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IN VITRO INCUBATION OF GILLS ISOLATED FROM THE GULF KILLIFISH, *FUNDULUS GRANDIS*

An Honors Thesis

Presented to

the Department of Biological Sciences

of the University of New Orleans

In Partial Fulfillment

of the Requirements for the Degree of

Bachelor of Science, with

Honors in Biology

By

Joseph Anthony Diaz

May 2014

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Most of all, thank you to my wonderful family. None of this would be possible without the love and devotion of my wife, Stephanie. Her unwavering support allows me to reach for the stars. And to my children, Allister and Aviana, thank you for being my ever-present muses.

Abstract

The use of viable and reliable tissue preparations allows for experimental approaches *in vitro* that would not be possible *in vivo.* In this experiment, gills were isolated from gulf killifish, *Fundulus grandis*, and subjected to various incubation conditions. A combination of two types of media and two incubation apparatuses were compared in their ability to sustain tissues, as measured by the vital stain trypan blue (TB) and percent lactate dehydrogenase (LDH) leakage. Trypan Blue analysis indicated no significant difference in tissue viability, regardless of the treatment or incubation time. However, percent LDH leakage analysis revealed that the two most influential variables were the medium and apparatus in which the gills were incubated. Interestingly, incubation time did not have a statistically significant influence on gill viability as measured by LDH leakage. The results of LDH leakage analysis suggest that incubation using SW in the 4 ml apparatus is the best combination tested.

Keywords: Gill, Incubation, Organ, Medium, Apparatus, *Fundulus grandis*

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Introduction

Fishes have proven to be a valuable resource with respect to physiological and biochemical studies. Due to the complex and varying conditions of the aquatic environment, many species of fish have developed compensatory mechanisms that allow them to survive in habitats that fluctuate drastically. Because of their development of these mechanisms, fish have become commonly used tests subjects for the exploration of physiology (Heath, 1995). One such genus of fish is *Fundulus* (Burnett, *et al.* 2007), which includes the gulf killifish, *F. grandis*.

Studies on whole animal subjects can often prove to be challenging (Ritchie, *et al.* 1971). Introducing an animal to hypoxia, for instance, may lead to behavioral and whole organismal compensatory mechanisms. Conversely, using isolated cells may prove to be less valuable than whole tissues in physiological studies because isolating cells negates intratissue compensatory mechanisms that are seen *in vitro* (Groneberg, 2002). One of the issues with the tissue isolation studies done by Groneberg (2002), Ritchie (1971) and Schreiter (2012) are the costs and skill associated with perfusing whole tissues, both of which are exacerbated by the use of the small organs often used in comparative biology research. For these reasons, developing techniques that are both cost effective and easy to accomplish on isolated organs are of great importance for physiological studies.

A key factor in tissue culture is the medium in which the tissue is incubated. When developing a medium that can sustain viability of a tissue, one must take into consideration osmolatiry, ion concentration, a source of energy, and contamination of both the medium and the tissue. Isoosmolarity between the tissue and medium is

important because a tissue will expend a great deal of its energy trying to maintain its volume and internal concentration to that of the levels which will support its metabolic activity (Bradshaw, *et al.* 2012 and Willenborg, *et al.* 2012). In an isolated tissue preparation this becomes very important because that tissue's energy sources are not being replenished at the same rate as *in vivo*, if at all.

Similarly, much consideration must be given to the fact that microbial infection leads to rapid tissue death (Stanbridge, 1971). This is of particular importance for killifish gill incubation because gills are in direct contact with the estuaries from which the fish came and these estuaries are teeming with microbes (Crump, *et al.* 2004). It would be very common for these microbes to be carried over from tissue dissection to the *in vitro* incubation apparatus, where the microbes would be able to multiply and lead to rapid degradation of the tissue.

The aim of the present study is to develop an easy and low cost method of maintaining viability of gills isolated from the gulf killifish. Most techniques for tissue isolation include perfusion of the tissue using variations of physiological saline (Pierrot, *et al.* 1995 and Ohtsu, *et al.* 1989). In these cases, a tissue was isolated, followed by canulation of the afferent vasculature. Various types of physiological saline can then be perfused into the tissue through the canulation. This technique requires considerable skill and the required equipment is often expensive. Here, the approach was to use entire gills bathed in a solution without canulation and perfusion to assess whether tissue remained viable without these additional steps.

Materials and Methods

Husbandry

Fish were acquired from either Joe's Landing Marina in Barataria, LA or Rigolet's Bait and Marina in Slidell, LA. They were treated with API anti-bacteria (Ab) and anti-parasitic treatments (Furan-2 and General Cure, respectively) and allowed to acclimate to the laboratory for at least two weeks before experimentation. The fish were divided into 38 l tanks that supported no more than 7 fish at a time in dechlorinated New Orleans tap water to which was added Instant Ocean in order to reach a 1/3 concentration of seawater, as was measured by specific gravity. Water temperatures were kept between 17°C and 22°C and the fish were on a 16:8 light:dark cycle. The fish were fed approximately 0.1 % body mass of commercial dried fish flakes five to six times per week. Food was withheld for 24 h prior to euthanasia. Fish were sacrificed in an overdose of MS-222 (0.25 g of MS-222 and 1.0 g of $NaHCO_3$ per l of 1/3 seawater). All research conformed to national and institutional guidelines for research on vertebrate animals (protocol no. UNO-13-005).

Incubation Apparatus

There were 2 designs for the incubation apparatuses. Apparatus 1 consisted of a four well cell culture plate holding between 1.5 and 2 ml of the appropriate medium and tissue and then was placed in a Billups-Rothenburg Modular Incubation Chamber that was continuously supplied humidified air via a standard aquarium air pump. The incubation chamber was placed on an orbital shaker set to 60 rpm (Figure 1a).

Apparatus 2 consisted of capped 15 ml conical tubes in which gills were individually incubated in 4 ml of media. The tubes were fed ambient air via an aquarium air pump through aquarium tubing. On the end of the tubing, were plastic pipettes which had a 5 mm hole drilled approximately 10 mm from the tip. The pipettes were inserted through a hole in the cap such that the open end of the pipette was resting on the bottom of the conical tube (Figure 1b). The target bubbling rate of the conical tubes was approximately 10 bubbles per second, or approximately 60 ml per minute per tube.

Dissection and Incubation Procedures

After euthanasia, fish were blotted with a paper towel and weighed. Gill arches were then dissected and placed in a petri dish containing $\frac{1}{3}$ SW. Individual gills (eight per fish) were isolated and put in their respective incubation medium. The average time between euthanasia and the start of *in vitro* incubation was 5 min.

Viability Analyses

Gills were sampled at either 2 or 24 h of incubation and analyzed in one of two ways. They were either stained with TB or the tissue and media were separated and snap frozen using either a 95% ethanol and dry ice bath or liquid nitrogen. Both medium and tissue were then stored at - 80° C for later analysis of LDH content. For media samples of 1.5 ml, the medium was concentrated using Amicon Ultra-4 10 K Centrifugal Filters before freezing.

Trypan Blue Staining

Staining with TB was achieved by adding TB stock to the medium to achieve a final concentration of 0.04% TB. The gills were left in the TB stain for no more than 7 min, at which time they were extracted from the stain and briefly rinsed with $\frac{1}{3}$ SW. After this, residual mucous was removed from the gills and gills were photographed using a Leica MZ75 dissection scope and camera connected to a computer using the Leica Application Suite software.

The photographs were then analyzed for percentage stained using ImageJ. The percent stained was calculated by using the Color Threshold option to isolate the gill itself from noise in the photograph. After this, the gill arch was cut out from the picture. Last, the Color Threshold option was again used to select all pixels in the photograph that appeared blue and divided this number by the number of total pixels in the gill. This was done to both sides of each gill and the percent TB staining of the gills was calculated as the mean of the two sides.

Lactate Dehydrogenase Analysis:

Lactate dehydrogenase content analyses were done in both gill and medium by conducting assays as outlined in Martinez *et al.* (2006). When incubating in 1.5 ml of medium, the medium was first concentrated using Amicon Ultra-4 10 K Centrifugal Filters to approximately 700 ul. Briefly, the tissues were homogenized in 100 mM HEPES (pH 7.4), 10 mM KCl, 0.1 mM DTT, and 0.2% Triton X-100. Next, the homogenate was centrifuged for 15 minutes at 2400 x g. The supernatant was then used for LDH assays. Each assay included 100 mM HEPES (pH 7.4), 10 mM KCl, 0.17 mM

NADH, and tissue supernatant or medium in 0.95 ml. After reading the background change in absorbance, 50 ul of 20 mM pyruvate was added the get a final pyruvate concentration of 1 mM. The average change in absorbance over the first 30 sec (60 data points) after pyruvate addition was used to calculate LDH activity using the equation:

$$Units\ of\ LDH = \frac{change\ in\ absorbance \times volume\ of cuvette}{absorbance\ coefficient\ of\ NADH \times path\ length\ of\ cuvet}$$

The assays were conducted using a Beckman DU 640 spectrophotometer.

Percent LDH leakage was calculated using the following equation:

$$Percent\ LDH = \frac{Units\ LDH\ in\ Medium}{Units\ LDH\ in\ Medium + Units\ LDH\ in\ Gill}$$

Total Protein Analysis:

Protein analysis was done as outlined by Brown *et al.* (1989), using bovine serum albumin as the standard. Specific activity was calculated as units LDH/mg total protein.

Data Analysis:

The data for the gills were tested with two tailed t-tests in order to determine which factor had the greatest influence on tissue viability. Next, a three way analysis of variance (ANOVA) was conducted for measures of tissue viability to test for differences between incubation time, incubation medium, apparatus and the interaction between these variables. All results are expressed as means ± one standard deviation. The results of LDH leakage from one fish were excluded because 3 out of 4 of the results from that fish were outliers.

Results

Optimization

Results of early experiments clearly indicated a need for optimization of the experimental design. Both LDH leakage and Trypan Blue staining showed more than 90% death of the tissue after 24 h of incubation (Figure 2). By combining the methods of previous studies and developing my own protocols, I was able to optimize the experimental design of the current study by testing a variety of incubation media. The first of which consisted of dechlorinated New Orleans tap water that was treated with Instant Ocean such that its salinity was 1/3 that of ocean water, which I will now refer to as 1/3 seawater. This resulted in a significant amount of tissue death after 24 hrs of incubation. The next step in optimization was to test the saline from Genz, *et al.* (2011) and add 5.5 mM glucose to the 1/3 sea water medium. This resulted in a significant improvement in tissue viability shown by TB staining (Figure 3).

However, both of the media appeared murky post treatment and LDH analysis suggested that there was significantly more cell death than was evidenced by TB staining. Furthermore, both the 1/3 seawater and Genz, *et al.* (2011) physiological saline medium developed what appeared to be significant bacterial growth after two days of storage at room temperature. In response to these observations, I filter sterilized both the 1/3 seawater and the modified Genz, *et al.* (2011). Immediately prior to experimentation, I would then add penicillin, streptomycin, amphotericin and glucose from a filter sterilized stock to reach a final concentration of 5.5 mM. I will now refer to the combined

solutions as $\frac{1}{3}$ SW for the 1/3 sea water based solution and PS (Table 1). This resulted in low TB staining (Figure 4) and relatively low LDH leakage.

General Results

Seven fish (4 male and 3 female) were used in the course of this study. Table 2 summarizes the mean ± 1 standard deviation for all fish used in for data analysis. The table includes fish mass, gill mass, gill protein content normalized for gill mass, gill LDH content normalized for gill mass, and LDH specific activity. Table 3 is a summary of the affects each variable of incubation had on the viability of gills as indicated by LDH leakage and TB staining. The variation in sample size is due to the exclusion of samples from one fish because three out of four of these samples were outliers in LDH analysis. It is noteworthy that there was a large discrepancy between tissue viability between the two measurements.

Results of Means Comparisons

As is illustrated by Figures 5 and 6, analysis of LDH leakage revealed significantly less tissue death when using $\frac{1}{3}$ SW as the incubation medium ($p \leq 0.05$). Conversely, when using TB as the marker, there was no significant difference in tissue viability between the two types of media ($p = 0.26$). Comparisons between LDH as a function of apparatus showed that there was a trend toward less tissue death when using Apparatus 2 ($p = 0.07$). However, the t-test of TB staining showed no difference between the Apparatuses ($p = 0.70$). Interestingly, neither LDH nor TB staining indicated any

difference in tissue viability over the course of the 22 hr incubation ($p = 0.32$ and 0.12, respectively).

Analysis of Variance

Comparisons using ANOVA resulted in similar results. The only statistically significant result was the influence of medium on tissue viability as measured by percent LDH leakage ($F_{1,20} = 6.223$, $p = 0.021$). Near significant values for apparatus ($F_{1,20} = 4.20$, $p = 0.054$) and a apparatus and time interaction ($F_{1,20} = 3.912$, $p = 0.062$) are worth noting. Incubation time itself did not have a significant influence on tissue viability according LDH measurements ($F_{1,20} = 0.98$, $p = 0.33$) (Table 4A).

Analysis of TB staining revealed no significant difference in tissue viability regardless of medium ($F_{1,20} = 1.13$, $p = 0.30$), apparatus ($F_{1,20} = 0.049$, $p = 0.83$), or duration of incubation ($F_{1,20} = 2.63$, $p = 0.21$) (Table 4B).

Discussion

Optimization

The early results of TB staining led me to believe that there was significant tissue death (> 90%). This was likely due to a combination of factors, including an ion imbalance between the media and tissue, bacterial contamination of both the media and tissue, and a lack of energy source for the tissue to fulfill critical metabolic pathways. By combining the techniques developed in this experiment, namely the use of an isoosmotic medium, and those that included Ab and Am (Kelly, *et al.* 2000) and glucose (Genz, *et al.* 2011), a viable medium was devised. When taking into account the nearly-significant effect of apparatus and significant preference for $\frac{1}{3}$ SW, this study suggests that Apparatus 2 with $\frac{1}{3}$ SW is the preferred method of incubation.

There are some considerations that must be taken into account when using Apparatus 2. Primarily, inconsistencies inherent to the bubbling mechanism can lead to premature death if too low or agitation of the tissue if too high. There were a couple of instances when, after being set to the rate described above, the apparatus would significantly increase or decrease over time. I believe that this may have contributed to the high variation of the data collected from Apparatus 2.

This may be avoided by using a different source of air flow. Store bought aquarium pumps are not of particularly high quality. This means that the output of these pumps can be expected to vary in their rate. This may be avoided in the future by replacing the aquarium pump with a compressed air source. Another concern inherent to Apparatus 2 that must be taken into account is the relative difficulty of use

when compared to Apparatus 1. The design of Apparatus 2 lends itself nicely to incubating many tissues at once, with consistent exposure to all of the tissues because 4 tissues per plate and many plates can be incubated in the same incubation chamber simultaneously.

This consistency among tissues is not as true with Apparatus 2. The separate environments of the conical tubes and air sources in the Apparatus 2 can lead to variation among the exposures of the incubated tissues, whereas the Billups-Rothenburg Modular Incubation Chamber is able to hold many incubation plates, with multiple tissues per plate. This means that every tissue within that chamber is exposed to the same environment.

Applications

The primary focus of this study was to identify methods of maintaining viability of isolated whole gills. The target viability was arbitrarily set at 10% death. That is, if a tissue was shown to have experienced 10% or less death after 24 hrs of incubation, that tissue would still be considered viable. As is shown by Table 3, both the LDH and TB markers indicate that the mean tissue death was less than 10% when using $\frac{1}{3}$ SW in Apparatus 2, making this combination a viable option for future studies requiring whole gill incubation.

Other Considerations

Many studies that focus on biochemical responses to stressor are done with insults that last well under 24 hrs. The present study suggests that exposures using these

apparatuses may last up to 24 hrs. This is important because many cells and tissues have mechanisms of compensation in place that act to buffer the effects of stressors such as hypoxia that are not depleted until long after exposure. For instance, Jibb, *et al.* (2008) showed that brain, heart and gill tissues sampled from whole fish exposed to 12 h of hypoxia did not have measurable molecular responses and that skeletal muscle just began to exhibit those responses at the 12 h mark. By showing that keeping tissues alive for a long period of time is possible, future studies can use them as a control against those exposed to longer-lasting stressors than is typical, leading to broader understandings of those mechanisms.

While both LDH leakage and TB staining are widely used as viability markers, the present study showed that there is not a direct correlation between the results of the methods. This may be explained by taking into consideration that TB is typically used as a cell culture viability stain. This means that TB blue is typically introduced onto a two dimensional culture, resulting in all cells in that culture becoming exposed to the viability stain. In this study, TB was only introduced to the superficial cells of the mucosal side of the gills. This means that the only cells that had an opportunity to take up TB were those that were in direct contact with the solution. This would exclude a great majority of cells that make up the overall composition of the tissue, yielding artificially low levels of TB staining.

On the other hand, leaked LDH levels may have been read as higher than what were actually released by the gills' cells. This may be accounted for by a few considerations. First, the rate of contamination of the gills themselves is most likely high (Heath, 1995). A major portion of these contaminants are likely to have been from

bacteria and parasites, both of which are known to have LDH in their cells. Therefore as the bacteria were exposed to the Ab and Am of the $\frac{1}{3}$ SW and PS, they would die and release LDH that would leak into the medium, raising the percent LDH, inducing an artificially high leakage amount.

Similarly, the likelihood for parasitic infection of the gills is high and was observed in some of the gills sampled. The increased stress placed on the gill tissue would likely cause depletion in the nutrient production from which parasites feed. This would eventually cause death of the parasites and a release of their LDH into the medium. This additional contribution of LDH would result in an increased estimation of gill tissue death. Lastly, the dissection of the tissues inherently resulted in damage to those tissues. This damage would have resulted in an exacerbated death rate to adjacent cells.

Conclusion

The present study set out to identify an easy and affordable means of whole tissue incubation. As is evidenced by the LDH leakage, the more stringent of the two measures of viability, it was successful. With a mean death rate of 6.1 ± 7.7% after 24 hrs of incubation (Table 3), and a probable explanation of the high error explained by a vast improvement of dissection over the course of the study, I can confidently recommend Apparatus 2 with $\frac{1}{3}$ SW for future studies aimed at whole gill analyses, as long as compressed air is used as an air supply.

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Table 1. Physiological saline composition modified from Genz, *et al.* (2011). * signifies addition of solutions was immediately before use.

Compound	Concentration
NaCl	144.1 mM
KCl	5.1 mM
$CaCl_2 \times 2H_2O$	1.6 mM
$MgSO_4 \times 7\ H_2O$	0.9 mM
$NaHCO_3$	11.9 mM
NaH_2PO_4	2.9 mM
*Glucose	5.5 mM
*Penicillin	1000 I.U./ml
*Streptomycin	1000 I.U./ml
*Amphotericin	0.25 ug/ml

Table 2. Summary of experimental fish and gill measurement used for final data collection. Values are expressed as means ± S.D.

Sex (Male : Female)	Fish Mass (g)	Gill Mass (g)	Gill Protein Content (mg/g)	Gill LDH Content (U/g)	LDH specific activity (U/mg)
4:3	9.38 ± 6.41	0.0169 ± 0.0070	24.5 ± 9.5	51.8 ± 22.6	2.26 ± 0.85

Table 3. Means for viability measurements in the various experimental treatments.
Values are expressed as means ± S.D. (n = 3 or 4 per treatment)

Medium	Apparatus	Incubation Time (hr)	Percent LDH Leakage	LDH Specific Activity (U/mg)	Percent TB Stain
PS	1	2	17.2 ± 1.7	2.21 ± 0.65	0.8 ± 0.7
PS	1	24	21.1 ± 6.7	1.98 ± 1.45	0.9 ± 1.2
PS	2	2	18.5 ± 13.2	2.10 ± 1.08	0.8 ± 0.2
PS	2	24	10.8 ± 9.1	1.32 ± 1.09	1.6 ± 0.7
$\frac{1}{3}$ SW	1	2	6.2 ± 6.6	2.23 ± 1.03	1.0 ± 0.4
$\frac{1}{3}$ SW	1	24	20.4 ± 10.6	2.30 ± 1.01	2.0 ± 1.5
$\frac{1}{3}$ SW	2	2	4.4 ± 2.4	1.81 ± 0.72	1.1 ± 0.8
$\frac{1}{3}$ SW	2	24	6.1 ± 7.7	2.48 ± 0.49	1.6 ± 1.3

Table 4. Results of three way ANOVA on measures of tissue viability.

A. Lactate dehydrogenase leakage

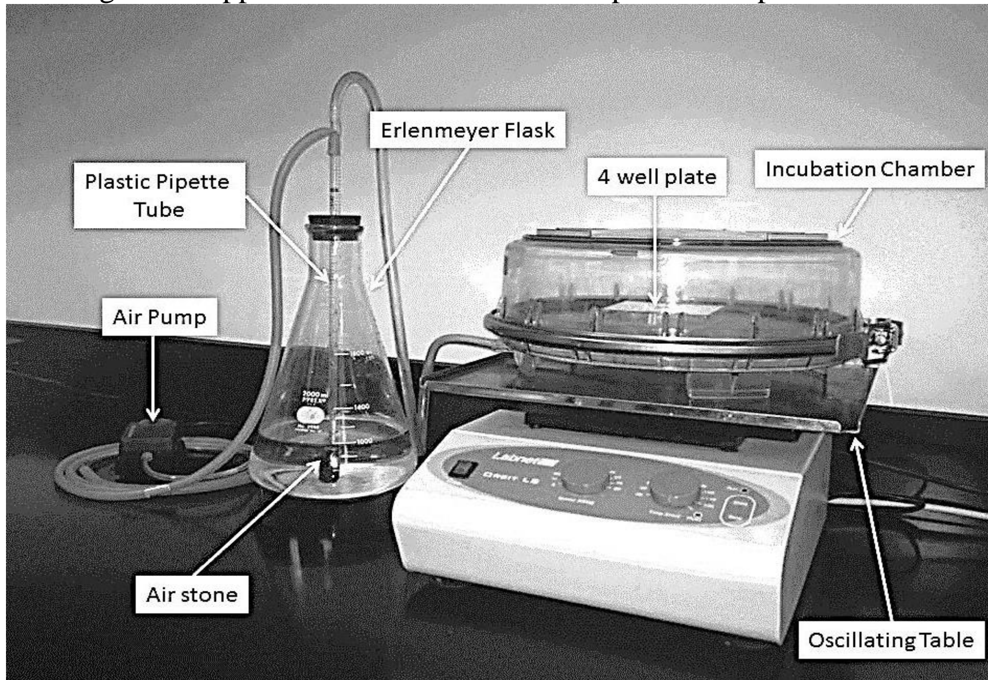
Source	Sum of Squares	Degrees of Freedom	Mean Squares	F-Ratio	P value
Medium	395.799	1	395.799	6.223	0.021
Apparatus	266.830	1	266.830	4.195	0.054
Time	62.536	1	62.536	0.983	0.333
Medium X Apparatus	20.928	1	20.928	0.329	0.573
Medium X Time	165.824	1	165.824	2.607	0.122
Apparatus X Time	248.822	1	248.822	3.912	0.062
Medium X Apparatus X Time	0.416	1	0.416	0.007	0.936
Error	1,272.060	20	63.603		

B. Trypan blue staining

Source	Sum of Squares	Degrees of Freedom	Mean Squares	F-Ratio	P value
Medium	1.033	1	1.033	1.126	0.301
Apparatus	0.045	1	0.045	0.049	0.826
Time	2.411	1	2.411	2.628	0.121
Medium X Apparatus	0.389	1	0.389	0.424	0.523
Medium X Time	0.151	1	0.151	0.165	0.689
Apparatus X Time	0.007	1	0.007	0.008	0.931
Medium X Apparatus X Time	0.724	1	0.724	0.789	0.385
Error	18.347	20	0.917		

Figure 1. Diagram of the two incubation apparatuses

A. Diagram of apparatus 1. See text for a complete description.



B. Diagram of apparatus 2. See text for a complete description.

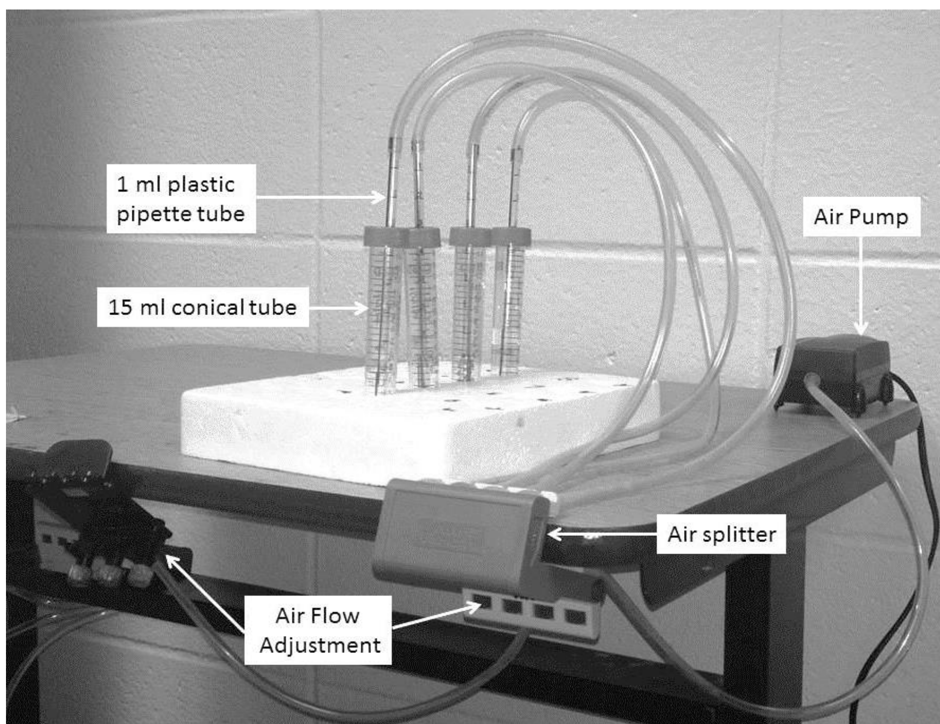


Figure 2. Trypan Blue staining of a gill incubated for 24 hrs in $\frac{1}{3}$ SW without glucose, antibiotics, or antimycotic added to the solution. Lower image corresponds approximately to boxed area in upper image.

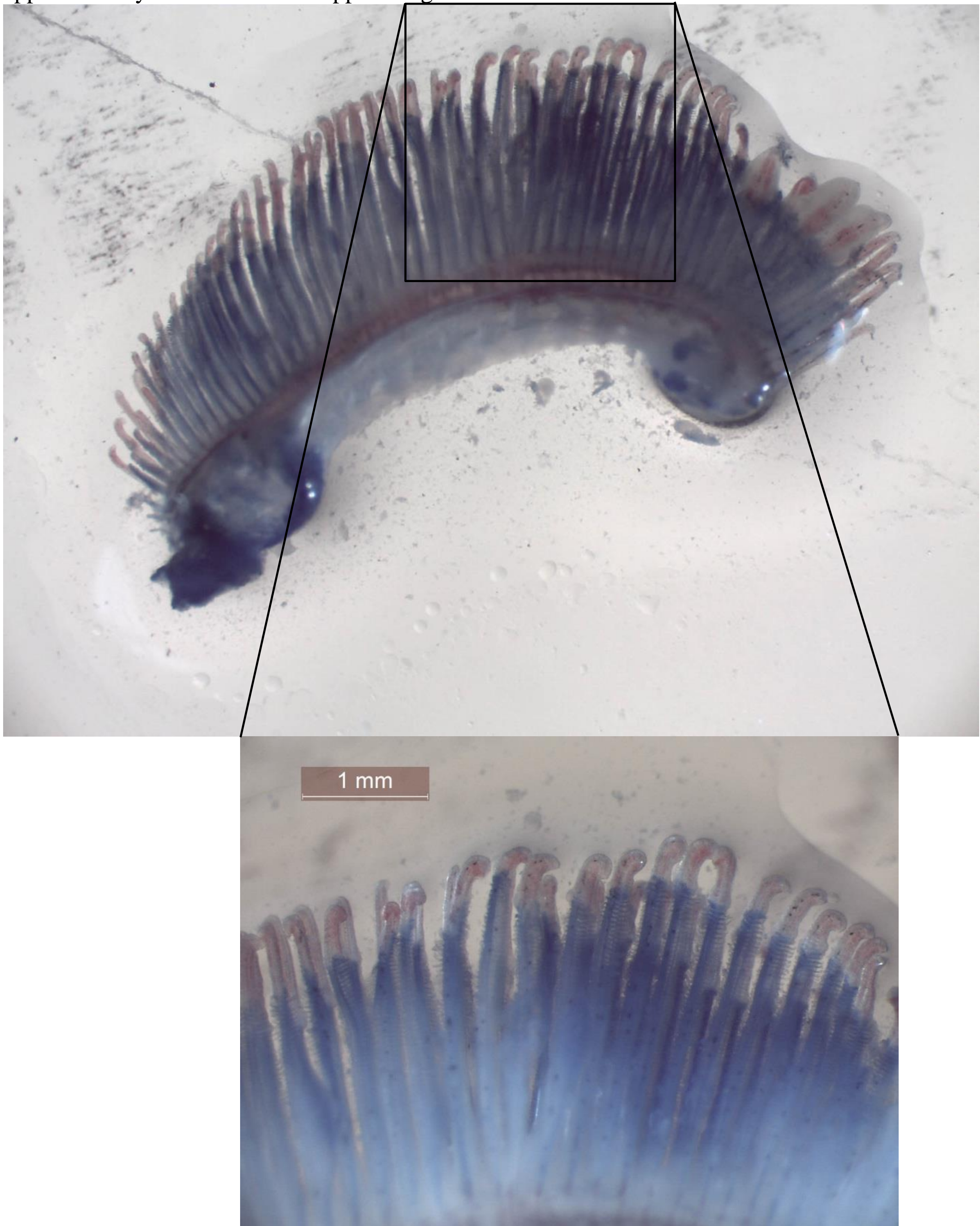


Figure 3. Trypan Blue staining on a gill incubated for 24 hrs in $\frac{1}{3}$ SW with glucose but without antibiotics or antimycotic added to the solution. Lower image corresponds approximately to boxed area in upper image.

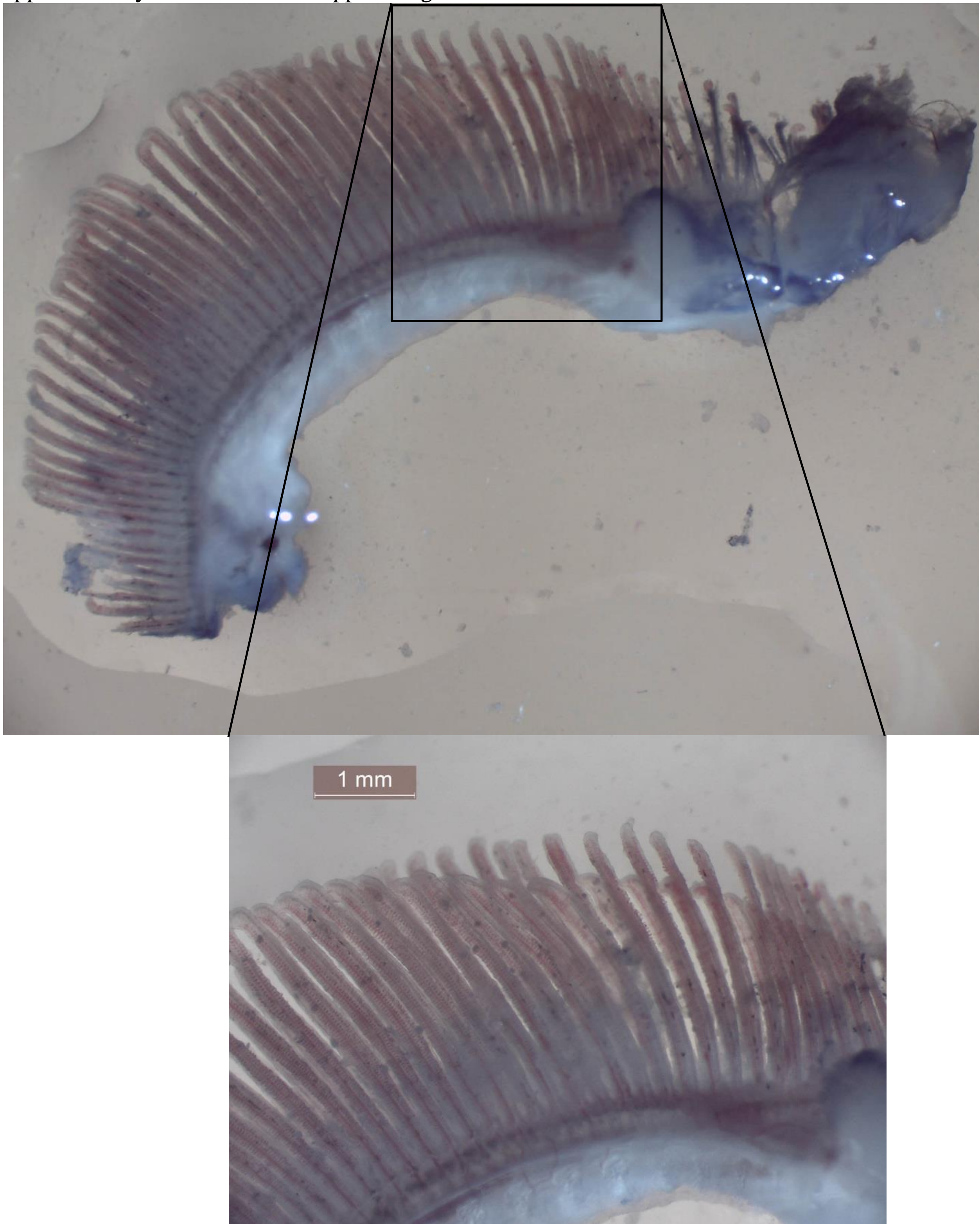


Figure 4. Trypan Blue staining on a gill incubated for 24 hrs in $\frac{1}{3}$ SW with glucose, antibiotics, and antimycotic added to the solution. Lower image corresponds approximately to boxed area in upper image.

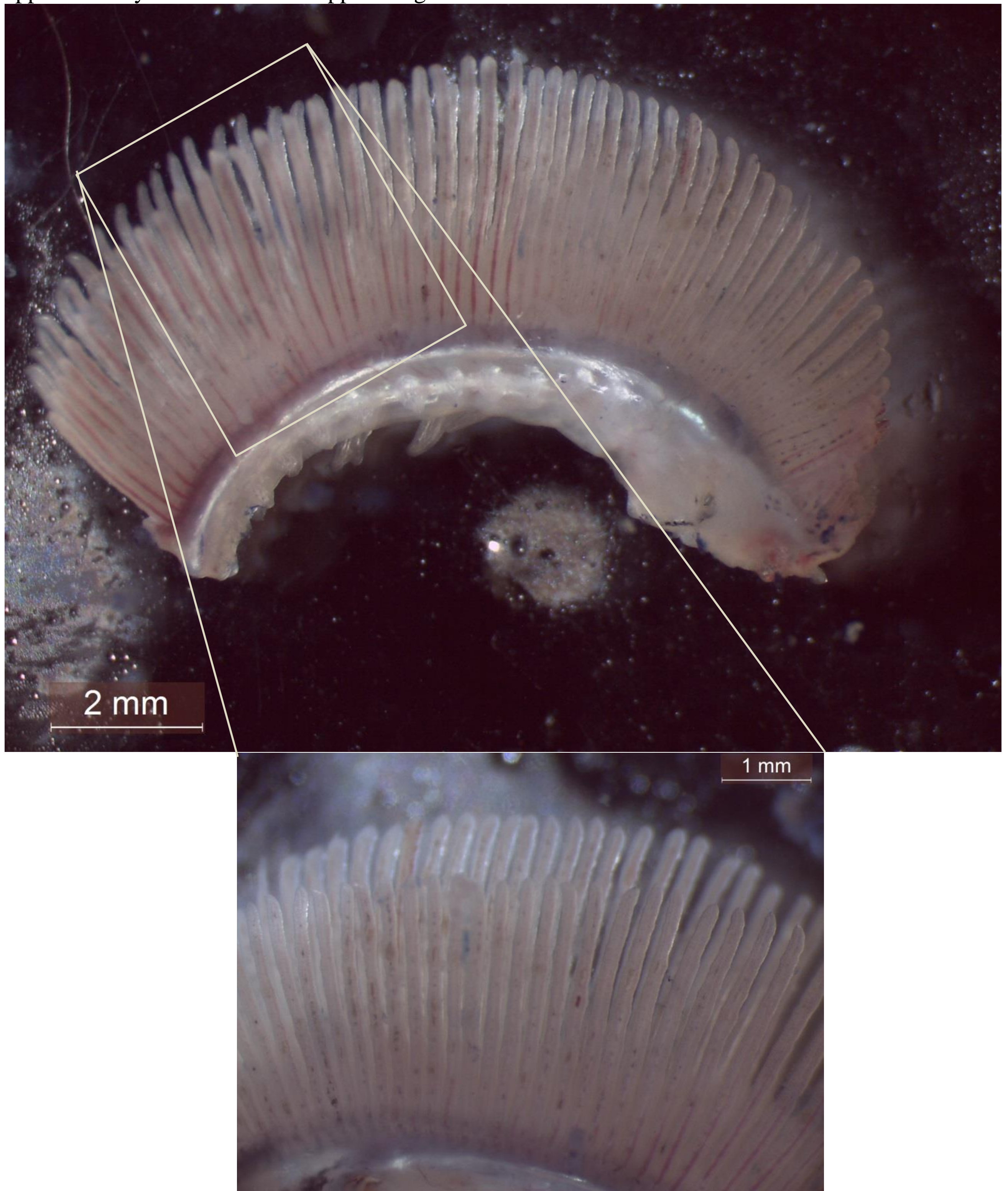


Figure 5. Effects of the treatments on the percent of tissue LDH leakage. Three or four gills per treatment. Bars are SD. * Signifies a statistically significant difference ($p \leq 0.05$).

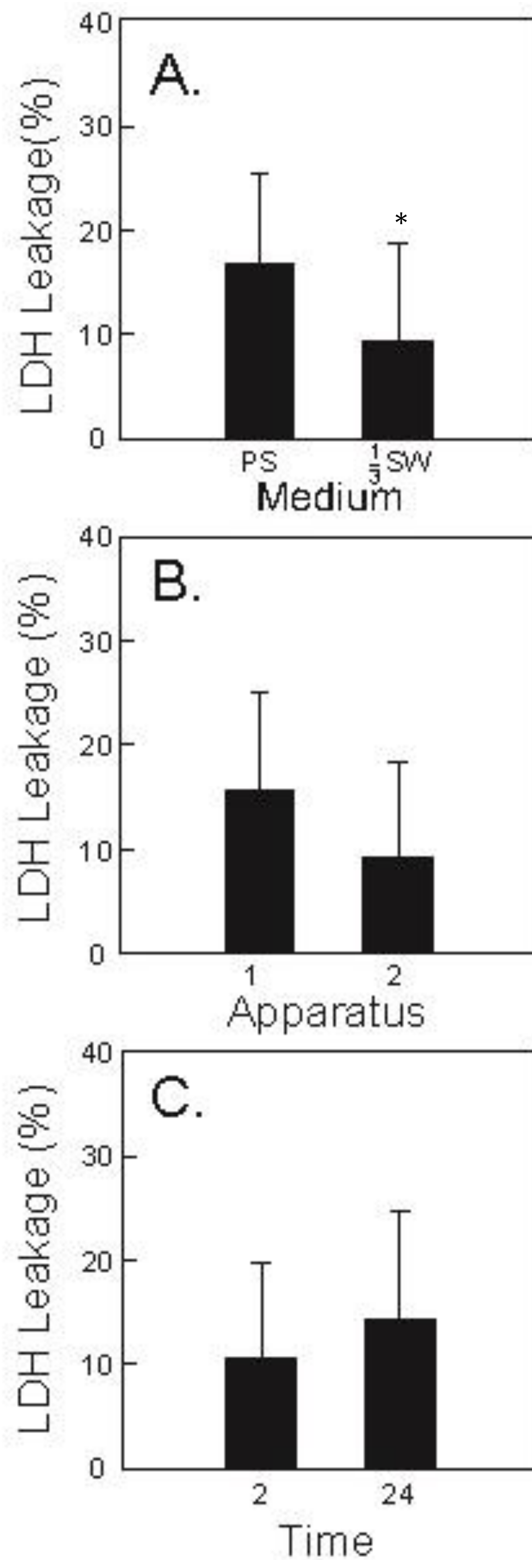
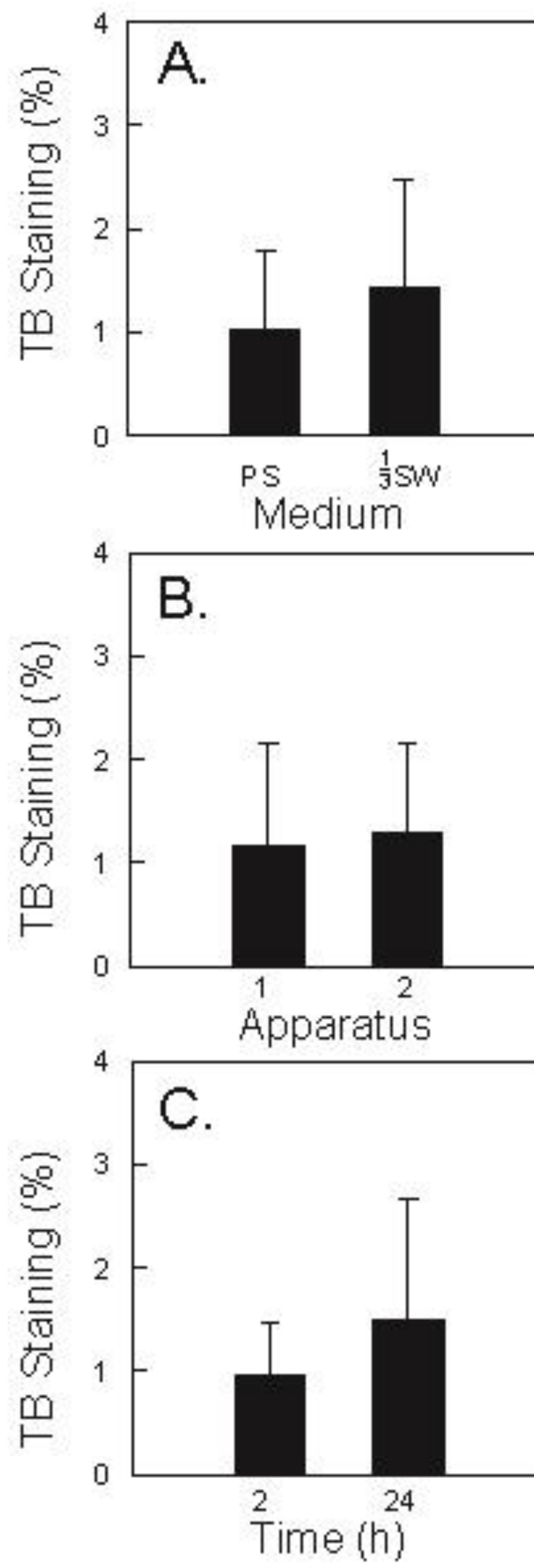


Figure 6. Effects of treatments on the percent of tissue staining experienced by the gills. Three or four gills per treatment. Bars are SD.



APPROVAL SHEET

This is to certify that Joseph A. Diaz has successfully completed his Senior Honors Thesis, entitled:

In Vitro Incubation of Gills Isolated from the Gulf Killifish, Fundulus Grandis

Bernard B Rees
________________________ Director of Thesis
Bernard B. Rees

________________________ Second Reader
Jacqueline B. Nesbit

Abu Kabir Mostofa Sarwar
________________________ for the University Honors Program
Abu Kabir Mostofa Sarwar

April 30, 2014
Date