Short-term continuous cultures of *Gammarus* sp., *Tigriopus californicus,* and *Nannochloropsis* sp.

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Abstract

The key components of my Honors College thesis addressed the following questions: can stacked rectangles of corrugated plastic sheeting make suitable habitat for a continuous culture of amphipods (*Gammarus sp.*), can a small scale semi-continuous culture of *Nannochloropsis sp*. be reliably maintained in the laboratory for a 3-month period, and can a commercial, non-viable microalgae (*Nannochloropsis sp.*) be used as an alternative to laboratory-grown microalgae to feed and maintain a continuous culture of copepods (*Tigriopus californicus*)? There has been ample research and practice of long-term commercial culturing of these organisms, however my research will address the short-term culturing of *Gammarus sp., Nannochloropsis sp.,* and *Tigriopus californicus* for use in a laboratory employing a more practical and simplified approach for use in the laboratory. Amphipods were reared in a standard 38 L aquarium, copepods were cultured in two 19 L plastic buckets, and the phytoplankton was grown in three inverted 2 L clear plastic bottles. These organisms are intended for short-term use by students in a prospective fall 2020 marine biology laboratory at Oakland University. The results of my investigation established protocols for the practical maintenance of these three different cultures over a stipulated 3-month trial period. Amphipods were successfully maintained, and the initial population actually increased by more than 73% by the end of the trial period. I was successful in maintaining a continuous culture of copepods. Although copepod populations underwent cyclic oscillations, by maintaining two separate cultures, there were always sufficient numbers of copepods available. I was also successful in developing techniques for sustainable cultures of microalgae in optimal densities over the same trial period.

Introduction

This research thesis was prepared to help my mentor, Professor Gary Miller, to determine if it was possible to culture certain marine organisms in a laboratory setting for a short term, three-month period. These marine organisms are intended for use in a proposed marine biology laboratory course to compliment the marine biology lecture class currently offered at Oakland University. Traditionally, marine biology laboratory courses are taught in institutions near oceans, but since Oakland University is not near an ocean, a marine biology laboratory here would need to have live aquarium specimens. In essence, my thesis tested the feasibility of maintaining specific cultures, amphipods, copepods, and microalgae, as they will be important organismal components in the laboratory course.

I first researched how these organisms were reared and maintained commercially. After learning about these techniques, I developed my own protocols which were simplified and more suitable for short term, or temporary cultures needed for only a single semester.

Current Research

Amphipods

Amphipods are mostly small, benthic, shrimp-like crustaceans. Additionally, they are omnivores and feed on detrital material. They are found from shoreline to deep water. Amphipods are an important food source for many marine organisms. They live in burrows in the sediment. Furthermore, amphipods are sometimes used in sediment toxicity bioassays. Amphipod reproduction was an important component of this experiment because you need reproduction in order to ensure stable populations. It takes one to three weeks for amphipod young to hatch (entomology.ifas.ufl.edu). After about another week, the juveniles leave the

brood pouch resembling the adults (entomology.ifas.ufl.edu). In less than a year, most amphipod young from various species become adults (Smith and Whitman 1992).

Baeza-Rojano et al. (2013) used three different kinds of plastic mesh with differing complexity levels as artificial substrates for the attaching and sheltering of amphipods. The three different kinds of plastic mesh included one folded into a ball, the second folded but more compact with smaller pores (diameter), and the final one was upright without folding (Baeza-Rojano et al. 2013). However, there were no significant differences found in the total amount of individuals on each mesh (Baeza-Rojano et al. 2013). Female and male adults were found to be more abundant in folded meshes that had large pore diameters (Baeza-Rojano et al. 2013).

The amphipod, *Amplesica abdita*, had been held in the laboratory setting for several generations with the feeding of algal food daily, however, culturing success has not been consistent (Redmond et al. 1994). Algal food included: *Chaetoceros calcitrans*, *Phaeodactylum tricornutum*, and *Pseudoisochrysis paradoxa* (Redmond et al. 1994). Amphipods became sexually mature after about 20 days at 25 °C, and young were produced after 34 to 36 days (Redmond et al. 1994). Short term tests quantified growth of *Amplesica abdita* in approximately 10 to 14 days (Redmond et al. 1994).

Over five years after the introduction of another amphipod species, *Caprella mutica*, into the Port of Nagoya Public Aquarium, Japan, this amphipod has been successfully raised through many generations per year for public show in a 500 L tank (Nakajima and Takeuchi 2008). Nakajima and Takeuchi (2008) prepared the tank using: a circulation system, a coral sand bed filter, and a heater/chiller unit. Reef Crystals (which are artificial seawater) were also used by Nakajima and Takeuchi (2008). Food for *Caprella mutica* included: hatched juveniles of *Artemia* and the diatom *Phaeodactylum* (Nakajima and Takeuchi 2008). The sex ratio of

females/(females + males) was 0.50 two years after the introduction (Nakajima and Takeuchi 2008). The mean number of embryos/female was 33 allowing the production of multiple generations in one year (Nakajima and Takeuchi 2008).

My research centered on whether was feasible to rear and maintain *Gammarus sp.* in a laboratory setting at Oakland University for a three-month period. This amphipod is available for purchase from certain biological supply facilities and is suitable for laboratory studies. I developed protocols that would be used to rear and maintain *Gammarus* in a small 38 L aquarium for future laboratory use at Oakland University utilizing an artificial habitat made from a stack of corrugated plastic sheeting. The corrugated plastic sheets provide ample hiding spaces for the amphipods. The plastic sheeting was translucent, so it was easy to visualize the amphipods for counting purposes.

Copepods

Copepods are small crustaceans found in both saltwater and freshwater. They can even be found in intertidal pools/zones. Copepods feed on microalgae including diatoms. They have various developmental stages including: nauplii, egg-carrying adult, and adult. They are an important food source for many marine and aquatic organisms. I measured various copepod life stages in this research. Copepods and other crustaceans all have similar lifecycles (biologydictionary.net). Like many organisms the lifecycle begins with an egg (biologydictionary.net). The egg hatches into a larva, called a nauplius, which has a head, tail, and an undefined abdominal region (biologydictionary.net). It takes multiple molts before the nauplius becomes an adult (biologydictionary.net).

Tigriopus californicus is the easiest saltwater copepod to culture (Greenley 2017). This copepod is available, adaptable, robust under a wide range of conditions, and willing to feed on non-viable algae (Greenley 2017). Greenley (2017) further mentions new, cheap, and easy algal feed products that can be frozen and then thawed when ready to use. Additionally, these phytoplankton mixtures have a lengthy shelf life in the refrigerator (Greenley 2017). These algal feed products come in a variety of different blends, however, *T. californicus* will accept most (Greenley 2017). According to Greenley (2017) enough food should be added so that you can just no longer see the bottom of the bucket and more food should be added when the water starts to clear (white buckets are beneficial for this because one can tell when to feed again based on the visibility of the bottom of the bucket). *Tigriopus californicus* is found in splash-zone tidal pools from southern Alaska to the coast of Baja California, Mexico (Greenley 2017). It ranges in size from 1,000 to 1,500 microns in length and is able to be successfully cultured at temperatures between 20°C and 27°C (Greenley 2017). Furthermore, temperature variations are tolerated by *T. californicus*, as long as they are not extreme (Greenley 2017).

I needed a way to quantify copepod population size in my cultures. I researched various methods used to culture copepods. One method (AlgaeBarn 2015), suggests that in order to estimate the number of copepods in a container, one must count the number of copepods from a small sample and then proportionally one would be able to calculate an estimate of the entire population in the container. An effective way to count copepods is to do so when they are immobilized (AlgaeBarn 2015). I was only interested in maintaining reasonable stable copepod densities. I did not need to know an absolute number of copepods in my culture.

The method I found the most suitable for culturing *T. californicus* in our laboratory was that used by Gordon Greenley. Greenley (2017) asserted that four items were necessary for his

method: saltwater, a culture vessel, an algal food source, and a starter culture of *T. californicus*. The culture vessel did not necessarily need to be of a certain type; it only needed to be able to hold water and non-toxic (Greenley 2017). For larger cultures, five-gallon buckets were commonly used (Greenley 2017). Greenley (2017) uses white five-gallon buckets because they were easy to obtain, easy to clean, inexpensive, and offered a contrast for seeing the copepod population. *Tigriopus californicus* will tolerate a range of salinities (32-40 ppt), but it is best to start at the lower end of this salinity range so that fresh water does not have to be added as frequently owing to evaporation (Greenley 2017).

A culture of *T. californicus* can be started by attaining a starter culture and culture vessel (Greenley 2017). The starter culture is allowed to acclimate to the temperature before its addition to the culture vessel (Greenley 2017). Finally, Greenley (2017) states that a sustainable food source will be required to maintain healthy, breeding copepods. Approximately every two weeks, the copepod culture is split and the about half the water replaced with fresh saltwater (Greenley 2017). Greenley (2017) reports that this allows the culture to continue increasing without stalling or reaching carrying capacity. To capture all copepods in their various life stages (nauplii to adult), a sieve with a mesh size around 70 microns was used (Greenley 2017). Artificial light induces *T. californicus* to become red-orange in color making them more visible (Greenley 2017). This is advantageous if copepods are going to be used as live feed for other animals as it increases their visibility. To help keep food suspended in the water column and aerate the culture vessel, a rigid, open-ended airline was used (Greenley 2017).

I needed a way to measure copepod density in order to monitor and compare my cultures. I consulted with Gordon Greenley (Husbandry Assistant at Sea Life Aquarium) about measuring copepod density upon which to base my methods. Greenley provided me with some important

tips such as taking samples from the center of the culture to avoid confounds, using known volumes of water, and the use of counting chambers.

I used a modified methodology for culturing, counting density, and monitoring copepods for my purposes. I cultured *Tigriopus californicus* and successfully established a simplified protocol for a continuous culture suitable for future marine biology laboratory use. Although I was able to maintain copepod cultures by feeding with commercial, non-viable microalgae, live phytoplankton appeared to increase copepod density.

Microalgae

Microalgae or phytoplankton are single-celled, photosynthetic organisms present in freshwater and saltwater systems. They form the base of the food web in both freshwater and saltwater environments. Phytoplankton are the basic synthesizers of organic material in marine environments. Additionally, microalgae play an important role in nutrient cycling in these aquatic habitats (Hoff and Snell 1987). Like every other organism the life cycle of microalgae is made up of growth, reproduction which is important to ensure stable populations, and death (sciencing.com). However, specific to phytoplankton is a period of dormancy which can occur regularly or only when growth conditions are poor (sciencing.com).

There are several thousand species of known microalgae, but I primary worked with one species (*Nannochloropsis sp.*) because it is commonly used in laboratory settings. When reared in the laboratory, phytoplankton is often referred to simply as green water. Growth in wild phytoplankton populations are normally nutrient limited. However, dense, eutrophic populations can be cultured in the laboratory by supplementing with nutrients in the form of a specially formulated growth medium.

In culture tanks, phytoplankton aid in sustaining water quality by regulating pH and removing excess nutrients (Hoff and Snell 1987). Ideal growth temperature depends on many things including: the algal species, nutrient concentration, acclimation temperature, and light intensity (Fogg 1966). Small fluctuations throughout the day within the temperature range of the algae are not problematic, however, lower temperatures stunt the growth of algae (Hoff and Snell 1987). Temperatures above 94°F or 35°C kill several phytoplankton (Hoff and Snell 1987). Ideal light intensity for phytoplankton ranges from 2500-5000 lux (lumens) or 250-500 foot-candles (Hoff and Snell 1987).

The procedure for culturing microalgae described by Hoff and Snell (1987) uses a starter culture. A clear cylinder was utilized for rearing microalgae (Hoff and Snell 1987). On a regular basis, cultures were diluted to avoid exceeding the carrying capacity (Hoff and Snell 1987). Cultures were occasionally restarted from a new culture (Hoff and Snell 1987). Hoff and Snell (1987) used increasingly larger sizes of culture cylinders for their microalgae. I did not follow the exact procedure of Hoff and Snell (1987) since their procedure is for maintaining long-term, large-scale pure cultures. I wanted to simplify the procedure to accomplish a short-term, small-scale, less rigorous methodology.

I viewed a website (Saltwateraquariumblog.com) that has a video showing how to raise microalgae in a home-setting. I used a few ideas from this video including using a reduced salinity of 30 ppt for the microalgae. Seawater cultures require about 25 cc of seasalts per liter, which produces a salinity of about 25 ppt (Hoff and Snell 1987).

This part of my research was to explore the practicability of culturing *Nannochloropsis sp.* in a laboratory setting at Oakland University for a three-month period. *Nannochloropsis sp.* is a readily available microalgae and is reportedly relatively easy to culture

(Saltwateraquariumblog.com). I established protocols that can be implemented for future laboratory use at the university. For example, green water cultures are prone to "crashing" when cultures are allowed to exceed their carrying capacity or get contaminated by zooplankton. To guard against these possibilities, I used a redundant system of three separate cultures so that if one culture failed the two remaining cultures could be used to re-start the failed culture.

In carrying out this research, I found practical methods and protocols for maintaining these cultures (amphipods, copepods, and microalgae) for a minimum of one semester. Ultimately, I used more of a holistic/qualitative approach to achieve success. The methodology described here will provide a guide for establishing and maintaining cultures of amphipods, copepods, and microalgae for educational use in the laboratory.

Methods and Materials

Amphipods

Gammarus sp. were reared in a 38-liter aquarium. I used normal seawater at 35 ppt. A submersible heater was used to keep temperature at a constant 25°C and I monitored water salinity by measuring specific gravity (1.024) at that temperature. The aquarium was partially filled to 30 liters. No filtration was used but an air-stone was placed in the tank to provide water movement. Habitat (pod hotel) was created for amphipods using corrugated plastic sheets cut to size and stacked in a basket made of plastic egg crate light diffuser material (approximately 15 by 10 by 6 cm). A piece of glass was placed on top of the pod hotel to weigh it down.

Amphipods were ordered from a supplier, acclimated, and then introduced to the aquarium. Amphipods were fed standard marine flake fish food that I ground up using a mortar and pestle. Approximately 0.2 g of ground flake fish food was added to the tank once each week.

A LED strip light was placed over the top of the tank with a timer that was set to 12 hours on and 12 hours off to simulate the natural day/night cycle. A glass cover on the aquarium was used to minimize the evaporation rate. RODI water was regularly added to compensate for any evaporation that occurred.

To maintain water quality, partial water changes (approximately 10 L every 3 weeks) were performed. Detrital materials at the bottom of the tank were removed using a siphon house during the water change procedure. I regularly tested for ammonia in order to monitor water quality in the amphipod tank. There was a slight increase in ammonia the first week, but none was detected thereafter.

Amphipod counts were performed at their introduction and again at the conclusion of the experiment. The pod hotel was removed from the tank by first placing it in a small plastic container inside the aquarium and then removing both from the tank that way amphipods were prevented from escaping. Each layer of the pod hotel was carefully inspected, and amphipods counted, which could be seen through the translucent corrugated plastic sheeting. I only counted amphipods that were residing in the pod hotel even though there were a significant number observed remaining in the culture tank.

Copepods

Tigriopus californicus were reared in two 19-liter buckets. Continuous lighting was supplied with LED lights that were fixed to the top of copepod cultures. I used normal seawater at 35 ppt. No heater was used (ambient room temperature ~ 23°C) and I monitored water salinity by measuring specific gravity (1.024) at that temperature. Culture buckets were filled to 15 liters. Very light aeration was provided by rigid airline tubing (without an air stone) that injected air

bubbles into the bottom of the buckets at a rate of 1 bubble/second. Copepods were ordered from a commercial supplier, acclimated, and then added to the buckets. Initially, the copepods were fed a commercial, non-viable microalgae. At week nine, this food was replaced by live microalgae (see *microalgae*). RODI water was regularly added to compensate for evaporation that occurred.

A secchi disk method was used to measure the density of microalgae (green water) visually. I differentially fed the copepod cultures such that copepod culture 2 (CC2) maintained a higher density of green water than copepod culture 1 (CC1) to see if copepod density would be affected. I was consistent in adding more food when the green water density dropped below established levels as determined by the secchi disk, which was based on previous experience of my mentor. The secchi disk was constructed by using a rod with a small black and white rectangle attached at a 90° angle to the bottom. The disk was lowered into the water and the depth at which it was no longer visible was used as a relative measure of green water density.

When detritus accumulated on the bottom of the cultures, I siphoned all the water down to just above the detritus and saved the siphoned water containing most of the copepods in a clean bucket. The detritus was thrown out and the culture bucket was cleaned. Then, the siphoned water with copepods was returned to the original culture bucket.

To mitigate population crashes, I siphoned approximately half of the water volume for copepod cultures through a 45 µl sieve when population density was nearing estimated carrying capacity. The water was returned to the culture bucket and the harvested copepods from the sieve were fed to live corals in a display aquarium that was not part of this research. While maximum carrying capacity was never formally established, I simply used my mentor’s expertise to judge when to perform this to avoid population crashes.

To standardize copepod counts, I removed exactly 235 ml of water from the center of the bucket each time by dipping a cylinder of that volume that had a 45 µm sieve glued to the bottom. I allowed the water to drain and the sieve was laid on a black-lined counting grid. Initially, I used a dissecting scope to determine differences in copepod life stages: egg-bearing females, adults [male/female], and nauplii. After this initial confirmation, I was able to visualize readily differences in the various copepod life stages by their size, color, and general appearance without the aid of a dissecting scope. I did not calculate the total number of individuals in each culture. The number in the 235 ml sample was used as a relative measure of the population density in each culture, which was sufficient for monitoring purposes.

Microalgae

Nannochloropsis sp. (green water) were reared in three two-liter clear plastic bottle. The bottoms were cut off and then the bottles inverted to create a roughly funnel-shaped culture vessel. The bottlenecks were stoppered and had two small holes: one for injection of air and the other for siphoning off dead cells. I used a slightly lower salinity for culturing the green water, approximately 30 ppt. No heater was used (ambient room temperature ~23°C) and I monitored water salinity by measuring specific gravity (1.019) at that temperature. Very light aeration was provided by rigid airline tubing (without an air stone) that injected air bubbles into the bottom of the two-liter bottles at a rate of 1 bubble/second.

Microalgae was obtained from a commercial supplier and added to the culture vessels. Micro Algae Grow™, an altered Guillard's medium from Florida Aqua Farms, was added at a volume of ½ to 1 ml per liter (Hoff and Snell 1987). I used 2 ml of commercial preparation of Guillard's medium (nutrients) to feed the microalgae each week. Additionally, continuous

lighting provided by LED grow lights was placed behind the culture vessels containing the algae that way all the algal cells would receive light not just the ones at the top of the culture vessel.

As the microalgae cells increase in number, the culture had to be diluted, or “split”, by removing half the green water and replacing it with fresh seawater. More nutrients were also added. Splits were required to prevent the microalgae culture from exceeding its carrying capacity. A dark green color was used as an indication that the culture was approaching capacity. At the beginning, the microalgae, was split once a week, but eventually the green water began to reproduce more rapidly, which necessitated splitting twice per week. The excess microalgae was fed to the copepod cultures.

Results

Amphipods

Amphipods were successfully maintained for the entire 3-month period. The number of amphipods received in shipment was 15 and by the end of the semester I counted 26 amphipods in the hotel (there were more actually outside the pod hotel). The initial population increased by more than 73% by the end of the 3-month period.

Copepods

I was able to maintain continuous cultures however the number of copepods varied over time. No discernable pattern was identified between egg-bearing females and nauplii and changes in total population. There appeared to be an increase in population size after I started using live phytoplankton as food from the splitting of my phytoplankton cultures. A significant difference (Chi-Square, $p<<0.001$) was observed in the number of copepods. CC1 had an

average total population density of 0.4 copepods/ml. CC2 had an average total population density of 0.66 copepods/ml.

Microalgae

At the beginning for the microalgae, a bad shipment was received the first time due to a weather delay and so it took a while to get a viable culture going, but after three months there were three stable cultures.

Table 1. Population structure of the two copepod cultures each week. Asterisks denote where no data was collected.

Week	Total CC1	% Adults CC1	% Gravid CC1	% Naup CC1	Total CC2	% Adults CC2	% Gravid CC2	% Naup CC2
1	100	*	*	*	52	*	*	*
3	18	100	*	0	165	13	*	87
4	20	100	*	0	190	11	*	89
6	60	7	*	93	100	80	*	20
7	46	85	*	15	26	69	27	4
8	30	77	13	10	290	18	5	77
9	5	20	40	40	235	18	6	76
10	111	47	4	50	222	73	9	18
11	214	26	7	67	246	90	10	0
12	278	34	8	50	25	80	20	0

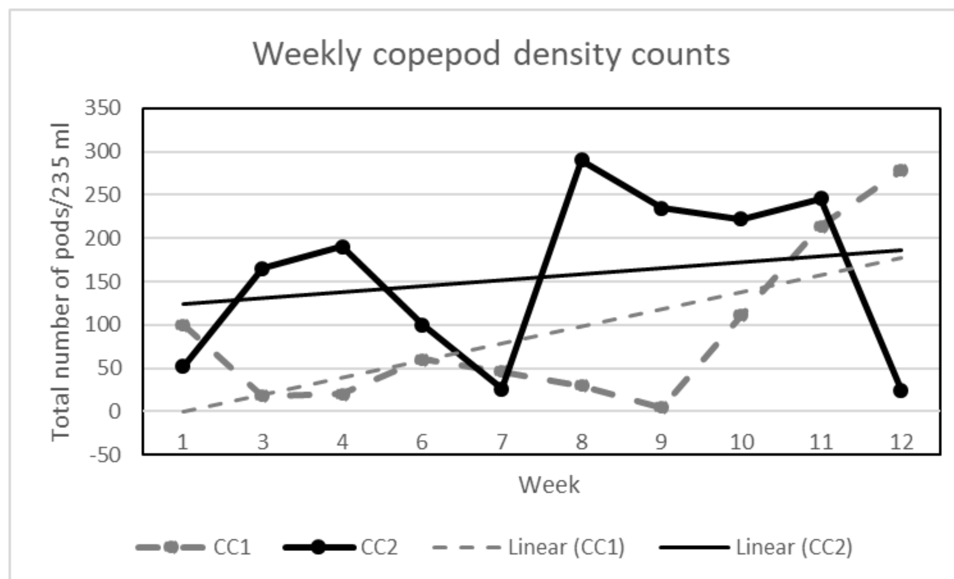

Figure 1. Relative density of copepod cultures for each week.

Discussion

Amphipods

I was successful in my research goal of maintaining a stable culture of amphipods. I noticed throughout this project that there was a far greater number of amphipods outside the hotel that were not measured, however, my research goal of growing and maintaining them was a success. It was especially evident during the breakdown of the amphipod tank that there were many more amphipods outside the pod hotel. The pod hotel served two purposes: one purpose was to provide suitable habitat and the second purpose was to quantify the increase in population size. More amphipods outside represented a more successful culture attempt than anticipated.

Copepods

Although there was fluctuation in the total number of copepods, I was successful in my research goal of maintaining adequate population densities of copepods. By feeding copepod

cultures higher densities of live algae, it appeared that higher densities of copepods could be maintained. In my study there were no discernible trends correlating the total number of copepods and copepod developmental stages (nauplii, egg-carrying adult, and adult). There was a difference in the average total population density of copepods in each culture in this experiment.

Microalgae

I was successful in my research goal of maintaining stable cultures of microalgae. Hoff and Snell (1987) suggest the top of the culture container being closed with cotton, a plastic cap, or aluminum foil including a hole for incorporation of the air tube, whereas I simplified the procedure by covering my cultures with plastic wrap and my air tube was injected from the bottom so it created a positive pressure that kept air from coming in. Additionally, one advantage I had was that I could siphon off dead cells that accumulated at the bottom of the inverted culture vessels more easily.

By having 3 culture of microalgae going at the same time, I had redundancy in case one were to crash. I had some difficulties with weather delays and getting non-viable cultures (due to microorganisms being present in the culture from the supplier), so I had to restart a few times, however once the cultures reached maximum growth rates, I was at first splitting them once a week and by the end of the experiment I was splitting them twice per week. There are more rigorous methods, however my goal of this study was to simplify them for more short-term, practical use.

Conclusions

Amphipods

A potential area of exploration regarding amphipod numbers is if they would increase with more pod hotels. It did not appear to be a problem; however, the amphipod tank became contaminated with green water. By putting on a hang-on outside filter, one could prevent this from happening and make the tank more stable.

Copepods

Since my data suggests that a live plankton culture supports higher densities of copepods, in the future I would recommend having a live viable microalgae culture before a copepod culture. The copepods were in 20-liter buckets in this research, however, a conical shape container could allow the siphoning of algae with less of a loss of copepods. I have anecdotal information stating that there is a correlation between the proportion of nauplii to adults so in the future I may be able to stabilize my cultures by manipulating the proportion of adults to nauplii through a selective sieve method. I would recommend feeding a greater density of green water due to the higher total population density achieved for CC2, however there was a crash at the end of the experiment.

Microalgae

Hoff and Snell (1987) used a far more rigorous cleaning method for ensuring avoiding contamination. I was not looking for such rigorous cleaning methods as this was a preliminary study, however I did experience contamination so in the future a more rigorous cleaning method similar to Hoff and Snell (1987) could be beneficial. Microalgae cultures could be made more

stable by using larger cultures (changes do not occur as quickly in larger water volumes). For example, I could change from liter bottles to 20-liter bottles in the future.

The procedures and protocols developed in this experiment indicate that it is possible to maintain sustainable cultures of amphipods, copepods, and microalgae required for a 3-month semester period. This is a viable method for future use. Furthermore, ways to improve this methodology I have suggested after exploring possible future improvements.

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