**Effects of desiccation practices and ploidy on *Vibrio* spp. abundances of cultured oysters, *Crassostrea virginica***

by

Stephanie M. Grodeska

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Approved by

William C. Walton, Co-chair, Associate Professor of Fisheries, Aquaculture & Aquatic Sciences
Jessica L. Jones, Co-chair, Research Microbiologist, FDA Gulf Coast Seafood Lab
Covadonga R. Arias, Professor of Fisheries, Aquaculture & Aquatic Sciences
Ruth H. Carmichael, Associate Professor of Marine Sciences, Dauphin Island Sea Lab

Abstract

The expansion of off-bottom aquaculture of the eastern oyster, *Crassostrea virginica*, to the Gulf of Mexico accompanied by high temperatures, which are correlated with *Vibrio parahaemolyticus* and *Vibrio vulnificus*, has raised human health concerns. Two routine oyster desiccation practices expected to elevate *Vibrio* spp. abundance in the oysters, 3 hour freshwater dip followed by 24 hour ambient air exposure and 27 hour ambient air exposure, were investigated in diploid and triploid *C. virginica* to determine the number of days of re-submersion prior to harvest necessary to drop *Vibrio* spp. levels back to ambient (non-elevated) levels. Results indicate that seven days of re-submersion allowed *Vibrio* spp. levels to return to submersed levels and decrease from elevated levels. This study found no consistent significant differences between ploidy, triploid and diploid, or desiccation practice, indicating no additional benefit or risk using either desiccation practice, with appropriate re-submersion time, or oyster ploidy.

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List of Abbreviations

| | |
|---|---|
| ADPH | Alabama Department of Public Health |
| ALS | Australian Long Line system |
| ANOVA | Analysis of Variance |
| AU AML | Auburn University Aquatic Microbiology Laboratory |
| CDC | Centers of Disease Control and Prevention |
| CFU | Colony Forming Unit |
| FAO | Food and Agriculture Organization |
| FDA GCSL | Food & Drug Administration Gulf Coast Seafood Laboratory |
| MPN | Most Probable Number |
| NOAA | National Oceanic and Atmospheric Administration |
| NSSP | National Shellfish Sanitation Program |
| PCR | Polymerase Chain Reaction |
| PSU | Practical Salinity Unit |

# Chapter 1: Introduction to off-bottom oyster aquaculture and the impact of *Vibrio* spp. on human health

## Background

Aquacultured oysters are often held high in the water column to increase food availability. However, this practice also increases the amount of biofouling found on both oysters and cages. Walton et al. (2012) deemed desiccation, removal of biofouling by air drying, critically cost effective to the oyster industry in the Gulf of Mexico. Oysters held at ambient air temperatures (26˚C), in such conditions as air-drying, are known to increase abundances of *V. parahaemolyticus* found in oysters (Gooch et al. 2002). Oysters held at ambient air temperatures of 18 – 34˚C, have also been found to have higher abundances of *V. vulnificus* than when initially harvested (Cook 1994). Warm air temperatures associated with the Gulf of Mexico, in addition to the act of air drying oysters, increase the abundance of *Vibrio* spp. in oysters (Johnson et al. 2010). This increase in *Vibrio* spp. abundances poses a potentially higher risk for those consuming raw oysters during warm summer months, when specific temperatures can put into effect state-specific control plans to minimize post-harvest growth of *Vibrio* spp. (NSSP, 2013).

*Vibrio parahaemolyticus* and *V. vulnificus* are naturally occurring marine and estuarine bacteria associated with filter-feeding molluscan shellfish. The ingestion by humans of raw seafood, such as oysters, that contain high abundances of *Vibrio* spp. increases the risk of rapid and fatal infections. *Vibrio parahaemolyticus* causes acute gastroenteritis and includes clinical features such as watery diarrhea, abdominal cramps, nausea, and vomiting (Daniels et al. 2000 & Levine and Griffin 1993), and is estimated to infect at an annual rate of 4,500 patients per year (Center for Disease Control and Prevention). *Vibrio vulnificus* symptoms include fever, chills,

nausea, abdominal pain, hypotension, and development of secondary lesions (Jones and Oliver 2009), and is responsible for 95% of all seafood-borne deaths (Oliver 2013). Most (85%) cases of *V. vulnificus* infections occur during the warmer months of May through October (Oliver 2013). The primary sources (93%) of both *V. parahaemolyticus* and *V. vulnificus* food borne infections are from consumption of raw oysters (Oliver 2013).

The manipulation of post-harvest oysters, which exposes them to ambient air temperatures, has been shown to increase *V. vulnificus* in un-chilled oysters (Cook 1994). A study conducted by Walton et al. (2013b) demonstrated oysters that were time/temperature abused, refrigerated, shipped, and packed prior to re-submersion saw an increase in abundances of *V. parahaemolyticus* and *V. vulnificus* after two days of re-submersion and reached levels below those of original harvest 1-2 weeks after re-submersion. However, this study was not performed on oysters subjected to routine aquaculture practices but on abused transplanted oysters, in an effort to determine the length of time it would take levels of *Vibrio* spp. in temperature-abused oysters to return to abundances at or below those at original harvest. Additionally, Walton et al. (2013a) studied whether the type of gear used to raise oysters affects the *Vibrio* spp. abundances. There was no significant difference among the gear types, which are associated with different types of desiccation practices, but with limited replication across years this study could not be used to make public health recommendations (Walton et al. 2013a). My research will not only determine effects of routine desiccation practices on *Vibrio* spp. but will also have replication across years using two laboratory methods, to evaluate the human health risk associated with common oyster desiccation practices.

With oyster aquaculture developing, there are more advancements being used by oyster farmers, such as the triploid oysters. The popularity of the triploid oyster is due to a faster growth

rate associated with the lack of production of gametes (Nell 2002). An additional benefit is that the triploid oyster also is more resistant to disease, such as multinucleated sphere unknown, MSX (Mathiessen and Davis 1992). There is also a possibility that triploid oysters are also more resistant to *Vibrio* spp. infections. A study conducted by Decker et al. (2011), revealed a positive correlation between *Vibrio* spp. abundances and gonadal tissue. The study was performed in *Crassostrea gigas,* with *V. splendidus* and *V. aestuarianus,* both of which induce mortality in the oysters but do not affect humans. My research will determine if triploid oysters are more resistant to *Vibrio* spp. than diploid oysters, thereby providing an additional benefit to use of triploid oysters in aquaculture.

**Significance**

The limited research on the effects of desiccation practices on *Vibrio* spp. abundances and the lack of comparison between diploid and triploid oysters leaves uncertainties to human health officials developing recommendations. Public health officials are concerned that the combination of extreme warm temperatures of the Gulf of Mexico and typical aquaculture practices to control bio-fouling could increase the risk of *Vibrio* spp. infections in humans without appropriate precautions. Currently, the Alabama Department of Public Health (ADPH) requires farmers to re-submerse oysters no fewer than 14 days prior to selling, after ambient exposure that exceed the mandated time/temperature window (W. Walton, pers. comm.). This time of re-submersion requirement may be putting unnecessary restrictions on oyster farmers, when routine practice is for farmers to desiccate oysters every seven days, and therefore there is a practical incentive to determine if a shorter re-submersion time would achieve the desired public health goals. The results of this study will provide data to human health officials to inform guidance on this form of oyster production without putting unnecessary restrictions on the oyster industry.

The first objective of this study (Chapter 2) will test how long it takes for *V. parahaemolyticus, V. vulnificus,* and *V. cholerae* abundances in oysters which are subjected to desiccation practices, a 27 hour air dry and a 3 hour freshwater dip followed by a 24 hour air dry, to return to levels not significantly different from *Vibrio* spp. abundances in oysters that were left submersed (never desiccated). I hypothesize that under routine aquaculture practices, *Vibrio* spp. abundances will return to levels found in oysters that were left submersed at or before seven days after re-submersion due to more frequent measurements and less abuse of the oysters compared to previous studies. The second objective (Chapter 3) will test the hypothesis that under routine

aquaculture practices, a 27 hour air dry and a 3 hour freshwater dip followed by a 24 hour air dry and oysters that were left submersed, triploid oysters will harbor fewer *V. parahaemolyticus* and *V. vulnificus* than diploid oysters over time. I hypothesize that the lack of gonadal tissues will be correlated with decreased abundances of *Vibrio* spp. found in triploid *C. virginica*. If there is a significant relationship, this would provide an additional rationale for the use of triploids in oyster aquaculture and could possibly influence public health guidance.

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## Chapter 2: Effects of desiccation practices on *Vibrio* spp. abundances within cultured oysters, *Crassostrea virginica*

### Introduction

*Vibrio parahaemolyticus* and *V. vulnificus* are human pathogenic bacteria commonly associated with food borne illnesses from the consumption of raw oysters, including *Crassostrea virginica*, the eastern oyster. The primary source (93%) of *V. parahaemolyticus* and *V. vulnificus* food borne infections is consumption of raw oysters (Oliver 2013). Symptoms of *V. parahaemolyticus*, which is estimated to cause illness in 4,500 patients per year, include acute gastroenteritis with clinical features such as watery diarrhea, abdominal cramps, nausea, and vomiting (Center for Disease Control and Prevention 2011, Daniels et al. 2000, Levine and Griffin 1993). *Vibrio vulnificus* is responsible for 95% of all seafood-borne deaths, and causes symptoms that include fever, chills, nausea, abdominal pain, hypotension, and development of secondary lesions (Jones and Oliver 2009, Oliver 2013). These pathogenic bacteria are correlated with warmer water temperatures, such as those found in the Gulf of Mexico (Johnson et al. 2010, Pfeffer et al. 2003). Most *V. parahaemolyticus* and *V. vulnificus* infections (85%) occur during the warmer months of May through October (Oliver 2013, Gooch et al. 2002, Kaneko and Colewell 1975).

The expansion of off-bottom oyster aquaculture along the Gulf Coast (Walton et al. 2013a) has raised concern for potential risks to human health associated with the production of oysters intended for raw consumption from the warm waters and air temperatures of the Gulf of Mexico. Regular desiccation practices such as extended ambient air exposure to reduce biofouling and freshwater dip followed by ambient air exposure used to reduce *Polydora* spp. worm are common and necessary practices used by shellfish farmers (Fitridge 2012, Calvo and

Schroer 2014). Oysters held in off-bottom culture are associated with lower *Vibrio* spp. abundances (Cole et al. 2015); however these biofouling removal practices, or desiccation practices, can greatly increase *Vibrio* spp. abundances (Hood et al. 1983, Cook 1994). Re-submersion of oysters after dry storage is known to reduce *Vibrio* spp. abundances to levels of oysters that have never been exposed (Kinsey et al. 2015, Walton et al. 2013a). This reduction of elevated levels of *Vibrio* spp. prior to harvest decreases human health risk and enables farmers to maintain a clean high dollar raw product. However, the lack of information on the effect of routine aquaculture practices on *Vibrio* spp. abundances prior to harvest may lead to compromised safety of the product.

The combination of extreme warm temperatures in the Gulf of Mexico and typical aquaculture practices to control biofouling could increase risk of *Vibrio* spp. infections in humans (CDC 2011). Currently, the Alabama Department of Public Health (ADPH) requires farmers to re-submerge oysters no fewer than 14 days prior to harvest after exposure to ambient conditions that exceed the mandated time and temperature window (W. Walton, pers. comm.). There is preliminary evidence to suggest that fewer than 14 days of re-submersion allow *Vibrio* spp. abundances to return to levels they would have had if kept submersed (Kinsey et al. 2015, Walton, 2013c). The current ADPH time requirement may be putting unnecessary restrictions on oyster farmers whose routine practice is to desiccate weekly, which provides a practical incentive for this study: to determine if a shorter re-submersion time would achieve the desired public health goals.

The goal of this research is to test the effects two different desiccation treatments, 27-hour ambient air dry and 3-hour freshwater dip followed by 24-hour ambient air dry, on *V. parahaemolyticus, V. vulnificus,* and *V. cholerae* abundances and to determine the length of time

required to return to ambient abundances in oysters after desiccation. The results of this research will aid public health officials to provide appropriate guidance to the oyster industry and allow oyster farmers to make informed decisions.

## Materials and Methods

### Sampling Locations and Conditions

The fieldwork was conducted at Auburn University's research field site in Portersville Bay, Coden, Alabama (Mississippi Sound), a shallow (1-2 m) firm mud bottom site with a small tidal range (0.5-1.0 m). At this site, mid-summer salinities typically range from 15 to 25 PSU and water temperatures range from 25-30°C (Walton et al., 2013b). These field conditions were expected to be favorable for proliferation of pathogenic *Vibrio* spp. (WHO-FAO, 2005). Environmental data during study were retrieved from mymobilebay.com using the Cedar Point station. These data included daily mean and daily minimum and maximum of salinity, water temperature, wind speed, precipitation across trials and mean and range of air temperature for the period of desiccation.

### Submersion and Treatment of Oysters

Data were collected during five two-week long replicate trials across two summers. Two trials in 2014 started July 13$^{th}$ and ended August 25$^{th}$. Three trials in 2015 overlapped and started July 15$^{th}$ and ended September 1$^{st}$. During each trial there were eight sampling points: $T_{pre\text{-}treatment}$, $T_{post\text{-}treatment}$, and 1, 2, 3, 7, 10, and 14 days post re-submersion.

Replicate baskets (BST Oyster Supplies, Australia), each stocked with 100-120 oysters from the same brood, were submersed on an Australian long-line culture system (ALS) at the study site and maintained at a depth where they were constantly submersed, even during extreme

low tides for 14-40 days, prior to sampling. This extended submersion period prior to any sampling allowed all oysters to reach ambient *Vibrio* spp. abundances (Kinsey et al., 2015).

In each trial, six randomly selected baskets were subjected to a 3-hour freshwater dip, and then allowed to air dry for 24 hours (referred to hereafter as 'freshwater dipped'). Another six randomly selected baskets were subjected to 27 hours of desiccation at ambient air temperatures (referred to hereafter as 'air dried'). The remaining six baskets were left in the water, as the control (referred to hereafter as 'submersed'). Samples of 15 oysters were taken from three randomly chosen baskets per treatment prior to any treatment (with additional oysters drawn from additional baskets if sufficient oysters were not present in the first three baskets); this sample ($T_{pre\text{-}treatment}$) was used to determine the initial *Vibrio* spp. abundances prior to treatment, and to confirm that the treatments significantly elevated *Vibrio* spp. abundances. Additionally, prior to re-submersion, 3 samples of 15 oysters were randomly selected from 3 baskets per treatment (hereafter referred to as '$T_{post\text{-}treatment}$'). During each replicate trial, a sample of 15 oysters was randomly collected from 3 baskets per treatment at each time point: 1, 2, 3, 7, 10, and 14 days after re-submersion (hereafter referred to as '$T_x$' where the sub-script x designates the number of days of re-submersion). All samples were packed in coolers with ice packs buffered by burlap sacks which maintained the oysters in a temperature of $\leq 15$ ˚C.

Due to the amount of time required for processing samples, samples were shipped to either Auburn University Aquatic Microbiology Lab (AU AML) in Auburn, AL or delivered to the FDA Gulf Coast Seafood Laboratory (GCSL) on Dauphin Island, AL. Prior this study, blinded samples were processed at each laboratory to demonstrate comparability of data (not shown). Oysters to be analyzed by the FDA GCSL were held in a cooler overnight to mimic shipping conditions required to deliver oysters to AU AML. AU AML processed $T_{post\text{-}treatment}$, $T_7$,

$T_{10}$, and $T_{14}$ samples. FDA GCSL processed $T_{pre\text{-}treatment}$, $T_1$, $T_2$, and $T_3$ samples, with the exception of trial V, when all samples were processed at the FDA GCSL.

**MPN and BAX® PCR**

Samples were processed following standard protocols according to NSSP guidelines (http://www.issc.org/2009GuidePDF.aspx), followed by BAX® PCR confirmation. Upon opening the cooler, 12-15 of the 15 oysters were cleaned and shucked under clean and aseptic conditions and homogenized in a sterile food blender. The homogenate was then inoculated into alkaline peptone water using a 3-tube Most Probable Number (MPN) strategy. Tubes were enriched overnight at 35 ± 2°C. The DuPont Qualicon BAX® system was used, following the manufacturer's instructions, to determine the presence of *V. parahaemolyticus, V. vulnificus,* and *V. cholerae* in MPN tubes with turbid growth. Positive MPN tubes were used to estimate the density of each *Vibrio* spp. in each sample based on standard MPN tables (Blogett 2010). For each *Vibrio* spp. the results were reported as MPN/g of oyster.

**Statistical analysis**

Environmental data were collected from mymobilebay.com using the Cedar point site. Environmental parameters that were analyzed included water temperature, salinity, wind speed, precipitation and air temperature. Air temperature was collected during the 27 hours oysters were subjected to desiccation treatments; these data were used to perform one-way ANOVA. Air temperature daily mean along with the minimum and maximum values were calculated. Data for the remaining factors were collected for the entire duration of each trial and the daily mean along with daily mean minimum and maximums were calculated. The daily means were used to perform one-way ANOVA to compare between trials, except precipitation. The daily mean maximum for precipitation was used to perform one-way ANOVA. Using the data that were

previously described for each one-way ANOVA, a main effects model was performed to determine environmental effects on *Vibrio* spp. during each trial.

Prior to any statistical analysis of *Vibrio* spp. data, *Vibrio* spp. abundance data were log transformed to meet assumptions of normality. To first assess whether the two manipulated treatments (freshwater dipped, air dried) successfully elevated *Vibrio* spp. abundances, $T_{post\text{-}treatment}$ levels were compared to the un-manipulated, $T_{pre\text{-}treatment}$, levels. Subsequently, for each trial a one-way ANOVA was performed with a post-hoc multiple comparisons t-test (alpha = 0.10) when significant effects were found. To ensure that any increases that could pose a risk to human health were recognized, we selected a liberal alpha of 0.10. A mixed model ANOVA was used to determine if there was an effect of aquaculture practices, trial, days since re-submersion, and any interactions between these factors (alpha = 0.05). Due to significant differences amongst trials (see results), two-way ANOVAs by trial were performed to determine differences among aquaculture practices, days since re-submersion, and any interactions between these factors (alpha = 0.05), with appropriate post-hoc multiple comparisons tests performed when significant effects were found. *V. cholerae* abundances were frequently (92%) below the limit of detection, so no statistical analyses of the data were performed. All statistical calculations were performed using the JMP statistical program (SAS Institute Inc., Cary, NC).

## Results

### Environmental

Although significant differences of environmental parameters were detected between trials (Table 2.1), there was no apparent effect on *Vibrio* spp. abundances except for *V. vulnificus* levels in trial I showed a positive correlation with water temperature ($p < 0.02$).

Table 2.1: Environmental data presented as means, with daily mean ranges, during each trial except precipitation which is the daily maximum mean. Superscript letters denote significant differences.

| Trials | Environmental data | | | | |
|---|---|---|---|---|---|
| | Water Temp (°C) | Salinity (PSU) | Wind Speed (knots) | Precipitation (cm) | Air Temp (°C) |
| I | 29.0 $^{D}$ (27.9-30.0) | 19.0 $^{D}$ (15.7-22.2) | 10.0 $^{A}$ (2.5-20.8) | 0.02 $^{A}$ | 27.0 $^{E}$ (23.8-28.7) |
| II | 30.4 $^{A,B}$ (29.6-31.5) | 22.0 $^{B}$ (19.1-24.7) | 9.1 $^{AB}$ (2.5-16.8) | 0.005 $^{B}$ | 27.2 $^{D}$ (23.7-29.7) |
| III | 31.0 $^{A}$ (30.1-32.0) | 19.9 $^{C,D}$ (16.3-24.2) | 9.6 $^{A}$ (1.8-19.8) | 0.003 $^{B}$ | 29.8 $^{A}$ (28.8-30.7) |
| IV | 30.2 $^{B,C}$ (29.3-31.1) | 23.7 $^{A}$ (21.1-26.8) | 10.4 $^{A}$ (3.3-19.9) | 0.009 $^{B}$ | 28.8 $^{B}$ (26.6-30.0) |
| V | 29.6 $^{C}$ (28.7-30.6) | 20.9 $^{B,C}$ (18.4-24.0) | 7.4 $^{B}$ (1.0-14.0) | 0.0006 $^{B}$ | 28.6 $^{C}$ (26.7-29.7) |

**Initial Effect of Manipulated Treatments**

For *V. parahaemolyticus* abundances in oysters at the onset of each trial (Table 2.2), there was an effect of the interaction of trial and treatment ($p < 0.01$). All abundances found in oysters that underwent air dried and freshwater dipped treatments were significantly higher ($p \leq 0.06$) than levels found in $T_{pre\text{-}treatment}$ oysters within each trial, using the alpha of 0.10 (Table 2.3), indicating that these two manipulations consistently elevated *V. parahaemolyticus* abundances prior to re-submersion (Figure 2.1). *Vibrio parahaemolyticus* abundances increased, from $T_{pre\text{-}treatment}$ to $T_{post\text{-}treatment}$, the least (0.6 log MPN/g) in trial I air dried and freshwater dipped oyster and the most (2.3 log MPN/g) in trials III and IV freshwater dipped oysters.

In two trials, I and II (Figure 2.3 and 2.4), abundances in oysters in neither manipulated treatment, air-dried [trial I (5.3 log MPN/g), trial II (5.3 log MPN/g)] or freshwater dipped [trial I (5.3 log MPN/g), trial II (5.2 log MPN/g)] significantly differed from the submersed oysters [trial I (5.0 log MPN/g), trial II (4.8 log MPN/g)] ($p \geq 0.11$). In trial IV (Figure 2.6), levels in air-dried oysters (4.7 log MPN/g) did not differ from submersed oysters (4.4 log MPN/g) ($p =$

0.41), while levels in freshwater dipped oysters (5.8 log MPN/g) were significantly higher ($p \leq 0.02$) than submersed (4.4 log MPN/g) and air dried oysters (4.7 log MPN/g). In trial II (Figure 2.4), *V. parahaemolyticus* abundances in oysters in submersed oysters (4.8 log MPN/g) were higher than levels found in $T_{pre\text{-}treatment}$ oysters (4.0 log MPN/g), indicating a significant ($P = 0.04$) increase in the ambient *V. parahaemolyticus* abundances over the 27-hr period of this trial.

Table 2.2: ANOVA tables comparing main effects of *Vibrio* spp. levels on $T_{pre\text{-}treatment}$ and $T_{post\text{-}treatment}$, including 3 hour freshwater dip plus 24 hour desiccation, 27 hour desiccation, and continually submersed for initial effects of treatments. Lines in bold represent significant differences (alpha = 0.05).

| *Vibrio* spp. | Source | DF | Sum of Squares | F Ratio | Prob> F |
|---|---|---|---|---|---|
| *V. parahaemolyticus* | **Trial** | **4** | **39.02** | **35.90** | **<0.01** |
| | **Treatment** | **3** | **30.92** | **37.93** | **<0.01** |
| | **Trial*Treatment** | **12** | **9.65** | **2.96** | **<0.01** |
| *V. vulnificus* | **Trial** | **4** | **15.98** | **7.76** | **<0.01** |
| | **Treatment** | **3** | **40.62** | **26.30** | **<0.01** |
| | Trial*Treatment | 12 | 5.73 | 0.93 | 0.53 |

Table 2.3: ANOVA post-hoc t-test comparison for *V. parahaemolyticus* levels on $T_{pre\text{-}treatment}$ (Pre) and $T_{post\text{-}treatment}$; 3 hour freshwater dip plus 24 hour desiccation (Freshwater), 27 hour desiccation (Air), and submersed. Lines in bold represent significant differences (alpha = 0.05).

| Trial | Level | - Level | Difference | Std Err Dif | Lower CL | Upper CL | p-Value |
|---|---|---|---|---|---|---|---|
| I | **Freshwater** | **Pre** | **0.59** | **0.23** | **0.07** | **1.11** | **0.03** |
| | **Air dried** | **Pre** | **0.58** | **0.23** | **0.05** | **1.10** | **0.03** |
| | Submersed | Pre | 0.19 | 0.23 | -0.33 | 0.71 | 0.42 |
| | Freshwater | Air | 0.02 | 0.23 | -0.51 | 0.54 | 0.95 |
| | Air | Submersed | 0.39 | 0.23 | -0.14 | 0.91 | 0.13 |
| | Freshwater | Submersed | 0.40 | 0.23 | 0.12 | 0.92 | 0.11 |
| II | **Freshwater** | **Pre** | **1.22** | **0.35** | **0.41** | **2.02** | **0.01** |
| | **Air** | **Pre** | **1.28** | **0.35** | **0.48** | **2.09** | **0.01** |
| | **Submersed** | **Pre** | **0.83** | **0.35** | **0.02** | **1.64** | **0.04** |
| | Air | Freshwater | 0.07 | 0.35 | -0.74 | 0.88 | 0.85 |
| | Air | Submersed | 0.45 | 0.35 | -0.36 | 1.26 | 0.23 |
| | Freshwater | Submersed | 0.38 | 0.35 | -0.43 | 1.19 | 0.31 |
| III | **Freshwater** | **Pre** | **2.19** | **0.35** | **1.37** | **3.00** | **<0.01** |
| | **Air** | **Pre** | **2.22** | **0.35** | **1.40** | **3.04** | **<0.01** |
| | Submersed | Pre | 0.52 | 0.35 | -0.29 | 1.34 | 0.18 |
| | Air | Freshwater | 0.04 | 0.35 | -0.78 | 0.85 | 0.92 |
| | **Air** | **Submersed** | **1.70** | **0.35** | **0.88** | **2.51** | **<0.01** |
| | **Freshwater** | **Submersed** | **1.66** | **0.35** | **0.84** | **2.48** | **<0.01** |
| IV | **Freshwater** | **Pre** | **1.91** | **0.38** | **1.03** | **2.80** | **<0.01** |
| | **Air** | **Pre** | **0.84** | **0.38** | **-0.05** | **1.72** | **0.06** |
| | Submersed | Pre | 0.50 | 0.38 | -0.38 | 1.39 | 0.23 |
| | **Freshwater** | **Air** | **1.08** | **0.38** | **0.19** | **1.96** | **0.02** |
| | Air | Submersed | 0.34 | 0.38 | -0.55 | 1.22 | 0.41 |
| | **Freshwater** | **Submersed** | **1.41** | **0.38** | **0.53** | **2.30** | **0.01** |
| V | **Freshwater** | **Pre** | **3.21** | **0.68** | **1.64** | **4.78** | **<0.01** |
| | **Air** | **Pre** | **2.33** | **0.68** | **0.77** | **3.89** | **0.01** |
| | Submersed | Pre | 0.78 | 0.68 | -0.78 | 2.34 | 0.28 |
| | Freshwater | Air | 0.88 | 0.68 | -0.68 | 2.44 | 0.23 |
| | **Air** | **Submersed** | **1.55** | **0.68** | **-0.01** | **3.11** | **0.05** |
| | **Freshwater** | **Submersed** | **2.43** | **0.68** | **0.87** | **3.99** | **0.01** |

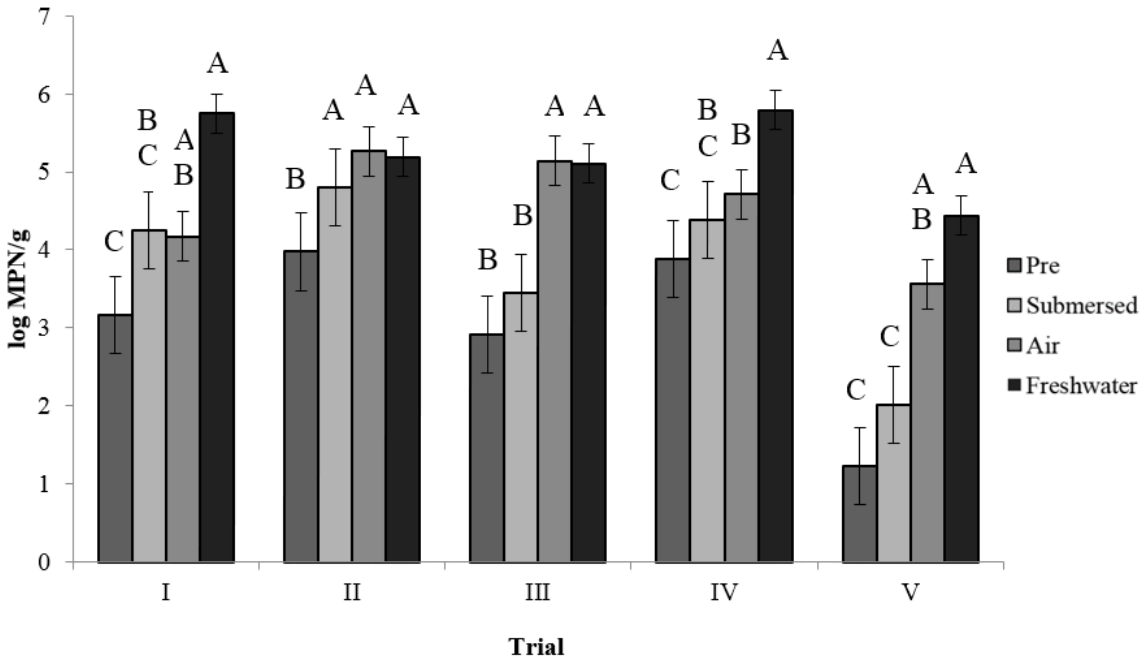


Figure 2.1: Mean *V. parahaemolyticus* abundances in oysters prior to any treatment ($T_{pre\text{-}treatment;}$ Pre), submersed controls (Sub), and prior to re-submersion after treatment: 24 hour desiccation (Air), and 3 hour freshwater dip with 24 hour desiccation (Freshwater). Bars represent standard error and different letters represent significant differences between mean levels as determined with post-hoc student t-test analysis performed by trial.

*Vibrio vulnificus* abundances were significantly affected by both trial and treatment, but there were no significant interactions between these two effects (Table 2.2). Looking at treatments (Table 2.4, Figure 2.2), each of the four differed significantly from each other ($p \leq 0.04$), in increasing order from $T_{pre\text{-}treatment}$ (3.5 log MPN/g), submersed (4.1 log MPN/g), air dried (4.9 log MPN/g), and freshwater dipped (5.7 log MPN/g). There were significant differences among trials, where only trial V (3.6 log MPN/g) was found to be significantly lower

than all other trials ($p < 0.01$), which range from 4.6 log MPN/g (trial I) to 5.1 log MPN/g (trial II), but no other trials differed ($p \geq 0.05$).

Table 2.4: ANOVA post-hoc t-test comparison for *V. vulnificus* levels on $T_{pre\text{-}treatment}$ (Pre) and $T_{post\text{-}treatment}$; 3 hour freshwater dipped plus 24 hour desiccation (Freshwater), 27 hour air dried (Air), and submersed. Lines in bold represent significant differences (alpha = 0.05).

| Level | - Level | Difference | Std Err Dif | Lower CL | Upper CL | p-Value |
|---|---|---|---|---|---|---|
| **Freshwater** | **Pre** | **2.19** | **0.26** | **1.66** | **2.72** | **<0.01** |
| **Air** | **Pre** | **1.34** | **0.26** | **0.81** | **1.87** | **<0.01** |
| **Submersed** | **Pre** | **0.56** | **0.26** | **0.03** | **1.09** | **0.04** |
| **Freshwater** | **Air** | **0.85** | **0.26** | **0.32** | **1.38** | **0.01** |
| **Air** | **Submersed** | **0.77** | **0.26** | **0.24** | **1.30** | **0.01** |
| **Freshwater** | **Submersed** | **1.62** | **0.26** | **1.09** | **2.15** | **<0.01** |

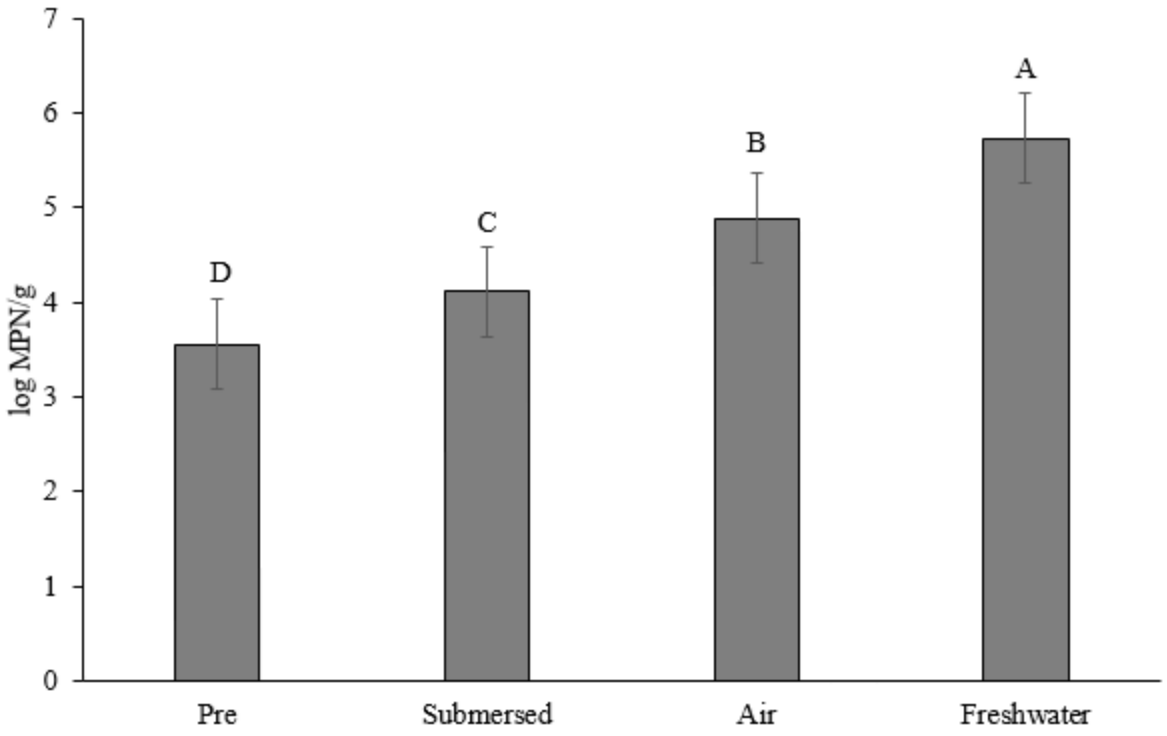


Figure 2.2: Mean *V. vulnificus* abundances with standard error bars across all trials of $T_{pre\text{-}treatment}$ (Pre) and $T_{post\text{-}treatment;}$ submersed (Sub), air dried (Air), and freshwater dipped (Freshwater). Bars represent standard error and different letters represent significant differences between mean levels as determined with post-hoc student t-test analysis.

**Effects of Treatment over Time**

***Vibrio parahaemolyticus***

While specifics varied within trials, *V. parahaemolyticus* abundances in oysters from manipulated treatments (air dried or freshwater dipped) did not significantly differ ($p \geq 0.05$) from the levels in continually submersed oysters by seven days post re-submersion (Table 2.7). In trial I (Figure 2.3), at $T_2$ *V. parahaemolyticus* abundances in air dried treatment oysters (3.8 log MPN/g) were similar to levels in submersed oysters (2.9 log MPN/g) and at $T_3$ abundances in freshwater treatment oysters (3.3 log MPN/g) were similar to levels in submersed oysters (3.7 log MPN/g). In trial II (Figure 2.4), at $T_1$ abundances in air dried treatment oysters (4.0 log

MPN/g) were similar to levels in submersed oysters (3.3 log MPN/g) and at $T_7$ abundances in freshwater dipped treatment oysters (3.4 log MPN/g) were similar to levels in submersed oysters (3.3 log MPN/g). In trial III (Figure 2.5), at $T_2$ abundances in air dried treatment oysters (2.8 log MPN/g) were similar to levels in submersed oysters (3.8 log MPN/g) and at $T_1$ day abundances in freshwater dipped treatment oysters (3.0 log MPN/g) were similar to levels in submersed oysters (3.5 log MPN/g). In trial IV (Figure 2.6), abundances in oysters reached those similar to levels in submersed oysters (4.8 log MPN/g) at $T_2$ for air dried (5.2 log MPN/g) and for freshwater dipped treatments (4.7 log MPN/g). During trial V (Table 2.6, Figure 2.7), treatment ($p=0.10$), days since re-submersion ($p = 0.73$), or treatment*days since re-submersion ($p = 0.14$) did not significantly affect *V. parahaemolyticus* abundances.

Similarly, the time point at which *V. parahaemolyticus* abundances began to significantly decrease from $T_{post\text{-}treatment}$ levels varied between trials. Abundances in oysters from air-dried samples significantly decreased at $T_1$, $T_2$, or $T_3$ depending on trial (Figure 2.3 - 2.6). Abundances in oysters from freshwater dipped samples began to significantly decrease at $T_1$ during trials I, II, and III and at $T_3$ during trial IV (Figure 2.3 - 2.6). For oysters from both treatments in trial IV, *V. parahaemolyticus* abundances did not begin to decrease from initially elevated levels, until after abundances returned to submersed levels (Figure 2.6).

Table 2.4: Mixed model ANOVA table for main effects on log transformed *V. parahaemolyticus* data. Lines in bold represent significant differences (alpha = 0.05).

| Source | DF | Sum of Squares | F Ratio | Prob> F |
|---|---|---|---|---|
| **Trial** | **4** | **45.05** | **14.73** | **<0.01** |
| **Days since re-submersion** | **6** | **49.71** | **10.83** | **<0.01** |
| **Treatment** | **2** | **17.89** | **11.70** | **<0.01** |

Table 2.6: ANOVA table for log transformed *V. parahaemolyticus* data by trial, testing effects of treatment and days since re-submersion. Lines in bold represent significant differences (alpha = 0.05).

| | | DF | Sum of Squares | F Ratio | Prob> F |
|---|---|---|---|---|---|
| Trial I | **Treatment** | **2** | **2.0** | **3.61** | **0.04** |
| | **Days since re-submersion** | **6** | **28.35** | **17.05** | **<0.01** |
| | **Treatment*Days since re-submersion** | **12** | **9.92** | **2.98** | **<0.01** |
| Trial II | **Treatment** | **2** | **5.67** | **12.03** | **<0.01** |
| | **Days since re-submersion** | **6** | **23.56** | **16.67** | **<0.01** |
| | **Treatment*Days since re-submersion** | **12** | **6.62** | **2.34** | **0.02** |
| Trial III | **Treatment** | **2** | **4.46** | **3.31** | **0.05** |
| | **Days since re-submersion** | **6** | **10.86** | **2.69** | **0.03** |
| | **Treatment*Days since re-submersion** | **12** | **16.89** | **2.09** | **0.04** |
| Trial IV | **Treatment** | **2** | **5.30** | **5.16** | **0.01** |
| | **Days since re-submersion** | **6** | **26.13** | **8.47** | **<0.01** |
| | **Treatment*Days since re-submersion** | **12** | **23.96** | **3.89** | **<0.01** |
| Trial V | Treatment | 2 | 4.20 | 2.41 | 0.10 |
| | Days since re-submersion | 6 | 3.14 | 0.60 | 0.73 |
| | Treatment*Days since re-submersion | 12 | 16.35 | 1.57 | 0.14 |

Table 2.7: Days since re-submersion at which *V. parahaemolyticus* abundances in each manipulated treatment (Air dried, Freshwater dipped) were not significantly different from levels in submersed treatments.

| Trial | Air dried | Freshwater dipped |
|---|---|---|
| I | 2 | 1 |
| II | 1 | 7 |
| III | 2 | 1 |
| IV | 2 | 2 |
| V | - | - |

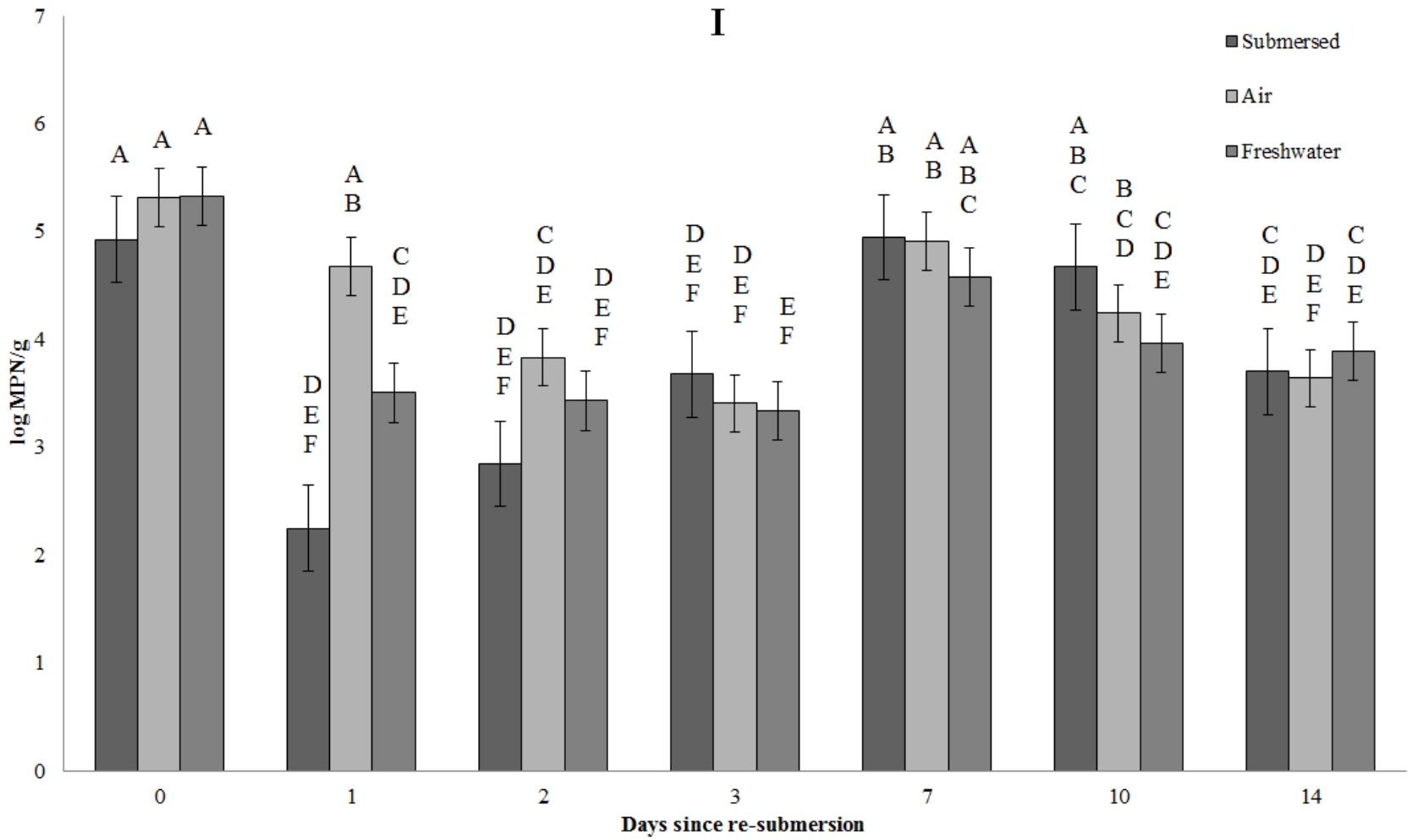


Figure 2.3: Mean log transformed *V. parahaemolyticus* with standard error bars for trial I, among three aquaculture treatments; control (Submersed), 27 hour desiccation (Air), and 3 hour freshwater dip with 24 hour desiccation (Freshwater). Days since re-submersion are shown on the x-axis, where $T_0$ is immediately prior to re-submersion ($T_{post\text{-}treatment}$). Different letters indicate significant differences as determined by the post hoc student t-test.

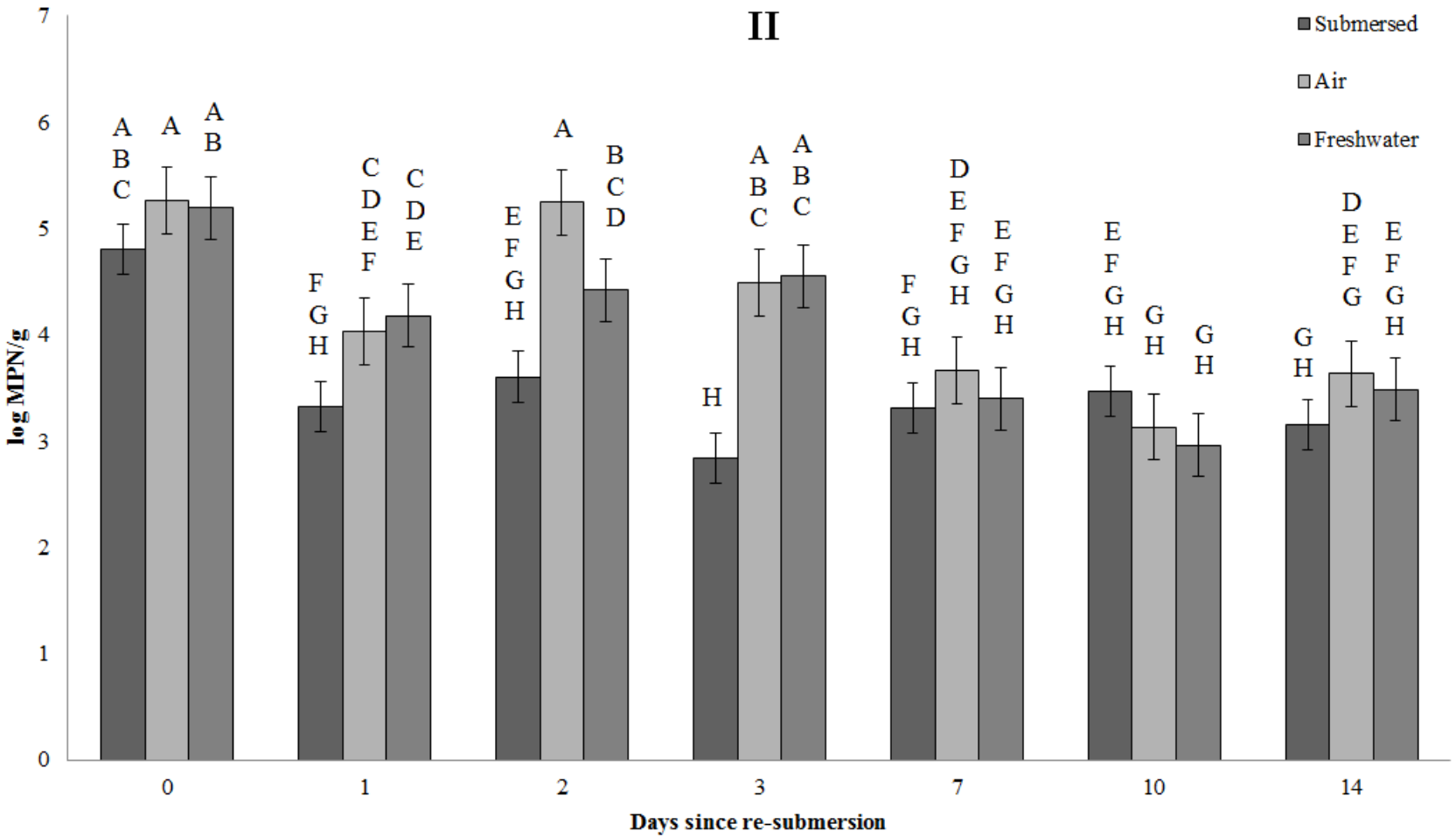


Figure 2.4: Mean log transformed *V. parahaemolyticus* with standard error bars for trial II among three aquaculture treatments; control (Submersed), 27 hour desiccation (Air dried), and 3 hour freshwater dip with 24 hour desiccation (Freshwater). Days since re-submersion are shown on the x-axis, where $T_0$ is immediately prior to re-submersion ($T_{post\text{-}treatment)}$. Different letters indicate significant differences as determined by the post hoc student t-test.

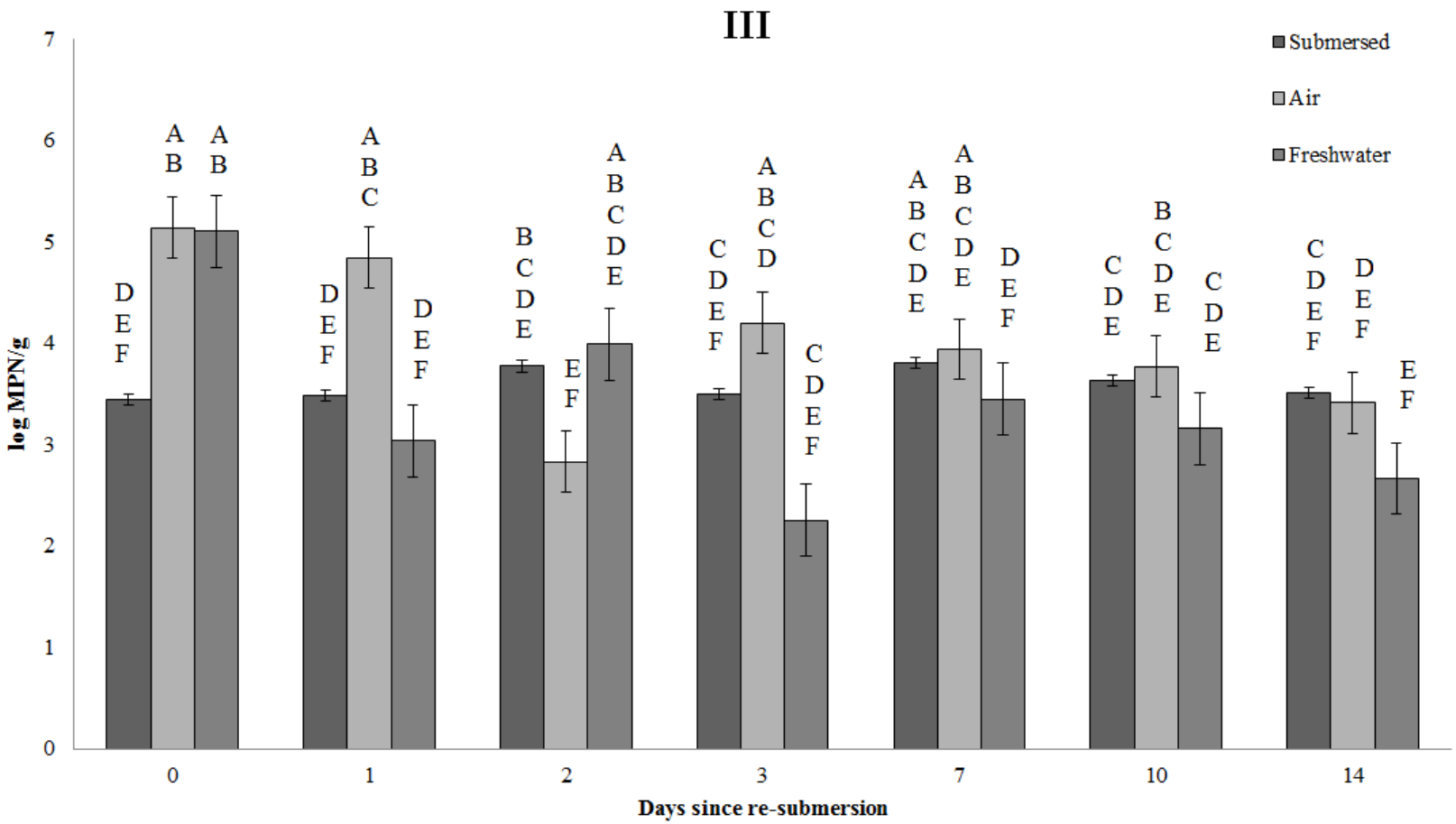


Figure 2.5: Mean log transformed *V. parahaemolyticus* with standard error bars for trial III among three aquaculture treatments; control (Submersed), 27 hour desiccation (Air dried), and 3 hour freshwater dip with 24 hour desiccation (Freshwater). Days since re-submersion are shown on the x-axis, where $T_0$ is immediately prior to re-submersion ($T_{post\text{-}treatment}$). Different letters indicate significant differences as determined by the post hoc student t-test.

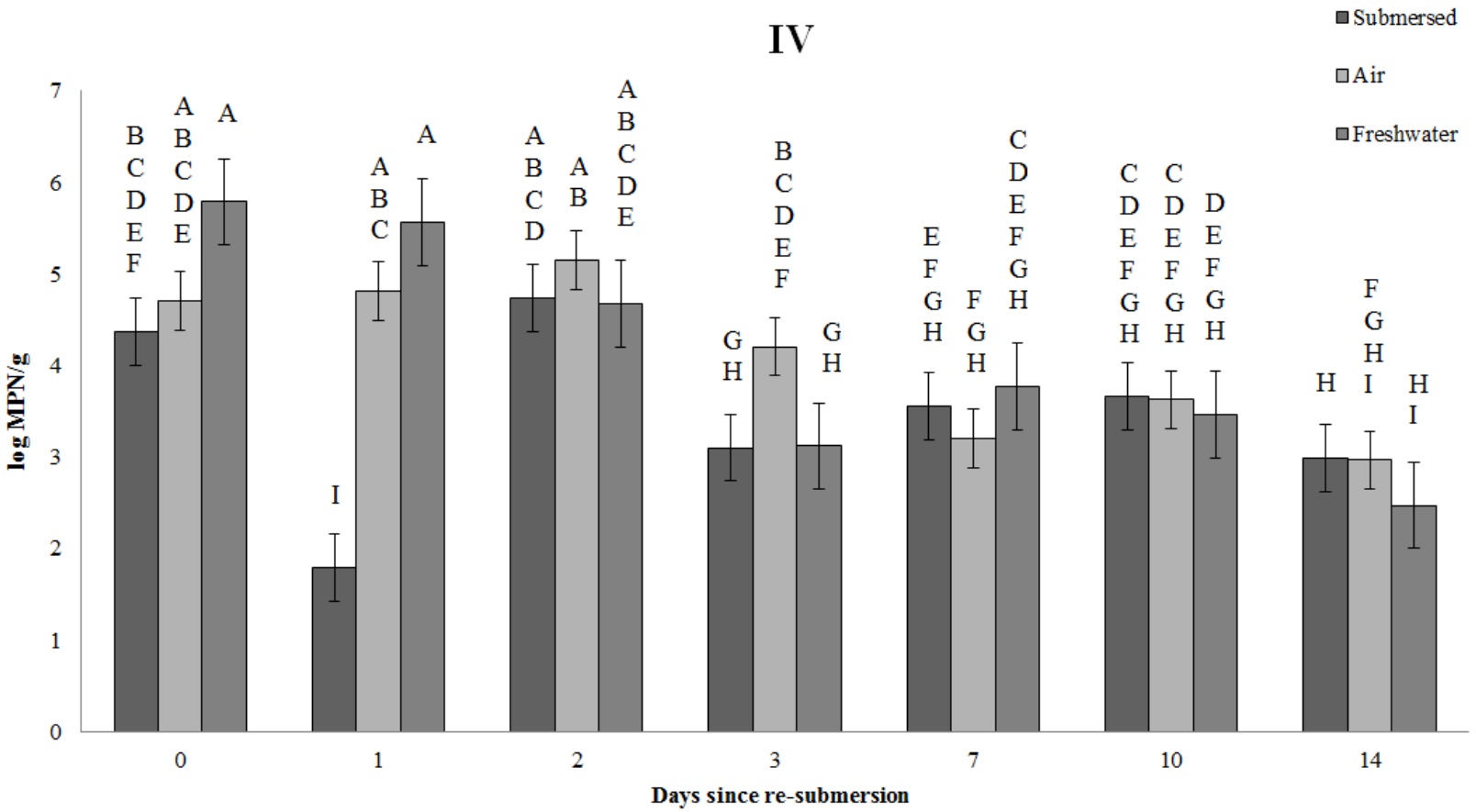


Figure 2.6: Mean log transformed *V. parahaemolyticus* with standard error bars for trial IV among three aquaculture treatments; control (Submersed), 27 hour desiccation (Air dried), and 3 hour freshwater dip with 24 hour desiccation (Freshwater). Days since re-submersion are shown on the x-axis, where $T_0$ is immediately prior to re-submersion ($T_{post\text{-}treatment}$). Different letters indicate significant differences as determined by the post hoc student t-test.

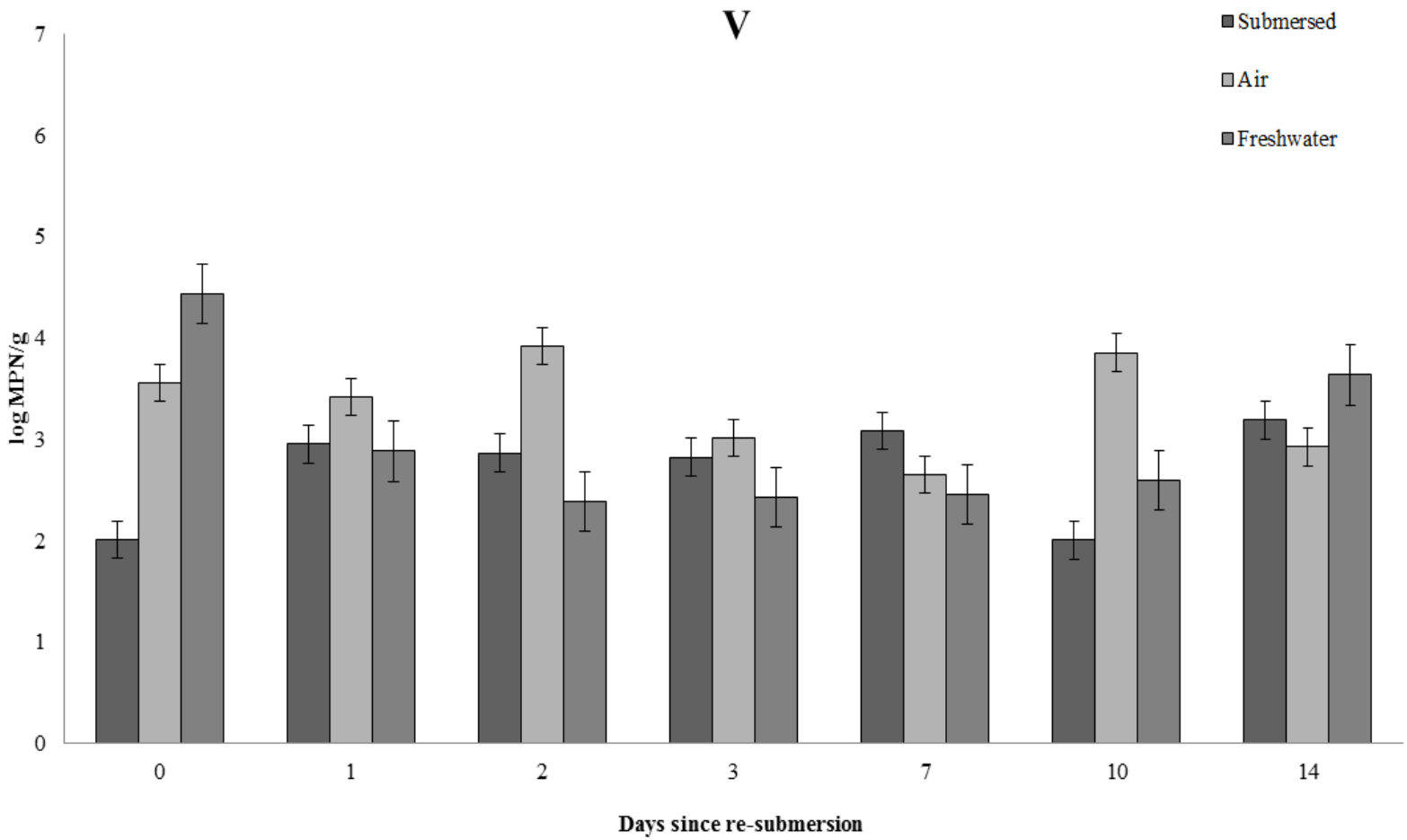


Figure 2.7: Mean log transformed *V. parahaemolyticus* with standard error bars for trial V among three aquaculture treatments; control (Submersed), 27 hour desiccation (Air dried), and 3 hour freshwater dip with 24 hour desiccation (Freshwater). Days since re-submersion are shown on the x-axis, where $T_0$ is immediately prior to re-submersion ($T_{post\text{-}treatment}$). No significance was found within trial V.

***Vibrio vulnificus***

While specifics varied within trials, *V. vulnificus* abundances in oysters from neither manipulated treatment (air-dried, freshwater dipped) differed from the oysters in the submersed treatment after 7 days or more (Table 2.10). In trial I (Figure 2.8), abundances in oysters reached those similar to submersed levels (2.9 log MPN/g) at $T_2$ for air dried (3.5 log MPN/g) and for freshwater dipped treatments (3.8 log MPN/g). In trial II (Figure 2.9), at $T_1$ abundances in oysters from air dried treatment (3.7 log MPN/g) were similar to levels in submersed oysters (3.4 log MPN/g) and at $T_7$ days abundances in freshwater dipped treatment oysters (2.8 log MPN/g) were similar to levels in submersed oysters (3.0 log MPN/g). In trial III (Figure 2.10), abundances in oysters reached those similar to levels in submersed oysters (4.1 log MPN/g) at $T_3$ in air dried (4.1 log MPN/g) and freshwater dipped treatments (4.5 log MPN/g). In trial IV (Figure 2.11), abundances in oysters reached those similar to levels in submersed oysters (3.6 log MPN/g) at $T_2$ in air dried (3.5 log MPN/g) and in freshwater dipped treatments (2.6 log MPN/g). During trial V (Table 2.9, Figure 2.12), effect of treatment ($p = 0.57$), days since re-submersion ($p = 0.13$), or treatment*days since submersion ($p = 0.32$) did not significantly affect *V. vulnificus* abundances. Additionally, in trial IV, there were instances where abundances in submersed oysters were significantly higher than levels in oysters that underwent desiccation treatments, freshwater dipped oysters ($T_{10}$ and $T_{14}$) and air dried oysters ($T_{10}$) ($P < 0.05$).

Similarly, the time point at which *V. vulnificus* abundances began to significantly decrease from $T_{post\text{-}treatment}$ levels varied among trials. Within each trial *V. vulnificus* abundances in oysters from manipulated treatments significantly decreased from $T_{post\text{-}treatment}$ levels by $T_2$, including trial I, II (only air-dried), and IV. The last abundances to begin to decrease were the levels in freshwater dipped oysters in trial III, where levels did not significantly differ from $T_{post\text{-}}$

$_{treatment}$ samples until $T_3$. In contrast, the abundances in oysters from trial II were the first to decrease, with levels becoming significantly different from $T_{post\text{-}treatment}$ levels at $T_1$.

Table 2.8: Mixed model ANOVA table for main effects on log transformed *V. vulnificus* data. Lines in bold represent significant differences (alpha = 0.05).

| Source | DF | Sum of Squares | F Ratio | Prob> F |
|---|---|---|---|---|
| **Trial** | **4** | **38.27** | **12.97** | **<0.01** |
| **Days since re-submersion** | **6** | **81.55** | **18.42** | **<0.01** |
| Treatment | 2 | 3.94 | 2.67 | 0.07 |

Table 2.9: ANOVA table for log transformed *V. vulnificus* data by trial. Lines in bold represent significant differences (alpha = 0.05).

| | | DF | Sum of Squares | F Ratio | Prob> F |
|---|---|---|---|---|---|
| Trial I | **Treatment** | **2** | **1.99** | **3.47** | **0.04** |
| | **Days since re-submersion** | **6** | **18.47** | **10.73** | **<0.01** |
| | **Treatment*Days since re-submersion** | **12** | **12.47** | **3.62** | **<0.01** |
| Trial II | **Treatment** | **2** | **5.27** | **9.51** | **<0.01** |
| | **Days since re-submersion** | **6** | **41.51** | **24.97** | **<0.01** |
| | **Treatment*Days since re-submersion** | **12** | **8.48** | **2.55** | **0.01** |
| Trial III | **Treatment** | **2** | **0.96** | **1.20** | **0.31** |
| | **Days since re-submersion** | **6** | **11.72** | **4.89** | **<0.01** |
| | **Treatment*Days since re-submersion** | **12** | **18.70** | **3.90** | **<0.01** |
| Trial IV | **Treatment** | **2** | **0.98** | **1.25** | **0.30** |
| | **Days since re-submersion** | **6** | **40.85** | **17.35** | **<0.01** |
| | **Treatment*Days since re-submersion** | **12** | **19.50** | **4.14** | **<0.01** |
| Trial V | Treatment | 2 | 1.24 | 0.58 | 0.57 |
| | Days since re-submersion | 6 | 11.40 | 1.76 | 0.13 |
| | Treatment*Days since re-submersion | 12 | 15.39 | 1.19 | 0.32 |

Table 2.10: Days since re-submersion at which *V. vulnificus* abundances in each manipulated treatment (Air dried, Freshwater dipped) were not significantly different from levels in submersed treatments.

| Trial | Air dried | Freshwater dipped |
|---|---|---|
| I | 2 | 2 |
| II | 1 | 7 |
| III | 3 | 3 |
| IV | 2 | 2 |
| V | - | - |

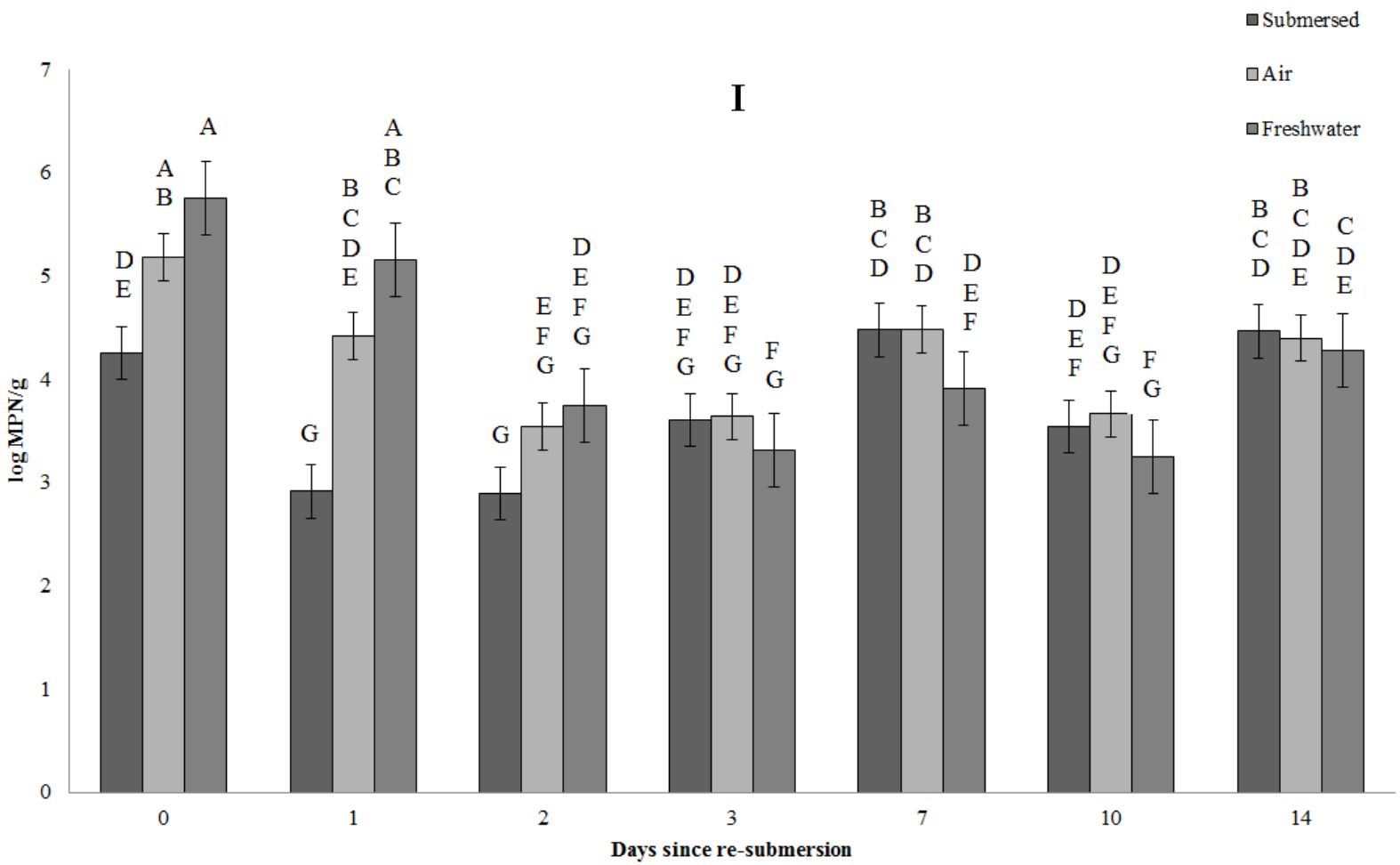


Figure 2.8: Mean log transformed *V. vulnificus* with standard error bars for trial I among three aquaculture treatments; control (Submersed), 27 hour desiccation (Air), and 3 hour freshwater dip with 24 hour desiccation (Freshwater). Days since re-submersion are shown on the x-axis, where $T_0$ is immediately prior to re-submersion. Different letters indicate significant differences as determined by the post hoc student t-test.

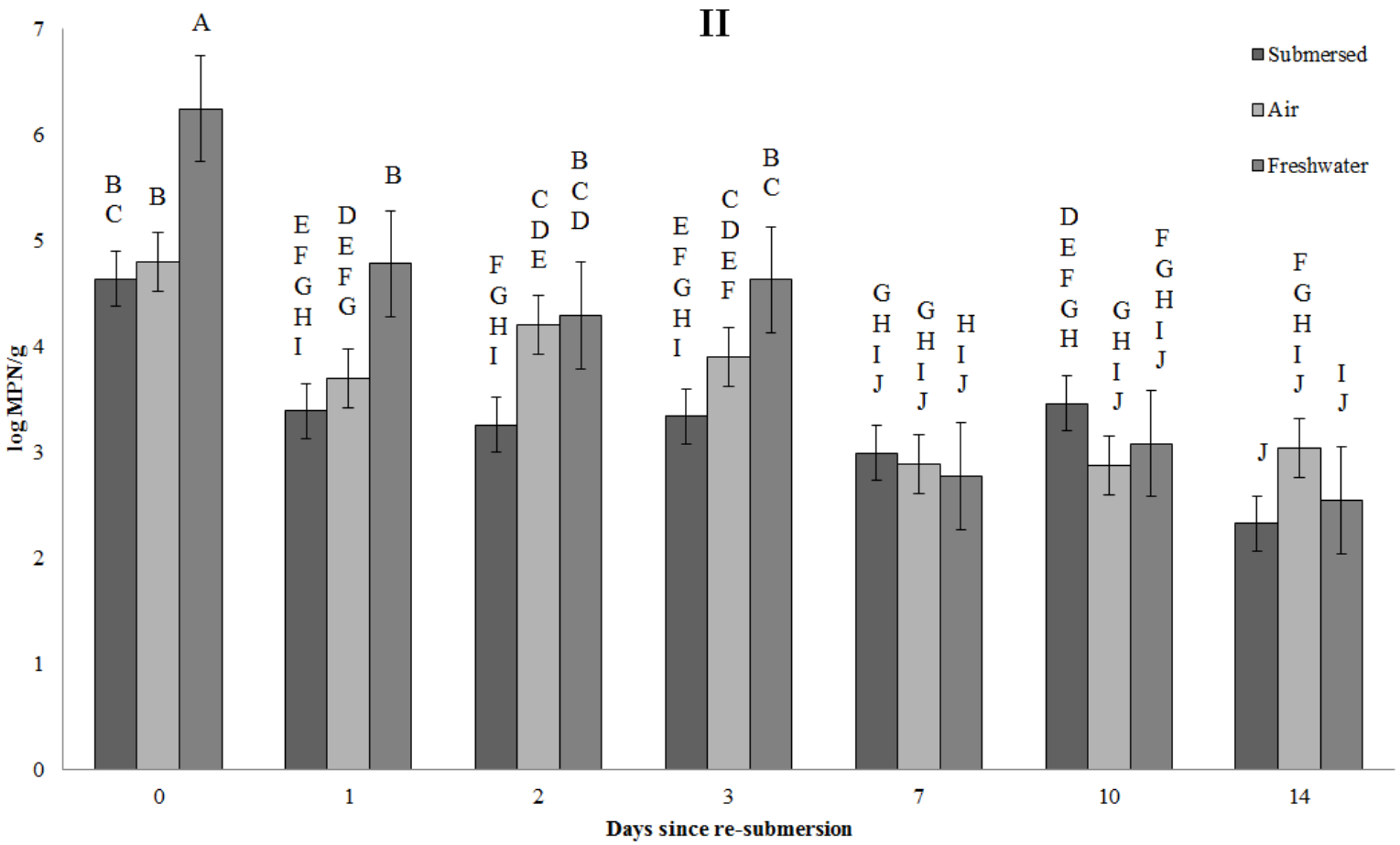


Figure 2.9: Mean log transformed *V. vulnificus* with standard error bars for trial II among three aquaculture treatments; control (Submersed), 24 hour desiccation (Air), and 3 hour freshwater dip with 24 hour desiccation (Freshwater). Days since re-submersion are shown on the x-axis, where $T_0$ is immediately prior to re-submersion. Different letters indicate significant differences as determined by the post hoc student t-test.

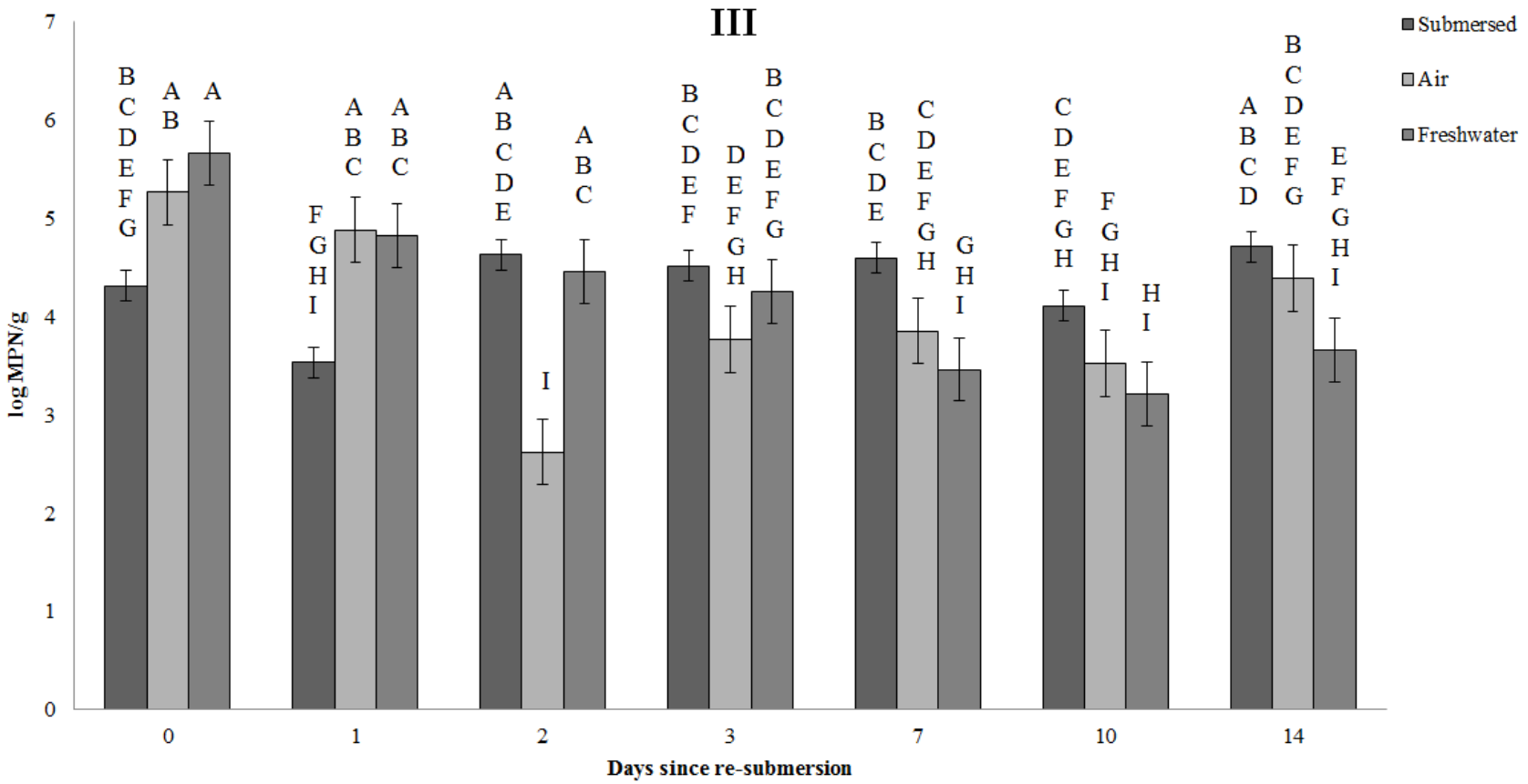


Figure 2.10: Mean log transformed *V. vulnificus* with standard error bars for trial III among three aquaculture treatments; control (Submersed), 27 hour desiccation (Air dried), and 3 hour freshwater dip with 24 hour desiccation (Freshwater). Days since re-submersion are shown on the x-axis, where $T_0$ is immediately prior to re-submersion. Different letters indicate significant differences as determined by the post hoc student t-test.

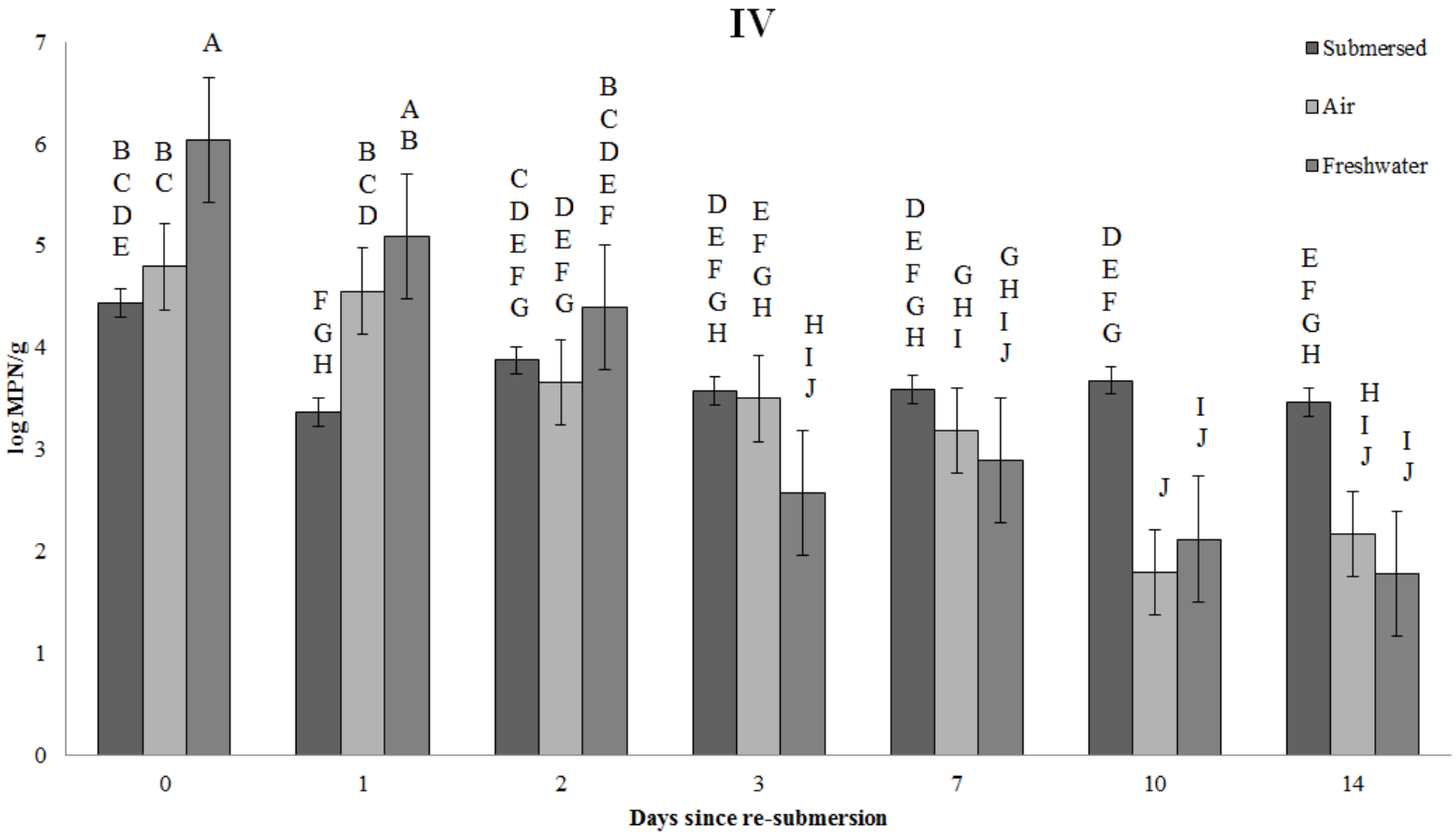


Figure 2.11: Mean log transformed *V. vulnificus* with standard error bars for trial IV among three aquaculture treatments; control (Submersed), 27 hour desiccation (Air dried), and 3 hour freshwater dip with 24 hour desiccation (Freshwater). Days since re-submersion are shown on the x-axis, where $T_0$ is immediately prior to re-submersion. Different letters indicate significant differences as determined by the post hoc student t-test.

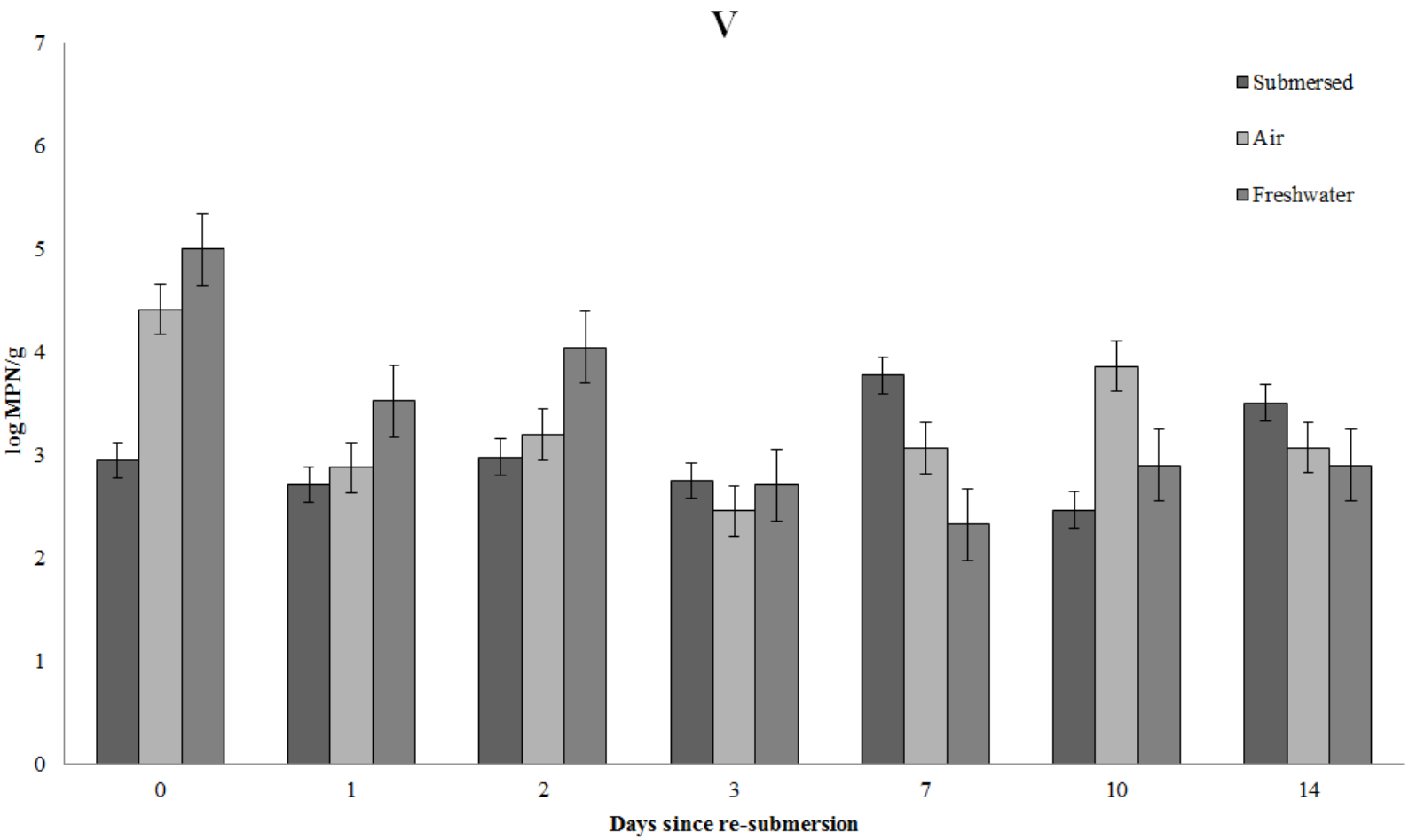


Figure 2.12: Mean log transformed *V. vulnificus* with standard error bars for trial V among three aquaculture treatments; control (Submersed), 27 hour desiccation (Air dried), and 3 hour freshwater dip with 24 hour desiccation (Freshwater). Days since re-submersion are shown on the x-axis, where $T_0$ is immediately prior to re-submersion. No significance was found with-in trail V.

**Desiccation Treatment**

When comparing *Vibrio* spp. abundances in oysters that underwent desiccation treatments, air dried and freshwater dipped, more than 85% of the time there were no significant differences in abundances between desiccation treatments. Out of 35 time point comparisons, among all trials, air dried and freshwater dipped oysters had no significant differences at 31 time points for *V. parahaemolyticus* levels ($p \geq 0.05$) and 30 time points for *V. vulnificus* levels ($p \geq 0.05$). Four time points with significant differences between manipulated treatments for *V. parahaemolyticus*, air dried oysters contained *V. parahaemolyticus* abundances that were significantly higher than freshwater dipped oysters at three of those time points ($p \leq 0.05$), and at one time point levels in freshwater dipped oysters were significantly higher than levels in air dried oysters ($p \leq 0.05$). At the five time points with significant differences between manipulated treatments for *V. vulnificus*, the levels in freshwater dipped oysters contained significantly higher levels than air dried oysters ($p < 0.05$).

**Discussion**

When temperatures are conducive to *Vibrio* spp. growth, such as water temperatures greater than 15 °C (Gooch et al. 2002, Murphy and Oliver 1992), the typical aquaculture practices of air drying or freshwater dipping followed by air drying can significantly increase *Vibrio* spp. abundances. On average, this increase was 0.6 to 2.3 logs greater than pre-treatment levels, indicating that oysters raised under typical off-bottom aquaculture methods could pose risks to human health.

Most treatments contained oysters whose abundances returned to submersed levels between two and three days. Trial II was the only trial in which *V. parahaemolyticus* and *V. vulnificus* abundances returned to submersed levels between 3 to 7 days. This may be due to submersed oysters in this trial having the lowest overall average *Vibrio* spp. abundances by 0.5

logs, excluding trial V where there were not significant differences among the treatments. However, during trial II *V. parahaemolyticus* and *V. vulnificus* began decreasing from initially elevated levels either at or before $T_2$. Our findings that 3 to 7 days are needed to return *Vibrio* spp. to submersed levels are shorter than previous studies finding that transplanted oysters took 14 days for *V. parahaemolyticus* abundances and 7 days for *V. vulnificus* abundances in oysters to return to submersed levels (Walton et al. 2013a). This research was supplemented by Kinsey et al. (2015), where oysters that underwent dry storage and were then re-submersed required 7 to 14 days to return to submersed levels. Walton et al. (2013a) conducted a study that involved transplanting oysters from Louisiana to Alabama. The potential added stress of transport, which caused 20% mortality, may have led to elevated *Vibrio* spp. abundances persisting as those oysters may have stayed closed for a longer period of time. It is also possible that, once re-submersed, they did not pump as efficiently. Kinsey et al. (2015) found oysters that underwent dry storage had total *V. parahaemolyticus* abundances return to submersed levels in only 7 days for 24 hour dry stored compared to 14 days for 5 hour dry stored oysters. The additional three hours of ambient air exposure in our study (27 h compared to 24 h) may have caused, what Kinsey et al. (2015) refers to as, the oysters to 'gasp' for both air and nutrients once returned to the water. This, in effect, could flush out the built up *Vibrio* spp. and may result in a shorter re-submersion time needed for *Vibrio* spp. abundances to return to submersed levels.

Variation of *Vibrio* spp. abundances were observed with-in trials including between desiccation treatments, when they decreased from initially elevated ($T_{post\text{-}treatment}$) levels, and between submersed oysters. In most cases, *Vibrio* spp. abundances in oysters that were air dried and freshwater dipped did not have significant differences. Where there were differences, air dried oysters tended to have higher *V. parahaemolyticus* abundances while freshwater dipped

oysters tended to have higher *V. vulnificus* abundances. These trends, although uncommon and inconsistent, are what would be expected of both *V. parahaemolyticus* and *V. vulnificus*. *Vibrio parahaemolyticus* is known to proliferate better at higher temperatures than *V. vulnificus* (Wright et al. 1996, Parveen et al. 2013) which may have occurred during 27 hour desiccation period when air dried oysters remained exposed in direct sun light during entire period whereas freshwater dipped oysters were placed in the shade during 3 hour freshwater dip. *Vibrio vulnificus* is known to occur at relatively low salinities (Wright et al. 1996, Kaspar and Tamplin 1993, Kelley 1982), during freshwater dip oysters may have opened and changed the salinity within the oyster. However, neither statistic nor biological comparisons reveal that these effects are consistent. Across all trials, *Vibrio* spp. abundances significantly decreased from $T_{post\text{-}treatment}$, suggesting that the submersion of oysters decreases *Vibrio* spp. abundances. While human health recommendations are generally based on abundances in manipulated oysters returning to levels in submersed oysters collected concurrently, it is important to note that *Vibrio* spp. in manipulated oysters can return to submersed levels but not be significantly decreased from $T_{post\text{-}treatment}$. This effect was seen with *V. parahaemolyticus* abundances during trial IV. This shows that despite *Vibrio* spp. abundances returning to levels found in oysters that have never been removed from submersed positions there is a possibility that the effects of the desiccation treatments may remain past that point.

Research has shown natural variability in submersed samples (Rhodes et al. 2013, Zimmerman et al. 2007) which are consistent with the results from this study. In most cases, except in *V. parahaemolyticus* abundances during trial II, abundances in submersed oysters varied significantly across some time points during individual trials on a day to day basis. This variation is also seen in comparison between submersed levels and manipulated treatment levels.

During trial IV, submersed oysters contained abundances that were higher than the manipulated treatment levels at $T_{10}$ and $T_{14}$. This day to day variation may have been due to natural inherent variability or unmeasured environmental parameters such as chlorophyll or turbidity (Zimmerman et al. 2007). Despite the day to day variation, a recommendation of seven days of re-submersion prior to harvest can still be made without an increased risk to human health due to regular desiccation practices.

**Conclusion**

This study was conducted on an ALS system in an approved aquaculture setting using anti-biofouling practices routinely used by oyster farmers. The abundances of *Vibrio* spp. observed are representative of the techniques and methodologies used within this experiment. While results may vary with the use of other farming techniques and methodologies, those used in this study are representative of the practices in the Gulf of Mexico. Based on this study, a 7-day re-submersion after the routine aquaculture practices of 27 hour air dry and 3 hour freshwater dip followed by 24 hour air dry is recommended as a best management practice for oyster aquaculture and handling to help protect public health, while minimizing the restrictions on the industry. A seven day re-submersion period would coincide with oyster farmers' weekly desiccation practice. The shorter re-submersion time allows less time for biofouling to occur thus requiring less time and money to clean oysters in order to receive a premium price at market. The results of this research will aid public health officials to provide appropriate guidance to the oyster industry and allow oyster farmers to make informed decisions while keeping a safe product for the consumer.

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## Chapter 3: Effects of desiccation practices and ploidy on *Vibrio* spp. abundances in cultured oysters, *Crassostrea virginica*

### Introduction

Shellfish aquaculture in the United States generates $323 million annually, with $45 million resulting from the production of the Eastern Oyster, *Crassostrea virginica* (FAO, 2016). In 2013, the global production of *C. virginica* was 107,917 tonnes of oyster in shell. Aquaculture methods such as off-bottom and cage culture are widely used in the Northeastern United States with much success. In a combined effort, Auburn University, Mississippi-Alabama Sea Grant Consortium and Louisiana State University are working with private growers to expand oyster aquaculture to the Gulf of Mexico (NOAA, 2015). In the Gulf of Mexico, oyster farmers have traditionally used on-bottom cultivation of oysters on leases, laying shell down as substrate for wild spat to settle and grow (Walton et al. 2013a). With concerns about the number of wild spat decreasing, oyster farmers have begun to adopt methodologies already established in the Northeast and in other parts of the world (NOAA, 2015). Some of these growing methods include suspended baskets, floating baskets, oyster cages, and bags suspended by legs (Walton et al. 2013a). Mississippi has begun the permitting process to allow these methodologies to be used in designated areas, while Florida, Louisiana, and Alabama have established private growers using these off-bottom methodologies (Northern Economics, Inc. 2014). The increase use of off-bottom cultivation, while beneficial to the Gulf of Mexico economy, is a concern due warm temperatures promoting the growth of *V. parahaemolyticus* and *V. vulnificus* (Hood et al. 1983, Johnson et al. 1973, Cook 1994, Gooch 2002, Johnson et al 2010). The warm ambient air and water temperatures accompanied by regular practices associated with aquaculture such as desiccation, which remove biofouling, and submersion of oysters into freshwater, to remove

*Polydora* spp., can lead to higher levels of *Vibrio* spp. in cultured oysters (Kinsey et al. 2015, Grodeska Chapter 2).

*Vibrio parahaemolyticus* and *V. vulnificus* are human pathogenic bacteria commonly associated with food borne illnesses, with most infections (93%) coming from the consumption of raw oysters (Oliver 2013). Consumption of raw oysters which contain high abundances of these *Vibrio* spp. can cause rapid septicemia, acute gastroenteritis, and even death in immune compromised individuals (Jones and Oliver 2009, Oliver 2013, Daniels et al. 2000, Levine and Griffin 1993). *Vibrio parahaemolyticus* and *V. vulnificus* abundances are correlated with warmer water temperatures, such as those found in the Gulf of Mexico (Johnson et al. 2010, Pfeffer et al. 2003). While aquaculture continues to expand, the production of the single set oyster for the raw bar market has increased (W. Walton, pers. comm.). The consumption of raw oysters from the Gulf of Mexico in conjunction with year-round warm temperatures has raised concern amongst human health officials due a higher risk of illness associated with consumption of raw oysters in warmer climates (Johnson et al. 2010, Pfeffer et al. 2003). Currently, single set triploid oysters are frequently destined for the raw market. The added cost of acquiring and raising these oysters deems it necessary that farmers receive a higher profit than when selling to a shucking house. If triploid oysters do in-fact harbor less *Vibrio* spp. this is an additional benefit for the use of triploid oysters.

In addition to adapting grow-out methodology, researchers have experimented with and modified the oysters themselves. Diploid oysters (two chromosomes) invest much of their energy into developing gonadal tissue rather than growth. Triploid oysters (three sets of chromosomes) are unable to reproduce, so expend more energy towards rapid growth (Nell 2002). The use of triploids also allows an extended summer market during the months that spawning diploid

oysters have “milky” meat that is undesirable to the consumer (Walton et. 2013a). Decker et al. (2011), revealed a positive correlation between *Vibrio* spp. abundances and gonadal tissue, indicating that triploid oysters harbor lower levels of certain *Vibrio* spp.. This study was performed with the Pacific Oyster, *Crassostrea gigas,* and with *V. splendidus* and *V. aestuarianus,* both of which can cause mortality in oysters, but have not been documented to cause disease in humans (Decker et al. 2011). While Decker et al. 2011 investigated *Vibrio* spp. that are not of particular interest to human health officials in the Gulf Coast, their findings lead to questions regarding certain *Vibrio* spp. that do have an impact on human health.

The objective of this study was to determine whether *V. parahaemolyticus* and *V. vulnificus* abundances differ between diploid and triploid oysters subjected to common aquaculture desiccation practices including 27-hour ambient air dry and 3-hour freshwater dip prior to 24-hour ambient air dry, followed by re-submersion. Measuring *Vibrio* spp. abundances across time may help determine the length of time needed to remove effects of such desiccation practices. A significant relationship between either ploidy, desiccation practices, days since re-submersion, and/or any interactions effect on *Vibrio* spp. could help provide information to the oyster farming industry and influence public health decisions.

## Materials and Methods

### Sampling Locations and Conditions

The fieldwork was conducted at Auburn University’s research field site in Portersville Bay, Coden, Alabama (Mississippi Sound), a shallow (1-2 m) firm mud bottom site with a small tide (0.5-1.0 m). At this site, mid-summer salinities typically range from 15 to 25 PSU and water temperatures range from 25-30 °C (Walton et al., 2013a). These field conditions were expected to be favorable to proliferation of pathogenic *Vibrio* spp. (WHO-FAO, 2005). Environmental

data during study were retrieved from mymobilebay.com using the Cedar Point station. These data included daily mean and daily minimum and maximum of salinity, water temperature, wind speed, precipitation across trials and mean air temperature for the period of desiccation.

**Submersion and Treatment of Oysters**

During this study, data were collected during three two-week long replicate trials in 2015. The trials started July 15$^{th}$ and ended September 1$^{st}$. Sampling over time was done to determine the length of time needed for *Vibrio* spp. abundances in oysters of both desiccation treatments and ploidy to return to levels not significantly different from those of submersed oysters, which will hereafter be referred to as returning to submersed levels.

Oysters were stocked in eighty replicate baskets (BST Oyster Supplies, Australia), each stocked with 100-120 diploid oysters, and another eighty baskets were each stocked with 100-120 triploid oysters from the same half-sibling brood. The triploid oyster brood used was verified using flow cytometry (Allen 1983). All baskets were submersed on an Australian Long-Line culture system (ALS) at the study site alternating ploidies by bay, and maintained at a depth un-exposed to air during even extreme low tides for a minimum of 14 days prior to sampling (and typically 45 days or more). This extended submersion period prior to any sampling allowed oysters to reach ambient *Vibrio* spp. levels (Walton et al. 2013b).

In any single trial, six randomly selected baskets per ploidy were subjected to a 3-hour freshwater dip, and then allowed to air dry for 24 hours (freshwater dipped). Another three to six randomly selected baskets per ploidy were subjected to 27 hours of desiccation at ambient air temperatures (air dried). The remaining six baskets per ploidy were left in the water, as the control (submersed). A sample of 12-15 oysters were taken out of three randomly chosen baskets for each ploidy prior to any treatment and represented as $T_{pre\text{-}treatment}$. This was used to determine

what the initial *Vibrio* spp. abundances were prior to treatment, and to determine the effect of treatment on *Vibrio* spp. abundances. Additionally, immediately prior to re-submersion, $T_{post\text{-}treatment}$, three samples of 12-15 oysters was randomly selected from three baskets per treatment for each ploidy. During each trial, a sample of 12-15 oysters was randomly collected from three baskets per treatment for each ploidy at the following time points: 1, 2, 3, 7, 10, and 14 days after re-submersion (hereafter referred to as '$T_x$' where the sub-script x designates the number of days of re-submersion). All samples were packed in coolers with ice packs buffered by burlap sacks which maintained the oysters in a temperature of $\leq 15$ ˚C.

Due to the amount of time that processing required and a parallel project, diploid samples were shipped to either Auburn University Aquatic Microbiology Lab (AU AML) or delivered to the FDA Gulf Coast Seafood Laboratory (FDA GCSL). To be consistent across labs, oysters destined for the FDA GCSL were held in a cooler overnight to mimic shipping conditions required to deliver oysters to AU AML. AU AML processed samples from $T_{post\text{-}treatment}$ and $T_7$, $T_{10}$, and $T_{14}$ days since re-submersion and FDA GCSL processed samples from $T_{pre\text{-}treatment}$ and $T_1$, $T_2$, and $T_3$ days since re-submersion. All triploid samples (due to funding constraints), along with all of trial III oysters, were processed at the FDA GCSL.

**Sample Analysis**

Samples were processed following standard protocols according to Bacteriological Analytical Manual (BAM). Upon opening the cooler, 12-15 oysters from each experimental group were cleaned, shucked, and homogenized in a sterile food blender. The samples were then processed for *V. parahaemolyticus* and *V. vulnificus* abundances by direct plating; samples were plated on T1N3 to isolate *V. parahaemolyticus* and VVA to isolate *V. vulnificus.* After colonies were lifted and lysed to filters, the remainder of processing was completed at the FDA GCSL

using alkaline phosphatase-labeled oligonucleotide probe colony hybridization for confirmation (McCarthy et al., 1999 Wright et al., 1993). Probe-positive colonies were counted and reported in CFU/g.

**Statistical Analysis**

Environmental data were collected from mymobilebay.com using the Cedar point site. Environmental parameters that were analyzed included water temperature, salinity, wind speed, precipitation and air temperature. Air temperature was collected during the 27 hours oysters were subjected to desiccation treatments; these data were used to perform one-way ANOVA. Air temperature daily mean along with the minimum and maximum values were calculated. The remaining factors data were collected for the entire duration of each trial and the daily mean along with daily mean minimum and maximums were calculated. The daily means were used to perform one-way ANOVA to compare between trials, except precipitation. The daily mean maximum for precipitation was used to perform one-way ANOVA. Using the data that were previously described for each one-way ANOVA, a main effects model was performed to determine environmental effects on *Vibrio* spp.

All *Vibrio* spp. data were log transformed and triplicate samples averaged so that replication was at the trial level. To assess whether the two culture treatments (freshwater dipped and air dried) successfully elevated *Vibrio* spp. abundances, $T_{post\text{-}treatment}$ levels were compared to un-manipulated $T_{pre\text{-}treatment}$ levels, a two-way ANOVA was performed to compare effects of culture treatment and ploidy on *Vibrio* spp. abundances with a post-hoc multiple comparisons t-test.

A three-way ANOVA was performed to compare the effects of ploidy, culture treatment, and days since re-submersion on *Vibrio* spp. abundances. A student's t-test was used for all post-

hoc comparisons where a significant effect was found. All ANOVAs and post-hoc comparisons were considered significant at $p < 0.05$. All statistical calculations were performed using the JMP statistical program (SAS Institute Inc., Cary, NC).

## Results

### Environmental

Although significant differences of environmental parameters were detected between trials (Table 3.1), there was no apparent effect on *Vibrio* spp. abundance ($p > 0.30$).

Table 3.1: Mean environmental data (salinity, water temperature, wind speed, and precipitation) with mean daily minimum and maximums over each trial and averaged air temperature for date of treatment (desiccation) with averaged minimum and maximums. Superscript letters denote significant differences.

| Trials | Environmental data | | | | |
|---|---|---|---|---|---|
| | Water Temp (°C) | Salinity (PSU) | Wind Speed (knots) | Precipitation (cm) | Air Temp (°C) |
| I | $31.0^{A}$ (30.1-32.0) | $19.9^{B}$ (16.3-24.2) | $9.6^{A}$ (1.8-19.8) | $0.003^{A}$ | $29.8^{A}$ (28.8-30.7) |
| II | $30.2^{B}$ (29.3-31.1) | $23.7^{A}$ (21.1-26.8) | $10.4^{A}$ (3.3-19.9) | $0.009^{A}$ | $28.8^{B}$ (26.6-30.0) |
| III | $29.6^{B}$ (28.7-30.6) | $20.9^{B}$ (18.4-24.0) | $7.4^{B}$ (1.0-14.0) | $0.0006^{A}$ | $28.6^{C}$ (26.7-29.7) |

### Initial Effect of Desiccation Treatment

At the onset of the trial, there was no effect of ploidy ($p = 0.20$), nor an interaction between culture treatment and ploidy ($p = 1.00$) on *V. parahaemolyticus* abundances (Table 3.2). The culture treatments (Table 3.2) had a highly significant effect on *V. parahaemolyticus* abundances ($p < 0.01$). Among the different culture treatments (Table 3.3, Figure 3.1), the abundances in the two manipulated culture treatments [air dried (4.6 log MPN/g) and freshwater dipped (4.5 log MPN/g)] were significantly higher ($p < 0.01$) than either concentration in the $T_{pre\text{-}treatment}$ oysters (3.0 log MPN/g) or the submersed oysters (3.2 log MPN/g), but did not differ

from each other (p = 0.83). Additionally, the submersed treatment did not differ from the pre-treatment (p = 0.37).

Table 3.2: ANOVA table for $T_{pre\text{-}treatment}$ and $T_{post\text{-}treatment}$ *V. parahaemolyticus* abundances; submersed, air dried, and freshwater dipped. Lines in bold represent significant differences (alpha = 0.05).

| Source | DF | Sum of Squares | F Ratio | Prob> F |
|---|---|---|---|---|
| **Culture Treatment** | **3** | **12.76** | **27.33** | **<0.01** |
| Ploidy | 1 | 0.28 | 1.80 | 0.20 |
| Culture Treatment*Ploidy | 3 | 0.01 | 0.02 | 1.00 |

Table 3.3: Post-hoc t-test comparison of *V. parahaemolyticus* levels (Table 1) for $T_{pre\text{-}treatment}$ (Pre) and $T_{post\text{-}treatment}$; submersed, air dried (Air), and freshwater dipped (Freshwater). Lines in bold represent significant differences (alpha = 0.05).

| Level | - Level | Difference | Std Err Dif | Lower CL | Upper CL | p-Value |
|---|---|---|---|---|---|---|
| **Air** | **Pre** | **1.58** | **0.23** | **1.10** | **2.06** | **<0.01** |
| **Freshwater** | **Pre** | **1.53** | **0.23** | **1.05** | **2.01** | **<0.01** |
| **Air** | **Submersed** | **1.37** | **0.23** | **0.89** | **1.85** | **<0.01** |
| **Freshwater** | **Submersed** | **1.32** | **0.23** | **0.84** | **1.80** | **<0.01** |
| Submersed | Pre | 0.21 | 0.23 | -0.27 | 0.69 | 0.37 |
| Air | Freshwater | 0.05 | 0.23 | -0.43 | 0.53 | 0.83 |

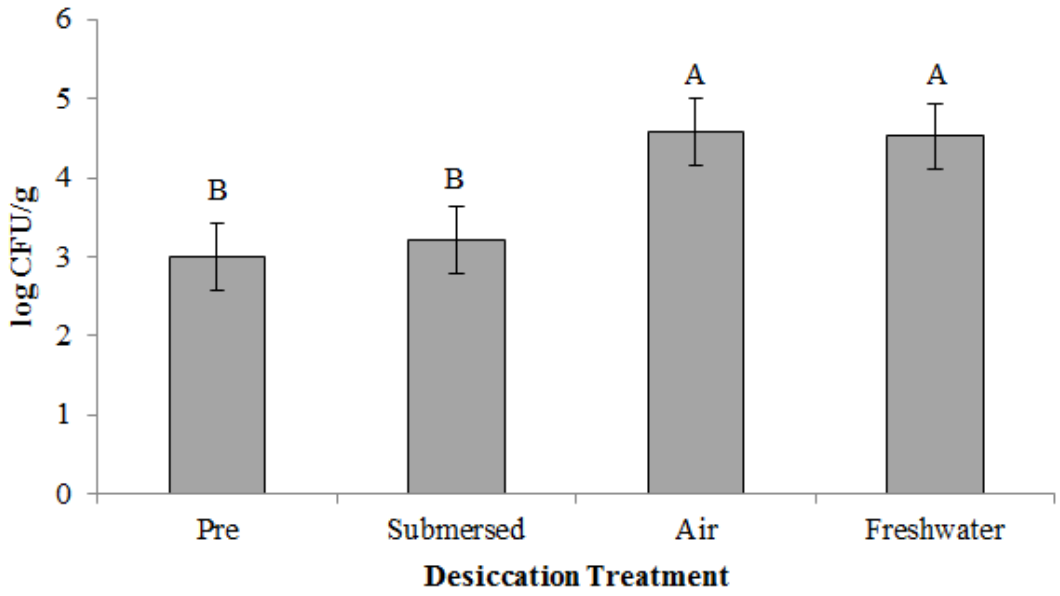


Figure 3.1: Mean *V. parahaemolyticus* abundances with standard error bars across all trials of culture treatment; $T_{pre\text{-}treatment}$ (Pre) and $T_{post\text{-}treatment:}$ submersed, air dried (Air), and freshwater dipped (Freshwater). Different letters indicate significant differences as determined by the post hoc student t-test.

Similarly, for *V. vulnificus* (Table 3.4), there was no effect of ploidy ($p = 0.52$) nor an interaction between culture treatment and ploidy ($p = 0.84$). There was an effect of culture treatment ($p = <0.01$). Among the different culture treatments (Table 3.5, Figure 3.2), the two manipulated treatments [air dried (4.9 log MPN/g) and freshwater dipped (4.9 log MPN/g)] were significantly higher ($p < 0.01$) than either the $T_{pre\text{-}treatment}$ oysters (3.8 log MPN/g) or the submersed oysters (3.9 log MPN/g), but did not differ from each other ($p = 0.99$). Additionally, the submersed treatment did not differ from the pre-treatment ($p = 0.89$).

Table 3.4: ANOVA table for $T_{pre\text{-}treatment}$ and $T_{post\text{-}treatment}$ *V. vulnificus* levels; submersed, air dried, and freshwater dipped. Lines in bold represent significant differences (alpha = 0.05).

| Source | DF | Sum of Squares | F Ratio | Prob> F |
|---|---|---|---|---|
| **Treatment** | **3** | **6.16** | **6.62** | **<0.01** |
| Ploidy | 1 | 0.13 | 0.43 | 0.52 |
| Treatment*Ploidy | 3 | 0.26 | 0.27 | 0.84 |

Table 3.5: Post-hoc t-test comparison of *V. vulnificus* levels (Table 1) for $T_{pre\text{-}treatment}$ (Pre) and $T_{post\text{-}treatment}$; submersed, air dried (Air), and freshwater dipped (Freshwater). Lines in bold represent significant differences (alpha = 0.05).

| Level | - Level | Difference | Std Err Dif | Lower CL | Upper CL | p-Value |
|---|---|---|---|---|---|---|
| **Freshwater** | **Pre** | **1.04** | **0.32** | **0.36** | **1.72** | **<0.01** |
| **Air** | **Pre** | **1.03** | **0.32** | **0.35** | **1.72** | **<0.01** |
| **Freshwater** | **Submersed** | **0.99** | **0.32** | **0.31** | **1.67** | **<0.01** |
| **Air** | **Submersed** | **0.99** | **0.32** | **0.31** | **1.67** | **<0.01** |
| Submersed | Pre | 0.04 | 0.32 | -0.64 | 0.73 | 0.89 |
| Freshwater | Air | 0.004 | 0.32 | -0.68 | 0.69 | 0.99 |

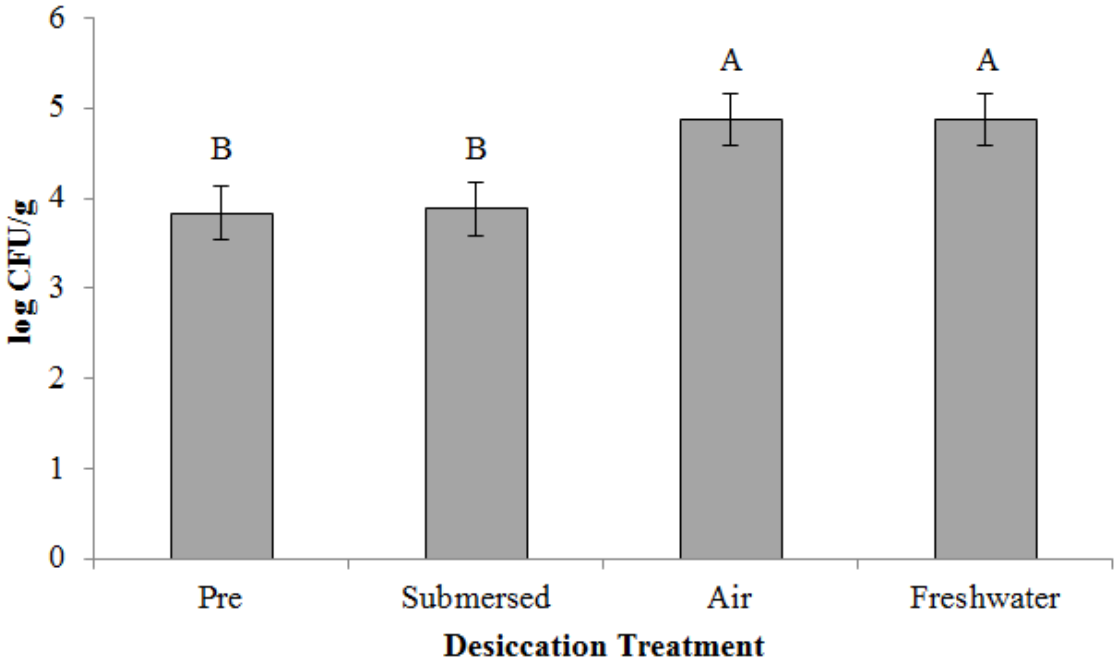


Figure 3.2: Mean *V. vulnificus* abundances with standard error bars across all trials of $T_{pre\text{-}treatment}$ (Pre) and $T_{post\text{-}treatment;}$ submersed (Sub), air dried (Air), and freshwater dipped (Freshwater). Different letters indicate significant differences as determined by the post hoc student t-test.

**Effect of Ploidy over Re-submersion Time**

For both *V. parahaemolyticus* and *V. vulnificus* (Tables 3.6 and 3.7 respectively), there was no effect of ploidy ($p \geq 0.06$), nor any significant interaction with ploidy ($p \geq 0.38$). Notably, however, for *V. parahaemolyticus*, triploids tended to have lower *V. parahaemolyticus* abundances than diploid oysters (Figure 3.3).

Table 3.6: ANOVA table of the test of effects of time, desiccation treatment, and ploidy on *Vibrio parahaemolyticus* abundances in oysters. Lines in bold represent significant differences (alpha = 0.05).

| Source | DF | Sum of Squares | F Ratio | Prob> F |
|---|---|---|---|---|
| **Model** | **41** | **38.24** | **4.99** | **<0.01** |
| Error | 84 | 15.69 | | |
| C. Total | 125 | 53.93 | | |
| **Time** | **6** | **17.82** | **15.90** | **<0.01** |
| **Treatment** | **2** | **7.13** | **19.09** | **<0.01** |
| Ploidy | 1 | 0.66 | 3.54 | 0.06 |
| **Time*Treatment** | **12** | **10.59** | **4.72** | **<0.01** |
| Time*Ploidy | 6 | 1.21 | 1.08 | 0.38 |
| Treatment*Ploidy | 2 | 0.18 | 0.49 | 0.62 |
| Time*Treatment*Ploidy | 12 | 0.65 | 0.29 | 0.99 |

Table 3.7: ANOVA table of the test of effects of time, culture treatment, and ploidy on *Vibrio vulnificus* abundances in oysters. Lines in bold represent significant differences (alpha = 0.05).

| Source | DF | Sum of Squares | F Ratio | Prob> F |
|---|---|---|---|---|
| Model | 41 | 22.67 | 2.14 | <0.01 |
| Error | 84 | 21.67 | | |
| C. Total | 125 | 44.33 | | |
| **Time** | **6** | **9.91** | **6.40** | **<0.01** |
| **Treatment** | **2** | **4.57** | **8.87** | **<0.01** |
| Ploidy | 1 | 0.30 | 1.18 | 0.28 |
| **Time*Treatment** | **12** | **6.01** | **1.94** | **0.04** |
| Time*Ploidy | 6 | 0.30 | 0.20 | 0.98 |
| Treatment*Ploidy | 2 | 0.17 | 0.33 | 0.72 |
| Time*Treatment*Ploidy | 12 | 1.40 | 0.45 | 0.94 |

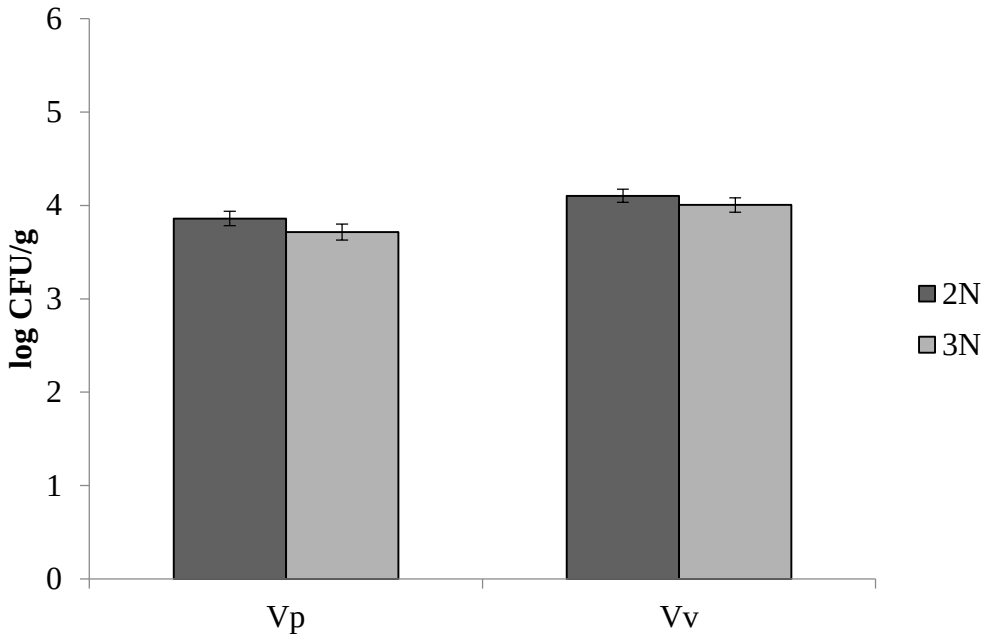


Figure 3.3: Mean ploidy comparison of log transformed CFU/g of *V. parahaemolyticus* (Vp) and *V. vulnificus* (Vv) on x-axis with standard error bars. The key describes ploidy: diploids (2N) and triploids (3N). No significant differences were found between ploidy.

**Effects of Time and Treatment Interactions**

For both *V. parahaemolyticus* and *V. vulnificus* (Tables 3.6 and 3.7, respectively), there were significant interactions between time and treatment ($p \leq 0.04$). For *V. parahaemolyticus* (Figure 3.4), abundances reached those similar to submersed (4.0 log CFU/g) at $T_3$ for air dried (4.3 log CFU/g) and freshwater dipped treatments (4.5 log CFU/g). Specifically, $T_{post\text{-}treatment}$, $T_1$ and $T_2$, abundances in oysters of the two desiccation treatments (air dried, freshwater dipped), which ranged from 4.0 log CFU/g ($T_2$ air dried) to 4.6 log CFU ($T_{post\text{-}treatment}$ air dried, $T_1$ freshwater dipped), were significantly higher than the abundances in oysters of the submersed treatment ($p \leq 0.01$), which ranged from 3.2 log CFU/g ($T_0$, $T_1$) to 3.4 log CFU/g ($T_2$), but from $T_3$ onward there were no significant differences among all three culture treatments within any

given number of days submersed. In addition, there were never any significant differences of *V. parahaemolyticus* abundances between the two manipulated culture treatments ($p \geq 0.05$) within any given number of days submersed (Figure 3.4).

Importantly, the abundances of *V. parahaemolyticus* in submersed oysters differed significantly among days (Figure 3.4); for example, the levels at $T_3$ (4.0 log CFU/g) were significantly higher than at $T_{post\text{-}treatment}$, $T_1$, $T_2$, $T_{10}$ and $T_{14}$ which ranged from lowest at $T_{10}$ (3.1 log CFU/g) to the highest at $T_2$ and $T_{14}$ (3.4 log CFU/g). Additionally, at $T_3$ the abundances in oysters in desiccated culture treatment samples returned to levels in submersed oysters [air dried oysters (4.6 log CFU/g) and freshwater dipped oysters (4.5 log CFU/g)], but did not decrease from initially elevated levels ($T_{post\text{-}treatment}$) until $T_7$ [air dried oysters (3.7 log CFU/g) and freshwater dipped oysters (3.6 log CFU/g)].

For *V. vulnificus* (Fig. 3.5), abundances in oysters that underwent desiccation treatments reached those similar ($p = 0.14$) to submersed oysters (3.7 log CFU/g) at $T_2$ in air dried (4.1 CFU/g) and freshwater dipped (4.2 CFU/g) treatment samples. Specifically, at days $T_{post\text{-}treatment}$ and $T_1$, the abundances in oysters from the two desiccated treatments (air dried, freshwater dipped) were significantly higher than the levels in submersed oysters ($p \leq 0.01$). By $T_3$ and for the remainder of the study, there were no significant differences in *V. vulnificus* abundances in oysters among the three culture treatments ($p \geq 0.05$). In addition, there were no significant differences between the abundances in the two desiccated culture treatments ($p \geq 0.05$) within any given number of days submersed.

The abundances of *V. vulnificus* in submersed oysters did not differ significantly among days ($p \geq 0.05$). Despite the lack of variation in abundances of *V. vulnificus* in submersed oysters, variation was observed with-in culture treatments among days. Abundances in oysters

from both desiccated culture treatments were significantly lower than elevated $T_{post\text{-}treatment}$ levels [air dried oysters (4.9 log CFU/g) and freshwater dipped oysters (4.9 log CFU/g)] at $T_2$. At $T_{3,}$ abundances in oysters from freshwater dipped treatments return to $T_{post\text{-}treatment}$ elevated levels (4.5 log CFU/g). The abundances in oysters from freshwater dipped treatments again decrease from $T_{post\text{-}treatment}$ levels at $T_7$ (3.8 log CFU/g) and do not return to elevated levels for the remainder of the study.

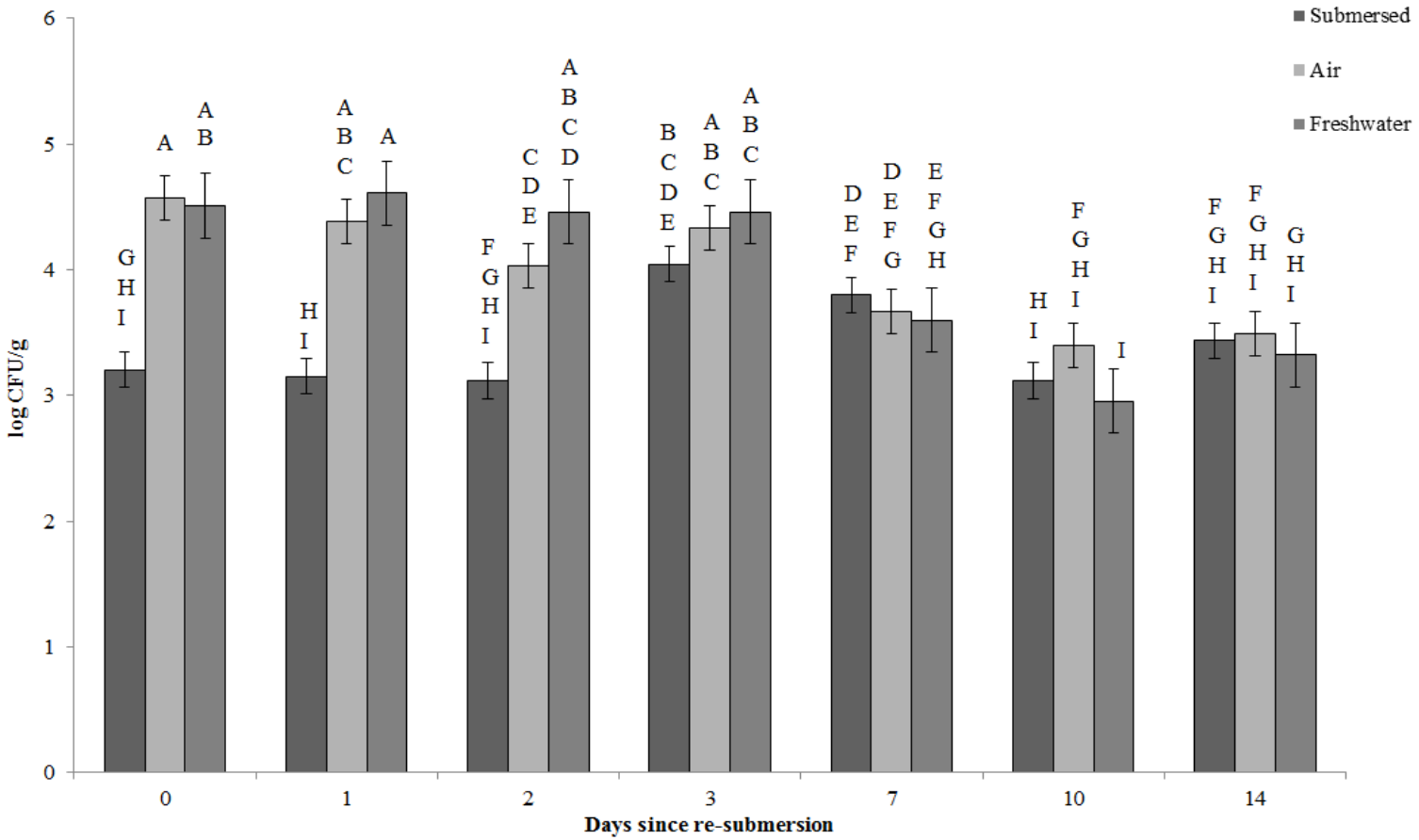


Figure 3.4: Mean log transformed *V. parahaemolyticus* (y-axis) with standard error bars. With a key describing desiccation treatment; submersed (Sub), 27 hour desiccation (Air), and 3 hour freshwater dip with 24 hour desiccation (H2O). Days since re-submersion are shown on the x-axis, where day 0 is immediately prior to re-submersion. Letters located above bars are representative of post-hoc student t-test results.

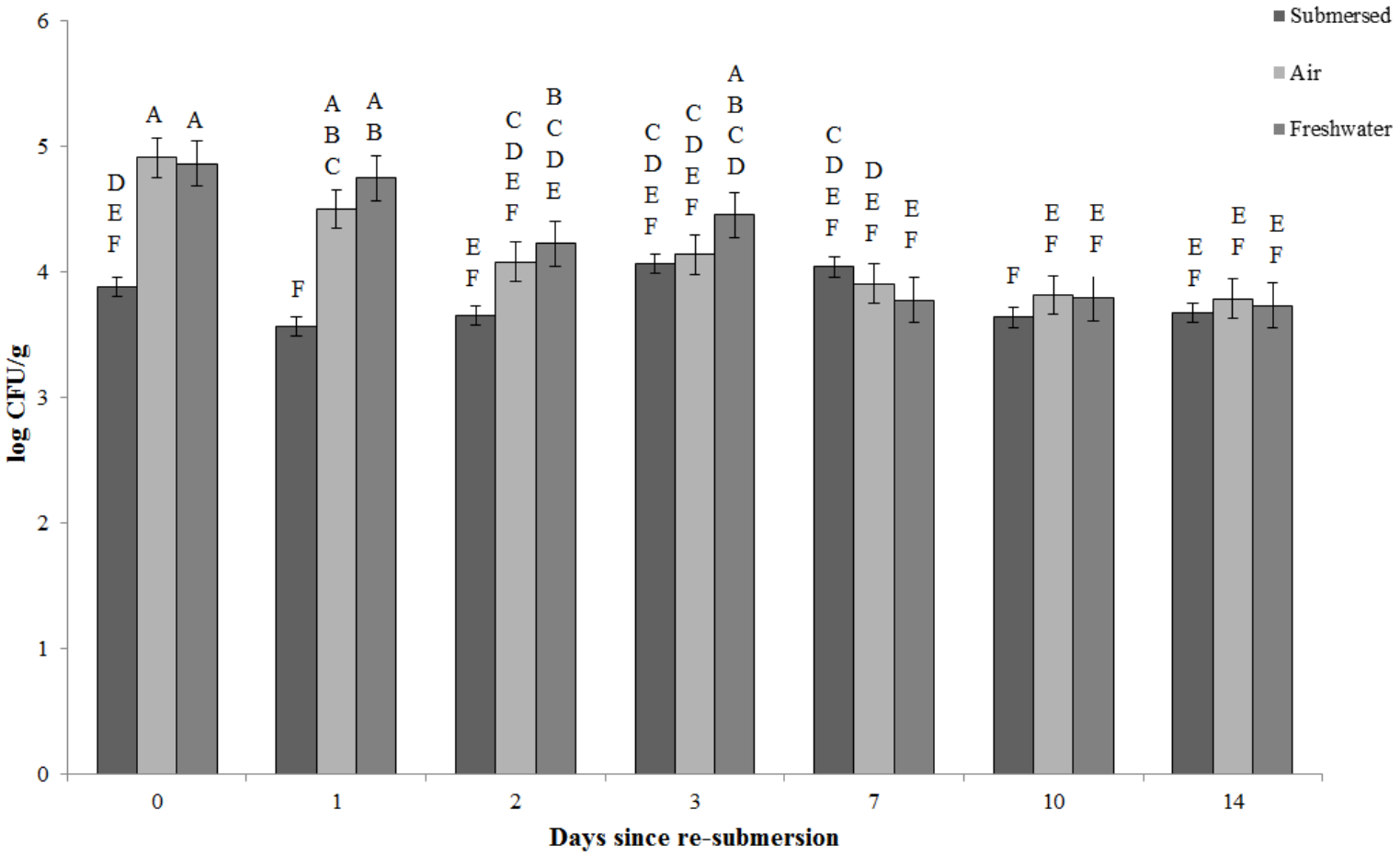


Figure 3.5: Mean log transformed *V. vulnificus* (y-axis) with standard error bars. With a key describing desiccation treatment; submersed (Sub), 27 hour desiccation (Air), and 3 hour freshwater dip with 24 hour desiccation (H2O). Days since re-submersion are shown on the x-axis, where day 0 is immediately prior to re-submersion. Letters located above bars are representative of post-hoc student t-test results.

**Discussion**

The lack of effect of ploidy shows no benefits for the use of either ploidy. Notably, however, for *V. parahaemolyticus*, triploids tended to have lower *V. parahaemolyticus* abundances than diploid oysters ($p = 0.06$); this trend was also seen in Walton et al. (2013). With no significant effect, ploidy will need to be further examined to determine its interaction with *Vibrio* spp. abundances.

Environmental parameters found in this study, such as water temperatures greater than 15˚C (Gooch et al. 2002, Murphy and Oliver 1992) and salinity between 5 and 25 (Bryan et al. 1999, Høi et al. 1998, Kaspar and Tamplin 1993) are conducive to *Vibrio* spp. growth. This is evident in the effectiveness of both routine desiccation practices, air dried and freshwater dipped, to significantly increase *Vibrio* spp. abundances compared to levels in continually submersed and $T_{pre\text{-}treatment}$ oysters. The increase of *Vibrio* spp. abundances by 1 to 1.5 logs show that there is in fact a higher human health risk associated with routine aquaculture practices, and indicates rationale for regulations being put on desiccation practices. When comparing between desiccation treatments, neither treatment air dried or freshwater dipped, had an increased risk or benefit. At each time point for both *Vibrio* spp. abundances no significant differences were found between the two manipulated desiccation treatments. These results are similar to Grodeska et al. (Chapter 2), where in more than 85% of manipulated desiccation treatment comparisons there were no differences. However, Grodeska et al. (Chapter 2) performed statistical analysis by trial which increased type I error, raising the probability that differences would be found.

The results of this study suggest a re-submersion time of three days will allow *Vibrio* spp. abundances in oysters that undergo manipulated desiccation treatments to return to submersed levels. These results were comparable to Grodeska et al. (see Chapter 2) that had *Vibrio* spp. abundances of samples that underwent routine aquaculture treatments returned to ambient levels

mostly between day 2 and 3, with one trial not returning to submersed until day 7. This information supports the previous recommendation (see Chapter 2) of seven days re-submersion and that further research into days 4, 5, and 6 could refine and reinforce this shortened recommendation.

Public health recommendations are generally based on abundances in oysters that are exposed to practices that may elevate abundances returning to submersed levels; however, it is important to note that in most cases *Vibrio* spp. abundances in oysters that underwent desiccation treatments did not decrease from initial elevated levels until after returning to submersed levels, except for air dried *V. vulnificus* abundances. It is important that *Vibrio* spp. abundances are given time to decrease from elevated levels because this, in theory, removes the effects of desiccation practices. While abundances returned to submersed levels by day three, most elevated levels do not significantly decrease from the initial elevated levels ($T_{post\text{-}treatment}$) until day seven. Seven days allows time for abundances to return to submersed levels and decrease from those initially elevated levels. This supports a recommendation of seven days of re-submersion prior to harvesting and is consistent with the recommendation resulting from the different analytical and statistical methodologies used in Chapter 2. The combined results of *Vibrio* spp. abundances returning to ambient between two to three days and levels significantly decreasing at or before day seven suggests that oysters subjected to routine desiccation practices may need fewer than seven days to remove the increased associated risk. Further investigation of the effects of desiccation practices, especially days four, five, and six, may result in a recommendation of less than seven days of re-submersion prior to harvest.

## Conclusion

Most studies of *C. virginica*, *V. parahaemolyticus*, and *V. vulnificus* have focused on dry storage and post-harvest methodologies that increase *Vibrio* spp.. Studies like these are used to determine the length of time it takes for *Vibrio* spp. levels to multiply. This information helps inform public health officials on the amount of time oysters can be held with or without refrigeration before they pose an increased risk. This study focuses on reducing *Vibrio* spp. abundances prior to harvest and to ensure that routine aquaculture practices do not increase the risk of illness to consumers. Future studies should be performed to support this research and to possibly look at other gear types such as floating cages, on-bottom trays, and floating bags. Expanding the knowledge of the impact of routine aquaculture practices on *Vibrio* spp. could decrease the length of time needed for re-submersion.

This study was conducted using routine aquaculture practices, in an approved aquaculture location in the Gulf of Mexico to determine if, under these experimental conditions, diploid or triploid oysters would contain significantly different *Vibrio* spp. abundances during time of re-submersion. We have concluded that, while there is a tendency for triploids to have lower abundances of *V. parahaemolyticus* than diploids, triploid oysters do not have significantly different *Vibrio* spp. compared to diploid oysters. When oysters underwent desiccation treatments, air dried or freshwater dipped, there was no apparent effect of treatment on *Vibrio* spp. abundances. Overall, it is important to note that, while *Vibrio* spp. abundances may have returned to submersed levels by day three, there is a possibility they are still affected by desiccation treatments until seven days after re-submersion. Allowing *Vibrio* spp. levels in oysters to return to submersed levels as well as decrease from initially elevated levels removes the increased risk associated with routine desiccation practices. For these reasons this study

supports a recommendation of seven days of re-submersion after routine desiccation practices prior to harvest.

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## 4.1 Summary

Oyster farmers make a number of choices when managing their operations, including gear types, harvest times, etc. Some of these choices may affect the safety of the harvested product by increasing the abundances of both *Vibrio parahaemolyticus* and *V. vulnificus*. This increase in abundances may occur when the oysters are removed from the water and exposed to elevated ambient air temperatures, which occurs during desiccation to control fouling. When oysters are removed from the water and subjected to temperatures above 15 ˚C, *Vibrio* spp. can proliferate inside oysters; these oysters are unable to eliminate *Vibrio* spp. through pumping. Once re-submersed, oysters are able to pump and exchange water, which may allow *Vibrio* spp. abundances to return to background abundances after seven days.

In this study conducted during the summer months when *Vibrio* spp. levels would be expected to be highest and there was the greatest likelihood of *Vibrio* spp. growth, we determined the following:

- Desiccation practices, 27-hour air dry and 3-hour freshwater dip followed by 24-hour air dry, did elevate levels of *V. parahaemolyticus* and *V. vulnificus*;
- A minimum of seven days re-submersion is recommended post-desiccation to allow *Vibrio* spp. abundances to return to submersed levels and decrease from elevated levels for either of the desiccation practices tested;
- *Vibrio* spp. levels in triploid and diploid oysters were not statistically different.

Oyster farmers should be aware that desiccation practices do raise *Vibrio* spp. abundances in their product, which raises risks associated with eating raw oysters. To ensure that harvested

oysters are as safe as possible, farmers need to be aware and allow at least seven days of re-submersion after the last period of desiccation prior to harvest.