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Extraction of Indirectly Captured Information for Use in a Comparison of Offline pH Measurement Technologies

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Highlights:

1. Measurements of pH were observed to vary by measurement technology.
2. A method for extracting indirectly captured information is demonstrated.
3. Causes of discrepancies are identified from indirectly and directly captured data.

Abstract

Understanding the causes of discrepancies in pH readings of a sample can allow more robust pH control strategies to be implemented. It was found that 59.4% of differences between two offline pH measurement technologies for an historical dataset lay outside an expected instrument error range of ± 0.02 pH. A new variable, $Osmo_{Res}$, was created using multiple linear regression (MLR) to extract information indirectly captured in the recorded measurements for osmolality. Principal component analysis and time series analysis were used to validate the expansion of the historical dataset with the new variable $Osmo_{Res}$. MLR was used to identify variables strongly correlated (p<0.05) with differences in pH readings by the two offline pH measurement technologies. These included concentrations of specific chemicals (e.g. glucose) and $Osmo_{Res,}$ indicating culture medium and bolus feed additions as possible causes of discrepancies between the offline pH measurement technologies. Temperature was also identified as statistically significant. It is suggested that this was a result of differences in pH-temperature compensations employed by the pH measurement technologies. In summary, a method for extracting indirectly captured information has been demonstrated, and it has been shown that competing pH measurement technologies were not necessarily interchangeable at the desired level of control (± 0.02 pH).

Keywords: variable extraction; ; ; , indirect data capture, technology comparison, historic dataset re-use

1 Introduction

In the biologics industry, mammalian (Chusainow et al., 2009), bacterial (Shiloach and Fass, 2005), and fungal cells (Durocher et al., 2002) are used to produce products such as antibodies, antibiotics, and vaccines. In 2014, these biotechnology products contributed USD 289 billion (Deloitte Touche Tohmatsu Limited, 2015) to the global pharmaceutical industry's USD 1 trillion revenue (Statista, 2016). Regardless of cell choice, understanding of the cell culture at the heart of a bioprocess is fundamental for ensuring production is safe, fit for purpose products and consistent. Reactor design (Varley and Birch, 1999), media formulation (Weuster-Botz, 2000; Zhang and Robinson, 2005), and processing strategies (Horvath et al., 2010; Senger and Karim, 2007) continue to be researched to increase cell culture knowledge. These efforts are increasingly supported by multivariate data analysis (MVDA).

MVDA techniques such as principal component analysis (PCA) and partial least squares or projection to latent structures (PLS) have aided scientists and engineers in reactor scale-up (Kirdar et al., 2007) and fault diagnosis (Gunther et al., 2007). One key use of MVDA is the retrospective interrogation of historic datasets from the routine monitoring of cell cultures through technologies including pH probes, osmometers, cell counters, and multi-metabolite bioanalysers.

1.1 pH Measurement

A fundamental controlled parameter in cell cultures is pH. It affects cell growth and metabolism (Schmid et al., 1990), production rates (Ozturk and Palsson, 1991), and product quality (Zanghi et al., 1999). Control of culture output could be improved by improved pH understanding and control (Trummer et al., 2006). To effectively apply a pH strategy, measurement technologies must give reliable and accurate readings.

Consistency between pH measurement technologies is frequently taken for granted. Between manufacturing sites and even within the same lab, individual scientists may use different technologies. If technology A is a Nova Bioprofile 400 with accuracy ±0.01% of the measured pH for a pH range 5.00 to 8.00 (Nova Biomedical, 2011) and technology B a Radiometer Analytical PHM220 with Metler-Toledo probe with accuracy ±0.01 for pH range 3.00 to 8.00 (Radiometer Analytical SAS, 2003), it would be assumed that the maximum expected difference due to pure instrument error is ± 0.02 pH units for a well-mixed sample. In this study, we found that for one cell culture robustness study, nearly 60% of differences in pH reading by two different offline technologies fell outside the allowable error band (Figure 1).

pH is neither constant nor directly proportional with respect to temperature (Barron et al., 2006; Mettler-Toledo AG, 2015; Radiometer Analytical SAS, 2007) and the relationship varies based on component concentrations (Rosenthal, 1948; Yoshimura, 1935). pH measurement technologies use built-in temperature compensation to prevent incorrect readings and incorrect corrective actions, however different measurement technologies apply different temperature compensation. If two technologies employ different pH-temperature compensations, they may give different readings for the same sample. The extent of this difference will be affected by the sample's chemical composition.

In the presented study, MVDA techniques were used to identify if the observed differences in readings were influenced by sample composition or temperature. Effects from sample handling (Evans and Larson, 2006), pH probe sterilization (Saucedo et al., 2011), probe age, or variations specific to individual probes are acknowledged as potential confounding variables, however these

were excluded as explicit variables for three reasons. First, these variables were not captured in daily monitoring datasheets. Secondly, as data were collected in a CMO study across 48 cell cultures, these variables were assumed to have a consistent effect across the activity of each cell culture from inoculation to harvest. Finally, an appropriate and statistically sound method for capturing and integrate such data for analysis is required for further exploration.

2 Materials and Method

Data were taken from a robustness study of a proprietary Chinese hamster ovary (CHO) cell line transfected with a vector containing a dihydrofolate-reductase (DHFR) selectable marker (Urlaub and Chasin, 1980) and the DNA sequence for a monoclonal antibody. Cells were grown using a proprietary chemically-defined medium in 15L glass stir tank reactors (STR) (Applikon Biotechnology) with a 10L working volume. Applikon i-Control controllers were used to monitor and control gas flows (Applikon Biotechnology, UK). Applikon i-Control controllers were also used for online monitoring and control of pH, dissolved oxygen (DO%), and temperature. Online pH was measured by a Mettler-Toledo pH probe connected to the controller. pH was controlled by CO_2 sparging and addition of sodium bicarbonate.

Nine control bioreactor cultures were initially maintained at pH 7.0 and 36°C. When a specified minimum viable cell concentration (determined by daily offline sampling) was reached, the pH and temperature setpoints were altered according to an experimental plan. Three bolus additions were made to each bioreactor culture. Bolus A was added when the pH and temperature setpoints were adjusted. Bolus B was added on Day 4 of the culture. Bolus C was added on Day 7 of the culture. Eleven experimental bioreactor cultures operated with deliberate deviations from standard operation. These included deviations directly captured in daily monitoring data, e.g. increased operating temperature, decreased operating temperature, omission of the shifts in pH and temperature setpoints. Other deviations were not directly captured in daily monitoring data and were collected as meta-data, e.g. use of expired medium or alterations to feeding strategy.

The cultures were monitored through daily offline samples. An offline pH reading was taken using the first pH measurement technology, a Radiometer Analytical PHM220 pH meter and a Mettler-Toledo pH probe coupled to a temperature probe. The Radiometer Analytical PHM220 was calibrated daily using standards of known pH; the standards' temperatures were used to create a pH-temperature compensation. When an offline measurement was made, the sample's temperature was also recorded and the compensation applied.

A second offline pH measurement was made using a NOVA Bioprofile 400. Here, the sample temperature (T) was entered at the user interface, and the unit heated the sample to 37 °C. The pH of the heated sample was measured, and then pH-temperature compensation was applied using a set equation (Eq. 1).

$$pH_{corrected} = pH + [-0.0147 + 0.0065 * (7.400 - pH)] * (T - 37)$$

Eq (1)

The NOVA Bioprofile 400 was also used to measure partial pressures of dissolved O_2 and CO_2 gases and the concentrations of lactate, glucose, glutamate, glutamine, Na^+, K^+, and NH_4^+. Total cell concentration and viable cell concentration were determined using a Vi-CELL XR (Beckman Coulter Inc., UK). From these values, an osmoalilty estimate was calculated using the in-built component calculator (Eq 2).

$$Osmolality = 1.86([Na^+] + [K^+] + [NH_4^+]) + \frac{[Glu]}{0.18} + \frac{[Lac]}{0.09} + constant$$

Eq. (2)

Finally, sample osmolarity was then measured directly using freezing point osmometry (FPO) with an OSMOMAT Auto (Gonotec GmbH, Germany).

2.1 Statistical Method

Multiple linear regression (MLR) is a widely applied regression tool. Given **X**, an $n \times (p + 1)$ dataset containing *n* samples with *p* inputs, and **Y**, an $n \times 1$ response vector, the relationship between **X** and **Y** can be modelled as:

$$\mathbf{Y} = \mathbf{X}\beta + \varepsilon$$

Eq(2)

with regression parameters $\beta = \begin{pmatrix} \beta_0 \\ \vdots \\ \beta_k \end{pmatrix}$ and errors $\varepsilon = \begin{pmatrix} \varepsilon_1 \\ \vdots \\ \varepsilon_n \end{pmatrix}$, where ε is normally distributed with a mean of zero and constant variance σ^2 (Upton and Cook, 2011). This model can then be applied to data to give:

$$\widehat{\mathbf{Y}} = \mathbf{X}\beta$$

Eq(3)

where $\widehat{\mathbf{Y}}$ is the vector of fitted (predicted) values. A limitation of MLR is that that noisy or co-correlated variables reduce model efficiency. A potential solution is iterative significance testing until all variables retained in the model have a *p*-value below a chosen threshold (here 0.05) (Streiner, 2003).

Principal component analysis (PCA) is a statistical tool suitable for the analysis of a large dataset ***X*** composing *n* samples and *p* variables (Wold et al., 1987). PCA captures the main source of variability in the dataset by creating new variables termed principal components (PC). Each PC is a linear combination of the original variables and orthogonal to the other PCs. A fewer number of PCs than original variables are required to be retained as the process is not operating in *p*-dimensions. In summary the data matrix, ***X***, is decomposed:

$$\boldsymbol{X} = \boldsymbol{T}\boldsymbol{P}^{\boldsymbol{T}} + \boldsymbol{E} = \sum_{i=1}^{k} t_i p_i + \sum_{i=k+1}^{p} t_i p_i$$

Eq (4)

where ***X*** is the original *n*x*p* data matrix, *k* is the number of PCs retained, ***T*** the *n*x*k* scores matrix, $\boldsymbol{P}^{\boldsymbol{T}}$ the *k*x*p* loadings matrix, and ***E*** is the *n*x*p* model residuals matrix (Wold et al., 1987). When the loadings for PCs are plotted in 2-dimensional space, positively correlated variables will cluster together. Negatively correlated variables will be diametrically opposed across the origin.

3 Calculation

A total of 785 sets of 12 daily sampling measurements were collected, covering 48 fed-batch cultures running for an average of 15 days under a range of conditions. Data were collated into a single 785x12 matrix. The dataset was analyzed using a three-step method (3.1 through 3.4 below). Minitab® Statistical Software was used for multiple linear regression (MLR) and principal component analysis (PCA). Additional figures were generated using Matlab®2010a from Mathworks®.

3.1 Extraction of Indirectly Captured Information

Osmolality is the concentration of solutes in a sample measured in osmoles of solute per kilogram of solvent (Osm/kg) (Advanced Instruments, Inc. 2011), however the measurement does not identify or specify quantities of individual components present in the sample. It was hypohesized that information concerning sample components not directly monitored in the dataset could be extracted from the FPO measurement by removing contributions from directly monitored variables.

A dataset-specific component calculator was created through MLR and iterative significance testing (Eq. 5).

$$Osmo_M = 236 - 0.298pO_2(mmHg) + 0.243pCO_2(mmHg) + 74.4Gln(g/L) - 14.6Gluc(g/L) + 254NH_4^+(g/L) + 0.263Na^+(mmol/L) + 4.27K^+(mmol/L) + 7.95TCC(10^6 cells/mL)$$

Eq. (5)

where $Osmo_M$ is the predicted osmolality and *TCC* is the total cell count for a sample. The model accounted for 83.6% of the variation in osmolality in the dataset. The indirectly captured data is in the form of the model's residual information (errors), $Osmo_{Res}$ (Eq. 6).

$$Osmo_{Res} = Osmo - Osmo_M$$

Eq. (6)

The historical dataset was expanded by treating the residual information $Osmo_{Res}$ as a new variable. Note that this residual information should not be confused with a surrogate variable where a difficult to measure variable *X* is replaced by a closely correlated and more easily measured variable *Y* (Upton and Cook, 2011).

3.2 Validation of Extracted Information Approach

PCA was performed using all recorded variables and either osmolality or $Osmo_{Res}$. The PCA model including osmolality captured 63% of cumulative X variance at the PC2 level. The PCA model including $Osmo_{Res}$ captured 57% of cumulative X variance at the PC2 level. The difference of 6% in cumulative X variance captured indicated a slightly more complex data structure for the $Osmo_{Res}$ model. The difference in cumulative X variance captured reduced with increasing model complexity, e.g. from 6% at PC2 to 2.5% at PC5.

The loadings for these models are displayed in Figure 2. In Figure 2A, osmolality is located between two clusters of variables. This is due to correlation with both groups of variables. In Figure 2B, the correlations between all variables, excepting $Osmo_{Re}$, are effectively unchanged. The variable representing indirectly captured information in this system, $Osmo_{Re}$, is now located in a postition indicating that, at the PC1 and PC2 level, $Osmo_{Res}$ contains information not as strongly correlated with other factors as in the original variable osmolality. $Osmo_{Res}$ is also closer to the origin indicating that the previously unextracted information has a lessened impact on the variance of the input dataset as a whole.

It was possible to identify patterns related to metabolism and culture processes when plotting $Osmo_{Res}$ against elapsed time (Figure 3 and Figure 4). During the first three days, $Osmo_{Res}$ decreased as expected from consumption of media compounds during the exponential growth phase. These media components were not directly measured; as they could not be accounted for explicitly the osmolality model, these components contribute to the osmolality model residuals.

For the reactors undergoing a change in operating temperature on Day 3, there is a further decrease after the addition of bolus A, then an increase following the addition of bolus B. This dip is not noted in the data for reactors undergoing the temperature change on Day 4, where bolus A and B were added at the same time. This indicates that the boluses' effects cancel out to some extent. A large increase occurs following the addition of bolus C; this is followed by a general reduction in the osmolality residuals, indicating consumption of components contributing to the osmolality model residuals.

3.3 Comparison of Offline pH Measurement Technologies

The variable osmolality in the daily monitoring dataset was replaced with $Osmo_{Res}$. The dataset was then subdivided based on when sampling took place relative to the pH and temperature setpoint

shifts as these datasets reflect two different process conditions: Before Shift (BS) and After Shift (AS). For data taken after the setpoint change was made (AS), cultures where experimental changes were not directly captured in the daily monitoring measurements were excluded, e.g. cultures run at high DOT were included whereas cultures testing modified feed strategies were rejected. MLR was used to model the differences between measurements by the Radiometer Analytical PHM220 and the NOVA Bioprofile 400 (B-N) for the BS dataset, AS dataset, and full dataset. Models were refined using statistical significance testing until all factors were $p<0.05$.

4 Results and Discussion

Factors identified as statistically significant ($p < 0.05$) in predicting differences in pH reading by the pH measurement technologies are indicated in Table I. Model R^2 are also indicated in Table I. These models indicate that sample composition and condition can affect agreement in pH readings by two pH measurement technologies.

$Osmo_{Res}$ was found to be statistically significant across all three models, indicating that certain components not directly monitored had a statistically significant impact on the differences in pH measurements.

A second key variable identified as statistically significant was temperature. Before the change in temperature and pH operating setpoints, temperature did not have a statistically significant impact on differences in pH readings. After the reactor operating temperature was reduced, the effect of temperature was statistically significant.

One possible explanation for this result is the different temperature compensations used by the technologies. The NOVA Bioprofile 400 used a set formula for pH-temperature compensation whereas the Radiometer Analytical PHM220 pH-temperature compensation was recalibrated daily. Furthermore, the NOVA Bioprofile 400 heated samples before measuring pH whereas the Radiometer Analytical PHM220 measured sample temperature and pH as is. The equipment were effectively applying compensations in opposite directions, which may also contribute to differences in a sample's recorded pH. The strength of this effect would increase proportional to ΔT.

4.1 Limitations of Extracted Information

An issue with the original osmolality measurement was that it could not identify which components contributed to the measurement. $Osmo_{Res}$ was a similarly indiscriminate factor as it captured a variety of components including bolus ingredients, culture medium, and by-products of cell metabolism. As the relative concentrations of these components were not constant through culture lifetimes or between cultures, $Osmo_{Res}$ may not have had a consistent impact on the discrepancies in pH readings, e.g. the effect of 40 mOsm/kg H_2O residual caused by bolus A may not have affected readings to the same extent as a 40 mOsm/kg H_2O residual caused by bolus B.

Additionally, use of the final model was restricted to consideration of the dataset from which the model was created. Hence the model was neither a generic nor predictive model for osmolality. Adaptations to allow predictive use and/or the creation of models tailored to specific formulations, cell lines, or products could result in a strong tool to increase cell culture knowledge from indirectly captured information. This includes both historic datasets and processes where more comprehensive measurement technologies (e.g. NIR) are not currently implemented.

5 Conclusions

Osmolality is a non-specific measurement of components; an osmolality reading of 100 mOsm/kg does not specify if it is 100 mOsm/kg of component A, B, C, or a mixture of all three. Information indirectly captured in a historic dataset was extracted by separating contributions to osmolality by directly monitored components from contributions to osmolality by components not directly

monitored. The new variable $Osmo_{Res}$ was a similarly non-specific factor, capturing a variety of components including bolus feed ingredients, culture medium components, and by-products of cell metabolism. The new variable $Osmo_{Res}$ was used to expand an historical dataset in a comparison of offline pH measurement technologies.

The demonstrated method for the calculation and verification of $Osmo_{Res}$ is recommended as a means to generating additional knowledge for a specific combination of cell line, product, and process platform. In addition to retrospective analyses, the method could be applied during Design of Experiment or process limit evaluation studies during process transfer with $Osmo_{Res}$ calculated as part of daily monitoring activities (Figure 5).

While much of what causes the discrepancies between the competing pH measurement technologies is still not understood, it was shown that the pH measurement technologies compared were not interchangeable at the desired level of agreement. These disagreements in pH readings could be attributed in part with sample composition and physical condition. From this it is recommended that offline and online monitoring technologies should be of the same design to prevent errors caused by differences in design. If two different designs must be used, it should be demonstrated that the technologies agree across a variety of conditions within the culture design space including temperature. Furthermore, the make and type of pH equipment used in a project should be recorded to ensure the similar equipment is used at all scales of reactor throughout a project (i.e. initial lab testing to full scale production) (Figure 5). In doing so, potential negative effects on product quality or titre are reduced and differences of technology eliminated from any subsequent gap analysis.

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References

Table I. Results of MLR and Significance Testing

X indicates variables identified as significant ($p < 0.05$) when modelling the difference in pH reading by two offline technologies for three datasets: All, Before Shift, After Shift. R^2 values for each model are also displayed.

Figure 1. Histogram of Differences in Offline pH Readings by Two Offline Technologies

Dotted lines indicate boundaries for differences due to instrument error (±0.02 pH units).

Figure 2. PCA Loading Plots

A. PC1 and PC2 Loadings for Data when including osmolality. Osmolality (circled) was located between two clusters of variables (●), indicating correlation with both clusters.

B. PC1 and PC2 Loadings for Data when using $Osmo_{Res}$. Spatial relationships between variables (●) are relatively unchanged compared to Figure 2A. $Osmo_{Res}$ (circled) was not located between two clusters of variables. PC2 loadings were multiplied by -1 to improve comparative visual analysis, due to inversion of the PC2 vector in the second PCA model produced.

Variable Numbers: [1] Temperature [2] Glucose [3] pO_2 [4] DOT [5] Lactate [6] K^+ [7] Glutamine [8] Glutamate [9] Na^+ [10] VCC [11] TCC [12] NH_4^+ [13] pCO_2 [14] Day [15] Osmolality [16] $Osmo_{Res}$

Figure 3. Boxplot of $Osmo_{Res}$ for Bioreactors Undergoing Change in pH and Temperature Setpoint on Day 3.

Boxplot of model residuals arranged by day of sampling with trendline overlay. Daily mean (Δ), outliers (*), and bolus additions (A, B, C) indicated.

Figure 4. Boxplot of $Osmo_{Res}$ for Bioreactors Undergoing Change in pH and Temperature Setpoint on Day 4.
Boxplot of model residuals arranged by day of sampling with trendline overlay. Daily mean (◊), outliers (*), and bolus additions (A, B, C) indicated.
Figure 5. Dataflow System for Improved pH and Culture Monitoring.
In the system, equipment maintenance records, equipment IDs, and offline monitoring data are captured in a single source (electronic laboratory notebook). Data are then used to create and maintain an osmolality model. The osmolality model residuals and other data feed into a apH monitoring model (1) and a culture growth model (2).

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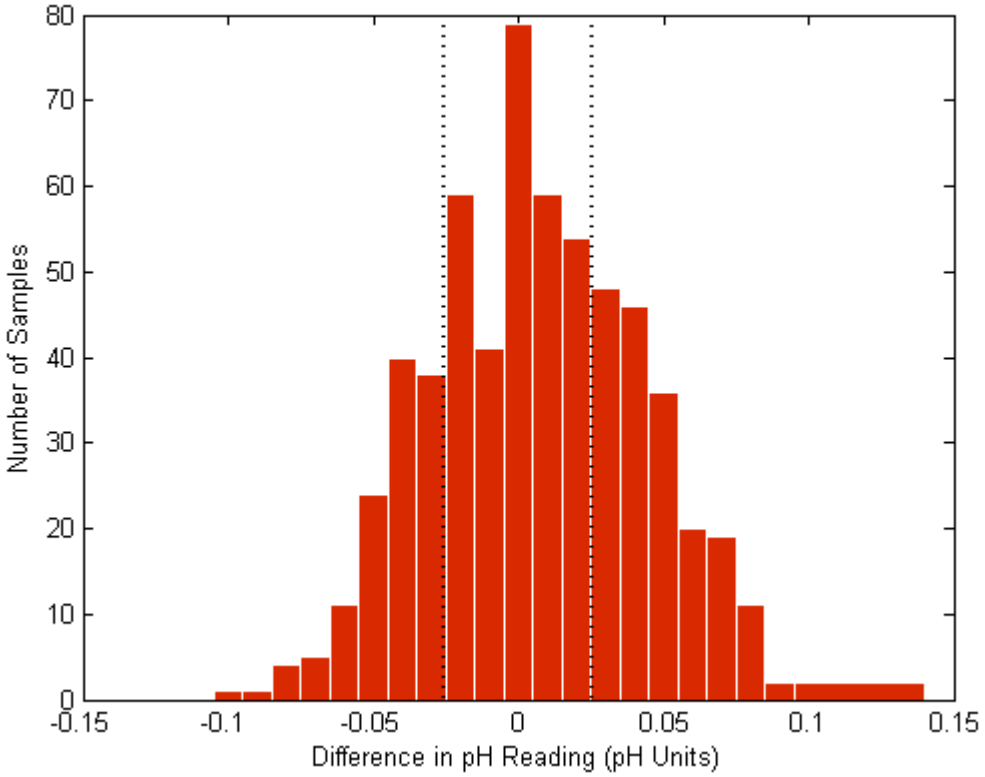

Figure 1

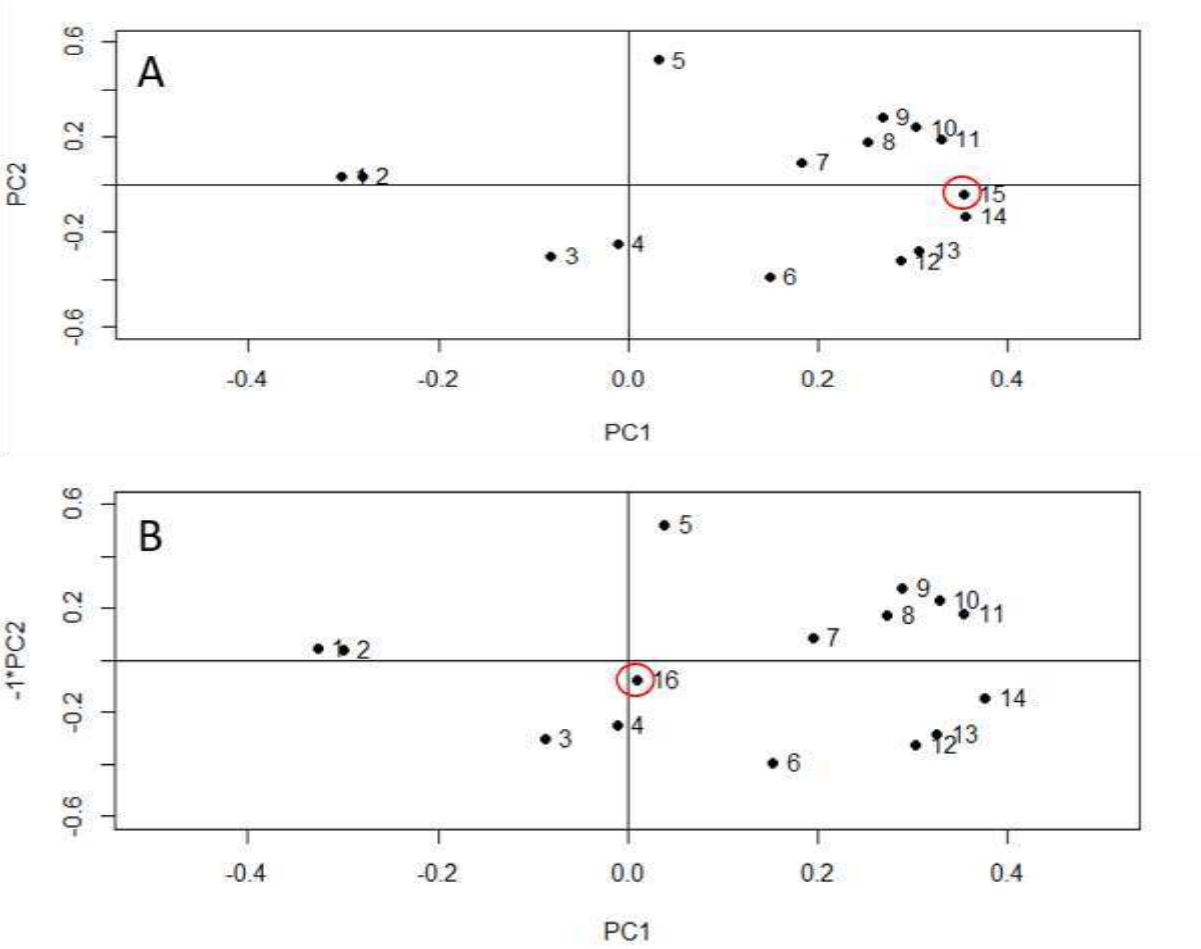

Figure 2

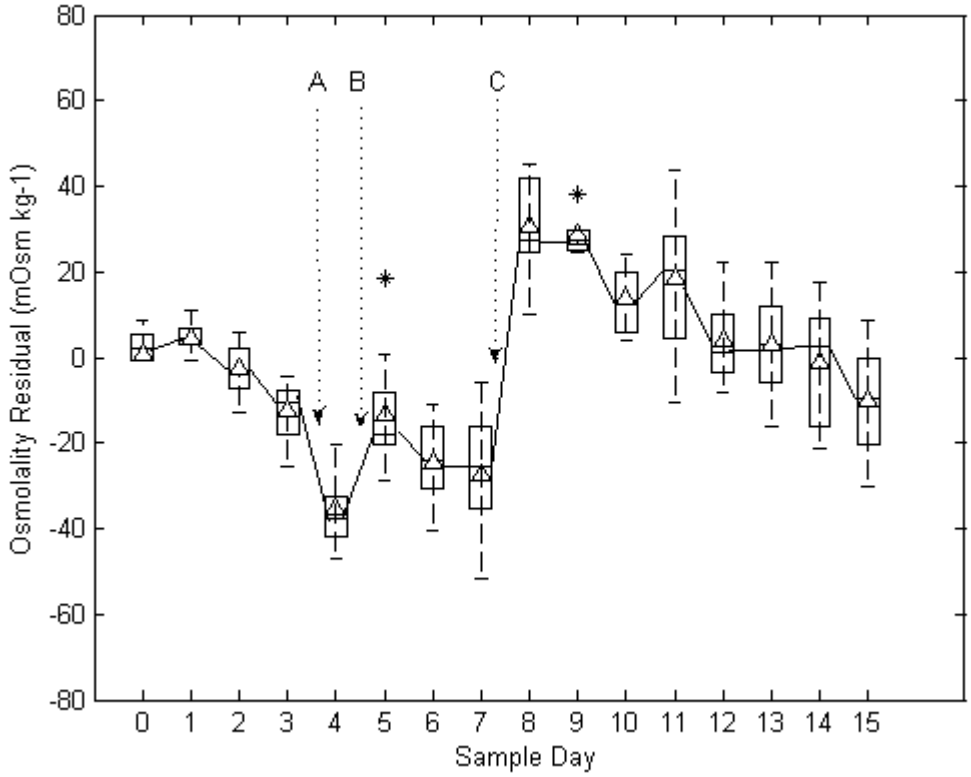

Figure 3

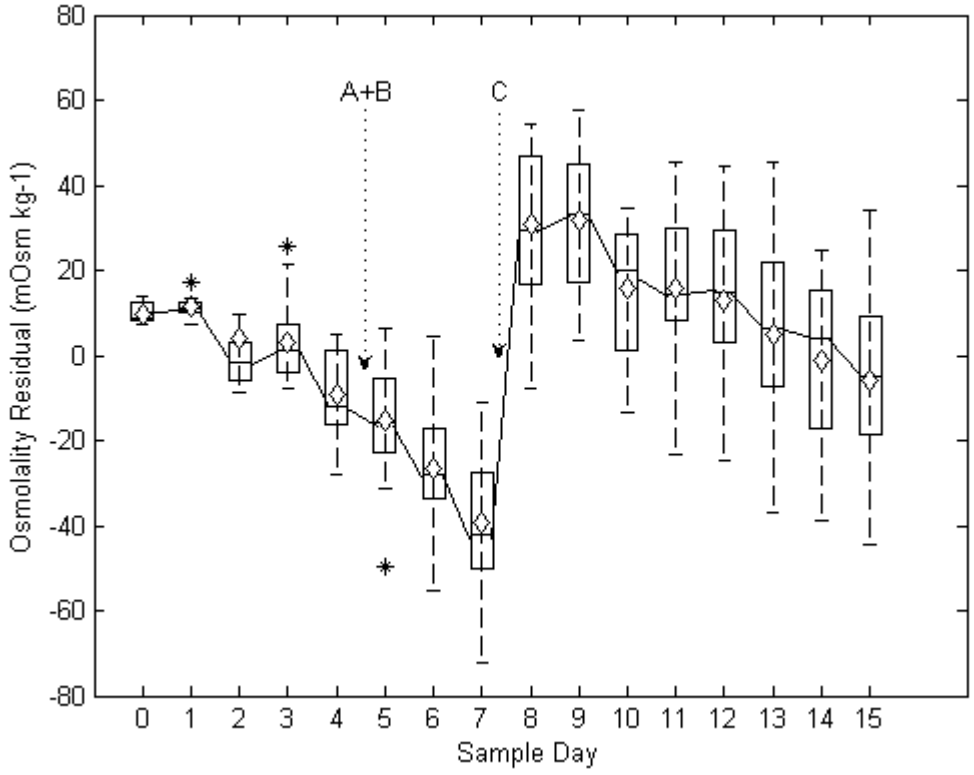

Figure 4

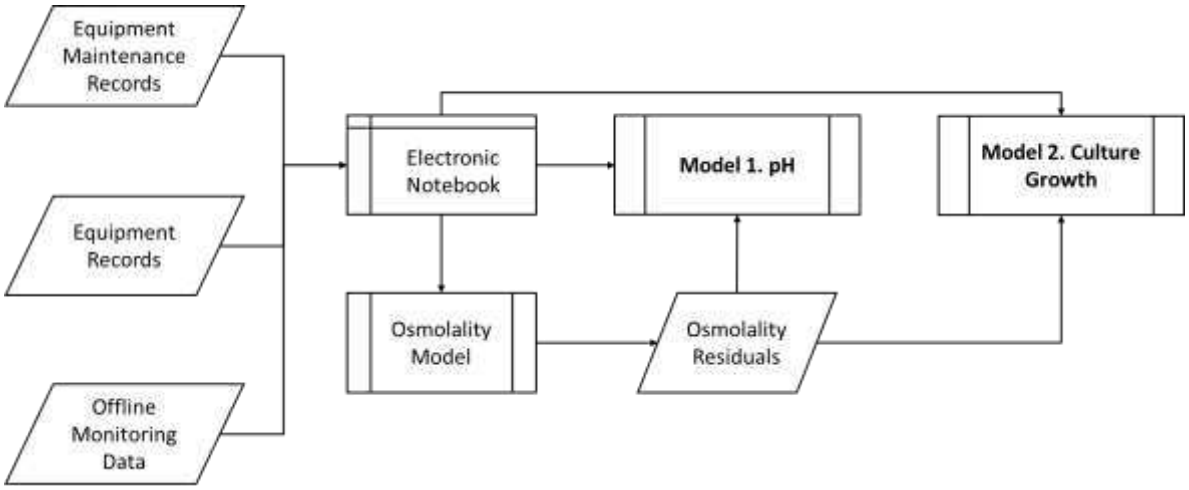

Figure 5

Table I: Results of MLR and Significance Testing

	Dataset Used		
Variable	All	Before Shift	After Shift
Constant	X	X	X
Time (h)	—	X	X
Temperature (°C)	X	—	X
DOT (%)	—	—	—
pO_2 (mmHg)	X	—	X
pCO_2 (mmHg)	X	—	X
Gln (g/L)	—	X	X
Glu (g/L)	—	—	—
Gluc (g/L)	X	—	—
Lac (g/L)	X	—	—
Na^+ (mmol)	X	X	X
K^+ (mmol)	X	X	—
Total Cell Concentration (10^6 cells/L)	X	X	—
$Osmo_{Res}$ (mOsm/kg)	X	X	X
Model R^2	0.389	0.458	0.465
Model *p*-Value	0.00	0.00	0.00