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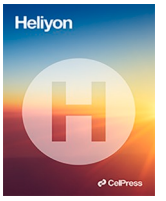

Research article

Evaluation of single-use optical and electrochemical pH sensors in upstream bioprocessing

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ABSTRACT

Culture pH is a critical process parameter during CHO cell bioreactor operations that is key for proper cell growth, protein production, and maintaining the critical quality attributes of a monoclonal antibody drug substance. The traditional means of measuring pH in bioreactors is with an electrochemical probe that can withstand and maintain accuracy through repeated sterilization cycles. An alternative technique for measuring pH is an optical sensor composed of a fluorescent dye that is sensitive to the hydrogen ion concentration. In this work we explore single-use electrochemical and single-use optical pH sensors in stirred-tank and rocking bioreactors, respectively, to understand how their overall performance compares to traditional electrochemical probes in benchtop glass stirred tank bioreactors. We found that the single-use optical pH sensors were generally less accurate than the electrochemical probes, especially in detecting large pH drifts from the setpoint. The single-use electrochemical probes were increasingly accurate as pH was increased from <7.0 to 7.5 but tended to decrease in accuracy as the batch age increased. In conclusion, single-use pH sensors offer a convenient means to measure pH during an upstream bioprocess, but the limitations of these sensors should be built into process control such that deviations in process pH, and consequently potential fluctuations in product quality, can be avoided.

1. Introduction

Control of pH during upstream biomanufacturing is essential to optimizing a bioprocess and ensuring product quality. Culture pH can affect critical cellular functions including, but not limited to, growth and metabolism, recombinant protein production, and post-translational modifications (e.g., glycosylation). There are two main types of pH sensors that can be used in bioreactors, electrochemical and optical. Glass membrane pH sensors are electrochemical sensors that generate a millivolt output corresponding to

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Table 1
Specifications for the pH Sensors used in this study.

	BP	CCA	STR EP	SU EP	SU OPT
Method	Electrochemical	Electrochemical	Electrochemical	Electrochemical	Optical
Location	At-line	At-line	In-line	In-line	In-line
Reusable/Single-Use	Reusable	Single-use	Reusable	Single-use	Single-use
Measurement Range	0–14	5.0–8.0	0–14	2–8.5	4.5–8.5
Control Range	NA	NA	NP	NP	6.0–8.0
Measurement Accuracy	±0.01 pH[a]	±0.05 pH	±0.01 pH[a]	±0.02 pH	±0.05 pH when within ±0.25 pH from offset calibration; ±0.1 pH when within 0.25–0.5 pH from offset calibration
Control Accuracy	NA	NA	NP	NP	±0.05 pH
Temperature range	0–100 °C	Approximately 37 °C[b]	0–140 °C	NP	NP
Probe material	HA glass	NA	Hamilton Type PHI glass	NP	Luminophore dye attached to a polycarbonate backing
Diaphragm	Ceramic	NA	Ceramic (HP-Coatramic)	NP	NA
Electrolyte	3 M KCl	NP	Pressurized Phermlyte	NP	NA
Reference System	ARGENTHAL™ with Ag^+-trap	NP	Hamilton Everef-F Ag^+ system	NP	NA
Autoclavable	NP	NA	Yes	NA	NA
Steam sterilizable	NP	NA	Yes	NA	NA
Gamma irradiated	NA	NA	NA	Yes	Yes

NA: Not applicable.
NP: Not provided within manufacturer specifications.
[a] Based on uncertainty of RICCA pH standards 4.0, 7.0, and 10.0 used for probe calibrations.
[b] Samples loaded onto the CCA are kept at or warmed to 37 °C prior to pH measurement.

changing pH values. As the name suggests, the measurement electrode portion of the sensor relies upon a specially formulated, pH-sensitive glass that reacts with the hydrogen ion concentration in the media. When the hydrated gel layer is electrified, the hydrogen ions in solution migrate to the outer gel layer. This creates a difference in potential between the outside gel layer and the internal gel layer, the latter serving as a reference potential and maintained at pH 7. Optical pH sensors are composed of a fluorescent dye embedded in a hydrophilic gel compound. This fluorescent dye is sensitive to the hydrogen ion concentration in the media. A blue light is directed on the sensing element using fiber optics, which emits light at a corresponding wavelength. Changes in pH result in a wavelength shift that can be measured through an optical detector. The ratio of wavelength change will correspond to the change in pH value.

Electrochemical probes have served as the industry standard for in-process pH measurement. Consequently, much of the existing commercial bioreactor hardware and software are designed for the millivolt input, which is a key advantage over newer optical detectors. Electrochemical probes are also robust and can withstand gamma sterilizations, autoclaving, steam-in-place (SIP) and clean-in-place (CIP) procedures. However, when compared to modern optical sensors, traditional electrochemical probes are large, costly, and not amenable to high throughput microbioreactors or other forms and scales of single-use technology. Traditional electrochemical probes also have established lifetimes, losing accuracy and responsiveness over time. The advantages of optical pH sensors or patches are that they can be small, inexpensive, readily disposable, free of shelf-life restrictions (which makes them amenable to times when there are supply chain issues), and they do not require calibration, saving time and simplifying process procedures. However, optical sensors do not offer the same level of process robustness that electrochemical sensors do and can have cross-sensitivity to certain chemicals (e.g., organic solvents that can damage the gel matrix and fluorescent dye). Furthermore, conductivity can impact measurement accuracy, which is limited in range to between pH 5 and 8. Optical probes also currently lack integrated temperature compensation. These limitations may explain in part why, despite being on the market for more than 20 years, optical probes are less widely used within upstream and downstream operations, although this may be changing due to the increase in single-use technologies.

Although electrochemical probes are most commonly reusable, electrochemical probes can be single-use, while optical sensors are typically single-use. Electrochemical single-use probes exist in some pre-assembled single-use microbioreactors and as sensors to be installed into single-use stirred-tank bioreactor bags. Optical pH sensors are found in some microbioreactors and benchtop single-use bioreactors, as well as on rocking bioreactor bags and even certain shake flasks [1,2].

Bioreactor pH is controlled through a combination of both gassing and acid or base additions to the culture. This is generally accomplished through a two-sided control loop [3,4] in which the bioreactor controller receives the measurements taken from the pH sensor and either increases the percentage of carbon dioxide in the gas mix if the pH is higher than the setpoint or turns on a pump to add base (e.g. sodium hydroxide or sodium bicarbonate) if the pH is lower than setpoint. With appropriate proportional integral

derivative (PID) control settings, based on what is detectable by pH sensors, tight control of pH can be maintained for the entirety of a bioprocess. However, pH feedback control becomes more challenging in larger bioreactors and inadequate mixing has been shown to cause significant pH excursions up to 0.8 pH unit difference when sodium bicarbonate was added from the liquid surface in an 8000 L bioreactor [5]. Similarly, pH excursion may not always be detectable by the pH probe/sensor, especially in very large bioreactors where mixing and location of base addition can impact the homogeneity of pH [5]. In one study, authors used two-compartment bioreactor systems to simulate pH inhomogeneities and intracellular pH measurement to demonstrate that even short-term exposure of Chinese hamster ovary (CHO) cells to elevated pH values (pH 7.8 and pH 9.0) affect cell physiology and overall process performance within 20 min, indicating that similar effects will arise during temporary pH gradients as they might occur in large-scale processes [6]. Others have noted that without optimized pH control, pH excursions can occur and lead to cell death [7]. Additionally, pH control becomes more challenging as the cell density is increased, such as in perfusion bioreactors [5,8]. While pH control in the biological range near neutral pH is widely recognized for proper mammalian cell growth and protein production, small changes in pH can sometimes be impactful for a given bioprocess and may influence product quality attributes of the protein therapeutic of interest. In some cases, a change in pH of 0.1 can result in a significant decrease in cell productivity [9] and for monoclonal antibodies, pH can impact protein glycosylation [10–14].

In this study, we compared pH control and accuracy among traditional reusable electrochemical probes in glass benchtop bioreactors (STR EP), single-use electrochemical pH sensors in single-use microbioreactors (SU EP), and optical pH sensors in single-use rocking bioreactor bags (SU OPT). We compared the online measurements to an automated cell culture analyzer (CCA), which uses an electrochemical microsensor, and a benchtop combination pH electrode. We compared the agreement between the bioreactor pH sensors and CCA for the entire data sets, and then grouped the data sets by pH, osmolality, and batch age to assess their correlation with sensor agreement. Lastly, we compared the cell growth, metabolism, productivity, and monoclonal antibody (mAb) relative N-glycan abundances from bioreactor batches using a pH 7.1 setpoint to those using a pH 7.3 setpoint to test the impact of pH excursion on our CHO cell bioprocess.

2. Materials and methods

2.1. At-line pH measurement

At-line pH measurements were taken using a benchtop combination pH electrode (Mettler-Toledo InLab® Routine Pro-ISM; 51344055) and a BioProfile® FLEX2 (Nova Biomedical) automated cell culture analyzer (CCA) (Table 1). Significant effort was made to avoid off-gassing of the culture sample between removal from the bioreactor by syringe and analysis by pH electrode and CCA by capping the sample and analyzing the samples within 5 min of removal from the bioreactor.

Following calibration, the benchtop pH electrode was qualified each day by measuring pH standard solutions (RICCA; 1480-16) of pH 4.0, 7.0 and 10.0 once or twice daily for 14 days. To properly apply statistical methods for pH values, which are logarithmic in nature, the measurements were transformed back to [H^+] from pH using the following Equation (1):

$$[H+] = 10^{-pH}$$

The mean [H^+] values were calculated, and accuracy was determined based on the discrepancy between the measured and theoretical mean using the following Equation (2), where the theoretical mean was defined as either the pH listed on the pH standard solution (for the benchtop pH electrode) or the mean of the pH range listed on the quality control (QC) standard (for the CCA):

$$Discrepancy\ (\%) = \frac{Measured\ mean\ [H+] - Hypothetical\ mean\ [H+]}{Hypothetical\ mean\ [H+]}\ x\ 100$$

Statistical tests for normality (D'Agostino-Pearson) and one sample two-tailed t-tests were run using GraphPad Prism 9.5.1.

The CCA automatically runs two QC pH standards (called Level 1 and Level 2) daily when in operation using a QC cartridge (Nova Biomedical BioProfile® FLEX2™ pH/Gas Auto QC Cartridge; 56073). We collected and evaluated the QC results for nine consecutive months of operation (Supplementary Fig. 1) by comparing the mean/median of the QC standard range for each standard lot to the mean and medians of the daily measurements. All QC pH data were transformed to [H^+] prior to statistical analysis and were analyzed by lot for normality (Anderson-Darling) and one sample Wilcoxon Signed Rank Tests. Discrepancy between the theoretical median and measured median for each lot are expressed in terms of percent.

2.2. Online pH measurement

The STR EP probes (Table 1) used in this experiment (Hamilton EasyFerm Plus; Sartorius BB-8848657) were used with the Sartorius Biostat® B-DCU II system using 5 L glass stirred tank reactors (STRs). Probes were calibrated ahead of autoclaving by performing a 2-point calibration slope using pH standard solutions of pH 4.0 and 10.0 (RICCA; 1480-16). A pH 7.0 standard solution was used to test the probe after calibration. SU EP and SU OP probes (Table 1) were already installed and integrated to the single-use bioreactor assembly. These probes were hydrated in culture media for $\geq$4 h prior to a pH offset being performed using an at-line CCA pH reading. Once the pH offset was performed, pH setpoint control was turned on and the culture allowed to reach pH setpoint. A minimum of one additional sample for pH was tested prior to inoculation.

Table 2
Bioreactor systems, pH sensor types and run information used in this study.

Bioreactor System Information	Vessel Information	pH Sensor type	Bioreactor Identifier	Cell Line	Operation Type	Run Duration (days)	pH Setpoint	pH Control CO_2 delivery
Benchtop Glass STR (Sartorius BioStat® B-DCUII)	Univessel® Glass 5 L	Autoclavable electrochemical probe	STR-1	VRC01	Fed-batch	8	7.1	CO_2 sparging
			STR-2	VRC01	Fed-batch	8		
			STR-3	TmAb	Perfusion	13		
			STR-4	TmAb	Perfusion	20		
Multi-parallel single-use STR (Sartorius Ambr® 250 Modular)	Modular Mammalian Vessel (001–2A23)	Single-use electrochemical probe	AMBR-1	VRC01	Fed-batch	8	7.1	CO_2 sparging and overlay
			AMBR-2	VRC01	Fed-batch	8		
			AMBR-3	VRC01	Fed-batch	8		
			AMBR-4	VRC01	Fed-batch	5		
			AMBR-5	VRC01	Fed-batch	11		
			AMBR-6	VRC01	Fed-batch	11		
			AMBR-7	VRC01	Fed-batch	11		
			AMBR-8	VRC01	Fed-batch	11		
			AMBR-9	VRC01	Fed-batch	10		
			AMBR-10	VRC01	Fed-batch	10		
			AMBR-11	VRC01	Fed-batch	10	7.3	
			AMBR-12	VRC01	Fed-batch	10		
Rocking bioreactor (Cytiva ReadyToProcess WAVE™ 25 rocker)	Cellbag BioClear 10, DOOPT II, pHOPT and screwcap (CB0002L10-33)	Single-Use optical sensor	WAVE-1	VRC01	Batch	6	7.1	CO_2 overlay
			WAVE-2	VRC01	Batch	8		
			WAVE-3	VRC01	Fed-batch	7		
			WAVE-4	TmAb	Fed-batch	14		
	Cellbag BioClear 10, DOOPT II, pHOPT and perfusion (CB0002L10-34)		WAVE-5	VRC01	Perfusion	21		
			WAVE-6	TmAb	Perfusion	10		

2.3. Cell lines and seed train expansion

These experiments used two CHO cell lines: (1) VRC01, a recombinant CHO–K1 cell line that expresses a model chimeric IgG1 [15] and (2) TmAb, a CHO–K1 cell line producing a recombinant mAb biosimilar of Trastuzumab (GenScript Biotech Corporation). For VRC01, a frozen cell stock (3×10^7 cells/mL) was thawed and seeded into a 125 mL shake flask containing 30 mL of cell culture medium (HyClone™ ActiPro™ cell culture media; SH31037). The shake flask was incubated at 37 °C and 8% CO_2 at an agitation speed of 150 rpm in an INFORS HT Multitron Incubator (Infors AG). The shake flasks were scaled up every 3–4 days. On the day of inoculation, cells were counted and the appropriate volume of inoculum to reach the desired initial cell density was added to each bioreactor via syringe (Ambr250 modular bioreactors) or pumped directly from the inoculum shake flask (Thomson Instrument Company Multiported Optimum Growth™ 5L Flask; 931116-PORT-TRT-F) using a tube welder (Sartorius Biowelder® TC; 16389).

For TmAb, a frozen cell stock (1×10^7 cells/mL) was thawed from the working cell bank into high-intensity perfusion CHO (HIP–CHO) medium (Gibco; A4230201) containing 0.1% anticlumping agent (Gibco; 0010057) in a 125 mL shake flask with a 40 mL working volume. The cells were grown at 37 °C, 130 rpm, and 8% CO2 in an INFORS HT Multitron Incubator (Infors AG) for 4 days and passaged twice to 0.5×10^6 cells/mL into a 250 mL shake flask and then into a 500 mL shake flask, and then grown for 4 days before inoculation into the bioreactors.

2.4. Bioreactor operations and in-process sampling

Twenty-two bioreactor runs in three bioreactor systems were evaluated in this study (Table 2).

Samples were removed from bioreactors at least once daily and run on the BioProfile® FLEX2 automated cell culture analyzer (CCA; Nova Biomedical) to measure pH, pO2, pCO2, osmolality, viable cell density (VCD), cell viability, cell diameter, glutamine, glucose, lactate, glutamate, ammonium, sodium, potassium, and calcium. Prior to inoculation of the bioreactors with cells, samples were taken daily for 3–6 days to monitor culture medium without cells over time. Cell specific consumption and production rates for a metabolite (M) were calculated using the following adapted formula [16] Equation (3).

$$q_M = \left[\frac{[M]_f - [M]_i}{C_f - C_i}\right] x \; daily\ growth\ rate$$

where

$$daily\ growth\ rate = \frac{\left(\ln(C_f) - \ln(C_i)\right)}{t_f - t_i}.$$

M_f represents the metabolite concentration in the bioreactor at time t_f and M_i is the metabolite concentration in the bioreactor at time t_i. C_f and C_i represent the final and initial number of viable cells for a given final and initial time.

During initial bioreactor runs, samples were measured using a calibrated benchtop pH electrode at the same time as the CCA, which were run in triplicate. Once the accuracy and precision of the CCA had been established, single measurements were taken on the CCA. For some bioreactor runs, additional sample volume was aliquoted, sterile filtered, and frozen at −20 °C for future titer analysis and N-glycan testing.

2.5. Titer measurement

Samples were thawed at room temperature and titer was measured using Protein A dip-and-read bio-layer interferometry (BLI) sensors (Sartorius, 18–5010) on the Octet® N1 system (Sartorius) and applied to a standard curve. Cell specific IgG1 production rates (Q_p, pg/cell/day) for each perfusion day were calculated using the following adapted formula [16] Equation (4):

$$Q_p(pg\,/\,cell\,/\,day) = \left[\frac{T_f - T_i}{C_f - C_i}\right] x\ daily\ growth\ rate$$

where

$$daily\ growth\ rate = \frac{\left(\ln(C_f) - \ln(C_i)\right)}{t_f - t_i}.$$

T_f represents the total IgG in the bioreactor at time t_f and T_i is the IgG in the bioreactor at time t_i. C_f and C_i represent the final and initial number of viable cells for a given final and initial time.

2.6. N-glycan analysis

N-glycan analysis was performed using AdvanceBio Gly-X N-glycan prep with Instant-PC (GX96-IPC, Agilent Technologies, Santa Clara, CA) following the manufacturer's instructions. Briefly, cell culture sample was removed from the bioreactor via syringe and the supernatant was filtered through a 0.22 μm filter and stored at −20 °C until analysis. After thawing at room temperature, mAb was then purified from spent culture using protein A affinity chromatography using 20 mM phosphate buffer pH 7.2 as a binding and wash buffer and 0.1% formic acid as an elution buffer. Eluted mAb was then neutralized to an alkali pH of 7.9 using 1 M HEPES pH 8.0 solution. The concentration of the mAb solution was then measured using UV-absorption at 280 nm and the extinction coefficient to determine whether it was within the analytical range for the assay. Afterwards, 20 μL of the purified mAb was mixed with 2 μL Gly-X denaturant and heated at 90 °C for 3 min to denature the mAb. After cooling, samples were subjected to deglycosylation by mixing in 2 μL of N-Glycanase working solution (1:1 Gly-X N-Glycanase:Gly-X Digest Buffer) with the sample and heating at 50 °C for 5 min. The sample was then diluted with 150 μL of load/wash solution (2.5% formic acid, 97.5% acetonitrile (ACN)). Load/wash solution (400 μL) was added to the Gly-X Cleanup Plate along with the diluted sample. The sample was then washed twice with 600 μL of the load/wash solution before being eluted with 100 μL of eluent. For LC-MS analysis, mobile phase A constituted 100% ACN while mobile phase B constituted 50% mM ammonium formate pH 4.4. The column used was an Agilent AdvanceBio Glycan Mapping column (2.1 × 100 mm, 2.7 μm). Using a flow rate of 0.4 mL/min, the gradient started at a 2 min isocrat at 80% ACN and dropped to 75% ACN at 2.5 min, then slowly decreased to 62% ACN until 50 min was reached. Afterwards the column dropped to 40% ACN between 50 and 53.5 min and went back to 80% ACN between 53.5 and 55 min and remained isocratic at 80% ACN until 60 min. Mass detection of the separated glycans was performed using an Ultivo QQQ Mass Spectrometer using the following measurement conditions: Agilent Jet Stream ESI source, any MS, positive mode, sheath gas 300 °C at 10.0 L/min, dry gas 150 °C at 9.0 L/min, nebulizer pressure 35 psig, VCap 2500 V, Nozzle 500 V, Fragmentor 120 V (if applicable), and m/z range 600–3000.

3. Results and discussion

3.1. Qualification of at-line pH measurement equipment

Prior to running any bioreactors, we first qualified our at-line pH measurement equipment, which consisted of a benchtop combination pH electrode with integrated temperature monitor and an automated cell culture analyzer (CCA). After a calibration of the probe on day 0, pH standard solutions (RICCA; 1480-16) of pH 4.0, 7.0 and 10.0 were measured once or twice daily for 14 days to evaluate day-to-day accuracy of our benchtop pH probe. To properly apply statistical methods for pH values which are logarithmic in nature, the measurements were transformed back to [H+] from pH, and accuracy was determined based on the percent discrepancy between the measured and theoretical mean. The probe was considered accurate and within specification if the percent discrepancy was between −10.87 and 12.20%, which correlates to ±0.05 pH. The mean pH measurements for pH 4.0, 7.0 and 10.0 over the 14-day period were 3.99, 7.02, and 10.02 for each standard, which equated to discrepancies of 3.30%, −3.72%, and −5.23%, respectively.

To evaluate accuracy and intermediate precision of the CCA, data from the integrated quality control (QC) module was used from nine consecutive months of operation (Supplemental Fig. 1). The mean of the QC standard range for each standard lot was compared to the mean and medians of the daily measurements and the ranges of the measured values were evaluated for intermediate precision. The lower QC standard ranges for the three lots were (1) 6.663–6.763, (2) 6.562–6.662, and (3) 6.634–6.734 and the measured ranges were 6.714–6.761 (0.047), 6.608–6.629 (0.021), and 6.683–6.697 (0.014), respectively. The discrepancies between the theoretical median and the median of the daily measurements were −3.41%, −1.35%, and −2.27%, for the three lots, respectively. The higher QC

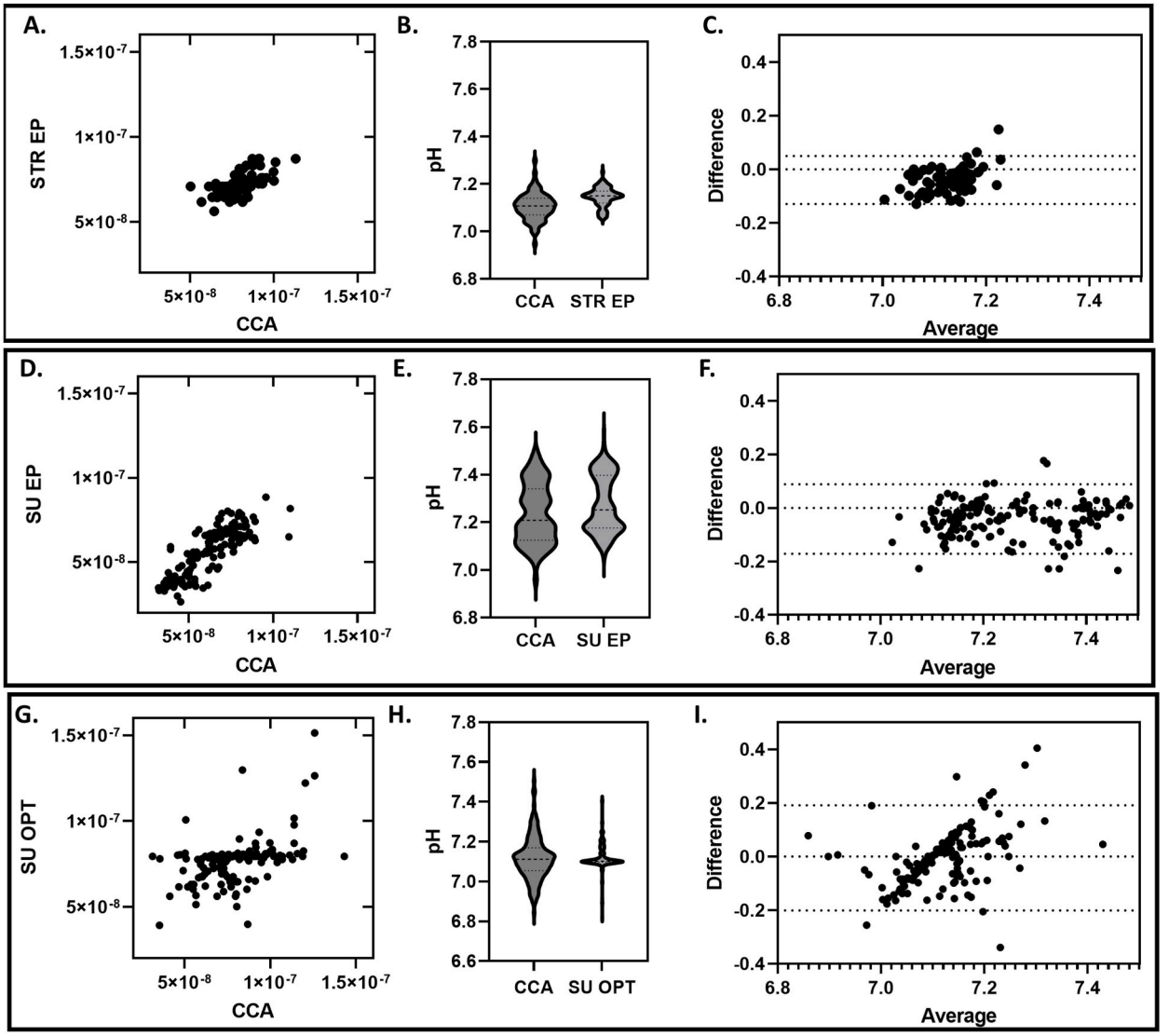

Fig. 1. The agreement of STR EP, SU EP, and SU OPT bioreactor pH sensors with an at-line CCA. Correlation plots are shown in insets A, D, and G. Frequency distributions, also known as violin plots, are shown in insets B, E, and H. Each plot depicts the full range of data points and the median and quartiles are shown in dashed and dotted lines, respectively, within each plot. Bland-Altman plots are shown in insets C, F, and I.

standard ranges for the three lots were (1) 7.286–7.386, (2) 7.238–7.338, and (3) 7.294–7.394 and the measured ranges were 7.319–7.374 (0.055), 7.280–7.288 (0.008), and 7.329–7.331 (0.002), respectively. The discrepancies between the theoretical median and the median of the daily measurements for the three lots were 1.95%, 1.07%, and 3.09%, respectively. Also of note, the CCA never measured an out of specification QC result during the 9 months of consecutive operation. These data gave us confidence in both the accuracy and intermediate precision of the CCA within the pH range of 6.6–7.3.

To evaluate repeatability of the pH measurements for the CCA, daily culture samples from a subset of bioreactor runs (STR-1, STR-2, WAVE-1, WAVE-2, AMBR-1, AMBR-2, AMBR-3, and AMBR-4) were measured three consecutive times. The average coefficient of variation was 0.098% (n = 108), indicating a very high repeatability for the measurements on the CCA when within the measurement range of pH 7.0–7.4.

Lastly, for the same subset of bioreactor runs during which CCA repeatability was evaluated, each daily culture sample pH was also measured using the benchtop combination pH electrode and compared to the average of the three CCA measurements. These data were transformed to [H+] and showed a linear correlation to CCA measurements with $R^2 = 0.86$. The average absolute difference between the [H+] measurements of the benchtop combination pH electrode and CCA was 5.85×10^{-9}. When we consider these data in terms of average difference in pH measurement, the average benchtop combination pH electrode pH measurement was 7.12 ($[H+] = 7.65 \times 10^{-8}$) while the average CCA pH measurement was 7.15 ($[H+] = 7.13 \times 10^{-8}$). The pH set point range used for mammalian cell bioreactors is typically at least 0.1 (i.e., ±0.05 pH units), and thus this average pH difference of 0.03 pH units supports very good

agreement between the benchtop combination pH electrode and CCA as comparable at-line pH measurement methods in the represented measured range of 7.0–7.4. Because the CCA performed with similar accuracy to the benchtop pH electrode and had very high repeatability and intermediate precision, we measured at-line pH using a single CCA measurement for subsequent bioreactor runs.

3.2. Agreement between CCA at-line pH measurements and glass stirred-tank bioreactor electrochemical pH probes (STR EP)

For four runs of our glass benchtop stirred-tank bioreactors (STRs), we collected at-line pH measurements once or twice daily using the CCA for up to 3 days prior to inoculation and for the duration of the runs after inoculation (11–20 days). For statistical purposes, all pH measurements were transformed to [H+] prior to analyses. The data were normally distributed ($p = 0.2202$) and thus a two-tailed paired *t*-test was used to compare the means of the [H+] values from the CCA to those from the in-line electrochemical probes in the bioreactors. The pairing was effective ($r = 0.64$; $p < 0.0001$) and the means are significantly different ($p < 0.0001$). However, the statistical significance of the difference of the means of the two data sets is not particularly useful in this case. Although there is a linear correlation between the at-line and in-line pH measurements (Fig. 1A), the slope of the linear fit is 0.41, indicating that the in-line probes were less responsive (slope <1.0) to changes in the culture pH compared to the CCA measurement method. Although all in-line probes passed calibration prior to autoclaving, all four probes had been subjected to repeated autoclave cycles prior to the runs shown and electrochemical probe accuracy and performance are known to decrease after repeated autoclave cycles.

When observing the frequency distributions of the CCA and STR EP measurements, the smaller range of the STR EP becomes clearer (Fig. 1B). In practice, this indicates that when the bioreactor pH was outside the pH setpoint range of 7.1 ± 0.1, the STR EP was not detecting that this deviation was occurring. However, the average absolute difference between the CCA and STR EP measurements was 0.047, which is generally acceptable for controlling the pH in bioreactors containing mammalian cells.

The agreement between the at-line CCA pH and on-line STR EP measurements were evaluated using a Bland-Altman analysis [17] to compare the two methods. The Bland-Altman plot (Fig. 1C) shows the difference of the measurements as a function of the average of the two measurements and provides calculations of bias, which is the average discrepancy between the measurements, and 95% limits of agreement between the methods. For CCA and STR EP, the bias was −0.040 pH units with 95% limits of agreement from −0.129 to 0.049 pH units. This can be considered good overall agreement between the CCA and STR EP assuming that inaccuracy of an average of 0.04 pH units of the bioreactor pH is not likely to impact product quality.

Lastly, for two of the four runs, as an additional test of the reusable electrochemical probes, we included a second electrochemical pH probe to monitor and compare probe to probe variation daily (data not shown). The average absolute difference between probes was 0.016 pH units and the sample Pearson correlation coefficient for the [H+] measurements was 0.92, indicating good linear correlation between the sets of probes. It should be noted that we expect newer probes (those that had been subjected to <10 autoclave cycles) would be more sensitive to changes in pH, and thus the data for STR EP can be viewed as a worst-case scenario for detection and accuracy.

3.3. Agreement between CCA at-line pH measurements and stirred-tank bioreactor single-use electrochemical pH probes (SU EP)

For twelve single-use STRs with single-use electrochemical probes (SU EP), we collected at-line pH measurements once or twice daily using the CCA up to 6 days prior to inoculation and for the duration of the runs (11–14 days). The data were not normally distributed ($p = 0.0001$) and thus the Wilcoxon matched-pairs signed rank test (two-tailed) was used to compare the medians of the [H+] values from the CCA to those from the SU EP. Like the STR EP analysis of means, the pairing was effective ($r = 0.83$; $p < 0.0001$) and the medians are significantly different ($p < 0.0001$). This result was expected as we do not anticipate the medians of pH data sets collected by the two different measurement methods to be the same. However, we do expect the measurements to vary together. There is a clear linear correlation between the at-line CCA and in-line SU EP pH measurements (Fig. 1D) and the slope of the linear fit is 0.72, indicating a greater sensitivity to pH fluctuations in the bioreactor by the SU EP than the aged STR EP used in this study. This can be observed in the frequency distributions of the CCA and SU EP measurements (Fig. 1E), which span a larger range of pH values in comparison to STR EP measurements (Fig. 1B). Overall, the SU EP has a higher distribution of pH readings than the CCA and did not capture some of the lower pH values (<7.0) measured by the CCA, but the distribution of data is the closest between the CCA and SU EP in comparison to the STR EP or SU OPT (discussed in the next section). The average absolute difference between the CCA and SU EP measurements was 0.044, which is equivalent to the STR EP and represents a larger pH range measured within the data set than the STR EP. The Bland-Altman plot comparing the CCA to SU EP (Fig. 1F) shows the difference of the measurements as a function of the average of the two measurements and that the bias (average discrepancy) is −0.041 pH units (the same as STR EP) with 95% limits of agreement from −0.172 to 0.089 pH units, which is larger than the limits between the CCA and STR EP (−0.129 to 0.049 pH units). This could be due to the larger sample size that is more representative of the population, which improves both the accuracy of the limits and predicting where to expect future differences between the two methods. Overall, we conclude that the agreement between the CCA and SU EP is comparable to that of the CCA and STR EP based on the average absolute differences.

3.4. Agreement between CCA at-line pH measurements and rocking bioreactor single-use optical pH sensors (SU OPT)

For six single-use rocking bioreactors using 2D cell bags with single-use optical pH sensors (SU OPT), we collected at-line pH measurements once or twice daily using the CCA up to 6 days prior to inoculation and for the duration of the runs (10–21 days). The data were not normally distributed ($p = 0.0239$) and thus the Wilcoxon matched-pairs signed rank test (two-tailed) was used to compare the medians of the [H+] values from the CCA to those from the SU OPT in the bioreactors. The pairing was effective ($r = 0.49$;

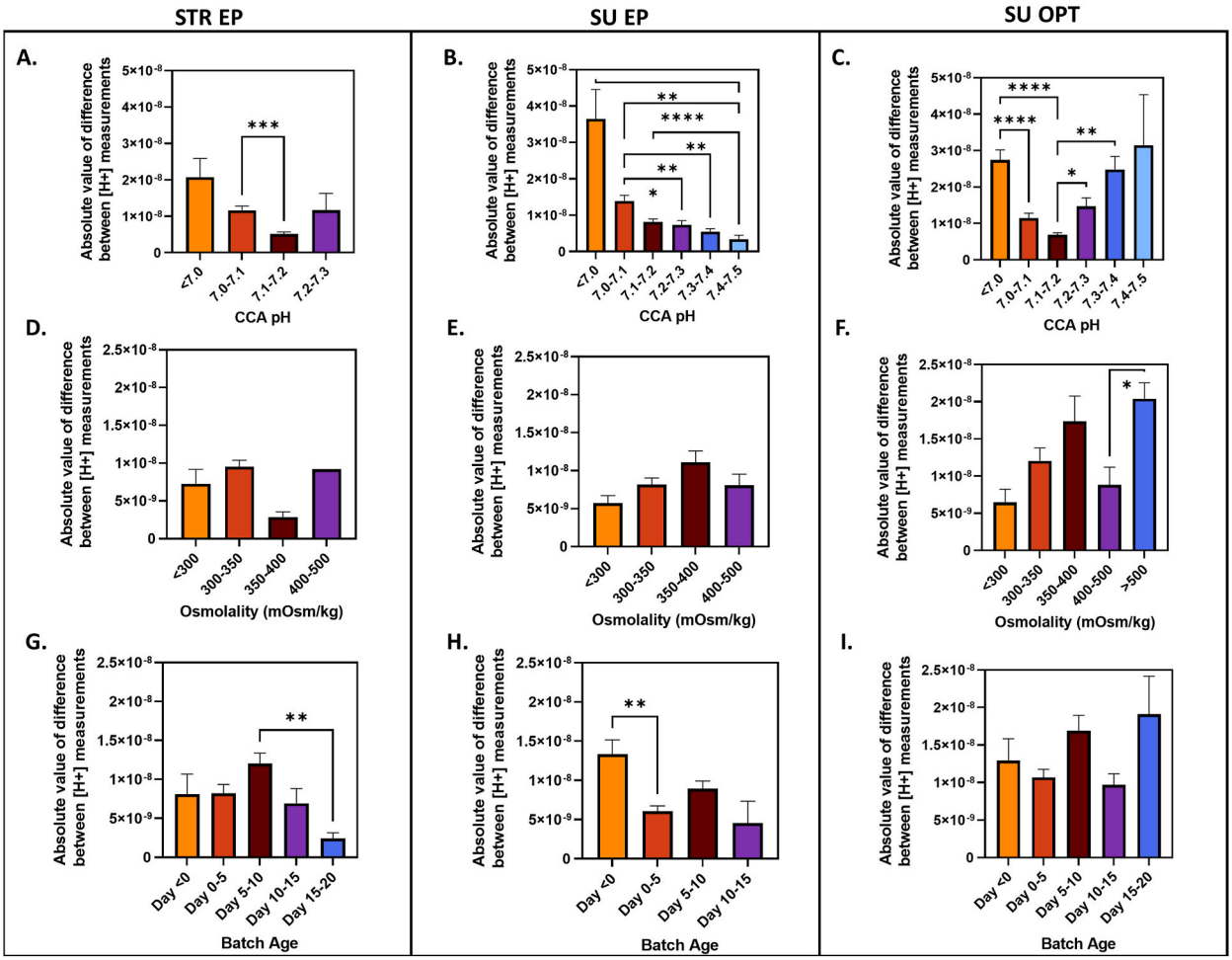

Fig. 2. Discrepancy between bioreactor pH sensors and at-line CCA measurements grouped by pH, osmolality, and batch age. Statistical significance was determined by Dunn's multiple comparisons tests. p value summaries are $p < 0.05$ (*), $p < 0.01$(**), $p < 0.001$ (***) and $p < 0.0001$(****). Error bars represent standard error of the mean.

$p < 0.0001$) and the medians are significantly different ($p < 0.0001$), like the SU EP median comparisons and STR EP mean comparisons. When we observe the correlation plot of the [H+] values (Fig. 1G), a linear correlation is not apparent as is the case for the STR EP and SU EP data sets. The clustering of the SU OPT data around the set point (pH 7.1) is clear in the frequency distribution plot (Fig. 1H), demonstrating that the optical pH sensors were not always responsive to actual changes in bioreactor pH. The SU OPT data do span nearly the entire range of the paired CCA data, which when observed as separate runs indicate that there may have been some lot-to-lot variability in the optical sensor performance.

The Bland-Altman plot comparing the CCA to SU OPT (Fig. 1I) shows the difference of the measurements as a function of the average of the two measurements and the bias (average discrepancy) is −0.005 pH units with 95% limits of agreement from −0.201 to 0.192 pH units. While the bias is the lowest for the SU OPT data set, the 95% limits of agreement are the largest. In this case, the bias is not capturing the true discrepancy between the CCA and SU OPT because the discrepancies occur evenly as either high or low values, which ends up canceling out the bias when averaged. However, the Bland-Altman plot offers another way of visualizing the lack of SU OPT sensor responsiveness as seen in the frequency distribution; where the data points appear to cluster in a line is due to SU OPT data being heavily centered about the mean (Fig. 1I). One can also see that the points that fall outside of the 95% limits of agreement (and are therefore significantly different from the CCA measurement by more than −0.201 or 0.192 pH units) are slightly more likely to occur above pH of 7.1, indicating that the SU OPT was not measuring the increase in pH in the bioreactor above setpoint. In summary, the SU OPT showed poorer performance than both the STR EP and SU EP.

3.5. Correlation of pH sensor accuracy and pH range

To assess any relationship between the pH being measured and the accuracy of the STR EP, SU EP and SU OPT, we grouped the absolute value of the difference between [H+] measurements by the CCA pH value (Fig. 2). We used differences in [H+] since pH units

are transformed from a logarithmic function, and therefore a 0.1 pH unit difference from a pH 6.7 to 6.8 ($\Delta[H+] = 4.1 \times 10^{-8}$) is five times more than a 0.1 pH unit difference from pH 7.4 to 7.5 ($\Delta[H+] = 8.2 \times 10^{-9}$). However, we grouped by CCA pH measurement ranges instead of [H+] to make the evaluation more applicable. For STR EP (Fig. 2A), since only the data set between pH 7.1–7.2 was normally distributed, a Kruskal-Wallis and Dunn's multiple comparisons tests were used to evaluate differences among groups. The medians of the grouped STR EP data were significantly different by Kruskal-Wallis (Kruskal-Wallis statistic = 21.52; $p < 0.0001$), where the Kruskal-Wallis statistic is the sum of the discrepancies among the rank sums, and the follow-up of the Dunn's multiple comparisons test indicated a significant difference between pH 7.0–7.1 and pH 7.1–7.2 ($p = 0.0003$). Why the STR EP measurements were more accurate between pH 7.1–7.2 versus pH 7.0–7.1 is not clear.

For SU EP (Fig. 2B), there was an interesting trend of increasing accuracy with increasing pH that is not apparent for the other pH sensor types. The medians of the grouped SU EP data were significantly different by Kruskal-Wallis (Kruskal-Wallis statistic = 37.87; $p < 0.0001$) and the follow-up of the Dunn's multiple comparisons test showed significant differences between several groups as indicated in Fig. 2B.

For SU OPT (Fig. 2C), there was a trend of decreasing accuracy with respect to increases or decreases from the setpoint pH of 7.1. The medians of the grouped SU OPT data were significantly different by Kruskal-Wallis (Kruskal-Wallis statistic = 53.23; $p < 0.0001$) and the follow-up of the Dunn's multiple comparisons test showed significant differences between several groups as indicated in Fig. 2C. Prior to bioreactor inoculation, a pH offset is always performed to ensure that the pH is at set point prior to the addition of cells. For these runs, the pH set point was 7.1 for all bioreactors and thus it makes sense that there are accurate measurements near the pH setpoint. The manufacturer also indicates that accuracy is better within 0.25 pH units from the offset calibration versus when it is 0.25–0.5 pH units from the offset calibration (Table 1) Inaccuracy as the pH increases or decreases from set point indicate a lack of responsiveness/sensor sensitivity of the SU OPT. While the inaccuracy did vary somewhat from sensor to sensor for the four cell bags tested, all bioreactor runs individually exhibited a trend of sensor drift to some degree. When using SU OPT in the future, especially when process pH is being controlled within a small range or when pH is known to affect product quality, checking pH at-line daily and performing regular offsets will be prudent practice to ensure that the SU OPT pH is not deviating too far from the actual pH.

3.6. Correlation of pH sensor accuracy and osmolality

Since increased osmolality is known to affect optical sensors, we were interested if high osmolality had a role in the SU OPT accuracy. To assess any relationship between the osmolality and the accuracy of the STR EP, SU EP and SU OPT, we grouped the absolute value of the difference between [H+] measurements by the osmolality (Fig. 2D–F). Like the data sets grouped by pH range, the data grouped by osmolality were not normally distributed for all groups and thus a Kruskal-Wallis and Dunn's multiple comparisons tests were used to evaluate differences among groups when appropriate. For STR EP and SU EP, as expected, there was no correlation between osmolality and sensor accuracy (Fig. 2D and E) and the medians of the grouped data were not significantly different by Kruskal-Wallis (Kruskal-Wallis statistic = 6.719 and 7.328, respectively; $p = 0.0814$ and 0.0621, respectively). However, for SU OPT the medians of the grouped data were significantly different (Kruskal-Wallis statistic = 15.03; $p = 0.0046$) and the follow-up of the Dunn's multiple comparisons test showed a significant difference between osmolality of 400–500 and > 500 ($p = 0.0131$). While there is a consistent trend of increasing difference in SU OPT and CCA [H+] measurements from <300 to 350–400 mOsm/kg, the increases were not significant. Although there is statistical significance between 400 and 500 and > 500 mOsm/kg, the lack of a clear trend of increasing inaccuracy aligning with increased osmolality does not allow us to conclude any relationship between osmolality and SU OPT accuracy from our data sets.

3.7. Correlation of pH sensor accuracy and bioreactor cell growth phase

Since osmolality increases during the average batch or fed-batch bioreactor run, and pH calibrations or offsets occur at the start of each run, we hypothesized that there may be covariance between pH range and/or osmolality with batch age. We grouped values for the absolute difference between [H+] by the CCA and bioreactor pH sensor in 5-day increments. Again, the data sets were not normally distributed for all groups and thus a Kruskal-Wallis and Dunn's multiple comparisons tests were used to evaluate differences among groups when appropriate. For STR EP (Fig. 2G), the medians of the grouped data were significantly different by Kruskal-Wallis (Kruskal-Wallis statistic = 17.00; $p = 0.0019$) and there was a significant difference between batch age groups by Dunn's multiple comparisons test between Day 5–10 and Day 15–20 ($p = 0.0011$). We hypothesize that the sensor accuracy appeared better between days 15–20 because the data were only from a single bioreactor run (STR-4) and the pH was very stable due to steady-state perfusion operation. Conversely, between days 5–10, the cultures were in flux. STR-1 and STR-2 were operated in fed-batch and the multiple feeds that occurred between days 5 and 8 resulted in system pH readjustments. For STR-3 and STR-4, perfusion and cell bleed rates were being adjusted daily. For SU EP (Fig. 2H), the medians of the grouped data were significantly different by Kruskal-Wallis (Kruskal-Wallis statistic = 15.13; $p = 0.0017$) and there was a significant difference between batch age groups by Dunn's multiple comparisons test between day <0 and days 0–5 ($p = 0.0018$). We believe this difference in sensor accuracy is due to the initial hydration step required for SU EP, which are stored dry unlike typical reusable electrochemical pH probes, requiring some additional time to soak in the culture medium for ≥4 h to equilibrate. For optimal performance, it seems ideal to let the probe soak overnight or even ≥1 day.

Lastly, the medians of the grouped SU OPT data (Fig. 2I) were not significantly different by Kruskal-Wallis (Kruskal-Wallis statistic = 6.781; $p = 0.1479$). This was surprising because we had hypothesized that the most significant sensor drift over time would occur with the SU OPT. There were trends of SU OPT signal drift during individual runs, but the drift was not enough to result in a statistically significant correlation between sensor accuracy and batch age.

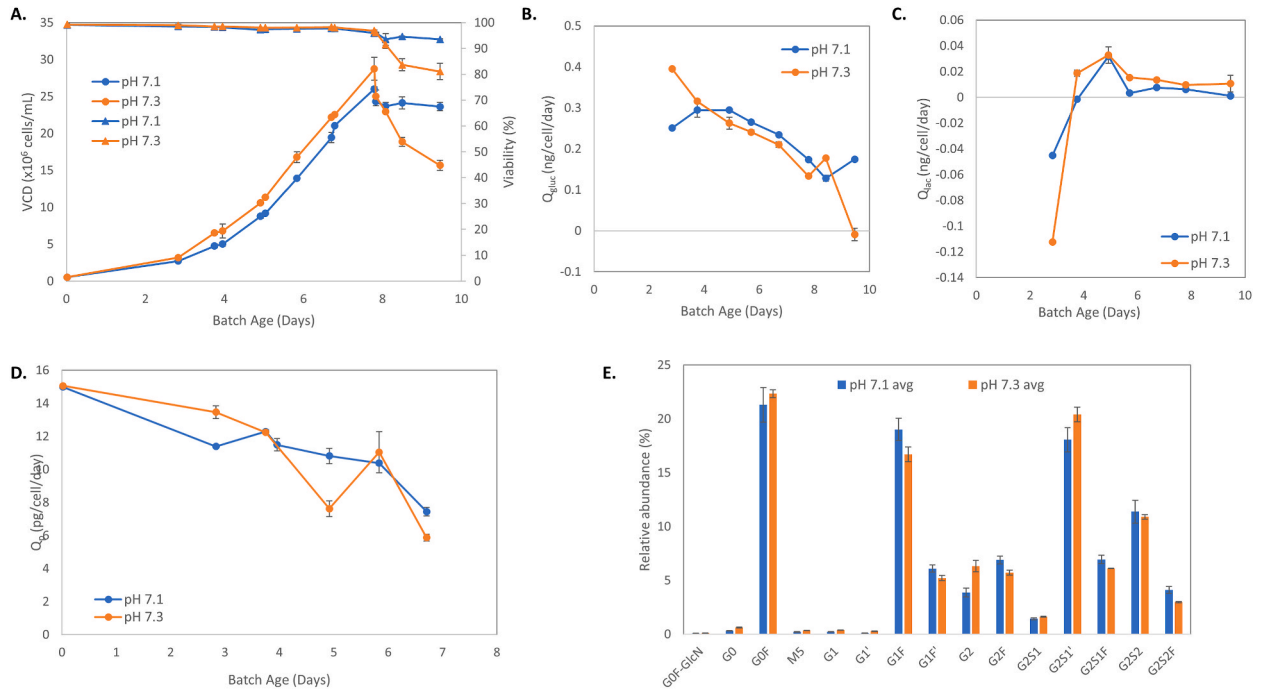

Fig. 3. Bioreactors generating mAb produced in VRC01 with set points of pH 7.1 and pH 7.3. (A) Cell growth and viability, (B) glucose consumption, (C) lactate production and consumption, (D) cell-specific productivity, and (E) mAb N-glycans under pH 7.1 and 7.3 conditions. Error bars represent standard error of the mean.

3.8. Cell growth, metabolite consumption, productivity and N-glycans under pH varied pH conditions

Since the 95% limits of agreement were as large as −0.201 and 0.192 pH units in the case of the SU OPT data set, we tested the effect of changing the pH setpoint by 0.2 pH units from 7.1 to 7.3 on the performance of the CHO cell culture in terms of its growth, metabolism, productivity, and mAb glycosylation. For this experiment we used our multi-parallel single-use STR system with SU EP because it was the most amenable to the experimental design of parallel bioreactor runs. The pH 7.3 condition led to slightly higher VCD beginning on day 4 compared to the pH 7.1 condition and resulted in a drop in cell viability after day 8 that was not seen at pH 7.1 (Fig. 3A). The specific glucose (Fig. 3B) and lactate consumption rates (Fig. 3C) were similar between the pH 7.1 and 7.3 conditions. This was not entirely expected for lactate as its metabolism is known to be affected by pH conditions [7,18]. However, the concentration of accumulated lactate is very low for this process with peak lactate concentrations ranging from 0.27 to 0.73 g/L, and concentrations below 0.1 g/L are out of analytical range for the CCA, making any differences among conditions difficult to discern for this process. The specific productivity was similar between the pH 7.1 and 7.3 conditions, with some slight variability from day-to-day (Fig. 3D). Reducing the pH setpoint during the production phase of CHO cells is a commonly used strategy to improve cell specific productivity [19]. However, to expect pH to affect titer, a pH below 7.0 may be necessary to discern a difference from pH 7.3 for this process. Lastly, we analyzed the relative abundance of N-glycans from the mAb purified from the harvested cell culture fluid between the pH 7.1 and pH 7.3 growth conditions (Fig. 3E). The patterns are very similar for overall relative glycosylation, sialyation, and high mannose species, with a slight trend of reduced fucosylation at the pH 7.3 condition (59.10% ± 2.34% versus 64.40% ± 3.75%). Additional replicates, and likely a lower pH condition, would be needed to test for significance of the effect of reduced pH on fucosylation for the VRC01 cell line.

Overall, these observations are like prior reports in the literature. A previous study showed miniature bioreactors with fluctuations between pH 7.0–7.5 had minimal impact on growth, productivity, and product quality, but the authors observed some changes in lactate metabolism [11]. In addition, product quality analysis yielded varied results for which two cell lines showed an increase in the G0F glycan and decrease in G1F, G2F, and Man5, but another cell line showed the opposite trend, suggesting that the response of CHO cells to the effects of fluctuating culture conditions is somewhat cell line-specific [11]. Some investigators note no significant changes in afucosylation levels in their CHO cell lines [10,20], while others have reported changes in afucosylation when applying either acidic or basic pH shifts [14,21,22]. In summary, the effect of a pH shift, whether intentional or unintentional, can impact cell growth and productivity, as well as product quality attributes, and the potential changes induced by pH excursions should be a well-understood aspect of any biomanufacturing process.

Table 3
Summary of pH sensor absolute discrepancies, limits of agreement, and correlations of [H+] discrepancy to pH, osmolality, and batch age.

pH Sensor type	Average absolute discrepancy (pH units)[a]	95% Limits of Agreement		Correlation[b] of discrepancy to:		
		From	To	CCA pH	Bioreactor Osmolality	Batch Age
STR EP	0.047	−0.129	0.049	Yes (***)	No	Yes (**)
SU EP	0.044	−0.172	0.089	Yes (****)	No	Yes (**)
SU OPT	0.072	−0.201	0.192	Yes (****)	Yes (*)	No

[a] Calculated using the average discrepancy in [H+] measurements and transformed into pH difference from pH 7.1.
[b] Correlation refers to the statistical significance by Kruskal-Wallis test; p value summaries are $p < 0.05$ (*), $p < 0.01$(**), $p < 0.001$ (***) and $p < 0.0001$(****).

4. Conclusion

In this study we evaluated three types of in-line pH sensors: traditional autoclavable electrochemical probes (STR EP), single-use electrochemical probes (SU EP), and single-use optical sensors (SU OPT). We found that the average absolute discrepancy between the electrochemical probes (STR EP and SU EP) and the at-line measurement from our cell culture analyzer (CCA) was less than that of the SU OPT (Table 3). For all three in-line sensor types, there was a correlation between the CCA pH and the discrepancy between the in-line sensor and CCA. In particular, the SU EP appeared more accurate as pH values increase from <7.0 to 7.5, while SU OPT were most accurate around the pH 7.1 set point with decreasing accuracy both as pH decreased or increased from set point. While there was a single correlation noted between the bioreactor culture osmolality and the discrepancy between the SU OPT and CCA at high osmolality, osmolality was not correlated to measurement discrepancies for STR EP and SU EP and overall was not a significant factor in sensor discrepancies. There was a correlation between the batch age and the discrepancies for STR EP and SU EP, but none identified for SU OPT. For the STR EP, we hypothesize that the sensor accuracy appeared better during late culture because those data were from a single bioreactor run and taken during steady-state perfusion operation, while between days 5–10, all cultures were undergoing rapid growth. For SU EP, the correlation with batch age suggests that the initial accuracy after the minimum recommended hydration may not be ideal.

Lastly, when subjected to highly similar processes that differed only in pH set points of pH 7.1 and 7.3, our VRC01 cell line and mAb exhibited only minor changes in cell performance and N-glycan modifications. Overall, this work demonstrates that understanding how tightly the pH range needs to be controlled for a given process is critical and implementing an appropriate pH control strategy that incorporates understanding of the accuracy of the bioreactor pH sensor is key to maintaining the desired monoclonal antibody product quality during upstream biomanufacturing.

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CRediT authorship contribution statement

Erica J. Fratz-Berilla: Writing – review & editing, Writing – original draft, Visualization, Project administration, Methodology, Investigation, Formal analysis, Conceptualization. **Casey Kohnhorst:** Writing – review & editing, Investigation. **Nicholas Trunfio:** Writing – review & editing, Formal analysis. **Xin Bush:** Investigation. **Aron Gyorgypal:** Investigation. **Cyrus Agarabi:** Supervision, Funding acquisition.

Declaration of competing interest

The authors have declared that no competing interests exist.

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Appendix A. Supplementary data

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