An Investigation of Possible Endosymbionts in *Nereis spp.*

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Abstract:

Symbiosis is a relationship found in nature in which two species benefit each other without any harm to the other. There are several types of symbiosis - one being chemosymbiosis in which bacteria complete specific chemical processes that aid the host. In this experiment the potential for chemosymbiosis and the presence of chemosymbiotic bacteria was examined in Nereid worms. By developing a system to create a contained anoxic environment, nerieds were tested in different conditions of anoxia, sulfide, and sediment type to determine if chemosymbionts were present and if so how they were acquired. After running the system for 8 days no worms were found alive in any of the anoxic treatments, however worms were found alive in the oxic environments. Examinations of these worms suggested the possibility that chemosymbionts may be present although further experiments need to be carried out.

Introduction:

In any ecosystem, the various interactions among organisms and their surrounding abiotic factors (e.g. chemicals, sediments, currents) are what keep the system active and the communities intertwined. Common biological relationships that occur in almost any environment include parasitism, mutualism, commensalism and symbiosis. One form of symbiosis, called endosymbiosis, occurs when chemoautotrophic bacteria live within the cells of a multicellular organism; another similar form, known as episymbiosis, occurs when chemoautotrophic bacteria reside on the outside of a host. The organism aids the bacteria's survival by hosting it within or on its own body while the bacteria perform functions which aid in the survival of the host. Endosymbiosis is a vital process for some marine organisms as it is the only way for them to thrive in otherwise hostile environments (Stewart, et al, 2005). One of the most extreme examples of endosymbiosis and episymbiosis occurs at hydrothermal vents in the deep sea benthos. In the late 1970s, a team of scientists from Woods Hole Oceanographic Institution discovered the presence of large (>3') marine vestimentiferan tube worms (*Riftia pachypilia*) within sulfide-rich waters at active hydrothermal vents on the ocean floor (Cavanaugh, et al. 2006). The bacterial endosymbionts living within the tissues of *Riftia* are able to utilize the sulfides abundant in the water column for chemical processes.

In this relationship the host organism supports the bacterium by assisting it in receiving chemicals needed for chemosynthesis while the endosymbionts use this substrate to support the development and health of the host. An example of the aforementioned substrate use is in carbon fixation. (Stewart, et al, 2005)

Since that discovery, scientists have found chemosymbionts living within the cells and tissues of a variety of marine organisms – many of whom live far removed from hydrothermal rift zones (Stewart, et al. 2005). In addition to Cavanaugh's research, Dr. Carmela Cuomo and her lab group (Cuomo, 2008) found that worms in organically rich sediment left without oxygen for a prolonged period of time grew significantly over the experimental period. At the end of the experiment, Cuomo (2008) found that the worms had also turned a deep red color. It was suggested that this might be the result of chemosymbionts taking residence within the organisms (Cuomo, personal communication). However, given that this was not the intent of the original study, it was not pursued at the time. The idea was examined further by King-Trudeau (King-Trudeau and Cuomo, 2010) in a study conducted on Nereid worms from New England coastal sediments.

The one environmental commonality that unites all of the known occurrences of chemosymbiosis in marine environments is the presence of a chemocline – a zone where oxygenated water and/or sediments are separated from waters and/or sediments rich in CO_2, H_2S, and other reduced metabolites. In coastal sediments, these reduced metabolites are the product of the bacterially-mediated anaerobic decomposition of organic matter contained within the sediments.

The organic-rich, sulfidic sediments of Western Long Island Sound (WLIS) are known to contain small bivalves and a variety of polychaete worms including Capitellids and Nereids. Previous

studies (Cuomo, 1985; Tsutsumi, et al., 1990; Miron and Kristensen, 1993, Tsutsumi, et al. 2001, Cuomo, 2008, King-Trudeau and Cuomo, 2010) have demonstrated that some members of these families appear to have an affinity for sulfidic environments and have suggested possible relationships with chemosymbiotic bacteria. The study presented here further explores the potential role that chemosymbiotic bacteria may play in the survival of Nereid worms in organic-rich, sulfidic sediments, such as those found in WLIS.

Methods:

Experimental chambers consisted of 8 Sterilite Ultra-seal 16.2 cup square containers (Dimensions 5/8" L x 6 5/8" W x 9 1/4" H). The watertight reinforced seal on the top lid allowed for each chamber to have a totally self-contained gas environment. The top lid was outfitted with 3 ports: one for water removal, one for water replacement, and one for gas infusion (either oxygen or nitrogen depending upon the treatment). Ports were drilled with a 7/32 inch diameter drill, completed with rubber tubing and sealed at the lid with liquid super glue to guarantee a complete seal. An air stone was attached to the bottom of the gas tubing to allow for more rapid distribution of the gases within the seawater. An airlock was also outfitted on the top of each container to prevent gas buildup within the chambers. Each container was filled with 10cm of either sediment or sand (depending upon the treatment) and 7cm of seawater. These containers were divided into oxic and anoxic conditions and placed in two fifty gallon Rubbermaid storage totes that were used as a water bath. The purpose of the water bath was to keep the temperature within the containers constant. The water baths were kept running via a pump which filtered, heated, and sterilized the water while keeping the water at the desired temperature of 22-2 ^{o} C. In the water baths a HOBO pendant temperature logger was kept throughout the experiment to constantly record the temperature.

The experimental design is shown in Figure 1. A total of 8 different conditions were tested: Oxic-Non-Sulfidic-Sand, Oxic-Non-Sulfidic-Mud, Oxic-Sulfidic-Sand, Oxic-Sulfidic-Mud, Anoxic-Non-Sulfidic-Sand, Anoxic-Non-Sulfidic Mud, Anoxic-Sulfidic-Sand and Anoxic-Sulfidic- Mud. The anoxic vs oxic treatments were designed to evaluate whether or not chemosymbionts were aiding the worms' survival; the sulfidic vs. non-sulfidic treatments were designed to evaluate if chemosymbiotic bacteria might be aiding their hosts by oxidizing sulfide to sulfate; the two different sediments were used to test whether or not the worms were picking up the symbionts from organic-rich mud.

Container 1: Oxic Conditions LIS Sediment No Sulfide Crystals	Container 3: Oxic Conditions LIS Sediment Sulfide Crystals Added	Container 5: Anoxic Conditions LIS Sediment No Sulfide Crystals	Container 7: Anoxic Conditions LIS Sediment Sulfide Crystals Added
Container 2: Oxic Conditions Sterile Playsand No Sulfide Crystals	Container 4: Oxic Conditions Sterile Playsand Sulfide Crystals Added	Container 6: Anoxic Conditions Sterile Playsand No Sulfide Crystals	Container 8: Anoxic Conditions Sterile Playsand Sulfide Crystals Added

Figure 1: Chart outlining each of the 8 conditions used in the experimental chambers over the course of the trial.

The Nereid worms were purchased from a bait and tackle shop in Milford, CT and came from Maine. It is likely that the worms are Nereis virens, however, due to similarities among the three main species of Nereid, the species could not be determined with absolute certainty. Therefore there is the small possibility that the worms may have been Nereis diversicolor or Nereis succinea.

Figure 2: The setup of the two water baths in which the experiment took place.

All worms were placed in individual containers of artificial seawater for a 24 hour starvation period prior to the start of the experiment. Water in the holding containers was changed once within the 24 hour starvation period. At the conclusion of the starvation period, the worms were patted dry and four worms were selected for each experimental chamber. All four worms were weighed and then placed into their respective experimental chambers where they were allowed to acclimate for 24 hours prior to the initiation of the experiment. Water samples were taken for dissolved oxygen and sulfide measurements at the start of the experimental period. Dissolved Oxygen was determined using a modified Winkler method. Water samples for sulfide analysis were taken, fixed with sodium hydroxide and zinc acetate, and set aside for later analysis using a modified version of the Fonselius colorimetric

method. Once the initial water samples were taken, nitrogen gas was bubbled into the anoxic tank for two hours to drive the water to anoxia. An additional set of dissolved oxygen and sulfide samples were taken at this time to confirm that the oxygen had been removed from the water in the chamber. Water temperature was recorded and general observations were made on the worms' behavior. This two hour bubbling and sampling routine was repeated every day for the entire 8 day experiment.

At the end of the experiment the experimental chambers were opened and the sediment was examined for living worms. All surviving worms were rinsed in artificial seawater, patted dry and weighed before being quickly preserved in a solution of 10% formalin. Preserved worms were sent out for sectioning and mounted as slides at McClain Laboratories. All slides were then stained with DAPI fluorescent stain and set with a mounting medium under a coverslip. Slides were photographed under a microscope at 20x magnification and fluorescent light to activate the stain.

Results and Discussion:

The results from this experimental trial were mixed. The system developed for this study worked well, as there was little to no fluctuation in the DO of the anoxic containers (Table 2). Creating a stable test environment is imperative to this investigation because it removes that possibility of error and variability within the experimental set up. This system is expected to be utilized again in several additional trials.

All worms in all of the anoxic chambers died, which is not completely unexpected since Nereids, especially *Nereis virens*, are not believed to be able to live in the total absence of oxygen without the aid of the chemosymbionts. Of the worms in the oxic chambers, 87.5% survived, whether or not sulfide was present. The majority of these worms, however, lost weight (Table 1), whereas worms in a previous research project (Cuomo 2008) grew after exposure to sulfide and anoxia. The weight-loss in worms from one of the containers with sulfide crystals was less than that of the worms from the containers without sulfide suggesting that there might be some chemosymbiosis occurring in at least one of the worms.

The containers with added sulfides were expected to have much higher sulfide levels than those without added sulfides. Yet, as Figure 3 clearly shows, sulfide levels were low in all containers, including those that were seeded with sodium sulfide crystals. The reason for this remains unknown at the present time.

Looking at the tissues of the surviving worms, there were some promising discoveries. If one examines the cross section they would see the outer epidermis and muscle within, but the majority of the worm would be comprised of an open coelom area. In this space you would not find nucleated cells, but other material because it is fluid filled cavity used as a hydrostatic skeleton. In figure 4 the outer layer of a neried cross section is depicted, with nucleated cells lining the epidermis. In figure 5, an area of the inner cross section was found to have nuclei within it. These nuclei belonged to bacteria which may very well have been the chemosymbionts we were looking for. This pattern of bacteria within the cross section was found in several of the slides in various clusters.

One possible confounding factor may be that the worms used in this study were a different species than the worms used in previous studies. Future trials will address this issue.

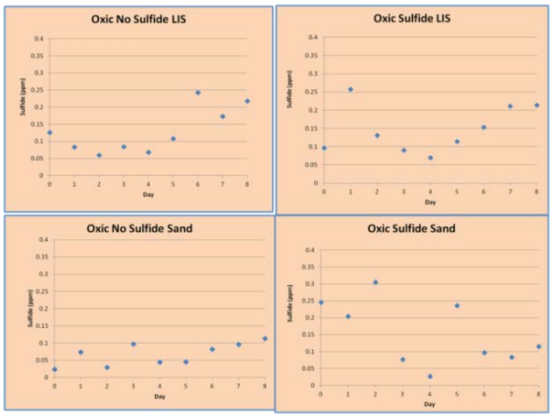


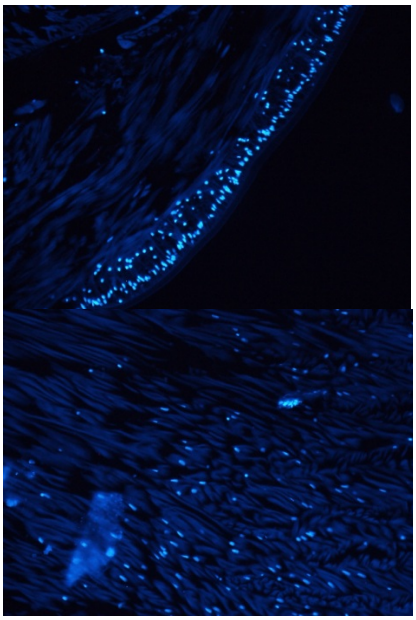
Figure 3: Sulfide levels within Containers 1-4, where worms survived the full experiment.

Container	Total WormWeight (g) Initial	Total Worm Weight (g) Final	Weight Change (g)
1	4.413 ± 1.982	3.504 ± 1.552	- 0.909
2	5.146 ± 2.635	3.990 ± 1.917	-1.426
3**	4.224 ± 1.077	4.354 ± 2.337	0.130
4**	3.808 ± 1.185	3.297 ± 1.917	- 0.511
5	4.071 ± 1.258	NA*	NA
6	4.291 ± 0.958	NA	NA
7	4.353 ± 1.006	NA	NA
8	4.098 ± 1.980	NA	NA

Table 1: Average worm weights (g) per container at start and end of experiment. All containers initially held 4 worms; all worms survived in Containers 1 and 2. ** only 3 worms survived in Containers 3 and 4 * no worms survived in Containers 5-8.

Dissolved Oxygen Levels: Measured in ppm									
Container	Initial D.O.	D.O. post N_2 addition	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8
1	7	NA	6	6	7	6	6	7	8
2	8	NA	8	7	8	9	7	8	8
3	7	NA	6	1	6	7	7	5	1
4	5	NA	5	7	7	6	6	7	7
5	7	0	0	0	0	0	0	1	0
6	8	0	1	0	0	0	0	0	0
7	6	0	0	0	0	1	0	0	0
8	5	0	0	0	0	0	0	1	0

Table 2: Dissolved oxygen levels (measured in ppm) taken at regular intervals throughout the course of the experiment.



Figures 4 and 5: Photos from cross sections of the head and gills of surviving worms

Conclusion:

In conclusion, the experimental system design worked well as evidenced by the fact that the anoxic containers maintained a dissolved oxygen level of zero to one throughout the experiment, showing very little deviation. Tissue sections from Nereids suggest the possibility that Nereid worms may, in fact, possess bacterial symbionts. This needs more study before it can be confirmed.

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Biography:

Ashley Winward is currently a sophomore at the University of New Haven, double majoring in Marine Biology and Environmental Science with a minor in Chemistry. Although she is unsure of her exact path right now, she knows that she would like to work in conservation and education outside of the typical classroom setting. This was Ashley's first experience in a research setting and it was a very eye opening experience to her which she thoroughly enjoyed.

Outside of the lab, Ashley can be seen out on the field performing with the UNH Charger Marching Band or at the Beckerman Recreation Center working at the front desk as well as at other clubs and activities.