Effect of hypo-osmotic stress on pedal disk diameter, volume regulation and concentration of Mg^{2+} in the sea anemone *Metridium senile*

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Abstract

The purpose of this study was to measure the effect of hypo-osmotic stress on pedal disk diameter, volume regulation and concentration of magnesium ions in the sea anemone *Metridium senile* subjected to 50%, 75%, and 100% seawater. These effects were measured by using a caliper to record changes in pedal disk diameter, a weighing balance to measure fluctuations in body volume, and a spectrophotometer to analyze the concentration of magnesium ions in *Metridium senile*. Chronic exposure to hypo-osmotic stress for twenty-one days resulted in initial conformation and then regulation of pedal disk diameter and body volume within two days. *Metridium senile* magnesium ion concentrations showed combined responses of ionoconformation for the first ten days of exposure and ionoregulation for the last eleven days of exposure to hypo-osmotic conditions.

Introduction

Sea anemones are predominantly marine, stenohaline osmoconforming animals (Schick, 1991). However, there are a few euryhaline species that occur in estuaries such as *Metridium senile* (Deaton, 1988). This anemone is ubiquitous along both the Atlantic and Pacific coasts of North America, attached to either protected pilings or substrata within the low intertidal and subtidal waters (Shumway, 1978). It has currently been found on the estuarine mudflats in Charleston, Oregon either attached to algal blades (both attached and drift algae) or simply on the mudflat with its pedal disk "attached" to the substrata. Within this habitat *Metridium senile* is subjected to frequent and sometimes large decreases in salinity levels resulting in hypo-osmotic stress. The osmotic concentration of the body fluids of osmoconforming sea anemones varies directly with

changes in the ambient salinity (Schick, 1991). The cells of osmoconforming euryhaline anemones exposed to decreased salinities are faced with a steepening of the osmotic gradient between intra-cellular and extra-cellular spaces, which can result in cell swelling (Pierce et. al, 1974). However, some euryhaline sea anemones are able to tolerate widely variant osmolarities by regulating body volume and ion fluctuations despite hypo-osmotic conditions (Oglesby, 1981). Consequently, estuaries inherently induce a variety of morphological and ionic stressors upon sea anemone physiology, specifically *Metridium senile*, due to the presence of an osmotic gradient (Shumway, 1978).

Euryhaline anemones have a variety of strategies for dealing with these stressors. This explains how they are able to tolerate low salinity ranges from 15-20 ppt (Benson-Rodenbaugh and Ellington, 1982). Sea anemones respond to hypo-osmotic stress by conforming their coelenteric fluid. Under hypo-osmotic stress the coelenteron is ventilated and its contents periodically replaced with fresh seawater (Fleming, 1967). This allows the coelenteric fluid to equilibrate with the ambient medium within three to six hours after a change in salinity (Schick, 1991). The same pattern is seen for concentrations of Na+, K+ and Cl- in the coelenteron fluid. Under hypo-osmotic conditions, the ion concentrations rapidly approach that of the external medium due to equilibration of the coelenteron with ambient salinity (Robertson, 1949).

Additionally, sea anemones utilize contraction and secretion of mucus to respond to hypo-osmotic stress. The former action minimizes the surface area in contact with the external medium, especially since ventilation of the coelenteron ceases, and reduces osmotic influx of water and efflux of ions and organic solutes (Schick, 1991). It is often assumed that the layer of mucus presents a barrier to water and solute movements, but its

diffusional permeability in sea anemones has not been studied (Pierce, 1982). It certainly causes an unstirred layer to develop at the ectodermal surface, which will decrease the flux of solutes. It is also suggested that the phosphonic acid of the phosphono-glycoproteins abundant in sea anemone mucus may act as ion exchangers (Schick, 1976). In sea anemones, this may provide a mechanism to maintain Ca^{2+} available to bind to the external membranes when the ambient osmotic concentration is lowered, which reduces the efflux of free amino acids (Fleming, 1967). Such a mechanism might allow anemones like *Metridium senile* experiencing short-term, tidal reductions in salinity to retain these organic solutes, especially since the trapped high-salinity coelenteric fluid and large extracellular space provide a reservoir against a decrease in environmental osmotic concentration (Deaton, 1988).

Euryhaline sea anemones also have the capacity to manage cellular amino acid concentrations by controlling the intracellular free amino acid pool (FAA), which increases in concentration with salinity (Webb et. al, 1972). This increase is an active regulatory process, not a passive equilibrium, for euryhaline sea anemones. Mechanisms of reducing intracellular concentrations of the FAA at low salinities include increasing the permeability of the cell membrane to them and thus increasing their efflux, which increases the rate of their oxidation (Shumway, 1978). An increase in the sea anemone's oxidation of FAA is accompanied by an increase in oxidative metabolism producing intracellular ATP synthesizing enzymes. Most of these ATP synthesizing enzymes are produced via the aid of ions, specifically magnesium (Mg^{2+}) (Deaton, 1988).

Magnesium is a very important macromineral to sea anemones. It is involved in hundreds of enzymatic reactions, many of which contribute to production of energy in the

form of ATP. It binds to ATP and activates the molecule for nucleophilic attack (Schick, 1991). Thus, in most cases, ATP must be bound to a magnesium ion in order to be biologically active (Rawlinson, 1934 and Shumway, 1978). Additionally, magnesium interacts with substrates or enzymes and is sometimes part of the active site. Therefore, reductions in magnesium concentrations could lead to decreased metabolic activity and eventually death (Schick, 1991). Due to the ability of euryhaline sea anemones to regulate their FAA concentrations, it has been suggested that it may be possible for anemones to regulate intracellular ions (Hoffmann, 1986). Consequently, the present study evaluates the ability of the sea anemone *Metridium senile* to regulate pedal disk diameter, body volume, and Mg^{2+} concentrations under hypo-osmotic conditions.

Materials and Methods

Collection of Metridium senile *specimens*

Twenty-one specimens of *Metridium senile* were collected from the mudflats of Charleston Harbor in Charleston, Oregon. All selected individuals were attached to algal blades and were approximately equal in size. Algae was removed and sea anemones were placed in a flow-through container immersed in running seawater (33 salinity) and allowed to acclimate for one week. Each individual anemone was allowed to attach to a piece of nylon mesh, measuring eight centimeters in diameter. Each attached individual was then placed in a fingerbowl, yielding eighteen fingerbowls with one anemone per fingerbowl.

Measurement of Pedal Disk Diameter and Body Weight

Measurements of pedal disk diameter and body weight were determined for each of the eighteen anemones by removing the mesh net from the fingerbowl. Each anemone's initial pedal disk diameter was measured non-invasively using a caliper. Pedal disk diameter was recorded in centimeters. Each sea anemone was then allowed to relax and release coelenteron fluid for three minutes. Individuals were then blotted on a paper towel and weighed wet on a Mettler AE 200 weighing balance. Weight was recorded in grams.

Salinity Treatments

One week after acclimation, three salinity treatments were prepared. Three tubs were each filled with one of the following treatments: 100% SW (33 salinity), 75% SW (25 salinity) and 50% SW (16.5 salinity). The diluent was reverse osmosis water and the salinity levels of each seawater treatment were confirmed with a refractometer.

Six anemones (each in a fingerbowl) were placed in 100% SW, 75% SW and 50% SW, yielding six individual anemones per seawater treatment. One hour after submersion in three salinity treatments, measurements of pedal disk diameter and body weight were obtained (as explained above) on the hour for four hours. After the fourth hour, pedal disk diameter and body weight measurements of each sea anemone were obtained daily for twenty-one days at twenty-four hour time intervals. After ten and twenty-one days, three sea anemones were removed from each salinity treatment and dissected for Mg^{2+} ion concentration analysis.

Preparation of Metridium senile *Tissue*

Anemones were dissected by cutting longitudinally through the mesentery tissue and mesogleal fluid using dissecting scissors. The mesentery tissue was snap frozen and stored in –80˚C for subsequent analysis of initial magnesium ion concentration.

Measurement of Magnesium Ion Concentration

Snap frozen *Metridium senile* tissue was homogenized. For each sample 0.3 grams of tissue was homogenized in 200ul of reverse osmosis water using a plastic mortar and pestle. 5ul of tissue homogenate and 1.25ml of 5% Trichloracetic Acid was added to a 1.5ml centrifuge tube. The cap was covered and mixed by inversion. The sample was then spun at 10,000rpm for 5 min in an Eppendorf centrifuge. 250ul of sample supernatant and 500ul of 20X Thiazole yellow working solution (diluted 1:20 with 0.015% Polyvinyl Alcohol) was added to a disposable cuvette. Cuvette contents were mixed by inversion using Parafilm to cover the top of the cuvette. 250ul of 2N LiOH was added to the cuvette. Then the sample absorbance was measured immediately at 540nm using a Beckman DU70 spectrophotometer. The concentration of magnesium for each sample was determined by comparing each sample's absorbance to a standard curve constructed by preparing 1M, 0.5M, 0.25M, 0.125M, 0.0625M, 0.0325M, 0.0156M and 0.0078M $MgCl_2$ standards. Magnesium concentration of tissue was measured in *m*M/g.

Results

Hypo-osmotic Stress Effect on Pedal Disk Diameter

Metridium senile showed an increase in pedal disk diameter with a decrease in salinity level during 24 hours of treatment. *Metridium senile* treated in 50% seawater showed the greatest increase in pedal disk diameter over the initial 24-hour period followed by 75% and 100% seawater treatments, respectively (Figure 1a). However, after approximately 24 hours of initial exposure to their respective salinity treatments, *Metridium senile's* pedal disk diameter in 50% and 75% seawater decreased to its initial size and stayed relatively constant, until the final measurement was taken 21 days later (Figure 1b).

Hypo-osmotic Stress Effect on Volume Regulation

Metridium senile showed an increase in percent body mass with a decrease in salinity level during 24 hours of treatment. Anemones treated in 50% seawater showed the greatest increase in percent body mass over the initial 24-hour period followed by 75% and 100% seawater treatments, respectively (Figure 2a). However, after approximately 24 hours of initial exposure to respective salinity treatments, percent body mass in 50% and 75% seawater decreased and stayed relatively constant, with a few minor fluctuations, until the final measurement was taken 21 days later. Percent body mass showed minor fluctuations in 100% seawater as well (Figure 2b).

Hypo-osmotic Stress Effect on Concentration of Mg^{2+} Ions

Magnesium ion concentrations in *Metridium senile's* tissue decreased with a decrease in salinity level over 21 days of treatment. Individuals treated in 50% showed a

larger decrease in magnesium ion concentration compared to individuals treated in 75% seawater. Those treated in 100% seawater retained constant levels of magnesium ions. After 10 days of treatment, magnesium levels in individuals treated with 50% and 75% seawater started to increase slightly until the last measurement was taken 11 days later (Figure 3).

Discussion

This present study on the effect of hypo-osmotic stress on pedal disk diameter, volume regulation and magnesium ion concentrations in the sea anemone *Metridium senile* supports the idea that this species may in fact be a euryhaline osmoregulator. Overall, *Metridium senile* shows evidence of being able to regulate pedal disk size under hypo-osmotic conditions (Figure 1a and 1b). *Metridium senile* also demonstrates the ability to regulate its body mass under hypo-osmotic conditions portraying an initial increase in body mass accompanied by relatively no change for the remaining 20 days of treatment (Figure 2a and 2b). Ionoconformation was expressed during the first 10 days of exposure to hypo-osmotic conditions. However, *Metridium senile* may show evidence of ionoregulation during the last 11 days of exposure to hypo-osmotic conditions, as the concentration of tissue magnesium ions in 50% and 75% seawater showed an increase of 20mM and 10mM respectively toward projected magnesium ion concentrations of the ambient seawater (Figure 3).

Metridium senile has been described as a euryhaline osmoconformer in regards to its response to hypo-osmotic conditions (Schick, 1991). However, the results of this study show that *Metridium senile* may be an osmoregulator. That is, this species is capable of

regulating not only its pedal disk diameter and body volume, but perhaps the concentration of its magnesium ions which are integral in many cellular enzymatic reactions involved in the production of ATP (Rawlinson, 1934). This was demonstrated by the compensatory changes in magnesium ion concentrations initiated after ten days of exposure to hypo-osmotic conditions, where there was a slight increase in magnesium ion concentrations of about 20mM and 10mM in 50% and 75% seawater, respectively (Figure 3). This response has been observed in other cnidarians, specifically in the mesohaline scyphomedusae, *Chrysaora quinquecirrha* which began regulating its ions fifteen days after exposure to hypo-osmotic conditions (Wright et. al, 1997) as well as other sea anemones, like the euryhaline osmoregulator, *Bunodosoma cavernata* which displayed a similar magnitude increase in ion concentrations after eight days of hypo-osmotic exposure (Benson-Rodenbaugh, 1982). Another sea anemone, *Diadumene leucolena* has been known to regulate its free amino acid concentrations (FAA) and potassium ions under hypo-osmotic stress (Steinbach, 1963). This is largely because *Diadumene leucolena* commonly inhabits the upper reaches of estuaries, where low-salinity water is fairly common (Schick, 1991).

There was a pronounced delay in response time in *Metridium senile's* body volume regulation (Figure 2b) compared to regulation of pedal disk diameter (Figure 1b). This was perhaps due to the fact that it takes longer for *Metridium senile* to adjust the quantity of coelenteron fluid as it responds to hypo-osmotic stress. Expansion and contraction of the coelenteron cavity has a relatively slower response time to external conditions compared to that of the pedal disk which maintains a rapid response time even under hypo-osmotic stress (Schick, 1991). Additionally, the measurement of magnesium

ion concentrations in the tissue may have some error since homogenization of tissue was not always perfect for each sample, especially for individuals treated with 50% seawater. Therefore, there may be inaccuracies of actual magnesium ion concentrations represented in Figure 3.

Overall, the study conveyed that hypo-osmotic stress on pedal disk diameter, volume regulation and magnesium ion concentrations causes *Metridium senile* to regulate to achieve optimum homeostatic balance. Additionally, *Metridium senile* may be using regulation as an adaptive method for surviving upon the estuarine mudflats. In particular, possible regulation of magnesium ions may be a way to conserve energy since regulating these ions increases the production of intracellular ATP (Schick, 1991, Benson-Rodenbaugh, 1982 and Rawlinson, 1934). It would be interesting to extend exposure time to four weeks to see if regulation continued or changed in response to external salinity fluctuations, since magnesium ion concentrations were still increasing on the last day of measurement. More importantly however, it would be interesting to measure other intracellular ion concentrations as well in addition to magnesium to see if *Metridium senile* regulates other ions under hypo-osmotic conditions. Ultimately, this better understanding of changes in the internal environment of an animal in response to acute salinity change helps one see how animals like *Metridium senile* have perhaps adapted their physiology to aid in survival within their fluctuating habitat.

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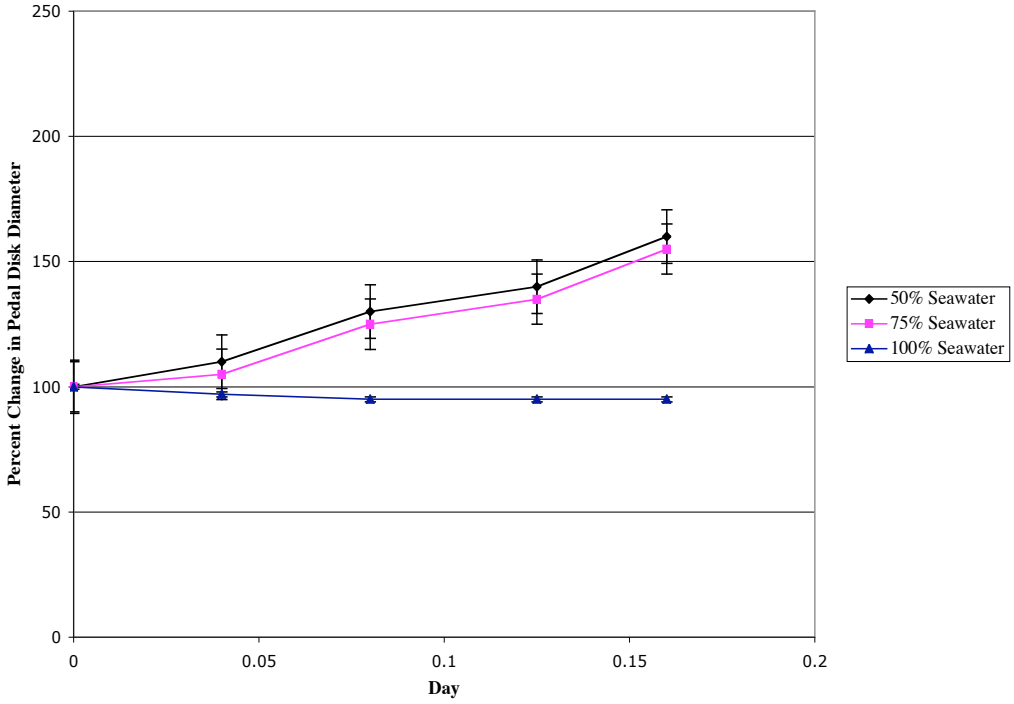

Figure 1a. Percent change in pedal disk diameter of *Metridium senile* exposed to three salinity treatments during Day 1.

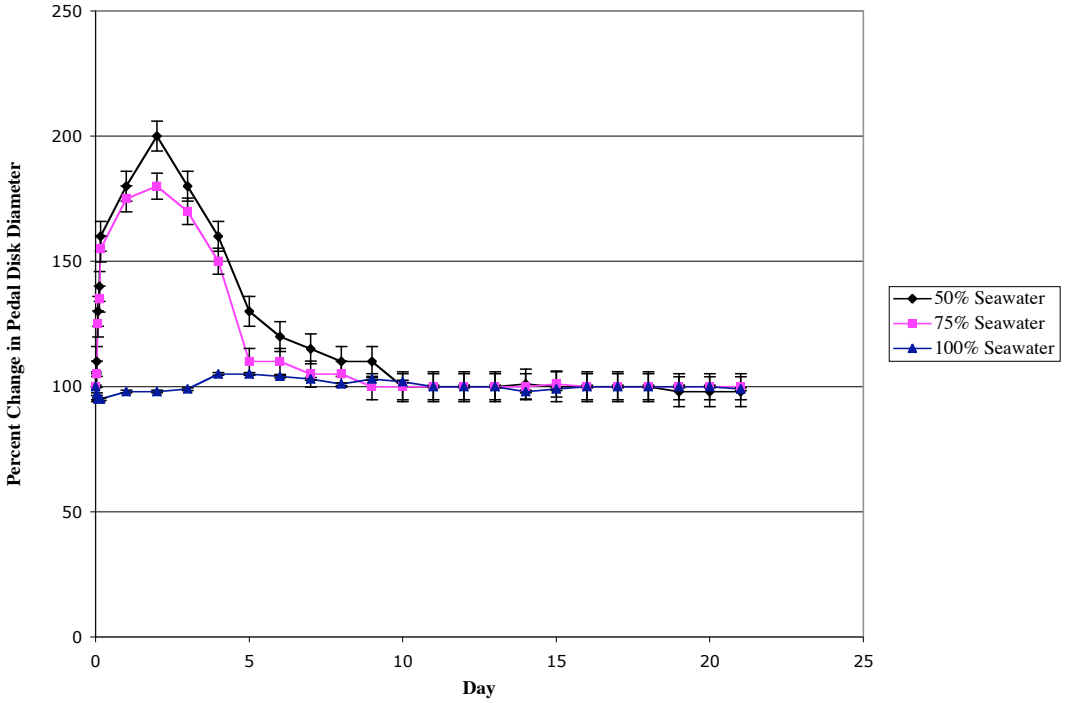

Figure 1b. Percent change in pedal disk diameter of *Metridium senile* exposed to three salinity treatments from Day 1 through Day 21.

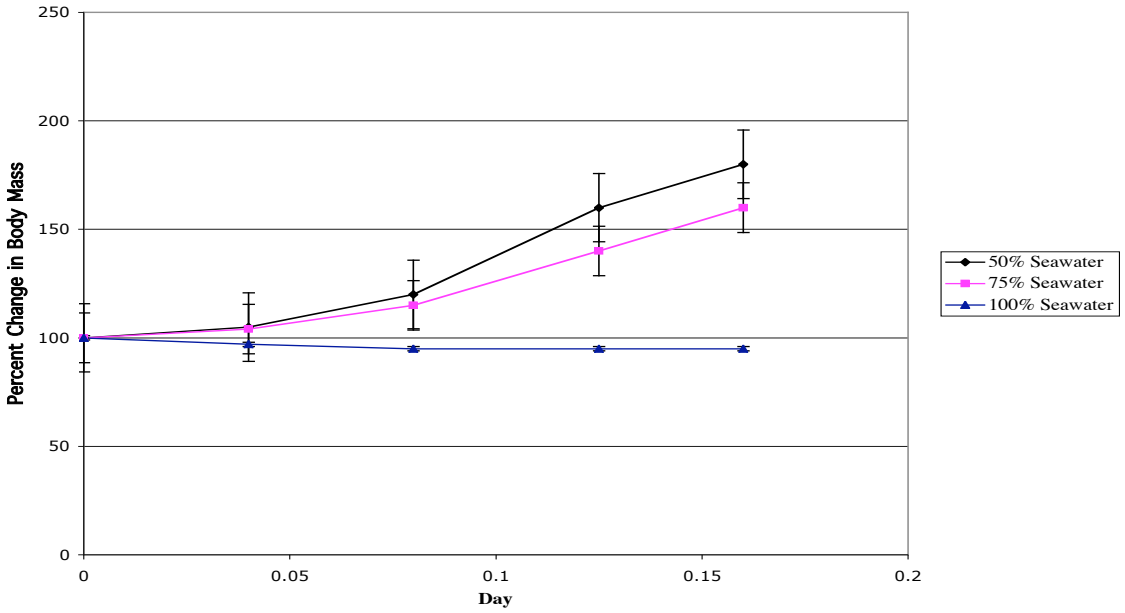

Figure 2a. Percent change in body mass of *Metridium senile* exposed to three salinity treatments during Day 1.

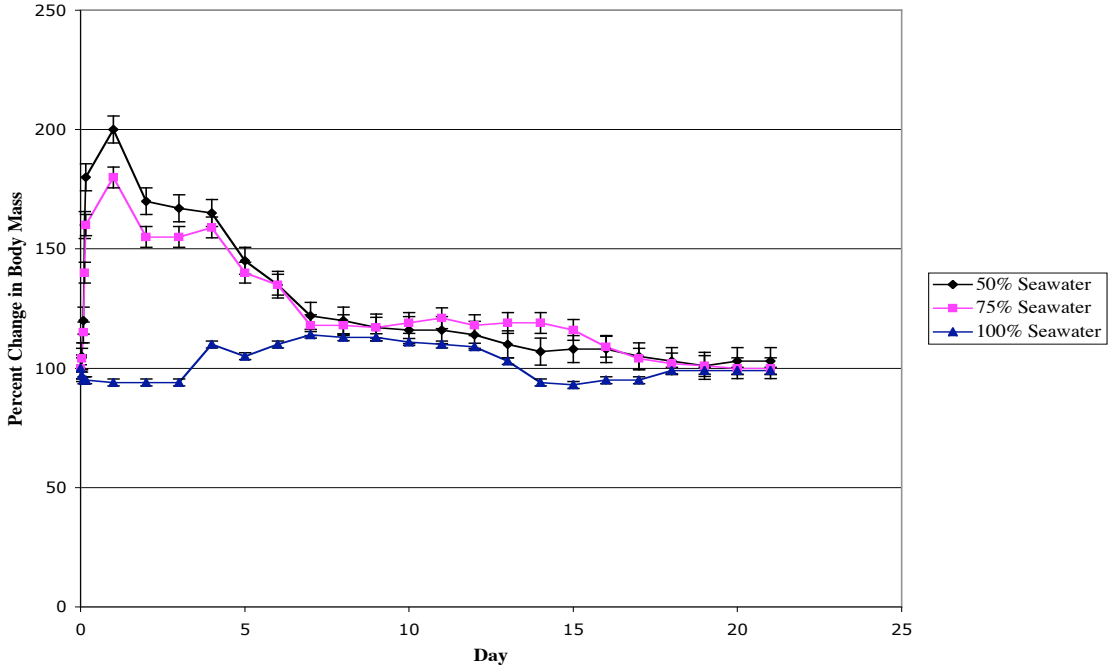

Figure 2b. Percent change in body mass of *Metridium senile* exposed to three salinity treatments from Day 1 through Day 21.

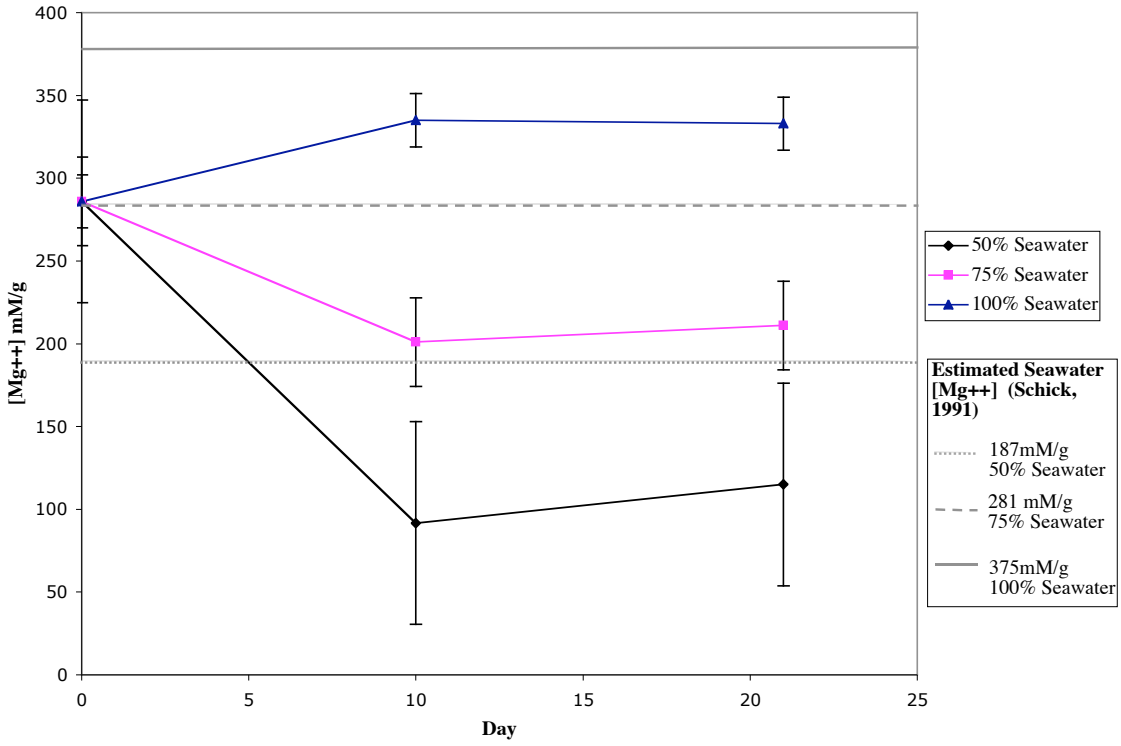

Figure 3. Magnesium ion concentrations in tissue of *Metridium senile* exposed to three salinity treatments**.**

Detailed Protocol

Effect of hypo-osmotic stress on pedal disk diameter, volume regulation and concentration of Mg^{2+} in the sea anemone *Metridium senile*

Collection of *Metridium senile* specimens

- 21 specimens of *M. senile* were collected from the mudflats of Charleston Harbor in Charleston, Oregon.
- Sea anemones were transferred to the lab in a bucket of fresh seawater.
- Algae and seaweed was immediately removed by hand.
- All sea anemones were placed in one flow-through container immersed in running seawater (33 salinity) and allowed to acclimate for one week.

Preparation of Salinity Treatments

- One week after acclimation, three salinity treatments were prepared.
- Three tubs were each filled with one of the following treatments: 100% SW (33 salinity), 75% SW (25 salinity) and 50% SW (16.5 salinity). The diluent was reverse osmosis water and the salinity levels of each seawater treatment were measured with a refractometer.

Preparation of Fingerbowls

- Each individual anemone was allowed to fully contract and attach to a circular nylon mesh net measuring eight centimeters in diameter.
- Each attached individual was then placed in a fingerbowl, yielding 21 fingerbowls with one anemone per fingerbowl.

Preparation of *Metridium senile* Tissue

- Three individual anemones were placed on a dissecting tray and were dissected by cutting longitudinally through the mesentery tissue and mesogleal fluid using dissecting scissors.
- 0.3 grams of mesentery tissue and mesogleal fluid was placed in a 1.5ml centrifuge tube and placed in liquid nitrogen to be snap frozen.
- The tube was stored in –80˚C for subsequent analysis of initial magnesium concentration.

Measurement of Pedal Disk Diameter and Body Weight

- Initial pedal disk diameter and body weight were recorded for the remaining eighteen anemones.
- Each anemone's pedal disk diameter was measured with a caliper without removing the anemone from the nylon mesh net. Pedal disk diameter was recorded in centimeters.
- Each sea anemone was allowed to fully relax and release its coelenteron fluid for 3 minutes.
- Individuals were then blotted on a paper towel and weighed wet on a Mettler AE 200 weighing balance. Initial weight was recorded in grams.
- After measurements were taken, each sea anemone was immediately transferred to its respective fingerbowl.
- Six fingerbowls were placed in 100% SW, 75% SW and 50% SW, yielding six individual anemones per seawater treatment.
- One hour after submersion in three salinity treatments, measurements of pedal disk diameter and body weight were obtained on the hour for four hours.
- After the fourth hour pedal disk diameter and body weight measurements of each sea anemone were obtained daily for twenty-one days at twenty-four hour time intervals.
- After ten and twenty days from initial measurement, three sea anemones were removed from each seawater treatment and the tissue was prepared as described above.
- The mesentery tissue was snap frozen and stored in –80˚C for subsequent analysis of magnesium concentration.

Preparation of Magnesium Standards and Reagents

1. Polyvinyl Alcohol (PVA): 0.015% w/v
    - Dissolve 0.015g in 100ml distilled water by warming in water bath or on hot plate with a magnetic stirrer. Some Thymol Crystal can be added to preserve the solution.

2. Thiazole Yellow: 20X
    - Dissolve 0.035g of Thiazole Yellow in 50ml of PVA and store in brown bottle in the dark.
    - For working solution dilute 1:20 with PVA. Working solution is stable for one day.
3. Lithium Hydroxide: 2N
    - Dissolve 0.84g in 10ml distilled water.
4. Trichloracetic Acid (TCA): 5%
    - Dissolve 2g in 40ml distilled water.
5. Magnesium Standards
    - Make 1M $MgCl_2$ solution, then dilute in half with distilled water in a series to make a standard curve of 0.5M, 0.25M, 0.125M, 0.0625M, 0.0325M, 0.0156M and 0.0078M $MgCl_2$ standards to achieve the correct molarity.

Measurement of Magnesium Ion Concentration in Tissue

- Snap frozen *Metridium senile* tissue was homogenized. For each sample 0.3 grams of tissue was homogenized in 200ul of reverse osmosis water using a plastic mortar and pestle.
- 5ul of tissue homogenate and 1.25ml of 5% Trichloracetic Acid was added to a 1.5 ml centrifuge tube.
- The cap was covered and mixed by inversion.
- The sample was then spun at 10,000rpm for 5 min in an Eppendorf centrifuge.
- 250ul of sample supernatant and 500ul of 20X Thiazole yellow working solution was added to a disposable cuvette.
- Cuvette contents were mixed by inversion using Parafilm to cover the top of the cuvette.
- 250ul of 2N LiOH was added to the cuvette.
- Then the sample absorbance was measured immediately at 540nm using a Beckman DU70 spectrophotometer.
- The concentration of magnesium for each sample was determined by comparing each sample's absorbance to a standard curve constructed by

1M, 0.5M, 0.25M, 0.125M, 0.0625M, 0.0325M, 0.0156M, 0.0078M $MgCl_2$ standards.

- Magnesium concentration of tissue was measured in *m*M/g.