I hope this proposal can be used as a guide to junior faculty. Note: blacked-out regions represent statements I did not write.

Statement of the Problem and Proposed Solution

About 90% of the cells in a healthy human adult body are microbial; the rest are human cells (8,58,60). When we die, the percent of microbial cells increases because necrotic human cells release their nutrients to the surrounding tissues, which supports the growth of microbes. To date, relatively few studies have investigated the human microbiome associated with death, which here we define as the "thanato-microbiome" (i.e., thanato-, Greek defn. *death microbiome*). The presumed reasons for the paucity of studies are: (i) lack of access to high-throughput DNA sequencing technologies, which is beyond the reach of most HBCU scientists, and (ii) problems with interpreting the massive amount data produced by the DNA sequencing technologies. Here, the Principal Investigator (PI) has solved these problems by establishing a collaboration with the Noble laboratory, which has a Roche/454 sequencer and a track record for interpreting microbial DNA sequence data in other studies (e.g., oil-impacted Gulf of Mexico surface waters and periodontal samples of canines and humans; see Letter of Support).

The PI proposes to study how microbial populations change in different organs in decomposing human bodies. We focused exclusively on humans because their microbiome has been well studied compared to other mammals. The reason for examining the abundances of microbial populations in decomposed bodies is that microbes likely reflect the post-mortem interval (PMI) (i.e., the elapse time since the mammal has died), which has been demonstrated in mouse and swine studies (37,48). We envision that specific microbial abundances can be used to estimate PMIs on the scale of hours to days with a 7-day maximum. The reason for examining microbial populations in different organs is because little is known about microbial communities involved in decomposing mammalian bodies. For example, where do these decomposing bacteria come from? Are specific types of bacterial species associated with specific organs? Are there successional changes in the bacterial community composition with time? From an evolutionary perspective, it would be interesting to investigate if there is a relationship between the microbes involved in decomposing mammal organs and mammalian species because it might indicate a "postmortem co-evolution".

To ensure success of this study, the PI's research team includes collaborations with Dr. Alex Pozhitkov at University of Washington, who is currently studying post mortem gene expression in mice and zebra fish (see support letter). Prof. Peter Noble, is an expert in microbial ecology, high throughput DNA sequencing and bioinformatics. Noble has been working with Pozhitkov (formerly his post-doc and a research scientist at Max-Planck-Institute for Evolutionary Biology) for 10 years with 10+ publications.

The PI asserts that this project:

- Will reveal new information, in terms of basic research, on how microbial populations change in different organs as human bodies decompose -- which is currently not known.

- Will help a junior STEM faculty member build research capability and effectiveness at a HCBU by improving research and teaching, and involving graduate and undergraduate students in "hands-on" research experiences.

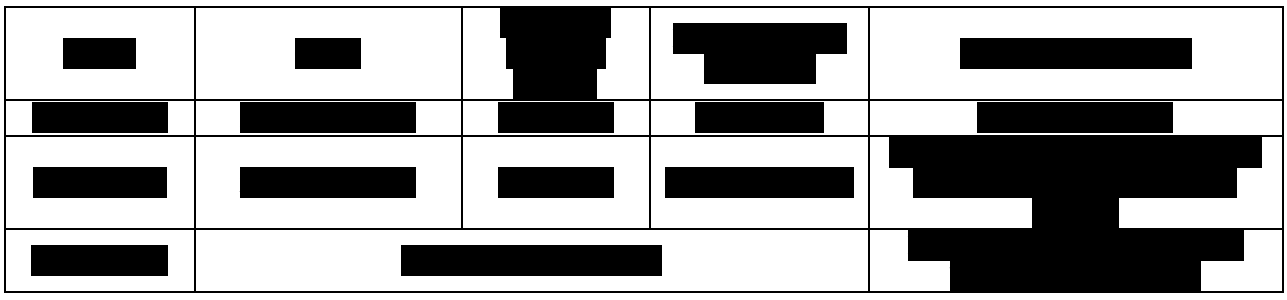
This proposal is consistent with the "Strengthening Forensic Science in the United States: A Path Forward" by the National Academy of Sciences, because it improves collaborations with broader science communities by linking forensic science faculty and students with experts in DNA sequencing, microbial ecology, and bioinformatics (6). The National Science Foundation (NSF) is the only Federal agency whose mission is to support basic research at the forefront of all fields of fundamental science. Few studies have examined the composition of microbial communities in different body organs as decomposition progresses. The PI asserts that the research proposed in this application is therefore appropriate for NSF support because it is basic research that will provide new information and education to forensic science at local (ASU), state (Alabama Department of [redacted]) and national levels (University of Washington).

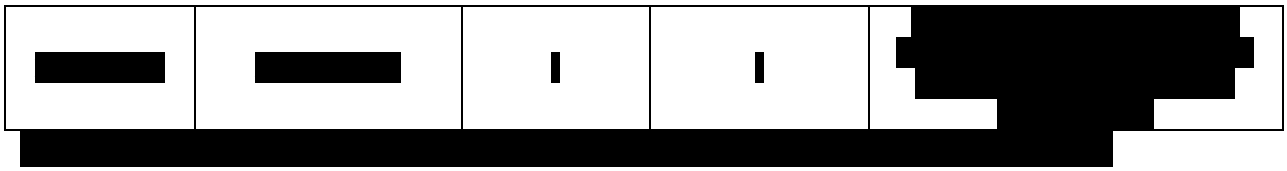
Background

Post-mortem Interval By definition, PMI is the elapsed time since a person has died and the post-mortem investigation begins. PMI can scale from hours and days to months and years. There are multiple approaches used to determine PMI such as changes in biochemistry (31,56, 57), entomological (36,66) and botanical processes (10,29). In addition, various formulae have been developed to estimate PMI based from multiple environmental and physicochemical conditions (e.g., 23,35). Table 1 shows the general characteristics used to determine PMI. In reality, most approaches only approximate PMI and they are not very accurate. To date, a universal approach to determine PMI remains an enigma (63).

Microbial populations and human death Recent scientific breakthroughs have resulted in a paradigm shift in the understanding of cells in the human body. Two significant findings, relevant to this proposal, are: most cells in the human body are microbial (60) and microbial cell abundance increases significantly after a person dies (14). Based on a survey of the literature, five sequential events occur after a person dies: (i) human cells become hypoxic because the heart stops and blood circulation ceases (18), (ii) hypoxia triggers the release of intracellular factors that causes the organized degradation of cellular organelles by proteolytic and autolytic enzymes (54), (iii) vesicles, formed by these enzymes, are released into the tissue fluid where they are phagocytized, (iv) membranes of human cells are lyzed by autolytic enzymes resulting in the release of cellular constituents (carbohydrates, amino acids, lipids, and minerals and water) to the tissue fluids, and (v) there is a massive increase in microbial abundance as they metabolize/utilize the cellular constituents (47).

Table 1. General characteristics used to determine PMI* based on references: 13,28,41.





We recognize that, at least initially, microbes cannot metabolize cellular constituents in the tissue fluid because they are suppressed by the human immune system (20). However, microbial cells eventually overwhelm the immune system (within 24 h) and proliferate. Ongoing research on post-mortem gene expression of the zebrafish (Dr. Alex Pozhitkov, see support letter) concurs with this scenario. A previous study suggests that in non-drowning cases, microbial proliferation occurs throughout the body beginning in the ileocecal area, spreading to the liver and spleen, and continuing to the heart and brain (3). Microbial colonization of the ileocecal area occurs by invasion of capillaries of the lymphatic and vascular system (47). Microbial proliferation also occurs in the respiratory system by the invasion of the mucus membranes (20). Yet despite information on increases in microbial abundances, there is a paucity of information on microbial community composition in different organs of the human body after a person dies and how these microbial communities change as the body decays as a function of time and temperature.

After a human dies, there is a massive proliferation of microbes in the gut, blood, liver, spleen, heart and brain. This proliferation occurs in a time- and temperature- dependent manner. Little is known about the microbes responsible for this phenomenon.

The RNA approach for measuring of microbial populations Most molecular methods for measuring microbial populations use ribosomal RNA (rRNA) genes because these genes occur universally in all living organisms and they contain highly conserved as well as highly divergent sequences. These sequences can be used to infer phylogenetic relatedness (68). Ribosomal RNA genes are present in one to several copies in a bacterial genome (1) and can be obtained from microbes by first extracting DNA using chemical and chemical disruption (e.g., 62) and then amplifying 16S rRNA genes using universal primers (27F and 1392R) and PCR (51). The amplicons can then be sequenced in a 454 pyrosequencer. Noble and Pozhitkov have extensive experience in determining microbial abundances in the human oral cavity using this approach (51,50).

The relative abundance of microbes in a biological sample can be approximated by the abundance of 16S rRNA genes.

Composition and abundance of microbial populations Output from the 454 Jr. typically consists of about 100,000 to 200,000 sequences per sample run. Each sequence has an average length of ~500 bp. The sequences can be uploaded to MG-RAST web site (38). The MG-RAST sequence-processing pipeline assesses the quality of the sequences and removes short sequences (multiplication of standard deviation of length cutoff of 2.0) and sequences with ambiguous bp (non-ACGT; max allowed number of ambiguous base pairs was set to 5). The pipeline annotates the sequences and allows data integration so that the sequences can be compared to other genomic samples. There are several databases (e.g., RDP, LSU) on the web site to annotate the 454 sequences. The annotated output contains information on the relative abundance of different microbial species at various levels of taxonomic resolution. By normalizing the samples to the total number of reads in a sample, different biological samples can be compared to determine the relative abundances of microbes by organ and in response to time/temperature effects.

454 pyrosequencing of 16S rRNA gene amplicons and MG-RAST can be used to infer the relative abundances of microbes at different taxonomic resolutions.

Standard analysis of rRNA gene reads Analysis of rRNA reads typically involves the determination of rarefaction curves and alpha diversity values, and the analysis of the data structure using principal component analysis and/or hierarchical clustering. Rarefaction curves determine if an adequate number of reads has been obtained from a biological sample. In a previous study of the human oral microbiome, Noble found that 5,000 to 20,000 reads were sufficient to reach saturation the rarefaction curves (Figure 1). In other words, further sequencing of the same sample would not result in a significant increase in the number of new microbial species discovered. Alpha diversity provides information of the diversity of the microbial communities in different organs and can be used to assess the effects of incubation time/temperature. Principal component analysis (PCA) reveals the dimensionality of the microbial abundances, i.e., how much variation in the data can be explained by principal components, as well as the possible relationships among the biological samples. PCA involves the orthogonal transformation of the relative microbial abundances into a set of principal components. Ordination plots reveal the grouping of samples (e.g., organ/temperature/time) sharing similar properties (e.g., microbes). We anticipate that the microbial populations will group by organ, temperature and/or time. Hierarchical clustering methods are typically used to back up the results obtained by the ordination plots. In theory, the robust groupings of biological samples indicate that the microbial populations are similar.

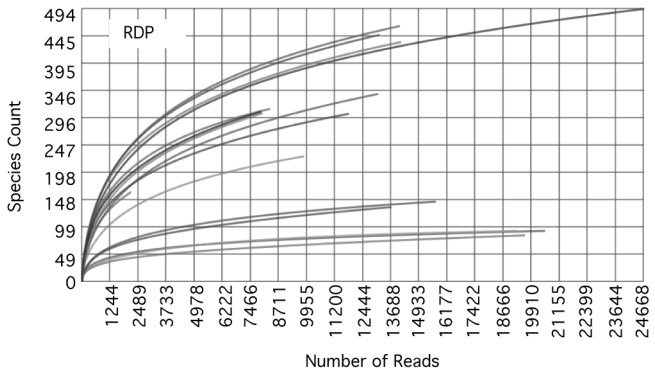


Figure 1. Rarefaction curves of 16S rRNA gene amplicons representing 5 different human oral microbiomes (51). Results obtained using RDP database with cutoffs of 97% similarity, min alignment of 100 bp and e-value of 10^{-5}.

Uncertainty in rRNA gene abundances We recognize that there is uncertainty in interpreting the sequencing data that is related to the taxonomic resolution. For example, annotation of the 454 reads is based on incomplete/limited data (e.g., RDP) and the similarity thresholds (97 to 99%) used to annotate the reads have a significant effect on taxonomic designations (27). In general, the accuracy of the annotations improves with taxonomic rank (species → genus → family → order → class → phylum → domain). We will deal with this uncertainty by performing PCA and clustering methods at multiple levels of taxonomic resolution.

Conventional statistical approaches can be used to link inputs (e.g., temperature, organ and relative microbial abundances) to outputs (e.g., PMI).

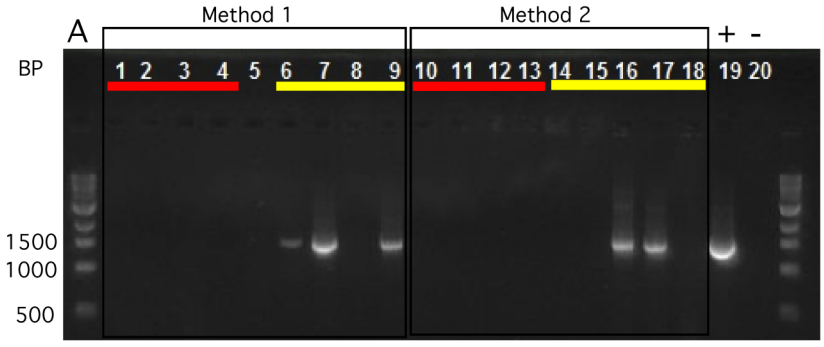
Preliminary results

We have successfully extracted DNA from the organs of three deceased humans (Cases 24, 25, and 35; Table 2) and amplified 16S rRNA genes using F27 and R1394 primers (Figure 2). The amplicons, that represent microbial rRNA genes, were only found in subjects with PMI of 50+ h. At present, we cannot draw conclusions about the earliest detection of microbes in organs

because we have sampled few subjects and the method to extract DNA from the organs has not been optimized (i.e., it is one of the objectives of this proposed study).
Currently, we are using two extraction methods: Method 1 involves extracting the DNA from the blood (~200 µL) and organs (1 cm^3) that is followed by conventional chemical (phenol/chloroform/ ethanol) and physical disruption (bead beating) methods (42). Method 2

Table 2. Frozen cadaver samples collected as of October, 2013. * GSW, Gun shot wound; N, Case number; C, Caucasian; A, African; L, Latino, M, Middle East. Table NOT SHOWN

involves using sterile cotton tip applicators to survey the blood and organs and transferring the tip to a centrifuge tube containing 500 µL of PBS. Rather than using physical disruption of the sample, the centrifuge tubes are vortexed, heated and then cooled (twice), centrifuged, and the supernant collected and processed by the conventional chemical extraction methods stated above. The key difference between the methods is that Method 1 follows the conventional approach used for processing environmental samples for DNA sequencing in the Noble laboratory, while Method 2 is a newer approach that might provide a broader survey of the microbes in the organs or in the blood.
The presence/absence of an amplicon in certain organs was found to be dependent on the DNA extraction method. In Case 25, for example, amplicons were obtained in DNA extracted from brain and spleen samples using Method 1 (Lanes 6 and 9) but no amplicon was obtained using Method 2 (Lanes 15 and 18). In the same case, an amplicon was obtained in a liver sample using Method 2 (Lane 17) but no amplicon was obtained using Method 1 (Lane 8). These apparent differences point to the DNA extraction methods as the source of these inconsistencies. These findings are further complicated by the fact that both extraction methods yielded amplicons for heart samples. Clearly, optimization of DNA extraction is needed before moving on with surveying of the microbes in decomposing organs and in the blood, which is the focus of Obj.1.



B

Gel	Method	Case	Lane	Product	Organ/Blood
A	1	24	1	-	blood
			2	-	brain
			3	-	heart
			4	-	liver
			5	-	blank
		25	6	+	brain
			7	+	heart
			8	-	liver
			9	+	spleen
	2	24	10	-	blood
			11	-	brain
			12	-	heart
			13	-	liver
		25	14	-	spleen
			15	-	brain
			16	+	heart
			17	+	liver
			18	-	spleen
B	1	35	2	+	heart
			3	+	liver
			4	+	spleen
	2		5	+	heart
			6	+	liver

Figure 2. A, Agarose gel shown 16S rRNA amplicons by organ and DNA extraction method. Red bar, Case 24 (42 h); Yellow bar, Case 25 (58 h).

Method 1, conventional chemical/physical disruption of cells; Method 2, Cotton tip, heating/cooling/centrifugation/precipitation. B, Table showing interpretation of the Gels A and B (Case 35; 83 h PMI, see Figure 3 and 4). +, 16S rRNA amplicon from *Salmonella enterica* LT2 genome; -, no template.

To investigate the microbial community composition in different organs, 16S rRNA genes were amplified from extracted DNA samples for Case 35 (Table 2). All samples yielded PCR amplicons of the correct size (~1200 bp) (Figure 3). Sequencing yielded about 175,000 sequences, with an average sequence length of 500 bp, that were uploaded to MG-RAST for annotation. The MG-RAST pipeline assesses the quality of sequences, removes artificial replicated sequences [27], removes short sequences (multiplication of standard deviations for length cutoff of 2.0), and sequences with ambiguous basepairs (non-ACGT; maximum allowed number of ambiguous base pairs was set to 5). The relative abundances of major bacterial groups by organ revealed that all samples were dominated by species in the genus *Clostridium.* To backup the MG-RAST results, we downloaded a 16S RNA database from NCBI (16Smicrobial.tar.gz) and blasted the DNA sequences against it. The retrieved GI numbers were then queried using Batch entrez, and taxonomic affiliations were determined using the Taxonomic Browser. To visualize the microbial community composition by organ, we created pie charts (Figure 4). Although our results are based on a single case, the MG-RAST output aligned with the output obtained by blasting the sequences against the 16S rRNA database. The results indicate differences in the microbial community composition by organ and DNA extraction method.

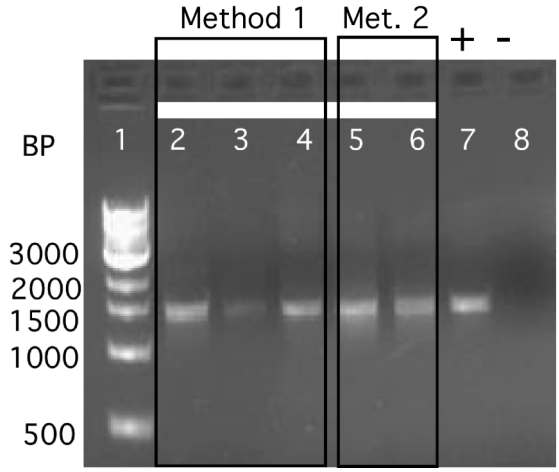


Figure 3. Agarose Gel B shown 16S rRNA amplicons by organ and DNA extraction method for Case 35 (83 h PMI). +, 16S rRNA gene amplicon from *Salmonella enterica* LT2 genome; -, no template. See Table in Figure 2 for interpretation.

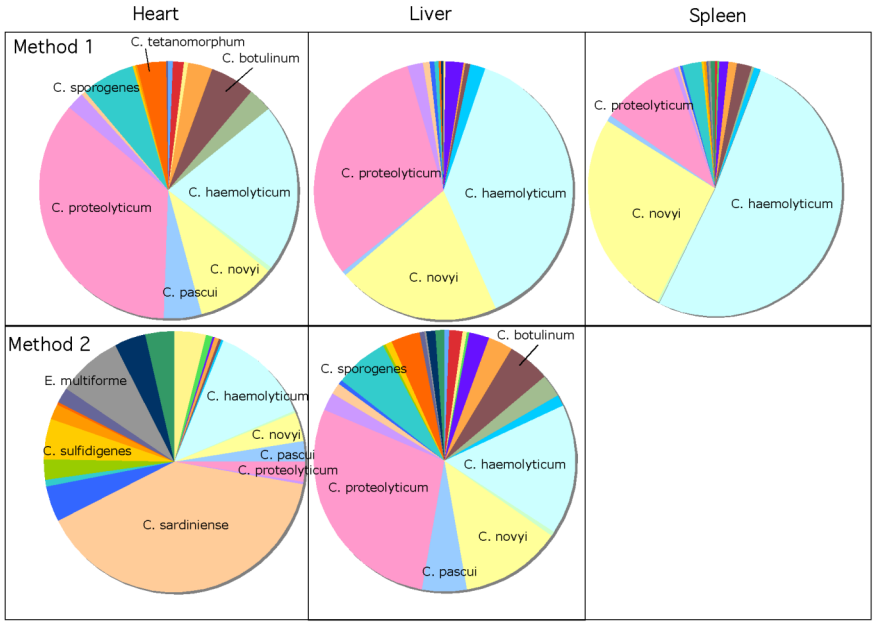


Figure 4. Relative abundance of major bacterial groups by organ from Case 35 (83 h PMI) (see Figure 3). Results show that the species from the genus *Clostridium* dominate the bacterial community and that there are differences in microbial populations by DNA extraction method and organ.

Research Objectives, Hypotheses, Rationale, Approach, and Anticipated Results

Objective 1. Select the optimal method to sample microbes in various organs and in the blood. **Rationale:** Our preliminary results revealed that two methods for sampling the organs and extracting the DNA yielded different microbial community compositions (see Figure 2 and Figure 4). These findings are also reflected in the rarefaction curves and alpha diversities (Figure 5). Method development is necessary to obtain consistent results with the highest microbial diversity possible.

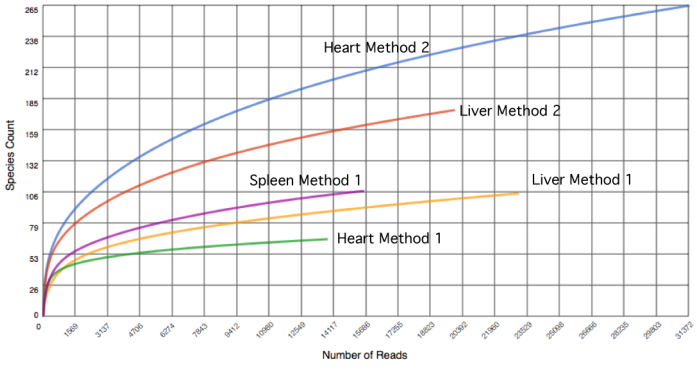


Figure 5. Rarefaction curves of Case 35 using two DNA sampling/extraction methods and the same parameters as in Figure 1. The alpha diversities of heart, liver and spleen using Method 1 was: 9.9, 6.8, and 7.1 respectively, while the diversity of heart and liver using Method 2 was: 26.0 and 16.1, respectively, indicating that Method 2 yields a greater microbial diversity than Method 1.

Approach: Samples of the blood and organs (spleen, liver, heart and brain) from 4 human cadavers will be obtained through autopsy examinations with PMIs of 50+ h (see Table 2). We have chosen these PMIs because the samples likely will contain microorganisms. We will

collect samples and extract DNA by using both methods (in triplicate). We with then compare rarefaction curves, alpha diversities, and pie charts to determine which of the two methods is optimal. We will then assess the optimal method by considering the means and standard deviations of the alpha diversities and performing Student t-test to determine if two sets of data are significantly different from each other.

Anticipated results: Our preliminary study revealed that Method 2 was optimal because it yielded a higher alpha diversity (21 ± 7) than Method 1 (8 ± 2), but the data are too sparse at the moment to move on to Objective 2.

Task 1.1 Extract DNA from the 4 sample sites of each cadaver by the two methods and determine the quality/quantity of DNA.

Task 1.2 Amplify the 16S rRNA genes using the GemTaq kit from MGQuest and universal primers, purify the amplicons, and determine the quality/quantity.

Task 1.3 Following Roche/454 standard sequencing protocols: build a Rapid Library and barcode each sample, pool the samples at equimolar concentrations, amplify the library using emulsion PCR, and then pyrosequence the samples in the 454 instrument.

Task 1.4 Split the sequence output by barcode, upload the sequences to MG-RAST for processing and annotation. The MG-RAST pipeline assesses the quality of the sequences, removes short sequences (multiplication of standard deviation for length cutoff of 2.0), and removes sequences with ambiguous bp (non-ACGT; maximum allowed number of ambiguous base pair was set to 5) (21,38).

Task 1.5 Analyze the data by producing rarefaction curves to determine if the thanatomicrobiome of different organs are adequately sampled in terms of number of total number of different microbes in a sample. Determine the alpha microbial diversities by method of DNA extraction.

Task 1.6. Produce pie charts to determine the effects of the DNA extraction on microbes at different levels of taxonomic resolution. Make a decision on the optimal method of DNA extraction based on obtained results.

Objective 2. Survey the thanatomicrobiome (blood, liver, spleen, heart and brain) of 30 cadavers that have a range of known PMIs to establish baseline data.

Hypothesis 1: As a human body decays, microbes proliferate in the blood, liver, spleen, heart and brain in a time- and temperature- dependent manner; therefore, the relative abundances of microbes will vary by respective body organ/temperature and PMI.

Rationale: The term "relative abundances" of microbes in Hypothesis 1 refers to the array of abundances occurring at different levels of taxonomic resolution for a given sample/time/temperature (henceforth referred to as the "microbial signature"). The relative abundance of a particular microorganism, or group of microorganisms within the same taxon (i.e., domain, family, order, class, genus), is determined by the number of copies of 16S rRNA genes in a sample and is dependent on the similarity (97% to 99%) thresholds of the annotation (27). The optimal level or levels of taxonomic resolution for accurately determining PMI can be determined by statistical analysis (e.g., linear/nonlinear regressions which Noble is an expert; see refs. 26, 43,42,45).

As mentioned earlier, as a body decomposes, human cells discharge their constituents into the fluid tissues, the immune system becomes overwhelmed, and there is a massive proliferation of microbes. A survey of the published literature reveals a paucity of information on the composition and abundances of microbes in the blood, liver, spleen, heart, and brain after death. To date, only one study has examined microbes in the blood and organs in two drowning victims

using high throughput sequencing (25). The study suggests that, in drowning cases, microbes entered the blood and organs through the lungs.
In the absence of drowning, one would expect microbes to enter the organs through the gut and respiratory systems and spread through the arterial/venous, lymphatic, and mucus system in a time/temperature dependent manner (47). A protrusion into the body (e.g., gun shot wound) or breakage of the skin might also introduce microbes and/or O_2 into the body. We would expect the microbial community composition and abundance in the blood and organs to have dependence on temperature and time because it is well established that bacteria have different growth optima (16); therefore, some bacteria will utilize cellular fluids more rapidly than others. Hence the microbial community composition and abundances would be expected to be time and temperature dependent.

Approach: We will only use samples from human cadavers that have a known PMI and the cause of death is homicide, suicide, car accident, and misadventure. We will not use cadavers from drowning cases because the microbial entry through the lungs significantly complicates interpretation (although it might be included in future projects). We have focused on the blood and liver, spleen, heart and brain in particular because we believe that the microbial proliferation in the lungs and gut tissues will be too rapid to be useful for relating to the PMI. Ideally, we will try to balance the number of males and females weight/height and age range used in the study but the primary criterion for selection will be the known PMI because we need a broad range (<1 day to 7 days) of samples to relate microbial composition/abundance to PMI.

The samples will be transported from the morgue to the laboratory on ice and immediately frozen to -80°C until processing. Processing involves extracting the DNA from the blood and organs following the optimized method determined in Objective 1. The quality and quantity of DNA will be determined by spectrophotometry (Nanodrop). We will amplify the DNA using PCR and universal primers (27F and 1392R). The amplicons will be purified and run out on an agarose gel to ensure that the amplicons are of the expected size and the quality/quantity will be determined by Nanodrop spectrophotometry. Each organ and blood sample will receive a unique bar code so that they can be multiplexed in the same 454 sequencing run. The bar code will be added to the samples during the 454 Rapid library construction. Equimolar concentrations of 4 libraries will be added to emulsion PCR (emPCR) reaction. The amplicons will be then be sequenced on the 454 Jr. instrument. The bar code will be used to sort out the samples after the sequencing run using Roche 454 software. The sorted sequences will be individually uploaded to MG-RAST for annotation and determining the relative microbial abundances at different levels of taxonomic resolution. We will explore the internal structure of the relative microbial abundance data and its dimensionality by principal component analysis (PCA). If the dimensionality of the data is sufficiently low, the samples will be plotted on ordination plots. Hierarchical clustering methods of normalized microbial abundances will be used to produce dendrograms that will support the ordination results. If there are significant differences in the groupings by organ/temp/time, we will identify the microbes responsible for these differences by performing Mann-Whitney tests. The Mann-Whitney is a non-parametric test that we have used in a previous study to determine statistical differences in microbial abundances between clinically–defined groups (51).

As in previous studies (44,51), we will explore the sequencing data using statistical tests. For example, in Figure 6, Noble and Pozhitkov found distinct differences in the oral microbes of subjects with two different clinically defined conditions (condition 1 and condition 2). To

identify the microbes responsible for these differences, we will perform non-parametric Mann-Whitney tests. In our previous study, the test identified 26 microbes that had higher % relative abundances in subjects with the first condition than those with the second condition. Moreover, subjects with the second condition had 10 unique microbes that were not found in the first condition. The results from condition 3 and 4 were ambiguous and not discussed here. We will use the same approaches to explore the relationships between the microbes from different organs, the microbes collected at different PMIs and different temperatures, and the microbes from different organs, different PMIs and different temperatures.

Anticipated results: We anticipate some commonalities in terms of microbial signatures (composition and abundance) in every human decomposition event – as demonstrated by peri-mortem chemical composition of soft tissue and bone, which is relatively stable (64). In other words, the PI asserts that certain microbes will have similar abundances in organs of cadavers provided that they are collected under the same conditions. We expect the microbial abundances to differ by organ in the same cadaver because organs have different biochemical compositions and it will take different times for the microbes to enter and proliferate in these organs and our preliminary results (although limited) support that (Figure 4). We also anticipate that the composition and abundance of the microbes to vary by the average temperature of the decomposition. We will determine the average temperature of the decomposition based on local information (e.g., weather) where the body was found (see Table 2 for example).

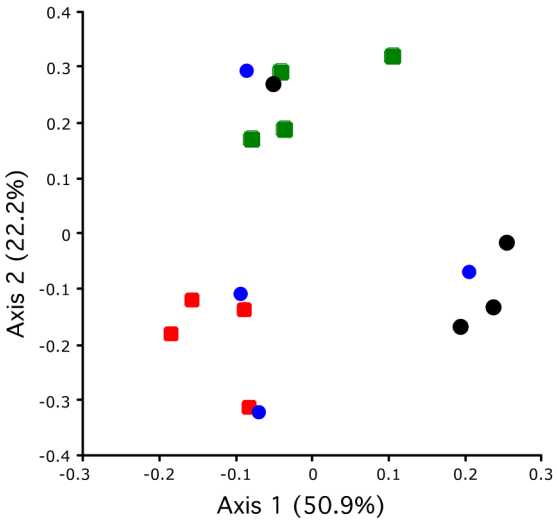


Figure 6. Ordination plot of human oral microbiome samples by condition based on the relative abundances of microbes by sequencing 16S rRNA amplicons. Condition: red, condition 1; green, condition 2; blue, condition 3; black, condition 4. From Ref. 51.

Although we will examine the microbes in the blood, liver, spleen, heart and brain of 30 cadavers, it is our intention to select only one to three organs for the next section of the project. We will choose the best organs for relating to the PMI based on the statistical analyses of the thanatomicrobiomes. From the PI's own previous forensic experience (i.e., the observed degradation rate), the spleen and liver will be optimal for relating to the PMI – but we do not know for certain.

Task 2.1 Extract DNA from the four sampling sites of each cadaver using the optimized method determined in Objective 1.

Task 2.2 Follow tasks 1.2 to 1.4.

Task 2.3 Produce rarefaction curves to determine if the thanatomicrobiome of different organs are adequately sampled in terms of number of total number of different microbes in a sample. Determine the alpha microbial diversities.

Task 2.4 Run PCA and create an ordination plot using normalized abundances of the microbes and Euclidean distance. Determine if there are any apparent differences in terms of known PMI and organ or cadaver sample.

Task 2.5 To determine which microbes are responsible for differences observed in the PCA ordination, download the annotated sequences, create a database, run Mann-Whitney tests on the normalized microbial abundances (at different levels of taxonomic resolution).

Task 2.6 Create histograms and pie charts of microbial abundances at different levels of taxonomic resolution by organ/cadaver/PMI in order to observe trends.

Objective 3. Select one to three of the microbiome sample sites that are optimal for determining PMI, and collect those sites (e.g. putatively, the spleen and liver) from an additional 70+ cadavers to determine their thanatomicrobiome.

Hypothesis 2: Microbial signatures in one to three sample sites in the same cadaver will be sufficient for determining the PMI.

Rationale: The survey of the microbiomes from the blood and organs (liver, spleen, heart and brain) of 30 cadavers will provide baseline data for this section of the study. We suspect that the microbiome of some organs will be better than other organs in terms of determining the PMI. Collecting the blood and 4 organ samples from 100 cadavers is costly in terms of personal time, costs of storing the samples, processing the DNA, and sequencing. Given that this is a two-year project, we will not have enough time and resources to conduct an in-depth survey of the thantomicrobiome of all sites (liver, spleen, heart and brain) in the human body. Reality dictates that we will need about 100+ cadaver samples to develop a mathematical model. Hence, we will narrow down the sampling from five sites (blood, liver, spleen, heart and brain) to one/three sites per cadaver, which would significantly reduce the costs. The specific sites to be investigated will be based on the results obtained in Objective 2.

Approach: We will follow the molecular methods and statistical analysis procedures outlined in the previous section. We will need about 100+ cadaver samples to have enough data to train/test/validate the mathematical models to accurately predict PMI. Like the previous section, we will try to balance the number of males and females and age range used in the study but the primary criterion for selection will be the known PMI because we need a broad range (<1 day to 7 days) of samples to predict the PMI. We emphasize that the key reason for developing models is to the establish the relationship between inputs (e.g., microbial species abundances at multiple levels of resolution, temperature) and outputs (e.g. PMI) is so that we can perform sensitivity analysis – which will reveal the specific microbial populations that are essential to predict PMI. The theory for and demonstrate of this approach is discussed in detail in Noble's two publications: Refs. 26, 45:

Anticipated results: Sensitivity analysis of the microbial signatures from the models will identify specific microbial populations that can be used to determine the PMI. We do not anticipate any problems with this section of the proposal because we have much experience in developing models and determining which inputs are key to predict outputs (see refs. 26, 43,42,45). We anticipate that microbial populations will different in various organs due to differences in biochemistry and time/temperature differences. The developed model will account for subtle differences in temperature as well as different levels of taxonomic resolution (i.e., species → genus → family → order → class → phylum → domain).

Task 3.1 Compare trends in terms of the abundances of specific microbiomes by organ and blood sample from Objective 2 to narrow down the number of collection sites in a cadaver. Select one to three sample sites for subsequent research (e.g. spleen and liver), depending on the statistical results.

Task 3.2 Collect and process the 70+ samples following tasks 2.1 to 2.6 with the exception that we will only process one (to three) of the selected sample sites in each cadaver. Up to 8 samples

will be bar coded and pooled in a 454 sequencing run depending on the results from the rarefaction curves.

Task 3.3 Train/test models. Determine the optimal architecture in terms of *x*-inputs (microbes at different levels of taxonomic resolution; organ; temperature), and *y*-outputs (known PMIs). Run sensitivity analysis to identify redundant inputs, so they can be removed from the training/testing/validation data sets.

Task 3.4 Using linear regression analysis, determine the R^2 of predicted PMIs (based on the validation data set) versus actual PMIs. We anticipate that the R^2 of the regression will be > 0.80 for the optimized linear or nonlinear regression models.

Task 3.5. Using the weights and biases of the model build the model in MS Excel and then code in C++ to make a stand-alone model that will be accessible to other scientists.

Task 3.6. Make an informative web page explaining the model and containing the C++ source code and example files. Both the source code and the example files will be downloadable so that other investigators can use the data (see examples at http://peteranoble.com/software.html).

Milestones

		Quarters							
Objectives	Tasks	1	2	3	4	1	2	3	4
1	1.1	X	X	X					
	1.2	X	X	X					
	1.3	X	X	X					
	1.4		X	X					
	1.5		X	X					
	1.6		X	X					
2	2.1			X	X	X	X		
	2.2			X	X	X	X		
	2.3			X	X	X	X		
	2.4			X	X	X	X		
	2.5			X	X	X	X		
	2.6			X	X	X	X		
3	3.1			X	X	X	X	X	X
	3.2			X	X	X	X	X	X
	3.3			X	X	X	X	X	X
	3.4			X	X	X	X	X	X
	3.5			X	X	X	X	X	X
	3.6			X	X	X	X	X	X
Manuscripts submitted					X				X

Capabilities and Competencies

Peter A. Noble, the PI's mentor, is an expert in microbial ecology, DNA microarrays, 454 sequencing and bioinformatics. Noble has worked with [redacted] for about a year, training her and her students in 454 DNA sequencing. Noble has worked with Alex Pozhitkov for 10 years in the field of understanding high throughput technologies, which has resulted in 10+ publications. Noble's laboratory is completely equipped for DNA pyrosequencing and DNA microarray development and he has a computer account at the Alabama Supercomputing facility.

Alex E. Pozhitkov, a consultant, is an expert in DNA microarrays, 454 sequencing, and bioinformatics. In collaboration with Max-Planck-Institute, Pozhitkov is lead author on a working paper dealing with gene expression upon zebrafish death (see support letter). He is an expert in bioinformatics and C++ programming.

Impact/Outcomes, Evaluation and Dissemination

A survey of the published literature using the Web of Science reveals a paucity of studies dealing with the microbiome after a human or any other mammalian dies. Hence, the impact of the outcomes in this project will likely be significant and novel. We anticipate the production of two high impact peer-reviewed papers and several presentations at national conferences (see Milestones). In terms of student training, this project will provide the supplies and travel for one MS and four undergraduate students in the [redacted] Science Program at ASU. Both [redacted] and Noble are currently involved in hands-on training of undergraduate/graduate students in molecular biology/bioinformatics. Hence, ASU students will be actively involved in extracting DNA, 454 sequencing, blasting the sequences on the NCBI web site, interpreting the annotations, summarizing the results, writing abstracts, and presenting research at national meetings. The laboratory work has brought forensic biology into ASU classrooms. Funding of this project would go a long way towards the development of inquiring minds and inquiring attitudes, and enhancing of scientific collaborations with local (ASU Forensic Science Program), state (Alabama Department of [redacted]), national (University of Washington) levels.
Management Organization

PI [redacted] will have overall responsibility for the project; she will be the contact person between the [redacted] offices and ASU and will organize bimonthly meetings. [redacted] will be involved in mentoring the graduate and undergraduate students (see undergraduate and graduate mentoring plans). Prof. Noble will be responsible for mentoring [redacted] (see Mentoring plan for the development of junior faculty in research). He will also be responsible overseeing the DNA sequencing and bioinformatics portion of the project. He will work closely with [redacted] and her

students and Dr. Pozhitkov at the University of Washington. Drs. [redacted], Pozhitkov and Noble will be involved in the writing of manuscripts and making presentations.

Implication of basic research for increasing knowledge

A recent PNAS publication (34) stated that: "the interactions between animals and microbes are not specialized occurrences but rather are fundamentally important aspects of animal biology from development to systems ecology". In keeping with this concept, microbes responsible for decomposing organs/bodies might also be a fundamentally important aspect of animal biology since decomposition is a universal phenomenon that has not been well studied. Funding of this project will increase knowledge and fill this void.

Determining if certain microbial signatures are indicative of PMI will significantly contribute to basic scientific knowledge because it might lead to practical applied applications (i.e., PMI meter). Specifically, the PMI meter will take inputs, such as the abundances of specific microbes (taxonomic resolution to be determined in this study) and using an algorithm (i.e., equation developed in this study) accurately predict the output, the PMI. Potentially, the generalizable algorithm (developed in this study) could be used for extracting legally binding evidence from a dead body.

Determining if there are specific microbial signatures in the organs of decomposing bodies might well tell us if certain microbes have co-evolved with humans (or mammals, in general). It has been well established that microbes have co-evolved with living humans because they dominate in terms of number of cells in our bodies and play important physiological roles in health and disease. For example, a recent study showed that the bacterium *Mycobacterium tuberculosis* has co-evolved with different populations of humans and spread to different corners of the planet (Comas et al. 2013). *If microorganisms have co-evolved with us in life, why wouldn't they co-evolve with us in death?* The primary reason may be as simple as microorganisms are acquire by us (the host) when we reach a certain age (e.g., puberty) and they are poised to proliferate when we die or when our immune systems collapse, which results in recycling nutrients back to the ecosystem. Alternatively, microbes might have evolved to transfer and propagate themselves into new hosts that consume internal organs. The consumption of organs is of much greater value to a host than muscle because organs contain more essential proteins, minerals and nutrients needed for host survival. This makes sense from the standpoint that many animals are known to be scavengers and even hunter-gathers and modern human societies are cannibalistic. It is essential to fund basic research in the thanatomicrobiome in order *to reveal these gray areas in the co-existence of microbes and humans -- in both life and death*.

Comas et al. 2013. Out-of-Africa migration and Neolithic coexpansion of *Mycobacterium tuberculosis* with modern humans. Nat Genet. 45:1176-1182. doi: 10.1038/ng.2744.

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