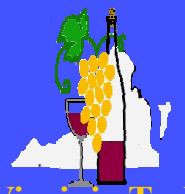
Enology-Grape Chemistry Group



Virginia Tech

Practical Monitoring and Management of Brettanomyces

Bruce Zoecklein
Head, Enology—Grape Chemistry Group
Virginia Tech

Information available at www.vtwines.info.
Click Enology Notes Index

This presentation is one originally presented by

Bruce Zoecklein
Head, Enology—Grape Chemistry Group
Virginia Tech

And

Lisa Van de Water
Pacific Rim Enological Services

Presentation Outline

- Overview of Brett research from my lab
- Practical conclusions for today's winemaking
- Review of others research
- HACCP-like Plans
- Review of practical Brett management issues

The faster the scientific advances, the greater the risk of widening the gap between what we know and what we do.

-Emile Peynand, 1984

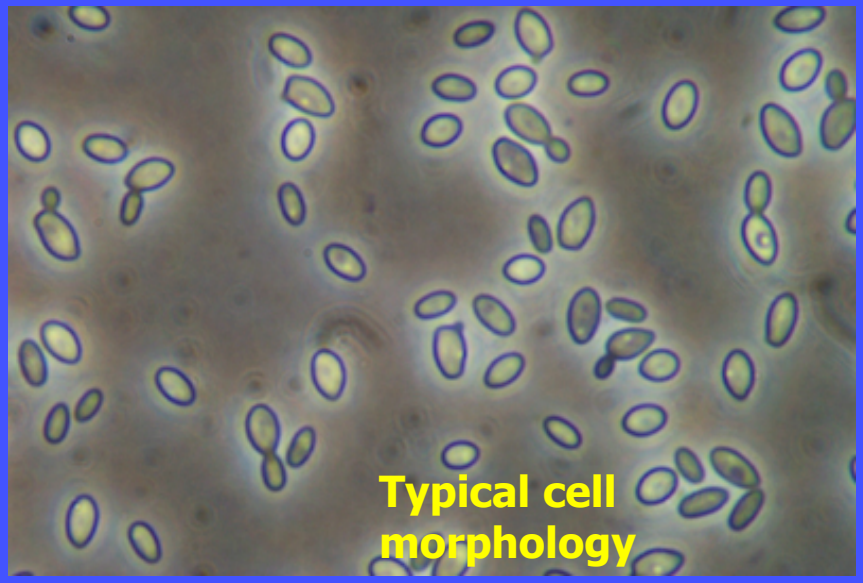
Misconceptions about Brett

- Some Brett is in all red wines
- Brett is not found in white wines
- Brett comes into wineries in new barrels
- Brett can only occur in barreled wines
- Brett is found only in dirty cellars
- All Brett is the same

Misconceptions about Brett

- Brett only develops in in dry wines
- Brett won't grow over 13.5% alcohol
- Controlling oxygen can control Brett
- Brett growth always results in high VA
- Brett is a characteristic of 'French style' wines

The many faces of *Dekkera*/*Brettanomyces*…



Source: Lisa Van de Water

Brett Descriptors

- Positive
  - Complex
  - Mature
  - Spicy
- Negative (partial list)
  - Animals
    - Sweaty horse/saddle
    - Wet dog
    - Manure
    - Barnyard
    - Mousy aftertaste
  - Plastic
    - Bandaids
    - Burnt plastic
  - Other
    - Burnt beans
    - Rancid
    - Metallic

Brettanomyces bruxellensis

- What is relationship between descriptors, cell growth and population densities?
- What are the specific chemical compounds responsible for these descriptors?
- What concentrations and ratios are need give a certain set of descriptors?
- What is the matrix/cultivar effect?
- What is the impact of strain variation?

Population dynamics and effects of *Brettanomyces bruxellensis* strains on Pinot noir wines

Ken Fugelsang
Department of Vitculture and Enology
California State University, Fresno

Bruce Zoecklein
Enology-Grape Chemistry Group
Virginia Tech, Blacksburg

For overview see www.vtwines.info
***Enology Notes* #92, Published in Am. J. Enol. Vitic. 54:294-300**

Brettanomyces bruxellensis: Comparison of Growth Profiles and Metabolites among Ten Strains in Pinot Noir Wine

- ***Question:*** ***Can differences in winemaker's experiences with*** **Brettanomyces** ***be attributed to strain, populations and/or metabolite differences?***

Experimental Design:

Ten genetically-characterized strains of *B. bruxellensis*

- **Pinot noir: 30 mg/L sulfur dioxide at crush. Ferment to dryness, press, clarify at 5°C (6 weeks).**
- **Rack to sterile containers, DMDC @ 700 mg/L.**
- **Bottled**
- **Initial inoculum: 50 CFU/mL (10 strains x 4 replications) + controls.**

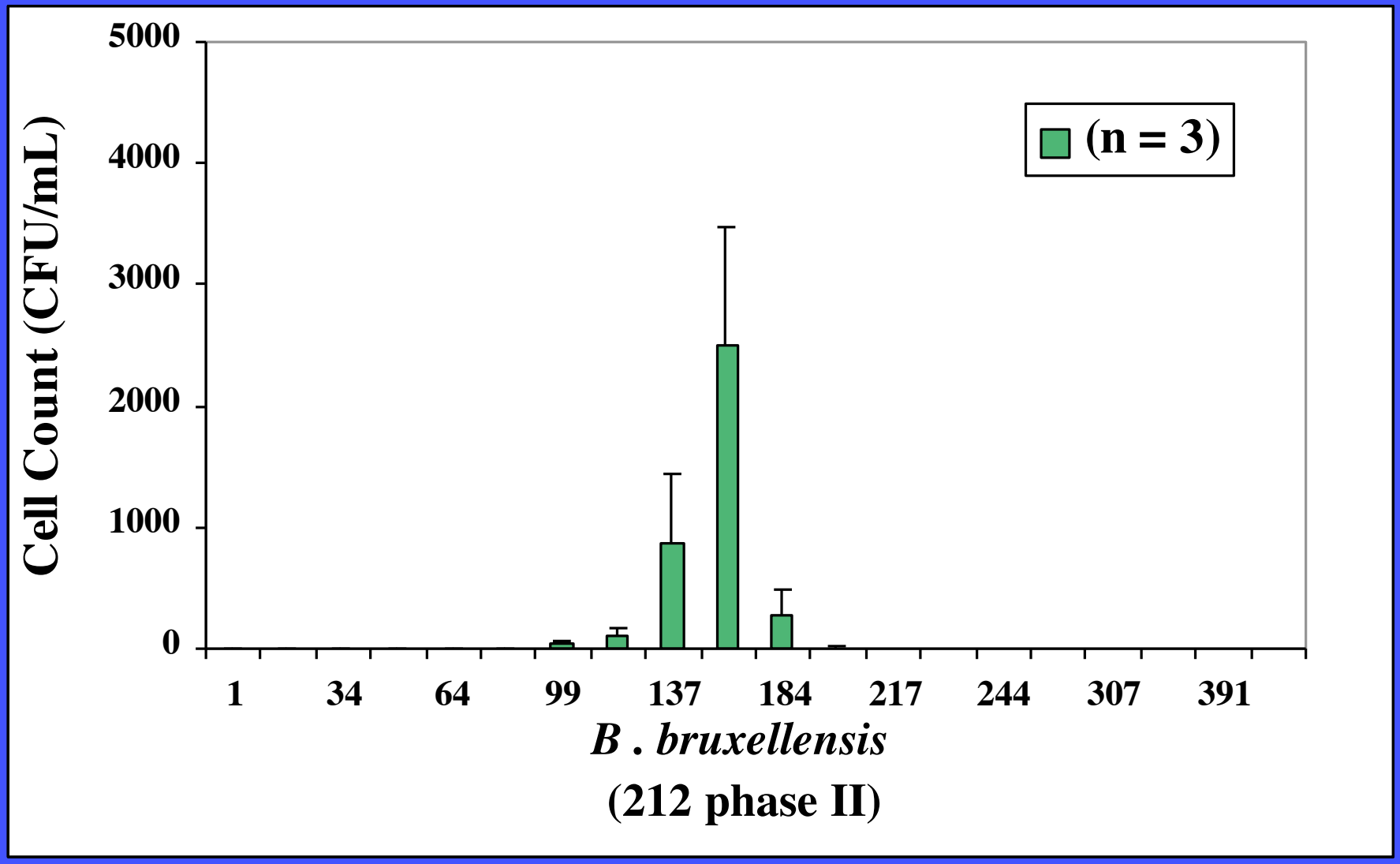
Sampling

Weekly plating for growth and chemical analysis for up to 712 days or until population declined to <30 CFU/mL.

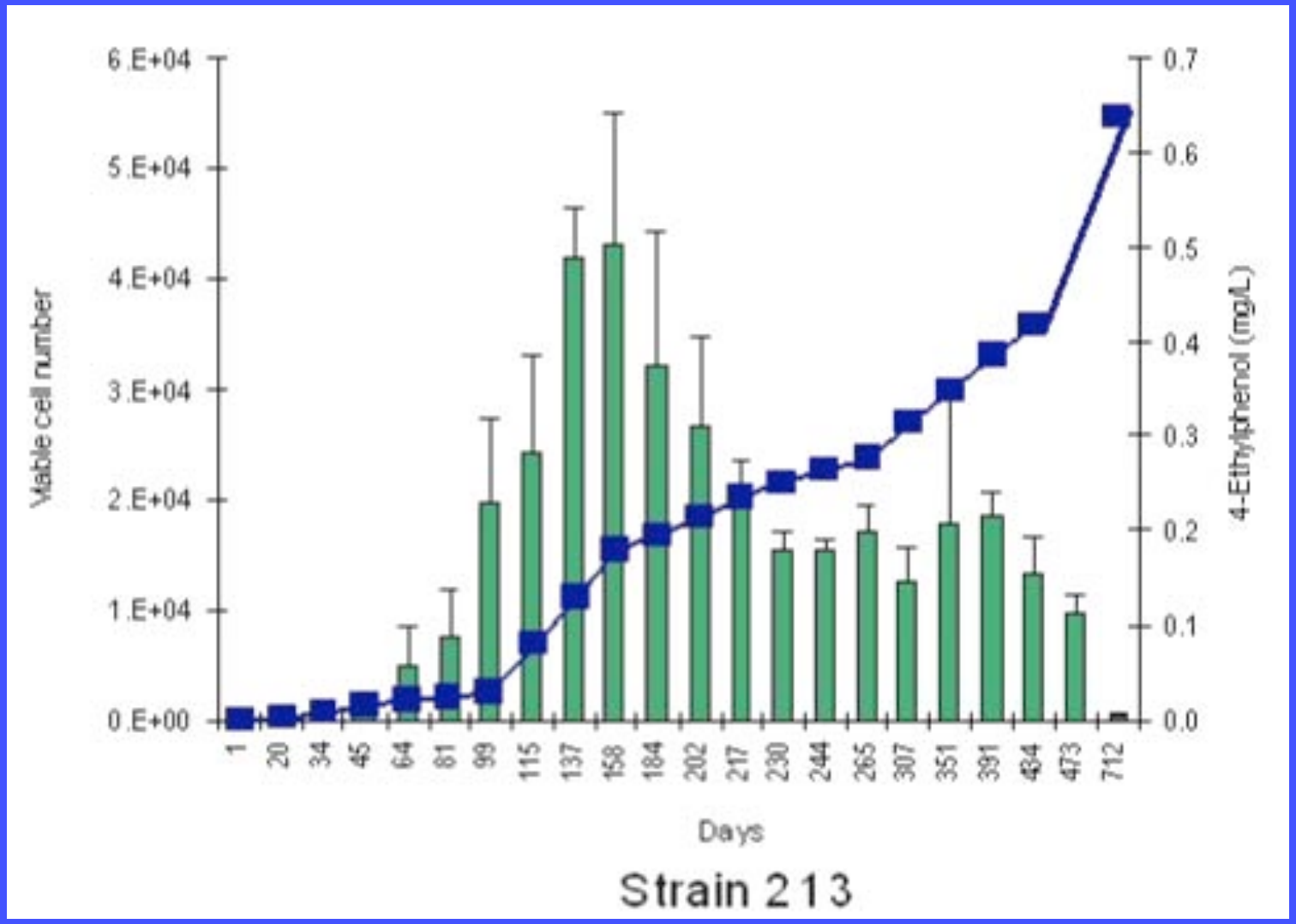
Analyte quantification by HE-SPME, GC/MS:

- 4-Ethylphenol (4-EP)
- 4-Ethylguaiacol (4-EG)
- 2-phenylethanol
- Guaiacol
- Isovaleric acid
- Ethyldecanoate
- trans-2-Nonenal
- Isoamyl alcohol
- Ethyl-2-methylbutyrate

Results


5000
4000
3000
2000
1000
0
Cell Count (CFU/mL)
(n = 3)
1
34
64
99
137
184
217
244
307
391
B . bruxellensis
(212 phase II)

Results (cont.)



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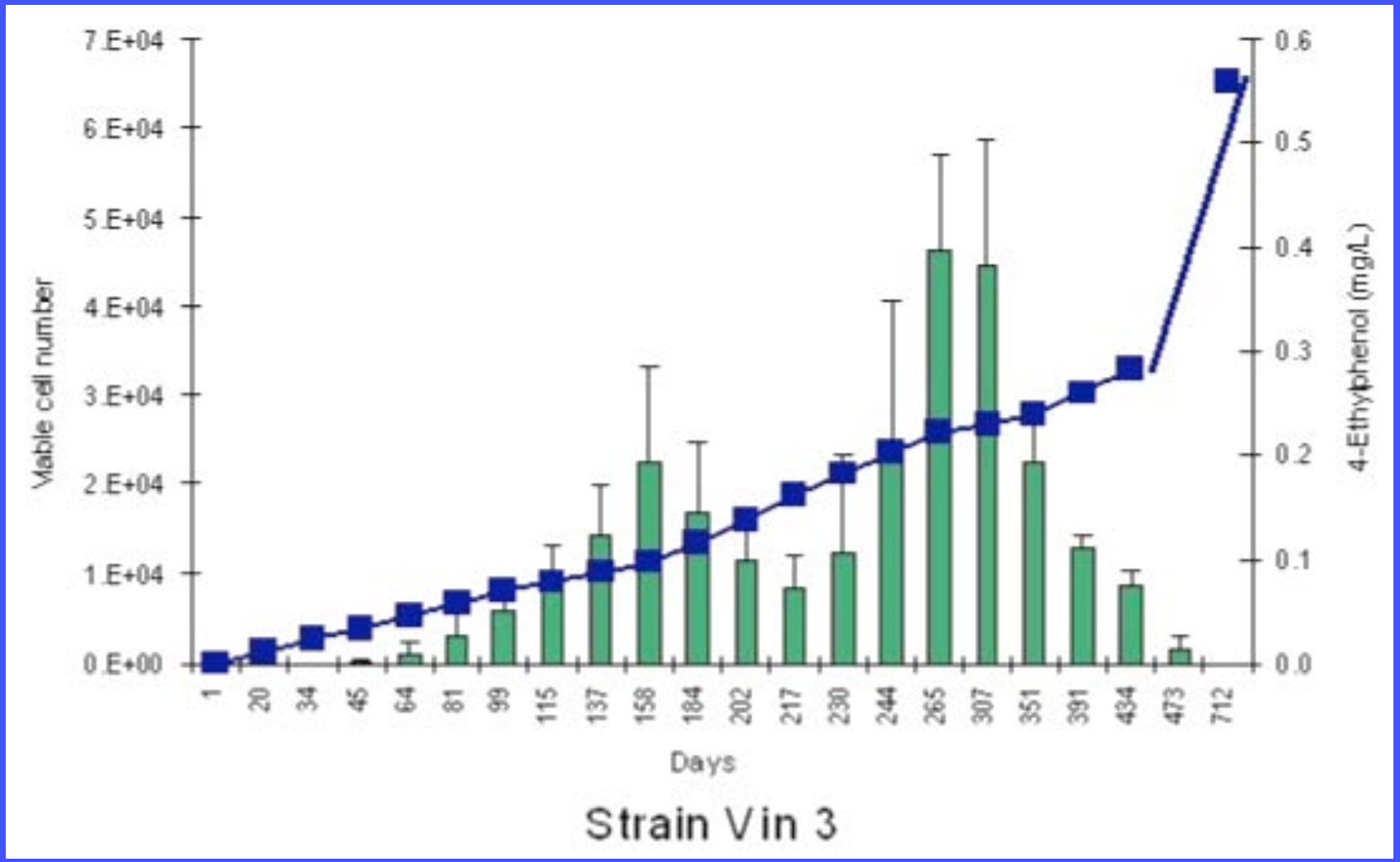

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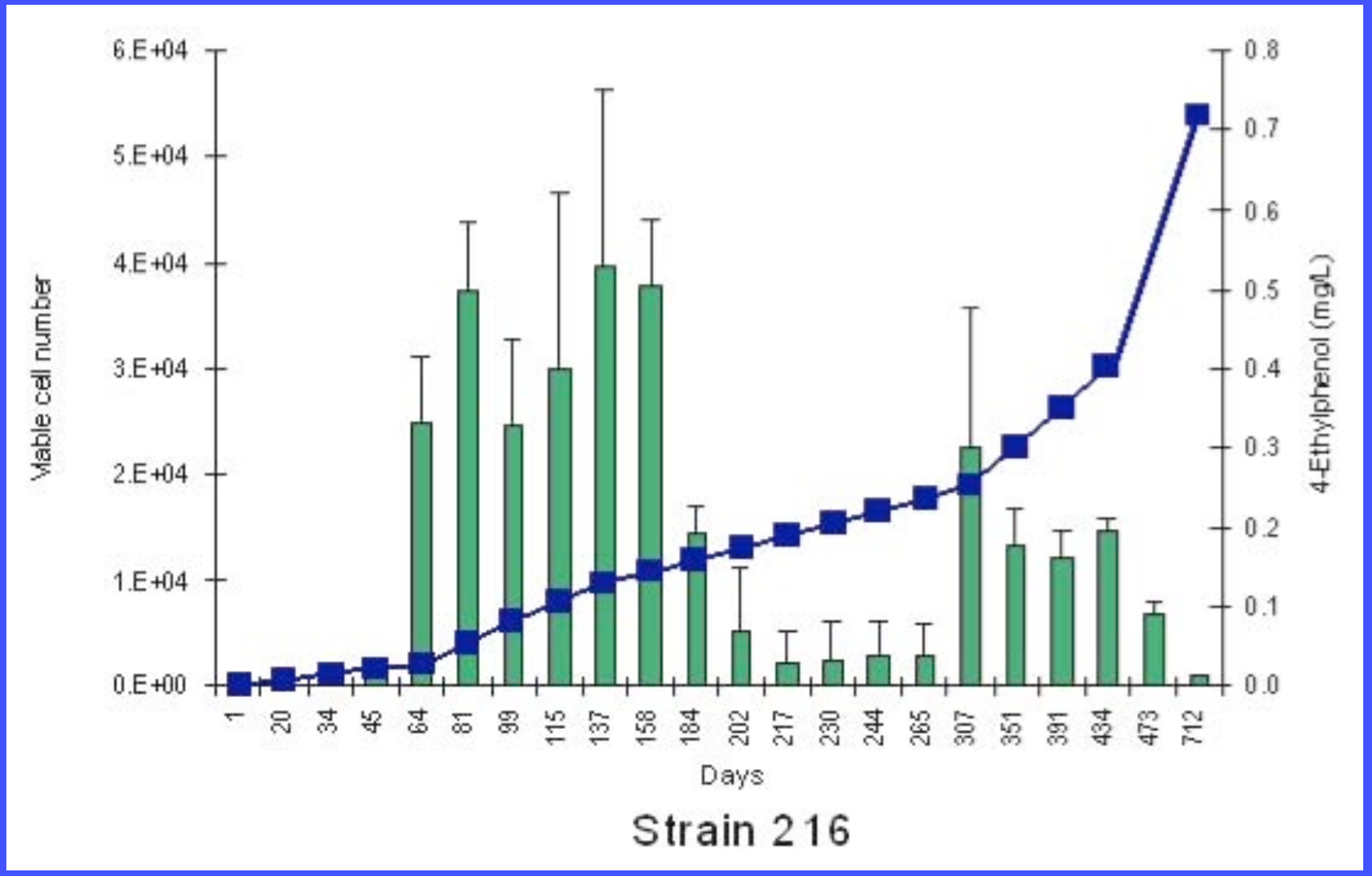
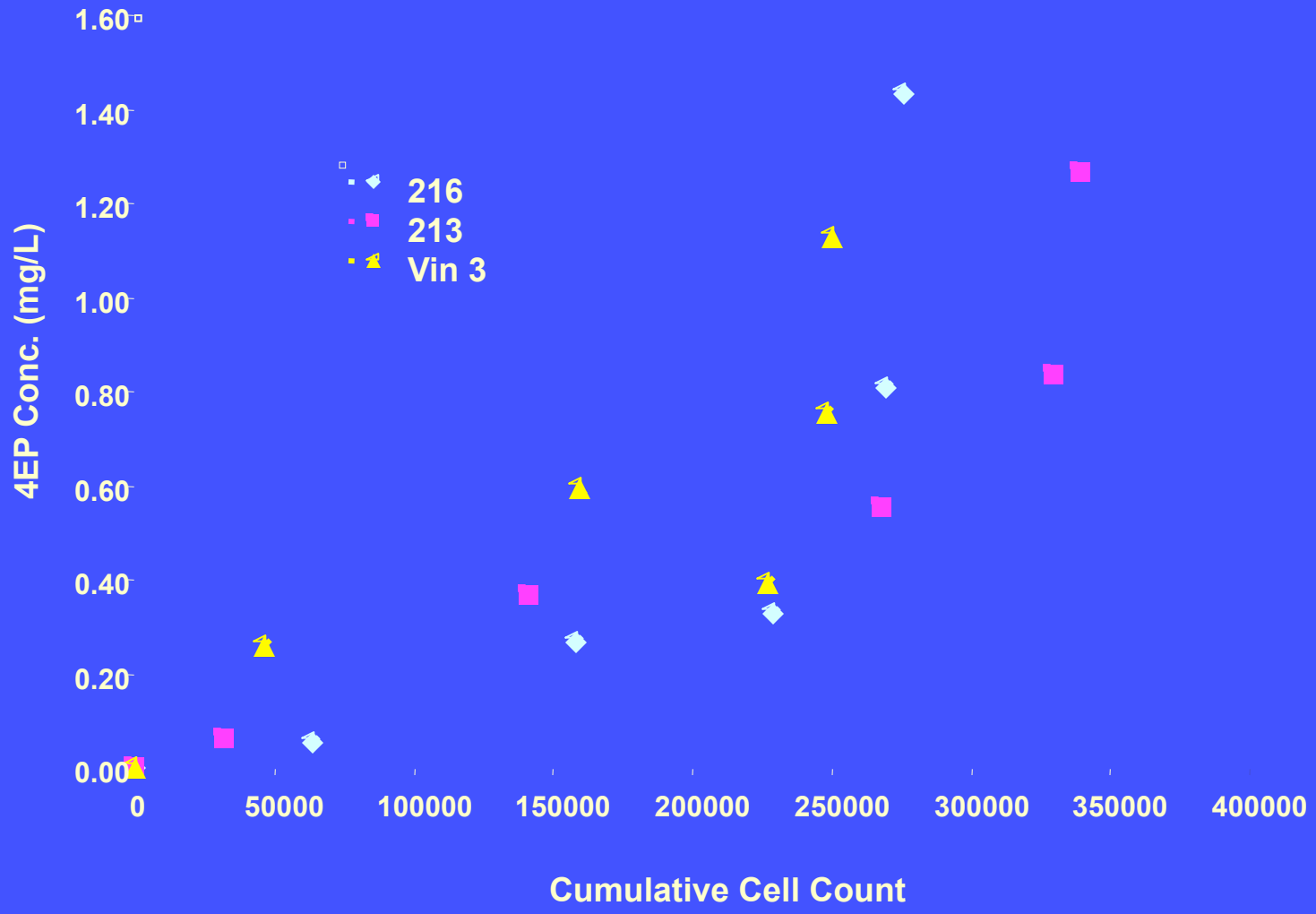


Viable But Not Culturable (VNC)

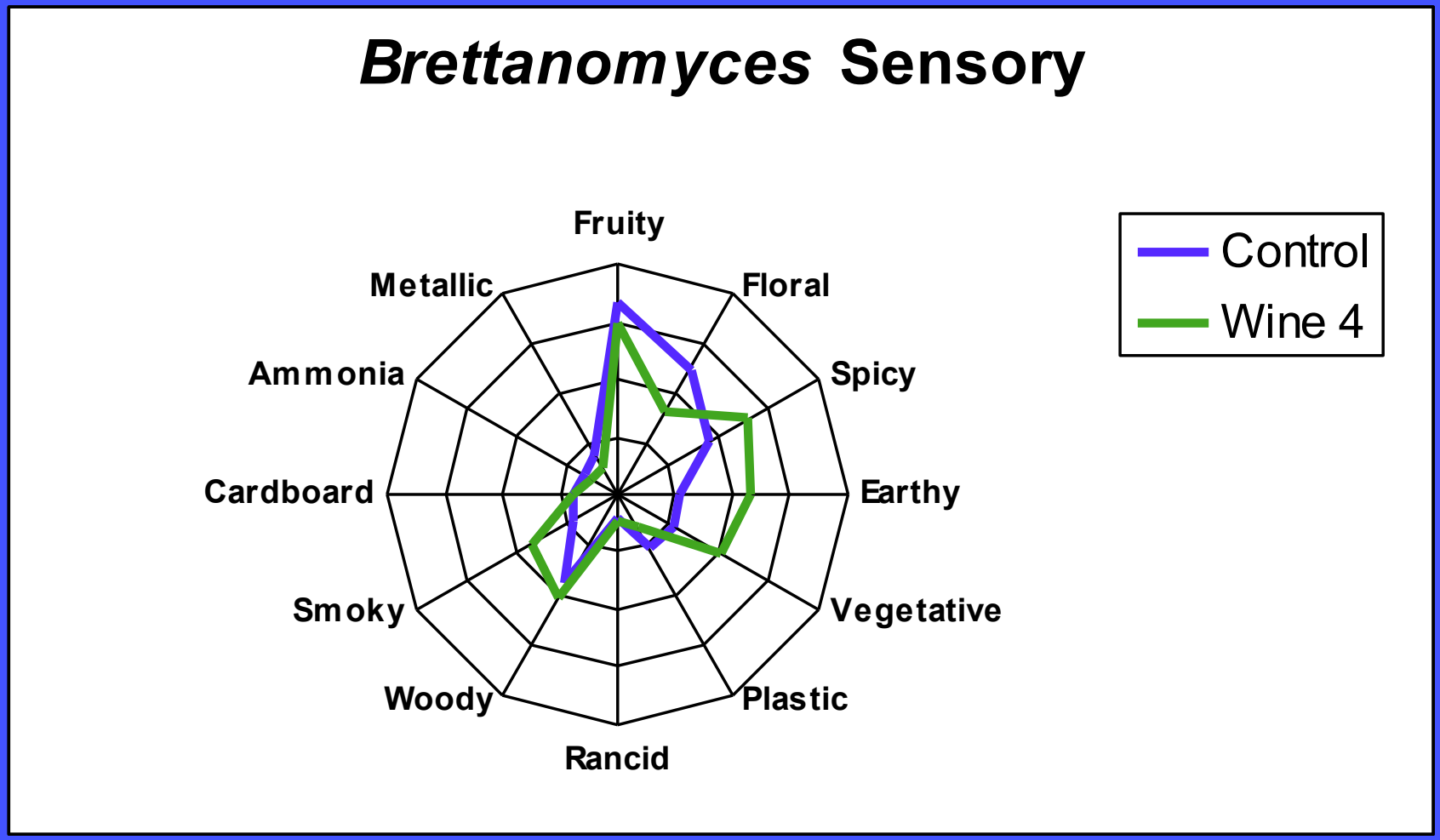
- Sublethally injured
  - Injury may be from any stress
    - Ethanol, pH, temperature, sulfite
  - May recover and still ferment and grow
- VNC
  - May still produce enzymes and metabolites
  - Associated with bacteria
  - Not studied extensively in yeasts

Brettanomyces

4-EP vs Cum. Cell Count, averaged



Pinot noir Sensory Evaluation



Conclusions

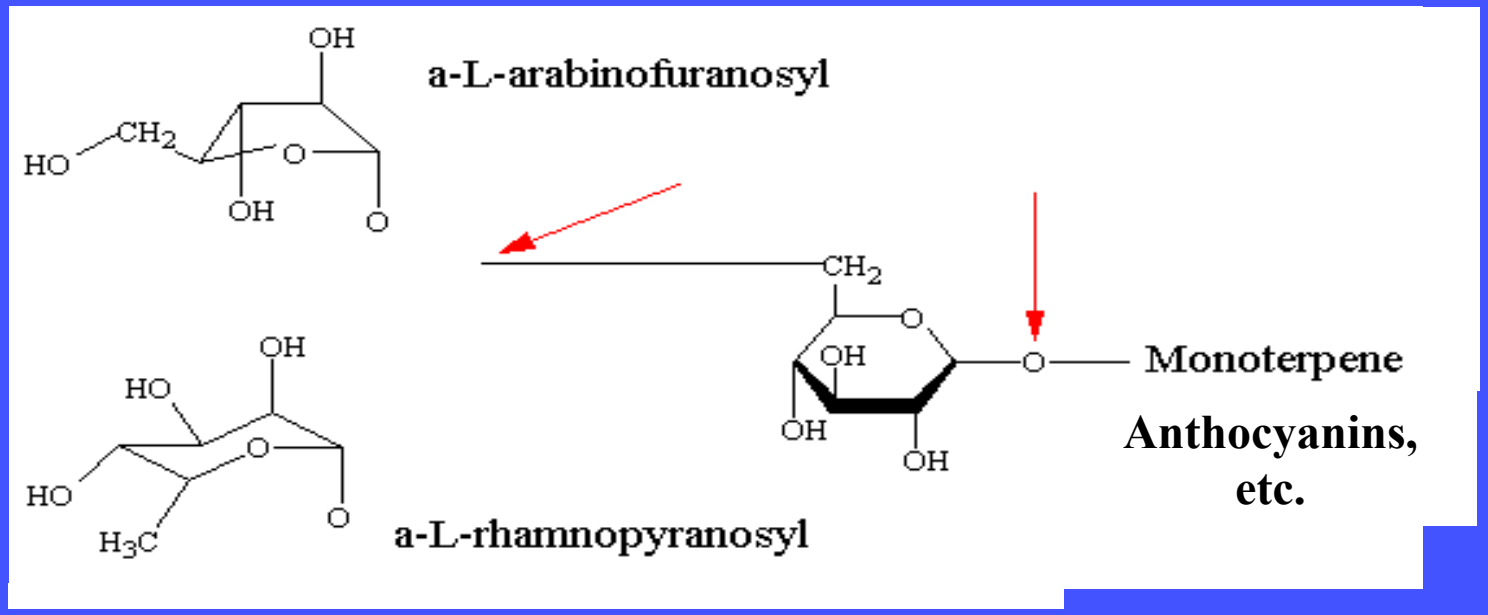
- Significant strain differences in length of growth cycle and peak population densities
- Blooms explained by VNC
- Large range of 4-ethylphenol (4-EP)
- Large range of 4-ethylguaicol (4-EG)
- 4-EP and 4-EG correlated
- 4-EP and 4-EG not correlated to isovaleric acid (IVA)

Conclusions

- With the exception of one strain, most 4-EP was produced after the population reached maximum cell density.
- The correlation between 4-EP and viable cell density was not as strong as the correlation with cumulative cell density.
- There were significant sensory differences among strains.
- 4-EP correlated to low glucose/fructose.

Important Enzymes: Esterases, Glucosidases

- Glycosidases
- Glucosidases



Glycosidase Activity in *Brettanomyces bruxellensis* strains

H.M. McMahon and B.W. Zoecklein.
***J. Ind. Micro. Biotech.* 23:198-203.**

A.K. Mansfield and B.W. Zoecklein.
***Am. J. Enol. Vitic.* 53:303-307.**

B. bruxellensis strain	Whole Cell	Permeabilized	Supernatant
211	27 c	142 e	11 bcd
212	5 d	341 a	9 bcd
213	34 c	105 f	14 bcd
214	19 c	110 f	6 cd
215	< LOD	74 g	11 bcd
216	59 b	321 b	24 a
Brux	26 c	182 d	11 bcd
Souche 'Ave'	14 c	138 e	7 cd
Souche 'O'	< LOD	< LOD	4 d
Souche 'M'	82 a	179 d	9 bcd
Vin 1	32 c	14 g	< LOD
Vin 3	22 c	232 c	9 bcd
Vin 4	65 b	25 g	4 d
Vin 5	4 d	21 g	11 bc

Conclusions

- Large variation among strains in total enzyme activity.
- Eight strains of *Brettanomyces bruxellensis* had high *beta*-glucosidase activity (670-2,650 nM/mL/g dry cells).
- Large variation in supernatant and permeabilized activity.
- Glycosidase activity of Brett is likely how the organism can survive in oak and perhaps some wines for very long periods

Results of Physiological Tests

L. Joseph, T. Henick-Kling, L. Conterno

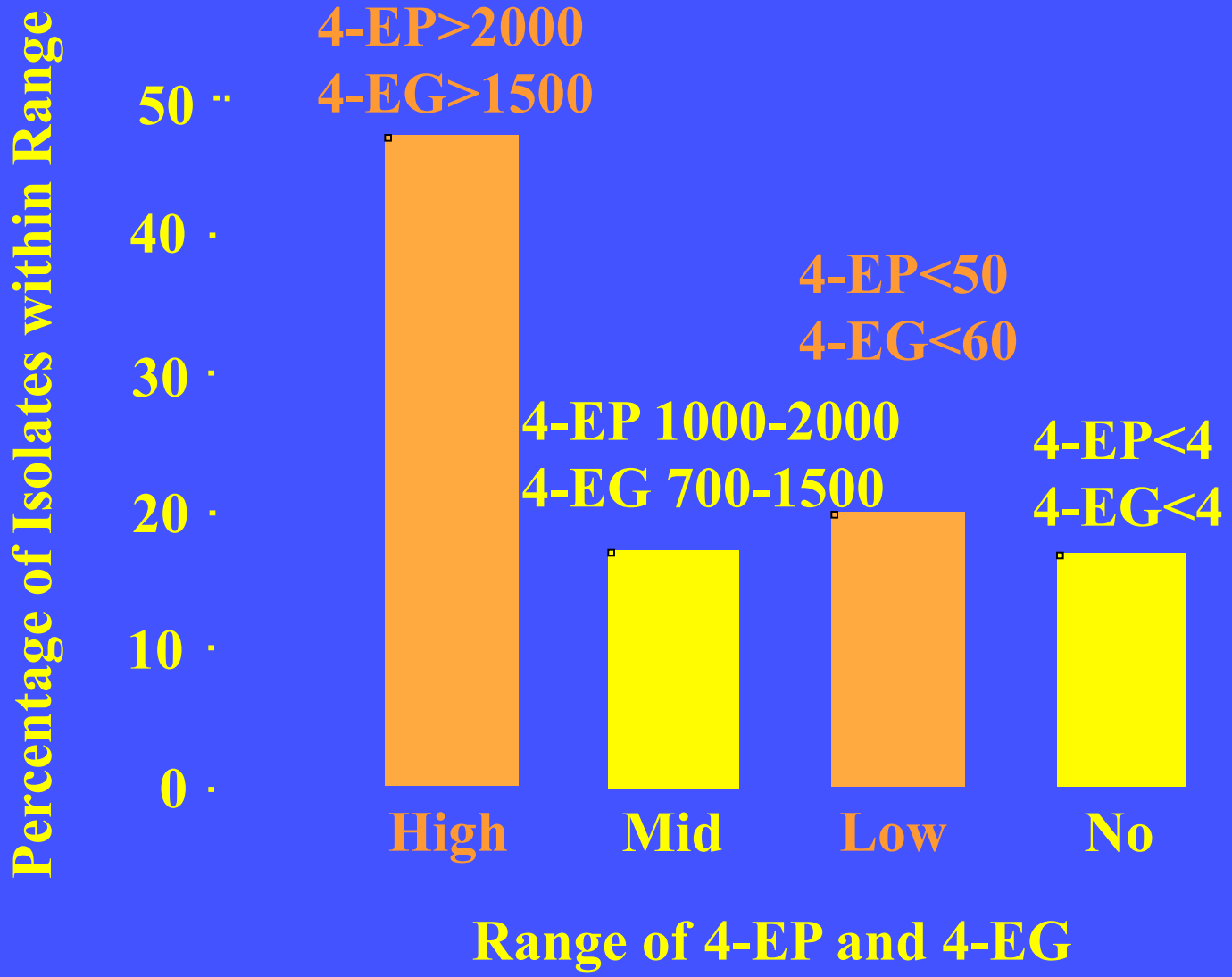
Regional differences in metabolism

- 75% of European strains used malic acid, 12% CA strains did
- All CA strains used nitrate, < 30% of European strains did
- 63% of European strains used ethanol, 18% CA strains did
- Most CA strains grew at 37 C, no European strains did

Physical Characteristics

- All isolates tolerant to 10% ethanol or higher.
- 33 isolates grew well at pH 2.
- More than 30% of isolates grew at 10^{o} C.
- More than 35% of isolates grew at 37^{o} C.
- 3 isolates (about 10%) grew at both temperature extremes.
- Almost 50% showed tolerance to 30 mg/L or greater free SO_2 at pH 3.4.

4-EP and 4-EG Production



Climate Impact on *Brett* Metabolites
Henschke (2004)

- 4-EP / 4-EG decrease in cool regions
- Malvidin-3-*p*-coumaryl glucoside may be precursor to 4-EP
- Malvidin-3-*p*-coumaryl glucoside in lower concentration in cool region, shaded fruit

Brett Growth

Physical effects

- **Usually grows slowly, over many months**
- **Can grow within weeks if conditions are favorable**
- **Grows in the wine, almost never as a surface film**
- **Growth is stimulated by oxygen, but very little is required**
- **Slight CO_2 gas**
- **Sediment in bottle**

Monitoring Brett

Methods:

- Metabolite analysis
- Sensory analysis
- Culturing, plate count
- Brett Sniff
- Antibody methods
- Genetic markers: PCR, Scorpions

The key to monitoring and management is to have a good HACCP-like plan in place

Minimize Substrates for Growth

Measure Fermentable N (ammonia and alpha amino acids)

(Formol titration, www.vtwines.info or *Am. J. Enol. Vitic.* 53:325-329.)

Excess fermentable N:

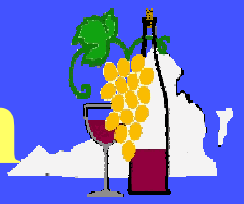
- Lowers the production of esters
- Increases the production of aldehydes
- Increases the likely hood of volatile sulfur compound production
- Increases the fermentation rate and lose of volatiles
- Increases substrates for Brett

All Brett strains require biotin and thiamin

All can use Arginine as an N source

Excess N including DAP may serve as ‘food’ for Brett

Elements of Sensory Evaluation



- Fully understand the objective (s)
- Evaluate representative samples
- Evaluate under proper conditions (temperature, TNSS, environment)
- Use trained evaluators with reference standards
- Minimize prejudice and bias
- Employ desirable and consistent tasting format
- Interpret results appropriately

Brett Aromas

Sensory threshold levels depend on the matrix

- 4 EP 120-1200 ng/L
  Bandaids, Plastic
- 4 EG 70-150 ng/L
  Smokey, Spice, Burnt Beans, Medicinal
- Isovaleric Acid +/- 1200 ng/L
  Rancid, vomit, barnyard
- Combination of these and other metabolites
  Provide the typical sweety horse, leather, horse blanket-type odors

Brettanomyces Sensory Detection

- Train to recognize danger signals using standards
  - When sensory effects are noticeable, it may be too late

- Matrix effect: cultivar, phenol composition Q and Q, metabolites:

  Tempranillo 4-EP 125, Cabernet Sauvignon 420 ug/L

- Synergistic effect on detection level:
  4-EP + 4-EG=426 ug/L
  4-EP alone=620 ug/L

- High 4-EP can mean High Brett character
- Low 4-EP can mean High Bret Character

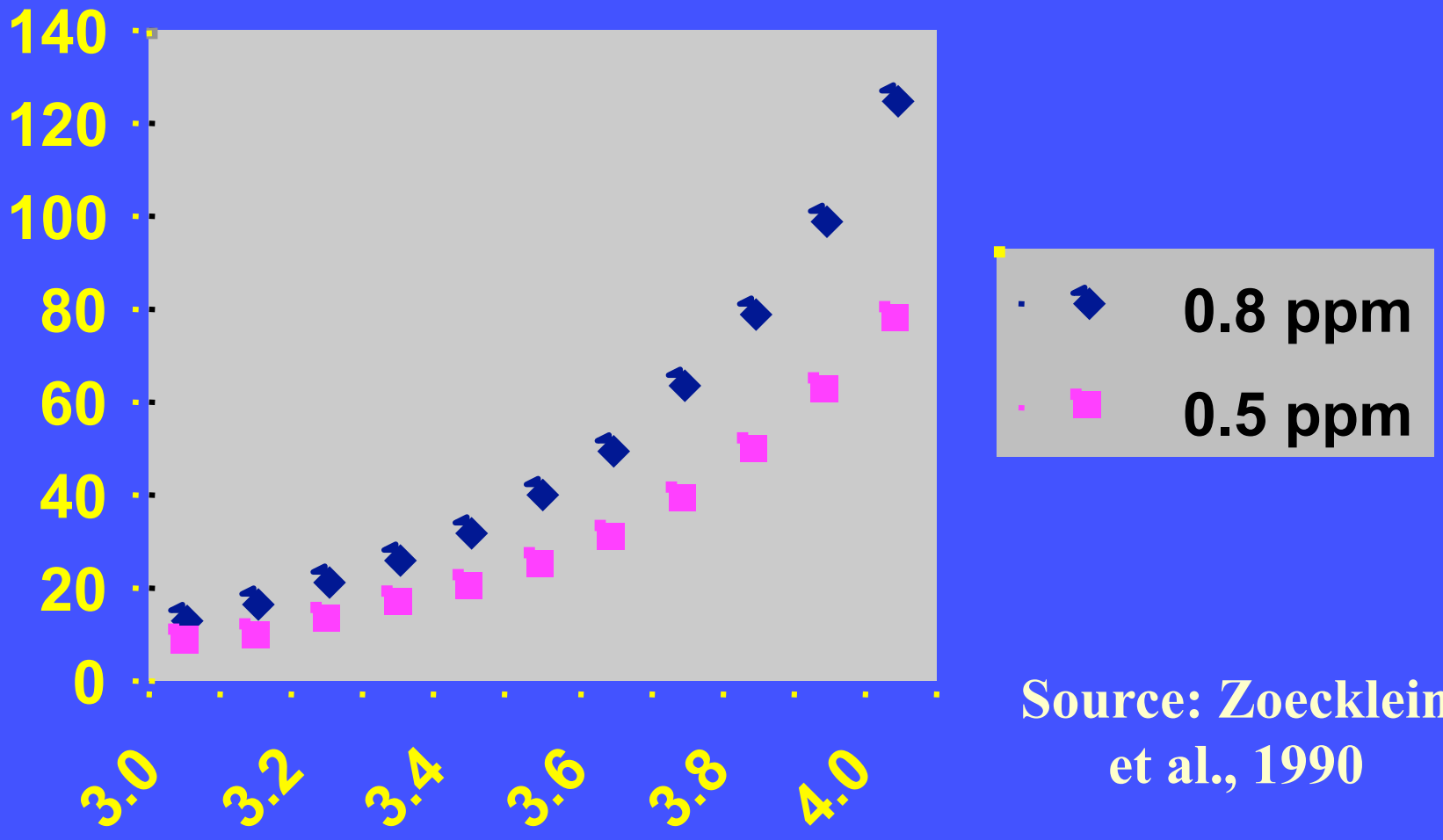
Wine Chemistry and Brett

- Alcohol synergistic but will not control
- Glucose and Fructose: 0.275 g/L = 1000 ug/L 4-EP
- VA concentration not correlated with 4-EP
- pH effects molecular FSO2

  Biofilm formation
- Sulfur dioxide and pH synergistic

  Fewer additions but larger concentration

Free SO_2 Needed to Achieve 0.5 and 0.8 ppm Molecular SO_2, at Different pHs



Source: Zoecklein et al., 1990

BBL Maturation

- Old wood vs. new wood
  cellobiose
  0.275 g/L can produce 1000 ug/L 4-EP
- Sampling
  representative
  avoid cross contamination
  use disposable plastic pipetts
  top with ‘clean’ wine (DMDC-**Velcorin** treated or filtered)

Brett and Sanitation

- Monitoring is key
- Understand differences between cleaning and sanitation
- Sanitation methods

Effect of Barrique Sanitation Procedures - Manuel Malfeito-Ferreira, 2004

- Barrel sanitation experiment
  - Cold rinse, then hot water rinse 3x 70 C
  - Same as above plus SO2 1 month (200 ppm pH3)
  - Cold rinse, fill with 90 C water 15 min
  - Cold rinse, 70 C rinse, steam low pressure 10 min
    - Most effective treatment

- *Brett / Dekkera* was found 8 mm deep in staves.

Barrels cannot be "sterilized" with SO_2, rinsing, or ozone.

Isolate *Brett*+ barrels.

Ozone Treatment

- High-pressure water wash barrel
  - Thorough blast with sharp stream of hot water
  - Rinse for 2-3 minutes
  - Must remove all organics
  - Cool down completely
- Treat with ozonated water
  - Filter and deionize water before ozonating
  - At least 2-2.5 mg/L ozone in barrel, 0.1 mg/L out
  - Time x Concentration

Ozone Summary

- Strong oxidizing agent
- No chemical residue
- Half-life at ambient conditions 10-20 minutes
- Degrades microbial bio-films
- Degrades natural rubber
- Is a surface active agent-does not penetrate

Brett and Biofilms

- Liquid / solid interface
- 17 / 35 strains form biofilms (Joseph, 2004)
- pH effect
- Impact of cleaning compounds on biofilms

Wine Bottling and Brett

- Sanitation
- Monitoring
- Filtration and filtration monitoring
- DMDC can be effective
- Synergistic with pH, sulfur dioxide, and alcohol
- Oxygen pick up

Monitoring *Brett*

- Have a HACCP-like plan (www.vtwine.info)
- Isolate contaminated barrels
- Sample barrels with disposable plastic pipets
- Top with *Brett*-free wine (filtered, pasteurized and/or Velcorin-DMDC)
- Keep barrels topped-up or not opened
- Monitor carefully before bottling

Brettanomyces Detection

- Direct Microscopic Examination
  - Difficult when < 1000 cells/ml
  - Requires skill in identifying cells
- Culturing
  - Sampling method is very important
  - Detects only microbes that are present and alive
  - Disadvantages:
    - Must select and prepare media properly
    - False negatives (VNC)
    - Takes time for growth (3-7+ days)
    - Requires skill in identifying colonies

HACCP Summary

- Define the production process, quality/style indicators, and their recommended values.
- Identify critical control points in the process where specific chemical methods can monitor quality indicators.
- Establish and carry out analysis methods that will give measures of quality/style indicators at each control point.
- Compare measured values with recommended values.
- Decide on action to modify any quality deficiencies.
- Carry out that action.
- Assess the result of that action by further analysis.