

REVIEW

Scale-down simulators for mammalian cell culture as tools to access the impact of inhomogeneities occurring in large-scale bioreactors

Katrin Paul^{1,2}  | Christoph Herwig^{1,2}

¹Institute of Chemical, Environmental and Bioscience Engineering, Vienna, Austria

²Christian Doppler Laboratory for Mechanistic and Physiological Methods for Improved Bioprocesses, Vienna, Austria

Correspondence

Christoph Herwig, Institute of Chemical, Environmental and Bioscience Engineering, TU Wien, Gumpendorfer Straße 1a, 1060 Vienna, Austria.

Email: christoph.herwig@tuwien.ac.at

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Abstract

During the scale-up of a bioprocess, not all characteristics of the process can be kept constant throughout the different scales. This typically results in increased mixing times with increasing reactor volumes. The poor mixing leads in turn to the formation of concentration gradients throughout the reactor and exposes cells to varying external conditions based on their location in the bioreactor. This can affect process performance and complicate process scale-up. Scale-down simulators, which aim at replicating the large-scale environment, expose the cells to changing environmental conditions. This has the potential to reveal adaptation mechanisms, which cells are using to adjust to rapidly fluctuating environmental conditions and can identify possible root causes for difficulties maintaining similar process performance at different scales. This understanding is of utmost importance in process validation. Additionally, these simulators also have the potential to be used for selecting cells, which are most robust when encountering changing extracellular conditions. The aim of this review is to summarize recent work in this interesting and promising area with the focus on mammalian bioprocesses, since microbial processes have been extensively reviewed.

KEYWORDS

2-compartment system, gradients, inhomogeneity, large-scale bioprocess, scale-up

1 | INTRODUCTION

The scale up of processes from development scale to production scale enables more cost-efficient manufacturing of products required in large quantities. Development or laboratory-scale reactors for process optimization and characterization usually range from 1 to 20 L. In recent years, miniaturized bioreactors, ranging from microliter to milliliter volume, have gained popularity to achieve high

throughput and decrease time lines for process development and characterization [1]. Particularly production processes for monoclonal antibodies (mAbs) that are in high demand, like adalimumab (HumiraTM) [2], are transferred to large-scale production reactors. These bioreactors can reach volumes of up to 25 000 L for mammalian cell culture processes [3]. Furthermore, it has been estimated that approximately 50% of the biologics will continue to be produced in bioreactor volumes of at least 5000 L [4]. There are different approaches

Abbreviations: CFD, computational fluid dynamics; CHO, Chinese Hamster ovary; CS, compartment system; PFR, plug flow reactor; STR, stirred tank reactor; VCC, viable cell count.

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for scaling up a process from development to production scale. Most commonly physical parameters, for example, the volumetric power input, oxygen transfer rate, or volumetric oxygen mass transfer coefficient, are kept constant throughout the different scales [5,6]. However, keeping one parameter constant results in the change of others parameters. The key example for this is the mixing time, which inevitably increases in larger bioreactors [7]. The poor mixing in turn causes the formation of microenvironments, which can vary in pH, dissolved oxygen (dO_2), partial pressure of CO_2 (pCO_2), and substrate concentration. Additionally more concentrated feedstocks are sometimes used at larger scales, which can exacerbate this problem [8]. Cells are therefore exposed to different microenvironments, which change based on the localization of the cell in the bioreactor. These fluctuations in the external environment can decrease cell growth, lead to the accumulation of side products, or impact product quantity and quality [8–11]. A first step toward the investigation of these effects is determining to which extent gradients are occurring in large-scale bioreactors. Experimental approaches include the characterization of large-scale vessels by installing multiple probe ports for, for example, pH or oxygen, and characterizing occurring gradients based on the different responses of the probes [12]. This is however not feasible for production bioreactors, since they would require modifications. Furthermore, even characterization of production reactors might not be possible due to the capacity demands for production [4]. However, there are published characterization studies of bioreactors ranging in volume up to 25 000 L [3,13]. Recently, a transparent 15 000 L cell culture reactor has been used to characterize the occurring flow patterns with the benefit of optical access into the reactor [14]. Computational fluid dynamics (CFD) simulations have also been widely used to characterize the flow fields of large bioreactors and provide information of the mixing dynamics in these reactors [13,15–17]. Based on these simulations, the time which a cell spends in a different microenvironment can be estimated and in turn be used to design scale-down simulators [18,19]. These simulators can either contain two or three separate compartments (2/3-CS, where CS is compartment system) with different conditions in each compartment or a single compartment (1-CS) with oscillating conditions [20]. Both types of simulators have different limitations and the choice of the system depends on the particular application. Based on the design, different residence times of the cells in the simulated gradient can be achieved and different reactor scales can be simulated. Therefore, scale-down simulators are valuable tools, which have the potential to elucidate the response of cells to different fast changing microenvironments. This can lead to an in-depth understanding of underlying causes for different process performance at large scale. Reviews about this topic usually focus on microbial cells, since the majority of literature exists in this area. Therefore, here the work with

PRACTICAL APPLICATION

The scale-up from development to production scale can still present challenges. Scale-down simulators aim to reproduce the large-scale reactor environment and have therefore the potential to elucidate culprits for impaired process performance at large scale. While these simulators are well established for microbial cells, only little research is available for mammalian cells. In this review, the current status of research is discussed, as well as the challenges associated with the establishment of these simulators for mammalian cells.

mammalian cells is reviewed, particularly the associated challenges and differences to established systems for microbial cells.

2 | DETERMINING THE EXTENT OF INHOMOGENEITIES IN LARGE-SCALE BIOREACTORS AS A BASIS FOR THE DESIGN OF SCALE-DOWN SIMULATORS

With the increasing volume of bioreactors, the mixing time in the reactor increases as well. While the mixing time of a laboratory-scale reactor with a volume of 3 L was determined to be approximately 10 s, a reactor with a volume of 15 000 L had a mixing time of 120 s [3]. Figure 1 summarizes the potential challenges, which can be encountered throughout the scale-up process from development to production scale.

Bioreactor mixing times are typically determined experimentally. There are a variety of approaches, which have been reviewed by Ascanio et al. [21]. However, most commonly colorimetry or tracer pulses are used [3,14,22]. Furthermore, these types of experiments are utilized to validate CFD simulations. Good agreement between experimental studies and CFD simulations has been demonstrated for mixing time studies [3,13]. Since CFD simulations describe the flow fields in the reactor, they can also be used to quantify the extent of heterogeneity, which can occur in the bioreactor. Based on these results, inhomogeneities, which have the potential to influence process performance, can be identified. For example, a combination of CFD simulations and experimental studies revealed dissolved oxygen (dO_2) gradients of only approximately 10% for a 15 000 L bioreactor [13]. Although it has been shown that glycosylation patterns of the product can vary with varying dO_2 levels [23,24], cells should not encounter hypoxia, when the process is run at

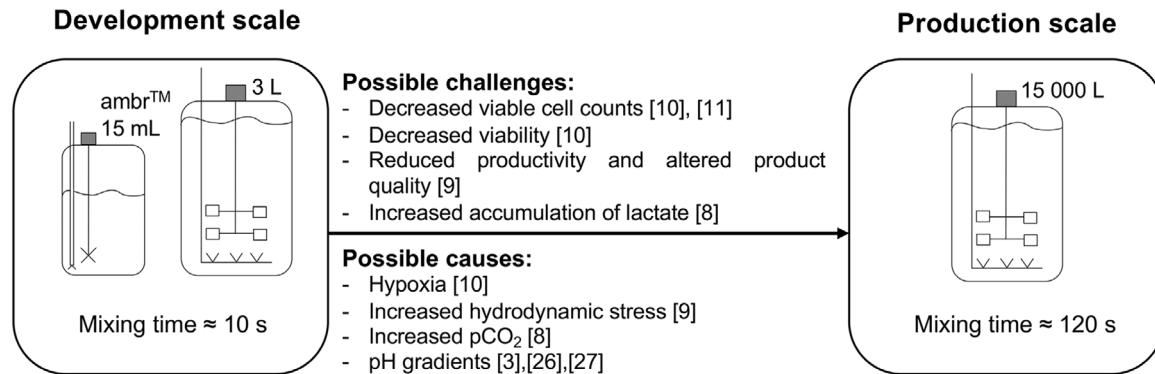


FIGURE 1 Possible challenges during scale-up

common operating $d\text{O}_2$ setpoints of around 50%. However, a previous study reported hypoxia as the root cause for impaired process performance when scaling up a process from 20 to 5000 L. Although the $d\text{O}_2$ profiles were similar in both scales, decreased viable cell counts (VCCs) and viabilities were observed in the 5000-L reactor [11]. The differing results show the benefit of characterization studies for bioreactors to anticipate possible inhomogeneities. These studies can potentially be the basis for the optimization of bioreactor operation and impeller configuration to improve mixing and therefore the volumetric oxygen transfer coefficient [3]. While bubble-associated shear stress has been mainly eliminated by the use of the polymer Pluronic F-68, there is a threshold at which the hydrodynamic stress can negatively impact process performance [10,25]. This limitation needs to be considered for bioreactor operation. Besides oxygen gradients, pH gradients have also been described in cell culture bioreactors. The experimental characterization of an aerated 8000-L bioreactor revealed pH amplitudes of up to 0.4 units, when 2 M sodium carbonate (Na_2CO_3) was added from the top of the reactor for pH correction [26,27]. CFD simulations have also been confirming the presence of areas with an elevated pH [13]. Substrate gradients have been extensively studied for microbial cells [28–31], while this area of research has not been pursued for mammalian cells. Since less concentrated feed stocks are used for mammalian cell culture and the cells take up substrate at a lower rate, large substrate gradients are unlikely. Therefore, the impact of substrate gradients on mammalian cell culture performance is likely minimal.

In addition to the extent of the occurring inhomogeneity, the exposure time of the cells to the different environment can be estimated by CFD simulations. This has been demonstrated for a *Penicillium chrysogenum* cultivation, where life-lines of the cells have been determined, which represent the residence time of the cells in a certain environment. The study showed that cells were exposed to inhomogeneity for approximately the circulation time of the bioreactor [32].

3 | MIMICKING INHOMOGENEITIES IN SCALE-DOWN SIMULATORS

Scale-down simulators aim to reproduce the large-scale environment, particularly its inhomogeneity, at the laboratory scale. They can be divided into systems consisting of a single (1-CS) or of multiple bioreactors (2-CS or 3-CS) [20]. An overview of the different systems and their applicability to different problems is shown in Fig. 2.

In a 1-CS, a process parameter is either oscillated or temporarily perturbed to simulate the heterogeneous large-scale environment. An example includes the oscillation of the $d\text{O}_2$ between 0 and 14% with different oscillation periods in a hybridoma culture. In comparison to the control, which was kept at a $d\text{O}_2$ of $10 \pm 1\%$, a decrease in VCCs was observed, which was more pronounced for longer oscillation periods. Furthermore, increased lactate levels and changes in product glycosylation were observed, when the cells were exposed to $d\text{O}_2$ oscillations [33]. The generation of pH excursions by bolus addition of 1 M NaOH to a Chinese Hamster ovary (CHO) culture is another example for a 1-CS scale-down model. pH perturbations up to a pH of 8.1 were generated in this case and increased lactate production and glucose consumption were observed in comparison to the control with continuous base addition [34]. However, this study also illustrates the limitations of a 1-CS to simulate pH inhomogeneity. Since the addition of increased amounts of pH corrective agent lead to an increase in osmolality, final osmolalities of $550 \text{ mOsmol kg}^{-1}$ were reported. This is problematic, since osmolalities above $400 \text{ mOsmol kg}^{-1}$ are associated with impaired cell growth [35] and can influence product quality [36,37]. Therefore, it is difficult to distinguish between effects caused by the addition of base and the effects caused by an increased osmolality. Although the study tried to circumvent this problem by running additional controls with an increased osmolality to separate the effects, it cannot account for interactive effects. Furthermore, previous reports have shown interactive effects, when CHO cells were exposed

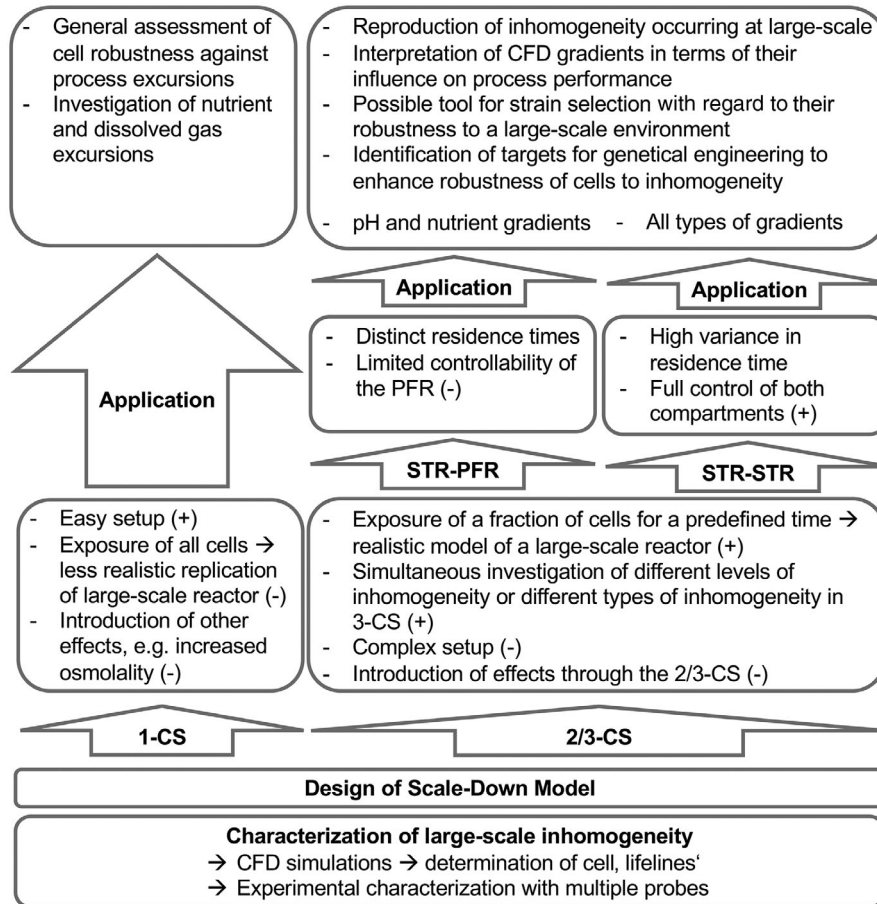


FIGURE 2 Overview of the different scale-down simulators and their applications, as well as limitations

to multiple stresses simultaneously, necessitating further studies to evaluate only the effect of pH excursions [38].

This challenge can potentially be circumvented by the use of a 2-CS, since an increased pH is only generated in a fraction of the bioreactor volume and therefore less base is needed. Furthermore, the 2-CS offers the benefit that only a part of the cell population, rather than all cells, is exposed to a different environment, making it a more accurate simulator of a large-scale bioreactor. In the case of pH inhomogeneity, a large stirred tank reactor (STR) represents the well mixed fraction of a large-scale bioreactor with the pH of the setpoint. This STR can either be connected to another small STR or plug flow reactor (PFR), which represents the zone where base is added and shows an increased pH. The volume of the compartment representing the inhomogeneous zone should ideally be derived from experimental studies or CFD simulations, where the extent of the zone was estimated. Studies investigating pH inhomogeneity have worked with volumes ranging from 5 to 14% of the total volume in the large compartment [39–41].

Brunner et al. used a 2-CS (STR-STR), where pH amplitudes up to a pH of 9.5 were introduced with 2 M sodium hydroxide (NaOH) in the smaller STR, exposing CHO cells to an increased pH for 90 s [41]. However, the high amplitudes caused an increase of almost 0.08 pH units in the large

STR, which was counteracted with 1 M hydrochloric acid (HCl). This resulted in an increased osmolality in the 2-CS up to $450 \text{ mOsmol kg}^{-1}$, presenting a similar problem as the previously discussed 1-CS study. For this reason, process performance was only compared until 120 h of the process, when a critical osmolality was reached. Until that point only a decreased maximal VCC was observed. A similar setup and strategy was used by Osman et al., who investigated the effects of pH amplitudes reaching a pH of 8.0 and 9.0 on hybridoma cells [40]. Cells were exposed to different perturbation frequencies as well as different perturbation lengths. With an increase in the number of perturbations, as well as their frequency, maximal VCC declined.

However, both studies utilized peristaltic pumps to circulate the cells between the two compartments. Nienow et al. identified the peristaltic pump of their 2-CS setup as the root cause for a reduced culture time and decreased product titer [39]. Peristaltic pumps have furthermore been shown to increase cell lysis, which is consistent with a study that showed shear stress up to 1000 Pa in a peristaltic pump [42, 43]. Centrifugal pumps, which generate less shear stress, have been shown to not adversely influence the cells [42]. A 2-CS, which was established to investigate the threshold of the maximal hydrodynamic stress for CHO and insect cells, used a

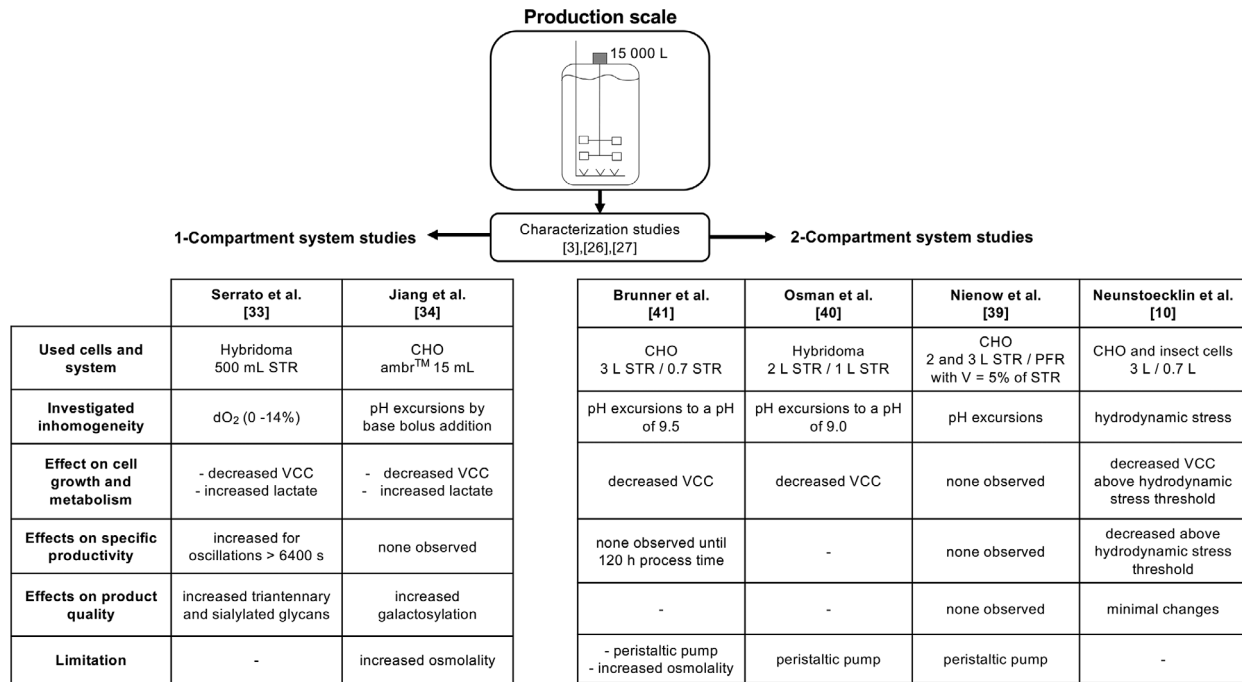


FIGURE 3 Summary of the different scale-down simulator studies

centrifugal pump. No adverse effects of the pumps on process performance were reported in this study [10]. A summary of the discussed studies is shown in Fig. 3.

Overall the choice of the scale-down simulator is a tradeoff between the advantages and disadvantages of the different systems. While 1-CS are easiest to setup, problems like increased osmolality of the medium for pH inhomogeneity studies can arise. Furthermore, the whole cell population is exposed to changing conditions, rather than just a fraction, as in a 2-CS. The different types of 2-CS systems also have distinct advantages. A STR-PFR setup can be equipped with multiple sensors and sample ports along the PFR, resulting in time resolved profiles. This setup also generates distinct residence times, but lacks the controllability of the small compartment, which can only be achieved with an STR-STR setup [44]. Additional STRs or PFRs can potentially be connected to generate 3-CS to investigate multiple inhomogeneities simultaneously. These types of systems have however only been established for microbial cells at this point [45,46].

4 | QUANTIFYING THE IMPACT OF INHOMOGENEITIES AND VALIDATION OF THE SCALE-DOWN SIMULATOR

In addition to choosing an appropriate scale-down simulator, suitable analytics are necessary to gain understanding of how large-scale inhomogeneities affect the cells. While standard cell culture analytics often already reveal an impact on

the cells, underlying mechanisms are difficult to elucidate without additional data. Particularly single cell analytics are well established for mammalian cells and can give information about cell cycle distributions and mode of cell death, for example [47]. These tools were used to determine the influence of single pH excursions on hybridoma cells, revealing a transient increase of apoptotic cells in response to the pH shift. However, the level of apoptotic cells returned to initial values, if the pH shift was not extensive [48]. While this shows that cells are able to adapt to an increased pH, if they are exposed to it for hours, pH inhomogeneity at large-scale occurs frequently and only for minutes. It still has to be determined whether or not cells are able to adapt in this scenario as well.

Omics approaches are another tool, which can enable an in-depth understanding of how cells respond to the large-scale bioreactor environment and aid in the validation of scale-down simulators. For example, proteomics have been used to investigate the difference in protein abundance between a 10 mL and 300 L bioreactor and found almost no difference between the scales [49]. A similar approach could be used to investigate differences to larger scales. Transcriptomics were used to identify the response of CHO cells to stress induced by agitation as well as sparging. The results showed different expression patterns for stress caused by agitation and sparging, with only little overlap between the two causes of stress [50]. Based on those differences in the transcriptome, agitation- or sparging-related stress during scale-up could be potentially identified. Furthermore, other relevant stress factors could be described by comparing transcription patterns

between the scales, which could also provide a basis for the validation of scale-down simulators. Novel tools, which can track epigenetic modifications in addition to changes in the transcriptome, have the potential to further solidify the understanding of the differences between reactor scales [51].

Eventually, it is necessary to judge whether the scale-down simulator is a good representation of the large-scale reactor. One way to assess this is by comparing the process performance of the large-scale reactor with that of the scale-down simulator. An example is the work of Neunstoecklin et al. where the process performance for the investigated 300 L reactor and the scale-down simulator were compared [25]. The study focused on the impact of hydrodynamic stress on insect cells (SP2/0). Therefore, the hydrodynamic stress resulting from different operating conditions of the 300 L was characterized. By varying sparger types, aeration rates, and stirring speed, it was possible to identify scenarios in which the large-scale reactor was operated at conditions, where the hydrodynamic stress exceeded the upper threshold of the SP2/0 cells. A comparison between the scale-down simulator and the 300 L reactor at similar levels of hydrodynamic stress showed similar VCCs, specific metabolic rates, as well as product concentration and quality, establishing the scale-down simulator as a valid representation of the 300 L reactor. The study furthermore showed that common indicators to assess whether or not cell damage occurs in response to hydrodynamic stress were not able to predict cell damage for the investigated scenarios.

5 | CONCLUDING REMARKS

With an increasing amount of research, which focuses on the characterization of flow fields in large-scale bioreactors, occurring large-scale inhomogeneities are well described. It is however still unclear how these inhomogeneities affect particularly mammalian cells. Although there are studies, which identify factors for poor process performance at large scale, no studies have investigated differences between the scales for a successful process transfer at large scale. While proteomics confirmed almost identical patterns between the 10 mL and 300 L scale, it is unclear whether this would hold up for larger scales as well. Since it has been shown that transcriptomics can be used to distinguish between different stress responses, an in-depth study of transcriptomic patterns at different scales could provide the basis for the design of rational scale-down simulators. Furthermore, new methods for the determination of epigenetic changes of the cells have the potential to illuminate how cells are adapting to the large-scale environment. More in-depth knowledge about these mechanisms has the potential to guide genetic engineering to generate cell lines, which are more robust in the large-scale environment.

At this point, multiple compartment scale-down simulators can only be designed to reproduce the large-scale environment in terms of the expected volume of the inhomogeneous zone and the residence time of the cells in that zone. Since the setup of such a system is however more challenging than for microbial cells, only little research has been conducted in this area. Mainly pH inhomogeneity has been investigated and rather extreme scenarios have been simulated. More work is needed to evaluate at which point pH inhomogeneity becomes critical. Furthermore, effects have been investigated for single cell lines and it has to be assessed whether there are differences in robustness between the different cell line lineages. A comparison between inherently more robust cell line lineages and their not so robust counterparts could further elucidate mechanisms of adaptation to fast changing environmental conditions. This knowledge could also guide the choice of which cell line lineage to use for development, if large-scale production is planned.

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CONFLICT OF INTEREST

The authors have declared no conflict of interest.

ORCID

Katrin Paul  <https://orcid.org/0000-0003-3114-5571>

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