Distribution of Yeast Cells, Temperature, and Fermentation By-Products in White Wine Fermentations

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Abstract: Yeast inoculation from the tops of tanks is common in white wine fermentations. Our objective was to examine whether inhomogeneities occur in tanks following yeast addition from the top with and without brief stirring after inoculation. We investigated whether inhomogeneities affect fermentation kinetics and formation of acetaldehyde, acetic acid, and pyruvate in Riesling juices with low turbidity. Samples and temperatures were taken at multiple tank heights in pilot-scale (105 L) and industrial-scale (2500 and 7000 L) tanks to investigate the distributions of temperature, yeast, and fermentation products in tanks of different volumes and height-to-diameter ratios. When the tank was not stirred following inoculation, cell counts were higher in the upper tank section for multiple days. Fermentation started earlier at the top of the tank than at lower levels, as indicated by specific gravity measurements. The top and bottom of the tanks differed by up to 8.6°C and 0.040 in specific gravity. The inhomogeneities lasted four days in the 7000 L tanks. These results suggest slow yeast sedimentation and poor mixing in the downward direction during the early fermentation phase. During vigorous fermentation, all parameters were homogenously distributed. Brief stirring after yeast inoculation provided uniform conditions during the early fermentation phase, except for temperature differences due to natural stratification. Stirring also shortened the fermentation duration. The final pyruvate concentration was significantly lower when the yeast was stirred in, presumably due to better nutrient availability throughout the tank, reducing sulfur dioxide demand. No differences were observed in acetaldehyde and acetic acid concentrations. When yeast is not stirred in following inoculation, tank sampling valves and temperature sensors do not reflect the entire tank during the early fermentation phase, which will negatively affect process control.

Key words: fermentation dynamics, *Saccharomyces cerevisiae*, sedimentation, spatial distribution, white wine, yeast cell distribution

During the vigorous phase of white wine fermentation, conditions in tanks are homogenous due to agitation by CO_2 formation (Guymon and Crowell 1977, Vlassides and Block 2000, Porret et al. 2007). However, CO_2 formation is low during the early and late stages of fermentation, and no additional external agitation is typically used during wine fermentations. Thus, yeast cells, nutrients, or fermentation products might be inhomogeneously distributed in wine tanks, and gradients in temperature might occur. Such inhomogeneities are thought to be more pronounced when yeast inoculation is performed from the top of the tank without subsequent stirring. Despite the recommendation to inoculate the yeast starter at the bottom of the fermenter before filling the tank with grape juice (Specht 2010), inoculation from the top of the tank after filling is a common practice. Inhomogeneities may affect the overall fermentation kinetics and reduce control over the process.

Winemakers need control over the fermentation process in order to assess fermentation progress. Fermentation control usually includes measurement of temperature and specific gravity on a regular basis (Jeffery and Wilkinson 2014). During the vigorous phase of white wine fermentations, the sampling valve and temperature sensor have been reported to be representative of the whole tank (Guymon and Crowell 1977, Porret et al. 2007). Yeast cells are also reported to show homogenous distributions during vigorous fermentation in pilot-scale tanks and in industrial-scale tanks of 1000 to 10,000 L (Vlassides and Block 2000, Porret et al. 2007). However, this might not be the case during the pre- and postfermentation phases.

Inhomogeneities in wine tanks during the pre- and postfermentation phases have not yet been fully studied for industrial wine fermentations, particularly not for low turbidity juices. Vlassides and Block (2000) observed high concentrations of insoluble solids (yeasts and grape solids) in the sediment layer

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during the first days of 1200-L tank fermentations and suggested that cell growth began from the tank bottom, becoming uniform when cell growth ceased. They suggested that the formation of these gradients was increased by incomplete settling of solids prior to yeast inoculation and by incomplete hydration of the active dry yeast before inoculation (Vlassides and Block 2000). The authors assumed that the common practice of yeast inoculation from the top of a wine tank without external agitation might result in gradients that are the reverse of those observed in their experiments.

In beer fermentations, an investigation of yeast inoculation from the top of a tank without subsequent mixing showed poor mixing in the downward direction at the beginning of the process in a 3.8-L tank with an extreme height-to-diameter ratio of 22.5 (Garcia et al. 1993). When inoculation was performed at other depths in the tank, the researchers found homogeneity for cell mass and pH values above, but not below, the inoculation point at the beginning of the process (Garcia et al. 1993). Further investigations using a 14-L cylindrical tank with a height-to-diameter ratio of 13.5 were performed, focusing on inoculations in the upper part of the tank. These trials also showed inhomogeneities in various chemical parameters and in the dry weight of *Saccharomyces carlsbergensis* cells at the beginning of the process (Garcia et al. 1994). The height-to-diameter ratios of beer fermentation tanks are usually between 1 and 2.5 (Narziß and Back 2001), which is also typical for wine fermentation tanks. Height-to-diameter ratio might influence fluid dynamics in the tanks. Meironke (2014) reported that the height-to-diameter ratio influences flow stability in cylindro-conical beer tanks.

Inhomogeneities have been documented for fixed inoculation points in beer fermentations but have not been studied in white wine fermentations. Considerable temperature gradients have been reported for red wine fermentations due to the high content of grape solids (skins and seeds), which can be controlled by cap management (Schmid et al. 2009). For white wine fermentations, inhomogeneities might also be relevant, possibly affecting the proliferation phase and fermentation duration. For example, in areas of the tank with higher yeast counts, local competition for nutrients may occur, affecting overall yeast metabolism and, thus, fermentation kinetics. Garcia et al. (1994) observed that beer fermentations finished rather slowly when inoculation was performed in the upper part of the tank and suggested that this was caused by poor agitation in the fermenter during the first days of the process. Wine experiments focusing on suspended solids in juice have also indicated that fermentation rates were fastest when yeast was suspended uniformly (as cited in Boulton et al. 1999).

It was the objective of this work to investigate inhomogeneities in tanks during white wine fermentations after yeast inoculation from the top with and without brief stirring. To examine the fermentation progress at various tank heights, we monitored specific gravity, temperature, and fermentation by-products. Yeast distribution and sedimentation were assessed by yeast cell count. Moreover, we aimed to evaluate whether a wine tank's sampling valve and temperature sensor are representative of the entire tank and to reveal how inhomogeneities manifest in tanks of different sizes. Pilot-scale tanks (105 L) with an extreme height-to-diameter-ratio were chosen to enforce wine-layering effects. Industrial-scale tanks (2500 L and 7000 L) with typical height-to-diameter-ratios were used to assess adaptability to practical conditions.

Materials and Methods

Pilot-scale tanks: Wine and experimental design. Riesling grapes (vintage 2017) were harvested from a vineyard of the Staatsweingut mit Johannitergut Neustadt (Pfalz region, Germany). After the grapes were crushed and pressed, the juice was clarified by flotation using FloraClair (Erbslöh). After the addition of perlite and diatomaceous earth, the juice was filtered with a lees filter. This was followed by two further filtration steps: a sheet filtration using layers of Seitz K100 filter sheets (Pall Food and Beverage) and a sterile cartridge filtration. Subsequently, the must was preserved according to the Seitz-Böhi process and stored with CO_2 in a pressurized stainless steel tank until the juice was used for the experiments four months later. The juice had the following characteristics: 189 g/L fermentable sugar, 8.4 g/L titratable acidity, 187 mg/L yeast assimilable nitrogen, pH 3.3, specific gravity (20°C/20°C) = 1.077, turbidity = 15 NTU.

Four Riesling fermentations were conducted in pilot-scale wine tanks in duplicate. Two fermentations were performed without stirring after yeast inoculation, and two fermentations were performed with stirring after inoculation. Yeast was rehydrated (0.25 g/L X5 Zymaflore, Laffort) according to the manufacturer's instructions. Fermentations were performed in four 105-L tubular tanks (Figure 1). For inoculation without stirring, 80 L of juice was poured into each of two tanks. Then, rehydrated yeast was added into the juice through an opening at the top of each tank. For the experiments including stirring, the rehydrated yeast was inoculated into 160 L of juice in a preparatory tank. The inoculated juice was stirred for 10 sec using a stirrer, which corresponds to 1 min/1000 L of juice. Subsequently, 80-L aliquots of juice were poured into two tanks using a flow meter. No additional yeast nutrients were added, thus ensuring that possible inhomogeneities in nutrient addition would have no effect on possible gradients in the distribution of yeast or fermentation by-products.

Temperature loggers (HOBO U22-001 Water Temp Pro v2 [Onset] and HOBO 64K Pendant [Onset]) were placed at various levels in the tanks as shown (Figure 1). The loggers recorded the wine temperature at 10-min intervals. Juice/wine samples were taken by syringes (Omnifix 250 mL, B. Braun Melsungen AG) from sampling constructions placed in the tanks (Figure 1). Each sampling construction was built from three hoses (John Guest 6 mm o.d. × 4 mm i.d., LLDPE) fixed to a thin stainless-steel chain and weighted at the lower end to ensure three fixed sampling points in the tank. Samples were taken after discarding the first 20 mL (top sample), 30 mL (middle sample), and 40 mL (bottom sample). To assess the influence of stirring on fermentation duration,

we ended each experiment when the specific gravity of the wine reached 0.995, which corresponds to ~5 g/L residual sugar. This allowed us to compare the fermentation durations of the unstirred and stirred tanks. The cellar temperature was 15°C at the beginning of the fermentation and 13°C at the end.

Industrial-scale tanks: Experimental design. Five Riesling fermentations (vintages 2016 and 2017) were conducted in two wineries in the Pfalz region in Germany in 2500-L and 7000-L tanks. The tank dimensions are illustrated in Figure 1. The 2500-L tank was equipped with a cooling plate inside the tank; the 7000-L tank was equipped with a cooling jacket. Table 1 shows the specific gravities of the Riesling musts, the yeast strains, and the day of diammonium hydrogen phosphate (DAP) addition, as well as the filling, cellar, and set temperatures. For all fermentations, yeast (0.25 g/L) was rehydrated according to the manufacturer's instructions. Inoculation was performed through an opening on the top of each tank, after which the yeast was either stirred into the juice by a stirrer (1 min/1000 L juice) or not stirred in (see Table 1). DAP was added from the top of the tank without subsequent stirring at concentrations of 0.4 g/L in the 2500-L tanks and 0.5 g/L in the 7000-L tanks (Table 1). A spot sampler (ALS 132, UK Sampling Gauges Ltd.) was used for sampling at different levels in the wine tanks. Additional samples were taken from the sampling valve after discarding the first 250 mL of liquid. Temperature loggers (HOBO 64K Pendant, Onset) were placed at various levels in the tanks to measure the wine temperature at intervals of 10 min. The levels for sampling and temperature measurements are shown in Figure 1. A temperature logger was located outside the tanks to determine cellar temperature.

Sampling frequency. One sampling was conducted before yeast inoculation. Following inoculation, sampling was performed at least each second day. As soon as vigorous fermentation commenced, sampling was performed every two to five days until the end of fermentation. The specific gravity, viable yeast count, and basic wine parameters were analyzed immediately after sampling. Samples for the analysis of acetic acid, acetaldehyde, and pyruvate were centrifuged for 5 min at 4500 rpm, and the supernatants were refrigerated at -23°C until analysis.

Basic wine parameters. Specific gravity was measured by a density meter (DMA 35, Anton Paar GmbH). Further wine parameters (glucose, fructose, pH, total acidity, and yeast-assimilable nitrogen) were measured via Fourier transform infrared spectroscopy (FT-IR) (WineScan FT120 Basic, Foss). Samples were centrifuged for 5 min at 4500 rpm and degassed prior to FT-IR analysis.

Turbidity. Juice turbidity was measured with a turbidimeter (2100Qis Portable Turbidimeter, Hach) calibrated with formazine standards of defined turbidity (10, 20, 100, 800 NTU) provided by the manufacturer. Sample cells filled with 15 mL of juice were shaken before measurement to ensure a homogeneous distribution of solid particles.

Acetic acid, pyruvate, and acetaldehyde. Concentrations of acetaldehyde, acetic acid, and pyruvate were measured by an automated photometric analyzer (Konelab 20i, Thermo Fisher Scientific Inc.) after centrifugation (8000 rpm, 5 min) and enzymatic conversion. For conversion of acetaldehyde

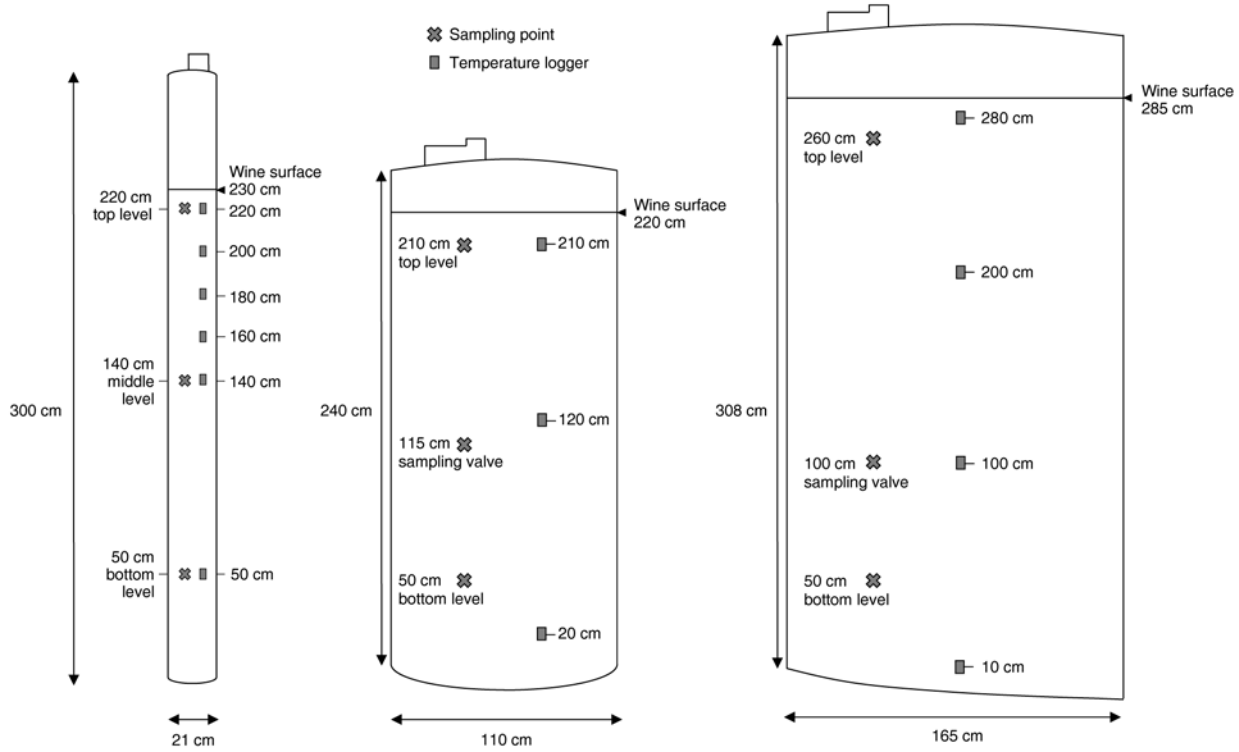

Figure 1 Sampling points and positions of temperature loggers in the 105-L pilot-scale wine tanks (left) and in the 2500-L (middle) and 7000-L (right) industrial-scale wine tanks.

and acetic acid, commercially available test kits (984347 and 984303, Thermo Fisher Scientific Inc.) were used. For enzymatic conversion of pyruvate, an enzyme suspension was prepared with 5 mg/mL lactate dehydrogenase suspension (Roche Diagnostics) diluted 1:10 with water. To provide a buffered cofactor reagent, 60 mg sodium hydrogen carbonate and 30 mg β-nicotinamide adenine dinucleotide reduced disodium salt hydrate were dissolved in 12 mL water. A buffer solution for enzymatic conversion was prepared by dissolving 14 g triethanolamine hydrochloride and 0.28 g Titriplex III (Merck) in 80 mL water and adjusting the pH to 7.6 with NaOH (5 mol/L). Subsequently, the solution was filled up to 100 mL with water. The water used in all preparations was purified by ELGA Purelab flex (ELGA LabWater). For the pyruvate test, an automated photometric analyzer pipetted 10 µL cofactor reagent into 50 µL buffer solution. Subsequently, 10 µL diluted sample (1:2 dilution with water) was added to the mixture. After 120 sec, 25 µL enzyme suspension was added for enzymatic conversion. After a reaction time of 420 sec, measurement was performed at a wavelength of 340 nm.

Viable yeast count. Cells were harvested by centrifugation (2400 *g*, 5 min) at 20°C and washed twice in phosphate-buffered saline (pH 7.2). After the addition of 20 µL fluorescein diacetate (5 mg/mL in acetone), the yeast suspension was incubated for 5 min in the dark and filtered through a 30-µm CellTrics Partec filter (Sysmex Europe GmbH). According to the fermentation stage, the filtered yeast suspensions were diluted to an appropriate cell concentration with phosphate-buffered saline, vortexed, and immediately analyzed by flow cytometry at a wavelength of 488 nm (CyFlow Cube 6, Sysmex). Data were analyzed using FCS Express 4 (De Novo Software).

Statistical analyses. Analytical data were statistically evaluated by analysis of variance followed by a least significant difference post-hoc test at a significance level of 5%. Data were tested for normal distribution by a Shapiro-Wilk test. Regressions were performed for the comparison of acetic acid, acetaldehyde, and pyruvate at an identical specific gravity of 1.053. The specific gravity of 1.053 was chosen as the first common specific gravity of the unstirred and stirred tank after the initial phase (when both tanks showed homogenous conditions). The comparison at an identical specific gravity is expedient to allow the comparison of fermentation-related metabolites at identical fermentation progress (Schwinn et al. 2019). Calculations were conducted using XLSTAT Version 2016.04.32229 (Addinsoft).

Results and Discussion

Pilot-scale experiments. We used 105-L tanks with an extreme height-to-diameter ratio of 14 that was intended to enforce wine-layering effects. Experiments were performed with and without brief stirring after yeast inoculation. The results showed differences in specific gravity between the three sampling heights between days 2 and 7 after inoculation in the unstirred tank (Figure 2A). At the top level, the specific gravity had declined on day 2, indicating that fermentation started early in the upper section of the tank. At the same time, higher viable yeast counts were detected at the top level (Figure 2A). At the middle and bottom levels of the tank, where viable yeast counts were significantly lower, no reduction in specific gravity was observed until day 6, indicating a delayed fermentation start. This suggests that inoculation from the top of the tank without stirring entails a higher number of yeast cells at the top level, resulting in an early fermentation start in the upper section of the tank. The delayed start of fermentation at the middle and bottom levels is likely a result of slow yeast sedimentation.

A higher oxygen content near the wine surface could also be responsible for higher viable yeast counts at the top level.

Table 1 Starting and surrounding conditions of fermentations in pilot-scale and industrial-scale wine tanks.

Vintage (year)	Stirring after inoculation	Specific gravity (20°C/20°C) grape juice	Yeast strain	Day of DAP[a] addition	Filling temperature (°C)	Set temperature (°C)	Cellar temperature (°C)
Pilot-scale tank (105 L)							
2017	Unstirred	1.077	X5[b]	–	14.5	–	15.3
2017	Unstirred	1.077	X5[b]	–	14.5	–	15.3
2017	Stirred	1.077	X5[b]	–	14.5	–	15.3
2017	Stirred	1.077	X5[b]	–	14.5	–	15.3
Industrial-scale tank (2500 L)							
2017	Unstirred	1.079	VB1[c]	4	11.3	19	17.2
2017	Unstirred	1.079	VB1[c]	4	11.3	19	17.2
Large industrial-scale tank (7000 L)							
2016	Unstirred	1.089	X5[b]	2	10.5	18	15.7
2016	Stirred	1.089	X5[b]	1	13.4	19	15.7
2016	Stirred	1.081	X5[b]	5	12.6	18	15.9

[a]Diammonium hydrogen phosphate.
[b]Zymaflore, Laffort.
[c]Fermivin, Oenobrands SAS.

Oxygen has been shown to increase yeast cell numbers and accelerate the initiation of fermentation (Ribéreau-Gayon et al. 2006). As explained later in this report, when the yeast was stirred in, the three tank levels did not differ in viable yeast count. Therefore, the influence of oxygen at the wine surface on yeast viability is not the most likely cause of the observed differences.

According to the literature, the calculated settling velocity of single yeast cells in grape juice is assumed to be less than 0.02 m/hr (as cited in Vlassides and Block 2000). On the basis of this and our own observations, we propose that the longer the distance from the wine surface to the bottom of the tank, the later fermentation starts in unstirred wine tanks when inoculation is performed from the top. In contrast to our findings, Vlassides and Block (2000) assumed that cell growth began from the bottom of the fermenter in 1200-L wine fermentations. The authors concluded this from high levels of total insoluble solids (yeast and grape solids) in the sediment layer after inoculation. Because the juices in our experiments were low in turbidity, we propose that the observations made by Vlassides and Block (2000) might be due to different concentrations of solids, which presumably influence the yeast sedimentation.

The specific gravity and viable yeast counts at the top and middle levels of the tank aligned on day 6 in the unstirred tank (Figure 2A). At the bottom level of the tank, a significantly lower viable cell count, and thus no progress in fermentation, was observed on day 6. These findings indicate mixing of wine layers from the middle level in an upward direction, which suggests that agitation in an upward direction occurs as soon as fermentation starts at a given level. The agitation is caused by the rise of CO_2 bubbles homogenizing all layers above the level of fermentation. However, no mixing in a downward direction was observed. Garcia et al. (1993) showed similar findings in experiments investigating different inoculation points in beer fermentations. The authors described poor mixing in the downward direction at the beginning of fermentation, whereas nearly perfect mixing was observed in the upward direction in relation to the inoculation point.

Theoretically, specific gravity curves constantly decrease during fermentation. However, an increase in specific gravity was observed at the top level in the unstirred tank between days 3 and 4 (Figure 2A). Another increase was observed between days 6 and 7 at the top and middle levels. These repeatedly observed increases in specific gravity, which are contradictory at first sight and not significant, could be caused by the mixing of unfermented, high-density juice from lower levels with more-fermented, lower-density juice from upper levels. The effect of mixing between lower levels, which just started fermentation, and upper levels, which are further fermented, might also explain the observations made in the yeast growth curves. Typically, viable yeast counts increase after inoculation and then reach a stationary phase, in which cell numbers remain constant (Zamora 2009). Yet, in the unstirred tank, a decrease in viable yeast counts was observed between days 4 and 6 at the top level, while numbers at the middle level increased (Figure 2A). The viable yeast count observed on day 6 at the top and middle levels may be a mean value resulting from viable yeast counts of all the wine layers between the middle level and the surface of the wine.

The specific gravity and viable yeast count at all three sampling levels in the unstirred tank aligned on day 7, indicating sufficient mixing and, thus, homogenous conditions. Garcia et al. (1993) and Porret et al. (2007) described homogenous conditions in wine tanks during the vigorous phase of fermentation due to agitation by CO_2. The phase from inoculation to homogeneity (days 0 to 7) is postulated as the "initial phase" of fermentation in our study. In contrast to the term "prefermentation", which suggests that no fermentation occurs at any level in the tank, "initial phase" takes inhomogeneities into consideration and includes levels that are already fermenting, as well as levels that are not yet fermenting.

In the stirred tank (Figure 2B), the three tank levels showed no differences in specific gravity or viable yeast count during the fermentation process. This suggests that stirring

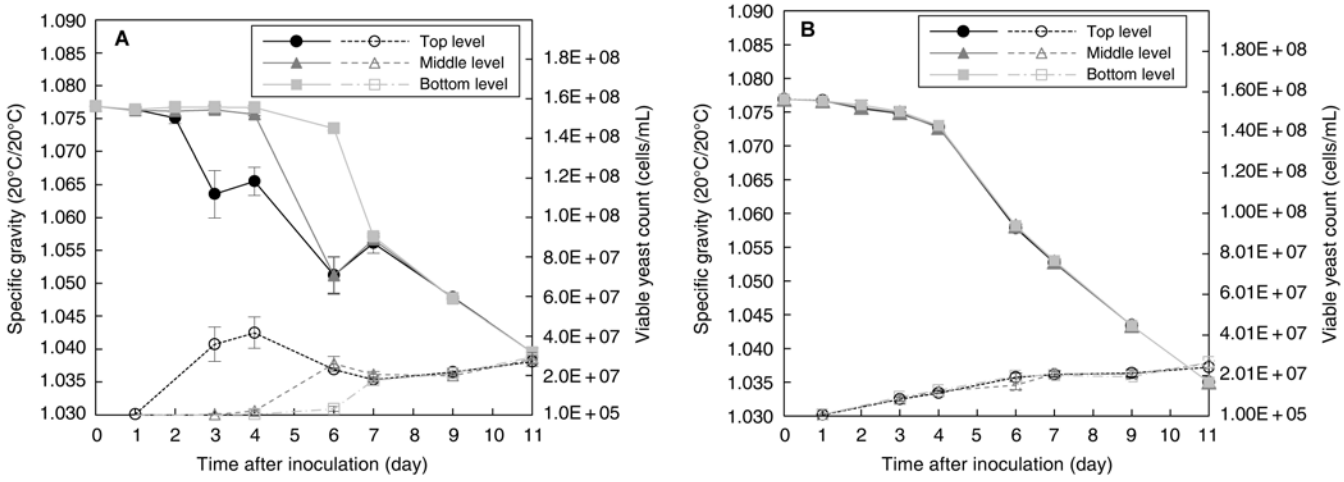

Figure 2 Specific gravity (solid lines) and viable yeast count (dotted lines) at three levels in pilot-scale wine tanks during the first 11 days after inoculation. Yeast was not stirred in (**A**) or was stirred in (**B**) after inoculation (mean ± SD, n = 2 ferments).

in the rehydrated yeast provides a uniform distribution of yeast cells and homogenous conditions, enabling a similar fermentation starting point at all tank levels.

Figure 3 compares the decline of specific gravity over the course of fermentation in the stirred and unstirred tanks. Confidence bars reflect inhomogeneity in the unstirred tank during the initial phase, whereas confidence bars in the stirred tank indicate homogeneity over the three sampling levels during the whole process. Fermentation duration was shorter by four days when the yeast was stirred in after inoculation. The shorter fermentation duration in the stirred tank is due to a faster fermentation rate in the initial phase (until day 7). The specific gravity was 0.004 lower in the stirred tank on day 7, indicating that fermentation was further progressed in the stirred tank than in the unstirred tank. After the initial phase, the fermentation rates were comparable. Thus, we propose that the initial phase governs the differences in the fermentation duration found in our study.

Our results support suggestions by other authors that inhomogeneities can affect overall fermentation kinetics (Garcia et al. 1994, Vlassides and Block 2000). We assume that the differences in fermentation kinetics between the stirred and unstirred tanks in the initial phase are due to the availability of nutrients. In the stirred tank, yeast cells are better distributed, and all cells have similar access to nutrients. In the unstirred tank, a high number of yeast cells in the top section must compete for nutrients within a smaller wine volume (Figure 2A). Nutrient limitations are associated with stuck and sluggish fermentations and reduced rates of cell growth (Bisson 1999). Oxygen, which might ingress during stirring, seemed to play a subordinate role in our experiment; otherwise, yeast numbers would have been increased in the stirred tanks rather than in the unstirred tanks. Oxygen can increase yeast cell numbers, especially in low-turbidity musts (Ribéreau-Gayon et al. 2006, Ochando et al. 2017). We found no significant difference ($p < 0.05$) in viable yeast cells between the unstirred and stirred tanks after the initial phase.

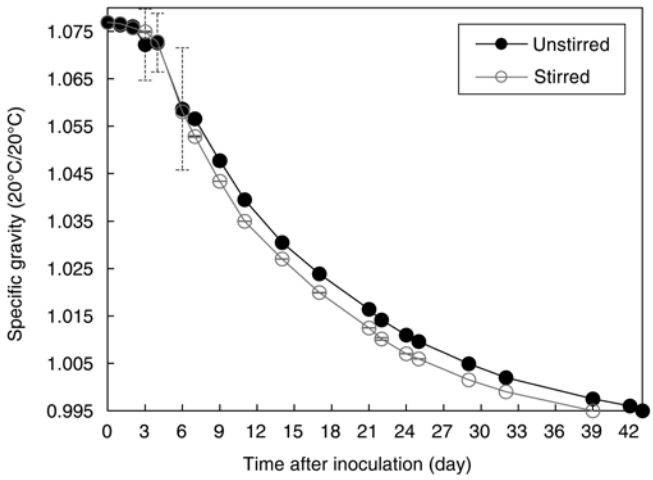

Figure 3 Specific gravity over the course of fermentation with and without stirring after yeast inoculation in pilot-scale wine tanks (n = 3 sampling levels × 2 ferments). The patterned confidence bars represent the standard deviation in specific gravity over the three sampling levels.

Acetaldehyde, acetic acid, and pyruvate, which are excreted in the juice especially during the proliferation phase and early fermentation (Ribéreau-Gayon et al. 2006), increased early at the top level of the unstirred tank (Figure 4A1 to 4C1). At the middle and bottom levels, fermentation by-products increased later, resulting in concentration differences in the vertical direction in the initial phase (Figure 4A1 to 4C1). The maximum differences between the top and bottom of the tank are compiled in Table 2. The maximum differences in acetaldehyde and acetic acid were observed on day 3 in the unstirred tank. The maximum difference in pyruvate between the top and bottom of the tank was observed one day later, on day 4. On day 6, homogenous distributions of fermentation by-products were observed at the top and middle levels of the unstirred tank (Figure 4A1 to 4C1), suggesting mixing of both levels. On day 7, concentrations of fermentation by-products were similar at all three tank levels. Concentrations at all levels remained similar until the end of fermentation, indicating appropriate mixing. In the stirred tank, acetaldehyde, acetic acid, and pyruvate showed no concentration differences in the vertical direction throughout the whole fermentation (Figure 4A2 to 4C2). As discussed earlier, higher viable yeast counts at the top level of the unstirred tank initiated an earlier fermentation start at the top (Figure 2A). Yeast counts were higher at the top level compared to the middle level until day 6 and higher at the top level compared to the bottom level until day 7. A high number of yeast cells may produce acetaldehyde, acetic acid, and pyruvate faster than a smaller number of yeast cells. Accordingly, the early increase of acetaldehyde, acetic acid, and pyruvate at the top level after inoculation can be explained by the higher number of yeast cells at the top level.

The influence of stirring after inoculation on the concentrations of fermentation by-products is shown in Table 3. No significant difference ($p < 0.05$) was observed for acetaldehyde after the initial phase on day 7. However, acetic acid was significantly lower in the unstirred tank on day 7, while pyruvate was significantly higher (Table 3). Yet, it has to be considered that specific gravity, and hence the fermentation progress, was different on day 7. When comparing the concentrations at a specific gravity of 1.053, which was the first common specific gravity of the unstirred and stirred tanks after the initial phase, no significant differences between the unstirred and stirred tanks were observed for acetaldehyde and acetic acid, but the differences for pyruvate were still significant (Table 3). Pyruvate remained higher in the unstirred tank until the end of fermentation, whereas no significant differences in the final concentrations of acetic acid and acetaldehyde were found (Table 3). High pyruvate concentrations may be adverse for wine quality because pyruvate is thought to be a precursor for diacetyl formation during malolactic fermentation. Recently, researchers showed that exogenous pyruvate induces diacetyl formation by *Oenococcus oeni* (Mink et al. 2014). Furthermore, an increased pyruvate concentration is undesirable because its binding capacity for sulfur dioxide (SO_2) results in an increased SO_2 demand for wine stability (Ribéreau-Gayon et al. 2006).

Figure 5 shows the pyruvate concentration over the course of fermentation in pilot-scale wine tanks with and without stirring after inoculation. Confidence bars reflect the inhomogeneity in the unstirred tank during the initial phase. In the unstirred tank, pyruvate increased strongly between days 2 and 6 of fermentation (Figure 5). After the initial phase (on day 7), a higher pyruvate concentration was observed in the unstirred tank than in the stirred tank. Thereafter, pyruvate decreased at a faster rate in the unstirred tank; however, it remained significantly higher until the end of fermentation.

Pyruvate is a key intermediate in metabolic pathways. Formed from glucose through glycolysis, it is decarboxylated to acetaldehyde by pyruvate decarboxylase during alcoholic fermentation (Pronk et al. 1996, Quirós et al. 2013). The strong increase in pyruvate concentration during the initial phase in the unstirred tank is likely due to the high excretion of pyruvate by yeast cells into fermenting wine. Yeast cells excrete pyruvate because pyruvic acid is a fairly strong organic acid (pK 2.46 at 25°C) that is highly dissociated at pH 6.0 to 6.5 inside the cell (Whiting 1976). To prevent a pH decrease in the cell, and thus cytotoxic effects, the yeast excretes the pyruvate anion with a hydrogen ion (Whiting 1976).

High levels of pyruvate excretion, as observed in our study at the top level of the unstirred tank, might be caused by nutrient limitations. A deficiency in thiamine has been reported to increase accumulation of pyruvate (Ribéreau-Gayon et al. 2006). Thiamine pyrophosphate is an essential cofactor for pyruvate decarboxylase (Pronk et al. 1996, Ribéreau-Gayon et al. 2006). Thus, the competition for thiamine at the top level of the tank may increase the

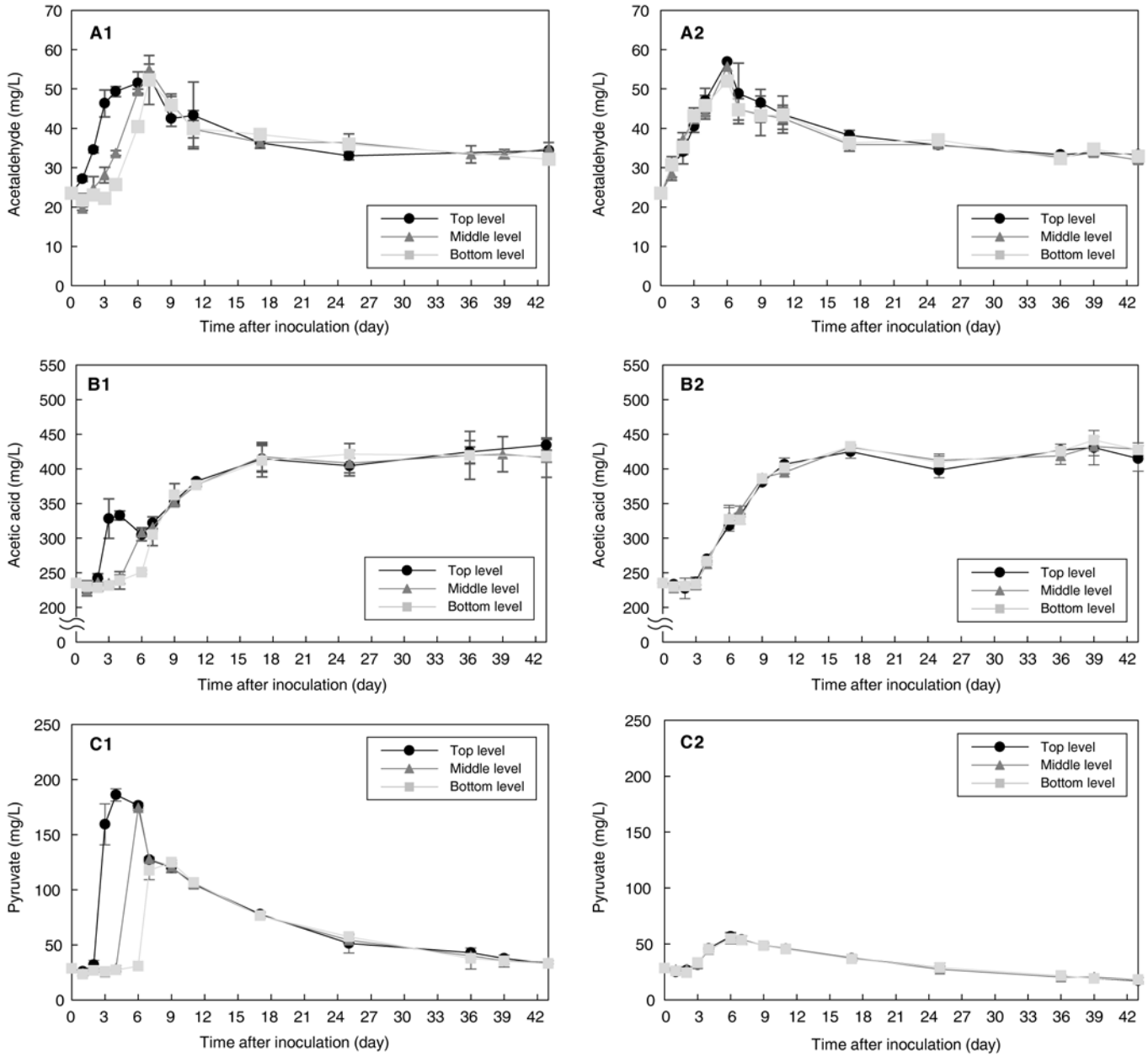

Figure 4 Concentrations of acetaldehyde (**A**), acetic acid (**B**), and pyruvate (**C**) at three levels in pilot-scale wine tanks over the course of fermentation without (**1**) and with (**2**) stirring after inoculation (mean ± SD, n = 2 ferments).

Table 2 Maximum differences in various wine parameters between the top and bottom levels of the pilot-scale tanks (mean ± SD, n = 2 ferments).

Stirring after inoculation	Maximum difference (top - bottom) Specific gravity (20°C/20°C)	Acetaldehyde (mg/L)	Acetic acid (mg/L)	Pyruvate (mg/L)	Maximum ratio (top:bottom) yeast cells	Maximum temp dif. (top - bottom) (°C)	Time until homogenous temp (days)
Unstirred	-0.022 ± 0.003	24 ± 3	105 ± 7	159 ± 7	115:1	2.2 ± 0.1	6.8 ± 0.1
Stirred	0.000 ± 0.0004	5 ± 0.4	15 ± 7	1 ± 6	0.9:1	0.9 ± 0.1	2.4 ± 0.3

Table 3 Concentrations of acetaldehyde, acetic acid, and pyruvate in pilot-scale tanks (mean ± SD, n = 2 ferments).

	Concentration after the initial phase, on day 7 (mg/L)[a,b]		Concentration at a specific gravity of 1.053 (mg/L)[a,c]		Concentration after fermentation (mg/L)[a,d]	
	Unstirred	Stirred	Unstirred	Stirred	Unstirred	Stirred
Acetaldehyde	53 ± 3 a[e]	46 ± 2 a	42 ± 1 a	46 ± 2 a	34 ± 2 a	34 ± 1 a
Acetic acid	314 ± 4 b	333 ± 3 a	377 ± 17 a	333 ± 3 a	420 ± 30 a	430 ± 10 a
Pyruvate	124 ± 2 a	56 ± 4 b	112 ± 2 a	56 ± 4 b	34 ± 1 a	20 ± 1 b

[a]Concentrations in the table are the mean values of all three investigated tank levels. The standard deviations are calculated from fermentation repetitions (n = 2).
[b]Day 7 is the first day that the unstirred and stirred tanks were homogeneous.
[c]First common specific gravity of the unstirred and stirred tanks after the initial phase.
[d]At a specific gravity of 0.995.
[e]Letters after the concentrations indicate differences for a significance level of $p < 0.05$ as determined by least significant difference post-hoc test.

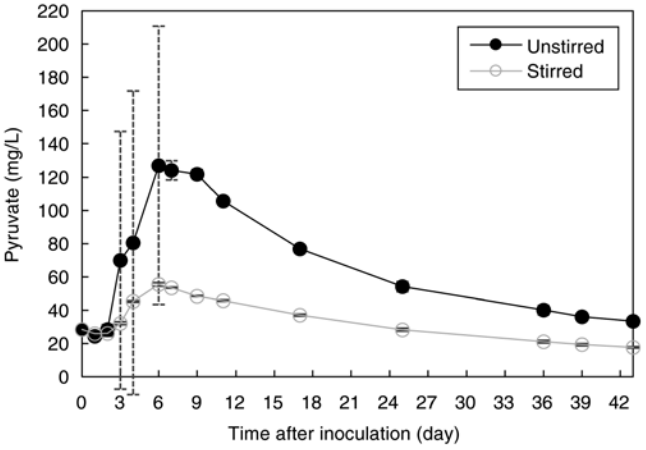

Figure 5 Pyruvate concentration over the course of fermentation with and without stirring after yeast inoculation in pilot-scale wine tanks (n = 3 sampling levels × 2 ferments). The patterned confidence bars represent the standard deviation in the pyruvate concentration over the three sampling levels.

excretion of pyruvate. Whiting (1976) reported that a lack of thiamine pyrophosphate, and hence excessive pyruvate inside the yeast cell, can be the reason for pyruvate excretion at a concentration greater than 100 mg/L. In our study, concentrations of almost 190 mg/L were measured at the top level of the unstirred tank (see Figure 4C1). In addition to nutrient limitations, elevated fermentation temperatures have also been reported to increase the production of ketonic acids (Ribéreau-Gayon et al. 2006). Temperatures were highest at the top level of the tank and therefore might have increased pyruvate concentrations.

Wine temperature measurement at six levels in the unstirred and stirred tanks allowed high-resolution description of temperature differences in the vertical direction and, therefore, an accurate interpretation of mixing processes within the tanks (Figure 6A and 6B). Table 2 compares the maximum temperature difference between the top and bottom levels, as well as the time required to reach a homogenous temperature for the unstirred and stirred tanks. While a maximum temperature difference of 0.9°C was observed between the top and bottom levels during the first two days when the yeast was stirred in after inoculation (Figure 6B), the temperature difference in the unstirred tank was larger (2.2°C) and lasted longer (almost seven days) (Figure 6A). The temperature differences in the vertical direction during the first two days in the unstirred and stirred tanks are likely to be density-induced due to natural temperature stratification, as no fermentation or poor movement is suspected. Temperature stratification is a well-known effect occurring during wine storage (Boulton et al. 1999), and temperature gradients are expected to be more pronounced with an increased tank height and in cellars with poor air circulation. Although temperature gradients in wine tanks are usually undesirable because of reduced process control, temperature stratification can be desirable in thermal storage applications used for energy solutions (Siegenthaler 2016, Li et al. 2017).

The conversion of sugars to ethanol is an exothermic series of reactions (Williams 1982, Boulton et al. 1999). On day 2, a stronger temperature increase, apparent by the increasing slope of temperature in Figure 6A, was observed in the top level of the unstirred tank. For the lower levels, this effect occurred delayed in descending order. The 50-cm level (bottom sampling level) showed the increase in temperature on day 6. Whereas the gradient during the first two days is likely a result of natural temperature stratification, the increasing slope of the temperature curve is explained by

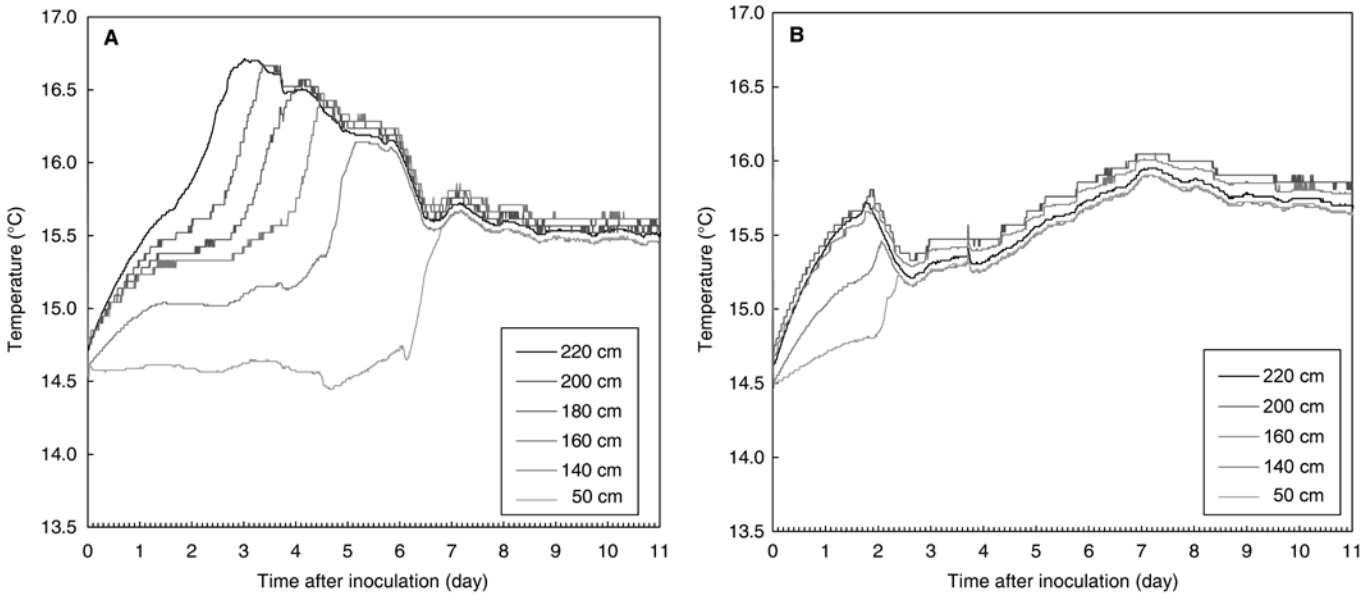

Figure 6 Wine temperatures at different levels in pilot-scale wine tanks during the first 11 days after inoculation. Yeast was not stirred in (**A**) or was stirred in (**B**) after inoculation (mean, n = 2 ferments). The cellar temperature was 15.3 ± 0.4°C.

Table 4 Maximum differences in various wine parameters between the top and bottom levels in industrial-scale tanks.

		Max. difference (top - bottom)						
Tank volume (L)	Stirring after inoculation	Specific gravity (20°C/20°C)	Acetaldehyde (mg/L)	Acetic acid (mg/L)	Pyruvate (mg/L)	Maximum ratio (top:bottom) yeast cells	Maximum temp dif. (top - bottom) (°C)	Time until homogenous temperature (days)
2500	Unstirred	-0.004	21	80	66	24:1	1.7	2.9
2500	Unstirred	-0.004	29	90	68	15:1	2.2	3.1
7000	Unstirred	-0.040	92	130	89	57:1	8.6	3.7
7000	Stirred	0.000	6	10	8	1:1	-0.9	0.3
7000	Stirred	0.000	4	10	14	0.9:1	0.3	0.6

the start of fermentation. The layer-by-layer increase of temperature revealed a delay in the start of fermentation of ~20 cm/day (0.01 m/hr) in the downward direction. This finding is in accordance with the sedimentation speed of yeast cells (less than 0.02 m/hr), as cited by Vlassides and Block (2000). Yet the delayed fermentation start in the downward direction is presumably determined not only by the sedimentation speed, but also by the time necessary for proliferation of yeast cells prior to fermentation start.

In the stirred tank (Figure 6B), the increasing slope of the temperature curve that indicates the beginning of fermentation was observed on day 2 for all tank levels. In combination with the finding that yeast cells were uniformly distributed over all sampling levels (Figure 2B), this suggests that fermentation started simultaneously at all tank levels.

In both the unstirred and stirred tanks (Figure 6A and 6B), increases in temperature were always followed by decreases in temperature. The temperature decreases might be explained by the mixing of low-density warm layers with higher-density cool layers. At the end of the initial phase (after 2.5 days in the stirred tank and after almost seven days in the unstirred tank), the conditions in the tanks were uniform. As soon as the conditions were uniform, no temperature differences in the vertical direction were observed in the unstirred and stirred tanks until the end of fermentation.

Industrial-scale experiments. In order to assess our findings' applicability in practical conditions, industrial-scale fermentations were carried out in tanks of 2500 L and 7000 L with a typical height-to-diameter ratio of 2. Five Riesling fermentations were examined with and without stirring after yeast inoculation. In the unstirred tanks of both volumes, differences in specific gravity between the top and bottom levels were observed during the initial phase (Table 4). The specific gravity was lower at the top level compared to both the bottom level and the sampling valve. Concentrations of acetaldehyde, pyruvate, and acetic acid were higher at the top level, resulting in differences between the top and bottom level in the unstirred tanks (Table 4). These results indicate an early fermentation start at the top level of the unstirred tanks, and thus support the observations made in the pilot-scale experiments. At the same time, higher viable yeast counts were detected at the top level of the unstirred industrial-scale tanks compared to the bottom level and the sampling valve (Table 4); this implies slow yeast sedimentation and poor mixing in the downward direction during the initial phase. Porret et al. (2007) also observed a higher yeast count at the top level of a

2000-L wine tank on the first day and explained this to be a result of inoculation from the top without subsequent stirring.

Temperature differences between the top and bottom levels of the unstirred industrial tanks were observed during the initial phase (Table 4) due to the early fermentation start at the top level. The initial phase took three to four days in the unstirred industrial tanks (Table 4). Large temperature differences might even have increased the concentration differences of yeast by-products between the top and bottom levels (Table 4). High fermentation temperatures have been reported to increase the concentrations of pyruvate and acetic acid (Whiting 1976, Ribéreau-Gayon et al. 2006). However, the influence of high fermentation temperatures on the formation of acetaldehyde remains controversial (Amerine and Ough 1964, Romano et al. 1994, Torija et al. 2003).

When yeast was stirred in, the specific gravity showed no differences between the top and bottom levels (Table 4). Moreover, no considerable differences were observed in concentrations of acetaldehyde, acetic acid, pyruvate, and yeast cells between the top and the bottom levels. These results indicate that stirring provides uniform starting conditions for fermentation. As in the pilot-scale fermentations, this suggests that stirring has a positive effect on the fermentation duration in industrial-scale tanks. In the unstirred tanks, a large number of yeast cells at the top level is thought to compete for nutrients and oxygen. In the stirred tanks, yeast cells were better distributed and equally supplied with nutrients, resulting in superior growing and fermentation conditions.

Differences between the top and bottom levels were more pronounced in the unstirred 7000-L tank than in the unstirred 2500-L tanks during the initial phase, as seen in all investigated parameters (Table 4). The 2500-L tanks reached a homogenous temperature more rapidly. Comparison of inhomogeneities in the two tank sizes is limited by the use of different commercial yeast strains and different Riesling juices. However, some conclusions are possible because the filling and fermentation temperatures were similar and *Saccharomyces* yeasts and the same grape variety were used for the experiments in both tank sizes. An earlier decline in specific gravity at the top level was observed in the 7000-L tank (Figure 7B), indicating an earlier fermentation start compared to the 2500-L tank (Figure 7A). At the sampling valve, the specific gravity was identical to that of the bottom level in both tanks.

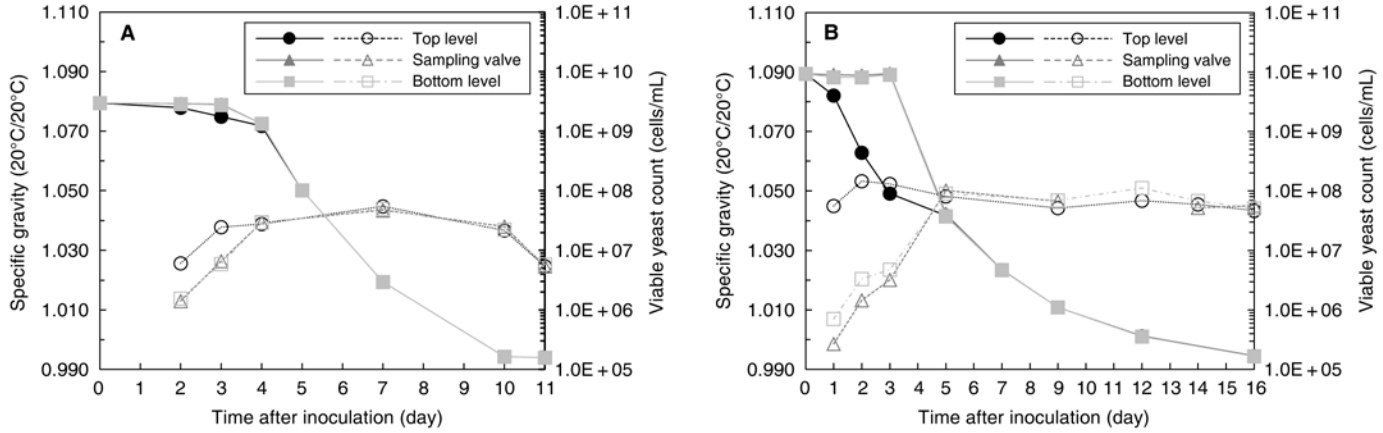

Figure 7 Specific gravity (solid lines) and viable yeast count (dotted lines) at different levels in an unstirred 2500-L tank (**A**) and an unstirred 7000-L tank (**B**) over the course of two Riesling fermentations.

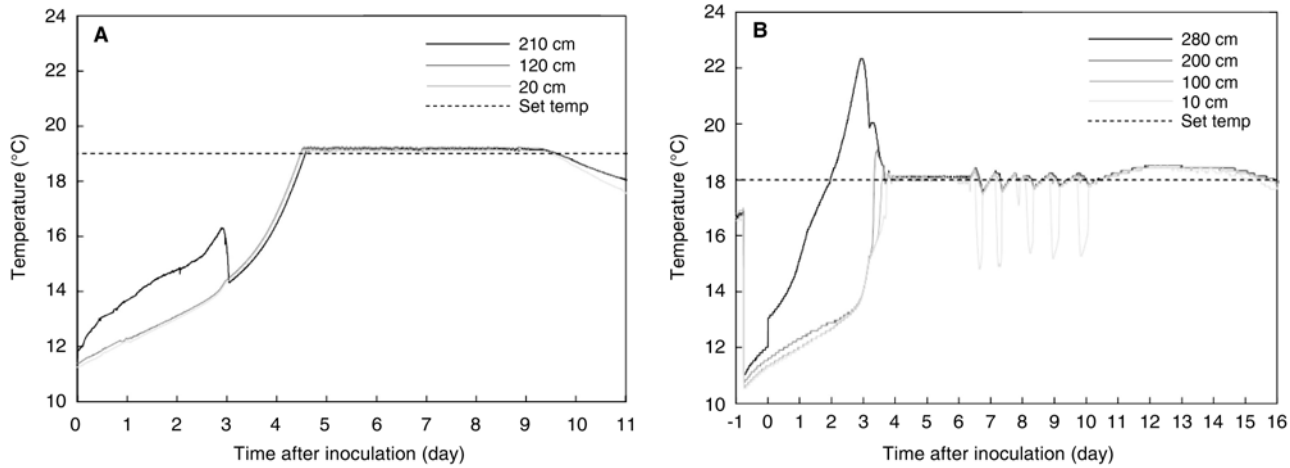

Figure 8 Wine temperature (solid lines) at different levels in an unstirred 2500-L tank (**A**) and an unstirred 7000-L tank (**B**) and set temperatures (dotted lines) over the course of two Riesling fermentations. The cellar temperatures were 17.2 ± 0.3°C and 15.7 ± 0.8°C.

As with the specific gravity, the difference in viable yeast count between the top and bottom levels was larger in the 7000-L tank (Figure 7B) than in the 2500-L tank (Figure 7A). The number of viable yeast cells at the top level was also higher in the 7000-L tank (Figure 7B) compared to the 2500-L tank (Figure 7A). The high number of yeast cells at the top level and the large difference between the top and lower levels were probably due to the larger volume of yeast suspension needed for the inoculation of the 7000-L tank: the yeast inoculation volume was 3.2 times larger in the 7000-L tank than in the 2500-L tank. Because the difference in tank diameter between the two tank sizes was disproportionately low (1.5-fold, see Figure 1 for tank dimensions), more yeast cells might have gathered in the upper section of the larger tank.

Moreover, the day of DAP addition may impact the fermentation progress at the top of the tank, thereby fostering larger differences between the top and bottom levels of unstirred tanks. DAP is known to accelerate the fermentation rate (Butzke 2010). DAP was added into the 7000-L tank on day 2 (Table 1). The early DAP addition at the top of the tank presumably intensified yeast proliferation and accelerated fermentation at the top. In the 2500-L tank, DAP was added on day 4, after the initial phase (Table 1). At this time, conditions were homogeneous due to agitation by CO_2.

In addition to the time of DAP addition and the yeast inoculation volume, the tank height might have been a factor in the inhomogeneities found in the unstirred tanks. Also, the yeast strain and the specific gravity of the grape juice may influence the sedimentation velocity of yeast cells. A final factor could be the gentleness with which the winemaker pours the yeast suspension into the top of the tank: the momentum of the yeast suspension hitting the wine surface can determine the initial penetration depth of the yeast into the upper levels of the grape juice.

The wine temperature during the initial phase was higher at the top level of the tanks, indicating ongoing fermentation (Figure 8A and 8B). The temperature increased strongly at the top level of the 7000-L tank (Figure 8B), whereas only a moderate increase was observed at the top level of the 2500-L tank (Figure 8A). This suggests a higher fermentation rate at the top level of the 7000-L tank compared to the 2500-L tank, a finding that supports the results of the specific gravity measurements (see Figure 7A and 7B). The higher fermentation rate at the top level of the 7000-L tank might be explained by this tank's higher viable yeast count compared to the 2500-L tank (see Figure 7A and 7B). Moreover, a multiplier effect is suggested: the high fermentation rate at the top level of the 7000-L tank further increased the temperature, which, in turn, further increased the fermentation rate. The maximum temperature difference between the top and bottom levels was 2.2°C in the 2500-L tank and 8.6°C in the unstirred 7000-L tank (Table 4).

However, only the temperature sensor at the top level in the industrial-scale tanks showed elevated wine temperature during the initial phase. All investigated levels below the top were lower in temperature and showed no temperature differences. This observation indicates that fermenting yeast cells were located in the top tank section, between 0 and 80 cm below the wine's surface, during the initial phase. This finding is in contrast to the pilot-scale fermentations, in which a layer-by-layer increase of temperature from the top to the bottom of the tank indicated a delayed fermentation start over the entire tank height. In the industrial-scale tanks, the similar temperatures in the lower levels suggest a simultaneous fermentation start in these sections. This might be explained by differences in the flow conditions between industrial-scale and pilot-scale tanks. Industrial tanks, which have a larger diameter than the pilot-scale tanks, may allow larger torus vortices (Meironke 2014). These vortices may accelerate the mixing of yeast cells in a downward direction. However, data on fluid dynamics in wine fermentation tanks are rare, so we can only hypothesize that the small diameter of the pilot-scale tanks impeded the mixing of different layers.

The maximum differences in temperature between the top and bottom levels were less pronounced in the unstirred pilot-scale fermentations (Table 2) compared to the unstirred 7000-L industrial-scale fermentations (Table 4). This might be mainly due to the high surface-to-volume ratio of the tube-shaped pilot-scale tanks. As a consequence, the cellar temperature influences the wine temperature more in pilot-scale tanks than in industrial-scale tanks.

After the initial phase, uniform conditions were observed at all tank levels in the unstirred industrial-scale tanks (Figure 8). The specific gravity, yeast cells, and fermentation byproducts were homogenously distributed, and the temperature was equal at all tank levels. These observations are in accord with previous reports regarding the vigorous phase of fermentation (Guymon and Crowell 1977, Vlassides and Block 2000, Porret et al. 2007). However, temperature loggers at the bottom level of the 7000-L tanks (10 cm above tank bottom) showed recurring temperature deviations of up to 3°C between days 6 and 11 (Figure 8B). This effect was observed in both unstirred (Figure 8B) and stirred tanks whenever the cooling system was active. The observed temperature deviations indicate poor mixing in the bottom section of the 7000-L tanks. It might be possible that the construction of the tank bottom does not allow optimal flow conditions at the bottom, even during the vigorous phase of fermentation.

Regarding process control, the sampling valve and the temperature sensors of the tanks can be viewed as representative of the entire tank during the vigorous phase of fermentation, but not during the initial phase.

Conclusions

The practice of yeast inoculation from the top of the fermentation tank without stirring had a larger-than-expected effect on fermentation kinetics in low turbidity juice. Fermentation started earlier at the top of the tank due to high viable yeast numbers, whereas the onset of fermentation was delayed by up to seven days at the tank's bottom. Slow sedimentation of yeast cells and poor mixing in the downward direction caused inhomogeneities that accounted for differences in specific gravity of up to 0.040 between top and bottom of the

tank. Thus, the fermentation progress recorded by the winemaker at the sampling valve presents a distorted picture of the actual process. Temperature differences of up to 8.6°C were not reflected by the temperature sensor of the tank, which must be considered disadvantageous for process control. Inhomogeneities negatively affected fermentation kinetics. Homogeneous starting conditions were created when yeast was stirred into the juice, and this reduced the final pyruvate concentration and overall fermentation duration. Low pyruvate concentrations decrease the SO_2 demand and may reduce diacetyl formation. Tank volume and height-to-diameter ratio of tanks were found to influence the yeast distribution and, therefore, the fermentation progress at different tank levels.

We suggest stirring the rehydrated yeast into the juice after inoculation from the top of the tank in order to ensure a uniform yeast distribution. This procedure prevents inhomogeneities and thus shortens the fermentation duration, reduces stress for yeasts, and increases process control. Although this study focused on the prefermentation and vigorous fermentation phases, future research concentrating on inhomogeneities during the postfermentation phase might be of interest.

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