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Project report

(final version)

Mechanical stress analysis of transfected CHO suspension cells in different pumps

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Table of contents

1 Introduction

In previous studies, we investigated hydrodynamic stress caused on CHO suspension cells by the Levitronix BPS-200 and MDP-200 centrifugal type pumps [ZHAW report 2011, ZHAW report 2011a]. Viability and viable cell numbers were compared to the two peristaltic pumps Masterflex® I/P Easy Load and Masterflex® L/S Cole Parmer. It was found that the Levitronix pump caused substantially lower mechanical cell damage realizing a constant flow rate of 3.4 L/min at back pressures between about 25 and 710 mbar.

The aim of the following study was the evaluation of mechanical stress caused on CHO suspension cells by the magnetically levitated centrifugal pump Levitronix BPS-600 which is designed to realize higher flow rates up to 75 liters/min at a maximum differential pressure of 3.2 bar. For this purpose, different operation points comparable to the previous studies were investigated. A 4-piston-membrane pump (Quattroflow 1200-SU) was applied as comparison system (see section 2.5). Additionally, CHO cells were incubated in shaken and non-shaken shake flasks (also called static culture), in which shear stress can be assumed as negligible.

2 Materials and Methods

2.1 Cell expansion in a wave-mixed system

The cell expansion was realized in similar manner to the previous study [ZHAW report 2011a]. Briefly, the CHO suspension cells (*CHO XM111-10*) were cultivated in the single-use wave-mixed system BIOSTAT® CultiBag RM 20L (Sartorius Stedim Biotech) with a maximum working volume of 11 liter. Chemically defined culture media (HP-1, Cell Culture Technologies, Switzerland) with additions of Pluronic F-68 and tetracycline was used. Tetracycline is not supplemented in order to prevent microbial contaminations, but is strongly required for cell propagation, because the cell line is based on the tet-off principle. This means that tetracycline is required for cell growth and its absence has to be guaranteed for successful product (SEAP) secretion. However, protein production was not part of this study.

The bioreactor was inoculated with cells from shake flasks (200 mL maximum working volume) at an initial working volume of 2.0 L and a cell density of about 0.5 x 10^6 cells/mL. The cultivation was realized in fed-batch mode with additions of fresh medium in steps of 3, 5 and 2 liters (see Fig. 1). The rocking motion was varied with increasing rocking angle (from 6° to 7°) and rocking rate (from 15 to 24 rpm) depending on the filling level to guarantee sufficient mixing and oxygen supply.

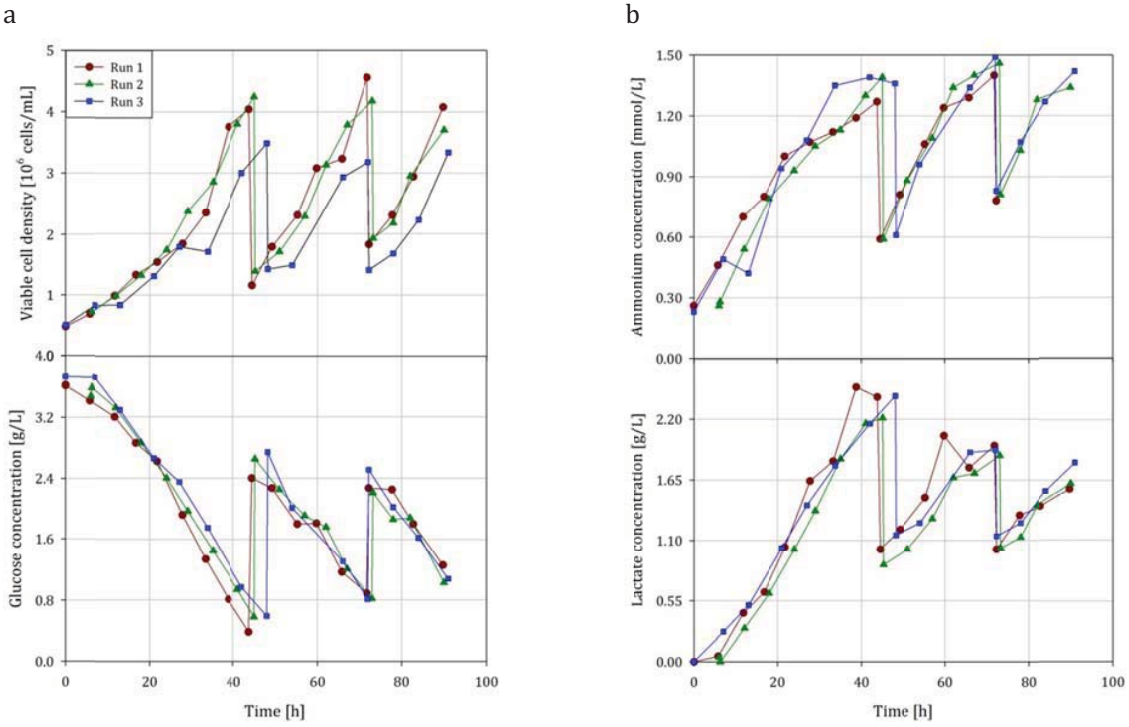


Fig. 1: Profiles of (a) vital cell density and glucose as well as (b) ammonium and lactate concentrations for the three cultivations with CHO suspension cells in fed-batch mode. Symbols: circles: run 1; triangles: run 2; squares: run 3.

For in-process control 2 mL samples were taken with a sterile syringe at least three times per day. Vital and total cell densities were measured automatically by cell counter NucleoCounter® NC-100 (ChemoMetec, Denmark) (see section 2.2). In addition, concentrations of glucose, lactate, glutamine, glutamate and ammonium as well as the pH value were quantified using a multi biosensor analysis system BioProfile 100 Plus (Labor-Systeme Flükiger AG, Switzerland). As shown in Fig. 1, concentration profiles of the three cultivations were rather similar and the final concentrations of both viable cells and main metabolites are well comparable.

2.2 Determination of cell concentration and viability

Total and viable cell densities were measured automatically with the cell counting device NucleoCounter® NC-100 (ChemoMetec, Denmark). The measurement is based on the fluorescence detection of the fluorescent dye propidium iodide (PI) which bounds to DNA. Results from the NucleoCounter® represent either total or non-viable cell concentration, depending on the sample preparation [Shah *et al.* 2006]. The viability is defined as the ratio of living (viable) cell density to the total cell density as indicated by Eq. (1), where VCD, DCD and TCD represent the viable, dead and total cell densities.

$$\text{Viability}\,[\%] = \frac{\text{VCD}}{\text{TCD}} = \frac{\text{TCD} - \text{DCD}}{\text{TCD}} \qquad (1)$$

2.3 Subcultivation

Subcultivations of the pumped cells were accomplished to investigate if the cells could further grow after high shear stress exposure. Cell growth as well as substrate consumption and metabolite production were evaluated. If the viability of the cell suspension was higher than 50 %, 10 mL of the pumped cell suspension were transferred to shake flasks with addition of fresh medium (HP-1, 40 mL). The cultures were incubated at 37 °C for about four days with daily sampling. Depending on cell growth and substrate consumption, fresh medium was added or cell cultures were passaged.

2.4 Reaction kinetics

For quantification and evaluation of mechanical stress, the kinetics of the viable cell density decay was modeled assuming first order kinetics, which can be described by the following relation, where VCD and k_D are the viable cell density and the cell death rate **(**h^{-1}) respectively.

$$\frac{dVCD}{dt} = -k_D \cdot VCD \tag{2}$$

Integration of Eq. (2) with LCD(t=0) = LCD_0 and subsequent linearization gives:

$$\ln(VCD(t)) = \ln(VCD_0) - k_D \cdot t \tag{3}$$

Thus, the cell death rate k_D can be obtained as the slope when plotting the logarithmic viable cell density as a function of time (see Fig. 2**)**. The quality of the predicted cell death rates was evaluated by the determination coefficient R^2 defined by Eq. (4), where y_i and $\bar{y}$ are the measured data and their mean value and f_i are the predicted values.

$$R^2 = 1 - \frac{\sum_N (y_i - f_i)^2}{\sum_N (y_i - \bar{y})^2} \tag{4}$$

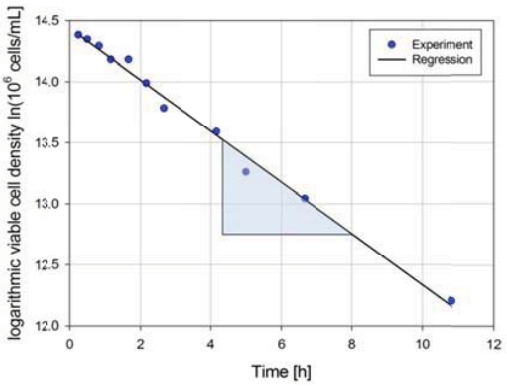


Fig. 2: Regression model for cell death kinetics. The cell death rate k_D is obtained by plotting the logarithmic viable cell density against the time and determining the slope of the obtained function.

2.5 Test setup and components

In the pump tests, three different pumps were used, as shown in Fig. 3 and Fig. 4. The two bearingless pumps Levitronix BPS-200 and BPS-600 as well as the 4-piston-diaphragm pump Quattroflow 1200-SU, which was applied as comparison system, were investigated. Additionally, two shake flasks – one unshaken and a second shaken at 120 rpm at standard cultivation conditions – were used as reference systems, where mechanical stress is negligible from our experience.

In the biological experiments, the back-pressures were varied between about 30 and 630 mbar at a constant flow rate of 3.4 L/min. These conditions were achieved at rotational speeds in the range of 1500 and 5000 rpm with the Levitronix BPS-200 and between 1300 and 4100 rpm with the BPS-600 pump (up to 40 and 50% of the maximum pump speed for the BPS-600 and BPS-200). The speed of the Quattroflow was set to about 360 rpm, which is only about 12 % of the maximum value. The back-pressure was varied by using tubing with smaller diameter (1/4") and a hose clip. Depending on the length of the constriction tubing different lengths of pump tubing were used to ensure a constant circuit volume of about 150 mL (see Tab. 1). The total liquid volume in the pump circuit was 3.4 L, which resulted in a mean residence time in the reservoir vessels of one minute. For controlling the flow rate and pressure, ultrasonic clamp-on flow sensor and single-use pressure sensor (provided by Levitronix) were used. All components with additional data are listed in Tab. 2.

The cell suspension was pumped in closed loops from temperature-controlled vessels with a total volume of 3.5 L (Chemap AG). The complete pump circuits including all tubing, connectors and the pump heads were steam-sterilized (121 °C, 30 min) in an autoclave (Systec V-150, Systec GmbH, Germany). Temperature was maintained at 37 ± 0.5 °C and monitored by a PC-based controller. The parameters for the three test cases are summarized in Tab. 1.

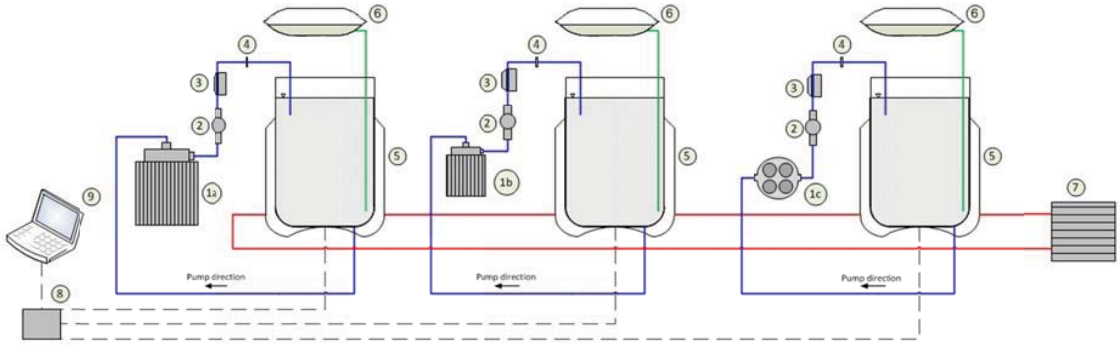
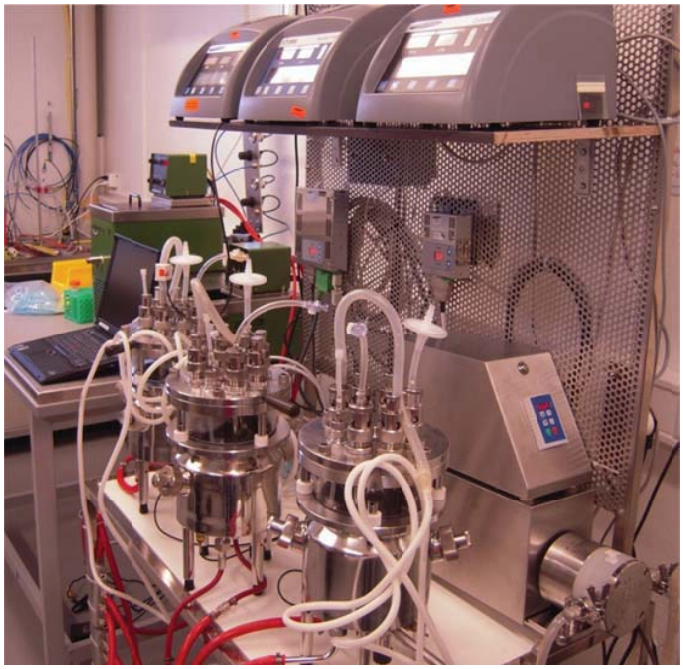


Fig. 3: Schematic of the test setup. The cell suspension is pumped with the three pumps Levitronix BPS-600 (1a), Levitronix BPS-200 (1b) and Quattroflow 1200-SU (1c) in closed loops from double-jacketed stainless steel tanks (5). The pressure and flow rate were monitored by single-use pressure sensor (2) and ultrasonic clamp-on flow sensor (3). The restriction of the tubing was realized by tubing with smaller diameter and a hose clip (4). Temperature was regulated by a thermostat (7) and monitored by a PC-based controller (8, 9).

a



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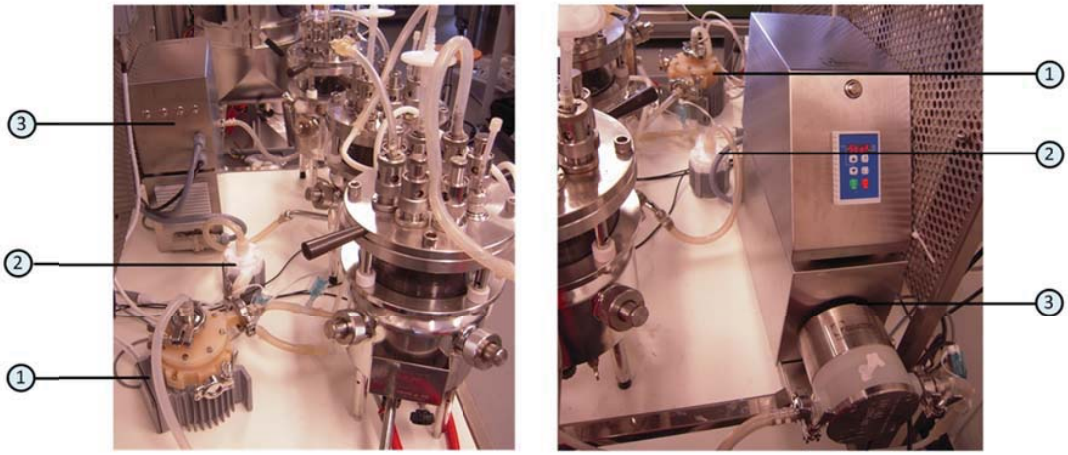


Fig. 4: Pictures of the test setup after sterilization. The two bearingless Levitronix pumps BPS-600 (1) and BPS-200 (2) were compared to the 4-piston-diaphragma pump Quattroflow 1200-SU (3).

Tab. 1: Descriptions and parameters of the investigated test cases

Characteristics	Symbol/ dimension	Test1	Test2	Test3	Notes
Flow rate	F / L·min^{-1}	3.4	3.4	3.4	- target flow rate; flow rate was controlled periodically during the experiment with clamp-on flow sensor; small variations (less than ± 2%) occurred
Back pressure	p / mmHg p / mbar	23 - 24 31 - 32	220 –228 293 - 304	457 – 475 609 – 633	- adjustment by use of constricting tubing and a hose clip
Pump speeds BPS-200 BPS-600 Quattroflow 1200-SU	N / rpm	 1500 1300 360	 3500 3000 340	 5000 4100 345	- values were kept constant
Tubing constriction (1/4")	L_R / mm	-	500	3000	- constriction of the tubing was readjusted periodically in order to keep pressure and flow rate constant
Pump tubing (3/8")	L_P / mm	2250	1800	900	
Total cell culture volume	V / L	3.4			- culture volume and volume inside the tubing circuit
Cell culture temperature	T / °C	37			- temperature was maintained at 37 ± 5 °C
Measured characteristics	--	Viable and total cell densities, viability, substrates & metabolites (glucose, glutamine, glutamate, lactate, ammonium)			- cell densities and viability were estimated by Cedex HiRes and NucleoCounter® NC-100 - concentrations of substrates and metabolites were analyzed by multi biosensor analyzer BioProfile 100 Plus

Tab. 2: Components of the pump setup

Pos.	Part name	Description
1a	Levitronix BPS-200	Re-usable pump head Bearingless motor Controller LPC-200.3 (firmware C2.25 R00) Manual panel
1a	Levitronix BPS-600	Re-usable pump head Bearingless motor Controller LPC-600.1 (firmware D6.25 R04) Manual panel
1b	Quattroflow 1200-SU	4-piston-membran-pump ¾" triclamp coupling 5° eccentric axle
2	Clamp-on flow sensor	Transonic systems Inc, H9XL, ultrasonic type
3	Pressure sensor	Netech Digimano 1000, piezo-resistive sensor
4	Hose clip	
5	Cell suspension reservoir	Stainless steel tank (Chemap AG) Temperature controlled via double jacket Total volume 3.5 L
6	Cell suspension transfer bag	Coupled to cell reservoir via sterile MPC coupling
7	Thermostat	THERMOMIX UB water bath liquid recirculating heater (B. Braun, Germany)
8	LabJack data acquisition device	8 single-ended, 4 differential 12-Bit analog inputs ±10 Volt analog input range USB 2.0/1.1 Low Speed Interface
9	PC	Data acquisition with software BORIS (v, 7.1)

2.6 Preparation and test procedure

The cell suspension was transferred into the sterile vessels using MPC couplings and the transfer tubing was welded to guarantee aseptic conditions. These working steps were done under a laminar flow chamber. The vessels were subsequently reconnected to the thermostat to achieve the target temperature of 37 °C. The pumps and tubing were filled with the suspension, the pressure sensors were calibrated and the tubing constriction was fine adjusted. Afterwards, the cell suspension was pumped for about two minutes to avoid cell sedimentation and to obtain homogenous suspension prior the start of the pump test.

Aliquots of 5 mL were taken with a sterile syringe from the three cell suspension reservoirs periodically, whereby the first 1 mL was discarded. The total volume of the samples was about 90 mL corresponding to about 2.6 % of the liquid volume. The shake flask cultures were sampled under a sterile work bench with a pipette. Due to the smaller culture volume of 50 mL, shake flasks were sampled less frequently. During the pump tests, viable and total cell densities as well as cell morphology and nutrient concentrations were determined.

2.7 Evaluation of the test setup

2.7.1 Flow rates

To guarantee reliable flow rates, the flow sensor was calibrated with the pump tubing (Masterflex® PharmaPure 96410-36). Therefore, the time required to pump a defined volume of 3 liters out of the reservoir vessels was measured. The calculated flow rate was compared to the value displayed by the clamp-on sensor. As shown in Fig. 5, a mean difference of 13 % between the two measurement techniques was obtained for all investigated cases. This value is marginal higher than in the previous experiments, which can be attributed to alteration of the tubing by steam sterilization. The values of the flow rate measured by the clamp-on sensor were thus corrected by a factor of 0.866 in all subsequent experiments.

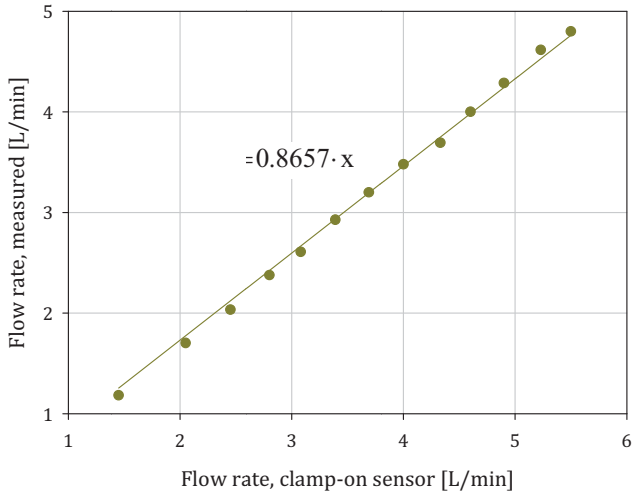


Fig. 5: Calibration of the flow rate indicated by the clamp-on flow sensor used in the pump experiments.

2.7.2 Characteristics of the BPS-600 pump

For the determination of the characteristics of the BPS-600 pump, the flow rate was measured with the clamp-on flow sensor at varying rotational speeds in the range between 1000 and 5000 rpm. Variation of the pressure was realized by constricting the pump tube with a hose clip. Due to the limited measurement range of the flow sensor, only flow rates below 10 L/min could be measured. In contrast to data provided by Levitronix, clearly decreasing flow rates were obtained with increasing back pressure (see Fig. 6). This could be a result of a modified pump head geometry as well as the simple measurement of back pressure instead of pressure difference between inlet and outlet. All data points are well described by a statistic model using a 4-degree polynom (see Eq. and Fig. 19 in the appendix):

$$F = \begin{aligned} & 0.6567 - 0.0391 \cdot N - 102.0884 \cdot p + 0.0197 \cdot N^2 + 151.9624 \cdot p^2 \\ & - 2.4954 \cdot 10^{-4} \cdot N^3 - 157.4087 \cdot p^3 + 1.196 \cdot 10^{-6} \cdot N^4 + 61.6017 \cdot p^4 \end{aligned} \quad (5)$$

where F is the flow rate (in L/min), N is the pump speed (in rps) and p is the pressure (in bar).

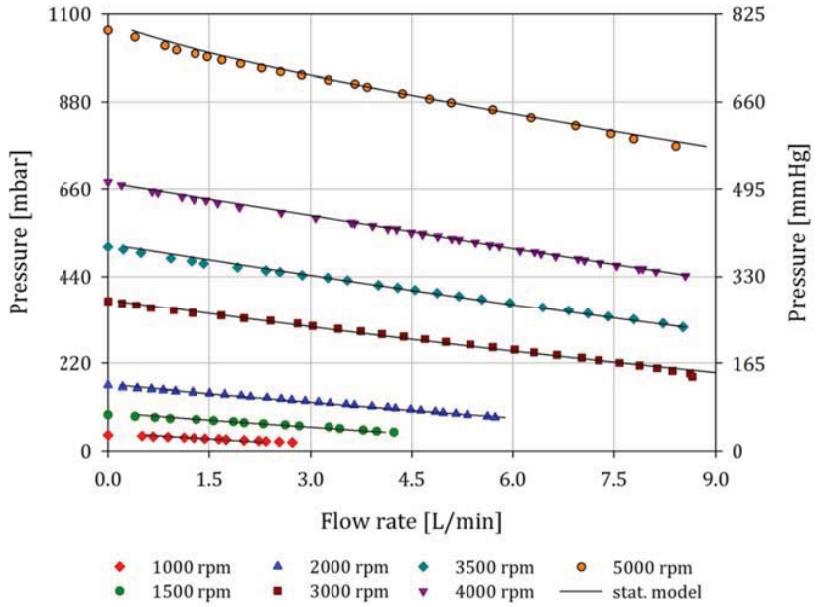


Fig. 6: Characteristics of the Levitronix BPS-600 centrifugal pump estimated for different rotational speeds between 1000 and 5000 rpm. Symbols indicate measured data and the lines were obtained by statistical model with 4-degree polynom.

3 Results

3.1 Determination of viable cell density

3.1.1 First test case

In the first test case, the rotational speed of the Levitronix BPS-200 pump was set to 1500 rpm and kept constant. Comparable pressure conditions at the desired flow rate of 3.4 L/min were obtained with the BPS-600 pump operated at 1300 rpm. The rotational speed of the 4-piston-diaphragm pump was set to 360 rpm (12 % of maximum value). Pressure in the pump tubing was adjusted to about 31 ± 2 mbar (see Tab. 1).

The mean initial living cell density at the start of pumping was about $3.68 \cdot 10^6$ cells/mL at a viability of 99 %. The glucose, glutamine and lactate concentrations were 1.57 g/L, 0 mmol/L and 1.46 g/L respectively (see Tab. 3). No glutamine was determined, although 2L fresh culture medium was added at the end of the cell cultivation. The medium addition results in a theoretical glutamine concentration of about 0.15 mmol/L, which is below the lower limit of the measurement range of the bioanalyzer used (see appendix).

Tab. 3: Initial values for cell densities and concentrations at the start of pumping in the first test case. The data given represent mean values and simple standard deviation estimated for the three cell reservoirs and the two shake flask cultures.

Component	Unit	Value
Total cell density	10^6 cells/mL	3.68 ± 0.54
Viable cell density	10^6 cells/mL	3.65 ± 0.55
Viability	%	99.0 ± 1.5
Glutamine	mmol/L	0.00
Glutamate	mmol/L	0.13 ± 0.09
Glucose	g/L	1.57 ± 0.04
Lactate	g/L	1.46 ± 0.03
Ammonium	mmol/L	n.d.

n.d. – not determined

The total and viable cell densities determined in the first test case are shown in Fig. 7. As expected, the viable cell densities of the pumped cultures decrease continuously and reached values between 0.582 and 2.68 x 10^6 cells/mL after 15 h. The 4-piston-diaphragm pump showed the highest decrease in both total and viable cell density with final values of 0.582 and 1.3 x 10^6 cells/mL respectively. No significant differences could be detected between the two bearingless pumps, where final viable cell concentrations between 2.43 and 2.68 x 10^6 cells/mL were measured at the end of the pump exposure. In contrast to previous experiments, the cell density in the static shake flask decreased during the first two hours but increased afterwards linearly from about 3.0 to 3.6 x 10^6 cells/mL. In the completed project, it was found that the cell density of the static culture remained constant without cell death or growth which was attributed to oxygen depletion caused by repeated cell sedimentation between the samplings. In comparison, the shaken flask culture showed a typical cell growth for about 8 hours until the cells density reached approximately 5 x 10^6 cells/mL. Afterwards the cell density remained almost constant which could be explained by limitation of glucose (concentration below 1.0 g/L) and amino acids as well as by the high lactate content above 1.8 g/L (see Fig. 9).

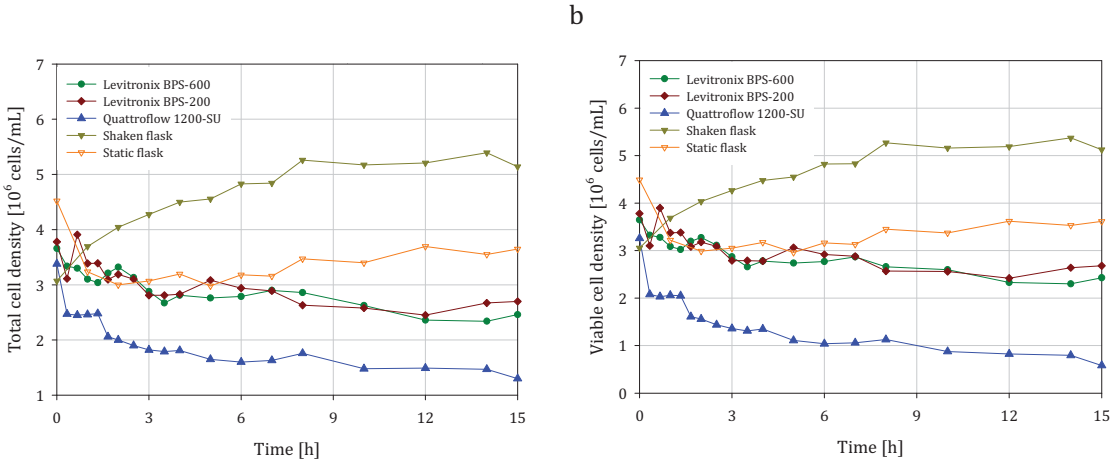


Fig. 7: Total cell densities (a) and viable cell densities (b) measured in the first test case (pressure of 31 ± 2 mbar).

In Fig. 8, the viability of the cell cultures during the first test is shown. The viability of the cultures pumped by the Levitronix systems remained high with values above 97 % over the complete process (with exception of one data point at 8h, which is probably a result of a measurement error). In contrast, the viability of the culture pumped by the 4-piston-diaphragm pump decreased to values below 50 % at the end of the experiment. Interestingly, the viability in this pump was already reduced by about 4 % at the beginning of the experiment which could be a result of the two minute pumping realized to avoid cell sedimentation (see section 2.6).

The lower viable cell density present in the Quattroflow 1200-SU pumped culture resulted in both lower glucose and lactate concentrations, as shown in Fig. 9. The glucose concentration was

about 1.2 g/L after 15 hours, but about 0.8 g/L were measured with the two Levitronix pumps. Here, no significant differences were obtained for the metabolite concentrations, which is not entirely surprising, since very similar cell densities were present in these cultures and the cell death rates were nearly identical (see section 3.2).

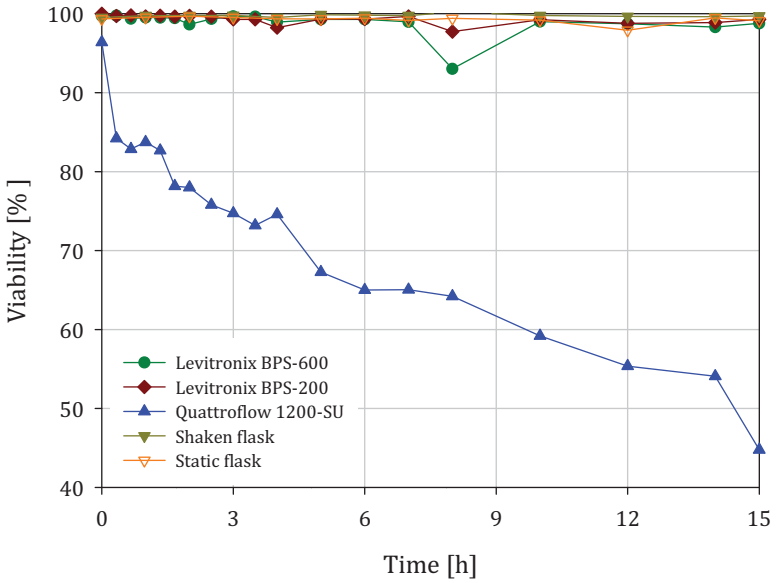


Fig. 8: Viabilities determined in the first test case (pressure of 31 ± 2 mbar).

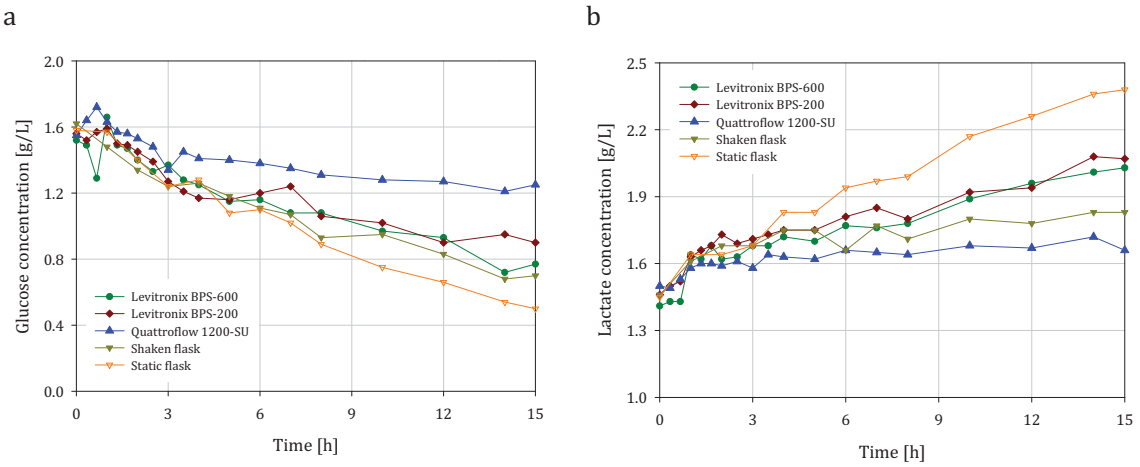


Fig. 9: Glucose (a) and lactate (b) profiles determined by the bioanalyzer Bioprofile 100 plus in the first test case (pressure of 31 ± 2 mbar).

3.1.2 Second test case

In the second test case, the rotational speed of the Levitronix BPS-200 pump was set to 3500 rpm and kept constant. Comparable pressure conditions at the desired flow rate of 3.4 L/min were obtained with the BPS-600 pump operated at 3000 rpm. The rotational speed of the Quattroflow 4-piston-diaphragm pump was set to 345 rpm (11.5 % of maximum value). Pressure in the pump tubing was adjusted to about 300 ± 5 mbar (see Tab. 1). The mean initial living cell density at the start of pumping was about $2.71 \cdot 10^6$ cells/mL at a viability of 97.8 %. The glucose, glutamine and lactate concentrations were 1.57 g/L, 0.14 mmol/L, and 1.52 g/L respectively (see Tab. 4).

Tab. 4: Initial values for cell densities and concentrations at the start of pumping in the second test case. Given data represent mean values and standard deviation estimated for the three cell reservoirs and the two shake flask cultures.

Component	Unit	Value
Total cell density	10^6 cells/mL	2.77 ± 0.06
Viable cell density	10^6 cells/mL	2.71 ± 0.07
Viability	%	97.8 ± 2.3
Glutamine	mmol/L	0.14 ± 0.04
Glutamate	mmol/L	0.00
Glucose	g/L	1.57 ± 0.04
Lactate	g/L	1.52 ± 0.05
Ammonium	mmol/L	1.23 ± 0.04

The total and viable cell densities obtained during the second test case are given in Fig. 10. Similar to the first experiment, the cell concentrations declined only in the pumped cultures and the maximum reduction of the cell count was found for the Quattroflow pump. Here, the viable cell density declined to $0.57 \cdot 10^6$ cells/mL while values of about $1.5 \cdot 10^6$ cells/mL were achieved with the Levitronix pumps. Only marginal differences between the BPS-200 and BPS-600 systems were observed. No clear trend of the cell density in the static culture was obtained with variations around $(3 \pm 0.5) \cdot 10^6$ cells/mL. This could be a result of cell sedimentation between the samplings although the suspension was shaken to obtain homogenous aliquots. The viable cell count in the shaken flask increased up to a value of $4.76 \cdot 10^6$ cells/mL after 12 h.

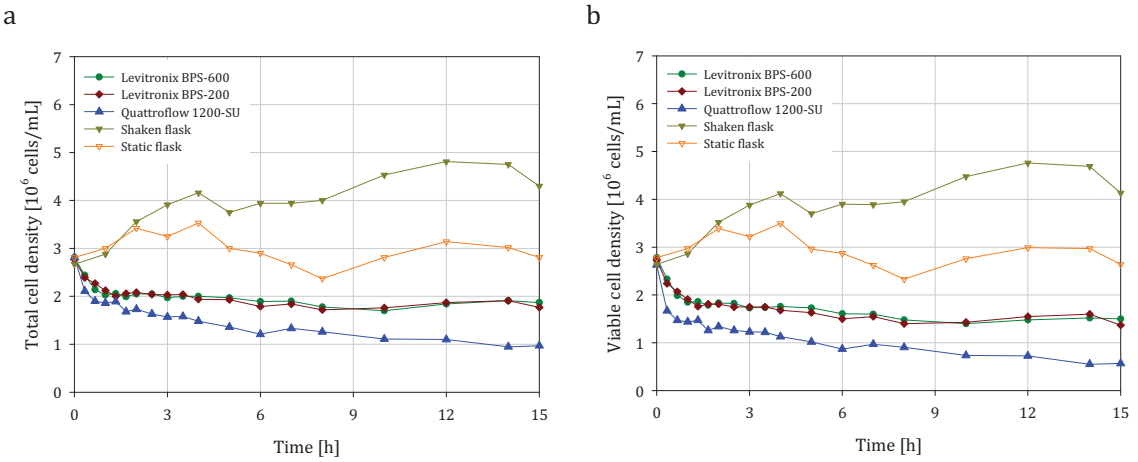


Fig. 10: Total cell densities (a) and viable cell densities (b) measured with NucleoCounter NC-100 in the second test case (pressure of 300 ± 5 mbar).

As indicated by the viable cell densities, only small differences in the viability were obtained between the two Levitronix pumps (see Fig. 11). The final viabilities were about 80 %, whereas the culture pumped by the comparison system had a value of 58 % after 15 hours. In agreement with the first test case, the viability in the 4-piston-diaphragm pump declined substantially within the first hour. This is not entirely surprising, since a nearly identical pump speed (345 rpm instead of 360 rpm) was used in both test cases. Interestingly, the viability of the Quattroflow pump remained rather constant between one and three hours but decreased steadily until the end of the experiment. In contrast, the viability of the flask cultures remained high (> 95 %) during the whole process.

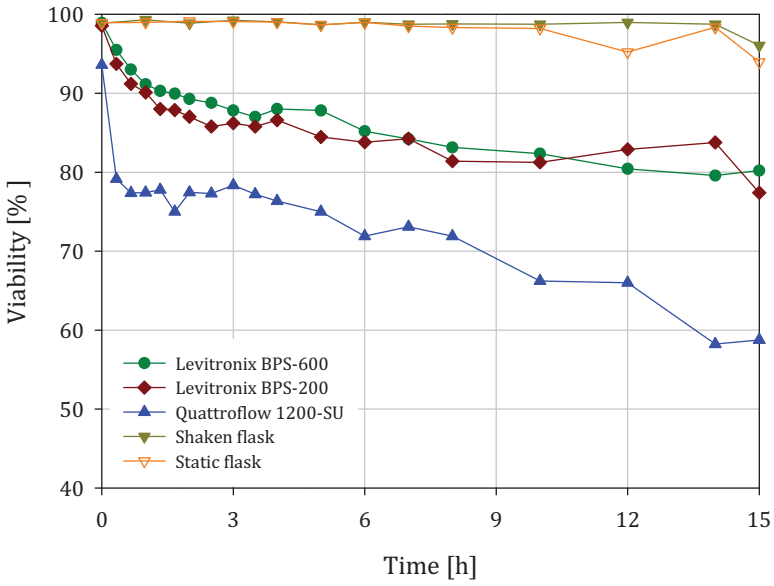


Fig. 11: Viabilities determined with NucleoCounter NC-100 in the second test case (pressure of 300 ± 5 mbar).

3.1.3 Third test case

In the third test case, the rotational speed of the Levitronix BPS-200 was set to 5000 rpm and kept constant. Corresponding pressure situation (420 ± 15 mbar) was obtained with the Levitronix BPS-600 at 4100 rpm and with the Quattroflow 1200-SU at 345 rpm (see Tab. 1). The initial viable cell densities at the start of pumping were about $2.90 \cdot 10^6$ cells/mL at a viability of 96.3 %. The glucose, glutamine, lactate and ammonium concentrations were about 1.44 g/L, 0.22 mmol/L, 1.75 g/L and 1.38 mmol/L respectively, which is comparable to the previous test cases (see also Tab. 3 and Tab. 4).

Tab. 5: Initial values for cell densities and concentrations at the start of pumping in the third test case. Given data represent mean values and standard deviation estimated for the three cell reservoirs and the two shake flask cultures.

Component	Unit	Value
Total cell density	10^6 cells/mL	2.90 ± 0.51
Viable cell density	10^6 cells/mL	2.80 ± 0.57
Viability	%	96.3 ± 3.9
Glutamine	mmol/L	0.00
Glutamate	mmol/L	0.22 ± 0.04
Glucose	g/L	1.44 ± 0.08
Lactate	g/L	1.75 ± 0.06
Ammonium	mmol/L	1.38 ± 0.03

In Fig. 12, the total and viable cell densities determined in the third test case are given. As expected, qualitatively similar results to the second test case were obtained. Again, the Quattroflow 1200-SU showed the highest decrease in both total and cell density. The final values were 0.693 and $1.28 \cdot 10^6$ cells/mL respectively. In contrast, the viable cell densities in the Levitronix BPS-200 and BPS-600 were 1.46 and $1.40 \cdot 10^6$ cells/mL respectively, which means about twofold higher values. Interestingly, the viable cell number of the BPS-600 was about $0.2 \cdot 10^6$ cells/mL lower than with the BPS-200 after four hours although the initial values was the highest ($3.26 \cdot 10^6$ cells/mL). These led to lower viabilities with differences between 2 and 12 % compared to the BPS-200, increasing with longer pumping time (see Fig. 13). The effect is also remarkable with respect to the cell death rate (see section 3.2 and Fig. 14), but the reasons for this deviation could be not clarified. However, the viabilities determined for the 4-piston-diaphragm pump were significantly lower again (at least 10 % over the complete test duration) and reached a final value of 54 % (see Fig. 13). In contrast, no cell death was observed in both flask cultures where the viability remained high (> 97 %) until the end of the experiment.

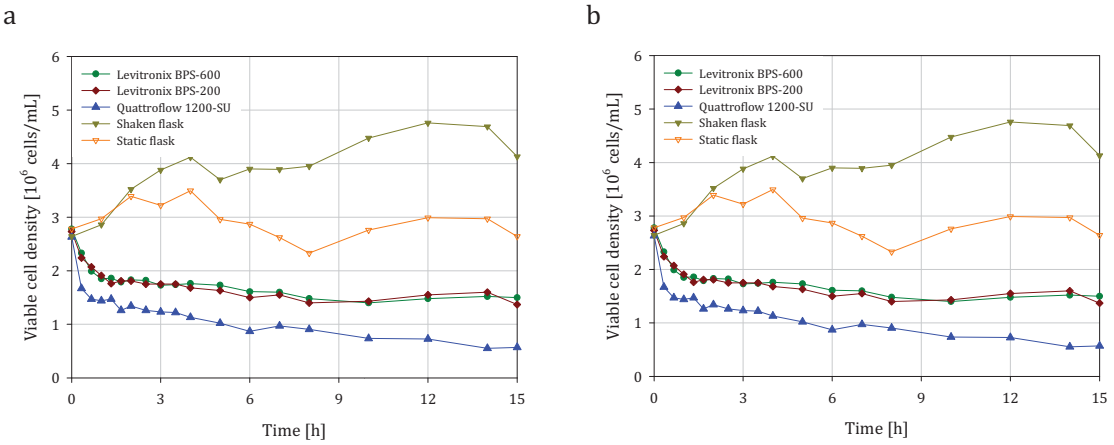


Fig. 12: Total cell densities (a) and viable cell densities (b) measured with NucleoCounter NC-100 in the third test case (pressure of 620 ± 12 mbar).

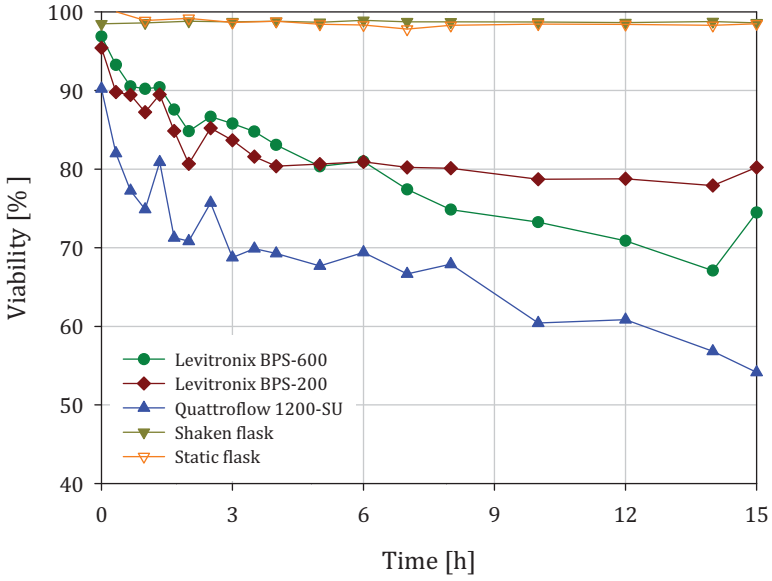


Fig. 13: Viabilities determined with NucleoCounter NC-100 in the third test case (pressure of 620 ± 12 mbar).

3.2 Evaluation of mechanical stress by cell death rates

In the following, the cell death rates obtained in the three experiments with the transfected CHO suspension cell line (CHO-XM 111-10) are summarized. First order kinetics of the cell death were assumed, as described in section 2.4. In general, good approximations have been found with determinations coefficients above 0.8. At low cell death rates the model regression became less certain, since the cell death rate represents the slope of the logarithmical VCD. However, only in the third test case R^2 was lower than 0.6 for the Levitronix BPS-200 (see Tab. 6).

It is obvious, that for all rotational speeds investigated the centrifugal-type pumps caused lower cell death rates than the 4-piston-diapgragm pump (see Fig. 14), which indicates lower hydrodynamic stress caused on the CHO cells under identical hydrodynamic conditions. As expected, an increase of the death rate was obtained at higher pump speeds of the centrifugal-type pumps – with the exception of the BPS-200 where identical death rates of 0.032 h^{-1} were calculated for 3500 rpm and 5000 rpm. However, it should be noted that the regression coefficient for the third test case (5000 rpm; 620 ± 12 mbar) is very small with a value of R^2 = 0.577, which means that the linear fit is very uncertain. Improved curve fitting could be achieved by modified regression models, which have not been applied due to comparison reasons. The cell death rates determined for the Levitronix BPS-600 rises with increasing pump speed from 0.023 h^{-1} to 0.046 h^{-1}, which means a twofold increase as well as a nearly linear relation of cell death and pump speed.

Comparable to results of the peristaltic pumps Masterflex® I/P Easy Load and Masterflex® L/S Cole Parmer investigated in previous studies, the death rates calculated for the Quattroflow 1200-SU remained nearly constant independent of the back-pressure. Although a higher rotational speed of the 4-piston-diaphragm pump was adjusted in the first test case (pressure of 31 ± 2 mbar) to achieve the desired flow rate of 3.4 L/min (360 rpm compared to 340 rpm in the second test case), no significant differences in cell death could be observed.

Tab. 6: Results of the regression analysis of the cell death kinetics.

Flow rate	Pressure	Cell death rate			Regression coefficient		
F [L/min]	p [mbar]	k_D [h^{-1}]			R^2 [-]		
		Levitronix BPS-600	Levitronix BPS-200	Quattroflow 1200-SU	Levitronix BPS-600	Levitronix BPS-200	Quattroflow 1200-SU
3.4	31 ± 2	0.023	0.022	0.068	0.745	0.616	0.907
3.4	300 ± 5	0.031	0.032	0.069	0.942	0.916	0.976
3.4	620 ± 12	0.046	0.032	0.065	0.820	0.577	0.816

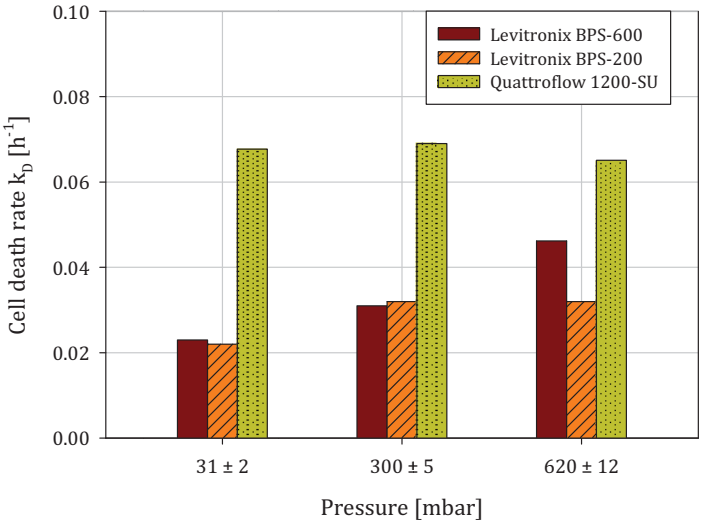


Fig. 14: Cell death rates determined for the different pressure situations.

3.3 Results of the subcultivation experiments

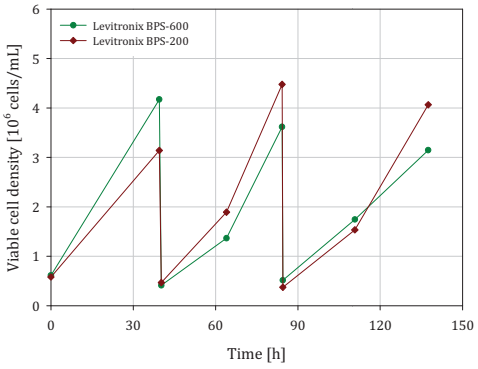
As described in section 2.3, aliquots of the pumped cell suspension were transferred from the pump reservoir to shake flasks, if viability of the cells was higher than 50 %. From our experience, it is very unlikely to regenerate cells with even lower viabilities, which is confirmed by the second trial (see below). Cells were incubated at 37 °C for about four days with cell passages or addition of fresh medium depending on cell growth.

In Fig. 15a, the viable cell density of cultures pumped by Levitronix BPS-200 and BPS-600 subcultivated after first test case is given. No cells pumped by the Quattroflow 1200-SU were subcultivated because of the low viability at the end of the pumping (44.8%). Starting from the initial cell density of about $0.6 \cdot 10^6$ cells/mL, cells grew with a mean growth rate of $\mu = 0.047\ h^{-1}$ (corresponding to a doubling time of $t_D = 14.7$ h) over three passage cycles and reached cell densities above $3 \cdot 10^6$ cells/mL in each cycle. Thus, the growth rate is comparable to the values obtained during cell expansion in the wave-mixed system (see section 2.1). Viability was above 99 % in all samples.

In contrast, the cells subcultivated after the second pump experiment (3.4 L/min; 300 ± 5 mbar) showed a different growth behavior. Cells of all pumps were subcultivated starting at cell densities between 0.2 and $0.47 \cdot 10^6$ cells/mL. Since a fixed volume of the aliquots was used, initial cell density depended on the final values at the end of the pump experiment resulting in the lowest concentration for the Quattroflow 1200-SU. No cell growth could be detected in the flask culture of the 4-piston-diaphragm pump. The viable cell density declined to $0.045 \cdot 10^6$ cells/mL within 45 hours, when the incubation was aborted. The other two cultures showed an increase of the viable cell density to maximum values of about $1.43 \cdot 10^6$ cells/mL, which means a nearly twofold doubling. The mean growth rates were 0.024 h^{-1} and 0.029 h^{-1} for the BPS-200 and BPS-600 respectively. These values correspond to doubling times of 28.6 h and

23.6 h respectively, which is significantly longer than those observed during cell expansion in the wave-mixed system as well as in the subcultivation of the first test case. This is in agreement with the higher cell death rates determined for the pumping process (see section 3.2). Surprisingly, the viable cell density started to decline after 60 hours although sufficient glucose content was available (above 1.1 g/L) and lactate was at high but acceptable levels (about 1.95 g/L). The viability of the last samples were 47 % (BPS-600) and 56 % (BPS-200) respectively and thus cultivation was aborted as well. The reason for the rapid cell death could not be completely clarified. A possible explanation could be the high ammonium level of 1.8 mmol/L reached after 60 hours (data not shown). Total concentrations of ammonia and ammonium as low as 2-3 mM have been reported to reduce cell growth considerably, depending on the cell line and culture conditions [Schneider et al. (1996)].

a



b

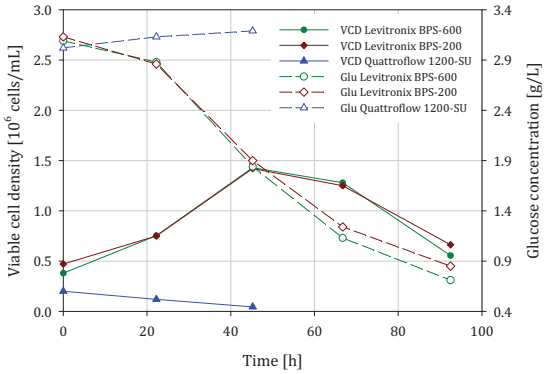


Fig. 15: Results of the subcultivation experiments. (a) Viable cell density of cultures pumped by Levitronix BPS-200 and BPS-600 during subcultivation in shake flasks; (b) Profiles of viable cell density (VCD) and glucose (glu) during subcultivation after the second pump experiment.

To avoid a negative effect of ammonia and lactate on cell growth during the subcultivation, 30 mL fresh medium (HP-1) was added after 60 hours of incubation in the third subcultivation experiment (see Fig. 16). Starting at an initial viable cell density of about $0.25 \cdot 10^6$ cells/mL, cells grew with mean doubling times of about 19 h, whereby the BPS-600 culture showed a nearly linear growth instead of the typical exponential found in the BPS-200 flask. The viable cell densities were $2.0 \cdot 10^6$ cells/mL (BPS-200) and $2.3 \cdot 10^6$ cells/mL (BPS-600) before medium addition respectively. Afterwards, cell density achieved maximum values of 2.8 (BPS-200) and $1.9 \cdot 10^6$ cells/mL (BPS-600) after 100 hours when glucose was exhausted. Ammonia levels were rather high again at concentrations of 1.64 mmol/L (BPS-200) and 1.75 mmol/L (BPS-600). However, the decrease in cell density at the end of the experiment is most probably related to the substrate depletion.

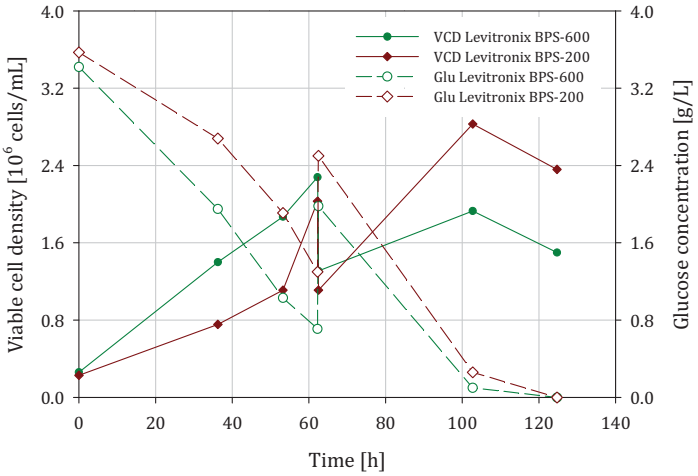


Fig. 16: Profiles of viable cell density (VCD) and glucose (glu) during the subcultivation after the third pump experiment. 30 mL fresh medium (HP-1) was added after 60 hours of incubation.

4 Discussion

In Fig. 17, viable cell density and viability of the three investigated pumps is given for the three test cases realized. It can be summarized, that the Levitronix magnetically levitated pumps had a higher viability over the complete process independent on the pump speed. This can be attributed to a lower mechanical stress caused to the CHO suspension cells compared to the Quattroflow 1200-SU system. The low mechanical stress is in agreement to previous findings, where the Levitronix BPS-200 system was compared to the two peristaltic pumps Masterflex® I/P Easy Load and Masterflex® L/S Cole Parmer [ZHAW report 2011; ZHAW report 2011a].

As expected, the most significant differences in the cell death profiles between the 4-piston-diaphragm and the centrifugal-type pumps were obtained for the lowest pump speed, where the viability remained above 95 % with the Levitronix systems, but dropped below 50 % in the comparison pump (see Fig. 8). Furthermore, a direct relation between pump speed and the cell death is obvious, which is in agreement to findings of previous studies [ZHAW report 2011a] as well as literature data [Zhang et al. (2006); Kamaraju et al. (2010)]. This can be explained by enhanced turbulence inside the pump head. In contrast, it can be concluded that the pressure difference is of minor importance on cell death, since the cell death rates obtained for the Quattroflow 1200-SU, which was operated at nearly constant rotational speed in all three experiments, was independent on the back-pressure. This in agreement with findings of Kamaraju et al. (2010), who found that cell breakage is dramatic for back pressures higher than 2.76 bar, which is far above the range investigated in the present study. However, it should be noted again, that the diaphragm pump was operated at only 12 % of its maximum speed. It is expectable that the mechanical stress in the Quattroflow pump is enhanced by increased pump speed as well.

The profiles of both viable cell density and viability differ between the three runs for the comparison system (see Fig. 17, third row) within a range of 10 to 20 %. However, we want to emphasize that each pressure situation was investigated only once due to the high cost and time efforts. If we assume, that the back-pressure has no influence on cell death, the cell death rates determined for the Quattroflow 1200-SU can be averaged. Thus, an average value of k_D = 0.067 h^{-1} with a standard deviation of 0.002 h^{-1} (corresponds to 3 % of the average value) can be calculated. Taken into account that the single death rates were obtained by linear regression with regression coefficients R^2 between 0.816 and 0.976, an acceptable reproducibility can be stated. This is also confirmed by comparison of the cell death rates determined in the present study with those of previous experiments (ZHAW report 2011a), as shown in Fig. 18. With the exception of a rotational speed of 3500 rpm, where k_D was two times higher compared to the second study, very good agreement was achieved. However, it should be noted that the k_D value of 0.016 h^{-1} for 3500 rpm (obtained in the ZHAW report 2011a) was lower than that for 1500 rpm, but the reasons for this result could not be completely clarified [ZHAW report 2011a].

(a) (b)

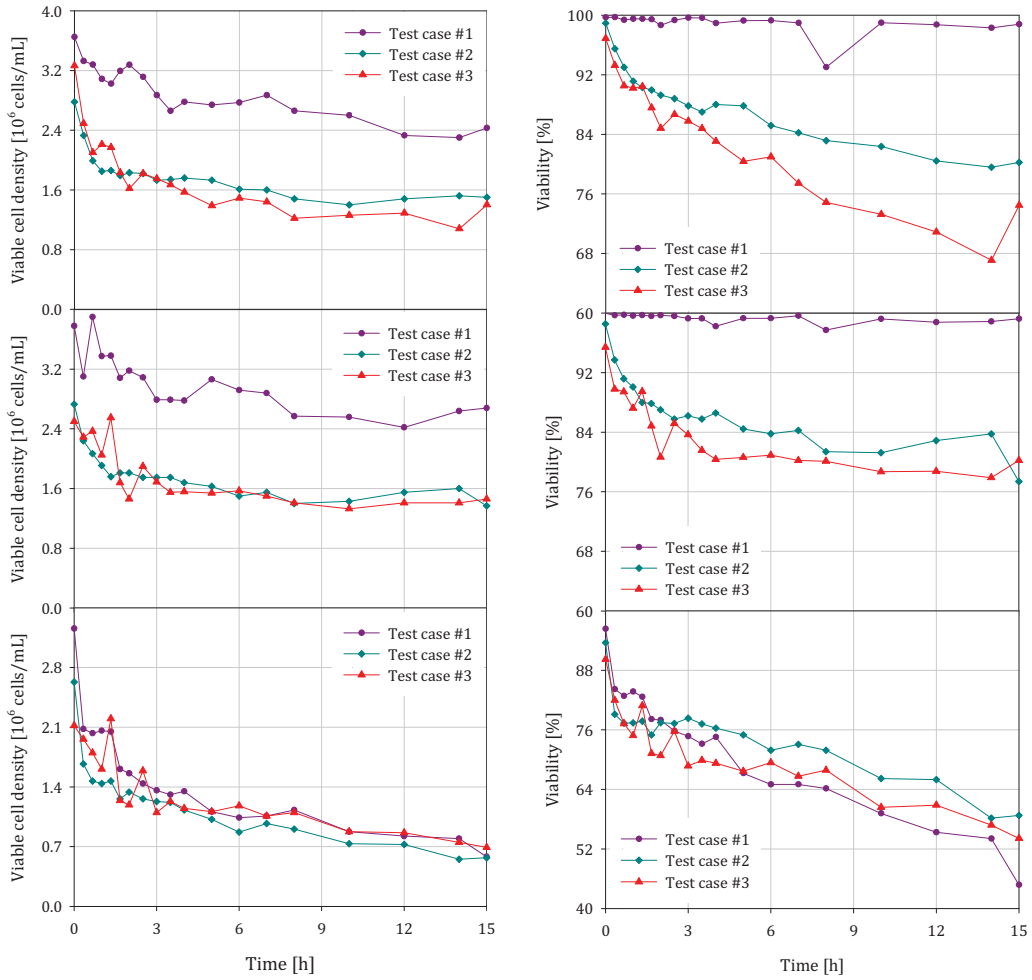


Fig. 17: Summary of viable cell density (a) and viability (b) obtained in the three test cases with the Levitronix pumps BPS-600 (first row) and BPS-200 (second row) as well as the Quattroflow 1200-SU (third row).

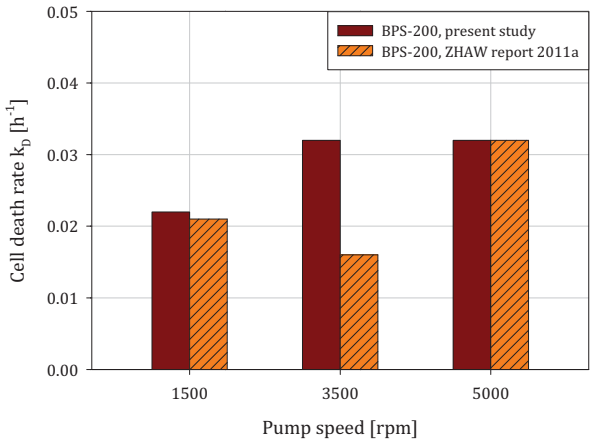


Fig. 18: Comparison of cell death rates determined for the Levitronix BPS-200 pump during the present and previous studies.

The results of the subcultivation experiments show in general different results between less and highly stressed cultures. Thus, comparable cell growth and metabolite profiles to the wave-mixed cultures, where mechanical stress can be assumed as low and homogenous, were obtained in the first subcultivation (after the pump test at pressures of 30 ± 2 mbar). For example, the mean doubling time was 14.7 h, which is the same as observed during cell expansion in the BIOSTAT® CultiBag RM 20L. In contrast, significantly longer doubling times above 20 hours (corresponding to growth rates below 0.035 h^{-1}) were determined in the two other experiments. Although the viability increased rather quickly (viability of the second sample was already above 95 % in all flasks), differences in the metabolisms were detected. The cells produced higher amounts of lactate and ammonia (data not shown) compared to non-stressed cultures. Lactate and ammonia are well-known to be toxic and inhibitory for mammalian cell cultures [Schneider et al. (1996)], which could be an explanation for the rapid cell death in the second subcultivation. However, for detailed investigations of metabolite consumption and cell growth during the subcultivation of mechanically stressed cells, a higher sampling frequency has to be realized. Additionally, the cultivation procedure should to be further standardized for better comparison.

5 Summary

In a previous study realized at the Zurich University of Applied Sciences (ZHAW), first experiments with the bearingless Levitronix BPS-200 pump and two peristaltic pumps were performed to investigate the hydrodynamic stress caused on transfected CHO suspension cells (XM111-10). The speed of the centrifugal-type pump was varied in a range between 1500 rpm and 5000 rpm at a constant flow rate of 3.4 L/min resulting in a defined exposure frequency of one per minute to high hydrodynamic stress. A method for evaluation of shear stress based on the decay of the viable cell density was established. Thus, one outcome of the previous study was the observation that the bearingless magnetically levitated pump Levitronix BPS-200 caused significantly lower cell death than the comparison systems. Furthermore, experiments with the Levitronix MDP-200, which has a modified pump head geometry, were realized. Again, results revealed low mechanical stress caused on CHO suspension cells.

The aim of the present study was to further investigate the hydrodynamic stress caused on CHO suspension cells by another bearingless centrifugal-type pump, the Levitronix BPS-600. This bearingless pump is designed to achieve higher flow rates of up to 4,500 L/h. The experimental setup installed in the previous study was applied. The three pumps are connected to cell reservoirs consisting of stainless steel vessel with double jackets for temperature control. The complete pump cycles including the pump heads were steam-sterilized prior the experiment to guarantee sterile conditions. The cell suspension was pumped at a constant flow rate of 3.4 L/min and various pressure situations were realized by adjusting different pump speeds.

The present study confirmed former results obtained with the Levitronix BPS-200 pump and revealed significantly lower hydrodynamic stress caused on the CHO suspension cells (CHO XM111-10) in comparison to the 4-piston diaphragm pump Quattroflow SU-1200. Depending on the rotational speed, the BPS-600 pumped caused similar hydrodynamic stress as its small-size counterpart BPS-200. Only at the highest pump speed at a pressure of 620 ± 12 mbar (5000 rpm with the BPS-200 and 4100 rpm with the BPS-600) a significantly higher cell death rate was determined with the BPS-600 system. However, even under such high pump speeds the cell death rate was about 30 % lower than in the 4-piston-diaphragm pump.

The results are a promising basis for further investigations. These could include the variation of the flow rate. All existing studies were conducted at a constant flow rate of 3.4 L/min, which led to a hydrodynamic stress exposure frequency of one per minute. However, taken the much higher flow rate into account, which could be realized with the Levitronix pumps, the effects of higher flow rates should be investigated. Besides variation of the pump speed, the exposure frequency could be further adjusted by variation of the cell suspension volume.

6 References

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ZHAW report (2011a). Mechanical stress analysis of transfected CHO suspension cells in magnetically driven pumps. Project report of the second working package.

7 Appendix

Tab. 7: Measurement ranges of the BioProfile NC-100 plus overtaken from [http://www.novabiomedical.com/products/biotech_analyzers/bioprofile_analyzers.php].

Parameter	Test methodology	Measurement range	Imprecision resolution*
Glutamine	Enzyme/Amperometric	0.2 – 6.0 mmol/L	5.0%
Glutamate	Enzyme/Amperometric	0.2 – 6.0 mmol/L	5.0%
Glucose	Enzyme/Amperometric	0.2 – 15 g/L	5.0%
Lactate	Enzyme/Amperometric	0.2 – 5.0 g/L	5.0%
Ammonium	Ion selective electrode	0.2 – 25 mmol/L	5.0%
pH	Ion selective electrode	5.00 – 8.00 pH units	0.01%
Na^+	Ion selective electrode	40 – 220 mmol/L	1.5%
K^+	Ion selective electrode	1.0 – 25.0 mmol/L	3.0%

* All imprecision values are within run

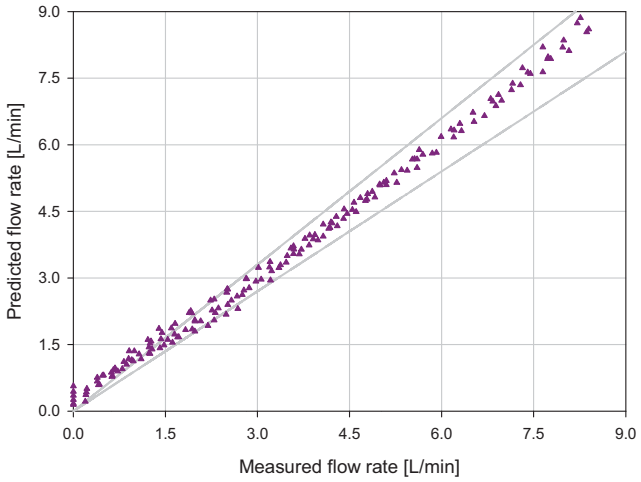

Fig. 19: Comparison of measured and predicted flow rate using statistical model. The lines indicate lower and upper limits of 10 % accuracy-