Exploring the earthworm gut as model for soil microbial ecology

Fatima Hussain

Department of Civil and Environmental Engineering
Massachusetts Institute of Technology
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Introduction

Soil microbial ecosystems are incredibly diverse, with every gram of soil containing ~1,000 unique bacterial ribotypes (1). High species richness, combined with extensive spatial structure, make soil a particularly challenging environment for studying microbial communities and their functions. Most of the organic matter in soil is concentrated to the top 10 cm associated with plant roots called the rhizosphere, which is oxygenated by plant root growth and high turnover by earthworms (2). Earthworms are responsible for the majority of turnover in the upper layers of soil. Their burrowing oxygenates the soil, while their digestion provides a transient anaerobic environment for soil microbes passing through them. The earthworm also helps us define system boundaries for studying soil microbial processes. Here, I explore using the earthworm gut as a model for soil microbial ecology, specifically focusing on denitrifying bacterial communities enriched within the worm.

Motivation and Approach

The goal of this mini-project was to investigate denitrifying bacteria in the earthworm gut using cultivation and imaging. Specifically, I asked two questions:

First, does the earthworm gut enrich for denitrifying bacteria that differ from the surrounding environment? To do this, I designed and tested enrichment media with either nitrate or nitrous oxide as the electron acceptor and acetate or formate as the electron donor in stoichiometric proportions.

Second, what is the spatial distribution of denitrifying bacteria along the worm gut? To visualize the gut bacteria, I explored different imaging techniques, including catalyzed reporter deposition fluorescence in situ hybridization (CARD-FISH), microbial identification after passive clarity technique (MiPACT), and scanning electron microscopy (SEM). My personal goals were to learn anaerobic cultivation and imaging techniques, as well as to explore soil microbial ecology for the first time.

Methods

Sample Collection

Compost samples and redworms (*Eisenia fetida*) were collected from a compost heap from Coonamessett Farm (41.6171N, 70.5751W) in Tupperware containers and kept in the lab with damp paper towels to maintain moisture until processing.

Enrichments

Selective media was designed using either nitrate or N2O as the sole nitrogen source, and either acetate or formate as the sole carbon source. Nitrate is the most oxidized form of nitrogen and thus allows us to test for complete denitrification. N2O is an intermediate in denitrification and can further be reduced to N2, however this pathway is far less studied. Acetate is a non-fermentable central intermediate in metabolism and thus should enrich for denitrifiers. While acetate metabolism is universal, formate utilization is restricted, allowing for selection of diverse metabolic processes. Selective media was designed in stoichiometric proportions according to the following balanced reactions:

$$2NO_3^- + 5CHOO^- + 7H^+ \rightarrow 5CO_2 + N_2 + 6H_2O$$
$$8NO_3^- + 5H_3CCOO^- + 13H^+ \rightarrow 10CO_2 + 4N_2 + 14H_2O$$
$$N_2O + CHOO^- + H^+ \rightarrow N_2 + CO_2 + H_2O$$
$$4N_2O + H_3CCOO^- + H^+ \rightarrow 4N_2 + 2CO_2 + 2H_2O$$

One enrichment per media-inocula combination was started (total of 8 samples), and each enrichment was transferred once.

Selective Media Recipes

1) Nitrate-Acetate Media

Solution	*Final Concentration*
*100x FW base**	1x
1M sodium sulfate	100uM
100mM potassium phosphate	100uM
1M MOPS buffer	20mM
1M sodium nitrate	5mM
1M sodium acetate	3.125mM
1M sodium bicarbonate	10mM
1000x vitamins	1x
1000x trace elements	1x
*soil extract***	1:1000 dilution

2) Nitrate-Formate Media

Solution	*Final Concentration*
*100x FW base**	1x
1M sodium sulfate	100uM
100mM potassium phosphate	100uM
1M MOPS buffer	20mM
1M sodium nitrate	5mM
1M sodium formate	12.5mM
1M sodium bicarbonate	10mM
1000x vitamins	1x
1000x trace elements	1x
*soil extract***	1:1000 dilution

3) N2O-Acetate Media

Solution	*Final Concentration*
*100x FW base**	1x
1M sodium sulfate	100uM
100mM potassium phosphate	100uM
1M MOPS buffer	20mM
1M sodium acetate	3.125mM
1M sodium bicarbonate	10mM
1000x vitamins	1x
1000x trace elements	1x
*soil extract***	1:1000 dilution
N2O	(14mL added to headspace of serum vials during setup***)

4) N2O-Formate Media

Solution	*Final Concentration*
*100x FW base**	1x
1M sodium sulfate	100uM
100mM potassium phosphate	100uM
1M MOPS buffer	20mM
1M sodium formate	12.5mM
1M sodium bicarbonate	10mM
1000x vitamins	1x
1000x trace elements	1x
*soil extract***	1:1000 dilution
N2O	(14mL added to headspace of serum vials during setup)

*100x Freshwater (FW) base (per liter) consisted of NaCl (1711mM), MgCl2.6H2O (197mM), CaCl2.2H2O (68mM), KCl (671mM)
**soil extract was made by combining 175g of compost and 175mL DI H2O in a kitchen blender and blending at full speed for 3 minutes, followed by centrifugation in 50mL Falcon tubes for 10 minutes at 3,000xg, and serial filtration through a Whatman GF/C microfiber filter and a 0.22um Millipore Stericup Complete Filter unit.
***Same N2O volume was used for formate and acetate enrichments

Inocula
Worm inoculum was prepared by surface sterilizing the earthworms with 75% ethanol and homogenizing their tissue using a glass tissue grinder. Compost was used directly with as the inoculum.

Serum vial setup
All media was flushed with argon gas for five minutes. Autoclaved 180mL serum vials were filled with 50mL flushed media and ~5g inoculum, sealed with butyl stopper and aluminum crimp, and flushed with argon gas for five minutes with a vent needle. For N2O, enrichments, 14mL of N2O was added after flushing, overpressurizing the vials. Enrichments were incubated at 30C without shaking until turbidity and gas formation was observed (~48 hours), analyzed for nitrate, acetate, and formate loss using IC and HPLC, and then transferred to like medium. IC and HPLC analyses were run on transfers after 4 days.

Ion Chromatography & High-pressure Liquid Chromatography
Enrichments, transfers, and media were sampled from serum vials for ion chromatography (IC) and high-pressure liquid chromatography (HPLC) analysis using a sterile needle syringe.

Samples for IC were dilution 1:100 (500uL sample + 4950uL DI water) and 0.22um-filtered prior to analysis. Dionex ICS AS-DV autosampler was used with Dionex ICS-2000 system for anions with the following: columns [Dionex IonPac AS22 Fast 4 µm 4 mm x 150 mm (analytical); Dionex IonPac AG22 Fast 4 µm 4 mm x 30 mm (guard)]; suppressor [Dionex AERS 500 4 mm]; eluent [1.2 ml/min; isocratic; 15 min
1.4 mM sodium bicarbonate (Dionex, Thermo Fisher Scientific)
4.5 mM sodium carbonate (Dionex, Thermo Fisher Scientific)]; suppression [29 mA]; standard [Dionex Seven Anion Standard II (Thermo Fisher Scientific)]; column temperature [30°C]; conductivity cell [35°C]. Data was processed using Chromeleon 7.2 SR4 software.

Samples for HPLC were acidified to a final concentration of 10% H2SO4 by volume (800uL sample + 89uL 5N H2SO4) and filtered prior to analysis. BioRad Aminex HPX-

87H column with BioRad 125-0129 guard cartridge was used with Shimadzu LC 2010C HPLC pumping unit. Measurements were made using UV/vis Detector and Refractive Index Detector.

Colony Isolation

Colonies were isolated from enrichment transfers under ambient oxygen by diluting each sample in its respective media and spread plating a dilution series (10^0 to 10^-5 dilutions; 150uL/plate) using glass beads on enrichment media-based agar plates (selective media amended with 1.5% Bacto Agar). Plates were dried in the biosafety cabinet and placed in anaerobic gas pack jars modified with gas lines on the lids, a petridish filled with calcium chloride, which served as a desiccant, and a gas pack pouch with an oxygen indicator. The Nitrate enrichment jars were flushed out with argon for 5 minutes and then plugged, while the N2O enrichments were first flushed with argon for 5 minutes and then pressurized to 2 atm with argon and evacuated to 1 atm for a total of 10 cycles before the chamber was amended with 360mL N2O (~10% final concentration) and then sealed. Plates were incubated at room temperature for 48 hours and then moved to 30C for 24 hours. Colonies from each enrichment were picked under ambient oxygen and directly placed into colony PCR master mix for 16s identification.

Colony PCR

Colonies from enrichments were directly picked into 50uL PCR reactions (25uL Promega GoTaq G2 Hot Start Green Master Mix, 2uL of 10uM 8F and 1391R 16s primers, 21uL H2O). The thermocycler conditions were: 5 min boil (95C), 2 min initial denaturation at 95C, 30 cycles of 30 sec denaturation 95C, 30 sec annealing 55C, 1.5 min extension 72C, followed by a final extension for 10 min at 72C.

Clone Libraries

5mL of nitrate-acetate-compost and nitrate-acetate-worm enrichments were concentrated prior to DNA extraction using Zymo Research Quick-DNA Microprep Kit. DNA was amplified with 8F and 1391R primers using Promega GoTaq Master Mix and 16s clone libraries were constructed using pGEM T-easy cloning system (all per manufacturer's protocol). Sequences were classified using the RDP pipeline (https://rdp.cme.msu.edu/pipeline/ with http://pyro.cme.msu.edu/).

CARD-FISH Imaging (bulk)

Published CARD-FISH protocol for sediment samples (3) was used on compost and worm gut samples with the following modifications: scraped worm guts and fresh compost were fixed overnight in 4% paraformaldehyde at 4C, sonicated for 1 minute, filtered on 0.22um polycarbonate filters, and stained with EUB388 (green) probes and DAPI (blue).

CARD-FISH Imaging (thin-section)
Thin-sections were prepared by fixing whole worms in 4% paraformaldehyde at 4C overnight, washing with PBS, and freezing in Tissue Tek using liquid nitrogen. Embedded worm samples were then sliced into 20um cross-sections using a Microm HM 505N Cryostat at the MBL Central Microscopy Facility, and mounted on poly-lysine covered glass slides with heat. Finally, samples were dried by incubating for 1 min in each 50%, 75%, 100% ethanol before staining with EUB594 probe and DAPI using methods described previously (4).

MiPACT-CARD-FISH imaging using confocal microscopy
Microbial identification after passive clarity technique (MiPACT) was performed as presented in DePas et al. (5) with the following modifications: worms were fixed using 4% paraformaldehyde overnight at 4C, washed 3x in PBS, and then embedded in polyacrylamide gel using 1.5mL eppendorf tubes as a mold. Specifically, fixed and washed worms were placed in 4% 29:1 acrylamide:bis-acrylamide and 0.25% VA_044 hardener in 1xPBS overnight at 4C, then incubated at room temperature in an anaerobic hood for ~5 m with the tube cap open to remove oxygen, and finally hardened by incubate in a 37C water bath for 12 hours. Embedded worms were carefully removed from eppendorf tubes and placed in 50mL Falcon tubes with 10mL of 8% SDS in 1X PBS for 5 days to clear lipids (only partial clearing was seen). Samples were trimmed and stained using Alexa EUB584 probe following CARD-FISH protocol described previously (4), and imaged using a Zeiss confocal microscope.

SEM Imaging
Longitudinally cut worm and compost aggregate were fixed in 4 % paraformaldehyde overnight at 4C, washed 3x in 1xPBS, and dehydrated by soaking for 20 minutes at room temperature in 25%,50%, 75%, 95% and 100% ethanol. Samples were further dried by critical point drying and spotter coated with platinum in the MBL Central Microscopy Facility.

Results

1) **Nitrate-acetate enrichments for both compost and worm samples show evidence for denitrification past nitrite.** Nitrate and nitrite measured by IC and acetate and formate measured by HPLC for the nitrate-based enrichments are shown in Table 1 along with the calculated expected losses based on stoichiometry. In the nitrate-acetate enrichment transfers, the nitrite produced combined with the residual nitrate did not account for all of the initial nitrogen, suggesting further denitrificaiton to N2O and/or N2 is taking place (Note: unfortunately, we did not have access to an N2O probe, so the loss in substrates was our main indicator for denitrification).

2) **Clone libraries derived from enrichments for cultivatable denitrifying bacteria suggest the earthworm is a selective environment for denitrifying pseudomonas.** When we examined the nitrate-acetate enrichments using light microscopy, they appeared to be mixed (Figure 1). To determine the community composition, we constructed a 16s clone library of each inoculum growing in the nitrate-acetate media. The clone library for the compost was more diverse than that of the worm gut. The compost consisted of pseudomonas and diverse firmicutes, while the gut was dominated by pseudomonas alone, suggesting that there is some level of selective pressure for denitrifying pseudomonas in the gut (Figure 2).
3) **Bacterial growth occurred under all cultivation conditions and strains specific to each enrichment medium were isolated.** N2O enrichments did not show significant loss of formate or acetate, suggesting any growth was a result of nutrient transfer from the inoculum. Nonetheless, specific colonies were seen on each of the N2O-based plate incubations. Representative colony morphologies and 16s rRNA-based classifications are shown in Figure 3 (full list in Appendix).
4) **Visualization of earthworm gut bacteria poses many challenges.** Few bacteria can be seen with CARD-FISH EUB388 (green) staining in the compost and worm gut bulk samples (Figure 4). CARD-FISH EUB594 (red) staining of worm cross-sections (Figure 5) only appeared to stain a small number of cells, and results are inconclusive due to auto-fluorescence of soil particles and chitin found in the worm. MiPACT-CARD-FISH confocal imaging (Figure 6) identified a few bacteria stained with EUB594 (red) inside of the worm on select z-stacks, but the thickness of the z-stacks was limiting in this case. Finally, SEM imaging (Figure 7) revealed superficial bacterial distribution on a compost aggregate and worm gut sample.

Conclusion and Outlook

Imaging bacteria in and along the worm gut presents many challenges. In particular, soil particles and the worm itself seem to be auto-fluorescent, interfering with the signal from FISH probes targeting bacteria in these samples. The contents of the worm gut are superficially indistinguishable from soil itself, meaning that fixation of these contents may require embedding in hydrogels to obtain sections with sufficient structural integrity for imaging. Optimizing fixation by one of these means prior to thin-sectioning and CARD-FISH has potential for future success.

We obtained preliminary results which suggest that the earthworm gut differs from the surrounding compost soil in terms of its bacterial composition. Culturable diversity both in the earthworm gut and compost denitrification enrichments suggested dominance of Gammproteobacteria in the family Pseudomonadaceae. However, the diversity of phylotypes in the earthworm gut was restricted in comparison to compost enrichments.

This result suggests that the earthworm gut potentially bottlenecks growth of soil-derived organisms for denitrification through selective pressures. Our analysis of culturable diversity gives some idea of the organisms that are likely to be actively growing within the sampled ecosystems, whereas culture-independent methods will identify both active and inactive organisms.

Additionally, in order to analyze specific processes *in situ* such as denitrification, enrichment techniques provide a relatively simple and inexpensive means to do so. By contrast, 16S rDNA sequencing or shotgun metagenomics, while potentially identifying organisms or genes likely to be associated with a process such as denitrification, will be insufficient on their own to connect organisms with specific processes. Techniques such as raman spectroscopy or NanoSIMS may be applied in the future for very high resolution of microbial processes *in situ*. For example, isotopic enrichments of 15NO3 to the soil sample could be followed with NanoSIMS to see where 15NO2 accumulates. However, these methods are limiting in throughput and potentially cost.

Moving forward, I can envision an approach in which earthworm gut contents and the surrounding soil material are subjected to short-term enrichments similar to those applied here, and organisms capable of using nitrate or nitrous oxide as electron acceptors could be identified via their uptake of deuterated water, or stable isotope probing using 15NO3 enrichments, which will specifically be incorporated into actively growing cells.

Together, the results from this exploratory study provide a route forward in terms of assessing the cycling of nutrients by microbial communities in the earthworm gut and compost. Transformation of nitrate past nitrite occurred to a measurable degree in a subset of enrichment cultures relatively quickly, but may have been delayed in others (see nitrate-formate enrichment with compost), suggesting that there are unobserved constraints on nitrate transformation under initial enrichment conditions. Again, radioisotopic methods could identify organisms that are involved in nitrate transformation even if there are other environmental signals limiting these processes. Further work should expand the replication of these enrichments to multiple worms and multiple compost samples to identify within and across-replicate variability. Culture-based approaches paired with culture-independent approaches can identify whether the organisms active in enrichments are the same as those active *in situ* in these environments, and potentially be informative as to if or how the earthworm gut selects for organisms from the soil in a variety of nutrient cycling processes.

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References

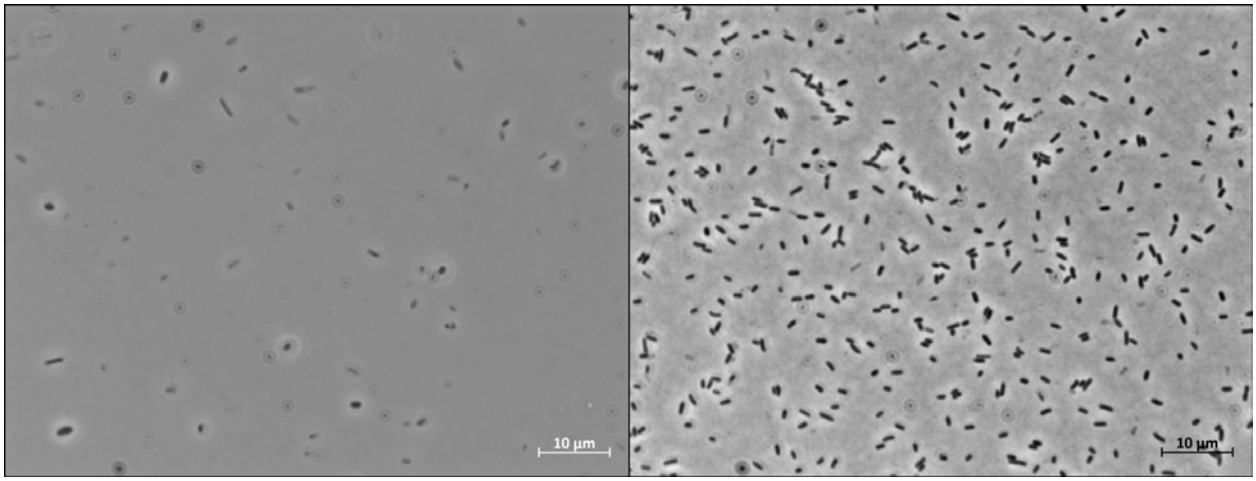
(1)Tringe, Susannah Green, et al. "Comparative metagenomics of microbial communities." Science 308.5721 (2005): 554-557.
(2)Drake, Harold L., and Marcus A. Horn. "As the worm turns: the earthworm gut as a transient habitat for soil microbial biomes." Annu. Rev. Microbiol. 61 (2007): 169-189.
(3)Ishii, Kousuke, et al. "An improved fluorescence in situ hybridization protocol for the identification of bacteria and archaea in marine sediments." FEMS Microbiology Ecology 50.3 (2004): 203-213.
(4)Pernthaler, Annelie, and Rudolf Amann. "Simultaneous fluorescence in situ hybridization of mRNA and rRNA in environmental bacteria." Applied and Environmental Microbiology 70.9 (2004): 5426-5433.
(5)DePas, William H., et al. "Exposing the three-dimensional biogeography and metabolic states of pathogens in cystic fibrosis sputum via hydrogel embedding, clearing, and rRNA labeling." mBio 7.5 (2016): e00796-16.

Tables and Figures

Table 1: IC and HPLC data suggest denitrification beyond nitrite occurs in nitrate-acetate enrichments both for earthworms and compost.

Sample	Nitrite	Nitrate	Formate	Acetate	Nitrate Loss	Formate/ Acetate Loss	Expected Nitrate Loss	Total N as NO3- & NO2-	Unaccounted N
Nitrate-Formate Worm Transfer	0.56	3.68	11.28	BDL	0.46	1.44	0.57	4.24	-0.1
Nitrate-Formate Media	BDL	4.14	12.72	BDL	NA	NA	NA	4.14	0
Nitrate-Formate Compost Transfer	1.75	1.62	3.78	BDL	2.52	8.94	3.58	3.37	0.77
Nitrate-Acetate Worm Transfer	1.14	0.25	0.13	BDL	4.02	3.08	4.92	1.39	2.88
Nitrate-Acetate Media	BDL	4.28	BDL	3.08	NA	NA	NA	4.28	0
Nitrate-Acetate Compost Transfer	0.92	1.25	0.09	BDL	3.02	3.08	4.92	2.18	2.1

Figure 1: Light microscopy of nitrate-acetate enrichments show transfers remain as mixed cultures.

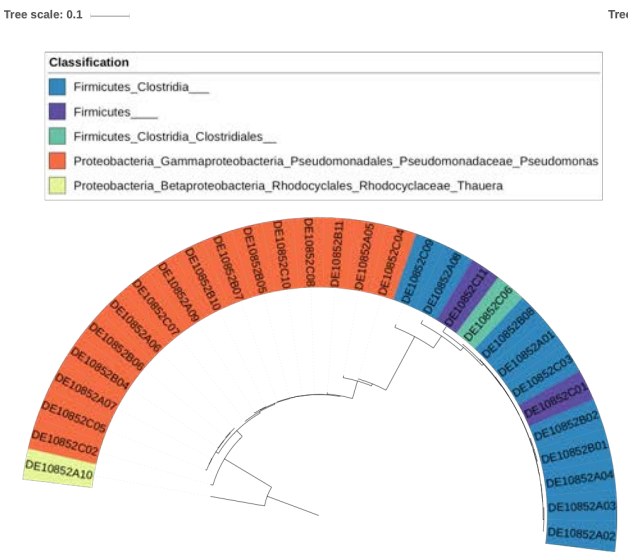


Nitrate-Acetate-Compost Enrichment

Nitrate-Acetate-Worm Enrichment

Figure 2: Phylogenetic trees of 16s clone libraries of compost and worm nitrate-acetate enrichments.

Nitrate-Acetate-Compost Enrichment 16s Clone Library



Nitrate-Acetate-Worm Enrichment 16s Clone Library

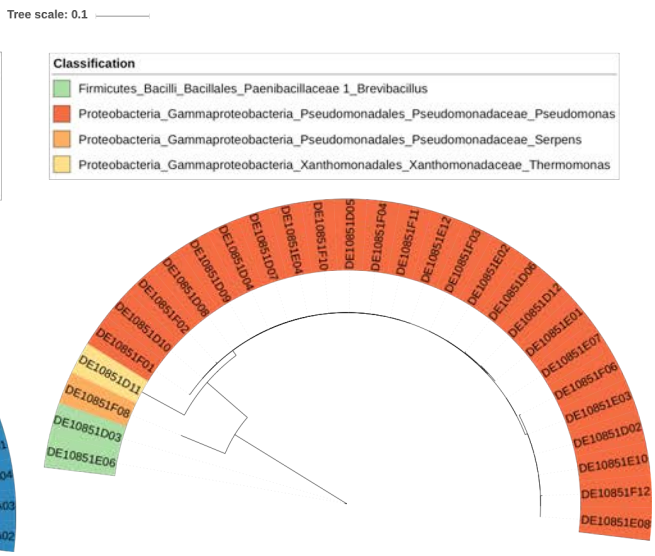


Figure 3: Colony isolate representatives from each enrichment

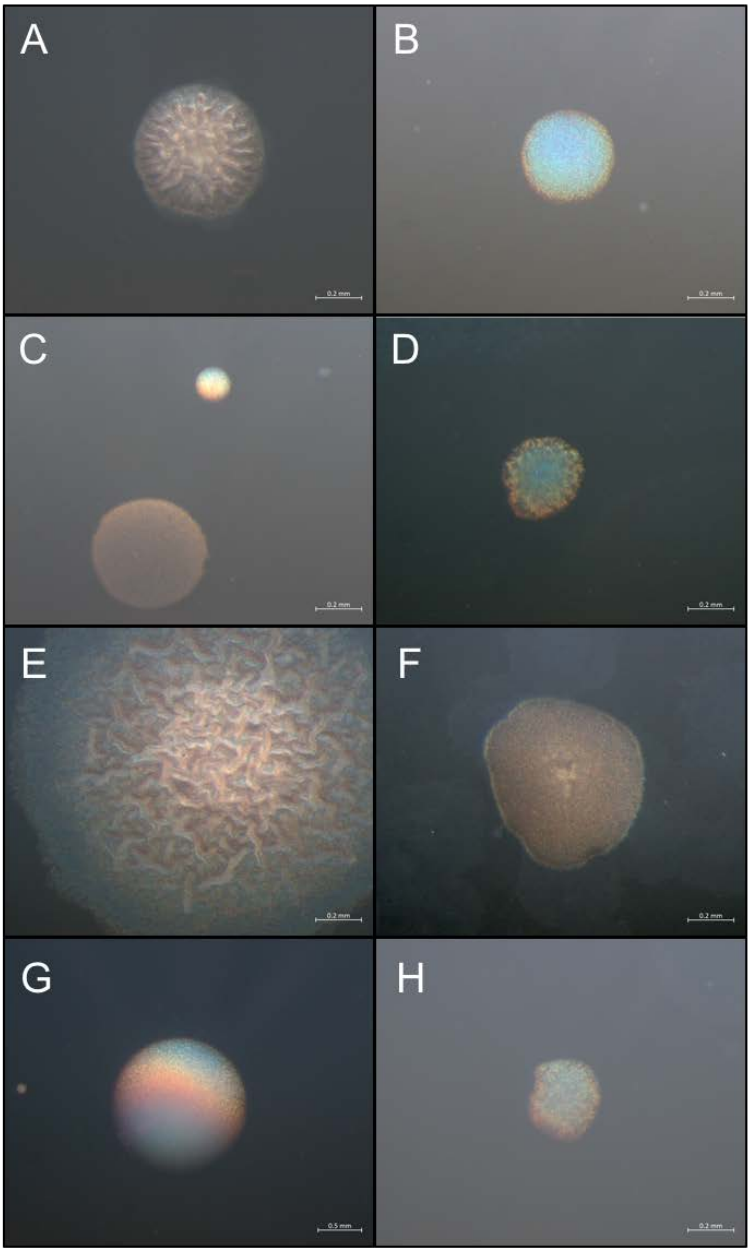
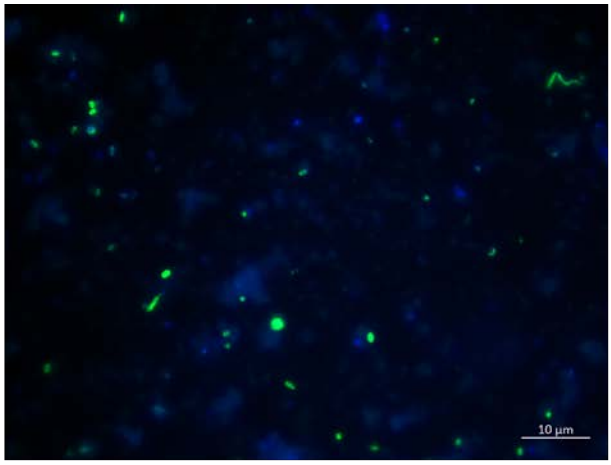
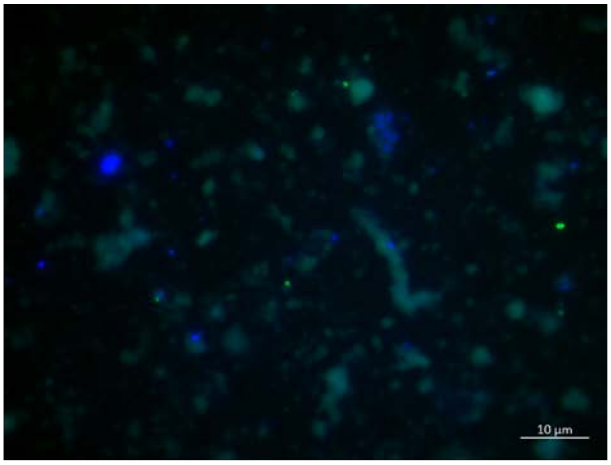


Image ID	Isolate	Isolation Plate	Top BLAST Hit	NCBI RefSeq	%ID	e-val	% query cover
A	17FHN2OAC5	N2O_Acetate_Compost_10-3	Pseudomonas songnenensis	NR_148295.1	99	0	98
B	17FHN2OAW6	N2O_Acetate_Worm_10-2	Aeromonas rivipollensis	NR_144574.1	99	0	98
C	17FHN2OFC3	N2O_Formate_Compost_10-2	Aeromonas hydrophila	NR_119190.1	99	0	98
D	17FHN2OFW2	N2O_Formate_Worm_10-2	Aeromonas caviae	NR_104824.1	99	0	98
E	17FHNAC4	Nitrate_Acetate_Compost_10-4	Pseudomonas songnenensis	NR_148295.1	99	0	99
F	17FHNAW3	Nitrate_Acetate_Worm_10-2	Pseudomonas multiresinivorans	NR_119225.1	99	0	98
G	17FHNFC2	Nitrate_Formate_Compost_10-4	Paracoccus pantotrophus	NR_114120.1	99	0	98
H	17FHNFW8	Nitrate_Formate_Worm_10-2	Pseudomonas multiresinivorans	NR_119225.1	99	0	99

Figure 4: CARD-FISH imaging of bulk compost and worm gut on filters

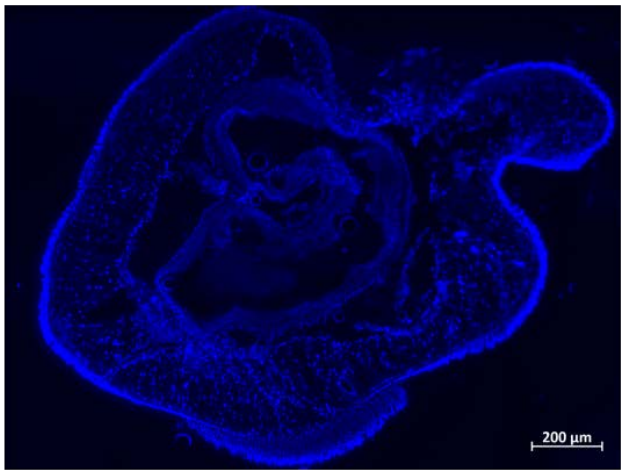


Compost stained with EUB388 (green) and DAPI (blue)

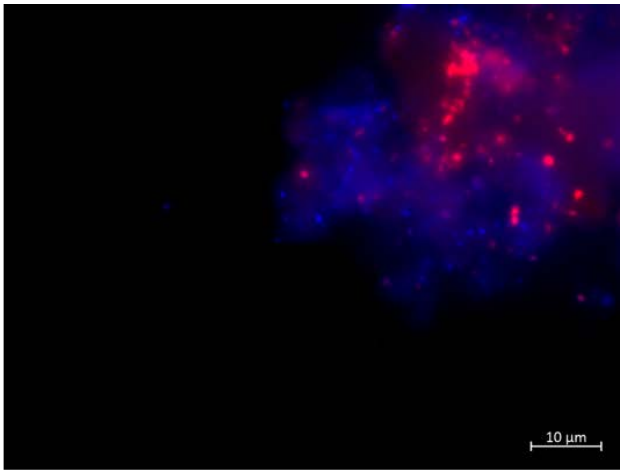


Worm gut stained with EUB388 (green) and DAPI (blue)

Figure 5: CARD-FISH on worm thin-sections



DAPI-stained worm cross-section



EUB594 (red) + DAPI(blue)-stained worm cross-section

Figure 6: MiPACT clearing (A) and confocal imaging (B) with CARD-FISH staining using EUB594 probe (red); auto-fluorescence from worm is shown in green

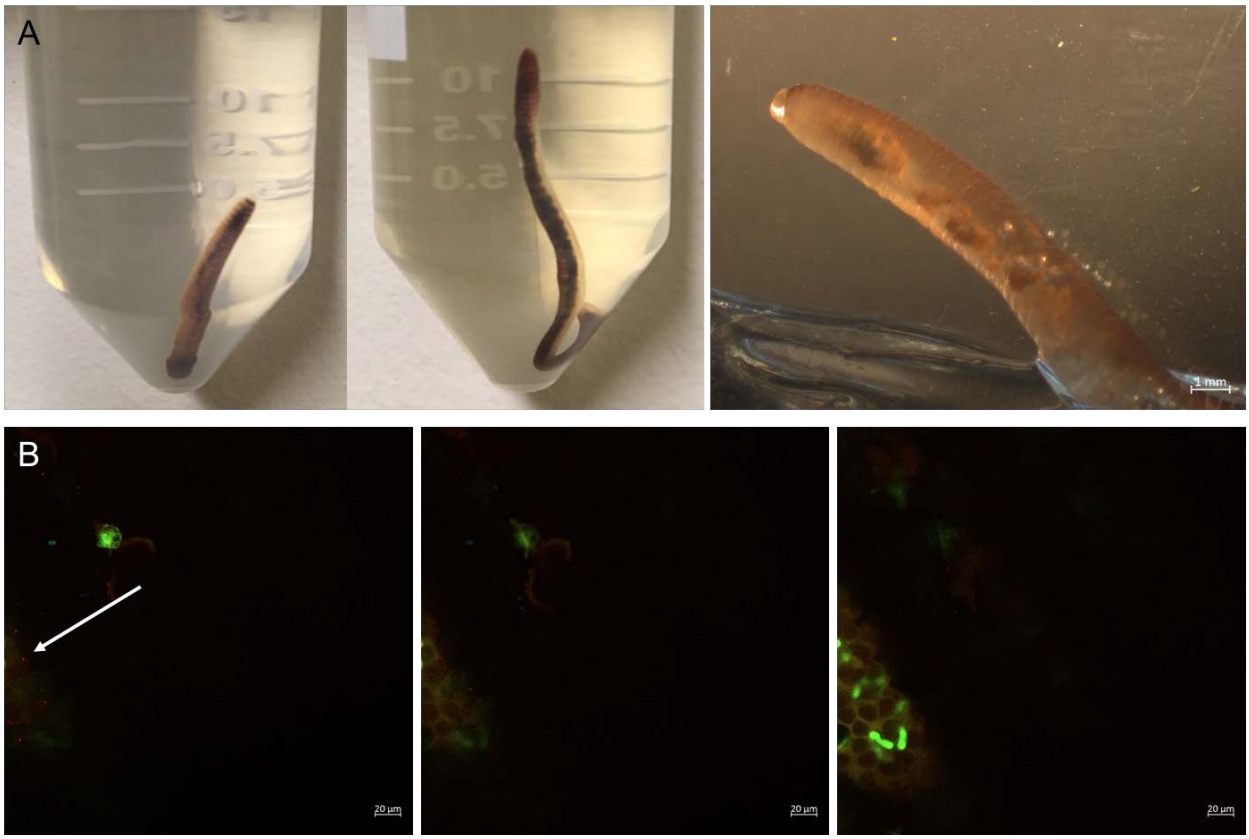
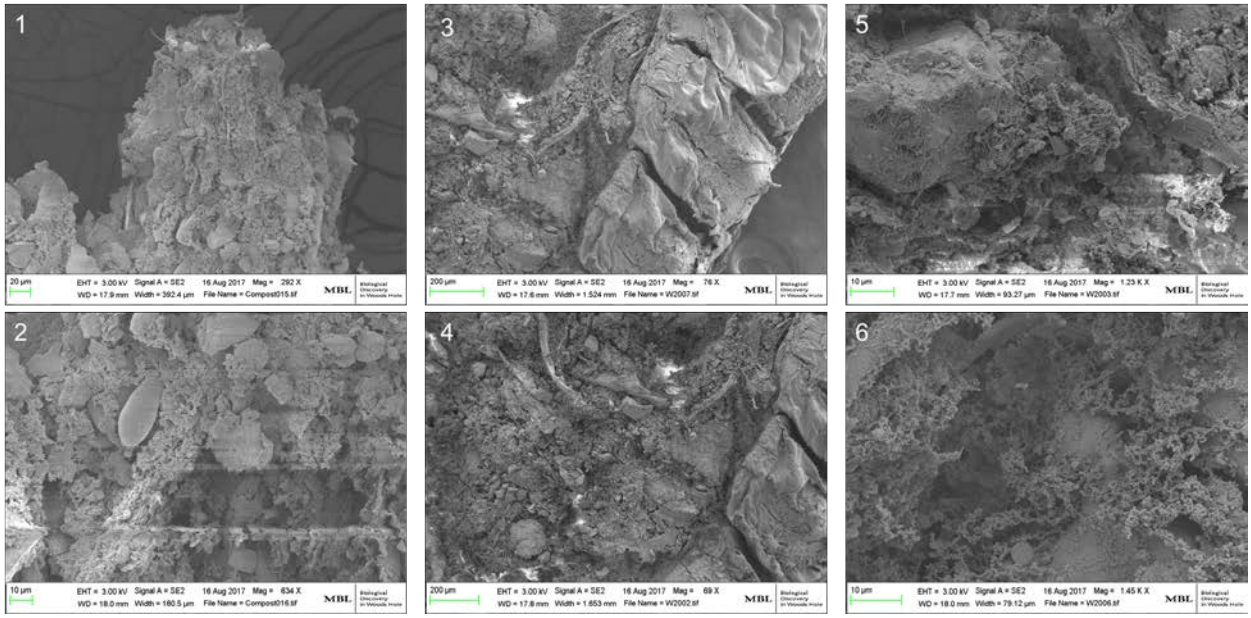


Figure 7: SEM images of compost (1,2) and worm guts (3,4,5,6)



Appendix: Isolate Table

Isolate	Isolation Plate	Top BLAST Hit	NCBI RefSeq	%ID	e-val	% query cover
17FHNAC1	Nitrate_Acetate_Compost_10-4	Pseudomonas songnenensis	NR_148295.1	99	0	99
17FHNAC2	Nitrate_Acetate_Compost_10-4	Pseudomonas songnenensis	NR_148295.1	99	0	98
17FHNAC3	Nitrate_Acetate_Compost_10-4	Pseudomonas songnenensis	NR_148295.1	99	0	99
17FHNAC4	Nitrate_Acetate_Compost_10-4	Pseudomonas songnenensis	NR_148295.1	99	0	99
17FHNAC5	Nitrate_Acetate_Compost_10-4	Pseudomonas songnenensis	NR_148295.1	99	0	98
17FHNAC6	Nitrate_Acetate_Compost_10-4	Pseudomonas songnenensis	NR_148295.1	99	0	98
17FHNAC7	Nitrate_Acetate_Compost_10-4	SEQ FAIL				
17FHNAC8	Nitrate_Acetate_Compost_10-4	Pseudomonas songnenensis	NR_148295.1	99	0	98
17FHNAW1	Nitrate_Acetate_Worm_10-2	Pseudomonas citronellolis	NR_114194.1	97	0	97
17FHNAW2	Nitrate_Acetate_Worm_10-2	Pseudomonas multiresinivorans	NR_119225.1	99	0	98
17FHNAW3	Nitrate_Acetate_Worm_10-2	Pseudomonas multiresinivorans	NR_119225.1	99	0	98
17FHNAW4	Nitrate_Acetate_Worm_10-2	Pseudomonas multiresinivorans	NR_119225.1	99	0	98
17FHNAW5	Nitrate_Acetate_Worm_10-3	Pseudomonas multiresinivorans	NR_119225.1	98	0	98
17FHNAW6	Nitrate_Acetate_Worm_10-3	Pseudomonas multiresinivorans	NR_119225.1	98	0	99
17FHNAW7	Nitrate_Acetate_Worm_10-3	Shewanella profunda	NR_104770.1	92	0	98
17FHNAW8	Nitrate_Acetate_Worm_10-3	Shewanella profunda	NR_104770.1	96	0	99
17FHNFC1	Nitrate_Formate_Compost_10-4	Paracoccus pantotrophus	NR_114120.1	99	0	98
17FHNFC2	Nitrate_Formate_Compost_10-4	Paracoccus pantotrophus	NR_114120.1	99	0	98
17FHNFC3	Nitrate_Formate_Compost_10-4	Paracoccus pantotrophus	NR_114120.1	99	0	99
17FHNFC4	Nitrate_Formate_Compost_10-4	Paracoccus pantotrophus	NR_114120.1	99	0	98
17FHNFC5	Nitrate_Formate_Compost_10-4	Azospirillum lipoferum	NR_117481.1	96	0	98
17FHNFC6	Nitrate_Formate_Compost_10-4	Pseudomonas jinjuensis	NR_114197.1	99	0	99
17FHNFC7	Nitrate_Formate_Compost_10-4	Pseudomonas aeruginosa	NR_117678.1	99	0	98
17FHNFC8	Nitrate_Formate_Compost_10-4	Pseudomonas aeruginosa	NR_117678.1	99	0	98
17FHNFW1	Nitrate_Formate_Worm_10-1	POOR SEQ				
17FHNFW2	Nitrate_Formate_Worm_10-2	Aeromonas hydrophila	NR_119190.1	98	0	98
17FHNFW3	Nitrate_Formate_Worm_10-2	Enterobacter aerogenes	NR_102493.1	99	0	98
17FHNFW4	Nitrate_Formate_Worm_10-2	POOR SEQ				
17FHNFW5	Nitrate_Formate_Worm_10-2	Aeromonas rivipollensis	NR_144574.1	99	0	98
17FHNFW6	Nitrate_Formate_Worm_10-2	Aeromonas hydrophila	NR_119190.1	99	0	99
17FHNFW7	Nitrate_Formate_Worm_10-2	POOR SEQ				
17FHNFW8	Nitrate_Formate_Worm_10-2	Pseudomonas multiresinivorans	NR_119225.1	99	0	99
17FHN2OAC1	N2O_Acetate_Compost_10-3	POOR SEQ				
17FHN2OAC2	N2O_Acetate_Compost_10-3	SEQ FAIL				
17FHN2OAC3	N2O_Acetate_Compost_10-3	POOR SEQ				
17FHN2OAC4	N2O_Acetate_Compost_10-3	POOR SEQ				
17FHN2OAC5	N2O_Acetate_Compost_10-3	Pseudomonas songnenensis	NR_148295.1	99	0	98
17FHN2OAC6	N2O_Acetate_Compost_10-3	Aeromonas jandaei	NR_037013.2	96	0	99
17FHN2OAC7	N2O_Acetate_Compost_10-3	SEQ FAIL				
17FHN2OAC8	N2O_Acetate_Compost_10-3	Aeromonas aquatica	NR_136829.1	99	0	98
17FHN2OAW1	N2O_Acetate_Worm_10-2	Aeromonas media	NR_036911.2	99	0	99
17FHN2OAW2	N2O_Acetate_Worm_10-2	Aeromonas caviae	NR_104824.1	99	0	98
17FHN2OAW3	N2O_Acetate_Worm_10-2	Aeromonas rivipollensis	NR_144574.1	99	0	99
17FHN2OAW4	N2O_Acetate_Worm_10-2	Aeromonas media	NR_036911.2	99	0	99
17FHN2OAW5	N2O_Acetate_Worm_10-2	Aeromonas rivipollensis	NR_144574.1	99	0	99
17FHN2OAW6	N2O_Acetate_Worm_10-2	Aeromonas rivipollensis	NR_144574.1	99	0	98
17FHN2OAW7	N2O_Acetate_Worm_10-2	POOR SEQ				
17FHN2OAW8	N2O_Acetate_Worm_10-2	Flavobacterium denitrificans	NR_042088.1	99	0	99
17FHN2OFC1	N2O_Formate_Compost_10-2	POOR SEQ				
17FHN2OFC2	N2O_Formate_Compost_10-2	POOR SEQ				
17FHN2OFC3	N2O_Formate_Compost_10-2	Aeromonas hydrophila	NR_119190.1	99	0	98
17FHN2OFC4	N2O_Formate_Compost_10-2	SEQ FAIL				
17FHN2OFC5	N2O_Formate_Compost_10-2	SEQ FAIL				
17FHN2OFC6	N2O_Formate_Compost_10-2	Aeromonas caviae	NR_104824.1	99	0	98
17FHN2OFC7	N2O_Formate_Compost_10-2	Aeromonas rivipollensis	NR_144574.1	99	0	98
17FHN2OFC8	N2O_Formate_Compost_10-2	Paracoccus pantotrophus	NR_114120.1	98	0	97
17FHN2OFW1	N2O_Formate_Worm_10-2	Aeromonas caviae	NR_104824.1	99	0	100
17FHN2OFW2	N2O_Formate_Worm_10-2	Aeromonas caviae	NR_104824.1	99	0	98
17FHN2OFW3	N2O_Formate_Worm_10-2	Aeromonas caviae	NR_104824.1	99	0	99
17FHN2OFW4	N2O_Formate_Worm_10-2	Aeromonas hydrophila	NR_119190.1	99	0	98
17FHN2OFW5	N2O_Formate_Worm_10-2	Aeromonas caviae	NR_104824.1	99	0	99
17FHN2OFW6	N2O_Formate_Worm_10-2	Aeromonas caviae	NR_104824.1	99	0	98
17FHN2OFW7	N2O_Formate_Worm_10-2	Aeromonas caviae	NR_104824.1	99	0	98
17FHN2OFW8	N2O_Formate_Worm_10-2	Aeromonas caviae	NR_104824.1	99	0	99