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The relationship between microbial community succession, decay, and anatomical character loss in non-biomineralized animals

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Abstract: A fundamental assumption of hypothesis-driven decay experiments is that, during decay, the loss of anatomy follows a sequence broadly controlled by the intrinsic compositional properties of tissues. Recent work investigating the succession of postmortem endogenous microbial communities (thanatomicrobiome) challenges this assumption in that the internal thanatomicrobiome exhibits a predictable succession in response to physical and chemical environmental changes that occur within a carcass. Thus, reproducible sequences of character loss during decay could be controlled by thanatomicrobiome succession dynamics. If so, exceptionally preserved fossil anatomy would reflect a succession of ancient contemporaneous microbial communities, about which we know nothing, rendering decay experiments uninformative. Here, we investigate two questions: (1) what is the role of exogenous and endogenous bacteria during formation of the thanatomicrobiome; and (2) do thanatomicrobiome successions control the sequence of anatomical character loss within a decaying carcass? Our analysis shows that the internal thanatomicrobiome is dominated by endogenous bacteria and that, even in the presence of inoculum, exogenous bacteria do not invade the carcass and replace native bacteria (while the carcass remains intact). This confirms that the use of environmental inoculum in decay experiments introduces a confounding variable. Our analysis also finds no correlation between thanatomicrobiome successions and the sequence of anatomical character loss, supporting the hypothesis that fossil non-biomineralized characters correlate with their propensity to decay in extant relatives. These findings indicate that the inability to model ancient bacteria does not invalidate decay experiments. We present a synthesis of the role of bacteria in non-biomineralized fossilization.

Key words: taphonomy, soft tissue fossil, decay, thanatomicrobiome, fossilization, bacteria.

UNDERSTANDING the taphonomic processes that affect organic remains postmortem is vital when interpreting fossil organisms, especially those that preserve highly informative non-biomineralized anatomy. Decay is perhaps the dominant control on fossilization of organic remains; the combination of autolysis and microbial metabolism acts from the moment of death and, under normal conditions, will proceed until all organic remnants are obliterated, preventing fossilization from occurring. In amenable environmental conditions, processes that promote preservation (maturation and mineralization) act to disrupt decay and stabilize organics over geological timescales to create fossils. Thus, to accurately interpret enigmatic non-biomineralized organisms from deep time, it is essential to understand that decaying organic remains are the substrate that preservational processes act upon during fossilization, and that these remains will never be completely intact. Consequently,

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investigating the process(es) of decay is of primary concern of palaeontologists (e.g. Briggs & Kear 1993a, 1993b; Sagemann *et al.* 1999; Donoghue & Purnell 2009; Sansom *et al.* 2010; Murdock *et al.* 2014; Purnell *et al.* 2018; Gäb *et al.* 2020; Gabbott *et al.* 2021; Harvey *et al.* 2021; Clements *et al.* 2022; Mähler *et al.* 2023; Cavicchini *et al.* 2025; Palmer *et al.* 2024; Vixseboxse *et al.* 2024; Waskom *et al.* 2025).

Decay experiments (hypothesis-driven experimental taphonomic investigations) are one of the most invaluable tools for analysing the decay of non-biomineralized tissues and gross-anatomy. Robust taphonomic investigations are typically lab-based and are designed to investigate specific aspects of decay, biostratinomic processes, environmental variables that impact decay, or individual preservation processes. Laboratory simulations that aim to replicate ancient environments or recreate the outcomes of fossilization fall outside the scope of experimental taphonomy, as the number of unconstrained and confounding variables typically involved in such simulations renders their results open to multiple equally plausible interpretations (equifinality; see Purnell *et al.* 2018 for a review of experimental design). In the last few decades, taphonomic experiments have significantly furthered our understanding of the preservation of non-biomineralized remains. With each experimental result, our understanding of key concepts improves: for example, transport and rapid burial mechanisms (e.g. Bath Enright *et al.* 2017), environmental controls on rate of decay (e.g. Briggs & Kear 1993a), how microbial decay drives the formation of geochemical conditions favourable for mineralization (e.g. Sagemann *et al.* 1999; Clements *et al.* 2022), that decay of different taxa and/or specific organs can bias soft tissue preservation (e.g. Clements *et al.* 2017, 2022), and that sediment composition plays an important role in maintaining the integrity of carcasses (e.g. Wilson & Butterfield 2014; Cavicchini *et al.* 2025). One of the most important observations revealed by experimental work is that during decay non-biomineralized anatomical characters are lost from the carcasses of organisms in an irreversible and reproducible, non-random sequence (e.g. Sansom *et al.* 2010, 2011; Murdock *et al.* 2014; Nanglu *et al.* 2015; Redelstorff & Orr 2015; Sansom 2016; Beli *et al.* 2017; Cavicchini *et al.* 2025). This is a fundamental concept as the procession of decay-modified characters are the anatomical substrate upon which stabilization and preservation processes can act, and the non-random nature of decay allows taphonomists to model and predict the continuum of character loss. Combining these models with our current understanding of the timing, interplay, and selectivity of information stabilization (preservational processes) across soft tissues, we can begin to model preservational regimes (e.g. Gabbott *et al.* 2021). Furthermore, as sequences of anatomical character loss have been demonstrated to be conserved between related organisms, combining the results of decay experiments with our understanding of preservational processes can shed light on the decay of fossil taxa which share homologous anatomical characters, a vital tool for interpreting enigmatic organisms (e.g. Sansom *et al.* 2011, 2013). Currently, the experimental approach is the only method that can generate detailed anatomical character decay sequence data.

Character-focused taphonomic experiments from which decay sequences can be derived are relatively recent. Historically, decay experiments have used qualitative observational data when describing morphological decay (e.g. Allison 1988a; Briggs & Kear 1993a, 1994a, 1994b; see Sansom 2014 for review). More recently, character-based methodologies have been developed, where the presence/absence of individual anatomical characters is encoded using predefined numerical categories (i.e. pristine = 1, decaying = 0.5, lost = 0; see Sansom *et al.* 2010; Sansom 2014). The value of this approach is that it provides quantitative data that are amenable to statistical testing and robust analysis of systematic biases (e.g. Sansom *et al.* 2010, 2011; Murdock *et al.* 2014; Cavicchini *et al.* 2025). Importantly, character-focused decay experiments have confirmed the underlying reproducible pattern of information loss outlined in older taphonomic work, generally in terms of 'stages of decay'. In many previous studies, it was assumed that soft tissues were lost to decay in a sequence determined by the relative resistance of different biomolecules to autolysis and decay (Allison 1988b; Tegelaar *et al.* 1989; Gill-King 1997; Briggs 1999, 2003; Briggs *et al.* 2000). Briggs (2003), for example, listed the sequence of organic materials as decreasing in their degrees of resistance to decay as follows: complex macromolecules > lipids > carbohydrates > proteins > nucleic acids (with some caveats; based on Tegelaar *et al.* 1989). At a gross level, this assumes that the intrinsic biochemical properties of soft tissues control their resistance to decay. However, experimental work has shown that reality is more complex: it is not possible to accurately predict sequences of character loss from composition alone. For example, lampreys possess several different anatomical characters composed of cartilage, but these differ in their resistance to decay and are not all lost at the same point in the decay sequence (Sansom *et al.* 2011, 2013). Notwithstanding the important subtleties of the sequence of character loss revealed by decay experiments, few taphonomists would argue against the view that the biochemical properties of tissues provide the fundamental control on their propensity to decay.

However, recent work within the field of microbial forensic science presents a potential challenge to this hypothesis and an alternative explanation for the repeated sequences seen in anatomical decay. Studies on humans, pigs and mice, in laboratory, terrestrial and aquatic

environments suggests that a distinct postmortem internal microbiome forms (termed the thanatomicrobiome; i.e. *thanatos*-, from Greek; death microbiome; Can *et al.* 2014), which colonizes and spreads through a carcass in a predictable succession controlled by changes in the physical and chemical environment (Dickson *et al.* 2011; Pechal *et al.* 2013; Metcalf *et al.* 2013, 2016; Can *et al.* 2014; Cobaugh *et al.* 2015; Guo *et al.* 2016; Javan *et al.* 2016, 2019; Lauber *et al.* 2014; Deel *et al.* 2020; Zhou *et al.* 2021; Aragonés *et al.* 2022). The formation of the thanatomicrobiome is suggested to be dynamically influenced by both the *ante mortem* internal (endogenous) microbiome and by exogenous bacteria that live on the carcass or in the surrounding environment (e.g. Dash & Das 2020). This raises the possibility that the reproducible patterns and sequences of anatomical decay seen in taphonomic experiments are controlled by microbial succession, potentially by various bacteria taxa specializing on different biomolecules and tissue types. This hypothesis, unless it can be rejected, poses a significant challenge to experimental analysis of the role of decay in fossilization: rather than reflecting the nature of exceptionally-well preserved fossil organisms themselves, the relative survival of soft tissues in fossilization would actually represent the succession of contemporaneous ancient microbial communities, about which we know nothing (and are unlikely to ever be able to model).

Here, we address two fundamental questions. First, what is the importance of exogenous and endogenous bacteria in the formation of the thanatomicrobiome within a carcass? It is possible that exogenous microbial species invade decaying carcasses before the carcass ruptures, and if they do so, they may dominate and/or replace the endogenous microbial communities. This question has a bearing on both the potential controls on decay (how important is the microbiota of the environment in which an organism decays) and the design of decay experiments. To our knowledge, no study has directly investigated whether exogenous bacteria invade an intact carcass immediately postmortem, yet the idea that exogenous bacteria are important drivers of decay provides the implicit methodological justification for the use of microbial inoculum in many previous investigations of decay (e.g. Briggs & Kear 1993a; Briggs *et al.* 1993; Hof & Briggs 1997; Sagemann *et al.* 1999; Duncan *et al.* 2003; Martin *et al.* 2004; McCoy *et al.* 2015). Despite the widespread use of inoculum, investigators generally know little about the community structures of the introduced microbiota or its role in subsequent decay.

Second, we address the key question outlined above: the hypothesis that the succession of the bacterial communities that develop as a carcass is broken down is correlated with the sequence of loss of anatomical information.

METHODS

The questions outlined above, expressed as null hypotheses, provide the structure for our experimental methods. This study was run in conjunction with an experiment to test hypotheses regarding the relationship between substrate entombment and character loss (DM unpub. data) and thus our methods are shared with this study. As outlined above, our experiments are designed to investigate specific variables that impact decay and preservation processes under controlled laboratory conditions. The experiments are not simulations of natural conditions and do not aim to mimic, recreate or represent particular environments or outcomes of fossilization (Purnell *et al.* 2018).

We selected amphioxus (*Branchiostoma lanceolatum*, phylum Chordata) as the organisms for this investigation. As a close living relative of early vertebrates, amphioxus has been the subject of several experimental decay investigations (Briggs & Kear 1994b; Sansom *et al.* 2010, 2011, 2013). The amphioxus were euthanized by overdose of tricaine methane sulphonate (MS-222; 2 mg/mL with buffer), following the same method as our previous work and in compliance with UK Government Guidance on the Operation of Animals (Scientific Procedures) Act, 1986, v.2014.

The null hypothesis regarding exogenous and endogenous bacteria is that the communities that develop within a decaying carcass through time are dominated by endogenous taxa; those that come from within the carcass itself (hypothesis 1). For this experiment, individual euthanized amphioxus (n = 10) were placed in clear polystyrene boxes (48 cm^3) containing artificial seawater (ASW; Tropic Marin mixed with distilled water; salt content 33–36 ppm, pH 8). An inoculum was then created using a sediment slurry collected from an estuarine setting. In many previous decay experiments, bacterial inoculums taken to be 'representative' of natural environments have been cultured from estuarine sediments (e.g. Briggs & Kear 1993a, 1994a, 1994b; Briggs *et al.* 1993; Hof & Briggs 1997; Sagemann *et al.* 1999; Duncan *et al.* 2003; Martin *et al.* 2004). Estuarine sediments were typically chosen as reported salinity tolerance of estuarine bacteria were thought to confer properties that are also useful to degradation and because these environments represent areas with elevated rates of organic matter degradation by both aerobic and anaerobic bacterial respiration (see Briggs & Kear 1993a). In these previous decay experiments, sediment and water mixtures have been collected from the Tay estuary, Scotland (e.g. Briggs & Kear 1993a, 1994a, 1994b; Briggs *et al.* 1993; Hof & Briggs 1997) and the Tamar River estuary, England (e.g. Sagemann *et al.* 1999; Duncan *et al.* 2003; Martin *et al.* 2004, 2005). We collected from Weir Quay, Tamar

River estuary, Devon. A small trench was dug close to the low tide mark, and seawater and sediment were collected per Martin *et al.* (2004) (this sediment presented as anoxic based on the clear transition from brown to black colour, accompanied by a strong sulphur smell); this sediment sludge was mixed at 50 mL per litre ASW per Briggs & Kear (1993a) and Martin *et al.* (2004). The polystyrene containers were sealed using silicon grease (Ambersil M494) to prevent gas exchange and invasion of bacteria external to the experiment. The containers were incubated at 25°C in a maturation chamber for the duration of the experiments, following our standard lab protocols, and were removed at approximately logarithmically spaced intervals (days 1, 28, 134 and 1138). For each sampling point there was one control (decayed in ASW without inoculum) and three inoculated amphioxus. At each sampling point, morphological character presence/absence data were collected from each amphioxus carcass using microscopy, and then each sample was destructively sampled for DNA extraction. Amphioxus organic material was removed from the polystyrene container, placed in a 2 mL microcentrifuge tube, and suspended in an amount of lysis buffer (Tris-1 mM EDTA buffer (pH 8.0) containing 5 mg/mL proteinase K and 10% (w/v) sodium dodecyl sulfate) needed to create a 2 mL sample. The sample was then vortexed until fully homogenized, flash frozen at −80°C and stored until required.

The null hypothesis regarding bacterial community succession and anatomical decay is that the communities that develop within a decaying carcass through time are not correlated with the sequence of character loss (hypothesis 2). The experiment was designed to test this hypothesis alongside an analysis of the relationship between character loss and sediment entombment (DM unpub. data), with samples of amphioxus decayed in three different sterile substrates: kaolinite (Puraflo China Clay, Clayman Supplies Ltd.), mica (muscovite, 2 μm to 1 mm, Imerys Ceramics), and bentonite (predominantly montmorillonite from Wyoming, Clayman Supplies Ltd.). Pure sterile substrates were chosen to limit uncontrolled variables, and to increase experimental replicability. The substrates were mixed with the ASW (Tropic Marin mixed with distilled water; salt content 33–36 ppm, pH 8) to form a sludge, each approximately equal in consistency and density (clay by weight: ASW – kaolinite, 3:4 = 1.5 g/cm^3; mica, 6:5 = 1.6 g/cm^3; bentonite, 2:3 = 1.4 g/cm^3). The slurries were then poured into clear polystyrene boxes (48 cm^3), filling them to the halfway mark. A stainless-steel mesh was laid on top of the sludge (to aid carcass exhumation at the sampling interval) and a freshly euthanized amphioxus was placed on the mesh. The container was then filled with one of the sludges, entombing the carcass completely, and incubated at 25°C for the duration of the experiment. Three samples of each substrate type were removed from the incubator at the following sampling points: days 2, 8, 21, 28, 35, 42 and 134.

Exhumation of samples entombed within a substrate presents a challenge, as removal can cause damage to the carcass. Each sample was flash frozen in liquid nitrogen immediately after the containers were removed from the incubator, and the frozen substrate block (with entombed carcass) was carefully removed from the polystyrene box. In sterile conditions, the substrate block was cracked open along the plane of the stainless-steel mesh, exposing the overlying carcass, which was then examined to identify morphological character states. The underlying substrate block was kept frozen. From this, a layer of approximately 1 mm depth was sampled from immediately beneath the carcass; this material included the substrate closest to the carcass during decay, and any persisting organic remains. This was placed in a sterilized beaker of lysis buffer, producing a slurry. As the experimental setup was sterile, any bacteria found in the slurry must be derived from the carcass. This slurry was vortexed and poured into a 2 mL microcentrifuge tube for DNA extraction and then flash frozen and stored until analysis. DNA extraction was performed on 53 viable samples across all substrates (see Clements *et al.* 2025, table 1.1). DNA extraction methods are outlined below. It should be noted that there was a high failure rate in DNA extraction, resulting in 10 successful extractions (see experimental limitations below).

Bacterial DNA extraction, amplification & sequencing

All DNA extraction, purification, and sequencing was performed in the Department of Infection, Inflammation, and Immunity laboratories at the University of Leicester. The samples were thawed and DNA extraction was carried out using the method appropriate for the sample type (e.g. sediment, tissue, solution) using the phenol–chloroform–isoamyl alcohol method as outlined in Nale *et al.* (2016), Qigen DNeasy Blood and Tissue kits, or DNeasy PowerMax Soil kits (see Clements *et al.* 2025, table 1.1). Once DNA was extracted from the samples, the 16S rRNA gene region was sequenced using Illumina HiSeq sequencing (e.g. Imam *et al.* 2019). The sequencing protocols used in this study followed the Illumina protocol for 16S Metagenomic Sequencing Library Preparation Revision B (Illumina Inc. 2013). Primers used targeted the V3–V4 region of the 16S ribosomal RNA gene and can be found in Clements *et al.* (2025, table 1.2). DNA sequencing allows bacteria within the samples to be categorized as operational taxonomic units (OTUs), and we used the USearch pipeline to process reads into OTU calls; the USearch sintax algorithm was used to designate

the OTUs using the Ribosomal Database Project (RDP; the reference database for 16S rRNA gene sequences). This allows calculation of the types of bacteria (referred to by OTU code; see Clements *et al.* 2025, table 5.1 for raw data including read counts), the bacterial community structure, relative, and absolute abundances (reported in Clements *et al.* 2025, table 2). qPCR was used to calculate the absolute number of bacterial cells in each sample by targeting the 16S rRNA gene using a Femto Bacterial DNA Quantification Kit (Zymo Research International) following the manufacturer's instructions. Kit controls, a phenol blank, and a negative control were run to test for any potential contamination in the methodology; contamination values can be seen in Clements *et al.* (2025 table 2.4). These values were subtracted accordingly from the final qPCR results in Clements *et al.* (2025, table 2.1). DNA sequencing was also carried out on a negative control (no contamination was found), and a positive control containing *Haemophilus* bacteria (OTU 2).

Analytical methods & utilization of comparative decay data

To test the similarity/dissimilarity of community structure and diversity between all samples where sequence data were successfully collected (i.e. hypothesis 1), we conducted a principal coordinate analysis (PCoA) using the software PAST v4.13 (Hammer *et al.* 2001); this allows analysis of bacterial community structure while removing abundance biases.

The analysis investigating the relationship between the change in the bacterial community structure through time and sequence of morphological character loss (i.e. hypothesis 2) was undertaken using detailed presence/absence decay data for anatomical characters in amphioxus. These data are comparable to data collected in previous analyses of chordate decay (e.g. Sansom *et al.* 2010, 2011). The bacterial communities within the decay environment on specific sampling days were directly compared with these morphological character data (see Clements *et al.* 2025, table 3). To investigate the potential correlation between character loss and bacterial community structure, we used a further principal coordinate analysis. As morphological character data in decay experiments are binary (presence/absence), we had to transform our bacterial data to be comparable, and so we converted the OTU data into presence/absence binary data for each sample. Spearman's Rank correlations were used to statistically compare the 'amphioxus decay space' and the 'bacterial community structure space' within the PCoA, to investigate any potential link between changes in bacterial community structure and the timing of loss of morphological amphioxus characters during decay.

The potential impact of tricaine methane sulphonate on bacterial communities

As noted above, we followed the methods of Sansom *et al.* (2010) in using tricaine methane sulphonate (MS-222; 2 mg/mL with buffer) to euthanize amphioxus. We observe here, however, that the statement by Sansom *et al.* (2010) that this 'does not adversely affect bacteria' is potentially misleading. The study they cite in support of this (Fedewa & Lindell 2005) actually found that at the concentrations of MS-222 used by Sansom *et al.* (2010) bacterial growth is inhibited. This does not necessarily mean our results are in question; the investigation of Fedewa & Lindell (2005) was designed to test the inhibitory effects of MS-222 on bacterial growth and the impact on analyses of bacteria using standard culture techniques. Consequently they *incubated* bacteria in media containing MS-222. For our analysis, on the other hand, amphioxus were immersed in MS-222 for only a short period. The subsequent decay took place in the absence of MS-222, so we have no reason to suspect that growth of bacterial communities would be impacted in our experiments. Fedewa & Lindell (2005) also acknowledged that the design of their experiments did not allow them to determine the effects of MS-222 on proliferation of internal bacteria, and that bacteria in tissue samples (closer to our situation) 'may remain unaffected at concentrations that inhibit external bacterial proliferation'. We also note that the majority of bacteria investigated by Fedewa and Lindell are Gammaproteobacteria, and although we did not identify the same species, Gammaproteobacteria are relatively abundant in a number of our samples, including the earliest, taken at day 28. Flavobacteriaceae, a species of which Fedewa and Lindell observed to be among the most sensitive to MS-222, are also relatively abundant in several of our samples (including day 28).

We are aware of little other work on the effects of MS-222 on bacteria, but perhaps the most relevant is Older *et al.* (2024). Their study included a comparison of bacteria in samples of skin, gills and gut contents from catfish euthanized by overdose of MS-222 (300 mg/L) with equivalent samples from catfish euthanized by cranial concussion and pithing. They found evidence that bacterial alpha and beta diversity differed between euthanasia methods, but only those detected in skin samples were statistically significant. Samples of gut contents, most relevant to our study, showed the fewest differences between samples from the fish. Older *et al.* (2024) suggested that further work was required to better understand how euthanasia methods might influence bacterial analysis in the context of aquaculture. We make a similar recommendation for research into the microbiome of decay. The available evidence, however, does not suggest that our use of MS-222 has significantly impacted our results.

Limitations of experimental design

It is important to highlight difficulties with collecting data for these types of experiments, to note the low replicate numbers presented here, and to frankly caveat the interpretation of our findings. Although we used appropriate DNA extraction kits for our experiment, we had a high rate of DNA extraction failures (see Clements *et al.* 2025, table 1.1; failures highlighted in yellow). We had a 94% failure rate of extracting viable bacterial DNA from amphioxus carcasses buried in kaolinite and mica sediments. There was also a high proportion of failures in bentonite (failure rate = 58%). Furthermore, the high level of failure means that on some sampling days there were complete extraction failures (e.g. days 8 and 42), while on others there were a low number of successful replicates. This limits our testing of the hypothesis regarding bacterial community succession and anatomical decay (i.e. hypothesis 2) to samples utilizing bentonite, reducing the temporal resolution of our results. DNA extraction failures are not uncommon when attempting to extract bacterial DNA from decaying carcasses, and this has been previously attributed to low microbial biomass (e.g. Metcalf *et al.* 2013). It is difficult to positively identify the cause of these DNA extraction failures, however, it is possible that variables such as refreezing may have contributed. Nonetheless, this work serves as proof of concept and the experimental design has been refined in our ongoing investigations.

RESULTS

Across all samples examined in this study, 575 bacterial OTUs were identified: 509 to phylum level, 375 to order level, and 196 to genus level (Clements *et al.* 2025, table 1.3). The samples varied in the total number of OTUs (average: 153 OTUs, range: 11–379; Clements *et al.* 2025, table 2.1).

Data from the PCoA analysis investigating similarity/dissimilarity of community structure (Fig. 1A; Clements *et al.* 2025, table 4) reveal several important findings pertaining to the bacterial community structures of inoculated and non-inoculated decaying carcasses. Firstly, inoculum created from sediment and seawater taken from the Tamar River estuary, UK, has a very different bacterial community structure to the native bacterial community found within a decaying amphioxus carcass. In fact, the community structure of the Tamar River inoculum is clearly distinct from all samples in this study, even samples where carcasses were decayed in inoculated ASW.

Secondly, the data from the experiments designed to test hypothesis 1 show that decaying amphioxus carcasses in inoculated ASW had the largest data spread, indicating that the bacterial community varied in structure through time as decay progressed. The variability in bacterial community structure seen between the inoculated samples is greater than the variability between samples decayed in non-inoculated ASW, or samples decayed within sediment without inoculum. The data show the bacterial community structure within amphioxus carcasses that decayed in non-inoculated ASW varied through time. In comparison, the bacterial communities within the amphioxus carcasses decaying in sediment show greater community structure variability through time than control samples, but far less than those samples decayed in inoculated ASW. In terms of coordinate space, there is some minor overlap between the inoculated samples, and the non-inoculated samples; interestingly this overlap only occurs with the inoculum sample from day 1138. It should be noted that the non-bentonite sediment samples (amphioxus decayed in kaolinite or mica) do not overlap with the inoculated samples, however, this result should be interpreted cautiously as the high failure rate of DNA extraction in these sediments means there are limited replicates.

The experiment designed to test hypothesis 2 allowed investigation of the link between changes in bacterial community structure through time within a decaying amphioxus carcass entombed in bentonite (Fig. 1B; Table 1; Clements *et al.* 2025, table 4) and the timing of the loss of anatomical characters during decay (Fig. 1C; Table 1). Spearman's Rank tests found a significant correlation between bacterial community structure (PCo 2 values in Fig. 1B) and 'time' (ranked time = sequence). Spearman's Rank correlation of sequence of character loss (PCo 1 values in Fig. 1C) and organismal completeness was also significant (see also Table 1). However, we find no correlation between PCoA values representing bacterial community structure and organismal completeness. This result suggests that the change in bacterial community through time is not linked to the sequence of anatomical character loss seen in a decaying amphioxus. There is nothing in our results that might lead us to reject the hypothesis that anatomical character loss is controlled by the intrinsic properties of the characters themselves.

Bacterial succession dynamics within decaying amphioxus carcasses

The succession of bacterial communities through time within amphioxus carcasses decaying in inoculated and non-inoculated conditions are shown in Figure 2. The data show that there is a succession of bacterial communities through time, although they are typically dominated by one or two OTUs.

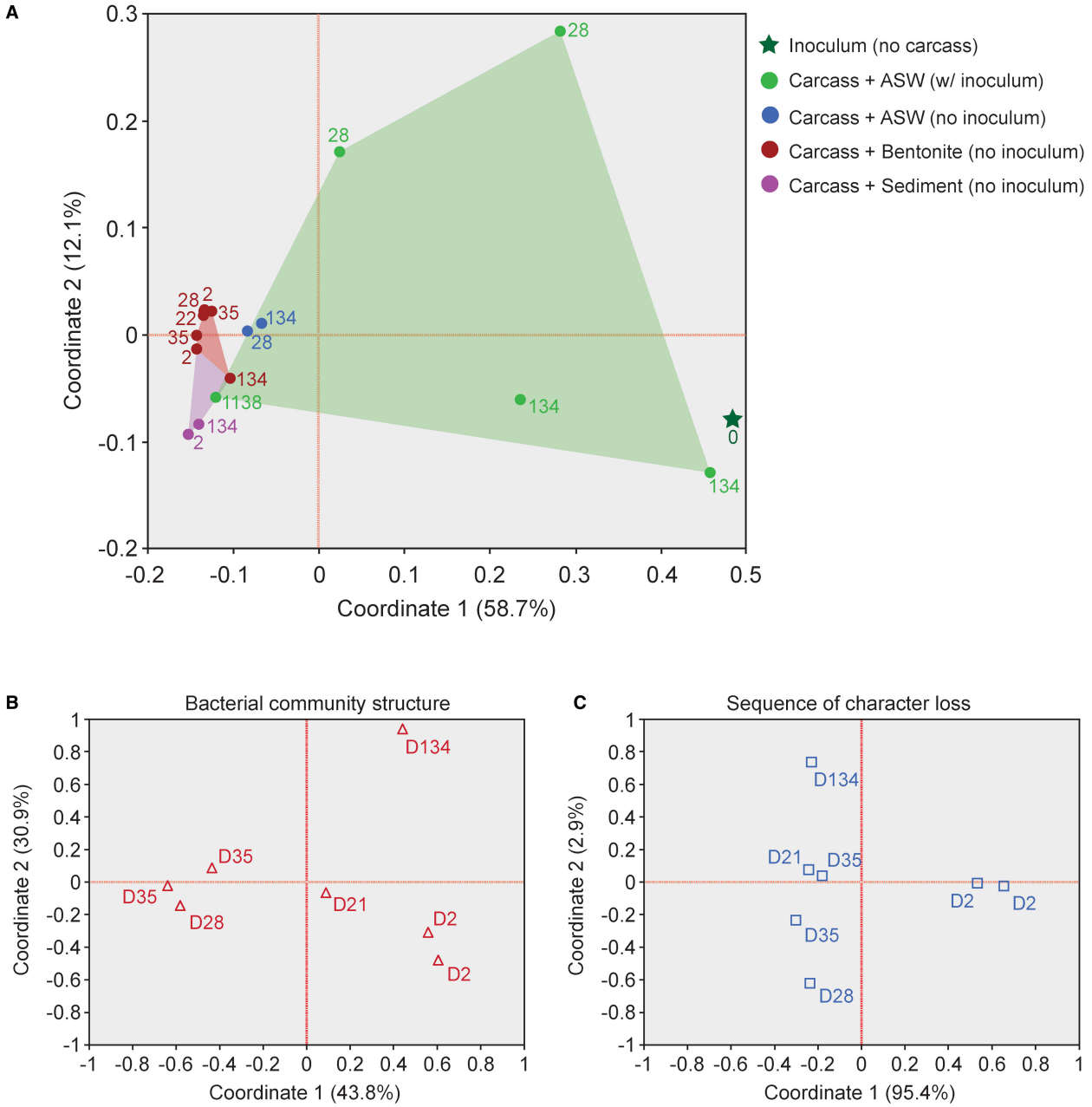


FIG. 1. Analyses of bacterial communities within decaying amphioxus (*Branchiostoma lanceolatum*) carcasses. A, principal coordinate analysis (PCoA) of presence/absence of bacterial OTUs in artificial seawater (ASW) controls, inoculated ASW samples, sediment samples, and pure 'natural' inoculum sample (without carcass), to display the disparity of bacterial community structure between each sample; purple points represent non-bentonite sediment samples (kaolinite and mica). B, PCoA of presence/absence of bacterial OTUs in bentonite sediment samples, to display the disparity of bacterial community structure between each sample. C, PCoA of presence/absence of morphological characters within the decaying amphioxus carcass at each sampling point. Coordinate variance is represented as a percentage. For eigenvalues and all coordinate values, see Clements *et al.* (2025, table 4).

The pure inoculum has a diverse bacterial community with 363 identified OTUs and the highest Shannon's equitability value of all samples (0.764; see Fig. 2; Clements *et al.* 2025, table 2.2). However, the majority of the OTUs identified represent tiny fractions of the overall community; 93% of OTUs each individually represent less than 1% of the relative abundance within the bacterial community. The inoculum's bacterial community is dominated by Chromatiales, commonly known as purple sulphur bacteria (Hunter *et al.* 2009). Other major components of the bacterial community are Desulfuromonadaceae, Desulfuromonadales, Rhodobacterales, as well as

TABLE 1. Spearman's rank correlations for PCoA co-ordinates relating to bacterial community structure (Bac Com; see Fig. 1B) and sequence of character loss (Dec Seq; see Fig. 1C).

Experiment	Variable 1	Variable 2	Spearman's rho	p-value
Bacterial Community Structure*	Coord 1 Bac Com	Time	−0.5637	0.1875
Bacterial Community Structure*	Coord 2 Bac Com	Time	**0.9456**	**0.0013**
Bacterial Community Structure*	Coord 3 Bac Com	Time	−0.0909	0.8463
Bacterial Community Structure*	Coord 4 Bac Com	Time	−0.2546	0.5817
Bacterial Community Structure*	Coord 5 Bac Com	Time	−0.0182	0.9691
Bacterial Community Structure*	Coord 6 Bac Com	Time	−0.0364	0.9383
Sequence of Character Loss†	Coord 1 Dec Seq	Completeness	**0.9636**	**0.0005**
Sequence of Character Loss†	Coord 2 Dec Seq	Completeness	0.0741	0.8745
Sequence of Character Loss†	Coord 3 Dec Seq	Completeness	0.0371	0.9371
Sequence of Character Loss†	Coord 4 Dec Seq	Completeness	0.0385	0.9348
Bac Com vs Seq Character Loss‡	Coord 1 Dec Seq	Coord 2 Bac Com	−0.6071	0.1482

Results highlighted in **bold** represent significant correlations.
*Test comparing the presence/absence of bacterial OTUs in bentonite sediment samples against sample day (i.e. time).
†Test comparing the presence/absence of morphological characters within the decaying amphioxus carcass at each sampling point with organismal completeness (see Clements *et al.* 2025, table 3).
‡Test comparing the coordinates displaying significant correlations between Figure 1B and C.

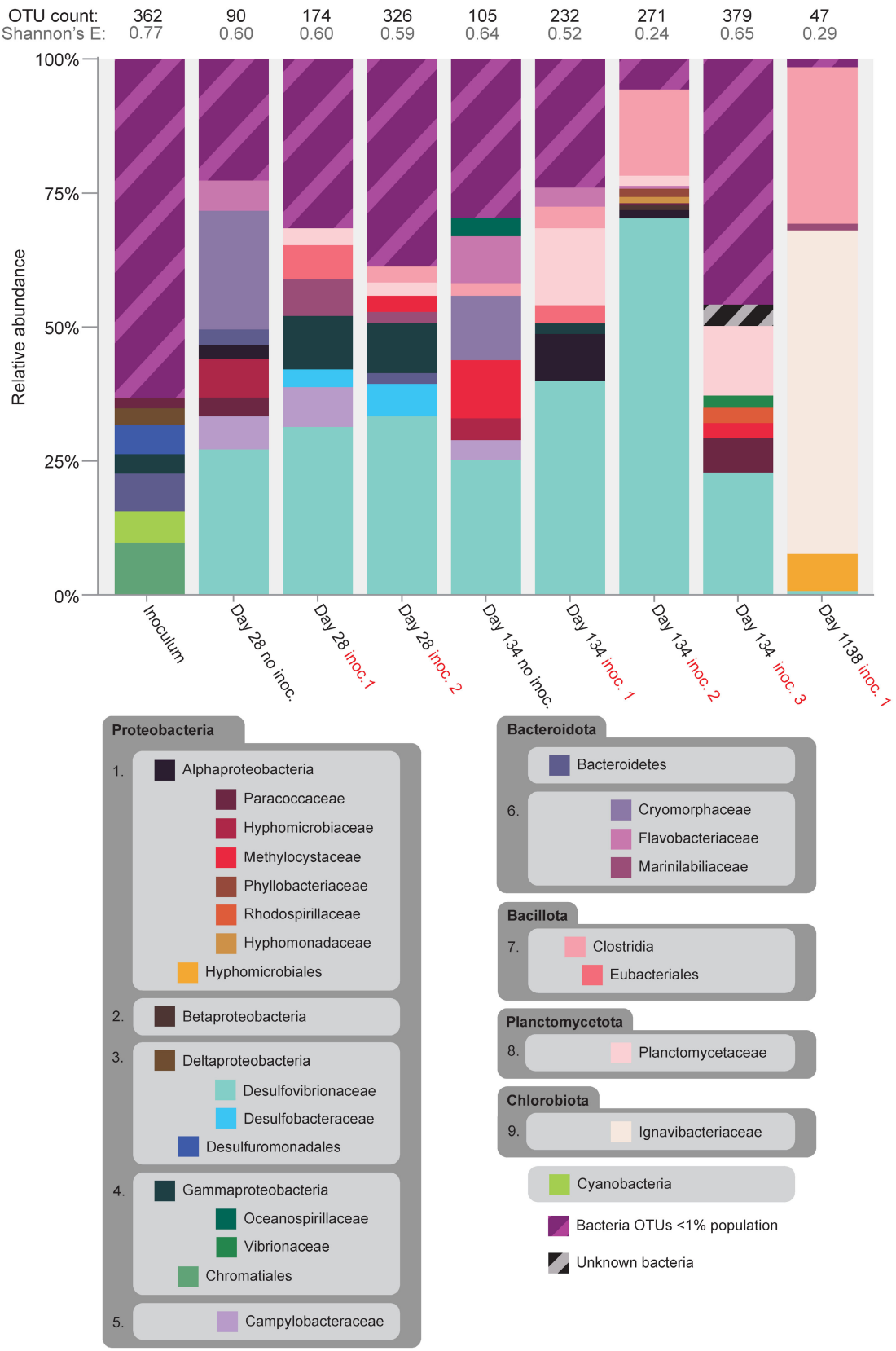
OTUs representing Cyanobacteria, Bacteroidetes, Gammaproteobacteria, Bacteroidetes, Deltaproteobacteria although these OTUs cannot be identified beyond the order level.

The number of bacterial OTUs in the population of the day 28 decaying amphioxus carcass in a non-inoculated medium is smaller, with 90 OTUs identified; only 17% of the OTUs individually represent a relative abundance greater than 1% of the bacterial community. The bacterial community structure differs from the inoculum, being dominated by Desulfovibrionaceae (OTU 5 in Clements *et al.* 2025, table 5; 27% relative abundance). At day 134 the number of OTUs in the bacterial community of the non-inoculated decaying amphioxus carcass is also smaller than the inoculum with 105 OTUs identified; again, only 17% of the OTUs have an individual relative abundance greater than 1% of the bacterial community. The bacterial community in this sample is also dominated by Desulfovibrionaceae (OTU 5; 25% relative abundance). Both of the bacterial communities of these samples are secondarily dominated by Cryomorphaceae (22% and 11% respectively); however, the other dominant bacterial OTUs vary between samples, potentially indicating some disparate bacterial community structural changes through time.

Importantly, our data show that, despite having much richer communities in terms of identified OTUs, the dominant OTU in all the inoculated samples is Desulfovibrionaceae (OTU 5; day 28, 31% and 32%; day 134, 39%, 70% and 21%). Desulfovibrionaceae (OTU 5) was not identified in the inoculum (although note the low number of inoculum samples), suggesting that in samples containing decaying carcasses, this bacterial taxon represents a native OTU from the carcass's endogenous bacterial population (although Desulfovibrionales are known to be found in the environment). Comparatively, with the exception of the second replicate of day 134 (70% relative abundance), the relative proportion of Desulfovibrionaceae is similar to the non-inoculated samples (*c.* 30%). Our data show that there are some bacterial community structure changes through time in the inoculated samples: samples on day 28 share high relative abundance of two Gammaproteobacteria taxa (OTU 90 and 73), whereas neither of these bacteria have abundance above 1% in the day 134 samples. The bacterial communities compared between inoculated samples on day 134 are varied, sharing few dominant OTUs outside of Desulfovibrionaceae (OTU 5).

OTU data were also collected on day 1138, well after the carcass had lost structural integrity and fully

FIG. 2. Relative abundance of dominant microbes within samples of pure inoculum and within decaying amphioxus (*Branchiostoma lanceolatum*) carcasses through time. Named bacteria in each sample are those with a relative proportion greater than 1% of the total population. All bacteria with relative abundances representing less than 1% of the community are agglomerated into the 'Bacteria OTUs <1% population' category. Bacteria taxonomy is reported to the family (or lowest possible) taxonomic rank based on Parte *et al.* (2020) and Oren & Garrity (2021), sub-organized by class: (1) Alphaproteobacteria; (2) Betaproteobacteria; (3) Deltaproteobacteria; (4) Gammaproteobacteria; (5) Epsilonproteobacteria; (6) Flavobacteriia; (7) Clostridia; (8) Planctomycetia; (9) Ignavibacteria.


OTU count: 362 90 174 326 105 232 271 379 47
Shannon's E: 0.77 0.60 0.60 0.59 0.64 0.52 0.24 0.65 0.29
100%
75%
50%
25%
0%
Relative abundance
Inoculum
Day 28 no inoc.
Day 28 inoc.1
Day 28 inoc. 2
Day 134 no inoc.
Day 134 inoc. 1
Day 134 inoc. 2
Day 134 inoc. 3
Day 1138 inoc. 1
Proteobacteria
1. Alphaproteobacteria
Paracoccaceae
Hyphomicrobiaceae
Methylocystaceae
Phyllobacteriaceae
Rhodospirillaceae
Hyphomonadaceae
Hyphomicrobiales
2. Betaproteobacteria
3. Deltaproteobacteria
Desulfovibrionaceae
Desulfobacteraceae
Desulfuromonadales
4. Gammaproteobacteria
Oceanospirillaceae
Vibrionaceae
Chromatiales
5. Campylobacteraceae
Bacteroidota
Bacteroidetes
6. Cryomorphaceae
Flavobacteriaceae
Marinilabiliaceae
Bacillota
7. Clostridia
Eubacteriales
Planctomycetota
8. Planctomycetaceae
Chlorobiota
9. Ignavibacteriaceae
Cyanobacteria
Bacteria OTUs <1% population
Unknown bacteria

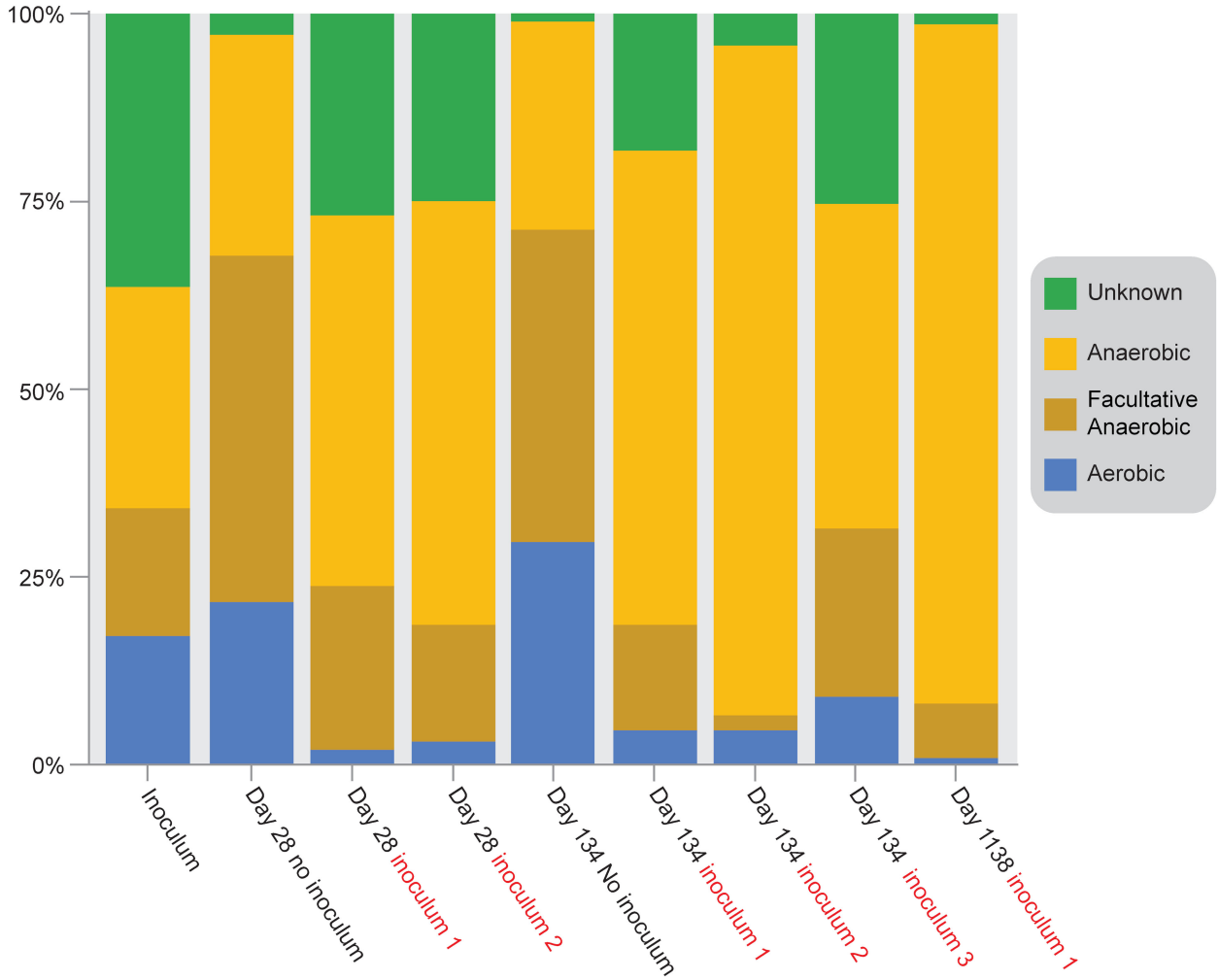


FIG. 3. Relative proportions of the respiratory mode of the bacterial communities within decaying amphioxus carcasses through time. See Clements *et al.* (2025, table 7).

disarticulated. Despite this sample having been inoculated at the onset of the experiment, its bacterial diversity on day 1138 was drastically different from all previous samples. The bacterial community was the smallest of any of the samples in this experiment stream with 47 identified OTUs and the lowest Shannon's equitability value of the whole experiment (0.14; see Fig. 2; Clements *et al.* 2025, table 2.2). Proportionally, this sample is dominated by Ignavibacteriaceae (OTU 434; 60%) and Clostridia (OTU 28; 28%), two bacterial taxa that do not present above 1% in any other previous sample. In fact, the day 1138 sample was dominated by four bacteria OTUs that had a relative abundance above 1%. The other 43 bacteria OTUs account for *c.* 3% of the relative abundance, and this includes Desulfovibrionaceae (OTU 5) which only accounted for a relative abundance of 0.6%.

Calculating the structure of the bacterial communities provides insights into the dominant mode of bacterial respiration in each sample, although we did not include chemolithoautotrophs in this assessment (Fig. 3). The bacterial community of the inoculum is dominated by bacteria that use anaerobic respiration (46%), but it also has the highest proportion of bacteria with an unknown mode of respiration (37%). The proportion of bacteria with an unknown respiratory mode is consistently higher in inoculated samples than in non-inoculated samples. Bacterial populations within the decaying carcasses are dominated by bacteria that respire anaerobically (average: 71%). Further examination of the anaerobic bacteria data does indicate that, in the non-inoculated samples, greater proportions of the bacterial community's diversity is made up of facultative anaerobes. This is not the case in the inoculated samples. Aerobically respiring bacteria are identified in all samples as small proportions of the community, although proportionally these communities are much lower in samples that were inoculated. The

day 1138 sample is overwhelmingly dominated by anaerobically respiring bacteria (*c.* 98%) with almost no aerobically respiring bacterial OTUs (>1%) and a very low component of unknown bacteria (*c.* 1%).

DISCUSSION

We are unable to reject our null hypothesis that the bacterial communities that develop within a decaying carcass through time are dominated by endogenous taxa; in both the inoculated samples and the control, the dominant bacterial OTUs of the thanatomicrobiome were not specific to, or found in the inoculum. The bacterial community of the inoculum was diverse, comprising in large part bacterial OTUs falling into the <1% abundance category, most of which are not found in either the control or inoculated samples. Adding an inoculum to decay experiments does not impact the endogenous thanatomicrobiome community structure, nor do exogenous bacteria seem to actively invade the carcass through the period of decay during which the carcass maintains its integrity. Our data show that in amphioxus carcasses, the internal thanatomicrobiome is typically dominated by the Desulfovibrionaceae: a family of anaerobic, sulphate-reducing bacteria which, while found in the general environment, are common components of the endogenous gut microbiota in a wide range of animals (e.g. Galushko & Kuever 2020; Sayavedra *et al.* 2021; Singh *et al.* 2023). Our data, therefore, suggest that endogenous anaerobic bacteria that inhabit an animal in life, proliferate and dominate the internal thanatomicrobiome within a decaying carcass (although the timing and propagation is poorly understood; see next section for detailed description of thanatomicrobiome formation). This result has implications for the design of decay experiments. An exogenous inoculum added to a decay environment contains a large number of bacterial taxa with low abundance that are not persistent in the thanatomicrobiome during decay. Inocula are not required to initiate decay (e.g. Briggs & Kear 1993a, Sansom *et al.* 2010; Clements *et al.* 2017, 2022) and in conjunction with our findings, the addition of a natural inoculum to an experiment investigating anatomical decay contributes nothing more than an uncontrolled and potentially confounding variable. Without detailed microbial community analysis, the use of such inocula creates significant replicability issues, especially as bacteria are only a part of any natural inoculum taken from the wild: Fungi and Archaea may also be present. This, and our results, leads us to recommend that for most decay experiments, inocula are not used.

We are also unable to reject our second null hypothesis, that the bacterial communities that develop within a decaying carcass through time are not correlated with the sequence of character loss. The community structure of the internal thanatomicrobiome is temporally dynamic through the decay process, but the lack of correlation is consistent with the hypothesis that, within a decaying carcass, it is not the succession of the bacterial communities that drives the patterns of anatomical character loss. But how far can we extend our results beyond amphioxus, and what do they mean for analysis of how non-biomineralized anatomy becomes fossilized? Gut microbiomes vary considerably between animals, even within a species (e.g. Luan *et al.* 2023; see references in Davenport *et al.* 2017), posing the question of whether this might lead to variation in sequences of character loss. Current evidence suggests not because: (1) sequences of anatomical loss are conserved between individuals of the same species and, where homologous characters allow comparison, between species (Sansom *et al.* 2010, 2011; Murdock *et al.* 2014; Cavicchini *et al.* 2025); and (2) postmortem, there is significant microbial turnover (dysbiosis) in a carcass, with anaerobic bacteria, normally found in small numbers in the guts of animals (see below), coming to dominate the internal thanatomicrobiome within hours of death. We are undertaking experiments to investigate this further, but considering our results here, it seems that despite the likely variation in initial endogenous microbiotas between specimens, internal microbial communities probably exhibit a high degree of convergence postmortem, particularly in terms of metabolic pathways to tissue degradation. Variation between living organisms' endogenous microbiotas is thus likely to be significantly reduced after death.

These factors, combined with our results, have significant implications for the field of experimental decay and taphonomy: lack of knowledge of the composition of ancient microbial communities that caused decay and character loss in organisms in deep time does not matter. Bacterial successions are not correlated with anatomical character loss; non-biomineralized characters preserved in fossils correlate with their propensity to decay in extant relatives (e.g. Gabbott *et al.* 2021); the sequences of character loss are conserved between individuals and taxa (e.g. Sansom*et al.* 2010, 2011, Murdock *et al.* 2014, Cavicchini *et al.* 2025) despite the likelihood of differences in gut microbiota between organisms in life. Together, this indicates that the specific taxa present in the microbiota are not likely to be significant in controlling the loss of characters. Crucially, this supports the foundational hypothesis that anatomical decay is an experimentally tractable route to understanding how exceptionally preserved fossils form.

Formation of the thanatomicrobiome, the microbial clock & implications for soft tissue fossils

The concept of the thanatomicrobiome as a distinct microbiome within and around a decaying carcass is relatively

new (Can *et al.* 2014). In forensic science, understanding the formation and succession of the thanatomicrobiome has attracted attention as a potential tool for criminal investigations, and more precise determination of 'time of death' or the postmortem interval (PMI) of cadavers than more traditional methods (see Javan *et al.* 2019; Metcalf *et al.* 2016; Deel *et al.* 2020). The rationale that postmortem interval can be resolved by investigation of the thanatomicrobiome comes from several studies that have identified a predictable 'clock-like' microbial community succession that occurs within and around a decaying carcass (e.g. Dickson *et al.* 2011; Pechal *et al.* 2013; Metcalf *et al.* 2013, 2016; Can *et al.*, 2014; Cobaugh *et al.* 2015; Guo *et al.* 2016; Javan *et al.* 2016; Deel *et al.*, 2020; Zhou *et al.* 2021; Aragonés *et al.* 2022).

The importance of the thanatomicrobiome in a taphonomic context has not previously been considered explicitly. The link between bacteria and soft tissue preservation is well established, and we know that bacteria directly and indirectly influence preservational processes such as mineralization (e.g. Sagemann *et al.* 1999; Clements *et al.* 2022; reviewed below), but what are the implications for the fossilization of non-biomineralized anatomy? Unfortunately, the relatively high number of DNA extraction failures in our study limits the temporal resolution of our view of the formation and dynamics of the thanatomicrobiome immediately postmortem. Nevertheless, our data show that, even in the presence of an inoculum, the internal thanatomicrobiome is dominated by endogenous bacteria until the carcass ruptures and exogenous bacteria can enter. This is vital for understanding soft tissue preservation: experimental results indicate that many soft tissue characters, including internal organs, are typically lost to decay before the carcass ruptures (e.g. Clements *et al.* 2022). How well does this accord with previous work on how the thanatomicrobiome develops and changes through time?

Before death, animals teem with bacteria; it is reported that the ratio of human cells to bacteria is approximately 1:1 (Sender *et al.* 2016; Walker & Hoyles 2023). In healthy living animals, while oral and skin microbiomes exist, only the guts and lungs have significant resident internal microbiotas. Most organs are considered to be largely sterile, with the immune system acting to restrict the organs and tissues that bacteria are able to inhabit (e.g. Can *et al.* 2014; Javan *et al.* 2016; Aragonés *et al.* 2022). Upon death, however, the immune system ceases to function and the mechanisms that control bacterial proliferation fail, allowing bacteria to rapidly colonize the carcass (Can *et al.* 2014; Aragonés *et al.* 2022). Where do these bacteria come from? In their review, Janssen *et al.* (2022) stated that both endogenous and exogenous bacteria contribute to decay and subsequent preservational processes, and while this is true, we note an important caveat, especially when considering the preservation of internal soft tissues: our data do not support the hypothesis that exogenous bacteria invade and colonize a carcass before internal tissues decay, unless the carcass ruptures or is otherwise compromised. In fact, our data, and other studies that decayed arthropods, mice, and human carcasses, indicate that gut bacteria proliferate and colonize the body cavity rapidly during decay, creating a distinct internal postmortem thanatomicrobiome (e.g. Metcalf *et al.* 2013; Tuomisto *et al.* 2014; Butler *et al.* 2015; Javan *et al.* 2019; Akutsu *et al.* 2020). The dominant bacteria found in our samples, Desulfovibrionaceae, is a group of anaerobic bacteria found commonly in the guts of animals (e.g. Kuever 2014; Galushko & Kuever 2020; Sayavedra *et al.* 2021). The formation of the thanatomicrobiome is still under investigation, but we know that during the first few hours of decay, the bacterial population within the gut is highly dynamic; high temporal resolution sampling of the thanatomicrobiome of decaying mice identified a distinct phase of gut bacterial community dysbiosis in the first 24 hours postmortem (Aragonés *et al.* 2022). In the first hours of decay, the 'normal' intestinal bacterial community collapses, probably due to the exhaustion of residual oxygen, and an ecological succession occurs where anaerobic bacteria outcompete and replace aerobic forms; this period, referred to as the adaptive phase, lasts for approximately 12 hours, until the anaerobic bacterial populations are established (Aragonés *et al.* 2022; our data support this). Experiments on human cadavers have shown a similar pattern of postmortem gut bacteria dysbiosis, demonstrating that the thanatomicrobiome becomes dominated by a few genera (Metcalf *et al.* 2013; DeBruyn & Hauther 2017; Zhou *et al.* 2021). This finding is pertinent for soft tissue preservation because the rapid switch from aerobic to anaerobic as the main mode of bacterial respiration is a vital step in the generation of the geochemical conditions required for some types of authigenic mineralization to occur (e.g. Sagemann *et al.* 1999; Clements *et al.* 2017, 2022; see below).

For bacteria found in the gut during life to dominate the internal thanatomicrobiome, they must somehow proliferate throughout a carcass. During the decay of *Artemia*, Butler *et al.* (2015) reported that bacteria escaped from the gut when the organ began to lose structural integrity. Clements *et al.* (2022) similarly reported that during the decay of European sea bass (*Dicentrarchus labrax*) small ruptures occurred at the stomach and anal terminations which, theoretically, could allow gut bacteria to escape. While this is a plausible route for gut bacteria to colonize the body cavity, Clements *et al.* (2022) reported that the gut was one of the last organs in a rotting seabass to lose structural integrity with visible breakdown of the gut wall occurring on the 11th day of decay. Therefore, it

seems less likely that gut breakdown can account for the rapid and sequential manner of organ colonization by anaerobic gut bacteria as seen in human cadavers (e.g. Javan *et al.* 2019; Zhou *et al.* 2021). An alternative explanation has been suggested: bacteria are typically highly motile (Janssen *et al.* 2022), and prior to the breakdown of the gut wall, as the immune system ceases to function, gut bacteria may colonize other organs via the capillaries of the vascular and lymphatic system (Noriko 1995; Javan *et al.* 2019; Dash & Das 2020). Logically, this must occur rapidly, because organ decay typically begins within hours of death (e.g. Gill-King 1997; Clements *et al.* 2022). We are currently undertaking a detailed investigation to establish empirically how anaerobic gut bacteria proliferate and become established throughout the carcass.

As decay proceeds, decay-related gases build up within the carcass. Commonly, this is most prominent in the gut region, presumably because anaerobic bacterial communities are quickly established in the gut, and the gut itself acts to restrict the movement of gases. Eventually, if gas cannot escape the gut can rupture, occasionally with enough force to breach the integrity of the carcass (e.g. Metcalf *et al.* 2013; Clements *et al.* 2022; Aragonés *et al.* 2022). Together with rupture of the dermis during general disarticulation of the carcass, exogenous bacteria can rapidly ingress, colonize, and replace endogenous populations (e.g. Metcalf *et al.* 2013; Javan *et al.* 2019; Aragonés *et al.* 2022).

While the carcass is decaying, exogenous microbial communities in the surrounding sediment also experience community shifts. Experiments have demonstrated that after burial, the low bacterial biomass on the skin of a carcass is overwhelmed by soil bacteria, creating a converging external decomposer community (e.g. Metcalf *et al.* 2013, 2016). Sediment type, burial depth, and other variable environmental conditions outside the carcass can make exogenous bacterial colonization hard to predict, limiting its use as a tool for postmortem interval estimations (Lemon *et al.* 2012; Javan *et al.* 2019; Deel *et al.* 2020). Our poor understanding of bacterial ingress after carcass rupture is complicated because many experimental designs involve decaying human cadavers (or other mammals, particularly swine) in poorly constrained, terrestrial, subaerial environments. This lack of constraint makes robust and repeatable analysis of post-rupture bacterial successions difficult (see Aragonés *et al.* 2022), but these studies typically demonstrate that ruptured carcasses rapidly become dominated by exogenous aerobic bacteria (e.g. Metcalf *et al.* 2013; Aragonés *et al.* 2022). This is likely to have limited applicability to the kind of environments where exceptional preservation of non-biomineralized tissues takes place. These environments, typically marine, enclose carcasses in sediments with limited pore water movement or limited oxygen exchange mechanisms and experiments show that decay-induced anaerobic conditions form rapidly (Sagemann *et al.* 1999) with external geochemical gradients that often persist long after carcass rupture (Clements *et al.* 2022). In our experiment we sampled on day 1138, long after the carcass lost its integrity (Fig. 2). In this sample, the thanatomicrobiome present in the previous samples had been replaced. The day 1138 sample was almost completely dominated by anaerobic bacteria (Fig. 3), and of the two OTUs that dominate the sample, Clostridia and Ignavibacteriaceae, the latter, which is the most abundant in sample 1138, is not found in the thanatomicrobiome of earlier samples (Fig. 2). Unfortunately, our experimental design and the time gap between the samples (1004 days) mean we cannot say if the rupture of a carcass in a sediment-enclosed marine setting would lead to ingress of exogenous anaerobic bacteria and a dramatic community turnover, but it is likely. Further experiments are required to test this and understand the nature and succession of the bacterial communities that develop in close proximity to a decaying, sediment-enclosed carcass compared to those within.

Review of the complex role of bacterial metabolism in soft tissue preservation

If an animal carcass is buried immediately postmortem in an environment amenable for fossilization, what factors govern the likelihood of internal soft tissue preservation, and what role do bacteria play in these processes? Three principal factors govern soft tissue preservation: rate of decay, the sequence of anatomical character loss, and processes of preservation (we focus on mineralization as it is the more common mode of preservation). All three of these factors are intrinsically linked to bacterial metabolism.

Almost immediately postmortem, the carcass begins to decay through a combination of autolysis and bacterial metabolism. Cellular autolysis starts within minutes of death: the functional processes of cells cease, and endogenous enzymes begin to act on the components of each cell itself, effectively 'self-digesting' the cell and surrounding tissues (e.g. Hyun *et al.* 2012; Zapico *et al.* 2014; Guo *et al.* 2016). Autolysis alone would eventually break down a carcass, but it is relatively slow. Critically, however, the biproducts of autolysis trigger blooms in the internal bacterial communities that metabolize the amino acids, carbohydrates, lipids, water etc. released from the rupturing cells (e.g. Can *et al.* 2014). As internal bacterial communities become established throughout the carcass (see previous section), bacterial metabolism rapidly becomes the dominant driver of organic matter breakdown (Raff *et al.* 2008; Janssen *et al.* 2022). In concert, these two

processes will break down the carcass, driving loss of anatomical information, disarticulation, and eventually, the complete destruction of organic matter. However, decay of organic material within a carcass is not random; experimental work has shown that anatomical characters decay in a repeatable sequence (Sansom *et al.* 2010). This is important, because the complex interplay between rate of decay and the sequence of anatomical character loss determines the organic template upon which preservational processes (e.g. mineralization) can act (e.g. Purnell *et al.* 2018; Gabbott *et al.* 2021). It is also important to note here that while some organic tissues may be geologically stabilized, all other tissues not stabilized by a preservational process will continue to decay until obliteration, including biomineralized tissues (see Purnell *et al.* 2018 for review; Clements & Gabbott, 2021; Gabbott *et al.* 2021).

Rate of decay is influenced by environmental variables that predominantly moderate bacterial metabolism. Rapid sedimentation and conditions at the water–sediment interface commonly characterized as 'inhospitable' suppress the activity of scavengers and sedimentary bioturbators, protecting the carcass from rapid disarticulation during the early phases of decay. It is a common misconception that environmental factors such as low temperature, high salinity, low oxygen content etc. stop decay. In fact, these factors only slow the rate of decay through modulation of bacterial activity (Briggs & Kear 1993a; Briggs 2003; Muscente *et al.* 2023; see Clements & Gabbott, 2021 for review). Even anoxia does not halt decay: many bacterial groups have evolved diverse metabolic adaptations that allow them to exist in low oxygen environments. These bacteria utilize electron acceptors other than oxygen to allow respiration to continue through a variety of anaerobic metabolic pathways (Allison 1988b; Briggs & Kear 1993a, 1993b; Sagemann *et al.* 1999; Briggs 2003; Janssen *et al.* 2022). Likewise, if burial occurs in an oxic sedimentary environment, with limited pore water movement ('closed' conditions), experiments show that the local supply of oxygen is rapidly depleted by bacterial respiration (e.g. Sagemann *et al.* 1999), forcing a swift transition in the dominant bacterial metabolic pathway from aerobic to anaerobic respiration (Briggs & Kear 1993a, 1993b; Sagemann *et al.* 1999; our results presented here).

Unless preservational processes act to convert or replace organic tissues into remains that are stable over geological timescales, decay will continue until complete carcass obliteration. What initiates soft tissue preservation? A growing body of evidence suggests that, somewhat paradoxically, the activity of anaerobic bacterial metabolism may trigger and sustain the replacement/replication of soft tissues by authigenic minerals (minerals that precipitate and grow *in situ*; see Briggs 2003). Anaerobic bacteria can induce mineralization indirectly and/or directly (referred to as bacteria-induced mineral precipitation; e.g. Hoffmann *et al.* 2021). Indirect mechanisms include: (1) the bacterial breakdown of organics liberates ions from tissues, fuelling mineralization through supersaturating the local environment and creating potential sites for mineral nucleation on decaying organic structures (e.g. Briggs & Kear 1993a, 1994a; Wilby & Briggs 1997; Clements *et al.* 2022; Janssen *et al.* 2022); (2) internal bacterial biofilms that form during decay act to pseudomorph soft tissues and mediate mineralization (e.g. Raff *et al.* 2013; Butler *et al.* 2015; Eagan *et al.* 2017); and (3) anaerobic bacterial respiration and metabolism fundamentally alter the geochemistry within and around the carcass (e.g. Sageman *et al.* 1999; Clements *et al.* 2017, 2022). It is well understood that many modes of mineralization, such as precipitation of calcium phosphate, pyrite etc., require specific geochemical conditions to trigger and/or maintain tissue replacement (e.g. Briggs & Wilby 1996; Sagemann *et al.* 1999; Clements *et al.* 2017, 2022), and these are also influenced strongly by bacteria. Experimental work has revealed significant geochemical gradients within a decaying carcass (Clements *et al.* 2022), and to a lesser extent in the local sediment surrounding the carcass (Sageman *et al.* 1999; Clements *et al.* 2017, 2022). The geochemical shift towards conditions amenable to mineralization is linked to increasing amounts of waste products of bacterial respiration within the localized area, including CO_2 (aq), sulfuric acid (H_2SO_4), and fatty acids (Sageman *et al.* 1999; McNamara *et al.* 2009; Clements *et al.* 2017, 2022). In solution, these chemicals create intense localized geochemical gradients within (and to a lesser extent around) decaying carcasses which can actively promote authigenic mineral precipitation (e.g. Sagemann *et al.* 1999; Clements *et al.* 2017, 2022). Moreover, the generation of reducing conditions has been shown to also suppress the impact of autolysis, retarding the rate of decay and potentially prolonging the window for mineralization to occur (Raff *et al.* 2006; Butler *et al.* 2015).

Burial plays a vital role in soft tissue preservation, especially in maintaining the geochemical gradients generated by decay: carcass entombment creates a closed environment that acts to limit diffusion of decay products away from the carcass (McCoy *et al.* 2015) speeding up the onset of decay (Cavicchini *et al.* 2025) while prolonging the duration of localized geochemical gradients needed for mineralization to occur (Sagemann *et al.* 1999; Clements *et al.* 2017). Entombment by sediment also plays an important secondary role: it acts to maintain carcass integrity, preventing disarticulation, and the escape of decay bi-products (e.g. Wilson & Butterfield 2014; Cavicchini *et al.* 2025; Waskom *et al.* 2025). The stabilizing effect of sediment can be

complemented by the formation of microbial biofilms that support epinecrotic bacterial communities (e.g. Darroch *et al.* 2012; Iniesto *et al.* 2015, 2021; Eagen *et al.* 2017; Corthésy *et al.* 2024; Palmer *et al.* 2024). However, it is unlikely that exogenous bacteria play a direct role in the preservation of internal soft tissues; our data do not support the hypothesis that they invade the carcass before internal organs are lost and there is growing evidence that sediment types often associated with soft tissue preservation, particularly clays, negatively impact bacterial growth (e.g. Wilson & Butterfield 2014; McMahon *et al.* 2016; Corthésy *et al.* 2024).

In the event that geochemical thresholds are met within a buried carcass, experiments have shown that mineralization does not occur randomly but is temporally and spatially restricted throughout decay; specific modes of mineralization discriminate between specific tissues within a carcass (e.g. Gabbott *et al.* 2021; Clements *et al.* 2022). This can be seen in the fossil record: patterns of soft tissue preservation clearly show that specific modes of mineralization are biased towards the preservation of specific tissues and organs (e.g. Wilby *et al.* 1996; McNamara *et al.* 2009; Jauvion *et al.* 2020; Clements *et al.* 2022). Previously, this has been explained by hypotheses of organ specific microenvironments (e.g. McNamara *et al.* 2009), but experimental investigations to date have failed support this hypothesis. Rather, the evidence suggests that pervasive geochemical environments develop throughout a carcass (Clements *et al.* 2022); once the bacterially induced geochemical conditions required for mineralization are established, it is the intrinsic biochemical and structural properties of tissues that govern the likelihood of mineral replacement (for a review see Clements *et al.* 2022).

The replacement of soft tissues by authigenic minerals reflects a delicate balance between competing processes. While bacterial metabolism is required to generate the geochemical conditions that allow mineralization to occur, too much metabolism will obliterate soft tissues, removing the anatomical information that defines exceptional preservation of non-biomineralized remains (Clements & Gabbott 2021). Clearly, as soft tissue remains in the fossil record are so rare, minute variations in any of these variables may prevent soft tissue preservation from occurring. Decay experiments over the last three decades have dramatically improved our understanding of how non-biomineralized tissues become fossilized, but many aspects, such as the triggers of mineralization, remain poorly understood. While fossil material gives us direct evidence of the end results, it is detailed and robust interdisciplinary experimental work that will provide the insights we need to address these gaps in our knowledge.

CONCLUSION

Bacteria play a direct and indirect role in influencing the preservational processes that geologically stabilize non-biomineralized tissues. Despite this importance, it has not been clear whether endogenous or exogenous bacteria dominate the bacterial communities that inhabit a carcass immediately postmortem, with many taphonomic investigations focusing on external bacterial populations. Furthermore, recent work in microbial forensic science has suggested that successional shifts in the internal thanatomicrobiome might be what controls the reproducible sequence of anatomical character loss seen in decaying carcasses. Here, we investigate the succession of the thanatomicrobiome within decaying amphioxus carcasses and present two key findings:

1. Even in the presence of inoculum, the internal thanatomicrobiome is dominated by endogenous anaerobic bacteria sourced from the digestive tract; exogenous bacteria do not invade the carcass and replace native bacteria species while the carcass is intact. Our data demonstrate that it is unlikely that external bacteria have a direct role in the preservation of internal soft tissues and confirm previous findings that adding an inoculum to a taphonomic experiment is not required for decay to occur. Without detailed microbial community analysis, naturally sourced inocula represent an uncontrolled and potentially confounding variable.
2. Our data do not support the hypothesis that temporal successions in the thanatomicrobiome control the sequence of anatomical character loss during decay. This further supports the hypothesis that the occurrence of non-biomineralized characters preserved in fossils correlates with their propensity to decay in extant relatives (e.g. Gabbott *et al.* 2021). Furthermore, combining our results with the evidence that the sequence of anatomical character loss during decay is conserved between individuals and across non-related taxa, we conclude that the inability to model ancient bacteria communities does not diminish the value of decay experiments.

Our experiment has highlighted some technical difficulties and limitations, but our results provide a valuable proof-of-concept, and further experimental work will be better able to address the gaps in our understanding of the formation and proliferation of the thanatomicrobiome during the early stages of decay.

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DATA ARCHIVING STATEMENT

Data for this study are available in the Dryad Digital Repository: https://doi.org/10.5061/dryad.8931zcs07

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