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Brettanomyces infection in wine

10.1 What is 'Brett'?

L. Bisson

'Brett' is a colloquialism referring to the yeast *Brettanomyces*. *Brettanomyces* is a budding yeast also called *Dekkera* and is perhaps the most controversial organism of wine production. *Brettanomyces* is frequently isolated from wines. If these isolates display sexual spore formation they are classified as *Dekkera*. If no spores are formed they are classified as *Brettanomyces*. DNA sequence comparisons indicate that *Dekkera* and *Brettanomyces* are identical, but the use of the two terms still persists in taxonomic classification.

Brettanomyces infection of wine is widespread and this yeast has been isolated from all wine-producing regions on six continents. This yeast is able to form biofilms, coatings of tanks, hoses and other winery surfaces that are difficult to eliminate and organisms in biofilms can resist sanitation agents and survive. Because wood is porous and provides wood sugars barrels are difficult to clean and sanitize and often host populations of *Brettanomyces*. Another reason *Brettanomyces* is such a problem is that it can exist in what is called a viable non-culturable (VNC) state. Most sanitation practices are monitored for effectiveness by determining if viable cells remain on the surface that was treated. Organisms in a VNC state do not grow under these conditions and give the false impression that the sanitation regimen was effective, but are in fact still viable and capable of growing later on.

Brettanomyces is known for growing under harsh environmental conditions and has an interesting metabolism. This organism makes both ethanol and acetate from sugar, and which form is produced depends upon the strain and the conditions of growth. More importantly, *Brettanomyces* is able to produce vinyl

phenols from wine phenolic compounds. The characters made by *Brettanomyces* are described as: sweaty horse, animal, earthy, wet wool, burnt plastic, Band-Aid, barnyard, smoky, soy sauce, spicy, mushroom, putrid, leather, mushroom, tobacco, lilac, wet dog, pharmaceutical, soapy, and baby diaper just to name a few. Not all strains produce all characters, and there is a great variation in the levels and spectrum of characters produced. It is difficult to predict how a given strain will perform in a specific wine, as the factors leading to the appearance of these compounds are not well known. This is the heart of the controversy surrounding this yeast – some winemakers feel that the conversion of grape varietal characters to specific *Brettanomyces* end products is central to the development of varietal character and the expression of terroir. Other winemakers consider the development of these characters to detract from true varietal character giving the wine an off-taste and defective aroma.

10.2 What is the history of *Brettanomyces* and where does it come from?

L. Van de Water

Taxonomy

With sincere apologies to Shakespeare, 'What's in a name? Would 'Brett' by any other name smell as nasty?' Some taxonomists use the name *Brettanomyces* (anamorph, nonsporulating) for these yeasts, others use *Dekkera* (teleomorph, sporulating) for all of them, and some use a combination. For simplicity here we will use the name *Brettanomyces* to mean *Dekkera/Brettanomyces bruxellenensis*, excluding *Dekkera anomala.*

Of the five *Brettanomyces/Dekkera* species currently recognized (*bruxellensis, custersianus, naardenensis, and nanus*, plus *D. anomala)*, only *bruxellensis* is consistently isolated from wine. Former species that were consolidated into *bruxellensis* after genetic and other tests include *B. custersii, B. intermedius, B. lambicus, D. abstinens*, and *D. intermedia* (Smith *et al.*, 1990; Smith, 1998; Barnett *et al.*, 2000; Egli and Henick-Kling, 2001). *Dekkera anomala* has been isolated in Europe from cider (Delanoe and Suberville, 2005) soft drinks (Smith and van Grinsven, 1984), beer and wine (Oelofse *et al.*, 2009). In the USA it has been isolated from cider and fruit juice (personal observation).

Because the same yeast's name changes when its taxonomy changes, and because different researchers continue to call the same yeast by different names, someone who is not a yeast taxonomist is likely to be confused. If you check the references cited here, you will find a plethora of different names, many of which have since been consolidated. However, a 'Brett-type' yeast in wine is almost certain to be *Brettanomyces/Dekkera bruxellensis* (it could be *D. anomala* but that is less likely), so you are dealing with the same yeast, no matter what its current taxonomic name(s) is/are.

Many different strains of *Brettanomyces/Dekkera bruxellensis* have been isolated from wine and studied to one extent or another. Genetic and physiological differences among strains are profound, and will be discussed in **10.3**.

History and distribution

In *Études sur le vin*, the original treatise on wine microbiology, Pasteur does not describe any malady that seems to refer to *Brettanomyces*, nor do his meticulous drawings include any yeasts resembling them microscopically (Pasteur, 1866). *Brettanomyces* was identified in 1904 in British beer (Claussen, 1904), and subsequently in beer and wine from other countries. 'Lambic beer' and some ciders are traditionally produced with *Brettanomyces* yeast. By now, *Brettanomyces* infection of wine has been confirmed in wines on six continents, and in nearly every region where wine is made (Licker *et al.*, 1998; Arvik and Henick-Kling, 2002). Our labs have cultured *Brettanomyces* from wines from Argentina, Australia, Canada, Chile, France, Italy, Moldova, New Zealand,

Thailand, Uruguay, and USA, which is to say, all countries whose wines were cultured.

Interestingly, the genetics of the strains studied by Conterno *et al.* (2006) indicated a loose geographical association among isolates, but there were many exceptions, with some New World strains in groups of Old World strains, and vice versa. For example, their largest grouping on the basis of 26S rDNA included strains from France and Belgium but also strains from California and Chile, plus a morphologically distinct strain from Thailand. Complicating the picture, physiological characteristics did not match the genetic groupings. Dias *et al.* (2003) and Curtin *et al.* (2005, 2007) also described strains belonging to one genotype found in wines from widely separated countries.

Recent history of *Brettanomyces* identification

In California, a 1972 Napa Valley Gamay spoiled in the bottle. The change was appalling; a fruity wine with lots of mid-palate flavors lost its fruitiness, developed strong horsey odors, became simple-tasting, and had a metallic aftertaste (personal observation, 1973). *Brettanomyces* was isolated and identified in 1974 from that Gamay by Dr Ralph Kunkee, microbiology professor at U.C. Davis (Kunkee, personal communication; Kunkee, 1996). Shortly afterwards, the yeast was cultured and identified in a number of other California wines (personal experience; personal communication, names withheld on request). Before then, *Brettanomyces* was called 'the Cucamonga stink' in California's Central Valley, because although it was recognized as a spoilage problem, they did not know what caused it. The classic 'Brett' descriptors (see **10.4**) were consistently noticed in all these wines, though there was no method at the time to identify the ethyl phenols and other compounds responsible.

Unfortunately, winemakers were reticent to discuss their experiences with other winemakers, which delayed recognition of the widespread nature of the *Brettanomyces* problem for some years. 'Brett' was actually called 'the clap' by some wineries, because of winery-to-winery transmission, because the infection was easy to miss (a slogan for STD testing at the time was 'you can have it and not know'), and because of the stigma attached to the infection. The result was that *Brettanomyces* was almost a taboo subject in California for a number of years. A prominent winemaker, reached by phone in 1974 because 'Brett' character had been detected in one of his wines, thundered, 'Why the hell would you want to talk about a thing like that?' and slammed down the receiver. Few winemakers acknowledged publicly (or even to their own sales and marketing people) that their wineries were infected, although most who were producing red wine knew they had a *Bretttanomyces* infection in their cellars. To this day, a 'don't ask, don't tell' dynamic lingers on.

During the 1970s and 1980s, wines were intensely scrutinized for *Brettanomyces* infection in California at wineries and commercial labs, but formal research was seldom if ever funded until the 1990s. USA research funding still struggles mightily, though some very important studies have been

performed already, and can continue if funded. More research is now carried out in other countries, because of funding considerations.

In some winegrowing countries, *Brettanomyces* was identified years earlier, but sometimes the awareness faded. For example, *Brettanomyces* was studied in South African wines in the late 1950s and early 1960s (van der Walt and van Kerken, 1960), and the genus *Dekkera* was named in 1964 (van der Walt, 1964). However, by 2004, many South African winemakers were unaware of *Brettanomyces* infections in their wineries (personal observation).

Winemakers in other countries also discovered relatively recently that *Brettanomyces* is the origin of certain sensory characteristics. Despite excellent Australian research on ethyl phenol production by *Brettanomyces* (Heresztyn, 1986), until the late 1990s it was assumed by many Australians that the so-called 'Hunter Valley stink' in Syrah was a regional or varietal characteristic. However, even in the 1970s, Californians visiting Australia recognized the telltale sensory characters of *Brettanomyces* in some of the wines, and some Australian winemakers were discussing the possibility that there was *Brettanomyces* in Australia (Carol Shelton, personal communication). Since 1999, when it was discovered that a large proportion of Australian reds had high ethyl phenol levels (Henschke *et al.*, 2004), the Australian Wine Research Institute have been extremely proactive in their research on *Brettanomyces* and in educating winemakers about the problem. By 2004, 1500 winemakers had attended their 'Institute Roadshows'.

While *Brettanomyces* was discovered in French wine in 1930 by researchers in Germany (Licker *et al.*, 1998), and studies continued in France over time (for example, Peynaud and Domercq, 1956), the link between *Brettanomyces* and 4-ethyl phenols in French wine was not observed until 1992 (Chatonnet *et al.*, 1992). Until then, *Brettanomyces* was assumed by French winemakers to produce only 'mousiness' (Ribereau-Gayon *et al.*, 2006). However, the other effects of *Brettanomyces* growth – horsey, barnyard, spicy descriptors – had been detected sensorily for many years in some French wines by winemakers and enologists in California, and *Brettanomyces* had been cultured from certain French wines since 1974 (personal observation). For example, a 1978 Bordeaux from a highly regarded château was cultured because of a complaint from a St Helena, CA, retailer, and was found to be heavily infected with *Brettanomyces*.

It is unfair to point to any one region as more thoroughly infected than another, however. Management practices usually make the difference in wines with 'Brett' character, not numbers of infected wineries. In the 1970s, Californians considered the Burgundy region in France to be a hotbed of *Brettanomyces* because of a miscommunication. Gerald Asher, then wine writer for *The San Francisco Chronicle*, asked Robert Mondavi why his 1971 Reserve Pinot Noir seemed so Burgundian, and he replied, 'Oh, that's from the touch of *Brettanomyces* we had in there.' In fact, that wine did not have *Brettanomyces*, it had *Pediococcus* (personal observation) which is very common in red Burgundies.

Does *Brettanomyces* come from the vineyard?

Whether or not *Brettanomyces/Dekkera bruxellensis* – the wine species – is commonly present in vineyards is very controversial. Some winemakers believe that *Brettanomyces* comes in on most grapes, therefore all red wines have *Brettanomyces*. Popular articles quoting wine writers and winemakers are filled with numerous references to *Brettanomyces* on grapes, but almost all of that evidence is anecdotal. A California winemaker declared in *Harpers Wine and Spirit Review* (Goode, 2003), 'Since Brett is largely ubiquitous, a rampant Brett infection is often more of a function of a large inoculum coming in on the grapes'.

Are these ideas about *Brettanomyces* substantiated by research? No. The opinion that *Brettanomyces* is part of the natural flora of all red wines is not borne out by testing of wines in hundreds of wineries over many years (personal observation). After reviewing formal research, independent lab results, and in-house studies performed by wineries, the inescapable conclusion is that not all wineries are infected with *Brettanomyces*, even when neighboring wineries are.

In fact, if *Brettanomyces bruxellensis* is present in vineyards, it is very difficult to find. A number of studies around the world over the past 35 years did not find the yeast on grapes (Fugelsang *et al.*, 1993; Oelofse *et al.*, 2008; also personal communication). In a 2007 French study, Renouf and Lonvaud-Funel (2007) developed a special liquid medium, EBB, to culture *Brettanomyces* yeasts from grapes, and then used polymerase chain reaction (PCR) to confirm the yeasts' identity. Similar studies in other regions would be helpful; a Napa Valley winery using EBB medium located no *Brettanomyces* on their grapes, but other yeast species (*Candida catenulata* and *Debaromyces hansenii)* grew. Those species also reacted with other tests presumed to be specific for *Brettanomyces* (personal communication, name withheld on request).

Many winemakers assume that *Brettanomyces* can populate vineyards if infected pomace is spread in the vineyard. This may indeed occur, but it has not yet been proven by controlled studies (a good topic for future research). The yeasts were found on airborne droplets within a winery (Connell *et al.*, 2002), and in insects in and around wineries by van der Walt and van Kirken (1960), though not on pomace. It has not been established that insects are actually vectors for infection of wine among different wineries. Species of *Brettanomyces* have been found in fruit orchards and in bees (Licker *et al.*, 1998), but it was not determined whether or not these belonged to *bruxellensis*; if not, they were unlikely to infect wine. All in all, *Brettanomyces* may be present at very low levels in some vineyards, but winemakers should not look to grapes as a primary source of *Brettanomyces* infection.

Brettanomyces in the winery

So how do the yeasts get into a winery? The most important vehicles of transmission of these yeasts from cellar to cellar are infected wines and wooden or plastic containers (Fugelsang *et al.*, 1993; Boulton *et al.*, 1996; also personal experience). Movement of bulk wine in a number of countries has spread

Brettanomyces yeasts internationally and domestically (Curtin *et al.*, 2007). When infected wine is purchased, unless the winery is prepared to deal with the infection before it enters the cellar, one wine can contaminate the entire cellar. Purchasing used red wine barrels from other wineries is economically very tempting, although *Brettanomyces* often comes along also (white wine barrels are much less frequently infected). There is no legal or moral requirement to disclose the *Brettanomyces* infection status of barrels for sale, even if known; it is definitely a 'buyer beware' situation. The practice of importing used barrels from Bordeaux wineries with the hope of capturing some of the 'gout de terroir' of that region has assisted in distributing *Brettanomyces* around the world. Used whiskey barrels have not been implicated in spreading *Brettanomyces*, presumably because the high alcohol content of whiskey prevents an infection from taking hold, even if somehow the yeasts were introduced.

Case histories abound of *Brettanomyces* entering a winery through wine or wood and taking up residence, infecting subsequent wines for many years thereafter. Three examples:

1. A new winery was built for the 2001 vintage. They were warned never to bring in anything from another winery, and had three vintages completely free of *Brettanomyces*, although the winery was in a valley full of infected wineries. Then, in 2004, the new winemaker bought 5000 liters of wine from another winery, but did not check it for *Brettanomyces* beforehand. The wine was infected, and as a result, the winery has been fighting *Brettanomyces* ever since (personal observation).
2. A winemaker whose small winery had been free of *Brettanomyces* unknowingly purchased an infected wine and used it to top all his red barrels, infecting the entire cellar.
3. The 1995 decision by a New Zealand winery to sell barrels after only three years sent *Brettanomyces* quickly around the country, into numerous wineries that previously were uninfected (personal observation).

Once in the winery, the yeasts are easily spread from wine to wine. Numerous studies have found infections in various places inside wineries, and as far back as 1960, it was recognized that these pools of *Brettanomyces* infection in the winery could lead to cross-contamination. 'The infection of wines and musts by *Brettanomyces* species is due to contamination spreading from latent foci of infection within the winery' (van der Walt and van Kirken, 1960).

Sanitizing measures such as SO_2, ozone, peroxycarbonate, and peracetic acid reduce the population in a barrel but do not kill all the yeasts, which can be 0.8 cm deep in the wood (Malfeito-Ferreira *et al.*, 2004). Shaving a barrel greatly reduces the infection (Pollnitz *et al.*, 2000) but does not always completely eliminate it. Asked why he was the only one in New Zealand with *Brettanomyces* in his Chardonnay, a winemaker immediately replied, 'That's because I use shaved red wine barrels for my Chardonnay' (personal communication, name withheld on request). The shaving got rid of the color but did not go down far enough to eliminate the infection deep in the staves.

Brettanomyces grows more readily in new barrels, but this is more likely to be related to the composition of new wood, rather than new barrels bringing in an infection. While some winemakers believe new barrels to be a primary source, this has not been confirmed by research (see **10.3**).

Avoiding *Brettanomyces* infection:

- Do not buy barrels, or wooden or plastic tanks, that were previously used for red wine in another cellar (white wine barrels could be infected but are much less likely to be).
- Do not buy bulk red wine unless it can be sterile-filtered before going into wood or plastic containers in your cellar.
- Do not buy equipment that cannot be sanitized with hot water or steam before use.

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10.3 How does *Brettanomyces* grow?

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Brettanomyces can live on other microbes' nutritional leftovers, earning themselves the nickname 'junkyard dog of wine yeasts'. They are surprisingly versatile in their nutritive requirements. At least some strains of *Brettanomyces bruxellensis* can live in almost any red wine, and some whites, below around 15% alcohol or so, bottled or not, dry or sweet. They can use a number of carbon-containing substrates, some of which are always present in the wine. Low wine pH is not a limiting factor except as it relates to molecular SO_2 (Conterno *et al.*, 2006).

Red wines are most at risk for *Brettanomyces* infection and growth. Red wine pH is higher than most whites, so SO_2 protection is not as effective. Reds are often kept warmer during at least part of their cellaring, and they often are aged in infected cooperage for a much longer time than whites would be. However, white wines are not immune to *Brettanomyces*; infections have been found in white wines fermented or aged in used wood (even if shaved), and in sparkling wine.

It has often been noted that *Brettanomyces* yeasts grow more extensively in new barrels compared to old ones. It was therefore speculated that this meant that new barrels were contaminated with *Brettanomyces*, but this has not been substantiated. Rather, it has been shown that most strains can use the disaccharide cellobiose, a component of toasted wood, as a sole carbon source (McMahon and Zoecklein, 1999; Mansfield *et al.*, 2002). This is a very important aspect of *Brettanomyces* growth, especially in new barrels. Cellobiose is more abundant in new oak, promoting more active growth if new barrels are filled with infected wines (Boulton *et al.*, 1996; Fugelsang and Edwards, 2007).

At normal cellar temperatures (13ºC/55ºF) and conditions the yeasts grow slowly over some months or may even become temporarily dormant, though at a warm temperature (over 20ºC/68ºF) they may grow significantly in a few weeks (Chatonnet *et al.*, 1995). For example, warming stuck wines to encourage fermentation also encourages *Brettanomyces* growth, if the wine has already become infected. Inconveniently, exponential growth may be stimulated by a rise in storage temperature when unfiltered wine is sent to market.

Brettanomyces can grow throughout the wine, but the cells tend to settle in barrels or tanks, so the population is often higher near the bottom, sometimes much higher (Boulton *et al.*, 1996). In barrels with ill-fitting bungs or other containers with ill-fitting closures, there may also be a population near the top, taking advantage of dissolved oxygen to broaden their range of substrates. Some strains make biofilms that can adhere to surfaces in contact with the wine, such as tanks, hoses, and other equipment (Joseph *et al.*, 2007). Despite some reports, the yeast does not normally make a film on the surface of cellared wine (Fugelsang *et al.*, 1993). Occasionally a very thin film may form (which can extend up the sides of a small glass container for several cm) if an infected lab sample of wine is continually exposed to air for several months (personal observation).

Micro-oxygenation is contraindicated for *Brettanomyces*-infected wines, because research shows that they take up the oxygen more quickly than the phenolics do, stimulating Brett growth (du Toit *et al.*, 2005, 2006). While stimulated by oxygen, they do not need it; the yeasts also grow in unfiltered, bottled wine (Chatonnet *et al.*, 1992; Fugelsang and Zoecklein, 2003; Romano *et al.*, 2008; Coulon *et al.*, 2010; also personal observation).

Brettanomyces appear to be less sensitive to CO_2 than *Saccharomyces* because they have been isolated from bottled sparkling wine (Ciani and Ferraro, 1997; Licker *et al.*, 1998). One *methode champenoise* sparkling wine had viable *Brettanomyces* cells after having been *en tirage* (on yeast lees, with a large buildup of CO_2) for four years (personal observation).

Strain diversity

Winemakers everywhere – and even wine microbiologists – tend to think of *Brettanomyces/Dekkera* as one yeast, but actually we should speak of the members of this species as 'them' rather than 'it.' There are many strains of this yeast, with extremely variable genetics and characteristics. Even chromosome size and number varies among *bruxellensis* strains (Oelofse *et al.*, 2008). Thus, whenever one discusses *Brettanomyces*, much of what one can say about a particular strain can be contradicted by the behavior of another strain (Egli and Henick-Kling, 2001; Dias *et al.*, 2003; Conterno *et al.*, 2006; Barbin *et al.*, 2008). Strain differences can account for some (though not all) of the misconceptions about *Brettanomyces* that abound in the popular literature.

Everyone does agree that strain diversity and distribution in *Brettanomyces* are quite extensive. Curtin *et al.* (2007) tested 244 isolates in 31 regions in Australia and identified eight genotypes; 207 isolates from 28 regions were one genotype, a few isolates fit each of two other genotypes, and five genotypes had one isolate each. Most wineries had only one genotype, but a few had more. Barbin *et al.* (2008) found 23 strains in 24 isolates from two French wineries over three years. Martorell *et al.* (2006) found the same molecular pattern in strains from Portugal and from the USA.

Strain differences in growth and metabolism

All 35 strains in a study by Conterno *et al.* (2006) utilized arginine and proline, the two major amino acids in grapes, as a sole nitrogen source. Proline is not normally used by *Saccharomyces* so it is available after fermentation. Arginine is used during yeast fermentation, but some can be left over because of high levels in grapes, overenthusiastic nutrient supplementation, and yeast autolysis during lees aging.

Those 35 strains also had an absolute requirement for the vitamins thiamine and biotin, which are also used by other microbes. Competition for these and/or other nutrients may be relevant to observations in wineries. One winery noticed that barrels infected with *Brettanomyces* struggled with malolactic fermentation

(MLF) while uninfected barrels of the same wine did not, and barrels that had completed MLF did not grow *Brettanomyces* as readily as barrels of the same wine that had not yet undergone MLF (personal communication, name withheld on request). Romano *et al.* (2008) proposed that MLF might increase toxicity toward *Brettanomyces*.

Conterno *et al.* (2006) confirmed that different strains can metabolize different substrates, show different growth patterns and morphology, and have different tolerances to alcohol, SO_2, and temperature. Some strains did not produce the classic sensory hallmarks, 4-ethyl phenol (4EP) and 4-ethyl guaiacol (4EG), in the wine used, though the researchers postulated that those same strains may do so in other wines (Lucy Joseph, personal communication).

Sugars

As seen in Table 1, all of the 35 isolates could grow on the hexose sugars, glucose and fructose and the disaccharide sucrose, and most could use galactose, maltose, cellobiose, and trehalose; some strains could also use other sugars and sugar alcohols. Dry wines, with combined glucose and fructose levels by enzymatic analysis of 0.2 g/L or less, are quite able to support *Brettanomyces* growth (Chatonnet *et al.*, 1995). *Brettanomyces* growth is too slow to compete successfully with *Saccharomyces* for sugar during yeast fermentation, but in stuck wines or bottled wines, *Brettanomyces* may help themselves to small amounts of various sugars along with other substrates.

Alcohol

Some strains (more European ones than US ones) can use ethyl alcohol as a sole carbon source in culture (Rodrigues *et al.*, 2000, Conterno *et al.*, 2006), and also in wine, reducing the alcohol level by a few tenths of a percent (personal observation). In aerobic conditions, many strains can produce alcohol and acetic acid from sugar. In anaerobic conditions acetic acid is not produced, and alcohol fermentation is at least temporarily inhibited, which is called the 'Custers Effect' (Ciani and Ferraro, 1997).

Variations in strain tolerance to alcohol have not been studied extensively, but this is definitely important. Barata *et al.* (2008) studied 29 strains, two of which tolerated almost 15% alcohol, but two did not even tolerate 14%. It may be significant the authors used mostly European strains, and included only two strains from California; many California wines 14% and over regularly grow *Brettanomyces*, and some strains manage alcohol levels at or over 15% (personal experience).

Growth patterns

There are also differences in growth patterns among strains. In a two-year study by Fugelsang and Zoecklein (2003), one strain reached a peak of cell growth, then declined (Fig. 1). Other strains grew, then declined so much that they were

Table 1 Summary of physiological characteristics of 35 *Brettanomyces* strains

Character tested	Frequency (%)	Isolates
Carbon source growth		
Arginine, cellulose, proline, tartrate	0	0
Adonitol	2	6
Arabinose, citrate, starch	3	9
Lactose, mannitol, raffinose	4	11
Ethanol	9	26
Glycerol	10	29
Lactate	12	34
Succinate	13	37
Malate	14	40
Galactose	28	80
Cellobiose, maltose	32	91
Trehalose	34	97
Sucrose	35	100
Nitrogen source growth		
Nitrate	25	71
Arginine, proline	35	100
Temperature growth		
at 37ºC	13	37
at 10ºC	11	31
Alcohol		
Tolerance >10%	35	100
Sulfite tolerance		
>30 mg/L at pH 3.4	17	49
pH growth		
at pH 2.0	33	94
4-EP and 4-EG (µg/L)		
High (>2000 4-EP; >1500 4-EG)	17	49
Medium (1000–2000 4-EP; 700–1500 4-EG)	6	17
Low (<50 4-EP; <60 4-EG)	7	20
None (<4.0 4-EP and 4-EG)	7	17

Source: Conterno *et al.* (2006).

not detectable by culturing, but later reached a second growth peak. Even when the culturable cell population had declined, the cells were still actively producing ethyl phenols. In another study (Barbin *et al.*, 2008), nine growth profiles of four types were found in 23 strains, including a two-stage growth by some strains. They wrote, 'One can assume that a compound necessary for growth was in default and that the yeast adjusted its metabolism before pursuing growth.' Whether or not the enzymes can continue to act in the wine after the cells have autolysed is not yet known. Much more research is needed on strain growth patterns to understand the behavior of these yeasts in wines.

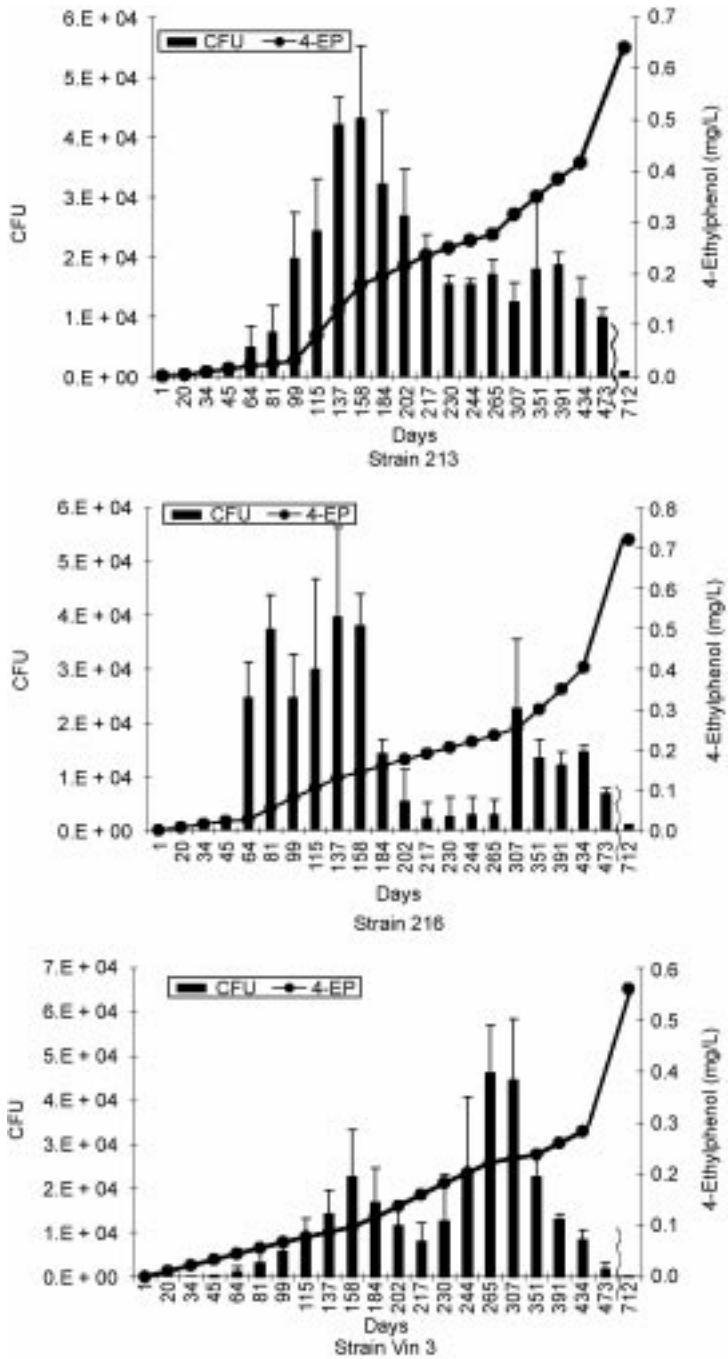

Fig. 1 Colony forming units/mL and concentration (mg/L) of 4-ethylphenol produced by three strains of *Brettanomyces bruxellensis* in Pinot noir wine. Source: Fugelsang and Zoecklein (2003).

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10.4 What do *Brettanomyces* do to wines?

L. Van de Water

Whether or not one appreciates the changes, it is obvious that *Brettanomyces/Dekkera* do affect the sensory characteristics of wine, sometimes only to a small degree, but sometimes profoundly. The more extensive the growth, and the longer the cells are present in the wine, the greater the sensory impact. However, it is essential to distinguish the sensory effects of *Brettanomyces* from other defects. For example, a 'barnyard' character may indeed be caused by *Brettanomyces*, but that descriptor is also used for H_2S or *Pediococcus*. Laboratory tests are necessary to distinguish different microbiological origins of sensory defects.

Production of ethyl phenols

Most *Brettanomyces/Dekkera bruxellensis* strains (perhaps all, in the right circumstances) produce the ethyl phenols 4-ethyl phenol (4EP), 4-ethyl guaiacol (4EG), and 4-ethyl catechol (4EC), by a two-step enzymatic process (cinnamate dehydrogenase followed by vinyl phenol reductase), from the hydroxycinnamic acids coumaric, ferulic, and caffeic, respectively (Hereszytn, 1986a; Chatonnet *et al.*, 1992, 1995, Hesford *et al.*, 2004; and many others).

Pichia guilliermondii, a yeast found on grapes and in wineries, can produce 4EP but has not been found to do so in wine (Stender *et al.*, 2001; Jensen *et al.*, 2009). Dias *et al.* (2003) remarked, '*D. bruxellensis* remain as the sole agents of "phenolic off-flavours" in wines.'

Production of ethyl phenols varies with *Brettanomyces* strain (Conterno *et al.*, 2006; Romano *et al.*, 2008; Oelofse *et al.*, 2009; Harris *et al.*, 2009; Coulon *et al.*, 2010), grape variety (Pollnitz *et al.*, 2000), and composition of the wine (lower alcohol leads to higher levels; higher residual sugar leads to higher levels), levels of precursors, temperature (more are produced at a higher temperature than lower), available oxygen (oxygen leads to higher ethyl phenols), and other variables (du Toit *et al.*, 2005, 2006; Suárez *et al.*, 2007; Romano *et al.*, 2008, Jensen *et al.*, 2009, Benito *et al.*, 2009), not all of them identified.

More 4EP usually is produced than 4EG, but ratios vary, causing pronounced sensory variations. Chatonnet *et al.* (1992) reported an 8:1 ratio for 4EP:4EG. In a survey of 61 Australian reds, Pollnitz *et al.* (2000) found average ratios of 4EP:4EG for Cabernet of 10:1, for Shiraz of 9:1 (highest 23:1) and for Pinot Noir 3.5:1 (lowest 1.6:1). A later Australian survey found 4EP:4EG ratios in Cabernet from 2:1 to 21:1 with higher ratios in cooler regions than in warm ones (Coulter *et al.*, 2003). An average ratio of 10:1 is often assumed (Romano *et al.*, 2009).

Sensory effects of ethyl phenols

The ethyl phenols 4EP and 4EC are usually described as having 'Band-Aid®' or 'plastic' odors, and 4EG smells 'medicinal', 'spicy' or like 'clove' or 'burnt beans.' The combination of these and other metabolites results in a character

with descriptors such as 'sweaty horse saddle', 'wet dog/wet wool', 'barnyard', 'manure', 'gamey', 'tobacco', and others (Fugelsang *et al.*, 1993; Boulton *et al.*, 1996, Licker *et al.*, 1998; Arvik and Henick-Kling, 2002). Fruitiness is reduced, the roundness and intensity in the mouth are diminished, and there is often a bitter, metallic finish.

Traditionally, levels of 640 parts per billion, (ppb, also written as micrograms/liter or nanograms/ml) or 425 ppb have been cited as the sensory threshold for 4EP in wine (Chatonnet *et al.*, 1992; Godden *et al.*, 2004; Ribereau-Gayon *et al.*, 2006; Romano *et al.*, 2009), but Peter Godden of the AWRI was quoted as saying, 'I've not been able to find an Aussie winemaker who doesn't find 100 micrograms/litre [4EP] negative' (Goode, 2003).

In fact, as many AWRI tastings have demonstrated, sensory detection levels for ethyl phenols vary greatly with wine composition, taster sensitivity, presence of other *Brettanomyces* metabolites, and even wine temperature (Curtin *et al.*, 2008; Cliff and King, 2009). In one study, the average detection level for a 10:1 mixture of 4EP and 4EG in Bordeaux wine was 92 ppb, but the levels in samples judged 'not tainted' by experienced tasters ranged from 5 to 1370 ppb, with an average of 403 ppb (Romano *et al.*, 2009). What is very important to understand is that the sensory perception of *Brettanomyces* character depends on many variables.

Fatty acids

Two fatty acids, isovaleric acid (IVA) and isobutyric acid (IBA) also contribute significantly to 'Bretty' odors (Fugelsang *et al.*, 1993; Licker *et al.*, 1998; Coulter *et al.*, 2003; Oelofse *et al.*, 2008; Romano *et al.*, 2009). Their production has not been correlated with ethyl phenol production, but they may tend to mask ethyl phenol perception somewhat (Romano *et al.*, 2009). The sensory effect of the combination, however, may be considered more unpleasant than ethyl phenols alone (Oelofse *et al.*, 2008; also personal observation) as IVA adds a 'rancid', 'vomit', 'sweaty feet', 'wet goat' or 'stinky cheese' component to the wine aroma (it is also a female pheromone).

Color degradation

Brettanomyces growth has been associated with color degradation in some wines. It has been postulated that the glycosidase activity found in many strains (Mansfield *et al.*, 2002) may lead to formation of colorless anthocyanins (Oelofse *et al.*, 2008), but this hypothesis has not been confirmed.

'Mousiness'

At least some *Brettanomyces* strains are capable of producing the 'mousy taint' compounds acetyltetrahydropyridine and acetylpyrroline from the amino acids ornithine and lysine (Hereszytn, 1986b; Costello *et al.*, 2001; Costello and Henschke, 2002; Grbin *et al.*, 2007; Snowdon *et al.*, 2006), which are liberated

by yeasts after fermentation. The affected wine has a spectacularly offensive aftertaste of mouse urine or rancid macadamia nuts (more familiar to some people than mouse urine). Despite numerous references to an intense odor, these compounds are only slightly volatile at wine pH, so 'mousy' wines in fact do not usually smell strongly of mice, but rather have a 'popcorn' or 'jasmine rice' aroma (Heresztyn, 1986b; Arvik and Henick-Kling, 2002; Snowdon *et al.*, 2006). 'Mousiness' is now so rare that many writers may not have experienced an actual 'mousy' wine themselves, or at least not for some years.

In the mouth, or if the wine is rubbed between the hands, the compounds become volatile at the higher pH of the body. So, when a 'mousy' wine is tasted, the nasty retronasal (palatal) aroma suddenly develops and is perceived as a horrible aftertaste after a few seconds' delay. The length of the delay differs for different people tasting the same wine (Paul Henschke, personal communication), and the varying ability of tasters to detect the off-flavor appears to be genetic, perhaps related to saliva pH (Snowdon *et al.*, 2006).

While it is well established that *Brettanomyces* can produce the 'mousy' compounds, especially in the presence of oxygen, *Brettanomyces* yeasts are not the only source. Lactic acid bacteria also produce the 'mousy' heterocycles, perhaps even more so than *Brettanomyces* alone (Costello *et al.*, 2001; Arvik and Henick-Kling, 2002). A 'mousy taint' can also occur after heating wine if yeast lees and/or lactic acid bacteria are present (personal observation).

Acetic acid

Most, If not all, *Brettanomyces* strains produce large amounts of acetic acid aerobically in culture (Freer, 2002), and in wine if sufficient oxygen is present (Ciani and Ferraro, 1997; du Toit *et al.*, 2005, 2006). However, *Acetobacter* bacteria are nearly universal companions of *Brettanomyces* in cellared wine, so a rise in volatile acidity is not necessarily attributable to the yeasts. A survey of bottled wines by the Australian Wine Research Institute did not find a correlation between levels of ethyl phenols and volatile acidity (Godden *et al.*, 2004).

Other effects

Brettanomyces can also produce biogenic amines (Caruso *et al.*, 2002), though strain differences have not been established. Some European countries put voluntary or compulsory limits on biogenic amines, so this is a topic for further research.

Brettanomyces metabolites can reduce the perceived fruitiness of wines, especially reds, by masking the fruity esters (Fugelsang *et al.*, 1993; Licker *et al.*, 1998; Cliff and King, 2009) or perhaps by producing esterases that metabolize some of them. As wines age, and fresh fruitiness diminishes, ethyl phenols may also seem more intense. Varietal character often becomes much less prominent after *Brettanomyces* growth (Boulton *et al.*, 1996), sometimes to

the point that experienced tasters may not be able to identify the variety, even if they do not find the wine to be spoiled (personal observation).

Spoilage or complexity?

This is a controversial question. At lower levels of ethyl phenols, many people often consider the effects of *Brettanomyces* to be a 'complexity' rather than spoilage, though levels of perception and preferences vary widely. Wines that some tasters consider completely spoiled are judged to be acceptable or even pleasant by others (Boulton *et al.*, 1996; Arvik and Henick-Kling, 2002; Goode, 2003; Curtin *et al.*, 2008; Romano *et al.*, 2008).

Some wine writers actively appreciate some 'Brett' character, going so far as to call people worried about the effects of *Brettanomyces* 'Brett nerds' (Lynch, 2004). Winemakers do occasionally blend wines with a small amount of 'Brettiness' to improve ratings (personal communications, names withheld on request). Prepared *Brettanomyces* cultures are available for use in beermaking, and may perhaps have been used in wine. However, it is wise to remember the admonition, 'never trust a microbe.' A strain of *Brettanomyces* that produced just a slight complexity in one wine in one situation may completely spoil another wine. Some winemakers seek 'good' strains of *Brettanomyces* which would add complexity but not spoil wine; however, the very real possibility that a strain might behave well in one wine, but badly in another, tends to discourage this search. At higher levels of ethyl phenols, their flavor begins to predominate, which many tasters dislike. A 'metallic' aftertaste may also develop, which is often perceived negatively even by those who are not offended by the aromas.

We tend to enjoy flavors to which we have become accustomed. In the past 30 years or so, there has been so much increase in 'Bretty' wines that for some people, that character is normal for red wine. At a wine industry conference in 2003, most tasters thought that a wine with 4000 ppb 4EP was disgusting, but one said that to him it just smelled 'normal' (personal observation). Indeed, 'Brett' character is sometimes thought to be a 'gout de terroir', although it is hard to understand how a microbe found worldwide could be considered part of a particular terroir. 'The same *Brettanomyces* characters will be imparted to all varieties in all climates. *Brettanomyces* metabolites are dominant and mask other flavors.' (Boulton *et al.*, 1996). Commercial winemakers who do not find 'Brett' character offensive should remember that many consumers consider very 'Bretty' wines to be unpleasant to undrinkable. A smaller winery may be able to target consumers who appreciate the 'Brett' character, but larger wineries may not have this option.

Interestingly, more than one winery making expensive wines in more than one country has based their signature style on the effects of *Brettanomyces*. Fugelsang *et al.* (1993) noted that 'some (not all) internationally recognized, award-winning wines had perceivable "'Brett character"'. However, other wineries have found less acceptance for their intensely 'Bretty' wines, and have had to withdraw some of them from the market at considerable expense (names

withheld on request). Few wine microbiologists, and winemakers whose wines have spoiled from the effects of *Brettanomyces*, would agree with Marc Perrin of Château de Beaucastel, who said, 'There are certainly some *Brettanomyces* in every natural wine because *Brettanomyces* is not a spoilage yeast (as many people think) but one of the yeasts that exist in winemaking' (Goode, 2003).

For a discussion of *Brettanomyces* growth in bottled wines, see **10.9**.

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10.5 How do I sample for *Brettanomyces* testing?

L. Van de Water

Record-keeping

When wine is tested for microbes, extensive records need to be kept or the results will not be very useful. Keeping the following information is highly recommended:

- wine identity
- container identity, including each individual barrel sampled
- sampling method and date
- date tested
- person or commercial lab doing the tests
- test methods
- test procedure if done in-house
- test results
- interpretation, and
- action taken.

Sampling procedures

Use new plastic or glass containers with new caps/corks, or resanitized containers and caps (autoclave, or run through a dishwasher with no soap, or rinse with 70% beverage alcohol; do not try to resanitize corks) for samples that are to be cultured. Do not rinse sampling devices or containers with unsterilized water. Sampling devices must be new or carefully resanitized between barrels to avoid contaminating the next sample, and also the barrel itself.

A recommended resanitizing procedure in the cellar is to rinse the device with water, then 70% beverage alcohol (not 95%, it is more effective if diluted to 70%), then with the next wine. If high-proof beverage alcohol is not available, 100-proof vodka will have to do. Do not use 'denatured' or 'reagent' alcohol, or 'meths'; these have additives that are poisonous to microbes as well as people. It is all right to put in an SO_2 rinse as well, but SO_2 is not a contact sterilant, so the alcohol rinse is important.

Except for chemical or sensory analysis, sampling is critical no matter which test method is used, because *Brettanomyces* cells tend to settle to the bottom of a barrel or other container (Barata *et al.*, 2008). Take a racking valve sample from a tank after letting at least a liter of wine run out of the valve (it can be returned to the top of the tank). Do not take top samples except for film evaluation. If a cell count that is representative of the wine is desired, the wine must be stirred or circulated. If the purpose is to find any *Brettanomyces* cells that may be present, a sample can be taken from the bottom valve of a tank, or from the bottom of a barrel with a sterilized hose. Another way to sample barrels is to use a device that takes a sample near but not at the bottom of a barrel, without a sucking action that disturbs lees.

Sampling new vs old barrels

New barrels bind free SO_2 at around twice the rate of older barrels (Ribereau-Gayon *et al.*, 2006). Also, cellobiose (used by many strains of *Brettanomyces*, see **10.3**) is higher in new wood (Fugelsang *et al.*, 1993), and there is also more trapped oxygen, so *Brettanomyces* tends to grow more quickly and extensively in new oak. If a wine is aging in a mixture of new and older barrels, the new barrels and old barrels should be sampled separately for SO_2 measurements and *Brettanomyces* tests.

For culturing *Brettanomyces*, it is extremely important not to take samples within one to three weeks of adding SO_2. Winemakers have observed for 30 or more years that at least some *Brettanomyces* strains respond to SO_2 by losing their ability to grow in culture (personal observation). The cells remain alive but temporarily do not grow on culture media (Millet and Lonvaud-Funel, 2000; du Toit *et al.*, 2005), resulting in false negatives based on plating (no visible colonies on agar = no *Brettanomyces* present). This state is sometimes called VBNC, although there are questions about whether this is the correct terminology. At least in some cases, the cells apparently can recover from this state and may reactivate in the wine (Umiker and Edwards, 2007). If SO_2 has been added recently to a wine, it would be best to use PCR-based methods of detection.

Unfortunately, it is still not possible to take samples for *Brettanomyces* testing from a barrel without destroying the barrel (Kenneth Fugelsang, personal communication), because the yeasts can penetrate deep into the wood (Malfeito-Ferreira *et al.*, 2004). Testing wine aged in a barrel is still the best way to test for *Brettanomyces* infection in that barrel.

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10.6 What methods do I have available to detect *Brettanomyces* infection?

L. Van de Water

There are several methods for detecting *Brettanomyces*, all helpful to the winemaker. Rather than selecting one method only, a combination of methods gives the most complete picture of the status of a *Brettanomyces* infection in a wine. Wineries are best served by utilizing all methods at their disposal. The key is to select the appropriate methods for the information you need.

Sensory detection

Sensory evaluation of wines should begin with tasting of fermentors every day, and continue every month or two until the wine is bottled. After bottling, wines should be tasted and analyzed every few months during the first two years, to detect any changes in the bottle.

With practice, winery workers can become proficient in recognizing the first signs of 'Brett' character developing in cellared wine. Even if no specific sensory character is perceived, if there is an unexplained difference between one container and others in the same batch, *Brettanomyces* or other microbial activity should be suspected. When the sensory effects of *Brettanomyces* growth are strong, the wine has been affected permanently, but even noticing it at this point is better than missing it entirely. Train everyone handling the wine, in the cellar, lab, and tasting room, to recognize 'Bretty' character. Use samples of 4-ethyl phenol (4EP) and 4-ethyl guaiacol (4EG), and other compounds such as isovaleric acid, plus a wine naturally spoiled by *Brettanomyces*.

- Levels of concern: any hint of 'Brett' character should be investigated by other detection methods.
- When to use sensory evaluation: any time that wine is handled, winery personnel should be alert to gassiness, cloudiness or odd aromas/flavors.

Chemical analysis

Routine chemical tests can indicate activity by a range of microbes, including *Brettanomyces*. Recommended tests include pH, free and total SO_2, volatile acidity, and glucose+fructose (separately, not added together). For a discussion of the meaning of the results, see **10.8**.

Measurement of the *Brettanomyces* metabolites 4EP and 4EG by gas chromatography-mass spectrometry confirms the sensory effects of *Brettanomyces*, and helps wineries correlate sensory observations with analytical data. As noted in **10.4**, sensory detection levels depend on wine composition, ratio of 4EP to 4EG, the individual taster, and many other variables, so measurement of ethyl phenols gives an objective reference point. Production of these ethyl phenols lags behind cell growth, and can continue to rise after cells have declined and are no longer detected in culture, so levels of ethyl

phenols do not correlate well with cell growth patterns (Fugelsang and Zoecklein, 2003).

- Levels of concern for ethyl phenols: levels of 4EP over four ppb indicate *Brettanomyces* growth at some point. A level of around 100 ppb 4EP may be detectible by 250–500 ppb 4EP and 20–50 ppb 4EG, a sensory effect should be noticeable (Godden *et al.*, 2004; Guerra, 2008). At levels over 1000 ppb 4EP and 100 ppb 4EG the aroma and flavor of most wines would be seriously impacted.
- When to test 4EP and 4EG: during cellaring to confirm *Brettanomyces* growth, and to correlate with sensory observations.

Microscopic exam

Cells can be seen microscopically if there are at least 1000–2000 cells/ml, preferably more. This is a large population, but *Brettanomyces* cell counts sometimes reach a million per ml or more. Samples can be centrifuged to concentrate the cells at least 10 times, but particulate matter can make it difficult to see cells after centrifuging. Wine samples are prepared for direct examination as wet mounts by putting a small drop on a microscope slide and covering it with a cover glass of #1 thickness; these preparations last for half an hour or so before starting to dry up.

Wet mounts are examined with a very good phase-contrast microscope. Costs start around US$2000–3000 for one with suitable resolution with plan or planachromat objectives of 10×, 40×, and 100× (oil). Eyepiece lenses are often 10×, but 15× is greatly preferred because the microbes appear larger and thus are easier to find. Phase-contrast permits observation of microbes and particles directly without staining, because light going through the cell is retarded compared with light passing through the wine. This sets up a diffraction pattern that makes the cell membrane dark and the cell contents darker or lighter than the background.

If cells look clear but ghostly under phase contrast, without much distinction between them and the background, the phase rings in the condenser may be out of alignment, or an incorrect phase ring, which does not match the objective may have been selected. Causes of fuzzy images are oil on the 40× objective (clean with lens cleaner and lens paper) or two cover glasses stuck together. Fingerprints on the cover glass and dried-up places on the slide can resemble yeasts or bizarre microbes. Look around for the edge of an air bubble; the microbes will be in the same plane of focus as the air bubble, and it will then be easy to tell a dried place from the wine sample.

Brightfield microscopes are not very useful for examining wine directly because the samples must be stained. White wines can be stained with Gram stain or 0.5% methylene blue and examined for yeast, but wine bacteria may resemble stain particles. Red wine pigment precipitates the stain, so red samples must be centrifuged and washed to remove the color, which may also remove some microbes. Phase-contrast microscopes are much preferable to brightfield for direct examination of wine.

Microscope operators must be trained specifically in wine microbe identification, not just general microbiology. Under the microscope, *Brettanomyces* cells are smaller than *Saccharomyces* cells, and are often apiculate (pointy). Some young cells may be ovoid (egg-shaped) but more will resemble olives or thin bowling pins. Bud scars tend to flatten the ends, so older cells begin to look like rowboats, barrels, or gothic arches ('ogive'), often with a bud on an angle at one of the edges. Identification cannot be confirmed by microscopic exam alone, so if any suspiciously apiculate yeasts are seen with the microscope, the wine should be checked for *Brettanomyces* by more definitive methods.

When growing in liquid (such as wine) *Brettanomyces* cells tend to make 'pseudomycelia', elongated structures resembling thin mold hyphae (Edwards, 2005). They range in length from 25 μm or so to 100 μm or much more. Small pseudomycelia can be seen in some cultures on Petri dishes, but they are much more extensive when growing in liquid.

- Levels of concern: any *Brettanomyces* cells (confirm by culturing or PCR) seen directly under the microscope indicate an extensive infection.
- When to examine wine microscopically for *Brettanomyces*: stuck wines (especially during reinoculation), wines that seem to be developing 'Bretty' characteristics, and any time that unexplained activity or sensory change (good or bad) occurs in one or more containers of the same batch. Topping wines should be examined for *Brettanomyces* and other microbes (*Acetobacter* especially) and cultured for *Brettanomyces*; microbiological disasters have happened because all the red wine in the winery was topped with wine infected with *Brettanomyces*.

Culturing

Culturing wines in Petri dishes is the traditional method of detecting *Brettanomyces.* This method is discussed in **10.7**.

Genetic methods: real-time PCR

In the past few years, tests based on polymerase chain reaction (PCR) for *Brettanomyces* and other wine microbes have become available (Phister and Mills, 2003; Cocolin *et al.*, 2004; Tessonnière *et al.*, 2009). The technology is very promising, representing a quantum leap from traditional methods. PCR methods take a few hours instead of days, and can often detect levels as low as 10 cells/ml, or possibly even lower if the wine is centrifuged to concentrate cells.

Real-time PCR is a molecular biology method, which amplifies DNA and links the amplification to fluorescence. In the first step, a small sequence of DNA (the 'target region') specific to the microbe of interest is isolated from the sample by lysing (breaking open) the cells, and this target is amplified using enzymes and primers, small pieces of DNA which bind to the selected target region. Then the primers and enzymes copy the target DNA billions of times

using cycles of alternatively heating and cooling in an instrument called a thermocycler. The DNA is linked to fluorescence of a dye added along with the primer or in some cases attached to it. The amount of DNA originally present in the sample is estimated by comparing fluorescence in the sample to prepared standards of *Brettanomyces* (Phister and Mills, 2003; Phister, personal communication).

The methods are not without pitfalls. Care must be taken during extraction of the DNA to prevent contamination from extraneous DNA. Phenolic compounds in red wine interfere with PCR assays and must be removed by adding PVPP before cell lysis. It is difficult to prepare standards with known concentrations, which are essential to quantitative results, partly because of *Brettanomyces*' tendency to form pseudomycelia, and because standards must be freshly prepared, or the cell numbers may change. Tessonnière *et al.* (2009) also recommended using another microbe and its primer as an internal control to monitor the success of the procedure from DNA extraction through amplification and detection, to prevent false negatives.

As well as active cells, PCR detects VBNC cells and other cells that are temporarily unable to grow in culture (Millet and Lonvaud-Funel, 2000). Thus, PCR can detect cells which would be missed by culturing but which may still be producing ethyl phenols, and which may also reactivate and resume growth later on (Umiker and Edwards, 2007; Coulon *et al.*, 2010).

Detection of dead cells by PCR-based tests in wine is controversial. Forensic PCR tests were designed to detect long-dead cells, including a 68-million-year-old dinosaur bone tested successfully a few years ago, but the time between yeast cell death in wine and disintegration of the nucleus, releasing DNA, is not clear. Dr David Mills remarked, 'As long as the targeted sequence of DNA, typically only a few hundred base pairs long, is still intact, a PCR will likely amplify that DNA.' The dye ethidium monoazide is being investigated to bind with dead *Brettanomyces* cells and eliminate them from the DNA preparation before testing with PCR (Mills and Neeley, 2006; Phister, personal communication). This is a promising way to ensure that dead cells are not detected.

Depending on the DNA sequence selected, the method can be extremely specific for the target microbe species, or it may be designed for broader reactions. Specificity is the greatest strength of PCR-based tests, if specificity is assured. There are several primers for *Dekkera/Brettanomyces bruxellensis* in use at labs around the world; ideally, these primers would react with all strains of this species, but with no other species. However, primers usually are checked against certain other species, but not all species which could potentially react. There is an unpublished report that a primer currently in use for *B. bruxellensis* reacted with other yeasts, including *Candida catenulata* and *Debaryomyces hansenii*, though it did not react with *Dekkera anomala*. Another primer for *B. bruxellensis* did not react with those non-*bruxellensis* yeasts, or with certain other species that may also be confused with *B. bruxellensis* in culture (unpublished results). A thorough testing of more *Brettanomyces* primers against numerous potential cross-reactive species is underway and will be beneficial to prevent false positive results.

While very useful, PCR tests do not completely replace culturing to determine numbers of cells are alive and active. Both methods provide very useful information. For instance, if colonies grow quickly on a Petri dish (three to four days or so, depending on media used), they are actively growing in the wine, so more aggressive management is appropriate than if some small colonies struggle up after six or seven days or more. On the other hand, PCR tests can alert the winemaker to the presence of VBNC cells that may reactivate later.

- Levels of concern: same as for cultured wines (see **10.7**).
- When to test for *Brettanomyces* by PCR (if available and economically feasible): stuck wines during reinoculation, routinely on a schedule during cellaring (such as every three months), when wine is moved, if 'Brett' character is suspected, or if any unexpected activity is noticed during cellaring. Test blend components and also the final blend at least one week before bottling. Test bottled wines if activity or bottle variation is noticed.

Gene sequencing

Some labs offer identification of purified colonies by sequencing around 300–350 base pairs of yeast DNA, usually from the 26S ribosome. The yeast is identified as to genus and species, or the closest species in an extensive databank. Costs are around US$100 per colony. Results include closest match, other species by percentage match, and a phylogenetic tree.

- When to send for sequencing: if a complete identification by genus and species is desired on a microbe that has been isolated and purified.

Other methods

Z-Brett, an antibody-based test, detects 1000 cells/ml. It can be swamped by large numbers of other species (such as during fermentation), so this test is best for monitoring large increases in *Brettanomyces* populations during barrel-aging. The test cross-reacts with some other winery-related species, including certain *Candida* (surface film yeast).

Research-level tests such as epi-fluorescence, flow cytometry, peptide nucleic acid probes (PNA), and restriction fragment linear polymorphism (RFLP) are not widely available commercially at this time.

Interpreting test results

How much *Brettanomyces* is too much? The answer depends on the type and composition of the wine, the stage of processing of the wine, and on how the result fits with previous results. Other factors to take into account are the intended filtration before bottling (or lack thereof), and the history of similar wines in the winery. Ideally, there would be fewer than 25 cfu/ml during

cellaring (see **10.7**), and zero before bottling, but this may not be achievable for all wines. It is wise to have wine periodically cultured for *Brettanomyces* or checked by PCR (preferably both) to establish a pattern for that particular wine.

Summary of tests

1. Sensory: detects 'Brett' character.
2. Microscopic exam: immediate result but at least 2000 cells/ml are required (less if sample is centrifuged).
3. Culturing: detects cells that are alive and not inhibited by SO_2. Direct culturing detects two to five cells/ml depending on sample amount used; membrane culture detects one cell in the amount filtered. Takes up to seven days in optimum conditions, longer in other conditions.
4. PCR-based tests: detects live cells, VBNC cells, and may or may not detect some kinds of dead cells. Can detect down to 10 cells/ml. Takes a few hours.
5. Sequencing: requires a purified culture. Yeasts are identified to genus and closest species in the databank.

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10.7 How can I culture the *Brettanomyces* strain in my wine?

L. Van de Water

Brettanomyces cells are grown by inoculating a Petri dish with wine and incubating it for a week or so, depending on conditions. Despite advice to the contrary, if the conditions are right, it is not difficult to culture *Brettanomyces/Dekkera*. Given optimum conditions, they can grow in seven days or fewer, and most colonies can be identified as *Brettanomyces* or not; ambiguous colonies can be sent to a wine analysis lab for confirmation.

Important: Do not culture wine for *Brettanomyces* within one to three weeks after adding SO_2 to the wine. The cells may become temporarily unculturable (Millet and Lonvaud-Funel, 2000; Umiker and Edwards, 2007), so false negatives could result (see **10.5**). Test these samples with PCR methods instead, or wait to take the sample.

Materials

Culture media can be purchased pre-sterilized (shelf life until opened is several years), or purchased dry and prepared in-house in an autoclave (Wisconsin Aluminum Foundry Electric Steroclave 50X has a large capacity and is cost-effective). Media can be sterilized in glass or narrow-mouth polypropylene bottles with caps. Also needed are a small 30ºC incubator, some sterile disposable plastic pipettes and disposable plastic 60 × 15 or 100 × 15 mm Petri dishes. To test filtered or bottled samples, pre-sterilized or resterilizable filter holders and nonsterile receiving flask, pre-sterilized membrane filters, vacuum pump, reagent alcohol, alcohol lamp, and metal forceps are also required.

Media

The general yeast media Wallerstein Labs Nutrient Medium (WLN), YM Green, and YM agar are often used. The first two have bromocresol green dye which yeast colonies pick up to some degree, aiding greatly in identification. Use only WLN produced in the USA; for reasons unknown, some brands produced elsewhere do not always grow *Brettanomyces* reliably, often resulting in much slower growth or even false negatives (personal observation). One Australian winery said that while their wines tested positive for 4EP/4EG, they could not grow *Brettanomyces*; a visit to the winery determined that in fact, they were growing *Brettanomyces* but the colonies were so slow-growing and so tiny (< 0.5 mm after more than a week) that they did not see them. Once they changed to a difference source of WLN, their cultures grew as expected. Culturing a sample known to have culturable *Brettanomyces* cells at the same time as the samples helps prevent false negatives.

Reducing the amount of dry WLN used from 80 g/L (the amount on the bottle) to 60 g/L promotes *Brettanomyces* growth (personal observation). Supplementing media with thiamine and biotin encourages growth of these yeasts. Adding p-coumaric acid, metabolized by *Dekkera/Brettanomyces* into 4EP, to the media

will result in development of the characteristic 'phenolic' odor of 4EP, which aids in identification.

Cycloheximide

Brettanomyces strains that have been tested so far grow on agar containing 100 ppm or more of the antibiotic cycloheximide, formerly known by the brand name Actidione. It is added after autoclaving the media, to suppress *Saccharomyces*, *Zygosaccharomyces*, and most (though not all) other yeasts (Edwards, 2005). Unless purchased media already contain 50 ppm cycloheximide, add 1 ml of a sterile solution of 0.5% cycloheximide per 100 ml of agar after autoclaving. Media with lower levels of cycloheximide (such as WL Differential Medium which has only 4 ppm) allow growth of too many other cellar yeasts, including some species of *Candida*, *Pichia*, and other genera (personal observation). Cycloheximide is a carcinogen and teratogen so in its pure form, it should be handled with great care, using gloves, dust mask, and lab coat.

Chloramphenicol or other bactericides are often added to media to suppress growth of bacteria, which are not sensitive to cycloheximide at all. Otherwise, they could swamp the *Brettanomyces* colonies, and acetic acid sometimes (not always) produced by *Acetobacter* bacteria in culture may confuse *Brettanomyces* identification.

Other cycloheximide-resistant yeasts

Kloeckera apiculata (teleomorph *Hanseniaspora uvarum*), the most populous yeast on grapes (Fleet and Heard, 1993), and a number of other vineyard yeasts are cyclohexmide-resistant. *Kloeckera* somewhat resembles *Brettanomyces*, and is often mistaken for it. Fortunately, *Kloeckera* dies during yeast fermentation or shortly afterwards, so it does not cause confusion in cultures of cellared wines. If juice or must is cultured, *Kloeckera* makes green colonies in one to two days on WLN with cycloheximide, as opposed to later-appearing white colonies of *Brettanomyces*.

There are also some non-*Brettanomyces*, cycloheximide-resistant yeasts that have been found in wine cellars and as incidental contaminants in wines. These yeasts can cause false positive results for *Brettanomyces* unless the colonies are examined carefully, macroscopically and microscopically. Unlike *Brettanomyces*, these yeasts grow quickly and do not produce acetic acid on the Petri dish, but the colonies may closely resemble *Brettanomyces*. The microscopic morphology of these yeasts is usually (though not always) quite different from *Brettanomyces*. Some of these yeasts identified so far through C. M. Lucy Joseph, Culture Collection Curator at University of California, Davis, are *Candida boidinii*, *C. cantarelli* (very common), *C. catenulata*, *C. ishiwadae*, *Debaryomyces hansenii*, *Lodderomyces elongisporus*, and *Pichia guilliermondii* (unpublished results). Further studies continue, including PCR tests (see **10.6**).

Other media

Other media for *Brettanomyces* growth have been developed. While popular in Europem DBDM uses ethanol as a sole carbon source, which not all strains can utilize, especially Californian ones (Conterno *et al.*, 2006), so false negatives may occur. Certain non-*Brettanomyces* yeasts associated with wine cellars also grow on this medium (Dias *et al.*, 2003).

The liquid medium BSM ('*Brettanomyces* Specific Medium', not the same as Bacterial Standard Medium, also called BSM) from Millipore allows growth of *Kloeckera* (Iland *et al.*, 2007) and some other cycloheximide-resistant yeast species (Louriero and Malfeito-Ferreira, 2003; Romano *et al.*, 2008; personal observation). *Brettanomyces* colonies take 10 days or more to appear on BSM, and they are difficult to see against a white filter, being white, cream, or slightly pink from red wine color. Calcium carbonate agar is sometimes used to confirm acetic acid production by *Brettanomyces* because a clear space forms around the acid-producing colony.

'Easy Blue', a pre-poured, blue/green-colored agar for *Brettanomyces* testing (Lebrun Labs, www.lebrunlabs.com), is offered for wineries without microbiology lab facilities. Cycloheximide and a bactericide are added to suppress growth of most other microbes. An odor of 4EP develops if *Brettanomyces/Dekkera* grow. The instructions do say that colonies should appear after four days or more, but do not state clearly enough that earlier-appearing colonies cannot be *Brettanomyces.* Recently, a winemaker (name withheld on request) panicked when colonies appeared the next day, but of course they were not *Brettanomyces*. As with other media for *Brettanomyces* culturing, *Kloeckera* and other cycloheximide-resistant yeasts will also grow on Easy Blue, though most (except *Pichia guilliermondii*) do not produce 4EP. The colonies should be checked microscopically for confirmation, either in-house or by a commercial wine-oriented lab (not general microbiology labs, which report only total colony counts and cannot identify specific wine microbes).

'Sniff-Brett' cultures involve smelling 4EP production by *Brettanomyces* colonies on a special medium instead of examining colonies under a microscope (for wineries without microscopes). Depending on population, it can take two to seven days to develop the smell.

Culturing procedure

To melt pre-sterilized, solid agar, heat the agar bottle in a container of water in a microwave, one minute at a time (do not allow to boil) until completely melted. Before pouring the agar, on the bottom (not the top) of disposable plastic Petri dishes, write agar type, lot number, and date poured with a permanent marker. Pour Petri dishes half full. Poured Petri dishes should be poured no more than one week before inoculating with a sample, and should be kept upside down in a closed plastic container in a cool place. Write the sample identity and date cultured on the bottom of the Petri dish before inoculating with sample.

If there are 10 to 20 cfu (colony-forming units) per ml or more, the wine can be pipetted directly on a Petri dish containing an agar medium. Centrifuging samples can help concentrate cells. If there are more than around 500 cfu/ml, the result will be a solid 'lawn' or TNTC (too numerous to count). To set up a direct culture, shake the sample and immediately use a sterile pipette to dispense 0.2 to 0.5 ml of wine onto a 60 mm or 100 mm Petri dish containing agar. Do not tip the dish to move the wine around; the cells tend to go to the edges. To move the wine around the dish, use a Pasteur pipette bent in a flame to resemble a hockey stick.

If the wine has been filtered, or if the population is expected to be quite low, the sample is filtered through a sterile 0.45 μm (micron) membrane and the membrane is cultured on agar or on a broth-soaked pad. For a membrane filtration culture, set up a sterilzed filter holder on a receptacle (which itself need not be sterile) to catch the filtered wine. Put a pre-sterilized 0.45 μm 47 mm filter in the holder.

If there is a cork in the bottle, dip a corkscrew worm into reagent alcohol and set it alight; remove the bottle capsule, dip the neck of the bottle into alcohol and flame it also. Open the bottle without touching the corkscrew worm or the bottle neck. If there is a screw cap, flame the neck of the bottle to be tested, and break the seal using a swab or tissues dipped in alcohol to cover your hand. Then pour wine carefully into the filter holder for vacuum filtration. Our labs filter 250 ml for yeast cultures, 100 ml for *Brettanomyces* only, and 100 ml for bacteria.

Flame metal forceps with alcohol and transfer the filter aseptically to an agar Petri dish, or the filter and broth-soaked pad to an empty sterile Petri dish. Incubate Petri dishes upside down at 28–31ºC. For direct cultures of wine, leave Petri dishes right side up overnight in an incubator, then turn upside down the next day. Using WLN and exactly the protocol described in this section, visible colonies appear in three to seven days. The faster the colonies grow, the more active the *Brettanomyces* cells are in the wine. If visible colonies appear in three or four days, different recommendations for handling the wine may be given than if small colonies struggle to grow after six or seven days. At lower temperatures, or if using other media, the colonies take longer to appear.

Identifying colonies

On WLN+C (cycloheximide) and other green media, *Brettanomyces* forms small, shiny, white colonies that turn olive green to light-green over time. The colonies produce acetic acid changing green media to yellow, which is helpful in identification. A slow-growing yeast making a strong acetic acid smell on a Petri dish of WLN+C is confirmed to be *Brettanomyces* or *Dekkera*.

After growing, some colonies will need to be checked microscopically, either in-house or sent to a wine analysis laboratory. Most *Brettanomyces* cells are smaller than *Saccharomyces*, and though some young *Brettanomyces* cells can resemble *Saccharomyces*, the majority will be more apiculate (pointed). A prominent feature of a *Brettanomyces* culture is extreme polymorphism (many different shapes), so the cells will be quite variable, even within the same culture

(C. M. Lucy Joseph, personal communication; Christian Butzke, personal communication; Zoran Ljepovic, personal communication; also personal observation).

Different strains of *Brettanomyces* cannot be distinguished by culturing. Although morphological differences can be observed among some strains, strain identity cannot be confirmed without extensive genetic and physiological research.

Even within one strain, cells vary greatly in size. Some are 3–4 μm in width to 5–8 μm in length (Millet and Lonvaud-Funel, 2000), but may be much longer. Some young cells may be ovoid (egg-shaped) but more may resemble olives. The first bud that a cell produces forms at one end, so the budding cell looks like a thin bowling pin, but it leaves a large bud scar, flattening the end somewhat (Fig. 1). Successive buds cannot form at the same place, so later buds are offset from the ends of the cell, and older cells begin to look like gothic arches ('ogive'), rowboats, watermelon seeds, or even barrels, with a bud sticking out from a corner of the flat end(s). Pseudomycelia (see **10.6**) may begin to form in culture but can become much more extensive in liquid, such as wine.

It is very important to note that identifying wine microbes microscopically, including *Brettanomyces*, takes some training. The very few texts about identifying wine microbes that are available are extremely useful (Edwards, 2005). Texts cannot replace hands-on training in identifying wine microbes, however, and attending a wine microbe identification session is recommended. A number of universities and commercial labs offer workshops on wine culturing techniques and wine microbe identification. These are best if one or more days are spent laying the foundation, then at least another day is spent working with the details of microbe identification (personal experience).

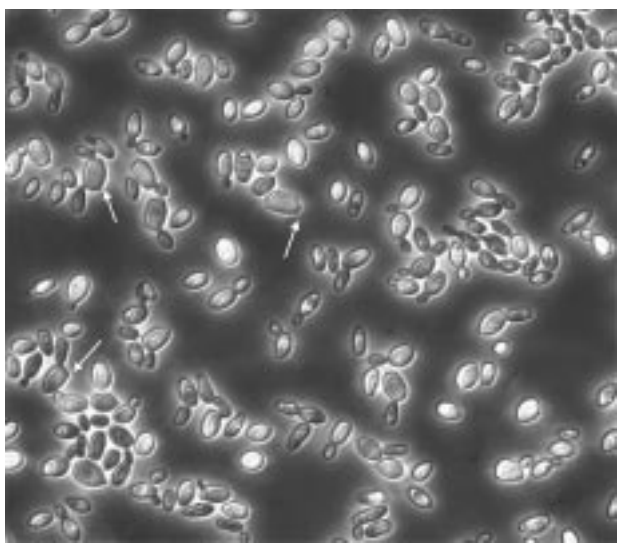

Fig. 1 *Brettanomyces* cells showing polymorphism. The three arrows indicate bud scars. Photo: © Lia Van de Water.

- Levels of concern: these vary depending on the situation and stage of production. In wineries believed to be free from *Brettanomyces*, even one colony is cause for alarm. In cellars with known infections, wines with 100 colony-forming units (cfu)/ml should be recultured frequently to monitor population increase; wines with 500 cfu/ml need better management; wines with more than 1000 cfu/ml require immediate attention to prevent spoilage, if the 'Brett' character is not already too intense.
- When to culture for *Brettanomyces*: routinely on a schedule (such as every three months) during cellaring, when wine is moved (before SO_2 is added!), if 'Brett' character is suspected, or if any unexpected sensory change occurs during cellaring. Culture blend components and also the final blend at least one week before bottling. Culture bottled wines if unexpected sensory change or bottle variation is noticed.

Summary of what winemakers should know about *Brettanomyces* culturing:

- Do not take samples within a few weeks after adding SO_2; false negatives can result.
- Following the exact procedures above, colonies should appear on WLN agar containing cycloheximide within three to seven days. On other media and under other conditions, colonies can take 10–14 days to appear.
- Culture wines periodically, not just once, to monitor patterns of growth in each wine over time.

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10.8 How can I manage *Brettanomyces* in the cellar?

L. Van de Water

Don't bring it in

The very **best** way to deal with *Brettanomyces* is not to bring it into the cellar. Once it is established in a winery cellar, managing it, not eliminating it, is the goal (Fugelsang *et al.*, 1993; Boulton *et al.*, 1996). While there are many anecdotal references for *Brettanomyces* entering winery cellars by various means, and it is possible that the yeasts may occasionally come in on grapes, overwhelming evidence from studies in a number of countries points to wine or wooden containers as the major vectors for *Brettanomyces* contamination of cellars (Boulton *et al.*, 1996; Fugelsang *et al.*, 1993). Refraining from purchasing red wine or wood used for red wine from other cellars (no matter how extremely inconvenient it is to pass up used red wine barrels for sale!) has protected many wineries from *Brettanomyces* infection, until a mistake was made and *Brettanomyces* was brought in with used barrels or infected wine.

Quarantine possibly infected lots

Before bringing wine into a larger cellar, keep the wine in quarantine until it can be cultured (and tested by PCR if available), or simply sterile-filter all received wines on the way into the winery from the tanker truck (Fugelsang *et al.*, 1993). Even then, put newly arrived wine into stainless steel, not wood, until it can be checked for residual *Brettanomyces*. Smaller wineries (and especially amateur producers) would be well advised never to bring in wine or any other item from another cellar. Do not top cellared wines with wine from another cellar, even bottled commercial wines. A purchased wine, no matter how tasty, may still have *Brettanomyces*, or it may have substrates (sugar, etc.) that could feed a *Brettanomyces* population if one is already resident in the winery.

Brettanomyces growth in the cellar

Danger signals for *Brettanomyces* growth in cellared wines include

- a wine that was quiet becoming active
- a wine that was clear becoming cloudy
- an unexpected change in aroma, flavor, or analysis results.

If any of these changes are noticed, take samples for analysis immediately, including pH, free and total SO_2, volatile acidity, and microbial testing. Examine directly under a phase-contrast microscope, but even if other microbes are seen microscopically, check for *Brettanomyces* by culturing or PCR, because they may also be present at levels too low to observe, but high enough to cause problems.

Wine composition influences *Brettanomyces* growth in cellared wines, especially nutrient depletion (sugar and nitrogen), pH and SO_2 management, and

turbidity. Cellar operations are also very important, including temperature control, minimizing oxygen contact, avoiding cross-contamination, population control, and sanitation.

Wine composition influencing *Brettanomyces* growth

Minimize substrates for growth (especially sugar and nitrogen)

Brettanomyces can grow on very small amounts of hexose sugars (0.2 g/L, well below what is considered 'dry'). However, they do avail themselves of more residual sugars if available. Stuck ferments are prime candidates for spoilage, especially from *Brettanomyces* and *Lactobacillus* (Godden *et al.*, 2004). Procedures to encourage resuming stuck fermentations encourage *Brettanomyces* growth, if the wine is already infected. The most important must adjustment to avoid stuck ferments is to reduce Brix to 25–25.5 if it is higher; others include reducing pH to below 3.6 during fermentation and adding at least 40 ppm total SO_2 before fermentation.

Supplementation with complex nutrients, not just diammonium phosphate (DAP), in two or three portions at appropriate times and levels is very important in preventing stuck ferments and in avoiding leftover nitrogen. Amounts and timing of nutrient supplementation depend on tests of yeast-available nitrogen (YAN) on the juice. Nitrogen supplements should be calculated carefully and added during the first half to two-thirds of fermentation, because the cells are unable to bring in nitrogen later on, so late additions provide nitrogen that *Brettanomyces* can use (Coulter *et al.*, 2003). Also, if too much DAP is added early in fermentation, utilization of amino acids can be inhibited, and some of these can be used by *Brettanomyces* as a sole nitrogen source (Conterno *et al.*, 2006).

Appropriate yeast strain selection and proper handling of yeast during rehydration and inoculation also help to prevent stuck ferments. Especially if grapes are damaged or diseased, be sure to add a vigorous dry yeast; do not ferment rotten or damaged grapes without adding yeast. To discourage growth of non-*Saccharomyces* vineyard yeasts before fermentation, which deplete nutrients and can inhibit *Saccharomyces*, minimize the length of time the must spends between 10 and 15ºC (50–60ºF). During fermentation, maintain strict temperature control so musts do not get too hot or too cold for *Saccharomyces* yeasts.

SO_2 and pH management

Regular sensory evaluation and analysis of SO_2 help winemakers watch for signs of microbial growth. Test for *Brettanomyces* population and SO_2 levels in wines in new barrels separately from older ones holding the same batch of wine; new wood binds SO_2 much more quickly than old wood (Ribereau-Gayon *et al.*, 2006).

The SO_2 in a wine exists as molecular, free, and bound. *Brettanomyces* yeasts are not sensitive to legal levels of bound SO_2 (Licker *et al.*, 1998). When

evaluating SO_2 effectiveness, it is very important to consider molecular SO_2, not just free SO_2! Over many years, certain levels of free SO_2 have been said to control *Brettanomyces* completely, but recommendations not based on molecular SO_2 are misleading. The free SO_2 level is meaningless unless it is taken with the wine pH to calculate molecular SO_2 (Margalit, 2004). For example, in *Handbook of Enology*, the authors say, 'a free SO_2 concentration of 30 mg/L SO_2 always results in the total elimination of all viable populations [of *Brettanomyces*] after 30 days', but then they clarify this statement as applying to 'red wines of normal pH levels (3.4–3.5)' and state that this level would be insufficient at pH 3.8 (Ribereau-Gayon *et al.*, 2006).

This brings up one of the most pervasive – and most consistently overlooked – problems with making recommendations about unfamiliar wines. The Bordeaux wines Dr Ribereau-Gayon refers to as 'normal' had a pH of 3.4 to 3.5, but a 'normal' wine in warmer climates – west coast USA, Chile, Argentina, South Africa, Australia, and others – may have a much higher pH unless it is strictly managed once the grapes are picked. Left to their own devices, some wines in those places may have a pH of 3.8 or higher. Thus, SO_2 management in Bordeaux, and in other cool climate regions as varied as (for example) New Zealand's South Island, Michigan, and California's Santa Cruz Mountains, is much easier because of naturally lower pH. Sadly, if winemakers do not understand the effects of climatic differences between their region and others, they may take advice (in vineyards as well as in wineries) that are appropriate for somewhere else, but inappropriate for their own grapes and wines.

In 1982 at the University of California at Davis, Clark Smith developed a formula and chart to assist winemakers in calculating molecular SO_2 from free SO_2 and pH (Fugelsang *et al.*, 1993). This discovery about wine pH was a revelation, explaining so much about the influence of pH that was not understood before. Indeed, in the 1970s, pH was often considered to be of little or no consequence to winemaking (personal observation). More than thirty years later, most commercial winemakers worldwide use the pH/SO_2 chart (Margalit, 2004), and those who do not, should do so.

For example, at 3.5 pH, 30 ppm free SO_2 calculates to 0.6 ppm molecular SO_2, sufficient to delay or inhibit growth of many *Brettanomyces* strains, though not all. But at 3.8 pH, 30 ppm is only 0.3 ppm molecular SO_2, too little to impact *Brettanomyces* growth. Note, however, that strain differences influence the response of *Brettanomyces* SO_2; a level of molecular SO_2 that will inhibit one strain may not inhibit another (Barata *et al.*, 2008; Umiker and Edwards, 2007).

Making larger SO_2 additions less frequently rather than smaller, more frequent ones, results in a higher percentage of free SO_2 rather than bound. Coulter *et al.* (2003) advise, 'Therefore it is beneficial to make a large addition of SO_2 to red wine after MLF, before aging in barrels, in order to reduce the populations of *Dekkera*/*Brettanomyces*.'

Brettanomyces is not especially sensitive to pH in the pH range of wine (Conterno *et al.*, 2006), but the lower the pH, the more molecular SO_2 is present at the same free SO_2 level, so the SO_2 is more effective. This is very

important when managing a *Brettanomyces* infection. Even one tenth of a pH unit makes a difference to the percentage of molecular SO_2, and to the management of the wine. If the pH is over 3.65–3.70, winemakers can reduce the pH to 3.6 or less with tartaric acid during cellaring for greater SO_2 effectiveness, then bring the wines to the desired pH with potassium carbonate or bicarbonate before bottling. This makes *Brettanomyces* management much more successful, and it uses only ions that are naturally found in wine. Alternatively, Wine Secrets (www.winesecrets.com) perform electrophoresis to reduce acidity as the same time as reducing pH.

Lower turbidity

SO_2 binds more readily in cloudy wines, leaving less free SO_2. If at all possible, clarify the wine (even just racking off gross yeast lees) before adding SO_2. Cleaning up wine as soon as it is finished yeast fermentation and MLF can help prevent spoilage. Chatonnet *et al.* (1995) noted that delaying racking, and thus delaying SO_2 addition and removal from solids, could result in significantly higher ethyl phenol levels. Lees contact also increases substrates for *Brettanomyces* growth through yeast autolysis.

Cellar conditions influencing *Brettanomyces* growth

Temperature control

Keeping the wine below 15ºC/60ºF greatly reduces *Brettanomyces* growth rate. Some strains (mostly European) can continue to grow at that temperature (Conterno *et al.*, 2006), but their growth will be slowed; other strains will cease growth (though not die). Special vigilance is needed in cellars whose temperature rises during the summer, encouraging a *Brettanomyces* 'bloom'.

Minimizing oxygen contact

The top surface of wine in tanks should be checked and topped at least once a week, and gassed regularly with N_2, CO_2, or argon. If a film is seen, this indicates that the headspace protection is not sufficient to prevent growth of aerobic microbes. *Brettanomyces* yeasts do not need oxygen, but their growth is stimulated by oxygen, which also allows them to use a wider range of substrates. Micro-oxygenation should not be performed on wines already infected with *Brettanomyces* because the yeasts take up the oxygen more quickly than the target molecules, wine phenolics (du Toit *et al.*, 2005). Each time wine is racked, some oxygen pickup occurs, though delaying racking must be balanced against the increase in micronutrients that occurs during yeast autolysis.

Avoid cross-contamination

Clean hoses thoroughly to remove debris, stain, tartrate deposits, residual wine, etc. Sanitize hoses, topping equipment, sampling devices and receiving containers between batches of wine, unless you know that both batches are already infected with *Brettanomyces*. Trading bungs among barrels can easily spread

infection (note: SO_2 solution does not penetrate into the bung to kill sequestered microbes). Check the *Brettanomyces* infection status of topping wine before use, or simply sterile-filter topping wine.

Do not ferment wines in *Brettanomyces*-infected wood, do not top wines that are not dry with infected wine, and do not put stuck or sweet wine into infected barrels, to avoid infecting wine with *Brettanomyces* while residual sugar is present. Do not put infected wine into new barrels; try to keep new barrels free of *Brettanomyces* as long as possible. Winemakers are often advised to 'keep the problem children together', that is, to keep infected wine away from uninfected wine. Zoran Ljepovic (QA/QC, Constellation Wines US) advises, 'We should put infected wines into quarantine because we cannot predict what the yeasts will do' (personal communication).

Population control if needed (fining/filtering)

Keep track of *Brettanomyces* population by culturing (and also PCR if possible). If the population is increasing rapidly, reduce the cell population by depth filter, diatomaceous earth, or crossflow filtration. Continue monitoring to watch for re-growth. Even if cell growth declines, the dormant cells may still be producing ethyl phenols. Monitoring 4EP and 4EG can track this, though it may be more economical to filter the wine to remove most of the cells than to check ethyl phenols frequently. If filtration is impossible, egg white fining and racking can remove a large proportion of the cells (personal observation). Other fining agents used to reduce yeast population are bentonite (not advised for reds because it tends to strip flavor), gelatin and PVPP.

Cellar and barrel sanitation

Sanitation is extremely important in preventing any microbial spoilage, including *Brettanomyces*, from spreading throughout the cellar. Effective cellar sanitizers include quaternary ammonia, peracetic acid, and peroxycarbonate. Chlorine is no longer recommended for use in wineries because of the potential for trichloroanisole formation (TCA, TeCA), strong moldy-smelling taints which can enter the wine through the air in the cellar.

Alas, there is no reliable way that is available to remove all the microbes from a barrel all the time (Coulter *et al.*, 2003; Fugelsang *et al.*, 1993; also personal experience) except perhaps steaming, which removes oak flavor. You can kill some of the microbes all the time, and all the microbes some of the time, but not all the microbes all the time. More microbial toxicity is assumed than actualized. Burning sulfur wicks/rings in between wines, and whenever barrels are stored empty, has been shown to be more effective against *Brettanomyces* than SO_2 gas (Ribereau-Gayon *et al.*, 2006). While burning sulfur reduces *Brettanomyces* population, it does not eliminate the yeasts, which may penetrate 0.8 cm deep in the wood (Malfeito-Ferreira *et al.*, 2004). The same is true for ozone, discussed below, and any other current treatment for infected barrels. Boulton *et al.* (1996) explained, 'We know of no sure way to sterilize infected barrels.'

A procedure to greatly reduce *Brettanomyces* contamination in barrels using high-power ultrasound has been developed in Australia and offered by Cavitus (Yap *et al.*, 2008). It is expensive, but warrants evaluation by wineries.

Ozone treatment of barrels, when carried out correctly, is very effective in reducing the *Brettanomyces* population (Guerra, 2008), and has been considered a godsend for some wineries battling *Brettanomyces* in barreled wines. *Brettanomyces* may re-grow in the wine, and/or in wines subsequently aged in the same wood. Ozone has no residual sanitizing effect, so if infected wine is re-introduced into an ozonated barrel, the yeasts will grow as readily as if the barrel had not been treated.

Effective (and safe) use of ozone requires adherence to strict procedures (Suárez *et al.*, 2007; Doug Manning, personal communication). The barrels need to be prepared with a high-pressure hot water wash so that there are no tartrates or other particles in them; they must be completely cooled after hot water treatment (by waiting, or using cold water rinse), before using ozone. Ozone should be charged into very cold, tightly filtered water. An ozone meter is essential; measure the ozone in the water going into the barrel (should be 2.0 to 2.5 ppm) and coming out (should be 0.1 to 0.2 ppm). Rinse barrels with cold water before filling with wine. In most cellars, the procedure should be followed outside to reduce ozone exposure of winery workers, but wearing a tag to check cumulative ozone exposure is strongly recommended anyway. Many companies offer ozone treatment of barrels as a service.

What is important?

Brettanomyces management depends on principles that apply to managing other microbes. The most important aspect of management of *Brettanomyces* – or any other microbe – in the cellar is to understand that an integrated, holistic approach is essential (Coulter *et al.*, 2003). These authors advise, 'In situations where only some of the suggested control strategies are implemented it is likely that reductions in 4-ethyl phenol concentrations will be sporadic.' Dr Charles Edwards of Washington State University compares the process of *Brettanomyces* management to putting a number of hurdles in its path. One hurdle is not enough; to stop its progress, at least several 'hurdles' must be in the way, combining to prevent rampant growth of microbes (Edwards, 2007).

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10.9 Can I bottle my wine unfiltered if it is infected with *Brettanomyces*?

L. Van de Water

It is well-established that *Brettanomyces* can grow in bottled wines (Chatonnet *et al.*, 1992; Fugelsang and Zoecklein, 2003; Cocolin *et al.*, 2004; Renouf *et al.*, 2007; Coulon *et al.*, 2010; also personal observation). So, the safest answer is 'no.' However, here are some recommendations to assess the relative risk.

Evaluating potential instability in the bottle

First, consider aspects of marketing and liability. Is bottle variation acceptable to your target market, or do your consumers expect a stable, consistent product? If the wine is being made for another company, and they have requested that the wine not be sterile-filtered at bottling, they need to sign a release absolving the bottling winery from all responsibility for microbial instability. These questions and any others related to bottle instability should be considered far enough ahead of time before proceeding to bottle wine infected with *Brettanomyces* without sterile filtration.

Then, ask the four questions:

1. Does it have residual fermentable sugar?
2. Does it have residual malic acid (if pH 3.3–3.4 or higher)?
3. Does it have *Pediococcus*?
4. Does it have *Brettanomyces*?

Testing for instability in the bottle

Assuming that the answer to the last question is yes, the others must also be investigated to prevent in-bottle spoilage from other microbes. Analysis by methods requiring a UV-VIS spectrophotometer, or at least a colorimeter (Megaquant, www.megazyme.com), are needed to assess stability. A winery that does not have the ability to run such tests should send samples to a commercial wine analysis lab.

Enzymatic glucose and fructose (not added together, tested separately), pH, free and total SO_2, and malic acid should be tested. If the wine is microbially unstable for other reasons such as residual fermentable sugar at 0.5 g/L or more, or malic acid over 0.1 g/L (in wine that underwent incomplete MLF), *Brettanomyces* may become irrelevant, and sterile filtration may be needed anyway. But if the other questions are answered 'no' (or if lysozyme is added to kill *Pediococcus*), then the focus can be on *Brettanomyces* infection status.

For most microbes, tests of levels of potential substrates are needed to help decide whether a wine can be bottled without sterile filtration. However, *Brettanomyces* can use a variety of substrates, so it is best to assume that cells can grow in the bottle if they are present. Because *Brettanomyces* can grow at levels of hexoses (glucose and fructose) well below those considered 'dry'

(Chatonnet *et al.*, 1992, 1995), the absence of significant residual fermentable sugar is not a limiting factor in *Brettanomyces* growth in the bottle. In the 1980s some wineries bottled wines unfiltered on the assumption that *Brettanomyces* could not grow in dry wine, but they discovered that this idea was incorrect.

Handling wines infected with *Brettanomyces* before bottling

White wines infected with *Brettanomyces* should be sterile-filtered at bottling with a membrane filter because even a small amount of yeast growth will render the wine unacceptably cloudy. Even for reds, the 'safe' answer is, of course, to filter out all the cells, so they cannot reactivate in the bottle. If they grow, they could potentially spoil the wine, or they could grow a little bit and then stop.

Many winemakers are reluctant to filter reds before bottling. However, they need to weigh the potential for *Brettanomyces* (and perhaps other microbes) to reactivate in the bottle against their preference to avoid pre-bottling filtration. This is not easy, no matter how much experience the winemaker or consultant has. Levels of *Brettanomyces* infection can range from almost undetectable to 10 million cells/ml (personal observation). While almost no one would bottle a wine that is visually cloudy with cells, it is difficult to set a lower limit on acceptable *Brettanomyces* populations in bottled wine. Sometimes, *Brettanomyces* does not grow in the bottle even though there are 100 cfu/ml or more, and sometimes they grow extensively from fewer than 1 cfu/ml. Culturing the wine can help in this assessment; healthy-looking colonies that grow sooner (in optimal culture conditions) indicate a more robust and active population of *Brettanomyces* than tiny colonies that appear later on (see **10.7**).

One way to evaluate a wine's spoilage potential is to hand-bottle three or four bottles straight from tank or barrel (preferably at a racking), without an SO_2 addition or filtration. Keep them in a warm place (in an office, or next to a hot water heater). Open one bottle a month, taste, and check the *Brettanomyces* population. If the 'Brett' character increases or the population rises, the wine is probably not a good candidate for bottling unfiltered.

Brettanomyces growth and survival in the bottle

When *Brettanomyces* grow in the bottle, there will almost always be bottle variation, sometimes quite dramatic (Chatonnet *et al.*, 1992; also personal observation). One bottle may have little or no effects, but another bottle in the same case may be completely spoiled. The lack of consistency means that repeat customers are likely to be disappointed, whether they prefer the 'Bretty' or the 'non-Bretty' bottles. When nutrients or vitamins are depleted, *Brettanomyces* becomes dormant but does not necessarily die. Live cells of *Brettanomyces* have been cultured from wines bottled for more than 30 years (personal observation).

4-ethyl phenol (4EP) in the bottle

If cells are present in the bottle, even if they do not grow extensively, they can continue to produce ethyl phenols after bottling (Fugelsang and Zoecklein, 2003; Coulon *et al.*, 2010). So, in a wine without recognizable 'Brett' character but bottled unfiltered, the ethyl phenols can continue to increase, sometimes dramatically (Fugelsang *et al.*, 1993).

In some wines that have been sterile filtered to remove all cells, the perception of 'Brett' character, particularly ethyl phenols, may increase quite dramatically even though analysis shows that the levels remained the same (personal observation). There is as yet no good explanation for this sensory phenomenon. Surely, as wine ages, its freshness and fruitiness diminishes, allowing other components to become more prominent, but the sometimes startling increase in perceived 'Brett' character without an increase in ethyl phenols so far remains a mystery.

Considerations about bottling unstable wine

Some winemakers decide not to sterile-filter their wine or use inhibitors even if it has a known *Brettanomyces* infection. While wine microbiologists are all too aware that *Brettanomyces* growth in the bottle can be a disaster, they also realize that winemakers sometimes choose the option of bottling unstable wine. It is hard for many winemakers to imagine that a wine which has been quiet for months in the cellar could become active once it is bottled, but this does indeed occur (personal observation).

The procedures recommended in **10.10** represent a conservative approach to *Brettanomyces* stability in the bottle. Every winemaker knows examples of wines that were bottled with viable *Brettanomyces* cells which did not grow in the bottle. Sometimes the yeasts' explosive growth completely spoils a wine, but sometimes they grow only a small amount or not at all. One of the most difficult and frustrating predictions to attempt to make pre-bottling is whether or not a small population of *Brettanomyces* will grow to a large one in the bottle. One never knows, but no matter how many instances a winemaker can cite when *Brettanomyces* yeasts failed to cause in-bottle spoilage, most wine microbiologists and consultants know of instances in which they did, and caused an economic calamity for the winery.

In one example, a Merlot from another country was featured at a fancy tasting in the Napa Valley. On its way to the USA, *Brettanomyces* grew extensively in the bottle, so the wine had developed a strong 'Brett' character. The distressed winemaker had no choice but to pour the wine, but as soon as he arrived back in his country he sought assistance to help prevent future problems in the bottle. The spoiled Merlot was already enough of a problem economically; they could afford no more microbial disasters.

It is the potential for disastrous activity in the bottle that leads wine microbiologists to lean heavily toward procedures to achieve stability rather than risk spoilage. Whatever negative effects filtration may have (and many

winemakers believe that the effects are only temporary), extensive *Brettanomyces* growth in the bottle is worse.

See **10.10** for information about inhibition of *Brettanomyces* at bottling.

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10.10 How should I prepare my wine for bottling if it has *Brettanomyces*?

L. Van de Water

Reducing ethyl phenols

Trials performed with yeast hulls, live yeast, and potassium caseinate/casein reduce ethyl phenols. Reverse osmosis can be used to remove some of the ethyl phenols, reducing the objectionable 'Brett' characters. Several companies provide this service.

Assessing stability potential

Test the level of *Brettanomyces* infection **before** making the final SO_2 addition, by culturing and also by PCR if available, which can detect VBNC cells. Culturing is also important because the shorter the growth time in culture, the more active the cells are likely to be in the wine.

Reducing population by fining and filtration

If the wine will be sterile-filtered through an absolute 0.65 to 0.45 μm membrane filter at bottling, then population reduction methods are recommended to reduce the cell load which the sterilizing filters may need to stop. The membrane filter should be preceded by a sterilizing depth filter or the sterilizing setting on crossflow or ultrafiltration. The final membrane filters should be just a precaution; they should not actually have to remove any cells. Membrane filters are very expensive, and the more cells they have to remove, the faster they will plug.

Egg white fining can precipitate around 90–95% of the yeast cells (personal observation), resulting in a much more manageable population. Other fining agents that can reduce yeast population are casein/potassium caseinate, PVPP, and gelatin. Bentonite fining also reduces yeast population, but it is usually considered to be unsuitable for red wines.

Filtering with diatomaceous earth (DE), crossflow, or a pad fliter reduces the *Brettanomyces* population dramatically, making it much easier to put the wine through the final membrane filters. The preparatory filters remove most of the yeasts, preparing the wine for sterile filtration.

One winery follows this procedure for reducing the *Brettanomyces* population: settle the wine for at least one to two months in the barrel, undisturbed. Rack the top 90% into a tank; filter the rest into the tank through DE or a pad filter. Fine the tank with egg whites, and settle for at least two weeks. Rack the top 90% into another tank; filter the rest into the second tank. By this time, the *Brettanomyces* population is down to only a few cells/ml, which are removed by sterile filtration at bottling.

It is very important to note that depth pads and cartridges may sport 'nominal' numerical designations matching those of membrane filters, such as '0.45 μm (micron)'. Understandably, winemakers often believe that the two

types of filters are equivalent. However, the 'nominal' micron designations refer only to an average retention size under certain circumstances, so they do not give the same protection as an 'absolute' membrane filter.

The difference between these two types of filters, both in cartridges, is critical. Depth filters depend on entrapment of particles (including microbes) in the matrix of the filter; particle size, particle load, pressure, and other variables determine what gets through and what does not. Wines with large amounts of small microbes are most likely to have microbes passing through depth filters. Depth filters remove a very large proportion of the *Brettanomyces* cells, and sometimes all of them, though not necessarily. A 0.45 μm membrane filter, however, is an absolute barrier unless its integrity is compromised (a hole in it), or unless cells are small enough to pass through the pores.

Inhibiting *Brettanomyces* growth

SO_2

A molecular SO_2 of 0.5–0.6 ppm is recommended for reds at bottling, to inhibit *Brettanomyces* growth temporarily, though it does not kill the cells. In most red wines, adding enough SO_2 to kill all the yeasts (at least 0.8 ppm molecular SO_2) would result in too high a level of total SO_2, because much of the SO_2 will bind to wine components.

If SO_2 is added just before bottling the 'Brett' cells may respond by going into a 'stunned' state which is sometimes called 'VBNC' ('viable-but-not-culturable'). The cells may shrink by as much as 30% (Charles Edwards, personal communication, 2007); some *Brettanomyces* cells are rather small anyway, around two to four microns by five to eight microns (Millet and Lonvand-Funel, 2000). These authors reported that starved lactic acid bacteria, acetic acid bacteria, and cycloheximide-resistant yeasts presumed to be *Brettanomyces* had passed a 0.45 μm membrane filter. In practice, however, for many years wineries have had extremely reliable success stopping *Brettanomyces* (and bacteria) with a membrane filter of 0.45 μm. Certainly, more work needs to be done on VBNC cells.

Sorbate/sorbic acid

The interaction of *Brettanomyces*/*Dekkera* and sorbate is not clear. There are quite a few references about resistance, at least in cultures (Chatonnet *et al.*, 1992; Licker *et al.*, 1998; Oelofse *et al.*, 2009); and a few references to sensitivity (Suárez *et al.*, 2007). More investigation of the response of different strains to sorbate in wine would be helpful.

Dimethyldicarbonate (DMDC, trade name Velcorin®)

Brettanomyces is sensitive to DMDC (Renouf *et al.*, 2008). Recent references suggest 100–150 ppm (Costa *et al.*, 2008) or 200 ppm (Oelofse *et al.*, 2009). Because DMDC degrades naturally within six to eight hours, there is no residual protection for wines that are reinfected after treatment. It is best used at bottling,

and a special (expensive) doser is needed to achieve the correct dose in each bottle. DMDC is a hazardous material, very harmful to skin, so it requires care to use safely. It is not permitted in all countries.

Other inhibitors

Chitosan, a polysaccharide derived from chitin, is reported to inhibit *Brettanomyces* at 3–6 g/L (Suárez *et al.*, 2007). It may be possible to use killer toxins made by the yeast strains *Pichia membranefaciens* (PMKT2) (Santos *et al.*, 2009), *Pichia anomala* and *Kluveromyces wickerhamii* to inhibit *Brettanomyces*. This competitive effect has been described as 'the big fish eats the little fish' (Guerra, 2008). More research is needed to determine whether these yeast toxins would work in wine being prepared for bottling. Also, inhibition of some *Brettanomyces* strains by peptides derived from bovine lactoferrin is under investigation.

Thermal inactivation

Of course, flash pasteurization is very effective at killing all microbes in the wine. It does require expensive equipment, and not all winemakers are happy to subject their wine to this procedure, especially their finer wines. At least one study found that simply heating wine to 35ºC/94ºF killed *Brettanomyces* (Couto *et al.*, 2005). However, heating could have serious effects on wine quality. Many years ago, a heating 'experiment' was carried out inadvertently by a Napa Valley Winery (Doug Manning, personal communication). Their 1978 Cabernet had won an important gold medal, but it had live *Brettanomyces* cells in the bottle; bottles were cultured every week to detect a 'bloom' if it began. But over the Fourth of July, the winery air conditioning failed and the wines became hot (no idea how hot); the bottles (miraculously) did not leak, but suddenly the *Brettanomyces* were dead. Many subsequent cultures grew no *Brettanomyces*, even 20 years later (personal experience).

Preparing the bottling line

After all the microbes have been filtered out of the wine, it is important that it does not become re-contaminated during bottling. The bottling line must be thoroughly sanitized to remove any resident microbes, including *Brettanomyces*, which could contaminate the wine. Sterilization, the complete removal of all microbes, can be achieved by running hot water (85–90ºC/180–185ºF, measured coming out of a filler spout) through the line for 20–30 minutes. Hot water may not physically penetrate far enough but the heat does, if it is applied for a long enough period. However, in bottling lines with blind spots and convoluted pathways, some microbes may be able to escape even being killed by the heat. Bottling lines can be sanitized with chemicals such as peracetic acid, quaternary ammonia, and ozone (a tricky procedure because of ozone's toxicity), though chemical sanitizers are not as effective as heat in killing microbes, especially

those sequestered in out-of-the-way places in the bottling line, including bends and T-joints.

Once the bottling line has been sanitized, it should be checked with a bioluminescence device, which detects ATP from cells (live or dead), to see if the procedure has eliminated all the microbes. If not, the bottling line should be re-sanitized before proceeding.

Without detracting attention from the extreme importance of bottling line sanitation, in most well-appointed commercial cellars *Brettanomyces* infections in the bottle nearly always come from failure to remove all the cells already present in the wine before bottling, rather than *Brettanomyces* contamination in the bottling line (however, bottling line contamination is very common for fermentative yeasts). Prevention of *Brettanomyces* growth in the bottle usually focuses on eliminating an existing infection.

After bottling

At least three bottles per bottling day (more for high-speed lines) should be checked by membrane filtration culture. PCR methods are not sensitive enough to detect the very low numbers of cells (a few cells per bottle, not per ml) required to test for fermentation stability of tightly filtered wine, though PCR could be used to check for *Brettanomyces* in wine that was not sterile-filtered.

Wines bottled without sterile filtration should also be cultured at 3, 6, and 12 months and tasted critically for any evidence of *Brettanomyces* (or other) growth. If any sign at all of activity, sensory change, or bottle variation is noticed, microbial growth should be suspected and the wine checked thoroughly.

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