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ARTICLE

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Mimicking CHO large-scale effects in the single multicompartment bioreactor: A new approach to access scale-up behavior

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Abstract

During the scale-up of biopharmaceutical production processes, insufficiently predictable performance losses may occur alongside gradients and heterogeneities. To overcome such performance losses, tools are required to explain, predict, and ultimately prohibit inconsistencies between laboratory and commercial scale. In this work, we performed CHO fed-batch cultivations in the single multicompartment bioreactor (SMCB), a new scale-down reactor system that offers new access to study large-scale heterogeneities in mammalian cell cultures. At volumetric power inputs of 20.4–1.5 W m^{-3}, large-scale characteristics like long mixing times and dissolved oxygen (DO) heterogeneities were mimicked in the SMCB. Compared to a reference bioreactor (REFB) set-up, the conditions in the SMCB provoked an increase in lactate accumulation of up to 87%, an increased glucose uptake, and reduced viable cell concentrations in the stationary phase. All are characteristic for large-scale performance. The unique possibility to distinguish between the effects of changing power inputs and observed heterogeneities provided new insights into the potential reasons for altered product quality attributes. Apparently, the degree of galactosylation in the evaluated glycan patterns changed primarily due to the different power inputs rather than the provoked heterogeneities. The SMCB system could serve as a potent tool to provide new insights into scale-up behavior and to predict cell line-specific drawbacks at an early stage of process development.

KEYWORDS
CHO fed-batch cultivations, gradients, large-scale heterogeneities, multicompartment system, scale-down, SMCB

1 | INTRODUCTION

The sector of biopharmaceuticals continues to increase its market share and the portfolio of recombinant therapeutic proteins is expanding rapidly (Kaplon et al., 2023). The global COVID-19 pandemic further accelerated innovations in this field and arose public awareness of the biotherapeutics' potential (Walsh & Walsh, 2022). Despite the commercial success of biotherapeutics, their development remains in the high-risk segment requiring high investments and extensive development phases before approval (Bhattacharya & Bhattacharya, 2023).

Within biopharmaceutical research and development, the efficient and cost-effective scale-up/scale-down of bioprocesses is a key aspect (Metze et al., 2020). Maintaining productivity and product quality across scales is crucial to fulfill the approved product specifications and keep production processes economically viable. However, understanding the mechanisms of reproducible and predictable scaling procedures remains in the focus of the biopharmaceutical research. Due to the fragility of mammalian cells, cell culture processes abstain from high power inputs and aggressive mixing (Nienow, 2015). The imposed constraints promote the formation of heterogeneities at larger scales which in turn prohibit the accurate prediction of industrial-scale performance based on lab-scale data (Lara et al., 2006). The increasingly heterogeneous environment during scale-up was reported to entail for example reduced productivities, enhanced by-product formation, and altered product quality attributes (Karimi Alavijeh et al., 2022; Xu et al., 2018). Consequently, tools are required to unravel the underlying mechanisms of observed scale-up effects and to reliably predict industrial-scale performance. Beside the computational analysis of so-called lifelines (Haringa, 2023; Hofmann et al., 2022; Kuschel & Takors, 2020), next generation scale-down devices for wet-lab analysis are needed to complete the simulation studies.

Over the last years, the number of studies addressing the issue of heterogeneities in industrial scale mammalian cell bioreactors increased. Many publications focused on the consequences of pH heterogeneities, using either oscillating pH conditions in a single bioreactor or multibioreactor set-ups to simulate alkali zones in production-size bioreactors (Brunner et al., 2017; Jiang et al., 2018; Langheinrich & Nienow, 1999; Nienow et al., 2013; Osman et al., 2001; Paul & Böttinger, Mitic, et al., 2020; Zakrzewski et al., 2022). Fewer studies applied similar scale-down systems to address the issue of oxygen heterogeneities at larger scales (Anane et al., 2021; Serrato et al., 2004; Zakrzewski et al., 2022).

Single bioreactor experiments applying oscillating conditions are comparably easy to setup. However, they might be limited in their realism since all cells are simultaneously exposed to the exerted effect and additional undesired effects might be introduced (Paul & Herwig, 2020). The often-used multicompartment systems, consisting of two or more connected bioreactors, are considered more realistic since only a fraction of the cells is exposed to the effect per time (Neubauer & Junne, 2010). Moreover, a more precise and isolated simulation of the effects is possible (Paul & Herwig, 2020). On the downside, apart from an increased contamination risk, the necessity of pumping the cells through those systems holds the potential to impose additional stress on the cells (Nienow et al., 2013). Furthermore, while these approaches provide interesting insight into the impact of single factor variations, they often lack the possibility to identify unknown impact factors or potential interaction effects that might influence real case scenarios.

In our previous study, we proposed a single multicompartment bioreactor (SMCB) as a new alternative including the multicompartment aspect while avoiding additional pumping stress (Gaugler et al., 2023). Advancing from the earlier approach of Schilling et al. (Schilling et al., 1999) compartment discs were designed and characterized to enable the tailored access to large-scale mixing times and compartment formation. Utilizing the findings from the preceding characterizations, this first cultivation study in the SMCB evaluates fed-batch cultivations with Chinese hamster ovary cell line CHO DP12 across three hypothetical scales. Since pH value variations have been studied abundantly by other authors, this factor was not included in this generic scale-up scenario. Instead, the goal of this study was to investigate cultivation environments under impaired mixing conditions from a more holistic perspective, allowing the identification of new potential access points to observed scale-up effects.

2 | MATERIAL AND METHODS

2.1 | SMCB and reference bioreactor set-up

Fed-batch cultivations were performed in Dasgip two-fold bioreactors (Eppendorf) with customized glass vessels (Laborgerätebau Ochs e.K.). In Figure 1 the set-up of the SMCB and the reference bioreactor (REFB) are illustrated.

The glass vessels were manufactured with five horizontal sampling ports in which additional glass tubes were inserted to ensure sampling from the moving liquid. The bioreactors were equipped with a cell culture-typical stirrer combination of Rushton turbine and pitched-blade turbine. The REFB represented a conventional cell culture lab scale bioreactor set-up without baffles and with inline probes to monitor dissolved oxygen (DO) and pH (InPro; Mettler-Toledo GmbH). In the SMCB, a compartment disc was installed inside the bioreactor below the second sampling port and above the second DO spot. The installation height of the disc was similar to the configuration in our previous characterization study (Gaugler et al., 2023) and created two similar-sized compartments at the final working volume of 3.25 L. In the attempt to further approach the large-scale environment in the SMCB, baffles were added to the reactor and optical sensor spots (PreSens) were used to monitor DO and pH. This way, nonscalable flow characteristics introduced by long probes were avoided and multiple measurement points for DO and pH could be realized.

2.2 | Cell line, seed train, and cultivation conditions

All cultivation experiments were carried out with a suspension-adapted CHO DP12 cell line producing an anti-interleukin-8 IgG1 antibody. The CHO cells were cultivated in chemically defined TC-42 medium (Xell AG) supplemented with 4 mM L-glutamine and for the first three subcultivations of the seed train with 200 nM Methotrexate. The seed trains were performed in five subcultivation steps in shake flasks (Corning Inc.) ranging from 125 to 1000 mL at 37°C, 5% CO_2 and 150 rpm (50 mm displacement) in a humidified incubator (Infors AG).

The fed-batch experiments were inoculated with a viable cell concentration (VCC) of 3×10^5 cells mL^{-1} and a starting volume of 2.6 L. The pH was set to 7.10 ± 0.05 using 1 M Na_2CO_3 as base and CO_2

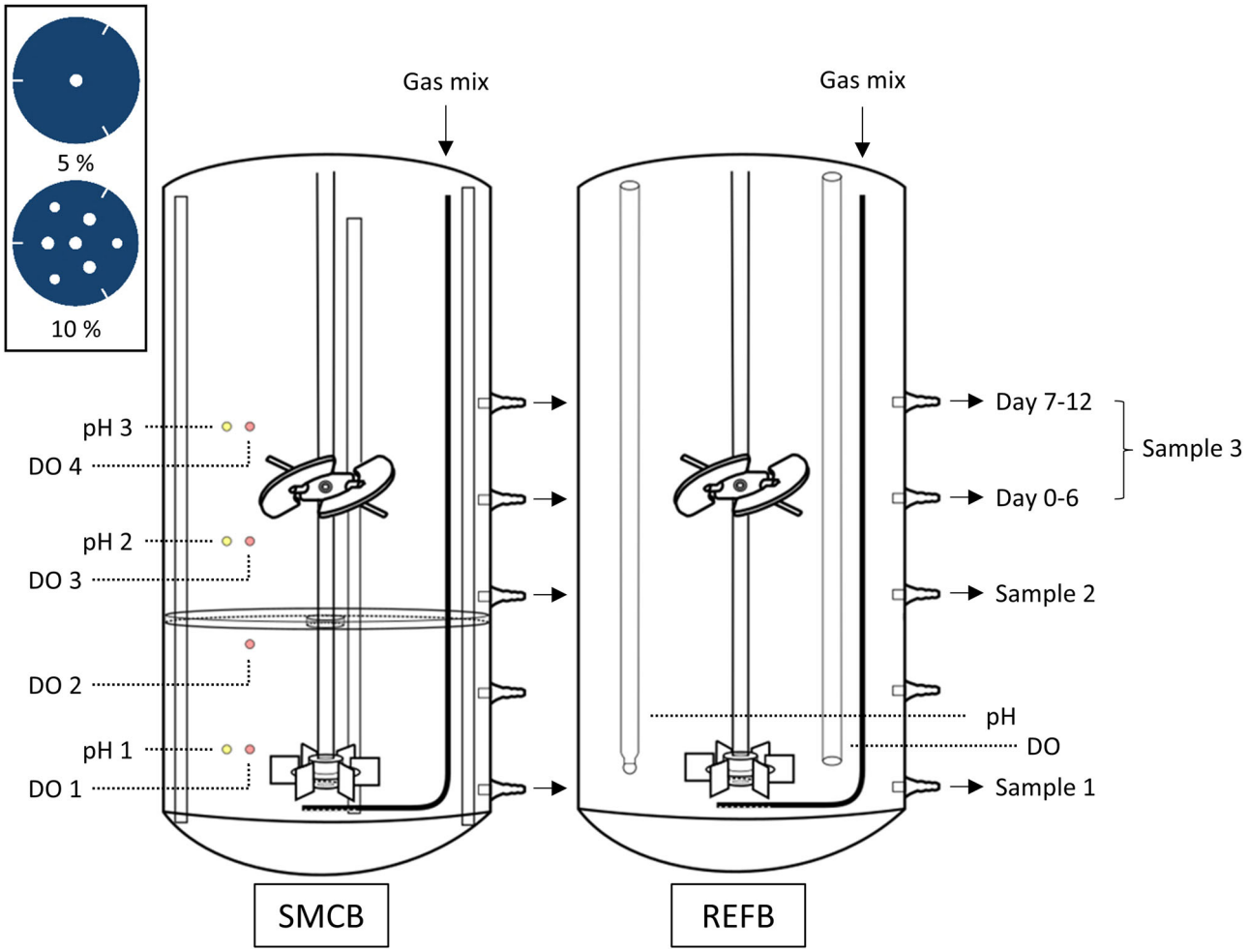

FIGURE 1 Schematic illustration of the single multicompartment bioreactor (SMCB) and the reference bioreactor. Rectangle: Compartment discs installed in the SMCB; percentages indicate the exchange area provided by the respective disc between the compartments.

as acid. The DO and the process temperature were controlled at 40% ± 2% and 37°C, respectively. 0.2 x Antifoam C (Merck KGaA) was added manually on demand. After an initial batch phase, continuous feeding with the feeding medium TCX2D + 40 g L^{-1} Glucose (Xell AG) was started 72 h after inoculation. After sampling, at glucose concentrations below 4 g L^{-1}, 500 g L^{-1} glucose was added as a bolus to replenish glucose levels of 4 g L^{-1}. The feeding was increased every 24 h corresponding to a daily feeding volume of 3.66% of the respective reactor volume after sampling. To account for incalculable volume additions due to base control, antifoam addition and glucose bolus feeds, volume compensation samples were taken. The fed-batch experiments were terminated after 12 days at a final volume of 3.25 L and a total volume increase of 20%, respectively.

2.3 | Scale-up study design

Based on previous mixing time and power input determinations in the SMCB (Gaugler et al., 2023), fed-batch cultivations were performed at three power inputs, simulating changing mixing times, and mass transfer across three hypothetical scales (Table 1). The mixing times represent macro mixing times and were evaluated via an optical pH-dependent method at an acid-to-base ratio of 1:5 (Fitschen et al., 2021; Godleski & Smith, 1962). SMCB cultivations were performed in biological duplicates and REFB cultivations as single runs.

For the condition with the highest power input, representing the smallest scale, a compartment disc providing an exchange area of 10% of the total cross-sectional reactor area was installed (Figure 1).

TABLE 1 Power inputs and mixing times applied in generic scale-up scenario.

	Mixing time $t_{95\%}$ (nongassed)	
Power input (W m^{-3})	SMCB (s)	REFB (s)
20.4	26	7
6.4	54	10
1.5	97	15

Abbreviations: REFB, reference bioreactor; SMCB, single multicompartment bioreactor.

For the other two conditions a disc with an exchange area of 5% was chosen to prolong mixing times and to mimic the decreasing mass transfer at larger scales (Xing et al., 2009). As described in our previous study, the combination of two similar-sized compartments and especially the macro mixing times at 1.5 W m^{-3} were designed to mimic the conditions observed in a 15 kL bioreactor, representing the largest scale simulated in this study (Rosseburg et al., 2018).

To enable the integration of potential DO heterogeneities into the simulated scale-up scenario, the optical spot furthest away from the point of gas entry was intended as control spot. Gas addition was realized at the reactor bottom. However, since the highest located spot DO4 remained above the liquid level for the first 4 days of the cultivations, the spot DO3 was chosen as DO control spot.

In the applied SMCB set-up with one compartment disc, the mixing of agents introduced from above proceeds comparably fast in the upper compartment. Since feeding and base control were realized via surface-addition the formation of an alkali zone was not expected. Nevertheless, to ensure the comparability of pH control in future SMCB studies that may include the creation of alkali zones, the choice of the pH control spot followed the same logic as for DO heterogeneities. Accordingly, the spot pH1, being furthest away from the point of base addition, was used for pH control. To compare the impact created by the SMCB to the general effects that originate from cultivating at different power inputs, single reference cultivations were performed in the REFB set-up.

2.4 | Sampling and extracellular analytics

Samples were taken once a day at three different heights (Figure 1) to enable the detection of potential differences across the bioreactor. To account for the rising liquid level during fed-batch, the highest sampling port was changed on Day 7. The upper-most port was chosen as top sampling point once permanently immersed in the liquid to take samples near the liquid surface over the whole course of the fed-batch experiment. VCC and viability were determined via holographic microscopy using a fluidlab R-300 device (anvajo GmbH). Glucose and lactate concentrations were quantified with a LaboTrace automatic analyzer (TraceAnalytics GmbH). Osmolality was determined by freezing point depression with an Osmomat 030 (Gonotec GmbH). Remaining samples were centrifuged at 230*g* and 4°C for 10 min and supernatants were stored at −70°C. From Day 4 ongoing, a part of each sample containing 200×10^5 cells was centrifuged separately and the pellets were stored at −70°C. Determination of antibody concentrations was performed by enzyme-linked immunosorbent assay (ELISA) as described by Pfizenmaier et al. (Pfizenmaier et al., 2015).

2.5 | Estimation of cell-specific parameters and statistics

Daily cell-specific rates were estimated from extracellular concentrations C, reactor volume before sampling V and VCCs over time according to the following equation.

$$q_{c_i} = \frac{V(C_i - C_{i-1})}{t_i - t_{i-1}} \left(\frac{V(\mathrm{VCC}_i + \mathrm{VCC}_{i-1})}{2} \right)^{-1}. \quad (1)$$

2.6 | Glycan analysis

Supernatants were thawed and the produced antibody was purified and concentrated using Protein A HP SpinTrap™ columns (Cytiva). Eluates were shipped to an external provider and glycan profiles were analyzed via the xCGE-LIF based high-performance analysis system glyXboxCE™ (glyXera GmbH).

2.7 | Fibronectin analysis

Cell pellets were thawed on ice, washed with ice-cold 1 x PBS and lysed in 200 µL cell extraction buffer (Thermo Fisher Scientific) containing 1 x protease inhibitor cocktail and 1 mM PMSF. The mixture was incubated on ice for 30 min with occasional vortexing and subsequently centrifuged at 13,000*g* and 4°C for 10 min. The cell extracts were stored at −70°C until analysis. The fibronectin concentrations in the cell extracts were determined by ELISA according to the manufacturer's protocol using the mouse fibronectin matched antibody pair kit (Abcam).

3 | RESULTS

Understanding and ultimately predicting changes in product concentration and critical quality attributes during scale-up remains a key area of interest in the biopharmaceutical process development. This study showcases a new approach to evaluate potential scale-up effects and their interplay in the SMCB system.

3.1 | Process performances in the SMCB scale-up study

To investigate the influence of scale-up effects on the process performance, fed-batch cultivations were performed at three different power inputs, enabling the simulation of changing mixing behavior and mass transfer at different scales. The two-compartment configuration in the SMCB was designed to mimic the conditions observed in a 15 kL bioreactor that was operated by collaboration partners (Rosseburg et al., 2018). The applied power inputs were chosen in accordance with the design space characterized in our previous study with 1.5 W m^{-3} representing the largest scale and approaching the mixing conditions in the 15 kL benchmark bioreactor (Gaugler et al., 2023).

Figure 2 shows a selection of key process parameters for the fed-batch cultivations with the cell line CHO DP12 producing an anti-interleukin-8 IgG1 antibody. The sampling at different heights revealed no clear differences or gradients inside the bioreactors (Supporting Information S1: Figure 1). Thus, for a better visual

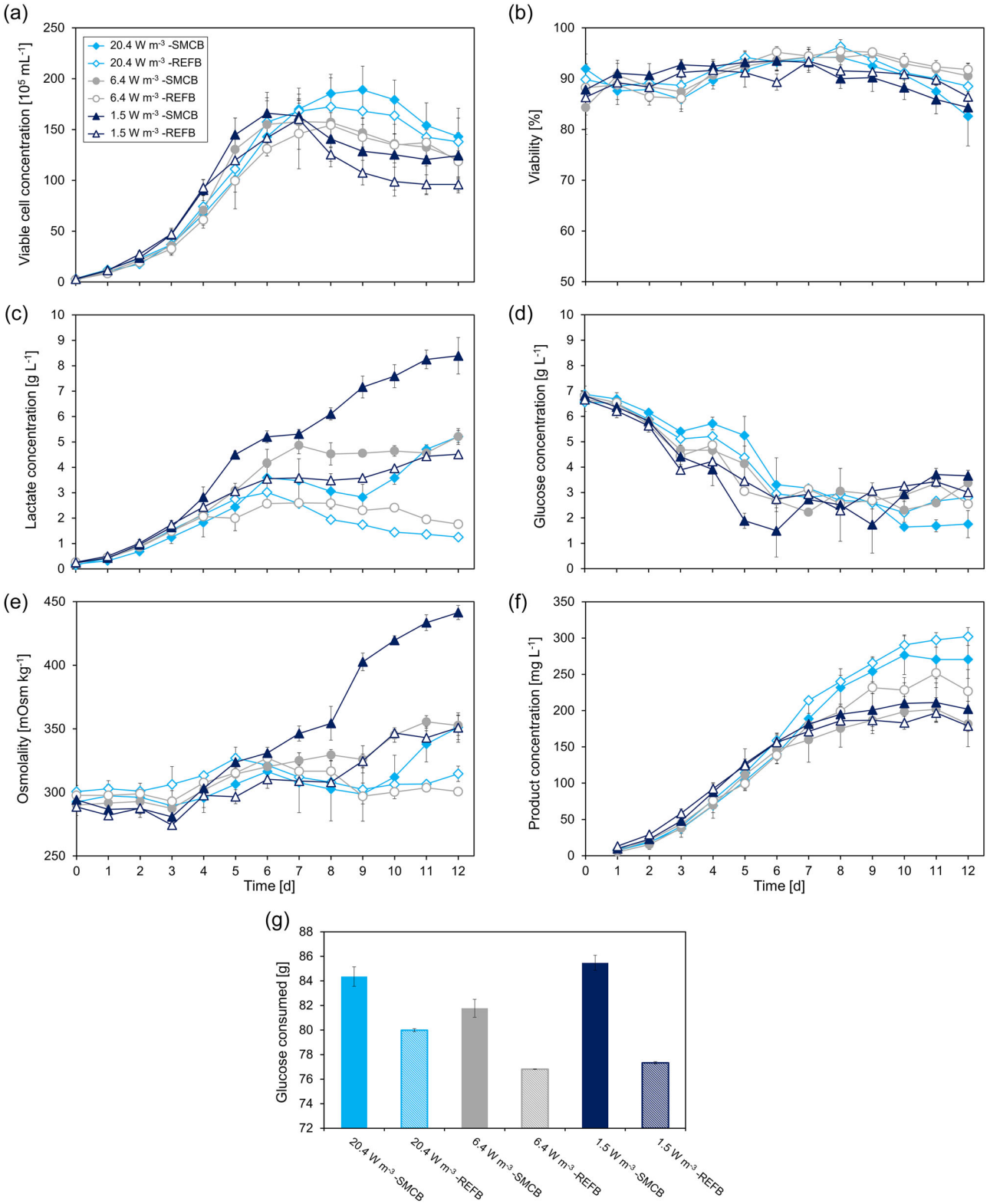

FIGURE 2 Process parameters of CHO DP12 fed-batch cultivations in the SMCB and REFB at three different power inputs. Profiles of VCC (a), viability (b), lactate (c) and glucose (d) concentrations, osmolality (e) and antibody concentration (f) as well as total amount of glucose consumed (f). SMCB runs were performed in biological duplicates. Samples were taken at three different heights and measurements were performed in triplicates. The error bars indicate the standard deviation. REFB, reference bioreactor; SMCB, single multicompartment bioreactor.

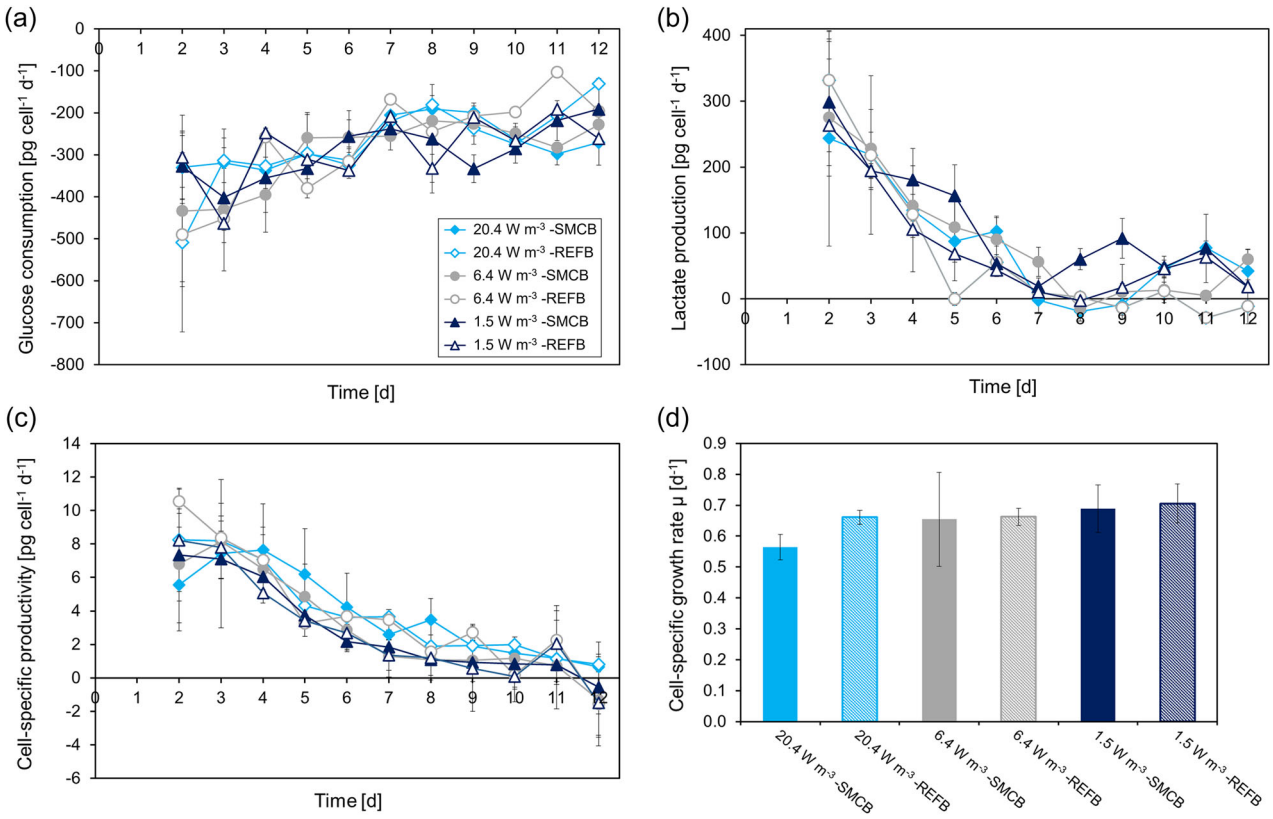

FIGURE 3 Process rates for fed-batch cultivations in the SMCB and REFB at three different power inputs. Daily cell-specific rates for glucose (a), lactate (b), and the produced antibody (c) and cell-specific growth rate (d) for the exponential growth phase (Day 1–4). SMCB runs were performed in biological duplicates. Samples were taken at three different heights and measurements were performed in triplicates. The error bars indicate the standard deviation. REFB, reference bioreactor; SMCB, single multicompartment bioreactor.

perception of the data, the mean values for the whole reactor per sampling time are presented in the following section.

The different power input settings impacted the growth behavior, the product and by-product formation as well as the substrate consumption in both bioreactor systems. While the viabilities remained on a high level for all evaluated scenarios (Figure 2b), the VCC profiles indicate an earlier decline of VCCs in the stationary phase with decreasing power inputs (Figure 2a). At 1.5 W m^{-3}, the initial growth seemed to increase slightly. Similar trends could be detected for the product formation (Figure 2f). For the initial hours of the cultivations, the increase in the product concentration was relatively similar for all conditions applied. From Day 7 ongoing, however, the product concentrations in the cultivations at 1.5 W m^{-3} and in the SMCB at 6.4 W m^{-3} remained on a similar level, while for the other conditions the product increase continued, reaching the highest antibody concentrations at 20.4 W m^{-3}.

The lactate production appears to be responsive to the changing power inputs as well, resulting in higher lactate concentrations with decreasing power inputs (Figure 2c). Furthermore, with the lactate production and due to the base control, the concomitant increase in the osmolality, key differences between the SMCB and the REFB can be found. After a similar initial lactate production phase in all cultivations, the lactate concentrations began to differ on Day 5. In the REFB at 20.4 W m^{-3} and 6.4 W m^{-3}, the cells switched to lactate net consumption for the second half of the process and lactate levels decreased for the rest of the cultivation. In contrast, in the SMCB at 20.4 W m^{-3} lactate was only temporarily consumed and after Day 9 lactate concentrations increased again. While similar end lactate concentrations were reached at 6.4 W m^{-3} in the SMCB, the overall lactate production was still higher, since no clear switch to lactate net consumption was visible. Ultimately, the cultivation conditions at 1.5 W m^{-3} led to a strongly enhanced lactate production in both systems, resulting in lactate concentrations above 8 g L^{-1} in the SMCB. Interestingly, the conditions in the REFB at 1.5 W m^{-3} seemed to have provoked similar lactate concentrations as the SMCB environment at higher power inputs.

Linked via the base addition for pH control, the osmolality profiles mirror the lactate profiles (Figure 2e). The SMCB cultivations at 20.4 W m^{-3} and 6.4 W m^{-3} and the REFB cultivation at 1.5 W m^{-3} reached similar final osmolality values. Consequently, the lactate concentrations in the SMCB at 1.5 W m^{-3} entailed the highest osmolalities, reaching final values near 450 mOsm kg^{-1}.

Owing to the daily glucose bolus feeds on demand, the glucose profiles appear similar for all conditions tested (Figure 2d). The only noticeable difference can be found in the SMCB at 1.5 W m^{-3}, exhibiting a comparably strong decrease in the glucose concentration from Day 4 to Day 6. However, when comparing the total amounts of

glucose consumed (Figure 2g), differences become visible. Apparently, the conditions in the SMCB led to an enhanced glucose consumption. Furthermore, differences in the glucose consumption for the different power input settings can be observed as well. However, contrary to the other evaluated parameters, with 6.4 W m^{-3} resulting in the lowest total glucose consumption, the differences do not directly correspond to the order of the applied power inputs.

When comparing the daily cell-specific glucose consumption rates, none of the different settings entailed a distinctly higher or lower rate (Figure 3a). Presumably, the smaller deviations were sufficient to exert a cumulative effect on the total amount of glucose consumed. Similarly, the calculation of the cell-specific productivity (Figure 3c) and the cell-specific growth rate (Figure 3d) revealed no profound differences between the evaluated conditions either. Thus, the differences in the product concentration seem to originate from the differing die-back behavior. Solely, the cell-specific lactate production rates (Figure 3b) differed for the second phase of the cultivations, agreeing with the pronounced differences in the lactate profiles. In general, all cell-specific rates decreased over time.

3.2 | DO heterogeneities in the SMCB

Notably, all evaluated process parameters were impacted by the varying cultivation conditions in this study. To further investigate the underlying reasons for the observed differences, potential heterogeneities were evaluated. Figure 4 shows the online DO and pH profiles in the REFB and at the different measurement points in the SMCB. The measurement signals of the upper-most DO and pH spot in the SMCB (DO4, pH3) were only included after 4.3 days once the spots remained permanently below the liquid surface.

In the REFB, the measured values for both DO and pH (Figure 4, purple lines) remained close to the setpoints of 40% and 7.1, respectively. Temporary drops and overshoots in the DO could be attributed to antifoam addition. As assumed previously, the pH signals in the SMCB (Figure 4d–f) revealed no overshoots or pronounced differences between the different measuring heights. Conversely, the combination of the compartment disc and the control spot in the upper compartment provoked pronounced differences between the DO levels in the upper and lower compartment of the SMCB (Figure 4a–c). While the DO values in the upper compartment were controlled close to the setpoint of 40%, the DO levels in the lower compartment decreased noticeably.

The difference between the two compartments intensified with decreasing power inputs and a reduced exchange area between the compartments. However, none of the measurement spots detected a full depletion of oxygen for the conditions tested.

Following the approach of Gao et al. (Gao et al., 2016) fibronectin levels were analyzed via ELISA to evaluate whether the observed reduction in the DO profiles was sufficient to trigger hypoxic cell responses (Figure 5). Fibronectin as a component of the extracellular matrix was reported to be upregulated upon hypoxia and thus serves as a biomarker for hypoxic stress reactions (Lokmic et al., 2012).

Fibronectin levels were analyzed for the lowest (SMCB/REFB1) and the highest sampling point (SMCB/REFB3). For the REFB run at 20.4 W m^{-3} no pellets were collected. Hence, the respective fibronectin data could not be provided. Increased amounts of fibronectin could be detected for the SMCB (Figure 5a) compared to the reference (Figure 5b). The comparison of the different ports indicated a relatively homogeneous fibronectin production across the reactor. The fibronectin production in the SMCB increased with higher power inputs, resulting in a considerable elevation of fibronectin levels at 1.5 W m^{-3}. Interestingly, the fibronectin levels for the REFB runs differed as well, exhibiting higher fibronectin values at 1.5 W m^{-3}.

3.3 | Deviations in the galactosylation at different power inputs

Glycosylation is considered the most relevant critical quality attribute of therapeutic protein production (Sumit et al., 2019). To check whether the investigated conditions impacted the product quality, protein A-purified end point samples were submitted to external glycan analysis. Figure 6 shows the most abundant glycoforms for the investigated scenarios. The complete glycosylation pattern including the rarer glycan species can be found in the Supporting Information S1: Figure 2.

The four depicted glycoforms account for more than 90% of all detected glycan structures. While the abundance of the G0F species decreased at lower power inputs, the G1F(1,6) and G1F(1,3) species became more abundant, indicating a rising degree of galactosylation at decreasing power inputs. From the analytical evaluation, the glycoforms G2F and Man9 cannot be distinguished. Based on the other highly abundant glycoforms, however, it seems reasonable to assume that the observed trend also encompassed the bigalactosylated glycan species.

4 | DISCUSSION

In this study, fed-batch cultivations with the IgG1 antibody-producing cell line CHO DP12 were performed at three different power inputs in the SMCB and the REFB set-up. The SMCB offers the possibility of a multifactorial scale-up evaluation in a multi-compartment reactor setup, while avoiding additional contamination risks or pumping stress. The REFB represents a conventional CHO cell culture lab scale bioreactor set-up.

4.1 | Differences originating from varying power inputs

The different power input settings provoked noticeable differences in both bioreactor systems, impacting the overall process performance

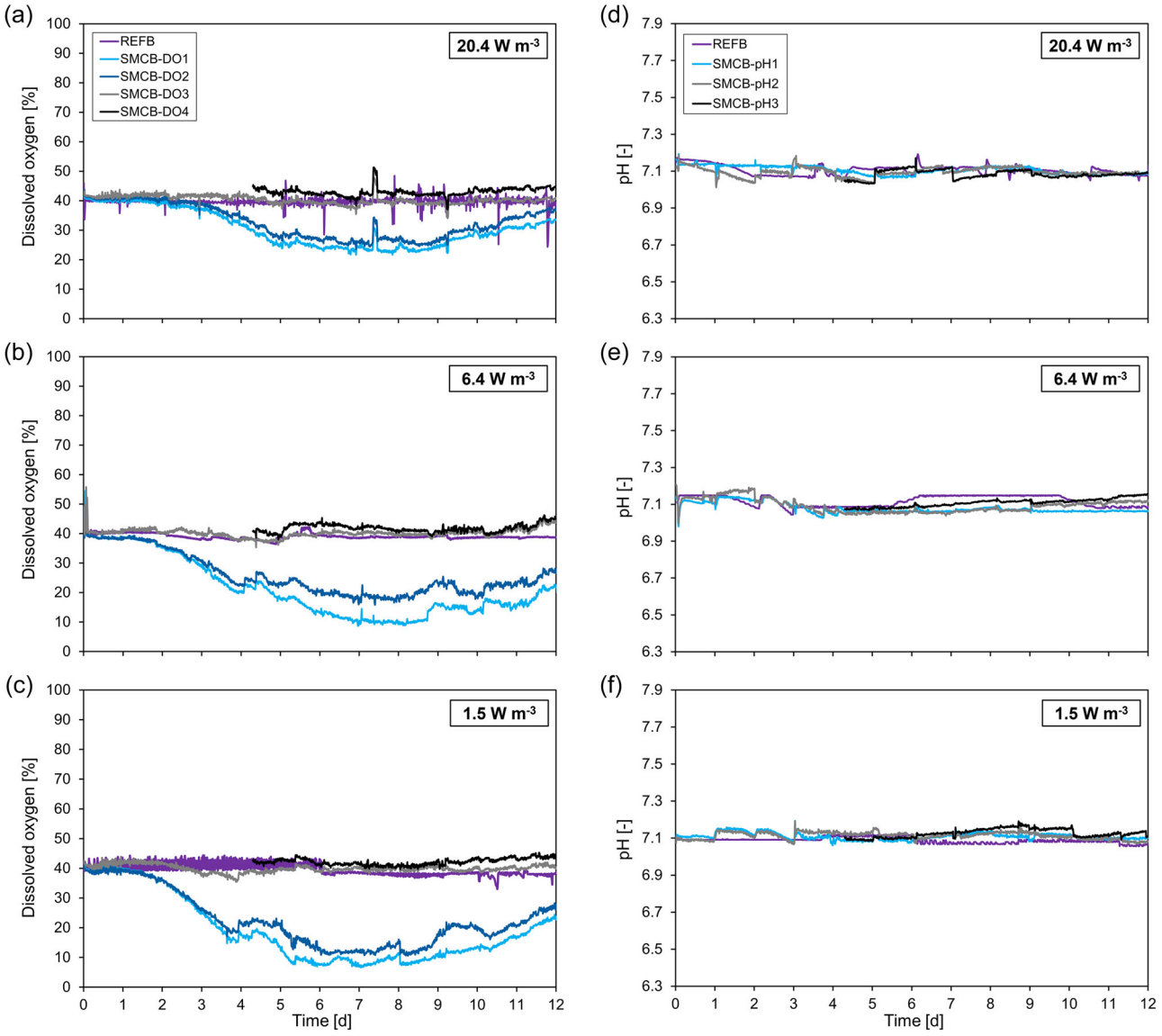

FIGURE 4 DO and pH profiles in the SMCB and REFB at three different power inputs. In the SMCB, DO (a) 20.4 W m^{-3}, (b) 6.4 W m^{-3}, (c) 1.5 W m^{-3}) and pH (d) 20.4 W m^{-3}, (e) 6.4 W m^{-3}, (f) 1.5 W m^{-3}) were monitored at different heights via optical sensor spots. The spots pH1 and DO3 were used as control spots. SMCB runs were performed in duplicates. Values for DO and pH were averaged over 10-min intervals. REFB, reference bioreactor; SMCB, single multicompartment bioreactor.

and the product quality. In general, CHO cells are known to be shear-sensitive, thus, varying power inputs and the concomitant differences in the exerted hydrodynamic stress could potentially alter process performance (Sieck et al., 2014). However, the power inputs applied in this study are comparably low. Considering that the local maximum energy dissipation rate can be up to two orders of magnitude higher than the volumetric average power input (Ståhl Wernersson & Trägårdh, 1999), the resulting values still remain below the reported thresholds of sublethal and lethal responses (Chalmers, 2015). Hence, it can be hypothesized that other factors connected to the power input than hydrodynamic stress entailed the observed process deviations.

4.2 | The absence of substrate gradients

The sampling at different heights revealed no gradients of the tracked components (Supporting Information S1: Figure 1). The formation of substrate gradients is a well described large-scale effect for microbial production processes (Kuschel & Takors, 2020). The occurrence of substrate gradients can be estimated by comparing the critical timescales τ for substrate supply τ_{supply} and consumption τ_{cons} (Sarkizi Shams Hajian et al., 2020). The timescale τ_{supply} can be approximated via the circulation time τ_{circ}, while τ_{cons} is calculated by dividing the average substrate concentration by the volumetric substrate consumption rates in the following equation.

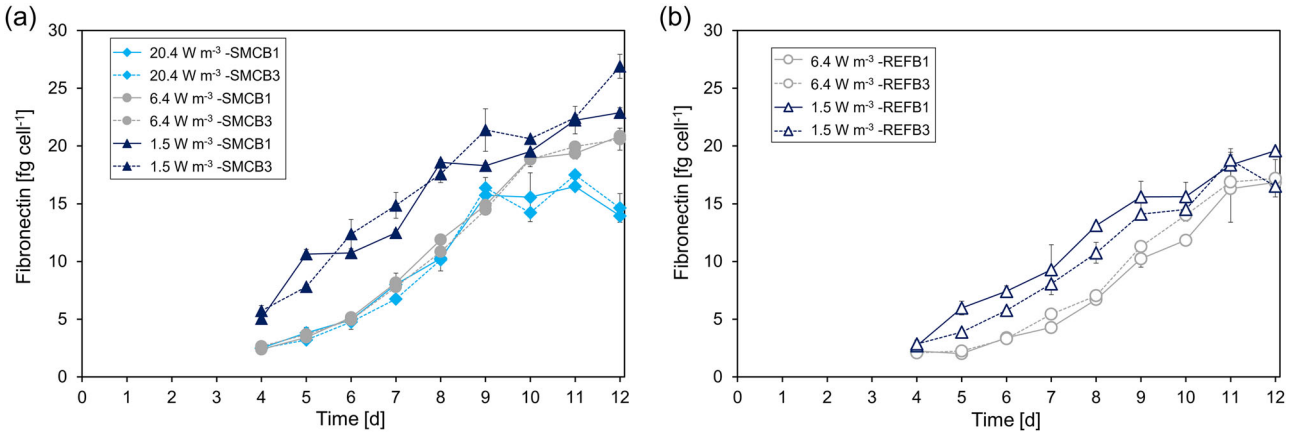

FIGURE 5 ELISA result for fibronectin levels in the SMCB (a) and REFB (b). Fibronectin levels were analyzed from cell extracts serving as indicator for a hypoxia-induced stress response. The error bars were calculated based on two biological replicates in the SMCB, single runs in the REFB and ELISAs performed duplicates. REFB, reference bioreactor; SMCB, single multicompartment bioreactor.

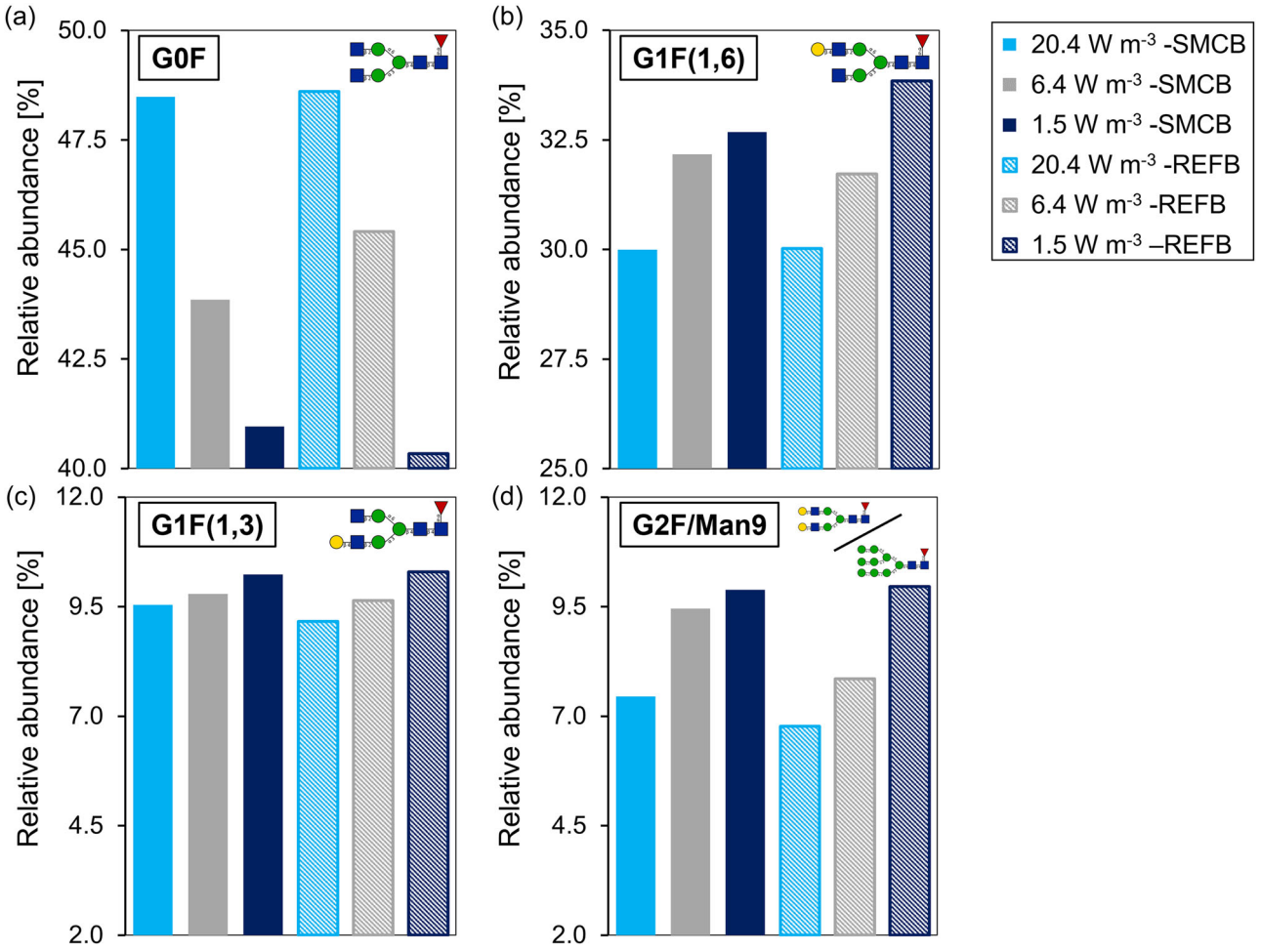

FIGURE 6 Highly abundant glycan species for SMCB and REFB fed-batch cultivations at different power inputs. Glycan analysis from end point samples was performed externally via the xCGE-LIF based high-performance analysis system glyXboxCE™ (glyXera GmbH). Most abundant glycoforms: (a) G0F, (b) G1F(1,6), (c) G1F(1,3), (d) G2F, or Man9. ■ D-*N*-Acetylglucosamine, ● D-Mannose, ▼ L-Fucose, ○ D-Galactose. REFB, reference bioreactor; SMCB, single multicompartment bioreactor.

$$\tau_{cons} = \frac{c_s}{q_s \cdot VCC} \quad (2)$$

Assuming τ_{circ} being one fourth of the mixing time (Lara et al., 2006), the theoretical probability for gradients can be calculated. The probability for gradients is high if $\frac{\tau_{cons}}{\tau_{circ}}$ is <1. At 1.5 W m^{-3} in the SMCB, the setting with the longest mixing times and thus, with the highest probability for gradient formation, τ_{circ} can be approximated with ≈24 s. When calculating the quotient $\frac{\tau_{cons}}{\tau_{circ}}$ for this setting, the resulting values remain clearly above 1000. Hence, with glucose being fed in excess and the low glucose consumption rates of CHO cells, glucose gradients can be ruled out.

4.3 | The effect of DO heterogeneities

The cultivation conditions in the SMCB provoked a pronounced reduction in the DO levels in the lower compartment that further intensified at decreasing power inputs and a reduced exchange area (Figure 4a–c). The observed reduction in the lower compartment seems reasonable. Presumably, gas bubbles that once entered the upper compartment were restrained from reentering the lower compartment by the compartment disc, leading to shorter gas residence times in the lower compartment. DO heterogeneities are assumed to be a common phenomenon in cell culture large-scale bioreactors (Xing et al., 2009). When comparing the timescales of oxygen supply and consumption as performed for glucose, while applying different literature values for oxygen uptake rates (Dahlmann et al., 2020; Maschke et al., 2022; Pappenreiter et al., 2019), $\frac{\tau_{cons}}{\tau_{circ}}$ assumes values between 5.0 and 0.1. Thus, the timescales for oxygen supply and consumption can be considered in a range where gradient formation is possible.

Anane et al. studied DO gradients in a STR (stirred tank reactor)-PFR (plug-flow reactor) system (Anane et al., 2021). Even tough, the monitored DO levels in the STR-PFR study were further reduced resulting in the complete temporary depletion of oxygen, the reported results agree with the findings of this study. Similar to the SMCB results (Figure 2), the authors reported a faster decline of the VCC in the stationary phase and increasing lactate concentrations under pronounced oxygen gradients. Moreover, Qian et al. described DO values of 15% as hypoxic conditions and also observed a reduced protein productivity and a stronger decline in the VCC due to hypoxia (Qian et al., 2014). Thus, presumably, the DO values in the lower compartment of the SMCB were low enough to trigger hypoxic stress responses.

Gao et al. suggested fibronectin as a biomarker for hypoxic stress with CHO large-scale production since DO gradients are difficult to measure at production scale (Gao et al., 2016). The authors compared fibronectin levels in a 20 L and 5 kL bioreactor and increased fibronectin concentrations at the 5 kL scale, indicating a cumulative effect of intermittent hypoxia. The deviations in the fibronectin levels in our study (Figure 5) seem to be in good agreement with the previous results. The hypoxic effect appeared to be cumulative as well and the conditions at lower power inputs that are assumed to approach large-scale conditions provoked similarly increased fibronectin levels.

Since temporary hypoxic conditions can hardly be prevented at production scale, it might be interesting to study the resilience against hypoxic stress of cell lines at an earlier stage of the process development in the SMCB. Zeh et al. went one step further and engineered CHO production cell lines to contain a hypoxia-inducible expression system, thereby utilizing the otherwise adverse effect to increase productivity (Zeh et al., 2021). Combining such cell engineering approaches with scale-down systems like the SMCB, might offer new possibilities to improve large-scale performance.

4.4 | Enhanced lactate production and glucose uptake

One of the most prominent differences between SMCB and REFB performance was the increased lactate formation. The characteristic of enhanced by-product formation is often described as an issue during scale-up and thus meets the requirement of mimicking large-scale behavior (Mulukutla et al., 2012). Furthermore, the switch in the lactate metabolism to lactate consumption is considered an important process indicator and its absence could be linked to impaired productivity (Le et al., 2012). Moreover, the accumulation of lactate to the concentrations reached in this study, exceeded the physiological level and could have adverse effects on for example productivity and growth (Mulukutla et al., 2010). Consequently, the observed decline in the VCCs and product concentrations might be connected to this phenomenon.

The combination of a high lactate production at an increased glucose uptake could be attributed to aerobic glycolysis or the Warburg effect. The observations also agree well with the previously stated intermittent hypoxia, since CHO cells are known to enhance glycolysis under hypoxia to compensate for the weakened oxidative phosphorylation (Mulukutla et al., 2015; Zheng, 2012). Additionally, the Warburg effect and increased lactate levels were associated with hyperosmolality (Da Veiga Moreira et al., 2021). Thus, at 1.5 W m^{-3} in the SMCB, the combination of high lactate concentrations and high osmolalities might have further stimulated each other.

However, additional triggers and mechanisms might yet to be identified, since the collected data lacks the explanation for the observed back-switch to lactate production in the SMCB at 20.4 W m^{-3} and in the REFB at 1.5 W m^{-3} after Day 9.

4.5 | Galactosylation differences

The glycan analysis revealed a link between the applied power inputs and the galactosylation index of the glycan patterns (Figure 6). At lower power inputs, the degree of galactosylation of the evaluated glycan structures increased. Notably, this finding holds true irrespective whether SMCB or REFB is applied. Both settings show the same galactosylation index. The power input deems to be the prime reason for modulated quality attributes which may be further propagated by

secondary effects such as resulting oxygen supply. Interesting enough, the SMCB-REFB setting allows to distinguish between impacts of gradients (substrates and dissolved gases) and physical parameters such as power input. Hence, the experimental settings enable a very specific investigation of stimuli-response interactions that is not supported in tests with mixed impacts.

Interestingly, the enzymatic activity of the galactosyltransferase GalT, the enzyme responsible for the galactosylation of the glycan structures, was reported to be particularly sensitive to changes in the extracellular environment (Hutter et al., 2017). This finding may support the independent observation that increased galactosylation occurs at DO extremes. Ivarsson et al. detected increased galactosylation at DO values of 10% and 90% compared to a physiological value of 50% (Ivarsson et al., 2014). Serrato et al. reported an increase in highly galactosylated glycoforms under extreme oscillatory DO conditions (Serrato et al., 2004). By analogy, studies of Godoy-Silva et al. and Sieck et al. described increasing galactosylation at higher energy dissipation rates, which may also be required to achieve high DO levels as described by Ivarsson et al. (Godoy-Silva et al., 2009; Ivarsson et al., 2014; Sieck et al., 2013). Taking into account that our studies with SMCB and REFB applied rather low power inputs leading to low and moderate DO levels (Figure 4), the observations agree with the other findings.

On another note, Villiger et al. observed that the addition of ammonia reduced any GalT activity to a minimum (Villiger et al., 2016). Furthermore, Synoground et al. observed decreased galactosylation under ammonia-stress (Synoground et al., 2021). Since ammonia is also known to accumulate during fed-batch cultures (Ahn & Antoniewicz, 2012), it could have played a role in the galactosylation differences observed in this study and thus, might be worth investigating in future studies. Consequently, whereas the power input could be identified as the primary source for the deviations in glycosylation, the secondary consequences of the latter still need further investigation.

5 | CONCLUSION

The SMCB was developed to copy large-scale conditions in a targeted manner while offering a flexible design that can be adjusted to the varying specifications of different production processes. In this first CHO cultivation study in the SMCB, we were able to mimic and study typical phenotypes of commercial scales at the lab scale. The possibility to largely decouple the volumetric power input from provoked heterogeneities offered a new angle to known large-scale behavior. For instance, the differences in the glycosylation appeared to be primarily influenced by the power input independent from the resulting mixing times and gradients. Apparently, the compartment disc concept holds the potential to not only create desired combinations of mixing times and power inputs, but also to add, remove, and dissect different scale-up effects. With additional discs in the SMCB and the considerate placement of both discs and control spots, a full detachment of power input and mixing characteristics as well as the design of specific cultivation environments should be achievable. Thus, perspectively, the SMCB could offer the possibility to individually design and study large-scale conditions ab initio in the lab. Follow-up studies in the SMCB will focus on extending the existing compartment model, incorporating additional large-scale information, and further investigating the mechanisms induced by varying power inputs and heterogeneities.

AUTHOR CONTRIBUTIONS

Lena Gaugler: Conceptualization; execution of experiments; data acquisition; data analysis and interpretation; manuscript writing. **Sebastian Hofmann**: Manuscript editing and revision; final approval. **Michael Schlüter**: Conceptualization; funding acquisition; manuscript editing and revision; final approval. **Ralf Takors**: Supervision; conceptualization; funding acquisition; manuscript writing; editing and revision; final approval.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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