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Understanding gradients in industrial bioreactors

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Abstract
Gradients in industrial bioreactors have attracted substantial research attention since exposure to fluctuating environmental conditions has been shown to lead to changes in the metabolome, transcriptome as well as population heterogeneity in industrially relevant microorganisms. Such changes have also been found to impact key process parameters like the yield on substrate and the productivity. Hence, understanding gradients is important from both the academic and industrial perspectives. In this review the causes of gradients are outlined, along with their impact on microbial physiology. Quantifying the impact of gradients requires a detailed understanding of both fluid flow inside industrial equipment and microbial physiology. This review critically examines approaches used to investigate gradients including large-scale experimental work, computational methods and scale-down approaches. Avenues for future work have been highlighted, particularly the need for further coordinated development of both *in silico* and experimental tools which can be used to further the current understanding of gradients in industrial equipment.
Keywords: Bioreactor, Gradients, Scale-down, Computational Fluid Dynamics (CFD), Compartment model, Scale-up, Sensor particles, Microbial physiology, Kinetic modelling, Industrial fermentation processes

Introduction

Industrial biotechnology is widely used for the production of a diverse range of compounds including food ingredients, chemicals and pharmaceuticals amongst others. Due to the wide range of industrial applications there is a need to understand and characterize the performance of industrial-scale bioreactors, particularly given the increasing need for sustainable production technologies (Noorman and Heijnen, 2017; Straathof et al., 2019).
Industrial-scale bioreactors can be much larger (more than 100 m^3) than laboratory equipment (typically 1-10 L); this difference in scale introduces challenges because the environment in the large-scale equipment can be much less homogenous than the lab-scale. Any inhomogeneity (gradient), e.g. in pH, substrate or dissolved oxygen concentration, can affect the process performance (i.e. yield, productivity, product quality, etc.) (Crater and Lievense, 2018).
Quantifying the extent to which gradients impact the process performance is particularly challenging. This is because it involves understanding the complex interaction between flow patterns inside the reactor and the physiology of the organism of interest. The study of gradients is further complicated by the difficulty involved in performing experiments, particularly at the largest scale. Despite the difficulties involved, understanding gradients is essential in order to improve the performance and hence economics of large-scale bioprocesses.

While gradients can occur in a range of processes (e.g. light gradients in photo-bioreactors (Bitog et al., 2011), gradients in anaerobic digesters (Dapelo et al., 2015; Van Hulle et al., 2014) and pH gradients in anaerobic batch fermentations processes with lactic acid bacteria (Spann et al., 2019b), this review will focus on large-scale, aerobic, fed-batch fermentation processes. This is because these processes currently have the widest range of industrial application. The causes of gradients will be examined, as well as their effect on microbial physiology. Finally, the current state of the art regarding methods to measure and quantify gradients will be discussed.

1. Causes of gradients

Generally speaking, gradients will occur if the rate of consumption or uptake is greater than the rate of transport. A common way of determining whether gradients will occur is to calculate the characteristic times for consumption (τ_C) and transport (τ). If the characteristic time for transport is less than the time for consumption gradients are not likely to occur, whilst if the characteristic time for transport is greater than the time for consumption then gradients are likely to occur (Sweere et al., 1987). Regime analysis can be applied generally to transport and uptake processes in bioreactors. Due to their importance substrate and oxygen gradients are the most widely examined (Enfors et al., 2001; Larsson et al., 1996), and this review will focus on these. The characteristic time for consumption is typically calculated by dividing the concentration of the substrate (S) by the rate of consumption (which is the product of the specific uptake rate (q_S) and the biomass concentration (X)):

$$\tau_c = \frac{S}{q_S X} \quad (1)$$

Examination of Equation (1) also makes it clear that gradients are more likely to occur at high biomass concentrations, and/or for microorganisms which have high substrate and/or oxygen uptake rates. Characteristic times for oxygen and substrate consumption for baker's yeast have been calculated to be 1 s and 10-100 s, respectively (Sweere et al., 1987). One of the reasons the characteristic time for oxygen consumption is relatively low is the poor solubility of oxygen in the fermentation medium (of the order 10^{-3} kg L^{-1} at atmospheric pressure) (Doran, 1995).

The characteristic time for transport largely depends on the design and operation of the reactor. Key design and operational characteristics affecting transport phenomena in aerobic fed-batch fermentation processes are outlined in Table 1. For aerobic fermentations, a key parameter is the oxygen transfer rate (OTR), and equivalent rates can be defined for other processes (e.g. those using gaseous feedstocks like carbon dioxide or carbon monoxide) (Ifrim et al., 2014; Siebler et al., 2019). Realistic oxygen transfer rates in large vessels are of the order 2-5 kg m^{-3} hr^{-1} (Humbird et al., 2017), giving τ values of the order 10-20 s. This analysis clearly demonstrates that dissolved oxygen will quickly become depleted in zones of the reactor where there is insufficient transport (e.g. there are no gas bubbles present).

The other important characteristic time is the time needed for substrate transport throughout the reactor. One way in which this can be determined is the mixing time. This is defined as the time needed for the concentration of an inert tracer to reach a pre-defined degree of homogeneity starting from an initial, totally segregated state. A value of ± 5% of the final, equilibrium concentration is typically used (Doran, 1995). A range of correlations exist to calculate the mixing time in bioreactors (J. Tramper and K. van't Riet, 1991; Kawase and Moo-Young, 1989; Nienow, 1998), with values being of the order 10-1000 s depending on the reactor design and operating conditions (Sweere et al., 1987). Alternatively, it is possible to calculate the circulation time (the time taken for the liquid to complete one circulation 'loop') and use this as the characteristic time for substrate transport. Use of the circulation time will give a lower value of τ

than the mixing time (Doran, 1995), typically by a factor of three to five. In practice, the values of both the circulation and mixing time depend on both the location where the substrate is introduced as well as the measurement location (Kawase and Moo-Young, 1989; McClure et al., 2015a), implying there are in fact multiple values of these parameters for a given reactor configuration. It is not immediately obvious which of these should be selected, but a conservative design approach would suggest using the highest value of the mixing time in the estimation of gradients using regime analysis.

At this stage it is also interesting to note some important differences between transport of oxygen and substrate. For both the substrate and oxygen, the length scale for transport is the distance between the source and the furthest point in the reactor. The difference between the two is that the substrate is typically added at a single point, whereas bubbles are much more uniformly distributed throughout the reactor. Hence, the length scale for substrate transport is of the order 10^0-10^1 m, while for oxygen transport it is of the order 10^{-2}-10^{-3} m. This implies much larger spatial variations in the substrate concentration. Secondly, in the case of oxygen it is not likely that cells will be exposed to sufficiently high concentrations to cause physiological problems (as the dissolved oxygen concentration will simply reach saturation). Hence, the major concern is maximizing the oxygen transfer rate. In the case of the substrate, a high concentration can lead to overflow metabolism (Sonnleitner and Käppeli, 1986), so the challenge is to keep the substrate concentration below a critical value.

Figure 1 shows a plot of characteristic times and transport times for experimental work reported in the literature (more details are given in Table 3). Using regime analysis, substrate gradients are expected in the majority of cases, which is in line with reported observations (more detailed discussion about the experimental and computational work is presented in Section 4.2). This illustrates an advantage of regime analysis, as it is possible to quickly determine whether gradients are likely to occur. However, the analysis does not provide any information about the effect of these gradients on relevant process metrics (e.g. the yield, product concentration and productivity). From a process perspective it would be desirable to predict how design and operational parameters affects the extent of gradients and hence the process performance. Interest in tackling this important question has driven research into understanding the effect of fluctuating conditions on cell metabolism, as well as the development of computational tools to model gradients, with both of these areas being examined in this review.

Table 1 – Summary of design and operational parameters and their effect on the incidence of gradients in large-scale reactors.

Operational factors	**Influenced process parameters**	**Challenges/Limitations**	**Refs**
Aeration rate	Increased aeration leads to (1) higher gas hold-up, which is directly correlated with the gas-liquid interfacial area and, therefore the **OTR**; (2) shorter **mixing times** because aeration may enhance mixing performance.	Increased energy demand. Potential increase in foaming. Impeller flooding.	(Abdullah et al., 2000; Garcia-Ochoa and Gomez, 2008; McClure et al., 2015b; Nienow,

			1998)
Agitation speed	Dictates flow pattern and flow intensity. Increased agitation speed (1) shortens the **mixing time;** (2) it decreases bubble size by promoting bubble breakage. Bubble diameter is inversely correlated with the interfacial area, ultimately improving **oxygen transfer**; (3) increases the volumetric power input, which is directly correlated with the $k_L a$, enhancing gas-liquid oxygen transfer.	Increased power input (power is proportional to the impeller speed cubed). Increased agitation can lead to damage to shear sensitive cells.	(Calderbank, 1958; Garcia-Ochoa and Gomez, 2008; Kawase and Moo-Young, 1989; Nienow, 1998)
Feed rate	The substrate concentration in the feed, the magnitude of the feed rate and the position where it is introduced can influence the **substrate distribution** in the fermenter. The magnitude needs to be such that one prevents the cells from starving or overfeeding.	Substrate limitation and overflow metabolism.	(Larsson et al., 1996; McClure et al., 2016; Tajsoleiman, 2018)
Headspace pressure	Increased headspace pressure improves **oxygen transfer** by two means: (1) decreasing the bubble diameter which is inversely correlated with the interfacial area assuming a constant gas volume fraction and increases the OTR, (2) increasing the value of the oxygen concentration at saturation, augmenting the oxygen gas-liquid driving force.	Increased energy demand to compress the gas. More complex and expensive vessel design to handle high pressures.	(Doran, 1995)
Impeller type and number	Both dictate the flow pattern and recirculation loops, which (1) influence the **mixing time** and (2) the bubble size which changes with the impeller type and decreases with a higher number of impellers due to enhanced bubble breakage, augmenting the **OTR**.	Increasing the number of impellers increases power input.	(Nienow, 1997, 1998; Pacek et al., 1999; Vrábel et al., 2000)
Reactor design	Besides the reactor geometry (height, diameter), reactor design involves the relative positioning and size of internal hardware in the vessel (impeller, sparger, baffles, coils). The (1) **mixing time**, (2) **OTR** and (3) **substrate distribution** can be affected.	Increasing the number of internals can complicate cleaning.	(Doran, 1995; Elson et al., 1986; Kawase and Moo-Young, 1989; McClure et al., 2016)

Reactor type (e.g. STR, BC)	The reactor type will (1) influence the flow pattern and the **mixing time**, as well as (2) the gas hold-up, which will directly influence the $k_L a$ and the **OTR**.		(Doran, 1995; Haringa et al., 2017; Kawase and Moo-Young, 1989; McClure et al., 2018)
Medium/broth physical properties	Surface active compounds in fermentation medium or broth can reduce oxygen transfer. Increased viscosity: (1) negatively influence mass transfer coefficients, decreasing the **OTR**. Mixing performance is also compromised with (2) the possible formation of cavities and an increase in the **mixing time**.	Avoiding viscous broth is impossible in some instances (e.g. filamentous fungi or in situations where the product increases the viscosity). There are practical and economic limitations in selection of the medium components.	(Gabelle et al., 2011; Noorman, 2011; Prins and van't Riet, 1987)

2. Biological response to gradients

As previously discussed, it is likely that cells will be exposed to fluctuating environmental conditions in large-scale reactors and the timescale of these fluctuations is often such that it can cause a biological response. From an engineering perspective, it is important to understand how such fluctuations impact key process parameters, such as the productivity, yield or product quality.

Figure 2 summarizes the response of cells to fluctuations in environmental conditions. The changes that can occur as a response to such fluctuations vary over a wide range of timescales (from seconds to hours). Changes in the metabolome (the type and intracellular concentration of small molecular weight (< 1000 Da) compounds) can occur at the second time scale. The most well-known example of this is overflow metabolism. Cells have a limited capacity for the aerobic utilization of substrate, with reasons for this being examined in detail elsewhere (Goel et al., 2012; Szenk et al., 2017). If there is insufficient dissolved oxygen available, or the level of substrate is above the maximum which can be utilized aerobically, excess carbon is used fermentatively with the concomitant generation of byproducts like ethanol or acetate (to maintain redox homeostasis) (Lengeler et al., 1999). This behavior is relevant as the biomass yield on substrate (Y_{XS}) is substantially lower for fermentative pathways. For example, the value of Y_{XS} for *Saccharomyces cerevisiae* for oxidation of glucose (0.49 g g^{-1}) is approximately tenfold higher than the fermentative value (0.05 g g^{-1}) (Sonnleitner and Käppeli, 1986), similarly the oxidative and fermentative values for *E. coli* are 0.5 and 0.15 g g^{-1}, respectively (Xu, Bo et al., 1999). Additionally, the presence of byproducts at sufficient concentration can also have negative effects on cell physiology (Roe et al., 2002) and hence process performance (e.g. high acetate concentrations can reduce the specific growth rate (Xu, Bo et al., 1999) and expression of recombinant protein in *E. coli* (Jensen and Carlsen, 1990)). Sub-optimal utilization of the feed

reduces the yield on substrate, which can negatively impact the overall economics of the process, particularly for commodity products where the substrate represents the largest portion of the total cost. Similarly, reductions in the product concentration can result in increased downstream processing costs and reductions in the productivity.

Exposure to gradients can also lead to changes in transcription. For example, Schweder et al. (1999) found that exposure to the fluctuating conditions in a large-scale (20 m^3 working volume) stirred tank bioreactor led to increased expression of genes associated with stress in *E. coli*. The extent of these changes was found to depend on the location within the reactor and the response occurred in seconds. Using a scale-down system, Lara et al. (2006b) found that cycling between anaerobic and aerobic conditions led to substantial changes in gene expression, as well as reduced heterologous protein production (due to post-transcriptional changes). One consequence of these transcriptional changes is an increased energy demand. It has been estimated that exposure to fluctuating conditions increases the ATP demand for maintenance by 40-50% in *E. coli* (Löffler et al., 2016). The practical consequence of this is that less carbon is available for product formation, leading to a reduction in the product yield.
These changes in the transcriptome also lead to changes in the proteome of the cell, which can lead to further changes in gene expression (e.g. induction of genes in response to stress) (Lencastre Fernandes et al., 2011; Lengeler et al., 1999). Again, such changes can impose a metabolic 'cost' on the cells, which leads to a decrease in the yield. For example, it has been shown that exposure to fluctuating conditions leads to a 10-fold increase in the incorporation of non-canonical amino acids in recombinant proinsulin produced using *E. coli* along with an 18% decrease in the product yield (Anane et al., 2019).
At longer time scales (hours to days), exposure to stressful environmental conditions may lead to an increased mutation rate (Maclean et al., 2013), and this can lead to heterogeneity in the population (Heins and Weuster-Botz, 2018; Lencastre Fernandes et al., 2011). In the long term, there will be selection for the sub-populations which are most capable of tolerating exposure to oscillations. In order to illustrate the effect of fluctuating conditions, the discussion has focused on exposure to fluctuating concentrations of a single carbon source. This case is also the most widely studied and may be the most relevant for industrial applications. In reality, cells can take up multiple components from the growth medium, and it may be necessary to consider the uptake of multiple components (e.g. oxygen and substrate, or multiple carbon sources) when considering the effect of gradients (see Table 4).
It has been noted that when *E. coli* was exposed to fluctuating environmental conditions the cells had higher viability (as quantified via flow cytometry), but a lower biomass yield than cells exposed to a uniform environment (Hewitt et al., 2000; Onyeaka et al., 2003). Similarly, exposing *S. cerevisiae* to fluctuating conditions was found to lead to a reduction in the biomass yield on substrate, but also to improved product quality (quantified using the gassing power of the yeast) (George et al., 1998). Finally, in aerobic fed-batch processes, *Aspergillus oryzae* has shown to develop morphological changes by reducing its size by half when pulse-feeding was used instead of continuous feeding (Bhargava et al., 2003a; Bhargava et al., 2003b). Although no significant changes in specific process rates were observed, broth viscosity was significantly reduced, enhancing oxygen transfer and facilitating process operation. These results raise two interesting issues. Firstly, they suggest that exposure to gradients may result in positive as well as negative outcomes. Secondly, it highlights the challenges involved with scale-down

experiments as it may be necessary to replicate multiple parameters (e.g. the yield and viability) in order to faithfully mimic industrial equipment.

From a bioprocess engineering perspective, these molecular and population dynamics effects lead to changes in substrate uptake, product formation and growth kinetics and in maintenance requirements by influencing the cell cycle, physiology and metabolism. These adaptations to environmental conditions and their accompanying changes in intracellular dynamics provide an additional challenge to the numerical modelling of microbial processes. The reason is that their mathematical description can become extremely complex due to the number of processes involved and the difficulty involved in obtaining accurate measurements of key parameters (Heins and Weuster-Botz, 2018).

In order to accurately model gradients, it is necessary to mathematically model the interactions between microorganisms and their environment. Approaches to do this can be classified as segregated or unsegregated and structured or unstructured. Unsegregated models treat the population of cells as uniform, while segregated models account for heterogeneity within the population. Structured models account for different intracellular components, while unstructured models do not. Like all modelling exercises, there is inevitably a trade-off between the desired level of accuracy and the complexity of the model; hence it is important to select a model type suitable for the desired application (Gernaey et al., 2010).

Unsegregated unstructured models have been widely used for a range of organisms including *A. oryzae* (Albaek et al., 2011), *Corynebacterium glutamicum* (Khan et al., 2005), *E. coli* (Anane et al., 2017; Xu, Bo et al., 1999) and *S. cerevisiae* (Sonnleitner and Käppeli, 1986) amongst others. These models are capable of providing good agreement with experimental data and they have the advantage of being relatively simple, and hence computationally efficient. The obvious disadvantages of this approach are that intracellular processes are neglected, and the population is treated as uniform.

Segregated models account for heterogeneity within the population; a very common way of doing this is through the use of population balance type approaches (Lencastre Fernandes et al., 2011). Here, the population is divided on the basis of one or more descriptor variables (e.g. the cell age, size or mass). Development and validation of such models necessitates the use of experimental techniques for the quantitation of population heterogeneity; such techniques have been examined in detail elsewhere (Heins and Weuster-Botz, 2018; Lencastre Fernandes et al., 2011). As previously noted exposure to gradients can lead to population heterogeneity (Heins and Weuster-Botz, 2018, Nieß et al., 2017), hence there is a need to couple population balance models with tools to predict spatial and temporal gradients (such tools are discussed in more detail in Section 4.1). Work in this area has examined the combination of CFD and population balance models (Morchain et al., 2013, 2014) in order to account for the adaptation of cells to the local environmental conditions. There is evidence (Brand et al., 2018) that the culture history of an organism affects its response to fluctuating environmental conditions. If such behaviour has a meaningful effect on the process performance, then a segregated model of microbial kinetics needs to be combined with some method of calculating the history of the local conditions experienced by the cells. This issue has been tackled with the use of Euler-Lagrange CFD approaches that model the Lagrangian trajectories of cells in the fermentation broth (see Section 4.1.1) and that can be used to estimate the frequency and magnitude of environmental oscillations that the cells experience. The combination of population balance models with Euler-Lagrange type approaches appears to offer a promising method of including the effect of culture history in gradient modelling. The bottleneck of their combination is still the large computational

cost of both approaches, which may be alleviated with the development of more representative computationally inexpensive methods to describe fluid dynamics (i.e. compartment models representative of large-scale behavior).

Rather than treating the cells as a black box, structured models account for intracellular behaviour. This can be done by modelling the intracellular concentrations of key metabolites or proteins (Tang et al., 2017) or through the use of more complex tools like genome-scale modelling which aim to represent all possible reactions within the cell (Gu et al., 2019; Liu et al., 2010). The use of such models has the potential to give far greater insight into how cells respond to gradients, while also vastly increasing the number of parameters which need to be measured and validated. Finally, the complexity of some models may make their integration with other tools (e.g. CFD) challenging from a computational perspective.

Selection of the appropriate degree of model complexity is likely to be both process and microorganism specific. For example, it was found that for *Streptococcus thermophilus* an unsegregated unstructured kinetic model provided good results both in Euler-Euler CFD simulations and compartment models (Spann et al., 2019a; Spann et al., 2019b). In contrast, for *P. chrysogenum* Euler-Lagrange CFD simulations, an unsegregated unstructured model showed a poorer prediction of the yield than an unsegregated structured model (Haringa et al., 2018). An interesting avenue for future research would be to determine whether there are guidelines that can be broadly applied in order to determine which level of model complexity is necessary to accurately predict the behaviour of large-scale bioreactors.

3. Tools for the investigation of gradients

Existing approaches for the quantification of gradients can be divided into three broad areas. The first of these are computational based approaches where a model of the fluid dynamics within the reactor is coupled with a kinetic model in order to calculate the concentration of substrate (or other relevant species) and hence determine the extent of gradients. Secondly, it is possible to perform large-scale experimental measurements to directly quantify the extent of any gradients. Finally, it is possible to construct scale down experiments, which aim to replicate the effect of gradients at a smaller scale in order to quantify their effect on process performance. This section will examine each of these approaches, discussing the current state of the art as well as the advantages and disadvantages of each approach.

3.1. Computational Fluid Dynamics (CFD)

Computational Fluid Dynamics (CFD) uses numerical methods to solve the Navier-Stokes equations in order to describe the hydrodynamics of a system. Such an approach has the advantage of providing a high-level of spatial and temporal detail about the flow within the system. Including appropriate physical models (i.e. those for mass transfer) and kinetic models enables the prediction of the local values of relevant parameters (e.g. the substrate concentration, specific growth rate, etc.) throughout the entire reactor volume. Furthermore, the utilization of CFD models allows the simulation and visualisation of systems that are difficult to run or access, such as large-scale bioreactors with operational settings far from the usual performance boundaries. In such cases, the utilization of CFD software compared with experimental procedures might become economically advantageous (Versteeg and Malalasekera, 2007).

The majority of CFD software uses the finite volume method for the solution of the equations. Detailed descriptions of the Navier-Stokes equations and the numerical methods used in their solution are presented elsewhere (Versteeg and Malalasekera, 2007); this review will focus on the most relevant aspects for the simulation of bioreactors. The first step in the solution is the

discretisation of the reactor geometry into smaller volumes (cells), i.e. generating a mesh which is used for the solution of the equations. Mesh generation is a compromise between numerical accuracy and computational demand; increasing the number of cells in the mesh necessitates additional computational resources to solve the model, while having an insufficient number of cells results in numerical errors. Larger or more complex geometries (e.g. the internals found in some bioreactors) increases the number of cells in the mesh, with a concomitant increase in computational demand. Solution of the equations also necessitates discretization in time; the smaller the time step, the more computational resources are necessary to simulate a given period of real time operation.

Due to their nature gradients are intrinsically transient phenomena, meaning that it is not possible to use a steady-state solution; this increases the computational resources needed. Similarly, simulation of rotating components (i.e. impellers) necessitates the use of small time-steps and hence adds to the computational demand.

Typical fermentation processes contain both a gas and liquid phase. The two major approaches which can be applied to the modelling of two-phase gas-liquid systems are the Euler-Euler and Euler-Lagrange approaches. In the Euler-Euler approach the phases are treated as inter-penetrating continua, with each phase having a volume fraction; the sum of all of the volume fractions must equal one. In the Euler-Lagrange approach the displacement of particles (here the gas bubbles) is calculated by integration of the particle momentum equation. The computational demand for the Euler-Lagrange approach is directly proportional to the number of bubbles, hence it is generally not used for modelling the gas-phase except at very low gas volume fractions (Rampure et al., 2007; Tabib et al., 2008). Both approaches require an accurate description of the inter-phase transfer of momentum, mass and turbulence. The challenges involved in selection of appropriate models is discussed elsewhere (Fletcher et al., 2017; Rampure et al., 2007; Tabib et al., 2008). Simulation of fermentation medium can be a particular challenge due to non-Newtonian rheology and the presence of surface-active compounds which affect both drag and mass transfer (Jamialahmadi and Müller-Steinhagen, 1992; McClure et al., 2014; McClure et al., 2015c; Prins and van't Riet, 1987). In order to accurately simulate realistic systems it is necessary to account for this behaviour in any CFD model (McClure et al., 2015d).

The simulation of multi-phase flows also necessitates the use of small timesteps (1-10 ms) needed to accurately describe the flows; this obviously increases the computational demand needed. Here it must be noted that selection of appropriate models and the use of appropriate solution methods is essential in providing an accurate description of the hydrodynamics; without this any results generated by the model are likely to be incorrect.

The Euler-Lagrange approach can also be used to model the transport of cells throughout the reactor, giving the history of conditions experienced. By including a large number of particles, it is possible to perform a statistical assessment of the number, type and length of fluctuations between varying reactor conditions. Such calculations may be the most representative of actual fermentation processes as they enable the time history of the cells to be accounted for (Haringa et al., 2017; Lapin et al., 2006; McClure et al., 2016).

The increasing availability of high-performance computing platforms has made it easier to solve large simulation tasks. However, the need for transient simulations with small time steps means that state of the art CFD models are currently capable of realistically simulating several minutes of a fermentation process, and not the entirety of a batch.

Table 2 summarises previous work using CFD to model bioreactors. Both stirred tanks reactors and bubble columns have been simulated. All of the authors in Table 2 have used the Euler-Euler

approach for modelling the fluid flow, with many using the Euler-Lagrange approach to model the trajectory of cells as they are circulated throughout the reactor. Interestingly, in addition to gradients in glucose concentration, gradients in pH, DO and carbon monoxide concentration have also been examined. Most work found that gradients were likely to be present, except for the work examining pH (Spann et al., 2019a; Spann et al., 2019b; Tajsoleiman, 2018) and dissolved oxygen (Bach, 2018) where no significant gradients were found. This may be due to the relatively small scale of the reactor (700 L) (Spann et al., 2019b) or the fact that the rate of oxygen uptake or lactic acid production is lower than the rate of transfer or mixing, meaning no gradients are likely to be found. Of the studies that have reported gradients both losses in yield (Haringa et al., 2018; McClure et al., 2016) and changes in transcription (Kuschel et al., 2017; Siebler et al., 2019) have been predicted. It was also found that the reported losses in yield were a function of the substrate addition point, highlighting the ability of CFD to be used as a tool for process optimisation.

Table 2 – Summary of CFD simulations published in the literature. Unless otherwise specified (Bach, 2018), the impeller(s) used were Rushton turbine disk.

Microorganism and parameter	**Approach**	**Operational settings**	**Results**	**Refs**
Clostridium ljungdahlii Substrate (CO) concentration	CFD/KM (Euler-Euler for fluids, Euler-Lagrange for cells)	BC; V_T = 125 m^3; τ_m = 27.3 ± 4.3 s; U_{sg} = 0.062[illegible] m s^{-1} (55% v/v CO); α_G = 0.2[illegible]-0.34; k_La = [illegible] h^{-1}; fluid properties of water; X = 10 kg m^{-3}.	S_{lim} = 8.4×10^{-5} kg m^{-3}; $q_S/q_{S,max}$ = 49%; 97% cells experience $S < S_{lim}$; 84% of the cells are likely to undergo transcriptional challenges after exposure of >70 s to the high substrate concentration stress-inducing zone.	(Siebler et al., 2019)
Escherichia coli Substrate (glucose) concentration	CFD/KM	STR; 0.07 and 70 m^3; 1 impeller; τ_m= 5 and 24 s; U_{sg}= 0.005 m s^{-1}; P_i/V_L = 1.8 kW m^{-3}; α_G = 0.05; k_La = 500 h^{-1}; F_S = 0.319 and 355/318.9 kg h^{-1} in top/bottom feed positions (value for μ_{set} = 0.5$\mu_{S,max}$); fluid properties of water; aerobic fed-batch; X = 10 kg m^{-3}.	τ_{SC} = 15 s; The physiological state of the cell population depends on the characteristic time of biological adaptation (T_a). The population specific growth rate strongly depends on the volume average concentration of the reactor ($T_a >> t_C$). On the other hand, the population specific uptake rate depends on the local concentration of glucose ($T_a \approx t_C$).	(Morchain et al., 2014)

Microorganism and parameter	Approach	Operational settings	Results	Refs
Penicillium chrysogenum Substrate (glucose) concentration	CFD/KM (Euler-Euler for fluids, Euler-Lagrange for cells)	STR; V_T = 54 m^3; 2 impellers; τ_m= 72.8 s; aeration neglected; P_i/V_L = 3 kW m^{-3}. Case studies: A) CFD snapshot. F_S= 240 kg h^{-1}. X = 55 kg m^{-3}. B) Dynamic CFD without volume addition for 60 h. F_S = 54 – 94.5 kg h^{-1}; fluid properties of water; X = 15 – 40 kg m^{-3}.	A) $q_{S,crit}$ = 0.95$q_{S,max}$; $q_{S,lim}$ = 0.05$q_{S,max}$; $q_{S,max}$= 0.20-0.29 kg kg^{-1} h^{-1}. 57% of cells undergo starvation ($q_S < q_{S,lim}$) and 7% experience exceeding glucose concentration ($q_S > q_{S,lim}$). B) Yield loss of 18-45% which can be reduced 9% by changing the feed rate location. Good fit with experimental data.	A: (Haringa et al., 2016) B: (Haringa et al., 2018)
Pseudomonas putida Substrate (glucose) concentration	CFD/KM (Euler-Euler for fluids, Euler-Lagrange for cells)	STR; V_T = 54 m^3; 2 impellers; τ_m = 27.3 ± 4.3 s; aeration neglected; P_i/V_L = 4.2 kW m^{-3}; F_S= 400 kg h^{-1} (corresponding to $q_{S,max}/2$); fluid properties of water; X = 10 kg m^{-3}.	$q_{S,max}$= 1.476 kg kg^{-1} h^{-1}; 72% of cells switched between standard and multifork (fast) DNA replication; 10% were likely to undergo massive transcriptional adaptations to starvation; 56% of cells replicated very fast with ($\mu \geq 0.3$ h^{-1}). 52.9% of cells with higher ATP maintenance demands; μ did not present significant changes to ideal mixing.	(Kuschel et al., 2017)
Saccharomyces cerevisiae	CFD/KM (Euler-Euler and Euler-Lagrange)	STR, 20-22 m^3 volume, 4 impellers, τ_m = 183 s; α_G = 0.18; k_La = 144 h^{-1}; F_S = 52 kg h^{-1}; fluid properties of water; X = 10 kg m^{-3}.	$q_{S,crit}$ = 0.2$q_{S,max}$; $q_{S,lim}$ = 0.05$q_{S,max}$; $q_{S,max}$= 1.70 kg kg^{-1} h^{-1}; 35% of cells undergo starvation ($q_S < q_{S,lim}$) and 24% experience exceeding glucose concentration	(Haringa et al., 2017)

Microorganism and parameter	Approach	Operational settings	Results	Refs
			($q_S > q_{S,lim}$).	
		BC; V_T = 0.24 m^3; U_{sg}= 0.16 m s^{-1}; α_G = 0.23; τ_m = 15 – 20 s; F_S = 3.96 kg h^{-1} at two locations: reactor top and below the sparger; fluid properties of water; aerobic fed-batch; X = 50 kg m^{-3}.	$q_{S,crit}$ = 0.61 kg kg^{-1} h^{-1}; $q_{S,min}$ = 0.03 kg kg^{-1} h^{-1}; 13 and 12% reactor volume experiencing overflow for top and bottom feed positions; starvation not reported; $Y_{XS}^{obs}/Y_{XS}^{true}$= 81-93% and 73-75% for top and bottom fed positions; substrate addition beneath sparger leads to a higher degree of heterogeneity (larger gradient and magnitude and frequency of oscillations between oxidation/overflow regimes).	(McClure et al., 2016)
Streptococcus thermophilus pH	CFD/KM (Euler-Euler)	STR; V_T = 0.7 m^3; 3 impellers; τ_m= 46 s; P_i/V_L = 0.13 – 0.79 kW m^{-3}; fluid properties of water; anaerobic batch; X_{max} = 6 kg m^{-3}.	No significant pH gradients were found.	(Spann et al., 2019a; Spann et al., 2019b; Tajsoleiman et al., 2019b)
Trichoderma reesei DO concentration	CFD/KM (Euler-Euler)	STR; V_T = 80 m^3; 3 Impeller configurations: 4 RTD; B) 1 RTD + 3 DP-PBT; C) 1 RTD + 3 UP-A310; $\tau_{m\,A}$= 191 s, $\tau_{m\,B}$= 172 s, $\tau_{m\,C}$ = 30.1 s; aeration accounted but rates are not specified; k_La = 125 – 350 h^{-1}; shear-thinning viscosity, surface tension of water; aerobic fed-batch.	$q_{S,max}$= 0.18 kg kg^{-1} h^{-1}; No significant DO concentration gradients were found.	(Bach, 2018)

3.2. Compartment models (CMs)

Compartment or networks-of-zones models consist of collections of ideally mixed fixed volumes with flow rates and connections between them such that their overall flow behaviour is able to represent the reactor fluid dynamics (Bezzo et al., 2004). Compartment models are less computationally demanding than CFD models (Jourdan et al., 2019) for two main reasons. First, CMs are normally run with just a few homogeneous volumes or compartments (2-70) (Pigou and Morchain, 2014), while the number of cells in a CFD mesh is rather large, e.g. ranging up to millions of elements for large scale bioreactors. Secondly, in compartment models, complex physical phenomena are not simulated, meaning that only species (e.g. substrate) mass balances are solved while governing equations describing fluid flow are excluded. These features result in very short simulation times (few seconds (Spann et al., 2019a)) without the need for High Performance Computing (HPC) hardware. These advantages make CMs very flexible and attractive for many practical applications such as optimization of the feeding point or of the location for acid or base addition. Additionally, it may be possible to implement more complex kinetic models in CMs due to their lower computational demand. Selection of the appropriate modelling approach involves a trade-off between the degree of simplification and computational demand and the most suitable choice depends on the research question.

The main challenge in the development of compartment models is their definition in terms of compartment volumes, flows and connections, which does not have a standard approach due to the variety of end-applications and required levels of accuracy of CMs (Jourdan et al., 2019). First, CMs were defined empirically (Bashiri et al., 2014) based on local hydrodynamic knowledge, theoretical models and empirical flow field predictions. With the growing utilization of CFD software, CMs have been built semi-automatically (Delafosse et al., 2014; Nørregaard et al., 2019; Öner et al., 2019; Pigou and Morchain, 2014; Spann et al., 2019a), meaning that CFD results are used for the calculation of flows but the differentiation of homogeneous zones is still based on the user-defined criteria. The fully automatic design of compartment models is finally accomplished with the development and exploitation of novel CFD-based tools (Tajsoleiman et al., 2019b), which allow the specification of homogeneity tolerance levels and the automatic definition of compartment volumes and connections and calculation of flow rates between compartments. This opens a new perspective regarding the easy construction and operation of models that can reproduce the fluid dynamic behaviour in complex systems satisfactorily and that are partially de-coupled from CFD models. In Figure 3, an example of an automated CFD-based compartment map based on the velocity profile of a bubble column simulation is depicted. In fermentation processes, CMs have been utilized for systems involving the simulation of pH gradients in a pilot-scale lactic acid bacteria fermentation process (Spann et al., 2019a; Tajsoleiman, 2018), glucose and oxygen concentration gradients in a 90 m^3 yeast fermentation (Tajsoleiman, 2018) and glucose concentration gradients for a 22 m^3 *E. coli* fermentation (Pigou and Morchain, 2014).

3.3. Experimental measurements

Direct measurement of gradients in industrial equipment is pursued as it provides the best evidence of the scale and impact of gradients on relevant commercial processes. Measuring gradients allows the validation of both modelling and mimicking approaches for their investigation. Furthermore, it can help at identifying limitations of both computational and experimental tools, aiming at their further improvement.

Despite their high value for those actively working in this field of research, gradient measurements at large scale are scarce. The first reason is the lack of available industrial reactors

for research purposes. Secondly, publication of realistic industrial data is rare due to confidentiality concerns. While pilot facilities might be accessible, gradients are not expected in such systems unless they are caused by operating at suboptimal settings. Furthermore, pilot data do not supply information about the real incidence of gradients in industry or about the potential issues that they cause at the real production scale. Thus, measurement of gradients in large-scale fermenters is the most interesting case for research and is also the most difficult to achieve.
The traditional method for the measurement of gradients in large-scale consists of the placement of multiple on-line probes and/or of sampling from several ports at different heights of the fermenter. Relevant examples in the literature include measurements of glucose (Bylund et al., 1998; Enfors et al., 2001; Larsson et al., 1996), the dissolved oxygen concentration (Bach, 2018; Oosterhuis and Kossen, 1984; Steel and Maxon, 1966) and pH (Spann et al., 2019b). Such measurements can require substantial alterations to be made to process equipment which can incur major costs (both directly, and indirectly in terms of lost production time). They also have the limitation in that they provide a relatively low resolution, as for practical reasons it is only possible to make measurements at relatively few (< 10) locations which are typically close to the wall of the reactor. More sophisticated approaches using lance-based multi-parameter (e.g. pH and temperature) sensors which can be moved within the reactor have been developed (Enseleit et al., 2017). Such approaches have the advantage of offering greater spatial resolution. However, substantial modifications to process equipment are necessary for their installation.
Table 3 summarises published work quantifying gradients at the pilot and commercial scale. All of the published work used stirred tank reactors, despite the fact that other fermenter designs (e.g. bubble columns) are used in other processes (e.g. baker's yeast production). Substrate (glucose) gradients were the most widely investigated, and it has been found that such gradients led to reductions in the biomass yield on substrate, as well as changes in gene expression.

Table 3 – Summary of large-scale experimental investigations into gradients in fermentation processes. Unless otherwise stated (Bach, 2018), the impeller/s used were Rushton turbine disks.

Microorganism or cell line and parameter	**Approach**	**Operational settings**	**Results**	**Refs**
CHO cells DO concentration	Single point measurements at large scale	STR; V_L = 3-5 m^3; $\tau_m > 100$ s; aerobic fed-batch; $X = 0.8 - 8 \cdot 10^6$ cell mL^{-1}.	Compared with a 20 L ideally mixed fermenter, hypoxia was detected in large scale, which led to the production of reactive oxygen species (ROS). By doubling the copper concentration in the medium at large scale, cell viability was also doubled, still not reaching the lab scale level. A 20 L reactor did not suffer hypoxia, therefore medium with	(Gao et al., 2016)

Microorganism or cell line and parameter	Approach	Operational settings	Results	Refs
			increased copper did not change significantly the cultivation evolution. Transcriptomic and proteomic studies were performed to identify hypoxia.	
Escherichia coli Substrate (glucose) concentration	Multiple-point measurements	STR; V_L = 8-9 m^3; 3 impellers; F_g = 0.25 – 0.75 vvm; 4 substrate feed phases: lag, exponential (μ_{set} = 0.3 h^{-1}); X_{max} = 30 kg m^{-3}	μ = 0 – 0.35 h^{-1}; q_S = 0.35 - 0.7 kg kg^{-1} h^{-1}; 20% lower Y_{XS} compared with bench scale; acetate formation; product formation rate unaffected by change in scale.	(Bylund et al., 1998)
		STR; V_L = 22 m^3; 4 impellers; τ_m = 60 – 120 s; F_S initially exponential to keep μ_{set} = 0.3 h^{-1} and subsequently constant; aerobic fed-batch; X_{max} = 35 – 40 kg m^{-3}.	Formate accumulation pointed out the development of oxygen limitation zones; reduced Y_{XS} due to repeated assimilation/production of acetate from overflow and mixed acid fermentation; concentration of mRNA of four stress induced genes was lowest at the sampling port most distant from the feed zone; flow cytometric analysis revealed reduced cytoplasmic membrane potential and integrity damage in large scale compared with lab scale.	(Enfors et al., 2001)
		Same as previous row	12% reduced biomass yield, formation of	(Xu, B. et al., 1999)

Microorganism or cell line and parameter	Approach	Operational settings	Results	Refs
			acetate (up to 300 mg L^{-1}) and formate (up to 50 mg L^{-1}).	
Pencillium chrysogenum Substrate (glucose) concentration	Single point measurements at large scale	STR; V_T = 54 m^3; 2 impellers; τ_m = 72.8 s; aeration neglected; P_i/V_L= = 3 kW m^{-3}. Case studies: A) CFD snapshot. F_S = 240 kg h^{-1}. X = 55 kg m^{-3}. B) Dynamic CFD without volume addition for 60 h. F_S = 54 – 94.5 kg h^{-1}; fluid properties of water, X = 15 – 40 kg m^{-3}.	$q_{S,crit}$ = 0.95$q_{S,max}$; $q_{S,lim}$= 0.05$q_{S,max}$; $q_{S,max}$ = 0.20-0.29 kg kg^{-1} h^{-1}. 57% of cells undergo starvation ($c_S < q_{S,lim}$) and 7% experience exceeding glucose concentration ($q_S > q_{S,lim}$).	A: (Haringa et al., 2016) B:
Saccharomyces cerevisiae Substrate (glucose)	Multiple point measurements	STR; V_L = 19.8 – 22.1 m^3; 4 impellers; τ_m = 147 – 166 s; U_g = 0.05 m s^{-1}; P_i/V_L= = 1.8 – 2 kW m^{-3}(?); α_G = 0.17; k_La = 180 h^{-1}; F_S = 7.5 – 54 kg h^{-1} (μ_{set} = 0.2 h^{-1}) at top and bottom positions; aerobic fed-batch; X = 0.15/0.18 – 25/20 kg m^{-3}.	A substrate concentration peak from approximately 40 to 80 mg L^{-1} was observed, being 80 mg L^{-1} the maximum local concentration of glucose reported. The pattern of the fluctuations depended on the turbulence level at the location of the feed.	(Larsson et al., 1996)
	Multiple point measurements	Bubble column, 215 m^3 total volume (17.5 m height, 4 m diameter), liquid volume ~ 120 m^3. 0.15 m s^{-1} superficial velocity, sample height 2.1 and 6.3 m.	Higher substrate (glucose and fructose) concentrations found closer to feed point.	(George et al., 1998)
Streptococcus thermophilus pH	Multiple-point measurements	STR; V_T = 0.7 m^3; 3 impellers; τ_m = 46 s; P_i/V_L= = 0.13 – 0.79 kW m^{-3}; fluid properties of water; anaerobic batch; X_{max} = 6 kg m^{-3}.	No significant pH gradients were found.	(Spann et al., 2019a; Spann et al., 2019b)
Trichoderma	Multiple-	STR; V_T = 80 m^3; 3	$q_{S,max}$ = 0.18 kg kg^{-1}	(Bach,

Microorganism or cell line and parameter	Approach	Operational settings	Results	Refs
reesei DO concentration	point measurements	Impeller configurations: 4 RTD; B) 1 RTD + 3 DP-PBT; C) 1 RTD + 3 UP-A310; $\tau_{m,A}$ = 191 s, $\tau_{m,B}$ = 172 s, $\tau_{m,C}$ = 30.1 s; aeration accounted but rates are not specified; $k_L a$ = 125 – 350 h^{-1}; shear-thinning viscosity, surface tension of water; aerobic fed-batch.	h^{-1}; No significant DO concentration gradients were found.	2018)

In order to increase the resolution of gradient measurements to the complete operating volume and, more importantly, to make gradient measurements available to all fermentation facilities, the development of free-floating sensor particles is gaining interest. These are small spherical devices ranging from a few millimeters to centimeters in size which contain one or multiple sensors that can measure relevant cultivation variables. The precursors of free-floating sensor particles are traditional flow followers (Mavros, 2001), which are traceable particles utilized to assess the flow pattern and/or to calculate the circulation time in both stirred and non-stirred mixing vessels (Fangary et al., 2000; Fields et al., 1984; Pant, 2000; Van Barneveld et al., 1987a, b). The addition of sensors and improvement of positioning techniques make free-floating sensor particles very attractive since they can provide spatio-temporal data of the fermentation process. The challenges in their development include the miniaturization and successful inclusion of sensors, ensuring the response time of the sensors is sufficiently fast to measure local values (i.e. the sensor response time is less than the timescale for transport), low power consumption or self-powering implementation, hermetic sealing, capability to be sterilized inside the fermenter, easy recovery, wireless communication in a hostile medium for real-time monitoring, buoyancy neutrality and positioning (Lauterbach et al., 2019; Reinecke et al., 2012; Reinecke and Hampel, 2016; Thiele et al., 2010; Todtenberg et al., 2015; Wadke et al., 2005). Despite the significant technical challenges involved, progress is being made in this area, with some companies (http://freesense.dk/) offering the spatio-temporal assessment of macro-mixing, pressure, temperature, pH and DO measurements.

Experimental measurements of gradients at commercial scale are necessary to obtain realistic understanding of the occurrence of gradients in fermenters, as well as for the validation of computational models. Such measurements still need to be combined with accurate kinetic models in order to determine how any gradients affect the cell physiology and hence provide accurate descriptions of the process performance.

3.4. Scale-down experiments

Scale-down experiments are used to expose microorganisms to defined fluctuations in order to quantify their response. The major advantage of such experiments is that it is possible to

precisely control the oscillations and sample collection and analysis is much simpler than for an industrial system. For the comprehensive characterization of the physiological response of cells to fluctuating conditions, scale-down experiments are generally combined with detailed analysis of cellular physiology. This can include quantifying the phenotypic variability (Dhar and McKinney, 2007), the budding index (Marbà-Ardébol et al., 2018), cell viability and membrane integrity (Hewitt et al., 2000), as well as metabolomics, transcriptomics and proteomics (Gao et al., 2016; Korneli et al., 2012; Lu et al., 2018).

Scale-down experiments can be divided into two major categories: pulse-feeding and multi-compartment simulators (Lara et al., 2006a; Neubauer and Junne, 2010; Neubauer and Junne, 2016) (Figure 4). Pulse-feeding simulators (Sunya et al., 2012; Wang et al., 2019) usually consist of a single lab-scale fermenter into which pulses of a variable of interest (e.g. carbon source, pH controlling agent) are applied at such frequency and magnitude that the simulator is able to mimic the oscillations that the cells experience at a particular location in a large-scale reactor. Similarly, variations in the dissolved oxygen concentration can be examined by varying the back-pressure or changing the composition of the inlet gas (Lara et al., 2006a). Multi-compartment simulators (Amanullah et al., 2001; Heins et al., 2015; Onyeaka et al., 2003; Simen et al., 2017) consist of two or more connected reactors operated at different conditions but with a combined overall performance capable of representing large-scale operation in terms of mixing performance and/or distribution of values of the studied gradient (e.g. substrate concentration, pH, etc.). Typical configurations of multi-compartment simulators consist of a plug flow reactor connected to a stirred tank reactor (PFR-STR) or two or more stirred tank reactors in series (STR-STR), while other configurations (e.g. two PFRs and one STR) have been used (Lemoine et al., 2015). For the study of glucose concentration gradients in PFR-STR systems, the PFR represents the feed zone of the reactor where accumulation of glucose may take place, while the STR resembles the well-mixed glucose non-limiting nor exceeding zone. This is similar with several STRs in series, where each of them aims to mimic a volume of the reactor experiencing different conditions.

From a practical perspective there are several challenges involved with the operation of scale-down systems. These include the ability to sample at a sub-second time scale (Schaefer et al., 1999), fast quenching of cellular activity and the development of methods for the extraction and quantitation of key compounds (e.g. metabolites, DNA, mRNA, etc.). While deployment and validation of such systems is challenging, there has been substantial progress in this area, and the availability of such technologies is not likely to limit scale-down studies (Schädel and Franco-Lara, 2009). Other practical issues can include avoiding biofilm formation, establishing stable recirculating flows, ensuring the rapid addition of feed(s) and avoiding having significant dead volumes (i.e. in tubing circulating between reactors). More serious challenges arise when attempting to replicate the physical phenomena found in large-scale reactors (Tajsoleiman et al., 2019a). Many of these issues are amplified when working with viscous fermentation broths (e.g. filamentous fungi broth). For example, the potentially unattainable large-scale Reynolds numbers at lab-scale can lead to the development of laminar or transitional rather than fully turbulent flows. Moreover, it is very complicated to reach industrially relevant tip speed values in small reactors due to equipment constraints. Tip speed affects the shear rate, causing changes in the rheology and morphology of filamentous fungi (Quintanilla et al., 2018). Other functional aspects to consider are the increased risk of vortex formation due to increased agitation speeds in

scale down systems, the differences in volume dynamics (e.g. different evaporation rates) and the differences in hardware which might generate new constraints such as the need to add a more dilute glucose feed because of constraints in the pumping system. Fundamentally it may not be possible to replicate all conditions found in large-scale operation, for example having good mixing necessitates a high agitation speed, which may lead to a higher shear rate or OTR than found in large-scale operation.

The second and more important issue is that while scale-down experiments have been demonstrated to be useful for metabolic analysis under fluctuating conditions (Neubauer and Junne, 2010), there is not a well-established approach that allows the direct scale-down of large-scale fermentation processes. Many of these experiments have been designed based on rules of thumb and educated guesses (Noorman and Heijnen, 2017), for instance by setting the re-circulation rate between scale-down reactor at the same value as the mixing time (Limberg et al., 2016). Small-scale systems may only be able to represent a portion of a large-scale reactor and not the entire volume which is likely to consist of multiple zones and not just the small number of compartments which have been generally examined (Table 4). Finally, in large-scale fed batch systems both the number and size of the zones will change with time and accurately replicating this at a small-scale represents a significant challenge. Hence, in order to accurately replicate industrial systems scale-down experiments need to be rigorously designed using large-scale experimental data or validated computational results.

With this principle in mind Haringa et al. (2017; 2016; 2018) have designed scale-down experiments based on Euler-Lagrange CFD simulations combined with microbial kinetic models. Haringa et al. (2016) followed a metabolic regime analysis approach for down-scaling by assessing the Lagrangian trajectories of cells, the per-regime residence time distributions and the substrate uptake dynamics when substrate limitation takes place. These methodologies have led to the successful design of a three-compartment and a pulse-feed scale-down experiment (Haringa et al., 2017) for the 22 m^3 *S. cerevisiae* fermentation process previously mentioned (Larsson et al., 1996), and a pulse feed scale-down experiment for a 54 m^3 *P. chrysogenum* fermentation process (Haringa et al., 2018).

Tajsoleiman (2018) took an automatic CFD/CM-based approach to distinguish different zones experiencing various metabolic regimes in 90 m^3 *S. cerevisiae* fermentation processes with varying glucose feed rate levels and calculated the compartment volumes and flows accordingly for the design of several multi-compartment scale-down experiments. Even though both computational studies have shown encouraging results to yield rational designs of scale-down experiments, experimental validation of the systematic design approaches and of scale-down experiment performance by comparing with large-scale operation is still required.

Despite these limitations scale-down experiments are a valuable tool in understanding how fluctuating conditions affect cell behaviour. Hence, they can be used to obtain increased scientific understanding, mimic large-scale systems and also as a tool for screening which strains are most suitable for industrial production (Noorman and Heijnen, 2017). Here it is essential to reiterate that useful outcomes from the last two applications will only be generated if the system is designed and operated in such a way that the imposed fluctuations correspond to those found at the large-scale.

As shown in Table 4 a wide range of industrially relevant microorganisms have been examined as well as the effect of a range of cultivation variables. A range of different systems have been

examined, and this has led to different conclusions being reached by researchers studying the same organism. For example, when examining *Corynebacterium glutamicum*, Limberg et al. (2016) found that substrate and DO concentration variations in a STR-STR and a PFR-STR scale-down bioreactor affected growth but not process yields for a batch process. In contrast, Lemoine et al. (2015) concluded that fluctuations in such process parameters affected the product yield in PFR-STR and 2PFR-STR fed-batch scale-down systems. Finally, other fed-batch studies from Käß et al. (2014a; 2014b) and again by Limberg et al. (2017) performed with PFR-STR and STR-STR scale-down systems, respectively, underlined the robustness of *C. glutamicum* to environmental oscillations by reporting no change in process metrics compared with control lab-scale fermentation processes. In all cases, residence times in the smaller reactors ranged between 78 and 180 s, so strong variations because of these values are not expected. On the other hand, batch STR-STR experiments from Limberg et al. (2016), were done at very high reactor volume ratios (1.6:1, 3.5:1), while the other cases were done with lower values (STR:PFR, 5.6:1, STR:PFR:PFR 5.6:1:1 (Lemoine et al., 2015); 4.6:1 (Käß et al., 2014b); 4:1 (Käß et al., 2014a)). Furthermore, they were the only experiments performed with batch rather than fed-batch operation. These two operational settings may have influenced the batch scale-down experiment to result into lower growth rate (Limberg et al., 2016). The work of Lemoine et al. (2015) found lower Y_{PS} in both the PFR:STR and the 2PFR:STR scale-down setups with comparable operational settings to the PFR:STR of Käß et al. (2014a; 2014b). This issue has been highlighted, (Lemoine et al., 2015) but is yet to be explained. In both studies, the *C. glutamicum* strains used were L-lysine-producing. Nevertheless, some differences between strains from the same species may provide variability regarding the impact of gradients. The stochasticity of gene expression (Heins and Weuster-Botz, 2018) also needs to be considered, leading to more negative consequences to non-homogeneous conditions in some cultures than in others.

Another example is the different results to DO concentration oscillations by two PFR-STR scale-down studies with *K. phaffii* (formerly known as *P. pastoris*). It was found that decreased growth and increased by-product formation was correlated to the time of exposure to low DO concentrations (Lorantfy et al., 2014). In contrast, another study did not see any correlation of process metrics with reactor residence time, but with the DO level (Jazini et al., 2014). Residence times in the PFR oscillated between 1 to 8 and 3.3 to 15 min, respectively. Furthermore, the STR:PFR volume ratios were 1:1 and 11.1:1, respectively. Thus, both setups have quite different residence time spans and volume ratio differences, leading to potentially conflicting results within the same experimental principle.

The last clear example reported here of different conclusions to scale-down experiments performed with the same microorganism correspond to the widely used yeast *S. cerevisiae*. Heins et al. (2015) ran two STRs in series and compared them with a single STR. They varied the glucose feed concentration and the dilution rate. In most cases, Y_{XS} and the biomass productivity were higher than in a single STR. In addition, ethanol yields were also lower because ethanol was re-assimilated in the non-feeding STR. On the other hand, scale-down work performed with PFR-STR, 2PFR-STR (Marbà-Ardébol et al., 2018) and STRs connected to a glass bulb or to two different pipes (Lejeune et al., 2010; Thonart et al., 2010) showed the opposite effect, as well as pulse-feeding experiments (Suarez-Mendez et al., 2014). Thus, it is likely that the operational setup did play a role in this case, where different conclusions arise from different reactor configurations.

Based on the above observations, it is concluded that the variation in design of scale-down experiments makes it challenging to provide comprehensive conclusions regarding the impact of gradients on microbial physiology. Nevertheless, a few microorganism-dependent observations have been established based on the scale-down studies reviewed (Table 4) involving substrate concentration fluctuations (i.e. those most widely studied). Exposure to substrate oscillations has led to a decrease in the glucose uptake rate in *Bacillus* sp. (Junne et al., 2011; Korneli et al., 2012), ultimately causing amino acid deficiency. In contrast, *E. coli* showed increased growth rates (Taymaz-Nikerel et al., 2011) as a result of an increased glucose uptake capacity (Neubauer et al., 1995). The formation of by-products (Sunya et al., 2012) and changes in the biosynthesis of amino acids derived from pyruvate (Soini, 2011) has also been reported as a result of exposing *E. coli* to substrate oscillations. The formation of by-products after substrate fluctuations has also been described for *A. niger* (Wang et al., 2019) and *S. cerevisiae* (Lejeune et al., 2010; Marbà-Ardébol et al., 2018; Thonart et al., 2010). Finally, *C. glutamicum* is unaffected by glucose concentration fluctuations (Käß et al., 2014a; Käß et al., 2014b; Limberg et al., 2016; Limberg et al., 2017), and *P. chrysogenum* activates the storage of central metabolites (De Jonge et al., 2014; Wang et al., 2018b).

Scale-down experiments can be used to gain detailed insight into how fluctuating conditions affect the metabolism of cells. However, the major challenge in the use of such systems is ensuring that they meaningfully represent the conditions found in large-scale bioreactors, an issue which is exacerbated by the relatively small amount of experimental data which can be used for the design and validation of scale-down systems.

Table 4 – Summary of the scale-down experiment setup and results reported in the literature for many industrially relevant microorganisms and cell lines.

Microorganism or cell line	**Parameter**	**Setup and results**	**Refs**
Aspergillus niger	Substrate concentration	Pulse feeding experiments. No influence on biomass or product levels. Up to 2-fold increase of organic acid by-products excretion (oxalate, citrate and pyruvate) in comparison with glucose-limiting steady-state conditions.	(Wang et al., 2019)
Bacillus megaterium	Substrate concentration	Intermittent excess feed supply. 40% reduced Y_{PS} via formation of acetate (overflow) and carbon dioxide. Caused by bottleneck at pyruvate level, which reduced the formation of the amino acids Trp, Asp, His, Gln and Lys and increased the level of Ala in comparison with a reference process. By supplementing deficient amino acids, Y_{PS} level was recovered to 100%.	(Korneli et al., 2012)
	DO concentration	Suboptimal DO-based substrate feed and two-compartment STR-STR scale-down experiments led to decreased product formation in comparison with a fed-batch fermentation process with optimal adjusted control (positive reference).	(Korneli et al., 2011)

Microorganism or cell line	Parameter	Setup and results	Refs
Bacillus subtilis	Substrate and DO concentration	Two-compartment PFR-STR scale-down experiment with feeding at the entrance of the PFR lead to decreased glucose uptake, increased ethanol formation and altered amino acid synthesis in comparison with feeding at the top of the STR (reference experiment under non-oscillating conditions). Carbon flux at excess glucose and low DO concentration triggers overflow metabolism. Consequently, the reduced carbon flux entering the TCA cycle does not support sufficient amino acid synthesis.	(Junne et al., 2011)
CHO cells	Fluid dynamic stress	Fed-batch bench scale culture with re-circulation loop to a microfluidic "torture" chamber where fluid dynamic stress is applied. At eddy dissipation rates significantly higher than typical commercial operations of 2.9×10^{5} and 1.2×10^{3} W m^{-3}, no significant effects on cell growth, viability and product quality were observed in comparison with duplicate control fermentation processes using standard conditions including low turbulent specific mean energy dissipation rates ($\varepsilon_T \approx 20$ W m^{-3}).	(Nienow et al., 2013)
	pH	With one-compartment batch fermentations with shifts to pH values of 9 and 7.9, it was reported that cells adapt faster to higher pH values. Two-compartment STR-STR experiments with pH = 9 in one zone lead to decreased growth in comparison with reference control cultures at constant extracellular pH = 7 performed with one STR, especially in the exponential phase. Consequently, lower viable cell number and product titer were achieved.	(Brunner et al., 2017)
	pCO_2	In high pCO_2 batch and fed-batch fermentations, the lactate metabolic shift (from production to consumption) did not occur compared with lower pCO_2 control values due to an imbalance in the production and re-generation of NADH at high pCO_2 levels.	(Brunner et al., 2018)

Microorganism or cell line	Parameter	Setup and results	Refs
Corynebacterium glutamicum	Substrate and DO concentration	Two-compartment STR-PFR and STR-STR batch experiments. Reduced growth rate, increased formation of by-products (L-lactate and L-glutamate) in comparison with one-reactor aerobic batch cultivations. Y_{XS} and Y_{PS} remained constant. No clear differences between scale-down setups besides different side product formation profiles.	(Limberg et al., 2016)
		Two- and three- compartment STR-PFR and 2PFR-STR fed-batch experiments. 2PFR-STR in comparison with STR-PFR has 2-fold production of lactate and succinate and of several amino acids (Gly, Thr, Glu and Gln). No pyruvate accumulation as in STR-PFR because of lactate production, enhanced by oxygen limitation. Compared with mixing one-compartment (STR) reference cultivation, higher polarizability of the cell membrane but not actual damage nor accelerated cell death and lower Y_{PS} in both scale-down cases.	(Lemoine et al., 2015)
		PFR-STR showed a higher turnover to side products and broth acidification in comparison with homogeneous STR cultivations without the PFR module. Lactate production due to oxygen limitation and re-assimilation. Besides that, no loss in process metrics in the oscillation minute range. A multi-omics study did not identify significant changes in response to PFR-STR operation.	(Käß et al., 2014a; Käß et al., 2014b)
		STR-STR cultivation with multi-omics analysis show that central metabolism is flexibly re-arranged via up- and down-regulation of genes. L-lactate formation and assimilation. Environmental changes had no effect on biomass and product formation in comparison with biological replicates in single STR acting as control.	(Limberg et al., 2017)
	Substrate (complex media)	2PFR-STR fed-batch experiments with complex media based on sucrose, molasses and corn steep liquor were performed. Compared with one STR acting as reference,	(Lemoine et al., 2016)

Microorganism or cell line	Parameter	Setup and results	Refs
		there was reduced volumetric product yield and accumulation of short fatty acids (L-lactate and acetate) and growth cessation and sucrose accumulation 10 h after the cultivation started. q_S reduced by 20%. No changes in cell vitality or lysis.	
	Substrate and DO concentration and pH	PFR-STR experiments with residence time in the PFR (τ_{PFR}) of 1, 2 and 5 min lead to decreased product formation of 26, 49 and 59% compared with one STR acting as control fermentation process. When τ_{PFR} = 5 min, CO_2 productivity was 3.1 fold. Population of viable non-culturable cells increased with the magnitude of the gradient.	(Olughu et al., 2020)
	CO_2/HCO_3^-	Cultivation in 3 STRs. No effect on growth and productivity in comparison with a reference single reactor process, 66 genes with differential expression showing fast transcriptomic response to fluctuations (after 3.6 min).	(Buchholz et al., 2014)
Escherichia coli	Substrate concentration	Pulse-feeding experiments did not lead to by-product formation. Within 30 s, the growth rate was increased 3.7 fold in comparison with steady state conditions. This did not lead to an increased level of amino acid formation. After 40-60 s, a new steady state was reached with higher metabolic fluxes and concentrations of metabolites.	(Taymaz-Nikerel et al., 2011)
		PFR-STR experiment. Cells which entered oscillations from a lower specific growth rate were more sensitive to oscillations than cells subjected directly after a batch phase (high growth rate).	(Brand et al., 2018)
		STR connected to a recycle loop where glucose is injected at the inlet. Cell membrane permeability and protein leakage were decreased in the STR with recycling in comparison to an ideally mixed STR.	(Brognaux et al., 2014)
	Substrate and DO concentration	Pulse-feeding experiments showed that glucose consumption and acetate and formate production rates had a linear relationship, increasing a few seconds after the perturbation (rapid adaptation). Independent responses to glucose pulse intensities ranging from 0.08 to	(Sunya et al., 2012)

Microorganism or cell line	Parameter	Setup and results	Refs
		1 g L^{-1}.	
		PFR-STR experiment. Biosynthesis of amino acids derived from pyruvate was affected (e.g. formation of non-canonical amino acid norvaline) in comparison with the reference single STR cultivation.	(Soini, 2011)
		PFR-STR experiment. Product formation unaffected, as in reference fermentation process performed in the STR without the PFR.	(Li et al., 2015)
	Ammonia concentration	PFR-STR experiment. Stringent regulation induction, 15% maintenance increase in comparison with a steady-state reference state without gradients in the STR.	(Simen et al., 2017)
	Dissolved CO_2 concentration	STR-STR system. At t_C = 375 s, 11% decrease of growth, 23% increase of acetate concentration in comparison with samples taken from the same culture just before the onset of dissolved CO_2 concentration gradients	(Baez et al., 2011)
	pH	STR-STR system. With increasing re-circulation time between reactors, the plasmid DNA and biomass yields, as well as the plasmid DNA final titer decreased in comparison with reference cultivations at constant pH values of 7.2 and 8. Differential mechanism to cope with pH fluctuations were found, depending on whether the fluctuations were transient or constant.	(Cortés et al., 2016)
Komagataella phaffii (Pichia pastoris)	DO concentration	PFR-STR system. Decreased growth and increased by-product formation with increasing time of exposure to low DO concentrations in comparison with non-induced cultures.	(Lorantfy et al., 2013)
		PFR-STR system. Multivariate study showed that the residence time in each reactor did not influence titer, productivity and physiology over the time span used (0 – 15 min). DO level influenced titer and specific productivity. Both residence time and DO level did not influence Y_{XS} or Y_{CO2S}.	(Jazini et al., 2014)
Penicillium	Substrate	Pulse-feeding experiments at 30 s, 3 min and	(Wang et

Microorganism or cell line	Parameter	Setup and results	Refs
chrysogenum	concentration	6 min showed accumulation of high levels of central metabolites during feast phase to cope with external substrate deprivation in famine phase. STR-STR experiment with mean residence time of 6 minutes showed that the storage pool of mannitol and arabitol constituted a large contribution of the carbon supply on the non-feed reactor. Product formation was decreased in both cases and it was inversely correlated to the intracellular glucose concentration level. The results were compared to a chemostat cultivations with continuous feeding.	al., 2018b)
		Pulse-feeding experiments. 38% of glucose was recycled once in storage metabolism. Thus, storage metabolism helps coping with environmental fluctuations and contributes to decreased volumetric productivity compared with reference steady-state cultivations.	(De Jonge et al., 2014)
		Pulse-feeding experiments. 2-fold reduction of penicillin production compared with control chemostat cultivations.	(De Jonge et al., 2011)
	Fluid dynamic stress	Chemostat fermentations at volumetric power inputs of 1 and 3.83 kW m^{-3} representing the bulk fermenter and the impeller zones, respectively. At 3.83 kW m^{-3}, 20% more cell lysis occurred and increased degeneration of penicillin in comparison with working at 1 kW m^{-3}. Higher-affinity glucose transport to the cell and metabolic re-arrangements also occurred.	(Wang et al., 2018a)
Pseudomonas putida	Iron availability, solvent exposure and DO concentration	Chemostat cultivations with distinct stress conditions in comparison with a chemostat cultivation with all nutrients supplied in excess besides glucose. Correlated acceleration of DNA replication with environmental stress as a coping mechanism. Growth rate kept at the same level and consequent longer cell cycle phases before and after replication to compensate.	(Lieder et al., 2016)
Saccharomyces cerevisiae	Substrate and DO concentration	One- and two-compartment STR experiments with varying glucose feed concentration (50-300 kg m^{-3}) and dilution rate (0.05 -0.2 h^{-1}) were performed and compared. In general, Y_{XS}	(Heins et al., 2015)

Microorganism or cell line	Parameter	Setup and results	Refs
		and biomass productivity were 50% larger in STR-STR cultivations. Ethanol yields were three-fold higher in single STR. CO_2 yields also had higher values in a single STR. No dependence between yields and dilution rate observed. Cells in a single STR had larger membrane robustness. Re-assimilation of ethanol in non-feeding reactor in STR-STR.	
		PFR-STR and 2PFR-STR experiments. Decreased growth rate and increased accumulation of carboxylic acids compared with single reference STR. Changes in sterol and fatty acid synthesis in response to varying cultivation conditions. Higher heterogeneity in cell morphology.	(Marbà-Ardébol et al., 2018)
	Substrate	PFR-STR experiment combined Markov chain modelling has shown that individual microbial cells are subjected to severe glucose starvation under a DO-control feed strategy.	(Delvigne et al., 2012)
		STR connected to a glass bulb or two different pipes resembled large-scale operation. Results showed a decrease on Y_{XS} and q_P and an increase in fermentation time and ethanol formation in comparison with a well-mixed reference reactor without re-circulation.	(Lejeune et al., 2010; Thonart et al., 2010)
		Pulse-feeding experiment with repetitive feast/famine regime lasting 400 s compared with reference chemostat cultivation. Y_{XS} reduced by 5%. Averaged substrate and oxygen consumption and CO_2 production rates were comparable to control levels. Delayed response to oscillations.	(Suarez-Mendez et al., 2014)
	pCO_2	Chemostat experiments with varying pCO_2 conditions lead to membrane depolarization, decrease in the intracellular pH and increases in HCO_3^- concentrations and changes in the ion balances. A peak in the ATP demand is also triggered. The experiments were compared to chemostat references with normal aeration.	(Eigenstetter and Takors, 2017)
	pCO_2 and pH	Low pH and high pCO_2 conditions (pH = 3, 50% CO_2) were tested with slow grown (0.0001 – 0.1 h^{-1}) chemostat and retentostat cultures and compared to cultures operated	(Hakkaart et al., 2020)

Microorganism or cell line	Parameter	Setup and results	Refs
		with control conditions (pH = 5, 0.04% CO_2). Higher maintenance requirements and death rates occurred mainly due to low pH. High pCO_2 strongly affected genome-wide transcriptional responses to low pH.	
Yarrowia lipolytica	DO concentration	STR-PFR with oxygen limitation in PFR. As a reference, a single STR fermentation process is performed with oxygen-enriched air to avoid oxygen limitation. For a mean residence time of 100 s, foaming was decreased and other parameters were kept at the same level. For a mean residence time of 200 s, foaming was also decreased, as well as Y_{PS}.	(Kar et al., 2012)
		Single STR with an automatic valve controlling the opening and closure of the air flow line. Oscillating DO concentration had a significant impact on the genetic expression level of the product (lipase) in comparison with a reference culture (single bioreactor) without DO concentration fluctuations.	(Delvigne et al., 2010)

4. Conclusions

Considerable research effort has focussed on developing tools to characterise the effect of fluctuating process conditions on industrially-relevant microorganisms, experimentally quantifying the presence of gradients in large-scale equipment and developing computational tools to model industrial bioreactors. This effort has been motivated by the desire to increase scientific understanding of the complex processes inside large-scale bioreactors and the industrial need to maximise process performance.

The advantages and disadvantages of different approaches to simulate and measure gradients are summarized in Table 5. Computational approaches have the ability to provide a high level of detail about the fluid flow and microbial physiology inside industrial bioreactors and to investigate conditions outside of the normal operating envelope. Another advantage of these tools is the ability to generate process information that is difficult to achieve experimentally data. However, generating accurate simulations relies on detailed and accurate models for both the fluid flow and microbial kinetics. Development, and more importantly validation of such models can necessitate considerable research effort. Thanks to advances in high-performance computing and cloud computing, the ability to access an appropriate computational infrastructure to run such models has greatly expanded. Often the limiting factor is the availability of skilled staff necessary for the development and testing of such modelling tools.

Experimental approaches, particularly those conducted at an industrial scale, are the most direct and useful way to realistically quantify the performance of industrial processes. However,

relatively little large-scale data is available in the open literature. This is often related to challenges in gaining access to industrial equipment, confidentiality concerns and the costs involved in performing a rigorous experimental program. Large-scale data is invaluable in the validation and design of scale-down experiments as well as computational models. Additional, large-scale experimental measurements building on the first pioneering studies (Enfors et al., 2001; George et al., 1998; Oosterhuis and Kossen, 1984) would provide invaluable data for the validation of both computational and scale-down tools. A clear avenue for future work in this field is the measurement and publication of large-scale data sets.

Table 5 - Summary of the advantages and drawbacks of the state-of-the-art tools to investigate gradients in large scale fermentation processes.

Tool	Advantages	Limitations
Modelling		
Computational Fluid Dynamics models with kinetics (CFD/KM)	- High level of detail in results. - Direct quantification of the impact of gradients on fermentation processes; it provides mechanistic knowledge. - Can be used to develop and evaluate new designs or operating conditions. - Can study setups with challenging access and/or off-limits operational settings.	- Expert knowledge/training required. - Only accounts for fixed-volume processes (batch or snapshots of fed-batch processes). - Long simulation times due to high computational cost; may need the use of HPC hardware. - Accurate model predictions rely on having accurate models of multi-phase physical phenomena and biological kinetics.
Compartment models with kinetics (CM/KM)	- Fast and flexible simulation with low computational cost. - Direct quantification of the impact of gradients on fermentation processes; it provides mechanistic knowledge. - Can study setups with challenging access and/or off-limits operational settings. - Can be used to develop and evaluate new designs or operating conditions.	- Need to provide accurate models of microbial kinetics. - May require the partial use of modelling (CFD) or measuring (sensor particles) of macro-mixing for their construction, i.e. expert knowledge may be needed.
Mimicking		
Scale-down experiments	- Microbial physiology can be directly assessed if off-line samples are taken (-omics studies, cell population heterogeneity).	- Design is not systematic and may not represent large-scale operation. - May not be able to reach comparable oxygen transfer rates, rheology and other physicochemical variables as in large-scale.
Measuring		
Multiple point	- Real knowledge of the process.	- Cost and complexity involved in the

measurements	- Validation of modelling and mimicking approaches. - Microbial physiology can be directly assessed if off-line samples are taken (-omics studies, cell population heterogeneity).	installation of instrumentation in industrial facilities. - Low spatial resolution (generally < 10 measurements located near the walls).
Free-floating sensor particles	- Real knowledge of the process. - Validation of modelling and mimicking approaches. - Non-invasive.	- Only for on-line measurements. - In development. - Expert knowledge necessary for data processing.

Extensive effort has been put into the development of tools and systems to quantify the effect of fluctuating environmental conditions on cellular behaviour using scale-down systems. These tools have illustrated how exposure to oscillations can lead to changes in the metabolome, transcriptome and proteome as well as lead to heterogeneity in the population (Figure 2). However, a major challenge with such systems is ensuring that the conditions in the laboratory mimic the environment found in a large-scale bioreactor. This remains a major challenge and future work should focus on the development of methods to ensure scale-down experiments are representative of real fermentations. Both experimental techniques (sensor particles) and computational tools (Euler-Lagrange models) are promising approaches, but more work in this area is needed. Additionally, there is a need for improved mathematical models to describe how organisms respond to fluctuating conditions. Such models should be designed with the aim of integrating them with either CFD or compartment models with the aim of obtaining a detailed description of large-scale bioreactors.

In conclusion, future work should aim at the further development of *in silico* tools and at model testing and gradient measurement in large scale facilities. Then, models that describe both fluid dynamics and microorganism behavior can be either validated or improved and implemented as a tool for fermentation process development. For process design and optimization purposes, computational tools can be used to screen the strain-dependent operational limits of each process parameter resulting from potential gradient development (operational factors from Table 1). It is only by doing this that gradients can be understood and tackled, ensuring process robustness despite a potential non-homogeneous fermentation environment.

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Figure 1 – Plot showing characteristic times for transport (mixing time) ($\boldsymbol{\tau_m}$) and substrate consumption ($\boldsymbol{\tau_{SC}}$) for published work in the literature. The solid line indicates the point at which $\boldsymbol{\tau_m = \tau_{SC}}$. Below this line gradients are expected. More details about the experimental work used to generate this plot are given in
Table 3.

Figure 2 – Schematic showing the effect of gradients on bioprocesses.

Figure 3 – Plot showing (A) CFD simulation contour plot result showing the velocity profile of a bubble column bioreactor and its accompanying compartment map on the right side developed with an automatic compartment map development method based on the axial and radial velocity fields (Tajsoleiman et al., 2019b). (B) Representation of the resulting network-of-zones with connections that can be used in modelling work for accounting for the fluid dynamics of the bubble column.

Figure 4 – Schematic of a traditional multi-compartment (A: PFR-STR; B: 3 STR; and C: pulse-feed) scale-down experiments to study glucose concentration gradients in aerobic fed-batch fermentation processes. Adapted from (Neubauer and Junne, 2010).