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Tunicate Invasiveness under Varying Conditions of Salinity and Temperature

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ABSTRACT

Sea squirts could be colonial or solitary and could easily adapt to many regions around the world because of their short lifespan, abilities to quickly gain energy supply, and relatively small biomass. An investigation was carried out to determine the degree of invasiveness of the tunicate through experimenting the viability of sea squirts under different salinities and temperatures designed to model the different environments of the globe where the sea squirt would potentially invade. Due to the inherently adaptive abilities of the sea squirt in their simple requirement of food, energy, and environment, sea squirts, whether colonial or solitary, have high chances of survival in anywhere with the minimal amount of energy and plankton food source [1]. The results of the experiment showed the high adaptability of the sea squirt in its thriving life functions under almost all the conditions given in the experiment, and a comparative competitive advantage over other filtering marine organisms.

Introduction

Invasive species had long been imposing threats and danger to native environments along with the species living within it. Cases including the Apple snail *Pomacea canaliculata* (in Chinese Fushou snail) found quite recently amongst the crops in the Eastern China fields [2], Burmese python released into the Everglades [3], and other species that were either purposefully or accidentally introduced into a new region, were all demonstrative of the destructiveness of the invasive species.

Tunicates belong to the phylum Chordata, subphylum Tunicata, and class Ascidiacea. In this experiment, the subject species belongs to the genus *Styela plicata*. [4] During a sea quirt's life cycle, they go through the process of metamorphosis. Specifically, they develop from the form of free-swimming larvae which resembled the shape of a tadpole to a sessile individual or colony that attached itself to the surface by an anterior sucker. This also inferred that they would be able to be found practically anywhere on all types of surfaces. They could practically reside on all forms of surface and therefore were allowed to live in all kinds of places, including the ropes on the fish farms or the sides of a fishing ship [4].

A major species of sea squirt that has been studied in the past that was known to be quite invasive belonged to the Genus *Didenum*. According to WHOI.edu, the tunicate *Didenum* "first appeared in New England waters," and was also "living in other temperate areas around the world" [5]. Its invasiveness was not unique to the Southeast China coast, but has been posing "a global threat," according to Mary Carmon, to the marine organisms that lived in environments which the tunicate may live in [5]. The tunicates have lived in habitats of sea weeds and most coral reefs in which required very specific water conditions that would be interrupted by the invasion of the sea squirt. Additionally, "the original distribution of *Didemnum* and other tunicate species is in dispute," for the previously studied origin of the sea squirt had been found to be an invaded area thousands of years ago [6]. Therefore, it became hard for scientists to determine the exact origin of the species, thus it was hard to determine the conditions of its invasiveness.

Their simple requirement of food also helped with their easily adaptable characteristic. According to Britannica, sea squirts have "two large pores" called siphons, with the inhalant one used "to guide water into the body cavity" and the exhaling one "serving as an exit" [7]. The process allowed the sea squirt to take in plankton from the water, and

through filtering the water out in the body cavity, they capture the plankton as their food supply and consume them as energy intake. This induced their heavy dependence on the quality of the water that they lived in, including the conditions of salinity, temperature, lighting, and abundance of plankton. However, according to the study by Lambert in 2007 [8], ascidians under the new aquaculture facilities of the recent years were able to spread rapidly with more access to attached surfaces such as ropes, nets, and etc. Lambert also indicated that sea squirts had high reproductive and growth rates, and could tolerate multiple environmental conditions, from the greatest depth to the lightest surfaces. This created the high invasiveness of the sea squirt, and continued to pose threats to areas and organisms in which they would invade. For example, they would pose enormous threats to aquaculture industries like shellfish farms. For instance, the invasive sea squirts were harming Connecticut's 30 million shellfish industry because their faster rate of prey consumption exceeded the shellfish's rate predation rate [9], which means that they were overexploiting plankton food supplies and outcompeted the shellfish populations around that region. While this has only been one case scenario, such similarities were experienced all over the world by fish farms, including the sampling site of this study. To further investigate the sea squirt's invasive capabilities, the plankton filtration rate of *Steyla* sp. under different salinity and temperature were investigated, in order to determine the regions of the world at which it would be able to invade and thereby the invasiveness of *Steyla plicata*.

Materials and Methods

Sampling of Sea Squirt

Sea squirt specimens of the species *Styela plicata*. were collected in a fish farm raft in the Dapeng Peninsula (22.564863, 114.535721). A water tank large enough to contain the samples of sea squirt was prepared. A water pump that does not require an electric wire and can consistently pump oxygen in the water containing the sample was also prepared, and its controller was attached to the side of outside of the tank. A hole was put through the cover of the box to allow the wire of the pump to enter through. A pair of gloves were also put in the tank for taking samples from rough surfaces. Upon arriving at the sampling site, Dapeng, the tank was filled with sea water until approximately 3/4 of the tank was filled. The water pump was then started to pump oxygen into the water. Then, around 10-20 sea squirt samples were collected from the ropes of the fish raft carefully without damaging their attached parts of their body. They were then put in the water tank with a lid on top of it to prevent water from spilling out, and the tank was taken back to the lab.

Experimentation on Tolerance to Salinity Changes

Three beakers of equal size were prepared, and 1000 mL of distilled water were poured in each beaker. After the beakers were set aside, a measurement paper was folded as a small cup and placed on the balance. 20 g, 30 g and 40 g of sea salt was poured into 1 L seawater in the prepared beakers to prepare 20 ppt, 30 ppt and 40 ppt seawater. A sea squirt was put in each of the beakers while the beakers were aligned with each other. A camera was placed next to the beakers on an upside-down box so that the siphons of the sea squirt would be taken into view. Once everything was settled, video recording was started and continued for around half an hour and the movements of the sea squirts, or precisely the squirting event, were counted. Figure 1 represented the way the first video was recorded and the orientation of each of the sea squirts in different salinity. They were first filmed on the side to detect their movements without looking at the siphon. Then they were filmed from the top as shown in Figure 2.

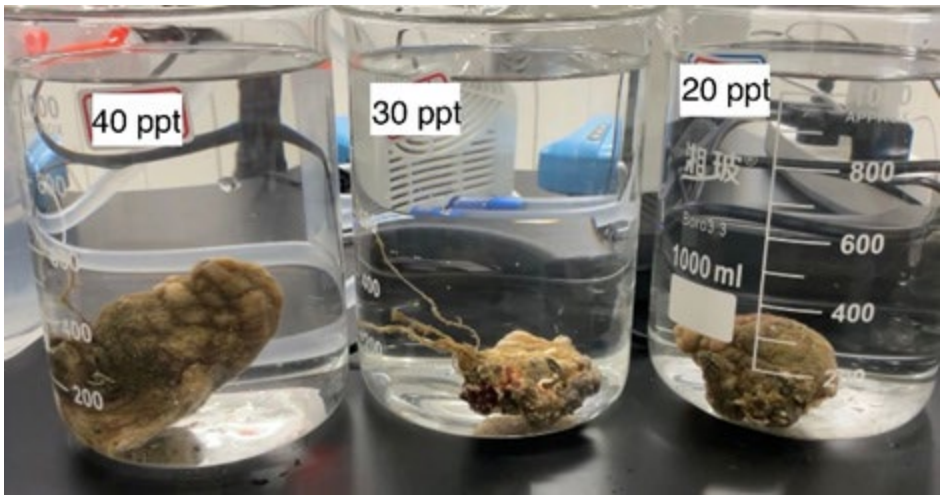

Figure 1. Arrangement of sea squirts in solutions of different salinity. The first video was filmed from the side of the beaker to observe its body movement.

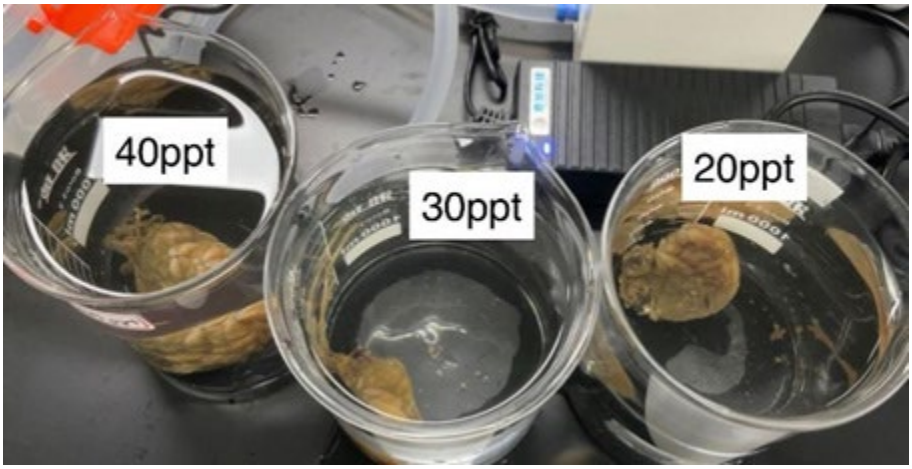

Figure 2. Arrangement of sea squirts in solutions of different salinity. The second video was filmed from the top of the beaker to observe the siphon.

Experimentation on Tolerance to Temperature Changes

A box that could contain at least two beakers within it was prepared, and about a half of the box was filled with distilled water to make a water bath. Two beakers that were filled with sea water with a salinity of 35 ppt were placed in the water bath. Their water levels were altered accordingly to prevent spilling water inside the box and outside of the beaker. The AL-160 Water Temperature Controller was then placed right next to the box in preparation for alteration of the temperature. The thermometer was responsible for recording the temperature that would be shown on the screen of the controller, and when the screen showed 20˚C, a sea squirt was placed in each of the beaker. The camera setting and during of video recording were the same as described above. Video recording of sea squirt behavior in 30 ºC, 35 ºC and 40 ºC water bath were also conducted as in 20 ºC.

Sampling of Oysters and Mussels

The sea squirts, oysters, and mussels were all once again collected in a fish farm raft in the Dapeng Peninsula (22.564863, 114.535721). This time, approximately 10 *S. plicata* sea squirt were collected from the ropes of the fish raft carefully without damaging their attached parts of their body. Another 10 green mussels (*Perna virdis*) were also collected from the wheels that stabilize the fish raft. The 10 oysters (*Crassostrea* sp.) were collected from under the fish raft. They were then put in the water tank with a lid on top of it to prevent water from spilling out, and the tank was taken back to the lab.

Dilution of Algae Samples

20 tubes of frozen green algae (*Nannochloropsis* sp.) sample were thaw in 4˚C. One 40 µL µL drop of the algae sample was placed on the microscope slide and put under the microscope. The exact number of algae cells in the 40 µL drop was counted on the magnified microscope screen with magnification of 40x. Then, two tubes of algae samples of approximately 2 mL together that would be used as the dependent variable was injected by the adjustable pipette into a 500 mL beaker filled with 500 mL of sea water with a salinity of 35 ppt from the same source as the sea water in the beaker. Then 40 µL of the diluted solution in the 500 mL beaker was placed on a microscope slide and observed under the microscope. The approximate number of algae cells was calculated according to the microscopic screen under 100x magnification. The sample of 500 mL would later be used to pour into the 1000 mL beakers for further dilution to allow clearer calculation of algae samples consumed by the target organisms.

Experimentation on Algae Consumption in Different Temperatures

Three beakers were prepared for each of the three species (sea squirt, oyster, mussel). Each of them was labeled with the designated species that would be put into the beaker. The three beakers were filled with 900 mL of 35 ppt sea water and were placed into the water bath system as mentioned above. The temperature was set to 20˚C. When the temperature of the water bath reached 20˚C, 100 mL of the diluted algae sample was poured into each of the beakers so that the total volume in the beaker was 1 LL. The solution was slightly stirred to ensure proper distribution of the algae in the diluted sea water. 40 µl of solution from each beaker were taken by the adjustable volume pipette and transferred onto three microscope slides. The pipette tips were changed after every transfer to prevent samples from mixing. The slides were placed under the microscope and the concentration of algae in each beaker was observed and calculated. Three such samples were evaluated under the microscope, and the average of the three data was used as the concentration recorded in the data. The algal concentration in each beaker was recorded, and this number serves as the initial algal concentration. Then the subject organism samples were placed in the beakers according to the beaker's label: sea squirts were placed in the beaker labeled "SS" (sea squirt), and oysters were placed in the beaker labeled "O," and the same was for the mussels placed in "M." The organisms were left in the beaker for 30 minutes when another 40 µl sample of the solutions in each beaker was taken and transferred on to the microscope slides before the coverslips were put on top of it. They were once again put under the microscope and observed and calculated for the concentration. The slides were disposed of after the concentrations were recorded. After another 30 minutes, the concentrations were calculated and recorded again. After the slides were disposed of, the species were taken out from the beaker and the solution in the beaker was disposed of as well. 0.9 L of sea water was then again poured into each of the three beakers, and another diluted solution of 500 mL was poured into the 900 mL sea water. The same procedure was repeated for different temperatures, mainly 20˚C, 25˚C, and 30˚C, and the initial and final concentrations in the beakers were recorded for each of the temperatures to measure the degree of change of these organisms under different conditions of temperature.

Experimentation on Algae Consumption in Different Salinity

Three 1 L beakers were prepared for three species that would be investigated. 514 mL of 35 ppt salt water was poured into each of the beakers, and 90% of the 1 L beaker was filled with 386 mL of distilled water to dilute the solution to 20 ppt. The solution was stirred carefully to allow proper mixing of the solutions. Then, after the three beakers had been filled entirely with the 90 mL 20 ppt solution, 100 mL of the diluted algae sample was poured into each of the beakers to allow approximately equal amounts of dilution with the salinity experiment. The solution was also stirred to ensure proper mixing. Then, the initial concentration of the beaker was recorded using similar methods as a 40 µL drop of the solution was placed on a microscope slide and the number of algae samples was calculated under the microscope and recorded. The microscope slide was disposed in the glassware waste container. Then, each sample of tunicate, mussel, and oyster were placed in the beakers labeled as the species indicated on each beaker, and the temperature of each beaker was also recorded. After 30 minutes, the algae concentration of each beaker was again recorded using the same method as recording the initial concentration. After another 30 minutes, the results were recorded the same way. The same procedure was repeated for different salinity, only for dilution of 25 ppt solution, 257 mL of distilled water was poured in 643 mL of 35 ppt salt water, and for dilution of 30 ppt, 129 mL of distilled water was poured in 771 mL of 35 ppt salt water.

Results

Salinity Effect on Ascidian Activities

It was seen from the first video that the sea squirt in 40 ppt presented the most frequent movement of inhaling and exhaling water, exhibiting a frequency of 21 squirts. The sea squirt in 30 ppt follows with a frequency of 13 squirts. The sea squirt in 20 ppt displayed the least amount of movement, with a frequency of 13 demonstrating it was alive. The data was summarized in Table 1. In the second video (Figure 2), which was recorded for 30 min, the sea squirts displayed similar trends of squirting frequency. As shown in Table 1, the frequency increased from 3 to 6, then to 7 as salinity simultaneously increased from 20 ppt to 30 ppt, then to 40 ppt. The line at which models the distribution of the frequency was shown in Figure 3. As depicted, the lines showed an inconsistent increase in the frequency of the movement of sea squirts, but the overall trend was the same for both the lines.

Table 1. Frequency vs. Salinity of the movement of the sea squirt in each of the differently oriented videos.

Salinity	Frequency (first video)	Frequency (Second Video)
20	13	3
30	14	6
40	21	7

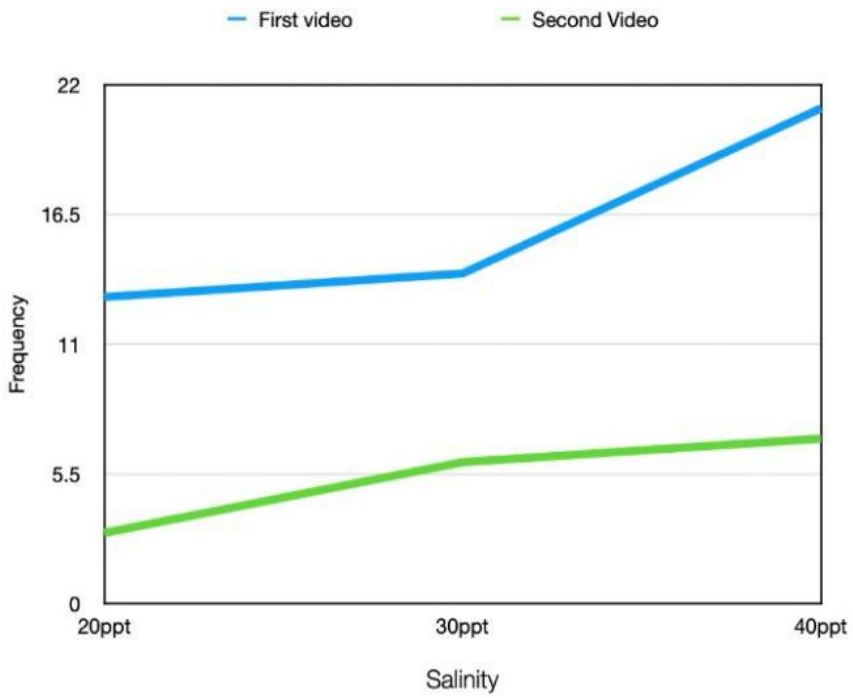

Figure 3. The frequency of squirting event in different salinity treatments.

Temperature Effect on Ascidian Activities

Sea squirts in 20 ˚C has normal frequency of squirting (Figure 4). Sea squirts in 30˚C has significantly higher frequency of squirting, with the frequency being 21 squirts. Sea squirts in 35 ˚C have similarly wide-opened siphon as sea squirts in 30˚C, and has higher frequency of squirting than the sea squirt in 30 ˚C. However, the sea squirts in 40 ˚C started with wide-opened siphon and squirted only 4 times before its siphon continued to decrease in size until the sea squirts eventually died off, or at least stopped inhaling and exhaling.

The results of this experiment also displayed similar outcomes with the salinity experiment, that the sea squirts are highly adaptable to different environments. Under temperatures ranging from 20 ˚C to 35 ˚C, the sea squirts are well-acquainted with the environment and did not show apparent signs of inhabit. However, a major change happened when the sea squirts were placed in 40 ˚C. Initially when the sea squirt was put in the beaker, its siphons were widely opened. Around 10-15 minutes later, the sea squirt became immobile, and all other organisms fouled on the sea squirt left its place. For example, the tubeworms that were within their residing places wiggled out and dropped themselves on the bottom surface of the beaker, then quickly became immobile. Therefore, it could be concluded that a temperature of 40 ˚C was hostile to most marine organisms instead of only specifically to sea squirts. Additionally, it could be seen from the graph in Figure 4 that the optimal temperature for the sea squirts' habitation was 35 ˚C, and the frequency dropped rapidly afterwards.

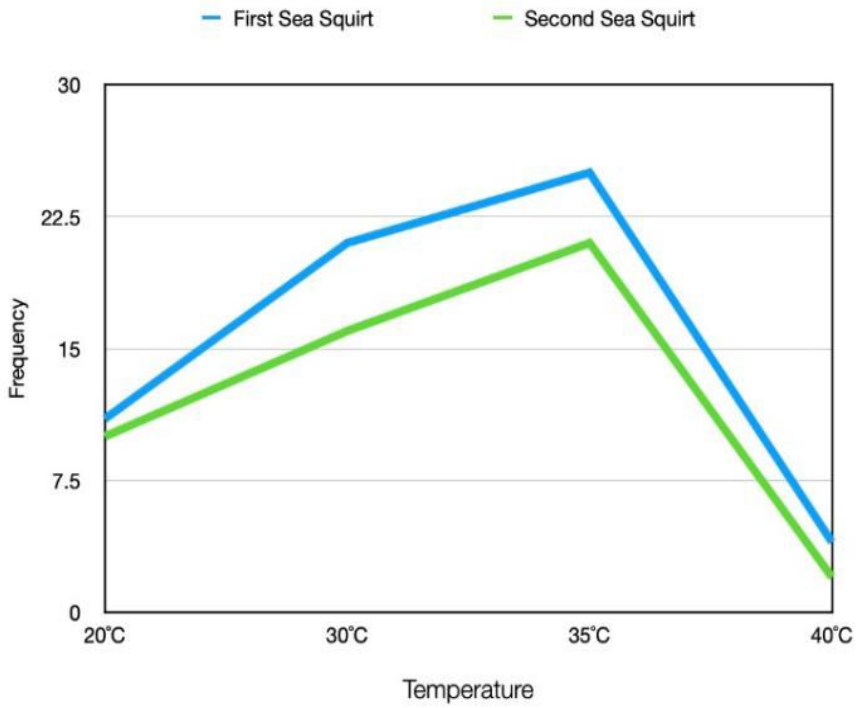

Figure 4. The frequency of squirting events in different temperature treatments.

Table 2. Frequency vs. Temperature. Frequency 1 and 2 represented each of the sea squirts in the beaker.

Temperature	Frequency 1	Frequency 2
20°C	11	10
30°C	21	16
35°C	25	21
40°C	4	2

Salinity Effect on Algal Consumption by S. plicata compared to other filtering Marine organisms

Figure 5 shows the rate of filtration for each examined organism under different salinities at different time points. It can be observed that the tunicate filtration rate in the first 30-minute interval of time for all salinities are higher than the filtration rate in the second 30 minute, but as salinity decreases the rate of the first 30 minute decreases as the rate during the 30-60 minute interval increased. However, this is different from the mussel filtration rate as shown in Figure 5B because there was a steady decline in the rate of the first 30 minutes, whereas the rate of the second 30 minutes was steadily increasing. Eventually the rate of filtration in the second 30 minutes for mussels surpassed the rate they had in the first 30 minutes and a steady increase was observed.

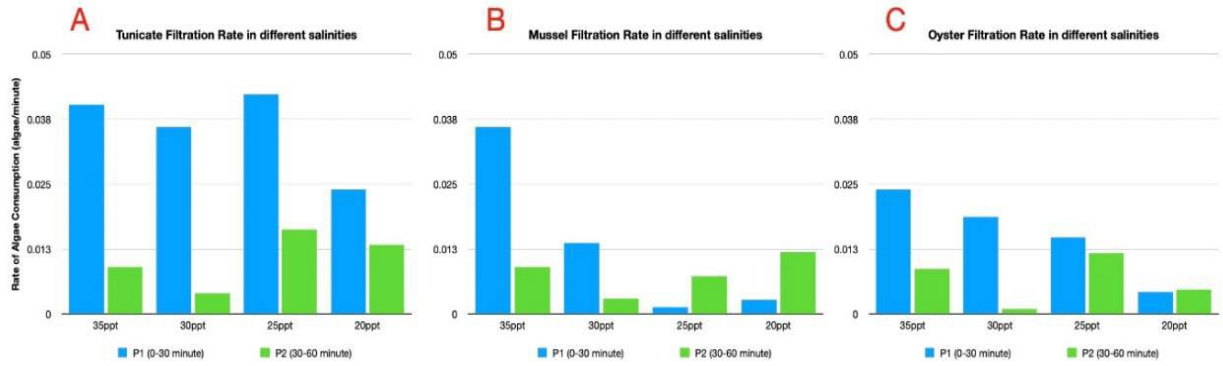

Figure 5. Rate of Algae Consumption in Different salinities of Different Organisms. Portion A represents Tunicate Filtration rate, portion B represents Mussel Filtration rate, and portion C represents Oyster Filtration Rate. P1 is the rate of consumption during the first 30-minute interval of each organism. P2 is the rate of consumption during the last 30-minute interval of each organism.

Oysters' filtration rate as shown in Figure 5C exhibit approximately the same trend with the mussels in the first 30 minute of the investigation, but the trend in the second 30 minute varies, but as opposed to the trend appearing in the first 30 minute, the filtration rate in the second 30 minute was lower than the rate of filtration in the first 30 minute. In 20 ppt solution, the rate of filtration in the second 30 minute surpassed that in the first 30 minute. This most likely shows the ability of sea squirts to consume plankton at a faster rate than the other marine organisms, because their initial rate of filtration and consumption of environmental organisms were higher in the first 30 minutes, while in the second 30 minute their filtration rate was also higher than the other organisms in all salinities. Their ability to adapt quickly as seen in the rising filtration rate of the second 30 minute allowed them to find all environments suitable as, according to Figure 5, the world temperature only fluctuates within salinity of 20-35, as recorded in the experiment. The high adaptability of sea squirts in all environments best demonstrated its invasiveness also because it was able to consume algae faster than any other filtering organisms that filter at a rate that would not exceed the limiting capacity of its own species.

Temperature Effect on Tunicate Consumption of Algae compared to other Filtering Marine Organisms

Figure 6 shows the rate of consumption of each species without looking at the specific rate of each species. The filtration rate of tunicates increases as temperature increases. In different temperatures, the rate of filtration in the second 30 min was higher than that in the first 30 minute (Figure 6A). Similar case applies to the filtration rate of mussels as indicated by Figure 6.B, but the numbers are around half of the rate of Tunicates and is thus slower than the Tunicate to filter the algae. However, oysters exhibit relatively different mechanisms in response to changing temperature. The filtration rate in the first 30 min surpassed the rate in the second 30 min when the temperature reached 25 ˚C, but as the temperature approaches 30 ˚C, the filtration rate of the first 30 minute was overly high until it dropped to extremely low points in the second 30 minute. Similar trends could also be shown in the behavior exhibited by the mussels and tunicates as they approached a temperature of 30 ˚C, for the three of the organisms all had relatively high filtration rates at the first 30 minute of 30 ˚C but dropped to depths of rates in the second 30 minute. This most likely meant that all three organisms found it harder to live in temperatures at around 30˚C or higher because though their initial rates might be high, their endurance in higher temperatures might be unsuitable and thus would not be able to live easily in higher climates. However, tunicates still exhibit an overwhelming advantage over Mussels and Oysters because despite the relative lowness of rates in the second 30 minutes, they still have the highest filtration rate. In figure 5, the world

temperature shows mostly the range from 22-38 ˚C, which was included in the experiment, and the majority of the world exhibits a temperature at around 30-35 ˚C. This most likely indicated the prevalence of sea squirts in these regions because they have a much higher rate of consumption of algae or preys around these regions as opposed to other marine organisms that were previously thriving in these regions without disrupting the natural food chain and energy trophic levels. This could also be attributed to their ability to maintain a high filtration rate through the course of adaptation and time consumption, enabling them to out-compete other organisms with their high filtration rate and better adaptive abilities.

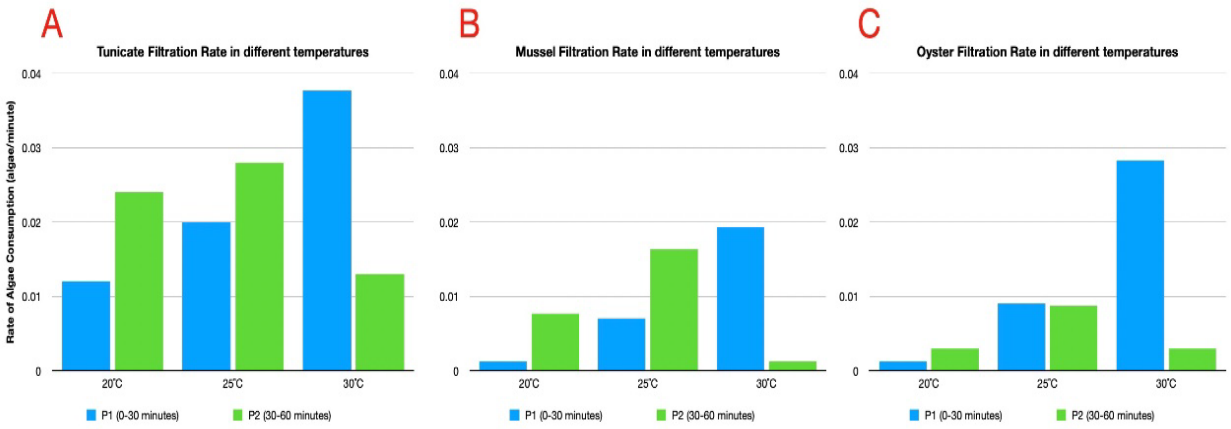

Figure 6. Rate of Algae Consumption in Different temperatures. Portion A represents Tunicate Filtration rate, portion B represents Mussel Filtration rate, and portion C represents Oyster Filtration Rate. [For P1 and P2's meaning, see the notes in Figure 5]

Discussion

There were two possible explanations for the outcome of the first experiment on the impact of salinity and temperature on *S. plicata* squirting frequency. One would be that the sea squirts altered their frequency under different salinity in order to better adapt themselves to the environment when they were first put in the beaker. The process of transferring them from the water tank containing all the samples to the experimental beaker would slightly affect the sea squirt's ability to move their siphon. Changing to the 40 ppt beaker was most drastic, which led them to move more squirting frequently to survive. The control group in the 30 ppt beaker was symbolic for the movement of the sea squirt normally in the ocean, which means that the frequency it displayed in the 30 ppt beaker was representative of the speed at which the sea squirts would be inhaling and exhaling in the ocean. In the 20 ppt solution, the sea squirt displayed less significant difference in squirting frequency with the 30 ppt solution. It could be inferred by thus observation that the squirting frequency increases with salinity, that it was directly proportional to salinity.

The other explanation for the outcome would be that sea squirts tend to inhale more often in environments with higher salinity because they needed more water intake to supply their plankton food reserve. Their squirting frequency increased not because they have excess energy to pump water in and out, but because under natural conditions sea squirts in lower salinity tend to require less energy for the intake of water because change in salinity may altered the community composition and led to a significant change of plankton diversity [10]. The need for pumping water in and out was significantly less than if they were put in waters with higher salinity. Therefore, even though there were no plankton in the beaker, their body detection of the salinity would enable them to alter their squirting frequency, thus allowing them to survive in various salinities.

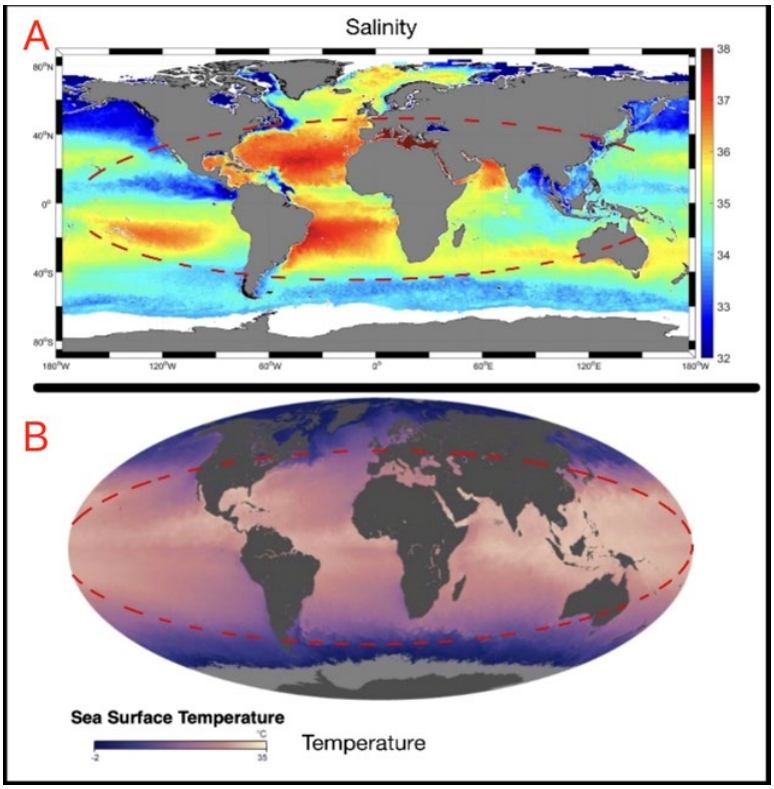

Figure 7. Comparative Image of the salinity and temperature graph. The red dotted lines show the area where they are quite similar in. A: ESA Global Map of Salinity [11]. B: NASA Global Map of Sea Surface Temperature [12].

Figure 7.A was an image taken from the ESA website of salinity that depicted an isoline map of the global trend of salinity ranging from 32 ppt to 38 ppt. Salinity in the Atlantic Ocean is relatively higher, ranging higher than 35 ppt to 40 ppt. For other parts of the world, the salinity was relatively lower, but no lower than 30 ppt. From the results of our experiment on the effect of salinity to S. plicata, it could be inferred that the *S. plicata* might be able to live in all parts of the world, whether it's the Pacific, Atlantic, or other bigger ocean environments and hence this species might have high invasiveness as a biofouling species. However, as shown by the data in Table 1 that as the salinity increased, the frequency of exhalation increases simultaneously. Therefore, in areas with higher salinity, the squirting frequency tended to be higher, meaning that if sea squirts were to settle in the Atlantic Ocean, they would require more energy for the frequent inhaling of water that they needed for plankton as food supplies, whereas in other places where the salinity wasn't as high, they wouldn't have to pump so often because there were abundant food sources.

Another examined factor affecting *S. plicata* invasiveness was temperature. In Figure 7.B [11], the global sea surface temperature was shown in the isoline map. The range that was depicted on this map was much broader than the range of the salinity map. It went from -2 ˚C to 35 ˚C. Because of laboratory limitations, the experiment was only set between 20-40 ˚C, which is about 1/4 of the temperature range in the map. However, the temperature in most of the regions labeled on the map, mostly the areas around the equator, are within the range of 20-40˚C. Based on our experiment results, the temperature around the equator would likely allow the presence of sea squirts because the optimal living temperature for the sea squirt was around 30 – 35 ˚C, which was fit temperature range around the equator. Therefore, it could be concluded that the sea squirt would be able to travel all along the equator and thrive in regions with relatively higher temperatures.

S. plicata has no known documentation as an invasive species so far. However, because the invasive *Didenum* species is a colonial species while *S. plicata* is solitary, meaning that it individually attached to surfaces. Therefore, the second experiment was designed to test the details of how the sea squirts would have been invasive. Specifically,

the experimental question how well *S. plicata* could acclimate to temperature and salinity changes with respect to the acclimation of oyster and mussel, which both have very broad geographic distributions and were known to have adapted easily to fluctuation in environmental factors.

To compete with the two prevalent organisms, *S. plicata* must possess several traits. Firstly, it must be able to compete with the two organisms for resources and food. In the second experiment, *S. plicata* demonstrated a dramatic difference in the rate of algae consumption compared to oyster and green mussel. *S. plicata* specimens were able to lead ahead in the initial filtration rate when introduced to a new environment as opposed to mussels and oyster's slow adaptation and could get accustomed to various salinities and temperatures faster than the other two. Knowing that the main source of energy and consumption of all three organisms was algae or phytoplankton, these marine organisms would be competing over their abilities to thrive with regards to their rate of food consumption. And under the knowledge that *S. plicata* has the highest rate of consumption, they could easily exceed the mussels and oysters in consuming food.

Secondly, they must have higher adaptability to various types of environments. Since *S. plicata* has constantly higher rates of consumption in the examined salinities and temperatures, and the filtration in the second 30 min exceeded that in the first 30 min, they are more adaptable to changes in salinity and temperature than the other two organisms, and thus would be able to propagate in new region faster than other organisms could.

There are limitations to these experiments. It might be rather superficial and insufficient to determine if *S. plicata* has high invasive potential simply based on the current data and temperature ranges around the world. In a more advanced experiment, the organisms would be allowed to adapt to the environment for a short amount of time, spanning from one day to a week. However, this experiment only tests on the acute and immediate response of the species to the experimental conditions, and thus would be susceptible to certain unavoidable flaws. Nevertheless, the results of the experiments presented in this study can serve as an indicator and represent a warning to the potential danger posed by *S. plicata*.

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