

Ecosystem type drives tea litter decomposition and associated prokaryotic microbiome communities in freshwater and coastal wetlands at a continental scale



Stacey M. Trevathan-Tackett^{a,*}, Sebastian Kepfer-Rojas^b, Aschwin H. Engelen^c, Paul H. York^d, Anne Ola^e, Jinquan Li^{f,g}, Jeffrey J. Kelleway^h, Kristin I. Jinksⁱ, Emma L. Jackson^j, Maria Fernanda Adame^k, Elise Pendall^f, Catherine E. Lovelock^e, Rod M. Connollyⁱ, Anne Watson^l, Inger Visby^m, Allison Trethowanⁿ, Ben Taylor^o, Tessa N.B. Roberts^o, Jane Petch^p, Lachlan Farrington^o, Ika Djukic^q, Peter I. Macreadie^a

^a Deakin University, Centre for Integrative Ecology, School of Life and Environmental Sciences, 221 Burwood Hwy, Burwood, VIC 3125, Australia

^b Department of Geosciences and Natural Resource Management, University of Copenhagen, Rolighedsvej 23, 1958 Frederiksberg, Denmark

^c Centre for Marine Sciences (CCMAR), University of Algarve, Campus de Gambelas, 8005-139 Faro, Portugal

^d James Cook University, Centre for Tropical Water and Aquatic Ecosystem Research (TropWATER), Cairns, Queensland 4870, Australia

^e The University of Queensland, School of Biological Sciences, St. Lucia, Queensland 4072, Australia

^f Hawkesbury Institute for the Environment, Western Sydney University, Penrith, NSW 2751, Australia

^g National Observations and Research Station for Wetland Ecosystems of the Yangtze Estuary, Ministry of Education Key Laboratory for Biodiversity Science and Ecological Engineering, School of Life Sciences, Fudan University, Shanghai 200438, China

^h School of Earth, Atmospheric and Life Sciences, GeoQuEST Research Centre, University of Wollongong, Wollongong, NSW 2522, Australia

ⁱ Coastal and Marine Research Centre, Australian Rivers Institute, School of Environment and Science, Griffith University, Gold Coast, Queensland 4222, Australia

^j Coastal Marine Ecosystems Research Centre, CQUniversity, Gladstone, QLD 4680, Australia

^k Australian Rivers Institute, Griffith University, Nathan, QLD 4111, Australia

^l School of Natural Sciences, University of Tasmania, Sandy Bay, TAS 7005, Australia

^m Derwent Estuary Program, 24 Davey St Hobart, TAS 7001, Australia

ⁿ RiverConnect - Greater Shepparton City Council, Shepparton, VIC 3630, Australia

^o Nature Glenelg Trust, PO Box 2177, Mt Gambier, SA 5290, Australia

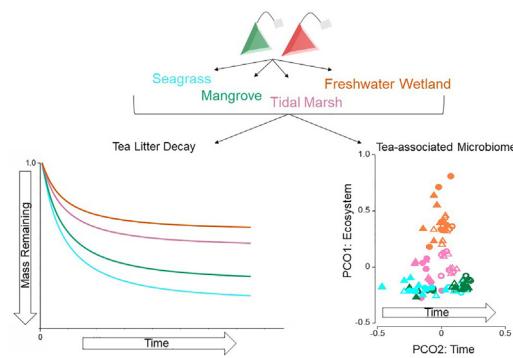
^p Melbourne Water, South East Regional Office, Worsley Road, Bangholme, VIC 3175, Australia

^q Swiss Federal Institute for Forest, Snow and Landscape Research WSL, Birmensdorf, Switzerland

HIGHLIGHTS

- We studied tea litter decay and microbiomes across four wetland types in Australia
- Ecosystem type had greatest effect on tea decay and prokaryotic community structure
- Decay rates in freshwater and tidal marsh wetlands were higher in warmer climates
- Microbiome community succession related to organic matter and oxygen availability
- Salinity and inundation conditions may have controlled belowground decay processes

GRAPHICAL ABSTRACT



* Corresponding author.

E-mail address: s.trevathantackett@deakin.edu.au (S.M. Trevathan-Tackett).

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ABSTRACT

Wetland ecosystems are critical to the regulation of the global carbon cycle, and there is a high demand for data to improve carbon sequestration and emission models and predictions. Decomposition of plant litter is an important component of ecosystem carbon cycling, yet a lack of knowledge on decay rates in wetlands is an impediment to predicting carbon preservation. Here, we aim to fill this knowledge gap by quantifying the decomposition of standardised green and rooibos tea litter over one year within freshwater and coastal wetland soils across four climates in Australia. We also captured changes in the prokaryotic members of the tea-associated microbiome during this process. Ecosystem type drove differences in tea decay rates and prokaryotic microbiome community composition. Decomposition rates were up to 2-fold higher in mangrove and seagrass soils compared to freshwater wetlands and tidal marshes, in part due to greater leaching-related mass loss. For tidal marshes and freshwater wetlands, the warmer climates had 7–16% less mass remaining compared to temperate climates after a year of decomposition. The prokaryotic microbiome community composition was significantly different between substrate types and sampling times within and across ecosystem types. Microbial indicator analyses suggested putative metabolic pathways common across ecosystems were used to breakdown the tea litter, including increased presence of putative methylotrophs and sulphur oxidisers linked to the introduction of oxygen by root in-growth over the incubation period. Structural equation modelling analyses further highlighted the importance of incubation time on tea decomposition and prokaryotic microbiome community succession, particularly for rooibos tea that experienced a greater proportion of mass loss between three and twelve months compared to green tea. These results provide insights into ecosystem-level attributes that affect both the abiotic and biotic controls of belowground wetland carbon turnover at a continental scale, while also highlighting new decay dynamics for tea litter decomposing under longer incubations.

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1. Introduction

Decomposition, or the breakdown of plant detritus, is a fundamental ecosystem process. It is essential for nutrient regeneration and the transformation of living biomass into soil organic matter, and therefore critical to rate and magnitude of carbon accumulation and mineralisation (Berg and McClaugherty, 2008; Wieder et al., 2013). In terrestrial ecosystems, decomposition metrics, such as litter mass, carbon and nitrogen loss, and microbial biomass and activity, are used to inform biogeochemical cycling in broad-scale earth system models (Bonan et al., 2013; Wieder et al., 2013; Bradford et al., 2017). As wetlands are critical to regulating atmospheric carbon concentrations, there is a need for analogous estimates of wetland carbon inputs and losses across a range of spatial scales to inform carbon cycling and climate models (Mitsch et al., 2013), and ecosystem management actions (Moomaw et al., 2018; Kelleway et al., 2020). Yet for wetland ecosystems, particularly for coastal vegetated ecosystems, there is a gap or a disparity in the types of observational decomposition data collected (Baird et al., 2020; Trevathan-Tackett et al., 2020b).

The environmental conditions unique to freshwater and tidal wetland ecosystems, such as hydroperiod, inundation, and allochthonous soil accumulation, shape the biogeochemistry and microbial ecology of the soils (Macreadie et al., 2017; Yarwood, 2018; Cragg et al., 2020). In the belowground compartment, water-logged soils are an important wetland attribute that enhances sequestration and preservation potential through reduced carbon remineralisation rates via anaerobic metabolic pathways (e.g. sulphate reduction, methanogenesis and fermentation performed by prokaryotic soil microbiota; Yarwood, 2018, Cragg et al., 2020). In coastal soils, sulphate reducers dominate organic matter turnover; however, through greenhouse gas flux measurements, methane cyclers have recently been shown to contribute to carbon emissions in tidally-influenced ecosystems (Al-Haj and Fulweiler, 2020). In contrast, methanogenesis is typically the prevalent carbon mineralisation pathway in freshwater wetland soils. Yet, methanogens co-exists with a smaller consortia of sulphate reducers that may divert carbon flow from methane to carbon dioxide (Pester et al., 2010). Additionally, salinity category, i.e. saline versus non-saline, is the strongest selector for prokaryotic microbiome structure on a global scale (Lozupone and Knight, 2007). The influence of salinity on community structure may be due to osmotic or ionic tolerance of marine taxa, as well as sulphate availability (Lozupone and Knight, 2007) that would

influence the predominant metabolic pathways under anoxic conditions.

In addition to the belowground physicochemical conditions, belowground biomass (roots, rhizomes) plays a key role in the accumulation of organic carbon stocks and peat formation. Belowground biomass directly contributes to the carbon stocks and soil stability (Kirwan and Megonigal, 2013; Lamont et al., 2020), with enhanced soil carbon accumulation through slow decomposition rates and the generally more recalcitrant organic matter quality of the detritus than other biomass types (Chimner and Ewel, 2005; Ouyang et al., 2017; Trevathan-Tackett et al., 2017). Further, compared to their aboveground counterparts, belowground biomass is subject to lower chances of herbivory, export and aerobic decay, all of which can enhance the amount of carbon available for sequestration *in situ* (Cebrian, 2002). Despite the importance of belowground biomass to carbon cycling dynamics, the variables affecting its decomposition in wetlands generally receives less attention than leaf litter breakdown, particularly in coastal wetlands (Ouyang et al., 2017; Trevathan-Tackett et al., 2020b). Consequently, there is a fundamental need to resolve the mechanisms underlying belowground decomposition processes, including the interaction between detrital organic matter, the microbial community involved in the decomposition process and the environmental conditions across wetland ecosystem types that are conducive or limiting to litter breakdown (Yarwood, 2018; Spivak et al., 2019).

Cross-ecosystem studies using standardised tea litter proxies have progressed our understanding of belowground organic matter cycling dynamics at regional-to-global scales. Tea litter decomposition has shown to be sensitive to ecosystem type and climate at a global scale, whereby warmer and more humid climates enhance decomposition in the first three months of incubation compared to cooler or drier climates (Keuskamp et al., 2013; Djukic et al., 2018). However, in some cases, the temperature-enhancing effects on organic matter breakdown may only be observed when also accounting for local- or microsite-based factors like inundation (Mueller et al., 2018) or vegetation (Petraglia et al., 2019) or when the data are aggregated at a biome or regional level (Djukic et al., 2018; Mueller et al., 2018). In addition to temperature effects on tea litter decay, a cross-ecosystem study of tidally-influenced freshwater and tidal marshes and mangrove ecosystems showed that lower elevations, and thereby more frequently flooded zones, decreased the tea organic matter stability (Mueller et al., 2018). This reduced stability could have potential consequences for soil

formation under sea level rise scenarios (Mueller et al., 2018). Furthermore, resolving the drivers of belowground decomposition using cross-ecosystem experiments may complement large-scale datasets that measure and model the drivers of belowground carbon stocks and sequestration rates, in order to identify opportunities for conservation, restoration and climate change mitigation efforts (for example, Nahlik and Fennessy, 2016; Serrano et al., 2019; Ewers Lewis et al., 2020).

The aim of this study is to assess belowground wetland decomposition using a cross-ecosystem design at a continental scale. Using standardised tea litter, we quantify the breakdown of organic matter in seagrass meadows, mangrove forests, tidal marshes and freshwater wetlands from 37 sites and four climate biomes across Australia. As with previous large-scale tea litter studies, we expect to show strong ecosystems differences in decomposition dynamics among the wetland types. Where possible, we tested the effects of climate biome within ecosystem type. We also described the tea-associated microbiota for each ecosystem type across a subset of these wetlands. We expect to find key differences in the prokaryote microbiome community structure and putative functional pathways between the saline and non-saline ecosystems. Our multidisciplinary approach combining ecological and microbiological techniques in a designed *in situ* experiment using standardised litter assays will advance our understanding of the belowground decomposition process in wetland ecosystems and contribute to improvements of integrative biogeochemical conceptual models.

2. Materials and methods

2.1. Tea litter, study area, deployment and sampling

We used nylon mesh tea litterbags with green tea and rooibos tea (Keuskamp et al., 2013). The teas represented two types of chemical recalcitrance: green tea as a relatively labile form of organic matter (OM) with higher water soluble content, and rooibos tea as a more recalcitrant or stable form of OM because of its higher lignin (acid insoluble) content (Keuskamp et al., 2013). These 'alien' litters are biochemically representative of local litter chemistries (Didion et al., 2016; Duddigan et al., 2020; Trevathan-Tackett et al., 2020a), and meant to be used at litter proxies and reference to understand decomposition and organic matter cycling (Keuskamp et al., 2013). Generally, the litter bag technique tends to exclude larger grazers from entering the mesh bag at the cost of retaining smaller particulate litter with the finer mesh sizes. Because of this, litter bag methods that use a small mesh size need to keep in mind that decomposition may be underestimated if faunal grazers are common to the study area (Bradford et al., 2002).

Tea litter bags were deployed as part of the global *TeaComposition H₂O* initiative investigating decomposition in wetland and marine ecosystems, and is an extension and co-initiative to the terrestrial-focussed *TeaComposition* (Djukic et al., 2018). Tea litter bags from the same batch as *TeaComposition* were deployed using a parallel methodology, with slight modification (Djukic et al., 2018). Tea litter bags were deployed in the summer between November 2017 and February 2018 to a total of 37 study sites encompassing four wetland ecosystems (seagrass meadow, mangrove forest, tidal marsh and freshwater wetlands), spanning 25–27° of latitude and four climates in eastern Australian (Walter and Breckle, 1999) (Fig. 1). The seagrass meadows were comprised of genera that have small to medium morphologies (i.e. *Zostera*, *Halodule*, *Cymodocea* and *Syringodium*). Most of the seagrass sites were subtidal, except *Zostera muelleri* in Gladstone (Table S1). Most of the mangrove sites were *Avicennia marina* forests, with some sites comprising *Rhizophora* spp. (sites 320, 327, 334, see Table S1). Both tidal marsh and freshwater marshes included a range of vegetation types from succulent-dominant (3, 364–369) to tree-dominant (355, 356, 358–360, 372, 375). Other sites were grass- or rushes-dominant (335, 374, 382, 385, 389, 390), while several sites were comprised of a mix of trees, grasses and rushes (376, 377, 401).

Initial mass was calculated by weighing the tea in the bag then subtracting the mean bag mass of 0.2000 g (averaged over 40 empty bags; 4-decimal balance). Within each site, litter bags were buried at 10–15 cm soil depth, ideally below the rhizosphere, and across two plots at least 1 m apart. This not only simulates the decomposition of belowground plant detritus, but protects the litter bags from excessive plant in-growth and disturbances at the soil surface. Within each plot there were two replicates for each tea type ($n = 4$ for each tea at each sampling time and site) (Djukic et al., 2018). For sites that assessed both mass loss (decay) and microbiome sampling, an extra set of tea bags was deployed for each site ($n = 4$ for each tea type), whereby the tea bags earmarked for decay and microbiome sampling were alternated in order to capture the potential heterogeneity within the plot.

Tea bags for decay analyses were collected at 3, 6 and 12 months for all sites. The tea- and soil-associated prokaryotic microbiome communities were analysed for eight of these sites (Fig. 1). Decay samples were cleaned of soil and dried at 60–70 °C until constant weight. Contaminating root biomass (i.e. root in-growth) was removed before weighing the final tea mass without the bag. For the microbiome samples, the tea litter bags were opened on-site and approximately 1 ml volume of tea litter was transferred to a 2 ml vial containing Zymo DNA/RNA Shield. Using a cut-off 3 ml syringe core, approximately 1 cm³ of bulk soils were sampled adjacent to and at the depth where the tea bags were incubating for comparative microbiome analyses. Microbiome samples were stored frozen until analysis.

2.2. DNA extraction, sequencing and bioinformatics

A subset of the microbiome samples from eight sites was selected for 16S rRNA gene amplicon analysis, i.e. the prokaryotic members of the tea- and soil-associated microbiome community. We targeted the 3- and 12-month sampling to capture the early and later stages of decay (Trevathan-Tackett et al., 2020b) from each ecosystem type representing two of the climates, temperate and warm temperate (Fig. 1). Bead-beating of the tea litter and soil samples (0.2–0.4 g fresh weight sample; 60 s and 5.5 m s⁻¹; FastPrep-24) preceded DNA extraction with the Zymo Mini Kit as per manufacturer instructions for soil (ZymoResearch). DNA was quantified using Qubit Broad Range before normalising to 5 ng µl⁻¹. Normalised gDNA template and Zymo Microbial DNA Standard were amplified using the V4 variable region of the 16S rRNA gene with the modified Eubacterial primers 515F (5'- GTGC CAGCMGCCGCGG- 3') and 806R (5'- GGAATCAGVGGTWTCTAAT- 3') (Walters et al., 2016). Triplicate PCR cycles were performed before pooling and cleaning with Zymo Clean and Concentrate Kit with modified elution using 10 mM Tris buffer (94 °C at 3 min, 25 cycles of 94 °C at 30 s – 50 °C at 30 s – 72 °C at 30 s, then 72 °C at 5 min). PCR products were indexed and sequenced on an Illumina MiSeq by Deakin University's Genomic Centre. All amplicon data are available at the European Nucleotide Archive (ENA) under projects PRJEB44018 and PRJEB44019.

Amplicon sequences were analysed in the QIIME2 DADA2 pipeline (Callahan et al., 2016; Bolyen et al., 2018). The resulting amplicon sequence variants (ASVs) were normalised to 54,200 reads per sample before alpha diversity (observed ASVs, Pielou's Eveness) and beta diversity (weighted UniFrac) metrics were calculated. This resulted in the removal of three samples: sediment sampled at 3 months in a temperate tidal marsh and two sediments sampled at a tropical mangrove at 3 and 12 months. Sequences were classified using the Silva v132 database at a threshold of 99% homology (Quast et al., 2012).

2.3. Data processing and statistical analyses

2.3.1. Decomposition analyses

Initial and final tea mass was used to calculate the proportion mass remaining for each of the time points. These data were used to assess the effect of time, substrate, ecosystem and climate on decomposition

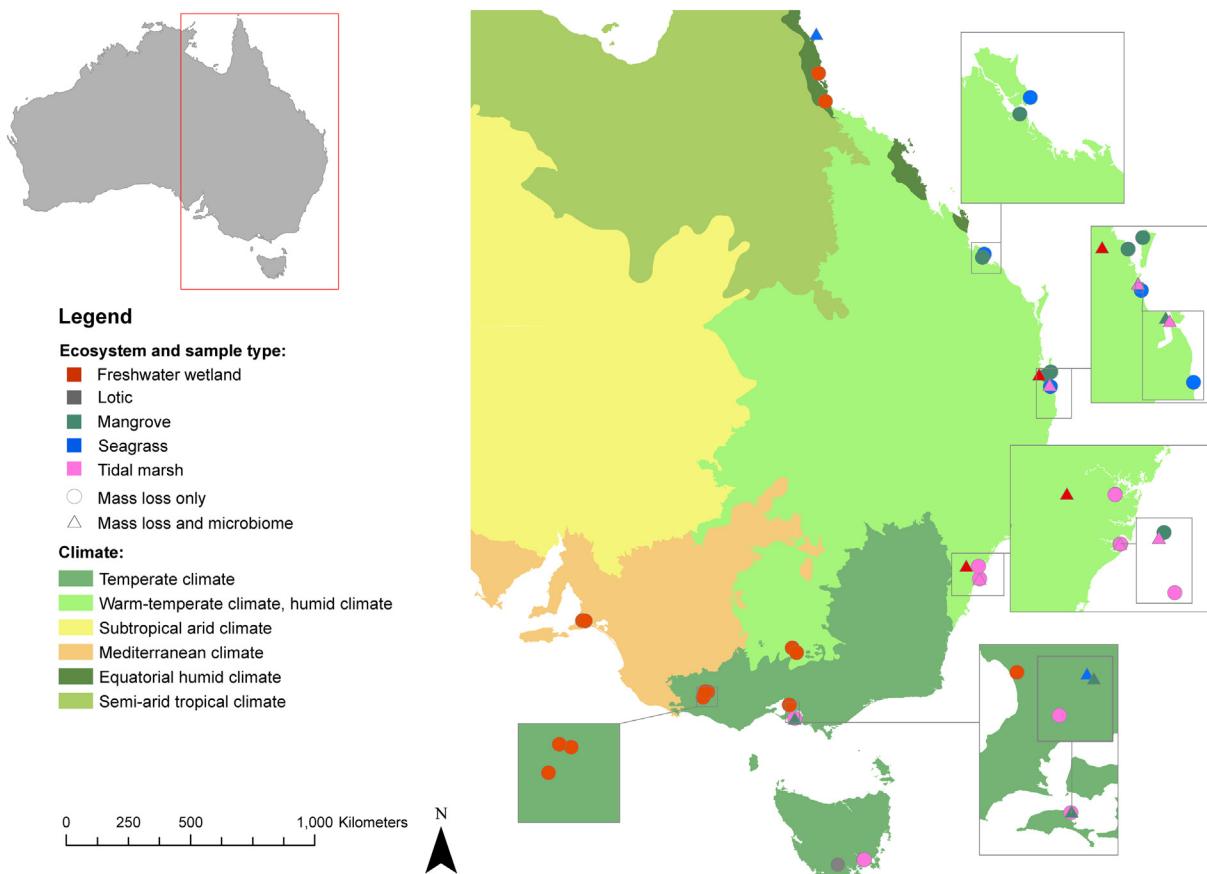


Fig. 1. Tea litter decomposition site map.

in two ways: mixed linear models (MLM) and exponential decay models. We used mixed effect models to assess the effects of tea type, ecosystem type and incubation time on the proportion mass remaining using the lmer package (Bates et al., 2015) in R version 3.6.2 (R Core Team, 2019). To test if the temporal trends in remaining mass were different among ecosystems and whether these depended on tea type, we fitted a full model including a 3-way interaction, all 2-way interactions and single terms as fixed factors. To account for the repeated sampling and for the spatial structure of the data, we included plot as a random factor nested within site. The model was reduced by removing non-significant terms, using backward model selection. Selection started with the 3-way interactions, followed by 2-way and single terms. In cases where two 2-way non-significant interactions or main terms were present, the term with the highest *p*-values were removed first. Only significant factors (*p* < 0.05) were included in the final model. Interactions between factors were explored with posthoc pairwise comparisons using the emmeans package (Lenth et al., 2018). For freshwater and tidal marsh ecosystems that had climate-level replication, we also tested whether litter decomposition within an ecosystem type depended on the climatic conditions of the site by comparing sites of the same ecosystem but from different climates. We used a model including climate, incubation time and tea type and the same random structure as previously stated. Similarly, we started with a full model and reduced until only significant terms remained.

Exponential decay rates are often used to describe the decomposition process over the entire observed period of time (Wieder and Lang, 1982), an approach used to calculate decay rates of natural litter. Single exponential decay rates were calculated using $W_0/W_t = e^{-kt}$, whereby W_0 was the initial mass, W_t was the mass at time t and k is the decay rate in proportion per day (d^{-1}), with the model inclusive of 100% mass at day 0. k was calculated separately for each tea type

for each site, each ecosystem type, and each ecosystem*climate combination. Sites sampled 3–6 months after the ideal sampling time were included in the initial visualisation and site-level calculations, but these data points, or in some cases whole sites, were excluded from further the statistical analyses in order to remove time-related biases on the mass loss data as a result of the longer incubation (see Table S1 for incubation time in days).

2.3.2. Microbiome analyses

Prokaryotic microbiome alpha diversity was analysed using the MLM approach described above to assess differences in substrate type (green tea, rooibos tea, soil). To assess alpha diversity of the prokaryotic members of the tea-associated microbiome during decomposition, a three-way model was used to assess the differences across tea type, ecosystem (seagrass, mangrove, tidal marsh, and freshwater wetland) and time (3 months and 12 months). Beta diversity was analysed with a three-way multivariate PERMANOVA, with ecosystem, substrate and time as fixed factors. Limited site-level replication for each ecosystem precluded the inclusion of a random plot factor in the design. The PERMANOVA test was repeated with only the tea samples to investigate the microbial community succession as decomposition progressed. For the PERMANOVA analyses, a Monte Carlo correction (P(MC)) was applied in cases where permutations were < 200 (PRIMER + PERMANOVA v 7) (Anderson et al., 2008). Indicator analyses on the filtered ASV taxonomy table (thereby removing rare ASVs) were performed using the indicspecies package (Dufrêne and Legendre, 1997; Cáceres and Legendre, 2009) to measure the association between the ASVs for the significant beta diversity results from the PERMANOVA analyses. An alpha of 0.05 was used for all statistical tests, except the indicator analyses which focussed on the most significant ASVs (*p* < 0.001).

2.3.3. Structural equation model

We used a structural equation model (SEM) with the piecewiseSEM package (Lefcheck and Freckleton, 2016) to test for direct and indirect relationships between the factors ecosystem type, incubation time (in days) and microbial community on remaining litter mass. The data used in this analysis were the tea-associated prokaryotic microbiomes samples (8 sites from the 3- and 12-month sampling; Fig. 1, Table S1) and the associated/paired mass loss data. The microbial communities were represented by the first two axes of the principal coordinates analysis (PCO). The factor 'ecosystem type' encompasses inherent environmental differences and occur across a range of climates zone that may affect the structure of microbial communities and litter decomposition rates (Ochoa-Hueso et al., 2019). A random effect identifying each plot and site combination was included to account for spatial and temporal dependencies in the data. We fitted separate models for each tea type to examine differences in pathways between the two tea types and removed non-significant paths from our final models, and Fisher's tests of directed separation were used to evaluate model fit (Lefcheck and Freckleton, 2016). To estimate the influence of the categorical variable ecosystem type, we used χ^2 likelihood ratio tests for each individual piecewise model, and compared among ecosystem types using post-hoc means adjusted Tukey tests at $\alpha = 0.05$ with the emmeans package (Lenth et al., 2018).

3. Results and discussion

3.1. Ecosystem-driven decomposition

During the first year of decomposition, the proportion of remaining tea mass was consistently lower for green tea than for rooibos tea litter.

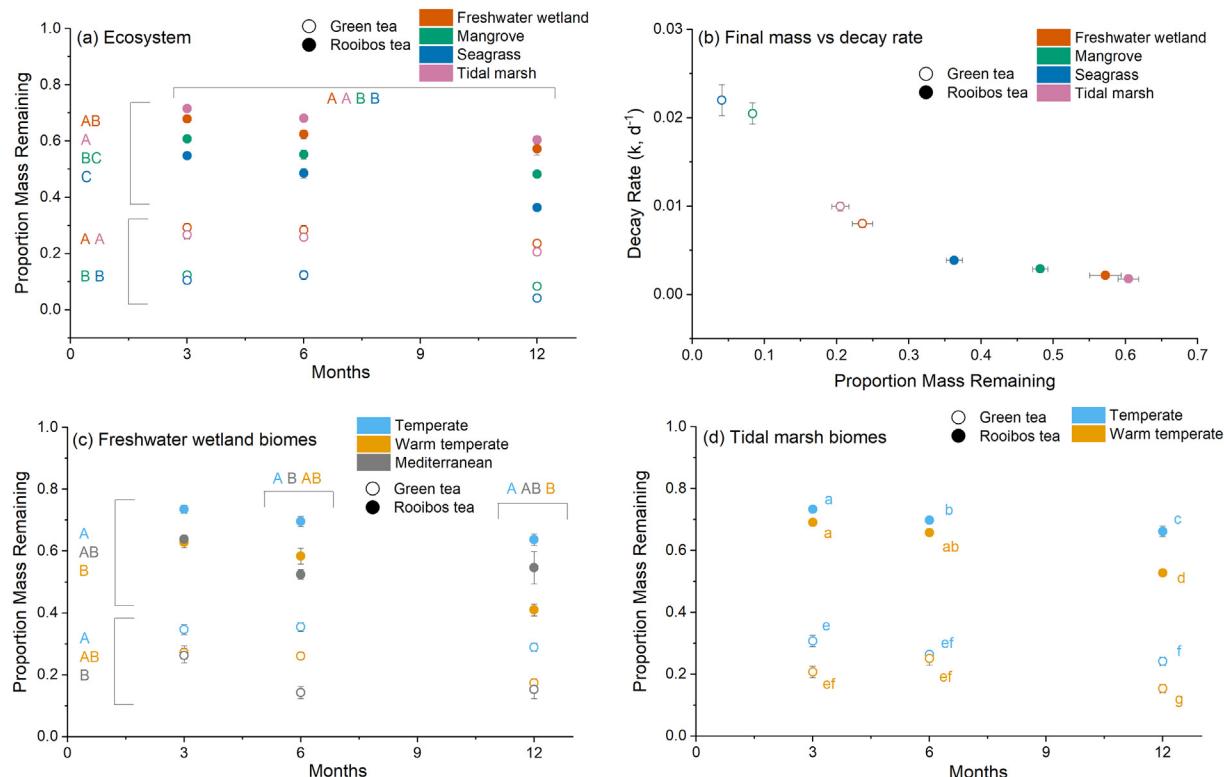


Fig. 2. Ecosystem and climate effects on tea decomposition in wetlands. Ecosystem type significantly ($p < 0.05$) affected the mass loss (a) and decay rates (b) of both the green and rooibos tea litter types. An analysis of a subset of sites that encompassed multiple climates revealed significant climatic effects on decomposition within freshwater wetlands (c; $N = 10$) and tidal marshes (d; $N = 12$). In all analyses, rooibos and green teas were significantly different from each other, so pairwise test letters indicate significant differences within each tea type. Capital letters represent pairwise differences for significant two-way interactions (tea*ecosystem/climate and time*ecosystem/climate), with horizontal and vertical brackets grouping the comparisons averaged across tea types and time, respectively; a, c). Lower-case letters represent pairwise differences for a significant three-way tea*climate*time interaction (d). Values are presented as the categorical time intervals and represent means \pm standard error for proportion mass remaining and decay rate. Raw mass remaining data are presented in Fig. S1. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

We hypothesise that the high decomposition rates in the mangrove and seagrass ecosystems are related to the greater inundation at these sites facilitating leaching-induced mass loss. In this case, instead of water-logged conditions creating an anoxic environment conducive to slower decay, porewater flushing led to high leaching, particularly for the green tea litter, and thereby an abiotic-driven reduction in mass. Similarly, water column incubations enhanced leaching compared to tea litter at the soil surface or buried (Seelen et al., 2019), while positions lower in the tidal frame have been found to have significantly higher tea litter stability compared to positions higher in the tidal frame (Mueller et al., 2018). Although, we do not have tidal or inundation data for all the sites in this study, all the seagrass sites were classified as subtidal, while mangroves in mainland Australia typically inhabit the intertidal zone from approximately mean sea level to upper intertidal elevations where they may be replaced by tidal marsh.

3.2. Climate effect within ecosystem type

Climate had a significant effect on decomposition dynamics for the subset of freshwater wetland and tidal marsh ecosystems analysed. While deployments across a broader range of sites within each climate are ideal for robust interpretation, our analyses highlight some preliminary trends. First, tea litter in the temperate climates generally decomposed more slowly than the warmer climates, shown by 7–16% higher remaining mass across both ecosystems and tea types (Fig. 2c,d, Table S3). For the freshwater wetland sites, temperate climates exhibited the least amount of decomposition, compared with the higher decomposition in the Mediterranean (green tea) and warm temperate (rooibos tea) climates after the year of decomposition *in situ* (Fig. 2c, Table S3). This resulted in 1.7- to 2-fold slower decay rate for the green tea and 1.6- to 1.8-fold slower decay rate for the rooibos in the temperate climates. For the tidal marshes, climate effects on green tea litter were significant at both 3- and 12-month samplings (Fig. 2d; $k = 0.00871$ and 0.0124 d^{-1} for temperate and warm-temperate, respectively), while the enhanced decomposition of the rooibos tea in the warm temperate climate was significant only after 12-months of burial (Fig. 2d; $k = 0.00153$ and 0.00214 d^{-1} for temperate and warm-temperate, respectively).

By accounting for climatic differences with ecosystem types over a continental scale, we showed that warmer climates may enhance below-ground litter decomposition. Enhanced tea decomposition rates due to elevated temperatures has been shown in the laboratory (Keuskamp et al., 2013; Trevathan-Tackett et al., 2020a) and in the field (Djukic et al., 2018; Mueller et al., 2018; Petraglia et al., 2019; Seelen et al., 2019). Similar to this study, (air) temperature only affected tea decay in temperate tidal marsh ecosystems within the same regional biome (Mueller et al., 2018) or globally when aggregated at the biome level (Djukic et al., 2018). Further, our use of a categorical climate approach integrates temperature and precipitation conditions that may be particularly important for freshwater wetlands that depend on the annual wet season for water. Petraglia et al. (2019) found that variable moisture conditions affected the impact of, or ability to detect, the effects of elevated temperatures on tea litter decomposition, likely due to changing oxygen availability. In contrast, under stable inundated conditions, e.g. lakes and subtidal environments, elevated temperature can enhance tea decomposition (Seelen et al., 2019; Trevathan-Tackett et al., 2020a). Further research aimed at teasing apart this potential temperature-inundation relationship would be useful for parameterising climate change effects across multiple ecosystems, particularly for ecosystems like seagrass meadows whose hydrology would be influenced by currents, tides and storms, but potentially less influenced by daily fluctuations in temperature in subtidal environments.

3.3. Ecosystem and decomposition effects on the prokaryotic microbiomes

Alpha diversity metrics, ASV richness and evenness, were 1.1–1.8-fold higher in the bulk soil compared to the two tea types ($p < 0.001$

for both; Fig. S2a,b), suggesting a degree of specialisation of the prokaryotic taxa colonising the tea litter (Pioli et al., 2020). Rooibos tea had higher richness and evenness than the green tea prokaryotic microbiome ($p < 0.001$; Fig. S2). Similarly, richness and evenness also increased from the 3- to 12-month incubation ($p < 0.001$ and $p = 0.003$, respectively; Fig. S2c,d).

3.3.1. Ecosystem effects

While we were limited in the number of sites from each ecosystem sampled for the prokaryotic microbiome community analysis, and have likely missed a level of within-ecosystem heterogeneity across sites (Behera et al., 2019; Palacios et al., 2021), we found that ecosystem type drove the differences in community composition (beta diversity; significant ecosystem*substrate and ecosystem*time interactions, Table S4). The ecosystem effect also explained 33.4% of the variation explained in the principle coordinates analysis, driven by the distinction between the freshwater wetlands and coastal wetlands and between tidal marsh and mangrove+seagrass ecosystems (PCO1; Fig. 3). Soil communities were stable over time (Table S4), whereas the tea-associated composition shifted and became more similar to the soil communities over time (PCO2; Fig. 3). In the pairwise analyses, the soil and tea litter communities differed in all ecosystems, except between the two tea litters within tidal marshes. Indicator analysis resulted in 650 highly significant ASVs ($p < 0.001$) from 132 families (Fig. S4; see detailed substrate results in Supplementary Materials). The Rhizobiales order, well-known for its nitrogen-fixing and rhizosphere members, dominated freshwater wetland microbiome communities, but were substrate-specific (Fig. S4). The prokaryotic microbiomes from tidal marsh and mangrove ecosystems consisted of putative sulphate-reducers and methane cyclers, though comprising of distinct families within each ecosystem. Syntrophobacteraceae ASVs, potentially involved in methane and sulphide production (Kuever et al., 2014), were indicative of both mangrove and seagrass soils. The tea-associated ASVs in the seagrass ecosystems consisted of a diversity of putative anaerobic functional groups from green tea, including methanogenic *Methanogenium* archaea, and rooibos tea litters.

Together these results indicate that ecosystem largely influenced the prokaryotic microbiome community. This was reflected in the overall community composition, as well as the putative metabolic pathways utilised by the indicator taxa, e.g. nitrogen-cycling and methylotrophy in freshwater wetlands and tidal marshes, and methanogenesis, sulphur cycling and fermentation in mangroves and seagrasses. These differences reflect general salinity conditions (e.g. marine taxa and sulphate

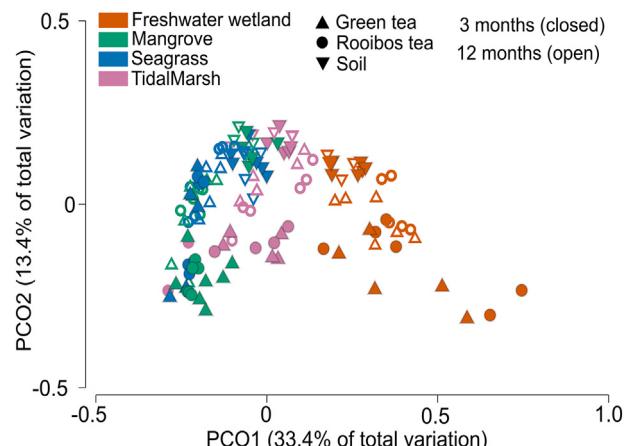


Fig. 3. Principal coordinates ordination of tea- and soil-associated prokaryotic microbiome communities. The soil-associated microbiome samples were collected at a similar depth as where the tea litter bags were decomposing. Samples from two sites were included per ecosystem, with replication of 2–3 samples for each site, substrate and time combination.

reducers), and potentially the fluctuating conditions in the tidal range allowing for different biogeochemical niches to be formed and utilised (Yin and Yan, 2020). However, within the putative functional guilds in each ecosystem, the taxa that colonised the tea litter from the soils were diverse, indicating association with the two tea types and suggesting a degree of functional redundancy in the capacity to degrade different OM compositions under predominantly anaerobic conditions (Pioli et al., 2020).

3.3.2. Shifts in the prokaryotic tea microbiome during the decomposition process

Focusing only on tea-associated samples to further investigate successional shifts in prokaryotic microbiome communities involved in tea litter decay, ecosystem type remained the key driver in the tea prokaryotic microbiomes with significant ecosystem*time and ecosystem*substrate interactions (Table S4). Indicator analysis of the ecosystem*time interaction resulted in 290 highly significant ASVs ($p \leq 0.001$; Fig. 4). Several taxonomic groups were identified as common to early or later stages of decay. For example, Anaerolineae family A4b and Spirochaetae were common early on in the freshwater wetland and tidal marsh ecosystems, while Deltaproteobacteria groups (*Desulfovibrio*, *Desulfuromonas*, *Myxococcales*) and Bacteroidetes family BD2-2 were indicative of early decomposers in the three tidal ecosystems. Tea decomposition at the later stages within the freshwater wetlands and tidal marshes were indicated by methane-oxidiser *Methyloceanibacter* and cellulolytic fermenter SBR1031 (Xia et al., 2016). Bathyarchaeota, known to consume a diversity of recalcitrant OM types (fatty acids, aromatics, carbohydrate polymers) using a variety of

anaerobic metabolic pathways (Zhou et al., 2018), were shared by all the ecosystems except mangroves. Furthermore, the tidal ecosystems were characterised by sulphur-cycling taxa at the 12-month sampling, but from different genera compared to the 3-month sampling, including *Desulfosarcina*, *Desulfatitalea*, *Desulfococcus* and *Sulfurovum*. This could suggest a potential difference in r- and k-strategist lifestyles within the Deltaproteobacteria.

Despite these similarities across multiple ecosystem types, the colonisation and successional shifts in indicator taxa were mostly distinct within each ecosystem (Fig. 4). For the freshwater wetlands, the indicator taxa shifted from aerobic Sphingomonadaceae and Bacteroidia (*Pedobacter* and *Sporocytophaga*) at three months to Xanthobacteraceae (*Bradyrhizobium* and purple sulphur bacteria *Rhodopseudomonas*) and Bacilli groups at 12 months. There were also ammonium-oxidising *Ellin6067* and *Nitrococcus* genera, as well as *Methanobacterium* and *Methyloceanibacter* ASVs in conjunction with *Syntrophobacter* in the later stages of decay in the freshwater wetlands, suggesting the use of multiple nitrogen- and methane-cycling pathways utilised one year into decomposition (Yarwood, 2018). Later decay in the tidal marsh was also represented by ammonium- and sulphur-oxidising groups *Nitrosococcaceae*, *Nitrosopumilus*, *Thiotrichaceae* and *Thioalkaliphila*. Mangrove tea communities shifted from anaerobic Bacteriodia indicator taxa to a community more indicative of sulphur cycling (*Sulfurimonas*, *Sulfurovum*, *Desulfobacteraceae*), as well as *Woesearchaeia*, recently implicated in assisting with methanogenesis in seagrass soils (Burkholz, 2018). In contrast, seagrass tea samples shifted from sulphate reducer-dominant Deltaproteobacteria to increased Gammaproteobacteria as

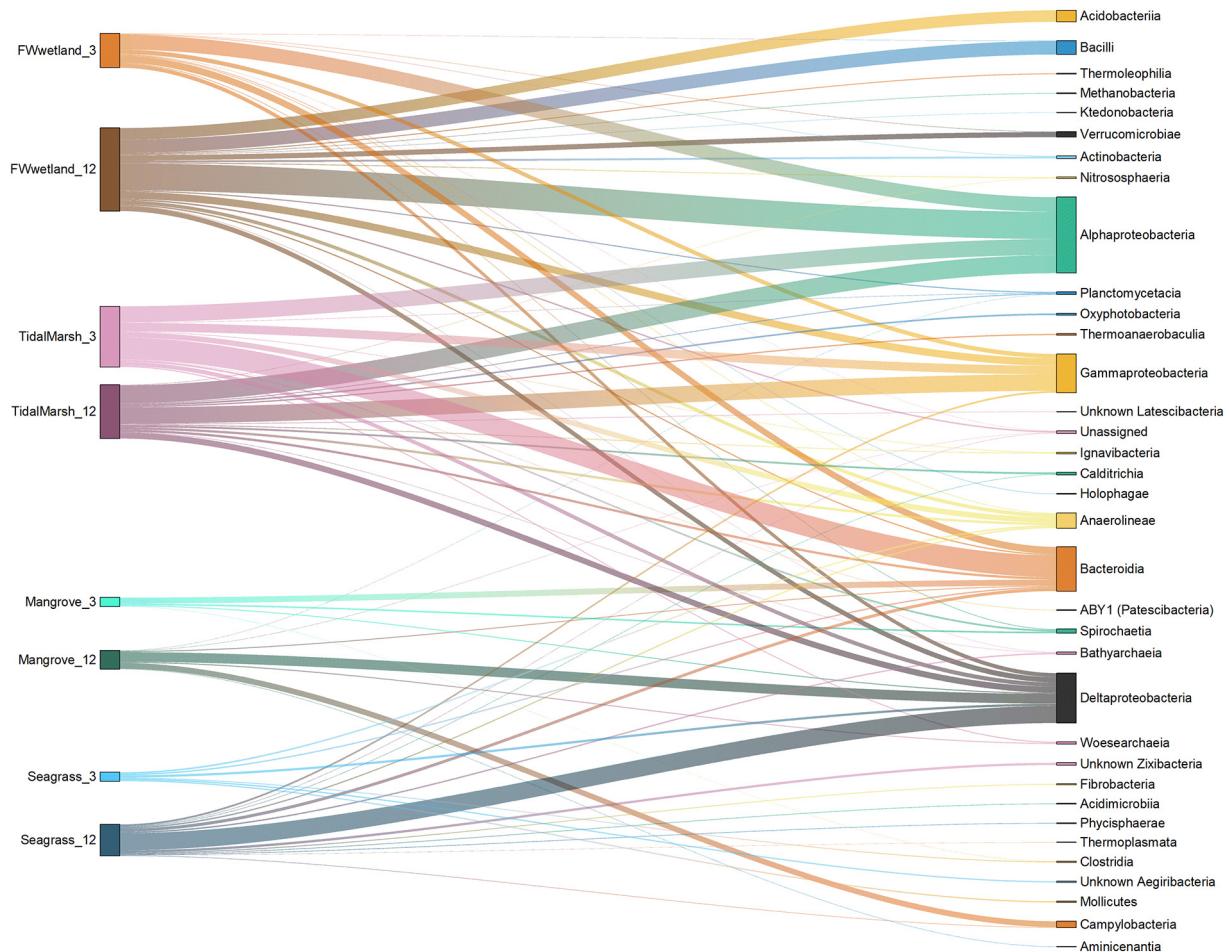


Fig. 4. Sankey diagram of the relative abundance of the class-level taxa significant to the Ecosystem*Time interaction for tea samples. Only Amplicon Sequence Variants that were highly significant within this interaction ($p < 0.001$) are shown. The 290 ASVs were summarised into 35 classes that encompass 132 families. Thicker lines indicate higher relative abundance.

decay progressed, including *Thiodiazotropha*, recently found to be a seagrass root endosymbiont involved in detoxifying sulphides within the rhizosphere (Martin et al., 2020b).

These results indicated shifts in key taxa contributing to early and later stages of microbial breakdown of the tea litter in wetland soils. We found that some of these shifts in indicator taxa were within similar putative functional groups, and potentially linked to changing availability and OM quality as decomposition progressed. Most notably, the sulphate reducers within the Deltaproteobacteria shifted from *Desulfovibrio* and *Desulfuromonas* indicating earlier tea decay, to *Desulfatitalea* and *Desulfosarcina* from Desulfbacteraceae indicating later decay. Additionally, we found a greater diversity in the metabolic pathways associated with the taxa during the 12-month sampling, including sulphur-oxidising, ammonium-oxidising and methane cycling. These results emphasise the key importance of multiple biogeochemical pathways for carbon degradation as decomposition progresses. For example, recent reports have highlighted the magnitude of methanogenesis and methane cycling in blue carbon ecosystems and the potential of these emissions to reduce net carbon preservation (Garcias-Bonet and Duarte, 2017; Rosentreter et al., 2018; Al-Haj and Fulweiler, 2020). Further, while the taxa colonising the tea litter were primarily anaerobic (Figs. 4, S4), the presence of taxa that utilised oxidative pathways for OM breakdown may be linked to root in-growth. The increased intrusion of roots into the litter bag over time may reflect the rhizosphere microbes and plants utilising the rich source of tea OM or contributing prokaryotic taxa to the tea microbiome itself, thereby highlighting the importance of the rhizosphere in nutrient cycling and detoxification for plant and soil health (Zheng et al., 2016; Martin et al., 2020a).

3.4. Determining direct and indirect effects of ecosystem type

Our model implies that ecosystem type affects remaining mass directly and indirectly through changes in microbial taxonomic

composition. The prokaryotic microbiome PCO shows differences in species composition among ecosystem types along the first axis (PCO1), whereas the second axis (PCO2) reflects changes in species composition between the 3rd and 12th month of incubation (as seen in Fig. 3). The bulk soil microbial communities did not change in a similar magnitude as the tea-associated communities over the incubation (Fig. 3), confirming that the PCO2 changes were likely related to the decomposition process rather than local or seasonal changes. Thus, the path between incubation time and remaining mass was included as a correlation, as there is no expected directional causality between them.

Mass remaining was affected by incubation time indirectly via the microbial community for both tea types, and by ecosystem directly but only for green tea (Fig. 5, Table S5-S6). For the green tea, we postulate that the direct ecosystem effect was likely a reflection of the influence of ecosystem-specific leaching prior to the first mass loss sampling at 3-month (Fig. 5a), an ecosystem-driven pattern that was maintained throughout the decomposition period (Fig. 5a). In contrast, rooibos tea mass loss was not dependent on ecosystem type in the SEM ($\chi^2 = 1.62$, DF = 3, $p = 0.65$; Table S5). Rather, the shift in microbial community composition as incubation period progressed was strongly related to the remaining mass of the tea, particularly of the rooibos tea (Fig. 5b). These results reflect that the mass loss between 3 and 12 months was greater for the rooibos tea (11–19% versus 4–6% for green tea), and that the associated prokaryotic microbial communities also shifted as decay progressed. We suspect that the green tea was more homogeneous after the leaching process, containing more recalcitrant carbohydrate and lipid/wax residues (Duddigan et al., 2020). Rooibos tea, conversely, may be representing greater diversity in (usable) OM over the year of decomposition, likely as polysaccharides, carbohydrates and cell-wall compounds (Duddigan et al., 2020). These potential shifts in OM quality over time for both tea litters match the indicator taxa at 12 months that have the putative functions for cellulose and recalcitrant OM degradation. Further, the greater amount and diversity of accessible

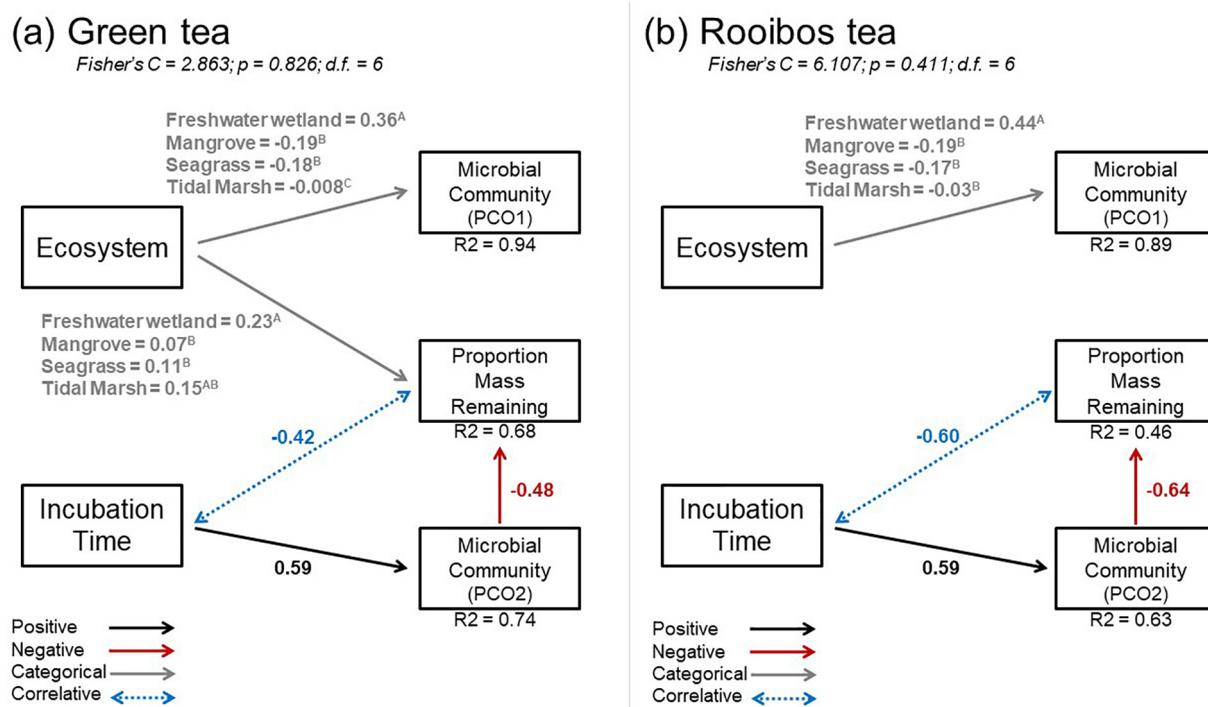


Fig. 5. Structural equation model using prokaryotic microbial community composition and tea biomass remaining for (a) green tea and (b) rooibos tea. Ecosystem (freshwater wetland, tidal marsh, mangrove, seagrass) and incubation time (in days) were the factors. Line colours indicate positive (black), negative (red) or categorical (grey) relationships, and numbers adjacent to lines are standardised path coefficients. For ecosystem type, unstandardized coefficients are given for each category, with letters indicating groupings based on posthoc Tukey tests. R² are conditional values that include both random and fixed effects. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

OM for the rooibos substrate may be linked to the higher community alpha diversity compared to the green tea litter (Fig. S2). This, in addition to the magnitude of change of the rooibos tea community during decomposition, likely underlies the tighter links to the microbial communities utilising this substrate.

3.5. Perspectives on microbial-driven decomposition of tea litter in wetlands

The key drivers in both tea litter decay and tea-associated prokaryotic communities were the differences between freshwater/tidal marsh wetlands versus mangrove/seagrass ecosystems. We hypothesise that the ecosystem differences in decay rates were in part from abiotic tidal flushing and inundation, whereas salinity and potentially inundation-linked anoxic conditions defined the prokaryotic microbiome structure and putative functions. The division due to marine influence is not surprising as salinity is shown to be a strong influence in the formation of the prokaryotic microbiome communities and diversity (Lozupone and Knight, 2007; Balmonte et al., 2020). When considering the decomposition process over a time series, salinity alone has been shown to be a smaller influence on the rate of aerobic organic matter breakdown than the quality and amount of organic matter available (Balmonte et al., 2020). In contrast, this study focussed on belowground organic matter turnover where the selective forces of both salinity and anoxic conditions (e.g. mangrove and seagrass soils) on the microbiome structure and functional groups, coupled with the differing quality of the tea litters, were important for the differential litter breakdown in wetlands. This combined effect has been shown to enhance litter decomposition in tidal marsh (Zhai et al., 2021) and mangrove (Kristensen et al., 2008; Ouyang et al., 2017) ecosystems, including belowground cellulose decomposition (Stagg et al., 2018). At the same time, the mangrove and seagrass ecosystems likely experiencing different inundation patterns also drove abiotic leaching early-on in the decomposition process, which is independent of the redox effects of water-logged soils (Godshalk and Wetzel, 1978). While we cannot tease apart the roles of salinity, oxygen and inundation separately, it is likely we are quantifying enhanced decomposition in the mangrove and seagrass ecosystems via multiple biotic and abiotic pathways in response to flooding (Stagg et al., 2017). Such combinations of flooding and salinity intrusion have been predicted to impact decomposition in tidal wetlands differently according to the position along the tidal frame (e.g. marine to freshwater tidal), and dependent on litter quality (Stagg et al., 2017; Stagg et al., 2018). Future studies using the standardised tea litters may be promising for teasing apart these impacts on decomposition along a salinity and tidal gradient.

We were able to identify cross-ecosystem commonalities (e.g. potential functional redundancies) and differences (e.g. community structure linked to salinity and oxygen) of microbial turnover of tea litter. The shift in the communities over time highlights the taxa that may be employing *K*-strategist lifestyles during the decomposition process, as well as the increasing presence of oxidising groups found in all ecosystem types. Both strategies may be important for the decomposition of more structurally complex forms of carbon, such as lignocellulose and lipids/waxes (Cragg et al., 2020). Further, the decay rates and putative metabolic functions of the prokaryotic microbiomes observed in this study could be reflecting the enzymatic activity of the microbiota across freshwater and coastal soils, which are influenced by functional and enzymatic diversity, temperature, pH and substrate availability (Arnosti, 2011; Arnosti et al., 2014). Linking decay rates with enzyme diversity and activity has been shown to be a key facet to explaining organic matter turnover and preservation in terrestrial ecosystems (Wieder et al., 2013; Bradford et al., 2017). Inclusion of these variables could help parameterise the carbon cycling and preservation models both for within- and cross-ecosystems comparisons.

In conclusion, this cross-ecosystem study on standardised tea litter decomposition and associated prokaryotic microbiomes showed that wetland type was a dominant indicator of microbial-driven tea litter decay.

Tea litter decomposition was driven directly by wetland type, likely via abiotic factors like inundation, whereas the tea-associated microbial communities and their putative functions, which were likely influenced by salinity, oxygen availability and shifts in OM quality, indirectly drove decay. We found preliminary evidence of a climatic effect across a continental scale, whereby the warmer climates exhibited enhanced mass loss over 12 months of decomposition consistent for both tea types. Together, the results presented here provides new insights into the factors influencing tea litter decomposition by prokaryotic microbiota, and advances our understanding of belowground processes in wetlands over a range of spatio-temporal scales and ecosystems.

CRediT authorship contribution statement

Stacey M. Trevathan-Tackett: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Visualization, Writing – original draft. **Sebastian Kepfer-Rojas:** Formal analysis, Data curation, Methodology, Writing – review & editing. **Aschwin H. Engelen:** Formal analysis, Funding acquisition, Methodology, Writing – review & editing. **Paul H. York:** Investigation, Writing – review & editing. **Anne Ola:** Investigation, Writing – review & editing. **Jinquan Li:** Investigation, Writing – review & editing. **Jeffrey J. Kelleway:** Investigation, Writing – review & editing. **Kristin I. Jinks:** Investigation, Visualization, Writing – review & editing. **Emma L. Jackson:** Investigation, Visualization, Writing – review & editing. **Maria Fernanda Adame:** Investigation, Writing – review & editing. **Elise Pendall:** Investigation, Writing – review & editing. **Catherine E. Lovelock:** Investigation, Writing – review & editing. **Rod M. Connolly:** Investigation, Writing – review & editing. **Anne Watson:** Investigation, Writing – review & editing. **Inger Visby:** Investigation, Writing – review & editing. **Allison Trethowan:** Investigation, Writing – review & editing. **Ben Taylor:** Investigation, Writing – review & editing. **Tessa N.B. Roberts:** Investigation, Writing – review & editing. **Jane Petch:** Investigation, Writing – review & editing. **Lachlan Farrington:** Investigation, Writing – review & editing. **Ika Djukic:** Conceptualization, Funding acquisition, Methodology, Writing – review & editing. **Peter I. Macreadie:** Conceptualization, Supervision, Writing – review & editing.

Declaration of competing interest

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

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Appendix A. Supplementary data

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