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BACTERIAL ASSEMBLAGES ASSOCIATED WITH CARBON SEQUESTRATION POTENTIAL IN MARINE WETLAND SEDIMENTS

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1. INTRODUCTION

1.1. Background

TeaComposition H2O (https://www.bluecarbonlab.org/teacomposition-h2o/) is a global aquatic decomposition initiative launched by the Blue Carbon Lab. The project involves experimental methods and international participation to quantify litter carbon turnover across ecosystems, continents and time. It uses local data to identify environmental, chemical and biological factors that affect decomposition processes. As part of this initiative, the Tea Bag Index (TBI) is calculated to quantify microbial-driven decomposition of plant material in a standardised and cost-effective way. The collected information is used to improve understanding of the global carbon dioxide cycle, specifically in regard to how carbon retention varies among wetland types.

The initiative's goal is to measure decay of plant material within wetland ecosystems by using two types of tea bags (green and rooibos tea, the latter hereafter referred as red tea) to represent standard plant litter (Keuskamp et al. 2013). Tea material in plastic mesh bags is placed in the sediments and weight loss is determined after three months. From the weight loss of red and green tea, two parameters are obtained: (i) the amount of material stabilised – being preserved in a recalcitrant (i.e. resistant to decomposition) organic carbon form (represented by stabilisation factor) and (ii) the decomposition rate. In carbon-accumulating environments, a higher stabilisation factor is expected, while degradation indicates release of carbon into the atmosphere.

Wetland ecosystems (seagrasses, coastal marshes and mangroves) are incredibly efficient at storing and sequestering atmospheric carbon dioxide (Adhikari et al. 2009). However, carbon sequestration potential depends on ecosystem-specific conditions, requiring robust empirical data to understand relationships between decomposition and environmental factors.

1.2. Scope of the study

A low-cost citizen science experiment has been designed by the Nelson City Council (NCC) as part of the TeaComposition H2O initiative, using two types of tea bags (green and red) planted across two Nelson estuaries (Waimea Inlet and Nelson Haven) and four wetland habitats (seagrass, patchy seagrass, mud and saltmarsh). This experiment is aimed to deliver important information on decomposition processes and release or sequestration of carbon, which may assist in identifying planting locations for future seagrass restoration projects to enhance blue carbon sequestration (i.e. capturing, securing and storing carbon in coastal and marine ecosystems, thus compensating carbon dioxide emissions into the atmosphere). It also provided NCC and Cawthron with a unique opportunity to study the microbial communities associated with this process.

Environmental DNA and RNA (eDNA/eRNA) metabarcoding is a molecular tool that can provide detailed information on species diversity from genetic fragments contained in small amounts of marine sediments (Cordier et al. 2020; Zaiko et al. 2022b). Organisms are identified without taxonomic expertise by matching short, high-throughput sequencing-derived gene fragments to a reference sequence library, allowing the composition of otherwise cryptic biological communities (bacteria, microalgae, micro- and meio-eukaryotes) to be deciphered. These organisms play an essential role in ecosystem functioning — carbon and nitrogen cycling, decomposition and energy transfer (Brasell et al. 2021; Marzocchi et al. 2021; Politi et al. 2021). Recently, this approach has been proposed as an effective tool for determining the source of organic carbon stored in sediments and it can be used to improve understanding of the carbon sequestration process in seagrass meadows (Reef et al. 2017). In this study, eDNA/eRNA metabarcoding analysis was applied to the samples collected as part of the NCC's tea bag decomposition experiment to:

- elucidate bacterial community composition in tea bag deployment locations and relate compositional differences in studied habitats to carbon sequestration potential (derived from the TBI parameters)
- estimate contribution of organic sources to different sites and habitat types using plant-specific molecular markers
- provide recommendations for further work to identify microbial indicator species for estuarine health assessment.

2. METHODS

2.1. Experimental setup and sample collection

The experiment was set up in two Nelson estuaries (Waimea Inlet and Nelson Haven) by NCC with the help of citizen scientist volunteers in late 2021. Briefly, 2 sampling quadrats representing 4 types of wetland habitats were replicated in 2 different locations within an estuary (16 quadrats per estuary). Each quadrat contained 15 tea bags of each tea type for a total of 30 tea bags per quadrat. These were left in place for three months (90 days) after which the tea bags were recovered. The details on the tea bag deployment, collection and processing for weighing is provided in Appendix 1, based on information provided by NCC.

The sediment samples for molecular analyses were collected by the NCC staff on a tea bag retrieval date, following the detailed protocols and guidelines provided in Appendix 2. In total,148 sediment samples were collected for processing. To discriminate bacterial communities (potentially associated with decomposition of specific litter material) from the background sediment microbiome, samples were collected from retrieved tea bags and undisturbed top sediments from the sampling site.

2.2. Molecular analyses

Samples were delivered on ice to the Cawthron Institute and processed for eDNA/eRNA isolation. Briefly, sediment samples were homogenised via bead beating (MiniG™ 1600) for 2 min, RNA and DNA were extracted simultaneously from ~2 g of sediment using the Zymo Quick-DNA/RNA™ Miniprep Kit (Zymo Research) and RNA reverse transcribed into cDNA as described in Zaiko et al. (2022a). All extract products were stored frozen (-80 °C) until further analyses.

For elucidating biodiversity of bacterial communities, the nuclear 16S rRNA fragment was PCR amplified (Clark et al. 2020). For estimating contribution of organic carbon sources, plant barcode of the *rbcL* gene was targeted in PCR amplifications (Reef et al. 2017). Negative extractions and PCR controls were also included as appropriate.

PCR products were visualised on 1.5% agarose gels stained with Red-Safe™ Nucleic Acid Staining Solution (Intron) to confirm the presence of target gene fragments. Where necessary, PCR troubleshooting was performed to acquire good quality amplicons. PCR products were cleaned and normalised using SequalPrep Normalisation plates (ThermoFisher Scientific, USA). This resulted in a cleaned DNA concentration of ~ 1 ng μl^{-1}. Amplicons were then processed for high-throughput sequencing on an Illumina Miseq™ at Sequench Ltd's facility. The amplicons were prepared for sequencing following the Illumina 16S metagenomics library prep manual.

2.3. Bioinformatics and statistical analyses

The raw sequence data were bioinformatically processed for quality control, trimming, denoising and taxonomy assignment using an established bioinformatics pipeline (Clark et al. 2020).

The tea bag weight data provided by NCC were collated into a single spreadsheet, quality checked for typing errors and used for estimating TBI parameters (stabilisation factor and decomposition) for different habitat types (Keuskamp et al. 2013). The non-parametric Wilcoxon tests were applied to test for a significant pairwise difference in tea bag weight before and after the 90-day deployment. Wilcoxon effect size was calculated to assess the magnitude of weight change for each experimental factor (estuary, tea type, habitat).

Significant differences between the bacterial assemblages for the habitat factor were tested using distance-based permutational analysis of variance (PERMANOVA), separately for each estuary (Anderson 2017). All analyses were performed in R software (version 3.6.1).

3. RESULTS AND DISCUSSION

Out of 960 tea bags planted in estuarine sediment for the NCC's experiment, 728 were retrieved. Many samples had unreadable labels post-retrieval, therefore pairwise before-after weight comparison could not be applied at an individual bag level, but was instead performed at a site or habitat level.

In agreement with earlier studies (Keuskamp et al. 2013), higher decomposition (weight decrease post-deployment) was observed for green tea bags compared to red tea bags (Figure 1). The magnitude of weight difference (the effect size calculated based on a Wilcoxon paired signed-rank test) in green tea bags was consistent between estuaries (average estimated effect sizes 0.79 ± 0.07 and 0.81 ± 0.08 for the Haven and Waimea samples, respectively) as well as between habitat types (0.77 ± 0.07, 0.76 ± 0.07, 0.83 ± 0.06 and 0.84 ± 0.07 for seagrass, mud, patchy and saltmarsh samples, respectively). The red tea bags data revealed lower magnitude of weight difference in the Haven samples (average estimated effect size 0.49 ± 0.20) compared to the Waimea Inlet samples (average estimated effect size 0.79 ± 0.09)[1].

[1] All pairwise differences were significant (Wilcoxon test, $p < 0.05$) except for red tea samples from Nelson Haven, saltmarsh and mud habitats ($p = 0.07$ and 0.11, respectively).

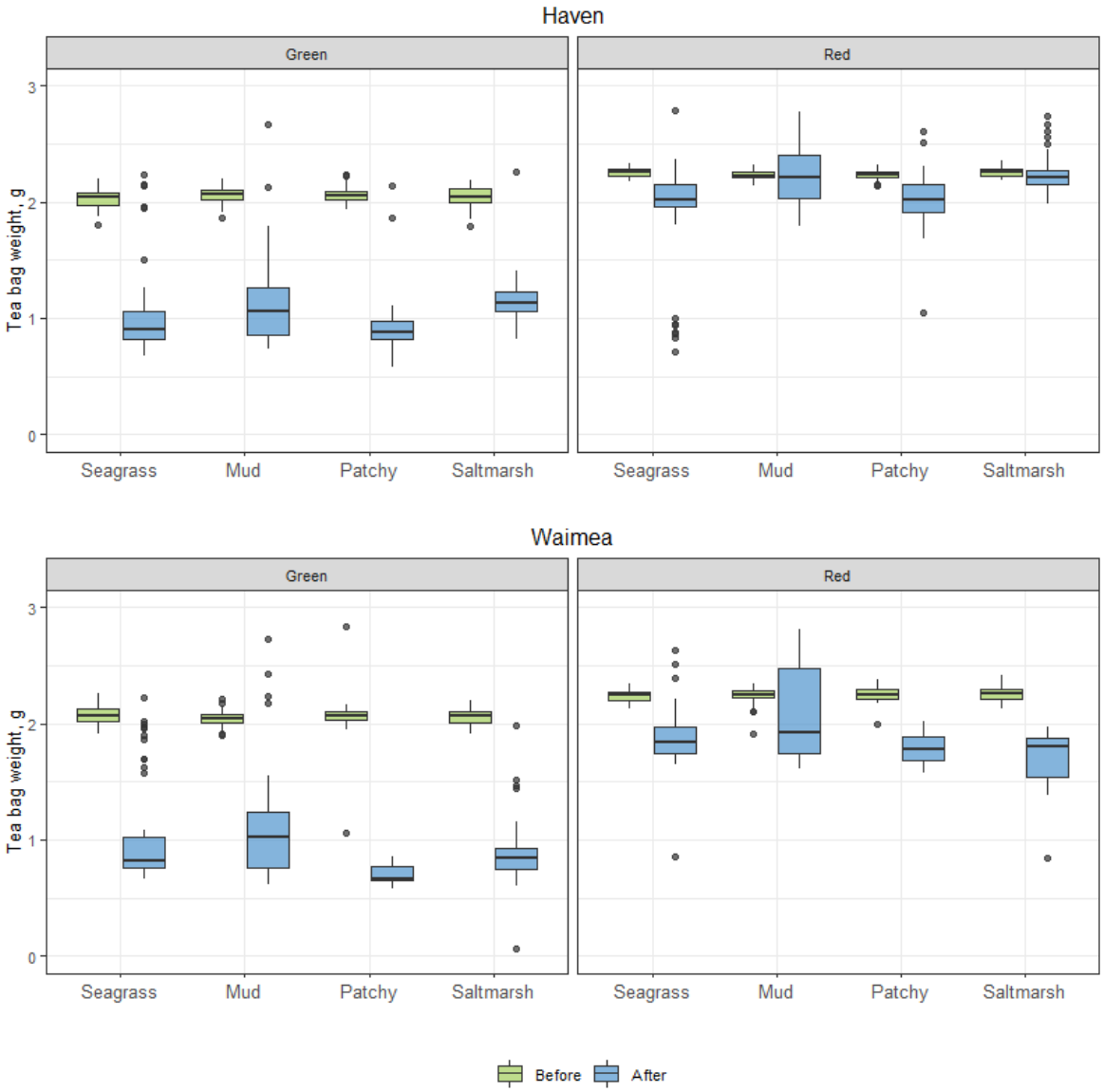

Figure 1. Green and red tea bag weights before and after the 90-day experimental deployment. Data range (whiskers), upper and lower quartiles (edges), the median (horizontal line) and the outliers (black dots) are represented for each considered habitat type.

It has been reported previously that in both terrestrial and aquatic systems, red tea decomposition might be suppressed by elevated nitrogen levels (Mori 2021). A possible explanation is that in a nitrogen-rich environment, microbes are not inclined to degrade more resistant compounds such as lignin, which are present in greater concentration in red versus green tea.

The TBI stabilisation factor (S) estimated from the combined change in green and red tea bags' weight (Keuskamp et al. 2013) and indicative of long-term carbon stability (Mori 2021), ranged from 0.14 to 0.65 in the Haven samples and from 0.14 to 0.56 in the Waimea Inlet samples. The only statistically significant pairwise difference

between habitats was reported for patchy and mud sites within the Waimea Inlet (Figure 2). However, the patchy habitat in both estuaries demonstrated overall lower S values, suggesting lower carbon retention compared to other habitat types.

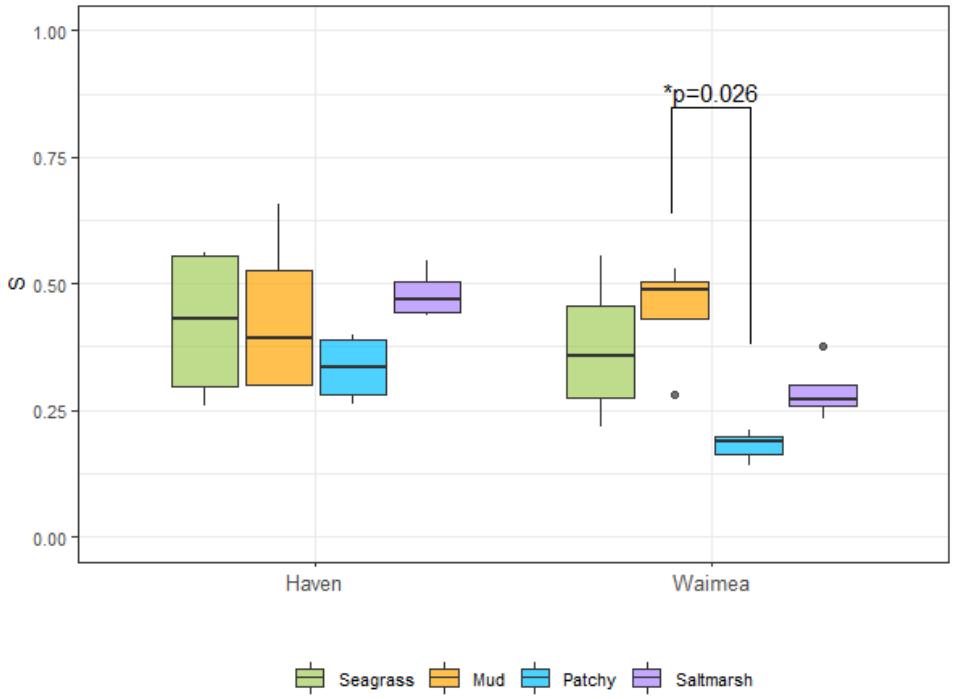

Figure 2. The TBI stabilisation factor calculated for 4 habitat types in 2 studied estuaries. Data range (whiskers), upper and lower quartiles (edges) and the median (horizontal line) are represented for each habitat type. Statistically significant pairwise within-estuary difference is indicated with horizontal brackets and corresponding p-value.

Although the current experimental data do not clearly reveal differential carbon sequestration potential between studied estuaries or habitats, the observed S values are generally higher than those reported from terrestrial and wetland ecosystems elsewhere (Keuskamp et al. 2013; MacDonald et al. 2018). This suggests that Nelson estuaries might be considered as prospective blue carbon ecosystems, i.e. coastal ecosystems that are important for their capacity to store carbon within the plants and in the sediments, thus contributing to sequestering atmospheric carbon dioxide—the nature-based solution to climate change.

The high-throughput sequencing data of bacterial communities comprised a total 1,345,335 good quality reads (on average, 14,948 sequence reads per sample—sufficient sequence yield for robust downstream analyses). A total of 24,458 amplicon sequence variants (ASVs, roughly corresponding to the lowest bacterial taxonomic rank, i.e. sub-species level), representing 76 phyla, were detected. The 22 most dominant phyla and their relative contribution to bacterial communities across different habitats and sample types are presented in Figure 3.

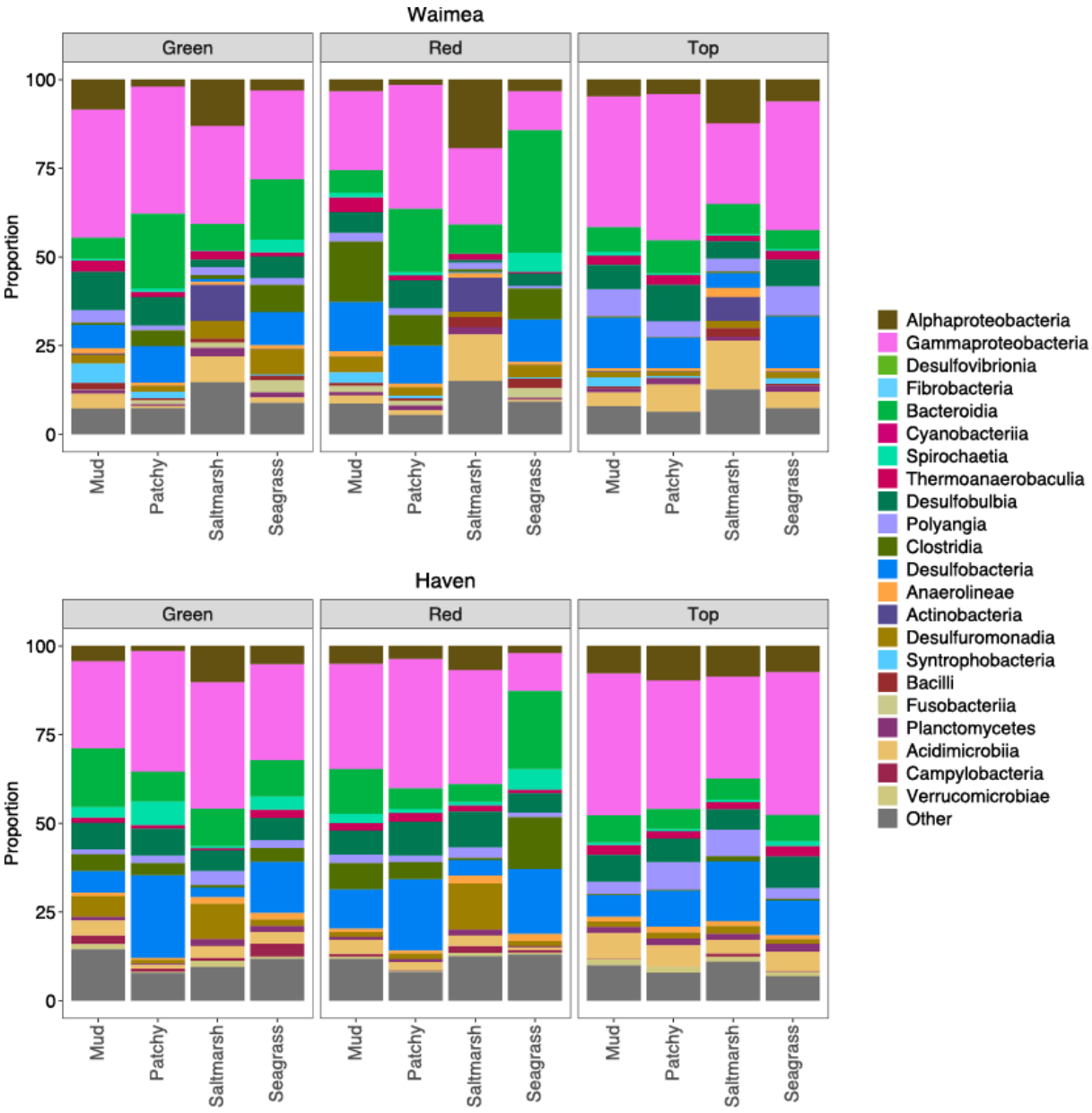

Figure 3. Relative read abundance of the 22 most dominant DNA-derived bacterial phyla among different habitat and sample types (green tea bags, red tea bags, top sediment) across the two studied estuaries.

At high taxonomic rank (i.e. phylum level), bacterial communities seemed consistent across sediment types of both estuaries, with the exception being the higher proportion of Clostridia in sediment samples collected from red tea bags (Figure 3). This anaerobic saprophytic bacterial group is known to ferment plant polysaccharides (Boutard et al. 2014). Rooibos tea is known to be rich in complex polysaccharide compounds (Nakano et al. 1997; Mori 2021), which might have attracted bacteria that are specialized in consuming this unique, rather persistent, carbon source. The

PERMANOVA analysis, however, revealed significant differences in bacterial community composition between all habitat types in the Waimea Inlet ($p \leq 0.01$) as well as between mud, saltmarsh and seagrass, and saltmarsh and patchy habitats in the Haven ($p < 0.05$). In the Waimea Inlet, the most prominent difference was in the relative contribution of sulphate-reducing bacterial groups (Desulfovibrionia, Desulffobulbia and Desulfobacteria, Figure 3), which were substantially higher in patchy and seagrass habitats (especially in samples collected from tea bags at ~8 cm depth) compared to saltmarsh habitats. These bacteria were previously reported in elevated abundances from enriched wetland and marine sediments (Clark et al. 2020).

The good-quality RNA, suitable for high-throughput sequencing and further analyses was derived from a subset (n = 12) of samples. The bacterial community composition of these samples indicated presence of the same main prevalent groups found in DNA samples (Figure 4), suggesting they were biologically active at the time of sample collection. Among the taxa that were substantially underrepresented in RNA samples, were Acidimicrobia (acidophilic actinobacteria), Desulfomonadia and Syntrophobacteria (both are anaerobic sulphur-reducing bacteria), and Campylobacteria (faecal-borne bacteria). Their high abundance detected in eDNA samples may be due to the capture of 'legacy' DNA from past communities now preserved in marine sediments.

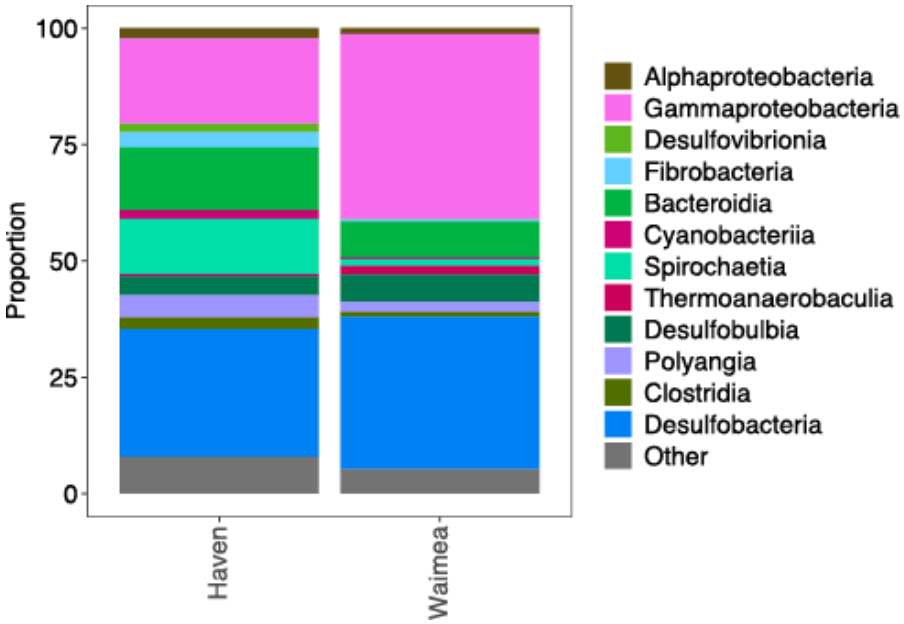

Figure 4. Relative read abundance of the 12 most dominant RNA-derived bacterial phyla in the two studied estuaries.

The high-throughput sequencing data of plant-based material contained in sediments comprised 3,390,003 good quality reads (on average 43,462 sequence reads per

sample). A total of 285 ASVs assigned to 81 species of plants (Streptophyta) were detected.

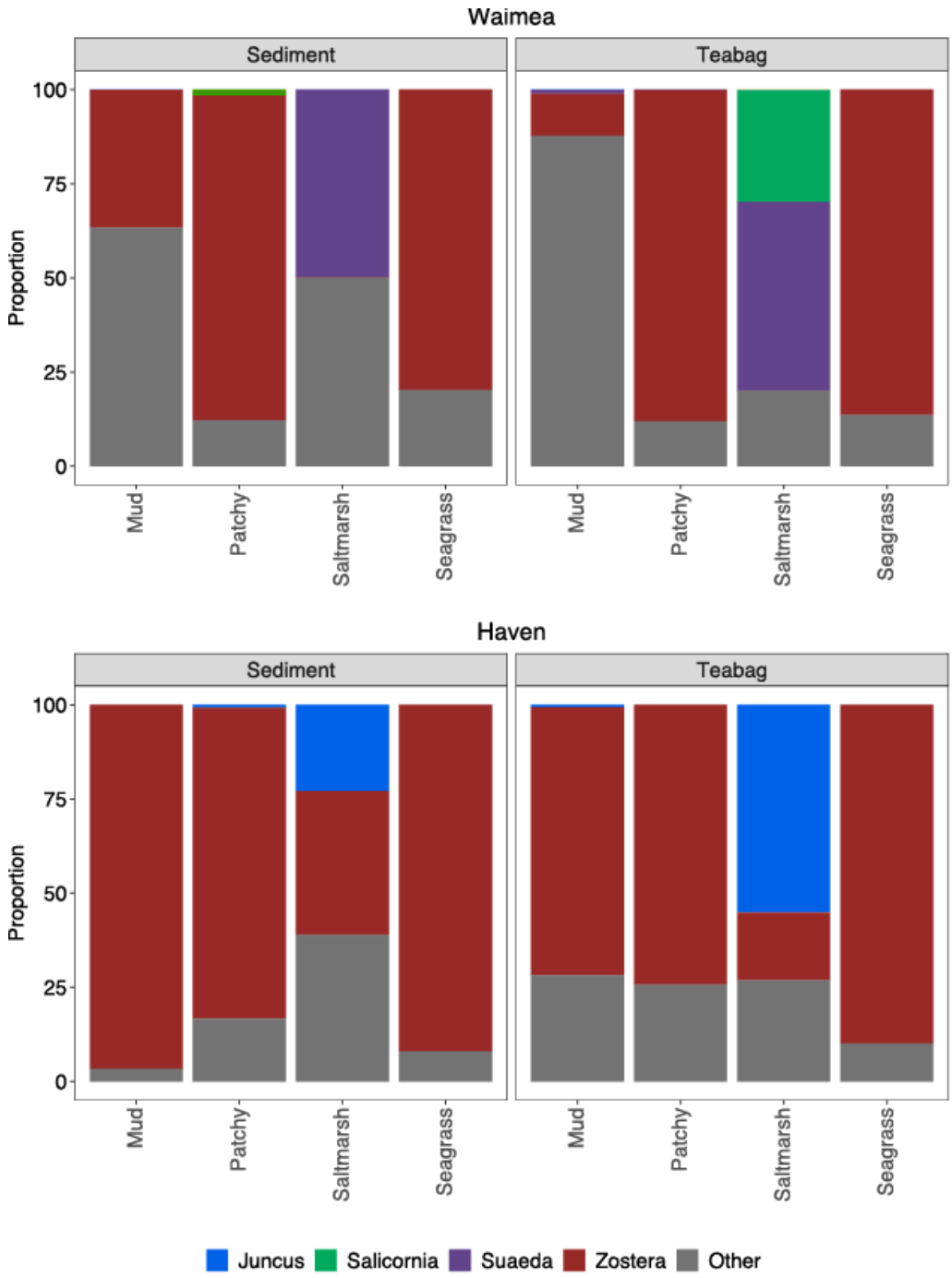

Figure 5. Relative sequence abundance of ASVs representing autochthonous wetland genera (i.e. seagrass, etc.) in relation to allochthonous (i.e. transported to the estuary from elsewhere, e.g. terrestrial sources) plant species (noted as 'Other').

The results suggest that wetland plant taxa (*Juncus spp., Salicornia spp., Suaeda spp.* and *Zostera spp.*) were the primary contributor to sedimentary carbon in all

studied habitats, except for mud in the Waimea Inlet (Figure 5). Overall, the detected compositional structure of the plant-based DNA signal, was consistent between samples collected around tea bags (at ~8 cm depth) and top sediments. The exception was the saltmarsh samples, where a higher contribution of non-*Zostera* sequences were detected at deeper layers (80% and 55% in the Waimea and Haven samples, correspondingly). The contribution of allochthonous plant material (i.e. transported to the estuary from elsewhere, e.g. terrestrial sources) to the DNA pool ranged from 12% to 88% in the Waimea Inlet (respectively, patchy and mud habitats) and 8% and 39% in Haven (respectively, seagrass and saltmarsh sediments). These were mainly terrestrial plants typically found in the adjacent areas (e.g. *Clematis* spp*., Eugenia* spp*., Schinus* spp.). The average contribution of allochthonous plant carbon sources observed in this study (27%) is higher than values reported earlier in Australia (Reef et al. 2017). This is consistent with the TBI results suggesting higher stabilisation potential of the intertidal sediments in Nelson estuaries compared to other ecosystems studied internationally (Keuskamp et al. 2013; MacDonald et al. 2018).

4. CONCLUSIONS AND OUTLOOK

The tea bag data derived from the experiment performed by NCC do not allow us to unambiguously classify the studied wetland habitats for their ability to sequester carbon. However, overall elevated stabilisation factor estimates (compared to those reported in other ecosystems) suggest the importance of both of the studied Nelson estuaries as blue carbon ecosystems. This is also supported by the results of the plant-derived eDNA analysis, which indicated substantial accumulation of allochthonous plant-based carbon in the upper sediment layer. Our study confirms that eDNA can be an effective, high-resolution tool for identifying major sources of sediment organic matter in blue carbon ecosystems. This technique may also have application for detecting long-term changes in contribution of different organic matter sources in marine sediments (Reef et al. 2017; Brasell et al. 2021). It should be noted that in this study for identifying plant material we used a molecular marker designed exclusively for detecting higher plant species. The primers used in this study did not amplify algal DNA fragments, which could also significantly contribute to sediment organic carbon (Reef et al. 2017). Combining several markers would allow for a more holistic identification of eukaryotic species and is recommended for future applications.

Since tea bag experimental data did not reveal significant differentiation in TBI estimates between habitats, the relatedness of bacterial community composition to carbon sequestration potential could not be determined in this study. However, bacterial groups indicative of nutrient enrichment were reported from the Waimea Inlet. It was demonstrated in earlier studies that eDNA-derived bacterial communities can be used to distinguish different enrichment levels (Clark et al. 2020), and more

importantly—discriminate smaller relative differences between pristine reference sites and moderately impacted sites. Detecting community change in response to low levels of impact is a crucial step in the advancement of modern biomonitoring as it would allow for implementation of management or remediation strategies at an early stage, increasing the effectiveness of these actions. This is crucial for detecting subtle changes in ecosystem health before it becomes too degraded.

Overall, eDNA-derived biodiversity information shows great promise for the development of sensitive and efficient estuary monitoring tools. To identify consistent responses in eDNA communities and developing robust indicators for routine estuary health assessment, a comprehensive validation study across larger temporal and spatial scales (as well as the range of pressure gradients) needs to be performed. Cawthron's recently-funded MBIE Smart Ideas project 'Transforming coastal monitoring: harnessing microbial communities to disentangle multi-stressor impacts' may provide an excellent opportunity to move this field forward nationwide.

5. ACKNOWLEDGEMENTS

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7. APPENDICES

Appendix 1. Estuarine decomposition (tea bag) experiment (A2886268) set up by Nelson City Council (provided by Vikki Ambrose, NCC).

Sites

There were 16 sites across each of the two estuaries (Waimea Inlet (WI) and Nelson Haven (H)). Each site has a GPS reference (A2886249). Each site is a 1 m^2 quadrat, with 6 planting rows. There are 4 habitat types:

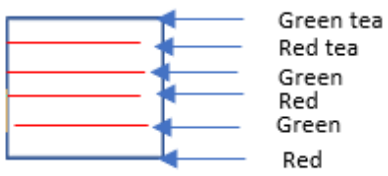
Soil type	Labelling code
Seagrass	S
Patchy seagrass	P
Saltmarsh	T
No growth/mud	N

Each soil type is planted in duplicate at a different location within the same estuary. This repetition addresses differences due to location/wind/waves/height, etc. between sites. There are two types of tea used: Lipton's green sencha (must be EAN 87 22700 05552 5 for original data or can now be EAN 87 10908 90359 5 to add to new data), and Lipton's Rooibos EAN 87 22700 18843 8. The original tea bag EANs contained plastic in the tea bag and so did not decompose under the conditions or time periods of this experiment. They stopped making these in 2017 (we found some old stock in Holland and Switzerland). The new EANs do not contain plastic and so TeaComposition H2O are still working out what difference that makes to the data.

Processing protocols

The tea bags were weighed (individually) prior to burial, with tags. [However, it was discovered that final weighing should be done without tags, so all original data require tag weights taken off. There are 2 different weights: one for green tags and one for red tags to be taken off "Before weight" columns in spreadsheet A2888211.] Tea bags were labelled according to tea/estuary/soil type/quadrat, e.g. WIS4 (Waimea Inlet, Seagrass, quadrat 4).

Tea bags were to be planted in alternating (tea type) rows of 5 tea bags each, starting with green e.g.:



Tea bags were planted 8 cm deep (depth of shovel part of trowel), and 15 cm apart (width of trowel shovel plus handle). Each site/quadrat therefore consisted of 6 rows with 5 bags, therefore 30 bags in all.

All sites at Nelson Haven were planted by volunteers. Only the Waimea Inlet sites off Martin Road, Monaco were planted by volunteers. Waimea Inlet sites off the airport peninsula and along the Eddyline underpass off Whakatū Drive were planted (and retrieved) by Council S&E staff due to difficulty getting volunteers to the sites.

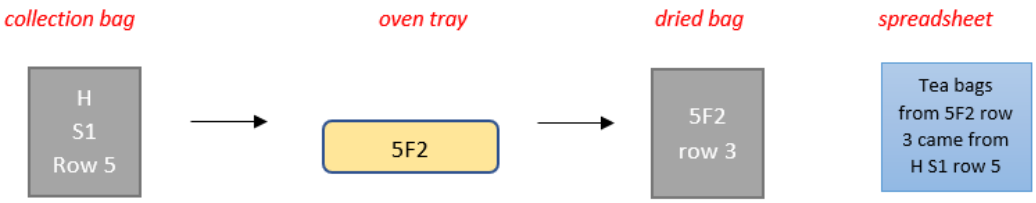
Tea bag retrieval

Airport peninsula and Eddyline underpass off Whakatū Drive samples were retrieved by S&E team NCC staff (Emma/Paul/Rowan/Jo/Stefan/Asita/Phil/Frizz/Janeen/Scott). Sites off Monaco and all of Haven were retrieved by volunteers.

Each quadrat was processed by one pair of volunteers. Each pair were provided with a box (bottom sealed so could sit in mud) with 6 waxed bags in it. Bags were labelled rows one to six. Volunteers were looking for five tea bags in each row. All tea bags from one row were placed into the bag labelled with correct site and row number.

Once collected, tea bags were placed in the NMIT oven at 70 ºC for 48 hours. Tea bags were placed into trays to go into the oven. A record was made of the location tea bags were collected from for each row in the oven tray, e.g.: Tray 1F1 – HS1 row 1 = H (Haven) S1 (seagrass quadrat 1) row 1 of the 6 S1 rows.

After drying tea bags were placed into waxed bags containing oven tray number and row number. Tea bags were then taken and cleaned very gently so not to split tea bags. Tea bags were then weighed. The tea bags still retaining legible labels could directly be recorded next to their original weigh and have the tea bag number highlighted in purple.



Tea bags without labels were cross-referenced by oven tray information and original collection bag information to determine where they came from; if one bag, or more had legible labels in a bag this confirmed the collection site. If the site differed between a legible label and the site recorded on bag, it was assumed all tea bags came from the same site as the labelled ones and the bag was incorrect.

Some labels were very difficult to read or almost looked as if there was no number on the label. Time was spent moving the label in different light angles where often the number became legible.

Tea bags had to be free of mud, seaweed, roots, etc. before weighing. At the start, tea bags were weighed whole, on the spreadsheet the weight of the green tea label, and red tea label must be taken off the rows of weights for red and green tea bags before weight. After drying, tea bags must be weighed with string but no label. peel string off label to weigh. It was recorded in spreadsheet if no string/half string/hole in bag.

Appendix 2. Sediment sampling protocol for molecular analyses

Sampling design

2 Estuaries (**WI** = Waimea and **H** = Haven)
4 Habitat types in each (**S** = seagrass; **P** = patchy seagrass; **N** = no growth/mud, **T** = saltmarsh)
2 Sites per habitat type
3 replicates of 3 sample types - the red tea bag surface (**Red**); green tea bag surface (**Green**) undisturbed top sediments in the proximity to the tea bag deployment (**Top**) collected from
4 field blank controls collected additionally (1 per sampling day)

Total number of sediment samples for processing = 148

Material needed (provided for each sampling event)

✓ 4 Chilly bins with pre-frozen ice packs
✓ 148 pre-labelled 5-mL sampling tubes (+2 extra) in cardboard boxes
✓ A box of latex gloves
✓ Rubbish bag for disposal of used gloves
✓ Field log sheet, field sampling protocol
✓ Marker pen
✓ Pen

NOTE: The main thing to remember is to not cross-contaminate the samples – use separate sterile tubes for every sample, handle sampling tubes with care, wear latex gloves when sampling, do not touch the inside/end of the sampling tube - and keep the samples chilled on ice immediately after collection.

Labels

Each tube will be labelled according to the following protocol:
Inlet name_Habitat type_Site#__Sample type_Sample replicate
e.g. WI_S_1_Red_3 would be the third sample replicate from the red tea bag, Waimea Seagras, site 1.
Take care to correctly collect samples into their corresponding tubes.

Sampling

1. On the sampling day, upon arrival to the first sampling site, prepare field blank control: put on gloves, open the sampling tube labelled as “Field blank” (“FB” on the cap), unscrew the cap, move the tube around (as like “collecting air”) and close the tube tightly. Place the tube in the box and on ice with other tubes.
2. Continue straight with the first sample. Check the label on the tube (pick the right one), carefully remove the lid of the tube without contaminating it, push the tube

~1cm into the sediment, shake the majority of the sediment into the tube. The sample should look similarly to the one on the picture below. Close the tube tightly and place on ice.

NOTE: For samples collected from the teabags – scrape the sediments directly from the teabag surface immediately after its retrieval from the sediments.

1. Repeat the sampling process as described in step #2 for all replicates from the site (n = 9; 3 from the green tea bag, 3 from the red tea bag, 3 from the undisturbed top sediments nearby).
2. At the next sampling site, change the gloves. Repeat step #3.
3. Repeat for each habitat type and estuary
4. Deliver the samples immediately after sampling to the laboratory: 98 Halifax St E, The Wood, Nelson 7010.