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WINERY CLEANING AND SANITIZATION: OPTIMIZED CHEMISTRIES FOR MANAGING FERMENTATION SOILS AND THE SULFUR DIOXIDE FUMIGATION OF WINERY COOPERAGE

By

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DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Food Science and Technology

in the

OFFICE OF GRADUATE STUDIES

of the

UNIVERSITY OF CALIFORNIA

DAVIS

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2021

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I. DEDICATION

To my lab, family, friends, and wonderful girlfriend:

Your support and guidance are why this thesis exists today. Let’s share a bottle sometime.

II. ABSTRACT

Cleaning and sanitization are essential operations in wine production. The use of chemical cleaning and sanitizing agents for managing waste in fermentation vessels and the sulfur dioxide fumigation of empty oak barrels are ubiquitous processes in commercial wineries but are poorly studied in academic literature. Increasing environmental pressures and concerns over the poor quality of winery wastewater have created a need for optimizing protocols by reducing chemical inputs and water usage while maximizing the efficacy of treatments and employee safety during application. To optimize the use of chemical cleaning and sanitizing agents for managing fermentor waste the performance of a wide range of commercially available cleaning and sanitizing chemistries was assessed using fermentation derived soils and spoilage microorganisms in planktonic and sessile physiologies in trials from bench scale to 2000-L fermentation tanks. Minimum effective antimicrobial concentrations of chemical treatments were determined for common winery spoilage yeasts using the minimum inhibitory concentration, minimum biocidal concentration assay, and a modified minimum biofilm inactivation assay. Propidium iodide fluorescent staining was used to determine the minimum effective contact time required for inactivating *Saccharomyces cerevisiae* cultures using peracetic acid. Results suggested that caustic cleaning agents were the most effective in removing fermentation soils, and that sanitizers were ineffective without thorough prior cleaning. Peracetic acid- and hydrogen peroxide-based sanitizer formulations were effective and have innocuous breakdown products compared to traditional chemistries. Manufacturers' suggested application rates were largely in line with the minimum effective antimicrobial concentration for the spoilage microorganisms tested. In practical settings, sanitary tank design may be the most important

factor in determining the success of cleaning and sanitizing efforts regardless of the specific chemicals applied.

The sulfur dioxide detection performance of colorimetric gas detection tubes, gas chromatography-sulfur chemiluminescence detection, and a novel electrochemical sensor apparatus was assessed. The electrochemical sensor was superior in linearity and precision versus the other detectors. American oak barrels were used to measure the persistence and antimicrobial efficacy of the sulfur dioxide fumigation of empty winery cooperage for pure gas application and the combustion of solid sulfur wicks. Prior to fumigation, the penetration rate and abundance of spoilage yeasts in barrel wood for cultures inoculated in grape must was determined for *Saccharomyces cerevisiae* and *Brettanomyces bruxellensis* cultures. *S. cerevisiae* cultures were recovered at 10 mm depth within one week of inoculation. *B. bruxellensis* cultures did not penetrate beyond surface samples within six weeks of inoculation. Measurable concentrations of sulfur dioxide existed after six weeks of storage for pure gas and sulfur stick fumigation, with concentration profiles closely described by power law functions regardless of the hydration status of the barrel wood. Both gas applications and sulfur stick combustion effectively eliminated culturable populations of *S. cerevisiae* and *B. bruxellensis* for all samples. Methods for assessing the mechanism of sulfur dioxide diffusion as a Fickian process according to the steady-state approximation poorly fit the measured gas data. Interactions between the diffusing gas and the stave wood and the heterogeneous structure of barrels may explain the discrepancy between the observed and predicted Fickian diffusive behavior. Together, this work presents the most comprehensive quantitative assessment of winery cleaning and sanitization to date in academic literature. These studies not only provide insight into the fundamental interaction of microorganisms, winery equipment, and chemical cleaners and sanitizers, but can

also act as a practical resource for winemakers to develop effective cleaning and sanitizing protocols and monitoring strategies.

CHAPTER 1: INTRODUCTION

1.1 Principles of winery cleaning and sanitization

Cleaning and sanitizing processes are fundamental to the success of any winemaking operation. A successful cleaning and sanitizing program minimizes the potential for spoilage throughout production and ensures the stability of finished wine leaving the facility. Grape processing, fermentation, and aging produce substantial quantities of organic and inorganic waste. This waste must be managed to ensure the success of future operations. Improperly managed soils can become endemic sources of contamination and can cause premature wear or damage to equipment. Spoilage results in a significant financial burden to production facilities and can harm the long-term reputation of a producer.

Wine production inherently relies on the controlled metabolism of bacteria and yeast. Spoilage results when microorganisms produce undesirable sensory character in fermenting juice or wine. Faulty characteristics are subjective, however, as each winemaker and drinker has differing personal expectations and preferences in finished wine[1]. For the purpose of this discussion microbial spoilage is considered from the perspective of production staff and results from the undesired activity of yeast and bacteria. Other forms of spoilage can result from chemical interaction with organic and inorganic residues on winery equipment. Examples include tartrate instability resulting from interaction with crystal precipitate and haziness from proteinaceous residue[2]. Wine faults related to the improper use of additives or human error in processing steps are legitimate concerns in industrial settings but are not considered here.

Cleaning and sanitization are often bundled together as a single process in discussion but are necessarily separate steps with different aims. Cleaning steps aim to physically remove gross

soils in and on vessels and processing equipment. Sanitization refers to microbial inactivation, and is specifically defined by the United States Food and Drug Administration as a process resulting in a five-log reduction in microbial viability (99.999% reduction)[3]. Cleaning is undoubtedly the more important step. Without proper initial cleaning, sanitization is extremely difficult. Physical soils harbor microorganisms and act as a barrier to sanitizers that can render sanitizing treatments ineffective[4]. Soils also readily react with many sanitizing agents, substantially reducing the antimicrobial action of the treatment[5]. Thermal sanitization strategies can even be deleterious to vessels that are improperly cleaned by baking soils onto surfaces, making them extremely difficult to remove without damaging the container[6].

Wine production generates significant volumes of waste, nearly three-quarters of which is produced during the short harvest period when grapes are ripe[7]. Solid grape waste includes pomace, stems, and lees, with smaller contributions from organic and inorganic mineral deposits and microbial residues from fermentation[8]. Cleaning operations seek to physically remove all forms of soil. Water rinses are used to remove the majority of loose or weakly-adhered soils, and are normally followed by a mechanical, chemical, or combination cleaning tactic[9]. Successful cleaning is normally judged by a lack of visible soil on the surface being cleaned, but this is a subjective and imprecise determination.

Wine is a low food safety risk product. The low pH, ethanol content, and polyphenol content of wine mean that pathogens do not normally present a risk to production[10]. There may still be minor health risks associated with unintended microbial activity in wine. Recent research has identified an association between environmental strains of lactic acid bacteria and elevated levels of biogenic amines in finished wines[11]. The concentration of biogenic amines normally found in wine are generally considered safe but can be linked to symptoms ranging from

headaches to gastric and pseudo-allergenic responses in sensitive groups[12]. Regardless, sanitizing efforts are not normally applied for health considerations specifically. Many wineries intentionally encourage the growth and metabolism of 'native' yeast and bacteria naturally occurring on the grapes or in the facility during fermentation and aging.

Sanitizing efforts instead target spoilage microorganisms. Because microbial spoilage character can be subjective, the classification of specific microorganisms as spoilage species is difficult. Some compounds that are used as indicators for microbial spoilage are even perceived as pleasant, or as adding to the complexity of a wine under certain thresholds[13]. Yeast and bacteria that are desired and even inoculated are considered spoilage species if present and active at an undesired stage in the winemaking process. In a review of the most-commonly implicated wine-spoilage microorganisms, Loureiro and Malfeito-Ferreira[14] classified spoilage yeast in four basic groups:

1. Fermenting strains (*Saccharomyces cerevisiae*) when refermenting bottled wines.
2. *Zygosaccharomyces bailii*, an osmotolerant species that produces sediment or cloudiness in bottled wines.
3. Film-forming and ester-producing yeasts (*Hansenula anomala, Kloeckera apiculata, Pichia* spp.*, Metschnikowia pulcherrima, Debaryomyces* spp.*).*
4. Off-flavor producing yeasts (*Brettanomyces* spp., *Schizosaccharomyces pombe,* and *Saccharomycodes ludwigii*).

Of all the species mentioned in the study, the authors concluded that *Saccharomyces cerevisiae*, *Brettanomyces bruxellensis*, and *Zygosaccharomyces bailii* present the greatest risk for spoilage in finished, bottled wine.

Lactic acid bacteria (LAB) and acetic acid bacteria (AAB) are commonly found on grapes and in wine[15]. Some strains of LAB are desired in certain wine styles, as in the case of *Oenococcus oeni* and occasionally *Pediococcus* spp. in malolactic-fermented wines[16]. AAB are generally considered spoilage microorganisms in any aspect of wine production, despite low levels of AAB-associated ethyl acetate contributing to increased perception of fruity flavors[17]. Of special concern in cleaning and sanitization is the formation of biofilms, sessile communities of microorganisms embedded in an exopolysaccharide matrix. Biofilms are implicated as having increased resistance to antimicrobial treatments versus planktonic communities of cells[18], and numerous wine spoilage organisms have been shown to form biofilms under enological conditions[19,20]. Effective sanitization methods must be capable of inactivating these spoilage organisms in sessile and planktonic physiologies in practical settings.

The goals of cleaning and sanitization are straightforward, but the topic is more complicated in practice. A wide range of chemical cleaning and sanitizing agents and equipment are readily available to the modern winemaker, however comprehensive quantitative support for the efficacy of these techniques is scattered and limited. Winemaking is a water- and chemical-intensive process so the situation is complicated by increasing concern over the resource demand and poor quality of wastewater output in winery operations[21,22]. Effective cleaning and sanitization strategies require a combination of the right tools (chemicals, application equipment, etc.) and efficient process execution to minimize resource consumption while maintaining a high sanitary standard[23], but resources for developing effective protocols are limited. Peer-reviewed research on the subject is often difficult to translate to production settings because many institutions lack winery facilities that emulate the scale and equipment used in industry[20,24]. Chemical vendor-suppliers provide recommendations for the use of cleaning and sanitizing

supplies, but data supporting these guides are scarce, if available. Technical winemaking texts discuss basic principles and validation methods[25,26], but supporting data are similarly lacking. The research to follow seeks to provide robust quantitative supporting data that will empower winemakers to develop efficient cleaning and sanitization protocols.

Any meaningful investigation of winery cleaning and sanitation is by necessity first divided into two categories. The handling of stainless steel (SS) and plastic tanks and equipment differs from the techniques that are relevant for wooden cooperage. Cooperage is expensive, porous, and confers desirable aromatic character to aging wine[27]. Chemical approaches to cleaning and sanitation normally used for metal and plastic materials could absorb into the staves, damage the wood, and leach desired aroma compounds[28]. Empty cooperage must also be kept hydrated to avoid cracking or losing its watertight properties, and preservation is necessary to avoid fouling of the vessel interior when barrels are emptied[29]. While wooden vessels have been used as fermentors for centuries, modern wineries overwhelmingly employ SS tanks and a combination of SS and plastic equipment for production[25]. Wooden cooperage is instead normally used for post fermentation aging and storage of wine in barrels. SS and plastic are referred to as 'fermentor materials' for the sake of brevity in this thesis, and wooden cooperage is specifically discussed in the context of barriques (225-L barrels). Cleaning and sanitization strategies for barriques are applicable to larger format wooden vessels, so the discussion on barrels is readily translated to larger systems. The following sections discuss the current state of knowledge for cleaning and sanitizing fermentor materials and barrels separately.

1.2 Fermentor materials

1.2.1 Vessel properties

Wine has been produced for millennia. A wide range of materials have been utilized to construct fermentation and storage vessels through history. The earliest storage vessels were clay amphorae, and the use of wooden barrels dates to around 350 BC[30]. Both types of vessel are still used today, but the use of SS tanks by far dominates modern wine production. The advent of temperature-controlled SS fermentors is undoubtedly one of the most important innovations in modern wine quality. SS is durable, chemically inert, and resistant to chemical and thermal damage. The nonporous nature of SS tanks makes vessels far easier to clean and sanitize versus any porous wood or clay progenitor[25].

Figure 1.1. Variability of inert tank sizes and materials used in wineries. *Left:* SS, lined concrete, and lined wooden vats in an Italian production facility. *Right:* A variety of SS tanks sizes and configurations in an American winery. *Photographs taken by author*.

Other tank materials are used in modern wineries. Concrete vessels are used across the globe and are especially valued for their thermal stability and ability to be poured into almost any shape[25]. Most concrete or stone tanks are lined with a nonporous epoxy coating (**Figure 1.1**). Chemical methods used for SS tanks apply for any inert, nonporous container so data are readily translated to epoxy lined tanks and plastics[31]. Unlined concrete or stone vessels are less common in the modern wine industry. The porosity of unlined vessels results in the same challenges to cleaning and sanitization as wood or clay[32]. Cement and stone have similarly low thermal conductivities to clay and wood versus SS, making thermal treatments much more challenging for any nonmetal vessels.

No two wineries are identical. The malleability of SS means vessels are fabricated in all sorts of sizes and configurations. A wide array of accessories, clamps, and connections further complicates the situation in practice. Tanks are commonly fabricated from grade 304-SS or the more expensive and corrosion resistant grade 316-SS. Most SS vessels and equipment are fabricated with a cold-rolled 2B mill finish, but a range of specialized physical and chemical polishes are commercially available[33]. These finishes aim to reduce the surface roughness of the steel for improved cleaning and sanitization efficiency. In reality, the impact of surface defects (scratches, imperfections, and welded junctions) appears to play a more significant role in the cleanability of steel than the surface finish[34]. Physical baffles such as temperature probes, screens, or ports and fittings present a major challenge to cleaning and sanitization so hygienic design may truly be the largest factor in the successful management of fermentor materials[35].

The choice of cleaning and sanitizing techniques is not straightforward. A wide range of chemical, physical, and thermal techniques are used in the wine industry today. Chemical cleaning and sanitizing agents are by far the most used in part because application requires little

to no capital equipment investment. A distinction is made between 'simple' and 'built' chemicals. Simple chemicals are those comprised of only one or two basic active chemical species, where built chemicals use surfactants, chelating agents, emulsifiers, or complex mixtures of active species to improve cleaning or sanitization performance[9]. Built chemicals are often substantially more expensive than the base chemistries they are formulated from, but few data exist on the comparative performance of the two groups. Nonchemical methods such as thermal treatment (i.e. hot water or steam), ultraviolet radiation, ozone, and high power ultrasound have all been studied in enological settings[36,37], but these studies have been applied in the context of barrel sanitization rather than stainless steel tank hygiene. Nonchemical methods are appealing in the quality and quantity improvements for winery wastewater, but the high equipment cost and design requirements for incorporating the technology limit the applicability of these techniques for most wineries. As a result, this thesis focuses specifically on the comparative efficacy of chemical cleaners and sanitizers.

1.2.2 Cleaning agents

Cleaning agents must be able to effectively remove residues adhered to storage vessels and equipment after preliminary water rinse cycles. Organic residues are derived from grape material and fermentation lees. Inorganic residues are primarily formed from tartaric acid precipitate, and to a lesser extent other mineral and metal deposits[38]. Cleaning chemicals are available in acidic and alkaline formulations. Acidic cleaners formulated from phosphoric and nitric acid can be useful to passivate and refresh the surface chromium layer of stainless-steel equipment annually, but in practice this is rarely performed. Alkaline cleaners are far more widely used as part of a regular cleaning program. Basic chemistries are more effective than acids at lifting soils from the effect of alkaline hydrolysis with fatty acid compounds[9].

Fermentation soils and most sanitizer formulations are acidic so using an alkaline cleaner also works to produce a more neutral-pH wastewater stream while also subjecting any spoilage microorganisms present to a rapid change in pH during the full cleaning and sanitizing process.

Caustic hydroxides of sodium and potassium salts were traditionally used as the base for most cleaning chemicals in industry. These compounds are strongly alkaline and capable of physically dissolving proteins and tartrates, making them extremely effective in removing fermentation soils. While strong alkaline caustic cleaners are still widely used they are increasingly falling out of favor due to health hazards associated with handling and environmental concerns in wastewater disposal[39]. Non-caustic cleaners based on sodium carbonate peroxyhydrate (SCP) are a popular alternative. SCP is an adduct of sodium carbonate and hydrogen peroxide. SCP functions as a stabilized form of hydrogen peroxide, which supplies active oxygen for bleaching and disinfection. SCP eventually breaks down into oxygen, water, and sodium carbonate[40]. Sodium carbonate is weakly alkaline and provides the benefits described above for alkaline cleaners. Calcium and magnesium ions can substitute for sodium ions in aqueous solutions of sodium carbonate, which acts as a water softener to prevent mineral buildup. Simple and built versions of sodium and potassium hydroxide and SCP-based cleaners are widely available. In recent years a new generation of biodegradable cleaners derived from natural materials like coconut and citrus have appeared on the market[41]. The diversity of cleaners provides winemakers with a variety of options, yet no data exists to compare the performance of any of these groups in enological settings. As a result, winemakers typically make choices based on cost, philosophy (environmental-friendliness or worker safety), or waste disposal limitations rather than cleaning efficacy.

One of the underlying challenges in studying the efficacy of cleaning agents is the ambiguity involved in measuring the cleanliness of a winery tank surface. Many cleaner formulations claim to function as dual cleaners-sanitizers, further adding confusion. Visual assessment is by far the most common method of assessing the success of cleaning operations, but this method is inherently qualitative and subjective[42]. Advanced spectroscopic and photometric methods have been used to assess the cleanliness of SS, but these methods cannot measure tank cleanliness *in situ*. Adenosine triphosphate (ATP) bioluminescence swabbing has been used to measure surface contamination of stainless steel equipment in a range of food and beverage industries, including wine[43,44]. Swabs are relatively inexpensive, simple to operate, and produce results in seconds. Surface contamination is measured according to the light producing reaction between ATP-bearing residues collected by the swab and the luciferase enzyme, which is evaluated using a handheld luminometer. Because ATP is present in both grape and microbial soil, swabs do not provide information about the contamination source. Traditional environmental culture swabbing is a natural compliment. Cell counts provide qualitative support for the source of the ATP load and can quantify the claims of sanitizing action for cleaning chemistries. Neither method can measure inorganic contamination so visual and tactile assessment remain a necessary component in evaluating surface cleanliness.

1.2.3 Sanitizing agents

Sanitizers play a critical role in operations as the last line of defense against spoilage. As with cleaning agents, sanitizing chemicals can be simple or built formulations and are based on a range of active chemistries. Acidic sanitizers are especially common in part due to the popularity of alkaline cleaning agents. Acidulated sulfur dioxide (SO_2) has traditionally been used as a sanitizer and storage solution, especially for empty cooperage[28]. Solutions are typically prepared

as a mixture of citric acid and potassium metabisulfite powder. While inexpensive and simple to prepare, employee health concerns over handling and the large volume of water required for storage solutions makes acidified SO_2 an unpopular choice in modern wineries for tank maintenance. Peroxyacetic acid (PAA) is widely used in modern wineries. Commercially available preparations are comprised of varying proportions of PAA, hydrogen peroxide, acetic acid, and water. PAA has the advantage of being biodegradable and decomposes to water, oxygen, and carbon dioxide with a half-life of 22 minutes in air[45]. No-rinse formulations of PAA are common. The efficacy of PAA as an antimicrobial has been studied in enological settings, but these studies are largely limited to the context of barrel sanitation[46,47]. A commercial winery trial was performed using a PAA-based sanitizer[43], however this study was limited to built sanitizers supplied from a single vendor with proprietary ingredients, limiting the applicability of the findings. The study also used 'naturally' contaminated vessels for treatment without any specific knowledge of the microorganisms present. As a result, the efficacy of simple PAA as a sanitizer in winery tanks remains largely unknown despite its widespread use.

Nonacidic sanitizers are used to a lesser extent in wineries. Chlorine dioxide (ClO_2) is a powerful oxidizer and deodorant used in a range of food processing industries. In production settings ClO_2 is typically generated onsite. The antimicrobial impact of ClO_2 on spoilage yeast and bacteria has been demonstrated in bench trials[48], but the use of ClO_2 is limited in wineries due to associations with chlorinated compounds and trichloroanisole (TCA) taint formation[49]. Capital expense and safety hazards associated with ClO_2 generation also limit use. TCA formation is related to the use of hypochlorite in household chlorine bleach. No evidence exists to support any link between TCA and ClO_2, nevertheless the chemical has been slow to be adopted in wineries. Other halogenated compounds based on iodine and bromine are used in

food processing facilities. These compounds have low toxicity for humans but can leave permanent stains on equipment and have relatively narrow spectrum activity[50]. Quaternary ammonium compounds (QUATs) are also widely used in food processing facilities. QUATs are generally nontoxic to humans and available in no-rinse formulations. QUATs are not broad spectrum, however, and some concern exists over the persistence and buildup of QUATs in waterways from wastewater disposal[51]. As with the more popular acidic sanitizer formulas, very little data exist to demonstrate the performance of these sanitizers for winery relevant spoilage organisms or conditions.

Evaluating the performance of a sanitizer is more straightforward than for cleaners. Numerous methods for microbial enumeration have been used for winery spoilage microorganisms[26,52]. The cost, complexity, and equipment requirements vary widely. Traditional culture methods are the most simple and most widely used for measuring cell viability[26]. ATP swabs are often used as a measurement of sanitation in practice. Polymerase chain reaction (PCR) based methods are also widely used. These methods can provide speciation information, however the user skill and equipment required for analysis are much more advanced than culture techniques[52]. Commercial enology laboratories offer liquid sample processing as a service to wineries that lack the necessary resources, but samples are too costly for routine measurement in most cases. Fluorescent staining has also been used to measure the viability of winery spoilage microorganisms[53,54]. Staining is often used in conjunction with flow cytometry for counting individual cells[55]. No commercial services exist for this type of measurement, and the operator skill and advanced equipment required for analysis means fluorescence is rarely used in industry. Antimicrobial susceptibility tests are well established for healthcare settings[56]. These tests provide a high-throughput method for assessing the efficacy of a range of chemicals at different

concentrations against yeast and bacteria cultures and are well suited for assessing winery antimicrobials. Minimum inhibitory and minimum biocidal concentration assays have been demonstrated for selected winery sanitizers and spoilage organisms, but the list is not comprehensive[57,58]. Regardless, *in situ* measurement of tank sanitation in industry is almost exclusively performed using ATP and culture swabbing. Any other methods of enumeration should be used in conjunction with common swab techniques so results can best translate to real-world operations.

1.3 Cooperage

1.3.1 Vessel properties

Wooden barrels have been used to store and age wine for well over a thousand years. Barrels have been made from a wide range of woods, but regional oak (*Quercus*) species have dominated production throughout history[59]. European oak (*Q. petraea, Q. robur*), American oak (*Q. alba*), and to a lesser extent local Hungarian and Slovenian oak species are most common in the modern wine industry. Oak wood is thermoplastic and watertight when properly harvested and dried, and most importantly contributes desired sensory

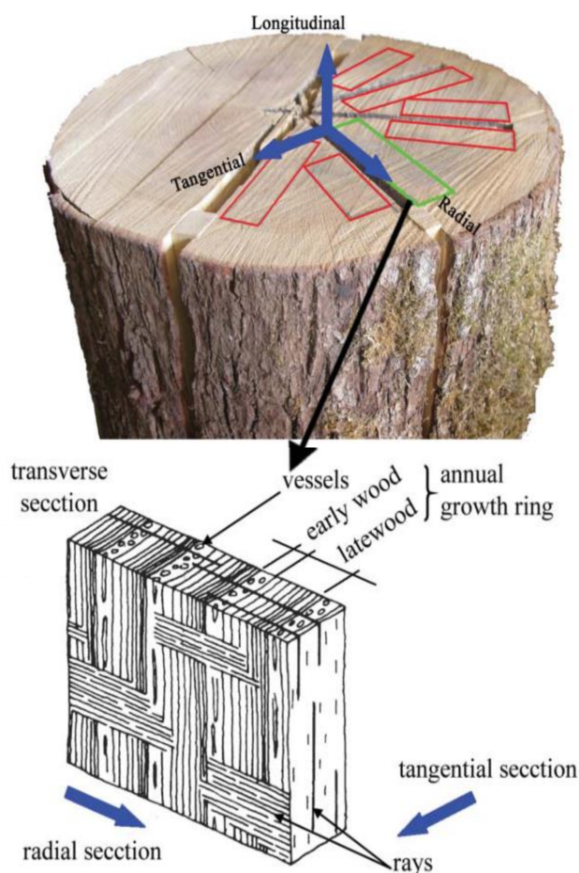

Figure 1.2. Orientation and cell structure of barrel staves relative to parent log material[45].

character to the finished wine[27]. The watertight properties of a barrel depend on the cellular structure of the parent material. Oak species are best characterized by their medullary rays, diffusive channels that run radially from pith to bark to transport nutrients in growing wood (**Figure 1.2**). Staves are sawed from logs so that the medullary rays remain parallel to the inner surface of the staves. This orientation prevents diffusion across the thickness of the stave. On average, a 25-28 mm thick stave (typical for a 225-L export barrique) will have five sets of rays perpendicular to the direction of wine penetration that prevent liquid from escaping through the wood[27].

American and European oak species can be distinguished by the abundance of tyloses in the cell structure of the American species *Q. alba*. Tyloses are occlusions in the vascular structure of angiosperms that occur during heartwood formation, strongly limiting the transport of gas and fluids across vessels[60]. Functionally, this means that American oak staves can be sawn without strictly following the medullary rays, leading to more efficient utilization of the parent log and a less expensive finished product. American oak wood also has a coarser grain structure versus European oak species and imparts different sensory qualities to aging wine[61]. Barrels are frequently constructed from a single harvest and forest, but the staves for a single barrel are rarely obtained from the same log[49]. The orientation of barrel staves relative to the parent log material is shown in **Figure 1.2**.

1.3.2 Cleaning and sanitization strategies

The same properties that make oak barrels effective for storing wine make them difficult to maintain. The porous nature of wood allows both liquid and microorganisms to penetrate staves, and aging wine can leave lees and tartrate deposits that must be scrupulously removed. Lees, tartrates, and other gross soil are removed in the cleaning process. Cleaning is almost

always accomplished by some form of pressurized water rinse, with a hot water or steam cycle to aid in tartrate removal[28]. The cleaning cycle must be thorough or any subsequent attempts at sanitization will be rendered ineffective in an unclean barrel[4].

Barrel sanitization cycles aim to manage populations of yeast and bacteria inhabiting the barrel wood. Numerous studies have attempted to describe the extent of yeast and bacteria penetration into barrel staves. *B. bruxellensis* is by far the most commonly studied genera of spoilage microorganism in this context, and is variably quoted as capable of penetrating barrel wood at depths up to 6-8 mm[62], 4-8 mm[63], 8 mm[4], and 9 mm[64]. Mixed cultures of yeast and bacteria have been observed to penetrate barrel wood up to 12 mm in alcoholic cider production[65], but *S. cerevisiae* barrel penetration specifically remains unstudied. Except for a study by Cartwright *et al.*[63], reports on penetration in enological settings all suffer from the same limitation for sample material and resort to the use of 'naturally contaminated' barrels. Such barrels are subjectively identified by cellar staff as having sensory defects, but there is little consistency among the cooperage, wine aging protocol, initial cleaning methodologies, and no specific knowledge of the microbe community in the barrels[46,62]. A renewed approach to investigating cell penetration using a controlled inoculation and sampling protocol is clearly needed if subsequent barrel sanitization is to be properly assessed.

A wide range of barrel sanitization methods have been studied, again typically employing *B. bruxellensis* viability as the measure for effective sanitization. The efficacy of hot water[64], steam[66], ozone[67], high-pressure ultrasound[64], microwave technology[68], and peracetic acid[46] have all been separately studied, among other less common techniques. While the findings are somewhat mixed, steam and ozone have consistently been the most effective sanitizing tools, but require treatment times well beyond what is feasible for real-world production settings (12 and

20 minutes for steam and ozone per barrel, respectively)[28]. The reality of wine sanitization in industrial settings is that protocols put in place are effective in cleaning soil and tartrates and sanitization of the inner surface of wood but are likely insufficient to inactivate yeast and bacteria at depth in the wood. If *B. bruxellensis*-derived phenolic character is detected in barrel, winemakers will most often discard the impacted barrel to limit the risk of spreading the infection.

1.3.3 Sulfur dioxide fumigation

Sulfur dioxide is ubiquitous in modern wine production. SO_2 is commonly used as an antimicrobial and antioxidant in aqueous form to protect aging wine and is nearly universally added to empty barrels as a sanitizer and preservative in modern wineries[69]. The use of SO_2 in empty barrels has been legally written into law for more than five hundred years[70], but the exact purpose and role of SO_2 in this context seems to have shifted over time. Originally, solid sulfur (S_2) was combusted in barrels to arrest the fermentation of infill wine and retard subsequent oxidation[69]. Winemaking texts from the late 19th century document the use of solid sulfur fumigation to prevent mold growth and "souring" of empty barrels[29], however only in the late 20th century has fumigation been studied in the modern context of sanitizing barrel stave wood in addition to protecting headspace oxidation[71]. The process remains poorly understood.

Sulfur dioxide is applied to barrels in part to sanitize the barrel staves. Sulfur fumigation is normally used in conjunction with steam, ozone, or one of the other techniques described above so the actual sanitization impact of SO_2 in fumigation is not known. In a study comparing different barrel sanitation methods, Aguilar Solis[4] evaluated SO_2 fumigation alongside four other barrel sanitization methods. The results were somewhat inconclusive because the study relied on naturally contaminated barrels and several of the treated barrels were found to have tartrate

residue from incomplete cleaning steps. Furthermore, cells were recovered by adding a liquid medium to the barrel for one hour, swirling the material in the barrel, and plating thereafter. Liquid penetration into barrels is slow so this recovery method is unlikely to interact with cells below the stave surface[59]. This work stands alone as the only published data on the antimicrobial impact of SO_2 fumigation in winery cooperage. The antimicrobial effect of gaseous SO_2 on yeast embedded in the barrel wood remains unknown.

Sulfur dioxide is largely applied to barrels for protection during prolonged empty storage. Free SO_2 in the barrel headspace prevents microbial growth and the development of undesirable oxidative aromas in the barrel. SO_2 is slowly lost from the barrel headspace during storage. Sulfur can be applied from a pure gas cylinder or by the traditional method of solid sulfur combustion. Recommended protocols for SO_2 application are readily obtained from educational outreach organizations, and in some cases involve regulation and certification by governmental bodies[72]. Very few quantitative data exist to support the recommended procedures, however, which are instead based on industry best practice advice. Little is known about the initial concentration of SO_2 post-fumigation or how the concentration decreases through storage thereafter.

1.3.4 Headspace sulfur dioxide detection

In their seminal technical winemaking text, Ribéreau-Gayon and Peynaud[71] published the only quantitative data for barrel headspace SO_2 concentration throughout storage available in scientific literature. This study was excluded from both translated and later revised editions of the text and can only be found in the original, French-language version of the book[73]. The authors compared the loss of SO_2 from the headspace of barrels in four different storage conditions (hydrated or dry barrels with or without a bung closure, respectively) and concluded

that SO_2 was at the limit of quantification for hydrated, bunged barrels after 30 days. Most published guidelines quote a similar re-dose interval, so it is not unreasonable to speculate that best practice guidelines could originally stem from an influential French publication. Unfortunately, the report lacks detail about the number of replicates, experimental procedure, or detection method used to produce the data. Considering the time of publication, SO_2 detection was almost certainly accomplished by a variation of the modern aeration-oxidation titrimetric method[74]. Measurement would be accomplished by passing a measured volume of gaseous headspace sample through a peroxide trap, and then titrating the resulting acidic solution with dilute sodium hydroxide and colorimetric indicator solution[75]. Regardless of the exact method the data suffers from a high limit of quantification (interpreted as 0.1 grams per 225-L barrel based on the text). Modern SO_2 detection technology presents an opportunity to significantly improve on these outdated and unclear measurements.

A wide array of suitable techniques exists for gaseous headspace SO_2 detection. Recent research has demonstrated that headspace measurements can even be used to improve the accuracy of data obtained from titrimetric methods for aqueous samples[76]. Headspace gas chromatography (HS-GC) and colorimetric gas detection tube (GDT) technologies have been used to measure gaseous SO_2 in enological settings[77,78]. Both methods are well suited for the measurement of barrel headspace SO_2 during storage. GDT are glass tubes packed with a colorimetric indicator that changes in response to contact with analyte gas. The tubes are graduated such that the length of staining directly indicates the measured concentration after a specific volume and flow rate of sample pass through the tube. Gas chromatography is used as the analytical reference in many fields[79]. Volatile sulfur compounds have been measured in wine samples using pulsed-flame photometric detection, mass spectrometry, and atomic emission

detection, but the development of a compound-specific sulfur chemiluminescence detector (SCD) with unparalleled sensitivity has supplanted other GC systems for this purpose[80]. GC-SCD has been demonstrated as an effective tool for measuring the sulfur dioxide content of wines from headspace samples and is a promising technique for the analysis of SO_2 in barrels[81].

Electrochemical sensor technology presents an alternative to traditional enological sampling methods. Modern screen-printed sensor technology has resulted in the proliferation of compact, sensitive electrochemical gas detectors. Printed electrochemical sensors have been used to measure a wide array of parameters in wine production[82], including the sulfur dioxide content in the headspace of liquid wine samples[83], but have yet to be used for measuring gaseous sulfur dioxide in the context of winery cooperage. Sensors are capable of continuous, real-time measurement with parts-per-billion level sensitivity. Screen printed electrochemical sulfur dioxide sensors present a major opportunity for advancing SO_2 measurement in the wine industry and are especially well-suited to barrel headspace measurement.

1.3.5 Diffusive sulfur dioxide loss

Barrels are not gastight. Oxygen permeability is a desired characteristic in the use of wooden barrels for aging wine, and other gaseous species can similarly diffuse through the wood. A wide array of techniques have been used to describe oxygen diffusion into aging wine though barrel staves[59]. Perhaps unsurprisingly no such studies exist for sulfur dioxide diffusion from the barrel headspace.

One common approach to describing oxygen diffusion though barrel involves modeling barrel staves as a semipermeable membrane and invoking Fick's laws of diffusion to mathematically describe gas transport[84,85]. Fick's first law of diffusion states that the rate of gas

transport, or flux, is proportional to the concentration gradient perpendicular to the membrane surface. Mathematically this relationship is represented as:

$$J = -D\frac{\partial C}{\partial x} \quad \textbf{(Eq. 1.1)}$$

Where the flux, *J*, represents the rate of transfer per unit area, *C* is the concentration of diffusing substance, *x* is the membrane thickness, and *D* is the Fickian proportionality coefficient. For unsteady state systems, if diffusion is restricted to one direction only the unidimensional fundamental diffusion equation can be derived[86]:

$$\frac{\partial C}{\partial t} = D\,\frac{\partial^2 C}{\partial x^2} \quad \textbf{(Eq. 1.2)}$$

Discreet solutions can be obtained for these equations if the boundary conditions are carefully specified and controlled. Often this is accomplished by use of the time-lag method or steady-state approximation. The steady state method has been used to analyze oxygen diffusion in oak staves[87,88]. These publications provide a template for analyzing the mechanisms of SO_2 loss though barrel staves from headspace gas measurements.

1.4 Objectives

The research conducted as part of this thesis had two primary objectives:

1) Optimize the use of chemical cleaning and sanitizing agents against common winery spoilage microorganisms and fermentation soils for fermentor materials.

2) Investigate the antimicrobial impact and loss of headspace sulfur dioxide in barrel fumigation practices.

These objectives are addressed in the four following chapters. In **Chapter 2** a wide range of chemical cleaning and sanitizing agents were evaluated against common spoilage microorganisms in sessile and planktonic physiologies in bench-scale trials. Effective treatments

from bench trials were used to develop protocols that were evaluated using 2000-L pilot scale tanks. The minimum inhibitory concentration and minimum biocidal concentration assay was validated as a high throughput method to determine the minimum effective antimicrobial concentrations of cleaners and sanitizers in **Chapter 3**. Fluorescent viability staining was used to determine the minimum contact time required to inactivate *Saccharomyces cerevisiae* cultures using peroxyacetic acid. **Chapter 4** investigated the penetration rate and abundance of *S. cerevisiae* and *B. bruxellensis* yeasts in American oak barrel wood using scanning electron microscopy and cell culture. The dosage and persistence of sulfur dioxide in barrel headspace during extended storage was measured, and inoculated staves were used to evaluate the antimicrobial impact of barrel fumigation. In **Chapter 5** a novel electrochemical sulfur dioxide module was constructed and compared to established headspace detection methods. The sensor was used to evaluate the diffusion of sulfur dioxide through barrel staves by modeling the barrel as a semipermeable membrane according to Fick's laws of diffusion.

Cleaning and sanitization processes are essential to the success of winery operations but comprehensive data to support the development of effective protocols are currently lacking. This work seeks to provide quantitative evidence that will empower winemakers to maximize operational efficiency while minimizing chemical input, and to demonstrate the use of novel gas detection technology for describing sulfur dioxide loss from barrel headspace.

CHAPTER 2: THE EFFICACY OF CLEANING AND SANITIZING CHEMISTRIES FOR THE INACTIVATION OF COMMON WINERY SPOILAGE MICROORGANISMS AND MANAGING FERMENTATION SOILS

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2.1 Abstract

Cleaning and sanitization are essential processes in winery production settings, but limited data are available demonstrating the comparative ability of chemical cleaning and sanitizing agents in removing fermentation soil and inactivating microbial contaminants in winery relevant conditions. In this study, the performance of 25 different cleaning and sanitation chemistries were compared using fermentation derived soils and spoilage microorganisms in planktonic and sessile physiologies in trials from bench scale to 2000-L fermentation tank management. Chemical screening was conducted in microtiter plates using direct inoculation and crystal violet techniques. Adenosine triphosphate bioluminescent swabs and environmental swabs for culturable cells were used to assess contamination on stainless steel coupons at bench scale and in 200-L replicate fermentations representative of winery conditions. The most effective treatments from these trials were used to develop cleaning and sanitation protocols that were compared in the processing of 2000-L fermentation tanks. Results indicated that caustic cleaning agents are the most effective in removing fermentation soils. Sanitizing agents were demonstrated to be ineffective without prior cleaning treatments. Peracetic acid- and hydrogen peroxide-based sanitizer formulations were effective and have less residual toxicity than other tested formulations. Tank design flaws are ultimately consistent sources of contamination, regardless of the cleaning and sanitizing protocol applied.

Key words: Cleaning, sanitation, microbial populations, yeast inactivation, water use, fermentation soils

2.2 Introduction

Cleaning and sanitization are essential aspects of winemaking. Wine production is a waste-intensive process and producers must manage this waste to ensure the stability and expected quality of subsequent fermentations and wines. Unlike most food and beverage processing industries, wine is a low food safety risk product because the acidity, alcohol, and sulfite content of the medium is unsuitable for the growth of pathogenic migroorganisms[10]. Instead, wine spoilage is normally associated with faults such as off-flavors or cosmetic defaults like turbidity or sediment formation in the finished product[49]. In the context of cleaning and sanitization, microbial and physical soil loads remaining from ineffective waste management protocols are direct sources of contamination. Wine is a highly scrutinized luxury good, so the commercial and financial viability of any production directly depend on a producer's ability to thoroughly and reproducibly clean and sanitize storage vessels and equipment.

Cleaning and sanitization are fundamentally separate processes with different aims. Cleaning cycles involve the removal of gross inorganic and organic soils[9]. Sanitization specifically refers to microbial inactivation and is defined by the United States Food and Drug Administration as a process resulting in a 5-log reduction in microbial viability (99.999% inactivation)[3]. The cleaning step removes the vast majority of contamination and is unquestionably the more important process. Cleaning treatments must be capable of removing inorganic and organic contaminants adhered to vessels and equipment that remain after preliminary water rinse cycles. Physical soils remaining from incomplete cleaning cycles harbor spoilage microorganisms and render subsequent attempts at sanitization impossible[4].

Modern wineries are commonly equipped with inert, nonporous stainless steel (SS), plastic, or epoxy fermentation vessels. Wooden and unlined vessels are used to a lesser extent,

but the porous nature of these materials makes thorough cleaning and sanitization extremely difficult[28]. Cleaning and sanitization for nonporous vessels are most commonly accomplished using alternating water and chemical wash cycles. Cleaning and sanitizing chemistries are marketed separately, however the distinction is somewhat muddied as some cleaning formulas are marketed as dual cleaner-sanitizers[89].

Though only a limited range of microorganisms can survive in the wine medium, numerous species can alter or ruin the sensory properties of the product. Yeast, bacteria, and molds all present spoilage risks in wineries that must be managed[90]. Fermentative yeasts may pose the greatest risk to production. *Saccharomyces cerevisiae*, *Brettanomyces bruxellensis*, and *Zygosaccharomyces bailii* yeasts are especially prevalent in the spoilage of finished wine in modern production facilities[14]. Acetic acid bacteria (AAB) and lactic acid bacteria (LAB) families are also frequently implicated in faulted wines[26]. Yeast and bacteria normally exist in a planktonic state in wine and juice, however numerous winery-associated species have been demonstrated as capable of forming biofilms, sessile colonies of cells embedded in a polysaccharide matrix[91,92]. Biofilms have been demonstrated as having increased antimicrobial resistance versus planktonic populations and may also require additional mechanical action to remove the physical biofilm structure[20]. Effective antimicrobial applications must therefore be capable of managing spoilage microorganisms in sessile and planktonic physiologies to successfully remove the spoilage threat to industry.

Dozens of cleaning and sanitizing chemicals are available to the modern winemaker. The pH, composition, and method of action for these chemicals vary greatly and there is little standardization across application protocols. Cleaning and sanitization are water-intensive processes that create an environmental burden to treat and dispose or reuse, depending on the

chemicals used[38]. Recent focus in sustainable winery operations has brought interest in optimizing cleaning and sanitization protocols with a goal of reducing water, energy, and chemical impact[7]. Additional focus has been directed towards the substitution of more benign chemicals for traditionally used products[24,93]. In some circumstances, legislation also exists to regulate and audit waste discharge from winery facilities[94]. Despite the wide choice in cleaning and sanitizing chemistries and waste disposal pressures, little quantitative evidence exists to compare the efficacy of traditional or novel chemicals in enologically relevant settings. Winemakers typically select products based on employee and environmental health considerations and empirical evidence (i.e. lack of observed contamination with established protocols) rather than direct evidence of antimicrobial and cleaning efficacy.

A significant limitation in the study of winery cleaning and sanitization is the lack of institutional research facilities capable of reproducing wastes of the same type and scale experienced in industry production settings. Limited research involving commercial-scale facilities does exist, however these studies have relied on ‘naturally’ contaminated vessels, without specific knowledge of the microorganisms present or uniform fermentation parameters[4,43]. Other studies have focused on the antimicrobial impact of specific winery chemistries and spoilage microorganisms in bench-scale trials[11,20,48], but the list is far from comprehensive. A third group of studies have studied the antimicrobial impact of winery-relevant sanitizers using pathogenic species as target microorganisms[95,96]. Results from the latter two groups of studies are difficult to translate to the context of cleaning and sanitizing production scale winery vessels and equipment.

Sampling techniques must also be carefully chosen to correspond to industry practice. In industrial settings, incomplete or ineffective cleaning and sanitation operations are often only

noticed when the sensory qualities of a wine are negatively impacted; however, there are numerous tools that are readily available for quantitative assessment of soil and microbial load[52]. Successful cleaning and sanitizing operations are normally determined by visually assessing surfaces for residues, but this is an inherently subjective judgement and does not truly assess sanitization efficacy. Adenosine triphosphate (ATP) bioluminescence swabs and traditional environmental swabs for culturable microorganisms are the most common methods for microbiological analysis in wine production, and are accessible and inexpensive tools for wineries to adopt[43]. ATP swabs are processed in handheld luminometers that produce readings in seconds. Swabs also provide some measure of cleanliness by reacting with ATP in grape cells in organic residues in addition to ATP in the cells of spoilage microorganisms. Traditional environmental swabbing is rarely used as the sole source of data for sanitization efficacy in wineries because of the time required for cell growth and lack of speciation data beyond colony morphology, but it is a simple and inexpensive technique that can directly assess sanitization impact. Cell culture methods are more commonly used as a periodic quality check for ATP swab samples in large wineries with in-house laboratory facilities or as part of commercially available sanitization audit services. Polymerase chain reaction (PCR) based methods are also common, but require specialized equipment and operator expertise[52]. Commercial laboratories offer PCR analysis as a service, but the relatively high sample cost limits the use of PCR sampling as a regular analysis in most cases, and assays are designed to analyze wine and juice rather than rinse water. Other methods for microbial enumeration have been demonstrated for spoilage microorganisms, including the crystal violet assay and fluorescence microcopy[19,97]. The crystal violet method is especially well suited to evaluate biofilm removal. Crystal violet stains biomass

irrespective of culture viability and can be used to directly measure the physical removal of sessile cultures[98].

This research assessed the efficacy of a wide range of cleaning and sanitizing agents with varying formulations, active ingredients, and concentrations against seven yeast and bacteria species commonly implicated in winery spoilage and typical fermentation soils. An effort was made to incorporate chemical formulations with innocuous breakdown products and formulas derived from organic byproduct streams for comparison against traditional chemistries. Bench-scale trials were used to screen a wide range of common winery chemicals against sessile and planktonic cell cultures. Effective chemistries from these trials were used to create cleaning and sanitization protocols that were validated and compared in commercial-scale SS tanks. Experimental trials were designed so results readily translate to industrial settings, with the goal of empowering winemakers to develop effective and efficient cleaning and sanitization protocols using quantitative evidence.

2.3 Materials and methods

2.3.1 Cleaning and sanitizing Agents

Twenty-five different cleaning and sanitizing agent formulations commonly used in commercial wineries were assessed in this study. Chemicals were prepared fresh for each trial using 1/8X phosphate-buffered saline (PBS) from Apex Chemical Co. (Arizona, USA) as a solvent and used within 30 minutes of preparation. Caustic alkali solutions were prepared using ACS-grade sodium hydroxide and potassium hydroxide from MilliporeSigma (Darmstadt, Germany) at two concentrations, respectively. Several proprietary formulations containing modifiers such as chelating agents, surfactants, and/or detergents were used. Proprietary caustic blends based on potassium hydroxide and sodium hydroxide supplied by California Soda Co.

(California, USA) were tested at manufacturer's recommended concentrations, as well as proprietary potassium carbonate and sodium percarbonate-based blends from AiRD Chemistry (Auckland, New Zealand). An environmentally friendly alkaline cleaner from Inventek Colloidal Cleaners (New Jersey, USA) and a cleaning agent designed to specifically target biofilms from Sterilex, LLC (Maryland, USA) were also used. Sanitizer treatments were a similar mixture of proprietary and nonproprietary formulations. Peracetic acid from Arcos Organics (New Jersey, USA), hydrogen peroxide, potassium bisulfate, and citric acid from MilliporeSigma (Massachusetts, USA), and chlorine dioxide from Selective Micro Technologies, LLC (Ohio, USA) were used as sanitizing treatments. A proprietary quaternary ammonium blend from ChemStation International, Inc. (Ohio, USA) and an iodine-based sanitizer from National Chemicals, Inc. (Minnesota, USA) were also tested. Treatment chemistries and concentrations are detailed in **Table 2.1**. Letter codes are included to aid the presentation of experimental results and to anonymize proprietary products used in trials. As trials increased in scale, a reduced subset of effective treatments from previous trials were used to facilitate experimentation. **Table 2.2** and **Table 2.3** detail the chemicals and protocols used for each trial in the subsequent sections.

2.3.2 Preparation of cell cultures

Saccharomyces cerevisiae, Brettanomyces bruxellensis, Zygosaccharomyces bailii, Acetobacter pasteurianus, Pediococcus parvulus, Lactobacillus casei, and Oenococcus oeni cultures were obtained from the University of California, Davis Viticulture and Enology Culture Collection.

Organism specific broth and agar from BD Biosciences (New Jersey, USA) were used for cell culture where indicated. YM nutrient was used for all yeast species. MRS nutrient was used

at 50% concentration for *P. parvulus, A. pasteurianus, L. casei* and supplemented with tomato broth from Campbell Soup Co. (New Jersey, USA) for culturing *O. oeni*. A sterile 50% grape juice medium was prepared for use as growth medium. Chardonnay juice was obtained from the UC Davis Teaching and Research Winery facility from fruit harvested in 2017 from the UC Davis Russel Ranch Vineyard. The juice was centrifuged at 5000 rpm for 10 minutes, and the supernatant was vacuum filtered to 0.22μM using a disposable bottle-top filter from Corning Inc. (New York, USA) into pre-sterilized containers for use. The grape juice was then diluted to 50% using sterile 1/8X PBS.

2.3.3 Chemical screening

An inoculum was prepared from isolated colonies and incubated in grape juice medium at 30°C for 1-5 days, depending on the microorganism growth rate by species. The cell suspensions were centrifuged and resuspended to an optical density of 0.5 at OD_{580} nm in grape juice medium. 96-well Nunc Microwell microtiter test plates from Thermo Scientific Inc. (Massachusetts, USA) were filled with 180 μL of inoculum and 20 μL of the treatment chemical. Eight replicates per treatment were performed. Treatments were conducted at five-minute intervals from five to 30 minutes, for a total of six contact times per organism and treatment chemistry. Samples were collected for viability by rinsing and resuspending wells in 1/8X PBS. The suspension was sampled onto appropriate agar plates and incubated at 30°C for up to 10 days to facilitate growth of fastidious cultures.

2.3.4 Biofilms by crystal violet assay

Inoculum were prepared as described above to an optical density of 0.2 at OD_{580nm}. 96-well plates were prepared by adding 150 μL of inoculum in the first six rows of the plate and

Table 2.1. Cleaning and sanitizing agents used. Ingredients in proprietary formulations are included when disclosed by producer.

	Cleaning Agents
A	10 g/L Potassium hydroxide [KOH]
B	20 g/L Potassium hydroxide [KOH]
C	20 g/L Sodium hydroxide [NaOH]
D	20 g/L Proprietary potassium caustic blend (85-90% potassium hydroxide [KOH])
E	20 g/L Proprietary sodium caustic blend (40-45% sodium hydroxide [NaOH])
F	170 g/L Proprietary cleaner blend (6% sodium carbonate [Na_2CO_3], 6% potassium carbonate [K_2CO_3], 4.9% ethylenediaminetetraacetic acid [$C_{10}H_{16}N_2O_8$], 6% alkyl dimethyl alkyl benzyl ammonium chlorides, 6.3% hydrogen peroxide [H_2O_2])
G	10 g/L Proprietary sodium alkaline detergent (sodium percarbonate [$C_2H_6Na_4O_{12}$]-based blend)
H	20 g/L Proprietary potassium alkaline detergent (potassium carbonate [K_2CO_3]-based blend)
I	17.5 g/L Proprietary alkaline bio-cleaner (blend of coconut fatty acid, coconut oil, palm oil, sodium benzoate [$C_7H_5NaO_2$], and organic silicone)
	Sanitizing Agents
J	0.1 g/L Peracetic acid [$C_2H_4O_3$] + 10 g/L hydrogen peroxide [H_2O_2]
K	0.2 g/L Peracetic acid [$C_2H_4O_3$]
L	0.1 g/L Peracetic acid [$C_2H_4O_3$]
M	2.7 g/L Potassium bisulfate [$KHSO_4$] + 10 g/L hydrogen peroxide [H_2O_2]
N	5.4 g/L Potassium bisulfate [$KHSO_4$] + 10 g/L hydrogen peroxide [H_2O_2]
O	10 g/L Hydrogen peroxide [H_2O_2]
P	2.0 g/L Proprietary quaternary ammonium blend (10% alkyl dimethyl alkyl benzyl ammonium chlorides)
Q	0.1 g/L Chlorine dioxide [ClO_2]
R	0.01 g/L Chlorine Dioxide [ClO_2]
S	0.005 g/L Chlorine Dioxide [ClO_2]
T	1.4 g/L Potassium bisulfate [$KHSO_4$]
U	2.7 g/L Potassium bisulfate [$KHSO_4$]
V	2.7 g/L Potassium bisulfate [$KHSO_4$] + 20 g/L citric acid [$C_6H_8O_7$]
W	5.4 g/L Potassium bisulfate [$KHSO_4$]
X	20 g/L Citric acid [$C_6H_8O_7$]
Y	1.5 g/L Proprietary iodine-based sanitizer (≤ 5% iodine [I_2], ≤ 5% hydroiodic acid [HI])

Table 2.2. Cleaning and sanitizing agents used by trial for bench scale and 200-L trials.

Trial	Cleaning Agents	Sanitizing Agents	Contact Time (min)
Chemical screening	All treatments (**A**-**I**)	All treatments (**J**-**Y**)	5, 10, 15, 20, 25, 30
Biofilms by crystal violet assay	All treatments (**A**-**I**)	All treatments (**J**-**Y**)	5, 10, 15, 20, 25, 30
Biofilm growth on SS coupons	**A-F**	**J, K, M, N, O**	5, 10
200-L trials	**A, C, D, E, F**	**J, K, L, O**	5, 10

incubated at 30°C for 10 days to form adherent colonies. The remaining two rows were left uninoculated as treatment blanks. Plates were then washed to remove growth medium and loosely adhered or suspended colonies. Two hundred microliters of treatment chemical was added per well at the concentrations indicated in **Table 2.1**. After a given contact time the plates were with 1/8X PBS to remove chemical residues and dislodged cells from the wells.

Crystal violet was added to each well and incubated for 30 minutes at 21°C. Crystal violet was removed from the wells by washing three times with 1/8X PBS. After drying for two hours, 200 µL of Koptek 95% ethanol from Decon Labs Inc. (Pennsylvania, USA) was added to each well and allowed to sit for five minutes to release residual dye from the colonies. Each well was pipetted to a corresponding well on a separate microtiter plate. The absorbance of the plate containing the transferred solution was read at 600 nm using a Synergy 2 microplate reader from BioTek Instruments (Vermont, USA).

2.3.5 Biofilm growth on SS coupons

Autoclavable Rainin pipette-tip refill boxes from Mettler Toledo Inc. (California, USA) were used to suspend eight 304-stainless steel coupons (3.25 cm * 8.125 cm) mounted vertically and evenly spaced without contact. To develop adherent cells on the coupons, 325 mL of inoculum was added to each box and placed in an incubator/shaker for 10 days at 30°C and 110 rpm. All seven test species were used separately in this trial, and an additional mixture of the

four bacteria in co-culture was tested. Prior to chemical treatment the boxes were emptied and rinsed a minimum of three times with sterile 1/8X PBS until no visible soil remained on the surface of the coupons. The coupons were transferred to pre-sterilized boxes for treatment.

Three hundred twenty-five milliliters of each respective cleaning or sanitizing agent was added to per box and allowed to soak for contact times of five and 10 minutes. The boxes were rinsed three times with sterile 1/8X PBS. Data were collected by swabbing both sides of the coupons either with an Ultrasnap ATP swab from Hygenia, LLC (California, USA) or flocked polyester swab from Puritan Medical Products Co. (Maine, USA). Four replicates were used for each swab type. ATP swabs were processed using a handheld ATP luminescence meter. Flocked swabs were processed by breaking the swab tip and adding to sterilized 2 mL microcentrifuge tubes filled with 1mL 1/8X PBS. The microcentrifuge tubes were vortexed to release cells from the flocks and plated on the appropriate media to develop cells.

2.3.6 200-L fermentation trials

The ability of cleaning and sanitizing agents to effectively remove fermentation soil and inactivate microorganisms when used in succession was assessed in four successive Cabernet Sauvignon fermentation trials. Grapes were harvested from the UC Davis Robert Mondavi Institute vineyard and were destemmed and crushed using a Delta E2 machine from Bucher Vaslin (California, USA) directly into 200-L fermentation vessels from Cypress Semiconductor Corporation (California, USA). One hundred fifty kilograms of grapes were used for each of the four fermentation replicates. The starting Brix of the must was measured using a handheld density meter from Anton Paar (Graz, Austria). Must was adjusted to a starting Brix of 25° Brix by adding sucrose from C&H Sugar (California, USA) if lower, or by adding winery process water if above. Titratable acidity was adjusted to at least 6.0 g/L with tartaric acid from Enartis

(California, USA). Must were inoculated with *S. cerevisiae* EC1118 yeast from Lallemand Inc. Quebec, Canada) according to manufacturer's specifications. One hundred and ten stainless steel coupons were suspended in the cap of the replicate fermentations using a custom-constructed holder (**Figure 2.1**). Wines were fermented to dryness, after which the coupons were extracted and added to sterilized pipette tip boxes for treatment. Coupons were treated by soaking in the test chemicals and rinsed with 1/8X PBS.

A screening of the cleaning agents alone was conducted using a reduced subset of chemicals shown to be effective in the previous trials for the first 200-L fermentation. Using a subset of the most effective cleaning agents from this screening fermentation, cleaning and sanitizing agents were tested in combination across triplicate fermentations. Each cleaner and sanitizer combination was performed with four replicate coupons for ATP and culturable cell swabs, respectively. Treatments for the screening fermentation and subsequent cleaner and sanitizer combination fermentations are detailed in **Table 2.2**.

Figure 2.1. Custom stainless-steel coupon holder with some coupons inserted. The height of the apparatus can be adjusted to latch onto the outside of fermentor and position coupons in the cap of the fermentation.

2.3.7 2000-L fermentation trials

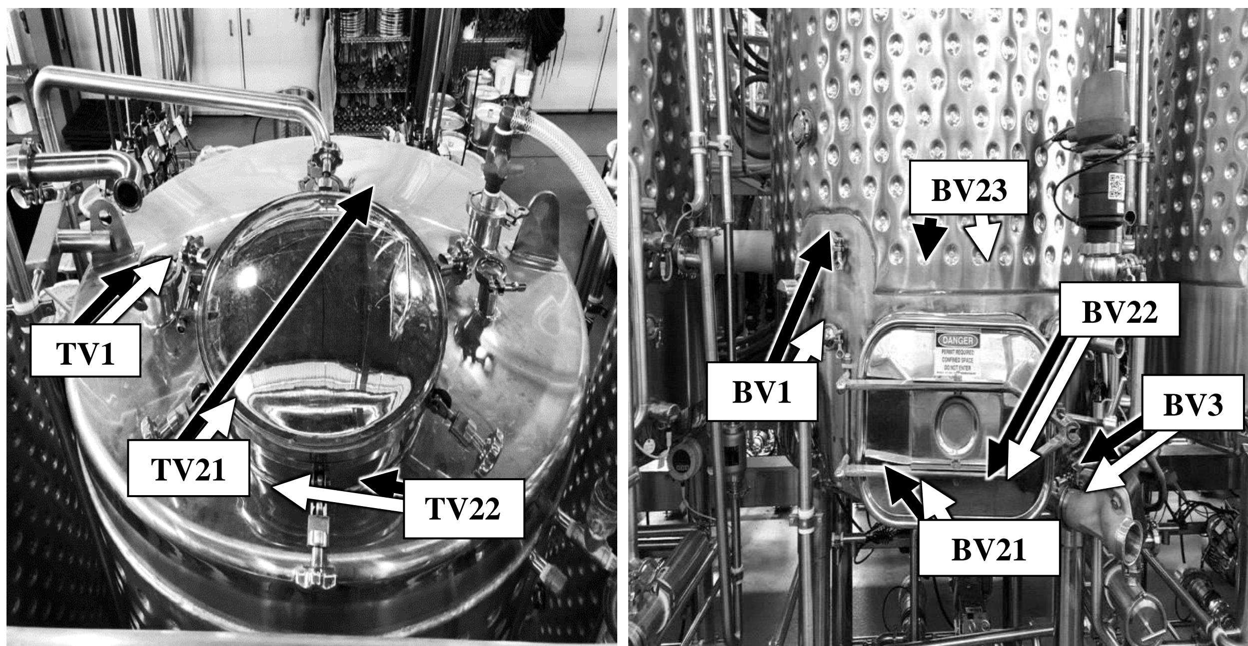
Ten successive cleaning and sanitation protocols were performed on 2000-L fermentation tanks after storing and draining finished wine. Five different cleaning and sanitizing protocols were tested in duplicate (**Table 2.3**). Four protocols were developed using the most effective cleaning and sanitizing treatments from 200-L trials by varying the contact time and application method. Sanitizers were applied either using a spray ball attached to the top of the tank or by using a fogger device from California Soda Company (California, USA) that creates a fine particulate mist of sanitizer to assess the effect of application method. The fifth protocol was developed to intentionally include a cleaner chemical and a sanitizer chemical that were less effective in earlier trials. This protocol was used to assess whether bench scale efficacy results translate to production winery configurations. Data were acquired using ATP swab and flocked swab samples collected from eight locations on the tank interior (**Figure 2.2**), as well as winery process water and ambient air blanks. An area equal to the size of the coupons used in the screening tests was swabbed and the samples were processed similarly. To remove sampling bias, staff performing the cleaning and sanitizing protocol remained unaware of sample location and frequency for the duration of the experiment. A mixture of red and white fermentation soils was used.

Table 2.3. Cleaning and Sanitizing protocols for 2000-L fermentor trials.

Cleaner	Cleaner contact time (min)	Sanitizer	Sanitizer application
D	5	K	Tank spray arm, 5 minutes contact
D	5	K	Fogger, 10 minutes contact
D	10	K	Tank spray arm, 10 minutes contact
H	5	L	Tank spray arm, 5 minutes contact

2.3.8 Statistical analysis

Multi-factor ANOVA with Tukey's HSD post-hoc test was performed on all data sets using R statistical software (Vienna, Austria)[99]. Significance is determined at $P < 0.05$.



Sample code	Description
TV1	Top tri-clamp accessory connection
TV21	Neck of top round manway
TV22	Tank ceiling
BV1	Upper (ATP) and lower (Culture swab) tri-clamp liquid transfer valves
BV21	Front rectangular manway gasket
BV22	Neck of front rectangular manway
BV23	Tank wall above front rectangular manway
BV3	Upper surface of diaphragm transfer valve
Process Water	Winery hose water
Ambient Air blank	Swab held inside tank without contacting surfaces

Figure 2.2. ATP and plate count swab locations. Black arrows indicate ATP swab samples. White arrows indicate environmental swab samples. Swabs are collected from tank interior.

2.4 Results

2.4.1 Chemical screening

To determine the antimicrobial impact of cleaning and sanitizing agents on planktonic cultures of yeast and bacteria, cell suspensions were exposed to treatment chemistries for contact

Table 2.3. Summary statistics for significant factors in experimental trials, as determined by ANOVA analysis. Significance is indicated as * = p < 0.05; ** = p < 0.01; *** = p < 0.001.

Variable	F value (degrees of freedom, residuals)	Significance
	Chemical screening	
Chemical Treatment	F(25, 994) = 2686.7	***
Organism	F(6,994) = 337.1	***
Interaction	F(150, 994) = 111.4	***
	Biofilms by crystal violet assay	
Uninoculated vs. Inoculated Wells	F(1, 6910) = 1526	***
	Biofilm growth on SS coupons	
Organism (ATP)	F(3,36) = 45.731	***
Chemical Treatment (ATP)	F(11,36) = 36.518	***
Chemical Treatment (Plate Count)	F(11,36) = 13.60	***
	200-L fermentation trials (screening)	
Chemical Treatment (ATP)	F(5,37) = 235.691	***
Chemical Treatment (Plate Count)	F(5, 36) = 23.059	***
	2000-L fermentation trials	
Sample Class	F(2,75) = 3.481	*

times ranging from five to 30 minutes and sampled onto agar plates to assess culture viability. Plate count data were grouped according to the chemical treatment, contact time, and organism tested. Statistical analysis indicated that the chemical treatment and organism variables were statistically significant (**Table 2.3**). Post Hoc analysis using Tukey's HSD test was performed separately for the organism and chemical treatment variables to identify potential clustering between the variables. The organism variable was not significant according to Tukey's test; however, the chemical treatment variable grouped cleaning and sanitizing agents into four significant groups, shown in **Figure 2.3**. Results indicated that group **a** treatments were effective in eliminating cell culturability for every combination of microorganism and contact time tested. Most of the group **a** treatments are marketed as cleaning agents. Caustic sodium and potassium chemicals (treatments **A**-**E**) and the proprietary blend of sodium and potassium carbonates and quaternary ammonium compounds (treatment **F**) are designed to be applied during the cleaning cycle in practical settings but performed as effective sanitizers under the conditions tested. Of the chemical treatments marketed as sanitizers, only a mixture of peracetic acid and hydrogen

peroxide (treatment **J**) was similarly effective. Group **ab** (treatment **K**) and group **b** (treatment **L**-**O**) sanitizers effectively eliminated cell culturability in roughly 75% of the microorganisms and contact times tested. These treatments all contain either peracetic acid or hydrogen peroxide in the formula. Group **c** treatments were not effective sanitizers. Many of the group **c** treatments were unable to eliminate cell culturability in any of the microorganisms or contact times tested (treatments **S-Y**). This group includes all of the potassium bisulfite concentrations assessed that were used in combination with hydrogen peroxide, the lowest concentration of chlorine dioxide, citric acid, and the iodine-based halogenated sanitizer. More concentrated applications of chlorine dioxide (treatments **P**-**R**) were only marginally more effective.

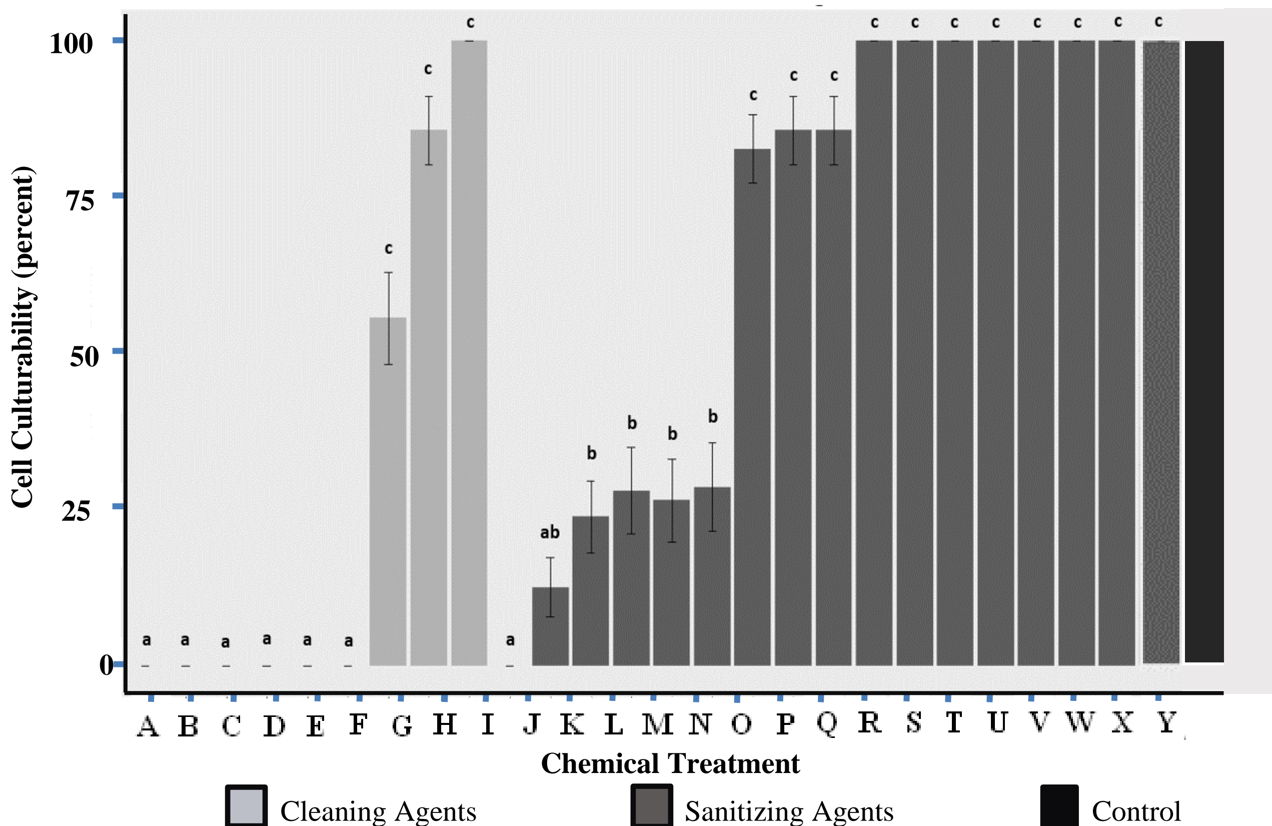


Figure 2.3. Results from chemical screening trial for planktonic cell cultures. Height of bars represents percentage of cells with growth (failed treatment). Letter codes **a, b**, and **c** indicate clustering based on Tukey's HSD post-hoc analysis. Error bars represent standard error of the mean (n = 8).

Group **c** cleaning agents (treatments **G-I**) did not perform well as sanitizers but are not normally marketed or intended to be used for this specific purpose.

2.4.2 Biofilms by crystal violet assay

Crystal violet staining was used to assess the performance of cleaning and sanitizing agents in removing sessile populations of yeast and bacteria from microwell plates. All seven microbial species assessed in the 96-well plate chemical screening experiment were included in the crystal violet assay, however none of the bacteria were able to form coherent biofilms in the well plates based on a lack of difference in absorbance between the uninoculated and inoculated control wells. All three yeast species produced significant differences in absorbance between control and inoculated wells, suggesting that the yeast species tested could form adherent

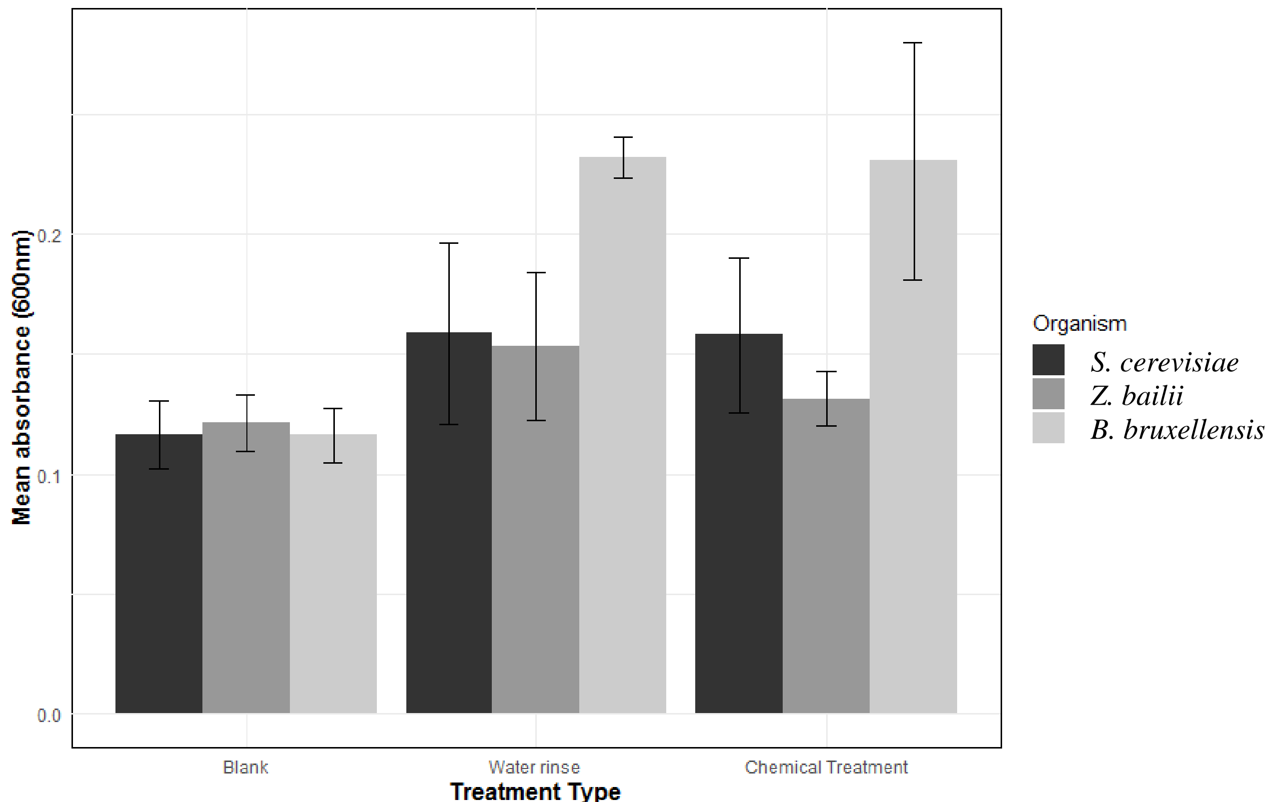


Figure 2.4. Mean absorbance values for yeast species by treatment type for crystal violet analysis.

colonies (**Table 2.3**). For all treatments, there was no significant difference between the cleaning and sanitizing agent treatments and the water rinse control treatment. **Figure 2.4** shows the mean absorbance values for the three yeast species tested for uninoculated, inoculated, and chemically treated biofilms. While the difference between blank wells and inoculated wells is readily apparent, water rinse control wells and chemically treated wells resulted in nearly identical absorbance values. The lack of significance between the cleaning and sanitizing agents and the water control rinse suggests that the chemicals were unable to successfully dissolve or loosen the adherent colonies from the well plate under the test conditions. Because none of the treatments were capable of significantly reducing biomass in the wells versus the water rinse control, the crystal violet data was not used to eliminate treatments from future testing. Instead, group **a, ab,** and **b** treatments from the chemical screening trial data (*Section 2.4.1*) were used for subsequent trials.

2.4.3 Biofilm growth on SS coupons

To assess the ability of cleaning and sanitizing agents to dislodge and inactivate yeast and bacteria cultures on SS surfaces, ATP and environmental culture swabs were collected from treated SS coupons that were previously soaked in inoculum to develop adherent colonies. ATP and environmental swab data for the treatment of stainless-steel coupons that were soaked in pipette tip boxes were assessed separately. For the ATP count data, the organism and chemical treatment variables were significantly different, but the contact time was not (**Table 2.3**). ATP swab data are displayed in **Figure 2.5**. This graph emphasizes the clear difference in the efficacy of cleaning chemicals (treatments **A-F**) versus sanitizing chemicals (treatments **G-K**) in treating soiled stainless-steel coupons. Cleaning agents were far superior in reducing the ATP load on the inoculated steel coupons than sanitizers. The significance of the organism variable in the ATP

data is most likely attributed to the inclusion of the bacteria mixture, which produced a far smaller ATP load on the SS coupons versus the yeast species across treated and control coupons. For the plate count data, only the chemical treatment variable was significant (**Table 2.3**). Plate count data support ATP results. Cleaning agents were superior in reducing microbial viability on SS coupons versus sanitizing agents. No cells were cultured from coupons treated with a cleaning agent, yet every sanitizing agent tested resulted in at least one species with culturable cells post-treatment (**Supplementary Figure 2.S.1**). As in earlier trials, the dual cleaning and sanitizing capability of the caustic cleaning chemicals (treatments **A**-**E**) and the proprietary carbonate and quaternary ammonium blend (treatment **F**) outperformed any of the sanitizing chemistries tested.

2.4.4 200-L fermentation trials

To assess the ability of cleaning and sanitizing agents to manage red wine fermentation soils, a custom SS coupon holder was constructed to suspend replicate coupons in the cap of 200-L fermentors during fermentation. Coupons were extracted and swabbed using ATP swabs and environmental culture swabbing after chemical treatment as previously described. To ensure the four fermentations were equally soiled, blank swabs were collected during each fermentation replicate and compared in a one-way ANOVA. The replicate factor was not significant so fermentation trials could reasonably be compared against one another. In the screening trial the cleaning agent variable was highly significant for both ATP and plate count data (**Table 2.3**). Both data sets suggest that the choice of cleaning agent is the primary driver of reducing fermentation soil. The importance of selecting an effective cleaning agent is readily apparent

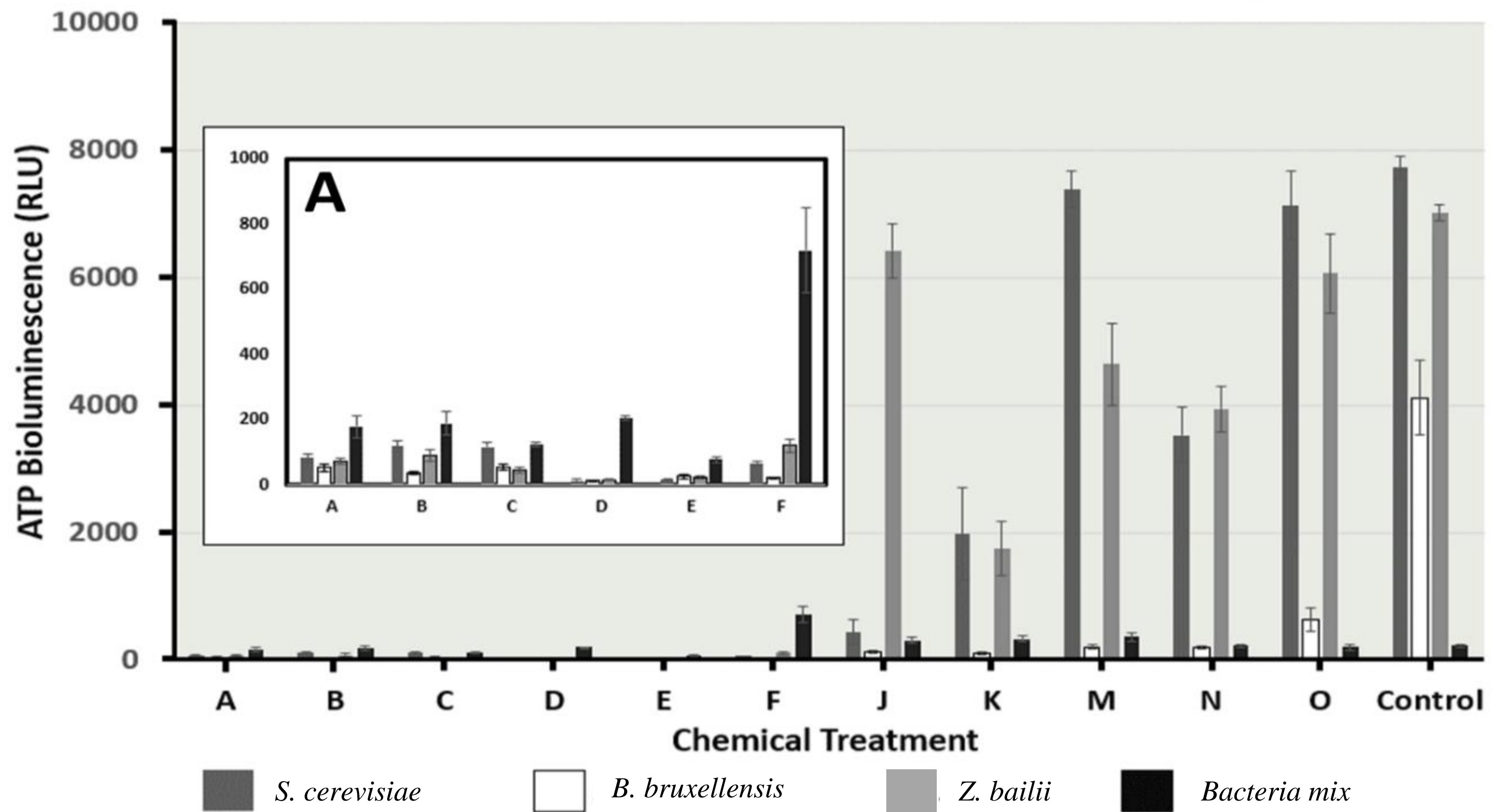


Figure 2.5. ATP swab results for treatment of biofilms grown on SS coupons. **A.** close-up of cleaning agent ATP counts to aid in visualizing results (n = 4).

when the ATP and plate count data are graphed together (**Figure 2.6**). Of the cleaning chemicals tested, proprietary caustic treatments (**D** and **E)** resulted in the lowest ATP values for treated coupons. The proprietary potassium caustic cleaner (treatment **D**) also significantly reduced cell culturability on treated coupons versus other cleaners, except for the proprietary carbonate and quaternary ammonium blend (treatment **F**). While treatment **F** successfully eliminated cell culturability for all treated coupons, this cleaner resulted in the highest ATP load of the chemicals tested. As a result, priority was given to ATP reduction as a measure of cleaning efficacy. Treatment **D** and treatment **E** were used as cleaning agents for the subsequent fermentations. For the remainder of the trials the main effect of cleaning agent, cleaning agent contact time, sanitizing agent, and sanitizing agent contact time were all assessed versus the ATP and plate count results. None of the variables were significant, implying that all the tested protocols were roughly equal in their ability to reduce soil levels and cell culturability. Since the

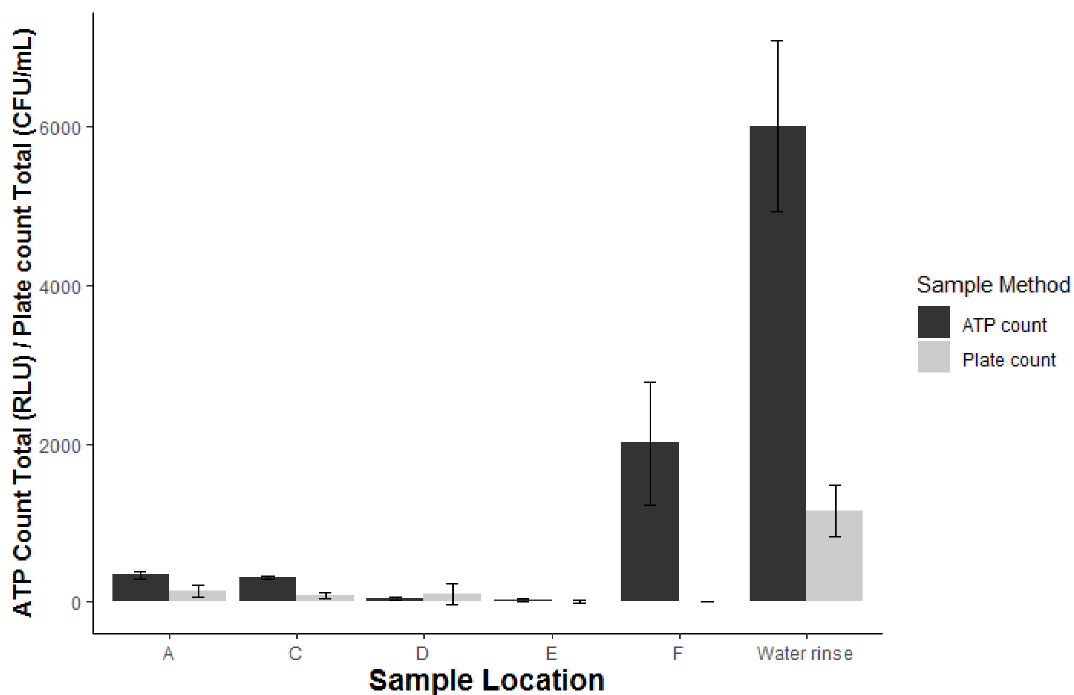


Figure 2.6. ATP and plate count data for 200-L fermentor screening trial by cleaner treatment (n = 8).

proprietary potassium caustic cleaner (treatment **D**) was the top performing cleaner in each of the trials, this was selected as the basis for protocols in trials using 2000-L fermentors.

2.4.5 2000-L fermentation trials

To assess the ability of cleaning and sanitizing protocols to manage fermentation soils and microorganisms in practical winery settings, 2000-L fermentors were swabbed with ATP and environmental culture swabs after winery staff performed cleaning and sanitizing operations using effective chemistries from the previous trials. Five combined cleaning and sanitizing protocols were assessed for the processing of 2000-L fermentors in duplicate. Four protocols were designed using the proprietary potassium caustic cleaner (treatment **D**). Peracetic acid was chosen as a sanitizer at a rate of 200 mg/L (treatment **K)** based on performance in earlier trials. A less performant potassium carbonate cleaner (treatment **H**) and lower concentration of peracetic acid (treatment **L**) were used to compare chemicals that were less effective in earlier trials at this large scale. ATP count and plate count swab data for the cleaning and sanitizing of 2000-L fermentors were grouped by fermentation replicate, cleaning regimen, and sample location. An additional sample class factor was added, which grouped sample locations broadly by position on the fermentation tank (top half of tank, bottom half of tank, and winery process water and ambient air blanks). For the ATP count data sample class was the only variable statistically significant, however in the case of the plate count data no variables were statistically significant (**Table 2.3**). The significance of sample class implies that the soil levels remaining in tank after cleaning and sanitizing operations is related to the location on the tank rather than the cleaning and sanitizing chemicals or contact time, with the valves and gaskets on the bottom of the tank possessing substantial culturable cell populations and ATP loads after treatment regardless of the protocol applied (**Figure 2.7**). This implies that the greatest gains in process efficiency may be

gained in giving additional attention and mechanical scrubbing action to areas with consistent contamination, especially if an effective cleaning and sanitization chemical regime is already followed.

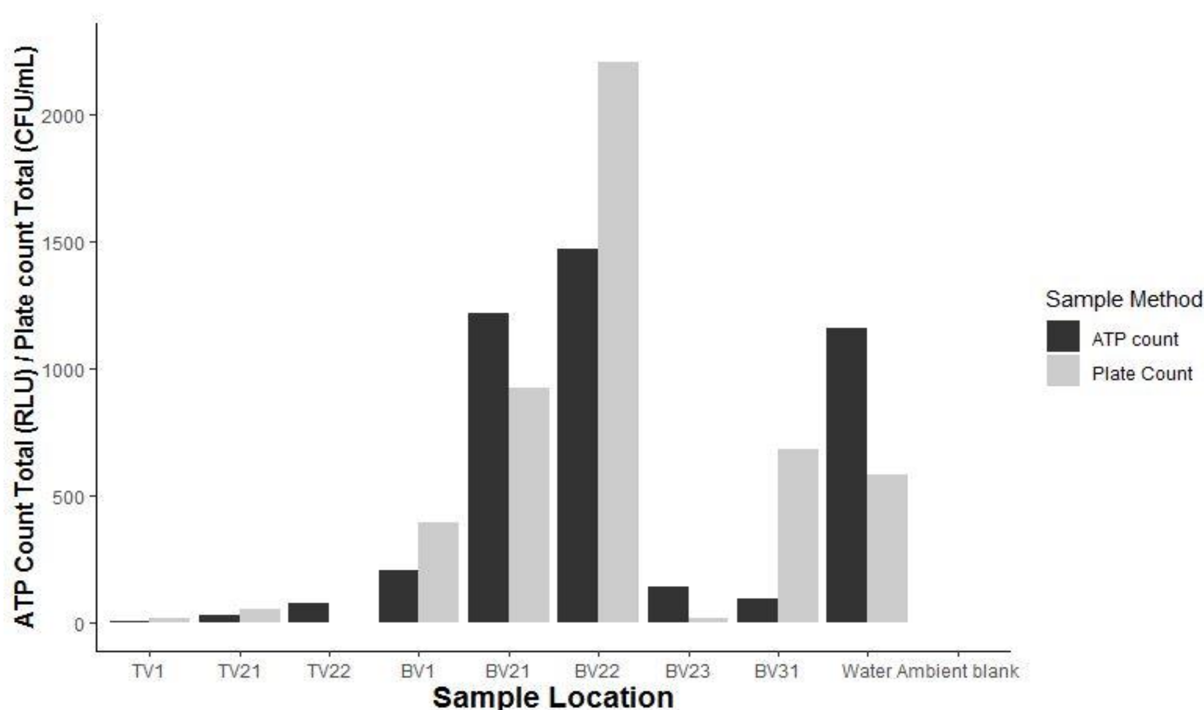


Figure 2.7. Total ATP and plate counts for 2000-L fermentor trials by sample location for all protocols (n = 10).

2.5 Discussion

The chemical screening trial proved to be a high-throughput method for reducing the number of chemical treatments that were investigated in subsequent experiments. Data analysis for this trial clearly suggest that the chemical agent was the driver of whether culturable cells were recovered after treatment, rather than contact time or the microbial species tested. Regarding the differences in the efficacy of individual cleaning and sanitation chemicals, statistical analysis clearly indicated which chemicals were effective in reducing microbial populations (group **a**, **ab**, and **b**, **Figure 2.3**), versus those that were ineffective (group **c**). Because cell culturability was used as the metric for screening the chemicals, this screening was

a test for sanitizing rather than cleaning action. Given this, it is interesting to observe that chemicals marketed and used cleaning agents were generally more successful at inactivating microorganisms than the products marketed specifically as sanitizers that were tested. As this test was truly a measure of sanitization, only products marketed as sanitizers were eliminated from subsequent trials based on their lack of efficacy (group **c** treatments **P-Y**). In practical settings, sanitizing agents are used in succession with cleaning treatments. Cleaning chemicals can clearly reduce microbial viability, so the increased pressure of successive chemical treatment may be sufficient to manage spoilage organisms with sanitizing treatments that were ineffective alone. Winemakers choosing to use these treatments should validate protocols using microbial sampling methods to ensure the complete cleaning and sanitizing protocol successfully reduces spoilage microbe viability.

Biofilm analysis via the crystal violet assay is a rapid and well-established technique for staining biomass remaining in well plates after chemical treatment[100]. In this study, the staining method was able to clearly differentiate between wells with biomass and control treatments for the yeast species. Commercial wine yeast have been documented forming biofilms on plastic materials[91], however bacteria species have only been documented as forming biofilms under enological conditions on barrel wood, and not in microtiter plates[92]. Thus, the lack of observable colony development for bacteria in this experiment may be caused by an inability to adhere to plastic microtiter plates under the conditions of this experiment rather than an inability to form sessile communities. The consistently higher dye retention in the inoculated wells for the yeast species indicated that these organisms were able to produce adherent colonies in microtiter plates under the conditions tested, however statistical analysis of the absorbance data indicated that the crystal violet method was unable to significantly differentiate between cleaning and sanitizing

treatments and water rinse control treatments. The cleaning and sanitation agents may have simply been unable to remove the biofilms from the well plates after 30 minutes contact time. As there is relatively little mechanical agitation within wells due to the capillary forces on such a small quantity of liquid, the cleaner treatment may need to dissolve the biomass rather than only lifting the soil from the surface to be effective under these experimental conditions. Since the crystal violet method applied here was unable to distinguish between viable and inactivated cells, cells may have been successfully inactivated despite biomass remaining[101]. The inability to determine whether the stained biomass is viable or inactive also means that this test was a measure of cleaning ability rather than sanitizing, opposite to the planktonic chemical screening trial. As a result, sanitizers were not eliminated from subsequent tests based on their efficacy in the crystal violet readings. Caustic cleaner treatments (treatments **A-E**, table **2.1**) produced the lowest average absorbance values for all three yeast species so these treatments were the focus of subsequent trials as they were the most efficient in removing biofilm soils and also had significant sanitizing performance evidenced in the planktonic chemical screening trials.

ATP bioluminescence swabbing and environmental swabbing for viable colonies of microorganisms are complementary methods employed in wineries for the assessment of effective cleaning and sanitation operations. In the biofilm growth on stainless-steel coupon trials the value of the swabbing techniques was clearly demonstrated. In this experiment, adherent microbial communities were developed on the stainless-steel coupons using Chardonnay juice, and any residues from the fermentation were rinsed with sterile water to a visually clean state. This is an important consideration, as visual cleanliness is often the only check performed after cleaning operations prior to proceeding with a sanitation step[102]. Both the ATP and plate count data showed a marked difference in cleaning versus sanitizing agents in eliminating viable

colonies and fermentation soil (**Figure 2.5**). Categorically, the cleaning agents performed in a superior fashion. These results show the importance and necessity of a cleaning step, even in the situation of a visually clean surface. If a sanitation step is directly applied without prior cleaning there is a real risk of developing endemic biofilms of the winery surfaces.

The inclusion of grape skins in the maceration of red wine adds a substantial soil load that must be cleaned post fermentation. In modern wineries cleaning and sanitizing operations are normally developed around a basic five-step protocol involving separate cleaning and sanitizing cycles sandwiched by water rinses[49]. This five-step process was incorporated in this study as the basis for testing cleaning and sanitizing agents in combination. In this scheme, the first water rinse removes gross soil and ensures that the cleaner does not react with heavy soils and lose cleaning power. The cleaning cycle dissolves or loosens soil, which is then removed by the second water rinse. The second water rinse is often acidified if an alkaline cleaner is used to neutralize the surfaces being treated prior to applying a sanitizer. The sanitizing cycle is used to inactivate any remaining microorganisms. This cycle is most often followed by a final water rinse to remove residual sanitizer, although some formulations may have innocuous breakdown products and not require a water rinse.

By building a custom device to mount stainless steel coupons in the cap of successive red wine fermentations, the soil and microbial load on fermentation tanks could be closely replicated. All of the cleaners in the screening were able to significantly decrease the amount of soils/microbial load versus water rinse controls (**Figure 2.6**). Proprietary caustic cleaners (treatments **D** and **E**) produced the greatest overall reduction in ATP load and microbial viability and were selected for subsequent trials. These chemicals were tested in succession with four sanitizing agents (**Table 2.2**) and the different cleaner and sanitizer combinations resulted in

similar levels of cleanliness and sanitation evidenced by a lack of significance in any of the variables tested. The lack of difference in the performance of the protocols tested may have been a consequence of the application method. The SS coupons were soaked in the test chemicals with no mechanical rinsing force. In practical settings rinsing apparatuses such as jets, sprinklers, and hoses are used to aid in physically lifting soils. Many cleaners are specifically formulated to improve the performance of pressurized rinsing operations, which may not be reflected in the soaking method used in this trial.

Trials at the 2000-L fermentor scale indicated that the location in the winery tank plays a more significant role in the ATP and plate count data than the cleaning and sanitation protocol applied (**Figure 2.7**). The most stringent cleaning regimen (10 minutes contact with 20 mg/L potassium caustic-based formula (treatment **D**) and 200 mg/L peracetic acid (treatment **K)**, respectively) produced similar results as the least chemical and time-intensive treatment (five minutes contact with 20 mg/L potassium carbonate cleaner (treatment **H**) and 100 mg/L peracetic acid (treatment **L**), respectively). This is an important takeaway for winemakers. Consistent contamination occurred on gaskets, valves, and other areas outside of direct influence of mechanical agitation from a spray arm mounted at the top of the tank. These problem areas must be the focus of manual cleaning and sanitation efforts. Automated chemical applications can result in consistent contamination in areas that are not mechanically impacted by the chemical stream or in areas missed altogether by the rinsing apparatus.

With the current focus on sustainability, a new generation of cleaning and sanitizing agents has appeared on the marketplace. Some of these chemicals are based on formulas that break down to yield innocuous compounds and others are made from organic constituents that biodegrade. In this experiment peracetic acid and combinations of peracetic acid and hydrogen

peroxide were generally the most effective sanitizing treatments. Both chemistries break down to innocuous products and are part of the movement towards more environmentally friendly practices. Their usefulness was demonstrated here, as these treatments performed better than the halogenated compounds, citric acid, and quaternary ammonium compounds in trials. Citric acid is not regularly used alone as a sanitizer, but instead as a rinse to remove and neutralize alkaline cleaner residue before the sanitization cycle. The results of the screening trial clearly demonstrated that citric acid was not an effective sanitizer and must be followed by a separate sanitization cycle.

Results from the 2000-L fermentation trials can also be interpreted in terms of sustainable operations. With a focus on saving chemicals, time, and water, it is noteworthy that the location on the tank played a more significant role in cleanliness than the cleaning/sanitation protocol applied. By reducing rinse cycle time and possibly even chemical concentration, and instead focusing worker attention on manually scrubbing areas in the shadow of spraying apparatus, savings could occur in all three of these factors. Further study using a revised protocol would shed light on possible improvements in method when using any cleaner and sanitizer combination. These types of savings can be achieved by designing cleaning and sanitation protocols as part of a broader Hazard Analysis and Critical Control Points (HACCP) system or International Standards Organization (ISO) 9000 family of certifications, established approaches to maintaining quality in the winery environment[103]. While much attention has been paid to winery wastewater valorization and reuse, ultimately reducing winery wastewater generation is the most efficient means of reduction and could be achieved by better designed cleaning and sanitizing processes[38].

2.6 Conclusion

This research investigated the efficacy of a wide range of cleaning and sanitizing agents for removing fermentation soil and inactivating spoilage microorganisms. Caustic based cleaning agents were superior in achieving both soil removal and antimicrobial action. Hydrogen peroxide- and peracetic acid-based formulations were effective sanitizing agents and break down into innocuous byproducts, making these good options for sustainable operations. ATP swabs and environmental swabbing for cell culture were useful strategies for evaluating the performance of cleaning and sanitizing protocols. Wineries can easily adopt these simple techniques to gain valuable insight into the efficacy of waste management operations. Improvements in tank cleaning operations, especially focusing on critical control points, may be a simple means for saving time, money, and chemicals in the winery.

Acknowledgments/Author disclosures

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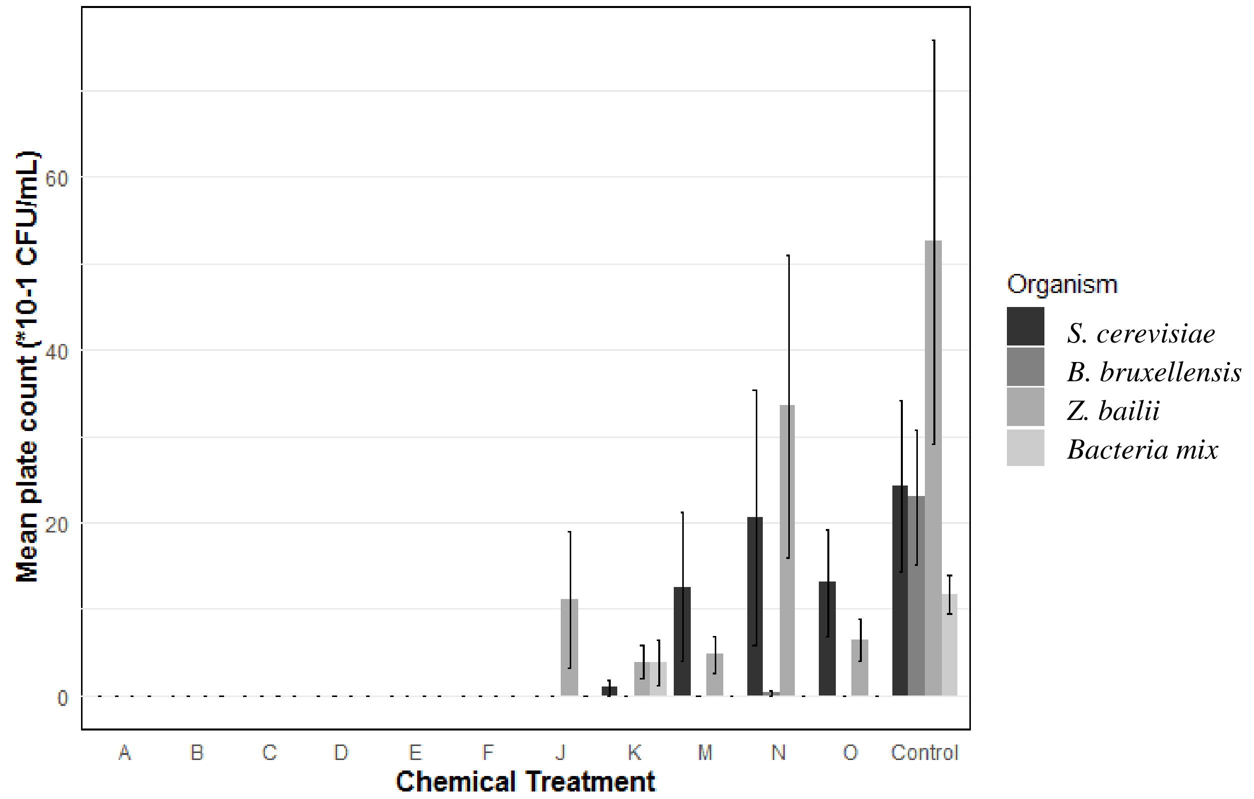
2.7 Supplementary



Supplementary Figure 2.S.1. Plate count data for treatment of biofilms grown on SS coupons. Error bars represent standard error of the mean (n = 4).

CHAPTER 3: OPTIMIZING THE CONCENTRATIONS AND CONTACT TIMES OF CLEANING AND SANITIZNG AGENTS FOR INACTIVATING WINERY SPOILAGE MICROORGANISMS†

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3.1 Abstract

Microbial management is one of the most critical aspects of winery operations and is normally achieved via chemical control. This study sought to optimize winery cleaning and sanitation protocols for the management of winery spoilage using a combination of antimicrobial susceptibility testing and fluorescence microscopy techniques. The minimum inhibitory concentration and minimum biocidal concentration assay and a modified minimum biofilm inactivation assay were performed using a range of common winery cleaning and sanitizing agents against *Saccharomyces cerevisiae, Brettanomyces bruxellensis, and Zygosaccharomyces bailii* spoilage yeasts. Results indicate that inhibitory and biocidal concentrations vary dramatically between organisms but are largely in line with established application rates for inactivation of all cells in planktonic and biofilm physiologies overall. Dual-channel fluorescence staining was employed to determine the minimum contact time for inactivating *S. cerevisiae* populations using two peracetic acid concentrations. Propidium iodide staining was validated as a live/dead proxy (R^2 = 0.99) and used to determine the contact time required to inactivate cell suspensions. Peracetic acid treatment trials indicate that *S. cerevisiae* populations are inactivated in five minutes or less at concentrations of 0.10-0.15 g/L. In conjunction, these experiments provide insight for winemakers to design effective and efficient cleaning and sanitization protocols.

3.2 Introduction

Microbial control is of paramount importance in wineries. Product quality and salability directly depend on a winemaker's ability to proactively prevent contamination from spoilage microorganisms during cleaning and sanitation operations[26]. Winery workers invest a significant quantity of time and resources cleaning and sanitizing winery surfaces and components, but processes are far from standardized or optimized. Quantitative data supporting the antimicrobial efficacy of typical chemical treatments versus winery spoilage microorganisms are limited so wineries typically independently determine the cleaning and sanitizing products to use, the protocol by which these products are applied, and a monitoring strategy for determining the antimicrobial impact of cleaning and sanitizing operations.

Wine production is a water and chemical intensive process[22,23]. Winery wastewater quality and treatment strategies are well studied[21,38,104] , but research on the minimization of chemical cleaning and sanitizing inputs is limited. Cleaning and sanitizing agents traditionally used in wineries include sulfur dioxide, citric acid, and caustic soda, all of which pose an environmental threat and financial burden in wastewater disposal[21]. With an increasing need for sustainable operations a new generation of environmentally friendly chemical agents is appearing on the market[4]. Common examples of chemicals in this category are peracetic acid and hydrogen peroxide, two sanitizing agents with wide spectrum oxidative antimicrobial action and innocuous breakdown byproducts[43]. Chlorine dioxide is another oxidizing sanitizer that rapidly degrades to nontoxic byproducts, and avoids the risks of residual toxicity and trichloroanisole 'cork taint' faults associated with the use of traditional hypochlorite-based sanitizers[105]. A sanitizing scheme based on a paired potassium buffer system that can be reclaimed for reuse via filtration has also been proposed[93]. While these chemistries are better alternatives to many of the

traditionally used sanitizers for residual toxicity and disposal logistics, quantitative data on antimicrobial efficacy of these treatments compared to traditional chemistries are lacking. Regardless of the chemical agents selected for the task, manufacturer's recommended concentrations often similarly lack quantitative evidence of antimicrobial efficacy of the suggested application rates.

Cleaning and sanitization are separate processes. Cleaning cycles are used to remove physical soils on winery vessels and equipment, where sanitization is specifically focused on antimicrobial action. Chemicals are separately marketed and formulated for these processes, yet many products traditionally sold as cleaning agents have been demonstrated as having significant antimicrobial properties at common application rates (**Chapter 2**). Sodium and potassium caustic chemicals typically used as cleaning agents were included in this study for comparison against chemicals marketed as sanitizers. A proprietary cleaner blend formulated specifically for biofilm removal was also included. The efficacy of chemical agents was measured in terms of microbial inactivation only, so all chemical treatments were assessed as sanitizers regardless of normal use case.

Zygosaccharomyces bailii, Brettanomyces bruxellensis, and unwanted *Saccharomyces cerevisiae* populations are commonly responsible for winery spoilage[14]. Of special concern are yeast populations existing as biofilms, surface-associated aggregates commonly reported to exhibit increased resistance to antimicrobial agents versus planktonic cultures[4,20]. Effective antimicrobial chemistries must be capable of inactivating winery spoilage microorganisms in planktonic and biofilm states to fully represent the spoilage threat to industry. The optimal use of any antimicrobial is the minimization of chemical concentration and contact time while maintaining effective antimicrobial control. Studies have been conducted on the comparative

efficacy of winery sanitizing agents[43,46,106] , but are limited in terms of the chemical treatments, application rates, and microorganisms tested. Antimicrobial susceptibility testing by serial dilution presents a method for assessing a wide range of treatments and organisms in planktonic and sessile physiologies with the specific aim of determining minimum effective application concentrations. Fluorescence microscopy and cell culture techniques can be used to determine the minimum contact time required for inactivating spoilage microorganisms using effective treatments from antimicrobial susceptibility tests. Together, these techniques present a method for optimizing the use of antimicrobials in enologically relevant conditions.

The minimum inhibitory concentration (MIC) and minimum biocidal concentration (MBC) assay is a well-established method for determining the minimum concentration of an antimicrobial agent required to inhibit or inactivate microorganisms[56,107]. Microbial suspensions are mixed with a series of dilutions of antimicrobial agents in microtiter plates and allowed to incubate overnight, after which a sample is collected on nutrient agar and the absorbance of the microtiter plate is read to assess in-situ cell growth. From these results it is possible to determine the minimum chemical concentration required for microbial inactivation (inability to culture) or inhibition (prevention of *in-situ* growth). Advantages of this method include its simplicity, ease of reproducibility, and the ability to perform high-throughput analysis of numerous antimicrobial agents with limited raw materials. The susceptibility of common spoilage yeasts to dimethyl decarbonate and of lactic acid bacteria to chlorine dioxide have been studied in the context of alcohol production using the MIC/MBC assay[48,58]. The assay is easily modified to study the susceptibility of sessile communities of microorganisms[20,108]. The MIC/MBC assay is an ideal method for cross-comparing chemical efficiency and was used in this study to examine whether a

range of cleaning and sanitizing agents can be applied below typical application rates to successfully inhibit and inactivate fermentative yeasts.

Dual-channel yeast fluorescence microscopy is a powerful tool for staining cellular components to observe changes in a population[109]. A common application involves the use of propidium iodide (PI), which is excreted by viable cells, but retained in inactivated cells[110]. When used in conjunction with a counterstain that stains all cells regardless of viability, the dual-channel fluorescence intensity can be used as a proxy for culture viability[111]. Numerous protocols and commercial kits that leverage this system are available, and the technique has been successfully applied to *S. cerevisiae and B. bruxellensis* in enological settings[91,112]. Fluorescence microscopy has yet to be applied to the context of chemical winery sanitization but can be a useful technique for determining the minimum contact time required for the inactivation of spoilage yeasts.

In this study, the MIC/MBC assay was used to identify minimum concentrations of a suite of chemical agents required to inhibit or inactivate three spoilage yeasts. The assay was modified and extended to determine the minimum concentration required to inactivate sessile communities of yeast. Dual-channel fluorescence using propidium iodide (PI) and SYBR Green 1 (SG1) was assessed as a proxy for cellular viability. PI staining was used to determine the contact time necessary for inactivation of *S. cerevisiae* with two concentrations of peracetic acid. This experiment presented a simple method for optimizing the use of chemical sanitizers that can aid winemakers in developing protocols with reduced chemical inputs and contact times.

3.3 Materials and methods

3.3.1 Yeast cultures, broth, and 50% grape juice medium

Saccharomyces cerevisiae, Brettanomyces bruxellensis, and *Zygosaccharomyces bailii* cultures were obtained from the University of California, Davis Viticulture and Enology Culture Collection.

Yeast-mold (YM) broth from BD Biosciences (New Jersey, USA) and a sterile 50% grape juice medium were used as culture media. YM broth was prepared according to manufacturer's instructions and autoclaved at 121°C for 20 minutes. To prepare the grape juice medium, Chardonnay grapes were harvested from the University of California, Davis Tyree vineyard at 25° Brix and directly pressed. The juice was centrifuged at 4500 x g for 10 minutes, filtered to 0.22µm using a sterile vacuum bottle top filter from Corning Inc. (New York, USA), and diluted to 50% with sterile-filtered water. Yeast-mold agar was prepared by adding 15 g/L agar from BD Biosciences (New Jersey, USA) to the YM broth formulation prior to autoclaving.

3.3.2 Chemical preparation

Ten different cleaning and sanitizing formulations were assessed in this study. Chemicals were prepared fresh for each trial using 1/8X phosphate-buffered saline (PBS) from Apex Chemical Co. (Arizona, USA) as a solvent and used within 30 minutes of preparation. Chemical species and maximum applied concentrations are detailed in **Table 3.1**. Several proprietary formulations were tested and are indicated where applicable. Caustic alkali solutions were prepared using ACS-grade sodium hydroxide and potassium hydroxide from MilliporeSigma (Massachusetts, USA), respectively. Proprietary caustic blends containing chelators, surfactants, and stabilizers based on sodium and potassium hydroxide were supplied by California Soda Co. (California, USA). A cleaning agent designed to specifically target biofilms from Sterilex, LLC (Maryland, USA) was used. Peracetic acid was sourced from Arcos Organics (New Jersey, USA). Hydrogen peroxide and potassium bisulfate from MilliporeSigma (Massachusetts, USA)

were used separately and in combination. Chlorine dioxide was generated by soaking a sachet from Selective Micro Technologies, LLC (Ohio, USA) in 1/8X PBS according to manufacturer's specifications.

3.3.3 MIC/MBC and biofilm assay

Separate 96-well microtiter plates were prepared for yeast inocula and challenge chemicals. Cell stocks were prepared in YM broth and 50% grape juice medium separately and diluted to 0.5 at OD_{600} nm. Two hundred microliters of inoculum was plated in each well of a 96-well microtiter plate. Challenge chemicals were prepared fresh at double the final test concentration for each trial. Four hundred microliters of the maximum concentration of each chemical was prepared in the first row of the microtiter plate and 200 µL of 1/8X PBS was plated in all other wells of the same plate. Dilutions were performed by mixing 200 µL from the first row into the 1/8x phosphate-buffered saline in the row below, mixing via pipette, and repeating until 400 µL of the lowest concentration remains in the last row with 50% dilution profile[107,113]. Two hundred microliters from each well of the challenge chemical plate was transferred into corresponding wells in inoculated microtiter plates, resulting in 400 µL final volume at test concentration in all wells of the 96-well microtiter plates. Details for the final dilution scheme are given in **Table 3.2**. Six growth check and six sterility check wells were included for quality assurance. Microtiter plates were incubated for 24 hours at 30°C. One hundred microliters from each well was transferred onto a corresponding YM agar plate to assess the biocidal effect of treatments. The absorbance of the microtiter plates was measured at 600 nm using a Synergy 2 microplate reader from Biotek Instruments, Inc. (Vermont USA). Agar plates were incubated for three weeks at 30°C to facilitate growth of less-rapidly dividing cells. Tests were performed in triplicate.

Table 3.1. Chemicals used in MIC/MBC assay.

Chemical Treatment	Maximum Applied Concentration
1. Potassium hydroxide [KOH]	80 g/L
2. Proprietary potassium caustic blend (85-90% potassium hydroxide [KOH])	80 g/L
3. Sodium hydroxide [NaOH]	80 g/L
4. Proprietary sodium caustic blend (40-45% sodium hydroxide [NaOH])	80 g/L
5. Proprietary cleaner blend (6% sodium carbonate [Na_2CO_3], 6% potassium carbonate [K_2CO_3], 4.9% ethylenediaminetetraacetic acid [$C_{10}H_{16}N_2O_8$], 6% alkyl dimethyl alkyl benzyl ammonium chlorides, 6.3% hydrogen peroxide [H_2O_2])	50% (v/v) *
6. Peracetic acid [$C_2H_4O_3$]	0.8 g/L
7. Hydrogen peroxide [H_2O_2]	90.4 g/L
8. Potassium bisulfate [$KHSO_4$]	10.89 g/L
9. Potassium bisulfate [$KHSO_4$] + **hydrogen peroxide** [H_2O_2]	5.44 g/L [$KHSO_4$] + 45.4 g/L [H_2O_2]
10. Chlorine dioxide [ClO_2]	0.25 g/L

*denotes pre-measured proprietary liquid.

Table 3.2. Dilution scheme used in MIC/MBC assay. Each entry represents a corresponding well in a 96-well microtiter plate.

1. Potassium hydroxide (g/L)	**2. Potassium caustic blend (g/L)**	**3. Sodium hydroxide (g/L)**	**4. Sodium caustic blend** (g/L)	**5. Proprietary cleaner blend (% v/v)**	**6. Peracetic acid (g/L)**
80.000	80.000	80.000	80.000	50.000	0.800
40.000	40.000	40.000	40.000	25.000	0.400
20.000	20.000	20.000	20.000	12.500	0.200
10.000	10.000	10.000	10.000	6.250	0.100
5.000	5.000	5.000	5.000	3.125	0.050
2.500	2.500	2.500	2.500	1.563	0.025
1.250	1.250	1.250	1.250	0.781	0.013
0.625	0.625	0.625	0.625	0.391	0.006
7. Hydrogen peroxide (g/L)	**8. Potassium bisulfate** (g/L)	**9. Potassium bisulfate + hydrogen peroxide** (g/L)	**10. Chlorine dioxide** (g/L)	**Growth Check**	**Sterility Check**
90.400	10.890	5.445 + 45.200	0.250	1	1
45.200	5.445	2.722+ 22.600	0.125	2	2
22.600	2.723	1.361+ 11.300	0.063	3	3
11.300	1.361	0.681 + 5.650	0.031	4	4
5.650	0.681	0.340 + 2.825	0.016	5	5
2.825	0.340	0.170 + 1.413	0.008	6	6
1.413	0.170	0.085 + 0.706	0.004		
0.706	0.085	0.040 + 0.353	0.002		

A modified form of the MIC/MBC analysis was developed to assess the efficacy of chemical treatments for the inactivation of sessile yeast populations. Biofilms were formed in 96-well microtiter plates according to the method described in Joseph, Kumar, and Bisson, 2007[19] using 50% grape juice medium. The supernatant was aspirated, and biofilms were washed with 1/8X PBS three times to remove residual growth medium. To conduct a trial, 200 µL of challenge chemical was plated in corresponding wells of the biofilm plate and allowed to sit for 24 hours at room temperature. The chemicals were aspirated, and wells were rinsed three times with 1/8X PBS to remove chemical residue. Two hundred microliters of 1/8x PBS was added to each well and the plates were sonicated for 30 seconds to release the biofilm from the microtiter plate surface before plating 100 µL from each well onto corresponding YM agar plates. Plates were incubated for three weeks at 30°C for growth and measured by plate reader as previously described. Tests were performed in triplicate

3.3.4 SYBR Green 1 / PI fluorescent staining

An ethanol kill curve was developed by mixing prepared stocks of live and dead *S. cerevisiae*. A sterile saline solution was prepared by mixing 8.5 g/L sodium chloride (NaCl) from MilliporeSigma (Massachusetts, USA) with deionized water and autoclaved at 121°C for 20 minutes. *S. cerevisiae* stock was prepared in 50% grape juice medium. Inactivated cells were prepared by centrifuging *S. cerevisiae s*tock and resuspending to 0.5 at OD_{600} nm in 70% ethanol from Decon Labs (Pennsylvania USA). After 30 minutes cells were centrifuged, washed three times with saline solution, and resuspended to 0.5 at OD_{600} nm in the saline solution. Live microbe stock was prepared by centrifuging the microbe broth, washing three times with saline solution, and resuspending to 0.5 at OD_{600} nm in the saline solution. The live and dead microbe stocks were mixed at 20% ratios to create a series of samples from 100% alive to 0% alive, for a

total of six mixtures. Fluorescent dye stock was prepared fresh for each trial. Dyes were prepared by adding 10 µl SYBR Green I from Thermo Fisher Scientific (Massachusetts, USA) and 0.2 mg propidium iodide from Arcos Organics (New Jersey, USA) to 10 mL of deionized water. One hundred eighty microliters of each alive/dead mixture was plated in a 96-well microtiter plate in triplicate. Twenty microliters of the stain stock was added to each well. Stained microtiter plates were placed on a shaker table and allowed to develop for 30 minutes at 200 rpm rotation. Plates were analyzed using a Synergy 2 microplate reader from Biotek Instruments, Inc. (Vermont USA) in dual channel mode. The fluorescent measurements were obtained using a 530/25 excitation filter and 590/35 emission filter for the red channel (PI) and a 485/20 excitation filter and 528/20 emission filter for the green channel (SG1).

Peracetic acid time-kill trials were conducted by mixing *S. cerevisiae* stock with peracetic acid solutions and quenching the reaction using a recovery medium after a specified contact time to limit further cell inactivation. *S. cerevisiae* stock was prepared at 0.5 at OD_{600} nm in 0.85% NaCl saline as described previously. Peracetic acid solutions were prepared at 0.10 g/L and 0.15 g/L concentration in 0.85% NaCl saline. Tests were performed by mixing 20 mL of *S. cerevisiae* inoculum with 5 mL peracetic acid solution. After a specified contact time the reaction was quenched by adding the inoculum-acid mixture into 225 mL saline solution supplemented with 1% fetal bovine serum (FBS) from MilliporeSigma (Massachusetts, USA). Samples were collected at contact times ranging from five seconds to ten minutes. A zero-contact time sample was created by adding the microbe inoculum and peracetic acid solution directly into the quenching media. Cells were centrifuged and re-suspended to 0.5 at OD_{600} nm in 0.85% NaCl saline solution. Trials were performed with n = 6 replicates for each peracetic acid concentration. Samples were collected for cell culture and fluorescent stain analysis. One hundred microliters of

sample were plated on YM agar to assess cell culturability. One hundred eighty microliters of sample was plated into a microtiter plate for fluorescent stain analysis, in triplicate. Cells were stained and analyzed as previously described.

3.4 Results and Discussion

3.4.1 MIC/MBC and biofilm assay

The minimum inhibitory concentration (MIC) and minimum biocidal concentration (MBC) assay was used to determine the lowest effective antimicrobial concentrations of ten common winery cleaning and sanitizing formulations versus *S. cerevisiae*, *B. bruxellensis*, and *Z. bailii*. Results for the MIC/MBC and biofilm assay are given in **Table 3.3**, along with typical application rates and manufacturer's suggested concentrations for comparison. The suggested chemical concentrations used in this experiment are either derived from the manufacturers themselves (for proprietary products) or taken as a consensus of several sources for basic chemistries[4,25,26,115]. MIC values were determined as concentrations that resulted in average absorbance measurements of 0.1 at OD_{600} nm or greater, a level which produces a light visible haze in the well plate by visual inspection[113]. MBC values were determined as the lowest concentration of a chemical that resulted in no colony growth on the agar plates. Average absorbance values and standard deviations are given in **Supplementary.**

There are several key takeaways from **Table 3.3**. First, the test medium clearly influences the concentrations required to inactivate or inhibit the yeast. For the MIC/MBC assay, higher concentrations of challenge chemicals were required to inactivate microorganisms in 50% grape juice medium for 56.7% of all organism and chemical combinations tested, compared to 30.0% of cases where the concentrations were equal and only 14.3% where the broth was higher. A possible explanation for the grape juice generally requiring higher chemical concentrations to

inactivate cells could be attributed to the increased dissolved species content of grape juice, which can react with the antimicrobials and slow or reduce their efficacy[115]. Possible explanations for higher concentrations required in YM broth could be related to the pH of the growth medium. The YM broth has higher pH than grape juice and could reduce the efficacy of acidic sanitizers versus trials using the 50% grape juice medium.

Biofilms are commonly reported to have increased resistance to antimicrobial agents versus planktonic suspensions of the same species[116]. In 40% of trials greater concentrations were required to inactivate biofilm populations versus planktonic cultures. However, in 36.7% of trials lower concentrations successfully inactivated biofilms versus planktonic cultures, so the theory of increased resistance is not well supported here. One possible explanation is due to the protocol, in which the biofilms are rinsed several times to remove chemical residues prior to sonication and sampling. If the chemical treatment is capable of physically dislodging the biofilm from the surface of the microtiter plate, cells would not be recovered in culture samples regardless of their viability. Cases in which the biofilm was inactivated at lower chemical concentrations than corresponding planktonic populations could be the result of this effect, which may be an inconsistency in the method applied as a measure of concentrations required for inactivation. In future study the method can be adapted to ensure biofilms are not physically detached by the treatment to be captured for culture analysis. A specialized microtiter plate specifically designed for assessing antimicrobial susceptibility of biofilm-forming bacteria (called the MBEC assay or Calgary Biofilm Device)[117] is available from Innovotech Inc. (Edmonton, Canada). This device is essentially a modified microtiter plate with pegs that are suspended in the growth medium to promote biofilm formation, and avoids potential issues associated with rinsing biofilms formed in the bottom of plates. This device is available in plain-

Table 3.3. Results for the MIC/MBC and biofilm assays. Reference concentrations included at bottom for comparison.

Organism	Medium	Indicator	1. Potassium hydroxide (g/L)	2. Potassium caustic blend (g/L)	3. Sodium hydroxide (g/L)	4. Sodium caustic blend (g/L)	5. Proprietary cleaner blend (% v/v)
S. cerevisiae	Broth	MIC	1.25 g/L	1.25 g/L	1.25 g/L	≤ 0.625 g/L	≤ 0.0039 %
		MBC	5 g/L	5 g/L	5 g/L	2.5 g/L	1.560%
	Grape Juice	MIC	2.50 g/L	5 g/L	2.5 g/L	2.50 g/L	≤ 0.0039 %
		MBC	10 g/L	20 g/L	20 g/L	10 g/L	≤ 0.0039 %
		Biofilm	20 g/L	2.5 g/L	10 g/L	5 g/L	≤ 0.0039 %
B. bruxellensis	Broth	MIC	5 g/L	1.25 g/L	2.5 g/L	2.5 g/L	≤ 0.0039 %
		Biofilm	5 g/L	1.25 g/L	2.5 g/L	2.5 g/L	≤ 0.0039 %
	Grape Juice	MIC	1.25 g/L	2.5 g/L	2.5 g/L	1.25 g/L	≤ 0.0039 %
		MBC	1.25 g/L	2.5 g/L	1.25 g/L	1.25 g/L	≤ 0.0039 %
		Biofilm	20 g/L	10 g/L	20 g/L	20 g/L	≤ 0.0039 %
Z. bailii	Broth	MIC	≤ 0.625 g/L	1.25 g/L	1.25 g/L	1.25 g/L	≤ 0.0039 %
		MBC	1.25 g/L	2.5 g/L	5 g/L	2.5 g/L	≤ 0.0039 %
	Grape Juice	MIC	≤ 0.625 g/L	1.25 g/L	1.25 g/L	1.25 g/L	≤ 0.0039 %
		MBC	2.5 g/L	5 g/L	5 g/L	5 g/L	≤ 0.0039 %
		Biofilm	10 g/L	20 g/L	2.5 g/L	10 g/L	≤ 0.0039 %
Recommended application rate			10 - 20 g/L	10 - 20 g/L	10 - 20 g/L	10 - 20 g/L	17% (v/v)

Table 3.3. (Cont.)

Organism	Medium	Indicator	**6. Peracetic acid (g/L)**	**7. Hydrogen peroxide (g/L)**	**8. Potassium bisulfate** (g/L)	**9. Potassium bisulfate + hydrogen peroxide** (g/L)	**10. Chlorine dioxide** (g/L)
S. cerevisiae	Broth	MIC	0.013g/L	≤ 0.71 g/L	0.681 g/L	≤ 0.043 g/L + 0.353 g/L	0.063 g/L
		MBC	0.05 g/L	≤ 0.71 g/L	5.44 g/L	≤ 0.043 g/L + 0.353 g/L	1.25 mg/L
	Grape Juice	MIC	0.05 g/L	≤ 0.71 g/L	0.681 g/L	≤ 0.043 g/L + 0.353 g/L	0.063 g/L
		MBC	0.1 g/L	≤ 0.71 g/L	10.89 g/L	≤ 0.043 g/L + 0.353 g/L	1.25 mg/L
		Biofilm	0.01 g/L	5.64 g/L	2.72 g/L	5.44 g/L + 22.6 g/L	0.063 g/L
B. bruxellensis	Broth	MIC	0.05 g/L	≤ 0.71 g/L	0.68 g/L	≤ 0.043 g/L + 0.353 g/L	0.063 g/L
		Biofilm	0.05 g/L	≤ 0.71 g/L	0.68 g/L	≤ 0.043 g/L + 0.353 g/L	0.063 g/L
	Grape Juice	MIC	0.1 g/L	≤ 0.71 g/L	1.36 g/L	≤ 0.043 g/L + 0.353 g/L	0.063 g/L
		MBC	0.1 g/L	≤ 1.42 g/L	2.72 g/L	≤ 0.043 g/L + 0.353 g/L	0.063 g/L
		Biofilm	0.1 g/L	≤ 1.42 g/L	0.68 g/L	2.72 g/L + 11.3 g/L	0.17 g/L
Z. bailii	Broth	MIC	0.025 g/L	≤ 1.42 g/L	0.34 g/L	≤ 0.043 g/L + 0.353 g/L	0.063 g/L
		MBC	0.1 g/L	≤ 1.42 g/L	0.34 g/L	≤ 0.043 g/L + 0.353 g/L	0.063 g/L
	Grape Juice	MIC	0.025 g/L	≤ 1.42 g/L	0.34 g/L	≤ 0.043 g/L + 0.353 g/L	0.063 g/L
		MBC	0.1 g/L	2.82 g/L	1.36 g/L	≤ 0.043 g/L + 0.353 g/L	1.25 mg/L
		Biofilm	0.1 g/L	11.3 g/L	1.36 g/L	2.72 g/L + 11.3 g/L	0.063 g/L
Recommended application rate			0.1 - 0.2 g/L	11.3 - 33.9 g/L	2.72 g/L	2.72 g/L ($KHSO_4$) + 11.3 g/L (H_2O_2)	0.1 - 0.2 g/L

This device is available in plain- or fluorapatite-coated plates to facilitate cell adhesion. In preliminary trials the three yeast species used in this experiment failed to form consistent, reproducible adherent colonies with plain or coated plates using the MBEC assay. For this reason, the MIC/MBC procedure was instead modified to incorporate biofilm formation as described by Joseph, Kumar, and Bisson[19] to assess biofilm susceptibility to antimicrobial agents.

Manufacturers suggest concentrations for effective application of their proprietary cleaning and sanitizing products. While in many cases a treatment was successful in inactivating some combination of organism and physiology at much reduced concentration, the treatments that resulted in complete inactivation of all specimens were in the range of manufacturer suggestions with the exception of the complex proprietary blend containing EDTA, carbonate blend, and quaternary ammonium compounds (QUATs), which was effective below one tenth of the recommended concentration. This is an interesting result, as the potential for chemical savings using this product could be large if extended contact time was feasible. The potassium bisulfate formulation, based on the green sanitation scheme presented from Boulton[93] was effective at or below the recommended concentration for all cases except *S. cerevisiae. S. cerevisiae* inactivation required substantially higher concentrations than indicated in the publication. This finding underlies the importance of the microbe species used in testing, since *E. coli* was used to develop recommended concentrations but was demonstrated to be a poor indicator of *S. cerevisiae* susceptibility in this study.

The MIC/MBC assay is a well-established, high-throughput method for determining the minimum concentration of antimicrobial agents required to inactivate yeast cells[56]. This method is commonly used in clinical heath settings but was adapted here to assess conditions relevant to

wine production. The assay is typically performed with Mueller-Hinton broth (MH), a media specifically designed for the purpose of antimicrobial susceptibility testing of nonfastidious organisms[113]. Preliminary analysis of growth rate for the three yeast species used in this experiment demonstrated that the cells did not reach sufficient population density to produce a visible haze in well plates after 24 hours incubation, a requirement for assessment of the MIC/MBC. YM broth was substituted for MH broth to meet this requirement and to correlate to results in **Chapter 1**. Fifty percent grape juice medium was used to replicate winery conditions and to assess the effect of growth and test media on antimicrobial susceptibility.

It is important to emphasize the difference between cleaning and sanitizing agents in practical settings, as products are separately marketed for cleaning and sanitation purposes with differing intended usage. Cleaning typically refers to the removal of soils, where the goal of sanitation is microbial inactivation. However, in this experiment products marketed as cleaners (potassium- and sodium hydroxide- based formulations and the proprietary cleaner mixture) effectively inactivate microbial populations at recommended concentrations. While there is no substitution for a separate sanitation cycle, it is important to acknowledge that the microbial population is at least somewhat diminished prior to beginning the sanitation cycle when designing protocols. The combined effect of a cleaner and sanitizer in succession is more effective than the sum of the cleaner and sanitizer action separately[46]. The numbers presented in this experiment are for use of the chemicals alone, so it is possible that lower concentrations may be effective for cleaner-sanitizer combination treatments in the practical winery environment.

Winemakers are not able to predict the spoilage microorganisms present inside tanks, so it is prudent to use chemicals at rates that are effective for all conditions. Cleaning and sanitizing chemistries should be applied at recommended concentrations, where effective, to ensure the

spoilage risk is properly mitigated. The treatments that required higher concentrations than suggested should be used with caution. In all cases, wineries should independently validate their chosen cleaning and sanitizing protocols by incorporating some form of monitoring strategy into routine cleaning and sanitization operations. Simple techniques like cell culture swabbing or adenosine triphosphate swabbing provide a quantitative indication of cleaning and sanitizing efficacy and can allow winemakers to objectively assess the success of these operations.

3.4.2 Fluorescent staining

Propidium iodide (PI) and SYBR Green 1 (SG1) fluorescent dyes were used to assess the minimum contact time required to inactivate *S. cerevisiae* cultures using peracetic acid at two concentrations. The ability of the PI/SG1 protocol to act as a proxy for cellular viability was validated using ethanol and a gradient of live versus dead cells inactivated via suspension in 70% ethanol. Linear regression analysis was performed using XLSTAT software from Addinsoft (Paris, France). Results are displayed in **Figure 3.1**, which illustrates two key points. PI alone acts as a reliable proxy for cellular viability with a high degree of linearity (R = 0.99). In contrast, SG1, which is included to stain all cells independent of viability, varies in fluorescence

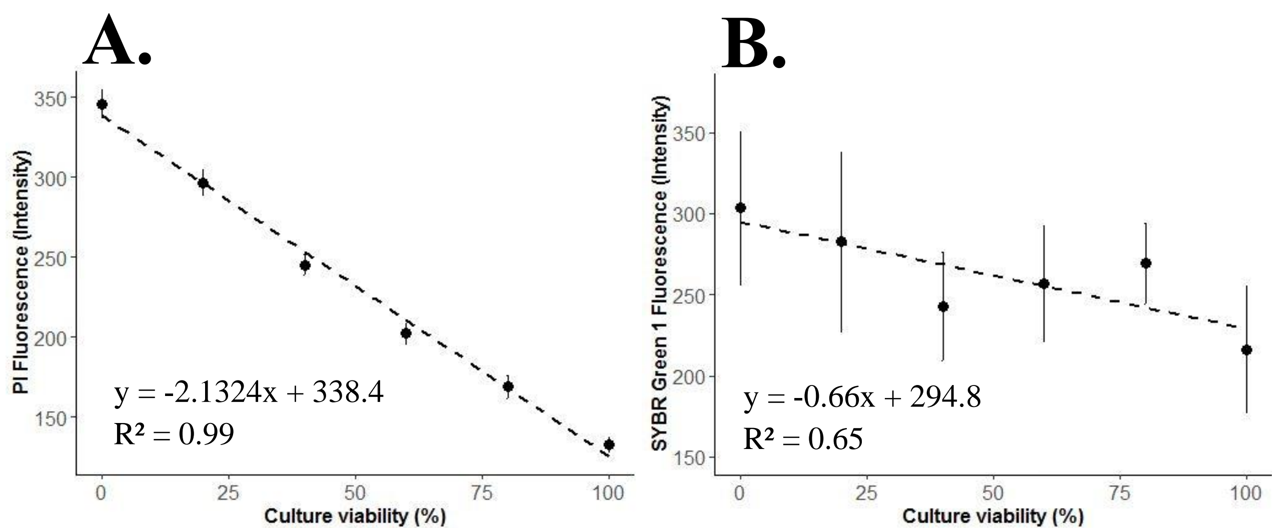

Figure 3.1. Fluorescent stain intensity for ethanol kill curve trials (n = 6). **A.** Propidium iodide fluorescence kill curve. **B.** SYBR green 1 fluorescence kill curve.

intensity substantially across the live/dead mixtures. Previous studies have indicated that the SG1 and PI can interact and compete for binding sites[118], however there isn't a clear trend for cells with higher PI fluorescence to exhibit lower SG1 fluorescence intensities, so this may not be the explanation here. In this case, the inclusion of SG1 intensity data would diminish the ability of PI fluorescence to reliably indicate cellular viability. For this reason, PI intensity was used alone in the analysis of peracetic acid inactivation rates.

Samples for time-kill analysis were taken over a range of 10 minutes. PI intensity data are shown in **Figure 3.2** and plate count data are displayed in **Table 3.4**. Results indicate that *S. cerevisiae* populations were effectively inactivated after five minutes contact time at both test concentrations of peracetic acid. PI fluorescence intensity data are closely modeled by logarithmic functions. These results suggest first-order, or apparent first order inactivation kinetics similar to patterns observed in classic enzyme inactivation or thermal inactivation models[119]. The close correlation between plate count and fluorescence data support the reliability of PI as a method for tracking cellular viability. Given these results, it could be suggested that

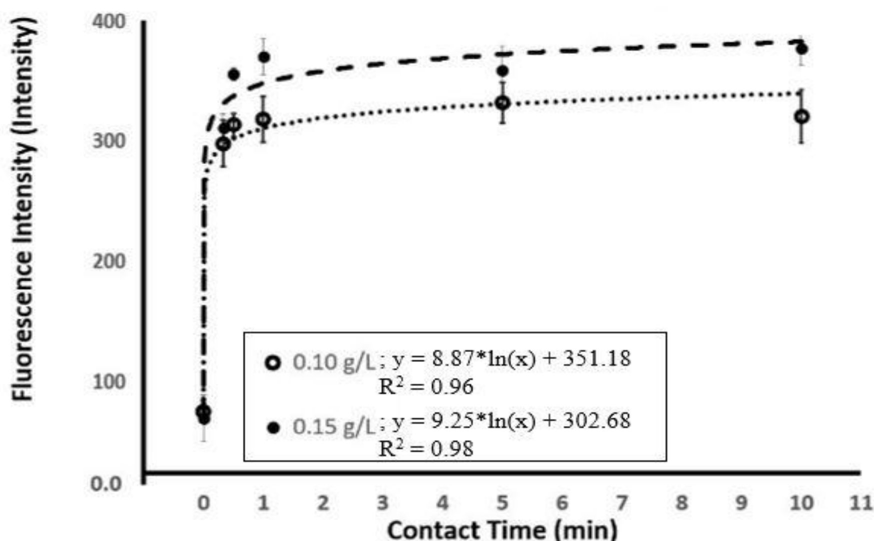

Figure 3.2. Propidium iodide fluorescence killing curves for Saccharomyces cerevisiae cultures exposed to two concentrations of peracetic acid (n = 6).

Table 3.4. Plate count results for minimum effective peracetic acid contact time trials (n = 6).

0.10 g/L peracetic acid								
Contact time (min)	Cell Count (*10^-1 CFU/mL)						Average (*10^-1CFU/mL)	Standard deviation
0.00	370	450	530	520	600	660	522	20%
0.08	58	79	64	112	80	62	76	26%
0.50	31	11	18	28	55	9	25	67%
1.00	1	10	0	0	8	3	4	118%
5.00	0	0	0	0	0	0	0	0%
10.00	0	0	0	0	0	0	0	0%
0.15 g/L peracetic acid								
Contact time (min)	Cell Count (*10^-1 CFU/mL)						Average (*10^-1CFU/mL)	Standard deviation
0.00	550	500	570	390	540	520	512	13%
0.08	66	49	81	72	60	59	65	17%
0.50	20	12	24	8	25	21	18	37%
1.00	0	5	7	9	0	8	5	82%
5.00	0	0	0	0	0	0	0	0%
10.00	0	0	0	0	0	0	0	0%

effective winery sanitation with peracetic acid may be completed with five minutes contact time at a concentration of 0.10 g/L. In real-world situations, wineries will encounter limitations in the ability of cleaning apparatuses to provide consistent chemical contact with contaminated surfaces, so sanitation cycles may need to be longer than this optimized contact time. Wineries should independently test and validate the antimicrobial efficacy of cleaning and sanitization protocols to ensure the suitability of any chemical treatment for their specific equipment and configuration.

Fluorescent staining is a natural complement to the MIC/MBC assay. The MIC/MBC assay provides a simple method for determining minimum effective chemical concentrations but requires 24 hours contact time between antimicrobials and cell cultures. This contact time is not feasible in practical winery settings during harvest where the minimizations of processing time is critical. Fluorescence spectroscopy can be employed in short-interval time-kill experiments and

provides a basis for assessing the minimum contact time needed for the concentrations obtained in the MIC/MBC assay. While traditional plate count data are still the standard approach in time-kill experiments, results can take days or even weeks for fastidious organisms[120]. Fluorescent stain data is rapidly acquired, requiring only minutes for scans using a microplate reader. Fluorescent staining with propidium iodide is a well-established method[121], but is not suitable to monitor the performance of every antimicrobial agent. The selective exclusion of propidium iodide depends on the loss of cellular membrane integrity and control[110]. Therefore, only a limited number of cleaning and sanitizing agents can be used that have modes of action primarily affecting membrane permeability. As such, the proprietary cleaner blend of EDTA, carbonates, and QUATs effective in the MIC/MBC could not be accurately assessed by this method. Nevertheless, PI fluorescence was still demonstrated as a useful method for monitoring peracetic acid inactivation of *S.* cerevisiae and could be expanded to include other test microorganisms and antimicrobials relevant to the wine industry.

3.5 Conclusion

In this experiment the minimum inhibitory concentration and minimum biocidal concentration assay was used to determine the minimum concentrations of common winery cleaning and sanitizing agents required to inactivate three common spoilage yeasts in planktonic physiologies. A modified version of the assay was used to investigate the difference in concentrations required to inactivate cells in biofilm physiologies. While certain combinations of organism, physiology, and chemical were effective below recommended concentrations, only a complex mixture of EDTA, carbonates, and QUATs was totally effective at concentrations below the manufacturer's suggested rate. Biofilms did not exhibit increased resistance to antimicrobial treatments in most cases. Fluorescent microscopy using propidium iodide was

validated as a proxy for cellular viability, and then used to assess the contact time required to inactivate *Saccharomyces cerevisiae* at two different concentrations of peracetic acid. Five minutes of contact time was effective for inactivating *S. cerevisiae* populations at the minimal effective concentration from the MIB/MBC assay (0.10 g/L). Wineries can use simple cell culture techniques to assess the efficacy of cleaning and sanitization operations and determine the minimum effective concentrations required for microbial control under their individual operational conditions.

Supplementary

Table 3.S.1. Absorbance averages for MIC determination (n = 3).

					***S. cerevisiae* broth**						
[NaOH]	**NaOH-blend**	**[KOH]**	**KOH-blend**	**Cleaner blend**	**Peracetic Acid**	**$[H_2O_2]$**	**$[KHSO_4]$**	**$[KHSO_4]$ + $[H_2O_2]$**	**$[ClO_2]$**	**growth check**	**sterility check**
0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.02	0.01	0.01		
0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.02	0.01	0.01		
0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.03	0.00	0.05	0.68	0.00
0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.05	0.00	0.22	0.61	0.00
0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.05	0.00	0.33	0.86	0.00
0.00	0.00	0.01	0.00	0.10	0.02	0.01	0.11	0.00	0.45	0.68	0.00
0.01	0.01	0.03	0.00	0.10	0.07	0.01	0.50	0.00	0.65	0.83	0.00
0.15	0.41	0.17	0.02	0.00	0.57	0.01	0.61	0.01	0.62	0.61	0.00
					***S. cerevisiae 50%* grape juice**						
[NaOH]	**NaOH-blend**	**[KOH]**	**KOH-blend**	**Cleaner blend**	**Peracetic Acid**	**$[H_2O_2]$**	**$[KHSO_4]$**	**$[KHSO_4]$ + $[H_2O_2]$**	**$[ClO_2]$**	**growth check**	**sterility check**
0.02	0.00	0.01	0.01	0.00	0.03	0.03	0.02	0.02	0.02		
0.03	0.00	0.03	0.00	0.00	0.03	0.03	0.02	0.02	0.02		
0.04	0.01	0.02	0.00	0.00	0.02	0.02	0.04	0.02	0.03	0.88	0.00
0.02	0.02	0.01	0.00	0.20	0.03	0.02	0.03	0.02	0.43	0.93	0.00
0.01	0.04	0.02	0.00	0.00	0.03	0.01	0.03	0.03	0.76	1.00	0.00
0.02	0.14	0.08	0.00	0.00	0.55	0.02	0.11	0.02	0.68	0.88	0.00
0.17	0.77	0.71	0.11	0.00	1.03	0.02	1.24	0.02	0.75	0.88	0.00
0.75	0.99	1.07	0.15	0.10	0.95	0.02	1.45	0.01	0.81	1.03	0.00
					***B bruxellensis* broth**						
[NaOH]	**NaOH-blend**	**[KOH]**	**KOH-blend**	**Cleaner blend**	**Peracetic Acid**	**$[H_2O_2]$**	**$[KHSO_4]$**	**$[KHSO_4]$ + $[H_2O_2]$**	**$[ClO_2]$**	**growth check**	**sterility check**
0.01	0.03	0.01	0.02	0.00	0.02	0.04	0.01	0.01	0.03		
0.00	0.02	0.02	0.00	0.00	0.02	0.01	0.01	0.00	0.02		
0.02	0.03	0.03	0.01	0.01	0.03	0.00	0.00	0.00	0.01	0.54	0.00
0.02	0.02	0.01	0.02	0.00	0.00	0.00	0.02	0.01	0.49	0.68	0.00
0.03	0.03	0.02	0.04	0.04	0.01	0.00	0.04	0.01	0.48	0.64	0.00
0.37	0.03	0.04	0.04	0.02	0.33	0.00	0.51	0.02	0.48	0.50	0.00
0.28	0.07	0.22	0.26	0.00	0.53	0.00	0.75	0.02	0.51	0.64	0.00
0.46	0.26	0.22	0.25	0.01	0.55	0.02	0.85	0.01	0.46	0.58	0.00

Table 3.S.1. (Cont.)

B. bruxellensis 50% grape juice											
[NaOH]	**NaOH-blend**	**[KOH]**	**KOH-blend**	**Cleaner blend**	**Peracetic Acid**	**[H_2O_2]**	**[$KHSO_4$]**	**[$KHSO_4$] + [H_2O_2]**	**[ClO_2]**	**growth check**	**sterility check**
0.01	0.00	0.00	0.00	0.02	0.01	0.01	0.02	0.03	0.02		
0.02	0.00	0.00	0.00	0.02	0.00	0.02	0.00	0.01	0.02		
0.02	0.00	0.00	0.00	0.03	0.01	0.03	0.03	0.02	0.01	1.32	0.00
0.01	0.00	0.00	0.00	0.00	0.00	0.03	0.04	0.02	0.12	1.30	0.00
0.02	0.00	0.00	0.00	0.02	0.21	0.02	0.16	0.02	0.53	1.12	0.00
0.01	0.00	0.00	0.00	0.07	0.22	0.02	1.10	0.03	0.91	1.31	0.00
0.03	0.16	0.11	0.03	0.00	1.21	0.03	1.15	0.02	1.05	1.10	0.00
0.12	1.15	1.23	0.95	0.09	1.44	0.03	1.06	0.03	1.07	1.21	0.00

Z. bailii broth											
[NaOH]	**NaOH-blend**	**[KOH]**	**KOH-blend**	**Cleaner blend**	**Peracetic Acid**	**[H_2O_2]**	**[$KHSO_4$]**	**[$KHSO_4$] + [H_2O_2]**	**[ClO_2]**	**growth check**	**sterility check**
0.03	0.03	0.02	0.04	0.02	0.03	0.03	0.02	0.02	0.03		
0.05	0.00	0.04	0.04	0.00	0.03	0.03	0.02	0.02	0.02		
0.03	0.01	0.03	0.04	0.00	0.03	0.03	0.02	0.01	0.01	0.29	0.00
0.02	0.03	0.02	0.02	0.00	0.04	0.03	0.01	0.01	0.11	0.47	0.00
0.02	0.04	0.02	0.02	0.02	0.04	0.02	0.02	0.01	0.23	0.56	0.00
0.02	0.04	0.02	0.02	0.07	0.31	0.03	0.04	0.01	0.35	0.55	0.00
0.04	0.04	0.03	0.04	0.00	0.50	0.03	0.34	0.02	0.49	0.58	0.00
0.03	0.51	0.58	0.21	0.09	0.50	0.03	0.54	0.03	0.53	0.61	0.00

Z. bailii 50% grape juice											
[NaOH]	**NaOH-blend**	**[KOH]**	**KOH-blend**	**Cleaner blend**	**Peracetic Acid**	**[H_2O_2]**	**[$KHSO_4$]**	**[$KHSO_4$] + [H_2O_2]**	**[ClO_2]**	**growth check**	**sterility check**
0.02	0.00	0.02	0.00	0.02	0.02	0.02	0.02	0.02	0.02		
0.04	0.00	0.02	0.00	0.02	0.02	0.01	0.01	0.00	0.01		
0.03	0.00	0.01	0.00	0.00	0.01	0.02	0.01	0.00	0.01	0.29	-0.01
0.02	0.02	0.01	0.00	0.00	0.02	0.03	0.01	0.00	0.13	0.47	-0.01
0.02	0.02	0.01	0.01	0.02	0.03	0.03	0.01	0.00	0.24	0.55	-0.01
0.01	0.03	0.01	0.01	0.00	0.31	0.02	0.04	0.01	0.36	0.54	-0.01
0.03	0.03	0.02	0.02	0.03	0.48	0.03	0.35	0.02	0.48	0.57	-0.01
0.02	0.48	0.57	0.20	0.04	0.48	0.01	0.53	0.01	0.52	0.59	-0.01

Table 3.S.2. Standard deviation of average MIC absorbance values.

S. cerevisiae broth											
[NaOH]	**NaOH-blend**	**[KOH]**	**KOH-blend**	**Cleaner blend**	**Peracetic Acid**	**[H_2O_2]**	**[$KHSO_4$]**	**[$KHSO_4$] + [H_2O_2]**	**[ClO_2]**	**growth check**	**sterility check**
0.003	0.003	0.085	0.001	0.018	0.015	0.007	0.007	0.002	0.004		
0.002	0.001	0.023	0.001	0.060	0.013	0.005	0.004	0.005	0.002		
0.002	0.002	0.003	0.002	0.045	0.008	0.002	0.005	0.005	0.069	0.383	0.004
0.001	0.002	0.001	0.001	0.010	0.007	0.002	0.002	0.002	0.094	0.182	0.005
0.002	0.001	0.002	0.001	0.002	0.008	0.002	0.002	0.003	0.073	0.374	0.004
0.004	0.006	0.001	0.002	0.088	0.003	0.008	0.003	0.002	0.151	0.272	0.004
0.010	0.040	0.002	0.006	0.121	0.008	0.008	0.235	0.001	0.065	0.241	0.003
0.129	0.186	0.003	0.102	0.193	0.010	0.005	0.165	0.003	0.154	0.233	0.002
S. cerevisiae 50% grape juice											
[NaOH]	**NaOH-blend**	**[KOH]**	**KOH-blend**	**Cleaner blend**	**Peracetic Acid**	**[H_2O_2]**	**[$KHSO_4$]**	**[$KHSO_4$] + [H_2O2]**	**[ClO_2]**	**growth check**	**sterility check**
0.006	0.005	0.006	0.022	0.007	0.037	0.005	0.002	0.002	0.004		
0.019	0.002	0.005	0.026	0.001	0.001	0.003	0.005	0.006	0.001	0.136	0.000
0.017	0.002	0.011	0.004	0.008	0.024	0.530	0.008	0.009	0.019	0.067	0.001
0.013	0.002	0.003	0.004	0.011	0.004	0.012	0.006	0.006	0.114	0.060	0.001
0.007	0.003	0.004	0.025	0.011	0.042	0.010	0.011	0.011	0.187	0.029	0.001
0.003	0.010	0.003	0.041	0.494	0.010	0.014	0.007	0.009	0.063	0.058	0.001
0.013	0.058	0.099	0.064	0.044	0.022	0.013	0.047	0.010	0.062	0.082	0.001
0.153	0.015	0.133	0.204	0.122	0.025	0.003	0.097	0.002	0.077	0.081	0.004
B bruxellensis broth											
[NaOH]	**NaOH-blend**	**[KOH]**	**KOH-blend**	**Cleaner blend**	**Peracetic Acid**	**[H_2O_2]**	**[$KHSO_4$]**	**[$KHSO_4$] + [H_2O_2]**	**[ClO_2]**	**growth check**	**sterility check**
0.002	0.000	0.085	0.001	0.007	0.002	0.014	0.001	0.001	0.001		
0.001	0.004	0.001	0.002	0.011	0.003	0.002	0.001	0.000	0.002		
0.001	0.001	0.002	0.001	0.005	0.006	0.014	0.001	0.001	0.010	0.003	0.001
0.001	0.001	0.001	0.001	0.006	0.004	0.010	0.001	0.002	0.007	0.019	0.006
0.001	0.001	0.002	0.004	0.002	0.003	0.023	0.001	0.001	0.004	0.031	0.005
0.001	0.003	0.002	0.001	0.002	0.000	0.023	0.003	0.003	0.010	0.044	0.003
0.002	0.001	0.001	0.001	0.001	0.004	0.069	0.014	0.005	0.035	0.018	0.001
0.002	0.002	0.002	0.001	0.001	0.000	0.019	0.264	0.003	0.023	0.021	0.002

Table 3.S.2. (Cont.)

[NaOH]	NaOH-blend	[KOH]	KOH-blend	Cleaner blend	Peracetic Acid	$[H_2O_2]$	$[KHSO_4]$	$[KHSO_4]$ + $[H_2O_2]$	$[ClO_2]$	growth check	sterility check
***B. bruxellensis* 50% grape juice**											
0.002	0.004	0.037	0.004	0.028	0.061	0.007	0.006	0.001	0.003		
0.001	0.002	0.122	0.006	0.032	0.003	0.017	0.004	0.005	0.004		
0.002	0.002	0.016	0.014	0.016	0.018	0.015	0.006	0.005	0.002	0.057	0.001
0.002	0.006	0.007	0.015	0.008	0.010	0.010	0.004	0.002	0.002	0.069	0.003
0.004	0.003	0.007	0.010	0.008	0.021	0.004	0.018	0.002	0.198	0.303	0.002
0.006	0.001	0.007	0.008	0.007	0.010	0.008	0.074	0.004	0.262	0.072	0.003
0.004	0.067	0.018	0.119	0.744	0.007	0.004	0.033	0.008	0.045	0.145	0.001
0.015	0.108	0.187	0.086	0.243	0.020	0.006	0.049	0.005	0.006	0.083	0.006
***Z. bailii* broth**											
[NaOH]	**NaOH-blend**	**[KOH]**	**KOH-blend**	**Cleaner blend**	**Peracetic Acid**	$[H_2O_2]$	$[KHSO_4]$	$[KHSO_4]$ + $[H_2O_2]$	$[ClO_2]$	**growth check**	**sterility check**
0.001	0.005	0.029	0.002	0.004	0.005	0.015	0.007	0.004	0.008		
0.003	0.005	0.028	0.014	0.011	0.014	0.013	0.015	0.009	0.005		
0.004	0.009	0.016	0.013	0.010	0.022	0.014	0.015	0.009	0.015	0.016	0.002
0.001	0.010	0.011	0.021	0.023	0.007	0.016	0.013	0.009	0.016	0.085	0.004
0.003	0.008	0.014	0.020	0.009	0.019	0.012	0.014	0.009	0.007	0.041	0.002
0.001	0.005	0.012	0.025	0.099	0.015	0.016	0.012	0.008	0.013	0.070	0.005
0.006	0.007	0.017	0.017	0.146	0.020	0.013	0.005	0.013	0.013	0.011	0.004
0.005	0.019	0.013	0.089	0.167	0.044	0.005	0.046	0.010	0.048	0.033	0.003
***Z. bailii* 50% grape juice**											
[NaOH]	**NaOH-blend**	**[KOH]**	**KOH-blend**	**Cleaner blend**	**Peracetic Acid**	$[H_2O_2]$	$[KHSO_4]$	$[KHSO_4]$ + $[H_2O_2]$	$[ClO_2]$	**growth check**	**sterility check**
0.001	0.005	0.029	0.002	0.004	0.005	0.015	0.007	0.004	0.008		
0.003	0.005	0.028	0.014	0.011	0.014	0.013	0.015	0.009	0.005		
0.004	0.009	0.016	0.013	0.010	0.022	0.014	0.015	0.009	0.015	0.016	0.002
0.001	0.010	0.011	0.021	0.023	0.007	0.016	0.013	0.009	0.016	0.085	0.004
0.003	0.008	0.014	0.020	0.009	0.019	0.012	0.014	0.009	0.007	0.041	0.002
0.001	0.005	0.012	0.025	0.099	0.015	0.016	0.012	0.008	0.013	0.070	0.005
0.006	0.007	0.017	0.017	0.146	0.020	0.013	0.005	0.013	0.013	0.011	0.004
0.005	0.019	0.013	0.089	0.167	0.044	0.005	0.046	0.010	0.048	0.033	0.003

CHAPTER 4: THE PERSISTENCE AND ANTIMICROBIAL ACTION OF SULFUR DIOXIDE IN WINE BARREL FUMIGATION

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4.1 Abstract:

Sulfur dioxide gas is ubiquitously used as a sanitizer and preservative for empty oak barrels in modern wineries, but the antimicrobial efficacy and diffusive behavior of sulfur dioxide fumigation is poorly understood. In this study, sixteen 225-L American oak barrels were used to measure the persistence and antimicrobial efficacy of the sulfur dioxide fumigation of empty winery cooperage. Pure gas was applied as 2.25 L and 4.5 L doses, respectively, and compared to the combustion of a five-gram sulfur stick. Prior to fumigation, barrel stave slices were inoculated with *Saccharomyces cerevisiae* and *Brettanomyces bruxellensis*. Samples were collected weekly to observe the penetration rate and abundance of cells at depth in the barrel wood over six weeks of inoculation. *S. cerevisiae* cells were cultured up to 8-10 mm depth in oak staves. *B. bruxellensis* cultures were largely limited to the 0-2 mm sample depth for all six weeks. After fumigation, sulfur dioxide measurements were collected every 48 hours for the first week and weekly thereafter for a total of six weeks of cellar storage. Results indicated that measurable concentrations of sulfur dioxide existed after six weeks of storage, with concentration profiles closely described by power law functions. Both gas applications and sulfur stick combustion effectively eliminated culturable populations of *S. cerevisiae* and *B. bruxellensis* on surface and depth samples. Scanning electron microscopy supported the observed limited penetration of *B. bruxellensis* versus *S. cerevisiae* at depth in oak staves. *B. bruxellensis* cultures expressed significant pseudohyphal features, forming biofilm-like masses that clog the barrel porosity and limit cell penetration.

4.2 Introduction:

Sulfur dioxide fumigation of empty wine barrels has been performed since antiquity, and the practice of burning sulfur-coated pieces of wood in barrels was first officially legally written into law in Germany in 1498[70]. The addition of sulfur dioxide to empty barrels is ubiquitous in the management of cooperage today, however the purpose of fumigation applications has seemingly shifted over time. Originally, sulfur would be burned in aging vessels just prior to filling with wine, with a primary goal of adding sulfur dioxide to the liquid in the process to arrest the fermentation. Sulfur was also burned in partially filled barrels for the same goal and to prevent headspace oxidation[69]. It is only in the late 19th century where fumigation would be explicitly used to protect empty wine barrels from microbial spoilage[29] and the impact of sulfur dioxide gas as a sanitizer for microbes embedded in the wood has only been considered since the late 20th century[71]. That process continues today, and sulfur dioxide is nearly universally added to empty wine barrels as a sanitizer and to prevent spoilage in long-term empty storage.

Fumigation is conducted either via the combustion of solid sulfur or by the addition of pure sulfur dioxide from a compressed gas cylinder. While the use of solid sulfur clearly predates pure gas application, both processes are still commonly used. Compressed gas use often requires additional licensing or permitting as well as equipment costs which partially explain the persistence of solid sulfur dosing today. Recommended dosing protocols for both methods of fumigation are readily available across institutional outreach organizations and certification boards, and there is considerable overlap in the recommendations[122,123]. Solid sulfur is available as five- or ten-gram sticks or disks of sulfur bound to a cotton or paper support, and these are simply ignited inside an empty barrel using a specialized holder to catch and allow the resulting ashes to be removed. Sulfur dioxide gas is produced as the solid sulfur combusts, consuming

some of the headspace oxygen in the process. Compressed gas cylinders are operated using attached spray wands which are inserted into barrels and sprayed for up to five seconds under an operating pressure of 15-45 psi[124]. Redosing is typically recommended at three to four-week intervals regardless of application type. The purpose of reapplication is to maintain antimicrobial concentrations of sulfur dioxide in the barrel headspace by replacing gas that has diffused from the barrel or is lost due to interaction with the barrel wood.

The antimicrobial properties of sulfur dioxide are only significant when the molecule exists as an uncharged species (SO_2), often referred to as "molecular" sulfur dioxide[49]. While gaseous sulfur dioxide will remain in the molecular form, aqueous sulfur dioxide exists in a pH-dependent equilibrium and the non-antimicrobial bisulfite ion form (HSO_3^-) outnumbers the molecular form above pH = 2. The saturation rate and profile in barrel wood with fluids is well studied[59], but the importance of the pH of the saturating fluid in the context of sulfur dioxide fumigation is rarely considered. Barrels that have stored wine with a higher pH or are saturated with winery process water may be more difficult to sanitize than barrels that have stored more acidic must or wine, so the composition of the liquid stored in the barrel may impact the efficacy of fumigation applications.

Because wood is porous, spoilage microorganisms are capable of penetrating into the structure of barrel staves. Sulfur dioxide must be capable of diffusing through the stave to effectively inactivate embedded cells, either in a gaseous state or as molecular SO_2 in aqueous equilibrium. The depth at which the spoilage yeast *Brettanomyces bruxellensis* is capable of penetrating barrel wood during extended storage in the maturation of wine is variably quoted between four and ten millimeters[62,63]. The depth and rate at which the commonly inoculated fermentative yeast *Saccharomyces cerevisiae* is capable of penetrating oak barrel wood in the

fermentation of must is unstudied, however. The microbial load embedded in the barrel staves is expected to be dominated by *S. cerevisiae* in the case of barrel fermentation or in the maturation of unfiltered wine post fermentation in practical settings. Therefore, a meaningful investigation of the impact of routine fumigation operations should evaluate the interaction of *S. cerevisiae* with barrel wood in the fermentation of grape juice. Methods for recovering treated stave pieces must also be capable of recovering cells below the surface of the wood to accurately assess the antimicrobial impact of treatments.

Despite the ubiquity of sulfur dioxide in barrel fumigation the process is poorly understood. The antimicrobial impact of sulfur fumigation has been studied[4,62], however these studies have relied on naturally contaminated barrels for samples and arrived at conflicting results. One major limitation in the analysis of barrel sanitation is the variability in the natural materials involved. The structure and porosity of stave wood can vary considerably based on numerous factors, including the species of oak, forest of origin, cooper, and toast level[59]. The composition of fermenting juice or aging wine also varies considerably by varietal, season, and a host of other factors. The variable composition of the barrel structure and liquid undoubtedly impact the abundance and behavior of cultures embedded in the wood during storage. The differences in starting materials, fermentation and maturation conditions, and microbial diversity may explain the discrepancy in the results of studies using naturally contaminated barrels. Contamination was sensorially detected by winery staff without specific knowledge of the microorganisms present, and the sanitation tests were also subject to prior cleaning by winery staff which may have created considerable additional variability in the sample pool. These studies also only investigated solid sulfur sticks as fumigants, and any other studies involving sulfur dioxide and barrel wood have used aqueous solutions of potassium metabisulfite as a

sanitizing medium[47]. The comparative antimicrobial performance of pure gas fumigation remains unstudied.

The initial dosage and headspace concentration of sulfur dioxide during storage is also poorly understood. Post-fumigation concentrations of sulfur dioxide in barrel headspace have been published[71], but the text lacks the detail required to fully understand the experimental design. More importantly, the titrimetric method used in this study has a high limit of detection that is easily improved upon with current technology and may have resulted in a large underestimate of the headspace sulfur dioxide concentration after thirty days of storage. Headspace gas detection tubes have been demonstrated as an effective tool for measuring sulfur dioxide concentrations in enological settings[77]. These colorimetric detectors have been traditionally employed in environmental safety applications but are well suited to measure the headspace concentration inside a wine barrel.

A renewed approach using controlled inoculation conditions, a more uniform barrel environment, and a comparison between pure gas and solid sulfur fumigation is clearly needed as the basis for assessing the impact of practical fumigation operations. In this study, the ability of *S. cerevisiae* and *B. bruxellensis* cultures to penetrate American oak barrel staves was assessed using traditional culture methods and SEM imaging. Inoculated staves were used to assess the antimicrobial impact of sulfur fumigation by solid sulfur combustion and pure gas application. The dosage and persistence of sulfur dioxide over six weeks of barrel storage was measured using colorimetric gas detection tubes. Ultimately this work seeks to empower winemakers to make more informed decisions in designing fumigation protocols and redosing intervals, and to better understand how yeast interact with barrel porosity in the fermentation of grape musts.

4.3 Materials and methods

4.3.1 Penetration and abundance of S. cerevisiae and B. bruxellensis in barrel staves

a) Stave inoculation

New medium-toast American oak (*Q. alba*) barrel staves from Cooperages 1912 Napa (California, USA) were first vertically sectioned into 2.5cm slices using a miter saw from Ryobi Ltd. (Hiroshima, Japan). A sampling guide was drawn on the cut side of the wood pieces to aid in future sample recovery. The barrel stave pieces were sealed on all sides except the inner face normally in direct contact with wine during storage using chemical and heat resistant silicone from Dow Chemical Co. (Michigan, USA) to prevent liquid and gas penetration in all other orientations.

Red grape concentrate from Global Vintners Inc. (Ontario, Canada) was first diluted to 24° Brix as measured by a handheld density meter from Anton Paar (Vienna, Austria) using municipal water. The pH of the juice was adjusted to 3.4 using tartaric acid from Cellar Science (California, USA). The grape juice was centrifuged at 5000 rpm for 10 minutes and sterile filtered to 0.22 µm using a disposable vacuum filter unit from Corning Inc. (New York, USA). Inoculum of *Saccharomyces cerevisiae* (ID: *UCD VEN 777*)and *Brettanomyces bruxellensis* (ID: *UCD VEN 2041*) were obtained from the UC Davis Viticulture and Enology culture collection and prepared separately in YM nutrient broth from BD Biosciences (California, USA). The cells were centrifuged and washed three times before resuspending in the grape juice medium to an optical density of 0.5 at OD_{600nm} as measured by UV-vis spectrometer (Shimadzu, Japan). Stave pieces were placed face-down in 0.5-L replicate glass containers from Corning, Inc. (New York, USA), and 350 mL of inoculated grape juice medium was added to each fermentor. Ten replicate glass containers were used for each species.

b) Cell recovery

Stave pieces were recovered weekly after inoculation for five weeks. An additional set of stave pieces was soaked for a sixth week to be used as a control in barrel fumigation tests. Five stave pieces were sampled for each yeast species per week. Samples were removed from the inoculation medium and rinsed with sterile water to remove loosely adhered surface cultures and fermentation soil. The sealed sides of the stave were wiped with 140 proof ethanol from Decon Labs Inc. (Pennsylvania, USA) and the silicone was removed using a sterilized scalpel.

To sample the barrel wood, stave pieces were first mounted on a drill press vise from WEN (Illinois, USA). Using a woodworking chisel, sweep gouge, and mallet from Pfeil Tools (Langenthal, Switzerland), 20 * 25 * 2 mm sections were chipped from the stave. A sterilized foil apron was used as a barrier between the vice and to catch the chipped samples. The samples were placed in sterile 50mL test tubes from Corning Inc. (New York, USA) before adding 25mL of recovery medium. The recovery medium was prepared as YM broth supplemented to 2% ethanol, and samples were incubated for 48 hours in the recovery medium at 30°C and 180 rpm rotation in a benchtop incubator/shaker from VWR International (Pennsylvania, USA). Samples were vortexed and diluted as necessary for culture counting prior to plating on YM agar. Plates were incubated for 48 hours at 30°C before obtaining cell counts.

4.3.2 Scanning electron microscopy of inoculated stave pieces

Scanning electron microscopy (SEM) of barrel stave pieces was conducted using a Thermo Fisher Scientific Quattro ESEM (Massachusetts, USA) operated under low vacuum mode at a 20.0 kV accelerating voltage. Stave pieces were inoculated as described previously for six weeks. Samples for imaging were collected using the same procedure as above, except the samples were limited to 10 * 10 * 1mm to facilitate imaging. Surface (top 1 mm), middle (4 mm

depth), and deep (8 mm depth) samples were imaged for *B. bruxellensis* and *S. cerevisiae* inoculated staves, respectively.

4.3.3 Persistence and antimicrobial action of fumigation treatments

a) Preparation of barrels and treatment

Sixteen new, unsulfured medium toast 225-L American oak barrels constructed from the same oak as used in the stave penetration experiment were used for fumigation. Barrels were stored at 70% relative humidity and 13.7ºC for the duration of the study. Inoculated barrel stave pieces were added to the barrels prior to hydration by removing and re-sealing the barrel heads. Two *S. cerevisiae* and two *B. bruxellensis*-inoculated stave sections were mounted upright in each barrel for treatment using the six-week incubated samples described in *Section 4.3.1a*. Additionally, five samples of each species were added to an untreated control barrel as an untreated control. The stave pieces were secured to the inner surface of the barrel using stainless steel wire prior to resealing the barrel. Barrels were filled with winery process water to hydrate and allowed to soak for 24 hours prior to draining to swell and ensure no leaks were present.

After draining for two hours the barrels were treated with sulfur dioxide. Five barrels were used per treatment. Sulfur dioxide was added either as pure compressed gas from Airgas Inc. (Pennsylvania, USA) or by the combustion of a solid sulfur stick from The Vintner's Vault (California, USA). The treatments were as follows: 2.25 L pure SO_2 gas, 4.5 L pure SO_2 gas, and combustion of a five-gram sulfur stick. The control barrel was left unsulfured. Pure gas applications were conducted using a custom flow meter, needle assembly, and a specialized silicon bung engineered to act as a self-sealing septum after piercing with the sulfur application needle from Airgas Inc. (Pennsylvania, USA) . Barrels were sampled over six weeks of storage.

b) Measurement of sulfur dioxide, sample recovery

Headspace sulfur dioxide concentrations were measured using colorimetric gas detection tubes from Drägerwerk AG (Lübeck Germany)[77,81]. The tubes were directly inserted through the silicone barrel bungs to take measurements and leveraged the self-sealing properties of the barrel bung to create an air-tight fit between the tube and bung. An unbroken tube was kept in the bung between samples to eliminate loss of sulfur from the sampling setup in between measurements. Measurements were taken at 48-hour intervals for the first week, starting 24 hours after fumigation. Samples were taken weekly thereafter. After six weeks, the stave piece samples were recovered in the same manner as before after removing the barrel heads to access the samples.

4.3.4 Data handling

Culture data were obtained by the standard plate count method[125]. Sulfur dioxide concentration data were processed and analyzed using XLSTAT software (Addinsoft, France). Error is calculated as standard error for culture data and sulfur measurements in all cases.

4.4 Results

4.4.1 Penetration and abundance of S. cerevisiae and B. bruxellensis in barrel staves

To assess the comparative penetration rate and abundance of *S. cerevisiae* and *B. bruxellensis* in American oak staves, stave pieces were soaked in inoculated must for six weeks and recovered weekly. It is important to recognize that *B. bruxellensis* contamination is typically associated with finished wines with elevated ethanol levels and limited residual fermentable sugars versus unfermented grape juice. *B. bruxellensis* cultures may also exhibit different penetration behavior in the context of aging wines. Unfermented grape juice was used as an inoculation medium to facilitate comparison with *S. cerevisiae* in this study, but the physiology of both yeasts may differ in the context of aging wine. Cell penetration data are shown in **Figure**

4.1 and *B. bruxellensis* and *S. cerevisiae* recovery are graphed separately to emphasize the different behavior of the two yeasts. *S. cerevisiae* cells penetrated to the deepest sample level (8-10 mm) after one week of inoculation, with fewer cells recovered in progressively deeper samples. Viability was highest in the first week, and generally decreased in subsequent weeks of sampling. *B. bruxellensis* cells were largely only recovered in surface samples. Cells were recovered in one 2-4 mm sample after two weeks of inoculation, however in very low numbers that are not visible on the scale of **Figure 4.1**. *B. bruxellensis* viability on surface samples decreased at similar rate to *S. cerevisiae* cultures over successive weeks of sampling.

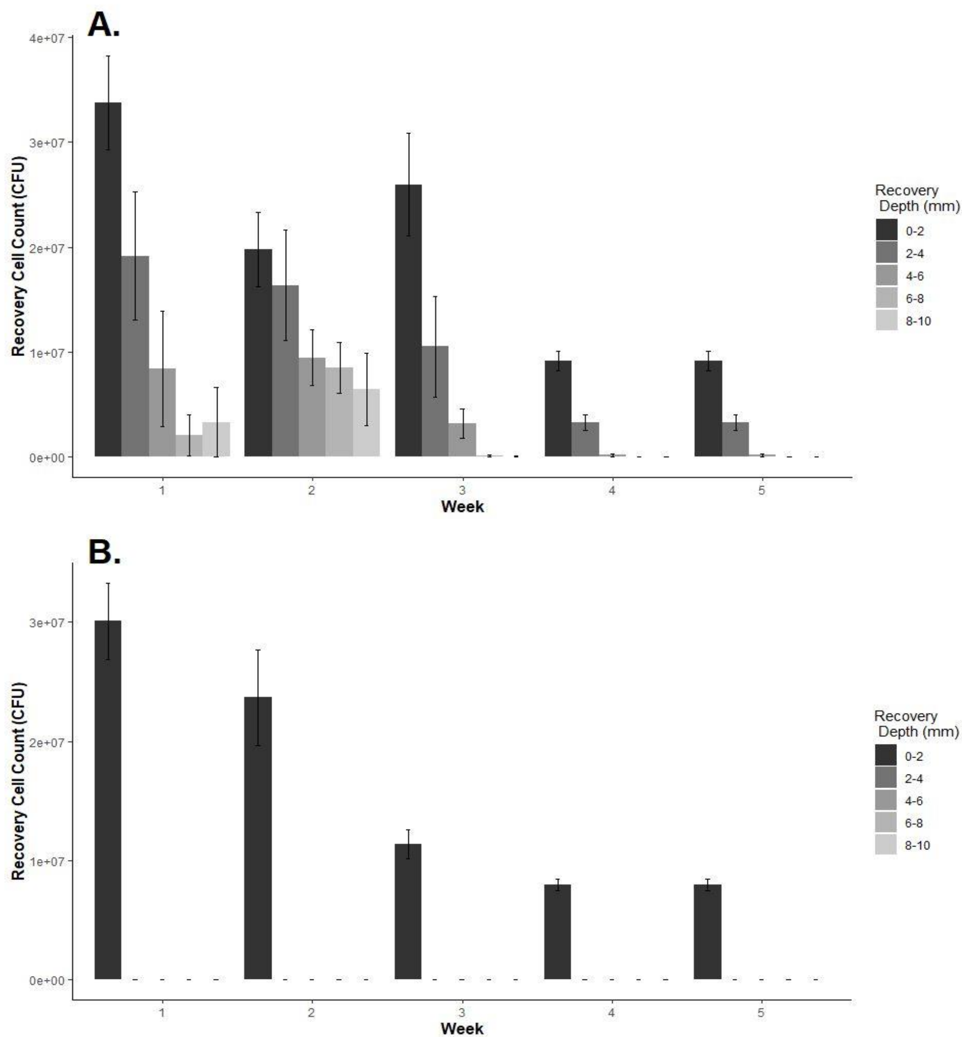


Figure 4.1. Cell penetration in barrel stave wood by recovery depth and week of inoculation. **A.** Penetration of *Saccharomyces cerevisiae* **B.** Penetration of *Brettanomyces bruxellensis*. Error bars represent standard error of the mean (n = 5).

4.4.2 Scanning electron microscopy of inoculated stave pieces

SEM imaging was used to investigate the physiology of *S. cerevisiae* and *B. bruxellensis* cultures embedded in barrel wood. *S. cerevisiae* and *B. bruxellensis* cultures exhibited dramatically different physiologies on barrel samples (**Figure 4.2**). *S. cerevisiae* cultures existed as separate cells scattered among the barrel pores (**Figure 4.2A**). *B. bruxellensis* cultures formed substantial extracellular structures (**Figure 4.2B**). Linear pseudohyphae were visible among the extracellular matrix and are clearly identifiable at higher magnifications (**Figure 4.2C**). *B. bruxellensis* appeared to form biofilm-like mats on surface samples that occluded barrel porosity.

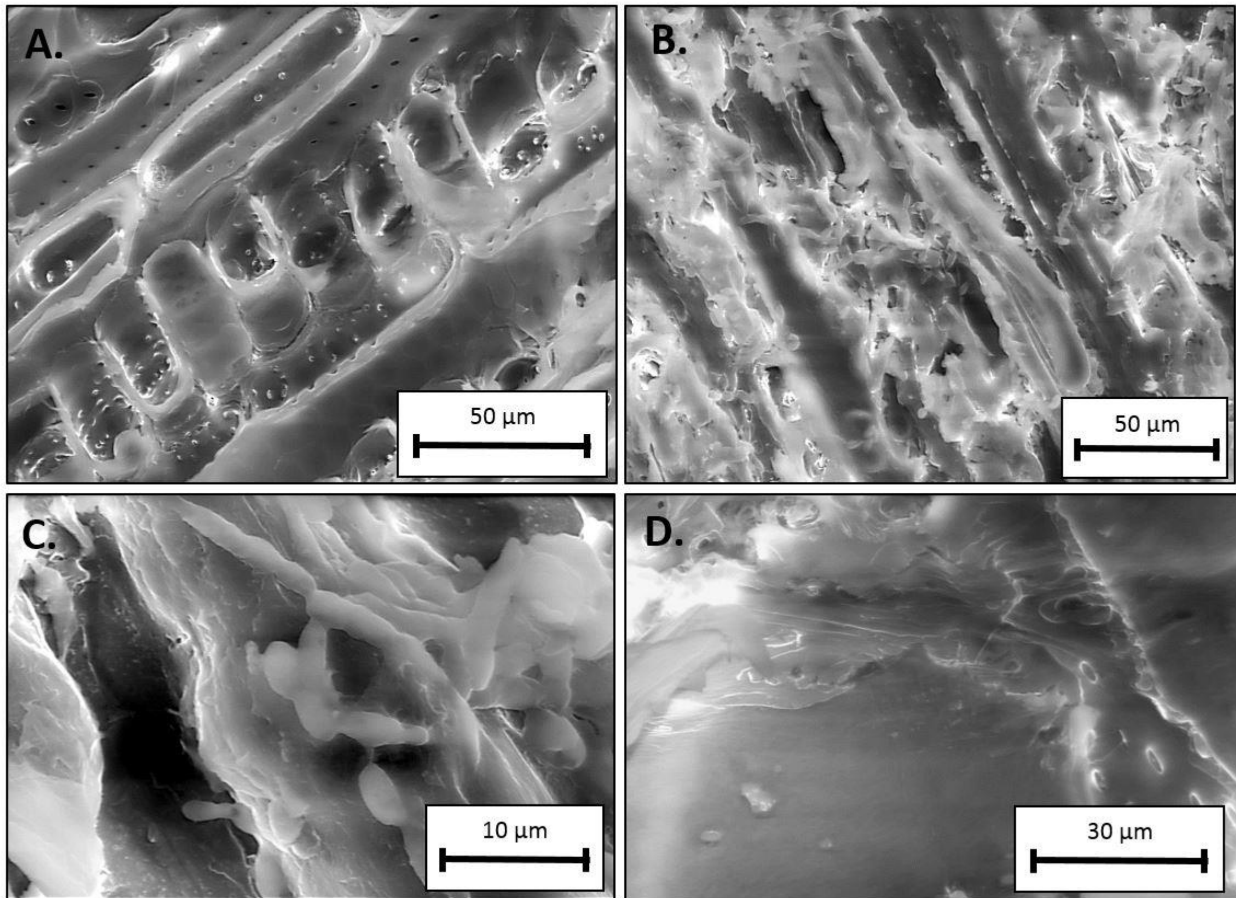


Figure 4.2. Contrasting physiologies of *Saccharomyces cerevisiae* and *Brettanomyces bruxellensis* in American oak barrel wood. **A.** *S. cerevisiae* cells scattered among larger wood pores on wood surface. Colony exists as separate cells inhabiting the wood. **B.** *B. bruxellensis* colonies form extensive elongate pseudohyphal structures and exist in a tangled extracellular matrix that occludes surface porosity. **C.** Close up of *B. bruxellensis* cells and pseudohyphae. **D.** 8 mm depth sample of *B. bruxellensis*-inoculated staves. No cells are found.

Table 4.1. Mean cell recovery from control staves in unsulfured barrel (n = 5). No cells were recovered from stave pieces in sulfured barrels.

Recovery Depth (mm)	*S. cerevisiae*		*B. bruxellensis*	
	Cell recovery (CFU/mL)	Standard error (%)	Cell recovery (CFU/mL)	Standard Error (%)
0-2	193600	26.9	138800	31.1
2-4	7280	38.7	0	0
4-6	480	89.9	0	0
6-8	0	0	0	0
8-10	0	0	0	0

SEM imaging strongly supported culture data, as no *B. bruxellensis* cells were detected below the surface samples (**Figure 4.2D**). The extracellular structure of *B. bruxellensis* cultures may have limited the depth at which cells were found.

4.4.3 Persistence and antimicrobial action of fumigation treatments

To measure the antimicrobial impact and persistence of sulfur dioxide gas in barrel fumigation, inoculated staves were loaded into barrels prior to fumigation and recovered after six weeks of cellar storage post treatment. Sulfur dioxide gas concentrations were measured using Dräger gas detection tubes. After six weeks of inoculation and six weeks of storage in an unsulfured barrel, the viability of both *S. cerevisiae* and *B. bruxellensis* decreased significantly in control samples versus the five-week inoculated samples in *Section 4.3.1.b* (**Table 4.1**). No cells were recovered in any samples from treated barrels for either yeast species, implying that all three fumigation treatments were successful in eliminating cell viability in the inoculated staves. In the unsulfured barrel, *S. cerevisiae* cultures were not recovered at 6 mm or deeper in any samples. *B. bruxellensis* were limited to surface samples, as in the inoculation trials described previously. Even with significantly reduced cell concentrations the yeasts pose a substantial threat of contamination[126]. As few as 10^3 cells/mL of *B. bruxellensis* contamination can produce detectable sensory faults in finished wine[127].

Measurable sulfur dioxide was detected in all fifteen study barrels over the treatment period. The burning of a five-gram sulfur stick and a 4.5 L dose of pure gas provide similar levels of headspace sulfur dioxide throughout storage (**Figure 4.3**). After 42 days of storage, the 2.25 L dose of sulfur dioxide resulted in free sulfur levels below $2.73 * 10^{-2}$ mg/L (10 ppm), which may warrant re-dosing. Sulfur dioxide concentration profiles during storage were closely described by power law functions using linear regressions of log-log transformed data.

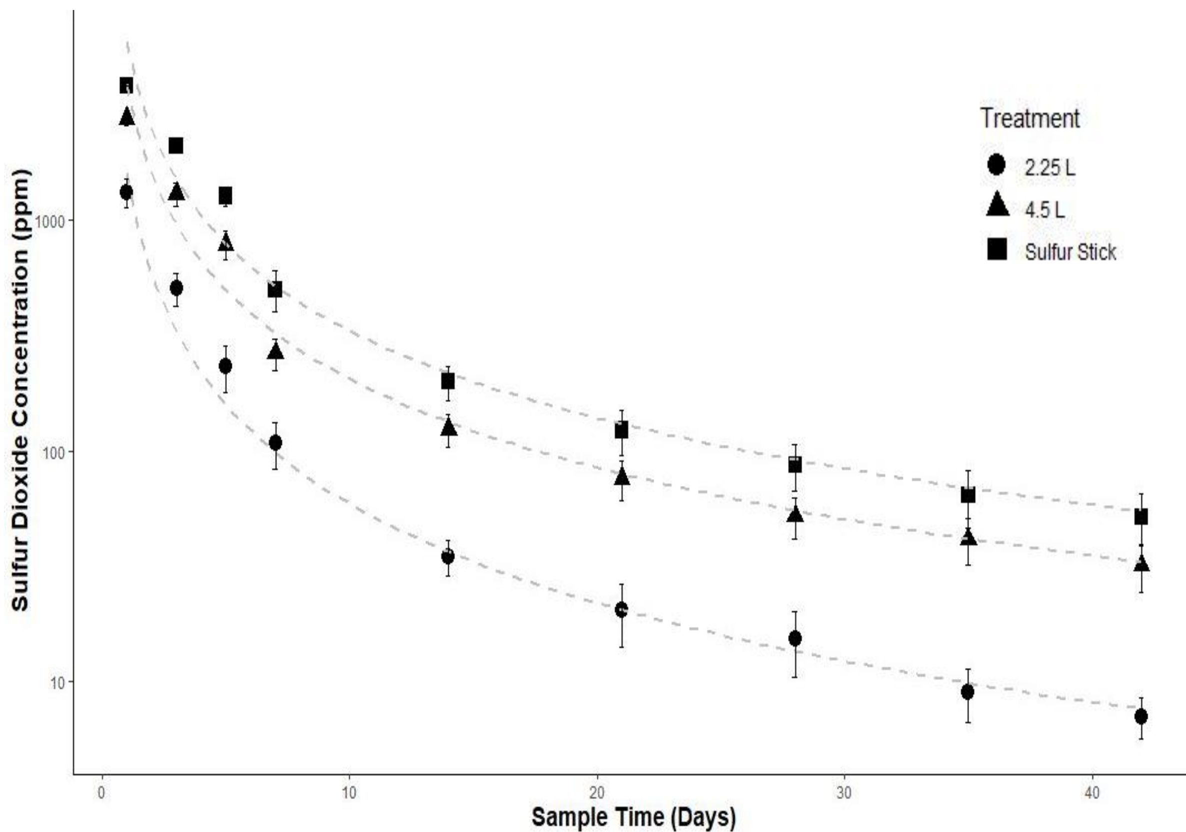


Figure 4.3. Concentration of sulfur dioxide measured over six weeks post-barrel fumigation for three different fumigation treatments. For each treatment n = 5 barrels. Dashed lines represent power law fit of results. Error bars represent standard error of the mean. $\mathbf{y}(\text{Sulfur Stick}) = 5935.9\mathbf{x}^{-1.252}$, $R^2 = 0.99$; $\mathbf{y}(2.25\text{ L}) = 1584.9\mathbf{x}^{-1.428}$, $R^2 = 0.99$; $\mathbf{y}(4.5\text{ L}) = 3871.1\mathbf{x}^{-1.275}$, $R^2 = 0.98$.

4.5 Discussion

S. cerevisiae and *B. bruxellensis* cultures displayed radically different behavior on and in barrel wood staves under the experimental conditions. *S. cerevisiae* cultures were able to quickly penetrate the barrel wood at depths up to 8-10 mm, whereas *B. bruxellensis* cultures were only found on surface samples in all but one sample throughout the experiment. The rapidity at which *S. cerevisiae* were able to penetrate to the deepest sample is somewhat surprising. Wine has been shown to penetrate barrel wood over a period of approximately 40 days, reaching an equilibrium between evaporation from and transport into the barrel wood at that stage[128]. Liquid penetration depth is often used as a proxy for microbe depth[129] so it is unexpected that *S. cerevisiae* would be present at the deepest sample after only one week of inoculation. Previous studies of yeast penetration in barrel wood have involved barrels that have stored finished wine as sample material, so the penetration behavior observed in this study could be partially attributed to the use of unfermented grape juice as a medium for inoculation. Because *S. cerevisiae* is not normally associated with spoilage there have been no studies on *S. cerevisiae* penetration in barrel wood specifically, so this behavior is previously unidentified. In aerobic and semi-aerobic environments, however, *S. cerevisiae* can produce significant quantities of acetic acid levels and result in off aromas inside barrels[130]. *S. cerevisiae* can therefore be a real spoilage threat and should not be ignored in barrel sanitation efforts, especially given the rapid and abundant penetration of the cells in the barrel wood observed in this study. *S. cerevisiae* may represent the most difficult target for sanitation, but off aromas produced by *S. cerevisiae* in an aerobic environment can be confused with faulty aromas from the spoilage genus *Acetobacter*[131], so this threat is often unrecognized. .

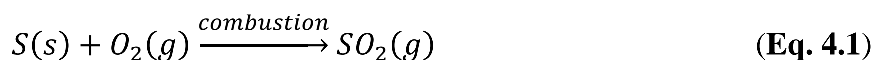
In contrast, *B. bruxellensis* is the most frequently implicated spoilage yeast for oak cooperage due to the production of unpleasant volatile phenols under aging conditions that can easily be identified[132]. *B. bruxellensis* has been cultured up to 9 mm deep in barrel wood in previous studies[64] but was limited to surface samples under the study conditions in this report. Several factors may explain this difference. First, these studies all involved substantially longer contact times between the liquid and wood and used finished wine with residual sugar as an inoculation medium[62,63,64]. It may be that fluid penetration depth is a poor proxy for microbe depth and instead cells are able to slowly migrate through the wood over a year or more of storage. Since these studies typically rely on naturally contaminated barrels for samples, interspecies competition may also have caused *B. bruxellensis* cells to exhibit different behavior than might be expected in monoculture. In fact, the only other study using a *B. bruxellensis* monoculture inoculum only recovered cells from the shallowest (0-4 mm) samples in American oak barrels using a supplemented wine medium[63]. Oak species may also play an important role in how far yeast can migrate through barrel wood. American oak contains abundant thyloses, outgrowths in the wood's cellular structure that run tangential to vessels and occlude porosity. American oak barrels can be sawn without following the medullary rays of the wood structure because the thyloses create a closed, liquid-tight cellular structure in multiple orientations[133]. It is argued here that the orientation of barrel staves relative to the medullary rays and presence of abundant thyloses may be a cause of the limited penetration of *B. bruxellensis* relative to studies utilizing French oak. It is also important to consider that the ability of *B. bruxellensis* yeasts to form biofilms has been demonstrated to be a strain-dependent phenomenon[19]. The limited penetration and pseudohyphal structuring observed in this study may not be observed with other

strains of *B. bruxellensis* in practical settings if the strain is incapable of forming adherent colonies in enological conditions.

SEM proved to be a useful tool for observing the physical relationship of cells and barrel wood. The two yeast species displayed vastly different colony morphologies on the wood surface (**Figure 2**). SEM imaging directly supported results from culture count data. SEM is a vital complement to culture methods, especially in the case of *B. bruxellensis* cells. *B. bruxellensis* are frequently implicated as entering a viable but not culturable state in the presence of environmental stressors, and this has been shown to occur specifically in the case of sulfur dioxide exposure[134]. Culture methods therefore cannot be used alone for determining the absence of cultures at depth and imaging is a direct way of obtaining that information. Imaging also provides an explanation for why *B. bruxellensis* are only found on surface samples. *B. bruxellensis* are frequently implicated for forming biofilms under enological conditions, sessile colonies of cells embedded in an exopolysaccharide matrix that confers additional resistance to antimicrobials[19]. Biofilms are characterized by pseudohyphal structures that create a web-like network on surfaces. Previous research has suggested *B. bruxellensis* may form pseudohyphal structures in barrel wood, and this study provides clear evidence of the formation of a robust extracellular network on the barrel surface samples. While this structure may be a stress response or protective mechanism for the *B. bruxellensis*, it may also clog barrel porosity and be a reason cells are not found deeper in the wood.

A 4.5 L dose of pure SO_2 gas produced roughly the same headspace concentration profile as the combustion of a five gram sulfur stick over six weeks of cellar storage (**Figure 4.3**). While the SO_2 concentrations produced by these methods are similar, several factors must be taken into consideration for the appropriate selection of a fumigation method. Pure gas application does not

require any combustion, and dosage can be carefully controlled using mass flow meters. However, pure gas cylinders must be safely secured and stored, and application requires specialized equipment. In some cases, purchasing and applying pure SO_2 gas from compressed cylinders is also subject to government licensing and audit[72]. On the other hand, solid sulfur strips are compact, inexpensive, and not subject to legislative control. When solid sulfur sticks are ignited, the combustion process consumes oxygen in the reaction:



$$S(s) + O_2(g) \xrightarrow{combustion} SO_2(g) \qquad \textbf{(Eq. 4.1)}$$

Two moles of oxygen are consumed per mole of sulfur dioxide, decreasing the molecular oxygen content of the barrel headspace significantly. This creates an aerobic environment that may add increased antimicrobial pressure obligate aerobes such as *Acetobacter* spp.[26]. Solid sulfur wicks must be stored in a dry environment, as studies have shown atmospheric moisture is capable of reducing the efficacy of solid sulfur fumigants significantly[69]. Solid sulfur wicks also contain cotton and other structural components that could produce side products during the combustion reaction. The results of this study indicate that solid sulfur combustion and pure gas application are both capable of successfully eliminating spoilage yeasts in wine barrels. Wineries can therefore choose application methods based on practical considerations such as cost, employee safety, and operational logistics while maintaining adequate antimicrobial protection in barrel headspace.

Our investigation into the persistence of sulfur dioxide in barrel fumigation indicated that measurable sulfur dioxide was present in all test barrels after six weeks of storage, with sulfur dioxide concentrations closely described by power law functions using a linear model to fit log-log transformed data. Power law functions are often derided by statisticians, as many real-world

processes can be fit to functions of the form $p(x) \propto x^{-\alpha}$. Many of these apparent power law relationships fall apart under closer scrutiny of more advanced nonlinear statistical models. This is important in population distribution or frequency studies, as log-log transformation can result in a skewed error distribution for the highest frequency observations and result in an artificially superior fit for the tail of the distribution. This supposed weakness in the log-log approach is argued for in this instance because a superior geometric fit is obtained to the tail of the function that is of most interest versus nonlinear regression models. In the context of wine barrel fumigation and redosing intervals, this fit allows for a better prediction for how long it would take to reach a certain lower limit of free sulfur dioxide in the headspace.

The power law relationship between storage time and sulfur dioxide concentration is important for two main reasons. First, the regression gives predictive power for SO_2 levels in the headspace as mentioned above. For instance, if the sulfur stick data is extrapolated to determine when an average of $2.73 * 10^{-2}$ mg/L (10 ppm) sulfur dioxide remains in the barrel it is predicted that this level would not be reached until more than 150 days of storage—far longer than the commonly stated recommendations. Second, the power law relationship gives some insight into the mechanism of SO_2 diffusion from the barrel. It has been shown that oxygen transport though a barrel is governed by Fickian diffusion, meaning gas loss is governed by the concentration difference between the barrel and surroundings and a so-called Fickian diffusion coefficient[135]. In contrast, power law relationships describe a non-Fickian process termed anomalous diffusion where the gas loss is governed by an additional, constantly changing external variable. It is argued here that the moisture content and heterogeneity of the medium are drivers of the anomalous behavior. The complex cell structure of barrel wood and natural variation in the tightness of staves could introduce non-Fickian diffusive behavior. The abundant tyloses in

American oak wood create pockets of nearly zero gas permeability, introducing significant heterogeneity to the diffusion medium[136]. Gas diffusion through oak has been shown to exponentially decrease with increasing moisture content[137], and the process of swelling the barrel prior to fumigation means the moisture content would decrease from saturation to eventually meet the humidity of the room during storage. It is likely the changing moisture content in the wood is driving the anomalous behavior, and sulfur dioxide diffusion from barrel wood may otherwise be a Fickian process[59].

All three fumigation treatments used in this experiment were effective in eliminating the culturability of *S. cerevisiae* and *B. bruxellensis* in all samples. This is an important finding, but it must be put into proper context of winery operations. New barrels were used in this experiment and the incubated stave pieces were rinsed of any visible soil, meaning the organic load in the barrel is lower than what might be expected in a barrel after aging wine for an extended period. The findings here can directly be applied to the storage of new barrels received at the winery, but successive reuse of a barrel may clog porosity or allow biofilms to become more robust if not scrupulously cleaned. The organic load could act as a physical barrier for sulfur dioxide diffusion or could react with the sulfur dioxide directly, leading to a loss of efficacy. Different oak species, cooperage, and toast levels are also expected to affect sulfur dioxide levels in storage, so it is encouraged that wineries establish baselines for their own barrel program and operations. Colorimetric gas detection tubes are an inexpensive capital investment and can help establish quantitative support for developing more efficient and accurate sulfur fumigation protocols. Ultimately, this work is an important step in understanding the efficacy of sulfur dioxide fumigation under controlled conditions, and how different yeast species can interact with barrel wood in very different ways. Winemakers should think critically about barrel cleaning and

sanitation, emphasizing thorough cleaning prior to any fumigation or other sanitation effort. Redosing intervals can likely be greatly increased while retaining free sulfur dioxide, but wineries must invest the time and effort to obtain baseline data for their cooperage to accurately determine redosing protocols.

CHAPTER 5: THE DIFFUSION OF SULFUR DIOXIDE GAS FROM WINERY COOPERAGE DURING CELLAR STORAGE

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5.1 Abstract

Sulfur dioxide is a widespread fumigant used to preserve empty wine barrels, yet the diffusive behavior of gaseous sulfur dioxide from barrel headspace remains largely unstudied. Modern detection methods are well suited to measuring sulfur dioxide gas in enological conditions. In this study, the sulfur dioxide detection performance of colorimetric gas detection tubes, gas chromatography-sulfur chemiluminescence detection, and a novel electrochemical sensor apparatus was assessed. The sensor apparatus was used to measure the loss of sulfur dioxide from barrel headspace for three American oak wine barrique over twenty days of dry storage. The electrochemical sensing apparatus was superior in linearity and precision versus the other detection methods during calibration trials. Sulfur dioxide loss curves were closely described by power law regressions. The mechanism of sulfur dioxide loss was analyzed by modeling the barrel as a semipermeable membrane according to Fick's laws of diffusion. The approximation by this method poorly fits the measured gas data. Interaction between the diffusing gas and the barrel and heterogeneous structure of that barrel may explain the discrepancy between observed and predicted diffusive behavior. The electrochemical sensor module presents an inexpensive and highly sensitive tool for sulfur dioxide measurement with the potential for numerous applications in the wine industry.

5.2 Introduction

Sulfur dioxide fumigation of winery cooperage is a ubiquitous practice for preventing spoilage in both new and used empty barrels. In this context, sulfur dioxide acts as an antimicrobial and antioxidant, preventing the proliferation of undesirable microorganisms and aromatic compounds that could negatively impact infill wine[28]. Sulfur dioxide is periodically re-applied throughout storage to replace gas that is lost to the surrounding environment from the headspace of the barrel. Recommendations for application and re-dosing protocols are widely available, and in some cases are subject to local government guidelines[72].

Despite the uniquity of this process, a lack of quantitative support exists to explain how sulfur dioxide is lost from the barrel headspace. In the late twentieth century, an experiment was conducted to measure the loss of sulfur dioxide in French oak barrels during storage[71]. The authors concluded that sulfur dioxide applied via the combustion of a ten-gram solid sulfur 'disk' would dissipate after roughly thirty days of empty storage and could not be detected thereafter. Despite lacking the detail necessary to fully understand the experimental methods and design this remained the only published data on the process for decades and may have influenced many of the protocols still in use today.

The situation has changed, however, as an assessment of the dosage, persistence, and antimicrobial impact of common fumigation protocols in barrels under cellar storage conditions using more-sensitive modern detection methods was recently conducted (**Chapter 4**). The authors showed that measurable sulfur dioxide persists well beyond thirty days of storage, with gas loss profiles described by power-law functions. Because this study was designed to mimic typical winery protocols, barrels were initially hydrated before fumigation. The initially saturated barrel interior slowly dries during storage. The role of moisture content on gas diffusivity in

barrels has been investigated for oxygen gas, and diffusivity is reported to exponentially decrease with increasing moisture content[85,138]. As a barrel dries, diffusivity is therefore expected to change drastically over time. Sulfur dioxide is readily absorbed into solution where the compound enters a pH-dependent equilibrium between ionic and molecular species, further complicating the diffusive behavior of sulfur dioxide though a barrel[139]. As a result, the authors postulated that the power-law behavior of sulfur dioxide may be indicative of an anomalous diffusive process owing to changing moisture content and the behavior of aqueous sulfur dioxide.

Under conditions of constant temperature and equilibrium moisture content, oak barrel oxygen permeability has been studied by modeling the barrel wood as a semipermeable membrane and invoking Fick's laws of diffusion[59,84,88]. Fick's laws state that the rate of uniaxial diffusion through a unit area of the membrane is proportional to the concentration gradient normal to that section, and can be expressed as

$$J = -D\frac{\partial C}{\partial x} \qquad \textbf{(Eq. 5.1)}$$

Where J is the rate transfer of substance, or flux, though the unit area, C is the concentration of diffusant, and x is the length of the diffusion pathway. The proportionality constant D is referred to as the diffusion coefficient, and describes the fundamental relationship between the driving force of diffusion (the concentration gradient on either side of the membrane) and the actual transfer of mass through the diffusion pathway (the flux)[140].

Discreet solutions to the diffusion equation can be obtained under certain conditions, often by use of the time-lag method or steady-state simplification[86]. In this experiment, the methods employed for oxygen diffusion were adapted to calculate a first order approximation of

sulfur dioxide diffusivity though American oak wine barrels under constant temperature and moisture content to better understand the mechanism of sulfur loss from cooperage.

Numerous analytical methods have been used to measure sulfur dioxide under enological conditions, widely ranging in terms of complexity and the equipment required for analysis. Simple iodometric titration-based assays have been used for decades, but more advanced chromatographic techniques are also commonplace today. Gas chromatography has successfully been used for the analysis of headspace sulfur dioxide in wine samples[141]. Sulfur chemiluminescence detectors can used in tandem for specific analysis of sulfur-bearing compounds[81]. These methods require user experience and expensive equipment but are powerful and accurate analytical tools.

Gas detection tubes are graduated glass tubes filled with a colorimetric indicator such that a measurement is made according to the length of the color change in the tube. These simple devices are commonly used to measure gas concentrations in environmental safety settings, and have successfully been used to measure headspace sulfur dioxide in enological conditions (**Chapter 4**)[77]. Sulfur dioxide measurement is typically based on the oxidation effect sulfur dioxide has on iodine complexes, which produces a bleaching effect. The tubes are operated with a calibrated bellows pump. The ease of use and published accuracy make the detector tubes a useful tool for gaseous sulfur measurement.

Electrochemical sulfur dioxide sensors are an attractive option for headspace gas detection[142]. Screen printed sensor modules are available that offer sensitive detection in a compact package. The sensors operate on the oxidation power of sulfur dioxide to produce a current that is converted to a digital signal by the module[143]. Inkjet-printed electrode technology

has been used to measure the sulfur dioxide content in aqueous sample of wine[144,145], but the technology has not been applied for the measurement of headspace sulfur dioxide in barrels. Screen printed sensors are well suited to the task of measuring gaseous samples directly. Commercially available screen printed electrochemical sensor sensors are easily operated by connecting to a microcontroller and present an inexpensive yet powerful tool for measuring sulfur dioxide gas[143].

In this study, the sensor module was incorporated into a flow chamber designed to pump gas across the sensor surface at a constant rate. the calibration performance of gas chromatography-sulfur chemiluminescence detection and gas detection tubes were first compared against the sensor apparatus. The sensor was then used to measure the diffusion of sulfur dioxide gas from three 225-L American oak wine barrels over twenty days of storage.

5.3 Materials and methods

5.3.1 Comparison of sulfur dioxide detection methods

Three sulfur dioxide detection methods were compared under laboratory conditions. Colorimetric gas detection tubes, gas chromatography-sulfur chemiluminescence, and an electrochemical sensor apparatus were assessed. The precision and linearity of detector response was assessed at three sulfur dioxide concentrations. Disposable gas calibration cylinders from MESA Specialty Gases & Equipment (California, USA) were employed at concentrations of 0.014 mg/L, 0.027 mg/L, and 0.068 mg/L, balanced with air. Gas flow was controlled at 0.5 L/min via a fixed flow regulator from MESA Specialty Gases & Equipment (California, USA) and 1-liter Tedlar gas sampling bag from SKC West Inc. (California, USA) were used for calibration, where applicable.

Throughout this report, the parts per million (ppm) convention is used for expressing results for the sulfur dioxide concentration, where applicable. This is to directly correspond with the graduations on the gas detection tubes, the electrochemical sensor output and manufacturer specifications, and the most recognized convention in the wine industry. The values are readily converted to units of milligrams per liter by invoking the ideal gas law and molecular mass of sulfur dioxide, using the following equations:

1) First, the molar volume of an ideas gas is calculated at the temperature of the system under consideration, ***T*** (in Kelvin), according to:

$$V\ (L\ mol^{-1}) = \frac{(1\ mol)(0.0821\ L*Atm*mol^{-1}*K^{-1})(\boldsymbol{T}\ K)}{1\ Atm} \qquad \textbf{(Eq. 5.2)}$$

2) The sulfur dioxide gas concentration, *C(x)* (in g/L), is then readily calculated according to this volume:

$$C(x)\ (g*L^{-1}) = X\ (ppm) * \frac{1}{10^6\ parts} * \frac{1}{V(L\ mol^{-1})} * 64.066\ (g*mol^{-1}) \qquad \textbf{(Eq. 5.3)}$$

Where *X* is the parts per million concentration of sulfur dioxide gas.

a) Gas Detection Tubes

Colorimetric Gas Detection Tubes (GDT) from Drägerwerk AG (Lübeck, Germany) were obtained with a pre-indicated detection range of 1-25 ppm (0.003^{-3} mg/L– 0.068 mg/L). Detection tubes are made of glass and initially sealed on either end. To perform a measurement both ends of the glass tube are broken to permit gas flow and a specified volume and flow rate of gas is pulled through the tube using a specialized handheld bellows pump from Drägerwerk AG (Lübeck, Germany). Tedlar bags were initially purged three times using calibration gas and then filled with the same test gas. GDT were directly inserted into the polypropylene valve-septum

assembly on the sample bag, and samples were pulled using the bellows pump at the manufacturer's specified stroke frequency. Sulfur dioxide concentration was directly read from the extent of bleaching and the pre-marked tube exterior. Measurements were performed in triplicate.

b) Gas Chromatography-Sulfur Chemiluminescence Detection (GC-SCD)

Gas chromatography analysis was performed using an Agilent 7890B gas chromatograph (GC) system coupled to a sulfur chemiluminescence detector (SCD) from Agilent Technologies (California, USA). A DB-Sulfur column (40 m length* 0.32 mm inner diameter * 3µm film thickness) containing a phenyldimethylsilyl stationary phase from Agilent Technologies (California, USA) optimized for use with the SCD system was used in the setup. Tedlar bags were prepared as described in *Section 5.3.1a* and samples were collected through the septum system. Twenty-five microliters of gas were collected per sample using a gastight syringe equipped with a trapping mechanism that allows samples to be transported short distances without significant gas loss from Hamilton Company (Nevada, USA). Samples were manually injected into the GC-SCD system. The GC was operated in splitless mode with a flow rate of 0.9 mL/min and a 40°C to 100°C linear temperature ramp. Temperature was ramped at a rate of 20°C/min and held at the final temperature of 100°C for one minute, for a total run time of four minutes per injection.

While the GC-SCD-DB Sulfur system is unparalleled for the separation of sulfur-bearing compounds, direct measurement of sulfur dioxide gas necessitates particular experimental design considerations to produce a stable output. First, the system must be powered on for several hours to stabilize the detector prior to injection. The GC-SCD was run in a low-power stabilization mode for 12 hours prior to sample analysis in this experiment. Unusual for capillary columns, the

DB-Sulfur column requires passivation at each concentration level. A stable output can thus only be obtained after a series of initial 'dummy' injections that are discarded in analysis. Preliminary method development indicated that a set of five such injections was required to produce a suitably stable output, which is incorporated in the data presented. After stabilizing, measurements were performed in triplicate.

c) Electrochemical sensor

A sensing apparatus was constructed using a screen-printed digital gas sensor (DGS) module from SPEC Sensors, LLC. (Massachusetts, USA). An initial sensor performance test was conducted by connecting the DGS module directly to a PC using the USB to UART interface integrated in the sensor module. In this configuration the sensor was operated using terminal emulator software from Tera Term (Tokyo, Japan). Using the calibration gas cylinders, the sensor was spanned and calibrated according to manufacturer's recommended setup procedure.

After preliminary calibration, the sampling apparatus was constructed by connecting the sensor module to a microcontroller/datalogger from Adafruit Industries (New York, USA) in serial communication. The microcontroller was programmed using the Arduino Integrated Development Environment from BCMI Labs SA (Chiasso, Switzerland). Power supply and datalogging were provided using the onboard USB and microSD connections.

A gas flow assembly was constructed to provide a consistent supply of analyte across the sensor surface (**Figure 5.1**). Gas flow was controlled using a 0.5 L/min micro diaphragm pump from CO2Meter.com (Florida, USA). Sensor housing was constructed by modifying 50 mL polyethylene sample tubes from Corning, Inc. (New York, USA) to fit the sensor body and sealed with chemical-resistant silicone sealant from Dow Chemical Company (Michigan, USA)

to provide a gastight fit. One-eighth inch inner diameter non-phalate PVC tubing from Thermo Fisher Scientific (Massachusetts, USA) was used as gas supply line. For calibration, this tubing was directly attached to the calibration gas cylinder and flow regulator described above. All measurements were collected over five minutes of gas flow at a one-second sample rate, averaging the last 100 samples to produce the recorded concentration. Calibration was performed in triplicate. Sensor performance was assessed in two configurations (**Figure 5.1**). Silicone barrel bungs from Airgas, Inc. (Pennsylvania, USA) were modified for sampling by threading the same PVC tubing through holes drilled in the bung. The tubing was arranged at different heights so gas would be drawn from the base of the barrel and returned to the top to facilitate mixing. Stainless-steel gas quick connects were affixed to the tubing above the bung. The connections allow the sensor to easily connect to barrels for sampling and prevent gas loss in between samples when the sensor is disconnected.

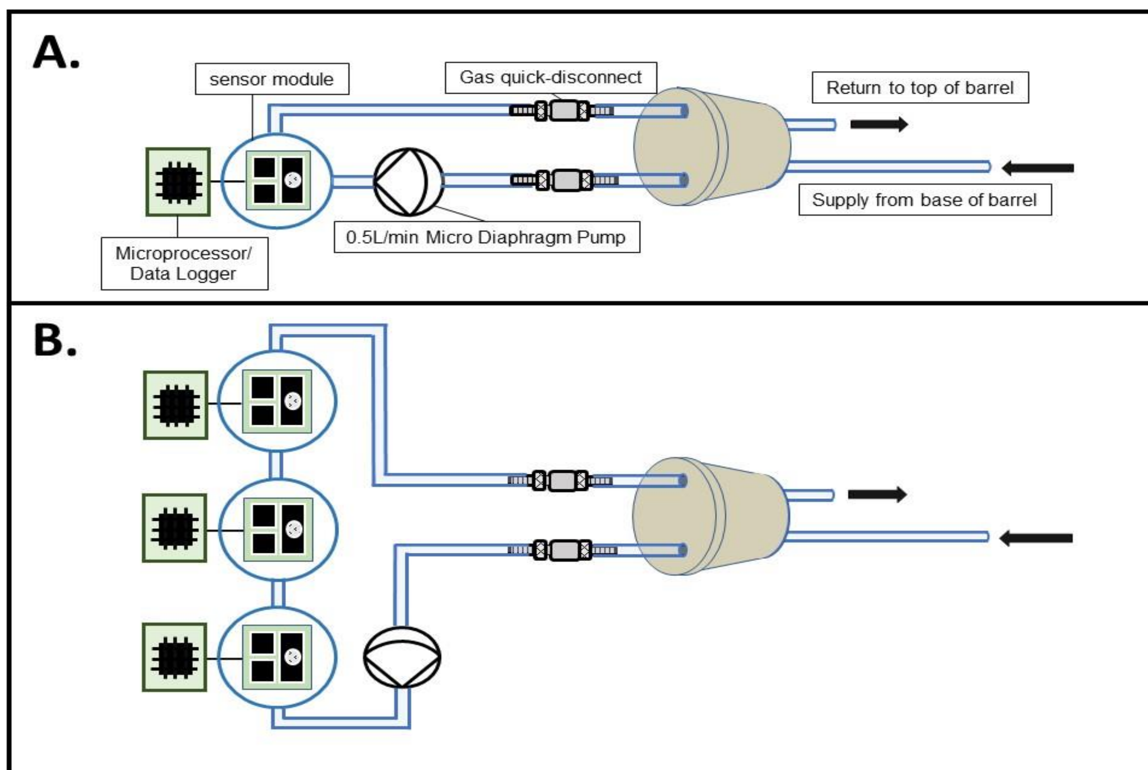

Figure 5.1. Sensor flow chamber configurations. **A.** Single-sensor configuration. **B.** Triple-sensor configuration. Each sensor is controlled by a separate microcontroller/datalogger unit.

5.3.2 Barrel measurements

The sensor apparatus described above was used in single-sensor configuration to measure the loss of sulfur dioxide over twenty days of storage in barrel. Three new, unsulfured 225-L American oak (*Quercus alba*) barrique from Cooperages 1912 Napa (California, USA) were used for the study. The storage environment was maintained at 70% RH and 12.7°C for the duration of the study to emulate typical cellar conditions. Sulfur dioxide was added to the barrels to a target of 25 ppm ($6.8 * 10^{-2}$ mg/L) using a custom concentrated gas cylinder-flow meter system from Airgas, Inc. (Pennsylvania, USA) via a syringe directly though the silicone bung. Sulfur dioxide measurements were collected daily for the first week of storage, beginning thirty minutes after dosage. Sampling was reduced to every four days for the remainder of storage. Measurements were performed in triplicate for each barrel. The sensor was periodically recalibrated throughout the study.

5.3.3 Data management

The precision and linearity of detector response in calibration trials was assessed by XLSTAT linear regression software from Addinsoft (Paris, France). Barrel data were similarly processed using linear regression of log-transformed data. The theoretical and measured diffusion coefficient were obtained according to the method described in Sorz and Heitz[88]. In this method, the diffusion coefficient is estimated by fitting the measured gas loss data to an exponential curve of the form

$$[SO_2] = a * e^{-b*t} \qquad \textbf{(Eq. 5.4)}$$

to sulfur dioxide data between 95 and 70% of the initial diffusant concentration, where *a* and *b* are coefficients determined by regression, and *t* is time (in seconds). Using the ideal gas law, the molar volume of sulfur dioxide can be calculated under the study conditions (**Eq. 5.1**), and the

exponential curve is integrated to determine the diffusive flux, J. The Fickian diffusion coefficient, D, can then be determined according to the equation:

$$D(m^2\ s^{-1}) = \frac{J(mol\ s^{-1}) * l\ (m)}{\Delta c\ (mol\ m^{-3}) * A\ (m^2)} \qquad \textbf{(Eq. 5.5)}$$

Where,

- J = diffusion flux, described above.
- l = diffusion pathway length. This is 0.025 m for export style wine barrels.
- A = surface area, estimated to be 2.01 m^2 for 225 L barrels[87].
- Δc = concentration gradient. External concentration is assumed to be zero, and the inner surface of the barrel is assumed to be in equilibrium with the barrel headspace.

The diffusion coefficient is also estimated without the exponential curve fit step, according to the method described in Nevares *et al.*[87] by linear fit to the tail of the data, where diffusion is sufficiently slow to approximate as a constant flux. Data from the last eight days of storage were analyzed by linear regression analysis using XLSTAT software from Addinsoft (Paris, France) to determine the Fickian diffusion coefficient by this method.

5.4 Results

5.4.1 Calibration assessment

A comparison between the detection methods used in this study is presented in **Table 5.1**. This figure details the performance characteristics of the GDT, GC-SCD, and sensor in initial calibration and flow chamber assemblies, respectively. Compared to the other detection methods assessed in this study the sensor is superior in linearity and precision.

Table 5.1. Calibration performance comparison of the detection methods investigated in this study. *S1*, *S2*, and *S3* are individual digital gas sensor modules. Three such sensors are tested in the UART/USB configuration for use in the triplicate flow chamber.

Detection Method	Linearity (R^2)	Standard Deviation		
		5 ppm (0.014 mg/L)	*10 ppm* (0.027 mg/L)	*25 ppm* (0.068 mg/L)
GDT	0.999	19.2%	3.0%	2.4%
GC-SCD*	0.999	3.7%	3.9%	7.3%
Sensors (USB to UART)				
S1	0.999	0.6%	0.2%	0.2%
S2	0.999	0.2%	0.5%	0.1%
S3	0.999	0.3%	0.3%	0.2%
Flow Chamber Assembly				
Triplicate	0.998	6.9%	3.5%	1.0%
Single Sensor	0.999	2.5%	2.4%	2.3%

GDT response for the calibration assessment is shown in **Figure 5.2**. The bleaching effect of sulfur dioxide on the tube packing is readily apparent. An exact endpoint of this bleaching can be unclear as the color fades across a gradient, and the user must make a judgement call for the final SO_2 reading. Further, the graduations on the tube are unequally spaced and scaled so the user must approximate readings that do not fall exactly on the graduations with some uncertainty. Considering the large standard deviation of readings at the lowest calibration level (5 ppm/0.014 mg/L), GDT response is highly linear overall and acceptably precise at the other calibration levels.

GC-SCD analysis of sulfur dioxide gas is complicated by the passivation requirement of the DB-Sulfur column. An increase in detector response over subsequent injections is readily apparent in **Figure 5.3**. Sensor output becomes progressively more stable over repeat injections. However, this is a clear limitation in the applicability of the manual injection method for practical use. Taking this requirement into account, the GC-SCD method produced an acceptable calibration curve in triplicate analysis under the study conditions.

The electrochemical sensor modules produced highly linear and precise calibration data under laboratory and cellar conditions. Sensor performance was superior in the USB to UART configuration, and both flow chamber configurations also outperformed the other detection methods. Calibration response data for the single-sensor flow chamber configuration are shown in **Figure 5.4**. Overall, the sensor output is highly consistent and reproducible across the replicates, with the sensor response lagging somewhat at the highest calibration level (25 ppm / 0.068 mg/L) in one replicate only.

Figure 5.2. Dräger GDT calibration response. The extent of bleaching indicates sulfur dioxide concentration using the pre-indicated scale (in ppm). Concentrations converted according to **Eq. 5.2** and **Eq. 5.3** for 12.7°C cellar conditions.

5.4.2 Barrel measurements

To study the diffusive loss of sulfur dioxide from barrel headspace over twenty days of cellar storage was monitored for three new American oak barrels using the electrochemical sensing apparatus. Regression analysis indicated that the trend of sulfur dioxide concentration versus storage time could be closely modeled by power law functions of the form

$$y = a * x^{-b} \quad \textbf{(Eq. 5.6)}$$

Where *y* is the concentration of sulfur dioxide (in ppm), *x* is the elapsed time after dosing (in hours), and *a* and *b* are coefficients determined via linear regression of log-transformed data. The resulting equations and R^2 data are shown in **Figure 5.5**. The trendlines produced by this regression are an excellent fit to the barrel measurement data.

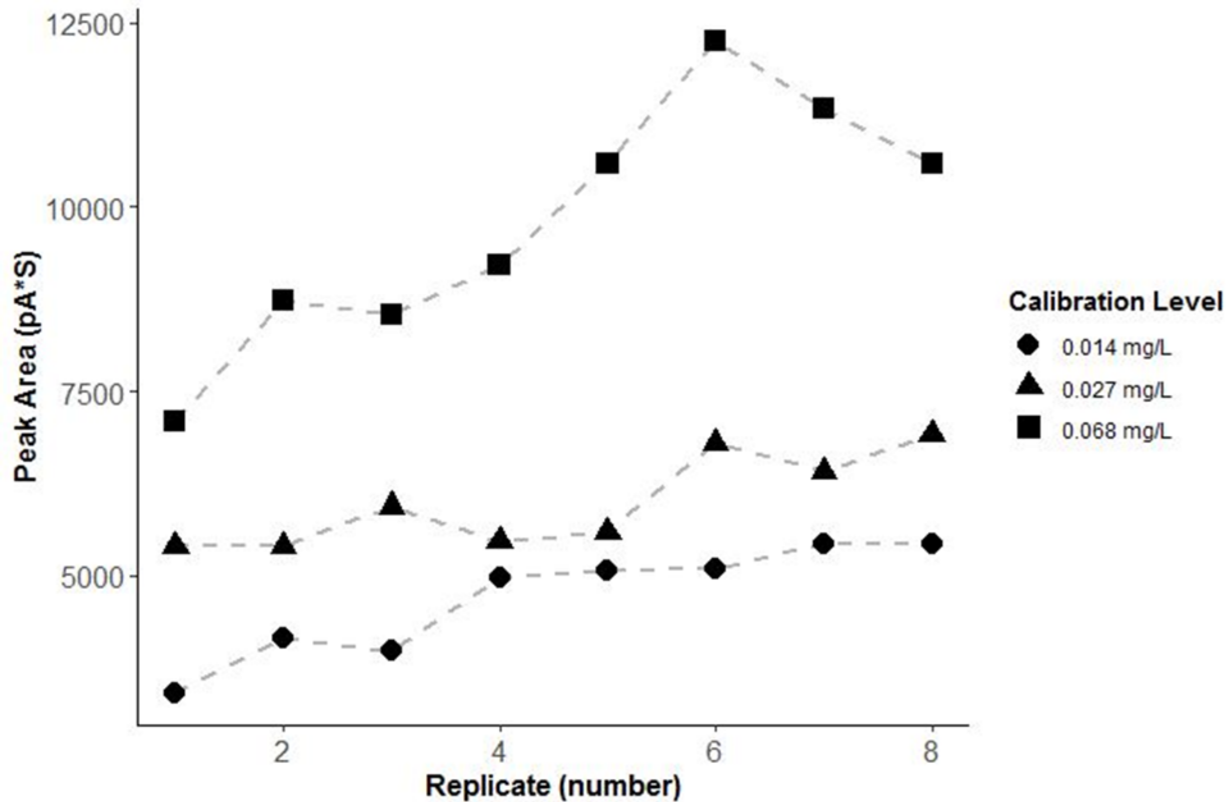

Figure 5.3. Passivation behavior of GC-DB-Sulfur setup. The detector signal increases over successive injections at a given concentration level before stabilizing.

Fickian diffusion coefficients were calculated according to the methods previously described. The values are compared in **Table 5.2.** The methods produce radically different diffusion coefficients. If the diffusion equation is directly applied to the power-law regression data, a wide range of D-values results. The observed effective D value ranged from $1.94*10^{-7}$ m^2 s^{-1} to $1.97*10^{-10}$ m^2 s^{-1}. Because the method described by Sorz and Heitz[88] relies on a fit to the earliest collected data and the method described by Nevares et. al[87] is a fit to the latest collected data, the D values produced by these methods are naturally at the upper and lower extremes of the observed effective D value, respectively. Regardless, these methods clearly do not accurately describe the observed sulfur dioxide diffusion.

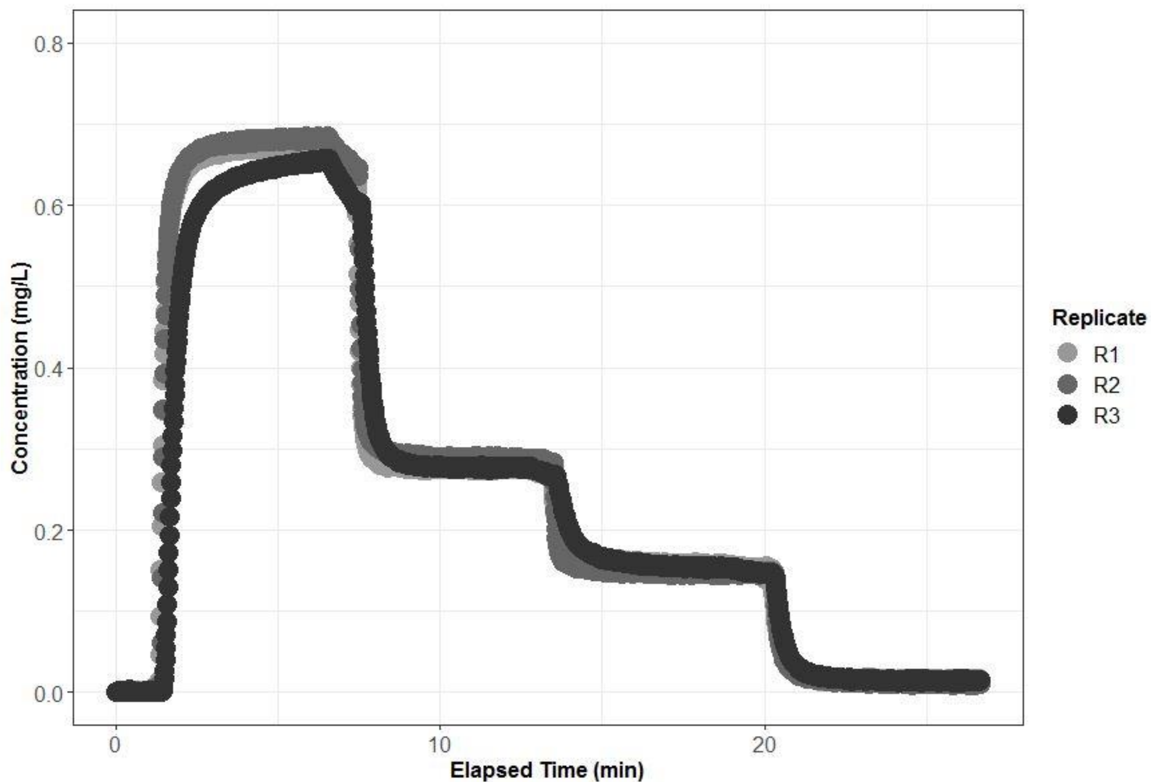

Figure 5.4. Sensor response to calibration gas in single-sensor flow chamber configuration. Chamber flushed with calibration gas for five minutes at each calibration level. Process repeated in triplicate for analysis.

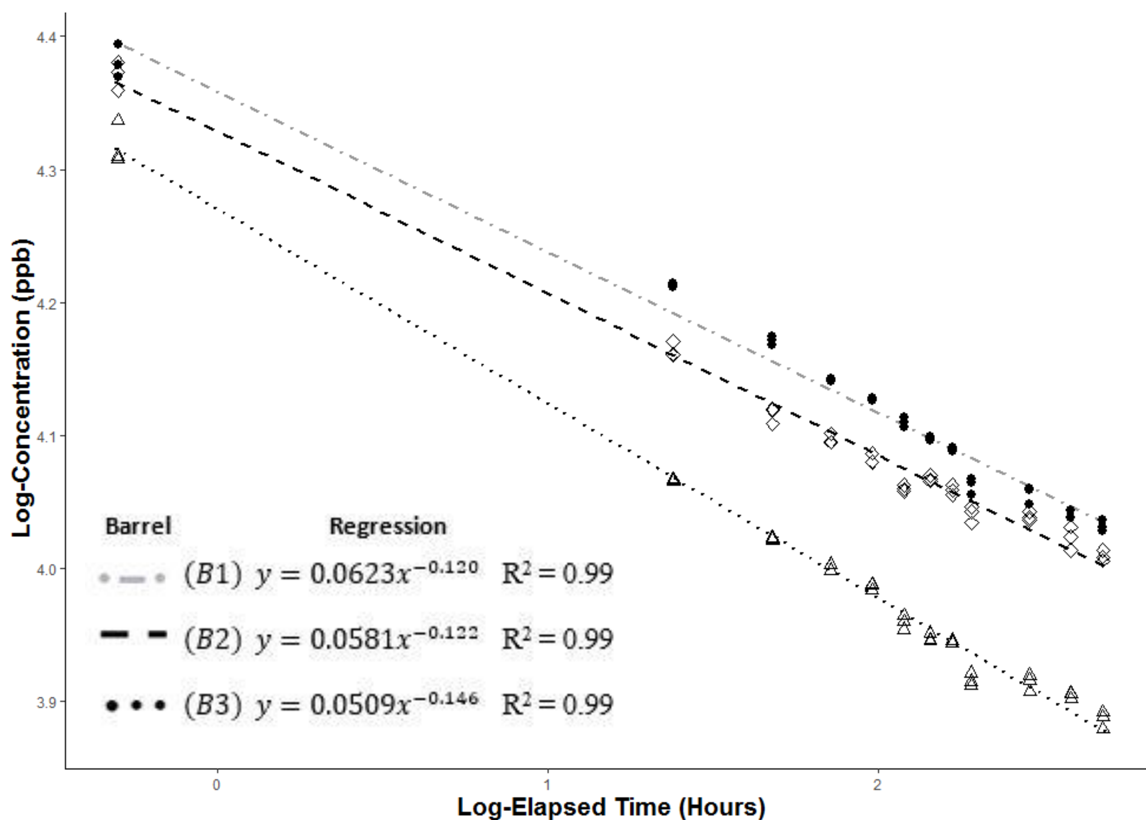

Figure 5.5. Linear regression analysis of log-transformed barrel sulfur measurements over twenty days of storage.

Sulfur dioxide is rapidly lost after initial dosing, but the flux is far slower than predicted by normal Fickian diffusion for most of the storage period. The estimation from Nevares et al.is essentially a fit to the tail of the data and fails to describe the full curve[87]. The results suggest that non-Fickian behavior plays a major role in the mechanism of sulfur transport from the barrel headspace and/or the process violates the assumptions implicit in the diffusion coefficient analysis.

Table 5.2. Diffusion coefficients obtained for steady-state analysis.

Method	Diffusion Coefficient (m2*s^{-1})
Sorz and Heitz 2006	3.50 * 10^-8
del Alamo-Sanza *et al.* 2017	2.21 * 10^-10
Measured	1.96*10^-10 ≤ *D* ≤1.94*10^-7

5.5 Discussion

In the wine industry, sulfur dioxide is rarely measured in the gaseous state. Winemakers instead regularly measure the sulfur dioxide content of aging wine using liquid samples via titrimetric methods. This may soon change. Recent research has shown that the aeration-oxidation and Ripper methods commonly used for aqueous samples are highly inaccurate for red wine samples, and a far more accurate measurement can be obtained by headspace analysis[141]. The quantity of sulfur dioxide in the liquid is then determined according to Henry's law and adjusted partition coefficients for alcoholic solutions. The results of this experiment further demonstrate the availability of accurate gas detection methods with a wide range of tools and corresponding operating costs. All three detection methods assessed in this study produced acceptable calibration curves and proved to be viable tools for the measurement of sulfur dioxide in barrel headspace. Each of the techniques used in this study has a unique set of pros and cons, and special considerations that must be weighed for proper selection and operation.

Colorimetric gas detection tubes are easy to operate and produce rapid readings with simple equipment. Separate GDT are available with a wide range of sensitivities and can be selected to measure concentrations from $2.7*10^{-4}$ mg/L up to 191 mg/L (0.1 ppm to 70,000 ppm) to suit many industrial production settings. Selecting the proper detection tube requires some knowledge of the expected concentration and range encountered so this study was specifically designed to correspond to the stated range of one detection tube. Little training is required to operate the calibrated bellows pump, and the overall accuracy observed in this study is on par with published results[77]. One disadvantage of this method is the potential for cross-sensitivity when measuring mixed gas samples, as sulfur dioxide cannot be measured accurately in the presence of hydrogen sulfide. Advanced knowledge of the gas species present in a sample is

therefore critical for accurate measurement so the tubes can only be used in certain environments. Nevertheless, at roughly half the cost of vendor analysis via titration GDT may become an increasingly popular choice for measuring sulfur dioxide in the winery.

Gas chromatography-sulfur chemiluminescence detection produced an acceptable calibration curve under the study conditions, however the applicability of this method is strongly hindered by the procedure required to produce a stable output. Sulfur dioxide gas can be difficult to accurately measure by chromatography due to the inherent reactivity and mobility of the compound, which can result in significant peak broadening for many capillary columns [81,146]. The peak shape issue is solved via the use of a sulfur-specific column and detector system like the DB-Sulfur/SCD used in this study but can result in a significant passivation phase for successive injections as evidenced above. The passivation requirement at each sample level means the number of throwaway 'dummy' injections that must occur for a proper experimental design quickly climbs to a level that is unmanageable for a manual injection method. GC-SCD is not without its merit or utility, however, as it is the only method in this study capable of providing speciation data. The GDT and sensor both suffer from cross-sensitivity with other gases so a preliminary assessment of the system via chromatography may be necessary if the exact gas species present are unknown. An experimental design that permits the use of an autosampler, internal standards, and easy creation of technical replicates can avoid some of the pitfalls experienced in this study. Measuring barrel headspace during storage without disrupting the system is difficult in such a scheme.

Electrochemical sensing technology is well established, and the compact design and sensitivity of modern modules presents a major opportunity for the wine industry. Sensor modules were superior in linearity and precision versus the other tested methods, and the sensor

could easily be incorporated into a flow chamber design to cycle samples from the barrel for analysis. The level of sensitivity demonstrated in the calibration trials rivals any published sulfur dioxide method currently employed in enological research[81]. Like the other methods used in this study, electrochemical sensors present their own set of challenges that must be incorporated for accurate sulfur dioxide measurement. Cellar environments are generally kept cool and humid, which can affect the sensitivity of the electrochemical cell. In the case of sulfur dioxide measured at 70% relative humidity, an increase in sensitivity of up to 20% can be observed over the first two weeks of measurement[148]. It is imperative that the sensor be stored at constant humidity for an extended period before stable measurements are obtained, and it is prudent to periodically recalibrate or re-span the sensor to ensure measurements are not affected by drift. Another limitation is the detection range of electrochemical sensors on the market, which are incapable of handling the typical sulfur dioxide concentrations produced during normal barrel fumigation (**Chapter 4**). The concentrations applied in this study were necessarily lower than typical industry standard as a result. Ultimately these sensors may find their best use in wineries as environmental safety monitors for cellar workers, or in the measurement of headspace sulfur dioxide for determining the aqueous sulfur dioxide content in wine samples.

Sulfur dioxide loss from barrel headspace was closely modeled by power law functions. Similar power law functions were used to describe the loss of sulfur dioxide from barrels under typical cellar storage conditions and fumigation protocols (**Chapter 4**). While it is tempting to conclude that an underlying diffusive mechanism is responsible for the similarity in the power of regressions obtained for sulfur dioxide loss, the correlation more likely results from the flexibility of power law fits to empirical data. Diffusion is significantly affected by moisture content so it is unlikely that diffusion in a dry barrel and saturated barrel would meaningfully be

described by a shared power integer. The significance of the power law may be more appropriately interpreted to suggest underlying anomalous transport processes for sulfur dioxide in dry wood. Anomalous diffusion is term used to describe processes where the mean squared displacement of diffusant changes over time according to a power law relationship but is also colloquially used to describe processes that deviate from the normal diffusion assumed by Fick's first and second laws. Anomalous-type diffusion is often attributed to heterogeneity in the diffusive system[148]. American oak barrel wood contains abundant thyloses that create extremely low permeability zones in the wood, and the rift sawing process means gas will diffuse through staves in a mix of orientations[59]. Along with the presence of a bunghole at the top of the barrel, both factors impose substantial heterogeneity in the diffusion medium. Interaction between sulfur dioxide and the barrel wood may also be causative for the observed anomalous behavior. Owing to the widespread use of sulfur dioxide to bleach wood pulp in the paper industry, it is well known that sulfur dioxide can bind and react with moist wood at high temperatures and even degrade cellulose[149]. The sorption is less well studied in dry wood at ambient conditions and this may also be contributing to or the cause of the observed anomalous behavior.

Fick's laws have been used to describe the migration of oxygen though barrel wood, however the methods cited in this study poorly describe the observed sulfur dioxide loss. This can be interpreted to support a conclusion that sulfur dioxide diffusion from barrel wood is an anomalous process, since the authors concluded that oxygen migrated through barrel wood via normal Fickian diffusion. Regardless of interpretation, some caution must be used when analyzing and applying the methods described in these studies. Both studies cite the steady-state simplification of Fick's law but fail to recognize that the boundary conditions of their respective experimental designs violate the assumptions required for the method. Most importantly, the

boundary conditions of the steady state method require the concentrations at each boundary to be time-invariant[150]. In both studies, however, the oxygen concentration at one boundary changes throughout the study. Moreover, any concentration function of the type $[y] = a * e^{-b*t}$ can be used to solve Fick's first law for a discreet diffusion coefficient. Derivatization (to determine the flux) returns a constant multiplied by the same exponential, and subsequent division by a concentration gradient that varies according to the same exponential will always return a constant value. Such an exponential function will provide a solution to Fick's law regardless of the boundary conditions. While the observed sulfur dioxide loss was not well described by the steady-state method, the effective diffusion coefficient range produced for sulfur dioxide in the analysis is useful for comparison with previously determined oxygen diffusion rates. Oxygen diffusion through *Q. robur* oak barrel wood has been found to range from $6.9 * 10^{-8}$ m^2 s^{-1} to $3.2 * 10^{-9}$ m^2 s^{-1}, depending on the orientation of gas transport[87,88]. The effective diffusion coefficient for sulfur dioxide gas loss data in the first week of sampling is on the same order of diffusivity, but dramatically decreases thereafter. Diffusivity after one week of storage is far lower than would be expected for gas transport through oak as a Fickian process.

Ultimately this study is an important step in beginning to understand the mechanism of sulfur dioxide loss from barrel headspace and demonstrates that gas loss can be described by a power law relationship versus time, regardless of the hydration status of the barrel wood. GDT, GC-SCD, and the electrochemical sensor module proved to be viable tools for measuring headspace sulfur dioxide in oak barrels. While GC-SCD performance relies on the use of expensive, specialized equipment and required a large number of injections for stable performance, winemakers can adopt simple techniques like the use of GDT to measure post-fumigation barrel sulfur dioxide concentrations during extended empty storage to determine

appropriate redosing intervals. The sensor apparatus was highly sensitive and may be useful in the future for wineries in safety and wine chemistry applications alike. As modern sensor technology continues to develop, the availability and performance of inexpensive, compact electrochemical sensors for the wine industry will only continue to improve.

CHAPTER 6: CONCLUSION

Cleaning and sanitization are critical processes in winery operations but are largely understudied in scientific literature. Ineffective management of winery waste can result in spoilage, negatively impacting the sensory character of finished wine and threatening the financial and commercial viability of a producer. Because wine spoilage is not normally associated with a threat to human health, winery cleaning and sanitization is far less controlled or regulated than most food and beverage industries. Practical cleaning and sanitization can be difficult to study because academic research institutions often lack the facilities and equipment necessary to replicate industry-scale production. As a result, there is a gap between the diversity of cleaning and sanitizing chemistries on the market and data to support the efficient and effective use of these tools. The preceding chapters addressed this knowledge gap by providing robust quantitative support for the most common cleaning and sanitization practices for fermentor materials and for sulfur dioxide fumigation of empty cooperage.

Winemakers have access to a wide range of chemical cleaning and sanitizing agents for managing fermentor waste. In **Chapter 2** and **Chapter 3** a representative selection of the most common cleaning and sanitizing agents were assessed against fermentation soil and spoilage microorganisms with the aim of producing effective cleaning and sanitizing protocols with minimal chemical input.

In **Chapter 2,** cleaning and sanitizing agents were first screened against seven spoilage species in planktonic and sessile physiologies. Effective chemistries were used in a series of increasing-scale trials using fermentor materials and eventually, 2000-L stainless steel fermentors. Caustic sodium- and potassium-based cleaning agents were superior in achieving both soil removal and antimicrobial action versus other cleaning formulations. Hydrogen

peroxide and peracetic acid were effective sanitizers and have innocuous breakdown products. These sanitizers are attractive for waste disposal considerations and are increasingly favored for sustainable operations in industry. Even in the case of visually clean surfaces, cleaning steps cannot be omitted as sanitizers were not effective without prior cleaning operations. Incomplete manual cleaning operations resulted in contamination on critical control points for tank surfaces and equipment, regardless of the chemical species used for cleaning and sanitization. Improvements in manual tank cleaning operations are likely the most effective means for saving time, money, and chemicals in the winery while effectively managing fermentor waste.

Chapter 3 sought to optimize the use of effective chemistries demonstrated in **Chapter 2** by exploring techniques for the minimizing the concentration and contact time of applications. The minimum inhibitory concentration and minimum biocidal concentration assay was demonstrated as a high-throughput method for evaluating the antimicrobial efficacy of winery cleaners and sanitizers against *Saccharomyces cerevisiae, Brettanomyces bruxellensis*, and *Zygosaccharomyces bailii* yeasts. A modified version of the assay was used to investigate the difference in concentrations required to inactivate cells in biofilm physiologies. Sessile cell cultures required equal or higher concentrations versus corresponding planktonic cultures in most cases. Specific combinations of organism, chemical treatment, and physiology were successfully inactivated below manufacturer's recommended concentrations, but only a complex built chemical comprised of percarbonate and quaternary ammonium compounds was totally effective below recommended levels.

Fluorescent staining was demonstrated as a useful technique for determining the minimum contact time required to inactivate populations of *S. cerevisiae* using peracetic acid. Fluorescent viability staining using SYBR Green 1 and Propidium Iodide is a well-established

technique, and commercial assays with prepared dye mixes using these two fluorophores are available. Interference between the fluorescent dyes and antimicrobial treatments limited the number of treatments that could be assessed by this method. Propidium iodide staining and corresponding culture data indicated that five minutes of contact time was required to inactivate *S. cerevisiae* at the minimum effective concentration of peracetic acid (0.1 g/L). This is an important consideration for practical winery settings, where equipment is regularly sanitized by quickly dipping parts into a container of peracetic acid. Winery workers must ensure that extended contact time between contaminated surfaces and sanitizers is essential for proper sanitization. Winemakers should consider using a modified form of antimicrobial susceptibility testing to determine whether their in-house procedures and protocols are effective for managing the fermentative species employed in their respective facilities.

Sulfur dioxide fumigation is universally used for empty for the preservation and sanitization of barrels, but quantitative data demonstrating the impact of fumigation are largely absent from scientific literature. **Chapter 4** and **Chapter 5** focused on measuring the dosage and persistence of sulfur dioxide in common fumigation applications, the antimicrobial impact of treatment, and the fundamental diffusive behavior of sulfur dioxide gas from barrels.

To study the antimicrobial impact of sulfur fumigation it is important to first understand how microorganisms interact with barrel wood. The penetration rate and abundance of *S. cerevisiae* and *B. bruxellensis* cultures in *Quercus alba* barrel wood were measured in **Chapter 4**. A method for recovering thin slices of barrel wood using a sweep gouge and chisel was developed for establishing the depth profile of penetrating yeast. This method was an improvement over the common approach of soaking treated staves in a recovery medium, which only effectively measure surface sanitization. *S. cerevisiae* cultures were observed to penetrate

Q. alba staves much more effectively than *B. bruxellensis* cultures in grape juice medium. *S. cerevisiae* cells were recovered at 10 mm depth within one week of inoculation. *B. bruxellensis* cultures were limited to surface samples in all but one stave sample throughout the six-week inoculation period. Scanning electron microscopy imaging supported the observed difference in species penetration in culture data and provided an explanation for the limited penetration of *B. bruxellensis* cultures versus *S. cerevisiae*. *S. cerevisiae* yeast were scattered throughout the stave structure as individual ellipsoid cells, whereas *B. bruxellensis* cultures exhibited extensive pseudohyphal structuring, forming a tangled web on the surface of inoculated staves. The network of pseudohyphae may have limited the penetration of *B. bruxellensis* cultures and could provide a physical barrier to subsequent chemical and thermal sanitization treatments.

The dosage, persistence, and antimicrobial action of common industry fumigation techniques were investigated in **Chapter 4.** Barrels were hydrated and treated by combusting solid sulfur wicks or with pure sulfur dioxide gas at two different concentrations, respectively. Measurable sulfur dioxide was detected in all barrels after six weeks of cellar storage. A 2.25 L dose of pure sulfur dioxide was the minimum rate for maintaining free sulfur dioxide in new American oak barrels for six weeks of storage at 70% relative humidity. The loss of headspace sulfur dioxide was closely modeled by power law functions using log-log transformed data. The power law relationship between sulfur dioxide loss and storage time is indicative of an anomalous diffusive process, in part owing to the changing moisture content of the initially saturated barrel staves during storage. All three fumigation treatments effectively inactivated *B. bruxellensis* and *S. cerevisiae* cultures in the test barrels. This study demonstrated that sulfur dioxide fumigation could be an effective sanitizing treatment in properly cleaned barrels. Free SO_2 gas persisted well beyond 40 days of cellar storage under typical application protocols.

Chapter 5 investigated the diffusive behavior of sulfur dioxide gas from cooperage further. Because gas diffusivity strongly decreases with increasing moisture content for porous solids, the changing moisture content throughout storage for the trials in **Chapter 4** was thought to be a primary driver of the observed anomalous diffusion. Oxygen diffusion though dry wood has been demonstrated as a Fickian process. **Chapter 5** analyzed sulfur dioxide diffusion from barrels below the fiber saturation point under conditions of constant moisture content as a semipermeable membrane according to Fick's Laws.

First, an electrochemical gas sensing apparatus was constructed. The accuracy and precision of this sensor module was compared against gas chromatography-sulfur chemiluminescence and headspace gas detection tube methods. All three techniques produced acceptable calibration curves. The electrochemical sensor module was superior in precision and accuracy versus the other detectors. Reliable detection via gas chromatography-sulfur luminescence system required a substantial passivation injection phase that was impractical for real-time barrel sampling. Colorimetric gas detection tubes are easy to operate and produced rapid readings but have poor sensitivity at low concentrations and can be costly to produce replicate measurements.

As in **Chapter 4**, sulfur dioxide concentration curves were closely modeled by power law functions in barrels with constant moisture content. The loss curves were poorly described by Fick's laws, suggesting that the loss of sulfur dioxide gas from barrels is a fundamentally anomalous process. Heterogeneity in the diffusive medium and interactions between the sulfur dioxide gas and barrel wood may explain the deviation from normal Fickian diffusion. Abundant tyloses in the structure of *Q. alba* barrel wood create closed diffusive pathways in the stave structure with close to zero gas permeability. Sulfur dioxide gas can also adsorb to wood.

Surface interactions may hinder the diffusivity of headspace gas by skewing concentration gradient in the stave towards the inner surface. Ultimately, this study is an important step in beginning to understand the mechanism of sulfur dioxide loss from barrel headspace and demonstrates that gas loss can be described by a power law relationship versus time regardless of the hydration status of the barrel wood. The sensor apparatus was highly sensitive and can be useful in the future for wineries in safety and wine chemistry applications alike.

Future research in the topic of winery cleaning and sanitization will undoubtedly be focused on the development of tools and strategies to address issues of increasing natural resource scarcity and environmental concerns over winery wastewater management. The use of nonchemical sanitizing strategies for fermentors like ultraviolet light and high pressure ultrasound present a major opportunity for reducing chemical inputs but are currently not cost effective options for most wineries. As this technology becomes more affordable and adaptable, studies will be needed to validate the performance and operational utility of these tools in practical settings to make an impact in commercial production facilities. Wastewater filtration and reuse in the context of winery cleaning and sanitization is a nascent technology that offers another path for reducing the environmental impact of winery operations but is similarly out of reach for most facilities with the current available technology. Cleaning and sanitizing chemistries must also be specifically formulated to be compatible with separation and filtration in this type of setup. Studies will be needed to assess the efficacy of any chemical strategies that are developed and whether filtration systems are capable of handling the highly variable waste loads associated with harvest operations in practical settings.

Nonchemical methods of barrel sanitization have also been developed but are currently impractical for routine use in production facilities due to the high equipment costs and treatment

time required for effective antimicrobial action. These technologies may become more relevant as the tools become more affordable and effective, but sulfur dioxide will unquestionably remain the preferred method for long term storage of empty cooperage for the foreseeable future. While the results presented in this thesis represent a major step in the quantitative understanding of fumigation, the data can only be considered to be numerically valid under the conditions tested. Additional studies are needed to determine how the loss of sulfur dioxide from barrel headspace varies by species of oak, the composition of saturating fluid and aging protocol, and barrel cleaning operation. The penetration depth and abundance of yeast and bacteria in barrel staves depends not only on the species present, but the aging protocol and composition of the must or wine in the barrel. Further research is needed to understand how these variables might affect the ability of sulfur dioxide to inactivate the community of microorganisms inside barrels to be directly applicable for a wider range of winery settings.

The loss of sulfur dioxide gas from the barrel headspace is a non-Fickian process, likely in part due to interactions between the diffusing gas and the oak medium. Further studies are needed to understand the kinetics of sulfur dioxide adsorption to oak staves, and any possible reactions that occur between the gas and wood, to fully understand the mechanisms of sulfur dioxide diffusion. Adsorption to barrel wood, and subsequent desorption into infill juice or wine could add unintended free sulfur dioxide to the liquid. Additional free sulfur dioxide could negatively impact fermentations or render wine unsuitable for certain classifications, such as organic labelling requirements in the United States. Further studies on the interaction of sulfur dioxide and oak wood will help winemakers to better fine tune the sulfur dioxide concentration of juice and wine during production and may enable winemakers to have more predictable fermentations and finished wine composition.

Sensors will become increasingly important tools in wine production in the coming years. Modern sensor technology has resulted in the proliferation of a wide range of inexpensive, compact sensors that can be used to measure physical and chemical parameters in juice and wine. Sensors are also useful for detecting employee health hazards from toxic substances in the winery environment. These tools offer the ability for wineries to conduct measurements in-house at a substantially reduced cost versus commercial laboratory analysis, often with a much higher degree of sensitivity versus traditional analytical methods. Despite the availability of these sensors, relatively few products that leverage the wide range of gas and liquid sensing technologies are available for purchase by wineries. Future studies are needed to assess the suitability of novel sensors for accurately collecting measurements in practical settings. Incorporating and manufacturing finished sensor apparatuses for commercial use can also require several years of additional effort after the technology is validated. Nevertheless, the field of digital sensor technology may ultimately represent the most important future innovations in wine production.

CHAPTER 7: BIBLIOGRAPHY

1. Goode, J. (2018) Flawless: Understanding Faults in Wine. *University of California Press, Berkeley.*

2. Van Sluyter, S. C. *et al.* (2015) Wine Protein Haze: Mechanisms of Formation and Advances in Prevention. *J. Agric. Food Chem.* **63**, 4020–4030.

3. United States Food and Drug Administration (2017) Food code : recommendations of the United States Public Health Service, Food and Drug Administration. *U.S. Dept. of Health and Human Services, Public Health Service, Food and Drug Administration.*

4. Aguilar Solis, M. (2014) Evaluation of common and novel sanitizers against spoilage yeasts found in wine environments. *PhD Dissertation, Cornell University Department of Food Science*.

5. Kim, J. & Huang, C.H. (2021) Reactivity of Peracetic Acid with Organic Compounds: A Critical Review. *ACS ES&T Water* **1**, 15–33.

6. Rutala, W. A. & Weber, D. J. (2008) Guideline for disinfection and sterilization in healthcare facilities. *U.S. Centers for Disease Control and Prevention*.

7. Oliveira, M. & Duarte, E. (2016) Integrated approach to winery waste: waste generation and data consolidation. *Front. Environ. Sci. Eng.* **10**, 168–176.

8. Zacharof, M.P. (2017) Grape Winery Waste as Feedstock for Bioconversions: Applying the Biorefinery Concept. *Waste and Biomass Valorization* **8**, 1011–1025.

9. Marriott, N.G., Schilling, M. W. & Gravani, R.B. (2018) Principles of Food Sanitation. *Springer International Publishing,* New York.

10. Azevedo, S., Battaglene, T. & Hodson, G. (2016) Microbiologically, wine is a low food safety risk consumer product. *BIO Web of Conferences* **7**, 04003.

11. Du Toit, M. & Pretorius, I.S. (2000) Microbial Spoilage and Preservation of Wine: Using Weapons from Nature's Own Arsenal-A Review. *South African Journal for Enology and Viticulture*, **21**, 74-96.

12. Ruiz-Capillas, C. & Herrero, A. (2019) Impact of Biogenic Amines on Food Quality and Safety. *Foods* **8**, 2:62.

13. Lytra, G. *et al.* (2014) Olfactory impact of dimethyl sulfide on red wine fruity esters aroma expression in model solution. *J. Int. des Sci. la Vigne du Vin* **48**, 75–85.

14. Loureiro, V. & Malfeito-Ferreira, M. (2003) Spoilage yeasts in the wine industry. *International Journal of Food Microbiology* **86**, 23–50.

15. Bokulich, N.A., Thorngate, J.H., Richardson, P.M. & Mills, D.A. (2014) Microbial biogeography of wine grapes is conditioned by cultivar, vintage, and climate. *Proc. Natl.*

Acad. Sci. **111**, E139 LP-E148.

16. Lisanti, M.T., Blaiotta, G., Nioi, C. & Moio, L. (2019) Alternative Methods to SO2 for Microbiological Stabilization of Wine. *Comprehensive Reviews in Food Science and Food Safety* **18**, 455–479.

17. Plata, C., Millán, C., Mauricio, J.C. & Ortega, J.M. (2003) Formation of ethyl acetate and isoamyl acetate by various species of wine yeasts. *Food Microbiol.* **20**, 217–224.

18. Mah, T.F.C. & O'Toole, G.A. (2001) Mechanisms of biofilm resistance to antimicrobial agents. *Trends Microbiol.* **9**, 34–39.

19. Joseph, C.M.L., Kumar, G., Su, E. & Bisson, L.F. (2007) Adhesion and biofilm production by wine isolates of Brettanomyces bruxellensis. *Am. J. Enol. Vitic.* **58**, 373–378.

20. Tristezza, M. *et al.* (2010) Susceptibility of wine spoilage yeasts and bacteria in the planktonic state and in biofilms to disinfectants. *Ann. Microbiol.* **60**, 549–556.

21. Christ, K.L. & Burritt, R.L. (2013) Critical environmental concerns in wine production: an integrative review. *J. Clean. Prod.* **53**, 232–242.

22. Costa, J. M. *et al.* (2019) Water as a critical issue for viticulture in southern Europe: sustainability vs competiveness. *IVES Tech. Rev.* 3182.

23. Musee, N., Lorenzen, L. & Aldrich, C. (2007) Cellar waste minimization in the wine industry: a systems approach. *J. Clean. Prod.* **15**, 417–431.

24. Zuehlke, J.M., Petrova, B. & Edwards, C.G. (2012) Advances in the Control of Wine Spoilage by Zygosaccharomyces and Dekkera Brettanomyces. *Annu. Rev. Food Sci. Technol.* **4**, 57–78.

25. Bird, D. (2011) Understanding Wine Technology, 3rd Edition: The Science of Wine Explained. Board and Bench Publishing, San Francisco .

26. Fugelsang, K. C. & Edwards, C. G. (2007) Wine Microbiology: Practical Applications and Procedures, *Springer Science + Business Media,* New York.

27. Singleton, V.L. (1974) Some Aspects of the Wooden Container as a Factor in Wine Maturation. Chapter 12: Chemistry of Winemaking, *American Chemical Society*, 254-277.

28. Stadler, E. & Fischer, U. (2020) Sanitization of Oak Barrels for Wine - A Review. *Journal of Agricultural and Food Chemistry* **68**, 5283–5295.

29. Rixford, E. H. (1883) The Wine Press and the Cellar: A Manual for the Wine-maker and the Cellar-man. *Payot, Upham & Co.*, San Francisco.

30. Work, H. (2014) Wood, Whiskey and Wine : A History of Barrels. *Reaktion Books*, London.

31. Work, H. H (2018) The Shape of Wine: Its Packaging Evolution. *Taylor & Francis*,

Oxfordshire.

32. Delfini, C. & Formica, J.V. (2001) Wine Microbiology: Science and Technology. *CRC Press*, Boca Raton.

33. Cronin, K. & Cocker, R. (2011) Plant and Equipment: Materials and Finishes for Plant and Equipment. *Encylopedia of Dairy Sciences* (2nd Ed.) 134–138.

34. Frank, J. F. & Chmielewski, R. (2001) Influence of surface finish on the cleanability of stainless steel. *J. Food Prot.* **64**, 1178–1182.

35. Lelieveld, H.L.M., Mostert, M.A. & Curiel, G.J. (2014) Hygienic design of food processing equipment. in *Hygeine in Food Processing* (2nd Ed.) 91–141.

36. Schmid, F., Grbin, P., Yap, A. & Jiranek, V. (2011) Relative efficacy of high-pressure hot water and high-power ultrasonics for wine oak barrel sanitization. *Am. J. Enol. Vitic.* **62**, 519–526.

37. Guzzon, R. *et al.* (2011) Survey of the yeast population inside wine barrels and the effects of certain techniques in preventing microbiological spoilage. *Eur. Food Res. Technol.* **233**, 285–291.

38. Chapman, J., Baker, P. & Wills, S. (2011) Winery wastewater handbook: production, impacts and management. *Sustainable Winegrowing British Columbia*, Penticton.

39. Mosse, K.P.M., Patti, A.F., Christen, E.W. & Cavagnaro, T.R. (2011) Review: Winery wastewater quality and treatment options in Australia. *Aust. J. Grape Wine Res.* **17**, 111–122.

40. Pambianchi, D. (2002) Techniques in Home Winemaking: A Practical Guide to Making Château-style Wines. *Véhicule Press,* Montreal.

41. Wise Soltuions (2021) Food Grade Cleaners: Ultimate Performance Non-Toxic Agricultural Inputs and Bio Lubricants. Available at: https://www.wisesolutions.net/food-beverage-processing/food-grade-cleaners.html. (Accessed: 20th February 2021)

42. Kohli, R. (2012) Chapter 3: Methods for Monitoring and Measuring Cleanliness of Surfaces. *Developments in Surface Contamination and Cleaning*, 107–178. *William Andrew Publishing*, Norwich.

43. Duarte, F.L., Lopez, A., Alemao, M.F., Santos, R. & Canas, S. (2011) Commercial sanitizers efficacy - a winery trial. *Ciência e Técnica Vitivinícola* **26**, 45–52.

44. Cais-Sokolinska, D. & Pikul, J. (2008) Evaluation of steel surface cleanliness level in dairies using the bioluminescence method. *Bull. Vet. Inst. Pulawy* **52**, 625–629.

45. National Center for Biotechnology Information (2021). Peracetic acid - Toxicity Summary. *WebWISER Substance List* Available at: https://webwiser.nlm.nih.gov/substance?substanceId=90&identifier=Peracetic acid&identifierType=name&menuItemId=69&catId=79. (Accessed: 25th January 2021)

46. Solis, M., Gadoury, D.M. & Worobo, R.W. (2016) An in vitro and in vivo evaluation of peroxyacetic acid as an alternative sanitizer for wine barrels. *Ciência e Técnica Vitivinícola* **31**, 41–50.

47. Wilker, K.L. & Dharmadhikarf, M.R. (1997) Treatment of Barrel Wood Infected with Acetic Acid Bacteria. *Am J Enol Vitic* **48** 516-520.

48. Meneghin, S.P., Reis, F.C., de Almeida, P.G. & Ceccato-Antonini, S.R. (2008) Chlorine dioxide against bacteria and yeasts from the alcoholic fermentation. *Brazilian J. Microbiol.* **39**, 337–343.

49. Boulton, R.B., Singleton, V.L., Bisson, L.F. & Kunkee, R.E. (1999) Principles and practices of winemaking. *Springer Publications*, New York

50. Chauret, C. P. (2014) Sanitization. In *Encyclopedia of Food Microbiology* (2nd Ed.) 360–364. *Academic Press*, Cambridge.

51. Hora, P.I., Pati, S.G., McNamara, P.J. & Arnold, W.A. (2020) Increased Use of Quaternary Ammonium Compounds during the SARS-CoV-2 Pandemic and Beyond: Consideration of Environmental Implications. *Environ. Sci. Technol. Lett.* **7**, 622–631.

52. Bokulich, N.A., Bamforth, C.W. & Mills, D.A. (2012) A review of molecular methods for microbial community profiling of beer and wine. *J. Am. Soc. Brew. Chem.* **70**, 150–162.

53. Vendrame, M., Manzano, M., Comi, G., Bertrand, J. & Iacumin, L. (2014) Use of propidium monoazide for the enumeration of viable Brettanomyces bruxellensis in wine and beer by quantitative PCR. *Food Microbiol.* **42**, 196–204.

54. Fernández-Pérez, R., Tenorio Rodríguez, C. & Ruiz-Larrea, F. (2019) Fluorescence microscopy to monitor wine malolactic fermentation. *Food Chem.* **274**, 228-233.

55. Bouix, M. & Ghorbal, S. (2013) Rapid enumeration of Oenococcus oeni during malolactic fermentation by flow cytometry. *J Appl Microbiol* **114**, 1075–1081.

56. Reller, L.B., Weinstein, M., Jorgensen, J.H. & Ferraro, M.J. (2009) Antimicrobial Susceptibility Testing: A Review of General Principles and Contemporary Practices. *Clin. Infect. Dis.* **49**, 1749–1755.

57. Rodríguez-Tudela, J. L. *et al.* (2003) Method for the determination of minimum inhibitory concentration (MIC) by broth dilution of fermentative yeasts. *Clin. Microbiol. Infect.* **9**, i–viii.

58. Costa, A., Barata, A., Malfeito-Ferreira, M. & Loureiro, V. (2008) Evaluation of the inhibitory effect of dimethyl dicarbonate (DMDC) against wine microorganisms. *Food Microbiol.* **25**, 422–427.

59. del Alamo-Sanza, M. & Nevares, I. (2018) Oak wine barrel as an active vessel: A critical review of past and current knowledge. *Crit. Rev. Food Sci. Nutr.* **58**, 2711–2726.

60. De Micco, V., Balzano, A., Wheeler, E.A. & Baas, P. (2016) Tyloses and gums: A review

of structure, function and occurance of vessel occlusions. *IAWA J.* **37**, 186–205.

61. Waterhouse, A.L. & Towey, J.P. (1994) Oak Lactone Isomer Ratio Distinguishes between Wine Fermented in American and French Oak Barrels. *J. Agric. Food Chem.* **42**, 1971–1974.

62. Barata, A. *et al.* (2013) Enumeration and Identification of 4-Ethylphenol Producing Yeasts Recovered from the Wood of Wine Ageing Barriques after Different Sanitation Treatments. *J. Food Res.* **2**(1), 140-149.

63. Cartwright, Z.M., Glawe, D.A. & Edwards, C.G. (2018) Reduction of *Brettanomyces bruxellensis* Populations from Oak Barrel Staves Using Steam. *Am. J. Enol. Vitic* **69**, 400-409.

64. Breniaux, M., Renault, P., Meunier, F. & Ghidossi, R. (2019) Study of High Power Ultrasound for Oak Wood Barrel Regeneration: Impact on Wood Properties and Sanitation Effect. *Beverages* **5**(10), 1-13.

65. Swaffield, C.H., Scott, J.A. & Jarvis, B. (1997) Observations on the microbial ecology of traditional alcoholic cider storage vats. *Food Microbiol.* **14**, 353–361.

66. Yao, R., Kwong, G., Miller, K. V & Block, D. E. (2020) Prediction of Effective Steam Sterilization Times for Wine Barrels Using a Mathematical Modeling Approach. *Am. J. Enol. Vitic.* **72,** 101-105.

67. Guzzon, R. *et al.* (2017) The impact of different barrel sanitation approaches on the spoilage microflora and phenols composition of wine. *J. Food Sci. Technol.* **54**, 810–821.

68. Gonzalez-Arenzana, L. *et al.* (2013) Microwave technology as a new tool to improve microbiological control of oak barrels: A preliminary study. *Food Control* **30**, 536–539.

69. Farkaš, J. *Technology and biochemistry of wine*. (New York : Gordon and Breach Science Publishers, 1988).

70. Maximilian I., Heiliges Römisches Reich, K. (1498) Ausschreiben betreffend die Verfälschung des Weins. Freiburg.

71. Ribéreau-Gayon, J. & Peynaud, É. (1982) Sciences et techniques du vin: Analyse et contrôle des vins. *Dunod*, Malakoff.

72. O'Connor-Marer, P.J. (2011) State of California Department of Pesticide Regulation Microbial Pest Control Study Guide, *California Department of Pesticide Regulation*, Sacramento.

73. Ribéreau-Gayon, P. (2006) Handbook of Enology, Volume II. *John Wiley & Sons,* Hoboken.

74. Office international de la Vigne et du Vin (2004) Compendium of international methods of wine and must analysis. *OIV*, Paris

75. International Organization of Wine and Wine (2009) OIV-MA-AS323-II-04A: Sulfur

dioxide (titrimetry). in *Compendium of International Methods of Analysis of Wines and Musts* 1–2, *OIV*.

76. Jenkins, T.W., Howe, P.A., Sacks, G.L. & Waterhouse, A.L. (2020) Determination of Molecular and "Truly" Free Sulfur Dioxide in Wine: A Comparison of Headspace and Conventional Methods. *Am. J. Enol. Vitic.* **71**, 222-230.

77. Coelho, J.M., Howe, P.A. & Sacks, G.L. (2015) A Headspace Gas Detection Tube Method to Measure SO2 in Wine without Disrupting SO2 Equilibria. *Am. J. Enol. Vitic.* **66**, 257-265.

78. Carrascon, V., Ontañón, I., Bueno, M. & Ferreira, V. (2017) Gas chromatography-mass spectrometry strategies for the accurate and sensitive speciation of sulfur dioxide in wine. *J. Chromatogr. A* **1504**, 27–34.

79. Dettmer-Wilde, K., Engewald, W. & Adachour, M. (2014). Practical Gas Chromatography : A Comprehensive Reference, *Springer Publishing*, New York.

80. Siebert, T.E., Solomon, M.R., Pollnitz, A.P. & Jeffery, D.W. (2010) Selective Determination of Volatile Sulfur Compounds in Wine by Gas Chromatography with Sulfur Chemiluminescence Detection. *J. Agric. Food Chem.* **58**, 9454–9462.

81. Jenkins, T. (2018) Determination of 'available' molecular and free sulfur dioxide in wine : a comparison of traditional and novel methods with headspace gas chromatography sulfur chemiluminescence detection. *Masters Thesis, University of California, Davis Department of Viticulture and Enology.*

82. Vasilescu, A., Fanjul-Bolado, P., Titoiu, A.-M., Porumb, R. & Epure, P. (2019) Progress in Electrochemical (Bio)Sensors for Monitoring Wine Production. *Chemosensors* **7**(4), 66.

83. Silva, K.R.B., Raimundo, M., Gimenez, I.F. & Alves, O.L. (2006) Optical Sensor for Sulfur Dioxide Determination in Wines. *J. Agric. Food Chem.* **54**, 8697–8701.

84. Kelly, M. & Wollan, D. (2003) Micro-oxygenation of wine in barrels. *Austrialian New Zeal. Grapregrower Winemak.* 29–32.

85. Junqua, R., Zeng, L. & Pons, A. Characterization of Oak Barrel Specific Oxygen Permeability. in *OENOIVAS* P13 (2019).

86. Suloff, E. C. Sorption Behavior of an Aliphatic Series of Aldehydes in the Presence of Poly(ethyleneterephthalate) Blends Containing Aldehyde Scavenging Agents. (Virginia Polytechnical Institute, 2002).

87. Del Alamo-Sanza, M., Miguel, L., Cárcel, C. & Nevares, I. Characterization of the Oxygen Transmission Rate of Oak Wood Species Used in Cooperage. (2017). doi:10.1021/acs.jafc.6b05188

88. Sorz, J., Hietz, P., (2006) Gas diffusion through wood: implications for oxygen supply. *Trees* **20**, 34–41.

89. LOGIC Incorporated. (2021) One Step Oxidizing Cleanser. Available at: https://www.ecologiccleansers.com/store/one-step/. (Accessed: 20th February 2021)

90. Ocón, E. *et al.* (2013) Screening of yeast mycoflora in winery air samples and their risk of wine contamination. *Food Control* **34**, 261–267.

91. Tek, E.L., Sundstrom, J.F., Gardner, J.M., Oliver, S.G. & Jiranek, V. (2018) Evaluation of the ability of commercial wine yeasts to form biofilms (mats) and adhere to plastic: implications for the microbiota of the winery environment. *FEMS Microbiol. Ecol.* **94**(2), 188.

92. Bastard, A. *et al.* (2016) Effect of Biofilm Formation by Oenococcus oeni on Malolactic Fermentation and the Release of Aromatic Compounds in Wine. *Front. Microbiol.* **27**(7), 613.

93. Boulton, R. (2015) Paired potassium-based buffers for sanitizing winery equipment and the carbon, nitrogen, sodium, and phosphorus footprints of winery cleaning practices. in *Advances in Wine Research*, 379–387, *Oxford University Press*, Oxford.

94. State of California (2021). California State Water Resources Control Board Winery Order 2021. Available at: https://www.waterboards.ca.gov/water_issues/programs/waste_discharge_requirements/winery_order.html. (Accessed: 1st February 2021)

95. Neo, S.Y. *et al.* (2013) Efficacy of chlorine and peroxyacetic acid on reduction of natural microflora, Escherichia coli O157:H7, *Listeria monocyotgenes* and *Salmonella* spp. on mung bean sprouts. *Food Microbiol.* **36**, 475–480.

96. Marques, S.C. *et al.* (2007) Formation of biofilms by Staphylococcus aureus on stainless steel and glass surfaces and its resistance to some selected chemical sanitizers. *Brazilian J. Microbiol.* **38**, 538–543.

97. Branco, P., Monteiro, M., Moura, P. & Albergaria, H. (2012) Survival rate of wine-related yeasts during alcoholic fermentation assessed by direct live/dead staining combined with fluorescence in situ hybridization. *Int J Food Microbiol* **158**, 49–57.

98. Stiefel, P. *et al.* (2016) Is biofilm removal properly assessed? Comparison of different quantification methods in a 96-well plate system. *Appl. Microbiol. Biotechnol.* **100**, 4135–4145.

99. R Core Team (2020) R: A language and environment for statistical computing.

100. Stiefel, P. *et al.* (2016) Enzymes enhance biofilm removal efficiency of cleaners. *Antimicrob. Agents Chemother.* **60**, 3647–3652.

101. Melo, A.S., Bizerra, F.C., Freymüller, E., Arthington-Skaggs, B.A. & Colombo, A.L. (2011) Biofilm production and evaluation of antifungal susceptibility amongst clinical *Candida* spp. isolates, including strains of the *Candida parapsilosis* complex. *Med. Mycol.* **49**, 253–262.

102. Christaki, T. & Tzia, C. (2002) Quality and safety assurance in winemaking. *Food Control* **13**, 503–517.

103. Aggelogiannopoulos, D., Drosinos, E.H. & Athanasopoulos, P. (2007) Implementation of a quality management system (QMS) according to the ISO 9000 family in a Greek small-sized winery: A case study. *Food Control* **18**, 1077–1085.

104. Ioannou, L. A., Puma, G. L. & Fatta-Kassinos, D. (2015) Treatment of winery wastewater by physicochemical, biological and advanced processes: A review. *J. Hazard. Mater.* **286**, 343–368.

105. Li, Q. *et al.* (2017) Preparation Process Optimization of Solid Chlorine Dioxide as an Environmental Friendly Disinfectant for Drinking Water. *Key Engineering Materials* **737**, 433–437.

106. Guzzon, R. *et al.* (2017) The impact of different barrel sanitation approaches on the spoilage microflora and phenols composition of wine. *J. Food Sci. Technol.* **54**, 810–821.

107. Wiegand, I., Hilpert, K. & Hancock, R.E.W. (2008) Agar and broth dilution methods to determine the minimal inhibitory concentration (MIC) of antimicrobial substances. *Nat. Protoc.* **3**, 163-175.

108. Girard, L.P., Ceri, H., Gibb, A.P., Olson, M. & Sepandj, F. (2010) MIC Versus MBEC to Determine the Antibiotic Sensitivity of Staphylococcus aureus in Peritoneal Dialysis Peritonitis. *Perit. Dial. Int.* **30**, 652–656.

109. Hašek, J. (2006) Yeast Fluorescence Microscopy. in *Yeast Protocol*, 85–96, *Humana Press,* Totowa.

110. Stiefel, P., Schmidt-Emrich, S., Maniura-Weber, K. & Ren, Q. (2015) Critical aspects of using bacterial cell viability assays with the fluorophores SYTO9 and propidium iodide. *BMC Microbiol.* **15**, 36.

111. Berney, M., Hammes, F., Bosshard, F., Weilenmann, H.U. & Egli, T. (2007) Assessment and Interpretation of Bacterial Viability by Using the LIVE/DEAD BacLight Kit in Combination with Flow Cytometry. *Appl. Environ. Microbiol.* **73**, 3283-3290.

112. Zhang, T. & Fang, H.H.P. (2004) Quantification of Saccharomyces cerevisiae viability using BacLight. *Biotechnol. Lett.* **26**, 989–992.

113. CLSI. (2008) Reference Method for Broth Dilution Antifungal Susceptibility Testing of Yeasts; Approved Standard — Second Edition Serving the World's Medical Science Community Through Voluntary Consensus. *NCCLS standards and guidelines* **22**.

114. Wine Institute (1961) Sanitation guide for wineries. *Wine Institute,* San Francisco.

115. Cosme, F., Pinto, T. & Vilela, A. (2018) Phenolic Compounds and Antioxidant Activity in Grape Juices: A Chemical and Sensory View. *Beverages* **4**(1), 22 (2018).

116. Patel, R. (2005) Biofilms and antimicrobial resistance. in *Clinical Orthopaedics and*

Related Research 41–47. *Lippincott Williams and Wilkins*, Philadelphia.

117. Ceri, H. *et al.* (1999) The Calgary Biofilm Device: New technology for rapid determination of antibiotic susceptibilities of bacterial biofilms. *J. Clin. Microbiol.* **37**, 1771–1776.

118. Davey, H.M. & Hexley, P. (2011) Red but not dead? Membranes of stressed Saccharomyces cerevisiae are permeable to propidium iodide. *Environ. Microbiol.* **13**, 163–171.

119. Huang, L. (2013) Determination of thermal inactivation kinetics of Listeria monocytogenes in chicken meats by isothermal and dynamic methods, *J. Food Control* **33**(2), 484-488.

120. Balouiri, M., Sadiki, M. & Ibnsouda, S.K. (2016) Methods for in vitro evaluating antimicrobial activity: A review. *J. Pharm. Anal.* **6**, 71–79.

121. Tawakoli, P.N., Al-Ahmad, A., Hoth-Hannig, W., Hannig, M. & Hannig, C. (2013) Comparison of different live/dead stainings for detection and quantification of adherent microorganisms in the initial oral biofilm. *Clin Oral Investig* **17**, 841–850.

122. Ribéreau-Gayon. P., Glories Y., Maujean A., Dubourdieu, D., (2006) Aging Red Wines in Vat and Barrel: Phenomena Occurring During Aging. in *Handbook of Enology* 387–428, *John Wiley & Sons*, Hoboken.

123. Bershaw, D. (2011) Managing Barrels for the Prevention of Common Wine Flaws. *Southern Oregon Wine Institute*, Roseburg.

124. California Department of Pesticide Regulation (2020). Laws and Regulations Study Guide, *California DPR*, Sacramento.

125. Spencer, J.F.T., Ragout de Spencer, A.L. (2014) Environmental microbiology : methods and protocols. *Springer Publications*, New York.

126. Krisch, J. *et al.* (2016) Latest about Spoilage by Yeasts: Focus on the Deterioration of Beverages and Other Plant-Derived Products. *J. Food Prot.* **79**, 825–829.

127. Chatonnet, P., Dubourdieu, D. & Boidron, J.N. (1995) The Influence of *Brettanomyces/Dekkera* sp. Yeasts and Lactic Acid Bacteria on the Ethylphenol Content of Red Wines. *Am. J. Enol. Vitic.* **46**, 463 LP – 468.

128. Feuillat, F. *et al.* (1994) Simulation expérimentale de l'expérience tonneau. Mesure des cinétiques d'imprégnation du liquide dans le bois et d'évaporation de surface. *OENO One* **28**, 227–245.

129. M. Malfeito-Ferreira *et al.* (2004) Effect of Different Barrique Sanitation Procedures on Yeasts Isolated from the Inner Layers of Wood. *Am. J. Enol. Vitic.* **53**, 304A.

130. Curiel, J. A. *et al.* (2016) Identification of target genes to control acetate yield during aerobic fermentation with Saccharomyces cerevisiae. *Microb. Cell Fact.* **15**, 156.

131. Bartowsky, E.J., Xia, D., Gibson, R.L., Fleet, G.H. & Henschke, P.A. (2003) Spoilage of bottled red wine by acetic acid bacteria. *Lett. Appl. Microbiol.* **36**, 307–314.

132. Romano, A., Perello, M.C., Lonvaud-Funel, A., Sicard, G. & de Revel, G. (2009) Sensory and analytical re-evaluation of 'Brett character'. *Food Chem.* **114**, 15–19.

133. Alamo-Sanza, M. del *et al.* (2016) Analysis of the role of wood anatomy on oxygen diffusivity in barrel staves using luminescent imaging. *Sensors Actuators B Chem.* **237**, 1035–1043.

134. Serpaggi, V. *et al.* (2012) Characterization of the 'viable but nonculturable'(VBNC) state in the wine spoilage yeast Brettanomyces. *Food Microbiol.* **30**, 438–447.

135. Junqua, R., Zeng, L., Pons, A. (2019) Oak Barrels: How moisture content influences oxygen permeability. *Oenoivas 2019,* III.P13.

136. Siau, J.F. (1995) Wood: influence of moisture on physical properties., *Dept. of Wood Science and Forest Products, Virginia Polytechnic Institute and State University*, Blacksburg.

137. del Alamo-Sanza, M. & Nevares, I. (2014) Recent advances in the evaluation of the oxygen transfer rate in oak barrels. *J. Agric. Food Chem.* **62**, 8892–9.

138. Nevares, I. *et al.* (2016) Ratiometric Oxygen Imaging to Predict Oxygen Diffusivity in Oak Wood During Red Wine Barrel Aging. *Food Bioprocess Technol.* **9**, 1049–1059.

139. Koliadima, A., Kapolos, J. & Farmakis, L. (2009). Diffusion coefficients of so2 in water and partition coefficients of so2 in water-air interface at different temperature and pH values. *Instrum. Sci. Technol.* **37**, 274–283.

140. Benallou, A. (2019) Fick's First Law: Diffusion Coefficients. in *Mass Transfers and Physical Data Estimation* 49–83. *John Wiley & Sons*, Hoboken.

141. Carrascon, V., Ontañón, I., Bueno, M. & Ferreira, V. (2017) Gas chromatography-mass spectrometry strategies for the accurate and sensitive speciation of sulfur dioxide in wine. *J. Chromatogr. A* **1504**, 27–34.

142. Khan, M. A. H., Rao, M. V & Li, Q. (2019) Recent Advances in Electrochemical Sensors for Detecting Toxic Gases: NO_2, SO_2 and H_2S. *Sensors* **19**, 905.

143. Stetter, J.R., Patel, V., Findlay, M.W. & Carter, M.T. (2017) Patent (#9,784,708 B2) Printed gas sensor. *United States Patent and Trademark Office*.

144. Schneider, M., Türke, A., Fischer, W.J. & Kilmartin, P.A. (2014) Determination of the wine preservative sulphur dioxide with cyclic voltammetry using inkjet printed electrodes. *Food Chem.* **159**, 428–432.

145. Ramos, R.M., Gonçalves, L.M., Vyskočil, V. & Rodrigues, J.A. (2016) Free sulphite determination in wine using screen-printed carbon electrodes with prior gas-diffusion microextraction. *Electrochem. commun.* **63**, 52–55.

146. Wang, H. *et al.* (2010) Effective and inexpensive gas chromatography method for detection of trace level SO_2 in H fuel. *J. Shanghai Univ.* **14**, 322–325.

147. SPEC Sensors LLC (2016) SPEC Sensor Performance in Extreme Environments. *Spec Sensors*, Boston.

148. Oliveira, F.A., Ferreira, R.M.S., Lapas, L.C. & Vainstein, M.H. (2019) Anomalous Diffusion: A Basic Mechanism for the Evolution of Inhomogeneous Systems. *Frontiers in Physics* **7**, 18..

149. Wang, Z., Wu, G. & Jönsson, L.J. (2018) Effects of impregnation of softwood with sulfuric acid and sulfur dioxide on chemical and physical characteristics, enzymatic digestibility, and fermentability. *Bioresour. Technol.* **247**, 200–208.

150. Crank, J. (1975) The Mathematics of Diffusion. *Clarendon Press*, Oxford.