5-2022

Investigating the potential role for the nervous system in controlling regeneration in Nematostella vectensis

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Investigating the potential role for the nervous system in controlling regeneration in *Nematostella vectensis*

An Honors Thesis Proposal submitted in partial fulfillment of the requirements for Honors studies in Biology

By

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Spring 2022

Biology

J. William Fulbright College of Arts and Sciences

The University of Arkansas

Acknowledgements

I would personally like to thank Dr. Nagayasu Nakanishi for putting his time and effort into helping me understand the world of research better. His guidance and support have allowed me to become more confident in my abilities to take on more independent studies. Additionally, I would like to thank all of the members of the Nakanishi lab for having patience with me and giving me helpful advice. Lastly, I would like to thank my committee members, Dr. Daniel Lessner, Dr. Nan Zheng, and Dr. Kim Stauss for taking time out of their day to attend my thesis. I am grateful to be given the opportunity to share my research with the University of Arkansas Honors College.

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Abstract

Nematostella vectensis is a marine sea animal that has become a model for developmental and evolutionary research. Included in the phylum Cnidaria, *N. vectensis'* was chosen as the model for this research. Not only can this animal go through asexual and sexual reproduction, but it also has the ability to regenerate. Although much research has been put forth in an effort to understand regeneration better, much is still unknown. The underlying mechanisms of regeneration in Cnidaria are illusive; however, studies within vertebrates have shown the substantial role of the nervous system. The objective of this experiment is to test if the activity of the nervous system, better referred to as a nerve net in Cnidaria, is responsible for the process of regeneration in *N. vectensis*. This paper outlines the process of using various chemicals to block neural activities within these animals then observing the effects on regeneration to further the knowledge on the underlying factors behind regeneration. Through this research it was found that both $MgCl_2$ and BTS slowed down the process of regeneration, but neither completely stopped regeneration from occurring. After three experiments were completed, it was concluded that the nervous system does not seem to be the main mechanism controlling regeneration. Evidence from the results show that both the nervous and muscular system seem to play a large role within regeneration. Further research could be performed to see whether anemone size, stress-responses, or chemical toxicity have specific effects on regeneration.

Introduction

Regeneration is a phenomenon that is seen in vertebrates and invertebrates. A long-standing interest in this unique ability has led to countless research projects. However, even with all the research provided on this topic, the mechanisms of regeneration are still not completely understood. Two theories dominate the origins of regeneration. The first being that regeneration arose as a trait in early organisms. Opposing this first theory, others have coined regeneration as a re-use of morphogenetic processes, such as asexual reproduction and embryogenesis. Although regeneration is highly conservative, the process of regeneration varies largely between vertebrates and invertebrates.[1] The nervous system has been shown to play a role in some of these regeneration process within rats and salamanders. It is important to research this controlling mechanism because it would determine if nervous system-dependent regeneration is vertebrate specific or if it is used in invertebrates as well. My research is focused on the potential role of the nervous system controlling regeneration within the invertebrate *Nematostella vectensis*.

Cnidarians, consisting of sea anemones, corals, and jellyfish, have become increasingly popular as a source of evolutionary and developmental knowledge due to their fully sequenced and easy manipulated genome.[2] Furthermore, they form a sister group to Bilaterians which consist of Protostomes and Deuterostomes.[3] Within the phylum of Cnidaria, two groups are present—Anthozoa and Medusozoa.[2] For the purposes of this research, the model organism, *Nematostella vectensis*, also known as the scarlet sea anemone, was chosen because of the pre-existing knowledge of the steps of regeneration within this animal.

Nematostella vectensis is an anthozoan cnidarian that predominantly presides in estuarine environments. These animals consist of two germ layers, an endoderm and ectoderm.[4]

Additionally, two wide-spread nerve nets exist within these two germ layers. Within these nerve nets, neurons communicate through chemical synapses and electrical synapses.[4]

Not only is *N. vectensis* a model for evolutionary purposes, but also regeneration. According to the National Institute of General Medical Sciences, regeneration is the process of rehabilitating or replacing vital cells, tissues, and organs that may have been damaged or completely removed. For regeneration to occur in *N. vectensis,* cell proliferation is required within these animals.[4] The study of regeneration in *N. vectensis* has been performed by implementing amputation. It has been found that initial wound healing, a separate process from regeneration, is completed by six hours post-amputation. Furthermore, mitotic events occurring within the cells of the animal were detected twelve hours post-amputation.[4] Since regeneration within *N. vectensis* is dependent on cell proliferation, further research was conducted to see how similar the patterning is between early development and regeneration. It was found that major patterning molecules, specifically Wnt, were present during regeneration and in embryogenesis.[4] In conjunction with this research, a study conducted by Johnston and colleagues has alluded to the activation of embryonic gene modules during regeneration. Through data collection it has been found that there are co-expression clusters between embryogenesis and regeneration; however, it still seems that after the 36 hours post-amputation mark, many of the genes expressed are unique to regeneration alone.[5]

The factors behind what controls regeneration have not been researched thoroughly. My research is focused on the potential role of the nervous system controlling regeneration within *N. vectensis*. I hypothesize that if chemicals that specifically block neurological functions are applied to anemones right after amputation, then regeneration will not occur. To test this hypothesis, chemicals such as magnesium chloride, benzyl-p-toluenesulphonamide (BTS), and

BAPTA-AM will be used to inhibit specific biological processes within *N. vectensis*. Magnesium chloride was chosen as it can be used as an anesthetic, which inhibits the nervous system. BTS was used because it inhibits cross-bridge formation between myosin and actin. In turn, power strokes cannot occur which will inhibit overall muscle contraction.[6] Lastly, BAPTA-AM was used as it lowers free calcium within cells which disrupts the nervous system. To observe the effects of these chemicals, anemones will be amputed and placed within specific mediums containing these chemicals and control mediums to see if regeneration occurs or not.

After completing three experiments it was concluded that the nervous system cannot be confidently determined as the main mechanism behind regeneration. Although regeneration was slowed by both $MgCl_2$ and BTS, BAPTA-AM treated animals regenerated as normal.

Materials and Methods

Six-well Plate Set Up

The conditions for each well (using 6-well culture plate) had to be set up before experiment could proceed. In the first crude experiment the different conditions were as follows; $MgCl_2$, BAPTA-AM (10uM, 100 μM, 1 μM), DMSO (dimethyl sulfoxide) control, and 1/3 Seawater negative control. The 2.43% magnesium chloride solution consisted of 1.67 mL of 7.3% $MgCl_2$ solution and 3.33 mL of 1/3 seawater. Three different BAPTA-AM solutions were created.

1. The 10 μM medium consisted of 5 mL of 1/3 seawater and 5 μL of BAPTA-AM.
2. The 100 μM medium consisted of 5 mL of 1/3 seawater and 50 μL of BAPTA-AM.
3. The 1 μM medium consisted of 5 mL of 1/3 seawater and 0.5 μL of BAPTA-AM.

The DMSO control was created by adding 50 μL of DMSO to 5 mL of seawater. The second experiment included the same set up with the exception of benzyl-p-toluenesulphonamide (BTS) being substituted for BAPTA-AM. Two different BTS solutions were made, one being 100 μM and the other 200 μM. The last experiment consisted of BTS, BAPTA-AM, $MgCl_2$, a DMSO negative control, and 1/3 salinity seawater control.

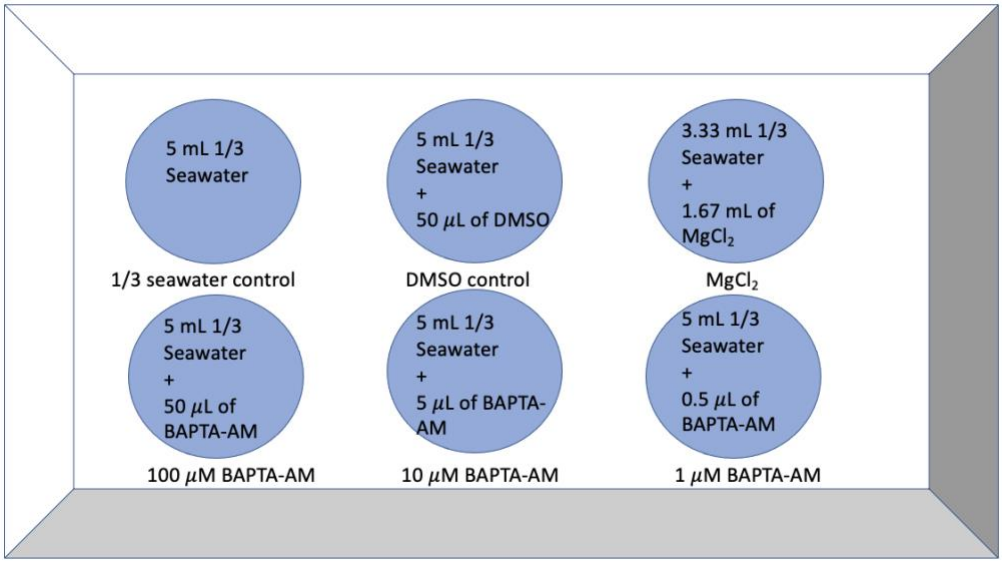

Figure 1: Schematic showing experimental set up of the six-well culture plate. This set up is specific to the first experiment; however, all future experiments are set up the same way, just using different chemicals.

Separating Oral and Aboral Halves

After separating out the wild-type animals into a petri dish with 1/3 seawater, the anemones must undergo relaxation. To limit the anemones movement, a 10 mL mixture of 7.3% $MgCl_2$ solution and 30 mL of 1/3 seawater was created. The animals were placed into this relaxation mixture and rotated slowly for 10-15 minutes. Once the anemones were relaxed, a clean sterile scalpel was used to make a transverse cut, effectively separating the oral and aboral halves. These halves were then placed into a corresponding well set up beforehand. During the first experiment, both oral and aboral halves were placed in the same well. However, in the second experiment the aboral and oral halves were placed in two separate wells containing the same solution. For the final experiment, only the tentacles were cut off with the use of a scalpel and placed into each well containing the different mediums.

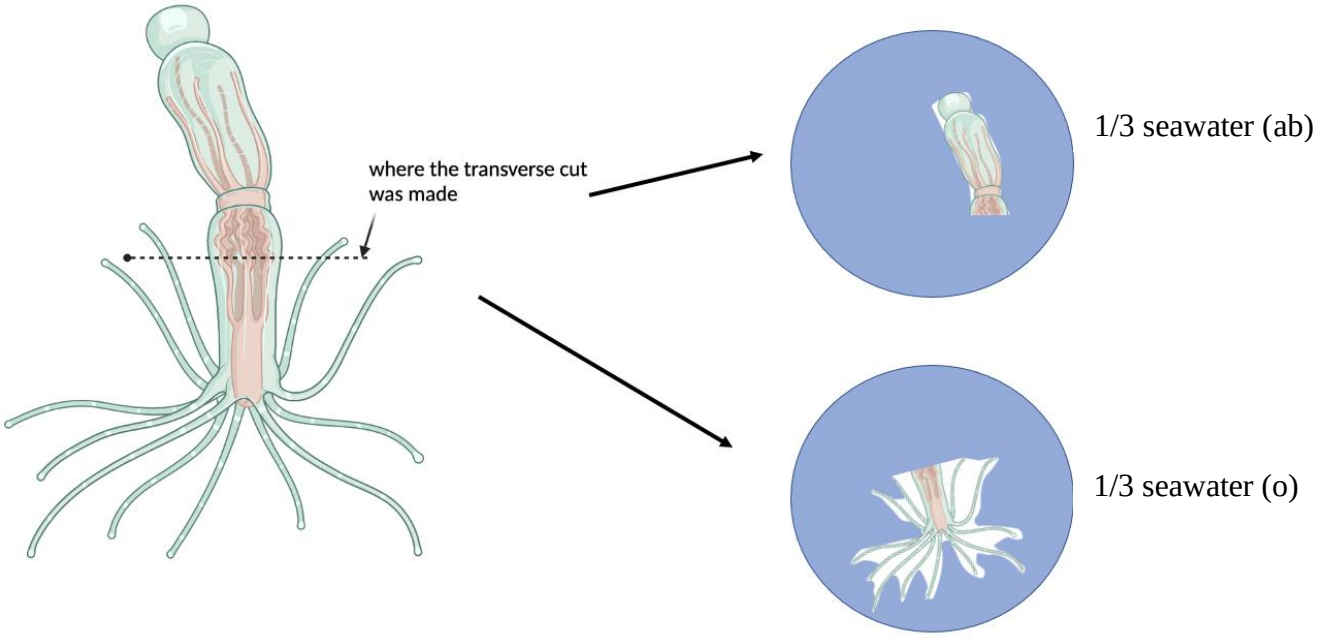

Figure 2: This figure shows where each anemone was roughly amputated using a sterile scalpel. The right side of the illustration shows how the oral and aboral halves of the anemone were split into two different well plates. This was how experiment two was run, the first experiment had both oral and aboral halves in one well.

Six-well Plate Maintenance

Each day after the original cut, one half of each solution in each well was replaced. This allowed the individual mediums to remain fresh while the experiment was running. Additionally, photos were captured each day showing the progress or lack of progress of regeneration in each medium.

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Results and Discussion

In the first experiment there was a clear lack of regeneration within the animals treated with $MgCl_2$. By day eight, animals within the magnesium chloride medium there was no physa elongation or tentacle buds, which hallmark regeneration. Additionally, it appeared as though the anemones had shrunk which brought up the question of whether the animals had died. Also, within experiment one, the animal treated with 100 µM BAPTA-AM, 10 µM BAPTA-AM, and 1 µM BAPTA-AM regenerated very similarly to both the negative control group (1/3 seawater) and the DMSO control. Many anemones, in both control groups as well as in the different concentration BAPTA-AM solutions regenerated by day eight.

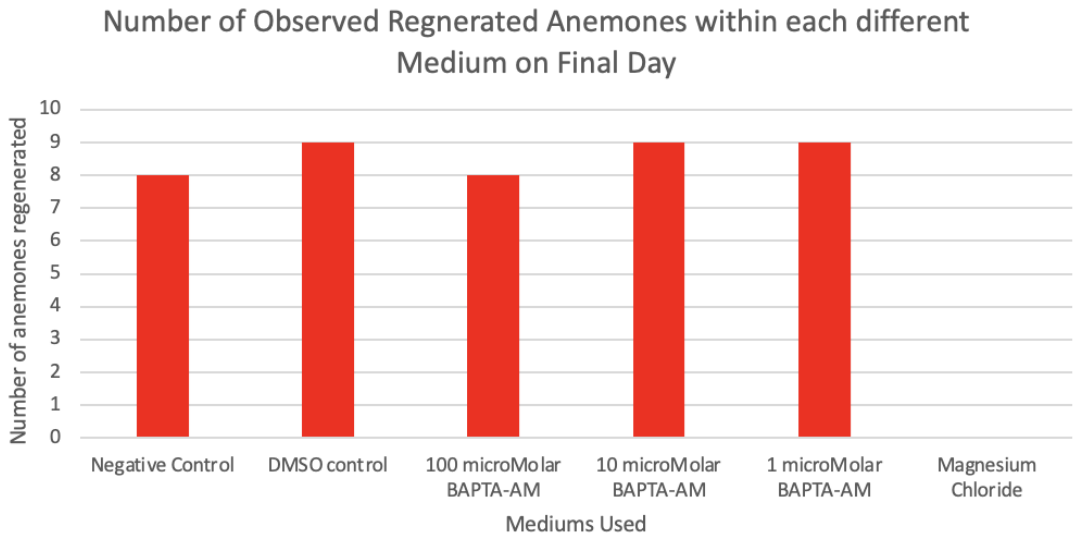

Figure 3: Bar graph detailing the results from experiment 1. Ten anemones total were placed within each medium. As shown in the graph, all mediums except $MgCl_2$ showed high levels of regeneration.

From the results of experiment one it could be concluded that BAPTA-AM did not halt regeneration. Consequently, BAPTA-AM was not included in the second experiment. In its place, benzyl-p-toluenesulphonamide (BTS) was used to observe if reduced muscular activities influenced regeneration. Since the animals within the $MgCl_2$ medium shrunk, but it could not be determined if the animals were alive or not, it was decided that the next experiment needed to be

modified. To solve this issue, the time that the animals were within each chemically treated medium were reduced for experiment two to see if the lack of regeneration is due to muscular and neural inhibition or chemical toxicity.

Results from experiment two differed from those of experiment one. First, regeneration was seen within the $MgCl_2$ treated animals. Although only seen in two out of the six animals, this still shows that regeneration is possible under such neuronal blocking. Wound healing was not seen in the other four animals within the $MgCl_2$ medium. On day five, all the mediums were removed and replaced with 1/3 seawater to see if regeneration would progress in animals that had not shown any growth. All mediums except 200 µM BTS showed an increase in regeneration.

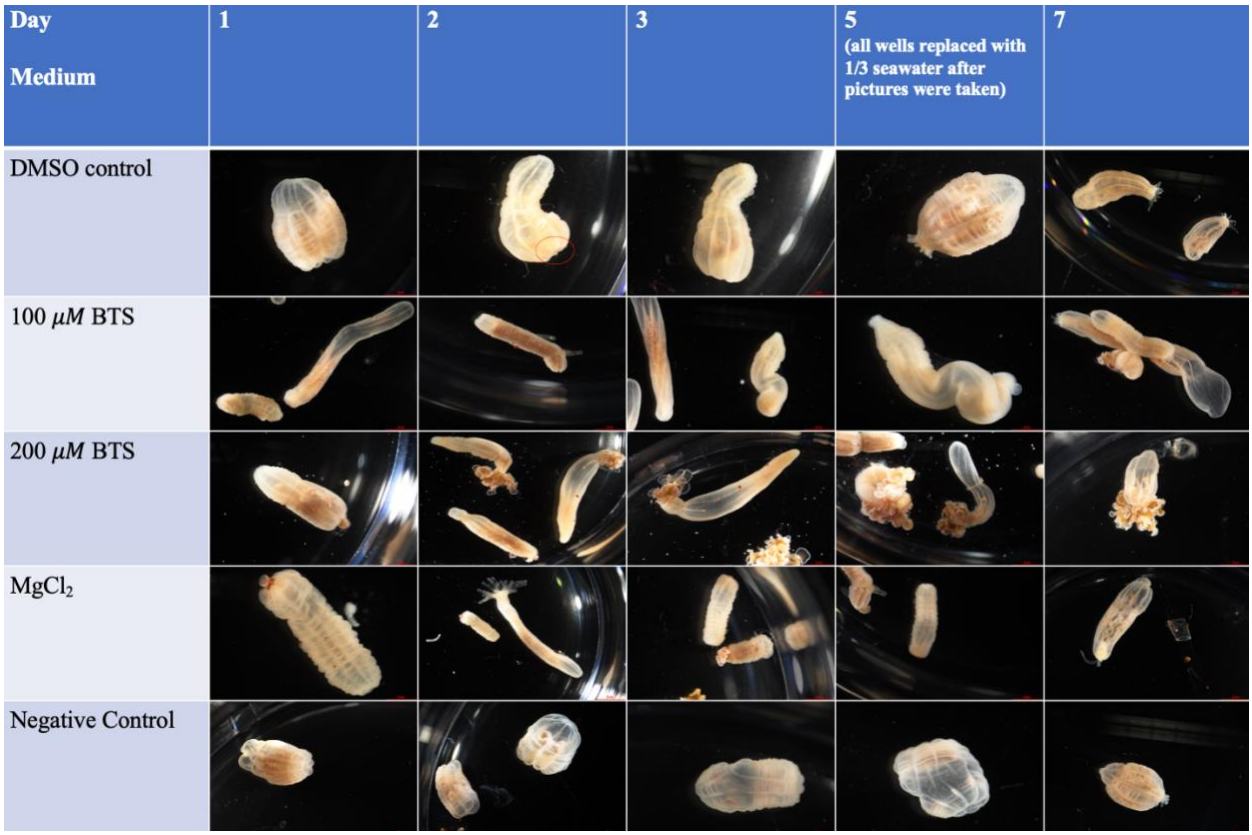

Figure 4: Table showing the progression of regeneration in each medium. This table also points out different phenotypes expressed in different mediums. Animals within the 200 µM BTS medium all showed signs of their mesenteries moving outside their bodies. Furthermore, some animals in the 100 µM BTS medium curled and twisted their bodies.

There were multiple disparities between the control animals when compared to the chemically treated animals. Abnormalities in regeneration were visualized, such as delayed wound healing, loss of tentacles, the lack of formation of oral-facing ridges, and polarity reversal. After 24 hours within the 200 μM BTS medium, two of the six animals that still had their oral ends intact grew tentacles out of the aboral end. This also occurred once in the $MgCl_2$ medium during experiment two. However, this phenomenon was not seen in any of control groups or any of the animals treated with BAPTA-AM.

Figure 5: Images taken of animals after 24 hours within the 200 µM BTS solution. Shown here are the two anomalies that were going under polarity reversal.

This asexual reproduction process is known as polarity reversal. In this process, a new oral crown develops where the physa should be elongating at the aboral end. As this process continues, the mesenteries elongate and eventually degrade while a common physa forms.[8] After weeks or months, the common physa contracts and separates the two halves.[8] However, in the following days these two unique animals in the 200 μM BTS medium and the animal in the $MgCl_2$ medium lost their tentacles. According to a study, polarity reversal was seen at a higher

frequency when the cut made was proximal to the pharynx.[8] This could account for the phenotypic abnormality seen in this experiment; however, the loss of tentacles could be due to chemical treatment. This could be tested more thoroughly by repeating the setup of this experiment but cutting all the animals specifically proximal to the pharynx. This increases the chance of polarity reversal in the animals but could also distinguish if this occurs only within the chemically treated animals or also in the controls. Lastly, if polarity reversal does occur in multiple mediums, one could track the processes and see if they differ from each other.

During the last experiment, only the tentacles were removed from the anemone. This immediately caused a variation in results from the first two experiments. Regeneration occurred much faster and, in all mediums, used. This could be due to the original cut. Relaxation may not have fully worked, as the anemones could still contract their tentacles into their bodies. This may have led to pieces of their tentacles being missed by the original cut. Overnight, these missed tentacles may have been un-contracted making it seem like regeneration happened very quickly. Shrinking also occurred during this experiment in both the $MgCl_2$ medium and the BAPTA-AM mediums.

All three experiments produced interesting and differing results. In the first experiment, there was a clear lack of regeneration in animals treated with the magnesium chloride solution compared to the control animals. However, the results differ in the second and third experiments where regeneration was present in magnesium chloride mediums. According to previous research, the nerve net within *Nematostella vectensis* resizes depending on the physical size of the anemone.[7] Accordingly, the nerve net will decrease in size when the physical size of the animal is decreased.[7] Research on salamander limb regeneration found that an adequate number of pre-existing nerves were vital to the process of regeneration.[8] Consequently, without the

needed number of pre-existing nerves, the limb would not regenerate at all.[8] Pertaining to the results found in each experiment, there may be a connection between the size of the anemone, therefore the nerve-net, and its ability to regenerate. Once the transverse cut is made, the size of the nerve net will decrease in correlation with the physical size decreasing. The animals used in these experiments were not always equal in original size before the cut occurred. This could account for some of the discrepancies in regeneration. To further test this idea, all anemones used would have to be of equal length before any cut could be made. Two different tests should be conducted, one using larger animals and another using smaller animals. Regeneration rate should be tracked closely to see if animals of smaller size regenerate slower than their larger counterparts. Furthermore, those animals treated with chemicals should be observed to see if their regeneration rate is even slower than that of the smaller animals within the 1/3 seawater control groups.

Within each experiment, the rate of regeneration was slower in both the $MgCl_2$ medium and the BTS medium. Both agents block important pathways, both neural and muscular, within *Nematostella Vectensis*. $MgCl_2$ acts as a general anesthetic which in turn limits the interactions between neurons. Since the interactions between neurons are limited while anesthetized, signals cannot be sent to direct muscle movement. Benzyl-p-toluenesulphonamide (BTS) directly reduces the number of attached cross-bridges between myosin and actin removing muscular ability.[9] It was found that the 200 μM solution of BTS was far more effective in limiting regeneration compared to its 100 μM BTS counterpart. According to a study done, muscle contractions may play a potential role in wound healing and regeneration.[10] Thus, having a stronger solution of BTS would inhibit more power strokes than that of the 100 μM BTS solution. From the data collected, it seems that both the muscular system and the nerve net play a

vital role in regeneration. However, chemical toxicity could also play a role in the lack of regeneration within these animals. *N. vectensis* induce stress responses by using efflux pumps and oxidative biotransformation to rid itself of toxins.[11] It could be hypothesized that in the prolonged presence of a chemical, the stress responses within the animal would increase which could divert the process of regeneration in favor of riding itself of toxicity.

Conclusion

Although no medium used in these experiments completely halted regeneration in *N. vectensis* each time, many showed results for slowing the process down immensely. BTS and magnesium chloride mediums had the largest effects on regeneration within the experiment, causing many abnormalities in the animals. These abnormalities could possibly be attributed to the lack of full function from their nervous and muscular systems or chemical toxicity. More research on stress-responses to each chemical used and size of the anemone nerve nets should be conducted to find out specifically what is causing these abnormalities to appear. Although no solid evidence was found for the nervous system controlling regeneration, such as the complete halting of the regeneration process, collected data showed it could possibly be both muscular and nervous systems working together to complete regeneration.

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