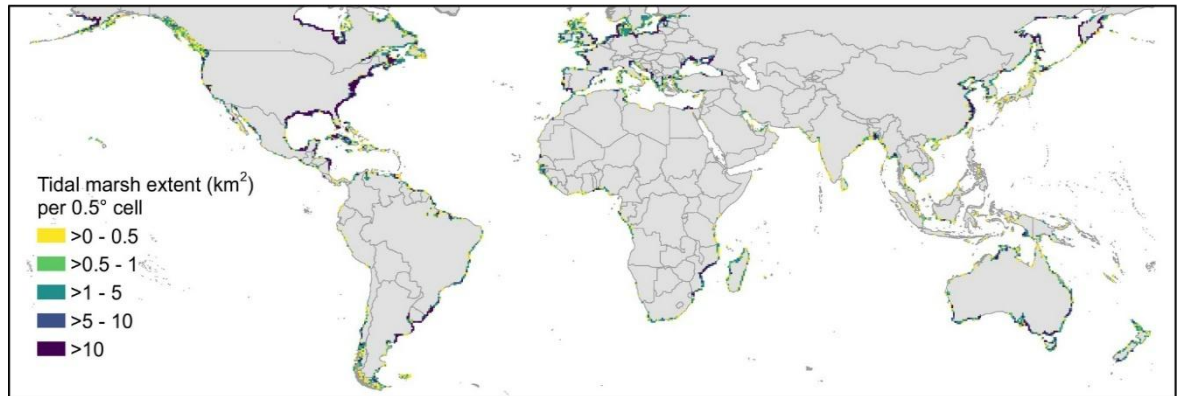


Figure 1.1. Adapted from Worthington *et al.* (2024), a map of global saltmarsh distribution in 2020.

Saltmarsh restoration

Saltmarsh restoration is implemented with the goal of counteracting the large global losses of this ecosystem currently underway (Murray *et al.*, 2022) and myriad benefits can result (Barbier *et al.*, 2011). Highly important to the communities residing in coastal areas is the flood defence provided by the ability of wetlands to decrease the energy of incoming waves (Morgan *et al.*, 2009), providing protection from rising sea levels and reducing the cost of damage caused by extreme weather events (Costanza *et al.*, 2008). Secondly, biodiversity is enhanced because habitat for a range of plant and bird species is provided (Lefeuvre *et al.*, 2003). Additionally, saltmarshes provide a refuge for an array of fish and crab species and thus play a role in promoting the survival of economically important fishery species, as well as in protecting biodiversity (Boesch and Turner, 1984). Due to the position saltmarshes occupy between land and sea, they often intercept nutrients drained from the terrestrial environment and control their release into the sea (Valiela and Teal, 1979). Furthermore, saltmarsh plants play a role in the cycling of important nutrients, such as nitrogen, phosphorus and carbon (Sousa *et al.*, 2010). In doing so, they contribute to the maintenance of ecosystem functionality and health by, for example, preventing coastal eutrophication and improving water quality (Koch and Gobler, 2009; Sousa *et al.*, 2010).

In addition to these roles, saltmarshes, along with mangrove forests and seagrass beds, are now recognised for their potential to sequester carbon and are listed among the so-called blue carbon

ecosystems (Macreadie *et al.*, 2021). These can be defined as coastal habitats capable of sequestering large quantities of organic matter (OM) originating from *in-situ* and external sources, thus aiding in the removal of atmospheric carbon dioxide (CO₂; Mcleod *et al.*, 2011). The regular tidal inundation characterising blue carbon ecosystems creates anaerobic conditions in their sediments, resulting in low levels of microbial activity that lead to slow rates of OM decomposition, allowing organic carbon (OC) to remain buried for millennia (Chmura *et al.*, 2003). The OC is stored in living biomass above- and below ground and in non-living material accumulated in the sediment (Duarte *et al.*, 2013). The two main sources of OC to blue carbon ecosystems can be defined as allochthonous and autochthonous (Saintilan *et al.*, 2013). The former refers to tidally delivered OM from external sources while the latter term describes the OM resulting from the productivity of saltmarsh plants. As a result of these mechanisms, blue carbon ecosystems come under the umbrella of natural climate solutions – natural strategies aimed at removing atmospheric CO₂ and thereby preventing a rise in global temperatures (Girardin *et al.*, 2021). Increasingly, restoration is carried out with the primary goal of creating additional OC sinks, with coastal protection and biodiversity gains seen as co-benefits (Jones *et al.*, 2024).

Organic carbon accumulation rates

In the past, global carbon budgets have often neglected to include wetlands but a review of studies investigating carbon dynamics in saltmarshes and mangrove forests around the world found the average OC accumulation rate of these ecosystems to be $210 \pm 20 \text{ g C m}^{-2} \text{ yr}^{-1}$ (Chmura *et al.*, 2003). Another, later review of the literature, that also included seagrass meadows, found the OC accumulation rate of global blue carbon ecosystems ranged between 18 and $1713 \text{ g C m}^{-2} \text{ yr}^{-1}$ (Mcleod *et al.*, 2011). When saltmarshes specifically were considered, McLeod *et al.* (2011) reported the average carbon accumulation rate to be $218 \pm 24 \text{ g C m}^{-2} \text{ yr}^{-1}$; this estimate was based upon the aforementioned study (Chmura *et al.*, 2003) and a subsequent analysis by Duarte *et al.* (2005). A more recent global review calculated a revised accumulation rate for saltmarshes of $245 \text{ g C m}^{-2} \text{ yr}^{-1}$ (Ouyang and Lee, 2014). In contrast, long term rates of OC accumulation in temperate, tropical and boreal forests were found to range between 0.7 and $13.1 \text{ g C m}^{-2} \text{ yr}^{-1}$ (Mcleod *et al.*, 2011). Clearly, therefore, the potential for blue carbon ecosystems to rapidly accumulate OC is substantially larger than that of terrestrial forests and, as a result, increased attention has been paid to these habitats over recent years, with the number of studies aiming to investigate various aspects of their functioning increasing rapidly (Duarte de Paula Costa and Macreadie, 2022).

The estimated, world-wide area potentially available for saltmarsh restoration is very large, ranging between 2,000 and 32,000 km² (Griscom *et al.*, 2017), meaning there is some uncertainty about the magnitude of the impact this particular natural climate solution could have upon climate change mitigation (Macreadie *et al.*, 2021; Jones *et al.*, 2024). A recent assessment of the blue carbon benefits arising from saltmarsh restoration stated that between 12.93 and 207.03 Mt CO₂ equivalents could be accumulated every year (Mason *et al.*, 2023). However, this estimate has been criticised as

a vast overestimate – instead of a mean accumulation rate of $64.70 \text{ t CO}_2\text{e ha}^{-1} \text{ year}^{-1}$, a value of $-0.8 \text{ t CO}_2\text{e ha}^{-1} \text{ year}^{-1}$ was calculated using the same data (Williamson *et al.*, 2024). This indicates a net climate warming impact from saltmarsh restoration, owing to methane (CH_4) and nitrous oxide (N_2O) emissions counteracting OC accumulation.

Saltmarshes in the UK

In the UK, saltmarshes are the blue carbon ecosystem of primary importance because of their wide distribution around the coastline (Smeaton *et al.*, 2022a). Within the UK specifically, average accumulation rates of $119 \text{ g C m}^{-2} \text{ yr}^{-1}$ (Parker *et al.*, 2020), $224 \text{ g C m}^{-2} \text{ yr}^{-1}$ for natural marshes and $363 \text{ g C m}^{-2} \text{ yr}^{-1}$ for restored marshes (Mason *et al.*, 2022) have been calculated previously, highlighting a great deal of variability between sites. This wide range of rates can be attributed to the numerous factors that can affect OC deposition and burial, which include plant species and soil type (Ford *et al.*, 2019), elevation (McMahon *et al.*, 2023) and the rate of sea-level rise (Rogers *et al.*, 2019).

Currently underway in the UK is the development of a Saltmarsh Code (Burden *et al.*, 2023), lending yet more relevance to these ecosystems. To provide funding for ecosystem restoration, a carbon code is required so that carbon credits can be marketed and purchased by organisations with the intention of offsetting their unavoidable greenhouse gas emissions (Freedman *et al.*, 2009). In addition to carbon trading schemes, the offsetting potential of saltmarsh carbon storage could be used as part of national climate policy to meet compliance obligations (Vanderklift *et al.*, 2019; Kelleway *et al.*, 2020). The restoration method usually employed in the UK context is that of coastal realignment – also called managed realignment and de-embankment – by breaching sea walls and allowing the tide to inundate areas that have previously been claimed, usually for agricultural purposes (Pethick, 2002; Fig. 1.2).

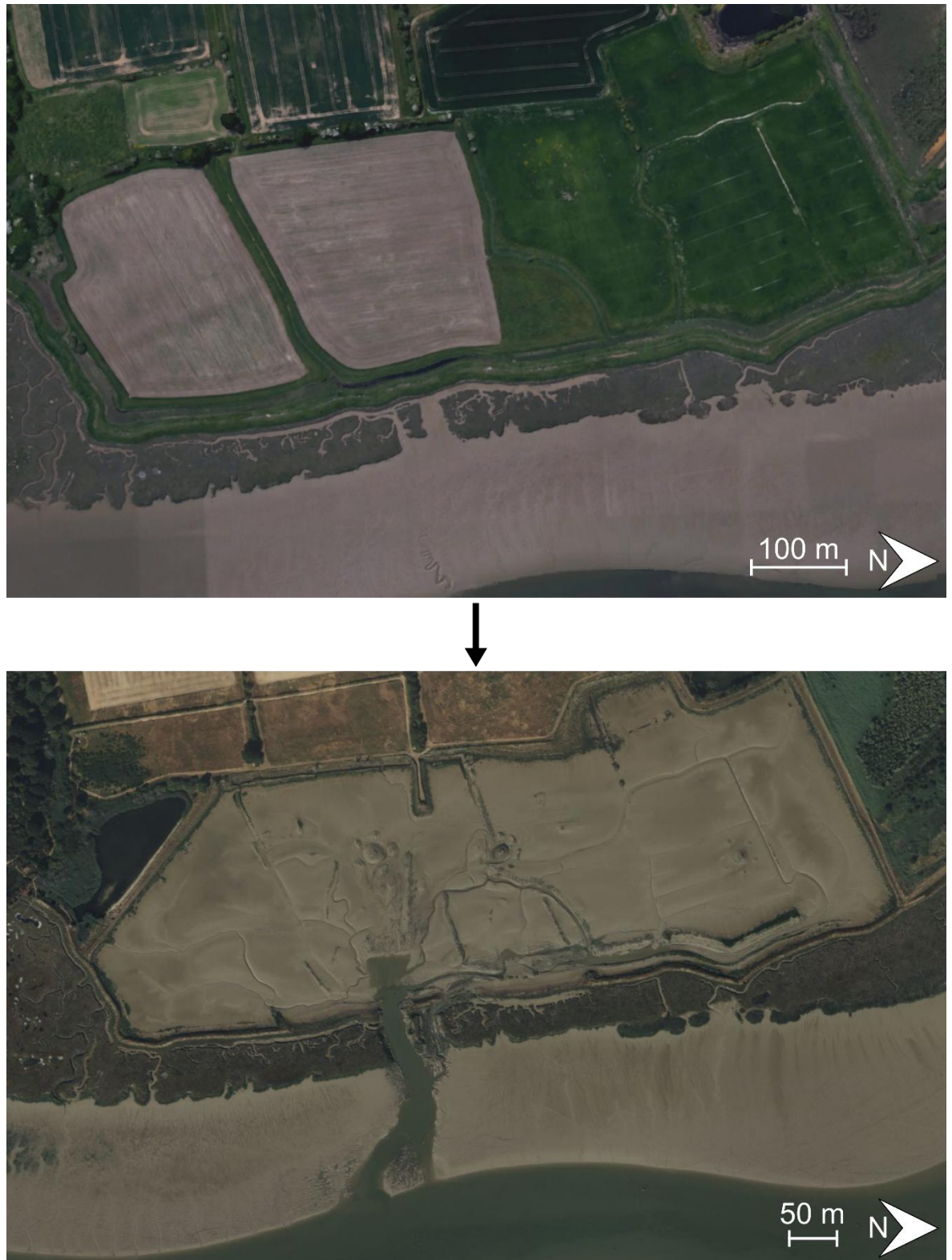


Figure 1.2. The same area before and after a breach was created in the seawall during 2015 to restore a saltmarsh in Fingringhoe Wick, Essex, UK.

Previous studies of restored saltmarshes

Recently, studies have investigated the rapid OC accumulation that occurs immediately after the breaching of the sea wall and the restoration of tidal inundation (Wollenberg *et al.*, 2018; Mossman

et al., 2022). The majority of OC deposited in the early stages of restoration was assumed to be allochthonous because plant colonisation can take several years (Garbutt and Wolters, 2008). In semi-natural marshes in the Wadden Sea, the large contribution of allochthonous OM to stored OC has been shown to continue for several decades after marsh establishment with brushwood groynes (Mueller *et al.*, 2019a). In both natural and restored marshes, debate surrounds the issue of whether the allochthonous portion of OC buried in a marsh should be included in the carbon credits derived from a quantification of OC stock (Houston *et al.*, 2024; Williamson *et al.*, 2025). It can be argued that allochthonous OC would not satisfy the additionality requirement of a carbon crediting schemes because, in the absence of a restoration project in a particular site, the external OM could have simply been buried elsewhere and/or this OM has often already undergone a degree of decomposition, meaning it is relatively stable (Needelman *et al.*, 2018).

Other studies of restored/created saltmarsh carbon dynamics are many but, given the variation known to exist between sites (Yando *et al.*, 2023), their conclusions often have only a limited applicability to the UK context. One study in the USA compared OC stocks and accumulation rates in marshes created by pumping dredged sediment into open water areas with measurements taken in nearby natural marshes (Kelsall *et al.*, 2023). They demonstrated that OC stocks in the created marshes were lower than in the natural equivalents. Furthermore, there was a higher proportion of labile carbon – the fraction of soil carbon most vulnerable to microbial decomposition and emission back into the atmosphere (Gulde *et al.*, 2008) – in the younger marshes, especially those only a few years old and not yet fully colonised by plants (Kelsall *et al.*, 2023). Similarly, McClellan *et al.* (2021) compared the OC and nitrogen (N) stores of marshes created in Louisiana using two different methods, the dredged sediment technique already discussed as well as the diversion of river sediment into deteriorating wetlands. This study concluded that the latter marsh creation technique was preferable as it led to more rapid OC accumulation and plant communities that better replicate those of natural marshes (McClellan *et al.*, 2021). They hypothesise that this is due to the greater mineral and organic inputs provided by the river diversion technique enhancing marsh productivity. Clearly, therefore, there exists a variety of studies that have aimed at comparing natural and restored/created marshes. It has been stated that one of the missing elements is a comparison of marshes restored through the breaching of sea wall defences (as opposed to the deliberate movement of dredged or river sediment) and their natural equivalents within the UK (Mason *et al.*, 2022), given that the latter two studies discussed were conducted in the USA. Consideration of whether these newly re-established sites function in the same way as their natural counterparts, and, therefore, whether they possess similar OC accumulation capabilities and provide the same co-benefits is essential. Furthermore, much of the work in restored UK marshes so-far has focused on certain flagship sites; for example, marshes of the Blackwater estuary in Essex and Steart marshes in Somerset (Burden *et al.*, 2019; Mossman *et al.*, 2022). Therefore, there remained a paucity of data relating to the carbon dynamics of a wide range of restored UK saltmarshes.

Recently, it has been shown that restored UK sites differ from natural marshes in several ways, which might in turn affect the carbon dynamics. First, the plant community composition of restored sites was found to differ significantly from natural reference sites, with early-successional species maintaining their dominance over time throughout the various marsh zones of the former (Fig. 1.3; Mossman *et al.*, 2012a & 2012b; Lawrence *et al.*, 2022). Different plant species vary in terms of structure and function, and this could affect OC accumulation because plants are the source of autochthonous OC but also control, to a degree, the trapping of allochthonous matter (Temmerman *et al.*, 2005). Furthermore, the existence of an underlying and impermeable layer of sediment, originating from before tidal inundation was restored and therefore usually resembling agricultural sediment, has important implications for the development of restored saltmarshes (Lawrence *et al.*, 2018). The presence of this impermeable layer has been shown to result in reduced creek development, water storage capacity, microporosity, pore connectivity and poor drainage (Fig. 1.3; Spencer *et al.*, 2017; Lawrence *et al.*, 2018). These differences could impact carbon dynamics by altering the abiotic conditions; namely, the sediment redox potential (Mossman *et al.*, 2012b), the determinant of oxygen availability. More waterlogged conditions are expected to reduce the quantity of OC broken down and released back into the atmosphere by creating more reducing conditions in which oxygen is scarce and decomposition rates low (Fig. 1.3). If saltmarsh restoration is to be used to tackle climate change, it is vital to investigate these differences further and develop a full understanding of the implications for OC accumulation and storage.

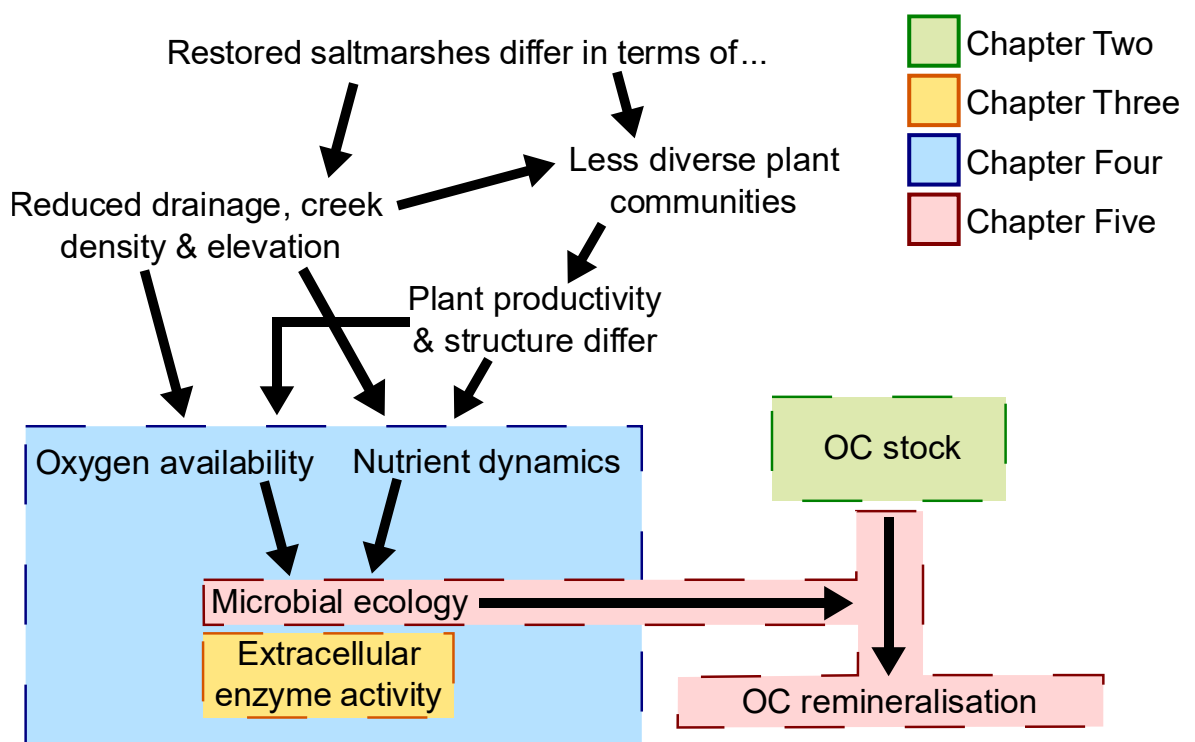


Figure 1.3. The underlying processes affecting saltmarsh carbon dynamics and how the different elements are grouped within this thesis.

After deposition in the sediment, OC sequestration by saltmarshes is dependent upon the microbial activity that degrades OM and results in the release of captured CO₂ back into the atmosphere (Wolters, 2000; Luetzow *et al.*, 2006; Macreadie *et al.*, 2025). Therefore, examining the microbial communities driving this process is another important undertaking (Pétillon *et al.*, 2023). With this goal in mind, a variety of studies to date have investigated the microbial ecology of these ecosystems (e.g., Sherr and Payne, 1978; Bowen *et al.*, 2012; Leadbeater *et al.*, 2021; Tebbe *et al.*, 2025). An investigation of temporal and spatial variability in saltmarsh microbial ecology found that, like the zonation of plant communities within marshes, microbial communities were strongly influenced by tidal inundation and the associated abiotic factors (e.g., sediment water content, salinity, oxygen availability; Rinke *et al.*, 2022). Several studies have also employed the teabag index (TBI) – a method that involves incubating the standardised OM found green and rooibos teabags to investigate decomposition (Keuskamp *et al.*, 2013). Relevant here are studies that assessed OM decomposition in saltmarshes under various experimental conditions (e.g., Mueller *et al.*, 2018; Tang *et al.*, 2020; Tang *et al.*, 2023). Connecting these two fields of study are investigations of the microbial communities colonising teabag litter introduced to saltmarshes (Trevathan-Tackett *et al.*, 2024; Neiske *et al.*, 2025). However, functional links and an assessment of restored saltmarshes remained knowledge gaps.

Microbial carbon cycling is a process of interest within the context of this study. Some microbes are capable of autotrophically fixing CO₂ which means assimilating molecules of this gas into cellular carbon (Berg, 2011). Conversely, they also drive the release of this gas back into the atmosphere through OM decomposition via direct oxidation to CO₂ or reduction to CH₄ (i.e., methanogenesis; Arndt and LaRowe, 2017). The former is an aerobic process, rates of which are expected to be low in saltmarshes because of the anoxic conditions produced by frequent tidal inundation (Wang *et al.*, 2019). During respiration, oxygen is the most energetically favourable electron acceptor but under anaerobic conditions microorganisms use less favourable alternatives such as sulphur or various N compounds (Sikora *et al.*, 2017). Saltmarshes and peatlands possess similarly anoxic conditions but CH₄ emissions from the former are often lower because tidal inundation results in the delivery of high levels of sulphate, allowing sulphate reducing bacteria to outcompete methanogens (Martens and Berner, 1974; Oremland *et al.*, 1982; Poffenbarger *et al.*, 2011). Despite this, CH₄ emissions from saltmarshes are possible and were shown to be enhanced by the introduction of plant species such as *Phragmites australis* and *Spartina anglica* (Kim *et al.*, 2020).

Another topic of interest involving saltmarsh microorganisms is N cycling. Denitrification occurs under anaerobic conditions and involves the reduction of nitrate (NO₃⁻) – which performs the function of terminal electron acceptor in the absence of oxygen – to a series of different compounds, the final one being nitrogen gas (Knowles, 1982). The intermediary compounds produced include

the greenhouse gas N_2O , which can be released to the atmosphere (Hall and Matson, 1999). This fact may be especially important in coastal ecosystems neighbouring agricultural fields, where anthropogenic NO_3^- inputs have been shown to change certain saltmarshes from N_2O sinks to sources (Moseman-Valtierra *et al.*, 2011). An investigation of N cycling microbial communities from a chronosequence of naturally developing marshes in the Netherlands included sites ranging from 0 (i.e., newly formed) to 100-years-old (Salles *et al.*, 2017). They found different microbial groups performing the same functions at each stage of soil development, suggesting an ability to respond and adapt to changing environmental conditions (Salles *et al.*, 2017). The variability present in saltmarsh ecosystems creates a range of niches and makes unpicking the different processes taking place a challenge. Gandy and Yoch (1988), for example, showed that, in the top 5 cm of sediment in their saltmarsh study site, sulphate reducing bacteria were responsible for around 70 % of N fixation. With increasing depth, this role was subsumed by fermenting bacteria.

This study

Integrating the different elements discussed in this section, from a large-scale assessment of OM stocks and sources to an examination of the specific chemical processes performed by the sediment bacteria that determine the fate of this OM, was the aim of this study. Doing so addressed several knowledge gaps within the field of blue carbon ecosystems. Carbon dynamics in marshes restored through creating a breach in the seawall (i.e. managed realignment) surrounding agricultural land had not been thoroughly investigated and an understanding of the origin of the OM within these sites was lacking. Although microbes were known to be the drivers of OM mineralisation, the specific taxa and processes involved within the sediment of these ecosystems needed further elucidation. Finally, a comparison of the abiotic conditions that select for different microbial taxa in natural and restored saltmarshes was required. Indeed, the overarching question in this thesis was whether the differences known to exist between the restored saltmarshes of the UK and their natural counterparts had consequences for their capacity to store OC and effectively mitigate climate change (Fig. 1.3).

1.2 Thesis outline

1.2.1 Organic matter composition and origin in restored and natural saltmarshes

In **Chapter Two** of this study the aim was to understand the carbon dynamics of four restored UK saltmarshes of various ages that had not previously been assessed in this manner by collecting and analysing sediment cores (Fig. 1.4). Furthermore, comparisons were made with natural counterparts in the same areas. Until recently, OC data for natural UK saltmarshes had been limited to very few sites, such as Dengie and Stiffkey marshes in Essex and Norfolk, respectively (Callaway *et al.*, 1996), and Hut marsh, also in Norfolk (French and Spencer, 1993). Data from restored saltmarshes also existed but this was primarily from large flagship sites and focused overwhelmingly on measuring carbon accumulation rates, as opposed to carbon stocks and sources (Burden *et al.*, 2019). This paucity of information was alleviated by recent studies that conducted thorough investigations of the

carbon dynamics of many natural saltmarshes around the British coast (Smeaton *et al.*, 2023 & 2024). However, there remained a need to gather equivalent data from restored saltmarshes to allow comparisons to be made. **Chapter Two** investigated the differences in the composition and origin of the OM in the sediment of restored saltmarshes, for which new data were generated, compared with that of the natural marshes examined by Smeaton *et al.* (2023 & 2024). Bulk elemental analysis was used to determine the OC and N content of the sediment, and this information was combined with $\delta^{13}\text{C}$ and thermogravimetric analyses to draw conclusions about the origin of the OM present in the sediment, whether it be predominantly allochthonous or autochthonous. Furthermore, to correctly interpret results from different depths in the cores, a novel approach using foraminifera – unicellular marine protists (Sen Gupta, 2003) – was developed to identify the breach horizon depth (i.e., the depth at which the transition from relic agricultural soil to saltmarsh sediment occurs).

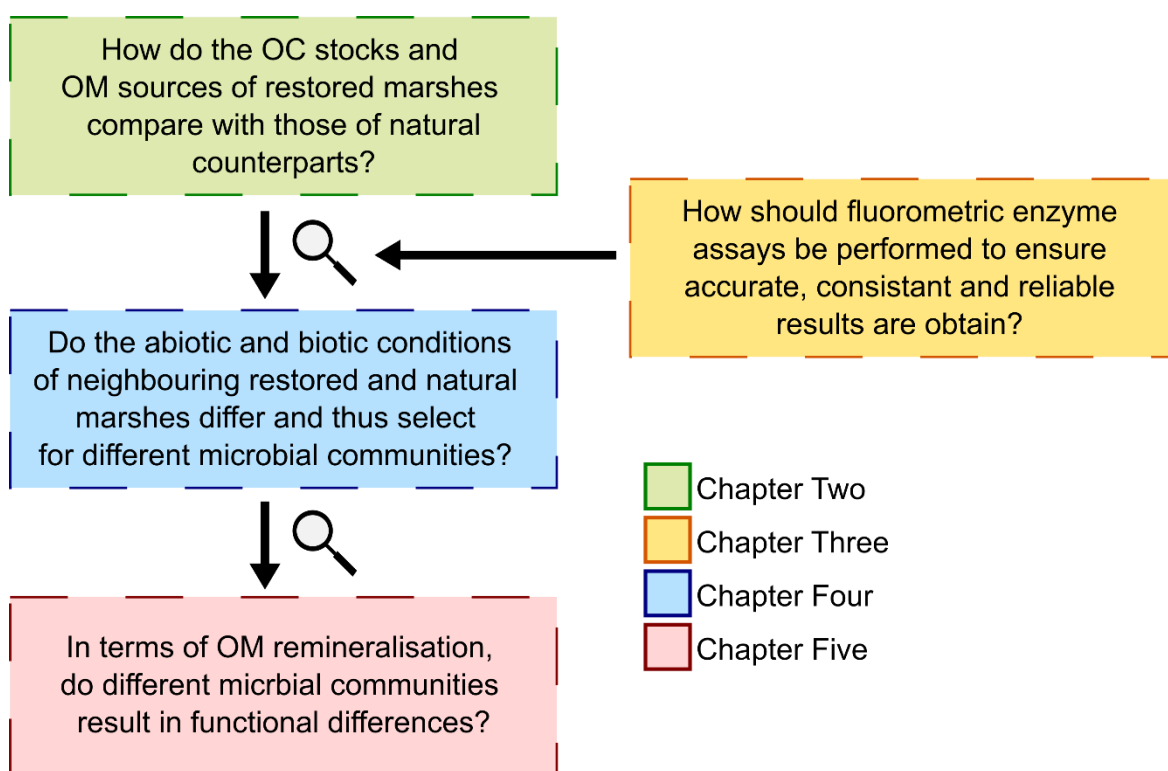


Figure 1.4. The research questions addressed in each part of this thesis.

1.2.2 Impact of storage temperature and diluent upon results of fluorometric microplate enzyme assays

Before delving into a comparison of the functionality of restored and natural marshes (Fig. 1.4), it became necessary to address various methodological questions relating to the fluorometric enzyme assays used to measure the extracellular activity of enzymes involved in OC, N and phosphorus cycling. The first of these questions aimed to establish the most suitable diluent to use for soil slurry creation. This slurry is used to conduct the assay and debate around the topic centres upon whether

a buffer is used as the diluent to prevent pH fluctuations or if distilled water alone is used to enable the sample to regulate its own pH, thus replicating the study environment more closely (German *et al.*, 2011). The question about the most suitable diluent to use feeds into a general debate within the field about whether assay conditions should either maximise the activity of the enzymes of interest or should attempt to closely replicate environmental conditions, thereby capturing the level of activity likely to occur in the study site (Sinsabaugh *et al.*, 2000). The points in favour of the former option include the fact that maximising enzyme activity will capture clear differences between samples, allowing for comparisons between studies, and that it is impossible to truly replicate environmental conditions in a 96-well plate (Nannipieri *et al.*, 2018). Buffer can be used to select a pH that either optimises enzyme activity or that matches the environment. The second question addressed by **Chapter Three** related to the storage temperature at which the aforementioned soil slurries are held until analyses can be conveniently conducted. Several previous studies used fresh soil while others deemed it acceptable to freeze slurries before performing the assays (Lee *et al.*, 2007). In **Chapter Three**, comparisons are made between the enzyme assays results obtained from soils collected from the same locations at the same time but prepared differently. Slurries were created with distilled water, phosphate buffer and creek water from the saltmarsh – in a bid to replicate environmental conditions yet more closely – and stored at either 4 °C or -20 °C prior to analysis.

1.2.3 Microbial diversity and enzyme activity in restored and natural saltmarshes

Based upon existing evidence of differences in plant community, hydrology and elevation between restored and natural saltmarshes, it was hypothesised that knock-on effects upon related variables such as oxygen availability and nutrient dynamics would change microbial community composition and functioning (Fig. 1.3 & 1.4). With increased waterlogging, more reducing conditions were expected to promote the survival of anaerobic microbial taxa as opposed to aerobic ones and extracellular enzyme activity was expected to be damped. **Chapter Four**, therefore, aimed to test this expectation. Microbial community composition in adjacent natural and restored saltmarshes was quantified, with spatial and temporal differences considered. As well as determining the microbial taxa present with 16S rRNA gene sequencing, functionality was also assessed by measuring extracellular enzyme activity with the refined assay methodology developed in Chapter Three. Furthermore, other factors, such as redox potential, sediment biogeochemical properties and plant community composition, were recorded so that the possible drivers of any differences could be uncovered.

1.2.4 Microbial community responses to teabag litter addition in restored and natural saltmarshes

To investigate the functional effects of the differences in microbial community identified in Chapter Four, results from the teabag index (TBI) were evaluated in **Chapter Five** (Fig. 1.4). Standardised

teabag litter allowed OM decomposition and stabilisation rates in neighbouring natural and restored marshes to be determined. Furthermore, the microbial communities colonising the tea litter were identified by extracting and sequencing DNA. In a recent review of the role microbes play in wetland OM decomposition, Yarwood (2018) stated that one key knowledge gap is the determination of which plant and microbe species contribute to OM accumulation. Although the TBI approach does not provide a direct functional link, the microbial groups potentially involved in OM cycling in saltmarsh sediment were uncovered based upon the taxa colonising OM introduced to the sediment. Furthermore, this allowed differences between marshes to be identified, and methodological conclusions to be drawn about the TBI approach.

Chapter Two

Organic matter composition and origin differ between restored and natural UK saltmarshes up to 100 years after the breach

2.1 Abstract

Atmospheric carbon dioxide uptake by restored saltmarshes can be converted to carbon credits that are sold to offset emissions and provide funding for restoration. While carbon stocks in restored marshes were found to reach equivalence with natural sites, the shares of autochthonous and allochthonous carbon sources entering these marshes are less well known. This question has consequences for carbon crediting because only the former can be attributed to the restoration and legitimately included. To determine if differences do exist, the conventional tools for determining organic matter source – elemental, stable isotope and thermogravimetric analyses – were applied to sediments from four restored saltmarshes of different ages. Comparisons were then made with existing data from nearby natural marshes. Breach depth horizons in cores were successfully identified using foraminifera, unicellular marine protists. The OC/N-ratio was persistently lower in restored saltmarshes compared with natural counterparts even one hundred years after the creation of a breach. Additionally, $\delta^{13}\text{C}$ signals indicated a mixture of organic matter sources to the restored sites and there was often a higher proportion, based upon thermal sensitivity, of recalcitrant material in the organic matter of the restored marshes. Together, these results indicated that a higher proportion of allochthonous organic matter was entering these restored marshes. Understanding the composition of the organic matter in restored marsh sediment will allow more useful and constructive climate change mitigation methods to be developed.

2.2 Introduction

Saltmarshes represent the interface between terrestrial and marine ecosystems. Breaching the seawalls surrounding areas previously claimed for agriculture (i.e. de-embankment) is a method of saltmarsh restoration (French, 2006; Garbutt and Wolters, 2008). Originally, the main aim of de-embankment in the UK was compensatory habitat creation with locations selected based upon coastal protection needs and nature conservation benefits also being emphasised (Pethick, 2001; Wolters *et al.*, 2005). More recently, attention is being placed upon the increased atmospheric carbon dioxide (CO_2) removal that results from this land use change (Connor *et al.*, 2001; Jones *et al.*, 2024). Currently, a ‘Saltmarsh Code’ is in development in the UK that will allow this increase to be quantified and converted to carbon credits that can be sold as a means of funding restoration and of offsetting the emissions of the buyer, emphasising the importance of this issue (Burden *et al.*, 2023). It is the capacity of saltmarshes to continuously accrete sediment and build elevation, combined with waterlogged conditions caused by tidal inundation and a high organic matter (OM) input from productive saltmarsh plants, that create an effective organic carbon (OC) sink. Part of the OM in saltmarsh sediment is preserved, thus ensuring the sequestration of the OC it contains. Rapid rates of OC accumulation have been recorded in the years immediately following the creation of a breach (Wollenberg *et al.*, 2018; Mossman *et al.*, 2022) and OC stock levels have recovered in as little as 4 to 20 years after restoration in some instances (Santini *et al.*, 2019; Poppe and Rybczyk, 2021). High

levels of variability within and between restored sites, however, mean there is much uncertainty about when and if restored saltmarshes come to replicate the conditions of natural long-standing habitats of the same type.

The differences shown to exist between restored saltmarshes and natural reference sites are manifold. Agricultural soil layers remain in restored saltmarshes after the creation of the breach, and these relatively compacted layers persist as saltmarsh sediment accumulates on top (Tempest *et al.*, 2015). Ultimately, water is unable to penetrate these layers, and drainage is impacted (Spencer *et al.*, 2017). In turn, the development of topographic variation is prevented and characteristics like elevation and creek density continue to resemble those of agricultural fields and lack the diversity present in natural marshes (Shafer and Streever, 2000; Brooks *et al.*, 2015; Lawrence *et al.*, 2018). Waterlogging and a lack of elevation gradients mean that plant community diversity is often lower in restored saltmarshes because the small-scale variation required for a mosaic of plant species with different inundation frequency requirements is missing (Mossman *et al.*, 2012a; Mossman *et al.*, 2012b; Lawrence *et al.*, 2022). Given these differences, the potential for differing OC dynamics in restored saltmarsh is strong.

One key factor in saltmarsh carbon dynamics is the origin of the OM. As well as sediment, OM from outside the marsh, be it from marine, coastal or riverine sources, is delivered by tides, and this is termed allochthonous (Zhou *et al.*, 2007). Whether or not this OM should be included in carbon crediting schemes is debated because its storage cannot be directly linked to the restoration action undertaken (Needelman *et al.*, 2018; Houston *et al.*, 2024; Williamson *et al.*, 2025). On the other hand, OM produced *in situ* (i.e., autochthonous) by the growth of saltmarsh plants provides an OC input that can only be attributed to the restoration. The contribution of allochthonous OM to saltmarsh stocks is highly variable (Williamson *et al.*, 2025). However, in many cases it represents a significant portion, especially in minerogenic marshes (e.g., Gebrehiwet *et al.*, 2008; Wang *et al.*, 2011; Martins *et al.*, 2022; Krause *et al.*, 2022; Fu *et al.*, 2023; Benjamin *et al.*, 2024). This is important for the reasons already outlined but also because the autochthonous input can be remineralised over a decadal timescale while the allochthonous input is more effectively preserved (Bartholdy *et al.*, 2014; Van de Broek *et al.*, 2016; Mueller *et al.*, 2019a; Komada *et al.*, 2022). Therefore, OM provenance is a central question to address when comparing restored marshes with natural counterparts.

Common tools to determine the possible mixture of allochthonous and autochthonous components present in saltmarshes are the ratio of OC to nitrogen (N) and the ^{13}C content of OC in the sediment (e.g., Jiménez-Arias *et al.*, 2020; Xia *et al.*, 2021; de los Santos *et al.*, 2022). The former is useful because the high level of protein in marine algae produces a large N content that lowers the OC/N-ratio compared with terrestrial plants that contain C rich cellulose (Meyers, 1994; Zhou *et al.*, 2007; Hulisz *et al.*, 2013). Specifically, OC/N-ratios below 10 are indicative of recently deposited marine OM (Hulisz *et al.*, 2013). Depending on the origin of the OM (i.e., higher plants or algae) and the photosynthetic pathway being used (i.e., C_3 or C_4), different amounts of the heavier carbon

isotope are incorporated into tissues during growth, producing distinctive signatures that can be identified with the latter method (Boutton *et al.*, 1998; Mueller *et al.*, 2019b). Three signals with relevance in saltmarsh ecosystems are marine particulate OM (POM)/algae, terrestrial plants (C_3 ; be they from a saltmarsh or agricultural field) and *Spartina* spp. (C_4). The $\delta^{13}C$ values of these sources tend to increase in the order listed. Untangling the results of this analysis is rarely a simple task, and consideration must be given to whether the signals at different depths along a sediment core are indicative of a change in OM source or if variation is simply due to OM decomposition (Luk *et al.*, 2020). Another important method for characterising the OM present in saltmarshes is via thermogravimetric analysis (TGA), whereby the fractions of labile or recalcitrant OM in a sediment sample can be differentiated and quantified (Smeaton and Austin 2022). In the context of TGA, labile OM refers to thermally sensitive molecules that burn at lower temperatures and are thus potentially more easily degradable in the environment (Schmidt *et al.*, 2011). On the other hand, recalcitrant OM is only lost at higher temperatures and so represents potentially more complex and stable molecules. OC is stabilized in marsh soils under reducing, oxygen-deficient conditions because the absence of O_2 as a highly efficient terminal electron acceptor constrains microbial decomposition (Megonigal *et al.*, 2003). In minerogenic marshes, additional stabilization mechanisms known from upland soils may also contribute, particularly the formation of mineral–organic associations. Although these associations may be weaker or less abundant than in well-aerated upland soils, recent studies suggest that they nevertheless contribute to OC stabilization in marsh sediments (Neisek *et al.*, 2025). When undergoing thermogravimetric analysis, the mineral associated OM is burnt off at higher temperatures and thus classed as recalcitrant while organic macroaggregates are unprotected when removed from their anoxic environment and burn in the labile temperature range (Pallasser *et al.*, 2013; Li *et al.*, 2023; Ortner *et al.*, 2025). These three methods are combined to achieve a more insightful picture of saltmarsh carbon dynamics and can be used to make comparisons between restored and natural marshes.

A crucial first step when measuring the properties of restored saltmarshes is to differentiate between the pre- and post-breach sediment layers. In addition to making accurate comparisons with natural marshes, this is particularly important when calculating OC stocks and accumulation rates because of the need to determine the land use of the site at the time of OM deposition (Smeaton *et al.*, 2023). Radiometric dating is often applied to saltmarshes but in the case of restored sites this is often not feasible due to the young age of a site and/or disruption of sediment layers during site construction. Previous efforts have successfully used diatoms and moisture content to determine the breach depth (Spencer *et al.* 2008). Problems with using diatoms, however, include poor preservation making it necessary to identify remains, instead of full valves, and the impossibility of any identification in samples containing coarse silt and sand (Spencer *et al.*, 2008). The foraminifera - unicellular marine protists (Sen Gupta, 2003) - present in saltmarshes have long been used to reconstruct past sea-level changes (e.g., Edwards and Horton, 2006) and were employed in one study to find the transition in sediment cores from tidal flat to saltmarsh ecosystems (Mueller *et al.*, 2019a).

Furthermore, foraminifera have previously been used as proxies to identify human disturbance in saltmarsh sediment (e.g., García-Artola *et al.*, 2016; Mueller-Navarra *et al.*, 2019) and the importance of salinity for most species makes clear the potential for using foraminifera to differentiate between the horizons in restored marsh cores. In this study, application of this technique presented an opportunity for its refinement and an assessment of its performance in saltmarshes restored via seawall breaching.

In this study, the variables quantified were OC, N and $\delta^{13}\text{C}$ signatures – the former two allowing OC/N ratios to be calculated – and the fractions of labile and recalcitrant OM, based upon TGA. The aim was to make comparisons across four different restored sites of different ages by applying this wide array of analyses that are not often brought together to produce a comprehensive discussion of saltmarsh restoration as a natural climate solution. I combined the new restored marsh data collected with an existing database of natural marsh reference sites. To achieve the overarching goal, the first objective was to determine the depth of the breach in the sediment cores using foraminifera, making it was possible to identify which core sections were representative of pre- and post-breach settings. As a result, the biogeochemical variables examined could be compared across sites and the possible reasons for any differences or similarities attributed to the stages of marsh development. Finally, I used the breach depth estimates to calculate OC stocks for the post restoration sections of the cores.

2.3 Study sites

2.3.1 Restored saltmarshes

The focus of the study is the east of England (Fig 2.1a & 2.1b), where saltmarshes are a common feature along the coastline that provide biodiversity and coastal protection benefits and where several restoration projects have already been implemented (Boorman, 2003).

As examples of older restored sites, previous studies have looked at marshes restored “accidentally” when storms created breaches in the surrounding sea walls, often as long as a century ago. Differences in the sediment characteristics between restored and natural sites – such as microporosity and pore connectivity – resulting from the presence of the agricultural soil layer were not identified in sites breached accidentally during storm surges (Spencer *et al.*, 2017); however, plant community differences, such as an absence of perennials and the predominance of the shrub *Atriplex portulacoides* have been identified (Mossman *et al.*, 2012b). Furthermore, recently created unmanaged sites were found to lack topographical diversity (Williams and Dale, 2023). Overall, this suggests that other persistent differences could exist in unmanaged sites that represent the future development of more recently restored marshes.

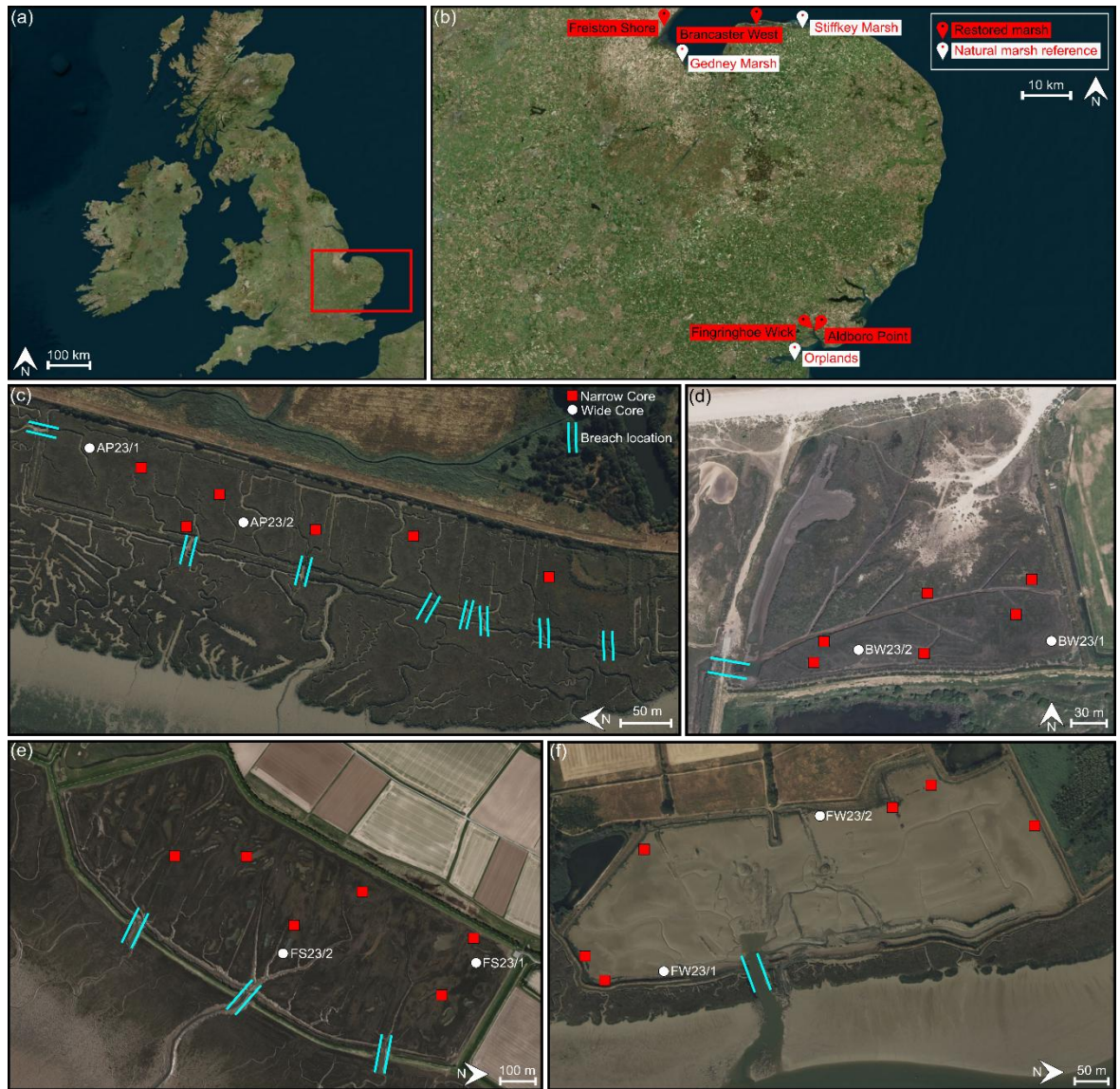


Figure 2.1. Locations of the sampling sites within (a) the UK and (b) the east of England and of the narrow (red squares) and wide (white circles) cores taken from (c) Aldboro Point, (d) Brancaster West, (e) Freiston Shore and (f) Fingringhoe Wick. The red markers in panel b represent the marshes that were sampled as a part of this study while the white markers are the natural marsh reference sites sampled by Smeaton and colleagues (2023; 2024). Breach locations in panels c-f are marked with turquoise lines. Map imagery © Microsoft Bing Maps; satellite imagery © DigitalGlobe (Maxar), 2020.

Aldboro Point

Aldboro Point (Fig. S2.1a) lies within the Colne estuary, approximately 12 km southeast of Colchester (51.832375, 0.986063). This marsh represents the oldest “restored” marsh included in the study, with tidal inundation reintroduced in 1921 by a storm-created breach in the seawall (Burd, 1992; Burd *et al.*, 1994; Wolters *et al.*, 2005). The reported history of the area prior to the final breach

suggested very inconsistent conditions. Enclosure with field boundaries occurred after 1840, by 1874 no seawall was present and the 1896 Ordnance Survey (OS) mapping indicated the area was again enclosed (Burd *et al.*, 1992). It is not definitively known when the final breach occurred, but the 1921 OS map showed a saltmarsh. For simplicity in this study, the breach was assumed to have occurred at the latest possible date, in 1921, as in other studies (e.g., Atkinson *et al.*, 2001; van Oevelen *et al.*, 2000; Wolters *et al.*, 2005). Therefore, at the time of sampling, the marsh was assumed to be 102 years old. The plant species recorded in 2023 at the selected sampling locations (Fig. 2.1c; Table S2.1) included *Puccinellia maritima*, *A. portulacoides*, *Limonium vulgare* and small patches of *Spartina anglica* (Table S2.2). The greatest distance recorded between a coring location and the closest breach was 103 m (Table S2.1). The tidal range is 4.5 m (Wolters *et al.*, 2005).

Monitoring of the vegetation at Aldboro Point in 1993 (72 years after the breach; Burd *et al.*, 1994) showed that species diversity was low, with only 18 % of the “target” species present (Wolters *et al.*, 2005). The most abundant species (41-60 % cover) were *Suaeda maritima* and *P. maritima*. Another study using data collected in 2004 (83 years after the breach) found that species richness in Aldboro Point did not differ significantly from a nearby natural reference marsh but that the plant community in the former was dominated by *A. portulacoides* and in the latter by *P. maritima* (Garbutt and Wolters, 2008). Data from the monitoring project of Burd and colleagues (1994) was used to calculate an initial sediment accretion rate of 3.00 mm yr⁻¹ and an average of 7.22 mm yr⁻¹ by another project (van Oevelen *et al.*, 2000).

Freiston Shore

Freiston Shore (Fig. S2.2b) is located within The Wash, ~8 km east of Boston in Lincolnshire (52.965328, 0.092937). The most recent artificial embankment in Freiston Shore was constructed in 1980 and the three breaches in the seawall were deliberately created in 2002, making the site 21 years old during sampling (Symonds and Collins, 2007). At the selected sampling locations (Fig. 2.1e; Table S2.1), the plant species present predominantly included *A. portulacoides*, *P. maritima*, and *Salicornia* spp., with smaller quantities of *S. anglica*, *L. vulgare* and *Aster tripolium* present (Table S2.2). The greatest distance recorded between a coring location and the nearest breach was 480 m (Table S2.1). The tidal range experienced by the site is 6.5 m (Wolters *et al.*, 2005).

Unlike the other study sites, Freiston Shore has been the subject of many investigations looking at a variety of factors. For example, it was reported that 88 % of the restoration site was vegetated only four years after breach creation (Freiss *et al.*, 2012), that substantial channel erosion had occurred at certain locations (Freiss *et al.*, 2014) and that the restored marsh had a wave amplification, as opposed to attenuation, effect (Kiesel *et al.*, 2019). Especially relevant to this study was a previous investigation of sediment accretion rates between 2002 and 2006 (Spencer *et al.*, 2012). The study by Wolters and colleagues (2005) referred to in the *Aldboro Point* section also included Freiston Shore and they reported that this site contained the same proportion (18 %) of

target species as Aldboro Point, but only one year after the breach. Furthermore, ten years after the initial breach was created in Freiston, topographic manipulation was carried out to increase habitat diversity and this led to an increase of plant species beta-diversity (Lawrence *et al.*, 2022).

Brancaster West

The third site, Brancaster West (Fig. S2.1c), is situated on the North Norfolk coast, around 15 km west of Wells-next-the-Sea (52.972107, 0.631014). Brancaster West was selected for restoration in 1996, work began in 2001 and was completed in 2002 (Doody, 2002; Myatt-Bell *et al.*, 2002; Wolters *et al.*, 2005). Like Freiston Shore, this site was 21 years old at the time of sampling. Prior to restoration, the area was used as a freshwater grazing meadow and differs from the other sites included in this study as the tide flows in via piped culverts, as opposed to a simple breach dug in a seawall (Doody, 2002). This difference does not impede comparisons, however, as the site still experiences regular tidal inundation that drives sediment and OM delivery, sediment redox conditions and plant species zonation. Development of the plant communities in the five years following the breach has been recorded and showed that *Salicornia europaea* and *S. maritima* were generally most dominant (Mossman *et al.*, 2012a). In addition, it was shown that the vegetation still differed significantly from the communities in natural reference marshes five years after restoration was implemented, which was put down to strongly reducing conditions in the Brancaster West sediment (Mossman *et al.*, 2012a). Most of the sampling locations (Fig. 2.1d; Table S2.1) were overwhelmingly dominated by *S. anglica* and *A. portulacoides* but there were more diverse patches in which *L. vulgare*, *Salicornia* spp., *A. tripolium*, *P. maritima* and *Triglochin maritima* were present (Table S2.2). The distance between coring location and the breach was no more than 290 m (Table S2.1). The tidal range in the area is 6.5 m (Wolters *et al.*, 2005).

Fingringhoe Wick

The youngest restored marsh (Fig. S2.1d) selected in this study sits within the Fingringhoe Wick Nature Discovery Park in Essex and, like Aldboro Point, lies within the Colne estuary (51.842106, 0.974856). The site is an area of former agricultural land to which tidal inundation was reintroduced by the creation of two breaches in the seawall in 2015, making it 8 years old during sampling (Vaughan, 2019). Prior to breaching the sea wall, the southern section of the area was used as an arable field while the northern part was a pasture (Cai *et al.*, 2022). This marsh largely resembles an unvegetated mudflat, but saltmarsh plant communities have begun to colonise the fringes of the site. Only three plant species were found across all the sampling stations (Fig. 2.1f; Table S2.1): *S. anglica*, *Salicornia* spp. and *A. portulacoides* (Table S2.2). The greatest distance recorded between a coring location and the breach was 510 m (Table S2.1). The tidal range is 4.5 m (Wolters *et al.*, 2005).

2.3.2 Natural marsh reference sites

Three natural saltmarshes, investigated in the studies conducted by Smeaton and colleagues (2023; 2024), were selected for their proximity to the restored saltmarshes (Fig. 2.1b) and were used as baselines to compare the biogeochemical variables of interest. Aldboro Point and Fingringhoe Wick, two sites almost opposite to one another in the Colne estuary, were compared with Orplands, approximately 16 km away. Freiston Shore was paired with Gedney Marsh, also ~16 km away, and Brancaster West with Stiffkey Marsh, ~20 km away. Detailed information about these natural saltmarshes is available elsewhere (Smeaton *et al.*, 2023, 2024; <https://catalogue.ceh.ac.uk/documents/5cc9ee72-ed88-4e71-bd8d-e3511bb9ed12>). To make comparisons possible, results from cores sampled at a 1 cm resolution were binned into the same depth intervals sampled by this study (see section 2.4.1).

2.4 Methodology

2.4.1 Sample collection

Fieldwork was conducted during June and August of 2023. From each of the four restored saltmarshes, eight sediment cores were collected – two wide diameter (5 cm) cores extracted with a Russian peat corer and six narrow diameter (2.7 cm) cores extracted with a gouge auger. The former cores were transported whole to the laboratory while the latter were subsampled in the field at the following resolution: every 2 cm in the top 10 cm and every 5 cm over the remainder of the cores. Within each of the two wide core sampling locations at every site, three replicate wide-diameter cores were always taken from within the same ~30 cm² area to ensure enough material was available for all the required analyses. One hundred cm deep cores were collected from Aldboro Point, and 50 cm deep cores were extracted from the other study sites, as it was anticipated that sediment accumulation following the breach in the more recently restored sites (i.e., those less than 21 years old) would be less than 50 cm. Although sedimentation rates of 7.5 cm yr⁻¹ have been reported for restored sites in the Severn estuary, far lower ranges (<2 cm yr⁻¹) have been recorded within east of England restored marshes (Mossman *et al.*, 2022). Furthermore, very high sedimentation rates usually only occur for the first few years following a breach, until the elevation of a site within the tidal frame has increased (Mossman *et al.*, 2022). Therefore, the assumption that no more than 50 cm of sediment had accreted in the younger restored sites was deemed legitimate. Usually, it was not possible to reach the maximum corer depth. In all cases, the plant species present within the 1 m² area surrounding each coring location were recorded. Cores were stored at -20 °C after being returned to the laboratory and until further analyses were conducted. Hence forth, cores from Aldboro Point are referred to as AP23/1-8, Freiston Shore FS23/1-8, Brancaster West BW23/1-8 and Fingringhoe Wick FW23/1-8. Wide cores are always numbered 1 and 2 (Fig. 2.1c – 2.1f).

2.4.2 Foraminiferal analysis

Foraminiferal analysis was used to identify the breach horizons. One replicate core from each of the two wide core sampling location were cut into 1 cm slices in the laboratory. Between 3.0 and 3.5 g of sediment was taken from each slice, and the precise wet mass of each recorded. These samples were then sieved and the fraction between 63 and 500 μm retained for analysis. Samples were refrigerated in 5 % isopropyl (HEXEAL) before analysis to ensure a high level of preservation. Rose Bengal staining was not used to differentiate between living and dead foraminifera, meaning that all counts represented the total foraminiferal population (Scott and Medioli, 1980). This was deemed acceptable because the main purpose of analysing the foraminiferal populations was to determine the breach depth and it has been shown that living foraminifera are overwhelming concentrated at the surface (Saffert and Thomas, 1998; Hayward *et al.*, 2014). Samples were split into eight equal aliquots and when foraminifera were present, the aim was to count a minimum of 50 individuals for each sample, following Kemp *et al.* (2020). If foraminifera were absent from the first aliquot, the entire sample was processed to confirm this was the case throughout. Foraminiferal species assemblage composition was only assessed in samples containing over fifty tests (Kemp *et al.*, 2020). The breach was deemed to have occurred at the lowest depth at which any number of foraminiferal tests (i.e., ≥ 1) were first encountered. This criterion was applied to Fingringhoe Wick, Brancaster West and Freiston Shore while the age-depth model results (see section 2.4.3) were relied upon for Aldboro Point. Murray (1979) was followed for taxonomic assignments to species level. In the cases of *Quinqueloculina* spp., *Elphidium* spp., *Ammonia* spp., *Rosalina* spp. and *Haplophragmoides* spp. only genus level identification was performed. Numbers of tests are given relative to the wet mass of the sample prior to sieving.

2.4.3 Radiometric dating

Radiometric dating was only conducted with one wide diameter core from Aldboro Point, the oldest site, because of the need for sediment old enough to achieve interpretable ^{210}Pb and ^{137}Cs profiles. When wet subsampling core AP23/1 in the laboratory, material from each centimetre wide slice from the two replicate cores, AP23/1A and AP23/1B, were combined to provide enough materials for radiometric analysis. Total (i.e., unsupported and supported) ^{210}Pb was determined indirectly via alpha spectrometry (ORTEC Alpha Ensemble) by measuring its decay product, ^{210}Po , and assuming secular equilibrium (Ebaid and Khater, 2006). In more detail, approximately 0.3 g of each 1 cm core slice between 0 and 40 cm was spiked with a known amount of ^{209}Po radiotracer (National Physical Laboratory) and digested using 12 mL of a mixture of 69% nitric acid (ARISTAR) and 47 %hydrogen fluoride (NORMATOM; 3/1 mixture) in a microwave assisted digestion system (CEM Mars 6). After neutralising any remaining free fluoride ions, each digest solution was transferred to a glass beaker, evaporated to dryness, and taken up to 25 mL using a 0.5 M HCl solution to allow for the spontaneous deposition of ^{209}Po and ^{210}Po on a silver disc for five hours at 90 °C. Each silver disc was finally counted via alpha spectrometry for up to five days to achieve an

adequate count. Selected adjacent slices (0-3, 8-10, 13-15, 16-18, 19-23, 24-26, 27-29, 30-32 and 38-40 cm) were combined to prepare a suitable geometry for gamma spectrometric analysis (ORTEC HPGe detector) and determine levels of ^{226}Ra (i.e., supported ^{210}Pb via ^{214}Pb) and ^{137}Cs at 352 and 662 keV, respectively (Woodward-Rowe *et al.*, 2025).

In R (version 4.4.1), the package *rplum*, version 1.0.0 (Blaauw *et al.*, 2023), was used to develop a Bayesian age-depth model generated from ^{210}Pb and ^{137}Cs data. This negated the need to select an equilibrium depth and allowed for the easy integration of the ^{137}Cs chronohorizon (Aquino-López *et al.*, 2018). Sediment accretion rates (SAR; mm yr⁻¹) were also obtained from the *rplum* model.

2.4.4 Bulk elemental and isotopic analyses

OC and N content, as well as dry bulk density, were measured in every sample from all cores, narrow and wide. Similarly to the replicate core used for the foraminiferal analysis, another replicate was sliced into 1 cm slices in the laboratory before being freeze dried along with the narrow core subsamples that were sliced in the field, as detailed in section 2.4.1. After freeze drying, samples were weighed and this dry mass was divided by the volume of the sample prior to drying to calculate dry bulk density (Dadey *et al.*, 1992). Moisture content was only calculated for the wide cores because it was possible to record the wet masses when subsampling in the laboratory; the dry masses were then subtracted from the wet masses and the products divided by the wet masses to calculate moisture content.

To approximately 1 g of every dried sample, I added 15 mL – split between three 5 mL aliquots – of 6 % sulphurous acid (Fisher Scientific) to remove carbonates by producing calcium sulphate dihydrate, water and carbon dioxide (Gibbs, 1977; Caughey *et al.*, 1995). Carbonates were removed to ensure that only organic carbon content was measured. Acidified samples were allowed to stand for 72 hours before being dried again in the freeze dryer. Finally, samples were homogenised for 30 seconds in a ball mill. From these homogenised samples, 5 mg were weighed into tin capsules and their OC and N content determined by elemental analysis with an CE440 elemental analyser (Exeter Analytical). It was sometimes necessary to repeat measurements for samples with conspicuously low N results by weighing 20 mg into a capsule, instead. A capsule containing approximately 1.6 mg acetanilide (Sigma-Aldrich) was run as a standard between every group of six sediment samples for quality control purposes. OC/N ratio was calculated by dividing the OC content of a sample by its N content.

Only the wide diameter cores were subject to bulk sediment $\delta^{13}\text{C}$ analysis and only every other centimetre was processed, except where the OC content showed high variability, in which case every centimetre was analysed in that section. To ensure that an appropriate amount of carbon dioxide gas was obtained after combusting the sediment sample for the Finnigan Delta plus XP Isotope Ratio Mass Spectrometer (Thermo Fisher Scientific), the amount of sediment weighed into the tin capsule

prior to this analysis was based upon the OC content and varied between 0.03 and 1.60 mg. Capsules containing approximately 0.5 mg of an internal laboratory standard (USGS89 collagen) were run in pairs before and after every 12 sediment samples to act as quality control and assess measurement drift throughout the measurement batches. The analytical precision of ^{13}C analysis was $\pm 0.10\%$ representing one standard deviation of all collagen analyses ($n = 16$). Values are given in δ notation versus VPDB (Vienna [Pee Dee Belemnite](#)).

2.4.5 Thermogravimetric analysis

Approximately 20 mg of the same dried and acidified sediment samples reported in section 2.4.4 were used to determine total OM, labile OM and recalcitrant OM content with a TRIOS SDT 650 (TA Instruments). The samples were heated from 40 °C up to 1000 °C under a constant stream of N_2 . To classify the OM, the methodology presented in Smeaton and Austin (2022) was followed where labile OM is lost in the temperature range between 200 °C and 400 °C, the recalcitrant fraction between 400 °C and 650 °C, and total OM was represented by the entirety of this range.

2.4.6 Organic carbon stock calculations

To calculate OC stocks, the narrow and wide core data were used separately, and the approach of Howard *et al.* (2014) was followed. First, OC density was calculated by multiplying the OC content of a sample by its dry bulk density (Fig. S2.2 & S2.3). Second, the carbon density of a core segment was multiplied by its length. Next, these values were summed across the length of a core that was deemed representative of a saltmarsh ecosystem. The sections of the cores taken to represent the post-breach marsh was determined upon a case-by-case basis: for Aldboro Point the radiometric dating results were used and for the other sites the foraminiferal analysis was the basis of this determination. In one case, the additional evidence provided by the OC and N analysis was used to adjust the breach depth range. With the narrow core results, averages of the totals of each core within a site were taken.

2.4.7 Statistical analysis

To determine whether statistical differences existed between the biogeochemical variables (OC, % N, OC/N ratio, proportion of recalcitrant OM) of the restored sites and those of the natural marshes, I used linear mixed effects models with sampling site and core identification being random variables. The lmer function from the *lme4* package in R was used (Bates *et al.*, 2015). OC and N data were log transformed prior to analyses to obtain normal distributions of residuals, which was determined by the visual inspection of diagnostic plots. Transformation was not necessary for OC/N ratios or proportion of recalcitrant OM. All statistical analyses were performed in R version 4.4.1 (R Core Team, 2024). Given the presence of random effects, denominator degrees of freedom were estimated with the Satterthwaite method (Kuznetsova *et al.*, 2017).

2.5. Results

2.5.1 Age-depth model

The core AP23/1 from Aldboro Point was the only one subject to ^{210}Pb and ^{137}Cs dating. These data were used to create a Bayesian age-depth model (Fig. 2.2a) in *rplum* (Blaauw *et al.*, 2023). The peak in ^{137}Cs levels clearly visible between 21 and 22 cm depth was only attributed to legacy discharge from the Bradwell Power Station, approximately 12 km from the site, which peaked in 1975 (European Commission, 1990; modelled as 1975 ± 5 years; Fig. 2.2b). Activity concentrations of ^{210}Pb decreased from the surface of the core down to 27-28 cm depth at which point concentrations stabilised (Fig. 2.2c). The level of supported ^{210}Pb activity concentration (i.e., ^{226}Ra determined via its decay product ^{214}Pb at 352 keV by gamma spectrometry) averaged at $24.5 \pm 1.9 \text{ Bq kg}^{-1}$, a value derived from the measurement of seven combined slices evenly spaced along the length of the core (Fig. 2.2c). The 2σ uncertainties produced by the model were less than 20 years up to a depth of 25-26 cm, below which point uncertainties increased down the core and reached 70 years in the lowermost sample (Fig. 2.2a).

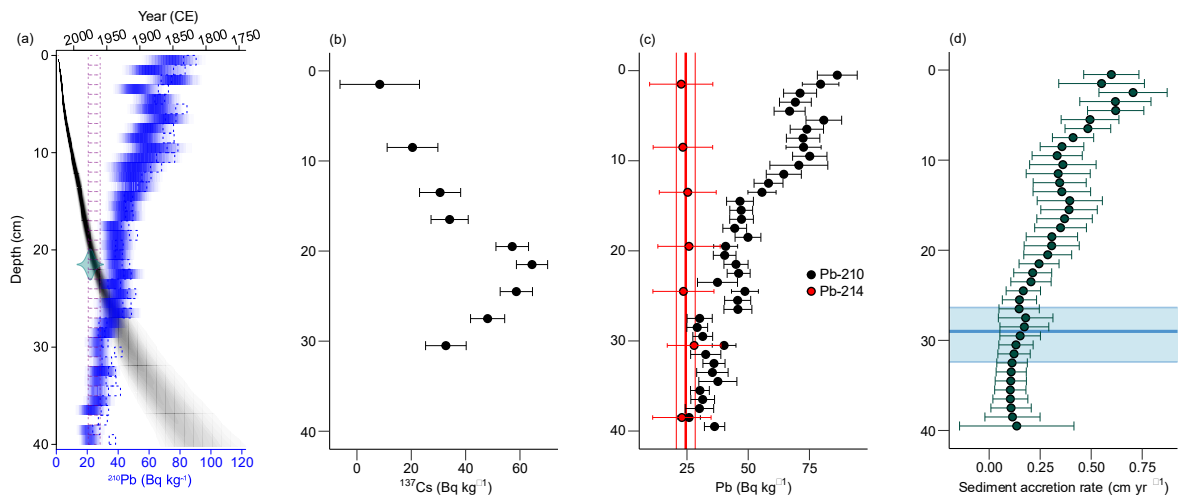


Figure 2.2. (a) The Bayesian age depth model for AP23/1, with the model represented by the black shaded area and the blue shaded area showing total ^{210}Pb levels. The green shaded area between 21-22 cm depth represents the ^{137}Cs peak at the calendar date 1975 ± 5 years. The model was developed with the *rplum* package (Blaauw *et al.*, 2023) using (b) ^{137}Cs and (c) ^{210}Pb data. A mean ^{214}Pb value was used for the model, and this is represented by the thick red line with the 2σ standard deviations represented by the thin lines. (d) Sediment accretion rates for each core slice were obtained from the model. The blue shaded area represents the estimated depth of the breach, based upon the ages assigned by the model to each centimetre of sediment. The thick blue line shows the depth estimated from the mean ages while the two thin lines show the depths calculated using the minimum and maximum ages (i.e., 2σ uncertainties). Error bars are $\pm 2\sigma$.

Using the estimated ages of deposition assigned to each section of the sediment core, it was possible to determine the depth that represented the year 1921, the year of the breach. From the mean ages, 29.0 cm was assigned as the breach depth, and using the 2 s uncertainty values, the maximum and minimum breach depths were assigned to 32.4 and 26.4 cm depth, respectively (Fig. 2.2d). These are the depths represented by the blue lines and shaded areas on all subsequent Aldboro Point figures.

Sediment accretion rate (SAR) values for each centimetre of sediment in the top 40 cm of the core were also derived from the age-depth model developed using *rplum* (Blaauw *et al.*, 2023; Fig. 2.2d). The average SAR between the bottom slice of the core (39-40 cm) up to 26-27 cm was 0.14 ± 0.28 cm yr⁻¹. Above 26-27 cm, the SAR increased gradually up to the top of the core (Fig. 2.2d). The beginning of this increase in SAR roughly coincided with the breach depth range estimated using the age-depth model. SAR increased gradually between 27 and 14 cm depth before remaining relatively constant until 7 cm. Values then continued to increase up to the top of the core, with a maximum SAR of 0.60 ± 0.14 cm yr⁻¹ recorded between 2 and 3 cm.

2.5.2 Foraminifera

Aldboro Point

Based upon the radiometric dating results, the breach depth was identified at 32.4 and 26.4 cm depth, and, to a degree, this was corroborated by the results from the foraminiferal analysis of core AP23/1. High numbers (>70 at minimum) of foraminiferal tests were found in almost every centimetre analysed from core AP23/1 (Fig. 2.3a). In samples from 31 to 33 cm depth, however, less than 40 tests were found, and these immediately proceeded and overlapped the lower limit of the breach depth range. Furthermore, there was a notable decrease in the numbers of tests per gram wet mass between 40-41, 35-36, 33-34 and 30-31 cm. Similar dips in numbers of tests per gram were detected in other parts of the core (i.e., between 90-91, 80-81, 70-71 and 55-56 cm) but these reductions in density were less sustained (Fig. 2.3a). The other core taken from Aldboro Point (AP23/2) followed a different pattern with several samples analysed that contained less than fifty tests (Fig. 2.3b). Additionally, there was generally a lower number of tests per gram in this core, with values no higher than 57 in all samples except for at the surface (398 tests) and between 81 and 75 cm depth (157 - 196). Within the estimated breach depth range, was one sample that contained 42 tests (i.e., 11 tests per gram; from 30-31 cm). Almost every sample examined from the Aldboro Point cores had assemblages overwhelmingly dominated by the agglutinated species *Entzia macrescens* (Fig. S2.4a & S2.4b).

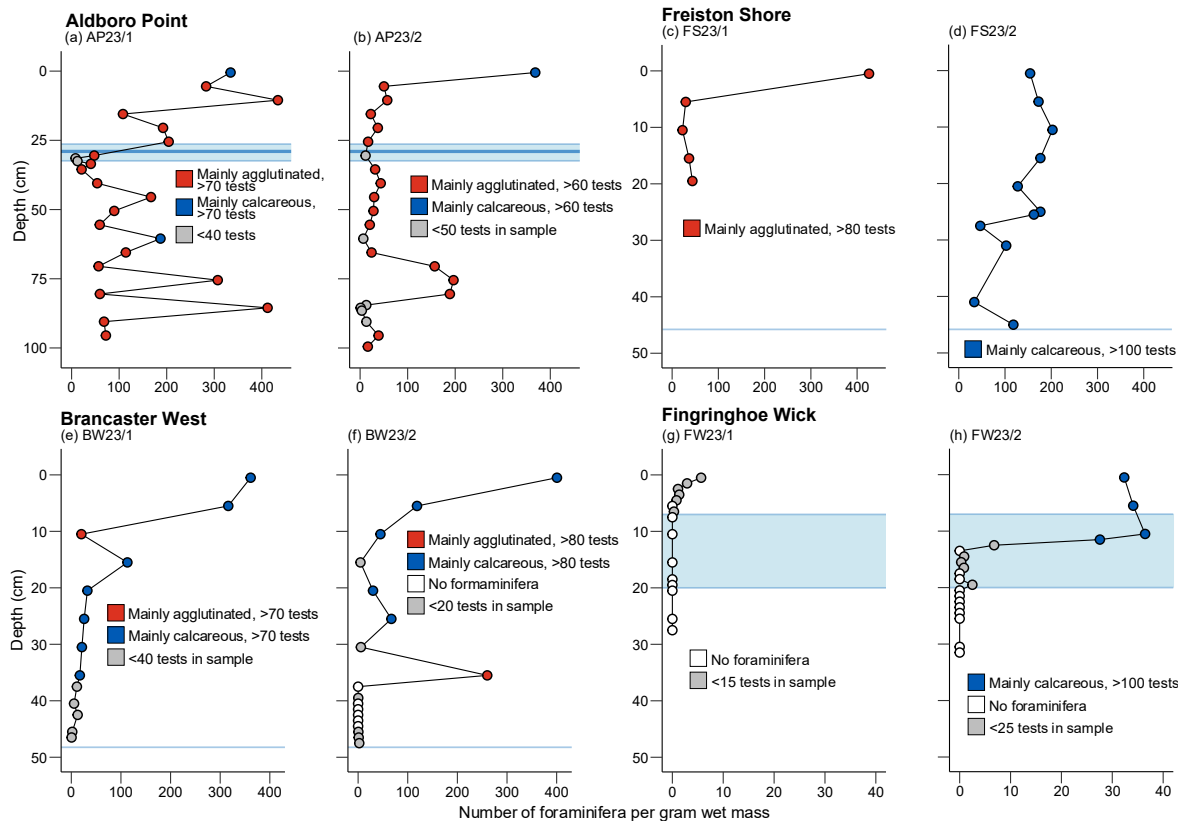


Figure 2.3. The numbers of foraminifera per gram of sediment in each centimetre of sediment processed from the two wide-diameter cores taken from (a-b) Aldboro Point, (c-d) Freiston Shore, (e-f) Brancaster West and (g-h) Fingringhoe Wick. The blue shaded areas represent the depth range in which the breaches could have occurred and were based upon the age depth model (Fig. 2.2) for Aldboro Point and upon the foraminifera results for the other sites (details are discussed separately for each site within section 2.5.2). In the Aldboro Point panels, the thick line shows the depth estimated from the mean ages of the age depth model, while the two thin lines show the depths calculated using the minimum and maximum ages (i.e., 2 σ uncertainties).

Freiston Shore

The cores from Freiston Shore contained high numbers (>80) of tests in all selected samples, including those from the lowest core depths achieved (19-20 and 45.0 cm in FS23/1 and FS23/2, respectively; Fig. 2.3c & 2.3d). Numbers of tests per g were lower in FS23/1, except for in the uppermost sample which contained the highest density value across both cores. The main difference between FS23/1 and FS23/2 was the overwhelming predominance of agglutinated species in the former and calcareous species in the latter. It was concluded that the breach depth was not reached and that the cores were representative of saltmarsh sediment only. As a result, the blue shaded area was placed just below the depth of the lowermost sample analysed (Fig. 2.3c & 2.3d)

Brancaster West

Brancaster West cores contained no or only very low numbers (<60) of foraminiferal tests below 35 cm depth (Fig. 2.3e & 2.3f). In BW23/1, low numbers of test were present in all five samples analysed between 47 and 38 cm depth (Fig. 2.3e). After this point, densities gradually increased up the core and samples were dominated by calcareous species. The one exception to this trend was a sample from 10-11 cm depth that contained a lower density of predominately agglutinated tests. BW23/2 contained less consistent patterns of test densities and assemblage compositions than BW23/1. The lowermost sample analysed contained a low number of tests and the next ~8 cm of sediment samples either contained similar amounts or were entirely empty of foraminifera (Fig. 2.3f). The core layer between 37 and 38 cm contained no foraminifera, but the layer above (36-37 cm) had 260 tests per gram, and these were predominately agglutinated species (Fig. 2.3f). The sample between 30 and 31 cm contained only 19 tests and all remaining samples from higher up the core contained predominantly calcareous species at far lower densities, except for in the uppermost sample (Fig. 2.3f). Similarly to Freiston Shore, the foraminifera data suggested that the breach depth was not reach in Brancaster West, meaning that the blue shaded area was placed below the depth of the lowermost sample analysed (Fig. 2.3e & 2.3f). Unlike Freiston Shore, the breach depth range of this site was later altered after examining the results of the biogeochemical analyses.

Fingringhoe Wick

There was a clear difference between the two cores from the youngest site, Fingringhoe Wick. FW23/1 contained no foraminifera until a depth of 6-7 cm, and above this point only very low numbers of tests, 13 at most (0-1 cm), were found (Fig. 2.3g). On the other hand, FW23/2 contained empty samples until a depth of 19-20 cm, low numbers (1-21 tests) in most samples between this point and 12-13 cm and then high numbers (>100) in all subsequent samples, bar one (Fig. 2.3h). Therefore, the possible breach depth for this site was between the first emergences of foraminifera in both cores, meaning a range between 7 and 20 cm.

2.5.3 Organic carbon and nitrogen content

In the Aldboro Point narrow core segments, OC and N content followed a pattern of gradually increasing values up the cores, similarly to the pattern in the corresponding natural marsh reference site, Orplands (Fig. 2.4a & 2.4b). In Aldboro Point values remained relatively stable between the bottom sample to 25-30 cm depth (i.e., near the estimated breach depth of 31 cm). Above this, values increased up the core, reaching an OC peak of 6.55 ± 1.40 % at 4-6 cm and a peak in N of 0.59 ± 0.08 % at 2-4 cm. In the Orplands cores, a similar, steady increase in OC and N content was seen, ending in 5.02 ± 0.71 % OC and 0.48 ± 0.14 % N in the surface sediment.

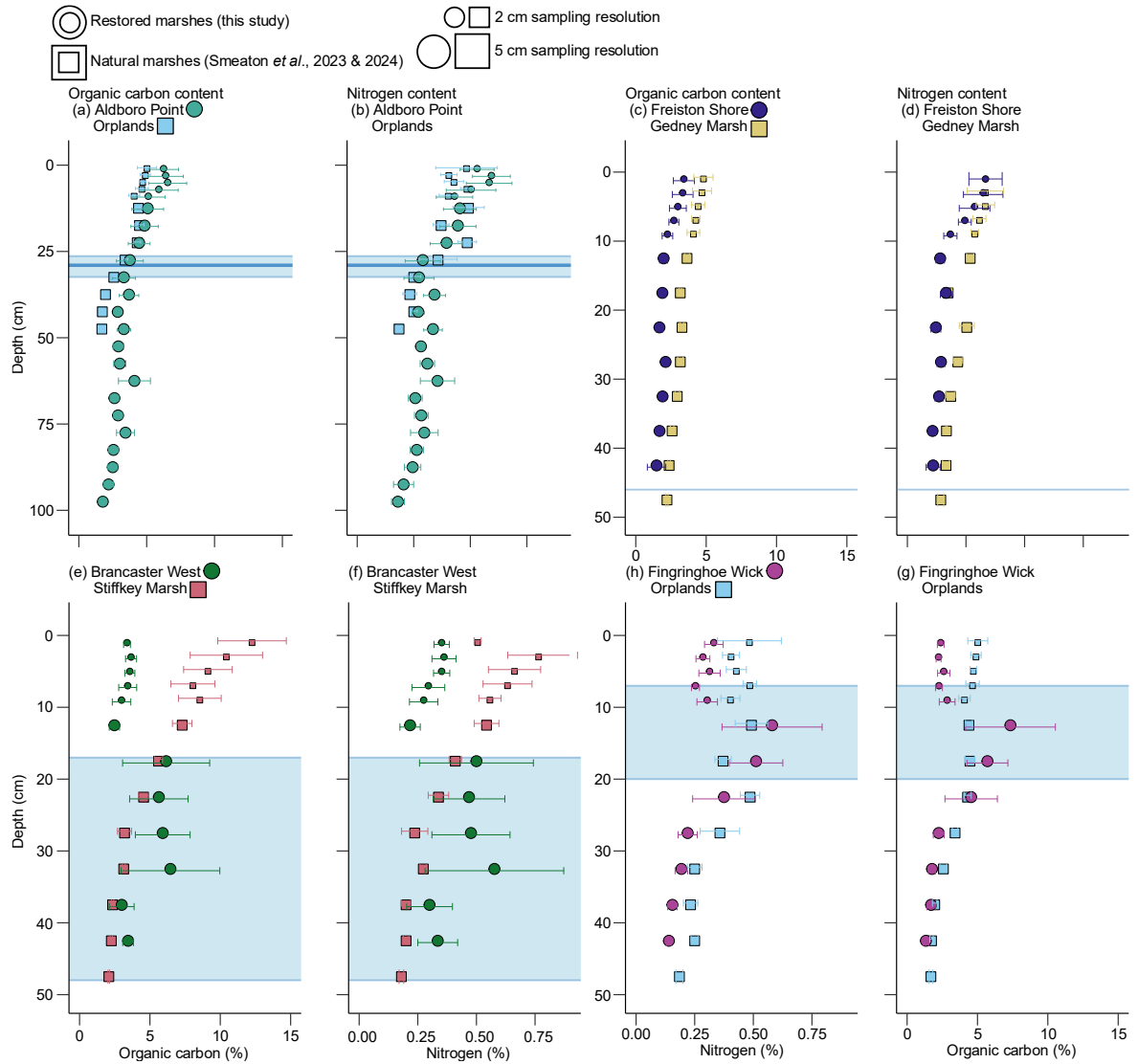


Figure 2.4. Organic carbon and nitrogen content of the narrow diameter cores and the corresponding natural marsh reference sites: (a-b) Aldboro Point and Orplands, (c-d) Freiston Shore and Gedney Marsh, (e-f) Brancaster West and Stiffkey Marsh and (g-h) Fingringhoe Wick and Orplands. Restored marsh datapoints are always represented by circles while natural marsh data, collected by Smeaton *et al.* (2023; 2024), are represented by squares. Smaller symbols represent 2 cm samples while larger ones represent 5 cm samples. The shaded blue areas denote the depth range at which the breaches may have occurred and is based upon radiometric dating for Aldboro Point and foraminiferal analysis for the other three sites. Additionally, the breach depth range for Brancaster West was expanded based upon the biogeochemical variables presented in this and subsequent sections.

Freiston Shore and Gedney Marsh cores also shared a similar pattern of OC and N content (Fig. 2.4c & 2.4d). In the Freiston Shore narrow cores, values remain constant from the bottom to a depth of around 9 cm and from this point an increase was seen, with the uppermost samples containing 3.41 ± 0.75 % OC and 0.33 ± 0.07 % N. The Gedney Marsh values continued to steadily

increase up the cores and reached peaks of 4.81 ± 0.69 % OC and 0.33 ± 0.08 % N in the uppermost samples.

In Brancaster West, OC and N content followed a distinctly different pattern (Figs. 2.4e & 2.4f). Again, and as was expected, the natural marsh reference site, Stiffkey Marsh, displayed a pattern of steady increase up the core, reaching 8.37 ± 1.3 % OC and 0.51 ± 0.02 % N in the surface samples. The lowermost samples (42-43 cm) from Brancaster contained 3.45 ± 0.38 % OC and 0.34 ± 0.09 % N. Immediately above the breach depth range, however, and in the three successive samples, there was a jump to a maximum of 6.46 ± 3.51 % in OC and 0.58 ± 0.30 in N. From 10-15 cm, values dropped down to 2.48 ± 0.37 % OC and 0.22 ± 0.04 N and small, successive increases were sustained to the surface (Fig. 2.4e & 2.4f).

Like Aldboro Point, Fingringhoe Wick was paired with Orplands for a natural reference site and was also compared against the same pattern of steady increase in OC and N up the cores (Fig. 2.4g & 2.4h). At the bottom of the Fingringhoe Wick cores (40-45cm), OC content was 1.34 ± 0.21 % and N content 0.14 ± 0.01 %. There followed an increase in both variables, with a clear jump in values from 20-25 cm depth and over the two subsequent depth categories (15-20 and 12-15 cm). Values peak at 7.34 ± 3.20 % for OC and 0.58 ± 0.21 % of N. In this case, the jump in OC and N content began below the estimated breach depth. At 8-10 cm – still within the estimated breach depth – values fell to 2.85 ± 0.56 % OC and 0.30 ± 0.04 % N and similar levels were sustained to the surface (Fig. 2.4g & 2.4h).

Overall, there was not a significant difference between the OC and N content of the restored marshes compared with their natural counterparts ($p = 0.17$ and 0.20 , respectively; Table S2.3). The interaction between marsh type and depth, however, was significant ($p = <0.001$, in both cases), meaning that differences between marsh types did exist at certain depth intervals (Table S2.3). Namely, and as already described, differences in OC and N content between restored and natural marshes were often strongest in the top ~10 cm of the cores.

2.5.4 Recalcitrant organic matter

The proportion of OM classed as recalcitrant was high across all sites, restored and natural, with most values being greater than 0.5 (i.e., 50 % recalcitrant; Fig. 2.5). In Aldboro Point and Freiston Shore, there was a small decrease in recalcitrant OM content up the cores, and, in both cases, this closely matched the patterns of the natural reference sites (Fig. 2.5a & 2.5b). Brancaster West samples below 15 cm depth also had values that overlapped with the natural reference site Stiffkey Marsh, but above this point, there was a divergence (Fig. 2.5c). A share of between 0.64 ± 0.03 and 0.67 ± 0.02 of OM was classed as recalcitrant in Brancaster West from 15 cm to the tops of the cores while in Stiffkey, the share was between 0.45 ± 0.04 and 0.56 ± 0.02 . Recalcitrant OM content was higher in the Fingringhoe Wick samples compared with those from Orplands throughout the length of the cores, but this difference became starker from 9 cm and upwards (Fig. 2.5d). The share of

recalcitrant OM in this top section was between 0.67 ± 0.02 and 0.68 ± 0.01 in Fingringhoe Wick and between 0.54 ± 0.02 and 0.55 ± 0.01 in Orplands. Proportion of recalcitrant OM did differ significantly between marsh type ($p = 0.047$) while depth ($p = <0.001$) and the interaction between depth and marsh type ($p = <0.001$) had far stronger effects (Table S2.3). This lends support to the observation that recalcitrant OM content differed most strongly between samples from the top ~ 10 cm of the marshes (Fig. 2.5).

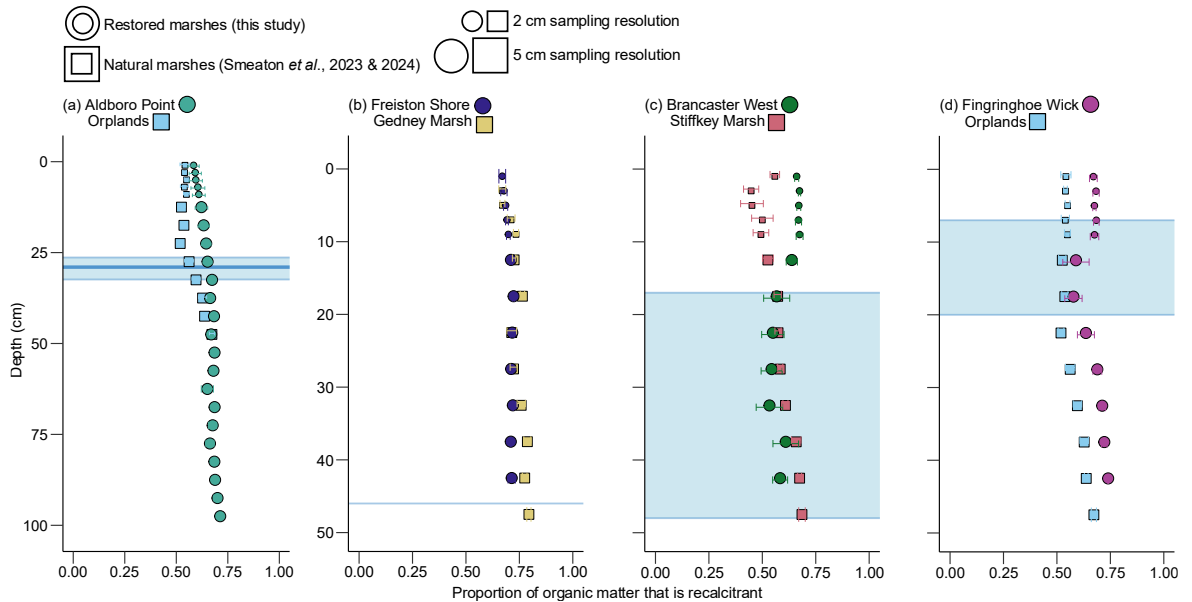


Figure 2.5. Proportion of total OM classified as recalcitrant by thermogravimetric analysis in the narrow diameter cores and the corresponding natural marsh reference sites: (a) Aldboro Point and Orplands, (b) Freiston Shore and Gedney Marsh, (c) Brancaster West and Stiffkey Marsh and (d) Fingringhoe Wick and Orplands. Restored marsh datapoints are always represented by circles while natural marsh data, collected by Smeaton *et al.* (2023; 2024), are represented by squares. Smaller symbols represent 2 cm samples while larger ones represent 5 cm samples. The shaded blue areas denote the depth range at which the breaches may have occurred and is based upon radiometric dating for Aldboro Point and foraminiferal analysis for the other three sites. Additionally, the breach depth range for Brancaster West was expanded based upon the biogeochemical variables presented in Figures 2.4, 2.6 and 2.7.

2.5.5 Organic carbon to nitrogen ratios and $\delta^{13}\text{C}$ signatures

Figure 2.6 showed that the OC/N values in restored saltmarshes were consistently lower than those observed in natural sites. Indeed, across all samples displayed in Figure 2.6, the mean OC/N for the restored samples was 9.95 ± 2.31 (1 s) and for the natural samples 14.30 ± 1.83 (1 σ). Statistical analysis showed the difference between marsh type to be significant ($p = 0.01$; Table S2.3).

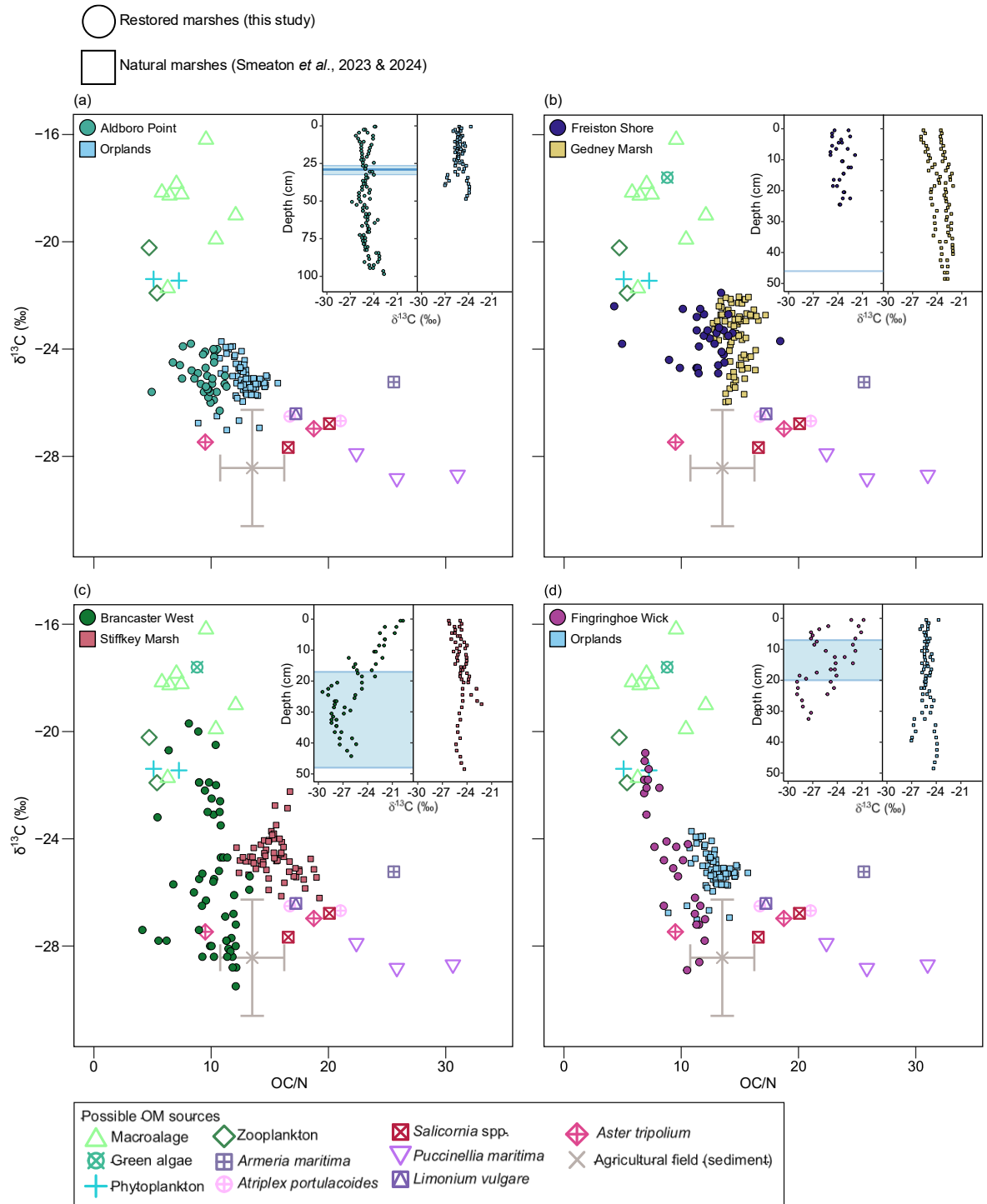


Figure 2.6. $\delta^{13}\text{C}$ and OC/N values of the post breach sections of the wide diameter cores taken as a part of this study and by Smeaton *et al.* (2024) from (a) Aldboro Point and Orlands, (b) Freiston Shore and Gedney Marsh, (c) Brancaster West and Stiffkey Marsh and (d) Fingringhoe Wick and Orlands. Restored marsh datapoints are always represented by circles while natural marsh data are represented by squares. Values of possible OM sources are adapted from Khan *et al.* (2015) and Smeaton *et al.* (2022). In-set plots within each panel show ^{13}C profiles along the lengths of the wide cores.

The OC/N ratios in the narrow cores (Fig. 2.7a – 2.7d) varied with depth. In Aldboro Point, there was a peak of 13.98 ± 1.42 at 25-30 cm that coincided with the breach depth range. Above this, ratios decreased to 11.44 ± 0.87 at the surface (Fig. 2.7a). Orplands, the corresponding natural marsh reference, also had increasing values up to a depth of 15-20 cm, at which point a peak of 13.60 ± 0.29 was reached before decreasing to 12.95 ± 1.15 at the surface (Fig. 2.7a). The OC/N ratios in Freiston Shore and Gedney Marsh decreased from the lowermost sample to the surface, although variability was greater in the former (Fig. 2.7b). In Brancaster West, the lowermost sample had a value of 10.70 ± 1.58 and ratios remained around this level, except for occasional peaks during the breach depth range. The Stiffkey Marsh OC/N ratios increased until 25-30 cm depth; above this point, values remained relatively constant to the surface (Fig. 2.7c). Between 45 and 20 cm in Fingringhoe Wick, values increased from 9.48 ± 0.79 to 11.50 ± 0.45 , with the peak occurring below the estimated breach depth range (Fig. 2.7d). Following this, values fell to 7.44 ± 0.48 at the surface.

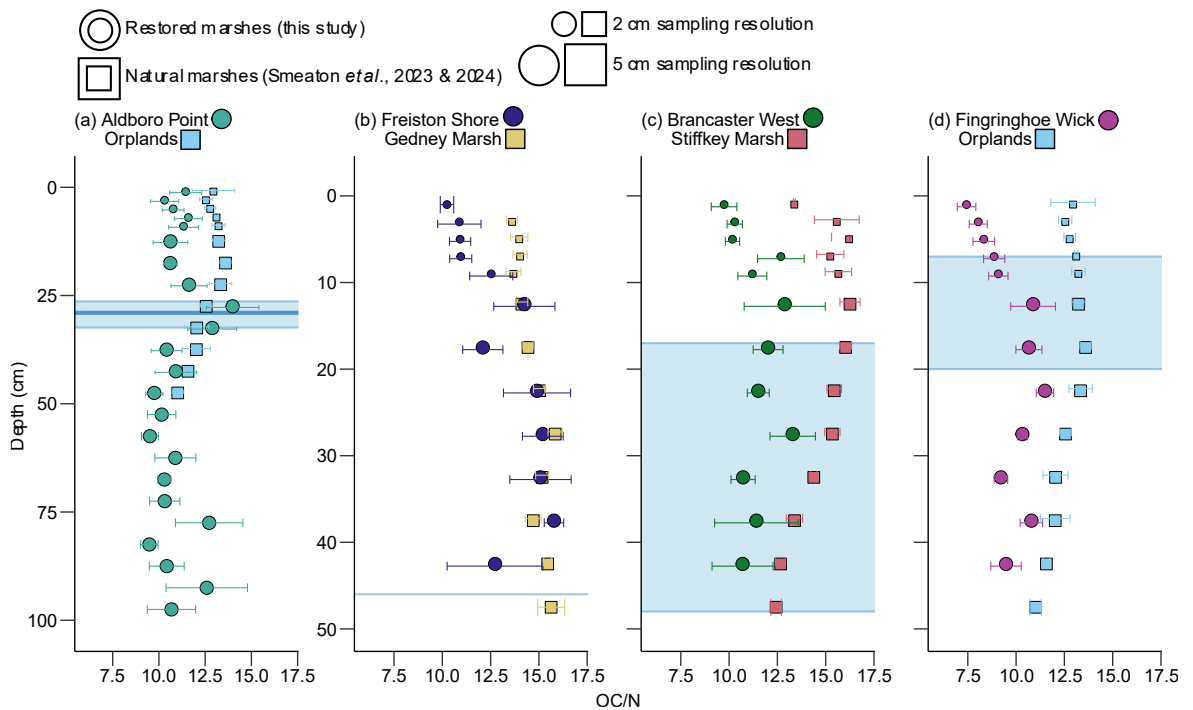


Figure 2.7. Ratio of OC to N in the narrow diameter cores taken as a part of this study and the corresponding natural marsh reference sites: (a) Aldboro Point and Orplands, (b) Freiston Shore and Gedney Marsh, (c) Brancaster West and Stiffkey Marsh and (d) Fingringhoe Wick and Orplands. Restored marsh datapoints are always represented by circles while natural marsh data, collected by Smeaton et al. (2023; 2024), are represented by squares. Smaller symbols represent 2 cm samples while larger ones represent 5 cm samples. The shaded blue areas denote the depth range at which the breaches may have occurred and is based upon radiometric dating for Aldboro Point and foraminiferal analysis for the other three sites. Additionally, the breach depth range for Brancaster West was expanded based upon the biogeochemical variables presented here and in Figures 2.4 and 2.6.

The $\delta^{13}\text{C}$ values in Aldboro Point and Freiston Shore were largely within the same ranges reported for their respective natural marsh reference sites (Fig. 2.6a & 2.6b). The former having values intermediate between the values recorded for C_3 saltmarsh plants and marine OM while the latter had values clustered more closely with the marine OM sources alone. In Brancaster West and Fingringhoe Wick, a wide range of $\delta^{13}\text{C}$ values was observed compared with the close clustering of the natural marsh sites (Fig. 2.6c & 2.6d). Brancaster West $\delta^{13}\text{C}$ values ranged between -29.50 and -19.70 ‰, while at Fingringhoe Wick the range was between -28.90 and -20.80 ‰. To contrast, the range in Stiffkey Marsh was -26.21 to -22.24 ‰ and in Orplands -27.02 to -23.72 ‰. Values in the former two sites, therefore, were closer to the distinctive range of *S. anglica* (-14.97 – -11.94 ‰; Khan *et al.*, 2015). Aldboro Point and Freiston Shore $\delta^{13}\text{C}$ values were consistent along the entire length of the cores while Brancaster West and Fingringhoe Wick values generally increased from the bottoms of the cores to the tops (Fig. 2.6; Fig. S2.5).

2.5.6 Estimating organic carbon stocks

By incorporating the different sources of evidence gathered during the study, it was possible to calculate several different OC stock values for each site (Table 2.1). Foraminifera analyses were conducted for wide cores alone, so it was possible to use the breach depth specific to each of these cores to calculate a stock using the OC values obtained for each centimetre of sediment (Fig. S2.6). With the narrow cores, it was possible to calculate mean and standard deviation values for each site because six of these cores were taken in each location. In the case of Fingringhoe Wick, two stock estimates were calculated using the narrow cores: one using the segments from depths above the lowest, foraminifera-based breach depth and another using only the segments above the highest possible breach depth. Two OC stock values were also calculated for Brancaster West but in this case the uppermost breach depth was based upon the additional evidence gleaned from the biogeochemical variables examined. It was assumed that the breach depth was not reached for Freiston Shore so only one narrow core stock estimate, using all core segments gathered, was calculated. For Aldboro Point, only the breach depth based upon the mean ages from the age-depth model was used and so only one narrow core estimate was calculated.

Table 2.1. Organic carbon stocks calculated using the evidence accumulated during the study. Stock estimates without standard deviation are based upon individual wide cores and the maximum depth used for these calculations are based upon the foraminifera results specific to that individual core. Autochthonous and allochthonous OM were not distinguished and so these estimates include OC from both sources.

Site	Age of site (years)	Core(s) used for estimate	Maximum length of core(s) used in calculation (cm)	OC stock $\pm$ SD (kg m ⁻²)
FW	8	Narrow cores from bottom of breach depth range	20	4.40 $\pm$ 1.45
		Narrow cores from top of breach depth range	8	0.90 $\pm$ 0.19
		Wide core FW23/1	7	1.95
		Wide core FW23/2	20	3.28
BW	21	Narrow cores from bottom of breach depth range	45	11.70 $\pm$ 2.55
		Narrow cores from top of breach depth range	15	2.80 $\pm$ 0.32
		Wide core BW23/1	44.5	14.01
		Wide core BW23/2	40.8	22.86
FS	21	Narrow cores from breach depth	45	7.52 $\pm$ 1.20
		Wide core FS23/1 (failed to obtain wide cores of a similar length to narrow cores)	26	3.37
		Wide core FS23/2 (failed to obtain wide cores of a similar length to narrow cores)	26	4.96
AP	102	Narrow cores from mean age depth model breach depth	30	5.98 $\pm$ 1.28
		Wide core AP23/1	29	4.03
		Wide core AP23/2	29	5.26

5. Discussion

Using the foraminiferal method to determine breach depth meant that it was possible to make comparisons between restored and natural marsh in terms of the biogeochemical variables that characterise the OM present in their sediment. The consistent differences identified can be used as indicators in restoration (Table S2.4).

2.6.1 Breach identification

The expected result from the foraminifera analysis was exemplified in core FW23/2 (Fig. 2.3g & 2.3h), in which foraminifera were entirely absent in all samples from the bottom of the core to a depth of 20-21 cm, representing an agricultural field with no tidal inundation. From 20 cm up to 12 cm, samples contained low numbers of tests (<21), and this was taken to represent the period during which the breach was created, and some foraminifera and/or propagules could have washed in by the tides (Weinmann *et al.*, 2019), supporting the existence of a time lag between breach creation and colonisation (Masselink *et al.*, 2017; Milker *et al.*, 2022). From 12 cm to the surface of the core, more than 100 tests were found in every sample examined, suggesting that foraminifera had successfully colonised this part of the site from this depth upwards. All the other cores analysed were harder to interpret and required greater nuance. Therefore, my results indicated that foraminifera could contribute to the identification of the breach depth in restored saltmarshes if a few key limitations and methodological refinements are borne in mind by future studies.

First, and most simply, to identify the breach depth range successfully, deep cores are required. For example, high numbers of foraminifera were found along the entirety of the cores taken from Freiston Shore and Brancaster West, and this was interpreted as an indication that the breach depth was not reached. In the case of the former site, using the mean sediment accretion rate of 1.06 ± 0.20 cm yr⁻¹ calculated by Spencer *et al.* (2012) between 2002 and 2006 for the interior of the restoration area suggested that approximately 20 cm of sediment could have accreted in the 21 years since the breach in Freiston Shore. This supported the suggestion that the breach depth in the high marsh core FS23/1 was missed. The higher rates of accretion recorded closer to the breach (e.g., a rate of 2.54 cm yr⁻¹ in the four years following restoration) would explain the consistent presence of foraminifera in FS23/2, a 46 cm core, taken 150 m from the closest breach, while FS23/1 was 376 m away from its nearest breach (Fig. 2.1d; Table S2.1). Conversely, the site had not been claimed from the sea for long before the creation of the breach, suggesting the possibility that these assemblages were representative of the pre-1980s saltmarsh. All the samples analysed from Freiston Shore contained high numbers of foraminifera, but the Brancaster West cores presented a more complex case. These did contain samples entirely absent of foraminifera, but they were interspersed with other samples that contained low numbers of tests. It is possible that the breach depth was just missed and that analysing samples from a greater depth would have meant reaching the agricultural layer from which foraminifera are normally absent. Therefore, the data from Brancaster West and Freiston Shore

demonstrated the importance of taking deep cores (>50 cm) - even if a relatively short time (~20 years) has elapsed since the breach - to ensure that the breach depth can be confidently identified. However, the challenge of taking deep cores from some sites, as was the case in the high marsh zone in Freiston Shore, could present a barrier.

A second factor that requires careful consideration is the high background variability across a site. In marked contrast to core FW23/2, foraminifera were absent from much of FW23/1, the other Fingringhoe Wick core. This implied that sediment accretion rates differed substantially between the two locations. Using the bottom of the breach depth range, the sediment accretion rate over the eight years since the breach in the vicinity of FW23/1 was 0.88 cm yr^{-1} while in that of FW23/2 it was 2.50 cm yr^{-1} . This result contradicts the sediment accretion rates recorded by another study in the first 15 months following the breach: these were higher in the formerly arable section of the marsh (where FW23/1 was taken) compared with the former pasture (where FW23/2 was taken; Cai *et al.*, 2022). In the years immediately following restoration, Pethick (2002) stated that accretion rates could be zero due to a lack of vegetation to hold the sediment in place, especially in the winter months and/or during storms. A possible explanation for the slower rate of accretion in FW23/1, therefore, is the removal of any sediment settling there by the tide due to the closer proximity of this coring location to the breach (i.e., Fig. 2.1d; Table S2.1). The breach depth range applied to the Fingringhoe Wick narrow cores was necessarily wide because of a need to reflect the results from both these cores. Variability in moisture content changes in the two cores tended to support this wide range (Fig. S2.7). Another example of within site variability comes from Brancaster West. As discussed, examining the foraminifera data in isolation suggested that the breach depth was below 50 cm depth. However, interpreting the OC and N peaks as representative of the pre-breach vegetation and/or root mat layer (see section 2.6.2 – *Age*) meant adjusting the upper limit of the breach depth range to 17 cm depth. Given that the OC and N was examined primarily by looking at the mean values across all the narrow cores at different depths and that only two wide cores were used for foraminiferal analysis, it is possible that giving greater attention to individual locations within a marsh would yield more closely constrained breach depth results.

Adjusting the foraminifera-determined breach depth range of Brancaster West based upon the OC and N data, points to another important conclusion drawn by this study – the desirability of employing multiple different methods to characterise sediment cores, where possible. The results from Aldboro Point also lend support to this point. The many and relatively short-term changes in use undergone by this site may have meant that there was insufficient time for the area to undergo the processes that lead to agricultural land directly behind seawalls being situated at elevations far below mean high tide (Pethick, 2002). As a result, the site could have quickly recovered after the final breach in 1921, and this explains the lack of empty samples around the time of the breach. Alternatively, storm surges could have been responsible for washing foraminifera into the site while it was still enclosed. In this case, it was possible to supplement the foraminifera results with

radiometric dating to identify the breach depth. Doing so allowed the breach depth to be identified with confidence. Furthermore, it showed that the fall in test densities in AP23/1 matched the breach depth estimated from the age-depth model, but this was not true of AP23/2, again pointing to within site variability.

Another conclusion drawn from this study was that defining zones, such as high marsh, low marsh and tidal flat, based upon foraminiferal assemblage data from restored marsh cores is not necessarily an accurate means of tracking the development of a site over time because difference may not extend beyond the highly sensitive foraminifera. Looking at the foraminiferal species assemblages alone strongly suggested that FS23/1 was representative of a high marsh environment while FS23/2 represented a tidal flat, owing to the dominance of agglutinated tests in the former and calcareous ones in the latter (Fig. 2.3e & 2.3f). However, the plant communities in both coring locations were overwhelmingly dominated by *A. portulacoides* (90 % cover; Table S2.2), meaning that defining the ecosystem type based upon foraminiferal species would bear no semblance to the reality. It is possible that the lack of topographical variation in restored marshes means the zonation of plants and foraminifera were uncoupled and/or that *A. portulacoides*, the plant species often seen to dominate in restored sites, was tolerant of a wider range of inundation frequencies than the foraminiferal community.

Other factors that could limit the usefulness of the foraminifera method are bioturbation and sediment mixing. These could mean that the foraminiferal assemblages found within a centimetre of sediment represent a mixture of populations formerly present upon the marsh surface during very different periods of its development, as opposed to representing just one period. Furthermore, bioturbation can enable the passage of electron acceptors (e.g. sulphate) into deeper sediment layers and thus allow for organic matter mineralisation, acid production and calcium carbonate dissolution (i.e., the destruction of foraminiferal tests; Berkeley *et al.*, 2007). Another possible limitation is that foraminifera can live down to 50 cm deep (or deeper) in the sediment/soil (e.g., Patterson *et al.*, 2004; Tobin *et al.*, 2005; Duchemin *et al.*, 2005; Milker *et al.*, 2015). This could influence the fossil assemblages if recent infaunal species lived deeper in the sediment. Most species are found at shallower depths and hence the impact of infaunal species should not be large, but this is nevertheless an important factor to acknowledge.

The OC stocks calculated using the restored saltmarsh sections of the cores from Freiston Shore, Brancaster West and Fingringhoe Wick varied widely depending on which cores (wide or narrow) and breach depth estimates were used (Table 2.1). This points to the limited usefulness of using foraminifera analysis alone to identify a breach depth that is then used as the basis for calculating a stock. A large reason for this variability, however, is elevation and accretion rate differences within a site and while radiometric dating might represent a more accurate means of identifying breach depth, its application could not overcome the issue of within-site variation without dating several cores from different location. However, the costliness of this analysis means this

approach is unlikely to be feasible and, therefore, a combination of different approaches that includes the foraminifera analysis applied in this study, could prove most effective.

2.6.2 Difference in OM composition between restored and natural saltmarshes

The clearest and most consistent difference between restored and natural marshes was the lower OC/N ratio values in the former (Table S2.3; Fig. 2.6), suggesting a difference in the sources of OM. Other studies have also found that restored saltmarshes had lower OC/N ratios than their natural counterparts and the differences were put down to a predominately allochthonous/marine input to the restored sites (Drexler *et al.*, 2020; Mueller *et al.*, 2020). While it is true that treating sediment samples with sulphurous acid can lead to losses of total N (Fernandes and Krull, 2008), if losses did occur and the N content of the samples was higher than recorded, this would only result in lower OC/N ratio values and thus further exacerbate the difference between natural and restored marshes.

Age

There were, however, exceptions to the tendency for lower OC/N ratios in restored sites. Clear peaks in OC, N and OC/N ratios were seen above and during the breach depth ranges of two younger sites: Brancaster West and Fingringhoe Wick. Peaks in OC content at breach depth were also identified in the sites investigated by Adams and colleagues (2012) and were assumed to represent the root mat of the agricultural fields present prior to restoration. The peaks identified in the Fingringhoe Wick cores could indicate the presence a similar layer but in Brancaster West it is likely that these peaks represent the terrestrial vegetation present prior to the breach. This vegetation was quickly killed by the reintroduction of the tide but was left in place to decompose and become buried in the accumulating sediment (Davy *et al.*, 2011). The large breach depth range applied to the Brancaster West cores meant that stocks were calculated that both excluded and included these non-halophyte OC peaks (Table 2.1). It can be argued that, had the area not been converted to saltmarsh, this OC would never have been buried in anoxic sediments and thus should be included in the stock calculation. The TGA analysis did not indicate that this terrestrial vegetation layer differed substantially, in terms of thermal stability, from natural marsh OM at a similar depth, meaning that its longer-term fate could be similar. The clearest conclusion to be drawn is that whether this layer is included in the calculation has a large impact on the magnitude of the stock, with a difference of almost 9 kg OC m⁻² between the two narrow core estimates (Table 2.1).

An alternative explanation for the OC and N peaks could be the large influx of OM shown to enter a marsh in the first ~five years following the creation of a breach, which necessarily comes from allochthonous sources because of the lack of saltmarsh plants in this early phase (Wollenburg *et al.*, 2018; Poppe and Rybczyk, 2021; Mossman *et al.*, 2022). However, the higher OC/N ratios of the peaks compared with the samples in the top 10 cm of the selected cores suggest this early OM was of a less strongly marine sources than the material entering the marsh later. In Aldboro Point, there were no peaks in OC and N during or near the breach but there was a clear peak in OC/N ratio

(Fig. 2.7a), suggesting that the OM had decomposed in the long time since the breach but that its origin was similar. The lack of OC peak is reflected in the relatively low stock estimates calculated for Aldboro Point, compared with those of the younger sites (Table 2.1). The higher OC/N ratios of restored marshes surface sediments could support the loss of the root layer by providing more nitrate to drive decomposition (Bulsecu *et al.*, 2019).

Elevation

The proportion of OM classed as recalcitrant was significantly higher in the top 10 cm of sediment from Brancaster West and Fingringhoe Wick compared with their natural equivalents (Table S2.3; Fig. 2.5). Often, labile OM content is higher in the surface sediments but decreases at lower depths due to the preferential decomposition of this material by microorganisms (Gore *et al.*, 2024). However, in these sites, the OM present in the surface sediments was thermally insensitive and so potentially more stable and complex. One possible explanation for this could be that a higher proportion of allochthonous OM entered the restored marshes. The labile component of this material could have been lost during decomposition in an external location before being washed into the restored sites. Alternatively, this OM could simply have been inherently more recalcitrant. Another plausible explanation is that both restored and natural marshes received broadly similar allochthonous inputs, particularly given their geographic proximity, with observed differences instead driven by site-specific factors such as elevation, hydrology, or sediment dynamics that influence OC retention. Elevation especially is a plausible driver because restored sites are often situated at lower elevation than their natural counterparts. Indeed, the plant communities present in Brancaster West and Fingringhoe Wick point to a low marsh setting that receives regular tidal inundation (Table S2.2).

Vegetation

In Fingringhoe Wick and Brancaster West *Spartina anglica* (70-90 %) was the only plant species present in the wide core sampling locations (Table S2.2) and the ^{13}C enriched signal of this C_4 plant explains the steadily increasing $\delta^{13}\text{C}$ values towards the surface of the cores (Fig. 2.6; Fig S2.5). Despite this, values were lower than the signal expected from this plant alone, which suggests a contribution from allochthonous material combined with the autochthonous *S. anglica* to produce a mixed signal (Middelburg *et al.*, 1997). Furthermore, most of the OC/N ratio values recorded for Fingringhoe Wick were below 10 (Fig. 2.6 & 2.7), the threshold reported for recently deposited marine OM (Hulisz *et al.*, 2013). This could indicate that allochthonous OM made a particularly large contribution to the stock of the youngest site, a possibility compatible with the fact that the area still largely resembles a mudflat (Fig. S2.1d).

Terrestrial plants potentially present before the breach have relatively low $\delta^{13}\text{C}$ values that are reported to range between -25.0 and -27.0 ‰ (Goni and Hedges, 1995) and sediment from agricultural fields has a mean value of -28.4 ± -2.17 ‰ (Smeaton *et al.*, 2022). A similar range was

observed within and above the breach depths of Brancaster West (-29.5 – -24.7 ‰ between the bottom and 20 cm depth; Fig. 2.6c; Fig. S2.5) and Fingringhoe Wick (-28.9 to -27.2 ‰ between 33 cm and 18 cm in FW23/2; Fig. 2.6d; Fig. S2.5). This, in combination with the higher OC/N values, corroborates that the peaks represented the vegetation and/or root mat of the agricultural fields and not OM entering the sites after restoration. In the case of Fingringhoe Wick, if the peak is assumed to represent the agricultural root mat, it would be expected to occur prior to the initial appearance of foraminifera in the cores. The fact that the peaks occur during the foraminifera identified breach depth range points to variability in sediment accretion rates and marsh development within the site. If the OC and N peaks in the Brancaster West cores are indeed representative of the pre-breach vegetation, their appearance at depths above the samples containing low numbers of foraminifera are to be expected, because sediment entering the site would initially settle around this vegetation and only gradually come to cover it completely.

Enrichment in ^{13}C with increasing depth was not seen in Aldboro Point and Freiston Shore (Fig. 2.6; Fig. S2.5). Values from the bottom of the breach depth ranges to the tops of the cores were relatively stable, ranging between -26.3 and -23.8 ‰ in the former and -24.9 and -21.9 ‰ in the latter. This implies that the expected changes to the OM components over time due to decomposition (Ehleringer *et al.*, 2000) had not occurred. This could mean the OM was relatively stable and had not broken down significantly. Alternatively, microbial processes could be occurring at certain depths that produce OC with a $\delta^{13}\text{C}$ signature that maintained the overall sediment $\delta^{13}\text{C}$ value despite changes to the OM from the surface (Goñi and Thomas, 2000). Most of the samples from Aldboro Point and Freiston Shore fell in-between the values reported for C_3 saltmarsh plant species and various marine OM sources, and this again points to a mixed signal. Therefore, Freiston Shore, a 21-year-old site, and Aldboro Point, a 102-year-old site, must also have received an input of allochthonous OM, despite plant communities that suggest a higher elevation setting compared with Brancaster West and Fingringhoe Wick (Table S2.2). The coring locations of the natural marshes were dominated by *A. portulacoides* and *P. maritima*, with *S. anglica* representing a minor contribution (<15 % cover) to the populations of one coring location within Gedney Marsh and another within Stiffkey Marsh (Ladd *et al.*, 2022). The presence of these C_3 plants explained the close similarity between the $\delta^{13}\text{C}$ profiles of the reference sites and those of Aldboro Point and Freiston Shore (Fig. 2.6a & 2.6b).

OM decomposition and stabilisation

OC, N and OC/N values were more clearly different between restored and natural sites in the top ~10 cm of the investigated cores. This is opposite to previously reported results (Havens *et al.*, 1995) and counterintuitive as newly accreting layers of marsh sediment often show signs of becoming increasingly “natural” because of vegetation modifying the surface sediment (Craft *et al.*, 1988; Craft *et al.*, 2002; Hulisz *et al.*, 2013). Lower OC/N ratios in saltmarshes can be put down to additional N being delivered by the tide and produced *in-situ* and/or a higher amount of algal biomass being

incorporated into the OM (Adams *et al.*, 2012). Except for Gedney Marsh, all natural reference marshes had OC/N ratios that decreased with depth (Fig. 2.6), indicating OM decomposition (Gebrehiwet *et al.*, 2008) and/or the incorporation of N from fungi and bacteria into the OM (Goñi and Thomas, 2000). The lower values of the surface sediments in the restored saltmarshes suggest that part of this signal was produced by OM entering the marshes that had already undergone this process of combination with fungal, bacterial or algal biomass. Another element that points to differences in the composition of the OM in restored site was the proportion of OC to OM (Fig. S2.8). Freiston Shore samples had lower values than those of the natural reference site throughout the cores while the other three sites had lower values primarily in the upper ~10 cm of sediment. Organic matter that is less OC rich could also be an indicator that the material in the restored sites has already undergone the initial stages of decomposition while the material in the natural sites was newer.

2.7 Conclusions

A total of thirty-two cores were taken from four restored saltmarshes in the east of England, ranging in age from eight to 102 years. Comparisons were made with the extensive natural marsh dataset developed by Smeaton and colleagues (2023; 2024). The most consistent difference between the restored sites compared with their natural counterparts was lower OC/N ratios in the former (Fig. 2.6; Tables S2.3 & S2.4). Furthermore, the proportions of recalcitrant OM tended to be higher in the top ~10 cm of restored site sediment than in the natural reference sites (Fig. 2.5; Table S2.3). These differences were interpreted as representing a greater input of marine, allochthonous and potentially partially decomposed OM to restored saltmarshes. Up to one hundred years after restoration, OC/N values had not recovered, which implies that the origin of OM remained different. Accordingly, determination of whether restored saltmarsh OC stocks represent CO₂ removal additional to that produced without the restoration is a crucial step in the carbon crediting process.

The vegetation and the root mat layers indicative of the agricultural field present prior to restoration were identified in two of the four sites from the OC/N ratio and the $\delta^{13}\text{C}$ signature. Based upon the breach depth determined with the foraminifera method, the OC peaks produced by the terrestrial vegetation and root mats were included in some of the stock calculations and thus led to far higher values than when they were excluded. Where deemed appropriate, caution should be taken to identify and discount this OC when stocks are being calculated for carbon crediting schemes. Conversely, pre-breach vegetation could represent a legitimate contribution to the OC stock because it would not have become buried in an anoxic sediment layer without the creation of the breach.

If the foraminifera method tested in this study is to be applied elsewhere it is vital to consider and account for within site variability by analysing several cores taken from locations widely dispersed across the site. Furthermore, deep cores are often necessary to reach the breach horizon because, despite a time lag between breach and colonisation, small numbers of tests can be washed onto a marsh immediately after tidal reintroduction. Additionally, defining zones, such as high marsh,

low marsh and tidal flat, based upon foraminiferal assemblage data from restored marshes is not necessarily an accurate means of tracking the development of a site over time because the difference may not extend beyond the highly sensitive foraminifera. Furthermore, in this study, the radiometric dating tools were very useful for identifying breach depth and may be more suitable if the goal is calculating accurate OC. However, to deal with variability within a site, a combination of methods, including the foraminifera analysis, is likely to be the best approach.

2.8 Supplementary Information

2.8.1 Supplementary tables

Table S2.1. Sampling date, latitude, longitude and this distance from the nearest breach of the cores taken by this study.

Marsh	Core ID	Sampling Date	Latitude	Longitude	Distance from nearest breach (m)
Aldboro Point	AP23/1	04/08/2023	51.834533	0.987312	50
	AP23/2		51.833077	0.986191	57
	AP23/3		51.834021	0.987137	103
	AP23/4		51.833562	0.986089	16
	AP23/5		51.833293	0.986758	68
	AP23/6		51.832377	0.986017	44
	AP23/7		51.831541	0.985929	76
	AP23/8		51.830220	0.985362	64
Freiston Shore	FS23/1	15/06/2023	52.970278	0.095722	376
	FS23/2		52.964833	0.095111	150
	FS23/3		52.969361	0.096861	248
	FS23/4		52.970361	0.094361	446
	FS23/5		52.967194	0.092111	480
	FS23/6		52.965056	0.093944	226
	FS23/7		52.963917	0.090500	379
	FS23/8		52.961944	0.090278	231
Brancaaster West	BW23/1	03/08/2023	52.971754	0.633353	290
	BW23/2		52.971694	0.630778	264
	BW23/3	05/06/2023	52.972167	0.631694	190
	BW23/4		52.971795	0.630168	79
	BW23/5		52.972306	0.633139	287
	BW23/6		52.971722	0.631611	173
	BW23/7		52.971583	0.630083	70
	BW23/8		52.972000	0.632889	263
Fingringhoe Wick	FW23/1	20/06/2023	51.839889	0.977083	154
	FW23/2		51.842139	0.972972	280
	FW23/3		51.839056	0.976953	245
	FW23/4		51.838556	0.976417	293
	FW23/5		51.839472	0.973806	267
	FW23/6		51.843167	0.972750	341
	FW23/7		51.843833	0.972278	415
	FW23/8		51.845389	0.973417	510

Table S2.2. Percentage cover of the plant species within the 1 m² areas surrounding the coring locations. AP - *Atriplex portulacoides*; AT - *Aster tripolium*; LV - *Limonium vulgare*; S – *Salicornia* spp.; SA - *Spartina anglica*; PM - *Puccinellia maritima*; TM - *Triglochin maritima*; BM – bare mud.

Core ID	AP (%)	AT (%)	LV (%)	S (%)	SA (%)	PM (%)	TM (%)	BM (%)
AP23/1	40	0	10	0	0	50	0	0
AP23/2	0	0	0	0	30	70	0	0
AP23/3	0	0	20	0	0	70	0	10
AP23/4	98	0	0	0	0	2	0	0
AP23/5	0	0	2	0	45	45	0	0
AP23/6	90	0	0	0	0	10	0	0
AP23/7	1	0	20	0	0	79	0	0
AP23/8	0	0	5	0	5	80	0	10
FS23/1	90	2	0	0	0	0	0	8
FS23/2	90	0	1	0	5	0	0	4
FS23/3	10	0	10	0	0	80	0	0
FS23/4	80	0	0	0	5	0	0	15
FS23/5	0	0	10	0	5	80	5	0
FS23/6	90	0	5	0	0	0	0	5
FS23/7	15	0	10	70	0	0	0	5
FS23/8	10	0	0	0	60	10	0	20
BW23/1	0	0	0	0	80	0	0	20
BW23/2	0	0	0	0	80	0	0	20
BW23/3	90	5	0	0	0	0	0	5
BW23/4	90	0	5	0	0	0	0	5
BW23/5	80	0	10	5	0	0	0	5
BW23/6	0	0	5	0	40	0	0	55
BW23/7	10	0	0	30	20	0	0	40
BW23/8	10	0	25	0	20	15	10	20
FW23/1	0	0	0	0	90	0	0	10
FW23/2	0	0	0	0	70	0	0	30
FW23/3	0	0	0	0	90	0	0	10
FW23/4	0	0	0	40	40	0	0	20
FW23/5	60	0	0	20	0	0	0	20
FW23/6	0	0	0	30	0	0	0	70
FW23/7	0	0	0	20	5	0	0	75
FW23/8	0	0	0	0	25	0	0	75

Table S2.3. Results of linear mixed effects models that tested the impact of marsh type (i.e., restored or natural), depth and the interaction between the two upon the key variables investigated by this study. All numerator degrees of freedom = 1. Denominator degrees of freedom were estimated using the Satterthwaite method.

Variable	Factor	<i>p</i>-value	Denominator <i>df</i>.	<i>F</i>-value
% OC	Marsh type	0.170	5.900	2.445
	Depth	<0.001	764.170	238.176
	Interaction	<0.001	764.170	30.735
% N	Marsh type	0.202	6.090	2.041
	Depth	<0.001	587.080	392.538
	Interaction	<0.001	587.080	47.600
Proportion of recalcitrant OM	Marsh type	0.047	6.030	6.218
	Depth	<0.001	597.220	168.243
	Interaction	<0.001	597.220	60.463
OC/N Ratio	Marsh type	0.014	6.070	11.733
	Depth	0.409	612.830	0.682
	Interaction	0.003	612.830	8.873

Table S2.4. Summary of the variables identified that differ between restored and natural saltmarshes that could be useful as indicators of restoration.

Factor	Natural marsh conditions	Restored marsh conditions
OC and N content	Values are lowest at the bottom of the core and increase at higher depths, peaking at the surface – this is due to OM decomposition.	A peak in values is seen during or soon above the restoration horizon and after this OC and N content fall to levels like those seen pre-restoration.
Ratio of recalcitrant OM	Labile OM content increases up the cores because a high level initially enters the sediment, but this is quickly broken down.	Soon after the restoration horizon, there is sometimes an influx of labile OM. The ratio remains very stable up the core, suggesting stabilisation happens through mineral association as in uplands.
OC/N ratio	Average across all natural marsh samples is 14.30 ± 1.75 .	Average across all restored marsh samples is 10.52 ± 2.65 .

2.8.2 Supplementary figures



Figure S2.1. Photographs of (a) Aldboro Point, (b) Freiston Shore, (c) Brancaster West and (d) Fingringhoe Wick, the sampling locations of this study.

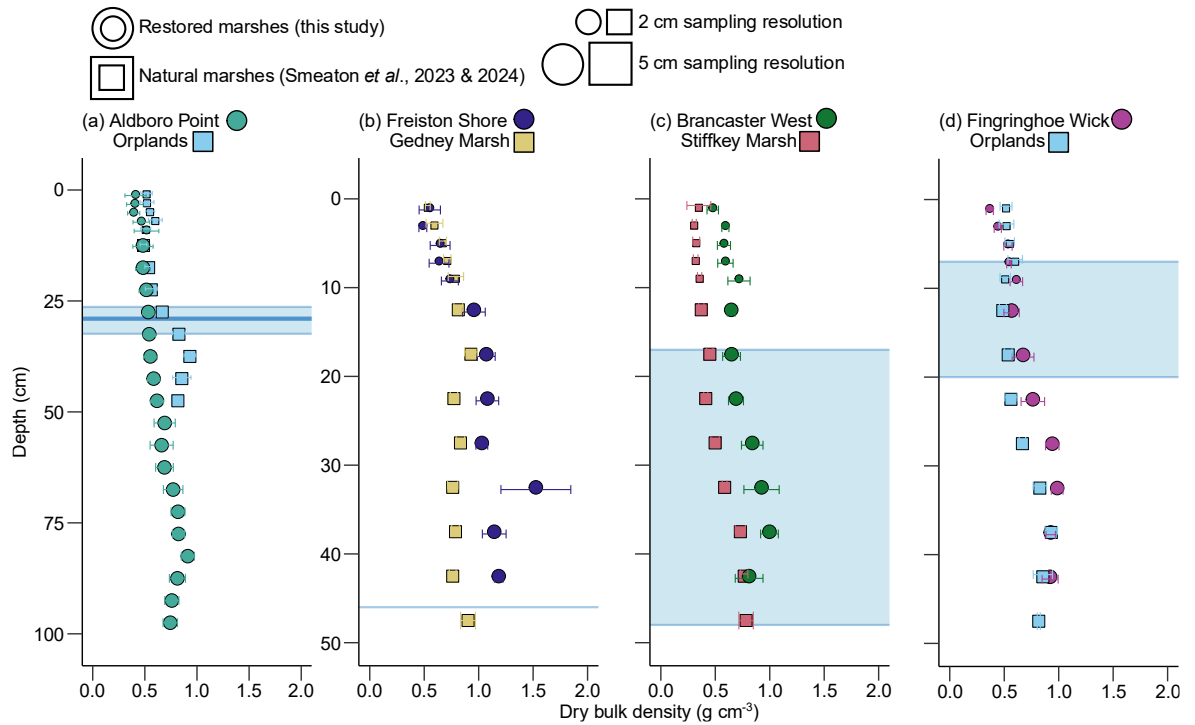


Figure S2.2. Mean dry bulk density in the narrow diameter cores and the corresponding natural marsh reference sites: (a) Aldboro Point and Orplands, (b) Freiston Shore and Gedney Marsh, (c) Brancaster West and Stiffkey Marsh and (d) Fingringhoe Wick and Orplands. Restored marsh datapoints are always represented by circles while natural marsh data, collected by Smeaton *et al.* (2023; 2024), are represented by squares. Smaller symbols represent 2 cm samples while larger ones represent 5 cm samples. The shaded blue areas denote the depth range at which the breaches may have occurred and is based upon radiometric dating for Aldboro Point and foraminiferal analysis for the other three sites. Additionally, the breach depth range for Brancaster West was expanded based upon the OC and N data presented in Figure 2.4.

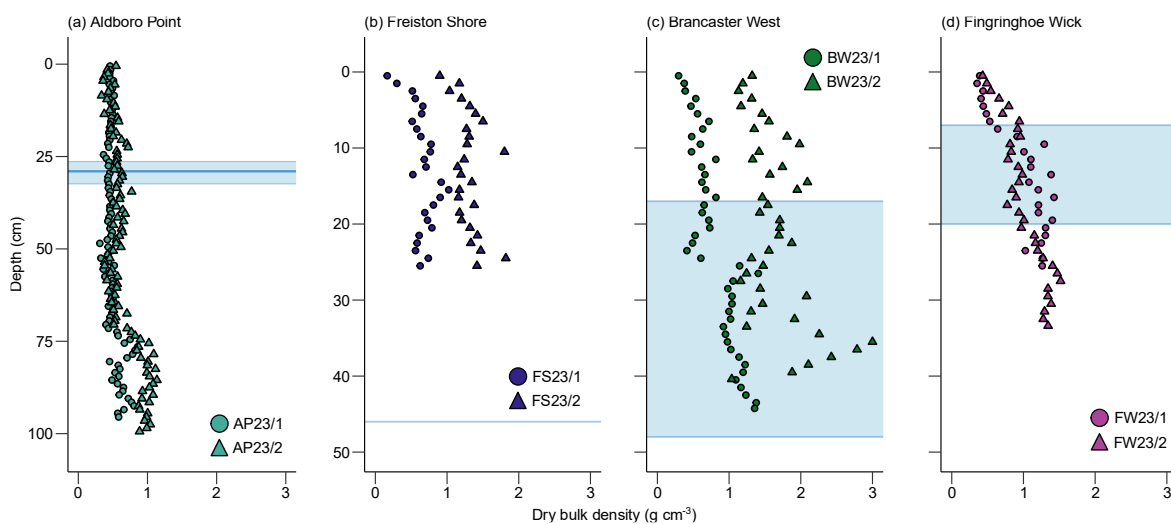


Figure S2.3. The dry bulk densities of each centimetre of the wide cores taken from (a) Aldboro Point, (b) Freiston Shore, (c) Brancaster West and (d) Fingringhoe Wick. The results from different cores are distinguished by different shapes.

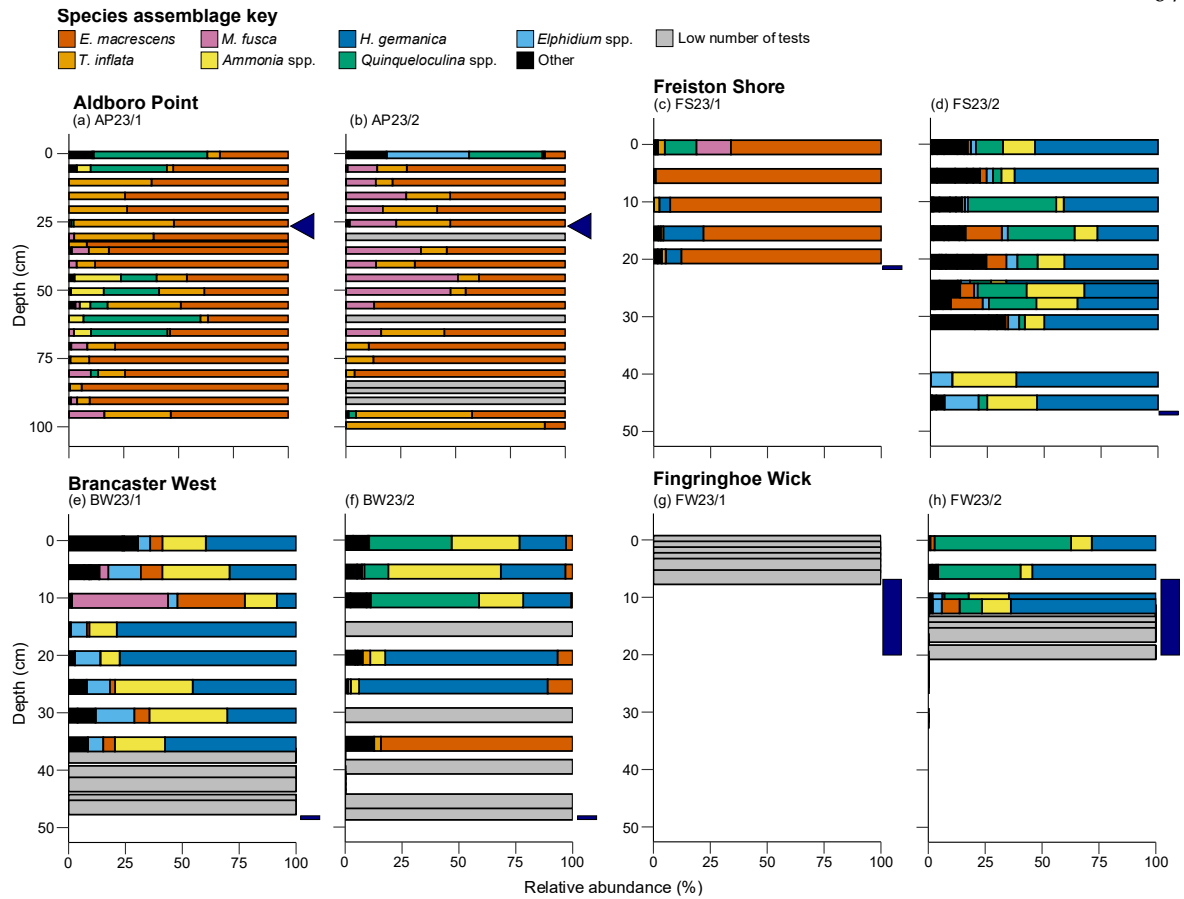


Figure S2.4. The relative abundances of the seven most abundant species in each centimetre of sediment processed from the two wide-diameter cores taken from (a-b) Aldboro Point, (c-d) Freiston Shore, (e-f) Brancaster West and (g-h) Fingringhoe Wick. All other species were lumped together and classed as “Other”. The dark blue triangles/boxes represent the depth range in which the breaches could have occurred. This was based upon the age depth model (Fig. 2.2) for Aldboro Point and upon the foraminifera results for the other sites (details are discussed separately for each site within section 2.5.2). In the Aldboro Point panels, the thick line shows the depth estimated from the mean ages while the two thin lines show the depths calculated using the minimum and maximum ages (i.e., 2 σ uncertainties).

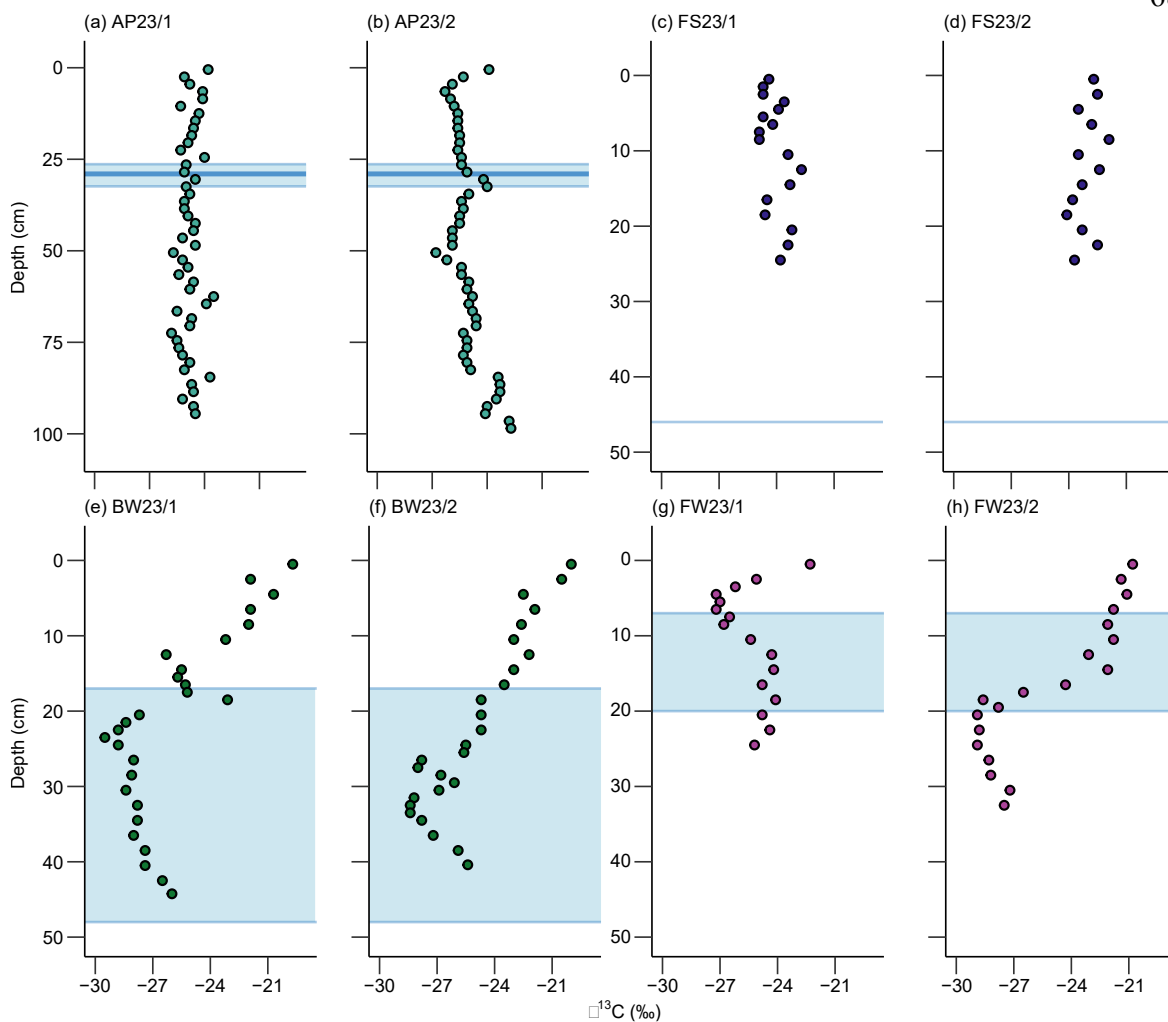


Figure S2.5. $\delta^{13}\text{C}$ signatures in the wide diameter cores taken from (a & b) Aldboro Point, (c & d) Freiston Shore, (e & f) Brancaster West and (g & h) Fingringhoe Wick. The shaded blue areas denote the depth range at which the breaches may have occurred.

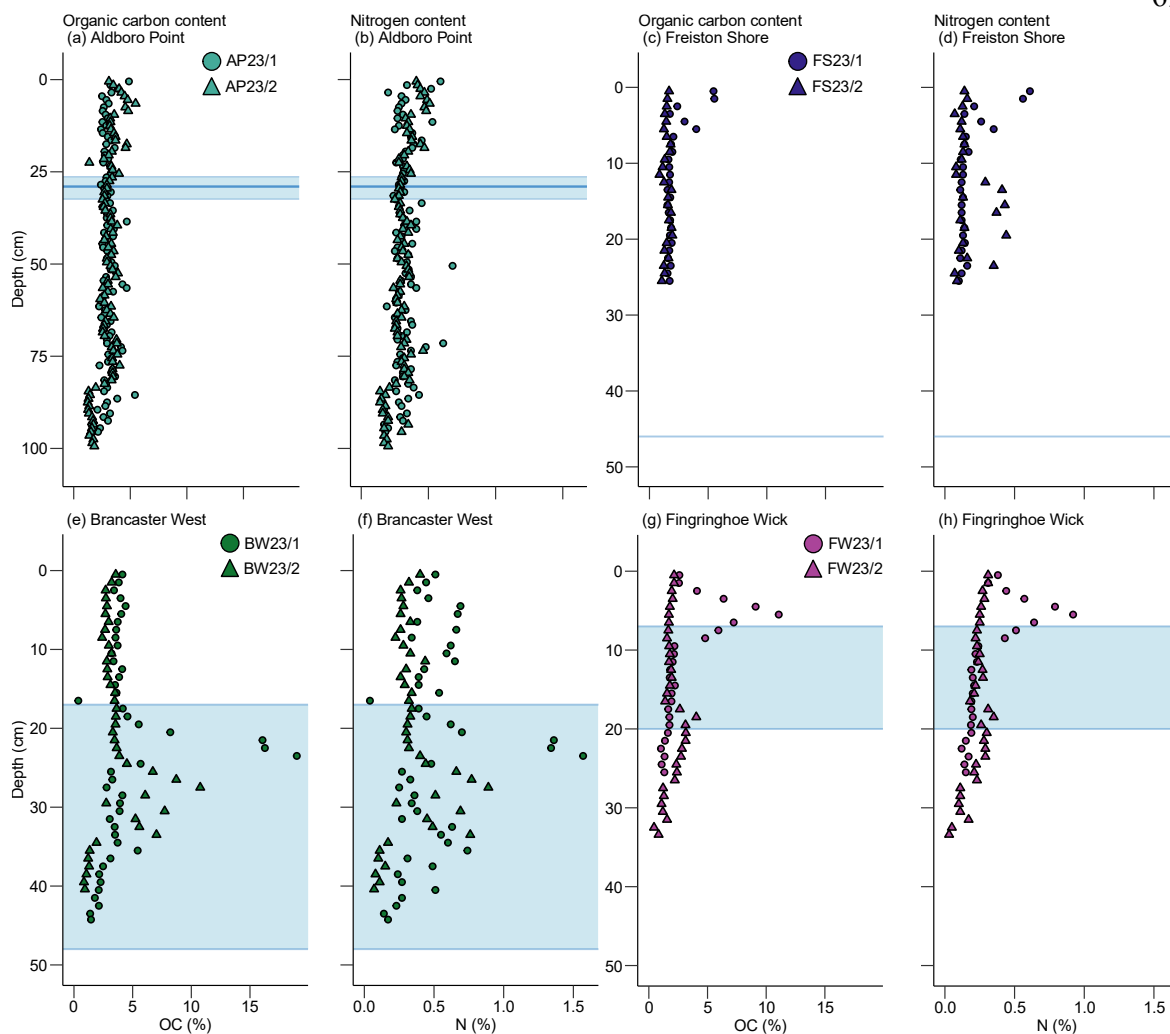


Figure S2.6. The OC and N content of each centimetre of the wide cores taken from (a) Aldboro Point, (b) Freiston Shore, (c) Brancaster West and (d) Fingringhoe Wick. The results from different cores are distinguished by different shapes.

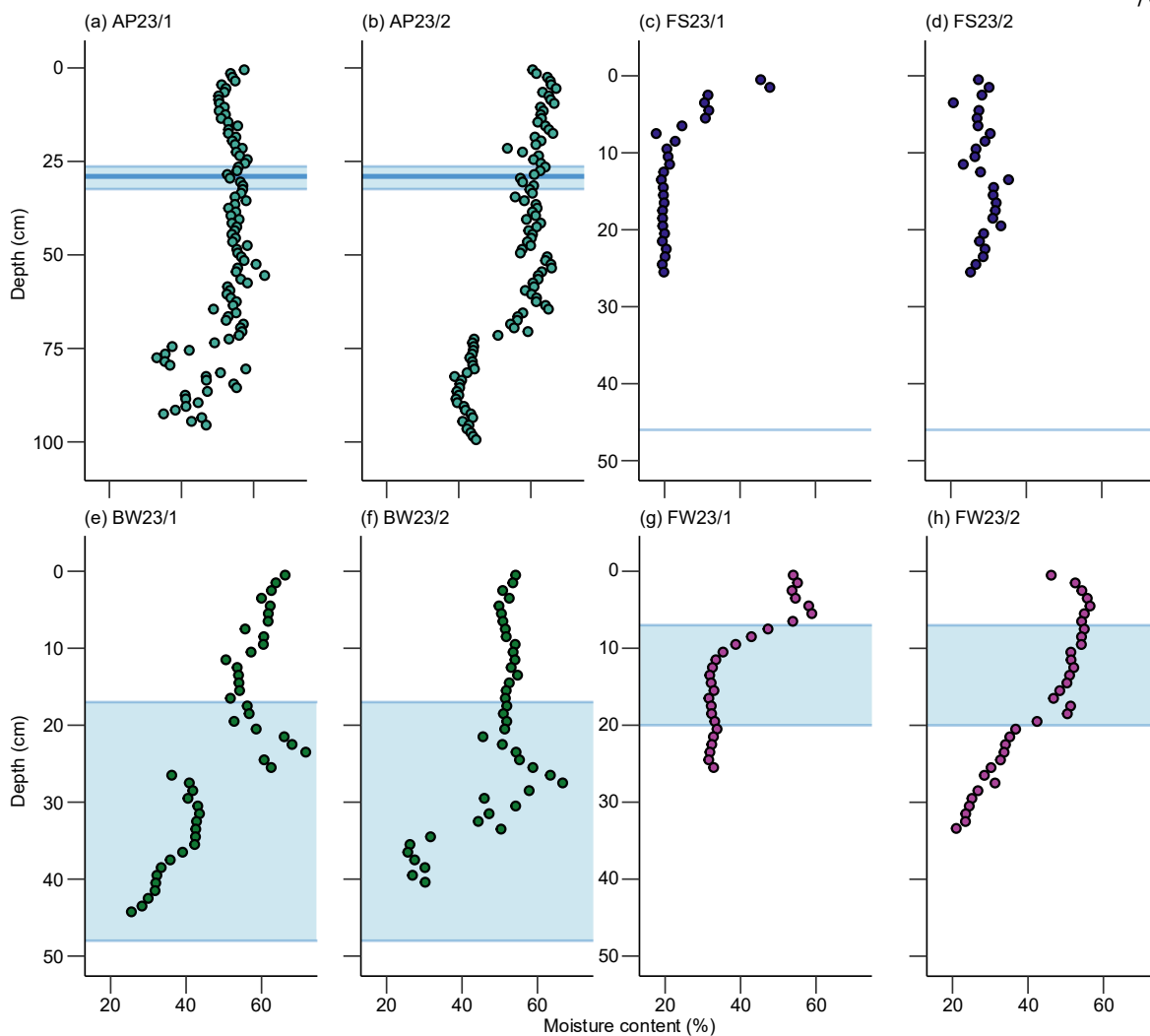


Figure S2.7. Moisture content of the wide diameter cores taken from (a & b) Aldboro Point, (c & d) Freiston Shore, (e & f) Brancaster West and (g & h) Fingringhoe Wick. The shaded blue areas denote the depth range at which the breaches may have occurred.

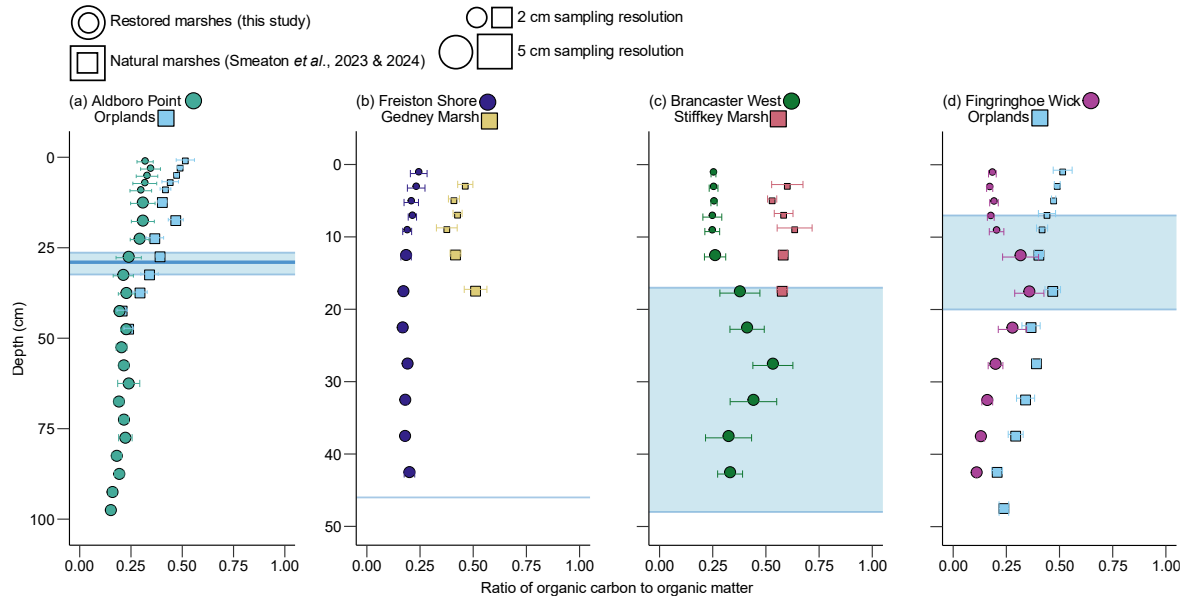


Figure S2.8. Proportion of total organic matter content that is organic carbon in the narrow cores taken from (a & b) Aldboro Point, (c & d) Freiston Shore, (e & f) Brancaster West and (g & h) Fingringhoe Wick. Restored marsh datapoints are always represented by circles while natural marsh data, collected by Smeaton *et al.* (2023; 2024) are represented by squares. Smaller symbols represent 2 cm samples while larger ones represent 5 cm samples. The shaded blue areas denote the depth range at which the breaches may have occurred and apply only to the restored saltmarshes, not the natural marshes. These ranges are based upon radiometric dating for Aldboro Point and foraminiferal analysis for the other three sites. Additionally, the breach depth range for Brancaster West was expanded based upon the OC and N data presented in Figure 2.4.

8.3 Detailed descriptions of foraminiferal species assemblages

Aldboro Point

Almost every sample examined from the Aldboro Point cores had assemblages overwhelmingly dominated by the agglutinated species of foraminifera *Entzia macrescens* (Fig. S2.4a & S2.4b). The cores differed, however, in terms of the less abundant species. Between 65 and 66 cm in AP23/1, the calcareous *Quinqueloculina* spp. were one of the most abundant in the sample (0.35), having only been present in another sample prior to this (Fig. S2.4a). The *Quinqueloculina* spp. population increased further in the sample from 60–61 cm, meaning that this was one of the few samples in these cores in which calcareous species predominate. This species was also present in the next three samples but was slightly less abundant before disappearing entirely until the top two samples (5–6 and 0–1 cm). Between the disappearance and reappearance of *Quinqueloculina* spp. and during the interval in which the breach was thought to have occurred, the agglutinated species *Trochammina inflata* became the second most abundant, having been present in low amounts throughout the lower core depths. The main differences in the assemblages of AP23/2 were the absence of the peak in

calcareous species around 60 cm and the relatively high numbers of the agglutinated species *Miliammina fusca* between 66 cm and 5 cm (Fig. S2.4b). The uppermost sample of this core was predominated by the calcareous *Quinqueloculina* spp. and *Elphidium* spp., which was almost entirely absent from AP23/1. There was no notable change in the assemblages in AP23/2 around the depths at which the breach was thought to occur (Fig. S2.4b).

Freiston Shore

Similarly to Aldboro Point, the agglutinated populations in FS23/1 consisted almost entirely of *E. macrescens* (Fig. S2.4c). At lower depths (i.e., 19-20, 15-16 and 10-11 cm), the second most abundant species was the calcareous *Haynesina germanica* while at the surface *M. fusca* and *Quinqueloculina* spp. were the second and third most abundant, respectively (Fig. S2.4c). In the samples from FS23/2, *H. germanica* and *Ammonia* spp. were present in every sample, with the former generally being the most abundant species (Fig. S2.4d). Between 27-28 cm depth, *Quinqueloculina* spp. reached higher levels of abundance (>10 %) in several samples and between 28 and 15 cm higher abundances (6 to 16 %) of *E. macrescens* were seen.

Brancaster West

BW23/1 contained a relatively straightforward assemblage compositions in which the calcareous *H. germanica* and *Ammonia* spp. predominated in all but one of the samples (Fig. S2.4e). The noteworthy exception was between 10 and 11 cm depth: agglutinated *M. fusca* and *E. macrescens* predominate in this sample and this coincided with a fall in test density to 20 tests per g (Fig. S2.4e). The dense population of foraminifera found in BW23/2 at a depth of 36-37 cm consisted almost entirely of the agglutinated species *E. macrescens* (relative abundance – 84 %; Fig. S2.4f). The remaining samples from higher up the core contained predominantly calcareous species at far lower densities. There were, however, clear differences in the species compositions of the calcareous assemblages at higher depths. Populations in samples between 25- 26 cm and 20-21 cm were overwhelmingly predominated by *H. germanica* while further up the core (10-11, 5-6 and 0-1 cm), there were high quantities of *Ammonia* spp., *Quinqueloculina* spp. and *H. germanica* (Fig. S2.4f).

Fingringhoe Wick

The assemblages in the samples from FW23/2 that contained a high number of foraminifera were initially predominated by *H. germanica*, but the abundance of this species decreased up the core and was replaced by an increasing quantity of *Quinqueloculina* spp. (Fig. S2.4g). *Ammonia* spp. remained at a similar level of abundance at all depths.

Chapter Three

Storage temperature and diluent cause inconsistencies in commonly used fluorometric microplate enzyme assays conducted with saltmarsh soils

3.1 Abstract

Fluorometric enzyme assays are a tool for determining potential enzyme activity in soils. A wide array of methodological approaches is adopted by different studies, making comparisons difficult. An important consideration is whether assay conditions replicate the environment of the samples or optimise potential enzyme activity. Related is whether buffer or distilled water is used as a diluent when creating the soil slurry used in the assay. Buffer is favoured for maintaining a stable pH while using distilled water more closely replicates environmental conditions by allowing the sample to regulate its own pH. Furthermore, sample storage before and after slurry creation is a factor that varies widely. Using soil from two saltmarshes, highly dynamic ecosystems experiencing extreme conditions such as waterlogged soils and high salinity, I compared the impacts of creating slurries with phosphate buffer, distilled water and saline saltmarsh creek water and of storing slurries at 4°C and -20°C before analysis. Assay outcomes were highly sensitive to these pre-assay treatments and that sensitivity was large enough to affect relative comparisons among samples. There were notable differences between diluents that could driver these outcomes. First, pH fluctuations occurred when buffer was not the diluent. Second, the actual pH of the assays differed by as much as 1.04. Third, when pH-dependent 4-methylumbelliferone was the fluorophore, quench effects were greater in distilled water controls than in assays with soil particles present because of pH differences arising in an unbuffered system. To conclude, the methodological differences tested here could compromise comparability among studies.

3.2 Introduction

Measuring extracellular enzyme activity provides a proxy for potential enzymatic reaction kinetics occurring in soils, enabling quantification and comparison between contrasting conditions. Soil enzyme assays have been conducted for over 50 years (German *et al.*, 2011) and are used to investigate the impacts of several different variables upon soil functioning, including questions as diverse as the impact of sea-level rise on wetland carbon stocks (Yang *et al.*, 2022) and the consequences of anthropogenic nutrient addition to different environments (Jing *et al.*, 2017; Qin *et al.*, 2024). These studies highlight that an ability to quantify soil microbial enzyme activity is especially important given the potential impacts of climate change and pollution upon soil functioning. More broadly, extracellular enzymes are majorly important in carbon and nutrient cycling, with implications for global organic matter storage (Sinsabaugh *et al.*, 2008). The fluorogenic microplate method is based upon using the fluorescence produced when fluorescent molecules bound to substrates are cleaved apart by the enzymes present in a sample to quantify the activity level of those enzymes (Pancholy and Lund, 1972; Freeman *et al.*, 1995). More fluorescence is indicative of greater enzyme activity (Foreman *et al.*, 1998). This is a commonly used assay, and an appealing choice compared with colorimetric assays (Dick *et al.*, 2018) because it is highly sensitive (Deng *et al.*, 2013). Furthermore, a high number of samples can be processed rapidly with a small amount of soil and there is the option of assuming a linear relationship between time and

fluorescence that allows one measurement to be taken at a single time point (Nannipieri *et al.*, 2012; Verlaine *et al.*, 2024). However, despite, or perhaps because, of the myriad different possible applications of this method, many inconsistencies in how the technique is applied have been highlighted (Deng *et al.*, 2017; Nannipieri *et al.*, 2018).

A previous effort to clarify the best approach to enzyme assays made a series of recommendations (German *et al.*, 2011). However, there remains a fundamental question of whether experiments should be designed with the goal of replicating the environmental conditions of the sample or of optimising the assay to allow the maximum possible rate of enzyme activity to be recorded. This difference makes it challenging to compare results across studies that use opposing approaches, but it could also mean that the results within individual studies are misleading (Nannipieri *et al.*, 2018). Some of the key parameters in sample collection and laboratory protocols are how and for how long samples are stored before analysis, whether buffer is used to create soil slurries to prevent pH fluctuations and, if buffer is employed, what pH is selected. Commonly used buffers include acetate, phosphate and sodium bicarbonate (e.g., Ross, 1987; Bandick and Dick, 1999; Sinsabaugh *et al.*, 2003; Sinsabaugh *et al.*, 2009).

The first step towards conducting enzyme assays after sample collection is the preparation of a slurry containing a certain mass of soil to a volume of a particular diluent (Saiya-Cork *et al.*, 2002). Often the diluent in question is a buffer, which allows the pH of the slurry and by extension, the assay to be controlled, preventing pH fluctuations that could impact the results. Therefore, the choice of buffer is one of the key determinants of whether the study is aiming to maximise the activity of the enzyme or trying to mimic environmentally relevant conditions (e.g., Sinsabaugh *et al.*, 2000; Yavitt *et al.*, 2003; Stursova *et al.*, 2006; Li *et al.*, 2009; Wallenstein *et al.*, 2009; Sun and Chen, 2024; Wang *et al.*, 2024). In addition to buffers, previous studies have used distilled water as a diluent in a further effort to replicate environmental conditions by allowing the soil alone to regulate the pH (Sinsabaugh *et al.*, 2000; Chaperon and Sauvé, 2007; Chaer *et al.*, 2009; Lessard *et al.*, 2013). Often this is done when the study involves testing the impact of metal toxicity in some capacity as commonly used buffers can alter results by, for example, chelating or changing the speciation of metal ions (Dussault *et al.*, 2008; Forero *et al.*, 2023). However, studies with goals unrelated to metal toxicity also use distilled water as a diluent in place of buffer (e.g., Caldwell *et al.* 1999) because when comparing samples, sites or treatments with variable soil pH choosing a buffer that can be used for all soils is difficult and using different buffers would invalidate comparisons between samples. There also exist cases in which distilled water was used to create the slurries, but buffer was pre-pipetted onto the microplate wells before running the assay (Marx *et al.*, 2001; Neubauer *et al.*, 2013). This emphasises the many inconsistencies that exist between the different methodologies applied in experiments aiming to measure the same things. Turner and colleagues (2010) have shown that the pH optimum of the same enzyme can differ between environments, further complicating matters. The next step in the bid to closely reproduce *in-situ* conditions is to use a diluent sourced from the

study site itself but, to my knowledge, this has not yet been attempted. This method could be especially applicable to soils experiencing extreme conditions, such as a saline or waterlogged soil environment.

Practical considerations when conducting fieldwork to collect samples that must be analysed in the laboratory include sample storage strategies to ensure the best possible preservation under the circumstances in which they are operating (Peoples and Koide, 2012). Often, studies give no or very little detail of how and for how long samples are stored before analyses. For example, approaches include preparing slurries on the day of the enzyme assay analysis, conducting assays within a week of sampling, merely stating that “fresh” soil was used to create slurries or that “immediate processing” was employed, or, finally, freezing fresh soil for an indeterminate length of time before analysis (Stursova *et al.*, 2006; Wallenstein *et al.*, 2009; Morrissey *et al.*, 2014a and 2014b; Tang *et al.*, 2020). To determine the storage method that provides the most accurate results, Lee and colleagues (2007) compared results obtained from commonly used techniques with those obtained from fresh samples and concluded, for enzyme assays, that storage at either 4 °C or -20 °C prior to analysis was acceptable as these treatments altered enzyme activities least. However, another study showed that different enzymes (e.g., N-Acetyl-glucosaminidase, phosphatase and phenol oxidase) were more sensitive than others to storage temperature and duration (DeForest, 2009). Conversely, Lane and co-workers (2022) recently found that freezing did not significantly affect the results of their enzyme assays. These contrasting results merit further investigation because of the possibility of interaction effects with the other factors involved in enzyme assays (Liu *et al.*, 2026b), such as buffer selection and pH changes. Furthermore, the potential usefulness of being able to freeze samples ahead of analyses with confidence in the fact that they will remain unaffected by the process highlights the importance of the question.

For the present methodological comparison, soil samples were collected from a saltmarsh. Saltmarshes are highly dynamic vegetated coastal ecosystems at the interface of the marine and the terrestrial environment. Their soils experience extreme conditions because regular tidal inundation brings an influx of salt water, creating a saline environment (Vernberg *et al.*, 1993). Beyond the issue of assay pH discussed above, conducting assays under salinity conditions that differ substantially from those of the native environment represents another major departure from *in-situ* conditions when the goal is to replicate the environmental context as closely as possible. Saltmarshes provide a means by which to investigate this problem, which could be overcome by using saline water from the site itself as a diluent. The relevance of these ecosystems to this study is further highlighted by the recent uptick in extracellular enzyme activity studies conducted with saltmarsh soils (e.g., Duarte *et al.*, 2012; Liu *et al.*, 2021; Tang *et al.*, 2021) because of their capacity for high rates of carbon accumulation (Smeaton *et al.*, 2024).

Here I compared different methodological approaches to assess the variability or potential inconsistencies introduced by diluent and storage choices. The same soil samples were subject to

preparation with different diluents (phosphate buffer, saline creek water and distilled water) and two soil slurry storage treatments (4 °C for a week and -20 °C for two weeks) prior to analysis. I used samples from two saltmarsh sites for the experiment. Assay pH was recorded at the beginning and end of the experiment. I expected that (1) storage and dilution conditions would significantly alter the measured activities owing to (2) unbuffered samples undergoing large pH fluctuations between the beginning and the end of the assay and (3) the freeze-thaw process and/or storage duration altering soil and enzyme dynamics. This study begins to elucidate the best practices for enzyme assays using saltmarsh soils, an ecosystem of growing interest.

3.3 Methodology

3.3.1 Study site

Fingringhoe Wick Nature Discovery Park in Essex is located approximately 12 km southeast of Colchester in the east of England. This site contains both a natural established saltmarsh (51.837373, 0.977097) and a restored marsh (51.842106, 0.974856), the two sampling sites of the study (Fig. 3.1a). The restored marsh is an area of former agricultural land to which tidal inundation was reintroduced in 2015 by creating two breaches in the seawall (Vaughan, 2019; Cai *et al.*, 2022). The natural marsh is densely vegetated while the restored site largely resembles a mudflat with vegetation growing only on the higher elevation fringes and seawall banks. Four sampling stations were established at the study site, two situated on the natural marsh (N3 and N5; Fig. 3.1b & 3.1c) and two on vegetated fringes of the restored marsh (R2 and R5; Fig. 3.1d & 3.1e).



Figure 3.1. The (a) saltmarsh study sites in Fingringhoe Wick Nature Discovery Park. Two sampling station, (b) N3 and (c) N5, were located on the natural marsh and another two, (d) R2 and (e) R5, on the recently restored marsh. Map imagery © Microsoft Bing Maps; satellite imagery © DigitalGlobe (Maxar), 2020.

3.3.2 Sample collection

On the 8th of November 2024, 10 cm² and 1 cm deep soil samples were cut with a bent spatula (Royal Eijkelkamp) from the surfaces of the four sampling stations. The spatula was wiped with blue roll

between stations. This was done twice: one to remove adhering soil and a second time with a fresh piece of blue roll. The spatula was not cleaned with ethanol. Soil samples were transported back to the laboratory and used to create soil slurries within 72 hours of collection. Additionally, water was collected from a creek crossing the natural marsh to use as a diluent. The creek water was stored at 4 °C and any sediment allowed to settle to the bottom until it was used further.

3.3.3 Determination of soil properties and plant biomass

To characterise the four sampling sites (Table S3.1), subsamples of soil of a known volume (25 cm³), extracted and measured with a modified syringe, (i.e., with the top part sawed off), were taken from each sampling station and freeze dried. These samples were first weighed to determine the dry weight, enabling the calculation of dry bulk density (mass of the dried soil divided by the volume of soil). Following acidification with sulphurous acid (6 %) to remove carbonates and a second freeze drying, samples were homogenised with a ball mill and organic carbon (OC) and nitrogen (N) content were determined by elemental analysis with a CE440 elemental analyser (Exeter Analytical). The same dried soil samples were used to determine total organic matter, labile organic matter and recalcitrant organic matter content with a TRIOS SDT 650, with the samples heated from 40 °C up to 1000 °C under a constant stream of N₂ (Smeaton and Austin, 2022). Classification of organic matter followed Smeaton and Austin (2022), with temperature ranges of 200 °C–400 °C for labile and 400 °C–650 °C for recalcitrant organic matter, and total organic matter represented by the entirety of this range.

Plant aboveground biomass was determined by harvesting all the vegetation in a 20 cm² area within each 1 m² sampling station, freeze drying and weighing the plants. Belowground plant biomass was assessed by taking 20 cm long - to capture the full root zone (Redelstein *et al.*, 2013) - and 5.7 cm diameter cores from each station and, following Smeaton *et al.* (2023) shaking the samples in 5 % sodium hexametaphosphate for 3 hours and then washing away the remaining soil through a 500 µm sieve. The remaining roots were then freeze dried and weighed. Finally, soil pH was measured in each sampling station with a Hanna pH Checker Plus.

3.3.4 Soil slurry preparation and storage

To make soil slurries, 4.5 g of soil were diluted (i.e., combined) with 50 mL of either 0.05 M, pH 7.4, phosphate buffer, distilled water or the creek water collected from the saltmarsh. Saltmarsh creek water was not filtered or heated before use, and I acknowledge that this meant introducing additional extracellular enzymes and microbes to these samples. The mean pH of the sampling stations was 6.8 ± 0.32 (Table S3.1). Phosphate buffer was chosen because a preliminary experiment showed that this choice would result in the least pH fluctuations between the beginning and end of an assay (0.02 ± 0.01 pH units), compared with other commonly used buffers such as bicarbonate (0.71 ± 0.06 pH units) and HEPES (0.18 ± 0.02 pH units; Fig. S3.1). Slurries were shaken by hand for 30 seconds to mix. Using the same soil sample collected from each sampling station, two slurries each were made

with the three types of diluents listed above. One from each of these sets was stored at 4 °C and assayed after a week of storage and the others were stored at -20 °C and assayed after two weeks.

3.3.5 Enzyme assays

The fluorogenic microplate enzyme assay method used here followed German *et al.* (2011), Mueller *et al.* (2017) and Mueller *et al.* (2020). Pre-pipetted into black Sterilin 96-well plates (611F96BK) was 50 μL of either the diluent used in the samples being run in a particular plate (i.e., phosphate buffer, saltmarsh creek water or distilled water), 100 $\mu\text{mol L}^{-1}$ fluorophore standard or 1.6 mmol L^{-1} substrate solution. Preliminary experiments showed this substrate solution concentration created saturating conditions. Separate standard curves were not generated for each diluent and fluorophore combination, and instead the single fluorophore standard concentration already noted was always used. Once all pre-pipetting was complete, either 200 μL of the soil slurry or 200 μL of the diluent in question was added. Plates were not randomised. Each soil slurry had eight technical replicates. Plates were then left to incubate in the dark for 16 h before fluorescence was recorded with a SpectraMax iD5 Microplate Reader (Molecular Devices) with an excitation value of 365 nm and an emission value of 460 nm. This incubation time was consistent with the method of previous studies of saltmarsh soil extracellular enzymes (Mueller *et al.*, 2017; Mueller *et al.*, 2020). Assays were conducted for β -glucosidase (EC 3.2.1.21), chitinase (EC 3.2.1.14), cellobiosidase (EC 3.2.1.91) and phosphatase (EC 3.1.3.1) using different substrates all conjugated to the fluorophore 4-methylumbelliferone (4-MUF) and for leucine-aminopeptidase (EC 3.4.11.1) with a substrate conjugated to 7-amido-4-methylcoumarin hydrochloride (7-AMC).

β -glucosidase is an enzyme that cleaves beta-glycosidic bonds and thus contributes to the breakdown of the cellulose that forms plant cell walls (Sinsabaugh *et al.*, 2008; Ngara and Zhang, 2018). Cellobiosidase is also involved in the breakdown of cellulose (Ejaz *et al.*, 2021). Chitinase is involved in the breakdown of chitin and other similar molecules and is thus often used as a reflection of microbial nitrogen demand (Sinsabaugh *et al.*, 2008; Mueller *et al.*, 2017) while also being recognised as a C-releasing enzyme (Mori *et al.*, 2023). Like chitinase, the activity of leucine-aminopeptidase is assumed to reflect microbial N demand as this enzyme removes the N-terminus group from amino acids (Matsui *et al.*, 2006). Phosphatases are the enzyme responsible for cleaving phosphate from different molecules (Chen *et al.*, 2019; Fan *et al.*, 2023) and, in the context of environmental studies, are taken to reflect microbial phosphate acquisition (Arnosti *et al.*, 2013).

To calculate rates of enzyme activity, the equations from German *et al.* (2011) were used. Activities were expressed per g of dry soil weight. The quench coefficients calculated and discussed reflect the ratio between the amount of fluorescence produced by the fluorogenic standard in the presence of the soil slurry and the fluorescence produced when only the diluent was present. This value therefore reflects the reduction in fluorescence caused by the presence of soil particles. Values closer to 0 indicate a greater degree of quenching or interference caused by the presence of soil.

3.3.6 pH fluctuations

To create an assay of sufficient volume to measure pH, separate assays were run in universal 30 mL containers (Thermo Scientific Sterilin). 0.5 mL of fluorophore-conjugated substrate and 2 mL of soil slurry were placed in each tube and run in parallel to the corresponding treatments in the microplates. Furthermore, larger volume standard control assays, containing 0.5 mL of the fluorophore standard and 2 mL of the diluent, were also conducted and their pH recorded. The tube rack was fully covered in aluminium foil to ensure complete darkness and pH was measured at the beginning (i.e., immediately after the addition of the soil slurry to the pre-pipetted substrate) and at the end of the 16 h incubation time (Table 3.1). The pH changes calculated and presented in this study represent the difference between the two pH values recorded at these time points.

Table 3.1. Start and end pH of the assays conducted with each enzyme and diluent combination. Means were taken across the four sampling stations and values are shown $\pm$ standard deviation. Also shown are the start and end pH of the fluorophore standard control assays (i.e., those containing the diluent and the fluorophore). These were measured only once, and so standard deviations could not be calculated.

Enzyme or standard	Diluent	Start pH	End pH
β -glucosidase	Buffer	7.53 $\pm$ 0.03	7.56 $\pm$ 0.03
	DI water	8.57 $\pm$ 0.15	7.98 $\pm$ 0.12
	Creek water	7.72 $\pm$ 0.12	7.49 $\pm$ 0.07
Cellobiosidase	Buffer	7.62 $\pm$ 0.04	7.61 $\pm$ 0.04
	DI water	8.56 $\pm$ 0.16	8.03 $\pm$ 0.13
	Creek water	7.85 $\pm$ 0.07	7.44 $\pm$ 0.07
Chitinase	Buffer	7.59 $\pm$ 0.03	7.62 $\pm$ 0.02
	DI water	8.54 $\pm$ 0.17	8.12 $\pm$ 0.18
	Creek water	7.73 $\pm$ 0.08	7.60 $\pm$ 0.10
Leucine-aminopeptidase	Buffer	7.53 $\pm$ 0.03	7.56 $\pm$ 0.03
	DI water	8.42 $\pm$ 0.10	8.18 $\pm$ 0.15
	Creek water	7.77 $\pm$ 0.05	7.65 $\pm$ 0.07
Phosphatase	Buffer	7.53 $\pm$ 0.02	7.55 $\pm$ 0.03
	DI water	8.21 $\pm$ 0.13	8.03 $\pm$ 0.15
	Creek water	7.60 $\pm$ 0.06	7.54 $\pm$ 0.07
4-MUF	Buffer	7.47	7.45
	DI water	7.64	7.49
	Creek water	8.15	8.08
7-AMC	Buffer	7.43	7.43
	DI water	7.63	7.49
	Creek water	8.29	8.38

3.3.7 Statistical analysis

I used linear mixed effects models, type III ANOVA tests and Tukey's post-hoc tests to determine whether the magnitude of activities differed between treatments (i.e., samples diluted with buffer, distilled water or creek water and stored at either 4 °C for a week or at -20 °C for two weeks). The formula used for the linear mixed effects models was: `lmer(enzyme activity ~ diluent * storage treatment + (1 | sample))`. Sampling station was treated as a random effect, meaning the non-independence of slurries created using subsamples from the same soil source was accounted for. These tests were performed separately for each enzyme. In the case of chitinase, including the

random effect led to over-parametrisation because there was close to no variation between sampling stations. Therefore, the following formula was used instead to analyse the chitinase assay results: $\text{lm}(\text{enzyme activity} \sim \text{diluent} * \text{storage temperature})$. Normality and homogeneity of variance was verified through visual inspection of residuals. Since only one biological replicate was taken from each sampling station, differences in activity between locations were examined by looking at the rank order of sampling stations. All statistical analyses and data visualisation were conducted in R Studio (version 4.4.1). Data was visualised using *ggplot2* (Wickham, 2016) while the packages *stats* (R Core Team 2024) and *lme4* (Bates *et al.*, 2015) were used for the statistical analyses.

3.4 Results

3.4.1 β -glucosidase enzyme assays

The three diluent treatments produced β -glucosidase activity results that differed significantly from one another ($p = <0.001$; $F = 85.0$), with buffered samples producing the highest activity, followed by distilled water and then by creek water (Fig. 3.2a; Table S3.2). Storage treatment also had a significant impact ($p = 0.03$; $F = 6.2$) but only the two sets of buffered samples differed from one another in terms of magnitude, hence the significant interaction between the two variables ($p = 0.001$; $F = 13.1$; Fig. 3.2a). Here, it was the 4 °C samples that produced the highest activity levels. The rank order of sampling stations did differ between treatments but there was some overlap (Fig. 3.2a; Table S3.3). Both buffered and both distilled water treatments produced similar outcomes. Samples containing these diluents and stored at 4 °C resulted in N3 having the highest activity, followed by N5, R5 and R2. In contrast, those stored at -20 °C resulted in N5 having the highest activity, followed by N3 and then either by R5 in the buffered samples or by R2 in the distilled water samples. On the other hand, creek water samples stored at 4 °C produced the following rank order: R5, N3, R2 and N5. Creek water sampled stored at -20 °C differed again, with N3 having the highest activity, followed by R5, R2 and N5. In β -glucosidase assays both the magnitude of activity and the rank order of sampling stations changed across treatments. Using creek water as the diluent most strongly altered the interpretation of the results.

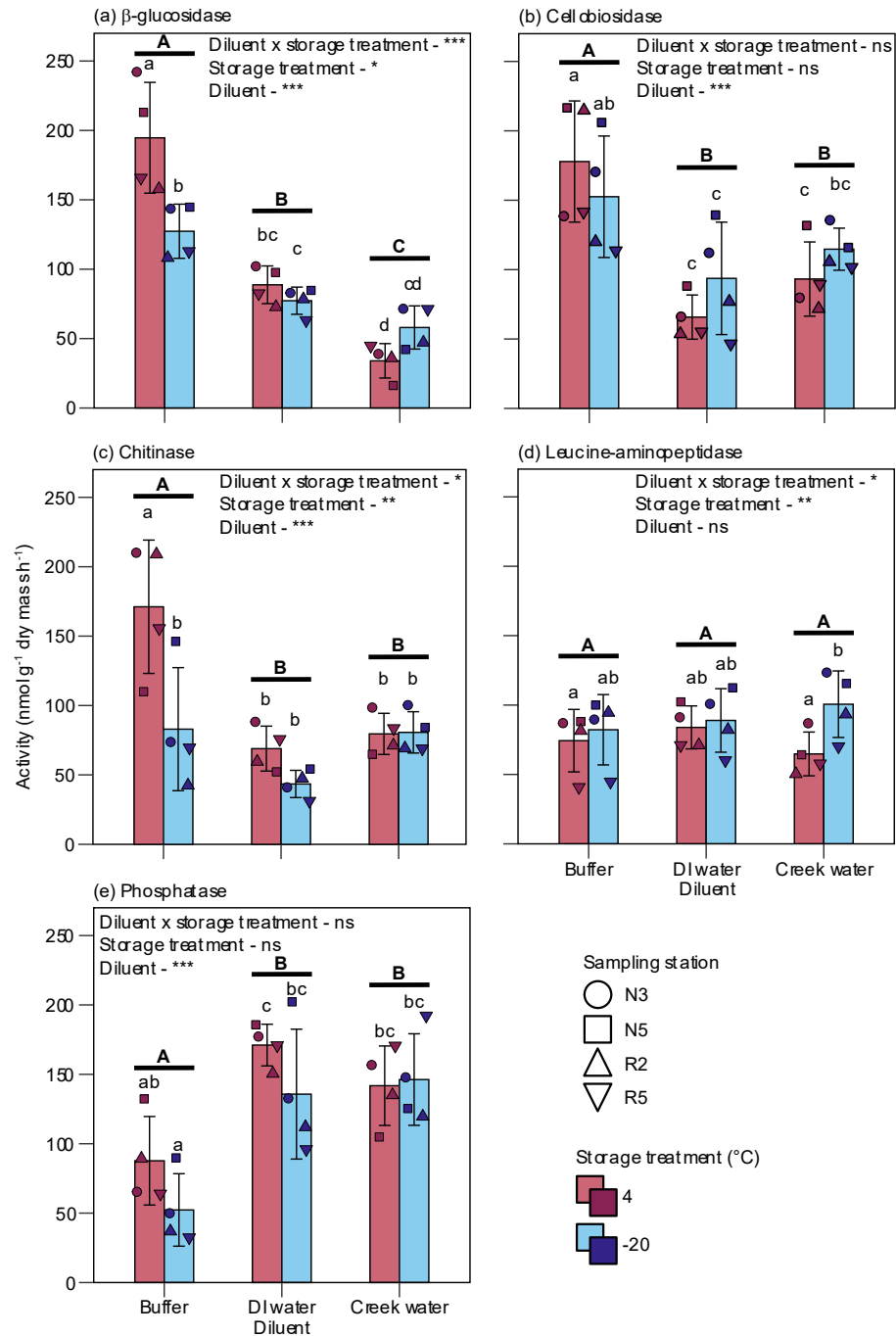


Figure 3.2. Mean activities (nmol g⁻² dry mass h⁻¹) across the four sampling stations of the enzymes (a) β -glucosidase, (b) cellobiosidase, (c) chitinase, (d) leucine-aminopeptidase and (e) phosphatase. Subsets of samples from each location were prepared using either buffer, distilled (DI) water or creek water as the diluent and sets of each were stored at either 4 °C (red) or -20 °C (blue). Error bars show $\pm$ standard deviation. Each sampling station is marked with a different shape so that the patterns of activity identified by the different treatments can be compared. Within each panel, the results of type III ANOVA and Tukey's post hoc tests are shown (*** - $P \leq 0.001$, ** - $P \leq 0.01$, * - $P \leq 0.05$ and ns - $P > 0.05$; Table S3.2). Lower case letters indicate differences between diluent and temperature treatments while upper case letters show the differences between diluents only.

3.4.2 Cellobiosidase enzyme assays

Diluent was the only variable that produced significantly different cellobiosidase activity results ($p = <0.001$; $F = 25.6$; Fig. 3.2b; Table S3.2). Buffered samples produced the highest activity levels while distilled water and creek water samples were grouped together and resulted in lower activities of this enzyme (Fig. 3.2b). N5 was ranked highest across all the treatments except for creek water samples stored at $-20\text{ }^{\circ}\text{C}$ (Fig. 3.2b; Table S3.3). The rankings of the other sampling station were far more mixed across the different treatments but one consistency across all samples stored at $-20\text{ }^{\circ}\text{C}$ was R2 always being ranked third and R5 always ranked fourth. Cellobiosidase assays were affected most strongly in terms of the different rank ordering of sampling stations produced by different treatments (Table S3.3), as opposed to in terms of the magnitude of the activity produced (Fig. 3.2a). However, there were no specific treatments that clearly produced a rank order that opposed those produced by the other treatments, given that they all produced different signals (Table S3.3).

3.4.3 Chitinase enzyme assays

Within the chitinase assays, diluent ($p = <0.001$; $F = 12.3$), storage treatment ($p = 0.005$; $F = 10.0$) and the interactions between these levels ($p = 0.019$; $F = 5.0$) were all significant variables (Fig. 3.2c; Table S3.2). Notably, the $4\text{ }^{\circ}\text{C}$ buffered samples produced much higher activity levels compared with the other treatments, none of which differed significantly from one another (Fig. 3.2c). Despite this, the buffered $4\text{ }^{\circ}\text{C}$ treatment produced a rank order of sampling stations that was very similar to those produced by the other two $4\text{ }^{\circ}\text{C}$ treatments. N3 was always ranked first and N5 was always ranked last, with some variation as to whether R2 or R5 were third or fourth (Table S3.3). On the other hand, $-20\text{ }^{\circ}\text{C}$ treatments resulted in N5 being ranked either first or second while N3 was first, second or third. There was more consistency when the other two sampling stations were considered as, again, they were almost always ranked in either the third or fourth positions (Table S3.3). Once again, it was the rank order of the samples as opposed to the magnitude of chitinase activity recorded, that was most strongly impacted by the different treatments. Here, it was the storage treatment that most strongly changed the outcome.

3.4.4 Leucine-aminopeptidase enzyme activity

Diluent did not significantly change the magnitude of leucine-aminopeptidase activity. On the other hand, creek water samples stored at $-20\text{ }^{\circ}\text{C}$ produced activities significantly higher than those stored at $4\text{ }^{\circ}\text{C}$ (storage treatment: $p = 0.003$; $F = 12.4$; interaction: $p = 0.03$; $F = 4.5$; Fig. 3.2d; Table S3.2). The values produced by the $-20\text{ }^{\circ}\text{C}$ creek water treatment, however, remained within a similar range to those produced by the other diluent-storage treatment combinations, none of which differed significantly from the $4\text{ }^{\circ}\text{C}$ creek water treatment. The rank order of the sampling station was relatively consistent across treatments (Table S3.3). In nearly every case, N5 and N3 were first or second while R2 was usually third and R5 fourth. The main deviation from this rule was buffered $-20\text{ }^{\circ}\text{C}$ samples that produced the following rank order: N5, R2, N3 and R5. Therefore, leucine-

aminopeptidase assays were most affected by the different treatments in terms of the rank order of samples while the magnitude of the activity recorded changed very little (Fig. 3.2d). Deviations in rank order were also minor and there was no particular treatment that led to startlingly different results.

3.4.5 Phosphatase enzyme activity

Diluent alone had a significant impact upon phosphatase activity ($p = <0.001$; $F = 16.9$; Fig. 3.2e; Table S3.2). In contrast to the results of the other enzymes, phosphatase activity was lowest in the buffered samples while distilled water and creek water samples were grouped together in a higher activity group. On the other hand, when the rank order of sampling stations was considered, buffered and distilled water treatments produced results that broadly agreed with one another: N5 had the highest activity and R5 usually had the lowest. Conversely, the creek water treatments ranked R5 first, N3 second and either R2 or N5 as fourth. Therefore, both the magnitude of activity and the rank order of sampling stations was impacted by the different treatments tested but the treatment that most strongly altered the interpretations of the result was buffer in the former and creek water in the latter.

3.4.6 pH changes during enzyme assays

As expected, buffered samples underwent only very minor pH fluctuations, with values ranging only between a decrease of 0.06 and a rise of 0.05 (Fig. 3.3). Distilled water samples, on the other hand, underwent the greatest pH fluctuations. These were always in the form of decreases in pH between the beginning and end of the assay, ranging between falls of 0.03 and 0.66. Creek water samples usually underwent pH fluctuations of a magnitude intermediate between those of the buffered and distilled water samples. Unlike the distilled water samples, this included increases in pH as well as decreases, although the latter was more usually the case. Creek water pH fluctuations ranged between a decrease of 0.55 and a rise of 0.14.

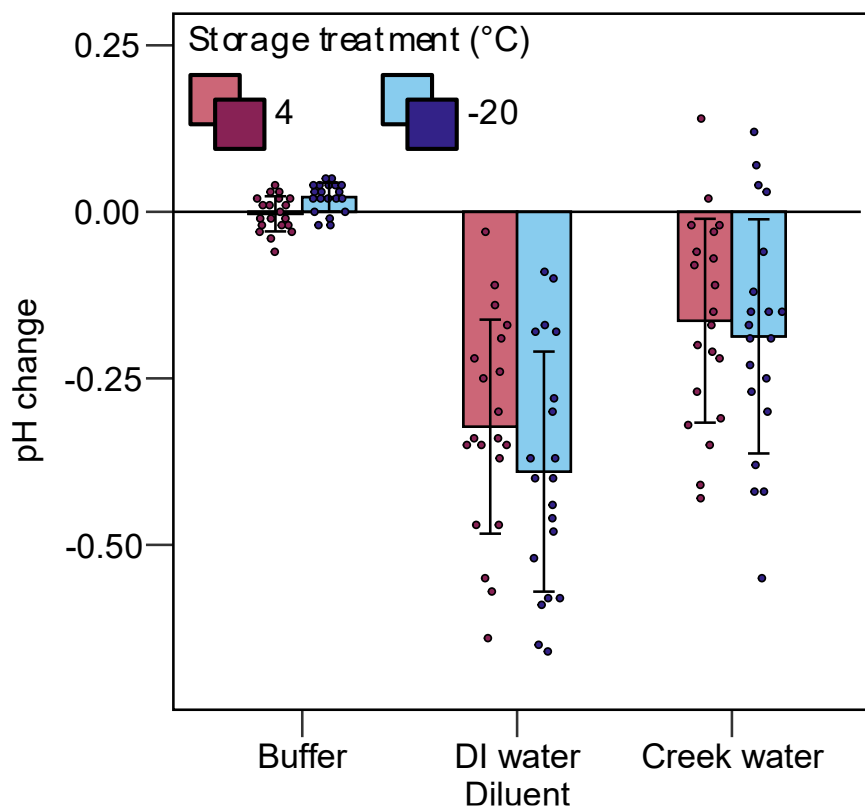


Figure 3.3. Mean pH changes recorded between the beginning and end of each assay conducted with soil slurries created with different diluents and stored at either 4 °C (red) or -20 °C prior to analysis. Error bars show $\pm$ standard deviation.

When the impact of the pH changes recorded upon the magnitude of the activity were considered, different impacts were apparent depending on the enzyme (Fig. 3.4). β -glucosidase, cellobiosidase and chitinase activities were generally lower in the distilled water and creek water samples (i.e., those samples that underwent pH fluctuations over the course of the assays; Fig. 3.4a – 3.4c). The opposite effect was apparent in the phosphatase assays, with relatively higher activity often being recorded for the unbuffered samples when compared with the buffered (Fig. 3.4e). In the case of leucine-aminopeptidase assays, there was very little separation between the samples undergoing different magnitudes of pH changes (Fig. 3.4d).

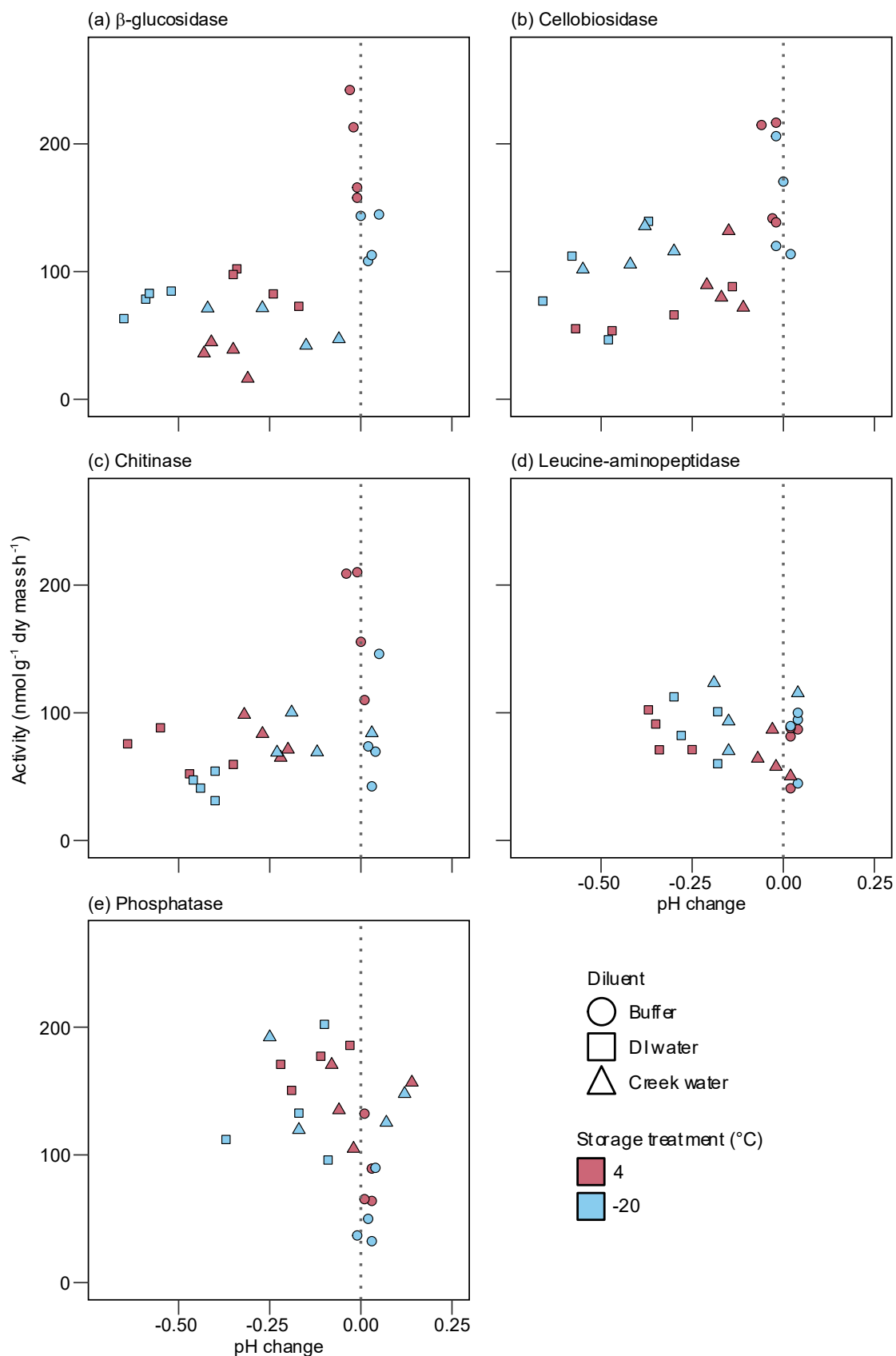


Figure 3.4. The activities of the enzymes (a) β -glucosidase, (b) cellobiosidase, (c) chitinase, (d) leucine-aminopeptidase and (e) phosphatase and the pH fluctuation recorded in each corresponding assay. The dotted line indicates the position of samples that underwent no pH change.

3.4.7 Quench coefficients

A stark difference between the leucine-aminopeptidase assays and those conducted for the four other enzymes being investigated were the quench coefficient values calculated in each case (Fig. 3.5). In leucine-aminopeptidase assay, the fluorophore used was 7-AMC and the quench coefficients produced were all below one, ranging between 0.29 and 0.80 (Fig. 3.5a). This represents the broadly expected result for this variable (German *et al.*, 2011). Conversely, β -glucosidase, cellobiosidase, chitinase and phosphatase assays were all performed with the fluorophore 4-MUF. The quench coefficients of samples prepared with buffer or creek water as the diluent were within the expected range, between 0.14 and 0.66 (Fig. 3.5b). However, distilled water samples had quench coefficients of a much greater magnitude ranging between 4.78 and 24.52 (Fig. 3.5b).

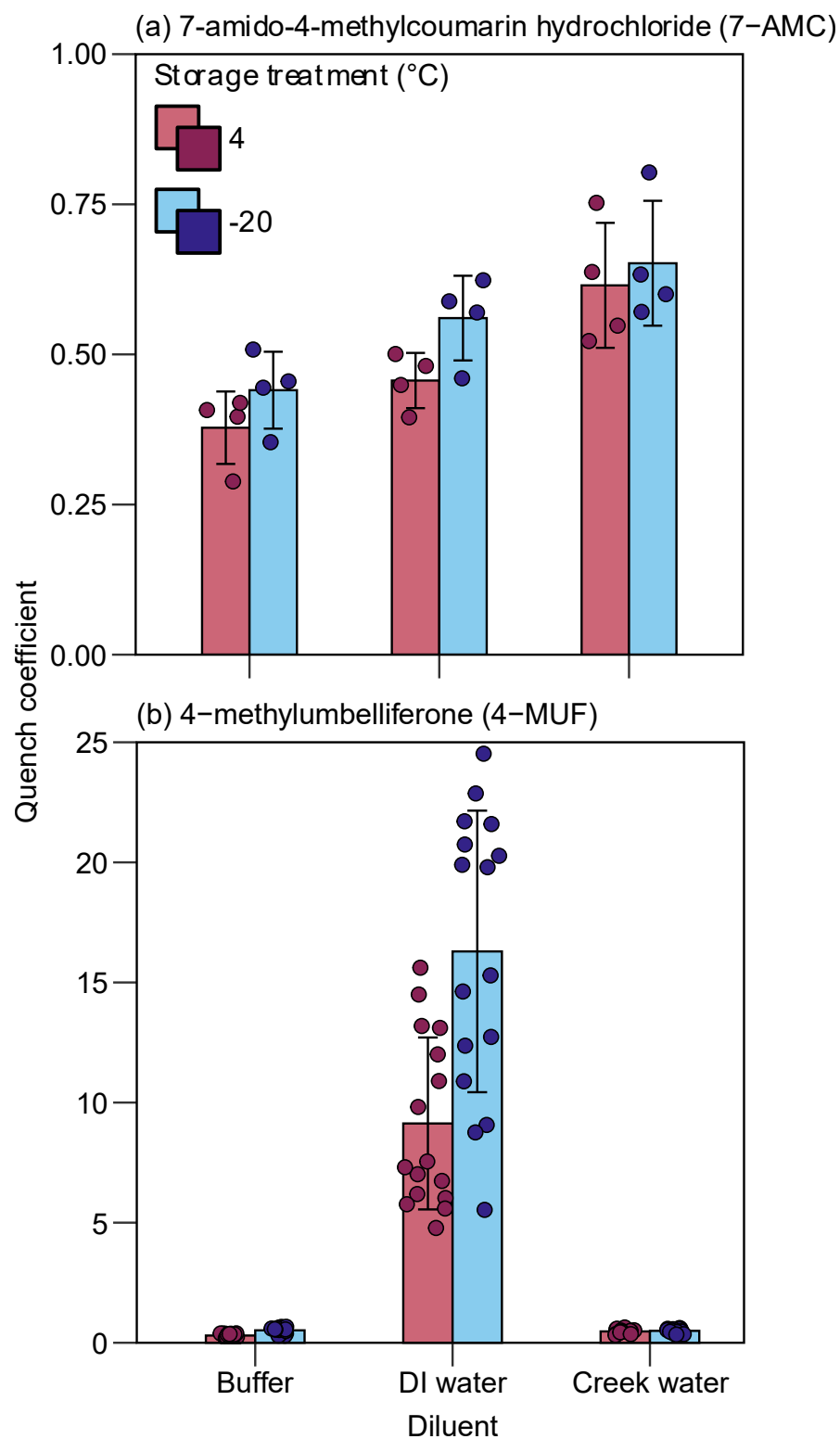


Figure 3.5. Quench coefficient values for assays performed with (a) 7-AMC as the fluorophore (i.e., leucine-aminopeptidase assays) and for those performed with (b) 4-MUF as the fluorophore (i.e., β -glucosidase, cellobiosidase, chitinase and phosphatase). Error bars show $\pm$ standard deviation.

3.5 Discussion

The aim was to assess the variability or potential inconsistencies arising from diluent and storage treatment choices made when preparing saltmarsh soil slurries ahead of enzymes assays. To do this, soils from the same locations were treated differently and recorded the pH fluctuations undergone by the assays. Many of the results obtained by the different methods tested in this study were highly inconsistent, with the same soils diluted and stored differently producing contradictory patterns of results. This meant that any conclusions drawn about enzyme activity levels were dependent upon the treatments used to prepare the samples prior to analysis. Furthermore, the magnitude of activity produced differed between treatments, but the direction of these differences depended upon the enzyme. Given that the environmental parameters measured did not range widely across the four sampling stations (Table S3.1), the enzyme activity levels within each location could genuinely have been very similar. This would explain the inability to detect a consistent rank order of sampling stations, which was true to greater and lesser degrees with all the enzymes tested in this study. Difficulties in obtaining a consistent pattern of results may have arisen from a failure to optimise the potential enzyme activity of each sample because the goal was to mimic *in-situ* conditions.

Neither of the storage treatments tested in this study can be said to represent “fresh” soil, but both are commonly used by studies due to a lack of time. Nevertheless, this meant that I lacked a benchmark against which to compare my results and therefore it was impossible to determine the treatment that produced the most accurate conclusions. Additionally, since both treatments were stored for different lengths of time, I could not unpick the impacts of storage temperature from those of storage time. Even so, showing the range of results that were produced when using different storage treatments and diluents is valuable and demonstrates the impacts these decisions can have upon study outcomes. It is recommended that future work test the variability produced using freshly collected samples so that concrete recommendations can be made about the best practices for enzyme assays.

3.5.1 The effects of diluents and pH changes

The saltmarsh creek water used as a diluent contained its own make-up of microorganisms and extracellular enzymes that could have influenced the results of the assays. For example, in the case of β -glucosidase, comparing the fluorescence detected in the substrate control wells (i.e., those containing the diluent and the fluorescent-conjugated substrate only) showed values from creek water plates were three orders of magnitude greater than those from buffer and distilled water plates, confirming that the creek water alone contained extracellular enzymes capable of breaking down the substrates provided. The rank ordering of sampling stations was most clearly affected by creek water addition in β -glucosidase and phosphatase assays, lending support to the idea that this diluent altered the composition of the soil slurry. In cellobiosidase assays, conversely, there was no obvious difference between the creek water results and those for the other two diluents, suggesting that the

impact upon cellobiosidase activity of the enzymes already present in the creek water was limited. One possible hypothesis is that there was simply less cellobiosidase activity in the creek water because this enzyme is known to be more specific to cellulose breakdown compared to β -glucosidase, which can breakdown a large range of the b-glycosidic polymers present in algae and plankton (e.g., Méndez-Líter *et al.*, 2018). Altogether, it was deemed that creek water results were not directly comparable with those produced when buffer and distilled water were used as diluents. Instead, the creek water treatment represented the consequences of adding a biologically active fluid. This does not necessarily invalidate the usefulness of this part of the experiment because the sampling locations on these saltmarshes regularly experienced inundation during high tides and thus the extracellular enzymes, microbes, dissolved organic matter, salinity and suspended particles introduced by this water mimic a natural process. To allow for direct comparisons with buffer and distilled water treatments, it is recommended that future studies introduce a filtering or heating step to remove or denature the enzymes and microbes present.

The goal of using different diluents to create soil slurries and run assays was to determine whether using creek or distilled water led to large oscillations in pH that would ultimately impact the results obtained. Indeed, pH changes of a greater magnitude were recorded when these diluents were used instead of phosphate buffer. Lessard and co-workers (2013) considered fluctuations of this magnitude to represent those naturally present in soils, but this does not exclude the possibility that enzyme activity was impacted. The impact of pH changes upon activity, when comparisons were made exclusively between buffered and distilled water samples, was not consistent across enzymes (Fig. 3.2). β -glucosidase, cellobiosidase and chitinase activities were lower in distilled water treatments, phosphatase activity was higher and leucine-aminopeptidase activity appeared unaffected (Fig. 3.4). Another factor to consider, as well as the pH changes, was the actual pH at which assays were conducted. Distilled water samples always had the highest starting pH (8.21 – 8.57), followed by creek water (7.60 – 7.85), and buffered samples had the lowest (7.53 – 7.62). It is therefore possible that particular diluents created conditions that were closer to the optimum pH of particular enzymes and thus drove the significantly higher magnitude of activity often seen for samples created with different diluents, as opposed to the changes seen being driven by the pH fluctuations, or lack thereof, that also characterised the diluents tested. Another study has shown that the impact of assay incubation temperature upon enzyme activity was modulated by the pH of the soil, suggesting that decisions about study design should be made with this in mind (Liu *et al.*, 2026a). Likewise, differences in background pH between diluents such as distilled water and buffer could be a key driver of enzyme activity levels.

It was not the case that those soil samples with a higher pH (N3 and N5) were better able to regulate their own pH when diluted with creek or distilled water because of a potentially higher Ca-carbonate content and resultant buffering capacity (Fig. S3.2). Natural marsh soil samples did tend to undergo slightly lower pH changes, compared with restored marsh samples, when diluted with

creek or distilled water, though this difference was not significant (Fig. S3.2). Therefore, when testing enzyme activities in samples with a pH range like those of this study (pH 6.4 – 7.2) it should not be assumed that buffer is unnecessary for controlling the pH.

Depending upon the fluorophore used in an assay, there was a startling contrast between the quench coefficients recorded for wells containing distilled water and those containing the other diluents tested (Fig. 3.5). That a higher fluorescence was produced when soil suspended in distilled water was mixed with 4-MUF than when distilled water alone was combined with the fluorophore was entirely contradictory to the general expectation that soil particles interfere with fluorescence readings by causing turbidity (German *et al.*, 2011). As an explanation, I point to the fact that distilled water fluorophore control wells had a higher pH than distilled water soil slurry wells (Table 3.1). The opposite was true when creek water was the diluent, while using buffer maintained a stable pH in control and assay wells (Table 3.1). This drove the quench coefficient differences because 4-MUF fluorescence is greater at more alkaline pH values (Mead *et al.*, 1955). German and colleagues (2011) recommended that samples be diluted further if the quench coefficient is below 0.5. There was no discussion, however, of what should be done if this benchmark is vastly exceeded and my results point to the conclusion that using a pH-dependent fluorophore in an unbuffered system will lead to chemically invalid single concentration standard values and/or standard curves. To calculate net fluorescence the assay fluorescence values were divided by the quench coefficient. Therefore, values greater than one would have reduced the net fluorescence that was carried forward to the final activity calculation. This would result in lower activity levels, and this was indeed the case for the β -glucosidase, cellobiosidase and chitinase assays performed with distilled water, as opposed to buffer (Fig. 3.2a – 3.2c). However, phosphatase activity was significantly higher in the distilled water samples compared with the buffered ones (Fig. 3.2e). It has previously been suggested that using phosphate buffer could impact the results of phosphatase assays (German *et al.*, 2011). Therefore, a possible explanation of this result is that providing an additional source of inorganic phosphate in the buffer led to competition with the phosphate bound to the fluorophore that must be broken down to produce a detectable signal. Thus, lower activity could be produced with phosphate buffer as the diluent than with distilled or creek water.

3.5.2 The effect of storage treatment

When the impact of freezing was tested previously it reduced activity in some cases (Wallenius *et al.*, 2010). Here, there were significant differences in the magnitude of activity caused by storage treatment in the cases of β -glucosidase, chitinase and leucine-aminopeptidase (Fig. 3.2; Table S3.2). Furthermore, there were significant interactions between storage treatment and diluent: β -glucosidase and chitinase activities were greater in 4 °C buffered samples than in -20 °C buffered samples while leucine-aminopeptidase activity was higher in -20 °C creek water than in 4 °C creek water. A possible explanation for the former effect could be that freezing and thawing and/or a longer storage period denatures the enzymes present in the soil. Conversely, a higher amount of activity in

the -20 °C samples could be the result of longer storage at a lower temperature causing cell lysis and the release of additional enzymes into the soil matrix. A recent study that compared β -1,4-glucosidase activities in soils stored either at -20 °C or by air drying, found that the impacts varied according to the other variables being tested, namely soil type (acidic, neutral or alkaline) and assay incubation duration (one, four or twelve hours; Liu *et al.*, 2026b). As a result, the authors challenge the conventional assumption that storage at -20 is always superior to air drying. Similarly, my result indicate that storage treatment effects vary by diluent and enzyme, meaning that blanket recommendations about best practice are not advisable.

3.6 Conclusions

The results of this study demonstrated that both diluent and storage treatment can significantly affect enzyme activity. However, this impact, whether stimulatory or inhibitory, differed between soil samples, and was more pronounced for some enzymes than others. β -glucosidase, chitinase and cellobiosidase activities were elevated by using phosphate buffer, as opposed to distilled or creek water, as the diluent, while phosphatase activity was suppressed. Conversely, leucine-aminopeptidase activity levels were not impacted by the choice of diluent. Furthermore, consistent rank ordering of sampling stations was rarely achieved across the different treatments tested.

The inconsistencies seen may be a result of the broadly similar conditions across the different sampling stations tested. Therefore, where this is the case, I emphasise the desirability of removing any potential for variability by creating assay conditions that are optimised with the enzyme being tested in mind, thus ensuring that clear, consistent patterns of activity are uncovered. This study showed that decisions to conduct enzyme assays in a particular way led to one set of results, but had different decisions been made, it is possible that different, even opposing, conclusions could be drawn. It is recommended that decisions are made based upon the research question being addressed in each case, however. Where different ecosystem types are being compared within the same study, attempting to replicate *in-situ* conditions could justifiably represent the best approach, revealing the locations likely to have the highest activity levels in the real world as opposed to under optimised conditions – the latter being an insight of little value. Even where samples are very similar, where resources allow, it could be valuable to attempt both approaches. Optimising conditions based upon the enzyme reveals which samples contain the highest quantity of that enzyme within its soil matrix but by no means reveals that these enzymes are active within the ecosystem itself.

pH fluctuations occurred when distilled and creek water were used as diluents but not when buffer was used. Another difference between the diluents were the high quench coefficient values calculated for distilled water assays when 4-MUF was the fluorophore, a result of the more alkaline pH of the standard control wells compared with the soil slurry wells. This suggests that single concentration standards, or indeed standard curves, developed using a pH-dependent fluorophore in an unbuffered system could lead to invalid results. Leucine-aminopeptidase activity levels,

determined with assays that did not use 4-MUF as the fluorophore, did not differ in magnitude between diluents, which was the not the case for any of the other enzymes tested, all of which were assayed with 4-MUF as the fluorophore. However, creek water activity results were often grouped with those of the distilled water assays while the quench coefficients of creek water assays were of a similar magnitude to those produced in buffered assays. This was likely the consequence of not denaturing or removing the microbes and enzymes already present in the creek water. Another factor that likely contributed to the differences in the magnitude of activity was the actual pH of the assays, which differed between diluents. The pH differences and the quench coefficient values revealed the complex consequences of the methodological decisions made when the enzyme assay method was employed. To determine a clear picture of the mechanisms by which these differences impact the magnitude of the activities calculated for different enzymes would require further investigation and efforts to isolate individual effects.

Freezing as a storage method is important because it allows samples to be collected and then put aside until analyses can be conducted more conveniently. Ultimately, this could mean that more analyses can be conducted at a higher standard because planning is made easier. Given that 4 °C samples stored for a week generated results just as inconsistent as those produced by samples stored at -20 °C for two weeks, conclusions could not be drawn in this study, as in others, about the suitability of either of these treatments as storage techniques. To determine this, the experiment would need to be repeated with buffered samples run at the enzyme optima and, to isolate the impact of storage temperature, samples stored for the same length of time at both temperatures. Experiments to test the impacts of different storage lengths at the same temperature could also prove insightful.

3.7 Supplementary Information

3.7.1 Supplementary tables

Table S3.1. Characteristics of the saltmarsh surface soil samples. N3 and N5 are from a densely vegetated natural marsh while R2 and R5 are from an area to which tidal inundation was newly restored in 2015 and that thus still largely resembles a mudflat. Results represent single measurements. OC – organic carbon; N – nitrogen; OM – organic matter; AGB – aboveground biomass; BGB – belowground biomass; DBD – dry bulk density; Lab. OM – labile organic matter; Recal. OM – recalcitrant organic matter.

Sampling station	OC (%)	N (%)	pH	OM (%)	ABG (g m⁻²)	BGB (g cm⁻³)	DBD (g cm⁻³)	Lab. OM (%)	Recal. OM (%)
N3	1.83	0.26	7.00	12.19	275.50	0.02	0.36	3.72	8.47
N5	5.30	0.56	7.20	15.29	272.60	0.12	0.42	5.97	9.33
R2	2.16	0.35	6.40	12.77	133.00	0.09	0.48	4.13	8.63
R5	3.15	0.43	6.60	12.61	149.45	0.04	0.41	4.84	7.77

Table S3.2. Results of two-way (chitinase only) and type III (all other enzymes) ANOVA tests performed for each enzyme. Significant *p*-values are highlighted in bold.

Enzyme	Level/Interaction	Numerator <i>df.</i>	<i>F</i> -value	Pr(>F)
β-glucosidase	Diluent	2	84.994	<0.001
	Storage treatment	1	6.167	0.025
	Diluent x storage treatment	2	13.071	0.001
Cellobiosidase	Diluent	2	25.626	<0.001
	Storage treatment	1	0.639	0.437
	Diluent x storage treatment	2	2.793	0.093
Chitinase	Diluent	2	12.288	<0.001
	Storage treatment	1	9.986	0.005
	Diluent x storage treatment	2	4.969	0.019
Leucine- aminopeptidase	Diluent	2	1.040	0.751
	Storage treatment	1	12.391	0.003
	Diluent x storage treatment	2	4.538	0.029
Phosphatase	Diluent	2	16.922	<0.001
	Storage treatment	1	0.105	0.105
	Diluent x storage treatment	2	1.071	0.367

Table S3.3. Rank order, from highest to lowest, of the extracellular enzyme activity recorded in each sampling stations using the different treatments tested.

Enzyme	Storage Treatment (°C)	Diluent	Ranking			
			N3	N5	R2	R5
β -glucosidase	4	Buffer	1	2	4	3
		DI water	1	2	4	3
		Creek water	2	4	3	1
	-20	Buffer	2	1	4	3
		DI water	2	1	3	4
		Creek water	1	4	3	2
Cellobiosidase	4	Buffer	4	1	2	3
		DI water	2	1	4	3
		Creek water	3	1	4	2
	-20	Buffer	2	1	3	4
		DI water	2	1	3	4
		Creek water	1	2	3	4
Chitinase	4	Buffer	1	4	2	3
		DI water	1	4	3	2
		Creek water	1	4	3	2
	-20	Buffer	2	1	4	3
		DI water	3	1	2	4
		Creek water	1	2	3	4
Leucine-aminopeptidase	4	Buffer	2	1	3	4
		DI water	2	1	3	4
		Creek water	1	2	4	3
	-20	Buffer	3	1	2	4
		DI water	2	1	3	4
		Creek water	1	2	3	4
Phosphatase	4	Buffer	3	1	2	4
		DI water	2	1	4	3
		Creek water	2	4	3	1
	-20	Buffer	2	1	3	4
		DI water	2	1	3	4
		Creek water	2	3	4	1

3.7.2 Supplementary figures

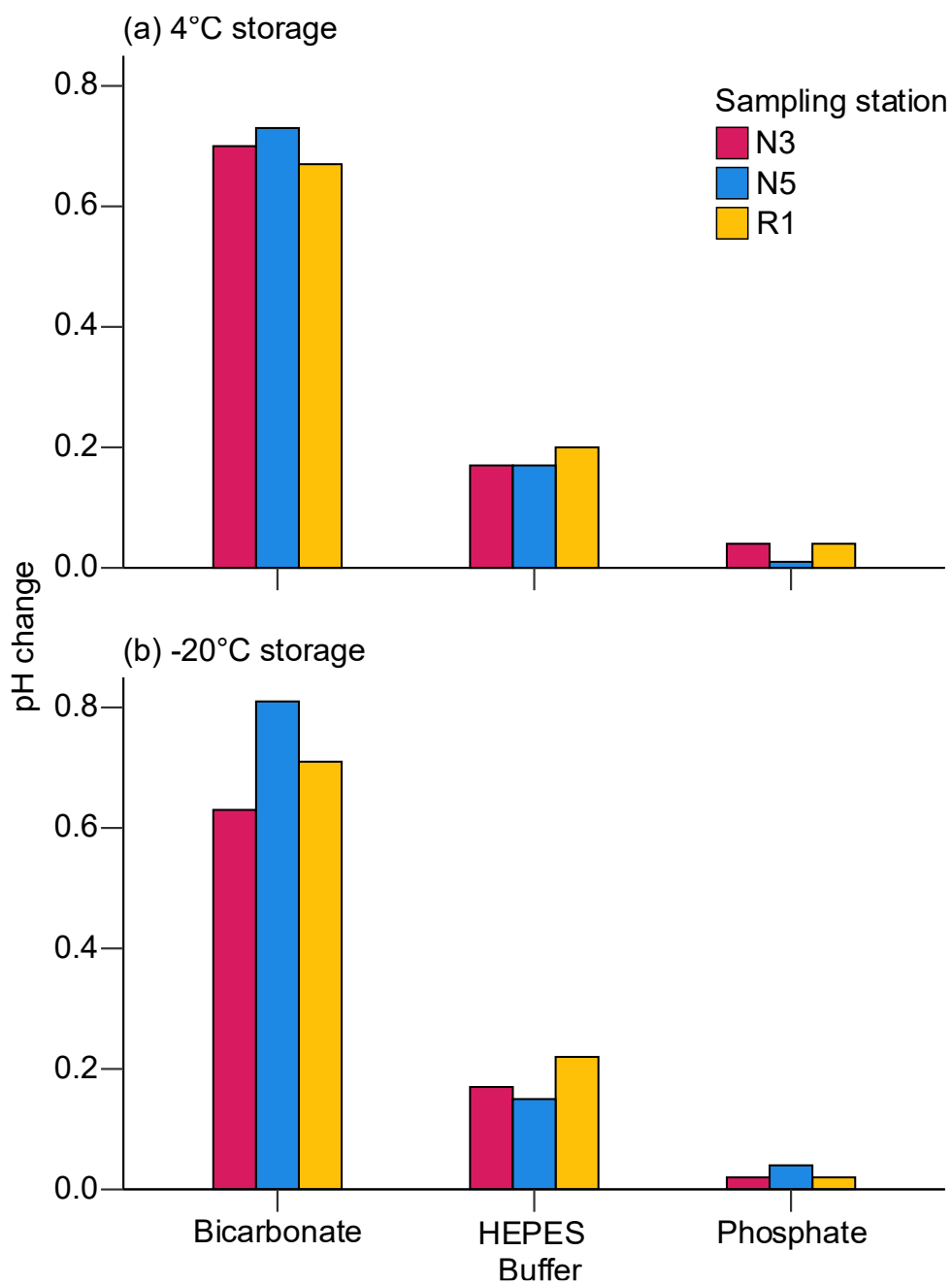


Figure S3.1. pH changes between the beginning and end of assays containing soil slurries stored at either (a) 4 °C or (b) -20 °C buffered by phosphate, HEPES and bicarbonate buffers. Subsamples of saltmarsh soil from each sampling station (N3, N5 and R1) were used to make soil slurries with each of these buffers.

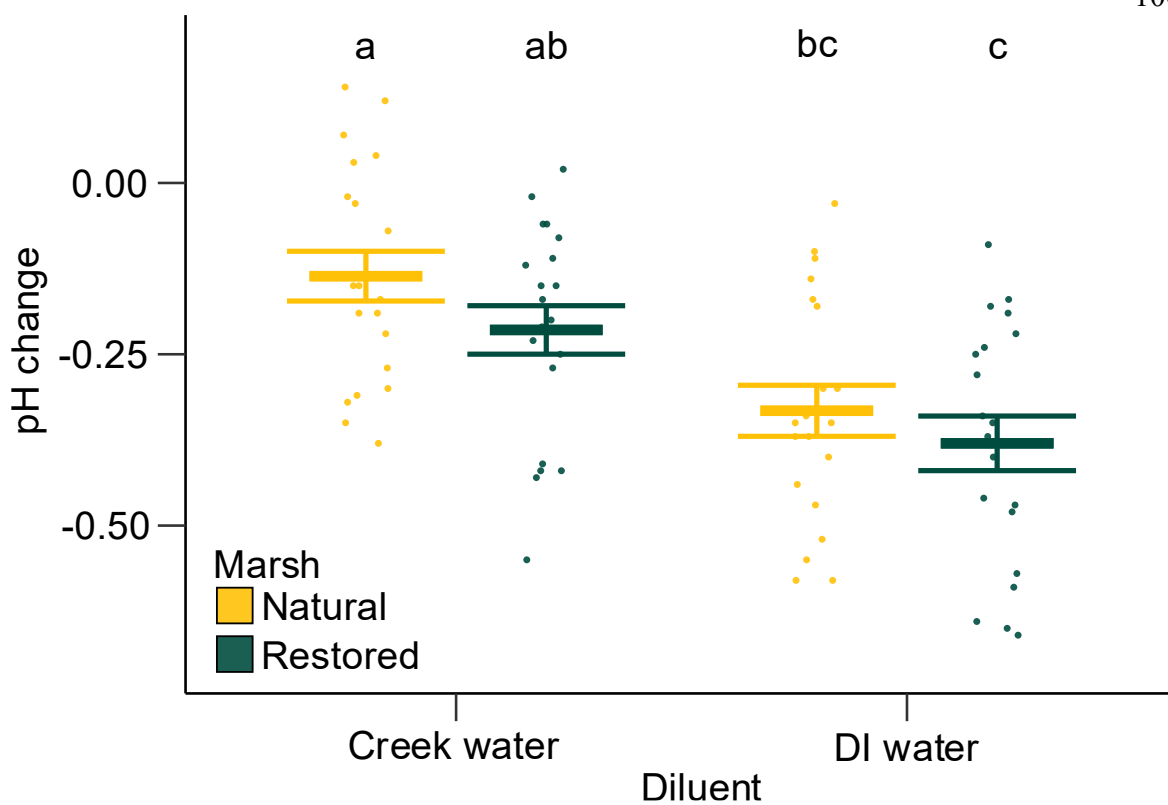


Figure S3.2. All the pH changes recorded for assays using soil slurries made from natural (N3 and N5) and restored (R2 and R5) soil for both samples diluted by creek water and distilled water, those expected to regulate their own pH levels. Letters represent the significance levels detected with a two-way ANOVA test. Error bars are $\pm$ standard error.

Chapter Four

Microbial diversity and extracellular enzyme activity differ strongly between restored and natural saltmarshes

4.1 Abstract

Saltmarsh organic carbon (OC) remains vulnerable to mineralisation by microorganisms even after entering the sediment. Waterlogged, low oxygen conditions help to slow decomposition rates, making saltmarshes suitable natural climate solutions. Restoring saltmarshes is a way of establishing new carbon sinks by breaching the seawall surrounding agricultural fields, allowing regular tidal inundation. However, this process results in saltmarshes that are less topographically, ecologically and hydrologically diverse than natural ones. Furthermore, restored sites are usually situated at a lower elevation within the tidal frame than natural ones, meaning they are more frequently inundated by the tide and thus have different drainage conditions. During the winter and summer, I profiled the surface and deep (~50 cm) sediment layers of two neighbouring saltmarshes – one natural and mature, another restored nine years ago. Microbial communities were characterised by 16S rRNA gene amplicon sequencing and extracellular enzyme activity measurements, sediment redox conditions were quantified with Indicators of Reduction in Soils (IRIS) sticks and the biogeochemical properties of the samples ascertained. The IRIS sticks results indicated that conditions were more strongly reducing in the restored marsh, and this was especially true during the summer months. It was in the deep sediment (40-50 cm) of the restored marsh that microbial communities differed most strongly from those of the natural marsh: communities were less diverse and sulphate reducing genera more abundant. Activities of the enzymes β -glucosidase, chitinase and phosphatase were higher in the deep sediment of the restored marsh than in the surface while in the natural marsh the activities of these enzymes became negligible at a similar depth. Taken together, this supports the continued existence of a different soil structure – namely, a relic agricultural field – beneath the steadily accreting restored saltmarsh.

4.2 Introduction

Over recent decades, saltmarsh ecosystems have been highlighted as important organic carbon (OC) sinks (Chmura *et al.*, 2003; Mcleod *et al.*, 2011). Microorganisms remineralise the OC trapped in organic matter (OM) within saltmarsh sediment and thus facilitate its release as organic compounds and as carbon dioxide (CO₂) back into the atmosphere (Macreadie *et al.*, 2025). In saltmarsh sediments, abiotic conditions – primarily, the low oxygen availability caused by flooding – are thought to restrict mineralisation rates by selecting for specific microbial communities and the processes they can perform (Yarwood, 2018; Spivak *et al.*, 2023; Erb *et al.*, 2024). Nevertheless, sediments release CO₂ and this matters when carbon credits are used to quantify the OC sequestered in a marsh following a particular action (Burden *et al.*, 2023; VERRA, 2023). If the OC sequestration represented by these credits does not truly constitute a long-term removal of CO₂ from the atmosphere, emissions are not offset, and the intended climate change mitigation goal is not met.

One strategy to remove more CO₂ from the atmosphere is restoring saltmarshes in embanked, agricultural areas where they were formerly present. This is usually done by breaching

the surrounding seawall (Jones *et al.*, 2024). However, many differences are known to exist between natural and restored saltmarshes and so it should not be assumed that the conditions known to slow down OC mineralisation in natural sites also function similarly in restored areas. First, newly accreting saltmarsh sediment covers layers of compacted agricultural soil, which water cannot penetrate easily, meaning drainage is reduced (Tempest *et al.*, 2015; Spencer *et al.*, 2017). This could mean differences in oxygen availability and sediment redox conditions. Secondly, restored marshes tend to lack the small-scale variations in elevation and the high creek densities that characterise natural sites (Shafer and Streever, 2000; Brooks *et al.*, 2015; Lawrence *et al.*, 2018), another factor that might affect local drainage and thus oxygen availability. In turn, plant community diversity tends to be lower owing to more uniform conditions across the marsh (Mossman *et al.*, 2012a; Mossman *et al.*, 2012b; Lawrence *et al.*, 2022). The biotic interactions that occur between plants and sediment microbes and the importance of environmental conditions for selecting the microbial assemblage, mean that these differences could also impact sediment microorganisms and thus have implications for OC remineralisation.

Several studies of saltmarsh microbial community composition have focused upon the sediment (e.g., Barreto *et al.*, 2018; Hurtado-McCormick *et al.*, 2022; Birnbaum *et al.*, 2025). It has been shown that anaerobic genera, such as *Tepidibacter*, *Caldithrix*, *Desulfovibrio*, *Fibrobacteres* and *Thiotrichaceae*, dominated the surface sediments of a marsh in Florida, USA (Barreto *et al.*, 2018). Others have investigated the strong impact upon microbial community composition that environmental variables like salinity can have (Zhang *et al.*, 2021; Gutekunst *et al.*, 2025). One of these investigations concluded that members of the classes *Gammaproteobacteria*, *Bacteroidetes* and *Firmicutes* became more abundant with increasing salinity (Zhang *et al.*, 2021). Other factors that are important determinators of saltmarsh microbial community composition are pH (Yu *et al.*, 2022) and OM quality (Leadbeater *et al.*, 2021). The taxa directly responsible for the decomposition of recalcitrant OM in saltmarshes were shown to belong to the classes *Gammaproteobacteria*, *Bacteroidetes* and *Deltaproteobacteria* (Leadbeater *et al.*, 2021). By controlling the microbial communities present these factors in turn determine the degree to which OC is preserved or lost (Erb *et al.*, 2018).

Studies of the impacts of saltmarsh restoration upon microbial communities are also numerous (e.g., Bernhard *et al.*, 2012; Negandhi *et al.*, 2019; Santini *et al.*, 2019; Lynum *et al.*, 2020; Keppeler *et al.*, 2023), but the projects differed greatly in terms of saltmarsh type (Yando *et al.*, 2023) and restoration method (Jones *et al.*, 2024). A comparison between the microbial assemblages in a restored saltmarsh constructed with dredged material from external sources and a natural reference site put the differences they identified down to the properties of the sediment used for the construction (Mavrodi *et al.*, 2018). Relevant to the functioning of saltmarshes as OC sinks is the finding that the genera *Desulfuromonas* and *Geobacter* were associated with high OC and nitrogen (N) densities in a restored Australian marsh and thus were related to the recovery of this function (Santini *et al.*,

2019). As well as marsh construction with dredged material, the restoration practices implement in the sites previously studied include reintroducing tidal inundation to freshwater wetlands and sediment diversion. To my knowledge, an examination of sediment microbes in saltmarshes restored through managed realignment (i.e. the creation of a seawall breach), a common technique used in NW-Europe, had not yet been attempted. Furthermore, it has often been true that, at most, only the top 10 cm of sediment was examined by previous studies (e.g., Barreto *et al.*, 2018; Santini *et al.*, 2019; Lynum *et al.*, 2020), meaning an understanding of the deeper, anoxic sediment layers was lacking.

Attempts to look beyond microbial assemblages and consider functionality are even more limited. One means of achieving this is quantifying extracellular enzyme activity (e.g., Mueller *et al.*, 2017; Mueller *et al.*, 2020; Tang *et al.*, 2020). Specifically, β -glucosidase, cellobiosidase, chitinase, leucine-aminopeptidase and phosphatase are often focused upon. These enzymes are released by microbes into the soil, play a crucial role in carbon and nutrient cycling and, therefore, affect OM storage (Sinsabaugh *et al.*, 2008). β -glucosidase and cellobiosidase both contribute to C cycling by breaking down the cellulose of plant cell walls (Sinsabaugh *et al.*, 2008; Ngara and Zhang, 2018; Ejaz *et al.*, 2021). The molecule chitin is broken down by chitinase, meaning it can provide a source of N but also of C (Sinsabaugh *et al.*, 2008; Mueller *et al.*, 2017; Mori *et al.*, 2023). Leucine-aminopeptidase likewise gives an indication of microbial N demand owing to its role in cleaving amino acids from peptide chains (Matsui *et al.*, 2006) while microbial phosphate acquisition is reflected by phosphatase activity (Arnosti *et al.*, 2013). A study that attempted to relate extracellular enzyme activity to restoration found that, in saltmarsh sediment to which suitable plant species were transplanted, activities of the enzymes acid phosphatase and urease were significantly lower a year after transplantation compared with sediment that was naturally colonised by plants (Duarte *et al.*, 2012). The explanation for this difference was that the rhizosphere microbial communities of the transplanted vegetation were not yet fully developed and so differed from those of the natural plants. Again, the impact of an underlying agricultural layer upon sediment enzyme activity caused by potential differences in abiotic conditions had not been considered, nor had the activities of the suite of enzymes listed been investigated in a restored marsh of this type.

Resource rich sediment containing high-quality litter can be distinguished by a combination of low dry bulk density (DBD) and high OC content (Smeaton *et al.*, 2024), as well as low OC/N values (Zhang *et al.*, 2008), while a resource scarce setting would have the opposite characteristics. Low DBD indicates sediment richer in OM that is less compacted, with a lower mineral content and possibly a higher water content (Wang *et al.*, 2017), while lower OC/N values suggest N-rich OM and the availability of this nutrient for microbial and plant growth (Craft, 2001). The latter can also be used as an indicator of OM origin because of the high N content expected of marine algae compared with the cellulose of terrestrial plants (Meyers, 1994; Zhou *et al.*, 2007; Hulisz *et al.*,

2013). Using these variables, sediment from the different marshes and depths can be characterised and the possible drivers of extracellular enzyme activity investigated.

In this study, the hypotheses were as follows: (1) Conditions in the restored marsh would be more waterlogged, meaning more strongly reducing conditions and indicating a more anoxic environment. (2) The restored marsh would contain more anaerobic microbial taxa and enzyme activity would be low, reflecting lower OM mineralisation rates, due to the lack of oxygen availability. (3) These differences between marshes would be more pronounced in the surface sediment but grow less apparent in the deep samples because conditions would become increasingly anoxic with depth in both sites. (4) Finally, by measuring OC, N and dry bulk density (DBD), in addition to the other variables discussed, I expected to identify the drivers of enzyme activity in the different marshes and depths. To address these hypotheses, I quantified sediment redox, measured physical and chemical sediment properties (i.e., DBD, OC, N and OC/N) and characterised microbial communities and enzyme activity levels in adjacent restored and natural marshes. Furthermore, these parameters were assessed across different sediment depths.

4.3 Methodology

4.3.1 Study site and sampling stations

Fingringhoe Wick Nature Discovery Park, Essex is located approximately 12 km southeast of Colchester in the east of England (Fig. 4.1). This site contains both a natural established saltmarsh (51.837373, 0.977097) and a restored marsh (51.842106, 0.974856; Fig. 4.1). The restored marsh is an area of former agricultural land to which tidal inundation was reintroduced in 2015 by creating two breaches in the seawall (Vaughan, 2019). Prior to the breach, the part of the marsh I sampled was used as an arable field (Cai *et al.*, 2022). The natural marsh is densely vegetated while the restored site largely resembles a mudflat with vegetation growing only on the higher elevation fringes and seawall banks, as a result samples from the restored site were representative of an area far lower in the tidal frame that experience different levels of inundation and drainage. The tidal range is 4.5 m (Wolters *et al.*, 2005).



Figure 4.1. The locations of the sampling site within (a) the UK and (b) the east of England, as well as the locations of the sampling stations within (c) Fingringhoe Wick Nature Discovery Park, Essex. Larger turquoise markers represent the main sampling stations focused upon throughout the study while the smaller white markers represent the secondary locations where additional samples were taken to verify whether microbial community differences were down to restoration status or variables related to tidal frame position of the marshes. Map imagery © Microsoft Bing Maps; satellite imagery © DigitalGlobe (Maxar), 2020.

Four primary, 1 m² sampling stations were selected, two each in the restored (R) and natural (N) saltmarsh (Fig. 4.1; Table S4.1; Fig. S4.1). On the restored marsh, station R-Corner was situated in the southeast corner while R-Breach was nearby but closer to the seawall breach. The first of the natural marsh sampling stations, N-Creek, was enclosed on two sides by the bend in a creek and the second station, N-Cliff, was very near the boundary between the marsh and the estuary, on top of a

small cliff. Of the secondary locations shown in Figure 1, N-low was at the bottom of the marsh cliff, N-wall was near the seawall that divides the two marshes and R-opposite was relatively far removed from the other two restored sampling stations, on the opposite side of the site (Fig. 4.1c).

4.3.2 Indicators of Reduction in Soils (IRIS) Sticks

Producing and using Indicator of Reduction in Soils (IRIS) sticks is an established method for determining the redox levels of soils at different depths (Castenson and Rabenhorst, 2006; Rabenhorst and Burch, 2006; Mittmann-Goetsch *et al.*, 2024). Briefly, to prepare the iron oxide precipitate used to coat the stick, 8 g anhydrous FeCl_3 was dissolved in 250 mL distilled water before adding 370 mL of 1 M KOH to increase the pH to 12. Six centrifugations at 1,000 rpm for five minutes each were used to remove ions from the resulting precipitate, which was then placed in a bath of distilled water for three days. After this, a final centrifugation for five minutes at 1,000 rpm was performed. This iron oxide precipitate was applied with a sponge to PVC film sheets that were subsequently cut into strips 50 cm in length and 10 cm in width. Using double sided tape, the strips were attached to 0.3 cm thick PVC foam sticks, 60 cm long and 10 cm wide. Sticks were prepared no more than three weeks in advance of placing them in the saltmarsh sediment. Fresh IRIS sticks were prepared before each visit to the site.

The first set of IRIS sticks was deployed on 19/01/24. Subsequently, sticks were collected and replaced every four to six weeks until December of the same year. Collections occurred on the following dates: 04/03/24, 25-26/04/24, 29/05/24, 09-10/07/24, 29/08/24, 09-10/10/24, 08/11/24 and 09/12/24. Sticks were inserted up to a depth of 50 cm, but it was not always possible, particularly in the restored marsh, to fully submerge the IRIS sticks because resistance was encountered below this depth. After returning to the laboratory, sticks were gently washed to remove adhering sediment before being scanned against a black background. The resulting images were analysed using a Random Forest model in the software R (version 4.4.1), following the method of Mittman-Goetsch *et al.* (2024). Each pixel was classified as either reduced (i.e., white), not reduced (i.e., orange) or as errors (i.e., the black background). For each ten-centimetre increment of the IRIS sticks, reduction indices were calculated that represent the proportion of pixels within each increment classed as reduced. Therefore, a higher reduction index value indicates that more paint was removed and thus represents conditions that were more strongly reducing and in which oxygen availability was lower. Furthermore, removal of the coating was shown in another study to be proportional to the abundance of metal-reducing microbes and thus also reflects the activity of these groups in the soil being investigated (Hino *et al.*, 2023). Results from sticks incubated from 19/01/24 to 04/03/24 and from 08/11/24 to 09/12/24 were combined to represent the conditions during winter and those incubated from 29/05/24 to 09-10/07/24 and from 09-10/07/24 to 29/08/24 were combined to represent summer conditions.

4.3.3 Sediment sampling

Sediment sampling took place on the following dates in the main sampling stations, marked by turquoise markers in Figure 1: 19/01/24 and 09-10/07/24, representing winter and summer conditions, respectively. Three replicate cores, 50 cm in length, were taken with a 2.7 cm diameter gouge auger from each of the primary sampling stations during these visits to the site. As with the IRIS sticks, resistance was sometimes encountered above 50 cm depth and, in these cases, shorter cores were obtained. It was previously shown that the relic agricultural layer in the restored marsh was between 7 and 20 cm below the surface in different parts of the site (Chapter 2). Replicate cores from a single sampling station were taken from within the same 0.5 m² area. Cores were subsampled in the field at the following resolution: 0-1, 1-2, 2-3, 3-4, 4-5, 5-10, 10-20, 20-30, 30-40 and 40-50 cm. Additional samples were taken from the secondary sampling stations indicated by white markers in Figure 1 on 08/11/24, as well as from the main sampling station R-breach. These were 10 cm² and 1 cm deep and therefore representative of only the surface sediment. These additional samples were only included in the microbial community analysis (see section 4.3.4). Samples were frozen at -20 °C until laboratory analyses were performed.

4.3.4 DNA extraction and sequencing

Not all core depths were used for DNA extraction and sequencing. The top and bottom depths of all three replicate cores from each sampling station and both sampling months were always analysed. Previously, it was determined that samples from this depth (i.e., 40-50 cm) in the restored marsh were representative of a relic agricultural soil, present in the site prior to breach creation (Chapter 2). In addition, where the IRIS stick data indicated variation in redox conditions that occurred at intermediate depths, these samples were also analysed. Each treatment had three replicates. The additional surface samples taken during November were also included, with three replicates being taken from each sediment sample. DNA was extracted from all these samples with the DNeasy Power Soil Pro Kit (QIAGEN) according to the manufacturer's instructions. One minor modification was required: for the surface samples, DNA was extracted from ~0.25 g of sediment, as was specified in the standard protocol, but it was necessary to use 0.40 g of sediment from the deep samples to ensure a sufficiently high concentration of DNA was obtained. DNA purity and quality was assessed with a Nanodrop spectrophotometer before samples were frozen at -20 °C before sequencing and agarose gel electrophoresis. Samples were sent to Novogene for PCR amplification and 16S rRNA gene Illumina sequencing. The 16Sv34 region was amplified using the primers CCTAYGGGRBGCASCAG and GGACTACNNGGGTATCTAAT. With a paired-end Illumina platform, libraries were sequenced and then the reads were assembled. After sequencing, the raw data was cleaned using DADA2 to reduce noise by removing duplicates with dereplication to 100 % similarity clustering (Callahan *et al.*, 2016; Li *et al.*, 2020). From this step Amplicon Sequence Variants (ASVs) were obtained. With the classify sklearn algorithm from QIIME2 (Bokulich *et al.*, 2018; Bolyen *et al.*, 2019), a pre-trained Naïve Bayes classifier was employed to provide species

annotation for each ASV. Silva 138.2 was the database used. To examine the alpha diversity, the Shannon index (Shannon, 1948) was calculated with the ASV data using the diversity function from the package *vegan* in R (Oksanen *et al.*, 2025). Information about the potential functions of the different genera identified was taken from the existing literature. Additionally, the FAPROTAX database was used to make predictions about the functions the microbial taxa present in the samples were capable of.

The top nine most abundant FAPROTAX predicted functions were focused upon because all other functions had only very low abundances. Furthermore, very similar functions were combined and thus the final seven categories closely examined were: (1) aerobic chemoheterotrophy; (2) anaerobic chemoheterotrophy (i.e., abundances in the category “aerobic chemoheterotrophy” subtracted from abundances in “chemoheterotrophy”); (3) fermentation; (4) nitrification (i.e., the sum of the categories “nitrification” and “aerobic ammonia oxidation”); (5) sulphate respiration (i.e., the sum of the categories “respiration of sulphur compounds” and “sulphur respiration”); (6) iron respiration; (7) methanogenesis.

4.3.5 Enzyme assays

Except for the additional surface samples taken during November, the same samples taken forward to microbial diversity analysis were used to perform enzyme assays. When samples were thawed for DNA extraction, a subsample was used to create a sediment slurry by adding approximately 2.5 g of sediment to 30 mL of 0.05 M phosphate buffer and shaking by hand for thirty seconds to mix. Sediment slurries were then frozen until enzyme assays could be performed.

Fluorometric microplate enzyme assays were conducted as previously described by German *et al.* (2011), Mueller *et al.* (2017) and Mueller *et al.* (2020) and similarly to the method describes in Chapter 3. Pre-pipetted into black Sterilin 96-well plates (611F96BK) was 50 μL of either 0.05 M phosphate buffer (pH of either 5.8 or 7.4), 100 $\mu\text{mol L}^{-1}$ fluorophore standard or 1.6 mmol L^{-1} substrate solution. Once all pre-pipetting was complete, either 200 μL of the soil slurry or 200 μL of the buffer were added to the wells. Each soil slurry had four technical replicates. Plates were left to incubate in the dark for 16 h, before fluorescence was recorded with a SpectraMax iD5 Microplate Reader (Molecular Devices) with an excitation value of 365 nm and an emission value of 460 nm. Assays were conducted for β -glucosidase (EC 3.2.1.21), chitinase (EC 3.2.1.14), cellobiosidase (EC 3.2.1.91) and phosphatase (EC 3.1.3.1) using various substrates that were all conjugated to the fluorophore 4-methylumbelliferone (4-MUF) and for leucine-aminopeptidase (EC 3.4.11.1) with a substrate conjugated to 7-amido-4-methylcoumarin hydrochloride (7-AMC). β -glucosidase, chitinase, cellobiosidase and phosphatase assay were conducted at pH 5.8 while leucine aminopeptidase assays were conducted at pH 7.4 to provide conditions that maximised possible enzyme activity to capture clear, consistent differences between samples (Chapter Three).

4.3.6 Determination of sampling station properties

To determine dry bulk density (DBD), organic carbon (OC) and nitrogen (N) content, subsamples were obtained with a modified syringe, (i.e., with the top sawed off) from each core sample used for DNA extraction and enzyme assays. The volume of each sample was recorded from the syringe and then samples were freeze dried. Once dry, samples were weighed to determine the dry weight, enabling the calculation of the dry bulk density (mass of the dried sediment divided by the volume of sediment; Dadey *et al.*, 1992). Following acidification with sulphurous acid (6 %) to remove carbonates and a second freeze drying, samples were homogenised with a ball mill and OC and N content determined by elemental analysis with an CE440 elemental analyser (Exeter Analytical). OC/N ratios were calculated by dividing the OC content by the N content.

4.3.7 Statistical analyses

Differences in the biogeochemical properties, IRIS results, Shannon Index values and enzyme activities between marsh type, sampling month and depth category (surface and deep) were determined with three-way ANOVAs. Middle depths were excluded as results from these depths were not obtained from every core. In some cases, response variables were log transformed to achieve a normal distribution and homogeneity of variance. This was ensured through the visual inspection of histograms and Q-Q plots. Often, the results of the Shapiro-Wilks tests remained significant after transformation but given that this test is known to be particularly sensitive when moderate to large datasets are used, this was deemed acceptable after the visual inspection step confirmed there was no major skewedness, for example (Ruxton *et al.*, 2015; Paramasivam *et al.*, 2024). Canonical Correspondence Analysis (CCA) was performed with ASV data. The formula used for the CCA was `cca(asv_df ~ OC + IRIS + Midpoint + C.N + DBD + Marsh_zone, data = env)`. Neither sampling month, sampling station nor marsh type were free of confounding by the fact that the sampling strategy was unbalanced, meaning that all the natural marsh primary sampling stations were representative of high marsh zones while every restored marsh stations were experiencing low marsh zone conditions. Furthermore, the secondary natural marsh sampling station that represented the low marsh zone, included to provide extra context for interpreting microbial community differences, was only sampled once. Therefore, I opted to use marsh zone the variable in this analysis as there was a relatively equal balance between high and low marsh zones and this more accurately reflected the likely cause of the differences in microbial communities (Table 4.1). Furthermore, when N content was included in the model, the variance inflation value (VIF) of this variable and that of OC content became greatly inflated (>10) so only one was included in the final model, a decision made acceptable by the close correlation between the two (Table S4.2). Correlation matrix analysis was performed to determine the relationships between the biogeochemical variables measured and the activities of the different enzymes recorded. For this analysis, enzyme activity values were activity per g OC m⁻² y⁻¹. When bacterial genera were examined, only the top twenty established accepted genera were considered so that conclusions could be drawn about the potential functioning

capabilities of the communities present. However, another top twenty that includes candidate genera is also reported (Fig. S4.2).

4.4 Results

4.4.1 Environmental conditions

Plant communities in the natural saltmarsh sampling stations, both of which represented a mature, established ecosystem, were far more diverse than those in the restored site, where *Spartina anglica* was the only species present in both sampling stations (Table 4.1). *Armeria maritima* and *Aster tripotum* dominated in N-Creek. N-Cliff contained an even split between these species, *Salicornia* spp. and bare sediment.

Table 4.1. Plant communities recorded in the four primary sampling stations during August of 2024. Bare sediment is defined as exposed substrate lacking any plant cover above the surface. Sampling stations named in bold text were the main sampling stations, focused upon throughout the study, while those named in non-bold text were secondary locations that were sampled to provide additional evidence in the microbial community analysis (Fig. 4.1; see section 4.3.3). The marsh zone assigned to each station in the canonical correspondence analysis (see section 4.3.7) is also detailed.

Sampling station	Ground cover category	Cover (%)	Marsh zone
N-creek	<i>Armeria maritima</i>	58.0	High
	<i>Aster tripotum</i>	30.0	
	<i>Salicornia</i> spp.	10.0	
	Bare sediment	2.0	
N-cliff	<i>Armeria maritima</i>	25.0	High
	<i>Aster tripotum</i>	15.0	
	<i>Salicornia</i> spp.	27.5	
	<i>Spartina anglica</i>	5.0	
	Bare sediment	27.5	
N-low	<i>Spartina anglica</i>	90	Low
	Bare sediment	10	
N-wall	<i>Armeria maritima</i>	70	High
	<i>Aster tripotum</i>	15	
	Bare sediment	15	
R-corner	<i>Spartina anglica</i>	90.0	Low
	Bare sediment	10.0	
R-breach	<i>Spartina anglica</i>	90.0	Low
	Bare sediment	10.0	
R-opposite	<i>Salicornia</i> spp.	75	Low
	Bare sediment	25	

The purpose of including the secondary sampling stations in the study design becomes clear when the plant community compositions are examined and viewed as proxies for tidal inundation frequency and drainage. N-wall closely resembles N-creek and N-cliff, with *A. maritima* dominating and *A. tripotum* present in lower amounts. On the other hand, the fourth natural marsh station, N-

low, is dominated exclusively by *S. anglica* and thus resembles the restored marsh sampling stations more closely than the other natural marsh stations (Fig. S4.1). This indicates that N-wall experience similar tidal inundation and drainage conditions to R-creek and R-breach. Finally, R-opposite, the only secondary restored marsh sampling station, differs from the other two restored marsh stations in being dominated exclusively by *Salicornia* spp. (Table 4.1).

The biogeochemical properties showed strong significant differences between marsh type and depth but not between months (Tables 4.2 & S4.2). OC and N content decreased with depth and tended to be lower in the restored marsh. DBD values remained relatively stable between the surface and deep sediment of the natural marsh but in the restored marsh values were far higher in the deep compared with the surface sediment. OC/N ratios were lower in the restored marsh surface sediment compared with the natural marsh but in the deep sediment restored marsh values rose and thus were like those of the natural marsh deep sediment.

Table 4.2. Results of the three-way ANOVA tests used to determine the impacts of marsh type, sampling depth, sampling month and the interaction between these variables upon the biogeochemical properties of the samples ($df = 1, 40$). Although values for intermediate depths are shown in Table S4.2, the statistical testing only involved values from the top and bottom depths. Significant results were never obtained for the following: Month ($p > 0.100$), Month x Marsh ($p > 0.120$) and Month x Marsh x Depth ($p > 0.510$). Highlighted in bold text are significant p -values. These analyses included only primary sampling stations, shown with turquoise markers in Figure 1c.

Variable	Level/Interaction	Adjusted p -value	F -value
OC	Marsh Type	<0.001	25.371
	Depth	<0.001	42.057
	Marsh x Depth	0.429	0.638
	Month x Depth	0.074	3.363
N	Marsh Type	0.003	10.000
	Depth	<0.001	118.734
	Marsh x Depth	0.059	3.776
	Month x Depth	0.016	6.323
OC/N	Marsh Type	<0.001	36.281
	Depth	<0.001	83.815
	Marsh x Depth	0.323	1.001
	Month x Depth	0.901	0.016
DBD	Marsh Type	<0.001	126.830
	Depth	<0.001	113.844
	Marsh x Depth	<0.001	76.818
	Month x Depth	0.399	0.727

4.4.2 Sediment redox conditions

In both marshes, the reduction indices recorded in winter were lower than those recorded in summer (Fig. 4.2; Table S4.3), indicating that conditions were more strongly reducing and oxygen availability was lower during the former. The different marsh types emphasised the contrast with values becoming especially low (<0.10) in the high elevation natural marsh during the winter (Fig. 4.2a & 4.2c; Fig. S4.3-S4.4) and especially high (>0.80 in R-Breach) in the low elevation restored marsh during summer (Fig. 4.2f & 4.2h; Fig. S4.5-S4.6). The IRIS data from the secondary sampling stations (Fig. S4.7-S4.9) were not included in the statistical analysis but the high reduction indices recorded in N-low (Fig. S4.8; $0.47 - 1.00$), make it clear that the redox conditions in this low elevation natural marsh sampling station more closely resembled the restored marsh conditions than those of the other natural marsh stations. Statistical testing showed that reduction indices differed significantly between season and marsh type, and there was a significant interaction between these two variables (Table S4.3). IRIS sticks incubated during spring and autumn showed that reduction index values increased gradually in the spring and remained high during the autumn before the winter decreases already described (Fig. S4.2 – S4.9). There were a few exceptions to this rule, however: the R-Breach sampling station had highly variable reduction index values in the winter, with some values close to zero and others close to one (Fig. 4.2g). N-Cliff reduction indices remained relatively low in the summer with means of less than 0.4, except for a few higher individual values (Fig. 4.2d). Along the depth gradients investigated, reduction index values tended to remain largely stable, but the clearest contrast between depths occurred in N-Creek during the summer, with values noticeably higher (>0.70) between 20 and 50 cm depth compared with the lower reduction index (<0.50) recorded between the surface sediment and 20 cm depth (Fig. 4.2b).

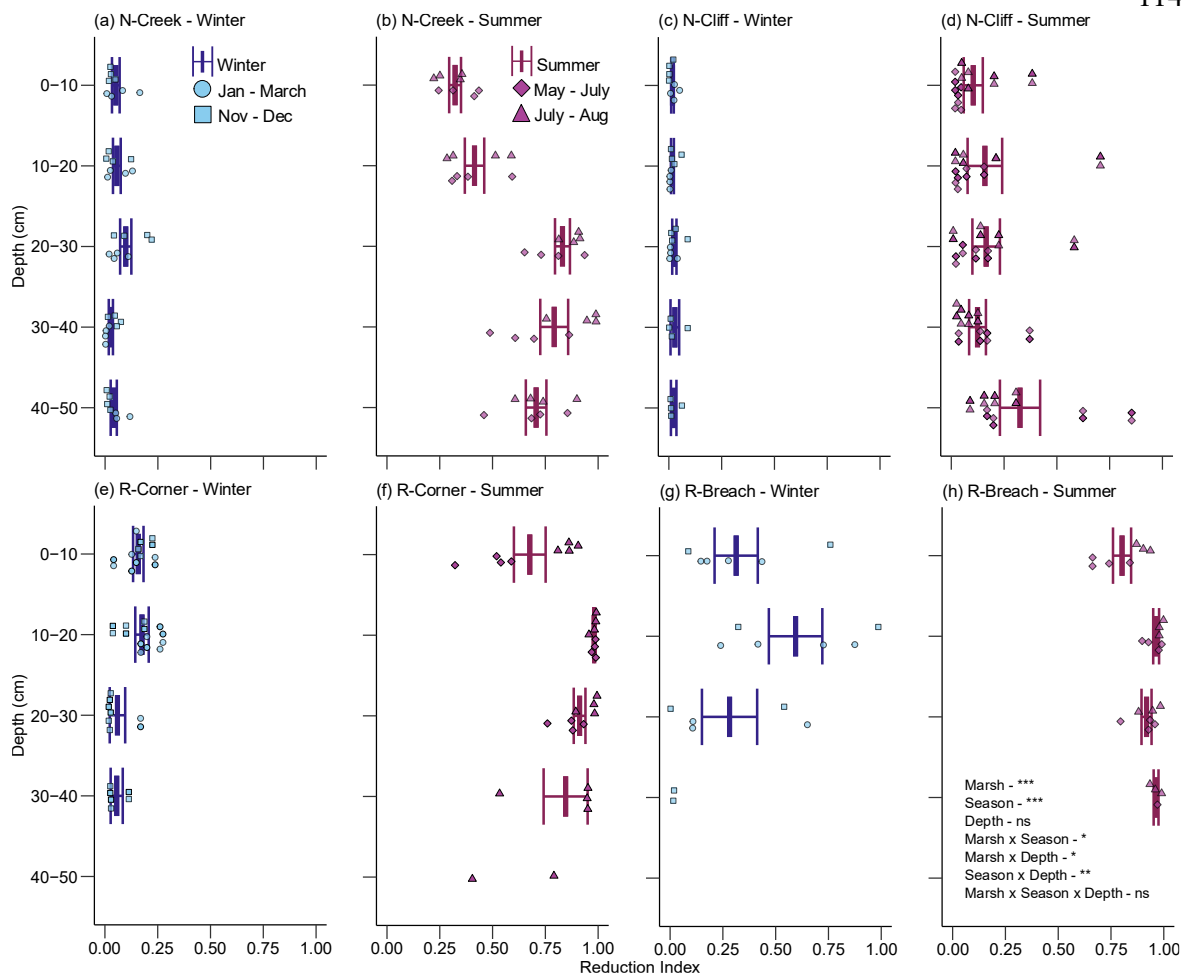


Figure 4.2. Indicators of reduction in soils (IRIS) sticks were placed (a-d) in the natural and (e-h) restored marsh sampling stations in two batches each over the winter (blue) and summer (red) months. Mean and error bars are only show when there are ≥ 3 values, otherwise only the individual data points are present. Error bars are $\pm$ standard error. Results of the three-way ANOVA are shown within the bottom right panel (***) - $P \leq 0.001$, (**) - $P \leq 0.01$, (*) - $P \leq 0.05$ and ns - $P > 0.05$; Table S4.3). Higher reduction index values are indicative of low oxygen conditions.

4.4.3 Microbial diversity

Canonical Correspondence Analysis with ASVs

The CCA model was significant ($p = 0.001$; $F = 2.58$; global permutation test with 999 permutations; Fig. 4.3a). The first two canonical axes explained 11.57 % of the total variance (CCA1 = 6.13 %, CCA2 = 5.44 %; eigenvalues were 0.77 and 0.69, respectively). Of the constrained variance, CCA1 explained 29.84 % and CCA2 26.51 %, meaning a cumulative total of 56.35 % was captured by these axes. Key environmental variables that were significant predictors were marsh zone ($p = 0.001$; $F = 2.51$), OC content ($p = 0.001$; $F = 2.17$), reduction index categorisation (i.e., values classed as either greater than or less than 0.5; $p = 0.002$; $F = 1.60$), OC/N ratio ($p = 0.001$; $F = 2.87$) and DBD ($p =$

0.001; $F = 2.45$). The other variable included, depth, was also close to be significant ($p = 0.09$; $F = 1.23$). The total constrained variance explained by these variables was 20.53 %.

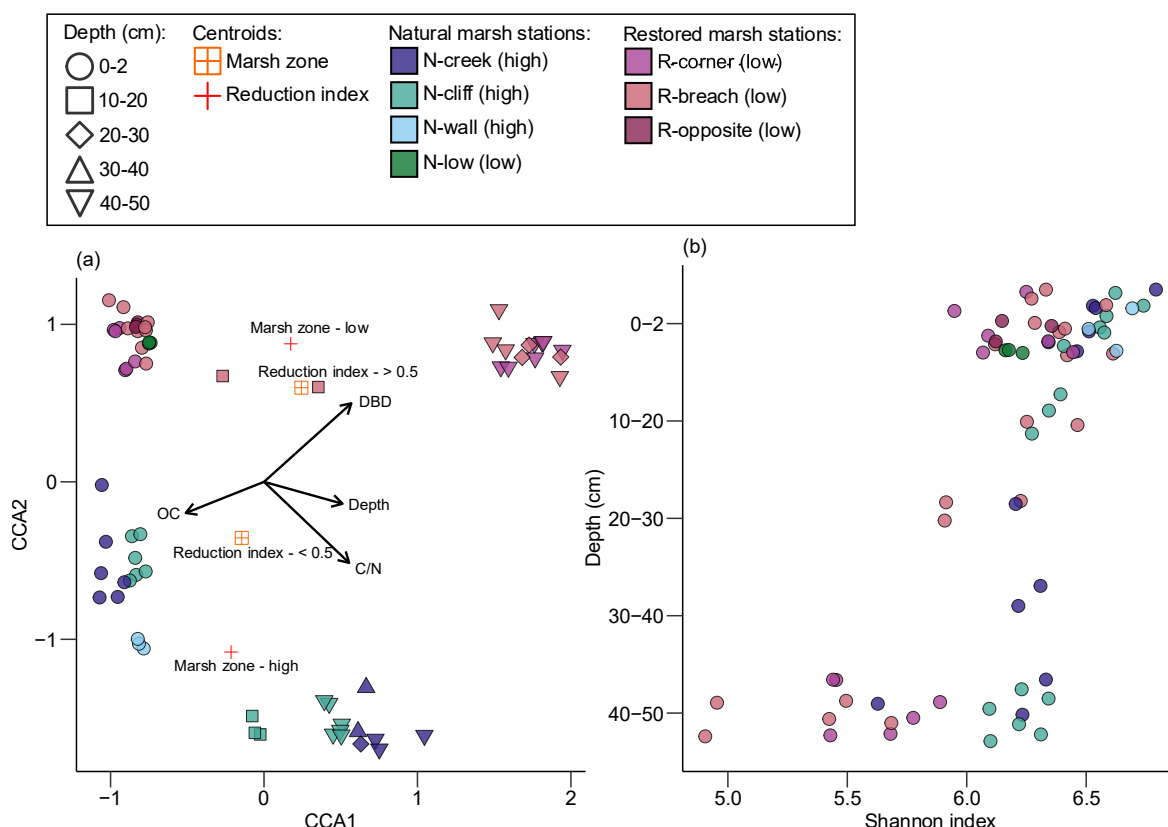


Figure 4.3. The 16S rRNA gene sequencing results were analysed using (a) canonical correspondence analysis (CCA) and (b) the Shannon diversity index, both calculated with ASV data. Shannon index values were analysed with a three-way ANOVA (** - $P \leq 0.01$ and *** - $P \leq 0.001$; Table S4.3). Month was not significant ($P > 0.36$) and neither were the following interactions: marsh x month ($P > 0.78$), month x depth ($P > 0.49$) and marsh x month x depth ($P > 0.89$). The depths of individual samples were divided into categories for visualisation, but this variable was a continuous one in the CCA analysis, with the midpoint depth of each core section analysed being used. The marsh zones represented by each sampling stations are detailed in brackets in the key.

The canonical correspondence analysis differentiated strongly between the microbial assemblages of the high and low marsh zones and between different depths (Fig. 4.3a). Surface samples were clustered by marsh zone, and the two groups were relatively close together while the deeper sediment samples were also in marsh-based clusters, but the two groups were located far apart. In descending order of influence, the variables that drove these differences in assemblages were marsh zone, DBD, OC/N ratio, OC content, reduction index category (i.e., values below or

above 0.5) and depth. DBD and OC content pulled in almost opposite directions. Reduction index and marsh zone centroids were closely clustered while the reduction index centroid and depth arrow were separated by close to 90°. Without the inclusion of N-low, the different clusters would have appeared to be separate cleanly by marsh type (i.e., restored or natural). However, N-low microbial communities resemble those found in the other low elevation sites from the restored marsh, as opposed to those present in the high elevation parts of the natural marsh (Fig. 4.3a).

Shannon index diversity

Shannon index values also showed a clear and significant difference between marsh zones, the low marsh being less diverse (Fig. 4.3b; Table S4.3). Furthermore, there was a depth effect, with the gap between marshes widening in the deeper sediment. For example, in samples from 40-50 cm, the deepest depth category, Shannon index values for the restored marsh ranged between 4.91 and 5.89 while in the natural marsh the range was 5.63 to 6.34.

Bacterial phyla

When the primary sampling stations were considered, there were differences between (1) the number and identity of phyla detected in the surface sediment samples compared with sediment from deeper down the cores (10-50 cm depth) and (2) between the natural and restored saltmarsh samples. In contrast, there was very little, if any, seasonal variation in the microbial diversity (Fig. 4.4). Pseudomonadota was almost always the most abundant taxon across the samples analysed but in some of the restored marsh samples Thermodesulfobacteriota was almost as or more abundant (e.g., Fig. 4.4d, 4.4g & 4.4h). Thermoproteota were absent or present at only very low abundances in the surface sediments of both marshes while their relative abundances were higher in the deep samples. Another difference between marshes was the relatively high abundance of Actinomycetota (4 – 31 % of the total reads) in the deepest sediment of the restored marsh while it was present in only very low amounts in the deepest sediment of the natural marsh (1 – 3 %). Myxococcota was present in very low abundances in the natural marsh (1 – 5 %) and values were even lower in the restored marsh (0.2 – 2.0 %).

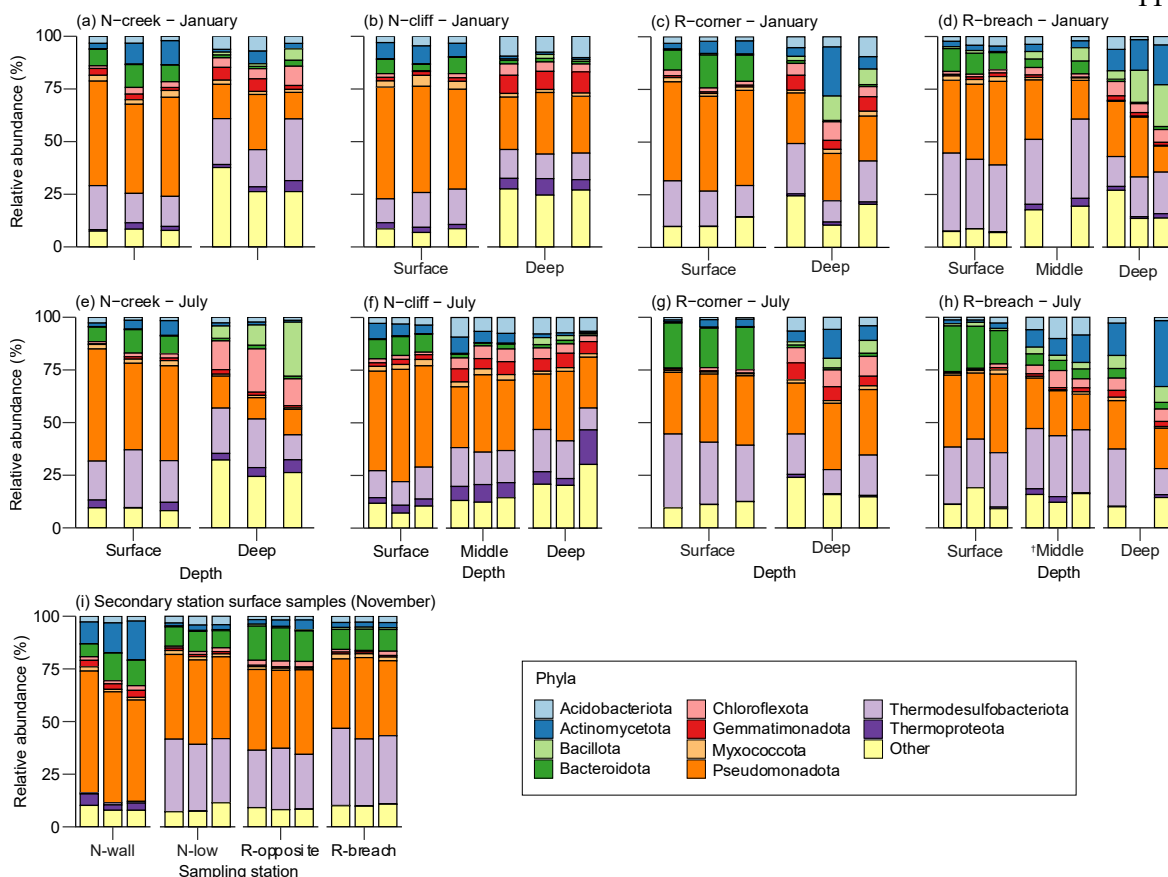


Figure 4.4. The bacterial phyla present during (a-d) January and (e-h) July in the (a-b and e-f) natural and the (c-d and g-h) restored marshes at different depth intervals. Also, (i) the phyla present in the secondary sampling stations during November. Values show the percentage of total reads represented by each phylum. The top ten most abundant phyla are shown with all remaining taxa classed together under the “Other” category (i.e., the pale-yellow bars that are always at the bottom of the stack). Replicate cores from the same month, station and depth are differentiated in the text with the labels A, B, and C, and these are always applied from left to right. *Deep refers to samples from 25-35 cm depth in this case and 40-50 cm in all others. †Middle refers to samples from 20-30 cm depth in this case and 10-20 cm in both other cases.

One difference between surface and deep sediment present across both marshes was the decrease in Bacteroidota abundance with depth (Fig. 4.4). If Bacillota were present, it was only in those samples from the middle and bottom depths, and while this was always true in the restored marsh, it was sometimes also absent from the deeper sediment of the natural marsh. Chloroflexota, Gemmatimonadota and, to a lesser degree, Acidobacteriota, increased in abundance with depth and were at times entirely absent from the surface sediments.

The additional samples taken from the secondary sampling stations during November were also dominated by Pseudomonadota (Fig.4.4i). In the three low marsh stations, N-low, R-opposite and R-breach, Thermodesulfobacteriota were always as or more abundant than Pseudomonadota.

Conversely, Thermodesulfobacteriota were almost absent from the high marsh station N-wall. Furthermore, N-wall had higher abundances of the phyla Actinomycetota, Verrucomicrobiota and Gemmatimonadota than the other three secondary sampling stations.

Genera abundances and predicted functions

Woeseia – an aerobe capable of facultative anaerobia commonly present in marine systems (Hoffman *et al.*, 2020) – was by far the most abundant of the established accepted genera in the surface sediments (Fig. 4.5a). In the natural marsh surface sediment, the mean abundance of this genus was 10.9 ± 1.0 % (calculated from all the natural marsh surface samples taken during winter and summer; the same principle is relevant to all subsequent mean values reported in the text and shown in Fig. 4.5). The abundances of *Woeseia*, however, were lower in the restored marsh surface samples, with a mean value of 6.6 ± 0.7 % (Fig. 4.5a). Genera that were more abundant in the restored marsh surface sediments compared with the natural were *Roseobacter*, *Lutimonas*, *Roseibacillus*, *Haloferula*, *Halioglobus*, *Luteolibacter*, *Seongchinamella*, *Lutibacter* and *Desulfosarcina* (Fig. 4.5a). Except for *Lutibacter* – some strains of which are facultatively anaerobic (Lee *et al.*, 2012) – and *Desulfosarcina* – anaerobic sulphate reducers (Harms *et al.*, 1999) – all of these genera are obligate aerobes (Shiba *et al.*, 1991; Yang *et al.*, 2007; Yoon *et al.*, 2008a; Yoon *et al.*, 2008b; Park *et al.*, 2012; Kim *et al.*, 2015; Kang *et al.*, 2020). A

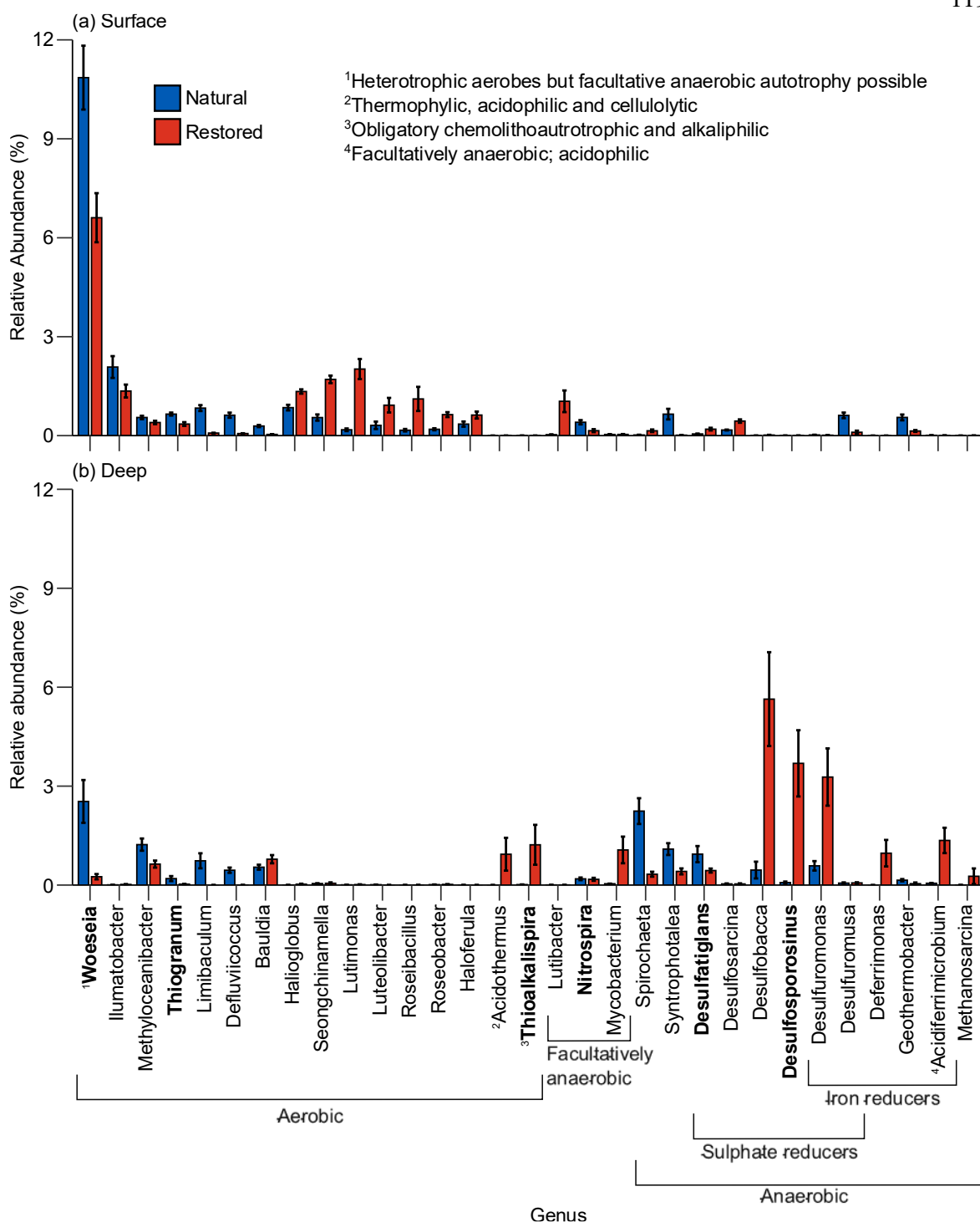


Figure 4.5. Mean relative abundances of the top thirty-one most abundant accepted genera in the (a) surface and (b) deep sediment of the natural (blue) and restored (red) marshes. Genera for which evidence of autotrophy exist are highlighted in bold along the x-axis (Watson *et al.*, 1986; Sorokin *et al.*, 2002; Mori *et al.*, 2015; Musmann *et al.*, 2017; Jochum *et al.*, 2018; Agostino *et al.*, 2020). Error bars show $\pm$ standard error.

Three genera known to be obligate anaerobes with sulphate and iron reducing capabilities – *Desulfuromonas*, *Desulfobacca* and *Desulfosporosinus* (Oude Elferink *et al.*, 1999; Pfennig and

Biebl, 1976; Pester *et al.*, 2010) – were far more abundant the restored marsh deep sediment than in the natural (Fig. 4.5b). The mean abundances of these genera were 3.3 ± 0.9 , 5.6 ± 1.4 and 3.7 ± 1.0 %, respectively, in the restored marsh deep sediment but only 0.6 ± 0.1 , 0.5 ± 0.3 and 0.08 ± 0.03 % in the natural marsh deep sediment. Other genera with higher abundances in the restored deep sediment compared with the natural were *Acidothermus*, *Thioalkispira*, *Deferrimonas*, *Acidiferrimicrobium*, *Mycobacterium* and *Methanosarcina* (Fig. 4.5). The presence of several of these genera in a saltmarsh is unexpected. For example, *Acidothermus* is strictly aerobic, thermophilic and acidophilic; its temperature growth range was reported to be between 37 and 70°C while its pH requirements are between pH 3.5 and 7.0 (Mohagheghi *et al.*, 1986). *Acidiferrimicrobium* is also acidophilic but is capable of iron reduction (González *et al.*, 2020). On the other hand, *Thioalkispira* is an alkaliphile, with a pH growth range that ranges between pH 8 and 10 (Sorokin *et al.*, 2002). This genus is also strictly aerobic but grows only under reduced oxygen concentrations of less than 2 % and is chemolithoautotrophic (Sorokin *et al.*, 2002). Less unexpectedly, *Mycobacterium*, *Methanosarcina* and *Deferrimonas* are a facultative anaerobe (Gengenbacher and Kaufmann, 2012) and two obligate anaerobes, respectively (Galagan *et al.*, 2002; Badalamenti *et al.*, 2016). *Methanosarcina* uses methane for respiration while *Deferrimonas* uses iron (Galagan *et al.*, 2002; Badalamenti *et al.*, 2016).

Genera more abundant in both the surface and the deep sediment of the natural marsh, compared with the restored, were *Limibaculum*, *Methyloceanibacter*, *Deffluviivovvus* and *Syntrophotalea* (Fig. 4.5). Respectively, these are an aerobic heterotroph (Shin *et al.*, 2017), an aerobic, facultative methylophile (Takeuchi *et al.*, 2014), an aerobe (Maszenan *et al.*, 2005) and a strictly anaerobe (Waite *et al.*, 2020). In the surface sediments of the natural marsh, *Ilumatobacter*, *Geothermobacter*, *Bauldia*, *Thiogramum*, *Nitrospira*, and *Desulfuromusa* were more abundant than in the restored marsh and, for the most part, became less abundant in the deep sediment. *Ilumatobacter*, *Bauldia* and *Thiogramum* are aerobic heterotrophs (Matsumoto *et al.*, 2009; Yi *et al.*, 2010; Mori *et al.*, 2015), while *Geothermobacter* is a thermophilic, mesophilic anaerobe capable of iron reduction (Pérez-Rodríguez *et al.*, 2021) and *Desulfuromusa* is a sulphate reducing anaerobe (Liesack and Finster, 1994). *Nitrospira* is primarily aerobic and a nitrite oxidiser (Watson *et al.*, 1986; Koch *et al.*, 2015). Other than *Woeseia*, which remained the genus with the highest mean abundance in the natural marsh deep sediment, genera more abundant in the deep sediment of the natural marsh than in that of the restored were *Spirochaeta* and *Desulfatiglans*. Strains of the former are either anaerobic or facultatively so (Harwood and Canale-Parola, 1984) while the latter is strictly anaerobic and capable of sulphate reduction (Suzuki *et al.*, 2014).

Of the six genera capable of autotrophy (Fig. 4.5), *Woeseia*, *Nitrospira* and *Desulfatiglans* were more abundant in the natural marsh while *Desulfosporosinus*, *Thiogramum* and *Thioalkispira* were more abundant in the restored. *Woeseia*, *Nitrospira* and *Thiogramum* were more abundant in the surface sediments while *Desulfatiglans*, *Desulfosporosinus* and *Thioalkispira* were more abundant

in the deep. Although *Woesia* was the most dominant of these species in the surface of the restored marsh, its abundance decreases dramatically in the deep sediment and *Desulfosporosinus* becomes the most abundant autotrophic genus. Conversely, in the natural marsh, *Woesia* was dominant across both depths. Overall, there was a relatively even division of primary producers between the two marshes and at different depths, but specific genera varied.

The abundances of the genera listed in Figure 4.5 in the secondary sampling station surface sediments showed clear divisions between the high elevation natural marsh station (N-wall) and the three other low elevation stations (N-low, R-opposite and R-breach; Fig. S4.10). *Ilumatobacter*, *Limibaculum*, *Bauldia* and *Nitrospira* were more abundant in the high elevation setting while *Thiogramum*, *Halioglobus*, *Seongchinamella*, *Lutimonas*, *Roseobacter*, *Lutibacter* and *Desulfosarcina* were abundant in the three low elevation samples. This represents considerable, though not complete, overlap with the division of genera between restored and natural (i.e., low and high elevation) primary sampling stations described above.

Predicted functions

Aerobic chemoheterotrophy was predicted to be the most abundant function in the surface sediments of the restored marsh, with mean values ranging between 4.3 and 5.6 % (Fig. 4.6a). A similar level of predicted abundance was apparent in one of the natural marsh stations (N-wall; 4.3 %) but levels were notably lower (1.7 – 2.4 %) in the other three natural marsh stations, two of which were representative of a high marsh setting while the other was situated at a lower elevation and thus more closely resembled the restored marsh. The most abundant predicted function in the natural marsh was nitrification; mean values ranged between 4.2 – 6.9 % in the three higher elevation natural marsh stations. Conversely, values in the low elevation natural marsh station and all three restored marsh stations ranged between 0.05 and 0.2 %. Sulphur respiration was predicted to be slightly more abundant in the restored marsh (0.2 – 0.8 %) than in the natural (0.01 – 0.3 %). Abundances of iron respiration and methanogenesis genes were predicted to be uniformly low in the surfaces of every sampling station (0.004 – 0.01 and 0 – 0.01 %, respectively).

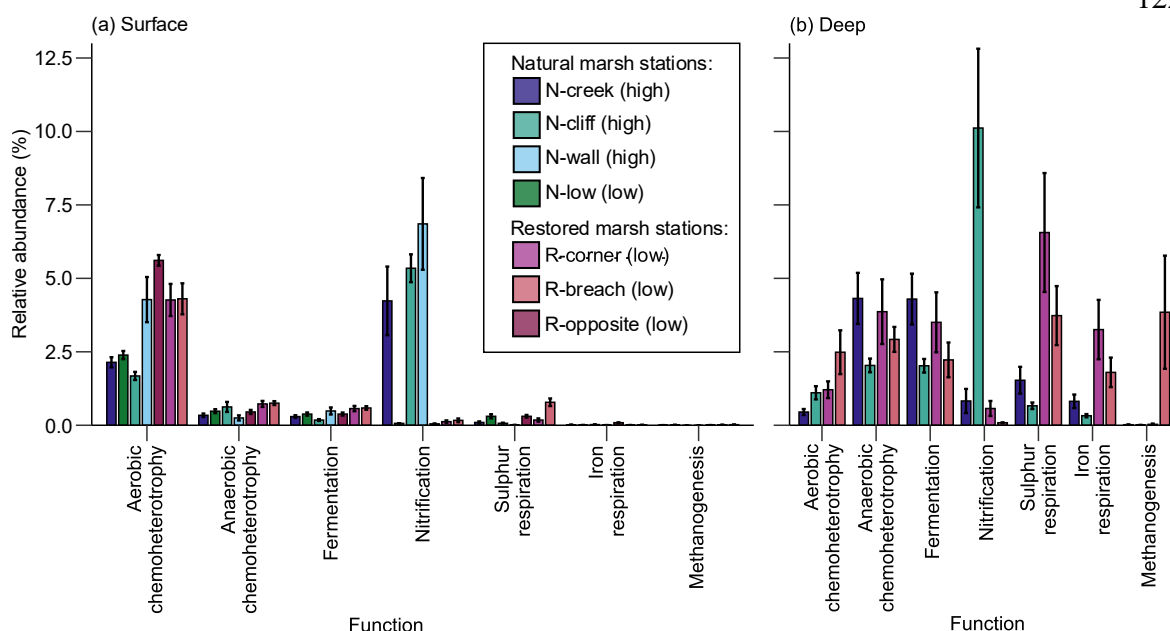


Figure 4.6. FAPROTAX predicted functions in the (a) surface and (b) deep sediment of the natural (blues and greens) and restored (reds and pinks). Only surface samples were taken from the secondary sampling stations and so these are only shown in panel (a). Error bars show $\pm$ standard error.

In the deep sediment, only samples from the primary sampling stations were available for analysis. There were no notable differences between the predicted abundances of aerobic chemoheterotrophy, anaerobic chemoheterotrophy and fermentation between natural and restored stations (Fig. 4.6b). Worth noting is that anaerobic chemoheterotrophy and fermentation were predicted to be more abundant in the deep sediment (2.0 – 4.3 % for both functions) than in the surface sediment (0.2 – 0.8 and 0.2 – 0.6 %, respectively). Predicted abundances of nitrification genes were very in one particular natural marsh station (N-cliff; 10.1 %), compared with the other three sampling stations (0.1 – 0.8 %). Sulphur and iron respiration were more abundant in the restored deep sediment than in the natural, with values in the former ranging between 3.4 and 6.6 % and between 1.8 and 3.3 %, respectively, while values in the latter ranged between 0.7 and 1.5 % and between 0.3 and 0.8 %, respectively. Finally, methanogenesis genes were highly abundant in one restored marsh station (R-breach; 3.8 %) compared with the other three stations (0.001 – 0.03 %).

4.4.4 Enzyme activity

β -glucosidase activity in the surface sediments was lowest in N-creek, especially during July (Fig 4.7a). Indeed, activity of this enzyme was lower during July across all stations. In the natural marsh deep sediment, β -glucosidase activity was lower than in the surface. In the restored marsh, values five and two times greater than the natural marsh deep sediment mean were recorded for R-corner during January and R-creek during July, respectively (Fig. 4.7b). Furthermore, in both cases, the mean values of the deep sediment were higher than the corresponding surface sediment means.

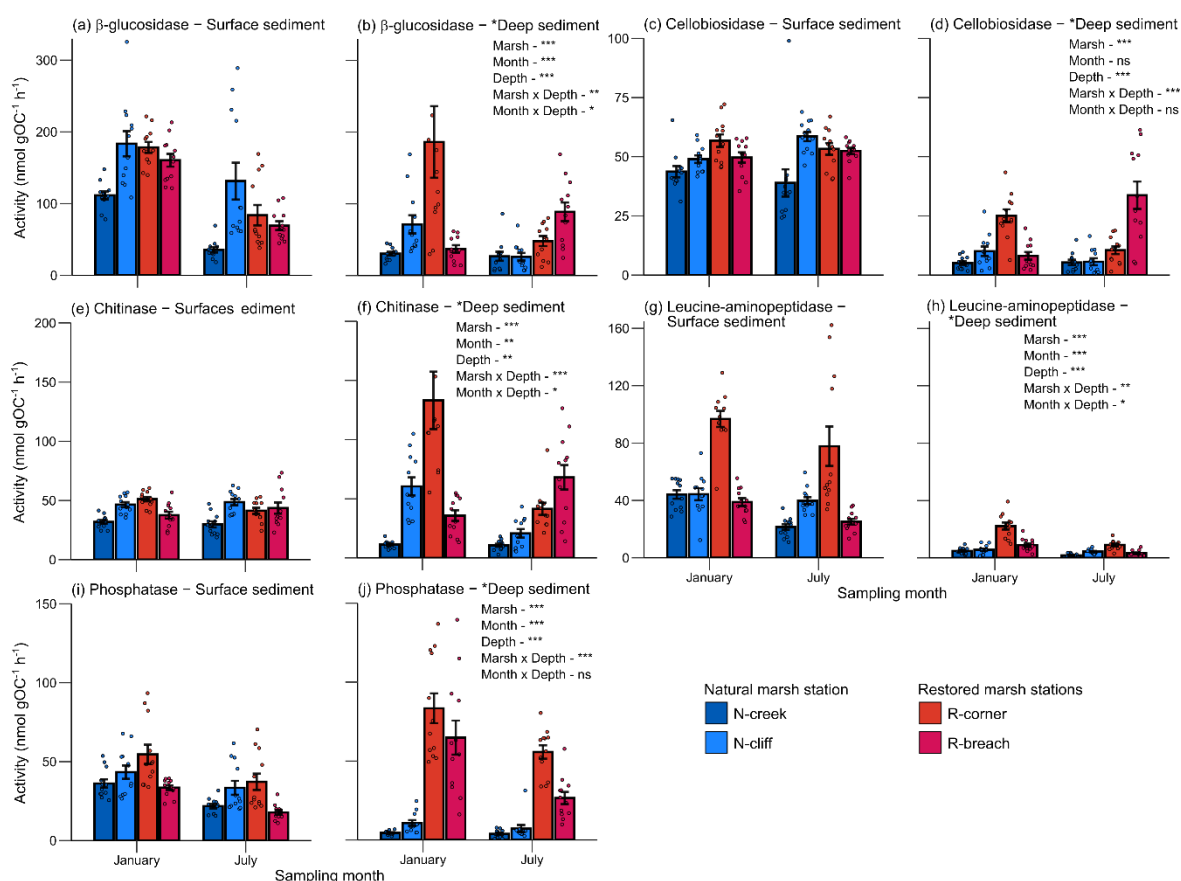


Figure 4.7. The activities of the enzymes (a-b) β -glucosidase, (c-d) cellobiosidase, (e-f) chitinase, (g-h) leucine-aminopeptidase and (i-j) phosphatase recorded in sediment sampled in January and July from the surface (first panel in each pair) and deep (second panel in each pair) sediment of the natural (blue colours) and restored (red colours) marshes. Individual values greater than $350 \text{ nmol g OC}^{-1} \text{ h}^{-1}$ were omitted from the plots for clarity but were included in mean and error bar calculations and in the statistical analysis. Results of three-way ANOVAs are shown within each pair of panels (i.e., one ANOVA per enzyme; *** - $P \leq 0.001$, ** - $P \leq 0.01$, * - $P \leq 0.05$ and ns - $P > 0.05$; Table S4.3). The following interactions were never significant: marsh x month ($P > 0.07$) and marsh x month x depth ($P > 0.07$). *Deep sediment comes from 40-50 cm depth in all cases except one (N-creek in January) in which samples came from between 25-35 cm depth. Results from samples of intermediate depths are not shown for simplicity.

Cellobiosidase activities did not differ significantly between sampling months, and all the surface samples had similar activity levels (Fig. 4.7c & 4.7d; Table S4.3). As with β -glucosidase activity, the lowest surface sample activities of cellobiosidase were recorded in N-creek. Compared with the surface samples, cellobiosidase activity in the natural marsh almost ceased in the deep sediment – the highest mean value in the former being $58.23 \pm 1.96 \text{ nmol g OC}^{-1} \text{ h}^{-1}$ and the highest in the latter $10.08 \pm 2.00 \text{ nmol g OC}^{-1} \text{ h}^{-1}$. Again, like β -glucosidase, there were peaks in deep

sediment cellobiosidase activity in R-corner during January and in R-breach during July (Fig. 4.7d), although in these cases the values did not exceed those of the surface.

In the surface samples, chitinase activity was relatively similar across all samples but N-creek, again, had the lowest mean activity (e.g., 29.81 ± 2.59 nmol g OC⁻¹ h⁻¹ in July; Fig. 4.7e). The highest mean chitinase activity value was recorded in the deep sediment from R-corner in January (133.13 ± 24.24 nmol g OC⁻¹ h⁻¹). N-cliff also produced higher values in the January deep sediment compared with the surface samples (Fig. 4.7f). In the deep sediment during July, the highest chitinase activity was 68.11 ± 10.31 nmol g OC⁻¹ h⁻¹ in the R-breach samples.

Compared to the other sampling stations, leucine-aminopeptidase activity was notably higher in the surface samples of R-corner during both sampling months, with a mean of 96.79 ± 5.56 nmol g OC⁻¹ h⁻¹ in January and 77.78 ± 13.79 nmol g OC⁻¹ h⁻¹ in July (Fig. 4.7g). The other samples were very similar during January while during July N-creek had the lowest activity followed by R-breach. In the deep sediment, leucine-aminopeptidase was very low across all sampling stations compared with the surface sediment (Fig. 4.7f). The mean activity of 22.24 ± 2.45 nmol g OC⁻¹ h⁻¹ in R-corner was the highest recorded for the deep sediments of any station.

Phosphatase activities were higher in the surface samples during January compared with July (Fig. 4.7i). R-corner had the highest activities during both months and N-creek and R-breach the lowest. Deep sediment phosphatase activities dropped dramatically in the natural marsh, from a mean as high as 43.33 ± 4.26 nmol g OC⁻¹ h⁻¹ in the surface samples to one of 10.75 ± 1.81 nmol g OC⁻¹ h⁻¹ in the deep (Fig. 4.7j). In the restored marsh, however, phosphatase activity in the deep sediment exceeded that of the surface samples. Taking the most extreme case as an example, R-corner mean surface activity in January was 54.60 ± 6.10 nmol g OC⁻¹ h⁻¹ while in the deep sediment it was 83.44 ± 9.44 nmol g OC⁻¹ h⁻¹.

For all enzymes examined, there were significant differences in activity levels between marsh type and depth categories (Fig. 4.7; Table S4.3). Activities also differed between sampling months in all cases except for that of cellobiosidase (Fig. 4.7; Table S4.3). Marsh type and depth category had significant interactions in all cases while month and depth interacted significantly for all but cellobiosidase and phosphatase (Table S4.3).

Drivers of enzyme activity

In order of descending strength cellobiosidase, leucine-aminopeptidase and β -glucosidase all had negative correlations with OC/N ratio and depth (Fig. 4.8; Table S4.4). The other two enzymes examined showed either weakly negative or no relationships with these variables. Dry bulk density, N and OC were the only variable with which different enzymes showed contrasting relationships. Cellobiosidase, leucine-aminopeptidase and β -glucosidase had negative relationships with DBD and

positive ones with OC and N content while the opposite was true of chitinase and phosphatase. Reduction index showed a weakly negative relationship with all enzymes except chitinase, which had no correlation with this variable.

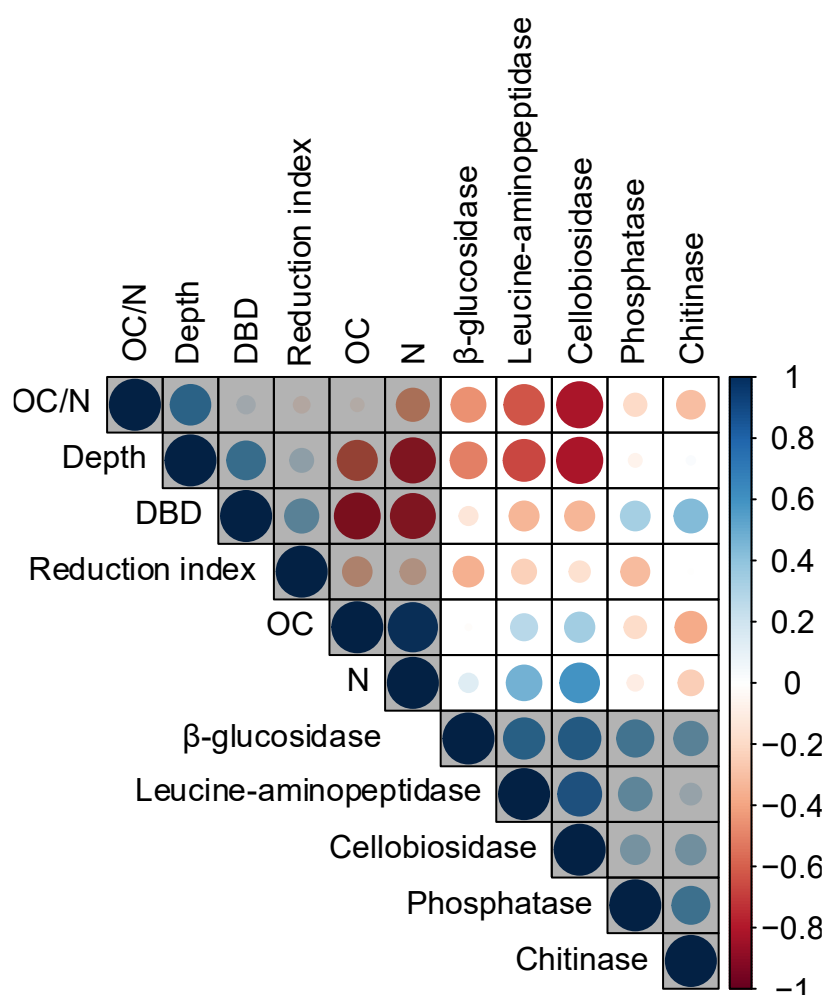


Figure 4.8. Correlations between enzyme activities and environmental variables. Shaded areas cover those interactions not focused upon in this study. The enzyme activity values analysed were in activity per g OC m⁻² y⁻¹. R² values are shown in Table S4.4.

4.5 Discussion

Differences in microbial community composition between marsh types (i.e., natural and restored) were likely driven by the lower elevation of the restored site, meaning the sampling stations situated upon this marsh experience increased tidal inundation frequency and reduced drainage. The small number of samples taken from a similarly low elevation setting in the natural marsh suggested that, despite originating from a natural site, these microbial communities were more similar to those of the restored marsh. Instead of decreasing with depth in the restored site, as was seen in the natural, extracellular enzyme activities were often highest in the deep sediment. This was true of chitinase, phosphatase and β-glucosidase and based upon the correlations between the activities of these

enzymes and the chemical properties of the associated sediment, it was concluded that their production was related to OC and N resource availability rather than redox conditions.

4.5.1 Redox conditions

The IRIS stick results can give an indication of microbial activity as well as of sediment redox. When more iron oxide precipitate was removed, this indicated a greater need for alternative electron acceptors, in the absence of oxygen, to enable respiration (Mittmann-Goetsch *et al.*, 2024). The reason for the much higher reduction indices – indicating more reducing conditions and lower oxygen availability – during the summer was likely increased microbial activity driven by higher temperature, a factor previously shown to increase microbial biomass levels in saltmarshes (Keith-Roach *et al.*, 2002).

Natural marsh reduction indices did not differ with depth during winter, with values remaining low from the surface to 50 cm depth (Fig. 4.2), suggesting that oxygen availability was sufficient to make using the iron oxide provided by the sticks unnecessary. Despite this, microbial communities differed strongly between the surface and deep samples and enzyme activities dropped off sharply in natural marsh deep samples compared with the surface samples. The expectation in saltmarshes is that organic matter cycling slows in lower depths because of low oxygen availability and here there was evidence of the former but not of the latter. Alternatively, it is possible that IRIS sticks provide a better indication of microbial activity than of redox conditions. In the restored marsh, reduction indices were consistently high across all depths in the summer while there was more variability across depths in the natural marsh (Fig. 4.2). Given that the restored site largely still resembled a mud flat (Fig. S4.1) and thus likely received regular tidal inundation, this was not unexpected.

4.5.2 Drivers of microbial diversity

One explanation for the differences between the microbial assemblages in the surface sediments of the marshes could be the direct impact of abiotic parameters such as elevation, tidal inundation frequency and drainage. Restored saltmarshes often sit at a lower elevation in the tidal frame because it can take time for sediment to accrete on the surface of the former agricultural field, meaning conditions are usually more waterlogged (Lawrence *et al.*, 2018). The IRIS stick results certainly suggested the restored marsh was under more strongly reducing conditions than the natural and the appearance of the area suggested limited drainage compared with the natural site (Fig. S4.1). Furthermore, a previous study that used artificially created saltmarsh islands to monitor the development of the sediment prokaryotic community over time, found that elevation was the key variable that shaped microbial community composition (Tebbe *et al.*, 2025). A related, indirect explanation could be the presence of different plant species in both marshes (Table 4.1), a factor previously shown to drive such variation (Ravit *et al.*, 2003; Rietl *et al.*, 2016). Restored marsh primary sampling stations and one of the natural marsh secondary stations (i.e., N-low) were

dominated exclusively by the C₄ plant *S. anglica* while the natural marsh primary sampling stations and N-wall had more diverse plant communities from which *S. anglica* was either entirely absent or present only in very low amounts (Table 4.1). Colonisation by *Spartina alterniflora* in a Chinese saltmarsh was found to greatly alter the bacterial assemblages (Shuang *et al.*, 2009). Another indirect, contributing driver could be the capacities of different plant species for trapping allochthonous OM (Mueller *et al.*, 2017), known to be an important carbon source for microorganisms in saltmarshes (Boschker *et al.*, 1999; Bouillon and Boschker, 2006).

The deep sediment marsh clusters were more widely separated in the CCA than the surface clusters (Fig. 4.3a), suggesting that the microbial communities differed more strongly at this depth. The restored marsh deep sediment layer was at the depth of the agricultural field present at the site prior to breaching the seawall and restoring tidal inundation (Chapter Two). Conversely, the deep sediment layers in the natural marsh were representative of a saltmarsh ecosystem, like the surface, but the decomposition of OM over time could have meant that the microbial OC source differed strongly from the natural marsh surface and favoured different species. Samples from 10-20 cm depth in the natural marsh were grouped separately but not widely distant from the surface samples, suggesting microbial assemblages that changed gradually with depth (Fig. 4.3a). In contrast, all restored marsh samples from intermediate depths were closely clustered with samples from the very lowest depths (Fig. 4.3a). The breach depth in this part of the marsh was thought to be relatively shallow, no more than 7 cm below the surface (Chapter Two). Therefore, it is likely that all but the surface samples from the restored marsh were representative of the pre-breach agricultural soil. Sediment will continue to accumulate upon the restored marsh surface, and the microbial communities present in the relic agricultural layer will be buried more deeply. The potential impacts of this relic community upon the development of the marsh and its functioning as an OC sink is therefore likely to diminish over time. However, it is possible that the agricultural soil layer did set the marsh on track to develop in a direction that diverged from that of its natural counterpart by selecting for different population of microbes and plants. In turn, these factors determined the origin of the OC being stored.

The microbial assemblages in this marsh did not change seasonally and this contrasts with previous studies of saltmarsh microbes that did find seasonal differences (e.g., Buchan *et al.*, 2003; Kearns *et al.*, 2016). Other studies concluded that temporal differences were minor and indicated that inundation frequency and the associated variables were the important determinators of microbial assemblages (e.g., Cleary *et al.*, 2017; Rinke *et al.*, 2022). In this case, it appears that abiotic factors likewise had a strong effect but that the OM sources available to microbes were also potentially important.

4.5.3 Predicted functions of the microbial communities

Sulphate reducing bacterial genera were more abundant in the restored marsh sediment than in that of the natural marsh (Fig. 4.5). A study of the changes in the prevalent OM mineralisation pathways in a freshwater marsh experiencing salinity intrusion found that sulphate reduction surpassed methanogenesis and iron reduction to become the most dominant pathway a few weeks after the introduction of saline conditions (Weston et al., 2006). Similarly, my results suggested that sulphate reduction was likely one of the dominant pathways of OM mineralisation in the restored site. This conclusion is supported by the FAPROTAX analysis, which showed a higher predicted abundance of genes relating to sulphate and iron respiration in the deep sediment of the restored marsh compared with the natural (Fig. 4.6). The lower abundances of the sulphate reducing genera in the natural marsh could reflect the less strongly reducing condition of these sediments, as indicated by the IRIS stick results (Fig. 4.2). Additional support for this interpretation is provided by the higher abundances of *Woeseia*, an aerobic genus capable of facultative anaerobiosis (Du et al., 2016), recorded in the natural marsh. Previous studies of saltmarsh and coastal sediments have also identified *Woeseia* as either one of or the most abundant genus present (e.g., Li et al., 2024 Rojas-Vargas et al., 2025).

Another study of restored and natural saltmarsh microbial communities conducted on Deer Island, USA, found that the sulphate reducing group in their samples were dominated by the order Desulfobacterales (Mavrodi et al., 2018). They also highlight the presence of the genus *Thermodesulfovibrio* from the order Nitrospirae. None of the sulphate reducing genera that were identified as most prevalent in the restored marsh of this study (i.e., *Desulfuromonas*, *Desulfobacca* and *Desulfosporosinus*) were of the same orders as those reported by Mavrodi and colleagues (2018). On the other hand, a study of the microbial communities in sediment taken from Stiffkey marsh, Norfolk, UK, reported the presence of *Desulfuromonas* (Jameson et al., 2019) while another study of a marsh in the San Francisco Bay, USA, found *Desulfosporosinus* in their samples (Moreau et al., 2010). Another study using a metagenomics approach found that sulphur cycling metabolisms were enriched in a restored marshes compared with a natural counterpart in the USA (Campbell et al., 2025). Taken together, this highlights the potential for variability between sites in terms of specific genera while also suggesting that a higher abundance of sulphate reduction generally is common across several restored sites.

4.5.4 Enzyme activity levels

Based upon the correlation matrix, β -glucosidase, cellobiosidase and leucine-aminopeptidase can be placed in one group while chitinase and phosphatase are placed in another (Fig. 4.7). Activity of the former group was higher at shallow depths where there was an abundance of high-quality OM with elevated OC and N content and lower OC/N ratios. From this, it appears that more investment was placed into the production of these enzymes when environmental conditions were favourable and food sources abundant. Conversely, the second enzyme group was more active when sediment was

compacted and less rich in easily degradable OM. If OC cannot be acquired from labile OM and N cleaved from proteins by leucine-aminopeptidase, producing chitinase to obtain these essential nutrients from more recalcitrant chitin molecules (Poshina *et al.*, 2018; Unuofin *et al.*, 2024) is an alternative. Likewise, when free phosphate ions are scarce, increased phosphatase production is necessary to free this nutrient from the available OM sources (Margalef *et al.*, 2017). Taken together, this suggests that the second group was released when high-quality OM was unavailable.

Variables that differed significantly between restored and natural marsh sediment were DBD in the deep sediment (averaging 0.27 ± 0.04 and 0.48 ± 0.03 , respectively; Table 4.2) and OC/N ratio in the surface sediments (averaging 8.17 ± 0.60 and 6.79 ± 0.41 , respectively; Table 4.2). Compared with surface activity, elevated activity levels of the enzymes chitinase, phosphatase and β -glucosidase were always evident in the deep sediment of one or both restored marsh sampling stations (Fig. 4.7). Conversely, leucine aminopeptidase and cellobiosidase activities were suppressed, which can be explained by the more resource scarce setting that was suggested by the higher DBD values. Although β -glucosidase belongs to the high-quality resource group, its relative elevation in the restored marsh deep sediment could be because this is a generalist enzyme capable of breaking down a vast array of substrates containing β -glycosidic polymers (Méndez-Líter *et al.*, 2018). The input of high-quality N rich OM suggested by the higher OC/N ratios did not result in higher β -glucosidase and cellobiosidase activity in the restored surface sediments compared with the natural. It has been demonstrated that, while the N content of decomposing OM does increase, the portion of extractable protein decreases, meaning that a low OC/N ratio does not necessarily indicate the presence of high-quality OM (Hemminga *et al.*, 1998). Therefore, the low OC/N ratios of the restored marsh surface sediment could indicate the presence of OM that has already undergone a degree of decomposition. On the other hand, leucine-aminopeptidase activity was considerably elevated in the surface sediments of R-Corner. The very low activities of all enzymes in the natural marsh deep sediment may have reflected the lack of a relic agricultural layer in this marsh.

4.5.5 Implications for carbon cycling and climate change mitigation

The restored marsh deep sediment was representative, not of a saltmarsh, but of a relic agricultural field. Therefore, the abiotic conditions and the OM sources available to the microbes differed from those of the natural marsh, where decreased OC and N content combined with low enzyme activities in the deep sediment suggested the existence of the expected of saltmarsh ecosystem conditions. In the surface sediments, the significantly lower OC/N ratio of the restored marsh also signified a different OM source, perhaps one of a more marine, and thus allochthonous origin. However, the main driver of differences between surface samples was likely the lower elevation position of the restored site compared with the natural.

The functional impacts of microbial community differences could be examined further with methods such as stable isotope probing and the taking of CO₂ flux measurements. Doing so would

help elucidate the fate of the OC in the restored saltmarsh system and create a link between microbial diversity and OC cycling. This could allow advice upon the usefulness of measuring microbial diversity in other or future restoration projects to be developed. Ultimately, microbial community composition and extracellular enzyme activity levels could be used as indicators of restoration success, from an OC accumulation perspective.

4.6 Supplementary Information

4.6.1 Supplementary tables

Table S4.1. Locations of the primary (bold text) and secondary (non-bold text) sampling stations.

Sampling station	Latitude	Longitude
N-creek	51.839444	0.977500
N-cliff	51.838056	0.977778
R-corner	51.838611	0.976944
R-breach	51.839167	0.976944
N-wall	51.838533	0.977323
N-low	51.838410	0.978099
R-opposite	51.843219	0.972766

Table S4.2. Biogeochemical properties of the primary (bold text) and secondary (non-bold text) sampling stations. Only the results from the primary sampling stations were included in the ANOVA analyses and only one sediment sample was taken from each secondary sampling station, meaning there were no replicates.

Month	Station	Replicate	Depth (cm)	OC (%)	N (%)	OC/N	DBD (g cm ⁻³)
January	N-creek	A	1	4.14	0.47	8.81	0.25
			31.5	2.92	0.31	9.42	0.24
		B	1	4.06	0.5	8.12	0.23
			25	3.54	0.33	10.73	0.26
		C	0.5	4.24	0.52	8.15	0.26
			32.5	3.26	0.34	9.59	0.25
	N-cliff	A	1	2.67	0.33	8.09	0.20
			43.5	2.03	0.23	8.83	0.29
		B	1	3.52	0.40	8.80	0.30
			45	2.82	0.28	10.19	0.31
		C	1	2.25	0.3	7.50	0.34
			45	2.25	0.24	9.38	0.31
	R-corner	A	1	2.80	0.44	6.36	0.28
			41	2.04	0.22	9.27	0.50
		B	1	2.27	0.32	7.09	0.27
			42.5	1.85	0.19	9.74	0.45
		C	1	3.15	0.45	7.00	0.25
			42.5	1.49	0.18	8.28	0.46
	R-breach	A	0.5	2.84	0.41	6.93	0.32
			15	11.4	0.85	13.41	0.21
			43.5	1.72	0.17	10.12	0.42
		B	0.5	2.88	0.39	7.38	0.26
			15	1.51	0.16	9.44	0.42
			44	1.00	0.11	9.09	0.46
		C	1	2.92	0.39	7.49	0.32
			15	4.08	0.37	11.03	0.24
			43	1.00	0.12	8.33	0.50
July	N-creek	A	1	6.13	0.68	9.01	0.20
			43.5	2.69	0.27	9.96	0.24
		B	1	5.54	0.67	8.27	0.22
			44	2.51	0.23	10.91	0.30
		C	1	5.46	0.61	8.95	0.22
			45	3.05	0.31	9.84	0.27
	N-cliff	A	1	2.68	0.35	7.66	0.31
			15	2.33	0.28	8.32	0.31
			43.5	2.61	0.27	9.67	0.30
		B	1	2.88	0.40	7.20	0.29
			15	2.81	0.30	9.37	0.27
			45	2.21	0.24	9.07	0.30
		C	1	2.98	0.40	7.45	0.29
			15	2.22	0.27	8.22	0.31
			43.5	2.21	0.23	9.61	0.28
	R-corner	A	1	2.98	0.43	6.93	0.31
			44	1.94	0.21	9.24	0.49

Table S4.2

			B	1	3.79	0.63	6.02	0.28
				42.5	1.69	0.17	9.94	0.48
			C	1	3.12	0.47	6.64	0.33
				43	1.76	0.2	8.8	0.46
			A	1	2.77	0.42	6.6	0.27
				25	1.31	0.14	9.36	0.48
				44.5	0.98	0.12	8.17	0.51
			B	1	3.32	0.51	6.51	0.28
				25	1.22	0.13	9.38	0.49
				35	1.57	0.18	8.97	0.45
				45	0.87	0.12	7.25	0.50
			C	1	2.94	0.45	6.53	0.26
				25	1.27	0.15	8.47	0.48
				35	1.57	0.18	8.97	0.48
				45	0.93	0.13	7.15	0.49
		N-wall	NA	1	5.30	0.56	9.46	0.42
		N-low	NA	1	1.83	0.26	7.04	0.36
		R-corner	NA	1	3.15	0.43	7.32	0.41
		R-opposite	NA	1	2.16	0.35	6.17	0.48

Table S4.3. Results of the three-way ANOVA tests to determine the impacts of marsh type, sampling depth and sampling month upon reduction index, Shannon index and enzyme activities. All the enzymes were tested with only two depths (surface and deep) in the Depth level while Reduction Index and Shannon Index were tested over four depth categories (i.e., the depths shown in figures 2 and 4b). The interaction Month x Marsh x Depth was never significant ($p > 0.070$). Highlighted in bold text are significant p -values.

Variable	Level/Interaction	Adjusted p -value	F -value	$d.f.$
Reduction Index	Marsh Type	<0.001	138.326	1, 227
	Month	<0.001	265.556	1, 227
	Depth	0.115	1.879	4, 227
	Marsh x Month	0.049	3.928	1, 227
	Marsh x Depth	0.014	3.444	4, 227
	Month x Depth	0.009	1.658	4, 227
Shannon Index	Marsh Type	<0.001	48.911	1, 42
	Month	0.355	0.874	1, 42
	Depth	<0.001	22.675	4, 42
	Marsh x Month	0.781	0.078	1, 42
	Marsh x Depth	0.003	5.608	3, 42
	Month x Depth	0.494	0.477	1, 42
β -glucosidase	Marsh Type	<0.001	23.615	1, 183
	Month	<0.001	51.146	1, 183
	Depth	<0.001	82.835	1, 183
	Marsh x Month	0.197	1.678	1, 183
	Marsh x Depth	0.002	9.666	1, 183
	Month x Depth	0.030	4.766	1, 183
Cellobiosidase	Marsh Type	<0.001	40.943	1, 184
	Month	0.499	0.459	1, 184
	Depth	<0.001	380.586	1, 184
	Marsh x Month	0.075	3.204	1, 184
	Marsh x Depth	<0.001	24.336	1, 184
	Month x Depth	0.503	0.450	1, 184
Chitinase	Marsh Type	<0.001	52.211	1, 183
	Month	0.004	8.491	1, 183
	Depth	0.002	10.209	1, 183
	Marsh x Month	0.225	1.485	1, 183
	Marsh x Depth	<0.001	34.581	1, 183
	Month x Depth	0.018	5.674	1, 183
Leucine-aminopeptidase	Marsh Type	<0.001	42.284	1, 184
	Month	<0.001	46.389	1, 184
	Depth	<0.001	559.750	1, 184
	Marsh x Month	0.297	1.092	1, 184
	Marsh x Depth	0.005	7.928	1, 184
	Month x Depth	0.025	5.112	1, 184
Phosphatase	Marsh Type	<0.001	213.452	1, 184
	Month	<0.001	41.316	1, 184
	Depth	<0.001	80.895	1, 184
	Marsh x Month	0.388	0.749	1, 184
	Marsh x Depth	<0.001	209.182	1, 184
	Month x Depth	0.647	0.210	1, 184

Table S4.4. R^2 values of the different combinations of variables shown in Figure 4.7. Negative values denote a negative correlation and positive values a positive correlation

First variable	Second variable	R^2 value
β -glucosidase	OC/N	-0.46
	Depth	-0.51
	Dry bulk density	-0.13
	Reduction index	-0.35
	Organic carbon	0.00
	Nitrogen	0.14
Leucine-aminopeptidase	OC/N	-0.62
	Depth	-0.67
	Dry bulk density	-0.34
	Reduction index	-0.23
	Organic carbon	0.27
	Nitrogen	0.48
Cellobiosidase	OC/N	-0.81
	Depth	-0.82
	Dry bulk density	-0.34
	Reduction index	-0.17
	Organic carbon	0.34
	Nitrogen	0.60
Phosphatase	OC/N	-0.20
	Depth	-0.07
	Dry bulk density	0.33
	Reduction index	-0.31
	Organic carbon	-0.19
	Nitrogen	-0.10
Chitinase	OC/N	-0.31
	Depth	0.00
	Dry bulk density	0.43
	Reduction index	0.00
	Organic carbon	-0.38
	Nitrogen	-0.24

4.6.2 Supplementary figures



Figure S4.1. The seven sampling stations that were included in the study, three (a-c) on the restored marsh four (d-g) on the natural. Within the black boxes in the upper left-hand corner of each panel, the name of station is given, and the markers correspond to those shown in Figure 1 of the main text. Larger turquoise markers represent the main sampling stations focused upon throughout the study while the smaller white markers represent the locations where control samples were taken to verify whether microbial community differences were down to restoration status or variables related to tidal frame position of the marshes.

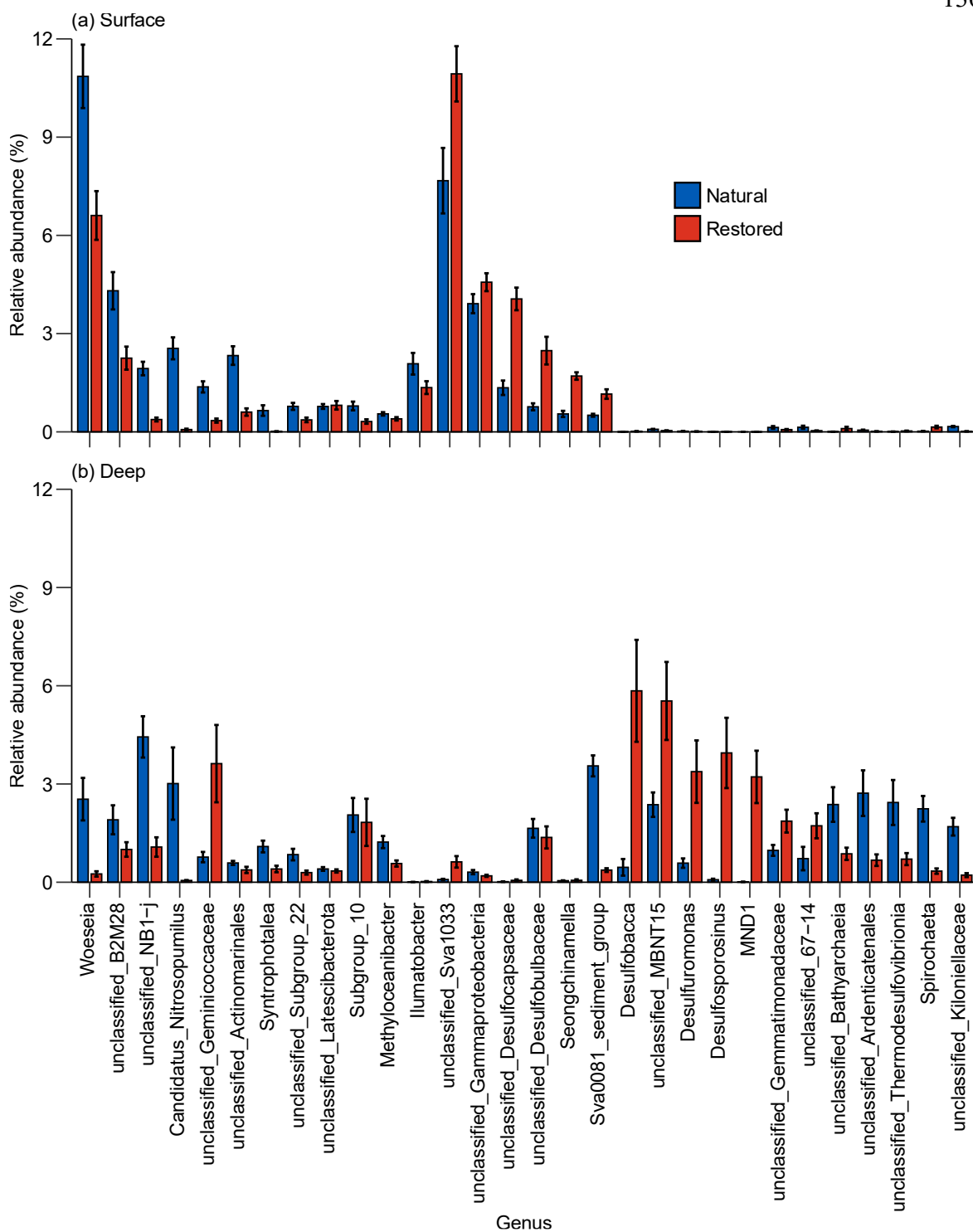


Figure S4.2. Mean abundances of the top thirty-one most abundant genera – including unclassified genera – in the natural (blue) and restored (red) marshes in (a) the surface and (b) the deep sediment. Error bars show $\pm$ standard error.

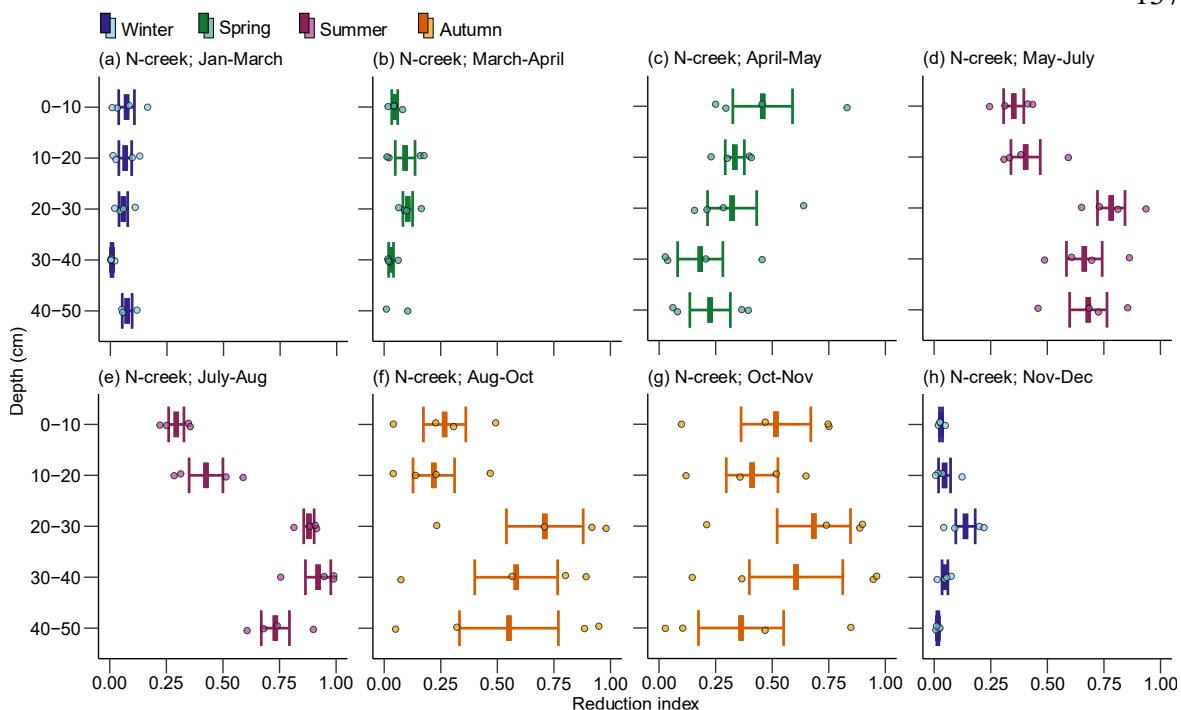


Figure S4.3. IRIS results from sampling station N-creek. Mean and error bars are only shown when there are ≥ 3 values, otherwise only the individual data points are present. Error bars are $\pm$ standard error. The title of each plot indicates the incubation interval of the sticks represented, and plots are ordered (a-h) from first to last batch deployed in the marsh. Higher reduction index values are indicative of low oxygen conditions.

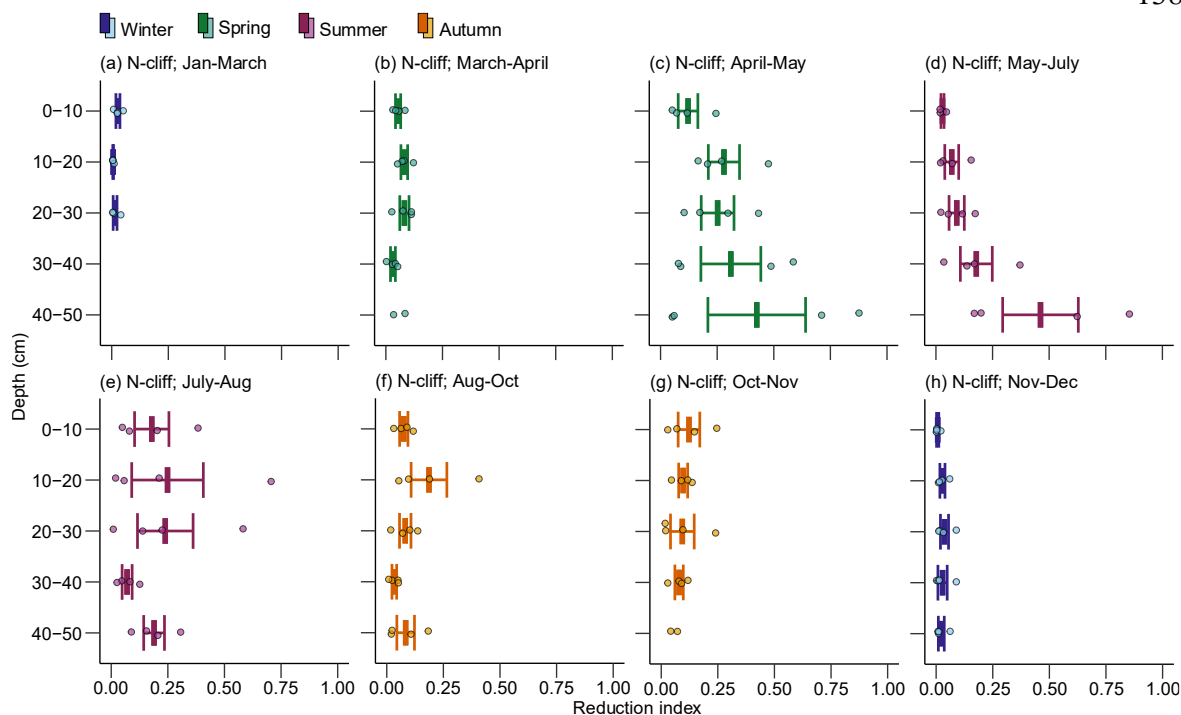


Figure S4.4. IRIS results from sampling station N-cliff. Mean and error bars are only shown when there are ≥ 3 values, otherwise only the individual data points are present. Error bars are $\pm$ standard error. The title of each plot indicates the incubation interval of the sticks represented, and plots are ordered (a-h) from first to last batch deployed in the marsh. Higher reduction index values are indicative of low oxygen conditions.

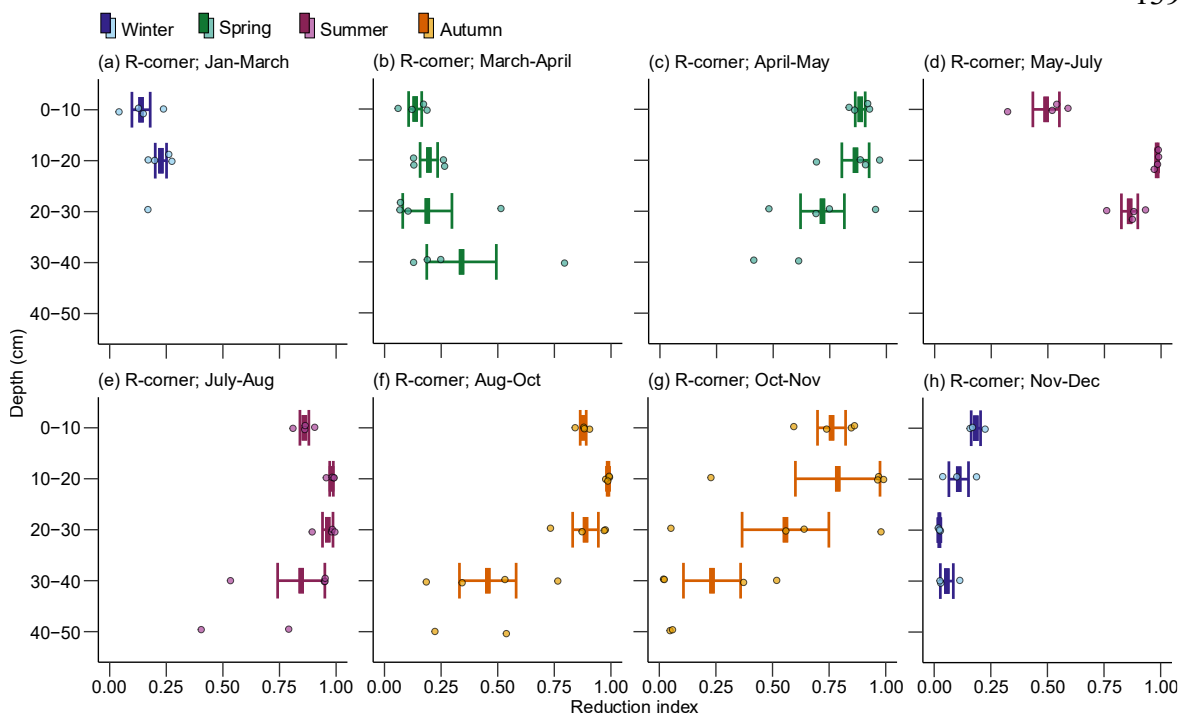
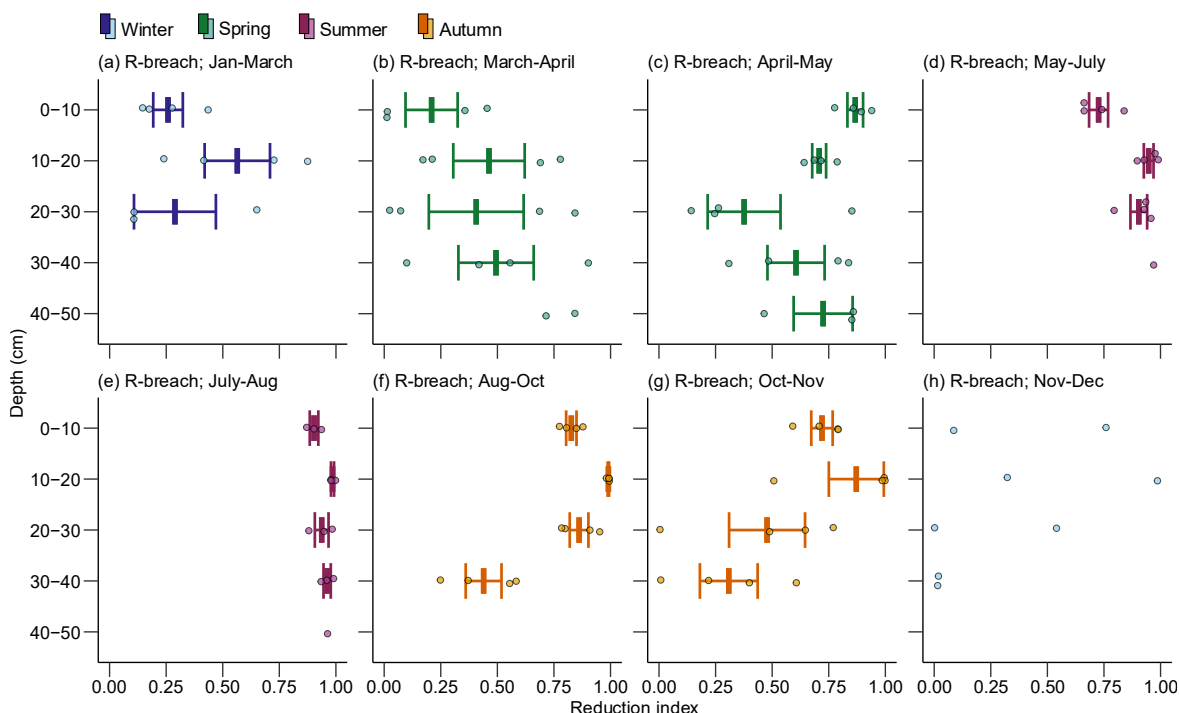


Figure S4.5. IRIS results from sampling station R-corner. Mean and error bars are only shown when there are ≥ 3 values, otherwise only the individual data points are present. Error bars are $\pm$ standard error. The title of each plot indicates the incubation interval of the sticks represented, and plots are ordered (a-h) from first to last batch deployed in the marsh. Sticks were often not recovered from the restored marsh because resistance was reached before they were fully submerged, making them vulnerable to the tides. Higher reduction index values are indicative of low oxygen conditions.



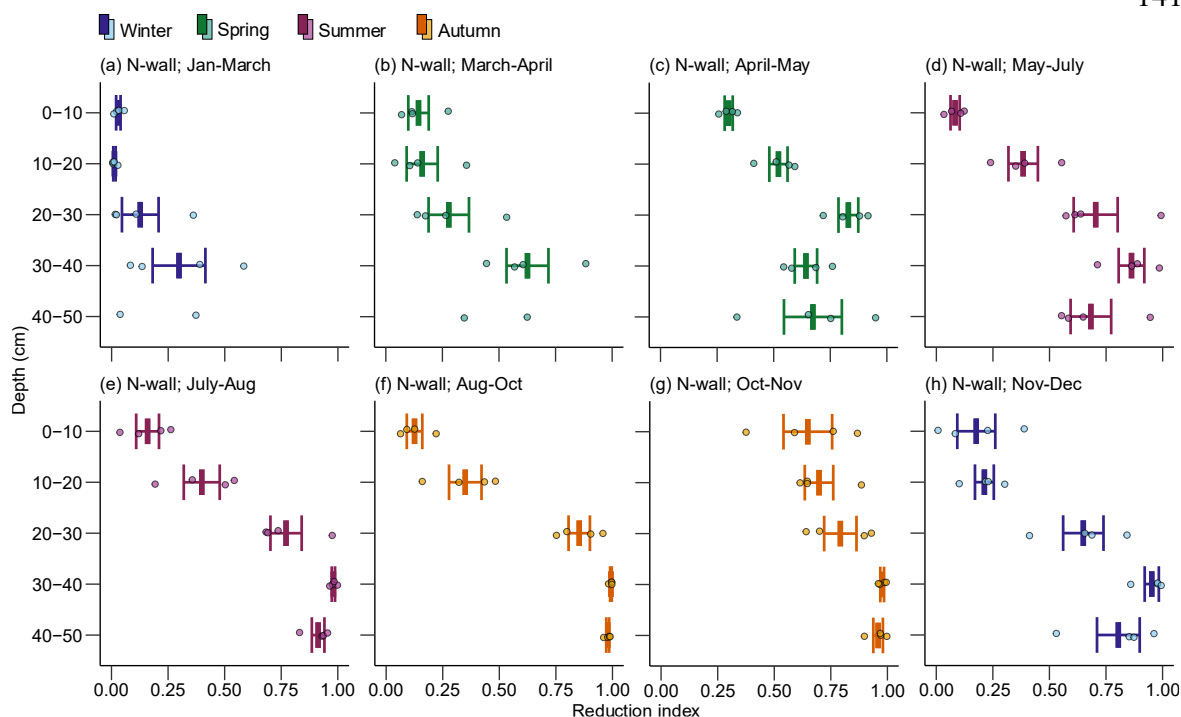


Figure S4.7. IRIS results from sampling station N-wall. Mean and error bars are only shown when there are ≥ 3 values, otherwise only the individual data points are present. Error bars are $\pm$ standard error. The title of each plot indicates the incubation interval of the sticks represented, and plots are ordered (a-h) from first to last batch deployed in the marsh. Higher reduction index values are indicative of low oxygen conditions.

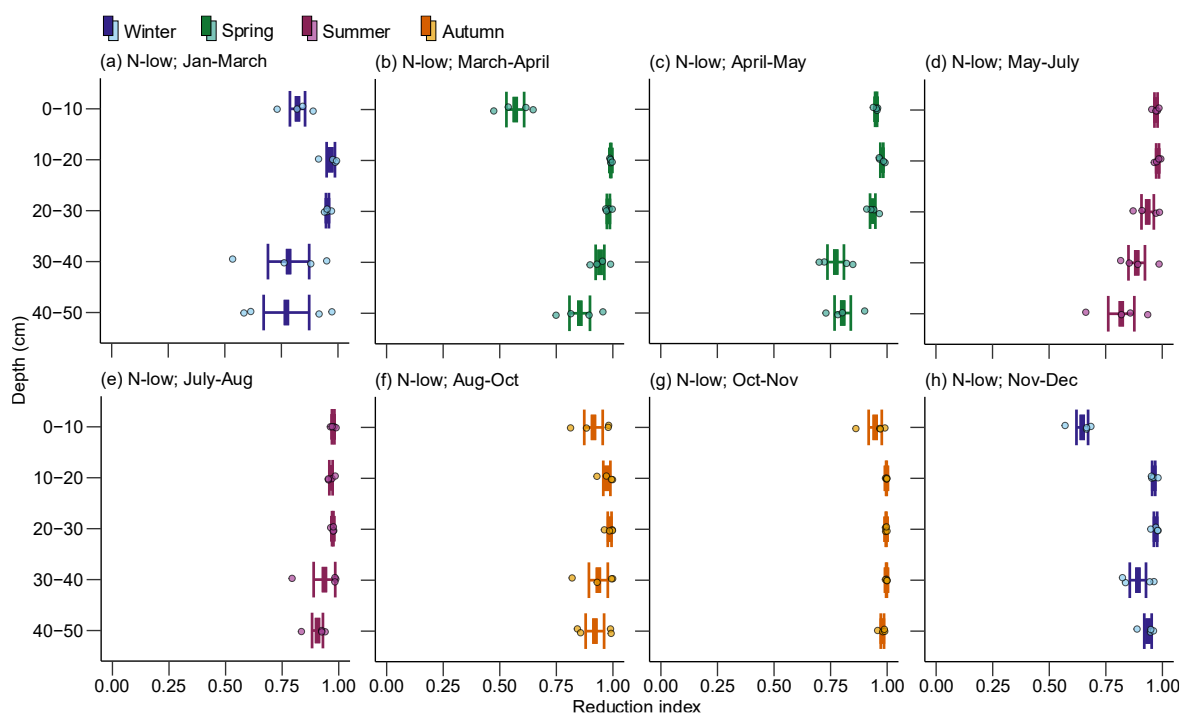


Figure S4.8. IRIS results from sampling station N-low. Mean and error bars are only shown when there are ≥ 3 values, otherwise only the individual data points are present. Error bars are $\pm$ standard error. The title of each plot indicates the incubation interval of the sticks represented, and plots are ordered (a-h) from first to last batch deployed in the marsh. Higher reduction index values are indicative of low oxygen conditions.

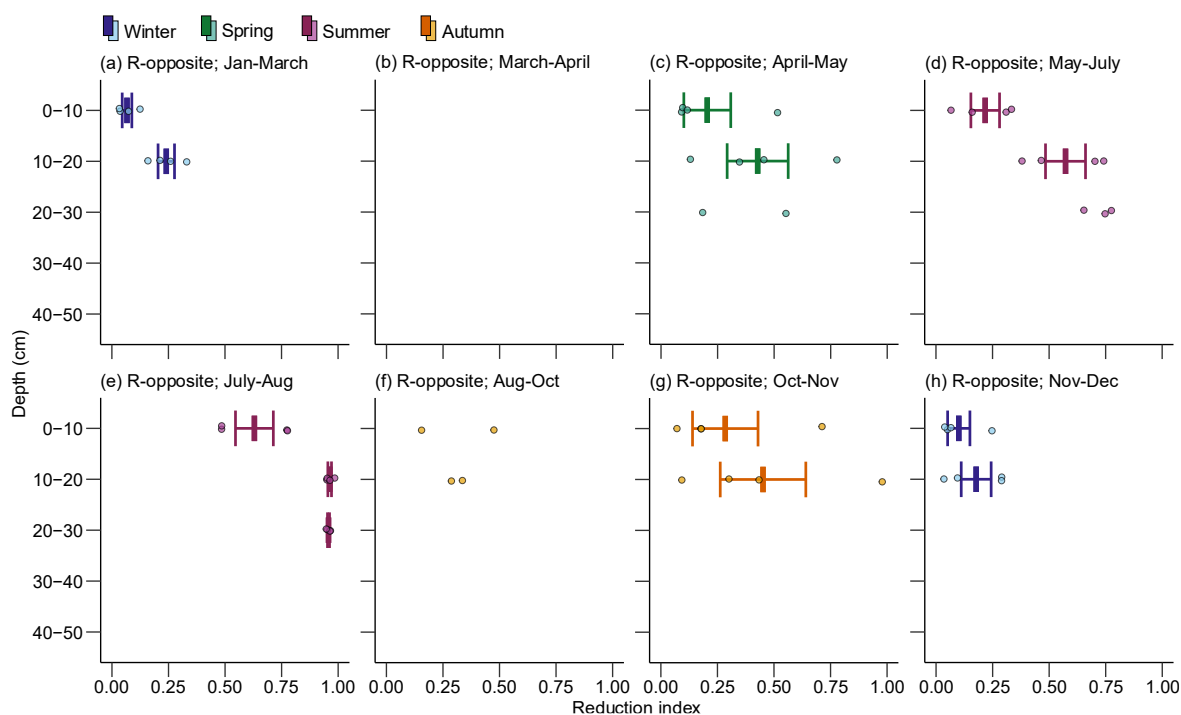


Figure S4.9. IRIS results from sampling station R-opposite. Mean and error bars are only shown when there are ≥ 3 values, otherwise only the individual data points are present. Error bars are $\pm$ standard error. The title of each plot indicates the incubation interval of the sticks represented, and plots are ordered (a-h) from first to last batch deployed in the marsh. Sticks were often not recovered from the restored marsh because resistance was reached before they were fully submerged, making them vulnerable to the tides. Higher reduction index values are indicative of low oxygen conditions.

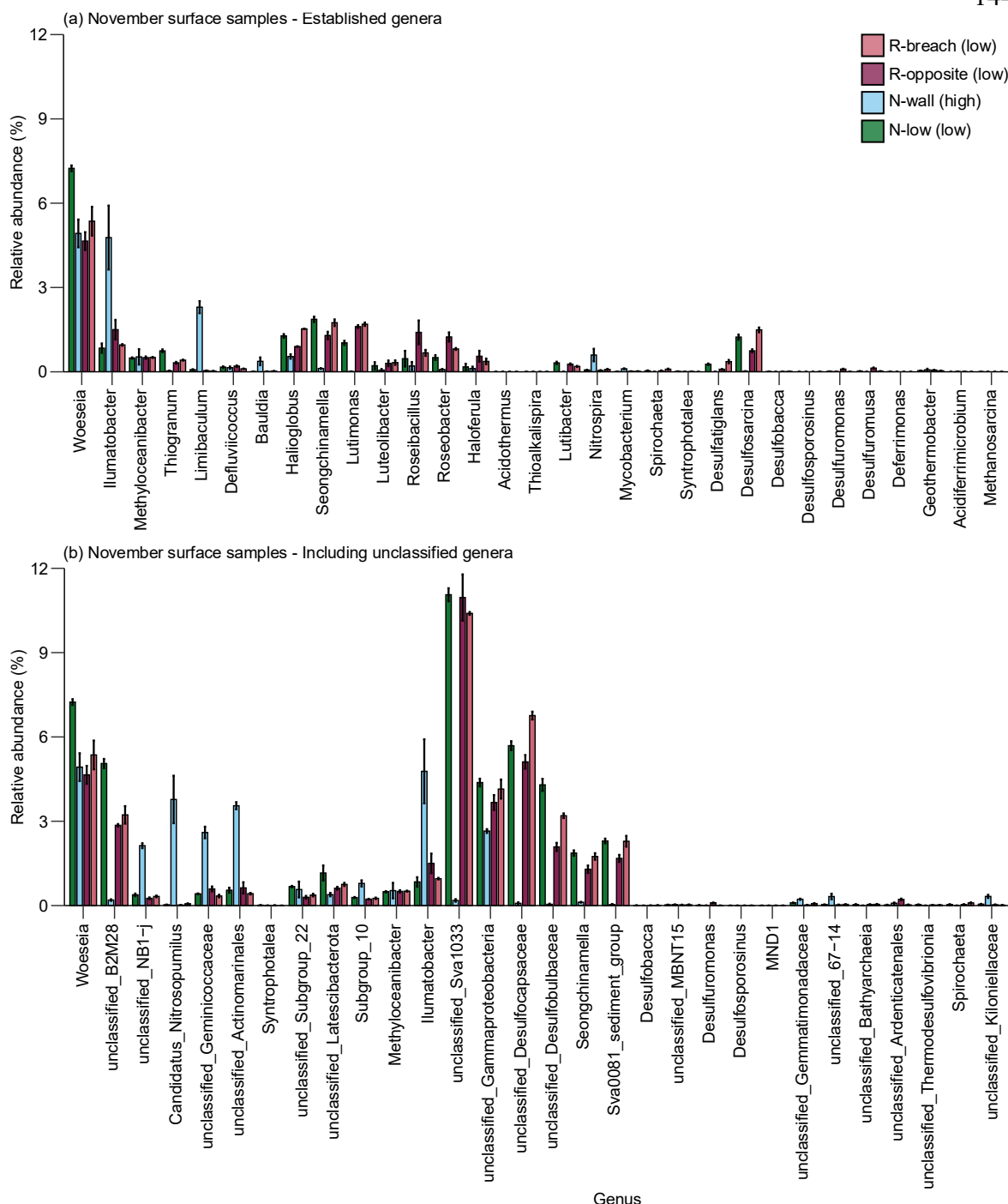


Figure S4.10. The top thirty-one most abundant genera in the additional surface samples taken during November of 2024. Abundance was calculated with (a) only the established genera and (b) with all genera, including those that are currently unclassified. Error bars show $\pm$ standard error. The key indicates the names of the sampling stations but also the marsh zone they represented.

Chapter Five

Decoupled microbial community composition and functioning responses to teabag litter addition in restored and natural saltmarshes

5.1 Abstract

Stability of organic carbon is crucial for saltmarshes used as natural climate solutions, making organic matter (OM) decomposition in these ecosystems an important process to understand. When additional carbon sinks are created via saltmarsh restoration, differences in hydrology and biodiversity result from converting former agricultural fields into marshes by breaching the surrounding seawall. In turn, these factors impact decomposition process by creating more strongly reducing conditions and changing the microbial communities present. Using the standard teabag index methodology, I calculated the decomposition and stabilisation rates in neighbouring restored and natural saltmarshes. Additionally, DNA was extracted from these teabags and microbial diversity examined using 16S rRNA amplicon sequencing to gain insights into the microorganisms driving decomposition processes. Furthermore, subsets of teabags were incubated for nine- and twelve-month incubation intervals to determine if successional changes occurred in the microbial communities involved in decomposition when later stages of OM decomposition were taking place. Teabags incubated in the restored marsh were dominated by anaerobic and fermentative groups of bacteria while natural marsh teabags predominately contained bacterial genera that were aerobic. Although microbial assemblages differed strongly between samples, OM decomposition and stabilisation rates were not significantly different, suggesting that different microbial groups were achieving the same effects. Continued decomposition of teabag litter occurred when teabags were incubated in the marsh for twelve months but successional changes in the microbial communities were not seen. This study begins to illuminate the specific microbial taxa and functions responsible for teabag litter decomposition in saltmarshes and highlights potential differences driven by factors like sediment redox conditions and plant community composition.

5.2 Introduction

Saltmarshes are sinks of organic matter (OM) that receive attention for their potential as natural climate solutions (Macreadie *et al.*, 2021). Waterlogged conditions often mean a lack of oxygen availability that slows decomposition and means that the organic carbon (OC) comprising the OM in the sediment is thought of as especially durable (Wang *et al.*, 2019; Houston *et al.*, 2025). As well as protecting saltmarshes, thus preventing existing OC stocks from being returned to the atmosphere and enabling these sites to continue burying OC, efforts are being made to restore saltmarshes and so create additional OC sinks (Connor *et al.*, 2001; Mason *et al.*, 2023). However, restored saltmarshes can differ from natural ones in terms of creek density (Symonds and Collins, 2007; Lawrence *et al.*, 2018), drainage (Tempest *et al.*, 2015; Spencer *et al.*, 2017) and plant community composition (Mossman *et al.*, 2012a; Mossman *et al.*, 2012b; Brooks *et al.*, 2015; Lawrence *et al.*, 2022). These are all factors that affect oxygen availability in the sediment, another variable known to differ between natural and restored sites (Mossman *et al.*, 2012b), creating contrasting abiotic conditions. Furthermore, sediment redox is one of the key determinators of microbial community composition (Rinke *et al.*, 2022), along with salinity, OM availability and pH (Zhang *et al.*, 2021;

Leadbeater *et al.*, 2021; Yu *et al.*, 2022). Indeed, the microbial communities present in the sediment of a low elevation restored saltmarsh have also been shown to differ from those found in a higher elevation natural marsh (Chapter Four). Microorganisms are the primary drivers of OC remineralisation (Macreadie *et al.*, 2025), meaning that these differences could impact the fate of the OM entering the sediment and thus the functioning of these ecosystems as natural climate solutions. A degree of saltmarsh OM decomposition over time is unpreventable, but it is the speed of this process matters when considering the stability of marsh OC stocks.

The Teabag Index (TBI) is a method used to measure the rate of OM decomposition and levels of OM stabilisation in the soils of many different ecosystems (Keuskamp *et al.*, 2013). The OM decomposition rate refers to the speed at which OM is broken down while OM stabilisation represents the amount of labile OM that is stabilised, becomes recalcitrant and remains in the bag at the end of the standard incubation period. To calculate these rates green tea, representative of labile material, and rooibos tea, a more recalcitrant material, are deployed in pairs for three months. A major advantage of the TBI is that using standardised litter is less labour intensive than creating litter bags with material from the study site (e.g., Lopes *et al.*, 2011; Carrasco-Barea *et al.*, 2022; Arnaud *et al.*, 2024). In most studies, the TBI is used only to calculate the two rates already discussed (e.g., Becker and Kuzyakov, 2018; Djukic *et al.*, 2018; Fanin *et al.*, 2020; Sandén *et al.*, 2020; Sarneel *et al.*, 2024). Often, it is a useful tool for determining the potential impact of experimental treatments, such as increasing temperatures and sea-level rise, upon the OM currently stored in soils (e.g., Mueller *et al.*, 2018; Sarneel *et al.*, 2020; Tang *et al.*, 2023; Trevathan-Tackett *et al.*, 2024). Therefore, it could be a useful tool for comparing OM decomposition in natural and restored sites.

A limited number of previous studies have gone a step further by investigating the microbial communities colonising teabag litter during incubation. These have focused upon forest, farm and wetland soils (Pioli *et al.*, 2020; Daebeler *et al.*, 2022; Neiske *et al.*, 2025; Trevathan-Tackett *et al.*, 2021; Gopakumar *et al.*, 2026). This is a valuable undertaking as it can give an indication of the dominant processes taking place during decomposition – in saltmarshes likely possibilities are sulphate reduction, metal reduction or fermentation because of the low oxygen conditions that often exist (Yarwood, 2018) – as well as providing insights, for example, into whether humification processes dominate over decomposition (e.g., Yan *et al.*, 2018). Of the studies focusing specifically upon saltmarshes, one conducted in NW Europe found an increase in the phyla Bacillota, Pseudomonadota, and Bacteroidota in the tea litter compared with the bulk sediment (Neiske *et al.*, 2025) while the other, conducted in Australia, found that Alphaproteobacteria, Gammaproteobacteria and Bacteroidia dominated their tea samples (Trevathan-Tackett *et al.*, 2021). The third previous study that focused upon saltmarshes is particularly relevant as it involved a comparison of restored and remnant, natural saltmarshes in Australia (Gopakumar *et al.*, 2026). They found that the bacterial families *Stappiaceae*, *Flavobacteriaceae* and *Demequinaceae* were more abundant in their restored sites while *Caulobacteraceae* was more abundant in the remnant natural sites. Not considered so far,

however, was the potential impact of differences in abiotic conditions between restored and natural marshes upon OM turnover rates and microbial OM colonisation. Restored saltmarshes are often more waterlogged than their natural counterparts and, given that this implies reduced oxygen availability and slower OM decomposition, this is an important difference to examine.

Another gap that remained to be filled was an examination of the microbial communities in teabags left in the marsh for longer than three months, which was only done in one of the previous studies (Trevathan-Tackett *et al.*, 2021). Usually, the standard incubation is for three months but recent work has indicated that using longer incubation periods can provide useful insights into the factors controlling the later stages of OM decomposition (Marley *et al.*, 2019). Furthermore, it has suggested that this could help overcome the potential bias towards the fast growing, quick dispersing taxa that colonise tea litter initially (Daebeler *et al.*, 2022). Additionally, as well as extracting DNA from the tea litter, previous studies had not, to my knowledge, examined the sediment or soil directly surrounding/adhering to the bags. Doing so could begin to suggest whether OM addition, especially that of the labile green tea, lead to a priming effect upon the native OM within the sediment in the vicinity.

In this experiment, I incubated green and rooibos teabags in neighbouring restored and natural marshes. As well as three-month incubation intervals over the summer and winter months, subsets of teabags were incubated for nine- and twelve-month intervals. Following collection, most of the incubated teabags were used to calculate decomposition rates and stabilisation factors (defined above) following the standard methodology while a smaller group (one from each of the replicate pairs incubated in the primary sampling stations) were subject to DNA extraction and sequencing. It was hypothesised that (1) decomposition rates would be lower and the stabilisation factor higher in the restored compared to the natural marsh due to differences in sediment redox conditions, (2) differences in sediment redox conditions would also lead to differences in the predicted functioning and composition of the microbial community, with fermentative, sulphate reducing and metal reducing taxa dominating in restored sites, (3) microbial communities would show clear successional changes when incubated in the marshes for longer than the standard three-month interval and the sediment surrounding the teabags would undergo similar microbial community functioning changes over time.

5.3 Methodology

5.3.1 Study site and sampling stations

Approximately 12 km southeast of Colchester, Essex, in the east of England, is the Fingringhoe Wick Nature Discovery Park. The study sites are within (Fig. 5.1): a natural established saltmarsh (51.837373, 0.977097) and a restored marsh (51.842106, 0.974856). Tidal inundation was reintroduced to the restored marsh in 2015 by breaching the existing seawall and prior to this the land was used for agriculture (Vaughan, 2019; Cai *et al.*, 2022). While the natural marsh is densely

vegetated, the restored site resembles a mudflat with vegetation only growing on the higher elevation fringes and remaining seawall banks. The tidal range is 4.5 m (Wolters *et al.*, 2005).



Figure 5.1. The neighbouring natural and restored saltmarshes that were the focus of this study. One primary sampling station (samples subject to DNA extraction and sequencing) was selected upon each marsh, and these are indicated by the larger, turquoise markers. The standard teabag index procedure alone was carried out with samples from the secondary sampling stations, indicated by the smaller, grey markers and labelled R1-R4 on the restored marsh and N1-N4 on the natural marsh. In November 2024, untreated control sediment samples were taken from the primary sampling stations but also from the two secondary sampling stations labelled with asterisks (i.e., N3 and R4). Map imagery © Microsoft Bing Maps; satellite imagery © DigitalGlobe (Maxar), 2020.

Primary and secondary sampling stations were established within both marshes, with each marsh having one of the former and four of the latter (Fig. 5.1; Table S5.1). The natural marsh primary sampling station (“Natural Primary” in Fig. 5.1) was dominated by *Armeria maritima* (70 % cover), with *Aster tripolium* (15 % cover) representing a smaller component of the plant community (Table S5.2). In the restored marsh, the primary sampling station (“Restored Primary”) was colonised by *Spartina anglica* alone (75 % cover; Table S5.2). Of the secondary sampling stations from which control sediment samples were taken (those marked with asterisks in Fig. 5.1), both the natural and restored marsh stations were dominated only by *S. anglica* (90 % cover; Table S5.2). In the remaining secondary sampling stations, only *S. anglica* and *Salicornia* spp. were recorded in the restored marsh while the natural marsh stations contained more diverse communities (Table S5.2).

5.3.2 Teabag index

The Teabag Index (TBI) methodology followed that of Keuskamp and colleagues (2013). Two replicate pairs of teabags per incubation interval were placed in each sampling station, be they primary or secondary (Fig. 5.1). Both replicates from the secondary sampling stations were used to calculate the TBI values, namely decomposition rate and stabilisation factor, while only one pair from the primary sampling stations was used for this purpose. Before deployment, pairs of teabags, one rooibos and one green tea, were sealed within wire mesh by folding the mesh in half with the teabags pressed between both halves to prevent the bags from breaking during recovery. The two halves were held together with cable ties cut short.

Following Marley *et al.* (2019), teabags were deployed for three different durations (three months, nine months, twelve months) during a period from January to December 2024. The three-month treatment was repeated twice, once each in summer and winter. This led to a total of four incubation intervals: namely, three-month summer, three-month winter, nine-month and twelve-month intervals (Fig. 5.2). The first set were deployed for three months from 25-26/04/24 until 09-10/07/24. This interval was taken to be representative of conditions during the summer and will henceforth be referred to simply as the summer incubation interval. The second set of three-month incubation interval teabags were deployed from 09-10/10/24 until 09/12/24. This set was representative of winter conditions and will be referred to as the winter incubation interval. The nine-month interval teabags were deployed between the 18/01/24 and 09-10/10/24. For the twelve-month pairs, the incubation interval was between 18/01/24 and 09/12/24.

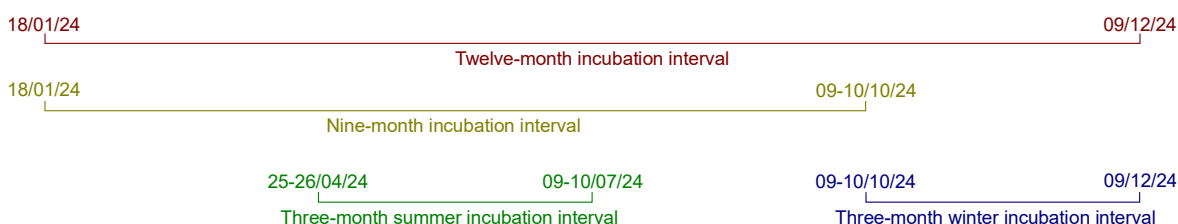


Figure 5.2. The teabag deployment timeline employed in this study to obtain results from four separate incubation intervals.

Following collection from the marshes, teabags were removed from the wire mesh, and any adhering sediment was gently washed away with distilled water. Teabags were then freeze dried before being weighed. The standard equations were used to calculate decomposition and stabilisation rates (Keuskamp *et al.*, 2013). One adjustment was made to the established technique: following Mason *et al.* (2025), I incubated green and rooibos teabags in distilled water for three hours – to mimic the first tidal inundation experienced by the teabags in a saltmarsh setting – before freeze drying them. Using the equations of Mason *et al.* (2025), I calculated and applied a leaching correction factor to the decomposition rate and stabilisation factor. This accounted for the fraction of

OM leached away during the initial tidal inundations that follow the beginning of the incubation interval, leaving a smaller quantity of OM to be decomposed by microorganisms.

5.3.3 DNA extraction and sequencing

The second replicate pairs of teabags from the primary sampling stations were used for DNA extraction and sequencing. As well as the tea inside the bags, the sediment surrounding the bags was collected and subject to the same procedure. As an additional control, untreated surface sediment samples were taken from the 1 m² areas that comprised the primary sampling stations and from the secondary sampling stations N3 and R4 in November (Fig. 5.1). It was shown that the microbial communities in the bulk sediment of these marshes do not differ between summer and winter (Chapter Four) and so it was deemed appropriate to only analyse one set of control sediment samples. These control samples were also subjected to DNA extraction and sequencing and three replicates – taken from the same ~10 cm² surface sediment – per sample were processed. The DNeasy Power Soil Pro Kit (QIAGEN) was used according to the manufacturer’s instructions to extract DNA from both the sediment and tea samples. A nanodrop was used to assess DNA purity and quality and samples were frozen at -20 °C prior to sequencing. Samples were sent to Novogene for PCR amplification and 16S rRNA Illumina sequencing. Using the primers CCTAYGGGRBGCASCAG and GGACTACNNGGGTATCTAAT, the 16Sv34 region was amplified. Libraries were sequenced using a paired-end Illumina platform and following this the reads were assembled. To reduce noise by removing duplicates using dereplication to 100 % similarity clustering, the resulting raw data was cleaned with DADA2 (Callahan *et al.*, 2016; Li *et al.*, 2020) and from this Amplicon Sequence Variants (ASVs) were obtained. A pre-trained Naïve Bayes classifier with the classify sklearn algorithm from QIIME2 (Bokulich *et al.*, 2018; Bolyen *et al.*, 2019) was used to provide species annotation for each ASV and the Silva 138.2 database used. Shannon index (Shannon, 1948) and richness index values – tools used to investigate the alpha diversity of a sample – were calculated with ASV data. This was done with the diversity function from the *vegan* package in R (Oksanen *et al.*, 2025). Furthermore, the FAPROTAX database was employed to perform function prediction analysis.

To make visualisation of the FAPROTAX predicted functions clearer, I focused upon the top nine most abundant functions and combined those that were similar, resulting in seven main groups: (1) aerobic chemoheterotrophy; (2) anaerobic chemoheterotrophy (i.e., abundances of the “chemoheterotrophy” category minus those of the “aerobic_chemoheterotrophy” category); (3) fermentation; (4) methanogenesis; (5) nitrate reduction; (6) nitrification (i.e., the sum of the abundances of the functions “nitrification” and “ammonia_oxidation”); (7) sulphur respiration.

5.3.4 Indicators of Reduction in Soils (IRIS) sticks

To determine the redox levels in the top 10 cm of sediment, I used the establish Indicator of Reduction in Soils (IRIS) sticks method (Castenson and Rabenhorst, 2006; Rabenhorst and Burch, 2006; Mittmann-Goetsch *et al.*, 2024). In 250 mL of distilled water, 8 g of anhydrous FeCl₃ was dissolved.

Next, 370 mL of 1 M KOH was added so that the pH increased to 12. The resulting precipitate was then subjected to six centrifugations, at 1,000 rpm for five minutes each time, to remove ions. Finally, the precipitate was placed in a distilled water bath for three days before undergoing a final centrifugation at 1,000 rpm for five minutes. A sponge was used to apply the iron oxide precipitate to PVC film sheets. The sheets were cut into 50 cm long, 10 cm wide strips, and attached with double sided tape to 0.3 cm thick PVC foam sticks. These sticks were 10 cm wide and 60 cm long. After being submerged in the marsh sediment for between four and six weeks, sticks were collected, scanned and the scans analysed in R with a Random Forest model following Mittmann-Goetsch *et al.* (2024). This analysis resulted in a quantification of the numbers of orange (i.e., not reduced) and white (i.e., reduced) pixels within each 10 cm segment of the sticks. For the purposes of this study, only the top 10 cm segment of each stick was considered, representing the depth of tea bag deployment. To ascertain the redox conditions in winter, sticks were incubated from 19/01/24 to 04/03/24 and from 08/11/24 to 09/12/24 while those incubated from 29/05/24 to 09-10/07/24 and from 09-10/07/24 to 29/08/24 represented summer conditions. These incubation intervals overlap with those of the teabags. For clarity, only IRIS data from the primary sampling stations and the secondary sampling stations from which untreated control samples were taken (i.e., N3 and R4 in Fig. 5.1) is presented.

5.3.5 Statistical analyses

Two-way ANOVA tests with interaction effects were used to determine: (1) if decomposition and stabilisation rates differed between marshes and the four incubation intervals (i.e., three months in summer, three months in winter, nine months and twelve months); (2) if reduction index values differed between sampling stations and seasons; (3) if Shannon and richness indices differed between substrate (i.e., tea, surrounding sediment and control sediment) and marshes. Given that control sediment samples were included in the diversity indices tests it was not possible to include incubation interval as a variable in the analyses because this category was not applicable to these samples. Given that visual inspection indicated differences in reduction index values within sites, sampling station was used as a variable instead of marsh type for the IRIS stick ANOVA test. Tukey's test was applied after the two-ANOVAs to determine which group means within a level differed significantly from one another. The aim with these tests was to identify if significant differences in abiotic conditions (i.e., IRIS stick results) were consistent with significant differences in decomposition rates, stabilisation factors and microbial community diversity. To further examine microbial community composition differences, two versions of a canonical correspondence analysis (CCA) were produced: one in which the controls were included and another in which they were excluded. Again, this was done because the control samples did not have an incubation interval, so this was only included as a variable in the CCA from which they were left out. The CCA was performed with ASV data.

5.4 Results

5.4.1 Decomposition rates (k) and stabilisation (S) factors

Within individual incubation intervals, there was never a significant difference between the decomposition rates and stabilisation factors of the natural marsh compared with the restored marsh (Fig. 5.3; Table S5.3). Stabilisation factors were relatively consistent across the summer, winter and the nine-month incubation intervals while the values from the twelve-month interval were significantly lower than those of most other groups (Fig. 5.3a & 5.3b), the exceptions being those groups with widely ranging values. Decomposition rates were significantly higher during the winter incubation interval, with slightly lower rates recorded during the summer interval and considerably lower rates during the nine- and twelve-month intervals (Fig. 5.3c & 5.3d).

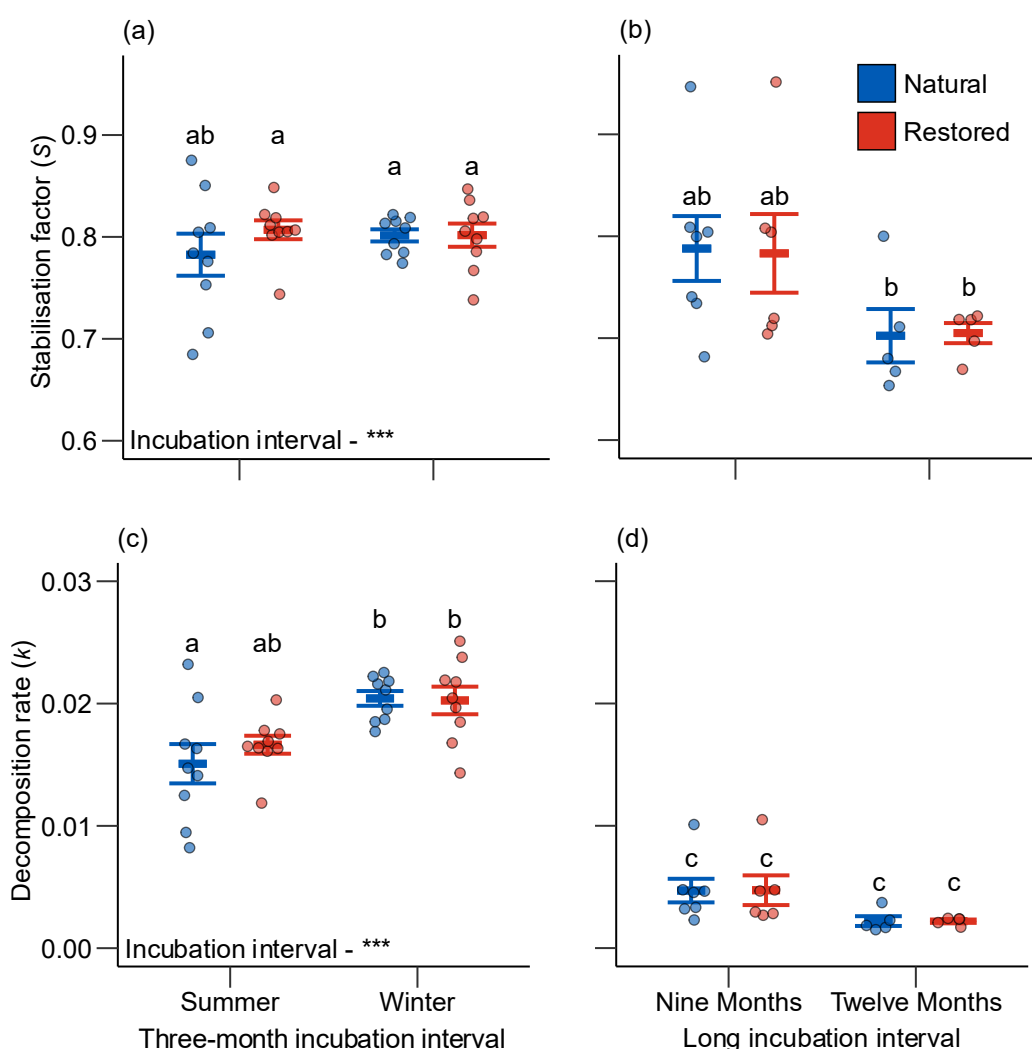


Figure 5.3. (a-b) Stabilisation factors (S) and (c-d) decomposition rates (k) of teabags incubated in natural (blue) and restored (red) saltmarshes during four different incubation intervals. The teabags were incubated in several different locations across both marshes (Fig. 5.1). Significance letters indicate the results of two-way ANOVA and Tukey's tests conducted for each pair of panels (i.e., a and b were one pair and c and d the other; *** - $P \leq 0.001$). Marsh ($P > 0.80$) and the interaction between marsh and incubation interval ($P > 0.50$) were not significant in either case (Table S5.3).

5.4.2 Sediment redox

There was a significant interaction between season and sampling station (Fig. 5.4; Table S5.3), with reduction indices higher in the summer compared with the winter (Fig. 5.4), representing conditions that were more strongly reducing and a lower level of oxygen availability. Across the stations, the secondary sampling station – where untreated control sediment samples were taken – on the natural marsh had the highest reduction index values in both seasons. In the primary natural marsh sampling station – where teabags used for DNA extraction and sequencing were incubated – reduction indices remained low across both seasons. The mean reduction index values of both restored sampling stations were intermediate between the two extremes represented by the natural marsh stations. The secondary sampling station in the restored marsh generally had higher reduction indices than the primary restored sampling station.

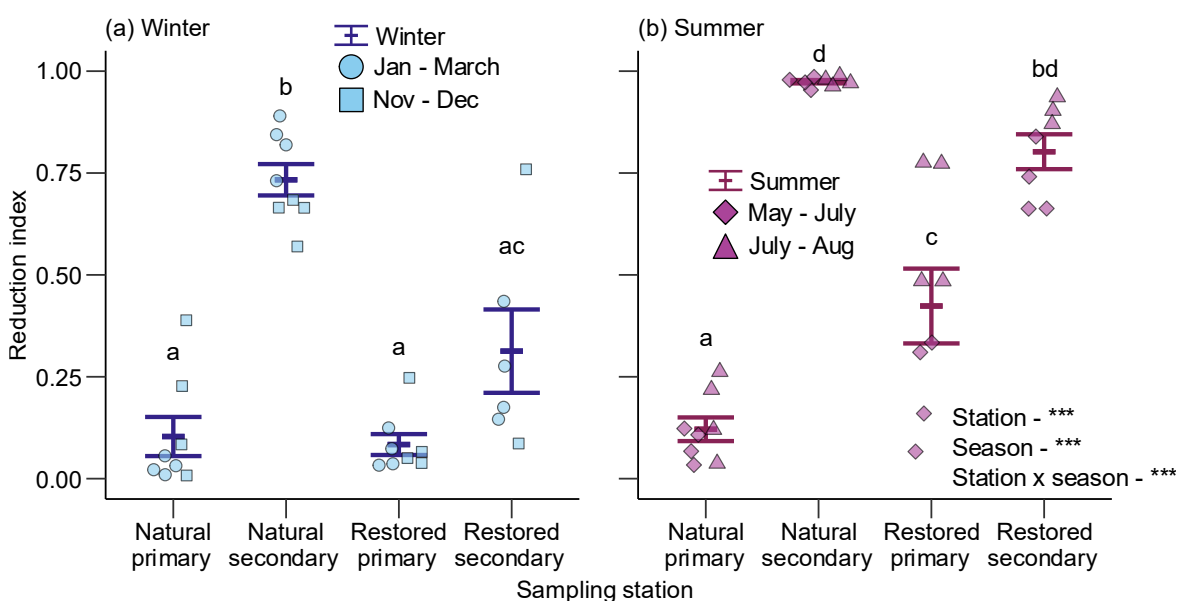


Figure 5.4. During the (a) winter and (b) summer months, IRIS sticks were deployed in both primary and two secondary (i.e., N3 and R4 in Fig. 5.1) sampling stations to determine sediment redox conditions in the top 10 cm. Error bars are $\pm$ standard error. Higher reduction index values are indicative of more strongly reducing conditions. Within panel (b), the results of the two-way ANOVA test are shown (***) - $P \leq 0.001$; Table S5.3) and significance letters across both panels indicate the results of a Tukey's test.

5.4.3 Canonical correspondence analysis

The CCA model that included the controls was significant ($p = 0.001$; $F = 2.1$; global permutation test with 999 permutations; Fig. 5.5a). The first two canonical axes explained 13.689 % of the total variance (CCA1 = 8.2 %, CCA2 = 5.5 %; eigenvalues were 0.8 and 0.5, respectively). Of the constrained variance, CCA1 explained 37.3 % and CCA2 25.2 %, meaning a cumulative total of 62.5 % was captured by these axes. Key environmental variables that were significant predictors were Substrate ($p = 0.001$; $F = 2.0$) and Marsh ($p = 0.001$; $F = 2.6$). The total constrained variance explained by these variables was 21.9 %.

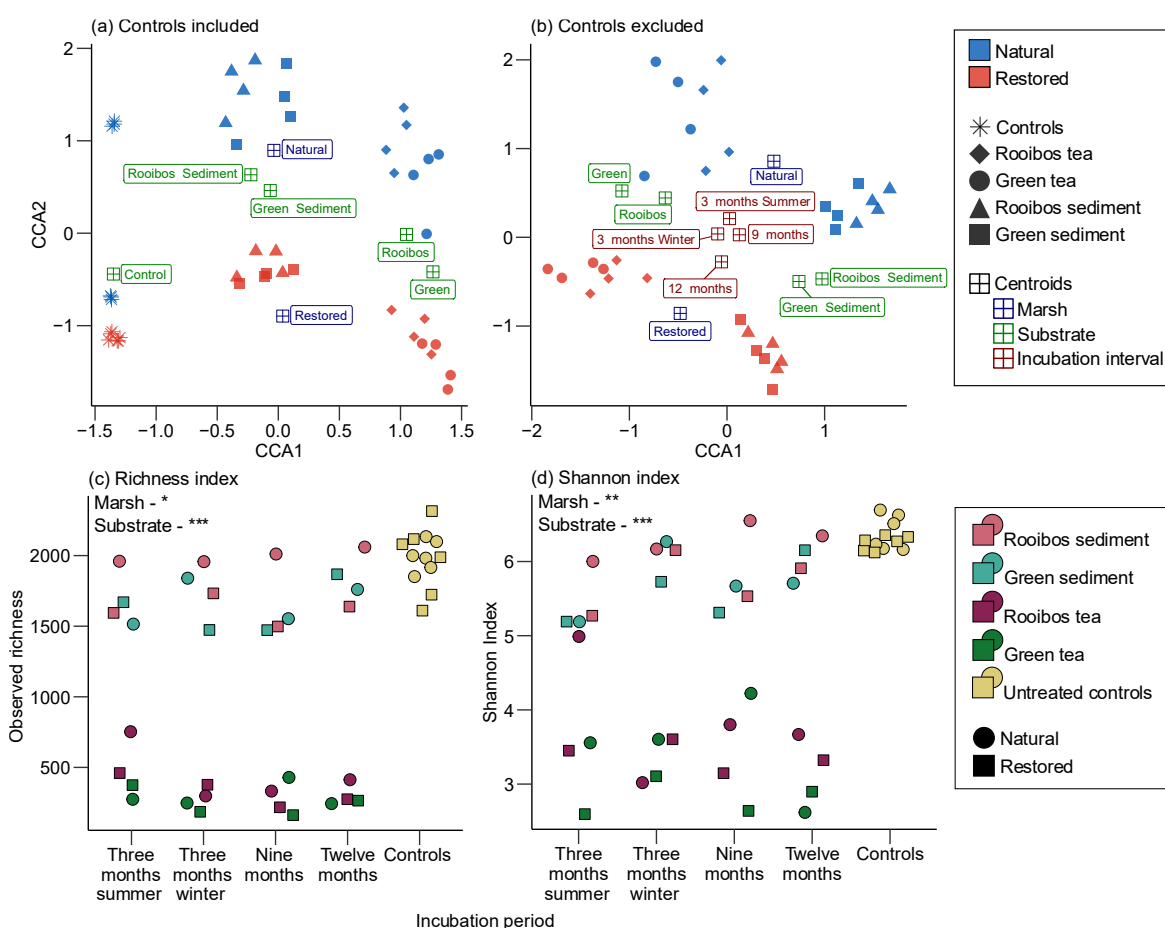


Figure 5.5. Canonical correspondence analyses (a) including the control samples and (b) excluding the controls, and (c) the richness index and (d) Shannon index values of all samples. Differences in observed richness and Shannon index values between marsh type and substrate (i.e., controls, tea or surrounding sediment) were analysed with a two-way ANOVA, and the results are shown within each panel (** - $P \leq 0.01$, *** - $P \leq 0.001$ and * - $P \leq 0.05$). The interactions between the two variables were not significant in either case ($P > 0.13$). Incubation period was not included in the ANOVA analyses because this category was not applicable to the control samples. In the CCA panels, centroids show the mean ordination positions of each level within a variable and these points are coloured by variable.

Also significant was the CCA model from which the controls were excluded ($p = 0.001$; $F = 1.7$; global permutation test with 999 permutations; Fig. 5.5b). The first two canonical axes explained 15.4 % of the total variance (CCA1 = 8.3 %, CCA2 = 7.1 %; eigenvalues were 0.6 and 0.5, respectively). Of the constrained variance, CCA1 explained 25.6 % and CCA2 21.8 %, meaning a cumulative total of 47.4 % was captured by these axes. Key environmental variables that were significant predictors were Substrate ($p = 0.001$; $F = 1.6$), Marsh ($p = 0.001$; $F = 2.6$) and Incubation period ($p = 0.001$; $F = 1.4$). The total constrained variance explained by these variables was 32.5 %.

In both CCA models, natural and restored marsh results were grouped separately. The exceptions to this rule were the communities found in the natural marsh primary sampling station controls (Fig. 5.5a). These were separated from the natural marsh secondary sampling station controls, which in turn were clustered near to the restored marsh controls (Fig. 5.5a). Tea and surrounding sediment samples were placed in separate clusters. In the second CCA (Fig. 5.5b), the incubation interval centroids were clustered relatively closely, with the twelve-month interval being most distanced from the other three, suggesting that differences between samples incubated for different lengths of time were relatively minor. Green tea and rooibos tea centroids were also closely clustered, as was the sediment surrounding the green teabags and the rooibos teabags, suggesting that only minor differences existed in the communities colonising the labile and recalcitrant material provided. The major differences were between the tea and the sediment and between the sampling stations.

5.4.4 Diversity indices

Both richness and Shannon diversity indices were significantly lower in the tea samples compared with the surrounding sediment and the controls (Fig. 5.5c & 5.5d). Differences were starkest when the richness index was considered (Fig. 5.5c): the control and surrounding sediment usually had similar values across all samples (1472-2315) while the tea samples had lower values (162-753).

5.4.5 Microbial community composition

Classes

The first difference to highlight between the tea (Fig. 5.6a-5.6d) samples and the controls (Fig. 5.6e) is the decreased abundances of the classes *Desulfuromonadia*, *Desulfobacteria* and *Desulfobulbia* in the former, where they were present only in either very low amounts (<3 %) or not at all. However, there were also differences within the control samples, with much lower abundances of these same classes in the natural marsh primary sampling station (0.003-0.600 %), in contrast with the other three stations (4-14 %; Fig. 5.6e). Furthermore, the natural marsh primary sampling station control contained higher abundances of *Alphaproteobacteria*, *Actinobacteria* and *Acidimicrobiia* (3-31 %) compared with the other controls (0.2-18.0 %). Similarly, *Actinobacteria* was usually more abundant in the tea compared with the controls and reached abundances far greater (1-60 %) than those seen even in the natural primary sampling station control. In the natural marsh, *Bacteroidia* was often the

most abundant class in both the tea and the surrounding sediment ($34 \pm 13 \%$) while in the control samples the relative abundance of this class averaged $11 \pm 3 \%$. *Clostridia* abundances were negligible in the controls but higher in the tea and surrounding sediment, though it remained one of the least abundant classes in these samples ($<12 \%$). *Gammaproteobacteria* was often slightly less abundant in the tea and surrounding sediment compared with the controls. Consistent differences between incubation intervals were less apparent. One exception was a consistently elevated abundance of *Bacilli* in the restored marsh tea and surrounding sediment during the summer incubation interval compared with the natural marsh in the same period, most other incubation intervals and the controls.

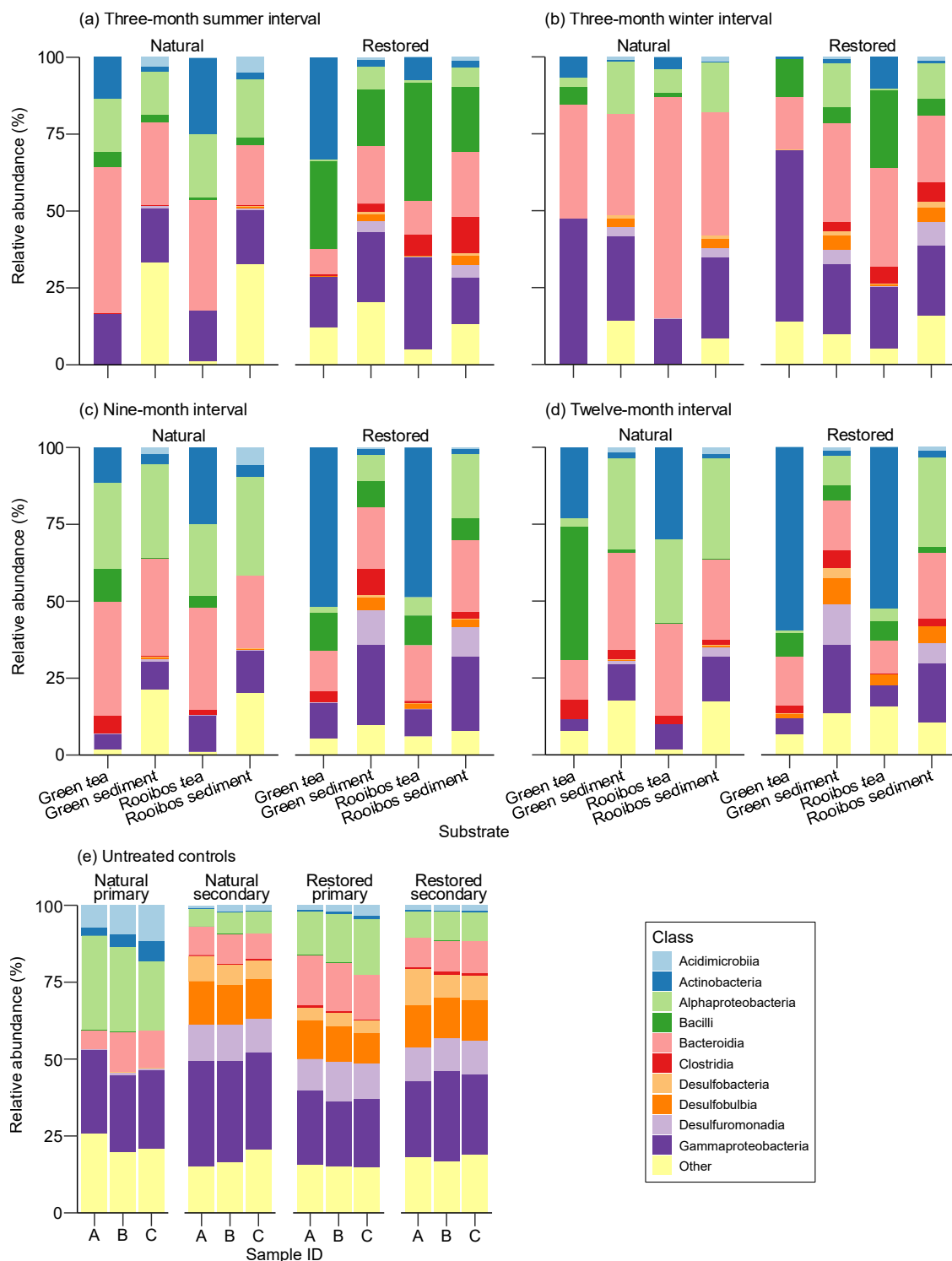


Figure 5.6. The bacterial classes present in teabags and the sediment surrounding the bags following (a) a three-month summer incubation interval, (b) a three-month winter incubation interval, (c) a nine-month incubation interval, (d) a twelve-month incubation interval, as well as results from (e) control sediment collect in November of the same year. Controls came from both primary sampling stations and from two secondary sampling stations (R4 and N3 in Fig. 5.1). The top ten most abundant classes are shown with all remaining taxa classed together under the “Other” category (i.e., the pale-yellow bars that are always at the bottom of the stack).

Genera

Given that incubation interval was found to be a less important driver of variation in the CCA, values were averaged across these intervals when looking at the abundances of different genera (Fig. 5.7). The top fifteen most abundant genera were determined when only the tea samples were examined (Fig. 5.7a & 5.7b) and when only the control samples are examined (Fig. 5.7c). Rooibos tea samples incubated in the restored marsh contained several genera with relatively high abundances (i.e., >5 %). Namely, in descending order of abundance, these were *Demequina*, *Acholeplasma*, *Cellulomonas*, *Marinobacter*, *Trichococcus* and *Saccharicinis* (Fig. 5.7a). *Demequina* and *Marinobacter* were also among the most abundant genera found in the rooibos tea incubated in the natural marsh but in addition to these, *Cellulophaga* and *Maribacter* also had mean abundances greater than 5 % (Fig. 5.7a). Like the rooibos tea samples, green tea in the restored marsh was dominated by the genera *Cellulomonas*, *Demequina* and *Acholeplasma*, as well as a genus not highly abundant in the rooibos tea samples, *Vibrio* (Fig. 5.7b). The natural marsh incubated green tea was dominated by *Acholeplasma*, like the restored green tea, *Maribacter* and *Cellulomonas*, like the natural rooibos tea, and *Pseudoalteromonas*, unlike either of the other treatments (Fig. 5.7b).

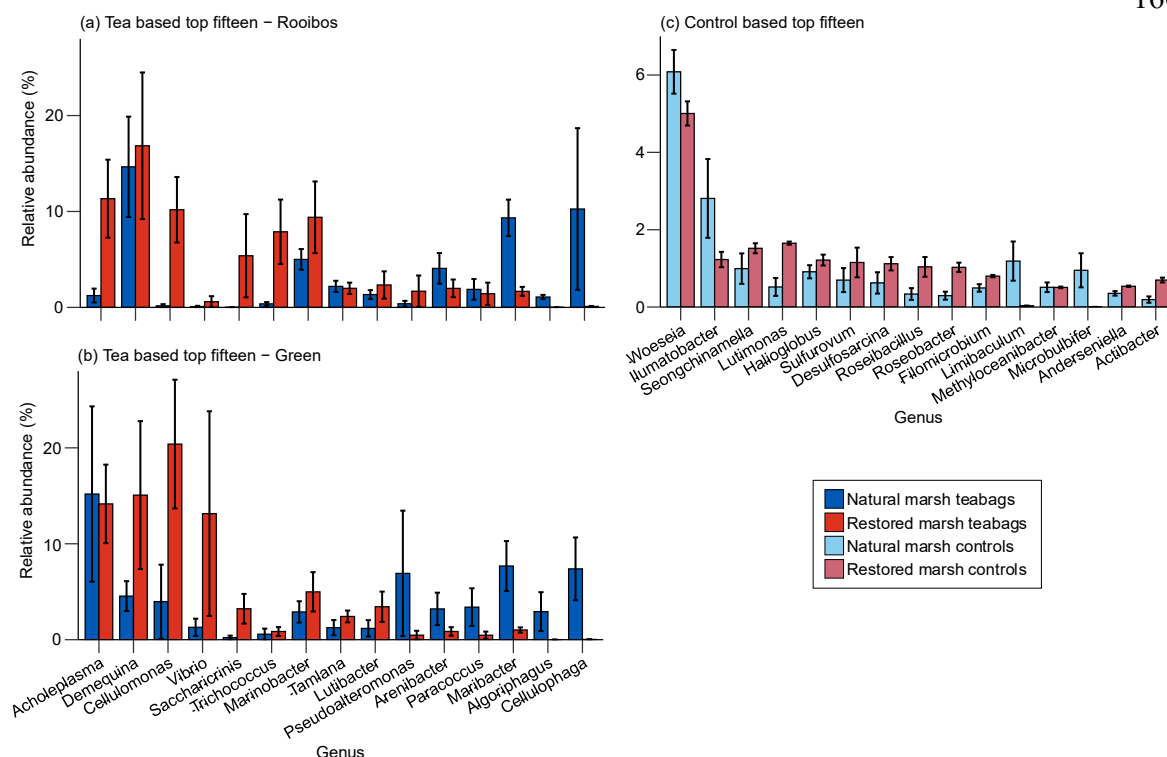


Figure 5.7. Abundances of the top fifteen most abundant genera in (a-b) the tea samples and (c) the control samples. (a) Rooibos and (b) green tea results are shown in separate panels. For the tea samples, the mean abundances of each genus across the different incubation intervals are shown. Error bars are $\pm$ standard error. For ease of visualisation, the surrounding sediment samples were not included. The genera most common in the teabag assemblages were entirely absent from or present in very low abundances in the control samples (<1 %). Likewise, the tea samples contained no or very low abundances (<5 %) of those genera that were most common in the control samples. Error bars show $\pm$ standard error.

Control samples from both the restored and the natural marshes were overwhelmingly dominated by the genus *Woeseia* (mean abundances >5 %; Fig. 5.7c). In the natural marsh *Illumatobacter* had the second highest mean abundance (3 %) while all the other genera in the restored marsh had similarly low abundances (<2 %).

5.4.6 Predicted microbial functions

In the sediment control samples, the abundances of all FAPROTAX predicted functions were low compared with the teabag and surrounding sediment samples (Fig. 5.8a). Aerobic chemoheterotrophy was consistently predicted to be one of the most abundant functions across all control samples (ranging between 2 and 6 % relative abundance) while the abundance of nitrification taxa was high (7 %) in the natural marsh primary sampling station compared with the other controls.

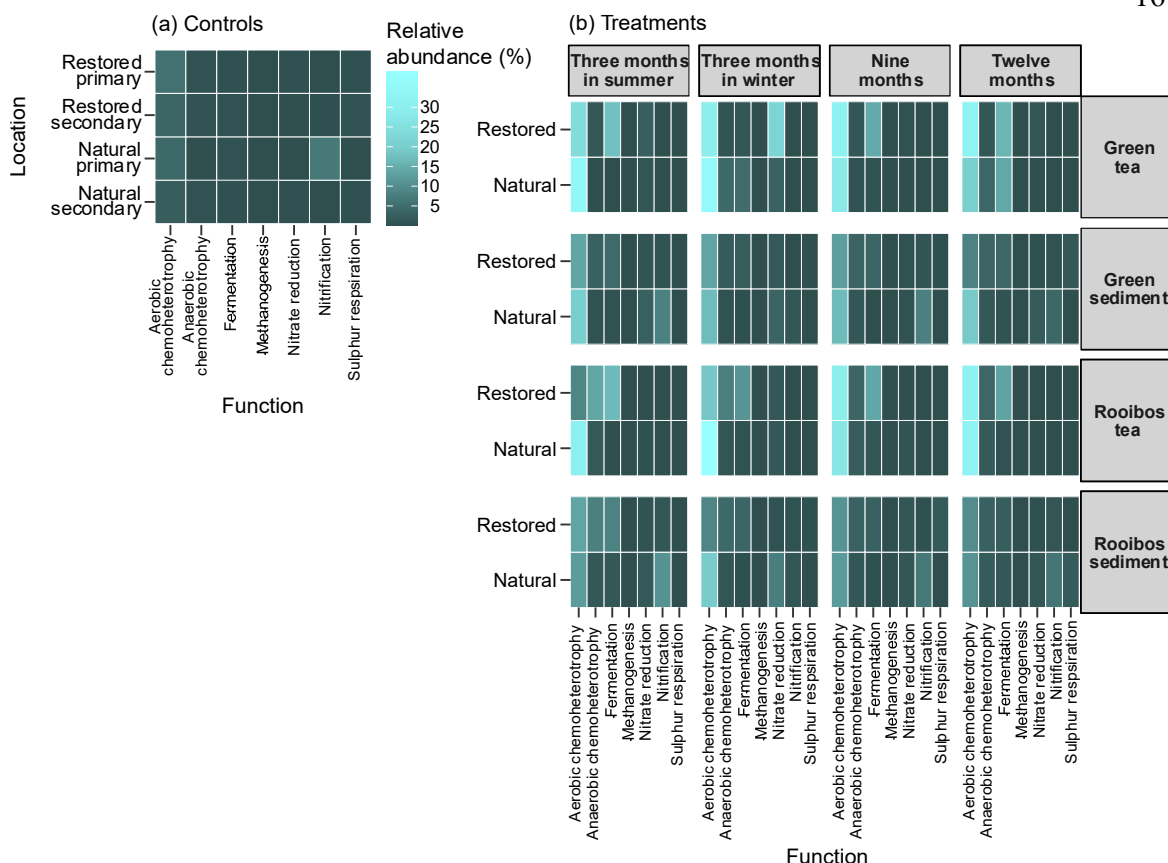


Figure 5.8. The FAPROTAX predicted functions present in (a) the control samples and (b) the tea and sediment surrounding the teabags. Control samples were collected in November 2024 while the teabags were subject to four different incubation intervals.

Aerobic chemoheterotrophy tended to be the most abundant function in the tea and surrounding sediment samples (Fig. 5.8b). Particularly in the natural marsh samples, this function overwhelmingly dominated (11-39 %). Conversely, restored marsh samples often contained other functions that were as or more abundant. For the green tea incubated in the restored marsh the second most abundant function was fermentation (1-18 %) in all cases bar one in which nitrate reduction was more abundant (22 %). Again, in the restored marsh rooibos tea, fermentation abundances (11-17 %) were a close second to those of aerobic chemoheterotrophy (9-31 %), the former even surpassing the latter during the summer incubation interval. Anaerobic chemoheterotrophy taxa were also more abundant in the restored marsh tea samples (1-14 %) compared with those of the natural marsh (0.1-4 %). Sediment surrounding the teabags incubated in the natural marsh was often more enriched in nitrification taxa (0.5-11 %) than the tea from the same marsh and incubation interval (0.00-0.03 %) while this was not the case in the restored marsh. Taxa able to perform methanogenesis and sulphur respiration were not predicted to be highly abundant, with all values ranging between 0.00-0.06 % and 0-2 %, respectively.

5.5 Discussion

Sediment redox did differ between sampling stations, with certain locations representing strongly reducing conditions, but despite this, OM decomposition and stabilisation rates did not differ between marshes. However, there were differences in the predicted functions of the microbial communities and in the composition of the communities. On the other hand, clear successional changes in microbial communities following longer incubation intervals in the marshes were not seen nor did the microbial taxa in the surrounding sediment correspond strongly with those present in the relevant teabags. The surrounding sediment microbial communities, however, did not correspond closely with communities of the untreated control samples.

5.5.1 Stabilisation factor and decomposition rate

Stabilisation factors and decomposition rates did not differ between natural and restored marshes during any of the incubation intervals and this implies that both sites function similarly in terms of OM decomposition (Fig. 5.3). Generally, and in saltmarshes specifically, a key determinant of decomposition rate is OM quality (Zhang *et al.*, 2008; Stagg *et al.*, 2018; Carrasco-Barea *et al.*, 2022) and, given that the OM provided to both marshes was the same standard teabag litter, this helps to account for the similar rates of decomposition recorded. According to Daebeler and colleagues (2022), stabilisation factor is more sensitive to the microbes growing during decomposition because the processes involved rely on several groups performing different functions. Therefore, the consistency in the stabilisation factors recorded in both marshes suggest that the different groups of microbes present in both marshes were ultimately creating the same effect, meaning there was functional redundancy.

The stabilisation factor of the nine-month incubation interval was not significantly different from the factors of the two three-month intervals (Fig. 5.3a & 5.3b; Table S5.3), suggesting that stabilisation was completed after three months and did not proceed further when teabags were incubated for longer than what is recommended by the standard methodology (Keuskamp *et al.*, 2013). However, the twelve-month incubation interval had significantly lower stabilisation rates, showing that additional OM was lost from the teabags during the tenth, eleventh and twelfth months of incubation. The decay of recalcitrant saltmarsh OM can continue into the second year following its deposition in a marsh (Valiela *et al.*, 1985) and these results suggest that this was also true of teabag litter, despite its very different origin. Unlike the stabilisation factor, decomposition rates for the nine- and twelve-month incubation intervals were similarly low and did not differ significantly (Fig. 5.3c & 5.3d; Table S5.3), suggesting that after the relatively quick decomposition that occurred during the first three months, considerably less material was lost in the subsequent period. This result highlights the importance of looking at the stabilisation factor, as well the decomposition rate, when using the TBI, as has been emphasised by Elumeeva *et al.* (2018).

5.5.2 Natural versus restored marsh microbial communities

Although decomposition and stabilisation rates did not differ between marshes, there were substantial differences in microbial community composition. Combined, these results suggest that both marshes functioned similarly in terms of OM cycling and that different microbial groups ultimately had the same impact. This result is in close agreement with the findings of another recent study that also determined both sediment microbial community composition and OM decomposition rates with the TBI in restored and natural marshes (Gopakumar *et al.*, 2026). They found that OM decomposition rates were similar in both marsh types but that microbial community structures differed, although they reported that the putative functions of these communities were similar.

There were four sulphur-reducing classes of bacteria that were either entirely or nearly absent from the tea and the natural marsh primary sampling station control samples (Fig. 5.6). The tea represented a resource hotspot introduced to the sediment while the natural marsh primary sampling station was representative of a high marsh setting. In contrast the natural secondary sampling station was representative of a low marsh environment, thus having more in common with the restored sampling stations (as indicated by the plant communities present; Table S5.2). High marsh areas tend to be relatively well oxygenated because of their high elevation position in the tidal frame (Mittmann-Goetsch *et al.*, 2024) and this was supported by the reduction index values recorded by the IRIS sticks incubated in the natural marsh primary sampling station (Fig. 5.4). The presence of oxygen produced conditions unfavourable for sulphur reduction and other anaerobic processes. Teabags incubated in the restored marshes experienced more reducing conditions (Fig. 5.4), but sulphur-reducing bacteria were still absent and the FAPROTAX analysis predicted that fermentation-related taxa were often far more abundant in restored marsh teabags than in those incubated in the natural marsh (Fig. 5.8b). In the presence of an OM source, fermentation may have become necessary if all other possible electron acceptors were quickly used up. Given that teabags incubated only in the high marsh zone of the natural marsh were used for DNA extraction and sequencing, this leads to the possibility that the differences in microbial taxa were the result of high versus low marsh conditions, as opposed to natural versus restored conditions.

The microbial genera most prevalent in the restored marsh also showed that conditions were more anaerobic in those sediments compared with the natural marsh. For example, *Trichococcus*, *Cellulomonas*, *Saccharicrinis* and *Vibrio* are all facultatively anaerobic genera (Liu *et al.*, 2002; Christopherson *et al.*, 2013; Yang *et al.*, 2014; Jiang *et al.*, 2021) that were considerably more abundant in the restored marsh (Fig. 5.7a & 5.7b). On the other hand, *Pseudoalteromonas*, *Maribacter* and *Cellulophaga* were more abundant in the natural marsh (Fig. 5.7a & 5.7b), and many representatives of these genera are obligate aerobes (Gauthier *et al.*, 1995; Nedashkovskaya *et al.*, 2004; Bowman, 2016). The fact that OM decomposition rates did not differ between these two redox conditions could have been because the anaerobic degraders of the restored marsh had a sufficient supply of labile OM from other sources, such as plant root exudates and/or washed in by the tides,

to allow the maintenance of a rate of decomposition similar to that seen in more aerobic conditions, even after the leaching phase of the tea itself was over (Kristensen *et al.*, 1995; Hulthe *et al.*, 1998).

Another difference between marshes in terms of the predicted microbial functions was the higher prevalence of nitrification taxa in the surrounding sediment of the natural site compared with the restored (Fig. 5.8b). The natural marsh sediment tended to possess a higher OC/N ratio than that of the restored marsh (Table S5.4), suggesting it was more N limited (Craft, 2001) and more nitrification activity was required to provide the N compounds necessary for microbial growth. The increased prevalence of nitrification taxa disappeared in the corresponding tea samples and aerobic chemoheterotrophy dominated. Both rooibos and green tea contain a relatively high level of N (1-4 %; Keuskamp *et al.*, 2013) when compared with the bulk sediment (0.21 – 0.56 %; Table S5.4), suggesting that N limitation was less of a problem within the tea, but this was not the case in the sediment immediately surrounding the bags.

Functional diversity analysis is a potentially useful tool for linking biodiversity to ecosystem functioning (Escalas *et al.*, 2019). It should be emphasised however, that the major caveat of using FAPROTAX to ascertain the functions being performed by a microbial community is that it merely provides a prediction of function, and the genes present in each sample – other than the 16S rRNA gene – have not been quantified or detected. To more robustly interrogate the microbial functions in the future, metagenomics could be used to provide a stronger prediction of the presence of genes while, to experimentally validate predicted functions, it would be necessary to measure process rates and transcript abundances.

5.5.3 Teabag microbial communities

There were also clear differences between the microbial communities growing upon the different substrates examined (i.e., tea, surrounding sediment and control sediment) but not between the communities colonising the green versus the rooibos tea nor the green surrounding sediment versus the rooibos surrounding sediment (Fig. 5.5a & 5.5b). Therefore, despite the differences in OM quality represented by the two tea types, this did not select for vastly different microbial communities. To contrast, a previous investigation of the microbial communities colonising teabag litter during incubation in soil, found large differences between green and rooibos communities, driven in part by the more labile nature of the OM present in the former (Pioli *et al.*, 2020). A strong contrast between the structure and function of the microbial communities colonising teabags compared with the bulk sediment has important implications for TBI research because this finding challenges the assumption that tea litter introduced to the site undergoes similar process to native OM. Furthermore, the fact that the tea altered the microbial communities of the surrounding sediment but that this did not result in communities that exactly matched those present in the teabags, suggests introducing an OM source has relatively far-reaching consequences. These could include a stimulatory or inhibitory impact upon the decomposition of the native OM present within the surrounding sediment.

Successional changes in bacterial community were expected to occur (Purahong *et al.*, 2016; Jiang *et al.*, 2019). While the microbial communities colonising the tea did not shift very much over the different incubation intervals, this variable was significant in the CCA and it was teabags incubated for twelve-months that contained assemblages that differed most strongly from the other treatments (Fig. 5.5b). This could suggest that successional changes in the community as the remaining material became increasingly recalcitrant only began to occur around a year after the OM entered the system, which is consistent with the results previously recorded by Trevathan-Tackett and colleagues (2021). The lower stabilisation rates of the twelve-month incubation interval also show that ongoing OM cycling processes were still occurring during this period and were partly captured by this modification to the standard methodology.

Of the diversity indices calculated, observed richness showed the starkest differences between tea and sediment (Fig. 5.5c), the former having lower values. From this it was concluded that the tea contained fewer rare taxa that were present in only very small amounts. This is consistent with the findings of all the previous studies that sequenced the DNA extracted from teabags incubated in an ecosystem – saltmarsh, forest or farmland – and untreated, bulk sediment (Pioli *et al.*, 2020; Trevathan-Tackett *et al.*, 2021; Daebeler *et al.*, 2022; Neiske *et al.*, 2025). This decreased diversity was likely a result of the OM source introduced to the ecosystem favouring the survival of a relatively small group of fast-growing bacteria.

5.6 Conclusions

The fact that teabag litter was still being processed twelve-months into the incubation interval has important implications for the long-term storage of the other OM entering the sediment, although further testing with native material would be required before concrete conclusions could be drawn. Furthermore, this shows the value of adjusting the standard teabag methodology and incubating teabags for longer than the usual three-month interval where it might prove insightful to do so. These results also showed that different microbial groups in the oxygenated, high elevation natural marsh sampling station compared with the reducing low elevation restored marsh station, broke down the teabag litter at similar rates using different processes. In the natural marsh, bacteria affiliated to typically aerobic genera predominated, while anaerobic bacteria were more abundant in the restored marsh. Furthermore, it was predicted by FAPROTAX analysis that aerobic chemoheterotrophy dominated in the natural marsh while fermentative processes were relied upon more heavily in the restored site.

5.7 Supplementary Information

5.7.1 Supplementary tables

Table S5.1. Locations of the primary and secondary sampling stations. Asterisks indicate the secondary sampling stations sampled to provide untreated control sediment samples for the microbial analysis and for which IRIS data is presented.

Sampling station	Latitude	Longitude
Natural primary	51.838333	0.977222
N1	51.839722	0.977778
N2	51.839444	0.977500
N3*	51.838333	0.978056
N4	51.838056	0.977778
Restored primary	51.843056	0.972500
R1	51.843611	0.972222
R2	51.839722	0.973333
R3	51.838611	0.976944
R4*	51.839167	0.976944

Table S5.2. Plant communities present in the sampling stations during August 2024. Asterisks indicate the secondary sampling stations sampled to provide untreated control sediment samples for the microbial analysis and for which IRIS data is presented.

Sampling station	Cover class	Cover (%)
Natural primary	<i>Armeria maritima</i>	70.0
	<i>Aster tripotum</i>	15.0
	Bare mud	15.0
N1	<i>Spartina anglica</i>	60.0
	Bare mud	40.0
N2	<i>Armeria maritima</i>	58.0
	<i>Aster tripotum</i>	30.0
	<i>Salicornia europaea</i>	10.0
N3*	Bare mud	2.0
	<i>Spartina anglica</i>	90.0
	Bare mud	10.0
N4	<i>Salicornia europaea</i>	27.5
	<i>Armeria maritima</i>	25.0
	<i>Aster tripotum</i>	15.0
	<i>Spartina anglica</i>	5.0
	Bare mud	27.5
Restored primary	<i>Spartina anglica</i>	75.0
	Bare mud	25.0
	<i>Salicornia europaea</i>	60.0
R1	Bare mud	40.0
	<i>Salicornia europaea</i>	80.0
R2	<i>Aster tripotum</i>	17.0
	Bare mud	3.0
R3	<i>Spartina anglica</i>	90.0
	Bare mud	10.0
R4*	<i>Spartina anglica</i>	90.0
	Bare mud	10.0

Table S5.3. Results of the two-way ANOVA tests with the relevant figures in the main text indicated in the first column. Significant ($p = < 0.05$) p -values are highlighted in bold.

Variable and figure	Level/Interaction	Adjusted p -value	F -value	df .
Stabilisation factor (Fig. 5.2a & 5.2b)	Marsh	0.628	0.237	1, 51
	Incubation interval	<0.001	7.964	3, 51
	Marsh x incubation Interval	0.873	0.233	3, 51
Decomposition rate (Fig. 5.2c & 5.2d)	Marsh	0.573	0.322	1, 51
	Incubation interval	<0.001	124.836	3, 51
	Marsh x incubation interval	0.805	0.328	3, 51
Reduction index (Fig. 5.3)	Sampling station	<0.001	80.054	3, 53
	Season	<0.001	47.406	1, 53
	Sampling station x season	<0.001	6.577	3, 53
Observed richness index (Fig. 5.5c)	Marsh	0.020	5.938	1, 34
	Substrate	<0.001	241.152	4, 34
	Marsh x substrate	0.138	1.871	4, 34
Shannon index (Fig. 5.5d)	Marsh	0.005	9.197	1, 34
	Substrate	<0.001	110.349	4, 34
	Marsh x substrate	0.499	0.858	4, 34

Table S5.4. Marsh biogeochemical properties measured in surface sediment samples collected during March 2024. Asterisks indicate the secondary sampling stations sampled to provide untreated control sediment samples for the microbial analysis and for which IRIS data is presented.

Sampling station	OC (%)	N (%)	OC/N	DBD (g cm⁻³)
Natural primary	5.30	0.56	9.46	0.69
N1	2.23	0.36	6.15	0.86
N2	5.86	0.56	10.46	0.59
N3*	1.83	0.26	7.04	0.74
N4	2.26	0.31	7.29	1.16
Restored primary	2.16	0.35	6.17	0.77
R1	2.10	0.22	9.69	0.89
R2	1.97	0.21	9.38	0.93
R3	2.19	0.32	6.84	0.84
R4*	3.13	0.43	7.28	0.58

Chapter Six

Discussion

6.1 Summary of the main results

6.1.1 Chapter Two

A clear and consistent difference between the restored marshes investigated in Chapter Two and their natural counterparts, was the lower OC/N ratio values in the former, even up to 100 years after the breach occurred. This lower OC/N ratio in the restored marshes points to a higher marine contribution to the OM stock because algae are N-rich compared with the C-rich cellulose of terrestrial plants (Zhou *et al.*, 2007). In addition, the sediment in top 10 cm of the youngest site, Fingringhoe Wick, and a 21-year-old site, Brancaster West, contained a higher proportion of recalcitrant OM compared with sediment from the same depths in the natural marsh, suggesting the presence of older OM that had already undergone a degree of decomposition to lose its labile OM fraction. Furthermore, these two restored marshes were colonised by the plant *Spartina anglica* alone in the locations from which cores were taken and this was reflected by the $\delta^{13}\text{C}$ profile along the lengths of these cores. However, the values recorded were insufficiently enriched to infer a contribution from this plant species alone. Instead, this pointed to a mixed signal, again, produced by contributions from OM of a marine origin. Taken together, the evidence was interpreted as indicating that the OM stocks of the restored marshes consisted of a higher proportion of allochthonous, marine OM. This was most obviously true of Brancaster West and Fingringhoe Wick, but lower OC/N ratios were a feature of Freiston Shore and Aldboro Point sediment, too.

To identify the transition between an agricultural field and a saltmarsh in sediment cores taken from restored marshes, it was concluded that the foraminifera technique developed in Chapter Two can be useful if key steps are included in the methodology and if it is used in combination with other approaches. First, instead of analysing two cores per site, it would be preferable to take many cores distributed widely across the study area. Doing so would ensure that within site variability in sedimentation rate is captured. Secondly, it is crucial to take deep cores to confidently ascertain the transition depth. Taking ~50 cm cores from the 21-year-old sites was insufficient and it is recommended that ~100 cm cores are taken from sites of a similar age if this method is employed in the future. Cores should be subsampled at a 1 cm resolution but, initially, samples from 5 or 10 cm intervals can be analysed for foraminifera. Additional samples from depth windows at which lower numbers of tests and/or clear transitions in species assemblages are seen can then be processed. This ensures that the process does not become too labour intensive.

6.1.2 Chapter Three

When assessing the enzyme assay methodology, it was clear that inconsistent results were achieved for samples collected from the same place at the same time when different storage temperatures (4 and -20 °C) and diluents (phosphate buffer, distilled water and saltmarsh creek water) were used for soil slurry preparation. There were three possible reasons for these differences. First, when creek water or distilled water were used, pH fluctuations during the assays were greater than when

phosphate buffer was the diluent. Second, the starting pH of the three diluents being tested differed, meaning that the activities of different enzymes would have been closer to optimisation in some assays than in other. Finally, assays using pH-sensitive 4-MUF as the fluorophore quench coefficient values that far exceeding one when distilled water was the diluent, meaning that the single concentration standards for these assays were invalid. It was concluded that enzyme activity should be maximised if identifying clear, consistent differences between samples is the goal and this means using a buffer to select an appropriate pH for the enzyme in question. This recommendation cannot be applied universally, and decisions should be made based upon the research questions being addressed by individual studies.

6.1.3 Chapter Four

Using Indicators of Reduction in Soils (IRIS) sticks to compare the functionality of a low elevation marsh, restored eight years ago in Essex, with a neighbouring, higher elevation, natural counterpart, showed that conditions were more strongly reducing in the former, indicating a lower level of oxygen availability in the sediment. 16S rRNA gene sequencing revealed that microbial communities differed most strongly between marshes in the deep sediment, with restored marsh deep assemblages that were less diverse and contained a higher proportion of sulphate reducing genera. Functions relating to sulphate and iron respiration were also predicted to be far more abundant in the restored marsh deep sediment compared with the natural. Another difference was the higher activities of the enzymes β -glucosidase, chitinase and phosphatase in the deep sediment of the restored marsh compared with the surface. Conversely, in the natural marsh, activities of all the extracellular enzymes examined were considerably lower in the deep sediment compared with the surface. Microbial communities also differed between marshes in the surface sediment, likely a consequence of the higher inundation frequency and reduced drainage experienced by the restored marsh sampling stations.

6.1.4 Chapter Five

In the same neighbouring restored and natural marshes studied in Chapter Four, the microbial assemblages colonising teabag litter differed substantially between marshes. The restored marsh sediment was dominated by anaerobic taxa, and fermentative processes were often predicted to dominate while the natural contain strictly aerobic taxa and aerobic chemoheterotrophy was always predicted to be the overwhelmingly predominant process. However, the decomposition and stabilisation rates calculated using the teabags incubated in both marshes were very similar and statistical testing did not show a significant difference. Therefore, different groups of microbes, performing different processes, were ultimately having the same impact upon the OM introduced to the sites. Additionally, tea litter decomposition continued when the bags were incubated for as long as twelve months but strong successional changes in the microbial communities in these teabags, compared with those incubated for three-month intervals, were not seen. Again, this suggests that

many of the microbial taxa present were contributing to both the initial and the later stages of OM decomposition.

6.2 Synthesis of results

The results of the foraminiferal analysis performed in Chapter Two indicated that the restored marsh deep sediment category in Chapter Four was representative of the relic pre-breach agricultural field. Additional confidence in this conclusion came from the fact that Fingringhoe Wick, the youngest marsh in Chapter Two and the focus of Chapter Four, was the site where the foraminiferal method proved most effective, producing results that offered a clear interpretation. In Chapter Four, therefore, the strong differences in deep sediment microbial community composition between marshes could be explained by the presence of an entirely different, relic ecosystem in the restored marsh at this depth. After the creation of a breach, the conditions created by the agricultural field onto which tidally delivered sediment was deposited could have selected for particular microbial taxa, and, in turn, this could also have affected the plant species that colonized the developing marsh because of the biotic interactions between microorganisms and plants in saltmarshes. A symbiotic relationship was suggested by the discovery that rhizosphere microbial community composition varied between the growth form and genotype of *Spartina alterniflora* present within sampling locations (Zogg *et al.*, 2018). Furthermore, evidence that saltmarsh plants recruit specific microbial taxa to promote salt tolerance was provided by a study that found differences between the microbial communities colonising the inside versus the outside of roots and leaves (Majeed *et al.*, 2024). In general, plants in saltmarshes determine the autochthonous OM input and the trapping of allochthonous OM, thus impacting the predominate sources of OM to the sediment (Duarte *et al.*, 2005; Temmerman *et al.*, 2005). However, in restored sites rapid rates of sedimentation in the first year – when plants are absent – can be caused by a combination of low elevation in the tidal frame and flow velocity rates low enough to allow sediment to settle, meaning that sediment and allochthonous OM deposition are not necessarily caused by dense vegetation (Mossman *et al.*, 2022). If the agricultural layer selected for particular microbial species, and the feedback between microbes and plants meant a continuing impact once the initial stage of marsh development had passed, these factors could ultimately be drivers of the higher proportion of marine OM entering the restored sites, indicated by the consistently lower OC/N ratios found in Chapter Two, even 100 years after breach creation. On one hand, this is an important conclusion because it was demonstrated that allochthonous OM was the main component of the long-term OC store in NW-European saltmarshes (Van de Broek *et al.*, 2018). However, the contribution of allochthonous OM to saltmarshes is highly variable between sites (Williamson *et al.*, 2025). Therefore, even if restored saltmarshes have an allochthonous OM input at the high end of the scale, this should not be taken to suggest that these ecosystems are highly effective natural climate solutions.

In Chapter Two, Brancaster West and Fingringhoe Wick samples from lower depths in the cores had relatively high OC values. By looking at the foraminifera, OC/N and $\delta^{13}\text{C}$ data in

combination, these peaks were attributed to the agricultural root mat previously identified in restored UK saltmarshes (Adams *et al.*, 2012). Using the foraminifera method to identify the breach horizon meant including these OC peaks in the stock calculations for these sites and thus lead to relatively high estimates. The high enzyme activity in the restored marsh deep sediment shown in Chapter Four, could be a result of the agricultural root mat, although the deep sediment category was from 40-50 cm, below the OC peaks. Furthermore, the enhanced microbial activity indicated by the presence of these extracellular enzymes could be indicative of the process by which this layer is broken down and lost. If this is the case, it could explain the lower OC stock estimate for Aldboro Point, where the agricultural root mat has broken down and been lost during the 100 years following the breach.

Alternative interpretations, however, are possible. Since Aldboro Point was used for agriculture for a relatively short time between embankment and the storm created breach, there may not have been enough time for a substantial root mat layer to build up in the first place. Another possible explanation arises from the methodological assessment of the enzyme assay technique presented in Chapter Three. After concluding that enzyme activity should be maximised to capture clear and consistent differences between samples, in Chapter Four, β -glucosidase, cellobiosidase, chitinase and phosphatase assays were run at pH 5.8 while leucine-aminopeptidase samples were run at pH 7.4. Therefore, it is possible that the high activities of β -glucosidase, chitinase and phosphatase were not actually occurring in the deep sediment of the restored marsh, but that a higher quantity of these enzymes were present in the sediment matrix and activity was prompted by the lower pH and/or the substrate saturation that is a requirement of all enzyme assay, regardless of whether the goal is to replicate environmental conditions. Again, a higher quantity of these enzymes could be a relic of the agricultural field present prior to restoration. Given that no clear conclusion about the best storage method was drawn in Chapter Three, samples collected for Chapter Four were frozen at -20 °C prior to analysis because fieldwork occurred over the course of a full year. Altogether, this suggests yet another impact of the agricultural layer upon the development of restored saltmarshes, in addition to those previously identified (e.g., Mossman *et al.*, 2012b; Spencer *et al.*, 2017; Lawrence *et al.*, 2018; see sections 1.1, 2.2 and 4.2).

Information ascertained from the IRIS stick analysis was used in Chapters Four and Five. More strongly reducing conditions in the restored marsh were associated with different microbial communities compared with the natural marsh in Chapter Four. In Chapter Five, the same low oxygen conditions in the top 10 cm of restored marsh sediment resulted in tea litter colonised by microbial communities different to those found within the material incubated in the less reducing natural marsh. In Chapter Four, sulphate-reducing genera were found to dominate, as expected of saltmarshes generally (Bahr *et al.*, 2005; Mavrodi *et al.*, 2018) and as was shown to be especially true of at least one other restored saltmarsh in the USA (Campbell *et al.*, 2025). Sulphate reducing genera in Chapter Five were present in the untreated controls (i.e., bulk sediment samples) but were often entirely absent from the teabags and from the high elevation, natural marsh untreated controls. Anaerobic

microbial taxa were more abundant in the restored marsh teabags while the natural marsh teabags had a higher proportion of strictly aerobic taxa. Together, these results elucidate the microbial community composition differences between natural and restored sites and support the expectation that more strongly reducing conditions will result in anaerobic taxa predominating. The abiotic conditions in restored sites have an impact upon microbial communities, the main driver of OC remineralisation, but TBI derived decomposition and stabilisation rates indicated that these differences do not have a functional impact. This finding agrees with a very similar study that compared OM decomposition and microbial communities between marshes restored more than ten years ago and remnant natural marshes in Australia (Gopakumar *et al.*, 2026). Despite differences in microbial communities, they reported that teabag litter decomposition rates did not differ between marshes.

One recent study considered whether increased understanding of saltmarshes functioning could mean improving the implementation of these natural climate solutions to enhance their contribution to climate change mitigation (McMahon *et al.*, 2023). They found that the elevation of restored saltmarshes in the tidal frame can have a large influence upon OC stock, with a two-fold increase detected above a benchmark elevation relative to mean high tide level (McMahon *et al.*, 2023). In this case, the authors concluded that the success of a restoration scheme – if the goal is burying substantial quantities of OC – can be improved either by selecting a site already situated at a suitable elevation or by artificially enhancing elevation through sediment dredging. However, based upon the findings of my study, selecting and/or creating sites with a higher elevation is unlikely to alter the potential impacts of the underlying agricultural layer upon the development of restored sites nor upon the origin of the OM that is predominantly stored within their sediments. Other actions suggested for enhancing blue carbon accumulation include reducing nutrient pollution, reintroduction of bioturbators and restoration of hydrology (Macreadie *et al.*, 2017). Determining whether these options could have an impact would require further study.

6.3 Future steps and wider implications of the study

6.3.1 Knowledge gaps and future work

This study has begun to build an understanding of OC dynamics in restored UK saltmarshes and, as a result, many areas of potential future enquiry have been uncovered. First, the elemental, isotopic and thermogravimetric analyses indicated a higher contribution of allochthonous OM to the restored saltmarsh OC stocks, but this could be quantified more precisely to improve understanding of the additionality of restored marsh OC. This could be achieved with a mixing model and/or biomarkers. A recent study that employed the latter in the form of microbial lipids, was able to trace the origin of OC in the sediment of two natural Irish saltmarshes and concluded that the primary source was marine (Akhmetkaliyeva *et al.*, 2025). This method is valuable because it allows a distinction to be

made between plant-derived and sediment-derived OM, and the proportion of the latter that originates from a marine, as opposed to a terrestrial, setting determined.

By synthesising the results of several chapters, I developed a hypothesis about the impact that an underlying agricultural soil layer can have upon saltmarsh development in terms of microbial and plant community colonisation and OC sources and accumulation. This could be tested and tracked over time by following saltmarsh restoration in a site from day zero. To corroborate the IRIS stick data gathered in this study, monitoring wells could be installed to allow groundwater samples to be taken (e.g., Van Putte *et al.*, 2020 & 2025). As an alternative to tracking the development of an entire site, mesocosm experiments could be employed to test these impacts. Such an experiment might involve installing a marsh organ (i.e., a device that simulates different levels of inundation frequency; e.g., Payne *et al.*, 2021; Arnaud *et al.*, 2024) containing a variety of sediment/soil types and/or sediment inoculated with specific microbial taxa. The marsh organ could be left in a saltmarsh over the course of a full growing season and the changes in the microbial communities and the colonising plants recorded, and the origin of the OM in the sediment determined.

While this study focused only upon OC stocks and accumulation, other crucial aspects of saltmarsh functioning to consider are methane (CH₄) and nitrous oxide (N₂O) emissions, given that these have the potential to detract from the net climate benefit of a restoration scheme (Williamson and Gattuso, 2022). The mesocosm experiments already discussed could incorporate GHG flux measurements using static chambers to capture a more complete picture of restored saltmarsh development.

In this study, the microbial communities in samples of saltmarsh sediment from different marshes, depths and seasons were identified but the presence of these microbes alone does not necessarily indicate that they are active within the system. Stable isotope probing (SIP) would enable the microbial groups actively responsible for OM decomposition to be identified. The SIP technique involves isolating the DNA of microorganisms grown on substrates enriched with ¹³C and then separating this DNA from that containing only the common ¹²C isotope using density-gradient centrifugation; the taxonomic and functional identity of this DNA can then be identified (Radajewski *et al.*, 2000). Application of the DNA-SIP technique to saltmarshes is limited to just one study, conducted using samples from the west coast of the USA and focusing upon sediment carbon utilisation (Darjany *et al.*, 2014). Clearly, therefore, it is necessary to apply this technique to samples from a range of geographical locations and to consider the fact that different carbon sources (e.g., glucose and cellulose) may be utilised by entirely different microbial groups. Such an experiment could involve microcosm laboratory experiments using sediment from restored and natural saltmarsh and ¹³C labelled plant material as a tracer. Specifically, plants common to UK saltmarshes could be grown in an atmosphere enriched with ¹³C-CO₂, and, after an incubation period, samples from these plants and/or the sediment taken, and the microbial DNA extracted. Plant species of particular interest

could include those found to predominate in restored saltmarshes, such as *Atriplex portulacoides* (Mossman *et al.*, 2012b).

The FAPROTAX analysis used in this study provides a prediction of function by comparing the 16S rRNA gene sequencing data with information about microbial functioning reported in the literature. Therefore, these results should be interpreted cautiously. A step to overcome this limitation would be using metagenomics, a method that involves sequencing all the DNA present in a sample as opposed to only the 16S rRNA gene. Doing so would provide more well-supported interpretations of microbial community functioning. After this, the rates of specific processes and the abundance of the RNA transcripts of the genes responsible for these functions, could be measured. For example, the reduction of sulphate to sulphide and methanogenesis could be determined in restored and natural marshes before making comparisons between the factors.

A large determinant of OM decomposition rate is the lability of the OM in question (Zhang *et al.*, 2008), providing a possible explanation for the similar rates of decomposition recorded in both the restored and natural marshes using the TBI method. Instead of tea, native marsh litter could be used to measure OM decomposition. For example, in Fingringhoe Wick, native litter bags would differ between natural and restored marshes because different plants dominated in each, thus giving a clearer indication of the rates of OM decomposition in both sites. A further step could be employing metagenomics to determine the decomposition processes taking place within these native litter bags. Also related is the horizontal export of OC from decomposing OM. While vertical export (i.e., CO₂ and CH₄ emission) can be measured with static chambers, horizontal export could be investigated by taking water samples from saltmarsh creeks. The latter is particularly relevant because lower creek density in restored marshes could impact the export of OC from these sites and into the open ocean.

6.3.2 Contextualisation

Blue carbon ecosystems are vaunted as natural climate solutions. However, more recently, these strategies have been labelled distractions from the systematic changes required if the net zero target is to be reached (Melanidis and Hagerman, 2022). In response to this criticism, one recent review of the efficacy of natural climate solutions concluded that a conservative estimate of their climate change mitigation potential is 11.5 Gt of CO₂ equivalents a year up to 2050, which amounts to approximately a 0.3°C reduction in global temperatures (Seddon, 2022). Therefore, natural climate solutions alone are not enough to sufficiently limit the mean global temperature rise. However, if appropriately implemented, this finding suggests that they do have a role to play, both in terms of supplementing wider climate change mitigation efforts through functioning as sinks of atmospheric CO₂ but also because of the other benefits they provide in addition to this function.

Under the umbrella of natural climate solutions, the restored saltmarshes investigated by this study can be used as important case studies. The conclusion that a higher proportion of allochthonous OM and OM that has potentially already undergone the processes of decay and stabilisation in an

external setting, enters these sites has serious implications. This OM could have been buried in a different ecosystem making the restoration irrelevant in terms of removing additional CO₂ from the atmosphere. Furthermore, OM that has already undergone decomposition, releasing quantities of CO₂ back into the atmosphere during the process, does not need to be protected within the anoxic sediment of a restored saltmarsh. It might be argued that carbon crediting is a means to an end to ensure the protection and restoration of these ecosystems but doing so will not contribute to climate change mitigation if the OC accumulation quantified does not truly offset emissions and/or represent a genuine contribution to the carbon budget of a nation.

Another important facet of this study is the focus upon the microbial communities of saltmarshes. This gives an indication of the processes taking place in the sediment and helps to highlight that the OM entering these ecosystems is not simply locked away but is still vulnerable to remineralisation. Even under the strongly reducing conditions of the restored marsh, anaerobic microbial taxa and fermentative processes produced tea litter decomposition and stabilisation rates that did not differ from those produced by the aerobic taxa of the more well oxygenated natural marsh. On the other hand, the domination of sulphate reducing genera in the bulk sediment of the restored marsh could be taken as a positive indicator that suggests that sulphate reducing bacteria are outcompeting methanogens and thus limiting the CH₄ emissions that can detract further from OC accumulation rates. There is a myriad of additional experiments that could be conducted to further elucidate these processes and more closely constrain the differences between natural and restored sites (see section 6.3.2), and this study helps to point the way towards the most promising avenues of enquiry.

Saltmarshes are highly dynamic ecosystems and even within the same marsh any number of competing and overlapping processes can take place that ultimately impact the OM in the sediment. These processes take place in the surface but also at lower depths, highlighting that vertical, as well as horizontal, variation exists. Although temporal impacts were not usually deemed a significant factor in this study, investigation in different marshes or even in the same marshes using some of the more detailed methods discussed in section 6.3.2 could contradict this finding. To conclude, all these elements highlight that using blue carbon ecosystems as tools to tackle climate change is immensely challenging to achieve with any degree of accuracy, let alone precision.

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