Maximizing yield of perfusion cell culture processes: Evaluation and scale-up of continuous bleed recycling

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ABSTRACT

Bleed recycling is a novel method to increase the yield of steady-state perfusion processes by concentrating process bleed to selectively remove biomass and recycle the liquid fraction. This results in significant product saving which otherwise would go to waste. As long as cells can be concentrated and separated, existing cell separation devices can be used for such an application. However, limited information comparing operation modes and efficiency for bleed recycling applications is available. For the first time, inclined gravity settling has been used as bleed recycling technology and was compared to acoustic separation. Except for lower debris removal, inclined gravity settling showed similar bleed recycling efficiency and no negative impact on cell viabilities, nutrient and metabolite levels and product quality. Additionally considering reduced system complexity and facilitated scale-up, inclined gravity settling was the preferred technology for further evaluation during a 42-day lab-scale perfusion process. Up to a 3.5-fold bleed reduction and an average harvest rate increase of 19% was achieved. Scalability was subsequently tested with a large-scale inclined gravity settler suitable for a 2000 L perfusion process confirming performance of lab-scale experiments. Bleed recycling characterization data from screening experiments combined with scalability demonstration facilitates decision making when considering bleed recycling for novel perfusion process settings to reduce perfusion waste, increase process sustainability and boost overall process yield.

1. Introduction

Recent reports about perfusion cell culture have shown increasing volumetric productivities, more homogenous product quality profiles, and benefits due to reduced product residence time in the bioreactor for labile proteins [1–4]. Continuous manufacturing of monoclonal antibodies consists of a steady-state perfusion cell culture process followed by a continuous capture step and multiple polishing steps [5,6]. To achieve a steady-state perfusion operation not only a cell retention device (CRD) for cell free harvesting, but also a waste stream (bleed) to remove excessive biomass is required. Bleeding is not a selective operation since the bleed has the same composition as the culture in the bioreactor. Assuming a process viable cell volume (VCV) of 10% means a

bleed composition of 10% biomass and 90% liquid fraction. As bleeding is required to maintain target cell density in a stable perfusion process, this causes a large amount of liquid (containing the product of interest) to be wasted along with excessive biomass.

When calculating process yield for perfusion processes, the product concentration (harvest titer) and the harvest rate must be considered [7]. Large process bleed rates are therefore undesirable in terms of process intensification as they lead to reduced harvest rates. Despite major improvements in harvest titer within the industry, little attention was put on perfusion bleed rate reduction.

A first strategy to improve process yield consists of reducing the overall process bleed rate. Growth inhibition by cell cycle inhibitors or temperature shifts were reported in literature [8]. Despite improved

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process yields, growth inhibition showed changes in product quality. Furthermore, the process bleed rate corresponds to the cell division rate. While low cell division rates might initially lead to improved process yields, this approach can jeopardize high long-term cell culture viability and stability.

A second strategy concentrates the bleed stream in order to reduce the liquid fraction that is wasted concomitantly with the biomass in the bleed stream. Only the concentrated biomass is directed to waste and the clarified liquid stream is directed back to the bioreactor, resulting in an increased perfusion harvest rate and process yield. The bleed stream coming from the bioreactor, corresponding to the cell division rate, is not reduced in this scenario, but a significant liquid fraction thereof is recycled back to the bioreactor. As such, a recent proof-of-concept study implementing a solid-liquid separation step using an acoustic separator to concentrate the biomass in the bleed stream of a perfusion process was conducted and specified as bleed recycling [9]. Good separation efficiency and no changes in product quality or metabolite profile were detected at a VCV of 5%, tested during a period of 4 days at 2 L scale. A liquid-solid separation device instead of growth inhibition could mitigate the risk of long-term process stability by keeping bleed rates sufficiently high without reducing culture performance. The study by Bielser et. al [9] was the first report of bleed recycling in the literature, and further research is needed to explore its potential benefits at higher VCV and under different perfusion process conditions. Furthermore, scalability of acoustic separation for bleed recycling has not been demonstrated.

Acoustic separators use an acoustic resonance field to cause individual cells to group together and form larger cell aggregates. These cell aggregates show enhanced settling properties due to their larger size and settle faster once the acoustic waves are interrupted [10,11]. Whereas very promising separation results could be achieved with acoustic settlers used as CRD in small scale [12–14] a limited amount of information is available about large-scale application of acoustic separators. Acoustic separators were successfully tested up to a clarification volume of 200 L/day and further scale-up by merging multiple smaller acoustic chambers was suggested [15]. Development of larger scale acoustic separators seems very difficult due to the significant power input required for cell separation, causing heat generation within the device [16,17]. A multi-chamber system might solve the problem of heat dissipation but increases system complexity drastically, raising a need to explore further technologies as bleed recycling alternatives. These technologies should be less complex and easier to scale, nevertheless showing similar performance characteristics.

Next to acoustic separation, inclined gravity settling has been successfully used as CRD to maintain stable perfusion processes [17–19] In contrast to acoustic separators, inclined gravity settlers were extensively studied and used for large-scale production bioreactors due to their relatively low complexity and good scalability [20,21], rendering them an interesting alternative to acoustic separation for bleed recycling. As the particles, in this case cells, settle down within the inclined channel, a higher density slurry is formed at the bottom of the channel. Driven by gravity, the slurry slips down the surface, generating a convective flow that enhances the settling process, described as "Boycott Effect" [22]. As no aggregation support by an acoustic resonance field is available, inclined gravity settlers tend to be larger than acoustic separators. This comes with an increased culture residence time within the inclined gravity settler, with unknown impact on culture performance when used for bleed recycling.

Even though settling technology does not guarantee 100% clarified culture harvest, which led to the widespread use of filter-based cell retention devices with a predominant use of tangential flow filtration (TFF) and alternating flow filtration (ATF) in combination with hollow fiber modules [3,17,23,24], the technological requirements for bleed recycling are different. In the case of bleed recycling, the main purpose of the bleed recycling device is to concentrate cells as much as possible rather than providing a complete cell free harvest stream. The clarified recycle stream, even if containing some remaining cells, will be directed back to the bioreactor and ultimately increase the harvest stream. Filtration for further downstream process operations is performed by the main cell retention device and not by the bleed recycling device. Since not only whole cells but also smaller particles such as cell debris can be recycled into the bioreactor, possible negative effects of cell debris on fouling of cell retention filters should be investigated [25,26]. Defining critical process debris levels and corresponding correlation to filter fouling has not yet been demonstrated and most likely changes depending on chosen process parameters, equipment, and operation mode.

The aim of this study is to demonstrate the application of bleed recycling for process intensification of industrially relevant perfusion processes. For the first time, inclined gravity settling is used as bleed recycling device and compared to acoustic separation with respect to bleed recycling efficiency, metabolite profiles, product quality attributes, cell debris removal, and ease of operation. A wide range of perfusion scenarios covering process VCV, process bleed rates and settler operation parameters were investigated at lab-scale, to quantify the potential of process intensification by bleed recycling for a wide range of cases. Scalability of bleed recycling was demonstrated by building a large-scale inclined gravity settler and recycling bleed streams of a 2000 L perfusion bioreactor with 1500 L filling volume. Details of the successful outcomes are reported, focusing on the performance of bleed recycling technologies, and even more importantly, on the impact of bleed recycling on cell culture performance and product quality.

2. Materials and methods

2.1. Lab-scale perfusion culture process

A proprietary CHO-K1 cell line producing a bispecific monoclonal antibody (mAb1) was expanded in an incubator (Multitron 4, Infors HT, Bottmingen, Switzerland) for 21 days using a proprietary chemically defined medium (Merck Serono SA, Corsier-sur-Vevey, Switzerland). Perfusion bioreactors (Labfors 5 Cell, Infors HT, Bottmingen, Switzerland) were inoculated with a seeding density of 0.6×10^6 viable cells/mL. Culture conditions were maintained at 36.5 °C with a dissolved oxygen (DO) setpoint of 50% (VisiFerm DO Arc, Hamilton, Bonaduz, Switzerland). The pH was controlled at 7.07 ± 0.17 (EasyFerm Plus Arc, Hamilton, Bonaduz, Switzerland) by CO_2 sparging and a 1.1 M Na_2CO_3 solution. Bioreactors were operated at 2 L working volume and perfusion was started on day 0 and kept constant at 1.3 reactor volumes per day (RV/day). Bioreactor harvests were gravimetrically controlled to maintain the bioreactor weight constant using alternating tangential flow filtration (ATF2H, Repligen, Waltham, Massachusetts, USA) with polyether sulfone hollow fibers having a pore size of 0.22 μm (Repligen, Waltham, Massachusetts. USA). After an initial growth phase, an online capacitance probe (Incyte Arc, Hamilton, Bonaduz, Switzerland) was used to keep VCV constant at 12%.

2.2. Pilot-scale perfusion cell culture

Another proprietary CHO-K1 cell line producing a bispecific monoclonal antibody (mAb2) was cultivated in a 200 L perfusion bioreactor (Mobius 200 L Bioreactor, Merck KGaA, Darmstadt, Germany). VCV was kept constant at 8% with an online capacitance probe (Incyte Arc, Hamilton, Bonaduz, Switzerland). Bioreactor harvests were gravimetrically controlled to maintain the bioreactor weight constant using either alternating tangential flow filtration (XCell ATF® 6 single use, Repligen, Waltham, Massachusetts, USA) with polyether sulfone hollow fibers having a pore size of 0.22 μm (Repligen, Waltham, Massachusetts. USA) to retain the cells. When the production was finished, the remaining cell culture was used to test the large-scale inclined gravity settler (Constructions Inoxydables SA, Châtel-St-Denis, Switzerland) with flowrates representative for a 2000 L perfusion bioreactor. During

the experiment, cell culture was agitated and maintained at 35 °C. The pH was controlled at 6.95 ± 0.15 (InPro3253i, Mettler Toledo, Greifensee, Switzerland) using CO_2 or 1.1 M Na_2CO_3 and DO was maintained at 50% (InPro6860i/12/120/nA, Mettler Toledo, Greifensee, Switzerland). Perfusion and capacitance controls were stopped.

2.3. Large-scale perfusion cell culture

A 2000 L bioreactor (Mobius 2000 L Bioreactor, Merck KGaA, Darmstadt, Germany) was inoculated at 0.67×10^6 viable cells/mL (mAb1) with a constant working volume of 1500 L. The pH was controlled at 6.95 ± 0.15 (InPro3253i, Mettler Toledo, Greifensee, Switzerland) using CO_2 or 1.1 M Na_2CO_3 and DO was maintained at 50% (InPro6860i/12/120/nA, Mettler Toledo, Greifensee, Switzerland). During the growth phase, the temperature was kept at 36.5 °C and was then shifted to 33 °C once the capacitance setpoint was reached. VCV was kept constant at 8% with an online capacitance probe (Incyte Arc, Hamilton, Bonaduz, Switzerland). A flowmeter (LFS-03SU-Z-SC1-G25, Levitronix, Zurich, Switzerland) was used on the process bleed line to monitor the flow and deduct the amount from the overall target harvest rate. Perfusion rate was gradually increased during growth phase until 1.3 RV/day from when it was kept constant until the end of the run. Product of interest was harvested through two single use alternating tangential flow devices in parallel (XCell ATF® 10 single use, Repligen, Waltham, Massachusetts, USA) with polyether sulfone hollow fibers having a pore size of 0.22 μm (Repligen, Waltham, Massachusetts, USA) to retain the cells. Harvest flowrates were controlled by flowmeters (LFS-03SU-Z-SC1-G25, Levitronix, Zurich, Switzerland) after each ATF10 (Repligen, Waltham, Massachusetts, USA) and addition of fresh media was controlled with gravimetric addition to maintain the weight of the bioreactor constant.

2.4. Inclined gravity settler lab-scale design and scale-up

Good separation efficiency of the settling device is a prerequisite for bleed recycling applications. Depending on the inlet flow rate the settler is designed for, the optimal settler size must be defined in order to reduce the residence time as much as possible whilst keeping high separation efficiencies. The inclined gravity settlers (Fig. 1B and Fig. 1D) were designed and scaled up based on the PNK (Ponder, Nakamura and Kuroda) theory. The design-acceptance criteria were chosen based on the theory-related suggestions (see supporting information). Settler dimensions and design parameter are summarized in Table 1. The

Table 1
Separator and settler specifications used to concentrate the bleed stream.

Parameter	Acoustic Separator (Lab-Scale)	Inclined Gravity Settler (Lab-scale)	Inclined Gravity Settler (Large-scale)
Number of channels	-	5	40
Plate length (cm)	-	6.8	30
Plate width (cm)	-	1.0	29.7
Distance between two plates (cm)	-	0.5	0.5
Horizontal settling area (cm^2)	-	17.0	17820
Settler inner volume (L)	$0.7 * 10^{-3}$	$24 * 10^{-3}$	30.4
Fluid clarification rate (mL/s)	$11.6 * 10^{-3}$	$4.8 * 10^{-3}$	4.6
Estimated settler residence time (h) *	0.03	1.15	1.46

*Residence time was calculated using inlet flow rates of 0.5 L/day for lab-scale operation and 500 L/day for large-scale operation.

constructional drawings for the lab-scale and the large-scale settler can be found in the supporting information section (SI Fig. 1 and SI Fig. 2).

2.5. Continuous bleed recycling and control strategy for 2 L perfusion process

Bleed recycling was realized by connecting the bleed stream coming from the bioreactor to the settler inlet, coupling the waste outlet of the settler to a waste container and returning the recycle stream to the bioreactor (Fig. 1A). To operate a bleed recycling device at a defined recycle rate, two Ismatec Reglo ICC peristaltic pumps were required to control the three flows (Ismatec SA, Barrington, USA). The first pump was placed between bioreactor and settler inlet (process bleed pump) and was controlled by a capacitance-based feedback-loop to regulate the process bleed stream. The second pump (recycle pump) was placed in the recycle loop, whereas the third flow was defined by the flow difference going into the waste container. The bleed rate in this setup was based on the capacitance signal. As a result, the control system had to account for fluctuations and automatically adapt waste and clarified stream flows to maintain a defined settler recycle rate. Therefore, the recycle pump was operated at a fraction of the process bleed pump. Given a certain recycle rate setpoint, the recycle pump speed was recalculated every 15 s by the bioreactor control system based on the process bleed pump speed.
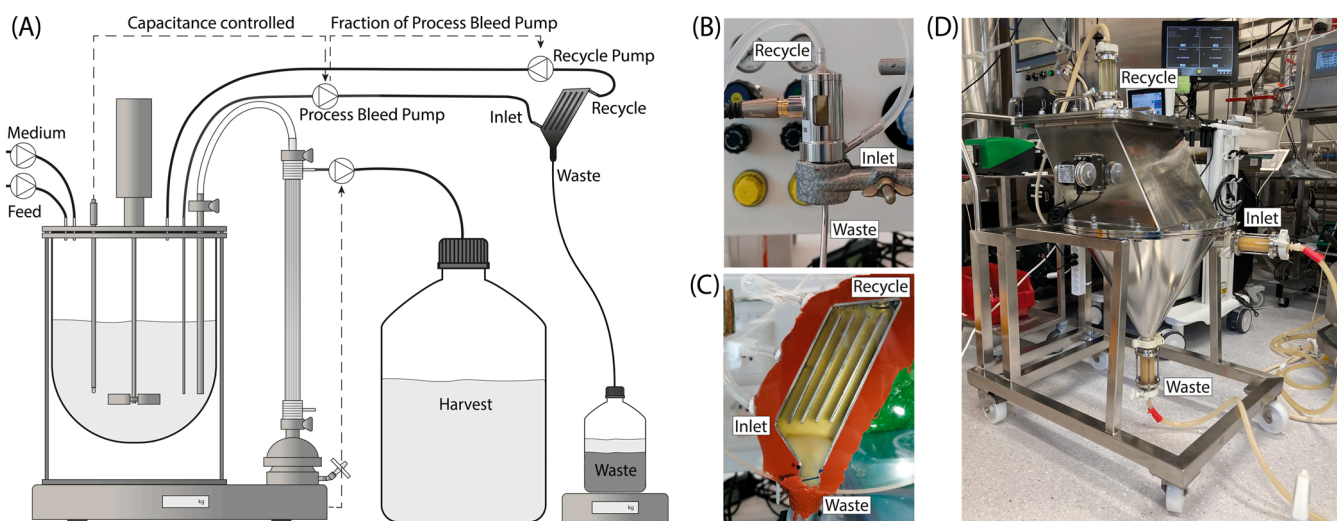

Fig. 1. Schematic representation of the bleed recycling setup (A), lab-scale acoustic settler (B), lab-scale inclined gravity settler (C) and large-scale inclined gravity settler (D).

2.6. Offline performance evaluation for inclined gravity settler and acoustic separator

Performance of the in-house developed inclined gravity settler (Fig. 1**B**) was compared in an offline setup to a commercially available acoustic cell separator BioSep 1 L with Mini BioSep Controller (Applikon Biotechnology, Delft, Netherlands) (Fig. 1**C**). Acoustic separation was driven by a control unit with power output 0.7 W, 3 min run time and 3 s stop time. Settler feed streams of both devices and the recycle stream of the inclined gravity settler were operated by Ismatec Reglo ICC peristaltic pumps (Ismatec SA, Barrington, USA). The recycle stream of the acoustic separator was operated by a MasterFlex L/S pump with model 07514–10 pump head (MasterFlex, Dinkelscherben, Germany). Both bleed recycling devices were fed from a common stirred vessel to ensure identical starting conditions. The entire experimental setup is illustrated in the supporting information (SI Fig. 3). Various inlet VCV ranging from 4% to 35% were loaded to the settlers by hourly sampling a steady-state 2 L perfusion bioreactor at 12% VCV and adjusting the VCV either by diluting with harvest or concentrating by centrifugation (800 g, 1 min) using a centrifuge 5810 R (Eppendorf SE, Hamburg, Germany). Cells were gently stirred during the loading to the bleed recycling devices to avoid sedimentation or aggregation using the IKA RET basic C magnetic stirrer (IKA Werke GmbH & Co. KG, Staufen im Breisgau, Germany). Experiments were performed at a stable inlet flow rate of 0.5 L/day, simulating 2 L perfusion process at 1.3 RV/day with approximately 20% bleed rate. The duration of each experiment was over 6 h to ensure a stable operation was achieved within the devices, after which all the settler streams (inlet, waste and recycle stream) were sampled for evaluation of settling performance, cell parameters, nutrients and metabolites, debris removal and product quality. For high debris experiments, sonication of cell culture was performed using a Branson sonifier 250 (Branson Ultrasonics, Brookfield, US) with a tapered microtip at maximum power output over 30 min prior to loading to the bleed recycling devices.

2.7. Large-scale inclined gravity settler operation

For performance evaluation, the inclined gravity settler (Fig. 1**D**) was connected to the 200 L bioreactor. A pump with a flowmeter (LFS-03SU-Z-SC1-G25, Levitronix, Zurich, Switzerland) was used to control inlet flowrate into the gravity settler. A calibrated pump (Serie 520 U, Watson-Marlow, Pendennis Court, Falmouth Business Park, Falmouth TR11 4SZ, United Kingdom) was placed on the bottom outlet of the gravity settler, to ensure no return of the concentrated cells back into the settler. Outlet flow at the top was monitored by a second flowmeter (LFS-03SU-Z-SC1-G25, Levitronix, Zurich, Switzerland). Performance of the large-scale gravity settler was evaluated by loading the 200 L culture volume to the inclined gravity settler at a speed matching an operation at 2000 L bioreactor filling volume at 1.3 vvd perfusion rate with bleed rates of 18% (470 L/day), 23% (600 L/day) and 28% (730 L/day). At the same time, settler recycle rates of 75% were targeted.

For at-scale evaluation, the inclined gravity settler was connected on the bleed line of the 2000 L bioreactor with a filling volume of 1500 L in the control state. The inlet flow (process bleed stream) was regulated by the capacitance probe. Clarified recycle (top of gravity settler) and concentrated waste (bottom of the device) were connected to two separated tanks. As another control system than for the 2 L perfusion run was present, the control strategy had to be changed. Instead of adapting the flow rates based on the process bleed stream, a fixed pump speed of the concentrated waste on the settler was set to reach a recycle rate of roughly 70%. Based on an average process bleed of 430 L/day, the concentrated bleed pump was set to 130 L/day. To stabilize the process bleed stream, an upper limit for the process bleed flow was set to 450 L/day.

2.8. Analytical methods

Cell density, viability, cell diameter, glucose, lactate, ammonia, and pH were measured using a BioProfile FLEX2 (Nova Biomedical, Waltham, USA) for lab-scale experiments. Lab-scale 2L bioreactors were automatically sampled by the FLEX2 On-Line Autosampler (Nova Biomedical, Waltham, USA) and samples were fractionated using a Teledyne Cetac ASX-7200 (Teledyne CETAC Technologies, Omaha, Nebraska, USA). For large-scale experiments, cell parameters were analysed using a Vi-CELL XR analyzer (Beckman Coulter, Brea, California, USA) and metabolites using a Vi-CELL MetaFLEX™ bioanalyte analyzer (Beckman Coulter, Brea, California, USA). VCV was calculated as follows [27]:

$$VCV = \frac{4}{3} \bullet \pi \bullet \left(\frac{D}{2}\right)^3 \bullet VCD \bullet 100 \tag{1}$$

Where D is the average cell diameter and VCD the viable cell density, assuming a spherical shape of the cells.

Separation efficiency (SE) for bleed recycling devices was calculated using formula 2, where VCV_R corresponds to the viable cell volume of the recycle stream and VCV_{In} to the viable cell volume of the inlet stream.

$$SE = 1 - \frac{VCV_R}{VCV_{In}} \tag{2}$$

Process titers were determined using a protein affinity high performance liquid chromatography device (PA-HPLC, Waters, Milford, Massachusetts, USA).

Protein glycosylation was quantified through a multi attribute method (MAM) which is based on high performance liquid chromatography-mass spectrometry (LC-MS, Vanquish™ Horizon UHPLC System and Q Exactive™ Plus, Thermo Fisher Scientific, Waltham, Massachusetts, USA).

Optical Density OD_{600} was measured in cell culture supernatant after centrifugation for 10 min at 3200 g using the UV-Vis spectrometer Genesys 10 Bio (Thermo Fisher Scientific, Waltham, Massachusetts, USA). For debris particle counting, the fluid imaging device FlowCam (Fluid Imaging Technologies Inc., Scarborough, Maine, USA) with Olympus UPLFLN 10X objective (Olympus, Shinjuku, Japan) was used to quantify particles with diameter > 1 μm. The samples were diluted 1:10 in deionized water and loaded to the flow camera at a flow rate of 0.15 mL/min and 15 frames/second without any size filter applied, resulting in an estimated capture efficiency of 20%.

3. Results and discussion

3.1. Offline performance evaluation of inclined gravity settler and acoustic separator

Bleed recycling on a perfusion bioreactor splits the bleed stream into a concentrated waste stream and a clarified recycle stream that is recirculated back to the bioreactor (Fig. 1**A**). As inclined gravity settling as technology for bleed recycling has never been used before, a comparison to a previously applied technology such as acoustic separation [9] in term of performance was necessary. During bleed recycling, the fluid in the process bleed and recirculation loop (before and after concentration) is not in a controlled environment (pH, DO, Temperature). Therefore, it was important to understand if the increased residence time of the inclined gravity settler compared to acoustic separation would impact the cell culture or some of the product quality attributes.

As cell culture fluids from different perfusion runs and different process times were used for settling experiments coming from a sampled steady-state perfusion bioreactor, inlet concentrations of analytes varied depending on the sampling day. On a specific day however, exactly the same culture was used to assess both acoustic separation and inclined

gravity settling as described in SI Fig. 3. Therefore, the data was normalized with respect to the inlet concentrations and Fig. 2 shows the difference of the waste and clarified stream compared to the respective inlet stream. Recycle stream concentrations for the acoustic separator and the inclined gravity settler were comparable to the inlet concentrations for glucose (Fig. 2A), lactate (Fig. 2B) and ammonia (Fig. 2C). Furthermore, there was no detectable shift in pH (Fig. 2D). Despite significant changes in the composition of the waste stream, there was no impact on the recycle stream for either settler type. Product glycosylation from samples of the inlet stream were compared to glycosylation measurements of clarified recycle stream samples. No significant changes in product quality between the inlet stream and the recycle stream for both the acoustic separator and the inclined gravity settler were detected, indicating that the increased residence time of the culture in the inclined settler had no impact on clarified harvest composition as well as on product quality (Fig. 3).

Cell debris as a concern for cell retention device clogging or cause for product sieving is desired to keep at low levels during the production process [25,28]. In a standard perfusion process, a fraction of the cell debris is constantly removed via the process bleed together with excess cells and harvest. When using a bleed recycling device, the waste bleed stream is significantly reduced and a large fraction of clarified harvest is returned to the bioreactor. Therefore, offline experiments were designed to investigate the particle clearance capacity of the acoustic separator and the inclined gravity settler.

Optical density measurements (OD_{600}) were used to quantify the smallest particles that were remaining in the liquid phase after sample centrifugation (see Section 2.8). These optical density measurements resulted in a slightly higher debris measurements for the inclined gravity settler compared to acoustic separation (Fig. 4A). Particle counts based on picture analysis with a minimal particle detection size of 1 μm in the clarified harvest streams confirmed that the acoustic settler favours

particle removal compared to the inclined gravity settler. A clear deviation from the dashed similarity line towards the inclined gravity settler further demonstrates that the inclined gravity settler leads to a higher residual particle concentration when using identical inlet streams (Fig. 4B). For a more detailed investigation of the difference between the acoustic and the inclined gravity settler, a concentrated high debris suspension was created by sonication of cell culture samples and inserted into both settling devices. Particles counts resolved to particle size are shown for the inclined gravity settler (Fig. 4C) and the acoustic settler (Fig. 4D). Identical inlet particle distribution resulted in much higher debris concentrations in the waste stream of the acoustic settler compared to the inclined gravity settler, consequently leading to reduced residual particle counts in the clarified recycle stream. Calculating the removal efficiency according to the particle size (Fig. 4E) reveals that both settler types completely remove particles larger than 10 μm, while separation efficiency drops for smaller particles. Whilst the acoustic settler still removed more than 60% of the smallest measured particles of 1 μm, the inclined gravity settler performed worse for smaller sized particle removal. Calculating the effectively removed particle fraction with a recycle rate of 80% (Fig. 4F), the acoustic settler removed roughly 84% of the particles, whereas the inclined gravity settler removed 47% of the particles for this particular debris enhanced scenario. Altogether, both settlers removed larger particles efficiently, but lost efficiency for smaller particles. Wang et al. [26] demonstrated that debris in the size of 20–200 nm can contribute to product retention and membrane plugging with similar range pore size. As the acoustic wave separator was predominantly better at removing larger particles with only slightly higher removal of submicron particles, it is questionable whether a clear benefit to avoid product retention for acoustic separation would be observable.

To balance additional operational complexity with benefits of increased process yield, many parameters such as process VCV setpoint,
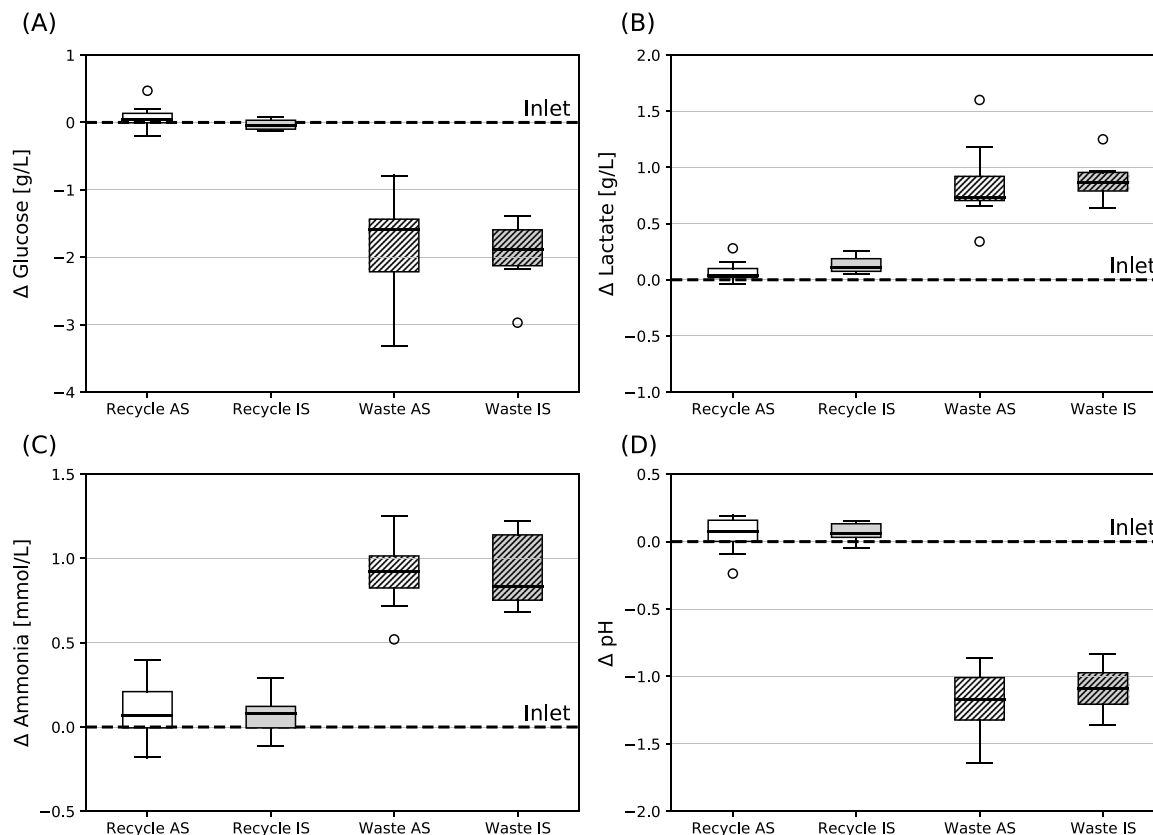

Fig. 2. Difference in glucose (A), lactate (B), ammonia (C) and pH (D) of the inlet stream into the settler (dashed zero line) compared the respective recycle and waste stream for acoustic separator (AS) and inclined gravity settler (IS) (n = 8).
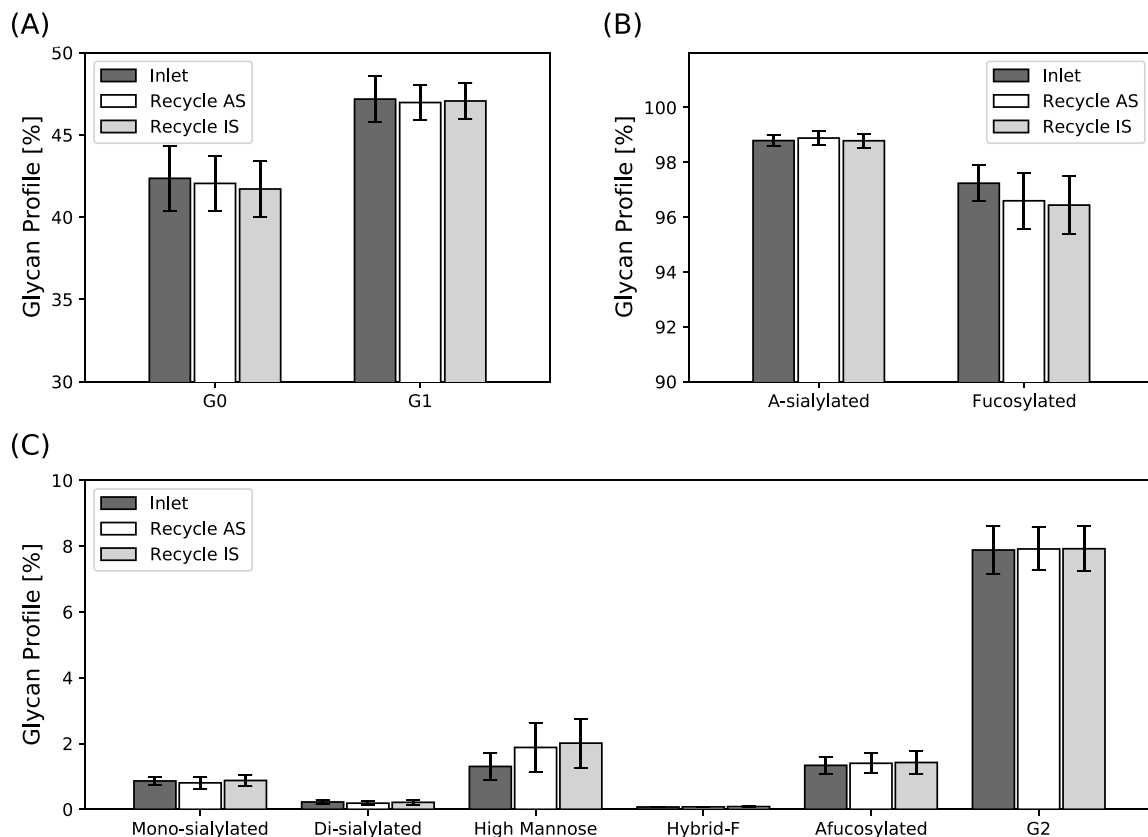
Fig. 3. Glycan forms of antibodies in the recycle stream of the acoustic settler (AS) and inclined gravity settler (IS) compared to the inlet stream for the offline experiment (n = 14).

process bleed rate and settler recycle rate must be considered. VCV as a key parameter for bleed recycling is defined by the VCD count and the average cell diameter (Fig. 5A). The grey point represents an example simulating a process bleed of approximately 20% (0.5 L/day for 2 L perfusion at 1.3 RV/day) for the utilized cell line producing mAb1. In order to maximize the process yield increase with a bleed recycling system, it is beneficial to operate the settling systems at higher recycle rates. However, operation at too high recycle rates can lead to accumulation of cells within the settling device due to limitations in compacting cells being removed with the smaller remaining waste stream. The acoustic separator and the inclined gravity settler where therefore evaluated at non-accumulating conditions and accumulating conditions with a bleed of roughly 20% to approximate the optimal operation conditions of each settling device. Inlet VCV values were chosen from 4% to 35%, covering a wide range of process setpoints (Fig. 5B). As no significant difference between the two bleed recycling technologies was detected, the optimal operation conditions were calculated by combining both data sets and performing a linear regression. Despite favoured debris removal, the acoustic separator did not achieve significantly higher recycle rates before entering the accumulating stage. This is a possible indication that although the cells tend to aggregate due to the acoustic waves, they do not result in a more compact cell suspension after gravity settling than with the inclined gravity settler. To calculate potential process yield increases, not only the gained knowledge about achievable recycling rates without cell accumulation, but also process bleed rates must be considered. It was therefore assumed that bleed recycling device volumes are scaled accordingly when changing process bleed rates to maintain high separation efficiency. Having a properly scaled separation device, yield increases were plotted as contour plots with either settler recycle rate (Fig. 5C) or process VCV setpoint (Fig. 5D) versus the respective process bleed rate. These plots help to estimate potential yield increases given a particular perfusion process

assuming optimal settler performances evaluated in Fig. 5B. For the example process indicated by the grey points, a potential process yield increase of 18% can be achieved. In general, the lower the process VCV setpoint and the higher the process bleed rate, the higher the yield increase can be expected.

In brief, the offline investigation to compare acoustic separation to inclined gravity settling showed no difference in nutrients, metabolites, product quality and settler efficiency. The acoustic separation results are in good agreement with Bielser et al. [9], demonstrating similar inlet stream and recycle stream characteristics. Despite the larger settler volume required for inclined gravity settling resulting in increased settler residence time (Table 1), no difference to acoustic separation was detected in this study. Acoustic waves seem therefore to facilitate cell aggregation and accelerate the settling process, which allows a reduction in the volume of the settling chambers, and subsequently reduced residence time within the separation chamber. But acoustic waves do not lead to higher compaction of the cell biomass in the waste stream, as similar performances were obtained with both technologies. Solely for debris removal, acoustic settling showed superior performance compared to inclined gravity settling. Due to the experimental data demonstrating that the increased residence time within the inclined settler compared to the acoustic separator had no impact on bleed recycling performance, culture parameters and product quality attributes, and for reasons of lower system complexity and simplified scale-up, all further experiments were conducted using inclined gravity settling.

3.2. Bleed Recycling in Lab-scale Perfusion Cultivation

A long-term steady-state perfusion run at 2 L scale was performed with a gravity settler connected to the process bleed stream to demonstrate improved process performance and operationality over prolonged
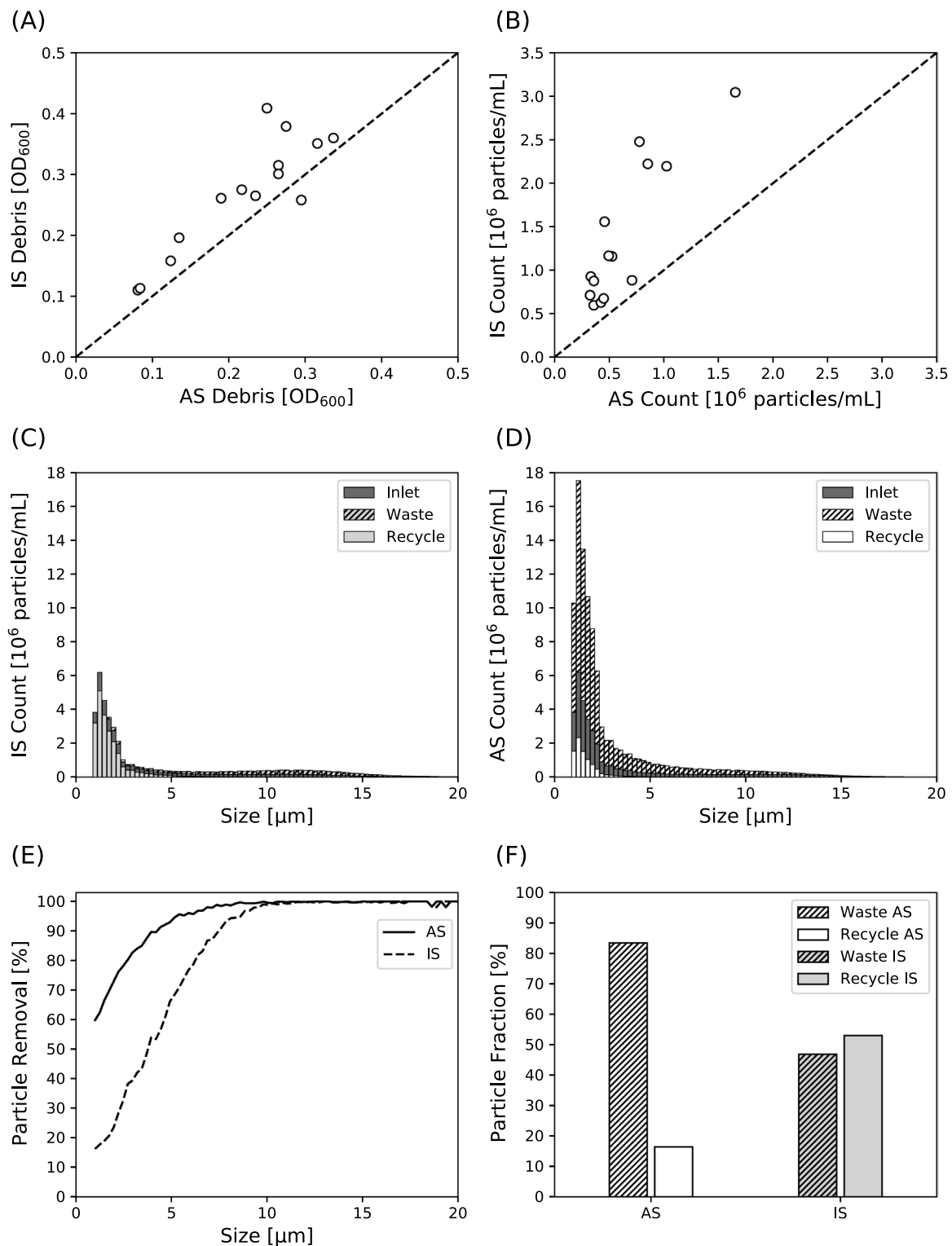
Fig. 4. Offline debris investigation of recycle streams using the UV-Vis spectrometer (A) and the fluid imaging device FlowCam (B). Layered histogram of intensified debris experiment showing the debris size distribution for the lab-scale inclined gravity settler (C) and the lab-scale acoustic settler (D), particle removal rate for specific debris size (E) and the calculated effectively returned particle amount considering a recycle rate of 80% irrelevantly of their size (F). Acoustic settler (AS), Inclined Gravity Settler (IS).

time. The inclined gravity settler was started on day 9 with a secure recycle rate of 50%, immediately increasing the process harvest rate from roughly to 90% compared to 72% in the control run (Fig. 6E). During 17 days of inclined settler operation, the recycle rate was step-wise increase up to 66% without accumulation within the settler

(Fig. 6C), keeping the harvest rate (on average 92%) significantly above the harvest rate of the control run (on average 77%) as demonstrated by the blue area in Fig. 6E. The process bleed decreased from initially 30% down to 20%, which is related to slower cell division rates. With an average bleed rate of 23% and an average settler recycle rate of 56%
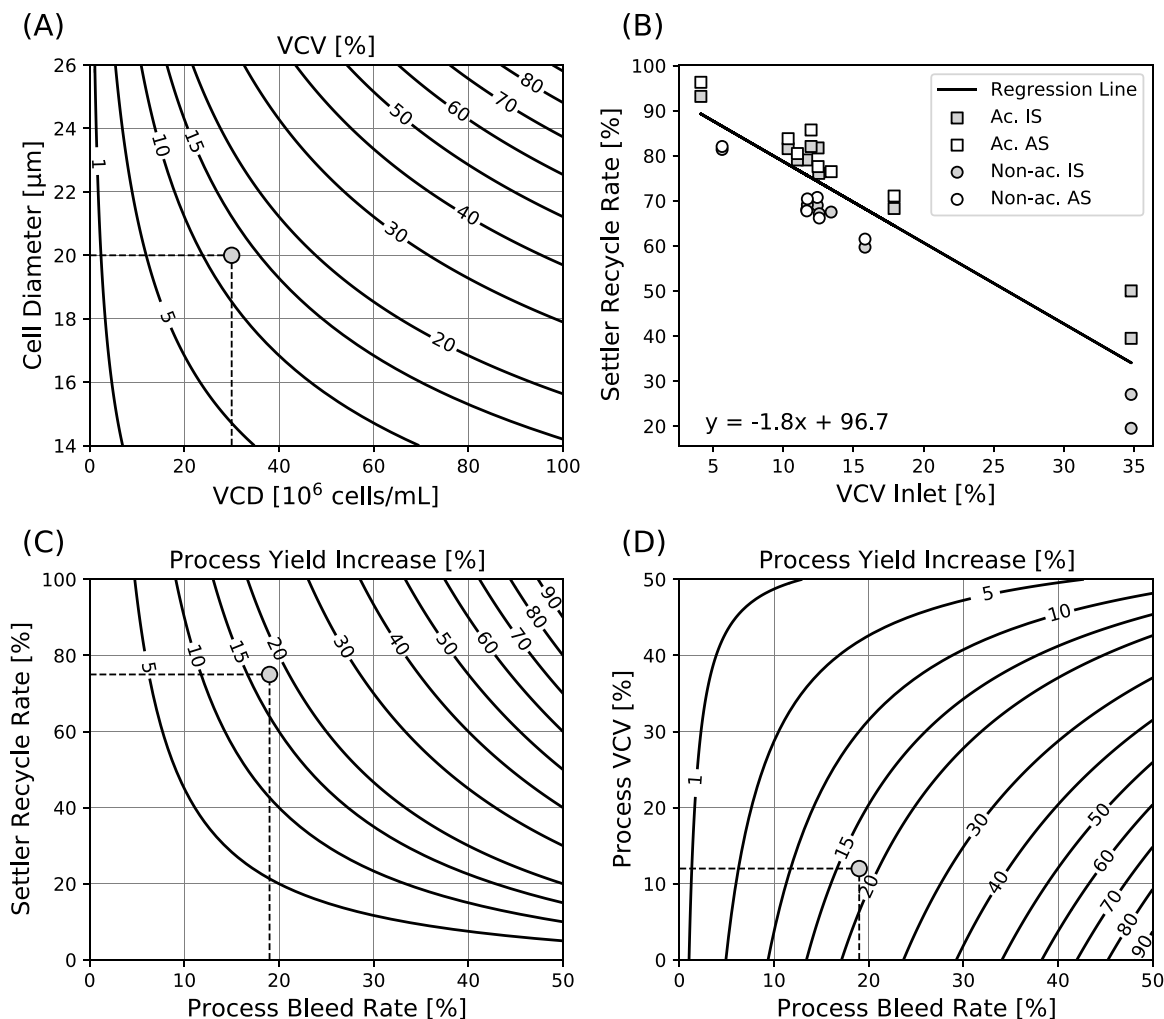
Fig. 5. Bleed recycling efficiency data and performance calculations. VCV contour plot showing the relationship of VCD and average cell diameter for VCV (A). Experimental data to evaluate optimal recycle rates by accumulating and non-accumulating settler experiments (B). The straight line represents an expected accumulation limit based on linear regression of all data points. Process yield increase contour plot relating process bleed rate to settler recycle rate (C) or process VCV setpoint (D) assuming optimal settler recycle rate as evaluated by the regression line in (B). The points in (A), (C) and (D) serve as an example for a process bleed of approximately 20% with the specified VCV of roughly 12%. Acoustic settler (AS), Inclined Gravity Settler (IS).

during these 17 days, a process yield increase of about 16% was predicted by Fig. 5C. It has to be mentioned that all predictions are based on the assumption that the harvest titer remains unchanged, and the process yield increase is solely increased due to higher harvest volumes. Experimental result showed good agreement with an accumulated harvest volume increase of 19% by bleed recycling (Fig. 6G). As process yield can not only be increased by increased harvest volume, but also by increasing harvest titers, the actual process yield increase during this bleed recycling period was higher. Harvest titer was slightly increased in the bleed recycling run leading to a process yield increase of 24% rather than the 19% coming from increased harvest volume (Fig. 6H). The reason for the titer increase cannot be explained by the presented data as bleed recycling should have no impact on harvest titer. Despite a slight titer increase and an increase of debris (Fig. 6F), all other process parameters such as VCV and viability (Fig. 6A), glucose and lactate (Fig. 6B), ammonia and pH (Fig. 6D) and product quality (Fig. 7) were comparable to the control run. This is in agreement with previous results from the offline screening, where no significant difference between settler inlet and return could be identified in terms of nutrients and metabolites (Fig. 2), as well as for product quality (Fig. 3). Further, the inclined gravity settlers showed reduced removal efficiency for smaller particles (Fig. 4), subsequently accumulating within the bioreactor

when operated for prolonged time. The experienced levels of debris did however not cause any problems with the cell retention device as harvest titers was not impacted compared to the control and showed even slightly higher concentrations.

Bleed recycling was stopped between day 26–34, leading to a decrease in cell debris. This debris reduction can be explained by the fact that once bleed recycling is stopped, all debris in the bleed stream goes to the waste as no bleed is recycled anymore.

The settler was restarted on day 34 to confirm accumulating conditions predicted by the regression line (Fig. 5B). At 12% biomass an accumulating recycle rate of approximately 75% was predicted. Therefore, the recycle rate was increased step by step (Fig. 6C), until it reached accumulating conditions at 75% on day 38, confirming the expected performance. An average bleed rate of 21% was observed in the control run during this second period of bleed recycling. A significant bleed rate reduction down to an average of 6% was achieved by applying an average settler recycle rate of 69%, leading to a predicted 18% process yield increase (Fig. 5C). This matched the experimentally determined 18% harvest volume increase (Fig. 6G) assuming no change in harvest titer. As harvest titer difference to the control got larger with proceeding run time, the product yield increase for the second bleed recycling phase increased even by 43% (Fig. 6H). The discrepancy in
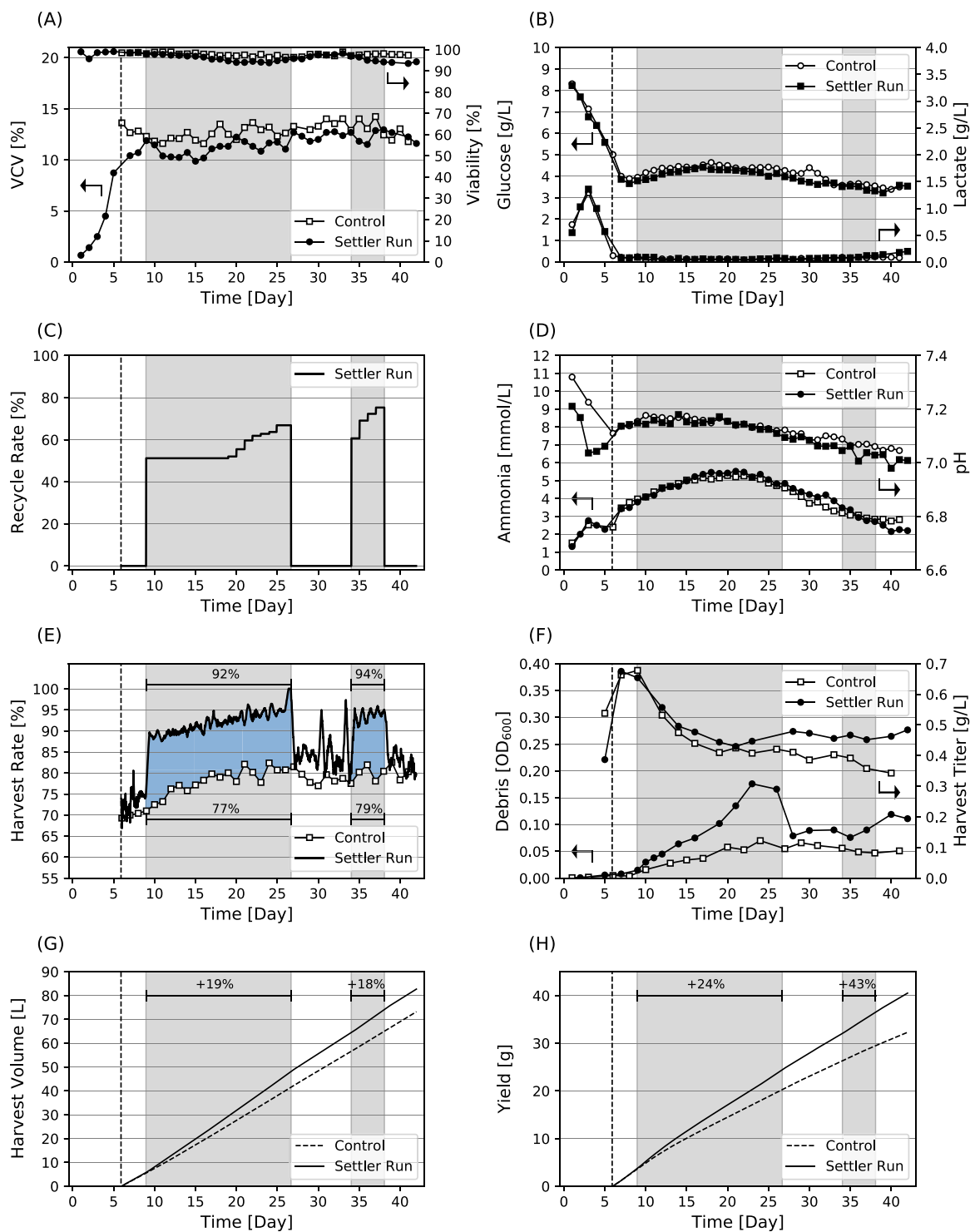
Fig. 6. Process data of lab-scale 2 L perfusion settler run compared with a standard control run without bleed recycling for mAb1. VCV and viability (A), glucose and lactate concentration (B), theoretical recycle rate setpoint (C), ammonia and pH profile (D), process harvest rate (E), harvest titer and debris trend (F), cumulative harvest volume (G) and cumulative process yield (H). Grey areas represent process phases were bleed recycling was performed, whereas bleed recycling was turned off during white process phases.

harvest titer cannot be explained by the presented data as all other process parameters were comparable to the control run and should not be overrated. Important is the fact that the harvest volume could significantly be increased during both bleed recycling phases without impacting the cell culture (Fig. 6) and product quality (Fig. 7), which is doubtlessly attributable to bleed recycling.

Overall, bleed recycling was successfully applied to a steady-state perfusion bioreactor run of 42 days and confirmed recycle rate and yield improvement estimation based on harvest volume increase. The automation setup around the settler enabled seamless operation. No operator intervention was necessary, the settler waste and clarified streams were self-adjusting to the process bleed fluctuations.
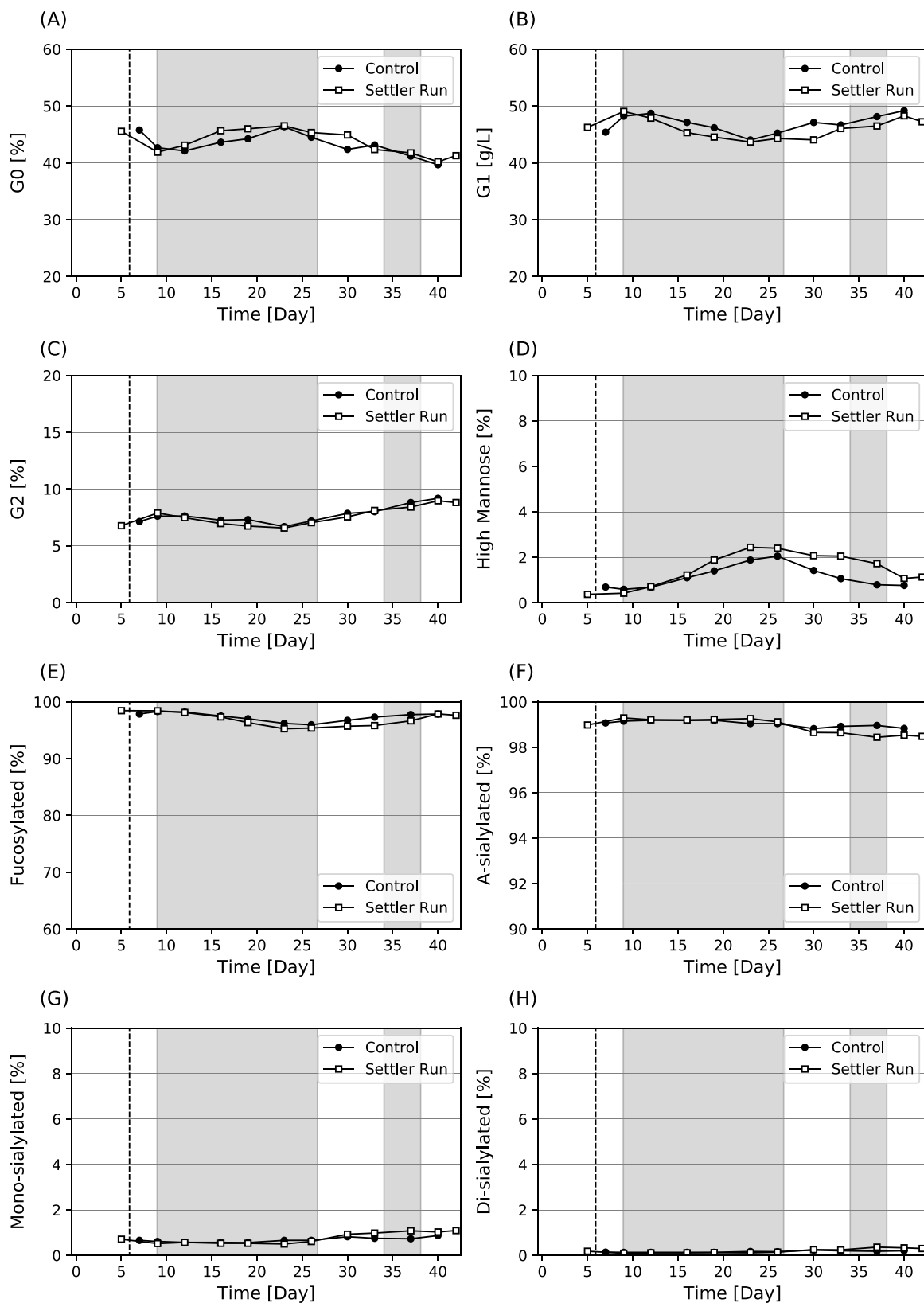
Fig. 7. CQA trends of lab-scale 2 L perfusion settler run compared with a standard control run without bleed recycling for mAb1. Galactosylation G0 (A), gal-actosylation G1 (B), galactosylation G2 (C), high-mannose (D), fucosylation (E), a-sialylation (F), mono-sialylation (G), and di-sialylation (H). Grey areas represent process phases were bleed recycling was performed, whereas bleed recycling was turned off during white process phases.

Additionally, to a previous bleed recycling proof of concept study performed with very high bleed rate and relatively low VCV setpoint of 5%, a best-case scenario for a proof-of-concept [9], this data shows that bleed recycling can be applied to more industrially relevant viable cell volumes above 10% and for extended periods.

3.3. Bleed recycling scale-up for 2000 L perfusion processes

The large-scale inclined gravity settler was designed to manage bleed recycling at a volumetric flow rate of 500 L/day (Table 1). This would correspond to a 2000 L perfusion process with a perfusion rate of 1.3 RV/day with roughly 20% of it as bleed rate. The evaluation was started with a process bleed rate of 18% (470 L/day), slightly below the foreseen maximal capacity of the inclined gravity settler. Recycle rates between 58% and 75% resulted, in the setup that was used, in an almost cell free recycle streams (Fig. 8A) with separation efficiencies above 97% (Fig. 8B). Subsequently, the process bleed rate was increased to 23% (600 L/day) and 28% (730 L/day), keeping high recycle rates of 76% and 75%, respectively. These process bleed rates were above the foreseen settler capacity and the separation efficiency decreased to 94% and 87%. Cell viabilities in the recycle stream however were not impacted. It is acceptable that the separation efficiency is not close to 100% as long as the process is not negatively impacted. Potentially, cells traveling through the bleed recycling loop could suffer from the absence of pH and pO_2 control. The high viability measured in the recycle stream gives confidence that the cells were not suffering from this (Fig. 8C). To avoid the presence of cells in the recycle stream at higher process bleed rates, the settler size should be increased respectively. Given a production process with a bioreactor volume of 2000 L, 1.3 RV/day perfusion rate, a recycle rate of 75% and a process bleed rate of 18–28%, this
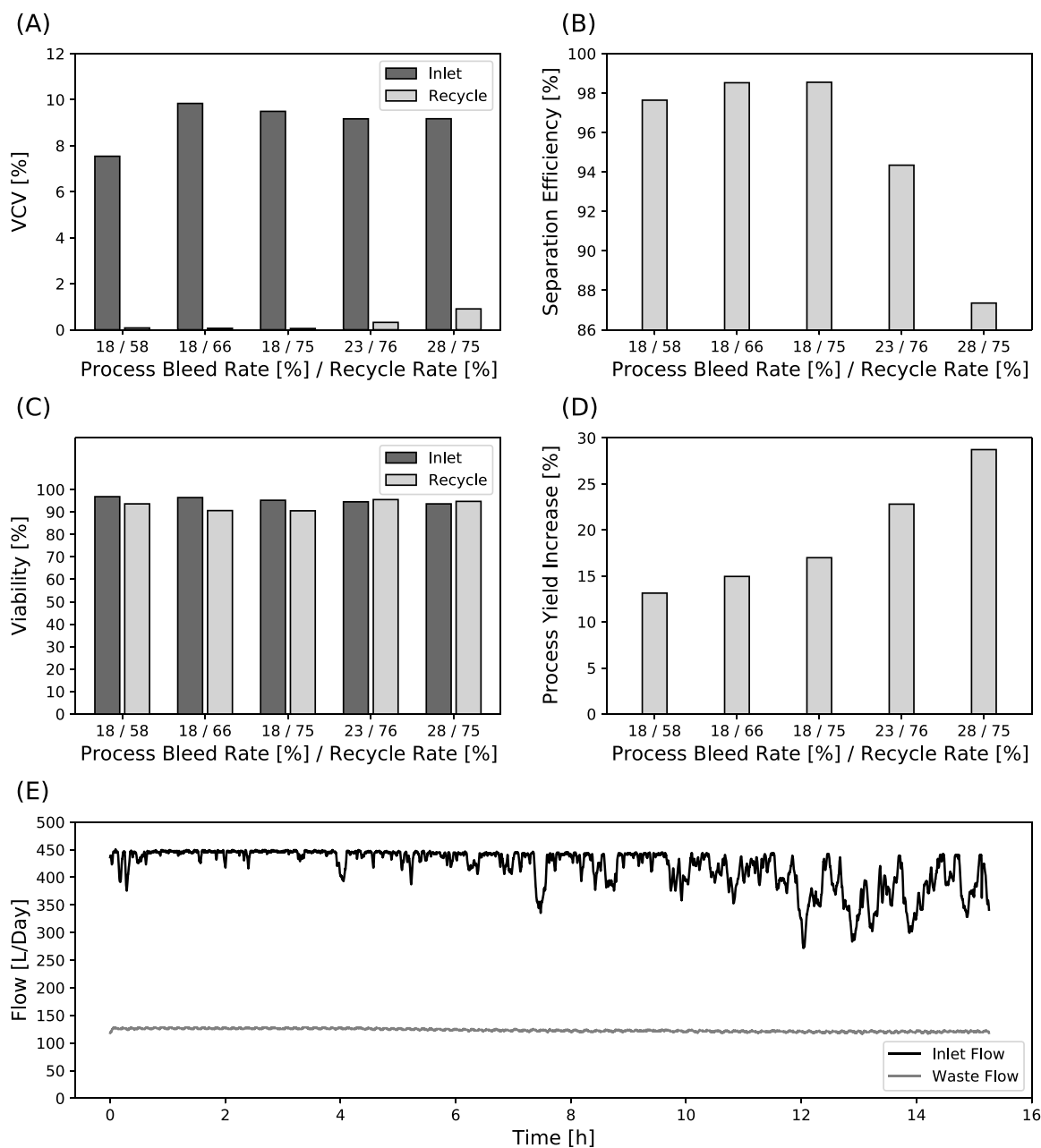

Fig. 8. Offline large-scale 2000 L perfusion process settler evaluation for various process bleed rates and recycle rates showing VCV of inlet and recycle streams (A), separation efficiency (B), cell viabilities (C) and process yield increase (D). Inline, real-time data of flow rates of inclined gravity settler connected to the 2000 L perfusion run (E).

experiment showed that a yield increase of 17–28% can be achieved with the large-scale inclined gravity settler (Fig. 8D). No significant changes in product quality were detected and data is summarized in the supporting information section (SI Table 1). Previously, inclined gravity settlers were used as cell retention devices in mammalian cell culture [20,21]. The main goal of this application is to retain cells within the bioreactor and keep high cell viability at the same time. Cells are ideally relatively quickly returned to the bioreactor after entering the uncontrolled environment within the inclined gravity settler to avoid negative impact on culture viability. This is usually achieved with rather high inlet and return (return stream on the bottom of inclined gravity settler containing cells) compared to the clarified harvest stream (on top of the inclined gravity settler). Here, it has been demonstrated that large-scale inclined gravity settlers can also be used as bleed recycling devices, enabling selective bleed operation. By operating the waste stream (on the bottom of the inclined gravity settler) at a relatively low flow compared to the inlet and recycle stream (clarified stream on top of the inclined gravity settler), it has been possible to concentrate biomass without impacting the recycle stream to the bioreactor (clarified stream on top of the inclined gravity settler).

The settler was then connected to a 2000 L perfusion bioreactor (filling volume of 1500 L) with fluctuating process bleed controlled by the capacitance signal and processed an average bleed of 430 L/day. The inclined gravity settler achieved an overall recycle rate of 70% and showed high separation efficiency of > 99% throughout the entire operation time (Fig. 8E). There was no quality drift detected from the start of the operation until the end of the operation (SI Table 2). Even though operation lasted only for approximately 15 h, the operating times corresponds to roughly ten times the calculated residence time in the large-scale inclined gravity settler (Table 1). Proof of concept for large-scale bleed recycling could therefore be demonstrated. To optimize the efficiency, an adaptive control strategy could be implemented as described for the 2 L perfusion system. This would reduce the concentrated bleed stream once the inlet stream decreases, which would ultimately lead to improved overall recycle rates.

4. Conclusions and outlook

This study for the first time used inclined gravity settling for bleed recycling applications in steady-state perfusion processes and compared it to the previously used acoustic separation technology. In lab-scale experiments, acoustic separation and inclined gravity settling showed similar bleed recycling efficiency and had no impact on nutrients, metabolites as well as on product quality. Solely for cell debris removal, the acoustic settler showed superior performance in removing smaller particles.

For reasons of simplified scale-up and reduced system complexity, inclined gravity settling was the chosen technology for long-term steady state perfusion bleed recycling. It was tested at lab-scale during a 42-day perfusion cell culture and was then scaled-up for a 2000 L perfusion process. An adaptive automation strategy was developed and tested to successfully operate the settling device at optimal setpoints despite fluctuations in process bleed rates.

For a perfusion process at lab-scale with a VCV setpoint of 12% and a bleed rate between 20% and 25%, the waste stream could be reduced by up to 3.5 times down to 6% effective bleed rate, resulting in a harvest rate increase of 19%. The large-scale inclined gravity settler connected to a 2000 L perfusion bioreactor with a filling volume of 1500 L was able to process an average bleed stream of 430 L/day with a recycle rate of 70%, constantly maintaining a separation efficiency of higher than 99%. Even higher bleed streams of 600 L/day were successfully concentrated with separation efficiencies above 94%, representing a perfusion run with 2000 L filling volume and 23% bleed rate. To the authors knowledge, this is the first time bleed-recycling has been performed at manufacturing scale using an inclined gravity settler. No impact on product quality was observed at both scales.

The insights gained on bleed recycling builds the basis of a rational support in decision making when considering this technology to improve process yield for an existing or new perfusion process with specific target process VCV, perfusion rate and process bleed rate. Similarly, this knowledge could be applied to other emerging technologies for bleed recycling such as continuous centrifuges, once they are available in right dimensions and allow stable operation throughout the entire runtime of a perfusion cell culture process.

CRediT authorship contribution statement

Patrick Romann: Conceptualization, Formal analysis, Investigation, Methodology, Visualization, Writing – original draft. **Jakub Kolar:** Data curation, Formal analysis, Investigation, Methodology, Writing – original draft. **Loïc Chappuis:** Data curation, Formal analysis, Investigation, Methodology, Writing – original draft. **Christoph Herwig:** Conceptualization, Methodology, Supervision, Writing – review & editing. **Thomas K. Villiger:** Conceptualization, Investigation, Project administration, Resources, Supervision, Visualization, writing – review & editing. **Jean-Marc Bielser:** Conceptualization, Investigation, Project administration, Resources, Supervision, Visualization, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

Data will be made available on request.

Acknowledgements

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.bej.2023.108873.

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