Exploring the Efficacy of Different Barrel Cleaning Procedures on *Brettanomyces bruxellensis* and *Acetobacter spp.* Populations and the Relative Financial and Environmental Benefits of Each Treatment.

Index

1. Summary

Barrel sanitation is an important topic for the wine industry because many wineries utilize barrels as part of their winemaking protocol. This study aimed to determine the best barrel sanitation technique for a medium-sized Napa Valley winery. This determination was based on microbial effectiveness, environmental, and financial impact of the separate treatments. A controlled experiment was conducted to compare the different treatments. After determining the effectiveness and cost of each treatment, several analyses were undertaken to determine the treatment that offered the best value to the winery. Steam proved to be the most effective against the three types of microbes measured in this study and had the highest value number when comparing costs to the effectiveness to cost ratio. Post-trial, a survey was conducted to determine the current winemaker perceptions and practices in Napa Valley for barrel sanitation. The goal was to examine the current perceptions in theory, after completing the barrel sanitation trial. The winemaker survey determined that although many wineries are using multiple treatment options, steam was by far the most preferred method for dealing with microbial contamination. This was then compared to the trial results to determine similarities and differences.

2. Introduction

The use of the wooden barrel as a means of transport or container dates back to Celtic times beginning in the 6th century BC (Robinson, 2006). Based on evidence from literature and art, use of barrels in winemaking appears to have become widespread by the 3rd century BC, when it began to replace amphorae as the container of choice for storage and transportation (Robinson, 2006). Today, oak barrels are still used for wine storage as well as for the aromatic and sensory enhancement of wines. However, since wood is a porous material, proper care and maintenance is vital in maintaining the vessel's usefulness over time (Marko et al, 2005). Two microbes are of particular significance, and threat, when a winemaker uses barrels to mature wine: *Brettanomyces bruxellensis* and *Acetobacter.*

Brettanomyces

Although *Brettanomyces* or 'Brett' can add complexity in small doses, in high concentration populations it can cause a number of unpleasant aromatic chemicals in a wine including 'animal,' 'barnyard,' 'stable,' and 'sticking plaster' (Chatonnet, Dubourdieu, and Boidron, 1995). Such aromas occur when Brett decarboxylates hydroxycinnamic acid into 4- vinylphenol and 4-vinylguaiacol and then finally into 4-ethylphenol (4-EP) and 4-ethylguaiacol (4-EG) (Zuehlke, Petrova, and Edwards, 2013). While there are five known species of *Brettanomyces*, currently only *B. bruxellensis* is associated with wine spoilage (Egli and Henick-Kling, 2001). While some winemakers believe that higher alcohol levels can prevent Brett contamination, Silva, Cardoso, and Geros (2004, p. 71) have proven that 'in the absence of glucose, *B.*

bruxellensis…exhibited a higher growth capacity at high ethanol concentrations with lower susceptibility to death from ethanol.' Brett also has the capacity to remain viable but non-culturable for long periods of time through spores, suggesting that removing its food source will not eliminate the danger of Brett contamination (Arvik and Henick-Kling, 2002). Sulfur dioxide will also cause Brettanomyces to enter a viable but non-culturable state which makes chemical control of the organism in wine challenging (Umiker and Edwards, 2007). Unlike other common wine yeasts, Brett is also capable of consuming cellobiose, a wood sugar found in barrels (Blondin et al, 1982). It is difficult to determine what precursors in the wine encourage the growth of Brett. Joseph et al. (2013, p. 240) found that 'coumaric acid [a pre-cursor to 4-ethylphenol] was shown to have inhibitory, neutral, or beneficial impacts on the growth and viability of Brett depending on both the level of aeration and the strain, making prediction of off-character formation challenging under production conditions.' 'Inadequate sanitation, high wine pH, and low [sulphur dioxide] (SO_2) concentrations' have all been identified as conditions in which Brett will thrive (Perez-Prieto et al, 2002, p. 3273). This conclusion is echoed in Heresztyn's (1986) study of mousy wines which emphasizes the importance of barrel sanitation. The complicating challenge of cleaning barrels contaminated with Brett is that it can be detected in depths of up to 8 mm into the wood in addition to forming significant biofilms (Malfeito-Ferreira et al, 2004) (Joseph et al, 2007).

Acetobacter

Acetobacter was long thought to be strictly aerobic and not a serious threat to the wine industry. However, research has shown that it is actually semi-anaerobic and still able to negatively affect wine quality beyond the production of acetic acid (Drysdale and Fleet, 1988). It has also been shown that while Acetobacter bacteria actively thrive in conditions above 70% dissolved oxygen and dies off below 50% dissolved oxygen, they can survive in wines at 50% dissolved oxygen suggesting that even if the wine does not show negative effects initially, latent bacteria may start to grow once oxygen is reintroduced (Drysdale and Fleet, 1989). Another study completed in France suggested links between the use of barrels to store wine and increases in acetic acid through both contamination and aeration from stored oxygen which collects in the pores of wood between barrel uses (Joyeux, Lafon-Lafourcade, and Ribereau-Gaynon, 1984).

3. Review of Research Context

Both *Brettanomyces* and *Acetobacter* are sources of concern for winemakers using oak to store wine. In a study undertaken to identify the quality and safety concerns in a winery setting, 'growth of […] *Brettanomyces, Dekkera*' and '*Acetobacter* in the wine' were listed as hazards for wine quality and Christaki and Tzia (2002, p. 512) mentioned 'careful barrel cleaning' and 'control of cleaning procedures' as preventative and controlling measures. Wedral, Shewfelt, and Frank (2010) explored options to control *Brettanomyces* induced flavor compounds stating that it was critical to avoid the use of contaminated barrels. The study by Garde-Cerdan and Ancin-Azpilicueta (2006, p.442), found that 4-EP and 4-EG 'were formed in higher quantities in wine stored in used barrels and that their concentration tended to increase during aging.' Shaving and re-firing the barrels resulted in an 80% decrease in 4-EP and 4-EG in the subsequently stored wines compared to pre-treatment; however, this treatment is extreme and not practical for the average commercial production setting (Pollnitz, Pardon, and Sefton, 2000). Burning sulfur blocks or 'wicks' has long been used to control microbial populations in barrels; however, this has been found to have qualitative ramifications since the elemental sulfur can be converted to hydrogen sulfide by yeasts during barrel fermentations (Thoukis and Stern, 1962). Suarez et al. (2005 (published in 2007), p 11) states that 'although cleaning methods are becoming more sophisticated, the shape and microstructure of wooden barrels afford undesirable organisms a great degree of protection'.

Currently, the methods that appear in published literature are steam, ozone, high pressure hot water with ultrasonics, and ultraviolet (UV) radiation. There have been numerous studies conducted; however, many have flaws in the experimental design which can make drawing definitive conclusions challenging. This highlights the need for an in-depth, controlled experiment comparing all of the main sanitation techniques in a single study.

In Rayne and Eggers's (2008) study the steam treatment followed by SO_2 gas was deemed to be the most effective, resulting in the lowest concentrations of 4-EP and 4-EG in the wines tested. It is important to note that they did not perform the treatments themselves, but relied on winemakers to report their hygiene procedures which 'could not be reliably reported for 32 of the 188 samples tested' (Rayne and Egger, 2008, p. 93). In the Wilker and Dharmadhikari (1997) study, hot water proved to be the most effective against *Acetobacter*; however, the study was conducted on wood samples in a lab setting, not production size barrels. This study also did not address any other treatments. Another study (Schmid et al, 2011) supported the idea that high pressure hot water (60°C) combined with ultrasonic treatment was able to effectively remove culturable Brett cells from the surface and up to four millimeters into the stave itself without harming positive aromatic compounds in the wood. It is important to note that this study did not compare this method to the other three methods in a production setting.

Ozone has also been studied as an effective sanitizer on stainless steel and was declared to be generally recognized as safe (GRAS) in 1997 which opened its use up to many industries (Hampson, 2000). One study compared ozone to hot water treatments to compare loss of positive volatile oak aromas. Yet, it failed to compare the efficacy of microbial sanitation of the two methods (Marko et al, 2005). Guzzon et al (2011) performed the most comprehensive published study to date by comparing UV radiation, ozone, and steam where they dismissed the use of UV radiation for barrels stating ‘the field of use for UV should be restricted to light-permeable matrices or the sanitation of surfaces’ (2011, p. 290). Both steam and ozone performed well during Guzzon’s (2011) study and were included in this experiment.

A citric acid and SO_2 solution is a relatively inexpensive treatment which rarely appears in research. This was introduced into this trial work as a result of a personal communication with Ed Killian, Director of Winemaking at Asti Winery, in Cloverdale, CA (2010, pers comm.). Since that original communication, many winemakers conversationally have also mentioned using this method for sanitation and for barrel preservation since it keeps the wood hydrated which prevents leaks and cracking staves. Since it has been touted casually as an effective method of microbial control in barrels, it was included in the trial for comparison to the more published methods of barrel sanitation.

4. Methodology

4.1 - Winemaking Research Procedure

Thirty-six used barrels were selected from a single lot of wine. The wine from individual barrels were analyzed using Polymerase Chain Reaction (PCR) and thus known to contain varying levels of Brett (Appendix 1). Barrels identified were grouped into six groups of six barrels each, (consistent with the methodology outlined in Guzzon et al in 2011), with total cell counts which were similar within the groups. Individual barrels were identified as one of six within each group. For example, barrel 1.5 was in the first group (control) and was the fifth barrel in the group (Appendix 1). The wine was pumped out, after which all the barrels had a thirty second cold water rinse to remove wine sediment. All barrels were sampled within each test group to establish baseline populations using the microbial analysis procedure for sampling and testing using the microbial analysis procedure outlined below. The six groups of barrels were separated into the following treatment groups:

1) Control – Neutral water (13.8°C) rinse for two and a half minutes using city water on the inline barrel washer.
2) Steam – Six minutes exposure to steam at 30 psi, three minutes bunged, then rinsed for two and a half minutes with city water (13.8°C) on the inline barrel washer.
3) Ozone – Rinse barrels with 82°C city water for one and a half minutes followed by two minute cold ozonated water rinse.

4) Hot Water – Rinsed with 82°C water for two and a half minutes using city water on the inline barrel washer.
5) Citric and SO_2 Solution – Prepared a solution of 84 g potassium metabisulfite and 182 g of citric acid in each barrel using city water. Allowed barrels to sit for two weeks then rinsed for two and a half minutes with city water (13.8°C) on the inline barrel washer.
6) Burning Sulfur - Burned sulfur sticks in each barrel for one hour then city water rinse for one minute (13.8°C) on the inline barrel washer.

4.2 - Microbial Analyses Procedure

Six hundred mL sterilized water was added to each barrel. The sterilized water was tested to confirm its sterility. The barrel was then sealed with a new, clean bung. All surfaces of the barrel were contacted by rolling six times on a barrel rolling rack and allowing the water to remain in the barrel for one hour. After this, 200 mL of water was removed using a sterile pipet to determine pre-treatment microbial populations. The balance of the water was emptied and discarded. At this point the treatment for the barrel was performed. Post-treatment, 600 mL of sterilized water was added to each barrel and sealed with a new bung. All the internal surfaces of the barrel were contacted by rolling six times on a barrel rolling rack and allowing the water to remain in the barrel for one hour after which 200 mL of water was removed using a sterile pipet. The barrels were resealed and after twenty-four hours an additional 200 mL of water was removed from each barrel to compare to the post-treatment sample. The remaining water was discarded.

Colony forming unit (CFU) analysis was conducted using plating on Difco™ Wallerstein Laboratory (WL) Nutrient media for Brett, *Acetobacter*, and yeast. PCR analysis was conducted using the Bio Rad MiniOpticon Real Time PCR system for Brett (Bio Rad VINEO extract DNA kit and Bio Rad Brettanomytest PCR kit for selectivity) to determine population numbers pre- and post-treatment. Although not the primary focus of the paper, *Saccharomyces* populations were also monitored as an additional check of sanitation success.

4.3 - Environmental Data Collection Procedure

The amount of water, gas, and electricity used was measured per treatment. A flowmeter monitored water going into the barrel sanitation line. A bucket collection system was used to capture water from steam and ozone treatments and then measured using large graduated cylinders. A voltmeter was attached to the power source for all equipment to determine electricity usage.

4.4 - Financial Analyses Procedure

For adequate and equal analysis of the microbial data, the quality of the different treatments needed to be calculated; however, there was no established way to quantify the quality of the different treatment methods. Therefore, this author developed formulas to quantify both the quality of the treatments, named the Average Effectiveness Number (AEN) and the value of the treatments, named the Value Number (VN).

The AEN of each treatment was determined by examining the difference between baseline populations and population post-treatment of each treatment. The formula developed by this author for the purpose of the analysis to determine AEN is as follows:

E= (B/A) + (D/C) + (G/F) + (I/H)

Average Effectiveness Number (AEN) = E

Average post-treatment* Brett population by PCR = B

Average pre-treatment Brett population by PCR = A

Average post-treatment *Acetobacter* by plating= D

Average pre-treatment *Acetobacter* by plating = C

Average post-treatment Brett by plating = G

Average pre-treatment Brett by plating = F

Average post-treatment *Saccharomyces* by plating = I

Average pre-treatment *Saccharomyces* by plating = H

* Refers to initial post treatment sample

The costs of all treatments were determined in US$, including capital expenses and general operating expenses (cost of water, electricity, and chemicals), and normalized over a three year time period to determine relative costs. The VN was determined by comparing relative costs to AEN of each treatment using the following formula also developed by this author:

V= E * X

V= Value Number (VN)

E= Average Effectiveness Number

X= Total Cost

4.5 - Winemaker Survey Procedure and Limitations

A survey was designed and distributed to 525 wineries in the Napa Valley (Napa Valley Vintners, 2017). These 525 wineries had contacts listed on the Napa Valley Vintners webpage and represent a high quality list of active wineries in the Valley. Of the wineries which received the survey request, 67 responded (13% response rate) to the questions. This represents enough responses to draw conclusions regarding current anecdotal observations and practices of barrel sanitation. This response rate resulted in a +/- 9% margin of error. All of the respondents have made, or are currently making, wine in the Napa Valley and had used barrels within the past 12 months as part of their winemaking process. The questions, which can be found in Appendix 4, covered many aspects including methods used, methods preferred, effectiveness, environmental impact, cost, previous microbial contamination experience, barrel destruction due to contamination, and wine spoilage. The results of this survey were compared to the barrel sanitation trial results to determine how close the current winemaker perception falls to the actual controlled experiment results. This was less a statistical exercise, and more a check and balance to determine if the experiment results matched the current perceptions.

There were two limitations regarding the survey which differed from the original plan set out in the Research Paper Proposal (RPP). The total winery number differs from the Research Paper Proposal (RPP) due to a website update at the Napa Valley Vintners from ‘over 400’ wineries when referenced in September of 2016 to ‘525 wineries’ when referenced in January of 2017. The statistics above represent the response rate for the updated number. Also limiting was the response rate. The RPP methodology set out that 120 wineries would be polled with a 50% response rate to result in 60 completed surveys. This population proved to be much more difficult to survey than originally anticipated. In total, all 525 wineries needed to be contacted multiple times to obtain the final 67 survey responses. The original goal number (60) of the RPP was surpassed; although, many more wineries had to be contacted to achieve that number than was originally planned.

5. Results

5.1 - Microbial Results

The raw microbial results can be found in Appendix 3. It is interesting that the number of Brett cells, measured by both PCR and plating, (Figures 1 and 2) and *Saccharomyces*, measured by plating, (Figure 4) were reduced on the control even with what should be considered a fast physical cleaning. The 24 hour post-treatment samples showed slight decreases in Brett population by PCR from the initial results but were not significantly different from the post-treatment sample in each treatment. It should be noted that *Acetobacter* numbers increased in 5 of the 6 control barrels indicating that *Acetobacter* numbers can rapidly multiply under unsanitary conditions.

As shown in Figures 1-4, steam performed very well for all microbial tests, resulting in zero populations on all *Brettanomyces*, *Saccharomyces*, and *Acetobacter* plates. There were extremely low populations by PCR shown in Figure 1 which may indicate 'injured' cells resulting in viable but non-culturable or recently deceased cells whose DNA is still able to be replicated by the PCR equipment. It should be noted in Figure 1 that steam had the overall lowest average Brett cell population by PCR post treatment of all the methods.

Ozone performed quite well overall. As shown in Figures 3 and 4, it showed zero growth on all six replicates for the *Acetobacter* and *Saccharomyces* plates; however, in Figure 2, one replicate on the Brett plates did show 1 CFU/mL. This indicates that ozone, while extremely effective, may not result in absolute cleanliness. There were also extremely low populations by PCR which may indicate 'injured' cells resulting in viable but non-culturable or recently deceased cells whose DNA is still able to be replicated by the PCR equipment (Figure 1).

The hot water treatment also performed favorably, with zero growth on all six replicates of both the *Acetobacter* and the Brett plates, as shown in Figure 2 and 3 and a single plate with 1 CFU/mL on the Saccharomyces plates shown in Figure 4. Of importance is that the PCR still showed high levels of Brett cells, suggesting the hot water does more injury to the cells than actual elimination (Figure 1). The high levels of cell counts would suggest numerous viable but non culturable cells rather than recently deceased cells. There was one anomaly in this PCR data, on replicate 4.1, which showed an extreme increase in Brett growth from a very small base. Upon rechecking the records this was shown to be the way it was reported from the machine. This led to the outlier replicate being rejected from the analysis data for that one test which is reflected in Figure 1.

The SO_2 and citric acid solution showed zero growth on all six replicates of *Acetobacter* and *Brettanomyces* (Figure 2 and 3); however, four of the six replicates showed *Saccharomyces* growth (Figure 4) suggesting that this treatment is not as effective as some of the others on the growth of *Saccharomyces*. The PCR results were nearly equal to that of the control; but, the starting point for the Brett cell populations for this treatment was uniformly lower across all six replicates than any of the other treatments (Figure 1). The barrels were sorted to have identical pre-treatment average Brett populations within groups, as is shown in Appendix 1, so this opens the question of this lot receiving a slightly longer initial rinse than the thirty seconds needed. Again, the Brett populations by PCR being nearly equal to the control would suggest that the absence of colonies on the plates is due to viable but non-culturable cells rather than recently deceased cells.

Unexpectedly, burning sulfur was not an effective treatment for any of the organisms other than *Saccharomyces* on the plates (Figures 2-4) and even the *Saccharomyces* still showed growth on two of the six replicates (Figure 4). Given that this method has been used throughout history for barrel sanitation, the lack of effectiveness is surprising. Even more concerning, the *Acetobacter* populations increased in all six replicates suggesting that this treatment may be doing more harm than good to the next wines in these barrels (Figure 3). This is an area which should be investigated further in depth.

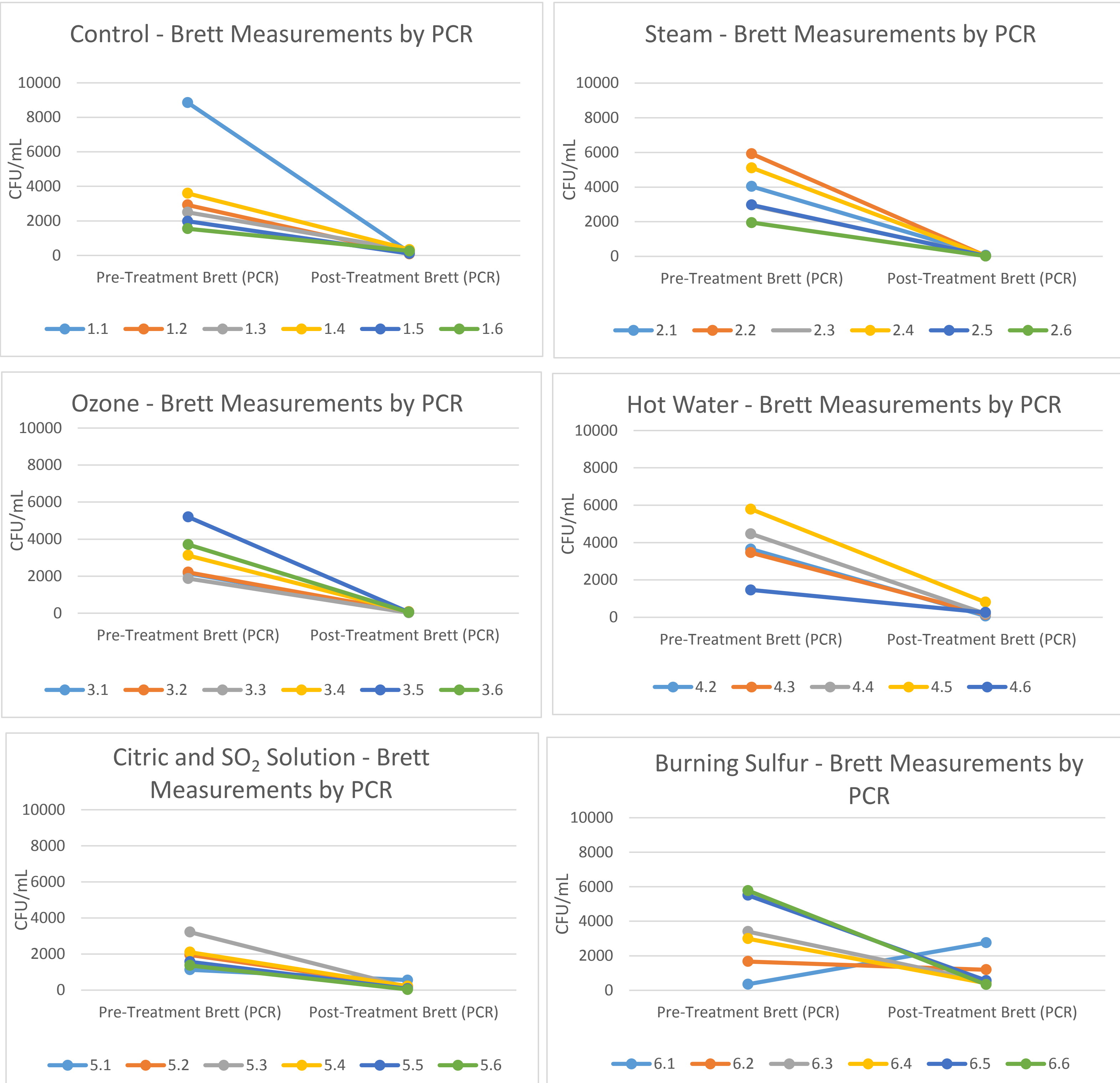


Figure 1 – Brett Population by PCR Pre and Post Treatments in CFU/mL

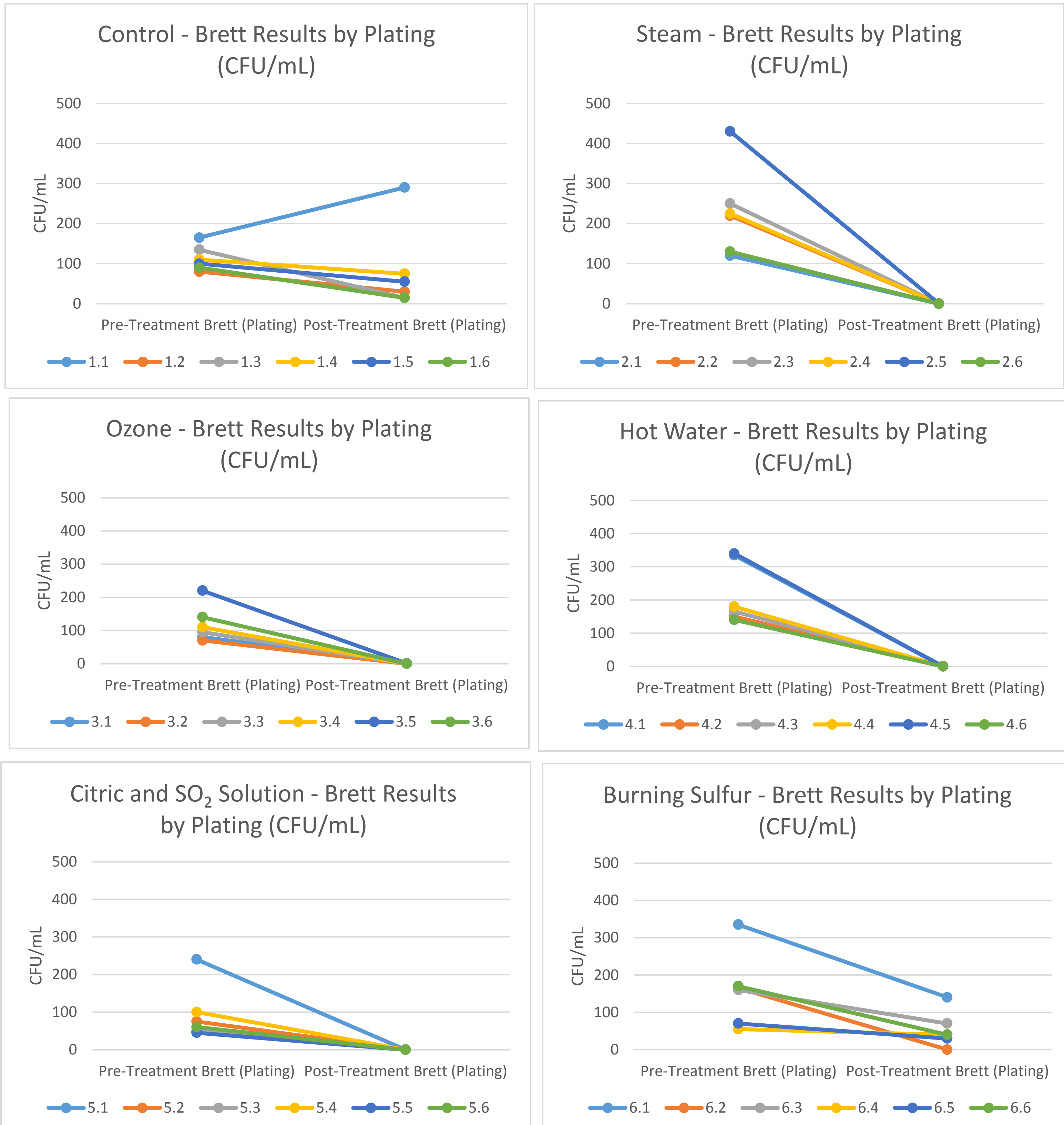


Figure 2 – Brett Population by Plating Pre- and Post- Treatments in CFU/mL

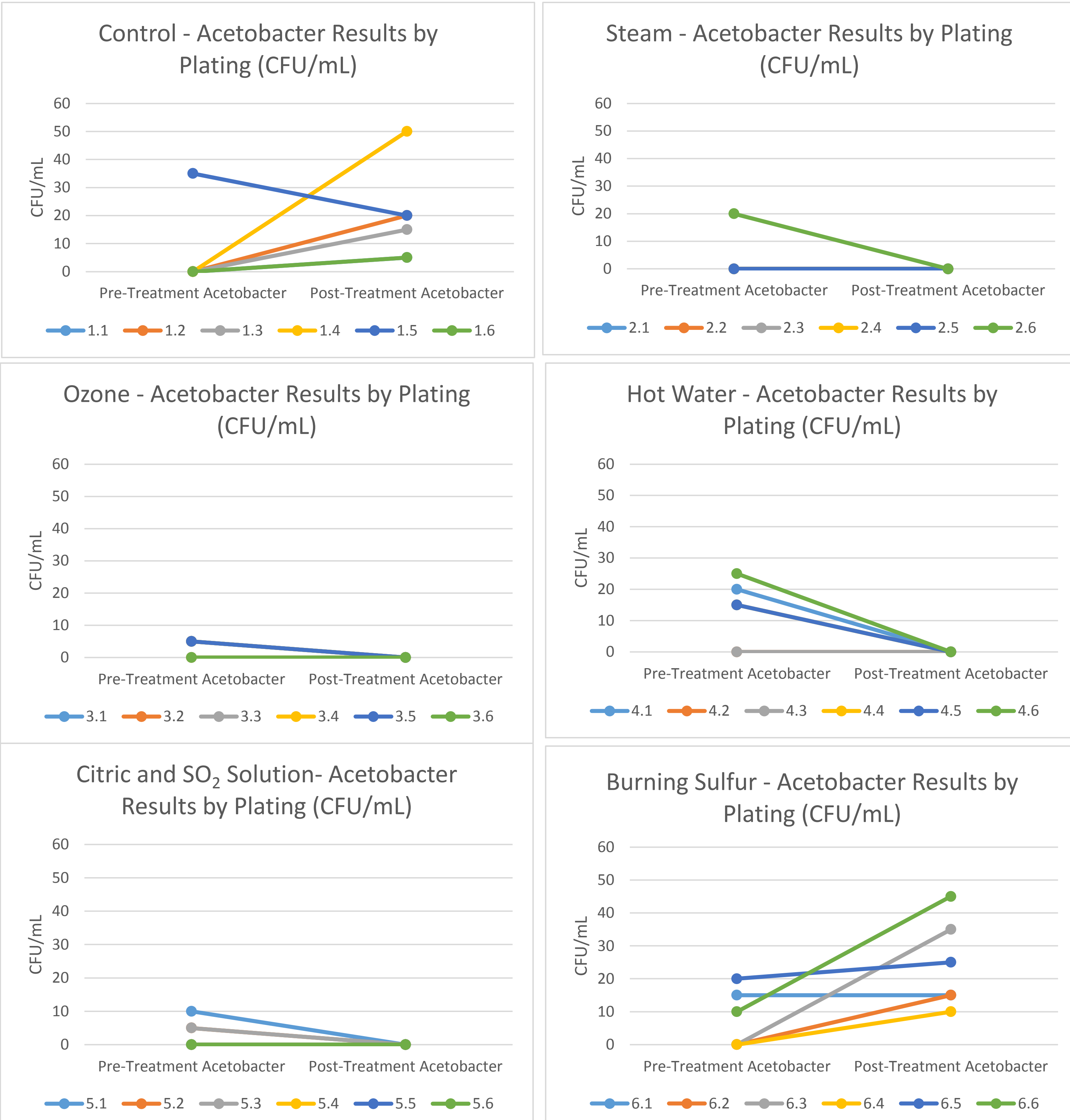


Figure 3 – Acetobacter Populations by Plating Pre- and Post- Treatment in CFU/mL

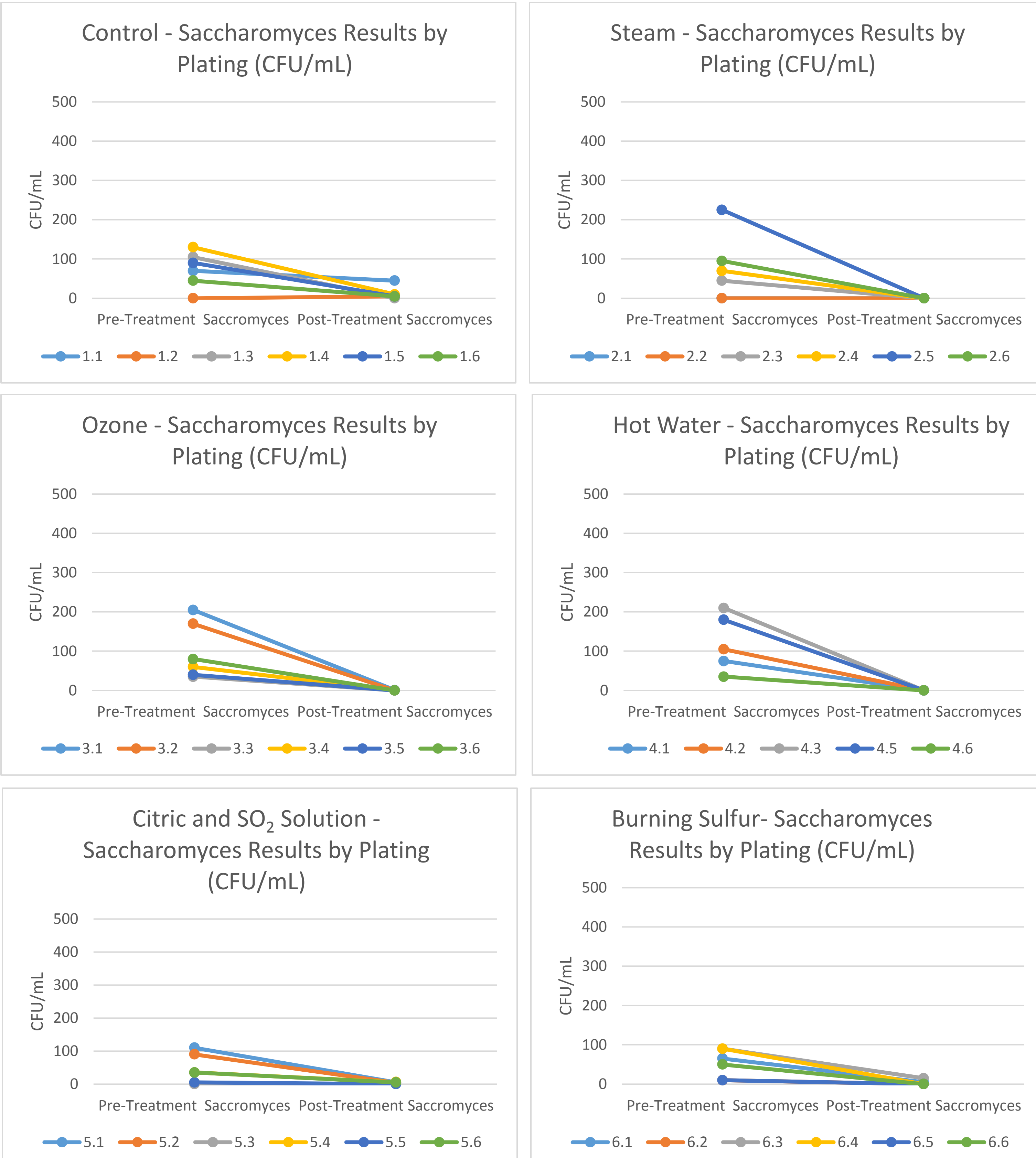


Figure 4 – Saccharomyces Population by Plating Pre- and Post- Treatment in CFU/mL

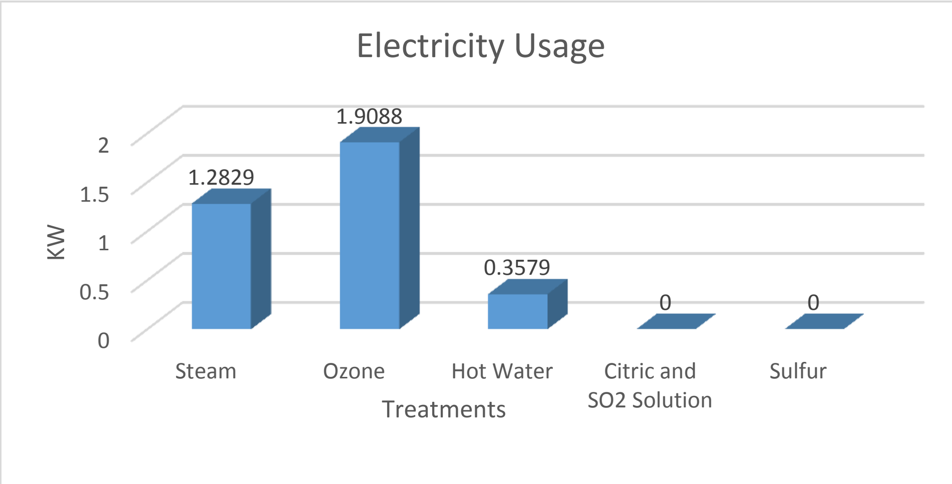
5.2 - Environmental Results

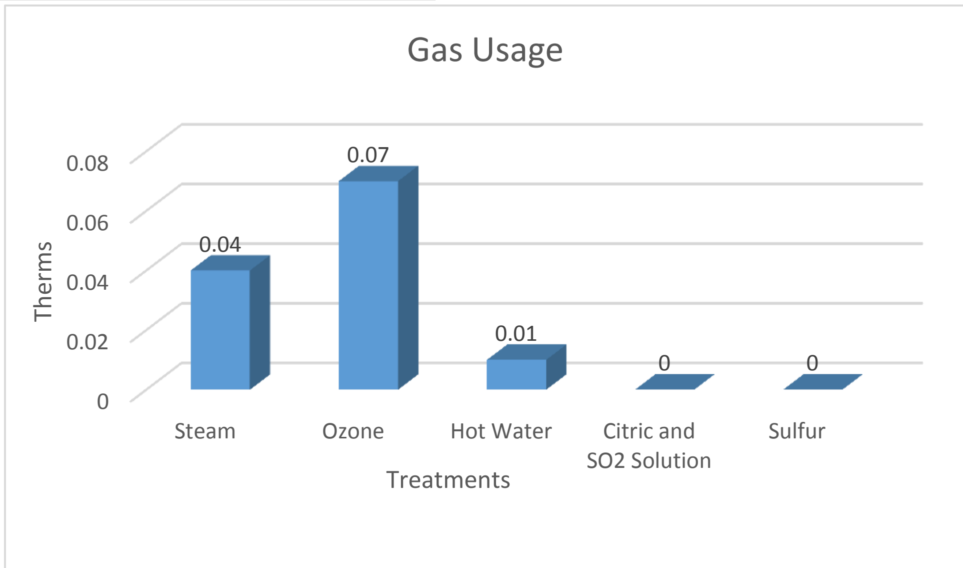
Table 1 shows the raw measurements of the utilities used by each treatment per barrel pair and Figure 5 shows the comparison of each treatment with respect to each environmental variable.

Table 1: Environmental results per 2 barrels for each treatment.

Environmental Results (per 2 barrels)				
	Electricity (BTU)	Electricity (KW)	Gas (Therms)	Water (Gallons)
Steam	4,377.74	1.2829	0.04	29.58
Ozone	6,513.42	1.9088	0.07	48.81
Hot Water	1,221.27	0.3579	0.01	26.6
Citric and SO2 Solution	0.00	0.0000	0	53.3
Burning Sulfur	0.00	0.0000	0	26.6

This was necessary due to the barrel line using a two barrel rack and rinsing two barrels at a time. The measurement of electricity was measured in British Thermal Units (BTU) then converted to Kilowatts (KW) to compare to the utility bill using the conversion 1 KW = 3412.142 Btu (Serway and Jewett Jr, 2014). The natural gas usage was measured in therms and was billed in therms on the utility bill. A therm is a unit of measure equivalent to 100,000 BTU (U.S. Energy Information Administration (EIA), 2016). The water results below all include a 26.6 gallon cold water rinse on the barrel washing line, post treatment, to remove any chemical or physical residue left from the cleaning process. This results in an additional 13.3 gallons per barrel per treatment. Ozone took the most electricity to run; however, it also took the most natural gas which was unexpected. This may be due to the efficiency of the utility use of the different equipment used rather than an issue inherent in the treatment itself. Of the treatments which used electricity or gas, hot water turned out to be the least impactful on energy use; however, it was also not as effective at cleaning as steam or ozone which will be explored further in the financial results section when determining value. Environmental impacts surrounding health and safety were not explored in this experiment.


Electricity Usage
KW
2
1.5
1
0.5
0
1.2829
1.9088
0.3579
0
0
Steam
Ozone
Hot Water
Citric and SO2 Solution
Sulfur
Treatments


Gas Usage
Therms
0.08
0.06
0.04
0.02
0
0.04
0.07
0.01
0
0
Steam
Ozone
Hot Water
Citric and SO2 Solution
Sulfur
Treatments

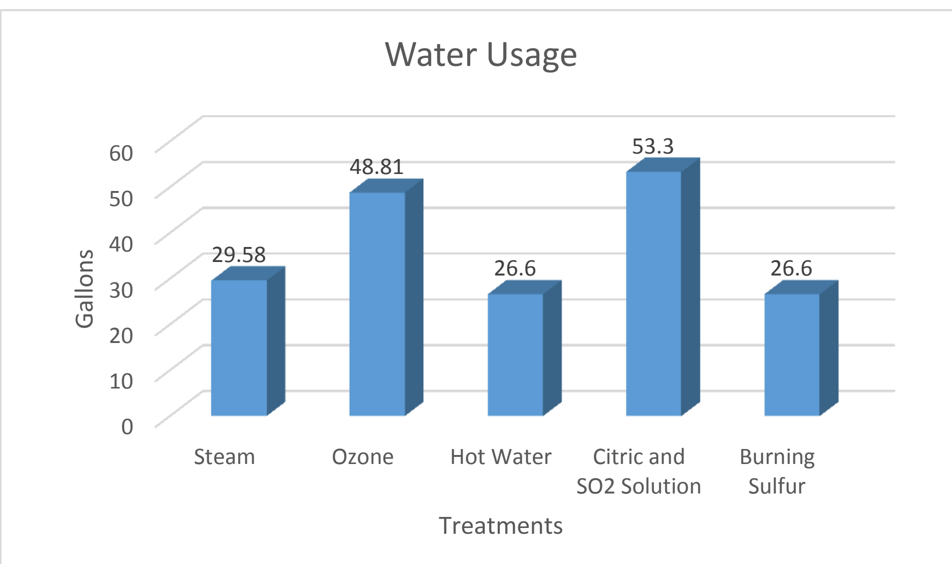

Water Usage
Gallons
60
50
40
30
20
10
0
29.58
48.81
26.6
53.3
26.6
Steam
Ozone
Hot Water
Citric and SO2 Solution
Burning Sulfur
Treatments

Figure 5 – Utility Comparison per Treatment Group

5.3 - Financial Results

Table 2 shows that as expected, when looking solely at the financial side of these treatments, steam and ozone are the most expensive in terms of total cost for treatment. This is primarily driven by the high utility costs of running the machines and secondarily by the capital costs of purchasing the machines (Table 2). Sulfur sticks are the least expensive of the treatments due to low labor costs and minimal material costs when compared to the other treatments. While more expensive than the sulfur sticks, the citric and SO_2 solution is quite inexpensive (21% less cost) when compared to the next most expensive treatment, hot water.

Table 2 – Total Cost of Treatment per Barrel for the First Three Years*

Financial Results	**Capital Costs****	**Electric**	**Gas**	**Water**	**Other Materials**	**Labor**	**Total Cost**
Steam	$0.35	$3.13	$0.68	$0.08	$0.00	$2.47	$6.71
Ozone	$0.58	$4.65	$1.01	$0.14	$0.00	$1.51	$7.89
Hot Water	$0.00	$0.87	$0.19	$0.08	$0.00	$1.08	$2.22
Citric and SO2	$0.00	$0.00	$0.00	$0.29	$0.17	$1.29	$1.75
Burning Sulfur	$0.00	$0.00	$0.00	$0.07	$0.20	$1.29	$1.56

*Financial assumptions located in Appendix 2

** Steam unit capital costs = $8,440.88 and ozone unit capital costs = $13,905.00 divided over 8,000 barrels treated each year for three years.

6. Treatment Analyses

In order to fully understand the impact of each of the treatments, a quantifiable comparison needed to be made to determine the quality of each treatment with respect to microbial control. To have a single quantifiable data point, allowing for each treatment's different effects on each microbe, these results needed to be combined into a single number. The starting point became the difference between baseline populations and population post-treatment. These were determined by averaging the results from each of the replicates excluding the one outlier from treatment 4.1. After the averages had been calculated, a simple ratio formula was developed by this author to calculate what was named an Average Effectiveness Number (AEN) of each treatment. This allowed comparison between each treatment and could then be used to further calculate treatment value. This formula determined a treatment AEN as follows:

E= (B/A) + (D/C) + (G/F) + (I/H)

Average Effectiveness Number (AEN) = E

Average post-treatment Brett population by PCR = B

Average pre-treatment Brett population by PCR = A

Average post-treatment *Acetobacter* by plating = D

Average pre-treatment *Acetobacter* by plating = C

Average post-treatment Brett by plating = G

Average pre-treatment Brett by plating = F

Average post-treatment *Saccharomyces* by plating = I

Average pre-treatment *Saccharomyces* by plating = H

Conceptually, if a treatment has zero effect on the average microbial population of a given barrel then the AEN would equal 4 when analyzing four different microbial tests. Numbers above 4 indicate a negative impact from the treatment resulting in an increase in the population of microbes. Numbers below 4 suggest a positive impact of the treatment resulting in a decrease in the populations. The AEN of each treatment is shown in Table 3. The AENs of each treatment are all below the control meaning that each treatment was, by itself, more effective than the control across all microbes which is to be expected. Steam and ozone were extremely close in average effectiveness with steam having a slight advantage. The next grouping of hot water and the citric and SO_2 solution was not as effective as the steam and ozone; however, they both showed significantly better than the burning sulfur or the control. These treatments both had less effectiveness due to the increase in *Acetobacter* seen.

Table 3 – Average Effectiveness Number for Each Treatment

Treatment	**Average Effectiveness Number**
Control	4.21
Steam	0.01
Ozone	0.02
Hot Water	0.14
Citric and SO2	0.18
Burning Sulfur	3.91

6.1 - Effectiveness to Cost Analysis

Taking into account the AEN, steam and ozone are very close in effectiveness; however, steam is 15% less expensive than ozone which introduces the concept of a value comparison. If a winery spends more money, do they necessarily achieve a greater kill rate proportional to the money they have spent? To further explore this concept after the AEN was calculated for each treatment, the prospect of determining the value became the next challenge. The total costs of each treatment needed to be taken into consideration, including capital expenses and general operating expenses (cost of water, electricity, and chemicals), and normalized over a three-year time period to determine relative costs (listed on pg. 23). Value was then determined by comparing relative total costs to the AEN of each treatment. The following formula was then developed by this author to do so:

V= E * X

E= Average Effectiveness Number

X= Total Cost

V= Value

By multiplying the AEN by the total costs, the value can be determined since the lower the AEN, the more effective the treatment and thus, the more effectiveness or higher quality treatment a winery can achieve for the money spent. Therefore, the closer to zero the resulting value number is, the more value the treatment offers. The results of this calculation are listed in Table 4.

Table 4: Value Number – Effectiveness to Cost

Treatment	Value number: Effectiveness to Cost
Control	N/A
Steam	0.07
Ozone	0.14
Hot Water	0.30
Citric and SO_2	0.32
Burning Sulfur	6.10

Using these numbers it is clear that steam offers the most positive effectiveness to cost with twice the value of ozone because it is slightly more effective as well as less expensive to operate. Hot water and the citric and SO_2 solution still offer a value due to their lower costs. Since both of these treatments were not as effective as steam or ozone, it would be up to the winery to determine how much contamination is acceptable for the costs of treatment. The AEN to Cost Value can be seen more clearly in Figure 6.

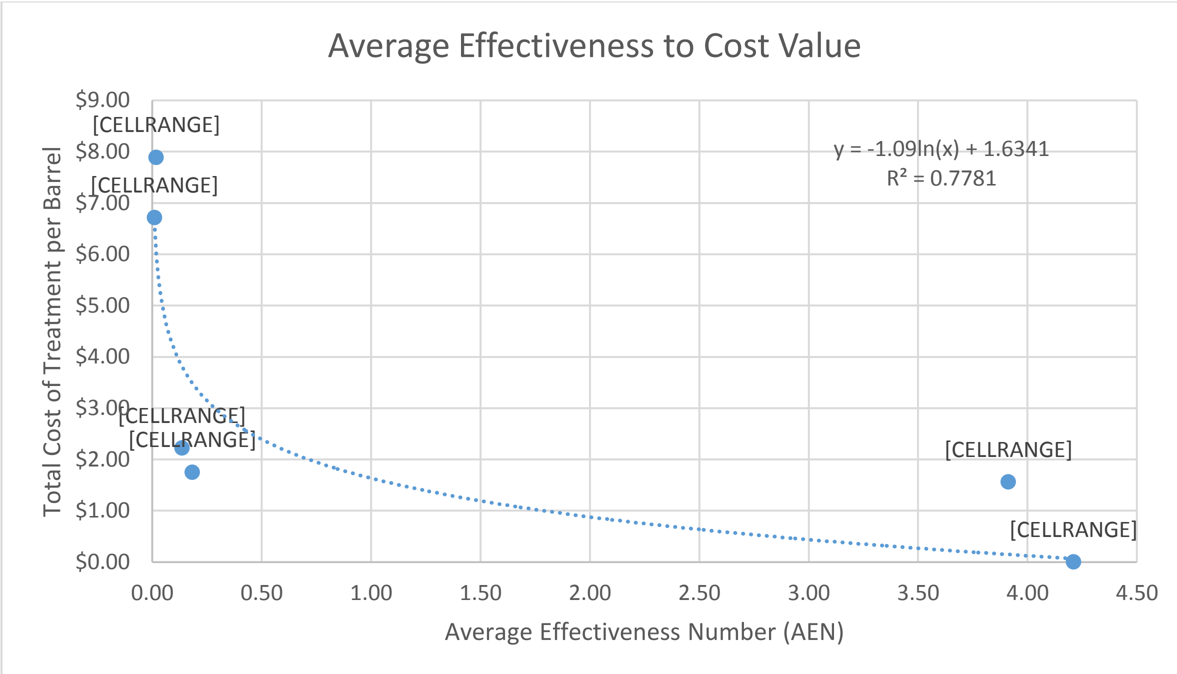


Figure 6: Average Effectiveness to Cost Value

Figure 6 shows the AEN plotted versus the cost of each treatment per barrel. The point of diminishing returns does become a factor here. The most effective treatments resulting in the highest kill rates also were the most expensive when treatments at one third the cost are still reasonably effective. There is a loose logarithmic correlation between the AEN and the total cost per barrel which does lend some credence to the idea that when a winery spends more, it achieves a more effective cleaning. Of course, it is up to the individual winery to determine if gaining a lower AEN is worth spending 67% more which is the difference between the cost of hot water per barrel and steam per barrel. It is clear that burning sulfur does not offer the effectiveness or value of the other four treatments and should not be considered as adequate sanitation of barrels. Figure 7 shows the same data with the burning sulfur data removed and the correlation moves into an 80% confidence range.

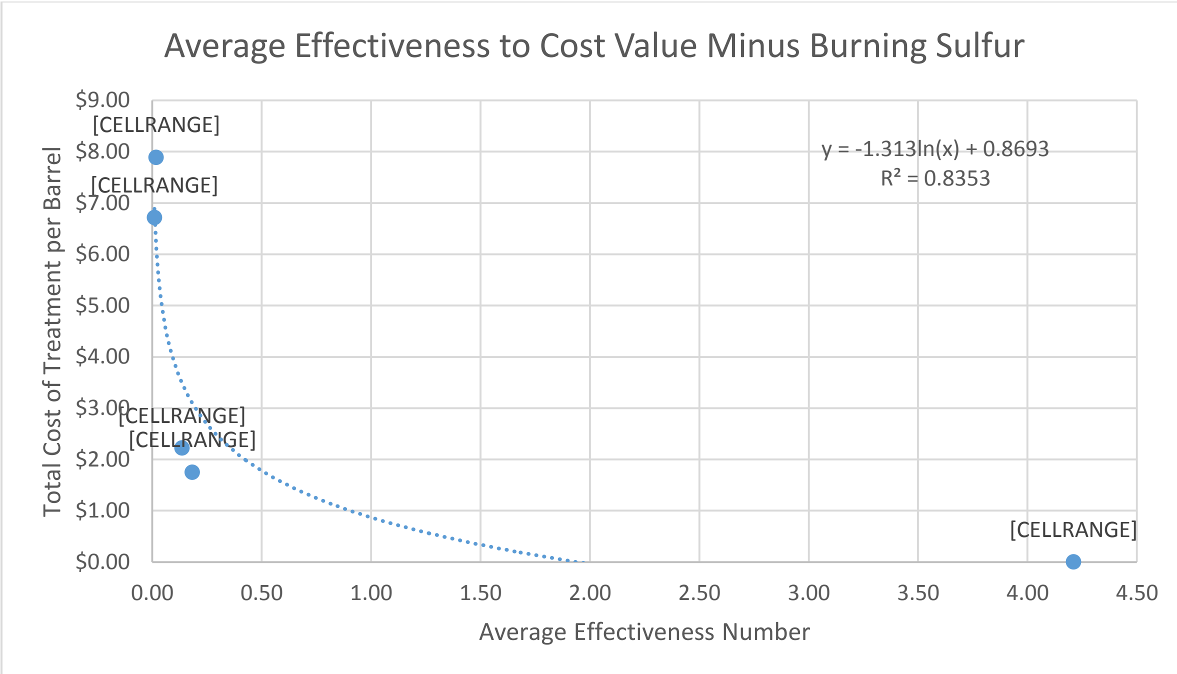


Figure 7: Average Effectiveness to Cost Value Minus Burning Sulfur

6.2 - Analysis for Lower Barrel Population

The above analysis was only for a moderately large size winery needing to treat 8,000 barrels a year. The next question to answer is at which point does a winery see diminishing AEN to cost value due to a smaller number of barrels needing treatment? The issue is that the treatments still carry the same level of capital outlay over three years. This results in a higher cost of treatment per barrel for those treatments which require high capital outlay, which is steam and ozone. Reapplying the higher rate per barrel to the financial analysis and then recalculating the value, causes the results to change as the barrel population drops. The analyses were run using less and less barrels to determine the point at which the value number of steam (0.30) was equal to the value number of hot water (0.30). The number of barrels where these two value numbers are equal is 120 barrels. Table 5 shows the financial results for the break point of 120 barrels per year, the costs of which are outlined below.

Table 5: Minimum Number of Barrels Treated per Year to Achieve Same Average Effectiveness to Cost Value as Hot Water (120 Barrels Treated per Year)

Financial Results	Capital Costs	Electric	Gas	Water	Other Materials	Labor	Total Cost
Steam	$23.45	$3.13	$0.68	$0.08	$0.00	$2.47	$29.81
Ozone	$38.63	$4.65	$1.01	$0.14	$0.00	$1.51	$45.94
Hot Water	$0.00	$0.87	$0.19	$0.08	$0.00	$1.08	$2.22
Citric and SO2	$0.00	$0.00	$0.00	$0.29	$0.17	$1.29	$1.75
Burning Sulfur	$0.00	$0.00	$0.00	$0.07	$0.20	$1.29	$1.56

Table 6: Value Number of Minimum Number of Barrels Treated Per Year from Table 5

Treatment	Value number: Effectiveness to Cost
Control	N/A
Steam	0.30
Ozone	0.84
Hot Water	0.30
Citric and SO2	0.32
Burning Sulfur	6.10

Using this analysis and assuming the winery wants the best quality for the cost of their treatment, steam continues to be the treatment of choice unless the winery is treating less than 120 barrels per year. It is at this point that the value number of steam and the value number of hot water are equal. Therefore, if only the cost to effectiveness, or value, is the deciding factor, then 120 barrels is the cutoff point beyond which steam makes the most sense by value and below which hot water offers sufficient effectiveness for cost.

6.3 - Cost of Multiple Machines

The main analysis above assumes one machine was purchased and used on 8,000 barrels. If there is a need for additional machines to treat the same amount of barrels due to some sort of unforeseen constraint (time, labor, etc.), the effectiveness to cost number may change unfavorably for the top treatments using the previous assumptions. Table 7 outlines the costs of purchasing two machines of both treatments that require capital outlay.

Table 7: Financial Ramifications of Purchasing Two Machines to Treat 8,000 Barrels per Year

Financial Results	**Capital Costs**	**Electric**	**Gas**	**Water**	**Other Materials**	**Labor**	**Total Cost**
Steam	$0.70	$3.13	$0.68	$0.08	$0.00	$2.47	$7.06
Ozone	$1.16	$4.65	$1.01	$0.14	$0.00	$1.51	$8.47
Hot Water	$0.00	$0.87	$0.19	$0.08	$0.00	$1.08	$2.22
Citric and SO_2	$0.00	$0.00	$0.00	$0.29	$0.17	$1.29	$1.75
Burning Sulfur	$0.00	$0.00	$0.00	$0.07	$0.20	$1.29	$1.56

Table 8: Value Number When Purchasing Two Machines to Treat 8,000 Barrels per Year

Treatment	**Value number: Effectiveness to Cost**
Control	N/A
Steam	0.07
Ozone	0.16
Hot Water	0.30
Citric and SO_2	0.32
Burning Sulfur	6.10

As shown in Table 8, purchasing two machines does not change the effectiveness to cost number on the steam units and only marginally raises the ozone number (0.16). The other three treatments remain unchanged since they do not require extensive capital investment.

7. Winemaker Survey Results

Following the completion of the barrel sanitation trial, it was important to explore the intersection of the theoretical and the practical. This exploration would determine if anecdotal evidence from winemakers would agree with the results of the trial. If so, this would lend substantial support to observational and practical practices of Napa Valley winemakers, even without previous comprehensive and comparative research to support them. The winemaker survey was used to investigate how closely the perceptions, held by Napa Valley winemakers of barrel sanitation, aligned with the trial results.

Of the winemakers who responded to the survey, 91% of them had experienced some level of microbial contamination in barrels during their career. Of those who had experienced contamination, *Brettanomyces* was the most common (91.8%) followed by acetic acid bacteria (82.0%) and lactic acid bacteria (54.1%). Mold (41%) and fermentation yeasts such as *Saccharomyces* (31.1%) were also concerns. A small number of respondents referenced 'other' microbes such as 2,4,6 trichloroanisole (TCA) forming microbes and film-forming yeasts such as *Pichia*. The majority of respondents (85.1%) felt that once a microbial contamination occurs there is some loss of quality, however the wine would still be usable or saleable. Only 4.5% of respondents felt that there would be a total loss of quality meaning the wine was unsaleable and unsalvageable. A majority (52.2%) of respondents said they would remove the affected barrel from their winery by selling or repurposing the barrel such as for planters or decoration rather than destroying it or trying to remediate it.

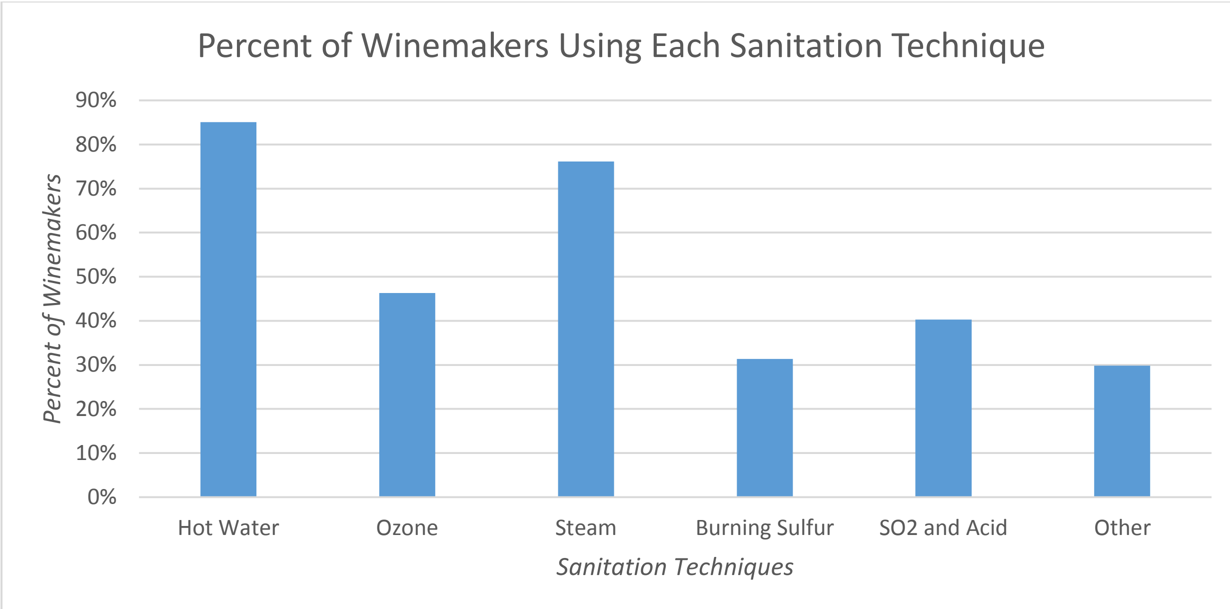


Figure 8 – Percent of Winemakers Using Each Sanitation Technique

As demonstrated in Figure 8, of the methods that have been used in the past 12 months hot water was the most widely mentioned with 85.1% of the respondents referencing using it. The second most widely used method was steam with 76.1% of respondents saying this method was used in the past 12 months. This reveals that many wineries in Napa are using multiple sanitation methods to clean barrels as shown in Figure 9.

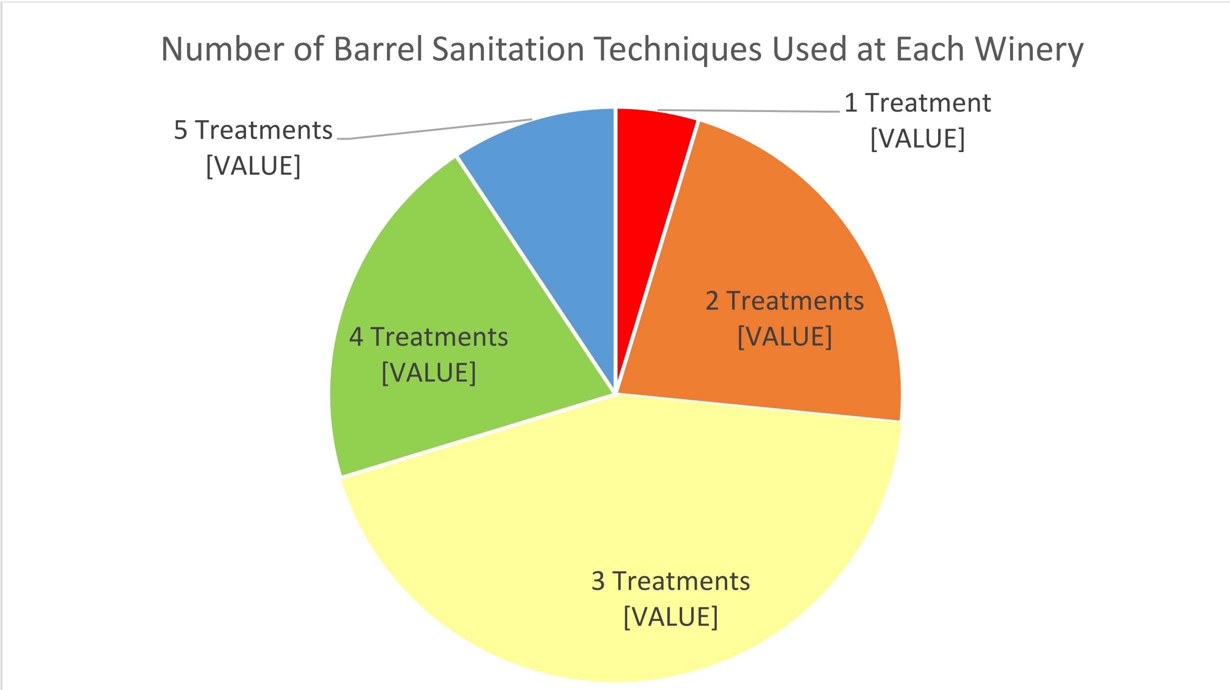


Figure 9 – Number of barrel sanitation techniques used by each winemaker

Of the tested methods, burning sulfur was the least mentioned but still utilized with 31.3% of respondents using it. There were two ‘other’ techniques mentioned including SO_2 gas and sodium percarbonate, the former of which is used in barrel storage but was not included in this experiment because it is not a sanitation method by itself but rather is typically used to maintain the sanitary environment once it has been established. Ozone (46.3%) and SO_2 and citric acid solution (40.3%) fell in the middle of usage frequency in the past 12 months. The survey also revealed that even if the respondents were not currently using one of the tested methods, they generally had used it in the past or had at minimum were familiar with all of the different methods.

When it came to rating the effectiveness against microbial growth of each method, hot water was rated by 71.6% of respondents as either ‘somewhat effective’ or ‘not very effective’ even though it was the most widely mentioned in use. Steam was considered the most effective of the treatments with 66% of respondents stating it was 'very effective' or 'extremely effective' on controlling microbial growth. Ozone and the SO_2 and citric acid solution were similarly favored with 69% and 68% of respondents respectively stating they were 'somewhat effective' or 'very effective'. Burning sulfur had a more moderate response with 42% of respondents ranking it as 'somewhat effective'. The results of these perceptions can be seen in Figure 10.

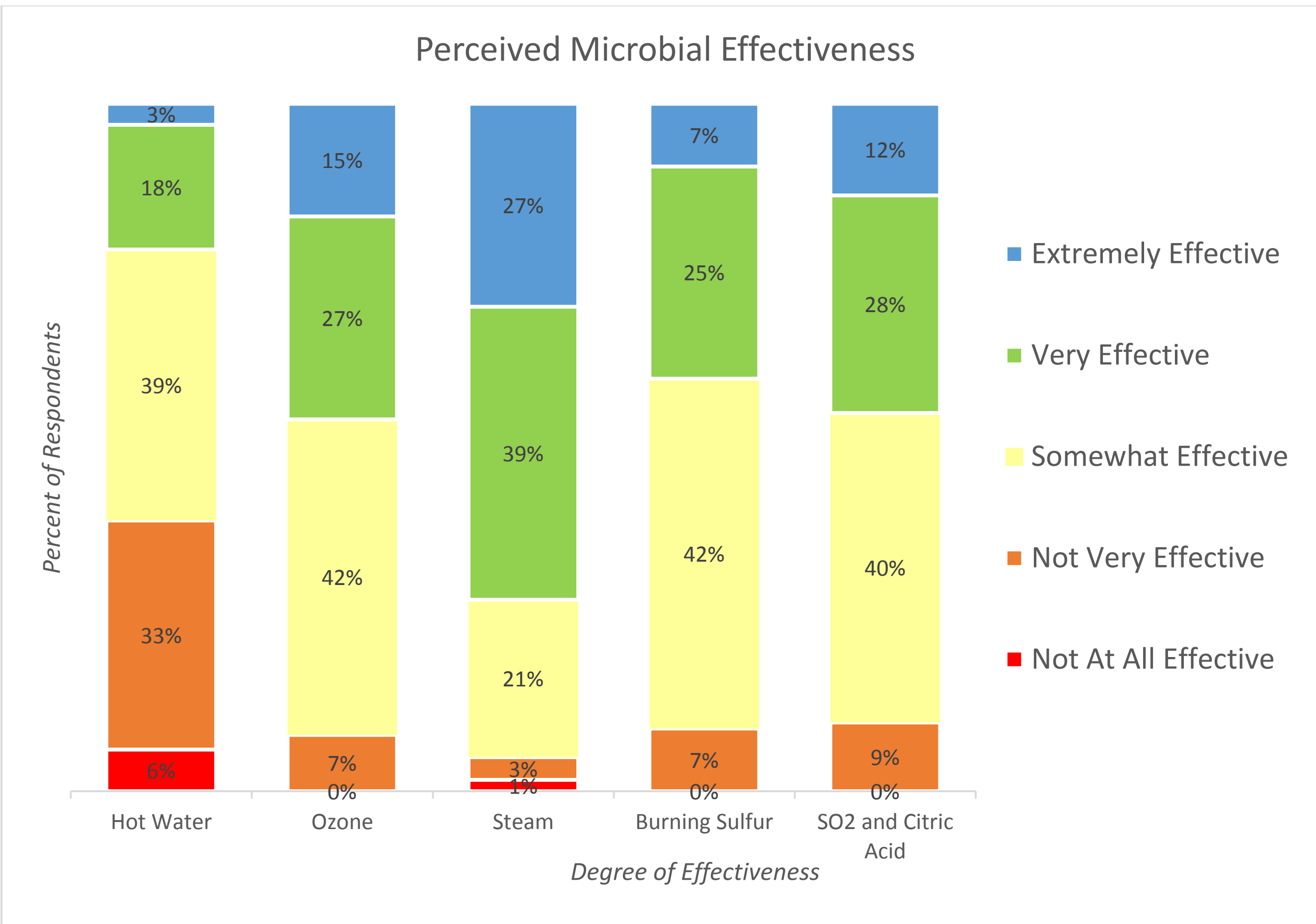


Figure 10 – Perceived Microbial Effectiveness by Treatment

As illustrated in Figure 11, when asked about environmental friendliness, respondents also seemed to look favorably on steam with 71% of respondents ranking it as ‘very friendly’ or ‘extremely friendly.’ Ozone was generally viewed as unfriendly to the environment with 52% of the respondents ranking it ‘not at all friendly’ or ‘not very friendly.’ Hot water had a normal bell curve shaped spread over all responses slightly favoring environmental friendliness. Burning sulfur had a similar bell curve trending slightly towards the unfriendly responses. SO_2 and citric acid solution had 72% of respondents call it ‘not very friendly’ or ‘somewhat friendly’.

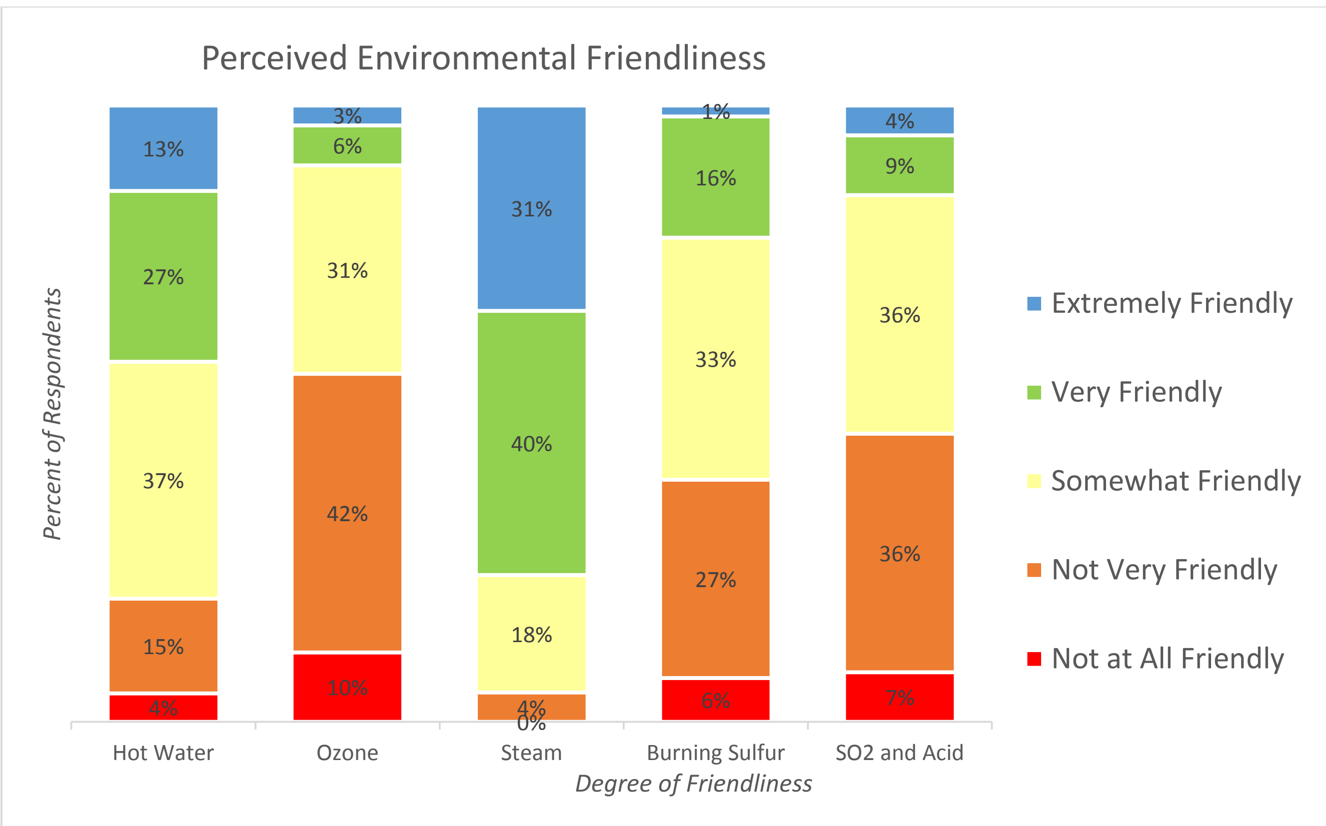


Figure 11 - Perceived Environmental Friendliness of Each Treatment

The perceived costs of each treatment were also asked of the survey respondents. The results appear in Figure 12. Interestingly, none of the treatments were ranked on the high end as ‘very expensive’ or ‘extremely expensive.’ Hot water saw 77% of respondents ranking it as ‘not very expensive’ or ‘somewhat expensive.’ Ozone was more widely spread in the middle with 48% of respondents stating it was ‘somewhat expensive’ with large groups stating it was ‘not very expensive’ (22%) and ‘very expensive’ (15%) on either side. Steam was ranked ‘not very expensive’ by 33% and ‘somewhat expensive’ by 42% suggesting the perceived costs of steam are slightly less than ozone. Burning sulfur and the SO_2 and citric solution were generally ranked with lower perceived costs with the majority of respondents ranking them as ‘not very expensive’ (45% and 54% respectively).

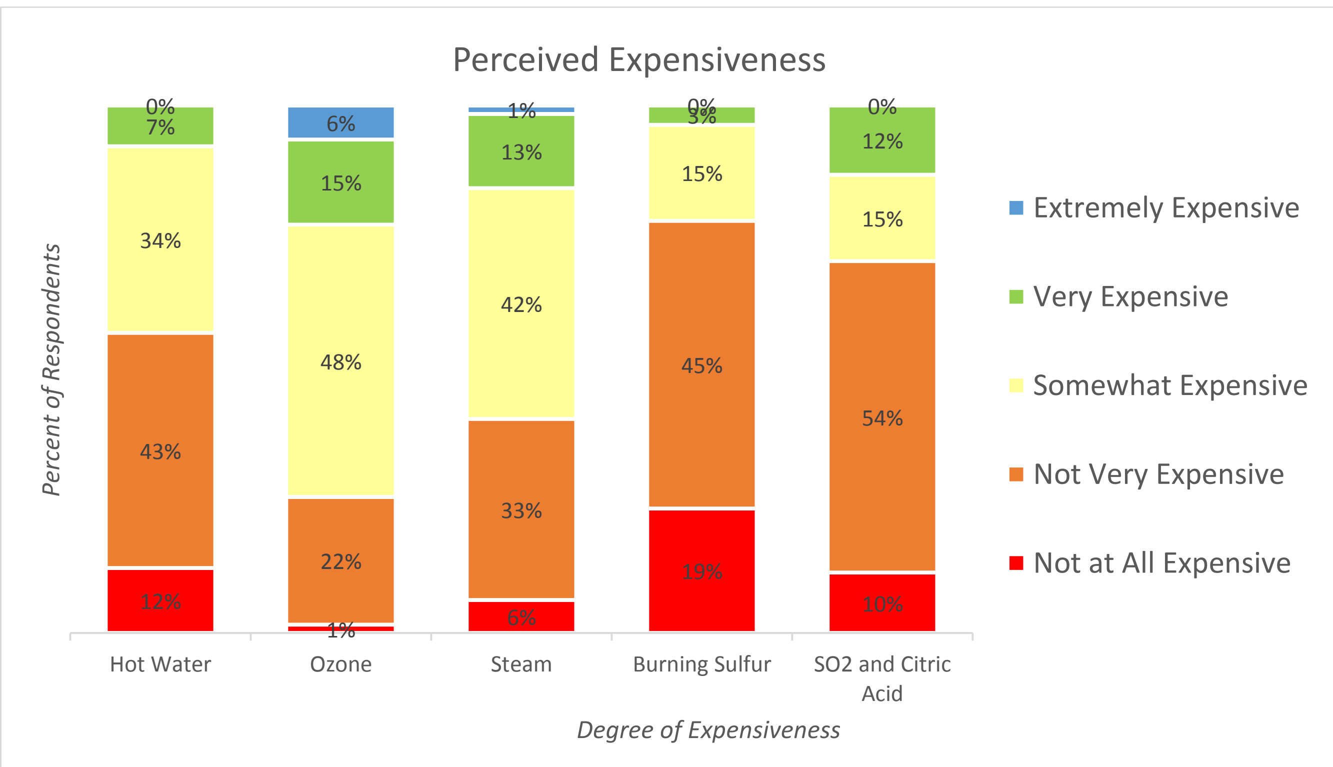


Figure 12 – Perceived Expensiveness of Each Treatment

Respondents were also asked about perceived effectiveness for the money of each treatment, the results of which are presented in Figure 13. Steam rated the highest on this with almost two thirds (67%) of respondents ranking it as 'very effective' or 'extremely effective'. Responses were mixed on the efficacy of hot water with nearly one third of respondents (31.3%) saying it was 'not very effective,' slightly less than one third (30%) calling it 'somewhat effective', and the remaining third (30%) stating it was 'very effective.' The SO_2 and citric solution had the majority (76%) of responses as 'somewhat effective' and 'very effective' which ended up being ranked higher than ozone (75%) or burning sulfur (63%) in the same categories.

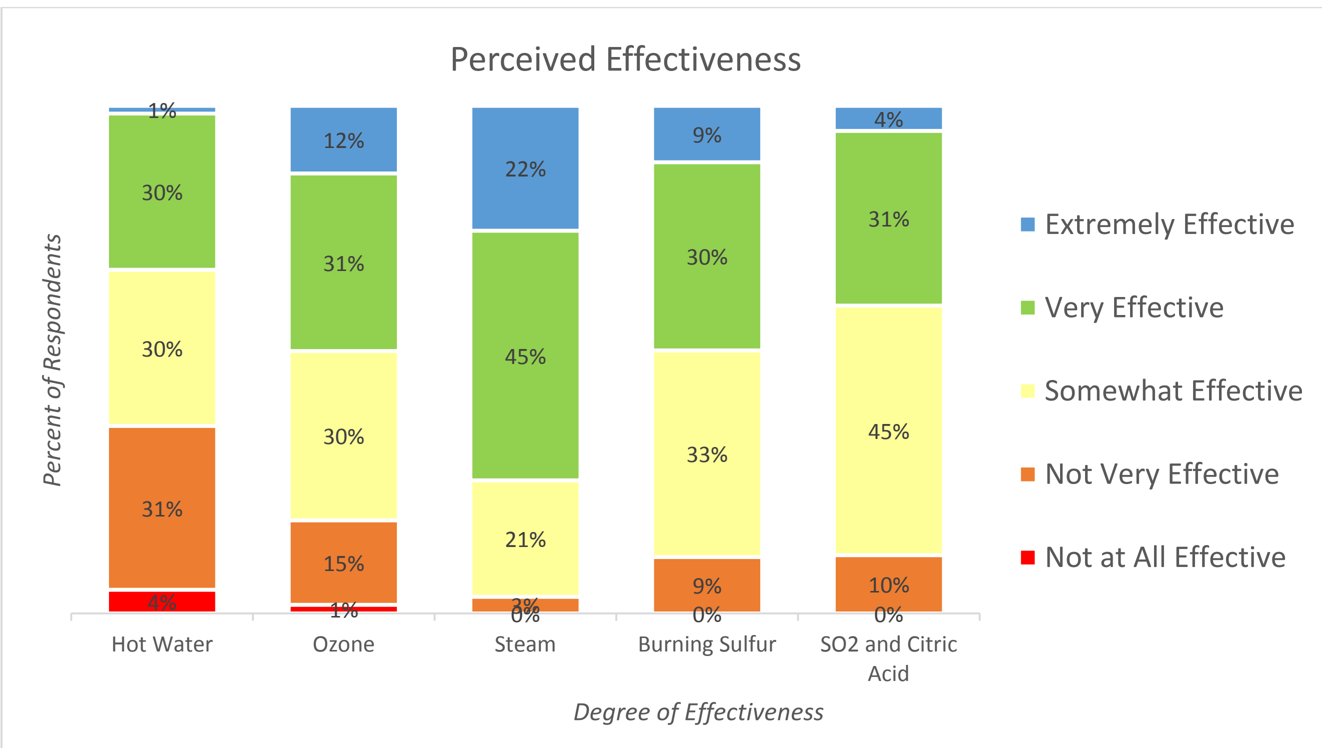


Figure 13 – Perceived Effectiveness of Each Treatment

Figure 14 shows the preference of the group of winemaker by barrel sanitation method. Overall, steam was the most preferred method with 59.7% of respondents favoring this method over other treatments. Many reasons for this were mentioned, but the top reasons were the vacuum action of the steam method, the deep penetration into the wood, low water use, no chemical residue, and ease of use. Ozone was the second most preferred with 13.4% of respondents favoring this; however, their written responses were less descriptive. Comments were that it was easy to use and does not leave a residual smell behind. Hot water was preferred by 10.4% of respondents but four of the written responses referenced using it in combination with another method. The SO_2 and citric acid solution was preferred by 9% of respondents and the general comments referenced that it is inexpensive and generally safe with the added benefit of keeping barrels hydrated. Burning sulfur had the lowest rate of preference at 7.5%. Comments referenced the low cost of the treatment as well as the ease of use making it possible to perform frequently.

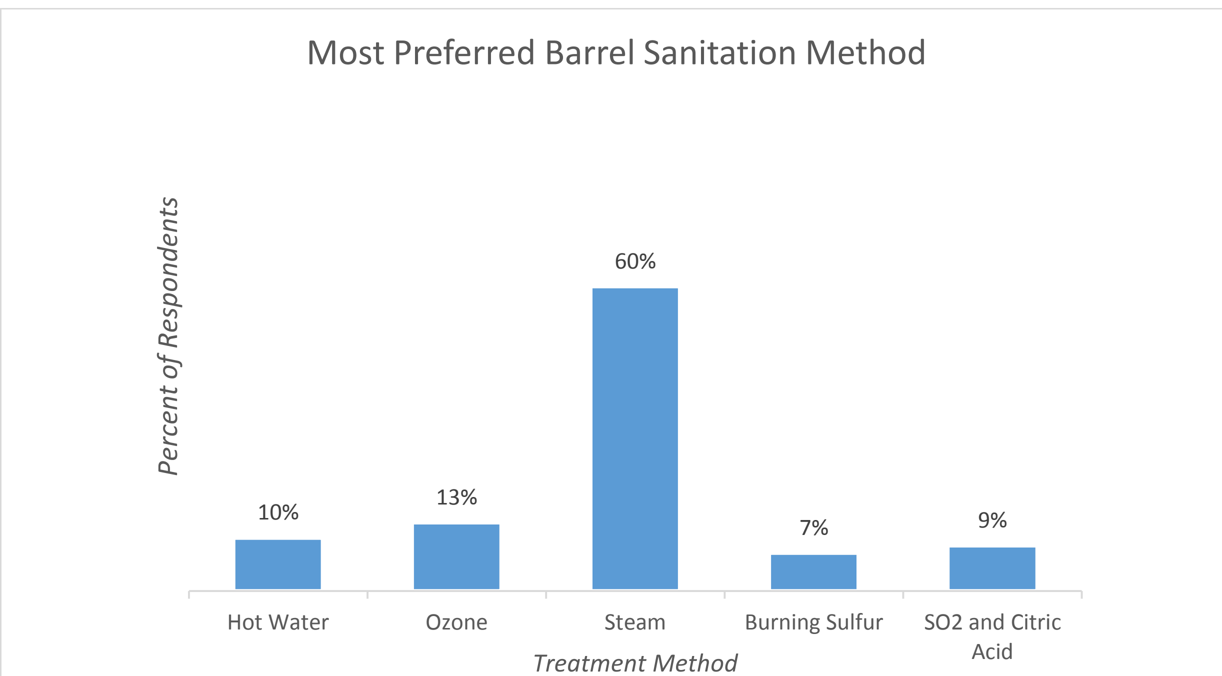


Figure 14 – Preference of Barrel Sanitation Method by Surveyed Winemakers

Overall, over 95% of respondents thought that barrel sanitation was at least ‘somewhat important’ with the majority stating that it was ‘very important’ to the wine industry. Even those respondents who had never experienced microbial issues in barrel rated this research ‘very important’ to ‘extremely important.’

8. Conclusions

8.1 - Survey Discussion

The survey results yielded very interesting insights into how winemakers in Napa are currently handling the process of barrel sanitation. Of the winemakers sampled, there were 207 responses stating which sanitation method was used at each winery. This confirms winemakers are using multiple methods of sanitation per winery. This was highly evident in the response section of how the winemakers would treat individual compromised barrels. Most responses had a minimum of two treatments and one had up to five different treatments to remediate a barrel. The majority of respondents (42%) showed three different treatments used. Many had three separate treatments suggesting that winemakers are trying multiple processes in the hope that one of them will remediate the issues associated with barrel sanitation. The three responses with a single treatment chose steam. This is in line with the results of the trial for this research that showed steam is the most effective treatment on microbes.

8.2 - Microbial Results Discussion

The microbial results of the different treatments were quite striking. The sensitivity of the PCR testing (Figure 1) was dramatically different than that of the plating methods suggesting that if DNA primers could be developed for *Acetobacter* and other spoilage organisms it would be helpful in identifying contamination prior to them becoming destructive. It is clear that 'viable but non-culturable' also plays a large role in the effectiveness of testing methods, although it is not guaranteed that viable but non-culturable cells will go on to cause detrimental issues. Further research would be needed to determine the extent to which ‘viable but non-culturable’ affects wine quality post-sanitation.

From a microbial effectiveness standpoint, both steam and ozone offer nearly identical kill rates for Brett, *Acetobacter*, and *Saccharomyces*. Hot water and the citric and SO_2 solution both show promise in terms of the plating however the PCR results suggest that the absence of colonies on the plate are not due to their elimination but due to the cells being injured to the point of viable but non-culturable. Based on these results burning sulfur should not be considered as effective and may be encouraging *Acetobacter* growth greater than what was seen in the control. These results echo the results of a study by W.J. du Toit, I.S. Pretorius, and A. Lonvaud-Funel (2005, p 869) which looked further into this concept and concluded that ‘the SO_2 did not completely eliminate *A. pasteurianus* […] and winemakers should therefore always use SO_2 in conjunction with other best practice winemaking procedures’. More study would be needed to determine if there was a causal relationship between the treatment and the *Acetobacter* growth. This is concerning since so many of the survey respondents ranked burning sulfur as ‘somewhat effective.’ This means that there is a commonly held assumption that sulfur is helping to address microbial issues when that may not be the case.

The survey results of the hot water treatment compared to the actual trial results also offer an interesting discussion point. Hot water was the most widely used of all the treatments; however, it was ranked as ‘somewhat effective’ or ‘not very effective’ by 71.6% of respondents. It also had very mixed responses on effectiveness for money which calls into question why winemakers are using it so widely. This is particularly true when the trial results are taken into account. Hot water may be resulting in many 'clean plates' which give winemakers a false sense of cleanliness when in reality the hot water is damaging the cells resulting in viable but non-culturable microbes. It could be that hot water is used more for general cleaning rather than microbial sanitation since the survey showed that many wineries are using multiple methods of barrel sanitation. Based on the results from the cold water rinse in the control groups, the physical act of cleaning even for a short period of time does have an impact on cell populations, particularly in the case of Brett.

8.3 - Environmental Results Discussion

From an environmental impact standpoint, the hot water treatment uses the least water, electricity, and gas. Steam was the next lowest water, electricity, and gas consuming treatment. This suggests that if a ratio could be developed to include environmental impact as well as effectiveness and cost of treatment, ozone and steam may not be positioned so closely on the value chart. Steam was perceived to be the most environmentally friendly in the winemaker survey as well. Ozone was found to be the least environmentally friendly using the most electricity, water, and gas of all the treatments. This was in line with current winemaker perception which ranked it as the lowest of the treatments regarding environmental friendliness.

8.4 - Financial Results Discussion

Steam has the highest overall microbial effectiveness with a very favorable effectiveness to cost ratio of all the treatments. This result aligned with current winemaker perception that steam offers the most effectiveness for the money. The effectiveness to cost ratio remained the same for the experimental assumptions in which a mid-sized winery was treating 8,000 barrels per year and purchased a single unit if needed (steam or ozone) to do so. The effectiveness to cost ratio also worked when assumptions were shifted to include a smaller barrel population of 120 barrels treated per year. Increasing the capital purchase to two machines instead of one for the experimental assumption of 8,000 barrels also had minimal impact on the effectiveness to cost ratio. Further analyses would be needed to determine if a winery with less than 120 barrels could achieve a similar value proposition by renting or leasing steam equipment therefore eliminating some of the intensive capital outlay of purchasing the equipment outright.

Tying the financial analyses back to the winemaker survey, it was interesting that none of the treatments ranked much above ‘somewhat expensive’ by the respondents. It may be that in a luxury priced wine region such as Napa, even the capital expense of steam or ozone seems like a small cost to winemakers in terms of the value of the wine and the barrels themselves. It could be concluded that if the survey were to be conducted with winemakers making wine in a more moderately priced region that those respondents may perceive steam and ozone as more expensive than hot water or citric and SO_2.

In summary, if a winery is seeking the most cost effective barrel sanitation method that can be purchased, that is also environmentally friendly; steam is the most effective choice. Wineries with less than 120 barrels would find the same value proposition with hot water sanitation alone; however, hot water does not offer the total microbial effectiveness that can be achieved with steam.

Bibliography

Arvik, T. and T. Henick.-Kling., 2002. Brettanomyces bruxellensis occurrence, growth, and effect on wine flavor. Practical Winery and Vineyard Management, May/June, pp. 117-123.

Blondin, B., R. Ratomehenia, A. Arnaud, and P. Galzy, 1982. A study of cellobiose fermentation by a Dekkera strain. Biotechnology Bioenginering, Volume 24, pp. 2031-2037.

Chatonnet, P., D. Dubourdieu, and J.N. Boidron, 1995. The Influence of Brettanomyces/Dekkera sp. Yeasts and Lactic Acid Bacteria on the Ethylphenol content of Red Wines. American Journal of Enology and Viticulture, 46(4).

Christaki, T. and C. Tzia, 2002. Quality and Safety Assurance in Winemaking. Food Control, Volume 13, pp. 503-517.

Drysdale, G.S. and G.H. Fleet, 1988. Acetic Acid Bacteria in Winemaking: A Review. American Journal of Enology and Viticulture, 39(2).

Drysdale, G.S. and G.H. Fleet, 1989. The Growth and Survival of Acetic Acid Bacteria in Wines at Different Concetrations of Oxygen. American Journal of Enology and Viticulture, 40(2).

du Toit, W.J., I.S. Pretorius and A. Lonvaud-Funel, 2005. The Effect of Sulphur dioxide and Oxygen on the Viability and Culturability of a Strain of Acetobacter pasteurianus and a strain of Brettanomyces bruxellensis Isolated from Wine. Journal of Applied Microbiology, Volume 98, pp. 862-871.

Egli, C.M. and T. Henick-Kling, 2001. Identification of Brettanomyces/Dekkera species based on polymorphism in the rRNA internal transcribed spacer region.. American Journal of Enology and Viticulture, Volume 52, pp. 241-247.

Garde-Cerdan, T. and C. Ancin-Azpilicueta, 2006. Review of Quality Factors on Wine Aging in Oak Barrels. Trends in Food Science and Technology, Volume 17, pp. 438-447.

Guzzon, R., G. Wildman, M. Malacarne, T. Nardin, G. Nicolini, and R. Larcher, 2011. Survey of the yeast population in side wine barrels and the effects of certain techniques in preventing microbiological spoilage.. European Food Research and Technology, Volume 233, pp. 285-291.

Hampson, B., 2000. Use of Ozone for Winery and Environmental Sanitation. Practical Winery and Vineyard Journal, Jan/Feb.

Heresztyn, T., 1986. Formation of Substituted Tetrahydropyridines by Species of Brettanomyces and Lactobacillus Isolated from Mousy Wines. American Journal of Enology and Viticulture, 37(2).

Joseph, C.M.L, G. Kumar, E. Su, and L. Bisson, 2007. Adhesion and Biofilm Production by Wine Isolates of Brettanomyces bruxellensis. American Journal of Enology and Viticulture, 58(3).

Joseph, C.M.L, L.W. Gorton, S.E. Ebeler and L.F. Bisson, 2013. Production of Volatile Compounds by Wine Strains of Brettanomyces bruxellensis Grown in the Presence of Different Precursor Substrates. American Journal of Enology and Viticulture, 64(2).

Joyeux, A., S. Lafon-Lafourcade, and P. Ribereau.-Gaynon, 1984. Evolution of Acetic Acid Bacteria During Fermentation and Storage of Wine. Applied and Environmental Microbiology, Issue July, pp. 153-156.

Killian, E., 2010. Director of Winemaking - Asti Winery, Cloverdale, CA [Interview] (April 2010).

Malfeito-ferreira, M., P. Laureano, A. Barata, I. D'Antuono, H. Stender, and V. Loureiro, 2004. Effects of different barrique sanitation procedures on yeasts isolated from the inner layers of wood: Abstract. American Journal of Enology and Viticulture, 55(304A).

Marko, S.D., E.S. Dormedy, K.C. Fugelsang, D.F. Dormedy, B. Gump, and R.L. Wample, 2005. Analysis of Oak Volatiles by Gas Chromatography- Mass Spectrometry after Ozone Sanitization. American Journal of Enology and Viticulture, 56(1).

Perez-Prieto, L.J., J.M. Lopez-Roca, A. Martinez-Cutillas, F. Pardo Minguez, and E. Gomez-Plaza, 2002. Maturing Wines in Oak Barrels. Effects of Origin, Volume, and Age of the Barrel on Wine Volatile Composition. Journal of Agricultural and Food Chemistry, Volume 50, pp. 3272-3276.

Pollnitz, A.P., K.H. Pardon, and M.A. Sefton, 2000. Quantitative ANalysis of 4-ethylphenol and 4-ethylguaiacol in Red Wine. Journal of Chromatography A, Volume 874, pp. 101-109.

Rayne, S. and N.J. Eggers, 2008. Research Note: 4-Ethylphenol and 4-Ethylguaiacol Concentrations in Barreled Red Wines from the Okanagan Valley Apppellation, British Columbia. American Journal of Enology and Viticulture, 59(1).

Robinson, J., 2006. The Oxford Companion to Wine. 3rd ed. Oxford: Oxford University Press.

Schmid, F., P. Grbin, A. Yap, and V. Jiranek, 2011. Relative Efficacy of High-Pressure Hot Water and High-Power Ultrasonics for Wine Oak Barrel Sanitization. American Journal of Enology and Viticulture, 62(4).

Serway, R.A. and J.W. Jewett Jr, 2014. Physics for Scientists and Engineers. 9th ed. Boston: Brooks/Cole Cengage Learning.

Silva, P., H. Cardoso, and H. Geros, 2004. Studies on the Wine Spoilage Capacity of Brettanomyces/Dekkera spp.. American Journal of Enology and Viticulture, 55(1).

Suarez, R., J.A. Suarez-Lepe, A. Morata, and F. Calderon, 2005 (published in 2007). The Production of Ethylphenols in Wine by Yeasts of the Genera Brettanomyces and Dekkera: A Review. Food Chemistry, Volume 102, pp. 10-21.

Thoukis, G. and L.A. Stern, 1962. A Review and Some Studies of the Effect of Sulfure on the Formation of Off-Odors in Wine. American Journal of Enology and Viticulture, 13(3).

U.S. Energy Information Administration (EIA), 2016. U.S. Energy Information Administration. [Online]
Available at: https://www.eia.gov/tools/faqs/faq.cfm?id=45&t=8
[Accessed 14 February 2017].

Umiker, N.L. and C.G. Edwards, 2007. Impact of Sulfur dioxide on Culturability and Viability of Brettanomyces in Wine.. American Journal of Enology and Viticulture, 58(3).

Vintners, Napa Valley, 2017. Napa Valley Vintners. [Online]
Available at: https://napavintners.com/wineries/
[Accessed 13 April 2017].

Wedral, D., R. Shewfelt and J. Frank, 2010. The Challenge of Brettanomyces in Wine. LWT - Food Science and Technology, Volume 43, pp. 1474-1479.

Wilker, K.L. and M.R. Dharmadhikari, 1997. Research Note: Treatment of Barrel Wood Infected with Acetic Acid Bacteria. American Journal of Enology and Viticulture, 48(4).

Zuehlke, J.M. B. Petrova, and C.G. Edwards, 2013. Advances in the Control of Wine Spoilage by Zygosaccharomyces and Dekkera/Brettanomyces. Annual Review of Food Science and Technology, Volume 4, pp. 57-78.

Appendices

Appendix 1 – Barrel Trial Barrel Identification Numbers and Original Brett Levels in Wine Prior to Treatment

Treatment	PCR Sample Id	Barrel #	Wine Brett Population (CFU/mL)	Group	Barrel Identifier
Control	22	M071642D	0.02	1	**1.1**
	36	M135770T	2050.00	1	**1.2**
	27	M135702T	605.00	1	**1.3**
	6	M135705T	34800.00	1	**1.4**
	13	M071485D	3040.00	1	**1.5**
	33	M135709T	635.00	1	**1.6**
Steam	1	M135713T	14200.00	2	**2.1**
	39	M135745T	656.00	2	**2.2**
	17	M070999D	1780.00	2	**2.3**
	40	M125889D	340.00	2	**2.4**
	7	M135706T	0.75	2	**2.5**
	43	M125239D	3160.00	2	**2.6**
Ozone	21	M125887D	1.95	3	**3.1**
	49	M125890D	1390.00	3	**3.2**
	12	M071486D	240.00	3	**3.3**
	38	M135740T	3500.00	3	**3.4**
	23	M071641D	13600.00	3	**3.5**
	44	M125240D	657.00	3	**3.6**

Hot Water	20	M071597D	11000.00	4	**4.1**
	24	M071598D	22.80	4	**4.2**
	47	M125237D	780.00	4	**4.3**
	45	M135679T	4730.00	4	**4.4**
	32	M135699T	1350.00	4	**4.5**
	29	M135710T	208.00	4	**4.6**
Citric and SO_2 Solution	19	M125888D	10600.00	5	**5.1**
	34	M125892D	182.00	5	**5.2**
	35	M135769T	1260.00	5	**5.3**
	46	M135680T	5240.00	5	**5.4**
	31	M134701T	37.60	5	**5.5**
	42	M135742T	788.00	5	**5.6**
Burning Sulfur	15	M060008T	1210.00	6	**6.1**
	2	M135714T	8450.00	6	**6.2**
	37	M135739T	104.00	6	**6.3**
	48	M125238D	73.50	6	**6.4**
	7	M135741T	847.00	6	**6.5**
	14	M135746T	7910.00	6	**6.6**

Appendix 2 – Financial Assumptions

Water:

$0.00546/ gallon

Source: This rate is based on the City of Napa Water fee of $5.46 per unit of water. One unit of water is equal to 1,000 gallons according to the City of Napa Water Department.

Electric Costs:

$4.87/KW

Source: This rate is based on averaging the Pacific Gas and Electric bills for RMW Bedford for the previous year to determine an average cost per kilowatt for the year. Actual costs will vary depending on the time of the year that the equipment is used.

Gas Costs:

$30.84/Therm

Source: This rate is based on averaging the Pacific Gas and Electric bills for RMW Bedford for the previous year to determine an average cost per Therm for the year. Actual costs will vary depending on the time of the year that the equipment is used.

Steam Unit:

$8,440.88

Source: Quote from Electro-Steam Generator Corporation Feb 14, 2015

Ozone Unit:

$13,905.00

Source: Quote from McClain Ozone Inc./EnviroEd April 30, 2014

Citric Acid:

$0.80/lb.

Source: Gary Midyette, Director of Operations, RMW. Actual unit costs.

Potassium Metabisulfite:

$0.88/lb.

Source: Gary Midyette, Director of Operations, RMW. Actual unit costs.

Sulfur Wicks:

$0.20/wick

Source: Gary Midyette, Director of Operations, RMW. Actual unit costs.

Appendix 3 - Micro Results from the Trial

Treatment	Barrel Identifier	Pre-Treatment Brett (PCR)	Post-Treatment Brett (PCR)	Pre-Treatment Acetobacter	Post-Treatment Acetobacter	Pre-Treatment Brett (Plating)	Post-Treatment Brett (Plating)	Pre-Treatment Saccharomyces	Post-Treatment Saccharomyces
Control	**1.1**	8850	189	0	5	165	290	70	45
	1.2	2920	82.2	0	20	80	30	0	5
	1.3	2500	298	0	15	135	15	105	0
	1.4	3600	341	0	50	110	75	130	10
	1.5	1980	109	35	20	100	55	90	5
	1.6	1550	260	0	5	90	15	45	5
Average	Control	3566.67	213.20	5.83	19.17	113.33	80.00	73.33	11.67
Steam	**2.1**	4040	63.8	0	0	120	0	0	0
	2.2	5920	25.9	0	0	220	0	0	0
	2.3	2930	65.2	0	0	250	0	45	0
	2.4	5110	27.5	0	0	225	0	70	0
	2.5	2970	30.4	0	0	430	0	225	0
	2.6	1950	21.2	20	0	130	0	95	0
Average	Steam	3820.00	39.00	3.33	0.00	229.17	0.00	72.50	0.00
Ozone	**3.1**	2160	52.6	5	0	80	0	75	0
	3.2	2210	61.2	0	0	70	0	105	0
	3.3	1870	18.8	5	0	95	0	210	0
	3.4	3120	72.7	5	0	110	0	65	0
	3.5	5200	57.5	5	0	220	1	180	0
	3.6	3710	47.1	0	0	140	0	35	0
Average	Ozone	3045.00	51.65	3.33	0.00	119.17	0.17	111.67	0.00

Treatment	Barrel Identifier	Pre-Treatment Brett (PCR)	Post-Treatment Brett (PCR)	Pre-Treatment Acetobacter	Post-Treatment Acetobacter	Pre-Treatment Brett (Plating)	Post-Treatment Brett (Plating)	Pre-Treatment Saccharomyces	Post-Treatment Saccharomyces
Hot Water	**4.1**	19.3	1070	20	0	335	0	205	1
	4.2	3650	64.3	0	0	150	0	170	0
	4.3	3460	149	0	0	165	0	35	0
	4.4	4470	189	15	0	180	0	60	0
	4.5	5790	807	15	0	340	0	40	0
	4.6	1460	253	25	0	140	0	80	0
Average		3141.55	422.05	12.50	0.00	218.33	0.00	98.33	0.17
Citric and SO_2 Solution	**5.1**	1130	550	10	0	240	0	110	5
	5.2	1950	222	5	0	75	0	90	4
	5.3	3220	158	5	0	50	0	0	0
	5.4	2100	217	0	0	100	0	35	6
	5.5	1570	75.8	0	0	45	0	5	0
	5.6	1370	33.2	0	0	60	0	35	5
Average		1890.00	209.33	3.33	0.00	95.00	0.00	45.83	3.33
Burning Sulfur	**6.1**	352	2760	15	15	335	140	65	5
	6.2	1670	1190	0	15	165	0	10	0
	6.3	3400	502	0	35	160	70	90	15
	6.4	2990	404	0	10	55	40	90	0
	6.5	5500	563	20	25	70	30	10	0
	6.6	5770	333	10	45	170	40	50	0
Average		3280.33	958.67	7.50	24.17	159.17	53.33	52.50	3.33

Appendix 4 – Survey Questions

Survey Questions

1) Are you a Winemaker, Consulting Winemaker, Assistant Winemaker, or Enologist in your current role?
    a. Yes
    b. No
2) Do you currently make or have you ever made wine in the Napa Valley?
    a. Yes
    b. No (Terminate)

3) In the past 12 months, did your winery use oak barrels as part of its winemaking process?
    a. Yes (Go to question 5)
    b. No (Go to question 4)

4) Have you ever worked with oak barrels in a previous position?
    a. Yes (Go to question 5)
    b. No (Terminate)

5) As part of the barrel sanitation program at your winery, which of these methods were used in the past 12 months? (CHECK ALL THAT APPLY)
    a. Hot Water
    b. Ozone
    c. Steam
    d. Burning Sulfur
    e. SO_2 and Acid solution

f. Other (PLEASE SPECIFY)

g. Do not have a barrel sanitation program

CHOICES ARE ANY METHOD NOT SELECTED IN Q5 (choices a – e)

6) What other methods have you ever used as part of a barrel sanitation program? (CHECK ALL THAT APPLY)

a. Hot Water

b. Ozone

c. Steam

d. Burning Sulfur

e. SO_2 and Acid solution

f. Other (PLEASE SPECIFY)

g. Have never used any other method

7) Thinking about other methods that you HAVE NOT used as part of a barrel sanitation program, with which of these are you familiar?

LIST ANY ANSWER NOT SELECTED IN Q5 and Q6

a. Hot Water

b. Ozone

c. Steam

d. Burning Sulfur

e. SO_2 and Acid solution

FOR ANY METHOD USED (Q5/6) OR FAMILIAR WITH (Q7) ASK:

8) In terms of effectiveness against microbial growth (yeast, bacteria, and mold), how would you rate these barrel sanitation methods?

(SCALE: EXTREMELY EFFECTIVE, VERY EFFECTIVE, SOMEWHAT EFFECTIVE, NOT VERY EFFECTIVE, NOT AT ALL EFFECTIVE)

a. Hot Water
b. Ozone
c. Steam
d. Burning Sulfur
e. SO_2 and Acid solution

FOR ANY METHOD USED (Q5/6) OR FAMILIAR WITH (Q7) ASK:

9) In terms of environmental friendliness, how would you rate these barrel sanitation methods?

(SCALE: EXTREMELY FRIENDLY, VERY FRIENDLY, SOMEWHAT FRIENDLY, NOT VERY FRIENDLY, NOT AT ALL FRIENDLY)

a. Hot Water
b. Ozone
c. Steam
d. Burning Sulfur
e. SO_2 and Acid solution

FOR ANY METHOD USED (Q5/6) OR FAMILIAR WITH (Q7) ASK:

10) In terms of the cost (materials and labor), how would you rate these barrel sanitation methods?

(SCALE: EXTREMELY EXPENSIVE, VERY EXPENSIVE, SOMEWHAT EXPENSIVE, NOT VERY EXPENSIVE, NOT AT ALL EXPENSIVE)

a. Hot Water
b. Ozone
c. Steam
d. Burning Sulfur
e. SO_2 and Acid solution

FOR ANY METHOD USED (Q5/6) OR FAMILIAR WITH (Q7) ASK:

11) In terms of effectiveness for the money, how would you rate these barrel sanitation methods?

(SCALE: EXTREMELY EFFECTIVE, VERY EFFECTIVE, SOMEWHAT EFFECTIVE, NOT VERY EFFECTIVE, NOT AT ALL EFFECTIVE)

a. Hot Water
b. Ozone
c. Steam
d. Burning Sulfur
e. SO_2 and Acid solution

12) Overall, which barrel sanitation methods is your most preferred?

a. Hot Water
b. Ozone
c. Steam
d. Burning Sulfur
e. SO_2 and Acid solution

13) Why do you say that? (OPEN Dialogue Box)

14) Have you ever experienced microbial contamination in any barrels at any time in your career?

a. Yes
b. No (SKIP TO 17)

15) Which type of microbial contamination have you experienced? (CHECK ALL THAT APPLY)

a. Fermentation Yeast – Saccharomyces species
b. Spoilage Yeast – Brettanomyces, etc.
c. Lactic Acid Bacteria – Lactobacillus, etc.
d. Acetic Acid Bacteria – Acetobacter
e. Mold
f. Other (PLEASE SPECIFY)
g. Don't know

16) In your opinion, when there is a low level of microbial contamination, to what degree is there a loss of wine quality?

    a. No loss of quality (wine is not affected by the microbial contamination)
    b. Some loss of quality (wine is still usable/sellable)
    c. Total loss of quality (wine can't be used/sold, must be destroyed)

17) Were you to have a barrel with microbial contamination, what would happen that barrel?

    a. Remove the barrel (Sell/Repurpose)
    b. Destroy the barrel
    c. Treat the barrel (Open Dialogue Box 'How would you/have you treat the barrels?')

18) In your opinion, how important is barrel sanitation research to the wine industry?

    a. Extremely Important
    b. Very Important
    c. Somewhat Important
    d. Not Very Important
    e. Not At All Important

Appendix 5 - Approved Research Paper Proposal

IMW Research Paper Proposal Submission Form			
Student ID	**22995**	**Date of submission**	
RPP Version No	**1**	**Name of Advisor**	**Wendy Cameron**

Note: RPPs must be submitted via your Advisor to the IMW

Proposed Title

Exploring the Efficacy of Different Barrel Cleaning Procedures on *Brettanomyces bruxellensis* and *Acetobacter spp* Populations and the Relative Financial and Environmental Benefits of Each Treatment.

Research Questions: Define the subject of your Research Paper and specify the specific research questions you plan to pursue. (No more than 200 words)

This research paper will focus on common barrel sanitation methods and their effectiveness in controlling the two main spoilage organisms found in barrels: *Brettanomyces bruxellensis* (Brett) and *Acetobacter spp*. During the tests, conducted at a well-known Napa Valley producer, other aspects will be measured and analyzed to determine the financial and environmental impacts of each method.

Primary Questions:

1) How effective are different methods of barrel sanitation for controlling these microbial populations in winery barrel populations?
2) What is the cost/benefit ratio of each of the treatments?

3) What are the environmental impacts of each treatment including utilities and water use?

4) Is the best method different for each organism?

Background Question:

1) To better understand current practices and to support and add depth to the analytical work undertaken, a survey will be conducted to understand how Napa winemakers' views of barrel sanitation compare to the results of the controlled experiment?

Specific Objectives:

1) Evaluate the effectiveness of each treatment on populations of *Brett* and *Acetobacter spp* per microbial testing procedures pre and post treatment.

2) Determine the cost of the experimental treatments and relative value based on treatment effectiveness.

Background and Context: Explain what is currently known about the topic and address why this topic requires/offers opportunities for further research. (No more than 200 words)

Millions of dollars are spent each year purchasing barrels for wine maturation and they represent a significant investment by wineries (Simental 2016, pers. comm.). There are many studies researching the negative effects of Brett and Acetobacter on wine, however very few studying comparatively the methods to remove these microbes from barrels. Furthermore, the studies which do compare methods in a controlled experiment using 225 Liter barrels do not compare more than two at a time nor do they look at the financial and environmental impact of each treatment. Using a practical winery setting, this research will compare and contrast five of the most commonly used sanitation methods against Brett and Acetobacter assessing their effectiveness, as well as financial and environmental costs.

Sources: Identify the nature of your source materials (official documents, books, articles, other studies, etc.) and give principle sources if appropriate. (No more than 150 words)

Source material will be:

Primary published research such as:

- Rayne, S. and N. J. Eggers. 2008. Research Note: 4-Ethylphenol and 4-Ethylguaiacol Concentrations in Barreled Red Wines from the Okanagan Valley Appellation, British Columbia. *American Journal of Enology and Viticulture*, *Vol. 59, No. 1.*
- Wilker, K.L, and M.R. Dharmadhikari. 1997. Research Note: Treatment of Barrel Wood Infected with Acetic Acid Bacteria. *American Journal of Enology and Viticulture*, *Vol. 48, No. 4.*
- Malfeito-ferreira, M. et al. 2004. Effect of different barrique sanitation procedures on yeasts isolated from the inner layers of wood. Abstract: American Journal of Enology and Viticulture. Vol 55:304A.

Secondary sources will be one-on-one interviews with winemakers and other researchers currently working on similar topics as well as equipment and product instructions. A winemaker survey will add further depth and evidence around methodology and practical application of the analysis.

Research Methodology: Please detail how you will identify and gather the material or information necessary to answer the research question(s) and discuss what techniques you will use to analyze this information. (No more than 500 words)

Winemaking Research Procedure:

36 used barrels are selected from a single lot of wine known to contain varying levels of Brett using Polymerase Chain Reaction (PCR) analysis from samples of each of the individual barrels. Barrels identified are grouped into 6 groups of 6 with total cell counts which are similar within the groups. The wine is pumped out, after which all the barrels have a 30 second cold water rinse to remove wine sediment. All barrels are sampled within each test group to establish baseline populations using the Microbial analysis procedure for sampling and testing. The 6 groups of barrels are separated into the following groups:

7) Control – Neutral water (13.8°C) rinse for 2.5 minutes using city water on the in line barrel washer.
8) Steam – 6 minutes exposure to steam at 30 psi, 3 minutes bunged, then rinsed for 2.5 minutes with neutral water on the in line barrel washer.
9) Ozone – Rinse barrels with 82°C city water for 1.5 minutes followed by 2 minute cold ozonated water rinse.
10) Hot Water – Rinsed with 82°C water for 2.5 minutes using city water on the in line barrel

washer.

11) Citric and SO_2 Solution – Prepare a solution of 84 g Potassium Metabisulfite and 182 g of Citric Acid in each barrel using city water. Allow barrels to sit for 2 weeks then rinse for 2.5 minutes with neutral water on the in line barrel washer.

12) Burning Sulfur - Burn sulfur sticks in each barrel for 1 hour then neutral water rinse for one minute using city water on the in-line barrel washer.

Microbial Analysis Procedure:

600 mL sterilized water will be added to each barrel and sealed. The water will be agitated to fully contact all surfaces, then remain still for one hour. 200 mL of water will be removed via sterile pipet for microbial analysis by PCR and plating on Difco™ Wallerstein Laboratory (WL) Nutrient media. The sterilized water is tested by itself as a control to confirm sterilization. Individual sterilized pipets are used for each sample to ensure there is no cross contamination between samples. The results of the 6 barrels in each treatment will be presented separately to determine significant differences.

Environmental Analysis Procedure:

Determine amount of water used via flow meter, natural gas via therm measurement, and electricity via Voltmeter.

Financial Analysis Procedure:

Determine the costs of treatments including capital expenses and general operating expenses (labor, cost of water, electricity, gas, and chemicals) normalized over a three year time period to determine relative costs. Compare relative costs to percent effectiveness of each treatment to determine value ratio.

Winemaker Survey Procedure:

120 winemakers from a population of 400 wineries in the Napa Valley will be surveyed assuming a 50% response rate, 90% confidence interval, and 10% margin of error to determine winemaker perceptions on barrel sanitation. Questions will cover many aspects including methods used, preferred, effectiveness, environmental impact, cost, previous microbial contamination experience, barrel destruction due to contamination, and wine spoilage due to contamination.

Potential to Contribute to the Body of Knowledge on Wine: Explain how this Research Paper will add to the current body of knowledge on this subject. (No more than 150 words)

Barrels have been traditionally used for wine storage; however current sanitation methods have not been fully explored and compared in a full scale production trial to give guidance to wineries looking to mitigate spoilage concerns. Many barrel trials in wineries only use 2-4 barrels for each experimental lot. Using six barrels for each test group will help increase the statistical significance of the experiment.

Several options for barrel sanitation are available; however, these options have differing costs and environmental impacts in addition to their effectiveness which must be explored as well.

This Research Project will explore the 5 main treatments for barrel sanitation by evaluating their efficacy in relation to one another and a control as well as evaluating the financial and environmental impacts of these treatments. Finding the best treatment for barrel sanitation will help remove or maintain spoilage microbes at levels that will not impact wine quality.

Proposed Time Schedule/Programme: This section should layout the time schedule for the research, analysis and write-up of the Research Paper and should indicate approximate dates with key deliverables. *Dates of submission to both Advisors and the IMW must be those specified by the IMW.*

Mid-September through November 2016:

- Submit completed RPP to institute via advisor – 3/11/2016
- Receive RPP approval – 27/11/2016
- Review current research
- Develop survey questions and design
- Contact Napa Valley Vintners to get contact information for Napa based winemakers

December 2016

- Begin building the Research Paper including Introduction and Methodology
- Send out survey to 120 Napa based winemakers to get current views on barrel sanitation

January 2017

- Review barrel treatments from sponsor winery in Napa, CA
- Review Microbial analysis post barrel treatment.

- Review financial data from winery and equipment producers
- Review survey data to compare anecdotal observations to data from the controlled experiment
- Finalize Introduction, Literature Review, and Methodology sections of the Research Paper

February 2017

- Evaluate and analyze data from experiments
- Analyze financial data and determine value ratio
- Outline conclusions
- Write first draft of the Results and Conclusion sections of the Research Paper

March 2017

- Finalize Research Paper including incorporation of comments by proof-readers, professors, mentors, and subject matter experts.
- Compile Appendices

May 2017

- Finalize Research Paper Final Draft
- Submit finished Research Paper to RP Advisor no later than 3/6/2017

June 2017

- Final Research Paper submitted to the Institute no later than 3/7/2017 via advisor