Laboratory Culture of *Didemnum* sp., an Invasive Colonial Tunicate

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Acknowledgements

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Abstract:

Didemnum sp. is an invasive colonial tunicate that is capable of out-competing native benthic species, resulting in loss of biodiversity, wreaking havoc on food webs, and having negative impacts on aquaculture (especially of other filter suspension feeders such as mussels). The purpose of this experiment was to culture *Didemnum* sp. in a controlled laboratory environment in order to determine the optimal conditions for growth and reproduction. Samples were collected from several locations on the coast of New Hampshire and Maine, and cultured at various temperatures and with various feeding regimes. Other factors, such as salinity, were controlled for the duration of the experiment. The samples died during every experimental treatment, preventing any growth data from being obtained. However, one set of samples were maintained for a short period (for the month of December), without undergoing any growth or reproduction before their death. As more is learned about *Didemnum* sp. and more becomes published, it becomes possible to begin determining what may have caused the samples to die. Possible causes of death may include desiccation, inadequate food, prolonged immersion in hyper-saline seawater, temperature shock, and nitrogenous waste buildup. The results of this project offer possible insight into future laboratory culture of *Didemnum* sp.

Introduction

This project focuses on the invasive species of colonial tunicate, *Didemnum* sp., which has recently been found off the rocky shores of the Northeastern United States. The species found off of New England have tentatively been identified as *Didemnum vestum*, although there is some controversy surrounding this (Daniel et al 2006), so in this report the species being studied will be referred to as *Didemnum* sp. Their high rate of reproduction, overgrowth of other benthic species, and rapid spreading makes *Didemnum* sp. an organism of interest that needs to

be investigated. *Didemnum* sp. is presumed to be native to Japan, however it has been found off both coasts of the United States, Pacific Canada, New Zealand, France, Ireland, and the Netherlands (Daniel et al 2006).

Didemnum sp. is a small colonial invertebrate of the Phylum Chordata with microscopic anatomical features, as each zooid averages just 1-2 mm in length (Daniel et al 2006). Juveniles possess a notochord during the larval stage of development, which is reabsorbed during metamorphosis (Pechenik 2005). Like other members of the Subphylum Tunicata, *Didemnum* sp. adults secrete a protective layer, the tunic, composed of polysaccharides and protein (Pechenik 2005). *Didemnum* sp. is a member of the ascidian Family Didemnidae, which are characterized by their anti-predatory chemical defense systems made by the sequestration of inorganic acids, called "didemnins" (Mercer 2003). These didemnins, as well as the tunicate's extremely low surface pH (3 or less), are probably used to deter predators such as fish (Mercer 2003). This has been observed in *Didemnum* species off the coast of North Carolina (Valentine et al 2007a).

Adult ascidians like *Didemnum* sp. lack true nerves and muscles, however some do have cells capable of conduction and contraction that allow minimal movement (Pechenik 2005). Individuals are hermaphroditic and ovoviviparous (Daniel et al 2006), meaning that individuals possess both male and female reproductive organs and fertilized, developing eggs are contained internally. Free-swimming larvae, also known as tadpoles, are then released, which eventually anchor themselves to a substrate and begin to colonize (Daniel et al 2006). Adults are filter feeders, with a buccal (or oral) siphon that draws water in via specialized, ciliated cells and an atrial siphon that pushes water out (Pechenik 2005). In colonies such as *Didemnum sp.*, multiple individuals have their own buccal siphons and a shared atrial siphon (Pechenik 2005) that often

make it appear as if they have clear, internal canals. It is currently believed that *Didemnum* sp. may subsist on a diet composed of "phytoplankton, suspended particulate matter, invertebrate larvae, and suspended bacteria" (Daniel et al 2006).

As colonial ascidians, *Didemnum* sp. individuals form colonies that are genetically identical as well as physically and functionally connected. This is due to colonies being founded by a single individual produced by sexual reproduction, which then buds asexually to produce many more individuals (Pechenik 2005). Experiments have shown that fragmentation may be another method of colony dispersal. This has been illustrated by reattachment tests performed by Bullard et al (2007), which showed that 75-80% of *Didemnum* sp. fragments could adhere to new substrates between just 6 and up to 30 hours in the lab. The ability to adhere to a surface in just 6 hours is important because most tides change within a 6 hour period (Mercer 2007) and pieces of colonies destroyed by storms, currents, or predators can be spread geographically to survive and grow as new colonies (Valentine et al 2007). Colonial ascidians usually survive for 1-3 years, and are capable of rapid growth that slows as the size of the colony increases (Daniel et al 2006).

Didemnum sp. colonies have a wide range of morphologies, from long and ropey to low, encrusting mats (Bullard et al 2007), however, the shape of the colony may not remain constant and can change over time as it ages (Daniel et al 2006). Colonies obtained in this experiment exhibited both the flat, carpet-like morphology as well as long and globular forms. Mat-like colonies can be found on the ocean floor growing over rocky substrate in areas with stronger currents (Bullard et al 2007). Eventually, pieces of colonies with rope-like structures, that tend to grow vertically, such as on ropes, can bud off, fall to the ocean floor, and grow if given the right environmental conditions (Mercer 2003). This is a critical issue because not only can the

newly settled fragments begin to bud asexually, but they may also contain developing larvae inside that can be released, causing the spread of *Didemnum* sp. to be even greater (Bullard et al 2007b). *Didemnum* sp. also vary in color, ranging from tan to cream, yellow, orange, or pinkish. These colors may depend on the environment and trace elements found throughout different seasons (Daniel et al 2006).

During the winter, *Didemnum* sp. exhibits seasonal regression in some areas. This includes "reducing mass to a dormant bud" until favorable conditions return and growth can begin again (Daniel et al 2006). This phenomenon is especially likely to occur in colonies located both tidally and in shallow sub-tidal waters, where "daily and seasonal temperatures vary appreciably" (Valentine et al 2007). In fact, Valentine suggests that seasonal decline may be closely linked to temperature change, "In October, a decrease in both water temperature and in the magnitude of daily temperature fluctuations coincides with the beginning of a gradual decline in health for some colonies" (Valentine et al 2007). Aside from the winter regression, colonies have been observed surviving in a range of conditions. In tidal pools where the species occur, temperature has been recorded ranging from less than -1 °C to over 24 °C, and in the sub-tidal zone species have been found to tolerate temperatures ranging from -2 °C to over 24°C (Valentine et al 2007a). *Didemnum* sp. also exist at on-shore and off-shore sites at depths ranging from less than 1m down to 81m (Bullard et al 2007b). Most ascidians, including *Didemnum* sp., are not usually found in salinities less than 25ppt. When the salinity is below a tolerable level the tunicates will close up their siphons, and may eventually die, if kept in these conditions for too long (Daniel et al 2006). Tadpole larvae do not favor low salinities either because it prevents them from metamorphosing at normal rates (Daniel et al 2006).

Invasive species like *Didemnum* sp. are of interest due to their significant disruption in habitat complexity, causing a loss of species diversity due to their overgrowth of native species in non-native habitats (Mercer 2003). They are also of interest because they may have considerable economic impacts (Daniel et al 2006). As good competitors with rapid growth and high reproductive rates, *Didemnum* sp. have been "observed over-growing mussels, oysters and scallops" (Mercer 2003). At multiple sites, encompassing an area of 230km^2 in the U.S. portion of George's Bank, *Didemnum* sp. have been recorded covering 50-90% of available space at depths of 45-60m (Bullard et al 2007b). *Didemnum* sp. grows on hard substrates, and have been found in pebble-cobble habitats, docks, pilings, rock outcrops, moorings, polythene plastic, steel chain, ship hulls and gravel (Mercer 2003; Daniel et al 2006; Bullard et al 2007b). This makes them a threat to both the ecology and economy of the New England coastline.

This invasive tunicate's ability to smother invertebrates such as sponges, mussels, oysters, scallops, barnacles, bryozoans, other ascidians, hydroids etc. (Bullard et al 2007b) may have a huge impact on many food webs. It may also begin to have an increasingly negative impact on shellfish aquaculture and farming (Daniel et al 2006). According to Coutts and Forrest (2007), a barge in New Zealand with growth of *Didemnum vexillum* was reportedly responsible for spreading the species to another harbor further south. Colonies were found around the barge's mooring, spreading to nearby artificial structures and the seabed in a previously uncontaminated area. This threat to nearby mussel farms instigated an eradication program that could not ultimately exterminate *Didemnum* from the area (Coutts and Forrest 2007). *Didemnum* sp. is spatially out-competing native species and preventing benthic larvae from settling into interstitial environments that wouldn't normally be covered up. Fish may resort to eating *Didemnum* sp., which has little nutritional value, and fish eggs or larvae may even die

because of the high acidity of its tunic (Daniel et al 2006). Due to their efficient filter feeding and vast amounts, they may even have a noticeable impact on the plankton community and in turn other animal species' in the food web (Daniel et al 2006). In New England, regressing colonies have been seen being scavenged by periwinkles, however, healthy colonies have no known predators (Valentine et al 2007a).

The goal of this experiment was to cultivate a sustainable reproducing colony of *Didemnum* sp. over a long-term period in a laboratory environment. After obtaining live samples, our objective was to determine some of the environmental factors that promote optimal growth and reproduction of *Didemnum* sp., as well as determining environmental tolerances for use in further studies. In order to achieve these objectives, colonies collected between October and March were maintained in filtered tanks in which salinity, food, temperature, and light exposure were carefully monitored and controlled. Gaining a better understanding of the factors that promote growth and reproduction of *Didemnum* sp. may aid in determining the best methods of managing their invasive movements in the future.

Materials and Methods

Over a 6 month period samples of *Didemnum* sp. were collected from Portsmouth Harbor, the Isles of Shoals, and Eastport, Maine. Large quantities of *Didemnum* sp. were obtained four times (as often as possible). Only two variables were tested for the duration of this project, temperature and feeding regimes. Other conditions, such as photoperiod and salinity, were maintained and monitored. To control photoperiod, fluorescent lights suspended above the tanks remained on at all times. The salinity range within the tanks (30-35 ppt) was maintained by daily measurements with a refractometer, and when necessary, de-chlorinated tap water was

added. For all aquaria used, the filter cartridge was removed at the time of the first feeding and replaced one hour after the last feeding of the day for the duration of the experiment. The first variable studied was temperature.

For the month of October, the tunicates were divided into six ten-gallon aquaria with whisper filters and one one-gallon plastic tub. Colonial samples were divided into small fragments and tied to microscope slides using monofilament. The slides were placed into enclosed containers, surrounded by wet paper towels, and left for three-hours before the slides were suspended in the aquaria. This technique has been seen to successfully result in adhesion when performed with *Botrylloides violaceus* (E. Westerman, personal communication). All aquaria were filled with seawater made with Instant Ocean salts to eliminate any of the possible variables associated with emersion in raw seawater (such as food, micronutrients etc.). The initial feeding regime consisted of 5 drops of Invertebrate Smorgasbord on Monday, Wednesday, and Friday twice a day for all tanks. Meanwhile, in order to determine the temperature that allowed for optimal growth, three aquaria were held at 21°C (room temperature), three at 10° C, and one plastic tub at 15°C.

For the remaining five months, various feeding regimes were tested. Each feeding regime was administered over a one-month period, all experiments were held at 15°C (the average temperature in Great Bay in June is between ≈ 16°C), and the aquaria were filled with seawater obtained from the Coastal Marine Laboratory at the mouth of Portsmouth Harbor, NH. Again, pseudo-replications were obtained for each treatment by suspending many fragments per aquarium.

During the month of November, new colonies of *Didemnum* were gathered and divided among five aquaria. The five aquaria were given varying food types twice a day. Aquarium 1

was given a combination of Marine Snow and Invertebrate Smorgasbord. A total of 20 drops was administered: five drops of Marine Snow and five drops of Invertebrate Smorgasbord in the morning, and then repeated in the afternoon. Aquarium 2 was given a total of 20 drops of Invertebrate Smorgasbord: 10 drops in the morning and 10 drops in the afternoon. Aquarium 3 was given a total of 20 drops of Marine Snow: 10 in the morning and 10 in the afternoon. Aquarium 4 was given a total of two drops of an algal paste of *Nannochloropsis*: one drop in the morning and one drop in the afternoon. Aquarium 5 was given a total of 2 drops of an algal paste called, "Tahitian Blend". This is a concentrated paste that consists of various types of algae, including Tahitian *Isochrysis, Tetraselmis, Nannochloropsis*, and *Pavlova*. One drop was administered in the morning and one drop in the afternoon.

The months of December began a new feeding regime that carried into January. The volume and type of food was kept consistent for all samples, one drop of Tahitian blend twice a day along with seven drops of Invertebrate Smorgasbord twice a day, and seven drops of Marine Snow twice a day. At the beginning of the month of December, the samples collected were distributed between the same five aquaria, which were emptied, washed, and refilled with fresh seawater. The samples used were not divided into small pieces as previous, but rather were placed on plastic panels and allowed to rest on the bottom of their respective aquaria. The aquariums were maintained until December 24. At this time all group members had left campus for Christmas, and it was only possible to have the samples fed once a day until January 4. The surviving samples were combined into a single aquarium.

New samples were obtained during the beginning of February, and divided between two clean aquaria with fresh seawater. On February 20th, a sustainable source of live phytoplankton was located, and the previous feeding regime was discontinued. The two aquaria immediately

began receiving 25 mL of solution containing both live *Isochrysis sp.* and live *Dunaliella sp.* twice a day. The plastic panels and samples were also suspended in the water column in plastic net baskets (See Fig 1a below). On March 6th new samples of *Didemnum* and a sample of *Botrylloides violaceus* were obtained and all samples were placed consolidated into a single aquarium. The same feeding regime (live phytoplankton solution) was administered. *Didemnum sp.* was cultured alongside *Botrylloides violaceus* to compare the growth rates of the two invasive tunicates under the same conditions.

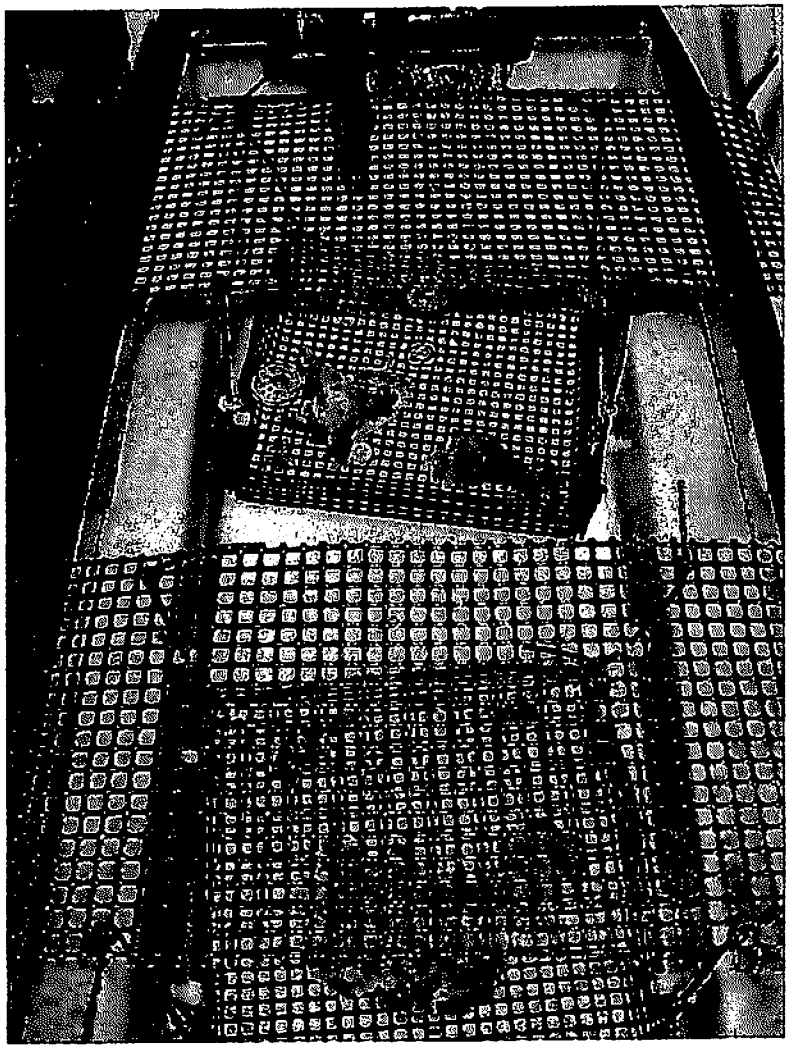

Figure1a: Tank set up for February 18th until the end of March. Didemnum suspended within the water column in wire baskets to promote optimal access to food and prevent sedimentary wastes from gathering siphons.

Results:

A sustained, reproducing colony was never established. The samples held at 20°C in both Instant Ocean and seawater began to deteriorate immediately, decreasing in size, and appearing hard and closed up. The samples would then begin to decay, looking similar to those seen in Figure 1. All the samples at 20°C appeared completely dead after five days and similar results were obtained from the samples held at 10°C.

The samples never bonded to microscope slides, despite various adhesion techniques. Adhesion to plastic panels was obtained only after larger samples (~ 7.5 cm^2) were placed onto panels and allowed to rest on the bottom of the tanks at 15°C. Colonies oriented in this manor were sustained from December 1 until December 23 (See Figure 2) when given a diet of Invertebrate Smorgasbord, Marine Snow, and Tahitian Blend and when held within a tolerable salinity range (Fig. 3). They seemed to be open and actively filtering, and retaining the same color and appearance as when they were originally collected. It is to be noted that while the samples appeared healthy, neither growth by asexual budding, or the formation of new colonies by larvae ever occurred. The cold room broke during the week of December 24, and this caused tank salinities to rise dramatically as a function of increased room temperature. And unfortunately, the majority of samples had already begun to decay when observed on January 4. Any samples that had not begun to decay were moved to a new tank with fresh seawater, and deteriorated shortly thereafter (See Figure 4) while held at a tolerable salinity range (Fig. 5). The February samples (both *Didemnum sp.* and *Botrylloides violaceus*) began to deteriorate within two weeks like all other samples, becoming closed up and hard looking (Figures 6 & 7, and Figures 8 & 9), despite being held at relatively consistent salinities (Fig. 10). It is important to note that the samples obtained on February 2 "blew up" in a manor never previously witnessed

(Fig. 11), and then deteriorated rapidly (Fig. 12). The average aquarium salinity was kept within the ranges of average salinity recorded at locations *Didemnum* sp. is known to inhabit along the Eastern Coast of North America (Fig. 13). No growth data was ever obtained because colonies survived only short periods of time in laboratory conditions.

Figure 1: Desiccated *Didemnum* (2/18/07)

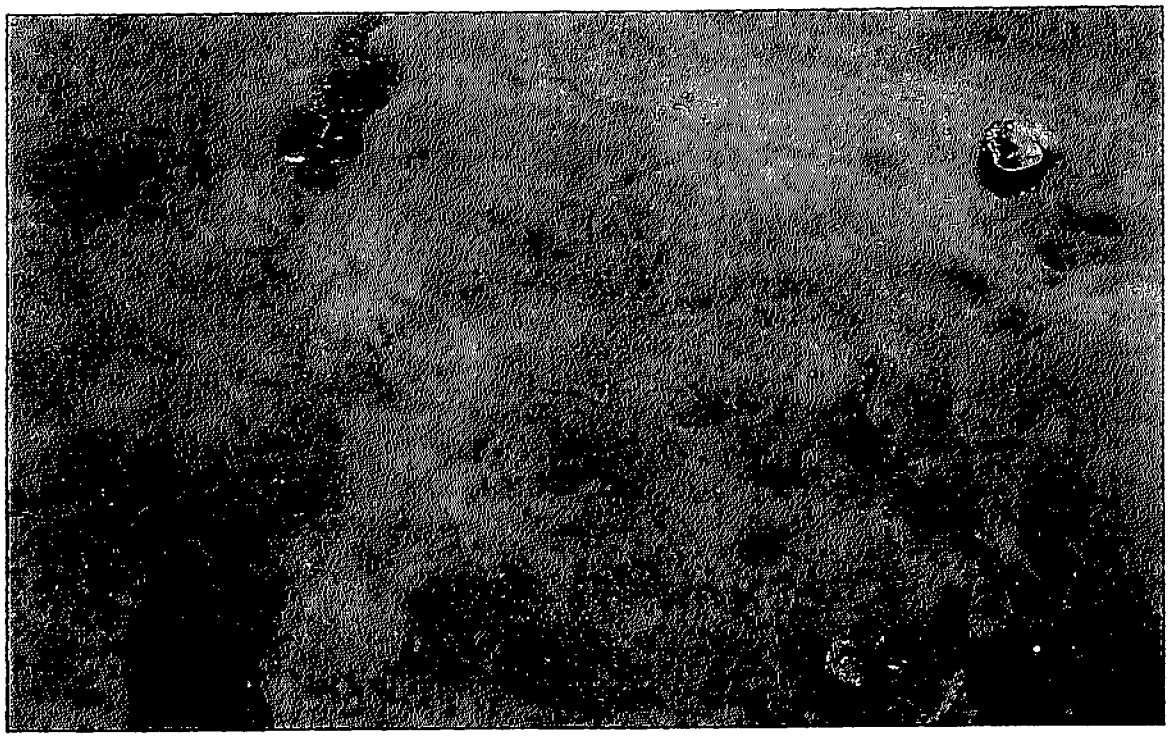

Figure 2: Healthy *Didemnum* sp. as seen upon collection from the Coast Guard pier in Portsmouth Harbor, NH (12/10/06)

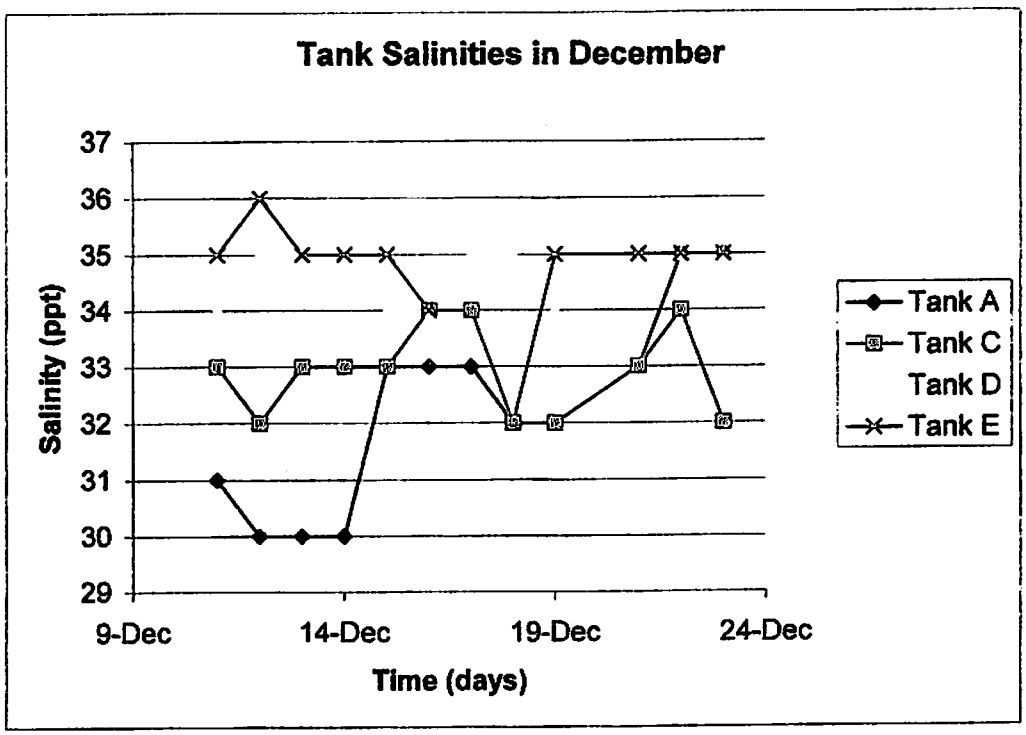

Figure 3: Tank salinities variations for the month of December. Increases in salinity were caused by evaporation of tank water, and decreases occurred after the addition of de-chlorinated tap water.

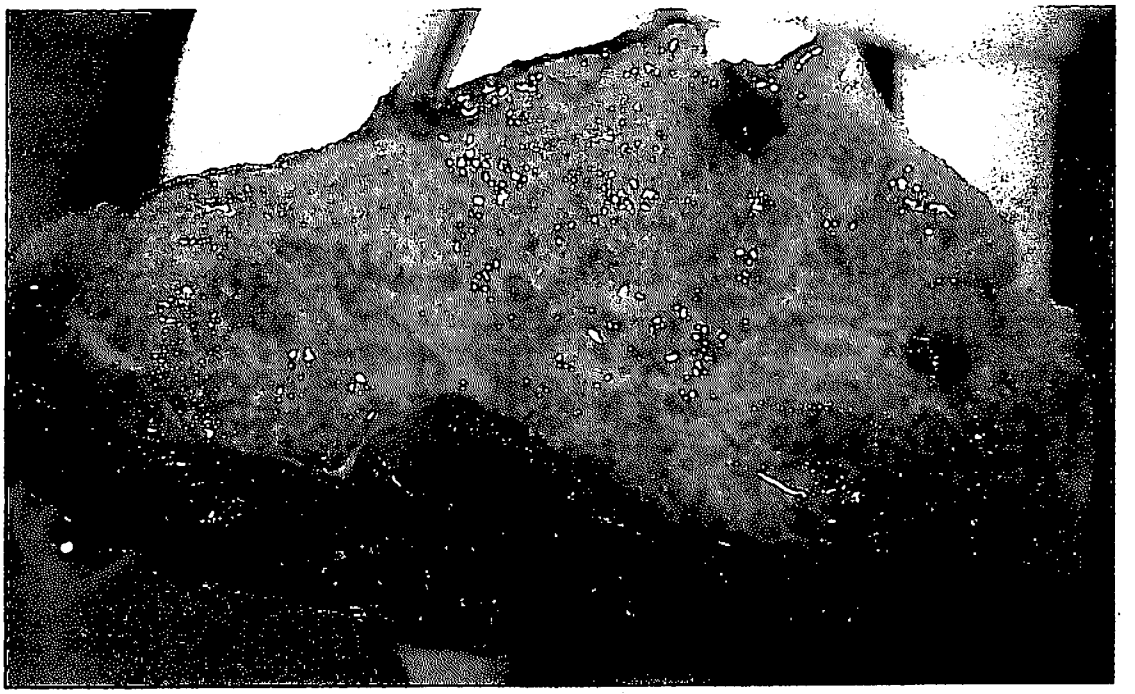

Figure 4: Blackened, curled up appearance of *Didemnum* sp. originally taken off plates obtained from the Coast Guard pier in Portsmouth Harbor, NH early December (photo on 2/6/07)
Food treatment: 1 drop Tahitian Blend algal, 7 drops of Invertebrate Smorgasbord and 7 drops of Marine Snow: all twice a day

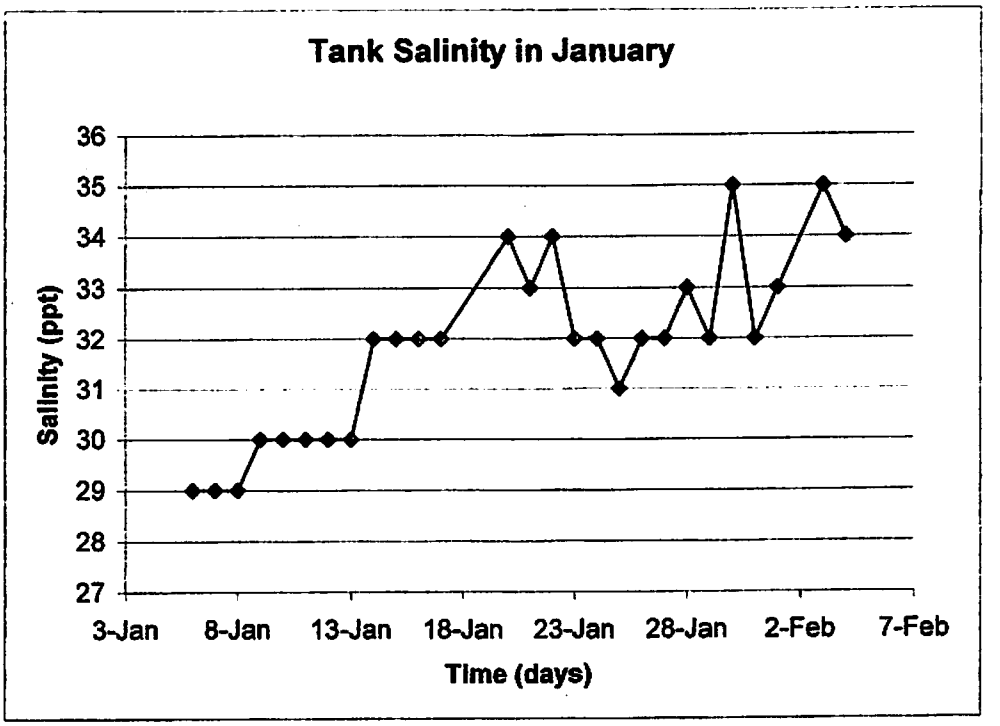

Figure 5: Tank salinity variations for the "survival" tank during the month of January. Increases in salinity were caused by evaporation of tank water, and decreases occurred after the addition of de-chlorinated tap water.

Figure 6 & 7: ***Botrylloides violaceus*** **growing on seaweed: top: healthy (3/6/07), bottom: recessed (3/20/06) Food treatment alongside** ***Didemnum*****: 25 ml live** ***Isochrysis*** **sp. and** ***Dunaliella*** **sp. twice daily**

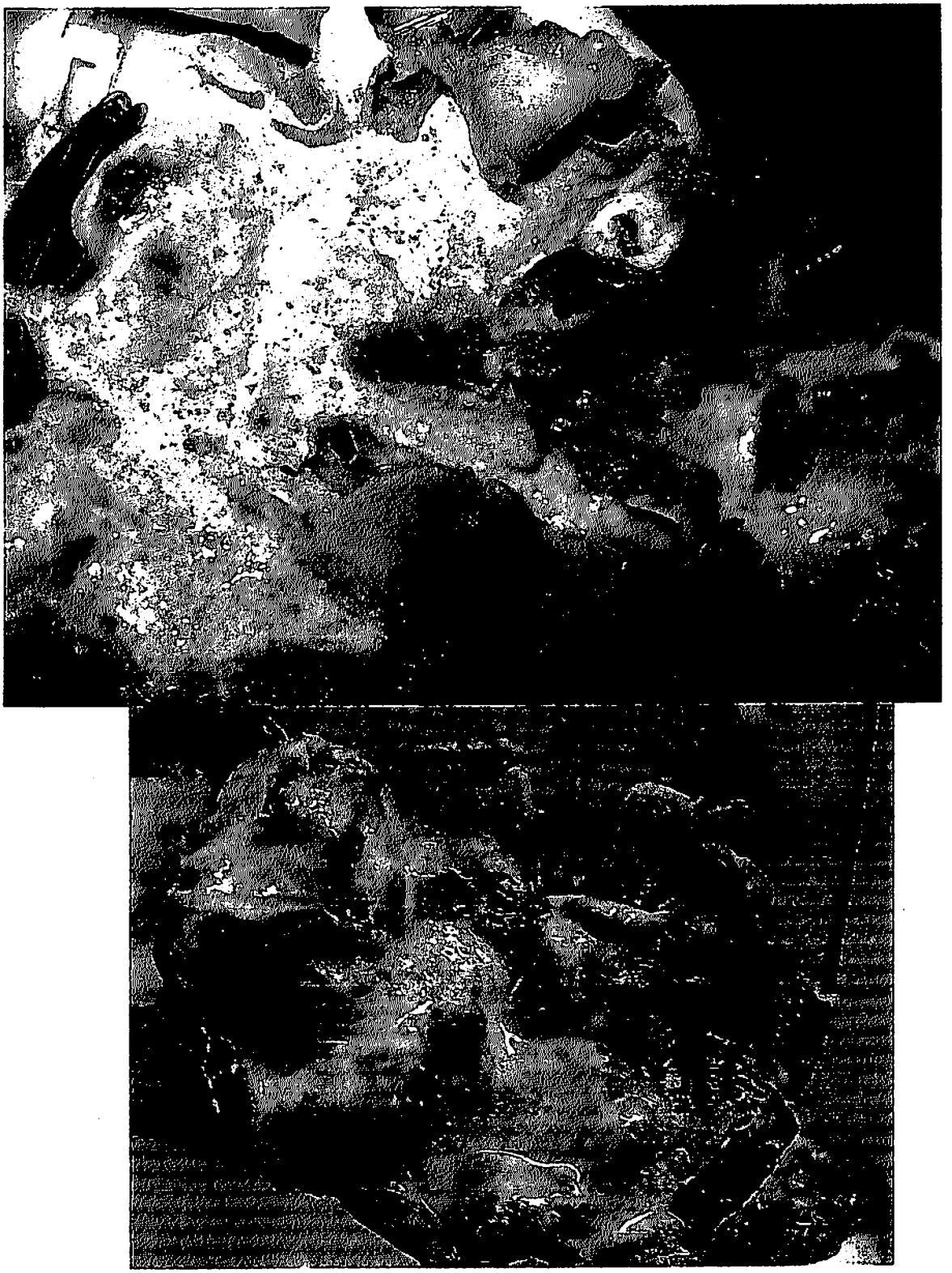

Figure 8 & 9: *Didemnum* sp. colonies from Eastport, ME, top: 3/6/07, bottom: 3/20/07
Food treatment: 25 ml live *Isochrysis* sp. and *Dunaliella* sp. twice daily

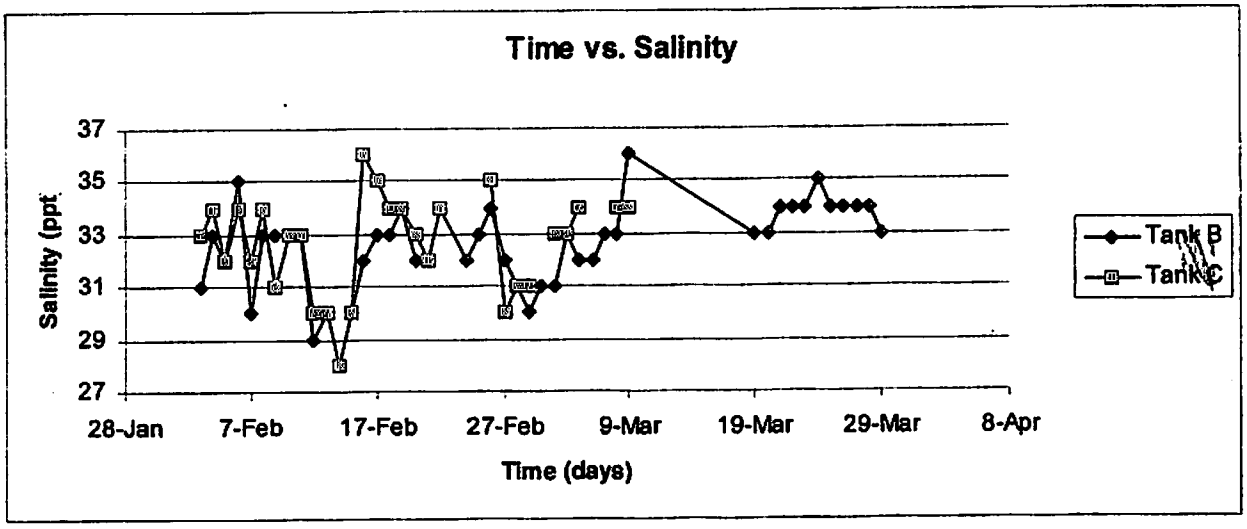

Figure 10: Tank salinity variations over the course of February, and through March (Tank B only). Increases in salinity were caused by evaporation of tank water, and decreases occurred after the addition of de-chlorinated tap water.

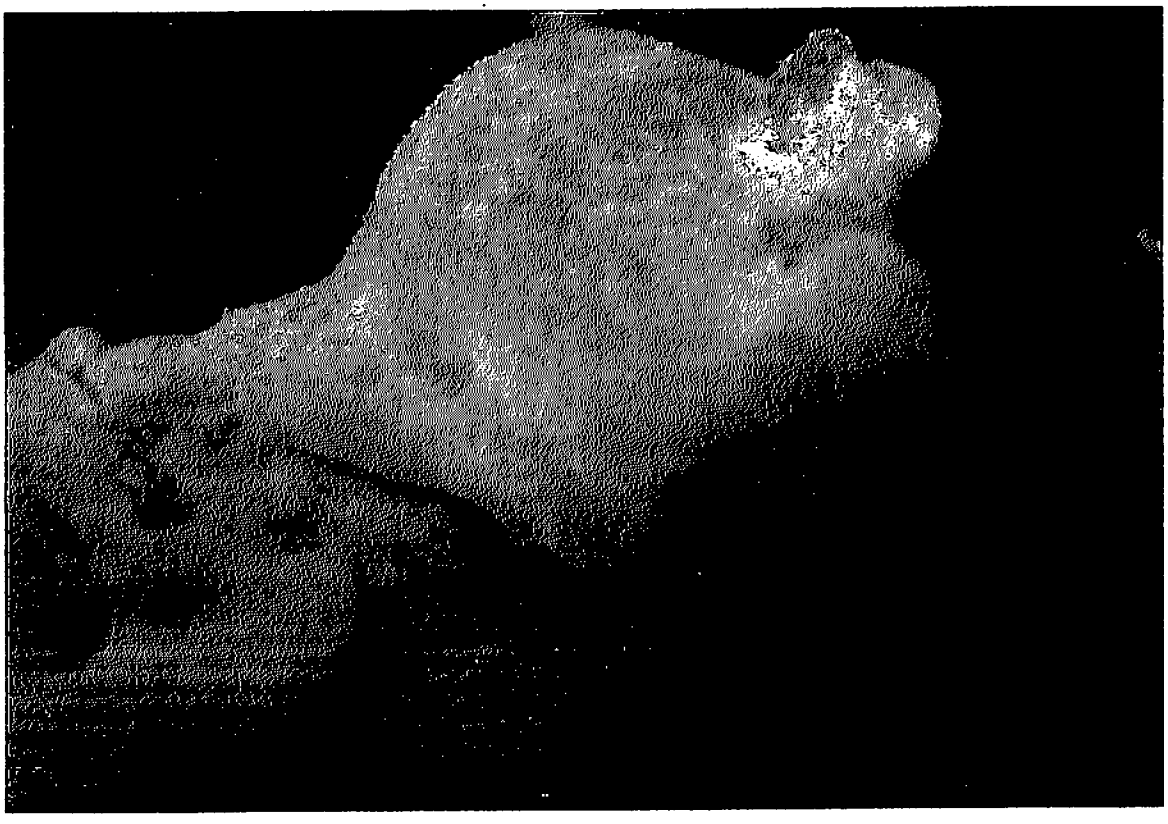

Figure 11: 'Puffy' state of *Didemnum*, exhibiting small orange dots under the tunic close to pore openings (2/11/07)

Figure 12: Desiccating and recessed *Didemnum* sp., deflated after previously being inflated (2/11/07)

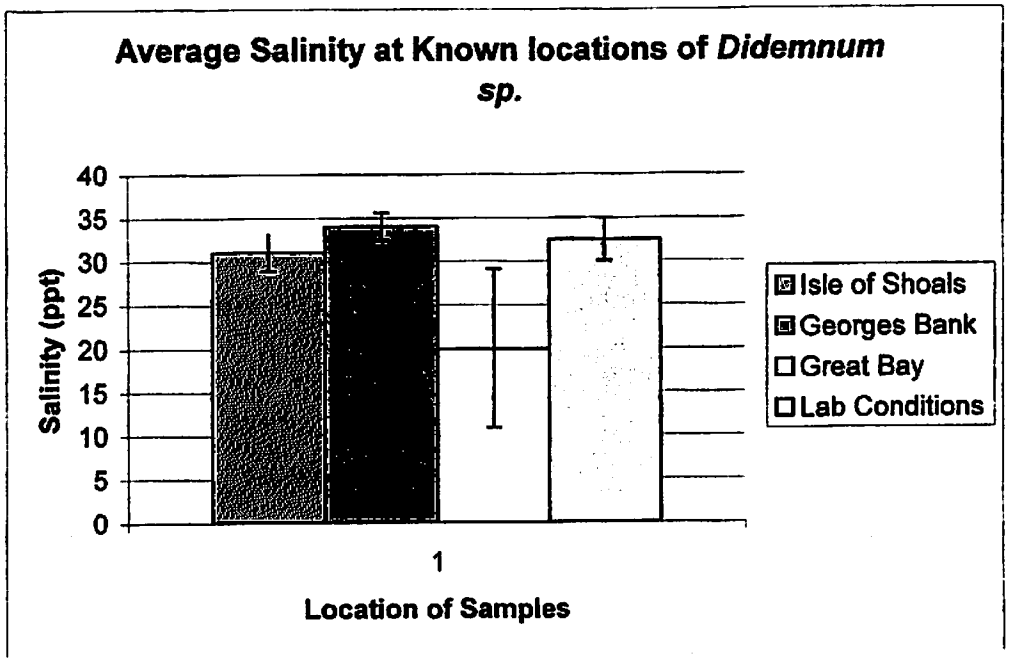

Figure 13: Average water salinity for various locations *Didemnum sp.* can be found along the Northeastern United States, as well as average laboratory conditions. The laboratory salinity concentrations (32 ppt) fall within the range *Didemnum sp.* is known to be successful at inhabiting as seen in the other locations (from 20 ppt at Great Bay to 34 ppt at Georges Bank).

Discussion:

No quantitative growth data was ever obtained because a living colony was never sustained. While the experiment did not progress as expected, it is to be noted that (to the best of our knowledge) no other experiments are being or have been conducted to culture *Didemnum* sp. under laboratory conditions for extended periods of time, and much can be learned from the techniques used and qualitative observations seen. While it is impossible to know what exactly caused the samples to die in every trial, recent publications and studies may provide some insight into possible causes of death, and provide background for more success in future attempts.

It was considered that a possible cause of death might have been the fragmentation of the samples in order to place them on the microscope slides. However, a recent comparative study illustrates that fragments of *Didemnum* sp. showed a strong ability to reattach to substrata (Bullard et al 2007). Furthermore, it has also been demonstrated that when colonial fragments of *Didemnum* sp. are placed in plastic bins and left in the "wild", they will reattach, initiating growth by asexual budding, and can increase by as much as 6 – 11 fold within the first 15 days (Valentine et al 2007).

While fragmentation seems an unlikely cause of death as illustrated by recent publications, a viable possibility of mortality early on may have been desiccation. As aforementioned, samples were initially cut into small pieces, placed on slides, and allowed to sit in empty plastic bins for a minimum of three hours. Scientists from the Woods Hole Lab in Massachusetts have observed that exposure to air for two to three hours will cause colonies to die, as seen in an uncontrolled 2004 experiment (Valentine et al 2007).

Another possible source of deterioration may have been the various feeding regimes. Colonial ascidians may consume phytoplankton, diatoms, invertebrate larvae, and bacteria

(Daniel et al 2006). Particles as small as 1 micron in diameter may be captured, and filtering rates, depending on the size of the tunicate, may be anywhere from 2 -3 liters of water per hour to 18.5 liters of water per hour (Daniel et al 2006). There is still little known about the morphology of *Didemnum* sp., and there is a distinct possibility the food sources we provided were too large (*Didemnum* sp. are quite small with each zooid averaging just 1-2 mm in length (Daniel et al 2006)), or given in inadequate amounts. This may have been the case with the samples in December. The volume of food administered may have been sufficient to sustain the colony but lacking in enough nutrition and energy to allow expansion.

There was some evidence that *Didemnum* sp. was utilizing an alternate food source. It was noted that the samples placed into Instant Ocean treated water died almost immediately, while samples placed in seawater lasted for a short period (no more than two weeks) before deteriorating. This may suggest that some micronutrient or microscopic food source found in seawater is necessary for laboratory culture. It was not possible to determine this "X" factor under the scope of this project. It is believed that the possibility of such an "X" factor (micronutrient, microscopic food source etc.) should be fully investigated in further studies, as knowledge gained may provide some insight into the life history and ecology of *Didemnum* sp.

Yet another possible cause of death could have been waste build up. Filter cartridges were rinsed in seawater and returned to filter daily, but tank water was seldom changed, and nitrogenous wastes were not monitored during the course of the experiment. It has been noted that in 2003 and 2004 *Didemnum* sp. was only present in fair water quality and moderate nitrogen levels in Massachusetts (Daniel 2006), and the main waste product of most ascidians is ammonia (Pechenik 2005). It is advisable that nitrogen levels be closely monitored and scrutinized in subsequent experiments.

Furthermore, the "controlled" elements, such as temperature, salinity, and photoperiod may have actually played a role in the samples' demise. It is known that *Didemnum* sp. can be found in tide pools and shallow sub-tidal areas, where it can endure vast changes in salinity and temperature (Valentine et al 2007). Subsequently, it was expected that maintaining a salinity range and temperature conducive to growth in the wild should allow for laboratory culture. However, during the first week of February, the refractometer broke, and was reading 5 ppt units below the actual salinity. This incorrect calibration was not discovered for about two weeks and the elevated salinities may have been deadly. Moreover, while the cold room was supposed to be 15°C, there were times when it broke. When it broke in December, this was especially significant because colonies began to deteriorate shortly after. Due to the fact that many other experiments are carried out in the cold room, it is an extremely high traffic area and the almost continuous opening of doors may have caused drastic heat loss. This temperature rollercoaster may have sufficiently temperature-shocked the samples enough to cause regression and death. Also, it is unknown how photoperiod may affect *Didemnum* sp. in terms of growth or reproduction, and it is believed that further experiments to examine this possible relationship should be performed. Much has been learned from the many attempts at culturing *Didemnum* sp. under laboratory conditions, and it is believed that after careful analysis of the results, future attempts to culture this mysterious animal will prove to be more successful.

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