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PAPER

PATHOLOGY/BIOLOGY

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The Potential of High-throughput Metagenomic Sequencing of Aquatic Bacterial Communities to Estimate the Postmortem Submersion Interval*

ABSTRACT: Human remains can be discovered in freshwater or marine ecosystems, circumstances where insects and other invertebrates have infrequently been used for understanding the time of postmortem submersion. In this study, the identification and succession of epinecrotic bacterial communities on vertebrate remains were described during decomposition in a temperate headwater stream during two seasons (summer and winter). Bacterial communities were characterized with 454 pyrosequencing and analyzed at phyletic and generic taxonomic resolutions. There was a significant increase in genera richness over decomposition during both seasons. Additionally, multivariate statistical modeling revealed significant differences in bacterial communities between seasons at both taxonomic resolutions and siginificant genera differences among sampling days within each season, suggesting a succession of these communities. These data are the first to describe aquatic bacterial succession using high-throughput metagenomic sequencing on vertebrate remains submerged in a freshwater habitat, and provide initial evidence for their potential use in forensic investigations.

KEYWORDS: forensic science, aquatic microbial communities, epinecrotic communities, high-throughput metagenomic sequencing, pyrosequencing, postmortem submersion interval, necrobiome

Over the last several years, there has been an increasing recognition of the importance of establishing a strong basic science foundation in the forensic sciences (1,2). With the development, reliability, and increasingly widespread use of high-throughput metagenomic sequencing (e.g., 454 pyrosequencing and Illumina MiSeq), the forensic sciences are poised to harness "-omics" technology to bridge basic science with medicolegal applications. This unique application of technology is especially timely for those forensic disciplines without traditional genomic foundations, such as taphonomy. This application of microbial communities also has the potential to expand into areas of forensic investigations involving human remains discovered outdoors, much like recent studies that have used high-throughput metagenomic sequencing of a human (3), swine (4), and mouse (5) microbiomes. The use of these next-generation technologies has the *potential* to enhance the ability of investigators to provide more accurate estimates and provide error rates associated with evidentiary interpretation, especially in quantitative timelines related to death.

Recently, metagenomic tools have become more widely available, thus allowing thorough investigations into epinecrotic bacterial communities during vertebrate decomposition (3–5). Pyrosequencing and other high-throughput metagenomic approaches (e.g., Illumina MiSeq) can taxonomically identify bacterial community changes throughout decomposition, generate large amounts of data, characterize sequences of unculturable or newly discovered microbial species, and are established genomic methods (6–8). Analyses using metagenomics provide the depth of results required to more comprehensively characterize and understand temporal and spatial bacterial community composition and succession on decomposing vertebrate remains (4). Given the technological advancements and increase in computing power and statistical modeling capabilities for metagenomic data, the characterization of environmental microbial communities using metagenomics is now becoming more widely available and cost-effective. The forensic sciences are positioned well to take advantage of the novel and innovative assessments and statistical modeling approaches for using these ubiquitous biological communities as evidence.

One task for medicolegal forensic investigators is the scientific estimation of the amount of time human remains have been submerged or partially submerged in aquatic environmental conditions, such as rivers, lakes, marshes, or the ocean, in cases involving suspicious drowning or aquatic body disposal. This

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time frame has been defined as the postmortem submersion interval (PMSI): the time between when a corpse enters an aquatic environment until discovery by authorities (9–11). The aquatic decomposition process of vertebrate remains has been described as initiating with submerged fresh remains proceeding to floating decay stages and ending with sunken remains (9–11). However, these broad categorizations and visual assessments of decomposition can vary based on water quality, depth of the remains, and other abiotic and biotic influences (9). Therefore, other biological indicators, such as microbial communities that are ubiquitous within environment, have the potential to better estimate a PMSI range.

In aquatic ecosystems, microbes are predominately found within biofilms rather than as free-floating planktonic forms (12,13). Biofilms are matrix-enclosed microbial communities that are both trophically (heterotrophs and autotrophs) and phylogenetically (prokaryotes and eukaryotes) diverse, containing algal, bacterial, fungal, and protozoan species (14,15). These microorganisms are encased in an extracellular polymeric substance (EPS) that is attached to surfaces in aqueous solutions (12,16). The EPS matrix is protective against changing abiotic conditions (17,18), traps and stores nutrients (14,19,20), and accrues enzymes that break down organic matter (21,22). Essentially, the biofilm is a micro-ecosystem that can be described using ecological theory and principles of landscape ecology (15). The type of surface classifies the biofilm and influences community composition and energy dynamics by dictating the dominant community. For example, epilithic biofilms that form on inorganic substrates like rocks are more autotrophic and represented by an abundant algal community, while the epixylic biofilms found on decomposing plant matter are more heterotrophic with a substantial fungal community (23–25). Within biofilms studied on decomposing piglet carcasses, diatoms (unicellular algae with a silica cell wall) demonstrated patterns of diversity and abundance throughout decomposition (9,26), but identification of these organisms is taxonomically intensive and requires habitat conditions with abundant sunlight. Bacteria diversity and abundance also change during marine decomposition, but cloning methods cannot effectively identify the entire community taxa composition (27). Biofilms on decomposing vertebrate carcasses, recently classified as epinecrotic (4), have been noted on salmon (28), waterfowl (29), rats (30), and pigs (11), but only a few studies have focused on this biofilm community for use in forensics. The commonality of these studies is that they are based on tracking community composition changes as epinecrotic biofilms develop during the decomposition process of a carrion substrate.

Biofilm development typically follows a pattern of succession, or the natural sequence of species change and replacement over time. Bacteria are the first colonizers and establish the pioneer community (31); these initial colonizers remain as the base layer for subsequent immigration and establishment of larger microorganisms (14,15). As the biofilm ages, larger and filamentous algae and fungi are incorporated until filamentous organisms dominate the biofilm at a mature stage with composition depending on the substrate and light conditions. Fungal organisms could play a role in the use of epinecrotic communities for forensics, but they have yet to be studied using high-throughput metagenomics. It is important to note that bacteria are always present throughout succession and are an important biological indicator of biofilm succession (27). Succession can range from weeks to months depending on abiotic and biotic conditions, and this sequence of bacterial taxa replacement during vertebrate decomposition could potentially be used to estimate the physiological time (e.g., accumulated degree days) in aquatic habitats, as it has been demonstrated in a terrestrial habitat (4). There are several factors known to affect microbial community succession during vertebrate carrion decomposition in terrestrial habitats including temperature (32,33), moisture and humidity (34), tissue type (27), surrounding vegetation (35,36), and soil pH (37). Likewise, there are a variety of environmental factors (e.g., temperature, dissolved oxygen, carbon, and nutrients) that researchers studying microbial assemblages of aquatic systems have identified as important drivers of microbial community succession (38).

In terrestrial environments, insects, with well-known life-history traits (e.g., development rates), along with geographical and seasonal distribution data, are often used to assist in minimum postmortem interval (mPMI) estimates, but only recently have microbial communities been investigated for similar applications (3–5). For submerged remains in aquatic habitats, literary consensus is that very few, if any, invertebrate species have evolved to consume vertebrate remains in a way that can reliably be used for estimating a PMSI range (10). However, several empirical studies using traditional sequencing approaches have provided valuable baseline information on the potential usefulness of using naturally occurring diverse assembly of microbes, such as algae, in aquatic systems to form evidentiary linkages among suspects, victims, and physical evidence (e.g., weapons) (9,11,39–44). Indeed, Keiper and Casamatta (45) suggested additional studies of algae and other aquatic flora for use in forensics. In response, two studies were conducted and each reported that algal and diatom communities showed promise for being used in estimating a PMSI (9,11), but both studies did not evaluate the prokaryotic taxa that are also part of aquatic biofilm communities. However, Dickson et al. (27) documented the bacteria community succession (but not the fungal communities) on decomposing remains in marine habitats using swine heads as a model of decomposition and employing capillary electrophoresis sequencing methods. While bacterial assemblages are ubiquitous, there have been no studies to date that have used metagenomic sequencing similar to what has been done in terrestrial settings (3,4). Thus, a description of the prokaryotic component of the microbial community throughout the decomposition process could be important in estimating a PMSI range of human remains in aquatic environments.

In this study, we employed high-throughput metagenomic sequencing to describe bacterial communities on the external surfaces of swine remains [as surrogates for human corpses (46)] to better understand the potential use of epinecrotic bacterial succession in aquatic death investigations. The objectives of this research were threefold: (i) characterize how the epinecrotic bacteria communities of decomposing vertebrate carcasses changed during decomposition in a stream habitat, (ii) assess whether this succession was consistent between two seasons (summer vs. winter), and (iii) provide data important for discussion of the potentials and limitations for using aquatic epinecrotic community succession in future studies of forensic application.

Methods

Study Site Description

The study was conducted from 26 June to 17 July 2012 (summer trial), and 9 November to 21 December 2012 (winter trial), in a first-order tributary to the west branch of Big Spring Run (39°59′29.1″N 76°15′49.0″W) within the Conestoga River watershed of Lancaster, Pennsylvania. Despite flowing through a

suburban/agricultural area of the watershed, the stream was bordered by 10–20 m of riparian forest and shrub vegetation. Canopy trees within the riparian zone that shaded 90% of the stream channel included silver maple (*Acer saccharinum* L.), box elder (*Acer negundo* L.), and sycamore (*Platanus occidentalis* L.) with a ground cover of multiflora rose (*Rosa multiflora* Thunb.) bordering both stream banks. The stream substrate consisted of a mixture of pebble and cobble throughout the study site. The swine carcasses (see below for details) were individually placed in run habitats of the stream to avoid being silted over in pools and the abrasive action of riffle habitats.

Water Chemistry

Water quality parameters of dissolved oxygen (mg/L), pH, conductivity (mS/cm), water temperature (°C), total dissolved solids (g/L), oxidation–reduction potential (mV), and salinity (ppt) were measured at a single location 30 m upstream of the uppermost carcass and 30 m downstream of the lowermost carcass on each sampling day during both seasons using a Horiba® (Kyoto, Japan) Multi Water Quality Checker (U-50 Series).

Qualitative Decomposition Changes

To visually describe the decomposition process for the carcasses, the duration of each decay stage was assessed and described using the framework of Zimmerman and Wallace (9). The summer trial ended the day when the sunken remains stage was sampled as there was minimal skin remaining for swabbing for epinecrotic biofilm, while the winter trial ended unexpectedly and abruptly as a result of scavenging events and cage vandalism from the public on Day 42 of decomposition. See below for more information regarding the sampling times.

Epinecrotic Bacterial Community Sample Collection

Stillborn *Sus scrofa* L. carcasses (1–1.5 kg; $n = 3$ per trial) obtained from the Penn State University Swine Research Facility (State College, PA) were used as the decomposition model for epinecrotic biofilms formation during summer and winter trials. Swine carrion has been evaluated as a suitable surrogate for human cadavers in decomposition studies (46), and swine skin has been used as a model for laboratory human skin bacteriological studies (47–50); however, piglet carcasses were used in this study and may not represent the same decay process of adult swine but do provide good models for research. Carcasses were placed on plastic drawer organizer trays (0.38 × 0.15 × 0.05 m) inside small, metal game traps (0.61 × 0.18 × 0.18 m, Havahart®; Animals B-Gone, Orrstown, PA) to facilitate sampling as the carcass disarticulated and to prevent scavenger removal of the carcasses. The cages also prevented the carcasses from breaking the surface, subsequently inhibiting colonization by terrestrial insects. Traps were anchored to the streambed on previously secured rebar, which allowed easy trap removal for sampling. During each trial, carcasses were placed ~15–20 m downstream from each other with one riffle/run reach between each one along the study segment of stream to minimize upstream effects of any carcass. This flowing nature of streams makes it difficult to have completely independent carcasses, and we acknowledge this as a limitation to this study.

Epinecrotic biofilm was sampled weekly at four consecutive time points during the summer trial [29 June (Day 0), 3 July (Day 7), 10 July (Day 14), 17 July (Day 21)] and biweekly during the winter trial [9 November (Day 0), 23 November (Day 14), 7 December (Day 28), 21 December (Day 42)] to account for the delayed decomposition resulting from cooler temperatures. To characterize the communities prior to submersion, samples were collected on "Day 0" prior to the carcasses being placed into the stream and thus represent the initial bacterial communities of the carcasses. The bacterial samples were collected from the external surface of the abdomen/rib cage using individually packaged sterile swabs; an area (approximately 5 × 1 cm) was swabbed with six strokes where the swab was rotated 180° after the third stroke and one direction counted as a stroke with care taken to not duplicate any previously sampled areas. The swab sample was individually placed into a sterile microcentrifuge tube and stored on ice for approximately 0.5 h until samples were placed in a −20°C freezer for further DNA extractions. The most downstream carcass was sampled first and sampling progressed upstream on each date. Carcasses were immediately submerged upon sample collection completion, and new gloves were worn for each sample collection.

DNA Extraction

DNA was extracted using a combination of methods from Miller et al. (51) and Zhou et al. (52) as suggested by Lear et al. (53). Samples were lysed in 1 mL extraction buffer made of 100 mM Tris–HCl (pH 8.0), 100 mM EDTA disodium salt (pH 8.0), 100 mM sodium phosphate (pH 8.0), 1.5 M sodium chloride, and 1% CTAB; 20 μL of proteinase K (10 mg/mL) and 25 μL of SDS (20%) were added to each sample in the extraction buffer prior to bead beating (0.25 g each of 0.1 mm and 0.5 mm glass beads) for 15 min on a horizontal vortex adaptor (MO BIO Laboratories, Carlsbad, CA) at full speed. The samples were incubated at 60°C for 30 min with gentle end-over-end inversions by hand after 15 min; 250 μL of supernatant was collected in a new microcentrifuge tube after centrifugation at 6,000 *g* for 10 min. The lysis process was repeated without vortexing with an additional 500 μL extraction buffer, 10 μL proteinase K, and 12.5 μL SDS to obtain a final supernatant volume of approximately 750 μL. DNA was separated from organic debris with a chloroformisoamyl alcohol (24:1 vol/vol) extraction and precipitated overnight at −20°C using isopropanol. Samples were removed from the −20°C and warmed to 37°C to dissolve salt precipitates, and the DNA was pelleted at 15,000 *g* for 30 m. Finally, the DNA pellet was washed twice with ice-cold 70% ethanol and dissolved in 50–100 μL ultrapure water (NANOpure II™, Thermo Scientific, Waltham, MA) water, depending on the DNA pellet size.

454-Pyrosequencing

Epinecrotic bacterial community composition was characterized by modified bacterial tagged encoded FLX amplicon pyrosequencing (bTEFAP) as described by Pechal et al. (4) for the characterization of bacterial communities from swine remains in terrestrial habitats. Briefly, extracted DNA (>200 ng) was sent to the Molecular Research Laboratory (Shallowater, TX). PCR amplification for bacterial V1-3 regions of 16s rRNA using Gray28F (5′TTTGATCNTGGCTCAG) and Gray519r (5′ GTNTTACNGCGGCKGCTG) primers was carried out, as previously described (54–58). Sequencing library generation began with a one-step PCR using a mixture of Hot Start and HotStar high-fidelity *Taq* polymerases. Following RTL protocols (www.researchandtesting.com), a Roche 454 FLX instrument

with titanium reagents and titanium procedures was used to perform the tag-encoded FLX amplicon pyrosequencing analyses. The sequences were deposited in the Sequence Read Archive (SRA) at the European Bioinformatics Institute with study accession numbers PRJEB5310 and PRJEB5311.

454-Pyrosequencing Analyses

The Q25 sequence data derived from the sequencing process was processed using a proprietary analysis pipeline (www.mrdnalab.com). Sequences were depleted of barcodes and primers; then, any sequences with <200 bp, ambiguous base calls, or with homopolymer runs exceeding 6 bp were removed. Finally, sequences were denoised and chimeras removed. Operational taxonomic units (OTUs) were defined after the removal of singleton sequences and with clustering at 3% divergence (59–63). OTUs were taxonomically classified using BLASTn against a curated GreenGenes database (64). All three carcasses during the summer trial had successful sequencing results, but unfortunately during the winter trial, there were insufficient reads for one of the carcasses and thus that indivual was excluded from subsequent data analyses.

Statistical Analyses

To test for water chemistry difference upstream (US) and downstream (DS) of the carcasses within each seasonal trial, a paired t-test was employed using Prism 5 (GraphPad Software, Inc., La Jolla, CA) by the measurements taken on each sampling date and the pairing date. To assess bacterial community structure change over decomposition, the taxon richness was calculated according to Simpson (65) and mean relative taxon abundance was determined at both phyletic and generic taxonomic resolutions for individual replicate carcasses and also averaged for each date. Further, Bray–Curtis distance with nonmetric multidimensional scaling (NMDS) and 999 permutations using vegan 2.0-7 library in the R statistical package (66) was used to visualize bacterial community differences between seasons and among sample weeks. For each ordination, the two axes that explained the most variation and the strongest orthogonality (lowest stress) were used for representing the data in multidimensional space (67). Differences in bacterial communities and variation over decomposition (i.e., sampling dates) were tested using Bray–Curtis dissimilarity values with permutational multivariate analysis of variance (PERMANOVA) with the adonis function using the vegan library in the R statistical package (66). PERMANOVA is a nonparametric technique used to differentiate groups of data based on a dissimilarity matrix (68). Significance was determined at $\alpha = 0.05$ for all analyses.

Results

Water Chemistry

Water temperature, pH, and total dissolved solids (TDS) were generally greater during the summer trial (Table 1), while dissolved oxygen (DO) and oxidation–reduction potential (ORP) were higher in the winter trial. The other variables showed consistency between seasons. Within each seasonal trial, the only water quality variable that was significantly different was DO ($t = 3.38$, df = 3, $p = 0.043$), which was higher upstream (US) than downstream (DS) of the carcasses only during the summer trial.

Qualitative Decomposition Changes

For the summer trial, the mean (±standard deviation) number of days to reach the first day of each decomposition stage was the following: submerged fresh began at 0.0 (±0.0) days, early floating began at 5.7 (±2.3) days, early floating decay at 9.0 (±1.7) days, advanced floating decay at 14.0 (±0.0) days, and sunken remains at 22.0 (±1.7) days (Table S1). During the winter trial, there was a delayed onset for each decomposition stage with submerged fresh, early floating, and early floating decay occurring at days 0.0 (±0.0), 5.0 (±5.6), and 21.0 (±0.0), respectively (Table S1); unfortunately, there was an unforeseen scavenging event and vandalism that caused the abrupt end of this trial and prevented further documentation of the decomposition progression.

TABLE 1—*Water quality parameters were collected at a single location 30 m upstream (US) and downstream (DS) of the swine carcasses on each sampling day for each trial. Paired t-tests were used to evaluate statistical differences between US and DS locations for each water chemistry parameter. DO was the only parameter that was found to be significantly different between US and DS: DO was higher US than DS only during the summer trial.*

Season	Day	Sampling Location	DO (mg/L)	pH	SC (mS/cm)	Temp (°C)	TDS (g/L)	ORP (mV)	Salinity (ppt)
Summer	Day 0	US	4.46	8.13	0.936	20.7	0.468	65.6	0.46
		DS	3.54	8.07	0.920	20.2	0.460	63.3	0.45
	Day 7	US	7.22	8.73	1.043	20.7	0.522	68.9	0.52
		DS	6.38	8.20	1.032	19.9	0.516	64.7	0.51
	Day 14	US	5.24	8.13	0.973	21.8	0.487	38.4	0.48
		DS	2.86	8.11	0.842	22.6	0.421	36.0	0.41
	Day 21	US	6.23	8.12	0.967	23.2	0.438	36.5	0.48
		DS	3.32	8.15	0.958	23.8	0.479	36.8	0.47
Winter	Day 0	US	3.23	–	1.010	10.6	0.413	95.6	0.41
		DS	3.12	–	0.878	10.2	0.439	711	0.44
	Day 14	US	10.51	8.24	0.941	13.6	0.603	207	0.50
		DS	9.72	8.21	0.930	11.6	0.596	196	0.50
	Day 28	US	9.44	7.79	0.976	11.7	0.626	242	0.50
		DS	10.17	8.01	0.972	10.3	0.624	258	0.50
	Day 42	US	9.26	7.93	0.498	9.6	0.321	250	0.20
		DS	9.5	8.05	0.820	9.6	0.525	257	0.40

DO, dissolved oxygen; SC, specific conductivity; TDS, total dissolved solids; ORP, oxidation–reduction potential.

The mean (±standard deviation) duration (number of days) the carcasses were in each decomposition stage during the summer trial was as follows: submerged fresh for 5.7 (±2.3) days, early floating for 3.3 (±0.6) days, early floating decay for 5.0 (±1.7) days, advanced floating decay for 8.0 (±1.7) days, and sunken remains for 3.3 (±0.6) days (Table S1). The sunken remains stage may have lasted longer, but our sampling was completed after a single collection of the sunken remains. Decomposition progression was slower during the winter trial with the replicate carcasses characterized as submerged fresh for 7.5 (±5.0) days, early floating for 16.0 (±5.6) days, and early floating decay for 28.0 (±0.0) days (Table S1); again, the unforeseen scavenging event and vandalism abruptly ended this trial.

Epinecrotic Bacterial Communities

The epinecrotic bacterial communities of the decomposing swine carcasses in the stream were represented by 17 phyla and 179 genera in the summer trial, while 13 phyla and 202 genera were documented in the winter trial (Tables S2 and S3). There were significant changes in taxon richness over decomposition with peak mean richness increasing from 21 to 82 genera within the first 21 days of decomposition during the summer, and peak mean richness increasing from 38 to 109 genera after 42 days of decomposition in the winter. Proteobacteria and Firmicutes were the predominate phyla during both summer and winter trials. However, the winter carcasses never reached the advanced floating remains or sunken remains stages, making it difficult to compare community differences seasons by stage. Over the summer trial, the mean relative abundance of Proteobacteria decreased from 91.4, 74.5, 60.4 to 30.5% over the four continuous weekly sampling periods, while Firmicutes displayed an inverse trend of increasing mean relative abundance from 2.4, 23.6, 36.3 to 58.0% over the same time period (Fig. 1). The relative abundance of the epinecrotic taxa for each individual carcass also demonstrated a similar inverse relationship, with Proteobacteria decreasing as Firmicutes increased over the decomposition process during both summer (Fig. 2) and winter (Fig. 3) trials.

At the generic level during the summer trial, the initial sampling period (Day 0) was dominated by *Pseudomonas* (42.3%), *Psychrobacter* (36.1%), and *Ewingella* (11.1%); by Day 7, *Klebsiella* (13.0%), *Psychrobacter* (12.5%), and *Citrobacter* (9.4%) were the predominate genera; on Day 14, there was a shift in community stucture with *Zoogloea* (17.2%), *Clostridium* (15.0%), *Dechloromonas* (14.2%), and *Desulfosporomusa* (8.2%) being the most abundant genera; and finally on Day 21, *Clostridium* (25.2%), *Enterococcus* (16.9%), and *Lactobacillus* (9.3%) were the predominant genera (Table S2).

A smiliar succession pattern was documented during the winter trial, as Proteobacteria mean relative abundance decreased from the intial sampling on Day 0 (81.9%) to Day 14 (52.3%) to Day 28 (36.7%), but was consistent on Day 42 (38.2%) of sampling (Fig. 1). Firmicutes mean relative abundance increased from the intial sampling on Day 0 (14.1%) to Day 14 (47.0%) to Day 28 (61.7%), but decreased slightly on Day 42 (56.5%) of sampling (Fig. 1).

At the generic level during the winter trial, the intial sampling time (Day 0) was dominated by *Psychrobacter* (41.7%), *Pseudomonas* (31.5%), and *Enterococcus* (9.1%); *Lactococcus* (27.8%) was predominant on Day 14; Day 28 was dominated by *Proteocatella* (30.3%), *Clostridium* (10.4%), and *Veillonellaceae* (10.2%); and finally on Day 42, *Veillonellaceae* (18.6%) and *Clostridium* (12.4%) were still the predominant genera (Table S3).

Using NMDS (Figs 4 and 5) and PERMANOVA (Table 2), the epinecrotic bacterial communities were significantly different

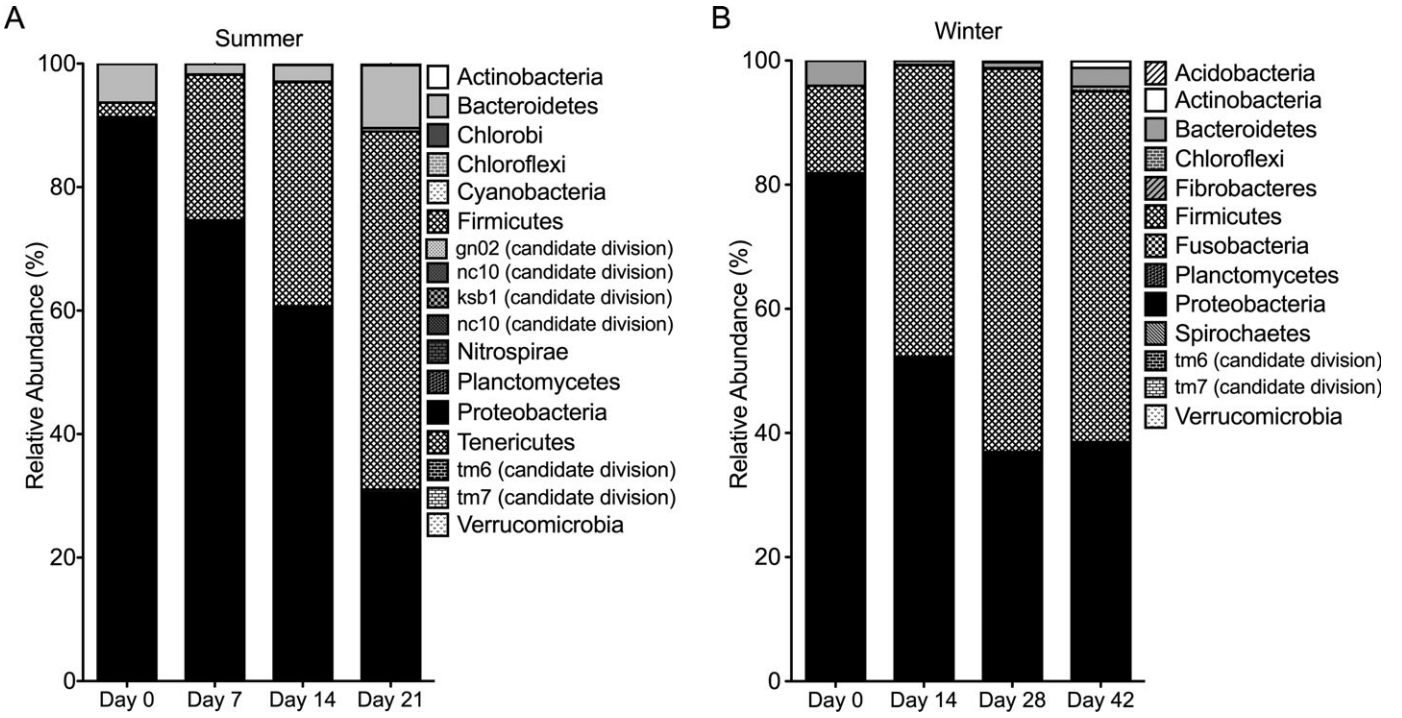

FIG. 1—*Epinecrotic bacterial community taxa mean relative abundances over decomposition of swine carcasses submerged in a stream: phyla are presented for summer (A) and winter (B) trials. Genera relative abundances are provided in Tables S1 and S2 for the summer and winter trials, respectively.*

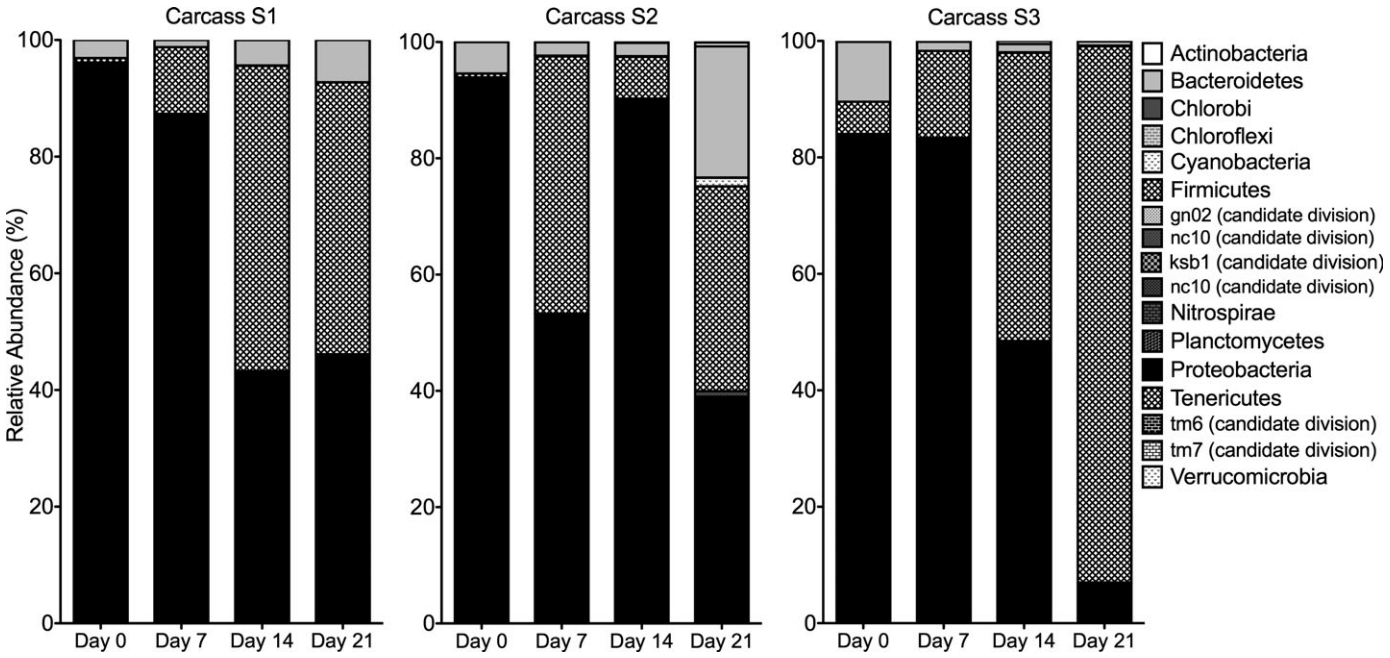

FIG. 2—*Epinecrotic bacterial community taxa relative abundances change over decomposition of individual swine carcasses submerged in a stream during the summer trial. Day 0 samples are the epinecrotic bacterial community of the carcass prior to being placed into the stream.*

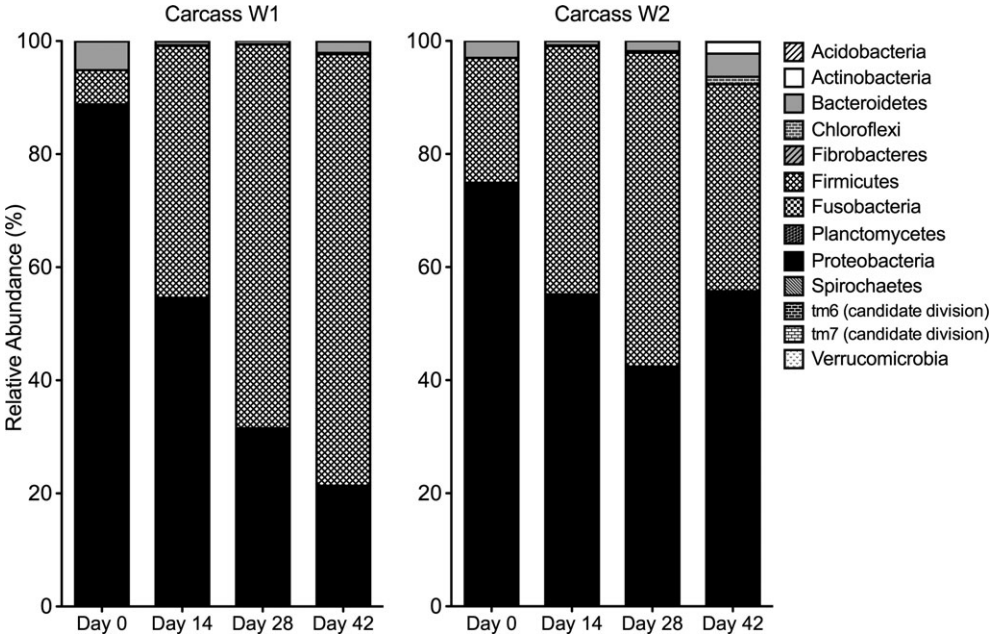

FIG. 3—*Epinecrotic bacterial community taxa relative abundances change over decomposition of individual swine carcasses submerged in a stream for approximately 42 days during the winter trial. Day 0 samples are the epinecrotic bacterial community of the carcass prior to being placed into the stream.*

between seasons ($p = 0.006$). During the summer, there were siginificant shifts in the bacterial communities at the phyletic and generic taxonomic levels of resolution among dates ($p < 0.05$). However, for winter, significant shifts in the communities were only significant among sampling dates ($p = 0.001$) at the generic level of taxonomic resolution.

Discussion

The focus of this study was to explore the use of high-throughput metagenomic sequencing of entire bacterial communities associated with decomposing vertebrate remains for further use and development in forensics. We successfully identified epinecrotic bacterial communities throughout the decomposition process of carrion placed in a freshwater stream during two seasons, providing preliminary support for the potential use of these communities in forensic investigations. There were significantly different bacterial communities between summer and winter seasons, and depending on the taxonomic resolution employed to characterize the communities, there was a significant shift in these communities throughout the decomposition process. The differences in communities between seasons may

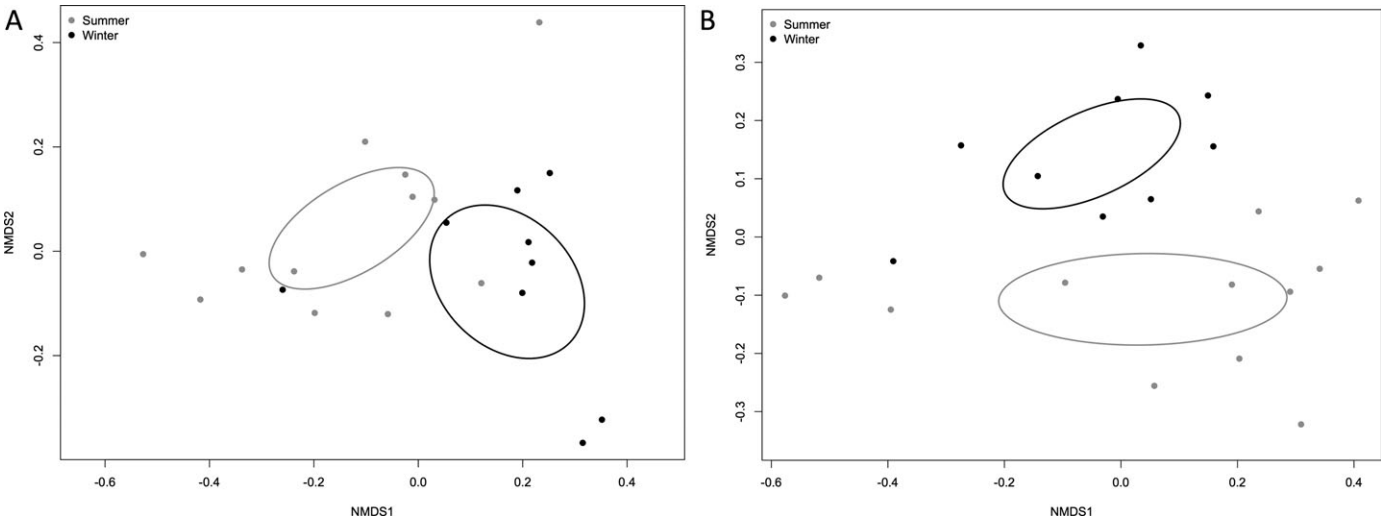

FIG. 4—*Nonmetric multidimensional scaling analysis ordination of bacterial communities with significantly different community structures between seasonal trials (summer and winter) at the (A) phyletic (stress = 0.063,* R^2 *= 0.98) and (B) generic (stress = 0.152,* R^2 *= 0.89) taxonomic resolution. The ellipses indicate 95% standard error of each season.*

be related to the abiotic conditions of the stream during those time periods, much like that reported for biofilms that form on inorganic substrates (69–71). Lower temperature and higher oxygen conditions during the winter trial may have selected for specific bacteria in the environment to initially colonize the carcasses, which were then replaced by bacteria that originally resided in the carcass (e.g., internal gut communities). It is well-known abiotic variables such as temperature, oxygen, and resource availability influence aquatic biofilm formation on nonliving surfaces (i.e., epilithic) in a way that affects community assembly (38,72–75). We suspect that this was the case in this study, shifting the bacterial taxa involved in the successional changes between winter and summer decomposition.

Further, carcass progression through decomposition during the summer followed an expected trajectory by taking an average of 22 days to decompose from submerged fresh to sunken remains. In the winter, the early floating decay stage was the last decomposition stage recorded prior to crayfish-scavenging events and vandalism, which prevented further characterization of the decomposition progress. However, winter carrion decomposition was approximately 2.3 times slower than the summer, requiring an average of 21 days until the early floating decay stage was reached, while in the summer it took an average of nine days. Based on these observations, the role of invertebrate scavenging on decomposing remains should be considered as a possible influence on evidence interpretation during investigations.

While there was intracarcass variation among carcasses, there were consistent bacterial community compositions for both summer and winter seasons; there was an inverse pattern of bacterial communities shifting from Proteobacteria as the predominant phyla in the early weeks of decomposition (submerged fresh to early floating decay) to the communities being dominated by Firmicutes in the latter portions of decomposition. In the summer trial, the predominant genera detected within Proteobacteria are known to be associated with vertebrate mammals and aquatic habitats. These genera include the following: *Pseudomonas* is common to aquatic systems (76) and has been found during the lipolysis process in adipocere formation (77); *Psychrobacter* has been reported from the nasal passageways of healthy swine (78); and *Ewingella* has been associated with meat spoilage (79,80). *Klebsiella*, which was detected during early decomposition in the summer, has been isolated from swine carcasses (81) and is commonly detected in aquatic habitats such as lakes (82). Other predominate genera detected during the decomposition process are typical of polluted aquatic systems: *Citrobacter* are coliforms that likely entered the watershed through septic systems (83); *Zoogloea* has been cultivated from vegetation, specifically filamentous algae, and is commonly reported from organically polluted watersheds (84); and increases in *Dechloromonas* may result from changes in anthropogenic land use (wastewater treatment plant effluent), as previously reported in suburban and urban rivers (85).

In the winter trial, there were several genera of notable interest. For instance, *Desulfosporomusa* are sulfate-reducing bacteria that have been detected in sediments of an oligotrophic lake (86). *Clostridium* has been detected in numerous environments (87,88) and is a common part of the human gut flora, which can be pathogenic (89). *Enterococcus* is a genera used worldwide as a fecal coliform indicator, and can be cultured from fresh lake water after 30 days at 4°C (90). Furthermore, in aquatic habitats with vegetation, there are significantly higher *Enterococcus* densities detected in the sediments than in habitats without vegetation (91). *Proteocatella* has been detected in penguin guano (92), in the canine oral microbiome (93), and in dairy wastewater storage ponds (94). *Veillonellaceae*, which is closley related to *Clostridium*, has been found in a variety of habitats ranging from the human oral microbiome (95) to crude oil (96). Thus, the bacterial communities reported in this study represent taxa that can be commonly found in aquatic habitats and those associated with common vertebrate species used in human food production. This is not surprising and may reflect the environmental circumstances of decomposition within the watershed. However, we do report significant changes in the overall communities that reflect time since submersion, successional descriptions that have potential use as metrics for estimating PMSIs in future studies or case work.

Numerous environmental factors, such as physical and chemical variables (e.g., temperature and oxygen content) and biological mechanisms (e.g., bacteria, fungi, and detritivores), play a substantial role in the decomposition of human remains in aquatic systems, thus directly influencing estimates of a PMSI range (10,11). Microbial communities vary by geographic

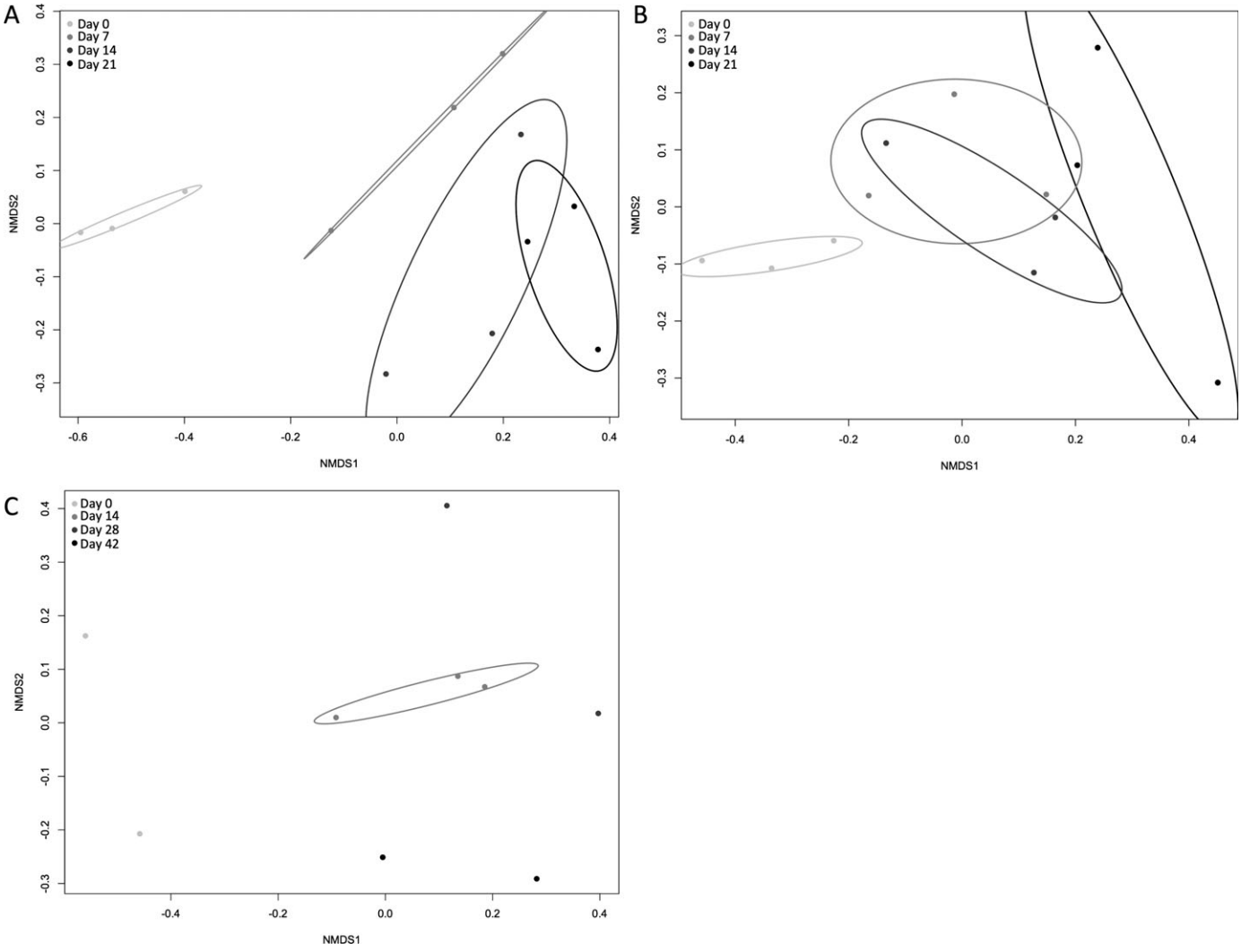

FIG. 5—*Nonmetric multidimensional scaling analysis ordination of bacterial communities among sampling days during each seasonal trial (summer and winter). (A) There were significantly different bacterial communities at both the (A) generic (stress = 0.089,* R^2 *= 0.96) and (B) phyletic (stress = 0.045,* R^2 *= 0.99) taxonomic resolution during the summer trial. There were only sufficient data points at the (C) generic level (stress = 0.0.111,* R^2 *= 0.92) to visualize significant differences among sampling days during the winter trial. The ellipses, when present, indicate 95% standard error of each sampling day.*

TABLE 2—*PERMANOVA results testing bacterial community structure responses at the phyletic and generic taxonomic levels between seasons, and among carcass decomposition days within each season (summer and winter). Significant effects are indicated by an asterisk.*

Taxonomic Level	Factor	Source	d.f.	*SS*	MS	*F*	*P*
Phylum	Season	Season	1	0.698	0. 698	5.53	0.006*
		Residuals	19	2.40	0.126		
		Total	20	3.10			
	Summer	Days	3	0.880	0.294	4.22	0.006*
		Residuals	8	0.557	0.070		
		Total	11	1.437			
	Winter	Days	3	0.419	0.140	1.29	0.273
		Residuals	5	0.543	0.109		
		Total	8	0.962			
Genera	Season	Season	1	0.942	0.942	2.77	0.002*
		Residuals	19	6.46	0.340		
		Total	20	7.40			
	Summer	Days	3	2.11	0.703	3.01	0.003*
		Residuals	8	1.87	0.233		
		Total	11	4.00			
	Winter	Days	3	1.35	0.449	1.99	0.001
		Residuals	5	1.13	0.226		
		Total	8	2.48			

region, season, and impacts of land use and land cover, thus possibly affecting how they could be used in forensic investigations, similar to the variability of using insects during investigations. And although the role of bacteria and fungi in both standing and flowing aquatic systems has been clearly elucidated (97–100), we are not aware of any study that has defined the role of stream or pond bacterial communities, the importance of specific taxa, or the succession of different microorganisms associated with the decomposition of carrion or human remains. Additional studies are warranted to more fully test and develop this potential metagenomic approach in forensic investigations.

While this study is novel and advances the knowledge of vertebrate remains decomposition in an aquatic habitat and the potential applications in forensics, there are limitations to the reference data set provided in this paper. Although conducted in a single stream (due to logistical constraints, carcasses were separated by at least one riffle/pool sequence in a single stream to minimize upstream influence of carcasses) and limited sample size, to the best of our knowledge this study documents the first high-throughput metagenomic approach identifying bacterial communities throughout carrion vertebrate decomposition in a freshwater habitat. Additionally, the use of pyrosequencing for

carrion decomposition has its own limitations, as previously discussed by Pechal et al. (4). The current bacterial taxa information provided would be an excellent beginning for future research directions to more comprehensively explore the application of aquatic epinecrotic microbial communities in the forensic sciences.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1 A visual assessment of the decomposition process for replicate swine carcasses (n=3) placed in a temperate headwater stream during two seasons (summer and winter) was determined according to Zimmerman and Wallace (9).

Table S2 Mean ± (standard deviation) bacterial taxa relative abundance as characterized by pyrosequencing and universal 16s rRNA primers over decomposition at weekly sampling intervals during the summer season.

Table S3 Mean ± (standard deviation) bacterial taxa relative abundance as characterized by pyrosequencing and universal 16s rRNA primers over decomposition at weekly sampling intervals during the winter season.