A Holistic Time-series Analysis of Stony Coral Tissue Loss Disease

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Final Report

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June 15, 2026

Completed in Fulfillment of PO #C5BC8C for
Florida Department of Environmental Protection
Coral Protection and Restoration Program
8000 N Ocean Dr.
Dania Beach, FL 33004

This report should be cited as follows:
Papke E, Ushijima B. 2025. A holistic time-series analysis of stony coral tissue loss disease. Florida Department of Environmental Protection. Florida. 1-34.

This report was funded through a contract agreement from the Florida Department of Environmental Protection's (DEP) Coral Protection and Restoration Program. The views, statements, findings, conclusions, and recommendations expressed herein are those of the author(s) and do not necessarily reflect the views of the State of Florida or any of its subagencies.

ACKNOWLEDGEMENTS

These authors would like to thank Grace Kennedy and the Richard M. Dillaman Bioimaging Facility at UNCW.

MANAGEMENT SUMMARY

Stony coral tissue loss disease (SCTLD) continues to be a threat to Caribbean coral reefs causing a massive decline in reef-building corals and reducing ecosystem function. A potentially novel disease, *Orbicella* acute tissue loss disease (OATLD), has emerged as an additional threat to *Orbicella* corals in Florida. These projects focused on identifying cellular-level changes associated with SCTLD exposure over time and comparing them with other stressors including OATLD, nitrogen enrichment, and temperature stress. Using transmission electron microscopy (TEM), physiological and morphological changes within the algal endosymbionts were examined to improve the understanding of disease pathology and identify potential diagnostic markers. The results indicate clear differences between SCTLD and OATLD. While potential bioindicators for SCTLD include the decrease in the number of starch granules in the endosymbionts in diseased tissue and the presence of nitrogen-related crystal structures, there was an increase in starch granules for OATLD diseased tissue and crystals seem to be unrelated to pathology. These findings may indicate that SCTLD and OATLD affect coral and algal physiology through different mechanism and support the hypothesis that these are two different diseases. Experiments examining environmental stressors showed limited effects of nitrogen enrichment on algal growth, photosynthetic activity, and cellular morphology. Temperature stress showed an increased in lipid droplets and greater starch granules, indicating physiological changes. SCTLD and OATLD create distinct morphological changes from each other as well as from nitrogen enrichment and temperature stress. Overall, this research continues to establish baseline cellular characteristics for corals of various health states, diseases, and stressors and highlight the importance of cellular-level diagnostics in coral disease research.

EXECUTIVE SUMMARY

Stony coral tissue loss disease (SCTLD) is one of the most devastating coral diseases on record, but the causative agent has not been identified. While SCTLD decimates Florida and the Caribbean, a potentially new disease, *Orbicella* acute tissue loss disease (OATLD), has emerged within the last few years. The lack of diagnostic tools available for SCTLD make is difficult to determine whether OATLD is a completely new disease or just a variation of SCTLD. Additionally, determining the differences between SCTLD, OATLD, and other stresses such as an increase in nitrogen or temperature has yet to be determined utilizing cellular pathology. Transmission electron microscopy (TEM) provides and invaluable diagnostic tool that can be utilized to characterize morphological changes within the coral and its algal endosymbionts and can inform physiological metrics to aid in determining pathology.

Key Findings:

SCTLD Time Series: The number of starch granules significantly decreases in diseased tissue over time compared to the controls. The endosymbionts may not be photosynthesizing as effectively or as efficiently, or it is possible that there is a breakdown in the coral-algal symbiosis which has been seen in previous studies. The number of starch granules could be a bioindicator for SCTLD pathology. The presence of crystal-like structures, hypothesized to be uric acid nitrogen storage, also significantly increase over time in diseased corals. This may indicate that SCTLD is affecting nitrogen cycling and the presence of these crystals could be a bioindicator for SCTLD pathology.

OATLD: The number of starch granules significantly increases in diseased tissue and crystal-like structures are not significantly present within the endosymbionts. This indicates that OATLD causes different morphological changes than SCTLD, suggesting that it is a different disease based on the cellular pathology as well as previous studies.

Symbiodiniaceae nitrogen: No concentration or form of nitrogen impacted the growth, photosynthetic activity, or ultrastructure of the algal cells. This may indicate that the concentrations of nitrogen were not high enough to cause an effect on the algal cells. The crystal-like structures were only present within two algal cells with an ammonia addition, but this result needs to be further explored. With these conclusions, it may be unlikely that algal cultures can accurately represent the effects of nitrogen on corals as it has been previously hypothesized that as little at 5 µmol nitrogen addition can cause detrimental effects to coral reefs while it did not impact these algal cells.

Symbiodiniaceae nitrogen and temperature: There were no significant changes in the growth or photosynthetic activity between the different temperatures or between the control and the 50 µmol ammonia addition. The number of lipid droplets was higher in the algal cultures experiencing temperature stress and the number of starch granules was greater in the controls experiencing algal stress. No crystal structures were seen for either the consistent or the increasing temperature experiments. It is likely that these cultures are more robust than newly isolated or algal cells in symbiosis and it is important to continue with verification of this potential surrogate system.

Conclusions:

The TEM datasets continue to build upon establishing baseline cellular pathology for a variety of health states and create baseline metrics for two potentially different coral diseases that can be used for diagnostics.

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1. BACKGROUND

Stony coral tissue loss disease (SCTLD) is currently devastating Florida's Coral Reef and has spread to at least 28 different countries and territories throughout the Caribbean. SCTLD affects at least 22 different species of reef-building corals, including species that are on the Endangered Species Act list, and can result in total colony mortality in weeks to months. Though this disease was first observed in 2014 near Miami, Florida and research on this disease has been ongoing, the pathogen(s) has yet to be identified. Previous research indicates that SCTLD is an infectious waterborne disease (Aeby et al., 2019; Dobbelaere et al., 2020; Muller et al., 2020) current hypotheses suggest that bacteria are involved in disease progression (Aeby et al., 2019; Neely et al., 2020; Ushijima et al., 2020). Additionally, the coral's algal endosymbiont, in family Symbiodiniaceae, is implicated in this disease as various work has described a breakdown of the algal-symbiotic relationship resulting in dysbiosis (Beavers et al., 2023; Deutsch et al., 2021; Meiling et al., 2021; Studivan et al., 2023). The genera differences of the algal symbiont within the coral are suggested to correlate to the coral's susceptibility to this disease (Klein et al., 2024), but additional work needs to be done regarding this when a pathogen is identified.

In the initial histological characterization of SCTLD, the algal symbionts showed evidence of vacuolization and exocytosis as well as lytic necrosis within the gastrodermal tissue of the coral (Landsberg et al., 2020). Through transmission electron microscopy (TEM), it was determined that SCTLD results in certain ultrastructure pathologies within the algal symbionts that include a decrease in starch reserves, thylakoid membrane breakdown, cavity formation, and chloroplast gigantism (Work et al., 2021). This study also identified the presence of viral-like particles (VLPs) within the algal symbionts, which were hypothesized to be in the family *Alphaflexiviridae* (Veglia et al., 2022).However, these VLPs were observed within diseased and apparently healthy tissues. These VLPs were also not present within the samples analyzed for the original case definition (Landsberg et al., 2020)and they have not been as prevalent in more recently observed samples from USVI (Work et al., 2024), so the connection between these VLPs and SCTLD has yet to be established. Recent work also indicates that these VLPs are present across multiple genera of the algal symbionts and have a global distribution (Howe-Kerr et al., 2023), and the mere presence of these VLPs may not correlate to SCTLD health state.

Current research from our group contradicts these findings regarding VLPs associated with SCTLD samples (Papke, Kennedy, & Ushijima, unpublished data). Our results indicate that the apparently healthy tissues have a significantly higher prevalence of VLPs compared to the disease lesions, though more works needs to be done to further elucidate the correlation between the presence of these VLPs and SCTLD health state with additional species and corals from different locations. Additionally, we identified the presence of crystal-like structures, which are hypothesized to be uric acid deposits based on previous literature (Clode et al., 2009; Kopp et al., 2013). It is well known that coral reefs can readily take up various forms of inorganic (ammonia, nitrate, etc.) and organic (urea) nitrogen that are dissolved in the surrounding seawater. These uric acid deposits in the algal symbionts provide a store of nitrogen for the coral-algal symbiosis in a low nitrogen environment and may be important under low nutrient conditions, such as when feeding is limited (Clode et al., 2009). Previous ultrastructure descriptions in

combination with NanoSIMS indicate that these deposits may be important for physiological functions for the coral and can form due to fluctuating dissolved nitrogen concentrations (Kopp et al., 2013), but it is unclear whether these are short term or long-term deposits as they have been commonly found within a significant portion of our samples. Specifically, about half of the algal symbionts in our SCTLD samples housed in aquaria had crystal-like structures, so it remains unclear how long these deposits are maintained within the algal symbionts before the nitrogen is utilized for other purposes. These data hint at potential inconsistencies when comparing captive and field samples as well as highlighting how little descriptions exist for how environmental conditions affect the ultrastructure of corals and their endosymbionts. These limitations can have serious consequences when trying to evaluate the health of corals facing current and future threats.

This project will build upon the current work of our research group, which is focusing on identifying the viruses and bacteria associated with SCTLD-affected corals, determining if they are specific to SCTLD lesions, and establishing if there are any associations between potential pathogens and the start of SCTLD lesions through time. Additionally, increasing nutrient loads in the seawater due to costal runoff could have catastrophic effects on the coral-algal symbiosis, which is a related aspect we have included in this proposal to investigate. This works aims to understand how an increase in nitrogen impacts the physiology of symbiotic algae in addition to temperature and determine whether these previously seen ultrastructure signs are related to SCTLD as a disease sign or are related to a decrease in nutrient availability further exacerbated by SCTLD. This data should allow us to separate the effects of SCTLD and pollution on cellular ultrastructure. This is a multi-phase project in which Phase I focused on sample collection and the beginning of sample processing, while Phase II focused on sample analysis. Phase III is the continuation of sample analysis as well as including experiments to determine changes in algal physiology in response to increase nutrients and stressors.

2. PROJECT GOALS

The goals of this project are to 1) document cellular pathologies associated with SCTLD over time using transmission electron microscopy (TEM), 2) document cellular pathologies of a potentially new coral disease (*Orbicella* Acute Tissue Loss Disease) affecting *O. faveolata* colonies in Florida using TEM, 3) catalog the cellular responses of algal symbiont cultures to nutrient pollution and heat stress. The data generated from this project will provide a comprehensive cellular view of what is occurring within the coral tissue during a SCTLD infection over time and will contribute to the holistic view of this disease from the analyses that were completed in Phase I and II. Additionally, this project aims to determine whether the potentially new *O. faveolata* disease differs in the cellular pathology from SCTLD. Finally, determining the cellular responses of the algal symbiont will aid in understanding how these symbionts are affected by nutrient pollution and temperature stress and will also help to differentiate between the effects of infectious diseases compared to environmental stressors, which allow for improved diagnostics and may provide insight into the coral-algal symbiosis.

- The project is a multi-phase project continuing from the previously funded Phase II (C3D89C), which focused on completing most of the analysis of naïve coral samples. Due to the extensive steps and amount of time TEM requires, not all TEM samples were imaged for this previous project phase.
- Phase III (current scope) focused on the processing and analysis of endemic coral samples and aided in establishing a cellular pathology by cataloging cellular changes associated with infectious diseases affecting Floridian corals (FY 2025 – 2026). Additionally, this phase investigated the effects of eutrophication and temperature on algal health and ultrastructure to determine the differences between environmental stress and infectious diseases.

<u>Task 1:</u> Quality Assurance Plan and Reporting.
<u>Task 2:</u> Image and analyze 10 corals from the time series project.
<u>Task 3:</u> Image and analyze 10 diseased *O. faveolata* corals of a potentially new coral disease observed in the Florida Keys.
<u>Task 4:</u> Test the effects of different concentrations of ammonia, nitrite, and nitrate on the health of Symbiodiniaceae cultures *ex situ* over time.
<u>Task 5:</u> Test the combined effects of temperature and nitrogen concentrations on Symbiodiniaceae cultures *ex situ* over time.

These results will provide a diagnostic tool for ultrastructure associated with SCTLD which will provide a catalog of images for comparative analyses for future disturbances. It will also aim to determine whether the *O. faveolata* samples have a different cellular pathology from SCTLD samples, providing insight into whether this is a new coral disease, which will significantly impact management and treatment options. Additionally, TEM can further investigate the presence of VLPs associated with different health states for SCTLD to identify a clear link. Finally, the nutrient and temperature experiments will help differentiate between the effects of the algal physiology from infectious diseases or environmental stressors, which will allow for improved diagnostics. It will also aid in determining the susceptibility or resilience of the algal symbionts to these structures and determine whether the stressors could be potential co-factors for SCTLD.

3. METHODS

3.1. Task 1 – Quality Assurance Plan and Reporting

There are no methods associated for this task.

3.2. Task 2 – Image and Analysis of Time Series Corals

TEM samples were previously fixed in a paraformaldehyde, glutaraldehyde, and instant ocean solution. The samples were embedded according to a previously created protocol (Papke et al., 2024) and were imaged at the UNCW Richard M. Dillaman Bioimaging Facility using a FEI Tecnai Spirit BioTwin TEM. The gastrodermis was targeted as the area of interest with a specific focus on the endosymbionts and VLPs within the endosymbionts. ImageJ was used for image analyses and the scale for each micrograph was set using the scale provided in each individual micrograph. The endosymbionts were

be analyzed based on signs of stress and previous literature for SCTLD (Camaya et al., 2016; Danovaro et al., 2008; Howe-Kerr et al., 2023; Work et al., 2021, 2024). Some of the parameters scored for the endosymbionts were the size, number of starch granules, number of lipid droplets, chloroplast breakdown, presence of VLPs, and presence of crystal-like structures. All statistical analyses were done in R. For numerical parameters, a Kruskal-Walis was used for significance while a Fisher's test was used for categorical parameters.

3.3. Task 3 – Image and Analysis of *O. faveolata* Corals

Task 3 is separate from Task 2 described above but is a complimentary project. The Ushijima Lab at UNCW previously collaborated with Dr. Karen Neely at NSU to analyze samples of a potentially new coral diseased affected *O. faveolata* colonies in the Florida Keys (tentatively named Orbicella Acute Tissue Loss Disease – OATLD). This task is a continuation of the previous proposal *Initial assessments of a potentially novel coral disease affecting key reef building corals on Florida's Coral Reef* (submitted by Dr. Karen Neely for FY 23-24). All methods were similar to task 2 and the ultrastructure was be compared to known SCTLD ultrastructure. All statistical analyses were done in R. For numerical parameters, a Kruskal-Walis was used for significance while a Fisher's test was used for categorical parameters.

3.4. Task 4 – Effects of Nitrogen on Symbiodiniaceae Cultures

Three genera of Symbiodiniaceae were used for these experiments (*Symbiodinium, Breviolum,* and *Durusdinium)* that were originally isolated in the 1990s by Dr. Mary-Alice Coffroth. The cultures are grown in 1x f/2 medium in Instant Ocean (35 ppt, pH 8) and are maintained in an algal incubator at 27°C and 35 $\mu mol/m^{-2}/s^{-1}$ light. For the nutrient experiments, additions of sodium nitrate, ammonium chloride, and sodium nitrite were added to each of the three genera in the concentrations of 2.5, 10, and 50 µmol/mL and and instant ocean addition was used as the control. The nitrogen type was added every three days for a duration of 13 days. Initial samples were taken before nitrogen addition to obtain the base nitrogen concentration, and initial cell count, and an initial TEM sample. Samples were taken for cell counts and nutrients every three days before the nitrogen addition and TEM samples were taken at the beginning, middle, and end of the experiment.

Growth measurements were obtained by cell counts using a hemocytometer. Photosynthetic activity was measured by first dark acclimating the cultures and then using the Z985 17 Cuvette AquaPen to obtain the fv/fm measurements to measure the quantum efficiency of Photosystem II. Nutrient samples were obtained and quantified using a colormetric assay. Lastly, samples were saved for TEM analyses and fixed with paraformaldehyde and glutaraldehyde. The TEM sample preparation was similar to the methods previously described for coral tissue preparation and the algal cultures were scored with similar parameters as stated previously.

3.5. Task 5 – Effects of Nitrogen and Temperature on Symbiodiniaceae Cultures

The methods for Task 5 were similar to the methods from Task 4 as described previously. The same measurements (i.e., cell counts, photosynthetic activity, nutrient analyses, and

TEM) were taken for each of these experiments. However, varying temperatures were tested in combination with nutrient stress to evaluate the potential synergistic effect of temperature and nutrients on algal ultrastructure. *Breviolum* was the only genus that was used for this experiment and ammonia was the only nitrogen source at an addition of 50 µmol/mL. Cultures were maintained at 27°C for the duration of the experiment and used as controls. For the experimental cultures, the temperature increased by 1°C every other day (Table 1) and ammonium was added every other day. Samples were taken for nutrient analyses at hours 1, 2, 24, and 48 while cell counts and photosynthetic activity measurements were taken before ammonium was added. TEM samples were taken at the beginning, middle, and end of the experiment. For the controls maintained at 27°C ammonium was also added, and samples were taken at the same time, however the temperature did not fluctuate. There were nitrogen controls used for each temperature, with an addition of instant ocean, and there were also media controls with instant ocean and ammonium as additions. All sample analyses were done as previously described.

Table 1. The experimental design for Task 5 including the timepoint and when each measurement was taken. The hour denotes when the ammonium was added for both the control and experimental cultures while the temperature denotes when it was increased for the experimental cultures. An x for each timepoint is when the sample was taken for each culture.

Timepoint	Day	Hour	Temperature	Nutrient	Cell Count	Fv/fm	TEM
1	1	Before addition	27	x	x	x	x
2	1	1	27	x			
3	1	2	27	x			
4	2	24	27	x			
5	3	48	27	x	x	x	
6	3	1	*28	x			
7	3	2	28	x			
8	4	24	28	x			
9	5	48	28	x	x	x	
10	5	1	*29	x			
11	5	2	29	x			
12	6	24	29	x			
13	7	48	29	x	x	x	
14	7	1	*30	x			
15	7	2	30	x			
16	8	24	30	x			
17	9	48	30	x	x	x	x
18	9	1	*31	x			
19	9	2	31	x			
20	10	24	31	x			

21	11	48	31	x	x	x	
22	11	1	*32	x			
23	11	2	32	x			
24	12	24	32	x			
25	13	48	32	x	x	x	
26	13	1	*33	x			
27	13	2	33	x			
28	14	24	33	x			
29	15	48	33	x	x	x	
30	15	1	*34	x			
31	15	2	34	x			
32	16	24	34	x			
33	17	48	34	x	x	x	x

4. RESULTS

4.1. Task 1 – Required Reporting Deliverables

4.1.1. ***Task 1:*** *Required reporting deliverables (UNCW).*

1) Manager-level summary.
The summary was included at the beginning of this report.
2) Meta-data uploaded to DataOne. To be included with final report.

4.1.2. Task 2: *Time Series Analysis*

1) Time Series excel document of all existing samples to date, including location of samples, and the products obtained from each sample. Listed as "Time Series Samples" within the "Time Series" folder.
2) Representative Time Series TEM images (5-10) (jpg, .png, or .tif) from at least 10 corals listed as "Time Series" folder.
3) Complete analysis of 10 Time Series imaged corals as an excel sheet with scored metrics and outline results in the final report. Excel sheet is listed as "Time26 image j" for raw analyses, "time26" for just the corals included within this reporting period, and "time" for all samples for this entire dataset.

4.1.3. ***Task 3:*** *O. faveolata Analysis*

1) OATLD excel document of all existing samples to date, including location of samples, and the products obtained from each sample. The excel sheet is named "OATLD Samples" within the "OATLD" folder.
2) Representative (5-10) OATLD TEM images (.jpg, .png, or .tif) from at least 10 corals with the "OATLD" folder.
3) Complete analysis of 10 imaged OATLD corals as an excel sheet with scored metrics. The raw data is within the "orb26 image j" sheet while the complete dataset is within the "Orbicella" sheet.

4.1.4. ***Task 4:*** *Nitrogen Symbiodiniaceae cultures*

1) Nutrient excel document of all existing samples to date, including location of samples, and the products obtained from each sample. Listed within "Nutrient Samples" excel sheet within the "Zoox Project" folder.
2) Nutrient growth curves of the endosymbiont growth over time for each experiment. Listed within the "zooxnitro" excel sheet.
3) Photosynthetic activity measurements provided in an excel document for each nutrient experiment. Listed within the "zooxnitro" excel sheet.
4) Nutrient experiment representative TEM images (5-10) (.jpg, .png, or .tif) from at least 2 samples per experimental condition and analyses of TEM images from the experimental conditions compared to the control will be provided in an excel sheet with the scored metrics. All images are included within the "Zoox Project" folder. All ImageJ analyses are included within the sheet "zooximagej" within the "Zoox Project" folder.

4.1.5. ***Task 5:*** *Temperature Nitrogen Symbiodiniaceae cultures*

1) Temperature-Nutrient excel document of all existing samples to date, including location of samples, and the products obtained from each sample. Samples are listed within the "Nutrient Samples" excel sheet.
2) Temperature-Nutrient growth curves of the endosymbiont growth over time for each experiment. The cell counts are listed within the "zooxtempnitro" sheet within the "Zoox Project" folder.
3) Photosynthetic activity measurements provided in an excel document for each temperature-nutrient experiment. Listed within the "zooxtempnitro" sheet within the "Zoox Project" folder.
4) Temperature-Nutrient experiment representative TEM images (5-10) (.jpg, .png, or .tif) from at least 2 samples per experimental condition and analyses of TEM images from the experimental conditions compared to the control will be provided in an excel sheet. All images are within the "Zoox Project" folder and ImageJ analysis is within the "zooxtempimagej" sheet.

4.2. Task 2 – SCTLD Time Series Results

The ten corals that were imaged within the 25-26 grant reporting period were added to the additional dataset to provide a complete picture of SCTLD over time. According to our previous data analysis, the number of starch granules and the presence of crystal-like structures were important parameters for SCTLD. Those parameters were the focus of these analyses along with other metrics such as average cell size, general cell and chloroplast breakdown, and the presence of viral-like particles (VLPs).

A Kruskal-Wallis with a bonferroni p-adjustment indicates that there is no difference in cell size between health states at any given time point. Though, the cells do decrease in average size at T3 and then increase slightly after (Figure 1).

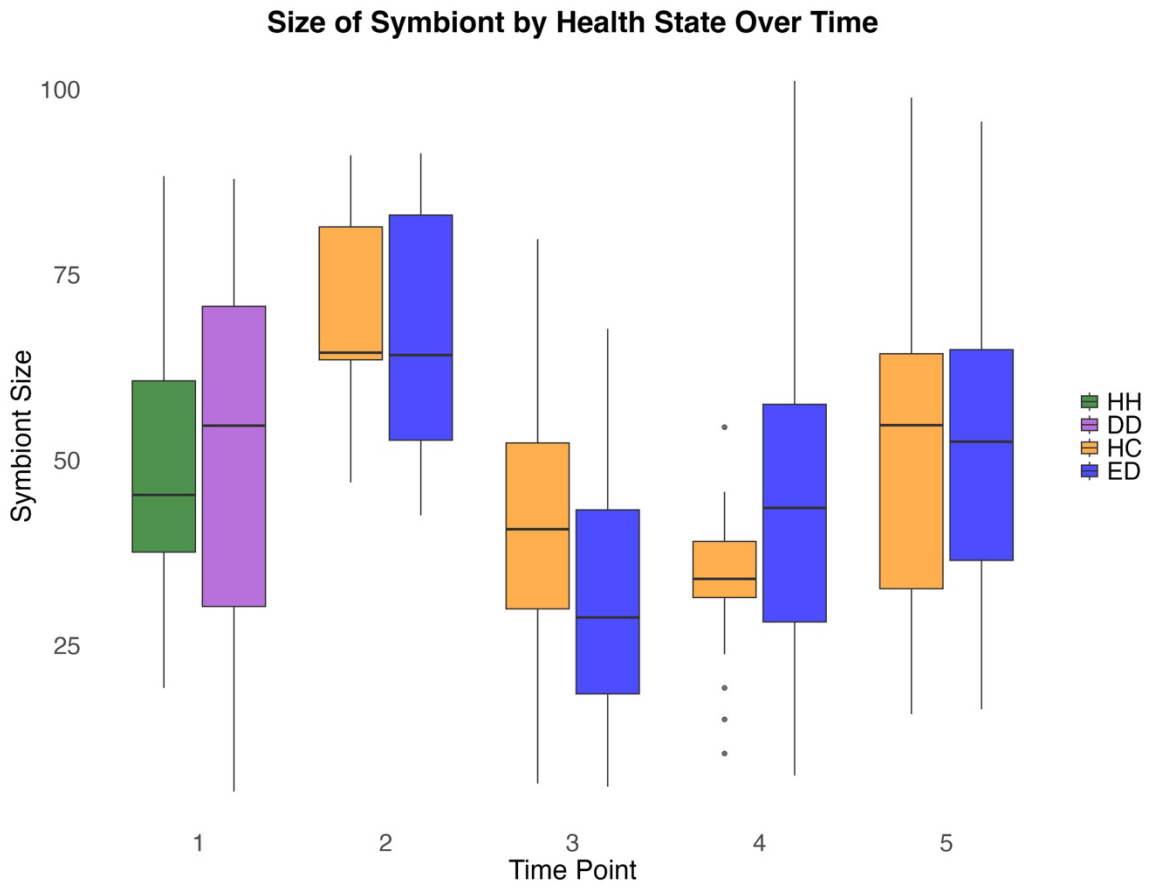

Figure 1. The average symbiont size over time by health state for the time series corals.

There was a great number of lipids in the beginning healthy corals compared to the beginning diseased donor corals collected from the field (KW: $p = 0.002$; Figure 2). There was no other difference in the number of lipid droplets between health state at a given time point except for T5, where the healthy controls had more lipids than the exposed to disease corals (KW; $p = 0.002$).

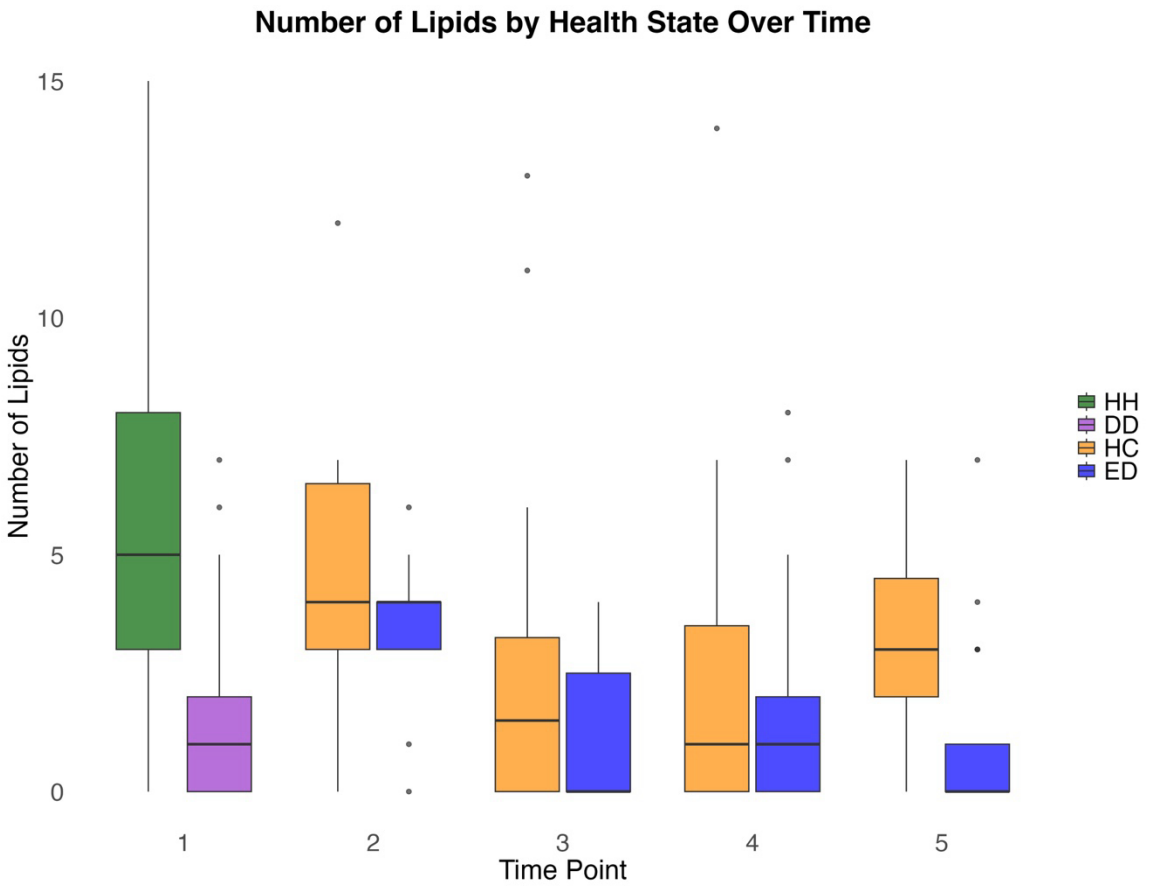

Figure 2. The number of lipids over time by health state for the time series corals.

The number of starch granules decreased over time in the exposed to disease coral (Figure 3). Initial naïve corals had significantly more starch granules than the diseased donor corals from the field (KW; $p = 0.114$). At T2 and T3 there was no difference in the number of starch granules by health state, but at T4 and T5 the healthy controls had significantly more starches than the diseased corals (KW; $p = 0.0013$ and $p < 0.00001$, respectively). This indicates that the number of starch granules may be a bioindicator for SCTLD pathology.

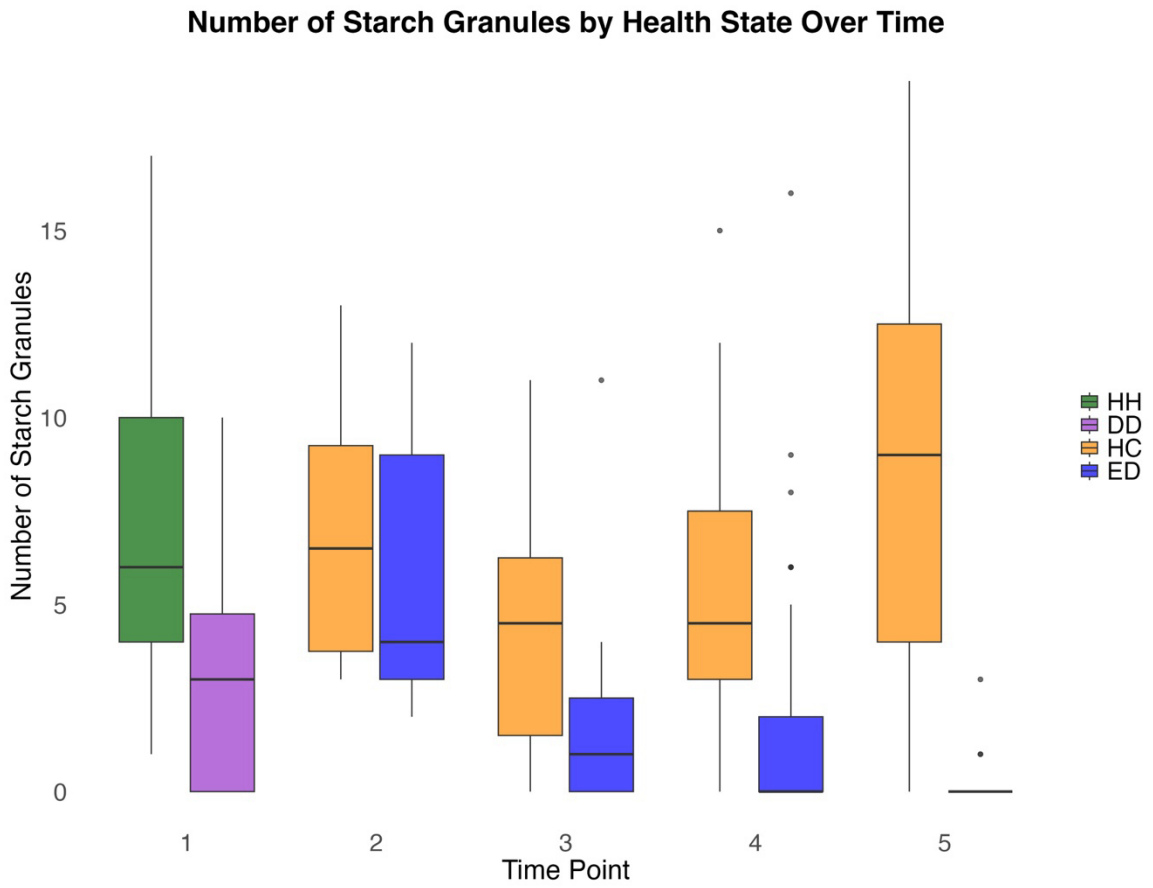

Figure 3. The number of starch granules over time by health state for the time series corals.

Crystal-like structures were identified in previous SCTLD samples and are believed to be uric acid deposits, a form of nitrogen storage. Within the naive corals, no symbionts have crystals while within the disease donor corals, crystals were present in 82% of the symbionts (Figure 4). The health control corals continue to have no crystals present within their symbionts, while the exposed to disease corals have an increase in the crystals present over time. Specifically, T4 is when the crystals are initially present and increase from 36% to 81% at T5. This may indicate that the presence of these crystal-like structures is a bioindicator for SCTLD.

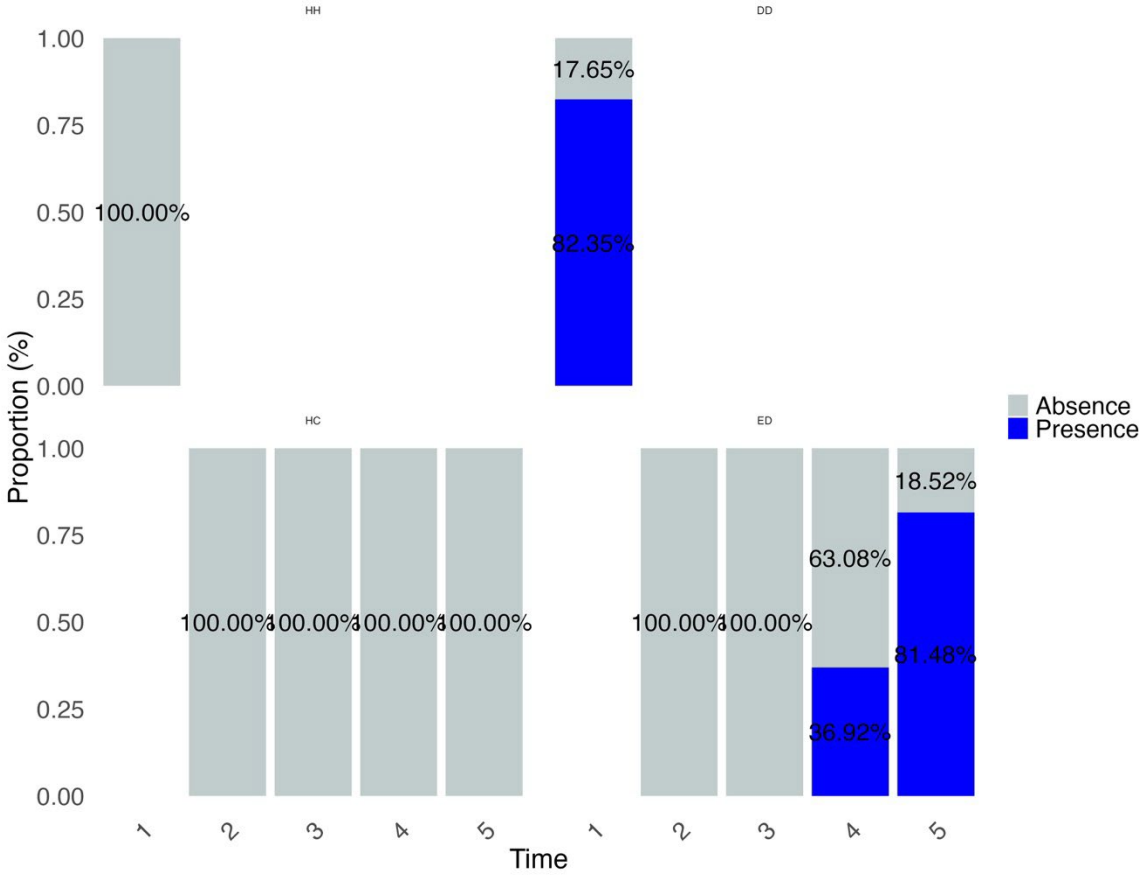

Figure 4. The presence of crystals over time by health state for the time series corals.

While the presence of VLPs was previously correlated to SCTLD, these data indicate that VLPs are not correlated to SCTLD health state (Figure 5). VLPs are present across all health states and times, indicating that they may not be related to SCTLD.

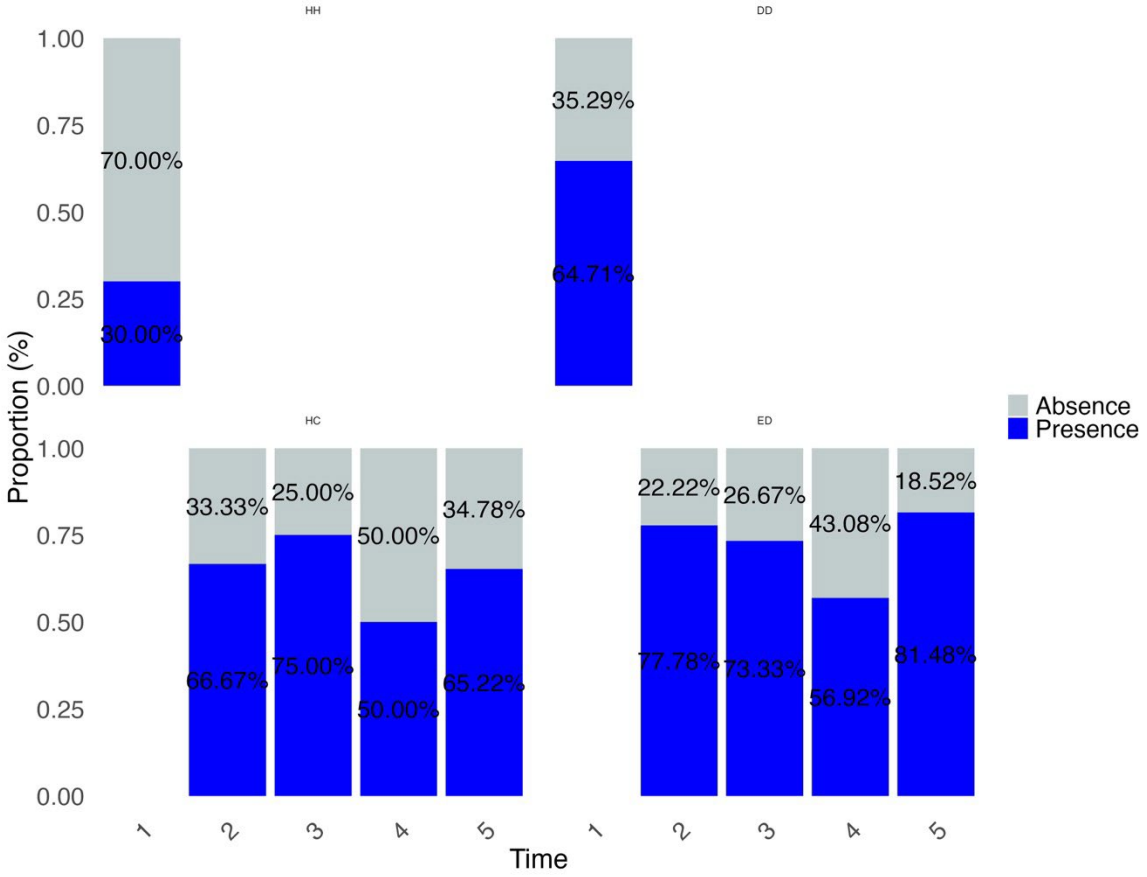

Figure 5. The presence of VLPs over time by health state for the time series corals.

4.3. Task 3 – OATLD Results

The main purpose of analyzing these coral samples was to compare these samples to previous SCTLD samples to understand whether or not these differ in their cellular pathology to determine if they are two different diseases.

The average symbiont size did not differ between health states (Figure 6).

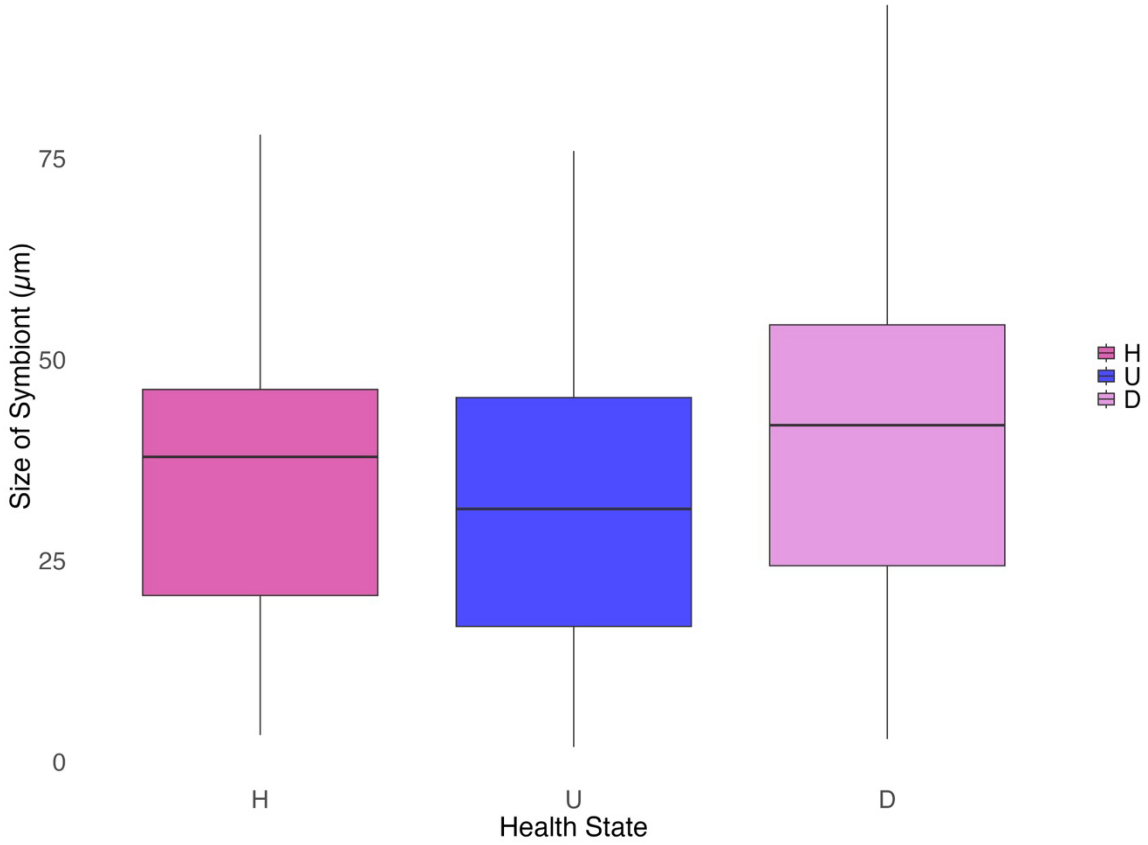

Figure 6. The average symbiont size by health state for the OATLD corals.

The number of starch granules was greater for the symbionts within the diseased corals compared to the disease unaffected (KW, $p < 0.00001$, Figure 7) and compared to the healthy (KW; $p < 0.00001$). There was no difference between the healthy and the disease unaffected samples. For SCTLD, the number of starch granules decreased within the diseased health state while for OATLD the number of starch granules increases.

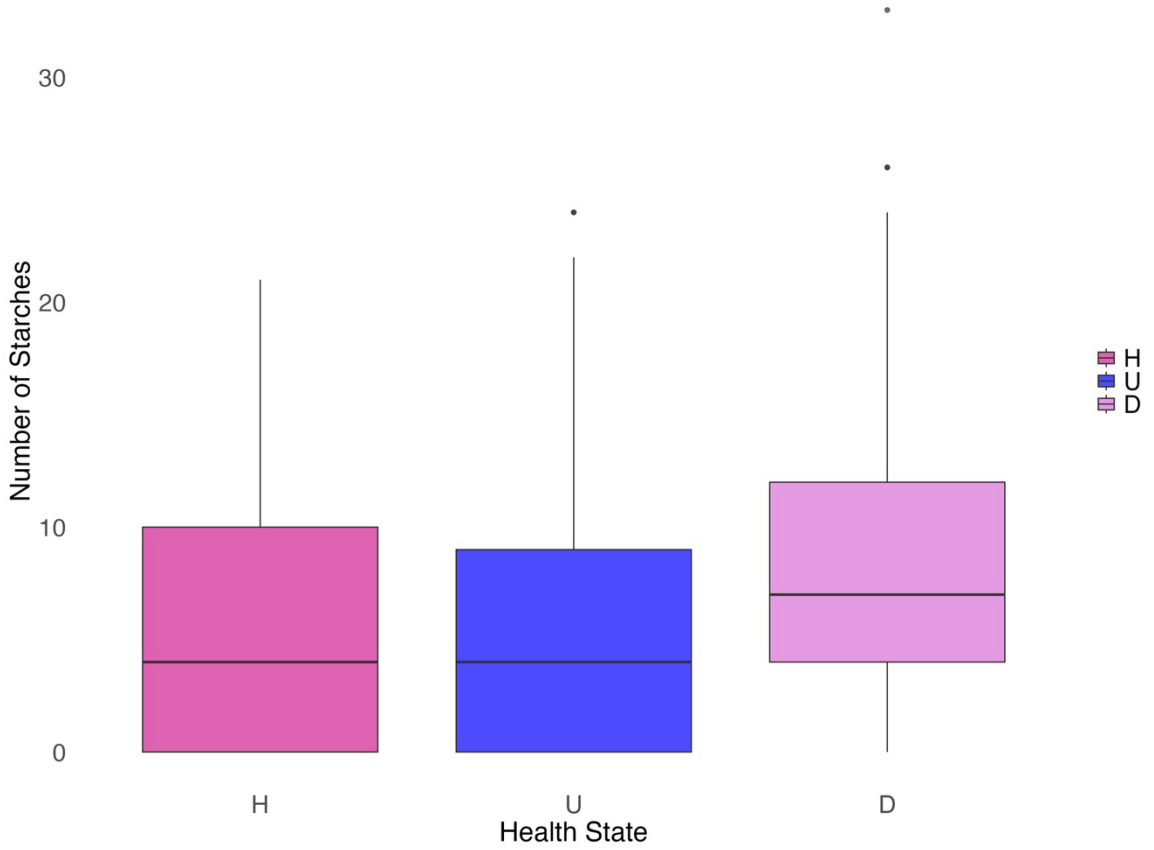

Figure 7. The number of starch granules by health state for the OATLD corals.

The number of lipid droplets also increased in the diseases corals when compared to the healthy (KW; p = 0.0005) and the disease unaffected (KW; p = 0.0067, Figure 8). While the number of lipid droplets did not change for SCTLD corals, in the OATLD corals the symbionts in the diseased samples are storing more lipids.

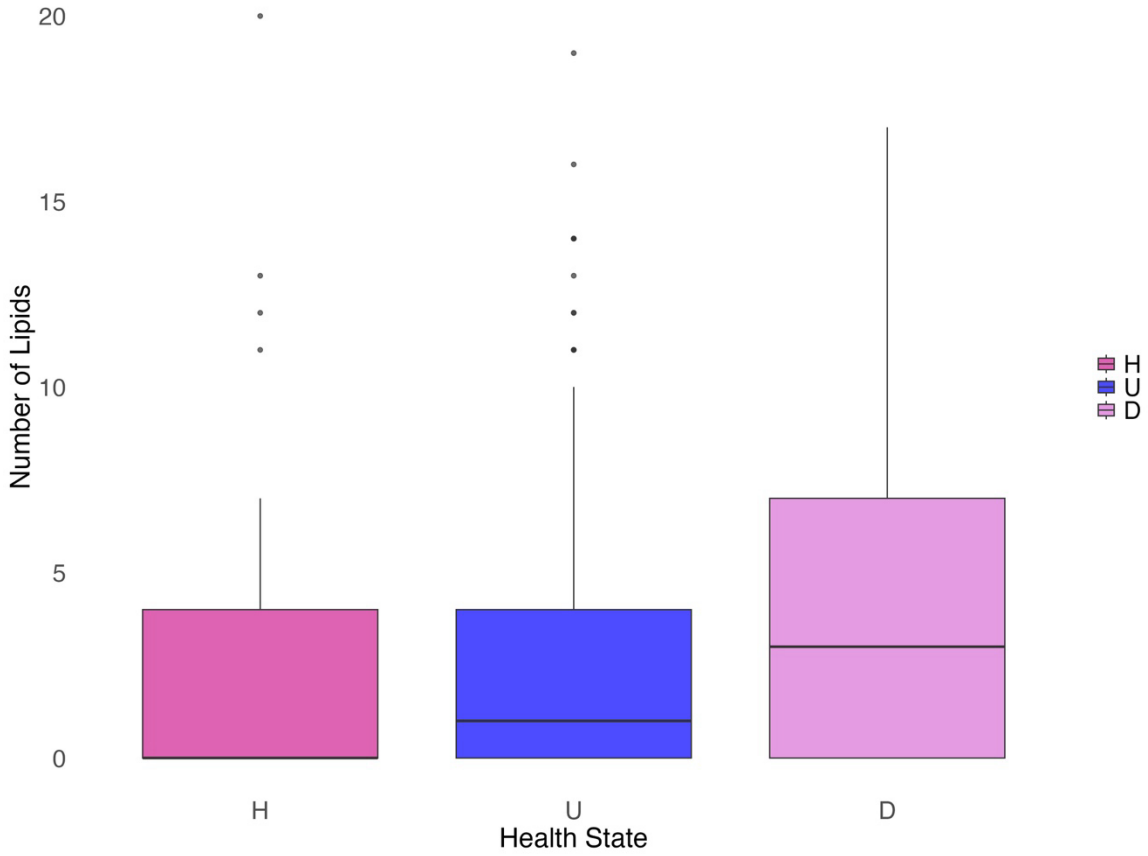

Figure 8. The number of lipid droplets by health state for the OATLD corals.

The presence of crystal-like structures is more prevalent in the diseased unaffected corals compared to the health (Fisher; $p = 0.03$) and the diseased (Fisher; $p = 0.02$). Overall, there is a low prevalence of the crystal-like structures across the dataset. Though these structures may be a bioindicator for SCTLD, they are not prevalent in the OATLD corals (Figure 9).

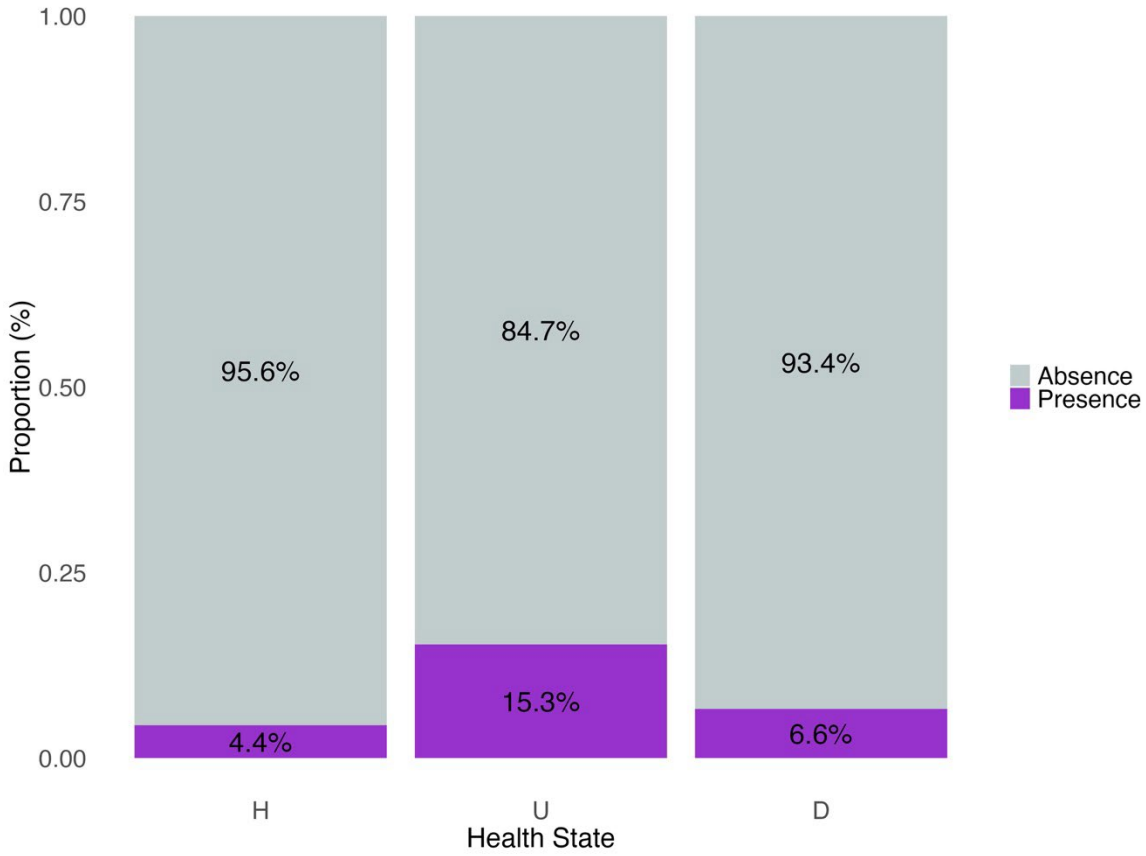

Figure 9. The presence of crystal-like structures across health states for the OATLD corals.

The presence of VLPs was more prevalent in the healthy samples compared to the disease unaffected samples (Fisher; $p = 0.003$). Also, the disease had more VLPs than the disease unaffected samples (Fisher, $p = 0.03$). The healthy and the disease health states had no statistical difference (Figure 10). This may indicate that there are less VLPs within the unaffected tissue on a disease colony, or it could potentially indicate that VLPs are not correlated to this disease.

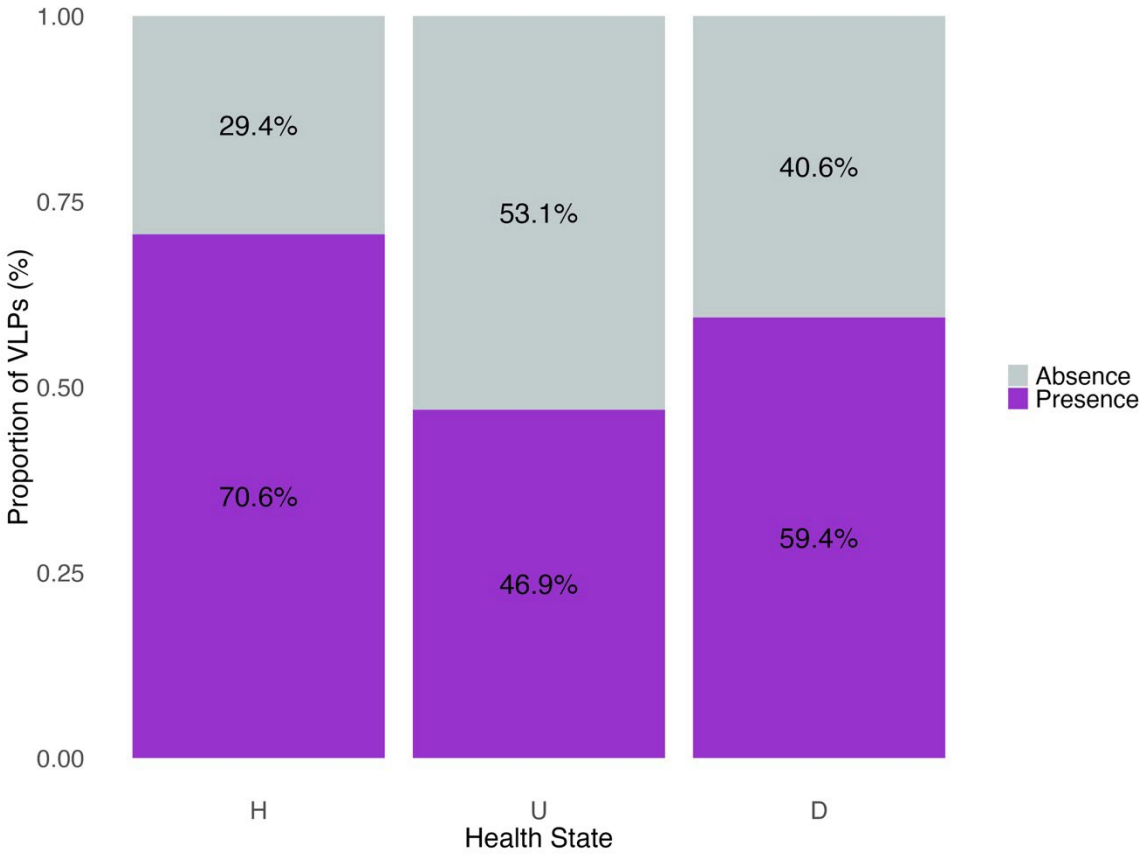

Figure 10. The presence of VLPs by health state for the OATLD corals.

4.4. Task 4 – Symbiodiniaceae Nutrient Results

The photosynthetic activity, quantum yield, indicates that there is no difference in the photosynthetic activity between concentration within an individual form of nitrogen within a genus (Figure 11). There may be a trend with increase photosynthetic activity in the 10 and 50 µmol nitrite addition only within *Durusdinium.*

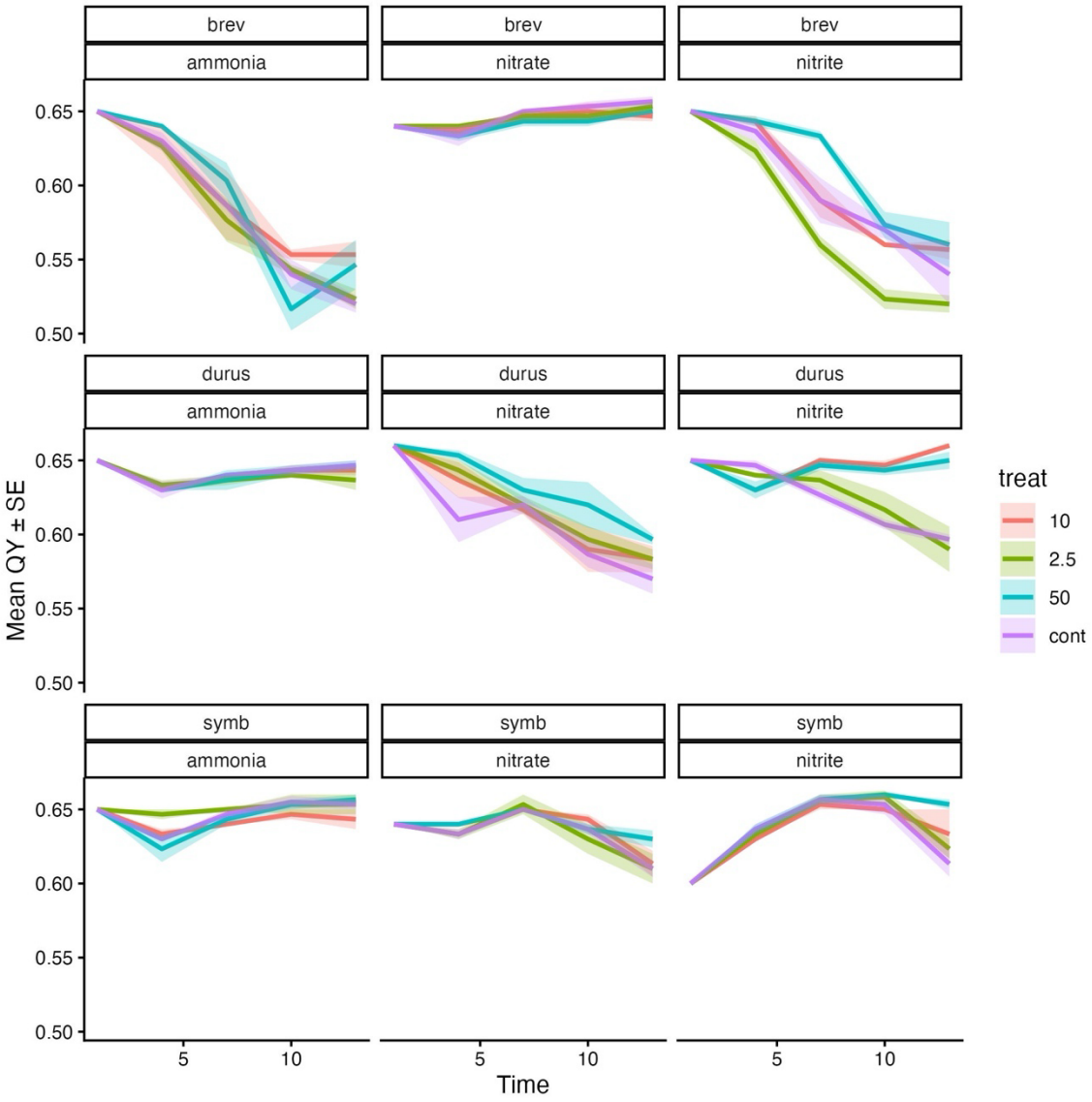

Figure 11. The quantum yield with the standard error over time for each genus and form of nitrogen. Colors denote the type of treatment, and the photosynthetic activity is plotted over time. Brev = Breviolum, durus = Durusdinium, symb = Symbiodinium.

Similarly, initial observations show that the growth rate of the algal cell did not change within treatment between concentrations, though there may be an increase within 10 and 50 µmol nitrite for *Durusdinium* and 10 and 50 µmol nitrate within *Symbiodinium* (Figure 12).

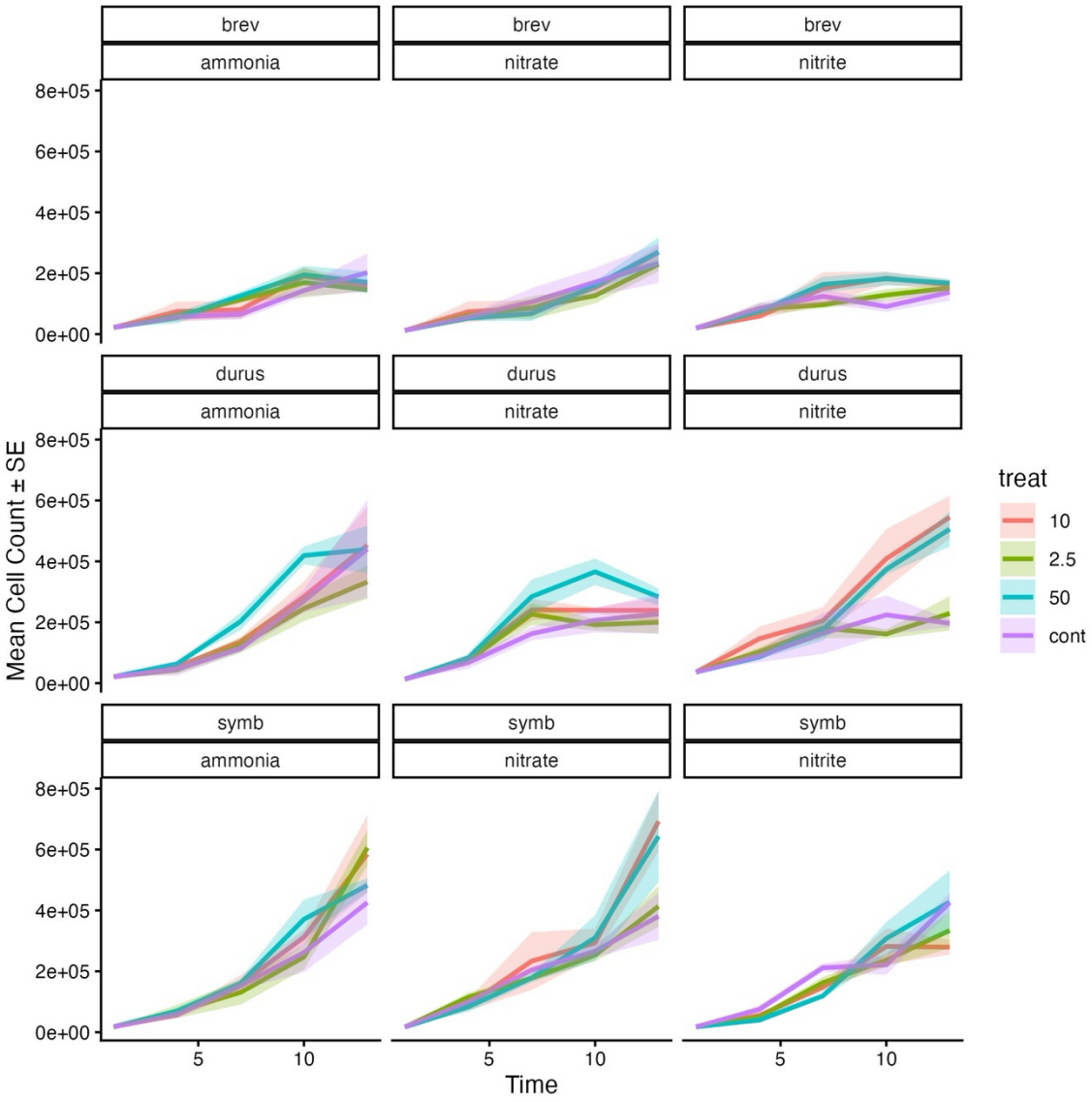

Figure 12. The mean cell count and standard error over time for each genus and nitrogen type. The concentrations are denoted by colors.

For all three genera, the ammonia presence was low regardless of the concentration (Figure 13). Nitrite levels were high for the 50 µmol addition and peaked in the middle of the experiment until it started to decline over time for all three genera. For nitrate, the levels increased at the end of the experiments for *Breviolum* and *Durusdinium* but did not change for *Symbiodinium.*

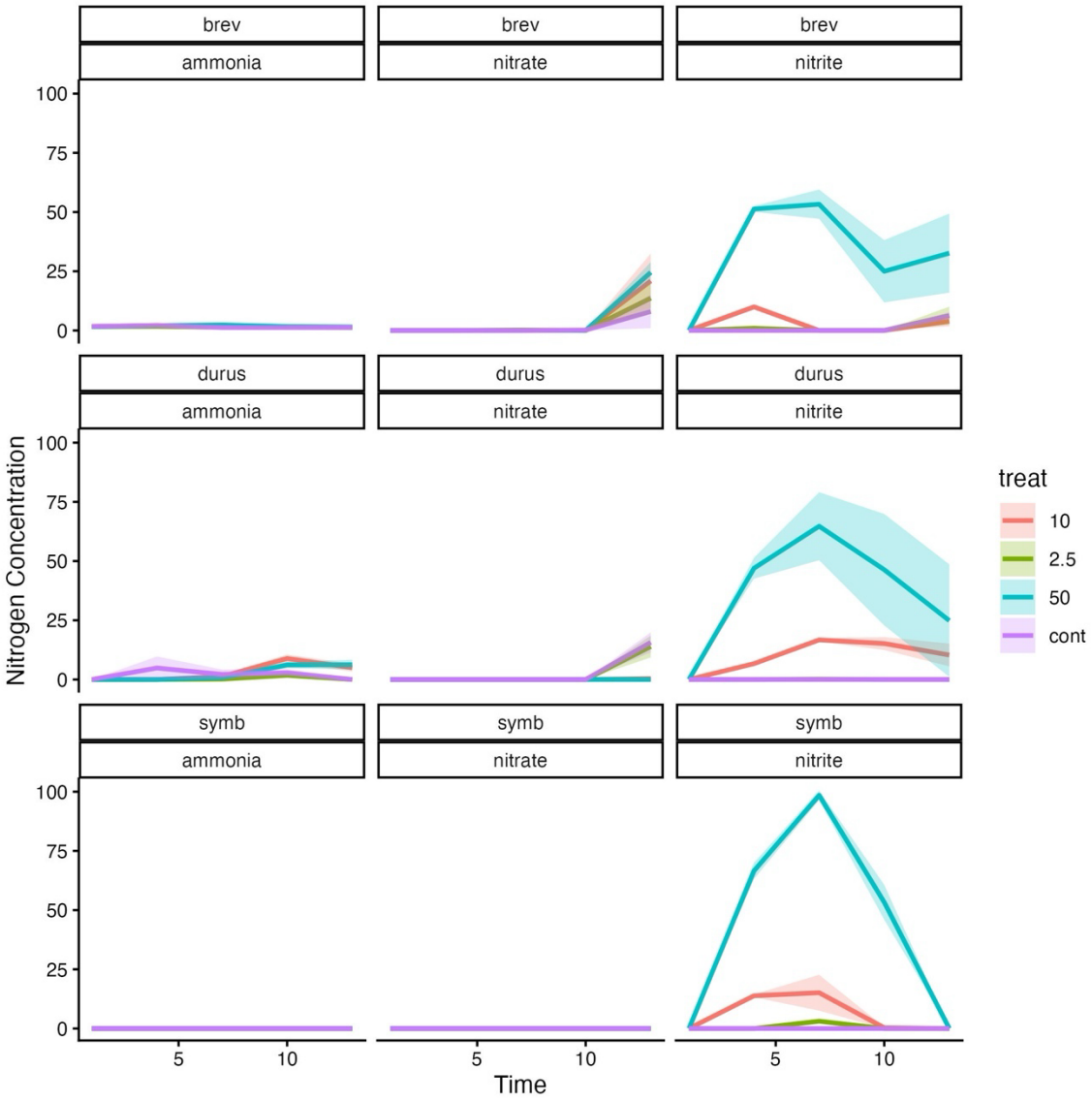

Figure 13. The three nitrogen concentrations by individual genera over time.

Within individual experiments, there are trends within the nutrient data. The ammonia addition within *Breviolum* indicated that most of the ammonia was being converted to nitrite regardless of concentration (Figure 14). It is also likely that the algal cells were utilizing the ammonia. Within the nitrite addition, the nitrate in the media was utilized within the first few days and the 50 µmol addition caused a potential conversion from nitrate to nitrate as the values become inverses of each other. The nitrate addition in *Breviolum* showed an increase in nitrate after day 10 and continued to increase over time until day 13. Additionally, nitrite production was low for the duration of the experiment until day 10 indicating that the conversation of nitrate to nitrite was occurring within these cultures.

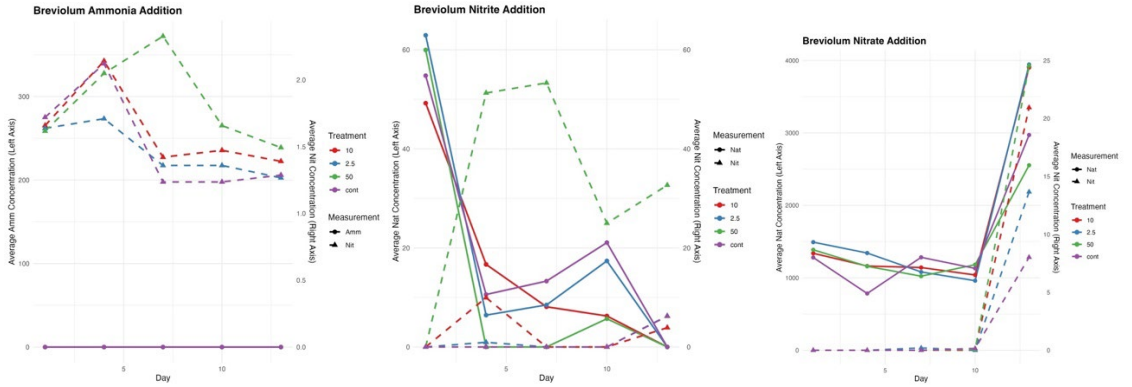

Figure 14. The nutrient concentrations of ammonia and nitrite in ammonia addition for Breviolum (left). The nutrient concentrations of nitrite and nitrate for the nitrite addition in Breviolum (middle). The nutrient concentrations of nitrite and nitrate within the nitrate additions for Breviolum (right).

For *Durusdinium,* the ammonia was utilized regardless of concentration and was not present until day 13 (Figure 15). There were small levels of nitrite present with an increase around day 10, which may indicate a conversion of ammonia to nitrite. For the nitrite addition, the nitrite was utilized and potentially converted into nitrate as seen at day 10 when the nitrate levels increase for the 10 and 50 µmol additions. For the nitrate additions, the nitrate was consumed steadily regardless of concentration and there was a slight increase in the nitrite production after day 10.

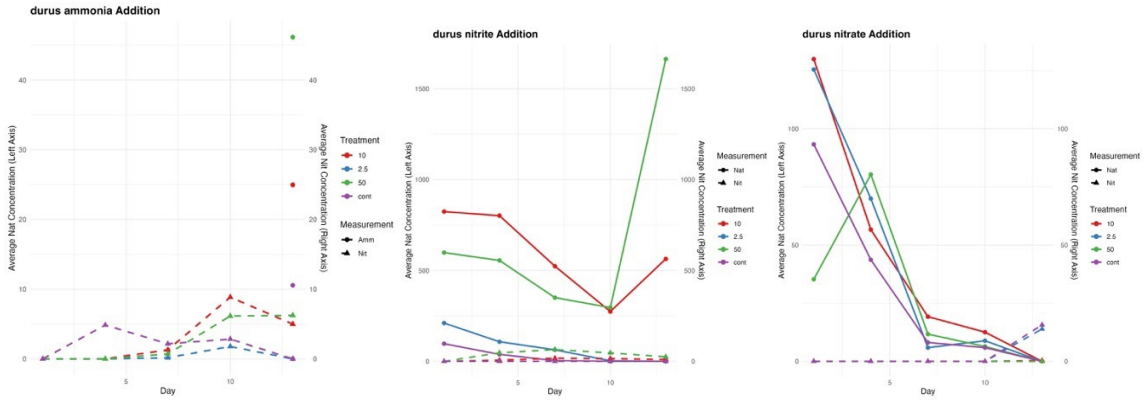

Figure 15. The nutrient concentrations of ammonia and nitrite in ammonia addition for Durusdinium (left). The nutrient concentrations of nitrite and nitrate for the nitrite addition in Durusdinium (middle). The nutrient concentrations of nitrite and nitrate within the nitrate additions for Durusdinium (right).

For *Symbiodinium*, the nitrite and ammonia levels remained at zero except at time 13 when the ammonia levels were higher (Figure 16). When both nitrite and nitrate were added, the levels of nitrate dropped over time indicating that the algal cells may be utilizing this form of nitrogen. There was very little presence of nitrite for either the nitrite or nitrate addition.

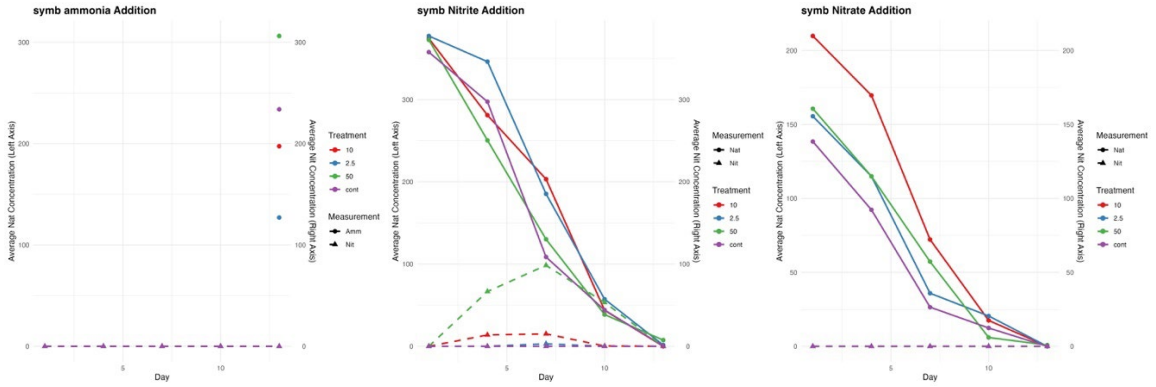

Figure 16. The nutrient concentrations of ammonia and nitrite in ammonia addition for Symbiodinium (*left). The nutrient concentrations of nitrite and nitrate for the nitrite addition in Symbiodinium* (*middle). The nutrient concentrations of nitrite and nitrate within the nitrate additions for Symbiodinium (right).*

Only *Breviolum* was imaged using TEM and all three forms of nitrogen were imaged. The last timepoint of 13 days was imaged for just the 50 µmol addition and it was compared to the control. The morphology of the algal cells is similar regardless of nitrogen addition or form of nitrogen added (Figure 17). The number of starches does not significantly differ between the 50 µmol addition and the controls for any forms of nitrogen. There are only crystal-like structures in the ammonia additions within the 50 µmol and none in the control or the other forms of nitrogen. Though the number of cells observed was small, this could be a trend that needs to be further explored. There were also no differences in cell size or number of lipids between treatments.

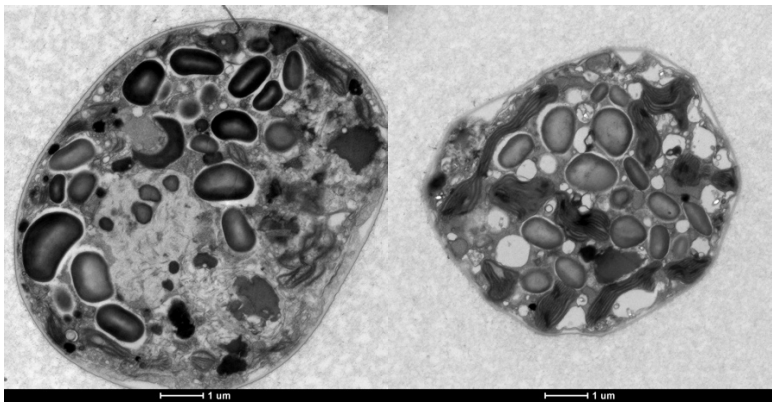

Figure 17. TEM images of algal cells in the control (left) and the 50 µmol ammonia addition (right). Scale bars = 1 µm.

4.5. Task 5 – Symbiodiniaceae Nutrient and Temperature Results

For this experimental design, only a 50 µmol ammonia addition was added to *Breviolum* cultures over a 17-day period. Within this experiment, there was a media control to understand the utilization of the ammonia without the algal cells present and there were also cultures maintained at 27°C while the other cultures increased 1°C every other day. The growth of the algal cells did not statistically differ when using a linear mixed effects

model between the control or the 50 µmol addition of ammonia for either the 27°C cultures or the temperature increase cultures (Figure 18). For the control 27°C cultures, the growth rate was not exponential and started to level off after 16 days while the ammonia additions continued to have exponential growth. For the temperature increase cultures, both the control and the ammonia addition continued to increase over time regardless of the temperature increase. This indicates that the temperature increase did not impact the growth rate. Additionally, there was no difference in the photosynthetic activity between treatments or between the control 27°C cultures and the increasing temperature cultures.

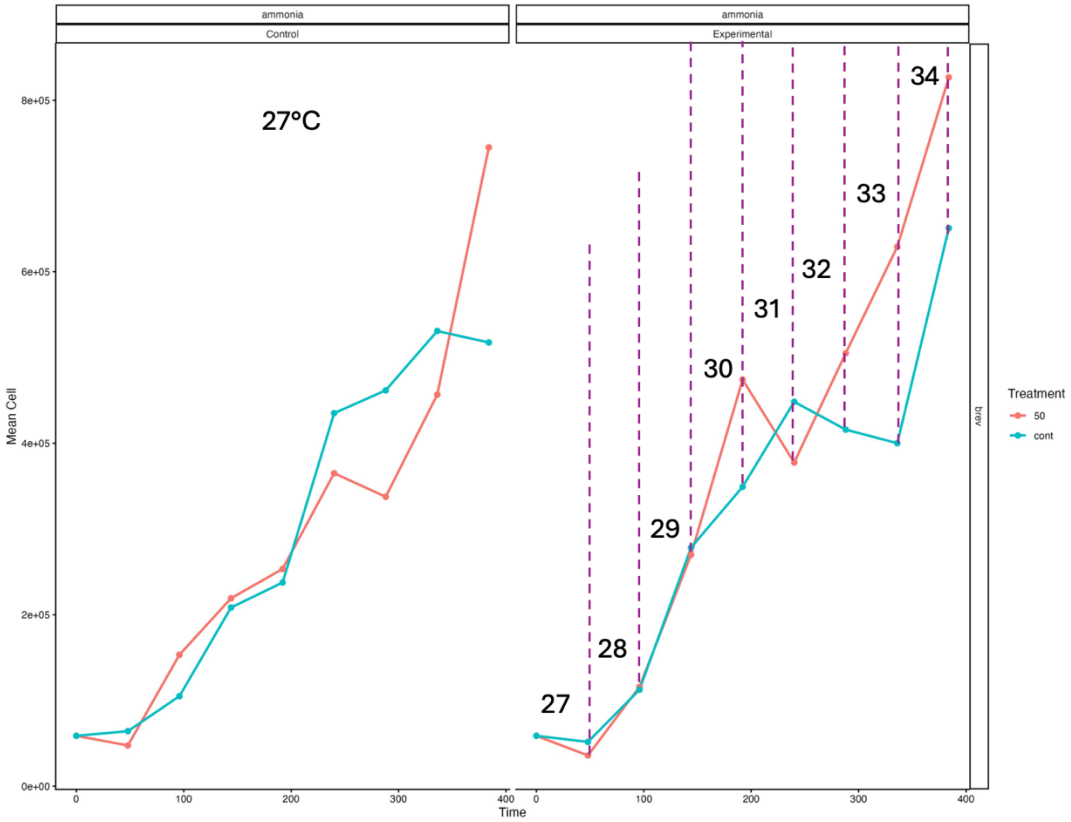

Figure 18. The mean cell count over time for the cultures maintained at 27°C and the cultures that had increasing temperature. The color denotes the treatment for either the control or the 50 µmol addition of ammonia.

The ammonia draw-down occurred within the first hour of addition and almost was completely utilized by 24 hours for both the 27°C cultures and the experimental temperature increase cultures (Figure 19). The ammonia concentration decreased over time, suggesting that the algal cultures were utilizing it more readily which could be due to the increase in cell count over time. The consistent temperature increase did not seem to affect the rate of ammonia consumption.

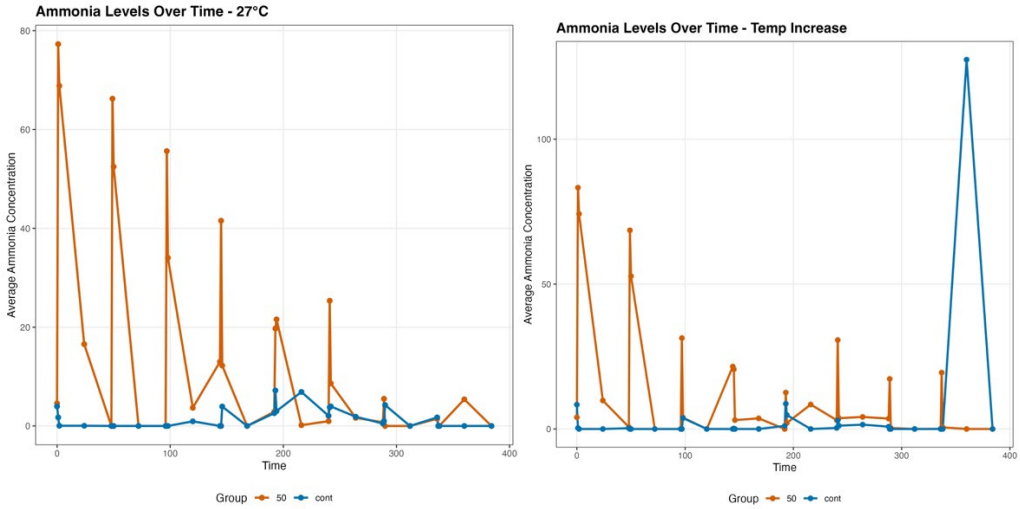

Figure 19. The ammonia levels over time for the 27°C cultures (left) and the experimental temperature increase cultures (right). The colors are either the control or the 50 µmol addition of ammonia.

When looking at the ultrastructure, the number of lipid droplets nor the number of starch granules differed between the controls and the 50 µmol ammonia addition. However, the number of lipids did differ between the controls and the 50 µmol cultures when comparing temperatures (Figure 20). This suggests that the consistent temperature increase causes an increase in lipid droplet accumulation. There was only a statistical difference in the number of starch granules within the control groups that did not receive an ammonia addition (Figure 21). The consistent increasing temperature cultures had more starches in their control algal cells than the 27°C controls. There was also large variation in the number of starch granules within both 50 µmol additions.

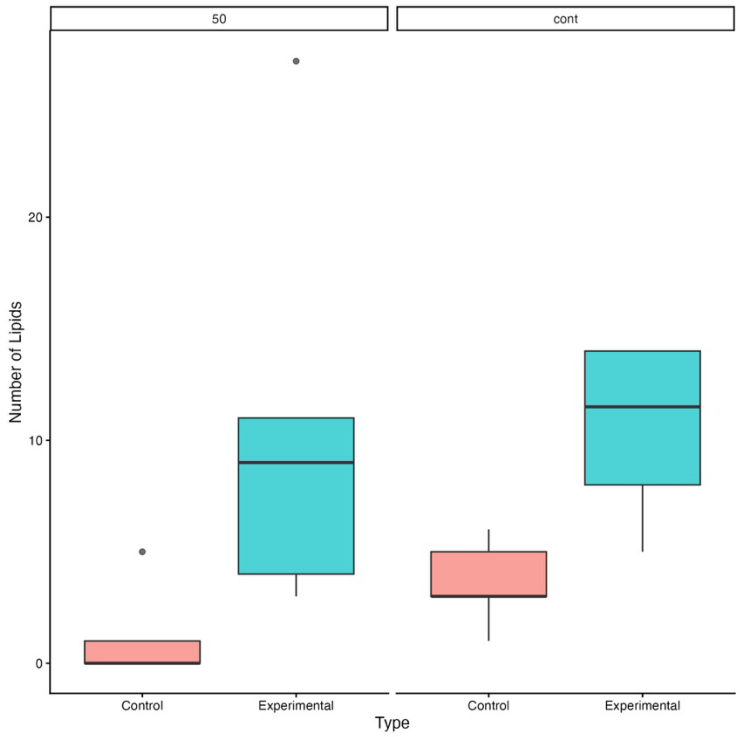

Figure 20. The number of lipid droplets separated by 50 µmol ammonia addition and the controls. The Control on the x-axis is the consistent 27°C temperature while the Experimental is the temperature increase.

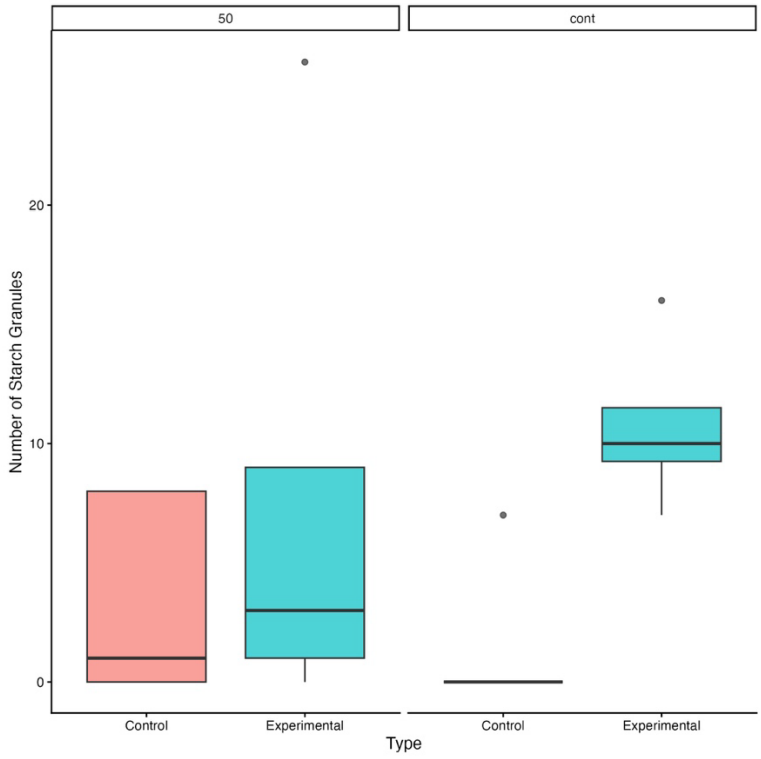

Figure 21. The number of starch granules separated by 50 µmol ammonia addition and the controls. The Control on the x-axis is the consistent 27°C temperature while the Experimental is the temperature increase.

Though there were crystals present in the 50 µmol ammonia addition for the previous experiments, there were not crystals within any of these samples. Additionally, VLPs were common within these samples which may indicate that they are present whether the algal cultures are in symbiosis or not.

5. DISCUSSION AND MANAGEMENT STRATEGIES

5.1. Time Series

The bioindicators for SCTLD could be the number of starch granules and the presence of crystal-like structures. The number of starch granules decreased in symbionts within disease corals, indicating that they may not be photosynthesizing at efficiently or as effectively. This was seen over time within the time series samples as the number of starch granules starched to significantly decreased in disease compared to the controls at T3 and continued for T4 and T5. Additionally, the presence of crystal-like structures increased in the diseased samples which could be a sign that the symbionts in diseased tissue are storing nitrogen during times of nitrogen limitation. These crystals increase over time starting at T4 and increasing at T5.

5.2. OATLD

The OATLD corals seem to have a differing cellular pathology within the endosymbionts compared to SCTLD diseased corals. The OATLD diseased corals have significantly more starch granules compared to the healthy which is the exact opposite from the SCTLD symbionts. Additionally, the presence of crystal-like structures is seemingly not correlated to OATLD while it is correlated to SCTLD. The number of lipids also increased for OATLD samples while they largely remain unaffected in SCTLD samples. These results indicate that the cellular pathology is difference, potentially creating evidence that these are two different diseases.

5.3. Symbiodiniaceae Nitrogen

Regardless of concentration or form of nitrogen added to any genera of the algal cultures, there were no drastic shifts in algal health or physiology. Nitrate may have impacted the photosynthetic activity and growth in just *Durusdinium* and did not affect the other genera. Additionally, each genus utilized the three different forms of nitrogen slightly differently. Each genus completely utilized the ammonia addition, but only *Breviolum* had a large production of nitrite while *Durusdinium* had minor production and *Symbiodinium* had no production. This could be due to these cultures not being axenic as there are some microbes that are associated with the cultures. For both *Breviolum* and *Symbiodinium* there is a utilization of the nitrate when the nitrite is added and there is a slight utilization for *Durusdinium* but then it is converted and there is a production towards the end of the experiment. *Breviolum* also differs when nitrate is added where both nitrate and nitrite are

produced at the end of the experiment while nitrate continues to be utilized over time for *Durusdinium* and *Symbiodinium* with very little production of nitrite. The algal cells may be utilizing the nitrate as it is very unlikely that nitrate is being converted to nitrogen gas as that is an anoxic process. Overall, there were no significant detrimental effects seen with any form of nitrogen and there were no differences between the additions and the control. Algal cultures may not be suitable for surrogate systems to test the effects of nitrogen on corals since they are not in symbiosis and could potentially handle much larger concentrations of nitrogen by themselves. The concentrations used in these experiments may not have been high enough to cause any visible effects to the algal cultures. In the future, higher concentrations of each form of nitrogen could be added and microbiome sequencing could be done on the cultures to figure out what microbes are present that could be involved in the nutrient cycling.

There were no significant differences in the ultrastructure between treatments for any form of nitrogen. *Breviolum* had the greatest change in nutrient concentrations, which is why it was chosen to be imaged. Since the concentration of the nitrogen did not yield any significant results within the photosynthetic activity or the cell count, the highest concertation was compared to the control to try to observe the greatest shifts in morphology. Though only *Breviolum* was imaged, it is possible that the other genera could show differences in their ultrastructure. Additionally, increases in nitrogen concentrations could potentially shift the ultrastructure more readily than the concentrations used in this study. Interestingly, the crystal-like structures are only present in the ammonia additions. Though the level of replication is low, this may indicate that ammonia could be related to the production of the crystals. More research needs to be done with a larger sample size and higher nitrogen concentrations to fully determine the impacts that nitrogen may have on these cells.

5.4. Symbiodiniaceae Nitrogen and Temperature

The cell count did not decrease with increasing temperature nor did the photosynthetic activity. Though it is expected that increased temperatures cause a decline in the health of the algal cells, this was not seen within these *Breviolum* cultures. Additionally, the ammonia addition did not negatively affect the algal cultures either at the constant or the increased temperature. These results suggest that these algal cultures may be more robust that previously expected and may not be representative of temperature stress when in symbiosis in the coral. It is also possible that a prolonged duration of temperature stress may cause a decline in the algal health rather than a consistent increase and it may take longer to see shifts in algal health. Future research should maintain a constant higher temperature with continuous influxes of higher concentrations of nitrogen to test this hypothesis.

The ultrastructure did not differ within experimental designs (i.e., 27°C or increasing temperature) between the controls and the 50 µmol additions. Interestingly, there was variation in the number of lipid droplets and starch granules between the temperatures within the same treatment group. A consistent increase in temperature may result in a storage of more lipid droplets regardless of ammonia addition while and increase in starch granules may only increase in the controls. The ammonia may be shifting the physiology of the cultures exposed to higher temperatures.

5.5. Management Recommendations

Nitrogen limitation may be correlated to stony coral tissue loss disease. The pathogen(s) may be causing a limitation or there is an initial limitation that then allows for colonization and physiological shifts in the coral to cause tissue loss. Further work is needed to understand the correlation of nitrogen to SCTLD, but it is possible that the presence of the crystal-like structures could be an identifiable feature used for SCTLD pathology. Nitrogen concentrations could be used to quickly identify SCTLD from other tissue loss diseases in the field.

It is likely that *Orbicella* acute tissue loss disease is a different disease from SCTLD. Due to this, OATLD may need different intervention strategies than SCTLD especially since initial research indicates that the amoxicillin treatment is not as effective against OATLD lesions. Further work is needed to determine transmissibility and rate of tissue loss in aquaria. Additionally, monitoring is needed separate from SCTLD monitoring to accurately observe these lesions.

The Symbiodiniaceae in cultures may not be an accurate representation for nitrogen cycling or temperature stress for Symbiodiniaceae in coral symbiosis. Though crystal-like structures were present in few ammonia addition samples, it is likely that these crystals form during nitrogen limitation. To understand the crystal formation and how they correlate to physiology, coral samples may be needed rather than algal cultures. Ultrastructure features indicate that SCTLD and OATLD create unique morphological features that could be tied to physiology and are different from nutrient or temperature stress. Though more challenging, TEM may be needed to distinguish features between different forms of stress.

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