

Amended Final Report – Year #1
11/12/2010

Bacterial consortia in swine waste lagoons: The role of purple phototrophic bacteria and anaerobic ammonium oxidation (anammox) in odor control and natural products synthesis

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Note: Year #1 funding was extended through Sept. 30, 2010. A report for the 12-month period July 1, 2009 through June 30, 2010 was submitted prior to approval and implementation of the no-cost extension. This amended report covers all activities through the period July 1, 2009 through Sept. 2010.

Research Objectives:

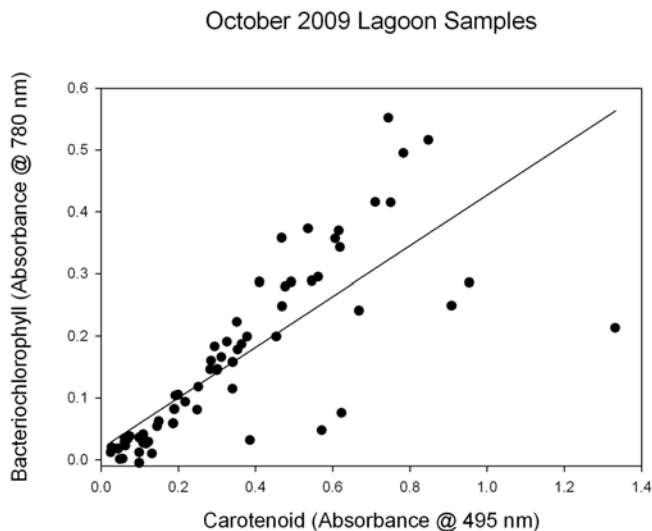
Our overall objective is to evaluate the microbiological processes supporting “healthy” swine waste lagoons, which we define as generally pink in color, emitting little odor and accumulating little sludge. We proposed to determine the factors favoring blooms of purple phototrophic bacteria in lagoons. We also proposed to detect and quantify the activity of anaerobic ammonia oxidizing (anammox) bacteria in swine waste lagoons under different conditions. We proposed to determine linkages among anammox activity, purple phototrophic bacteria, and swine waste properties. Ultimately, we proposed to quantify production of potentially useful natural products by the bacterial consortia typical of healthy waste lagoons and to conduct microcosm and mesocosm-scale studies to evaluate manipulations of swine waste to optimize lagoon health. Here we report progress toward these objectives with first year funding of a proposed 3-year investigation.

Waste lagoon sampling

We have continued coordination with Kraig Westerbeek at Murphy-Brown, Inc. in Warsaw to obtain samples of lagoon liquids as part of their routine waste analysis sampling program. We received approximately 75 samples in August and another ~75 in October, 2009. The first set was used to develop and adopt assay methods appropriate for use with such highly concentrated and variable samples, and to do initial screens on a subset for anammox activity. For example, chemical oxygen demand (COD) analyses specified by APHA (2005) for wastewater required some adjustments for swine waste, which is typically far more concentrated than human wastewater with respect to COD. The chloride content of swine waste requires use of mercuric sulfate to eliminate this

interference in the assay, among other such adjustments. Owing to heavy winter rains across eastern North Carolina, the normal lagoon sampling schedule was thrown off and we did not get another regular batch of samples (~45) until April, 2010. Analyses of these samples are in progress, with some results in (carotenoid and bacteriochlorophyll) and others (vitamin B12) awaiting assays.

The purple-pink color of “healthy” lagoons is conferred by blooms of purple phototrophic bacteria, which conduct bacterial photosynthesis using bacteriochlorophylls and associated carotenoid pigments. One of these carotenoids, which we have identified as spirilloxanthin (based on recent 800 MHz NMR analyses conducted by colleague Chris Halkides in the UNCW Chemistry and Biochemistry Dept., and on recently returned mass spectrometry results from collaborator George Dubay at Duke), has been a focus of our interest for some time. It has anti-oxidant properties equal or superior to lycopene, a commercially marketed carotenoid anti-oxidant. We have utilized simple spectrophotometry to quantify carotenoid contents of filtered lagoon samples extracted in acetone and have now related those data to HPLC-based quantification of spirilloxanthin in the same sample extracts. We have also quantified bacteriochlorophylls and compared those values to carotenoid contents in those extracts (see below).



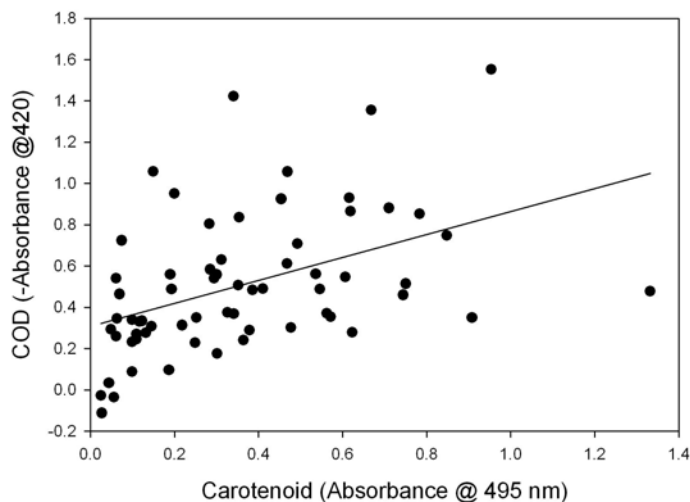
The data show a highly significant linear relationship between bacteriochlorophyll concentration and carotenoid concentration, supporting the bacterial origin of the carotenoid. The range of carotenoid concentrations is approximately 50-fold among lagoon samples, as is the range of bacteriochlorophyll values.

Average spirilloxanthin concentrations in swine lagoons sampled in October, 2009, estimated from conversion of spectrophotometric measures to total pigment via HPLC and use of the molar absorptivity for spirilloxanthin, were approximately 2 kg m^{-3} , with a maximum of 7.4 kg m^{-3} . An “average” lagoon of $20,000 \text{ m}^3$ volume would therefore contain approximately 40 metric tons of spirilloxanthin. We have previously determined that spirilloxanthin derived from swine waste lagoons is a potent anti-oxidant, equal to or better than lycopene (marketed as a diet supplement). Lycopene has a current retail value of approximately \$15,000 per kg as a dietary supplement. It is too early to extrapolate the potential commercial value of spirilloxanthin, but the comparisons with lycopene are interesting. We note also that spirilloxanthin is one of several natural

products synthesized by purple phototrophic bacteria. These same bacteria are known to produce vitamin B12. Retail prices for vitamin B12 range from \$15,000 to \$210,000 per kg, depending on formulation. Compounds used in human diets require varying degrees of approval, so the pathway to commercialization is necessarily long and potentially costly. However, carotenoids and anti-oxidants have other potential commercial uses. For example, commercial salmonid fish aquaculture utilizes the carotenoid astaxanthin to confer the desirable pink color in the flesh of these fish. Current astaxanthin costs to aquaculture are on the order of \$2,000/kg of astaxanthin. If swine waste-derived carotenoids can effectively substitute for astaxanthin, even a price point of \$1000/kg would generate substantial returns for swine waste producers. We will examine these questions further in additional research.

One of the relationships thought to control the tendency of lagoons to support purple phototrophic bacteria blooms is “volatile loading rate” – the concentration of labile organic matter loaded into a lagoon per unit lagoon volume. NRCS guidelines for lagoon design and management recognize a threshold loading rate. We analyzed chemical oxygen demand (COD) as a measure of volatile solids in lagoon liquids and compared the results to parallel measures of purple phototrophic bacteria biomass expressed as carotenoid content. The October 2009 samples yielded a positive relationship between these two parameters (figure below). This was not the expected result, but at least two factors must be considered. First, in lagoons with very significant bacterial blooms occurring, the bacteria themselves probably account for most of the organic matter and COD. Second, these results came from October samples, essentially at the end of the summer bloom period. Bacteria, particularly the purple phototrophic forms, grow very well at elevated temperature and would have likely metabolized a significant portion of total volatile solids at that time.

October 2009 Lagoon Samples



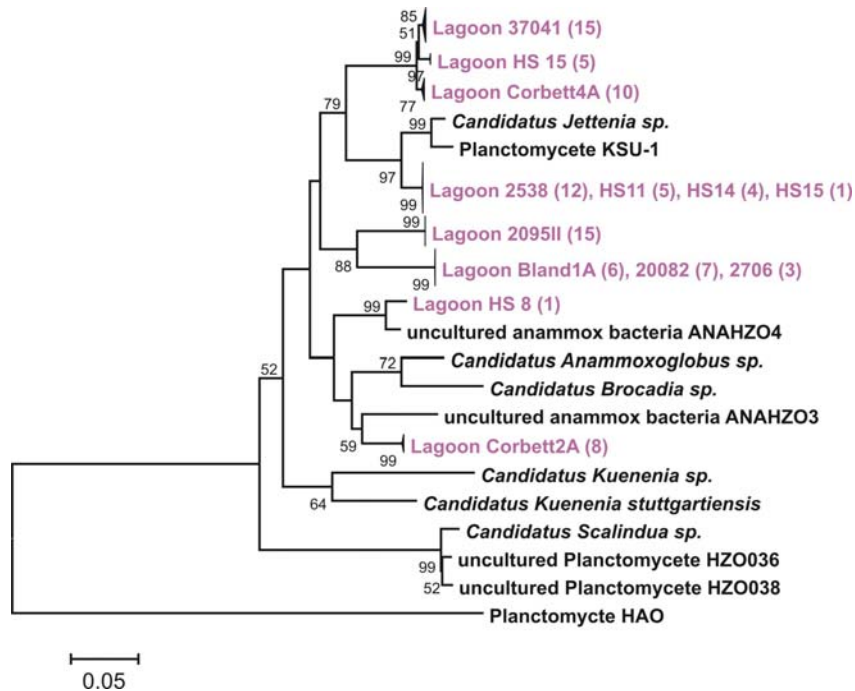
Analysis of data from swine waste analysis reports from NC Division of Agronomy for the same lagoon samples we have analyzed in our lab revealed several interesting patterns. Swine waste analyses performed by NC DA measure total nitrogen, phosphorus, potassium, calcium, magnesium, sulfur, iron, manganese, zinc, copper, boron, sodium, and pH. Using data from batches of samples collected in October, 2009

and from our previous study in 2008 (funded by UNC GA's Research Competitiveness Awards) we analyzed relationships between these parameters and other parameters we have measured in swine waste, including nitrite ion (substrate for anammox) and total reductants, vs. carotenoid and bacteriochlorophyll contents. Results of simple linear regressions showed several significant effects (Table 1), most notably between carotenoid content and sulfur, magnesium, and phosphorus in both batches, and between carotenoid content and sodium, calcium, and manganese in one of the two batches. The consistent (and positive) effects of sulfur, magnesium, and phosphorus on bacterial carotenoid concentrations make sense to us. Bacterial metabolism is thought to be rapid, requiring high turnover of phosphorus-rich ATP. Purple phototrophic bacteria would need magnesium to make the bacteriochlorophyll molecule. Perhaps most important, these same bacteria metabolize sulfur compounds, mostly sulfide ion, as an electron donor. Consequently, we will make sulfide measurements on waste samples using an ion-selective electrode and meter in the future. If these results are confirmed and extended, we can then test the idea that manipulation of phosphorus, magnesium, and sulfur could allow control of bacterial carotenoid production in swine waste lagoons.

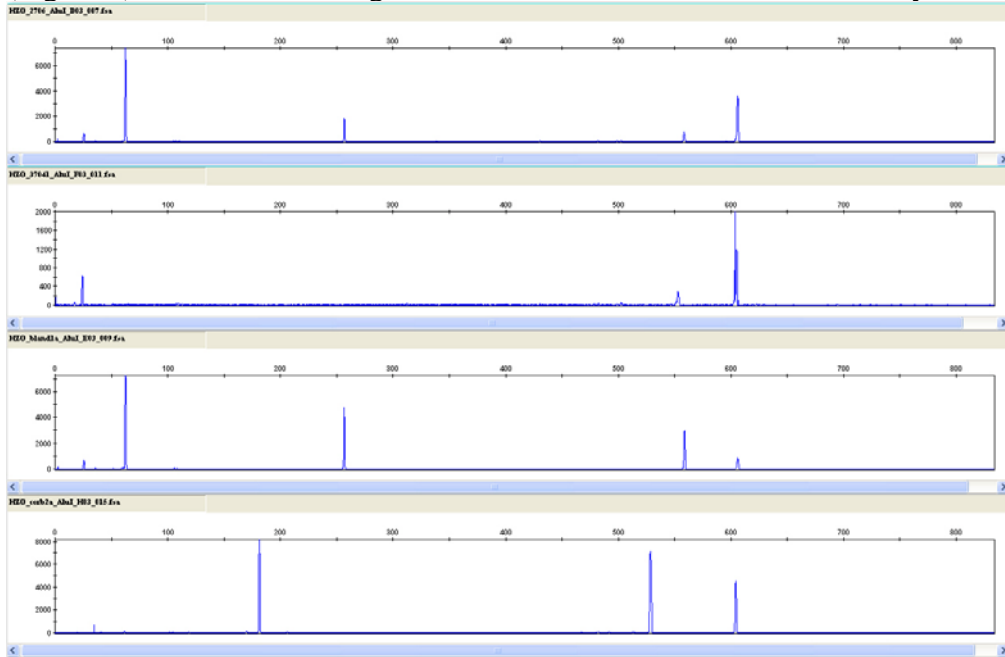
We have also conducted more sophisticated Principal Components Analysis of swine waste composition on carotenoid content, recognizing that many constituents covary. Results of this analysis revealed that the first two principal components (of 13 total) accounted for 66 and 73% of total variation in the two batches sample sizes of 108 and 58, respectively. Carotenoid content regressed significantly only against the first principal component, which was weighted most heavily (eigenvectors >0.25) by the values for nitrogen, phosphorus, calcium, magnesium, sulfur, manganese, zinc and copper, and for nitrogen, phosphorus, calcium, magnesium, sulfur, iron, manganese, zinc and boron, respectively. The consistency of these results supports the single factor linear regression results in Table 1, suggesting chemical controls on bacterial carotenoid concentrations, and the potential for manipulation of carotenoid production by chemical supplementation.

Anammox Bacteria Analyses

Anaerobic ammonium oxidation (anammox) was proposed as a microbial remedy to reduce gaseous ammonia emissions from hog lagoon systems. A total of 60 hog lagoon samples from August and October samplings were tested to determine the presence of anammox bacteria using a newly developed molecular method. DNA was extracted from each lagoon sample and was used to detect the hydrazine oxidase gene (*hzo*), a specific gene found only in anammox bacteria, by PCR amplification. We found that at least 20 lagoons had anammox bacteria in their microbial flora (one-third of all samples so far). Based on sequence analysis of the detected genes, at least 5 different species of anammox bacteria are present in different lagoon systems. We conducted sequence analysis of amplified *hzo* gene products (a marker for anammox bacteria) from 12 lagoon systems. Phylogenetic analysis of the detected *hzo* genes (below) showed the presence of at least 6 different types of anammox bacteria in hog lagoon systems.



Most lagoons have anammox bacteria closely associated with *Candidatus Jettenia* sp., while the bacteria in lagoons Corbett2A and HS 8 are related to *Candidatus Brocadia* and *Anammoxoglobus* spp. Additional analysis of DNA fingerprints of the *hzo* genes clearly demonstrated the presence of different anammox bacteria in different lagoon systems (Figure 2). We are examining which anammox bacteria more efficiently remove



From top: Lagoons 2706, 37041, Bland1A, Corbett2A

ammonia and nitrite in lagoon environments. We have established microcosms with 4 lagoon samples to measure and compare ammonia removal rates. Anammox bacteria grow very slowly, so results will take a while to obtain.

We have measured the concentrations of nitrite (NO_2^-) ion in swine lagoon samples, as nitrite is a key substrate in the anammox process. Nitrite concentrations were generally low (less than 2 micromolar, with high values of $\sim 7 \mu\text{M}$), and were not correlated significantly with COD or carotenoid content, nor was there any relationship between the nitrite concentration and presence of anammox bacteria. We suspect that nitrite synthesis is tightly coupled with anammox activity, so that nitrite concentrations themselves do not match up with anammox.

Flocculation/coagulation procedures have been tested over the summer to evaluate ways to extract bacterial products from swine waste. Several flocculants, including several commercial products tested previously by the “Smithfield” process as well as aluminum sulfate and ferric chloride, have been evaluated. Many of them effectively concentrated bio-solids from swine waste, leaving relatively clear supernatants. HPLC analyses of acetone extracts of the bio-solids showed that in almost all cases spirilloxanthin remained chemically intact in the concentrated bio-solids, taking us one step closer to a viable procedure for mass extraction of natural products from swine waste. We are now engaged in quantitative analyses of these flocculants and coagulants to determine efficiencies and costs.

Year 2 Research Plans

We received a spring sample set in April, received a summer sample set in mid-August, and are conducting similar analyses to capture the seasonality of the parameters we are measuring. We have adopted a vitamin B12 assay based on published HPLC techniques with fluorescence detection (a suitably sensitive technique), and will quantify B12 (cyanocobalamin) and its congeners (methylcobalamin, etc.) in frozen samples we already have from October, 2009 and April 2010 and in new ones coming in. We are working toward publishing the spirilloxanthin-antioxidant work soon; the manuscript is mostly written and awaiting a few contributions from co-authors. We will continue the anammox work as described above. We will also continue to examine the waste sample analysis reports available from the NC Division of Agronomy for the lagoons we have studied so far to support further statistical analyses of our results. We have added a few additional assays to the analytical work in response to statistical results in order to develop a more predictive understanding of the conditions favoring natural product synthesis and anammox activity, including direct measurements of redox potential, ammonium, sulfide, and chloride ions by selective electrodes.

Finally, we used NC Pork Council funding for our work to support a proposal to the US Dept. of Agriculture’s Conservation Innovation Grants program for a demonstration scale project aimed at evaluating a farm-scale system for extracting bacterial carotenoids and further treating swine waste to maximize its utility and added value. This project, if funded, would have provided a total of \$1 million for this work over a two year period. UNCW made a cash match commitment of \$250,000 toward this proposal, and that was matched by in-kind contributions by swine producer Jennings Humphrey of Pender County. Unfortunately, USDA declined our proposal this time around. We will seek additional funding as opportunities arise.

Table 1. Results of linear regressions of normalized carotenoid content (absorbance @495 nm) vs. chemical composition of swine waste lagoons. Results consistently significant across both batches indicated in bold.

Batch #1 (2008 samples, n= 108)

Carotenoid vs.	F	p	df	R ² _{Adj}
Nitrogen	ns			
Phosphorus	13.5	0.0004	1,106	0.10
Potassium	ns			
Calcium	ns			
Magnesium	11.5	0.001	1,106	0.09
Sulfur	16.9	<0.0001	1,106	0.13
Iron	ns			
Manganese	5.32	0.023	1,106	0.04
Zinc	ns			
Copper	ns			
Boron	ns			
Sodium	ns			
pH	6.58	0.0117	1,106	0.05 (negative effect)

Batch #2 (2009 samples, n=58)

Carotenoid vs.	F	p	df	R ² _{Adj}
Reducing power	ns			
Nitrite ion	ns			
Nitrogen	ns			
Phosphorus	5.95	0.018	1,55	0.08
Potassium	ns			
Calcium	5.83	0.019	1,55	0.08
Magnesium	7.95	0.0067	1,55	0.11
Sulfur	20.4	<0.0001	1,55	0.26
Iron	ns			
Manganese	ns			
Zinc	ns			
Copper	ns			
Boron	ns			
Sodium	5.72	0.02	1,55	0.08
pH	ns			
