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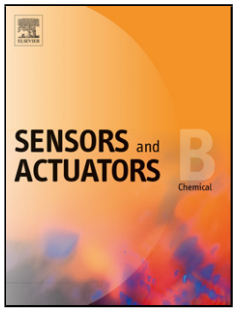

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New Perspectives in Shake Flask pH Control using a 3D-printed Control Unit based on pH online Measurement

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Abstract

Online pH control during microbial shake flask cultivation has not been established due to the lack of a practical combination of an online sensor system and an appropriate control unit. The objective of this investigation was to develop a minimum scale dosage apparatus, namely shake flask controller ("SFC"), which can control the pH during a complete cultivation and serves as technical example for the application of small liquid dispensing lab devices. A well evaluated optical, chemosensor based, noninvasive, multisensory platform prototype for online DO (dissolved oxygen)-, pH- and biomass measurement served as sensor. The SFC was designed as cap-integrated, semi-autarkical control unit. Minimum scale working parts like the commercial mp6 piezoelectric micropumps and miniature solenoid valves were combined with a selective laser sintering (SLS) printed backbone. In general it is intended to extend its application range on the control of enzymatic assays, polymerization processes, cell disruption methods or the precise dispense of special chemicals like inducers or inhibitors. It could be proved that pH control within a range of 0.1 pH units could be maintained at different cultivation conditions. A proportional-integral-derivative- (PID) controller and an adaptive proportional controller were successfully applied to calculate the balancing solution volume. SLS based 3D printing using

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polyamide combined with state-of-the-art micro pumps proved to be perfectly adaptable for minimum size, autoclavable lab devices.

Keywords: 3D-printing, pH-control, pH-monitoring, piezo pump, shake flask

1 Introduction

1.1 pH control in shake flasks

Automated process control in shake flask was little considered in the design of bioprocesses so far. The reason for this status lies in the absence of available, appropriate pumping systems and in a sensor system which is suitable for shake flasks. The pH changes which often occur during shake flask cultivations are (despite the use of buffer systems) sometimes measured and documented by sampling, but rarely controlled until today. There is clearly a great need for a fully automatic pH control in shake flasks since they are still employed in screening processes and also used in pre-culture preparation for larger scale cultivations, e.g. in bioreactors. In batch processes a missing pH control can, depending on substrates and cultivation temperature, result in a big deviation from the optimum condition by accumulation of metabolic by-products, which inhibit growth at acidic conditions [1,2]. Depending on the growth phase (exponential or stationary) of the pre-culture, the pH shift after transfer into fresh medium (inoculation) can be serious. Moreover pH dependent activation of certain genes which is related to a change in metabolism can negatively interfere with protein or compound production processes [3–5]. Another concern is the correct folding and stability of heterologously produced protein, especially if the protein is secreted into the medium [6]. Alternative solutions for the stabilization of pH in shake flasks are the application of more concentrated buffer solutions or buffer loaded polymer slices [7]. However, a higher buffer concentration may hinder cell growth, reduces cell viability in the early growth phase and influences metabolism [8,9]. Automatic pH control has been obligatory for stirred bioreactors since control units were available. In contrast, a passive pH control in shake flaks is only established in mammalian cell culture via diffusion of CO_2 through a membrane located in the cap. For microbial cultivations online pH measurement and control is not typically applied at this scale. Weuster-Botz *et al.* showed in 2001 that continuous feeding combined with pH control in shake flask can increase biomass yield of *E. coli* BL21 by 104 % compared to an uncontrolled batch. The system was composed of a syringe pump as well as pinch valves, tubing and pH probes for each flask [2]. Unfortunately, large, complex atline pumping systems did not proved to be adequate for an easy and fast controller setup. There has been also concepts to control pH by passive release of sodium carbonate from silicone rubber disks in microbial systems [10] or hydrogel in mammalian systems [11]. It was possible to keep pH values within a range of ± 0.3 pH units

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around the pH optimum during growth of *E. coli* BL21 in glucose, glycerol rich synthetic medium; in this way the media buffer content could be reduced from 0.2 M to 0.1 M [10]. Concerning mammalian culture the pH could be kept ± 0.2 pH units around the optimum for 7 days. However buffer loaded solids cannot precisely maintain a specific pH or adjust user defined pH gradients. A complete omission of additional buffer substances is not achieved. Up to now there is no commercial available product to perform online pH control in shake flask. Cultivations using shake flask are still far away from serving as a template or scale-down model for bigger bioreactor in terms of transferability. Therefore an active controller is still required.

1.2 Micropump micro-actuators for small lab devices

The first idea to apply conventional pumping systems like benchtop peristaltic- or syringe pumps to several shake flasks connected by tubing and a multiport valve is complicated in different ways and has a considerable space demand. Also important to consider is the way the correction solutions (base and/or acid solutions) reach the culture. Drops usually tend to be centrifuged against the flask wall and reach the culture delayed in time. Excessive use of pipes towards the liquid is not an option in terms of weight reduction and miniaturization. So solutions have to be dispensed as a jet. Focusing on dosage or injection purpose for volumes < 20 µL, pumps are needed which can pump at relatively high flowrates at a fraction of the size in comparison to their bigger counterparts. Minimum size peristaltic pumps which are based on tubing do not achieve these high flowrates as required to perform a jet injection. Micropumps like piezoelectric actuation pumps (more precisely "reciprocating displacement" pumps) cover all these properties and represent promising candidates [12,13]. They comprise even more advantages like less metal parts and lower energy consumption. The most successful design by Van Lintel *et al.* in 1988 is based on an electrical deformable piezoelectric disk attached to a thin diaphragm which seals a pumping chamber [14]. Since 2003 micropumps are available as commercial standard with different manufacturers launching products on the market. Today coin size micropumps (profile < 2.5 cm^2) are available up to 40 mL/min [15] and provide a net flow up to a backpressure maximum of 45 kPa [16].

1.3 3D printing technology for lab devices

3D printing has advanced to a common applied method in rapid prototyping in the last 10 years. Additive manufacturing exhibits major advantages compared to subtractive computerized numerical control (CNC) machining or injection molding. The main advantage is a very fast, tool free, implementation of complex components from a virtual model to the finished device. A fast reproduction and an easy modification of an existing device by reprinting accelerates the prototyping progress [17]. For the development of the SFC it was important to have the option

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to replace tubing by material integrated channels, because functional parts like valves cannot always be connected with tubing by standard, but have to be mounted. Another preference is the operators' safety. This implies that the tanks filled with acid or base have to be made as one peace (no gluing) which is possible with SLS in contrast to CNC machining [18,19]. Among different printing methods SLS reveals to be most suitable due to the good thermal, mechanical and chemical stability of compatible polymers like polyamide 12 (PA12) [20–22]. So far there is only little use of 3D printing for "macroscale" labware and devices in chemical and biotechnological laboratories. In 2013 Abe *et al*. showed that PA12, printed via SLS, has an excellent biocompatibility on fibroblast cells [23]. In 2014 Scheper and Beutel *et al.* proved its biocompatibility and autoclavability for labware in use. Well plates and shake flask caps were printed and tested successfully with yeast cells and mammalian cells [24,25].

2 Materials and Methods

2.1 Cultivation Methods and chemicals

The used microorganisms, cell lines, applied media and cultivation parameters are listed in Table 1. All media were prepared with deionized water produced by Arium® 661 Ultrapure water system (Sartorius Stedim Biotech AG, Göttingen, Germany).

2.1.1 Media preparation

Lysogeny broth (LB) unbuffered for *E. coli* K12: 5.0 $g \cdot L^{-1}$ yeast extract (AppliChem), 10.0 $g \cdot L^{-1}$ trypton/peptone from casein (Carl-Roth), 6.0 $g \cdot L^{-1}$ NaCl (Sigma-Aldrich), 10.0/20.0 $g \cdot L^{-1}$ glucose (Sigma-Aldrich). LB buffered for *E. coli* K12: 5.0 $g \cdot L^{-1}$ yeast extract (AppliChem), 10.0 $g \cdot L^{-1}$ trypton/peptone from casein (Carl-Roth), 5.0 $g \cdot L^{-1}$ K_2HPO_4 (Fluka), 3.0 $g \cdot L^{-1}$ KH_2PO_4 (Fluka), 1.0 $g \cdot L^{-1}$ NaCl (Sigma-Aldrich), 10.0/20.0 $g \cdot L^{-1}$ glucose (Sigma-Aldrich). ZYP-31 (F.W. Studier *et al.*, modified) for *E. coli* K12: 6.8 $g \cdot L^{-1}$ KH_2PO_4 (Fluka), 17.9 $g \cdot L^{-1}$ $Na_2HPO_4\,12 \cdot H_2O$ (Riedel de Haën), 3.3 $g \cdot L^{-1}$ $(NH_4)_2SO_4$ (Carl-Roth), 2.0 $g \cdot L^{-1}$ yeast nitrogen base (YNB) salts with amino acids (Y1250 Sigma), 0.06 $g \cdot L^{-1}$ protocatechuic acid (Fluka), 10 $g \cdot L^{-1}$ Glycerol (Rotipuran®, Carl-Roth). De Man, Rogosa, Sharpe (MRS) medium was prepared according to Ude *et al.* 2014 [26]. 200 $\mu l \cdot L^{-1}$ of TEGO® Antifoam KS 911 were added to each medium.

Table 1. Cultivation parameters used for the SFC evaluation.

Species	T. [°C]	Agitation [rpm]	Flask Type [mL]	Medium	Volume [mL]	Preculture [h]
E.coli K12	37	170	500 WB	LB, ZYP-31	100	15

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2.1.2 Equipment and pH measurement

The multisensory platform hardware specifications are according to Ude, Schmidt-Hager *et al.* [26,27]. X–ray sterilized, disposable shake flask (Corning® Inc., Corning, USA) equipped with DO- and pH sensor spots (PreSens GmbH, Regensburg, Germany) were used. The flaks were baffled (WB) or non-baffled (NB). The batch numbers are as follows:

1) SFS–HP5–PSt3–500–WB–VEC–v3 ID 13–06–01. (cultivations)

2) SFS–HP5–PSt3–500–NB–VEC–v3 ID 12–22–01. (controller performance tests)

The default sensor spot calibrations were:

1) Phase 0% air sat [°] 57.42, Phase 100% air sat [°] 24.18, Temp 0 [°C] 36.9, Temp 100 [°C] 36.9, P [mbar] 975.00, pH_{max} 26.85, pH_{min} 55.11, pH_{Temp} 36.90, dpH 0.6, pH_0 6.71.

2) Phase 0% air sat [°] 58.09, Phase 100% air sat [°] 24.65, Temp 0 [°C] 36.5, Temp 100 [°C] 36.5, P [mbar] 967.00, pH_{max} 21.43, pH_{min} 55.69, pH_{Temp} 36.5, dpH 0.59, pH_0 6.93.

Shaking was performed on an orbital shaker with 25 mm shaking diameter (Certomat® SII, Sartorius Stedim Biotech AG, Göttingen, Germany).

2.1.3 Correction solutions

Sodium hydroxide (Riedel de Haën) was dissolved in deionized water to produce 2 M or 3 M solutions. 37 % hydrochloric acid (Merck) was diluted with deionized water to receive 2 M or 3 M solutions. 200 µL·L^{-1} TEGO® Antifoam KS 911 were added to each solution. The solutions were filtered with 0.45 µm sterile filters (Wicom GmbH, Heppenheim, Germany).

2.2 Cell density measurement

The OD_{600} was calculated corresponding to Equation 1 [28]. A detailed description of the biomass sensor calibration process is given in a previous report [26]. The OD_{600} was measured in comparable cultivations beforehand with Libra S11 visible spectrophotometer (Biochrom Ltd., Cambridge, UK). The growth rate of microorganisms was calculated with Equation 2 and is described by a logarithmic gradient triangle [29]. The parameter *t* refers to the time span in which exponential growth takes place. The manual, offline OD_{600} measurement was replaced by the calculation of OD_{600} from the online biomass sensor amplitude since during application of the SFC no offline samples were taken. The parameters for different media are given in Table 2.

$$OD_{600}(amplitude, \alpha, \beta, c) = (\alpha + \beta \cdot amplitude)^{\frac{1}{c}} \quad (1)$$

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Table 2. Biomass sensor calibration parameters.

Species	LB α/ β/ c	ZYP-31 α/ β/ c
E. coli K12	-7.518 / 0.000398/ -1.896	-4,241/ 0.000275 / -1,693

$$\mu_{t1-t2} = \frac{\mathrm{Ln}(Amplitude_{t2}) - \mathrm{Ln}(Amplitude_{t1})}{t_2 - t_1} \quad (2)$$

2.3 3D modeling and printing

The SFC was modeled as 4 different parts using the software "Autodesk Inventor Professional 2013" (Autodesk, Inc., San Rafael, USA). The prototype parts were printed by Blue Production GmbH (Friedrich-List-Straße 49, Paderborn, Germany). PA12 (Eosint PA2200) (EOS GmbH, München, Germany) was used as polymer for SLS. All parts were printed in one powder bed (same batch). More information on the printing parameters is given as follows: Grain size of the powder (MEAN: 60 µm), layer width (100 µm), powder bed temperature (173°C), laser energy input (40 mJ/mm^2), printing speed (laser speed) (2000 mm/s). Channels inside printed parts were cleaned with pressurized air and smooth steel wire to remove all residual powder. The docking faces of the valve-mounts were abraded with 400 grit silicon carbide sandpaper to sustain a good sealing with the valves.

2.4 Controller algorithms

For the calculation of acid and base volumes 2 different types of controller were tested: 1. Adaptive proportional controller (Equation 3), 2. PID controller which is explained in supplementary section 1.3 and Equation S1. A specific software was written in C-sharp .NET 4.5 which made parameterization and online calculation of the dosage-volumes possible. For both controllers 2 basic parameters were defined: Set point [pH units] and waiting time [s]. The waiting time corresponds to the minimum time needed for the chemical equilibrium between pH sensor and the medium. Controller 1 is described by a linear correlation between the pH shift (ΔpH) after addition of pH correction solution and the added volume (Equation 3). Parameter *a* serves as gradient and parameter *b* as intercept. The equation was integrated into an adaptive controller algorithm by continuous recalibration of the parameter *a* while *b* remains constant by definition. During process a decrease of *a* leads to a bigger dosage at the same ΔpH. For further tuning 4 additional parameters were introduced: 1. Hysteresis [pH units], 2. Waiting time for the correlation between dosage and pH shift, 3. Minimum number of data points for the calibration,

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4. Maximum "age" of the calibration points. Especially parameter 4 provides the controller with a kind of big or small memory. Nonsensical correlations (e.g. base dosage is followed by a pH decrease) can be excluded from the calibration. Optional minimum and maximum values can be assigned for a, b and the dosage. A function for setting programmable pH gradients was integrated in the software.

$$dosage\ volume_i\ [\mu L] = \frac{\Delta pH_i - b}{a_i} \quad (3)$$

2.5 Controller design

2.5.1 Working principle

The SFC working principle is based on a tank (11.5 mL) connected to a valve and a piezoelectric micropump (mp6, Bartels Mikrotechnik GmbH, Dortmund, Germany), which is pumping the liquid through a nozzle into the shake flask (Figure 1A). A 2-way normally closed solenoid valve (LFNA 1250, LEE Hydraulische Miniaturkomponenten GmbH, Sulzbach, Germany) connecting pump and tank prevents any leakage of the pump in standby mode. The unit is divided in an autoclavable and non-autoclavable part (electronics and actuators) which is sterilized by sterilization in place (SIP) (section 2.7). They are separated by a flange/docking connection. In total the SFC comprises 2 working units (1x acid, 1x base) in which the valves have specific gaskets.

2.5.2 Hardware implementation

A detailed technical view of the SFC is illustrated in Figure 2A. The SFC is composed of 3 stages. Stage 1 is a modified screwable cap with a 0.2 µm fiber-reinforced polytetrafluoroethylene (PTFE) gas permeable membrane (Sartorius Stedim Biotech AG, Göttingen, Germany). Gas inlets sustain a constant gas exchange. Next to the membrane, 2 concave flanged 1/8'' PTFE capillaries (VWR International, Radnor, USA) are inserted into pipes and end with nozzles (not shown). Micro PTFE capillaries (0.82 mm outside, 0.35 mm inside, 6 mm long, Reichelt Chemie Technik, Heidelberg, Germany) served as nozzles to produce a thin jet, which enables a direct dosage into the culture (Figure 1B). Stage 2 contains two pumps located between two reservoirs. The reservoirs are equipped with a collection cone connected to the pumps inlets by tygon® tubing (3.0 mm outside, 1.0 mm inside, Saint-Gobain S.A., Courbevoie, France). The pumps outlets lead to docking connectors equipped with convex flanged 1/8'' PTFE capillaries. Stage 3 physically forms one part with stage 2 (Figure 2B). It contains refill ports for the tanks, pressure balances featuring polyethylene frits (25 µm,

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Omnifit, Diba Industries, Cambridge, UK) and one valve for each correction solution. The valves are mounted to blocks with integrated channels connecting pumps and tanks. The top part is composed of two circuit boards. It contains a processor (Propeller P8X32A-M44, Parallax Inc., Rocklin, USA), two mp6-OEM controller units and relays. The SFC is connected via RS-485-USB converter to a PC by cable which also contains a 12 V power supply. The different stages are kept together by a cap nut. Luer connectors were added to enable refill using disposable syringes and enabling manual pump flushing. The tare weight of the whole SFC is 111.33 g, while the loaded weight is about 132 g.

2.6 Controller calibration procedure

The controller was simply calibrated by precision scale with hydrochloric acid and NaOH solution. During service the controller is converting a volume command [µl] into the pulse count of the mp6 piezo elements. The calibration of the dosage volume against the pulse count was determined linear (supplementary section 1.5).

2.7 Controller sterilization procedure

Stage 1 was autoclaved at 121 °C and 200 kPa for 30 min mounted on the flask. The tubing of Stage 2 and 3 are self-sterilizing by filling the tanks and tubing with 2 or 3 M hydrochloric acid or sodium hydroxide solution. The underside of stage 2 (docking connectors) was sterilized in place using a UV-C station (supplementary section 1.6). The irradiation was carried out for 30 min. The sterility of the SFC was tested through a simple dosage routine with a LB medium filled 500 WB shake flask (supplementary section 1.7).

2.8 Controller performance tests

In order to test the performance of the SFC using an adaptive P controller or a PID controller algorithm, defined volumes of acid and base were dispensed into medium by the SFC (pulse perturbation). The time and the amount of correction solution required to balance the perturbation, hence readjusting the setpoint, was investigated. Two different complex media were used: LB (pH 7.0, unbuffered), MRS (pH 6.2, moderate buffered). The LB dosage program was: 2x 25 µL acid, 2x 50 µL acid, 2x 25 µL base, 2x 50 µL base. The MRS dosage program was: 2x 200 µL acid, 2x 400 µL acid, 2x 200 µL base, 2x 400 µL base. 500 WB shake flasks with 100 mL vol. were shaken at 150 rpm during dosage. The start parameters are listed in Table 3. The start parameters *a, b* of the adaptive P controller depend on the used medium and could be easily determined by prior titration experiments in LB and MRS. For this purpose variable amounts of acid and base (25-70 µL, 5 µL step) were added to 100 mL of medium and the pH shift was measured via pH sensor. The parameter *a* and *b* were determined by the linear

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regression of pH shift against volume. K_R, T_N, T_V of the PID controller were optimized empirical in LB medium step by step.

Table 3: Adaptive P controller parameters implemented for the performance test in typical microbial media.

parameter	LB medium	MRS medium
set point pH	7.0	6.2
hysteresis ±pH	0.05	0.05
waiting time [s]	90	90
actuation range [µL]	5-300	5-500
calib. intercept y (*b*)	0	0
calib. gradient (*a*)	0.0030	0.00040
waiting time correlation [s]	85	85
minimum data points	1	1
maximum age [s]	3600	3600
minimum gradient (*a*)	0,000050	0,000010
exclude nonsensical correlations	true	true

Table 4: PID controller parameters implemented for the performance test.

parameter	LB medium	MRS medium
set point pH	7.0	6.2
hysteresis ±pH	0	0
waiting time [s]	90	90
actuation range [µL]	5-300	5-500
K_R	120	120
T_N	300	300
T_V	40	40

2.9 pH control during microbial cultivation

The SFC was evaluated under realistic conditions by pH control during growth of *E. coli* K12 in LB medium with 10 $g \cdot L^{-1}$ / 20 $g \cdot L^{-1}$ glucose as carbon source and ZYP-31 medium with 10 $g \cdot L^{-1}$ glycerol. The first type of cultivation (LB) is a typical scenario at which *E. coli* K12 is excessive acidifying the low buffered medium resulting in a pH decrease of 1 pH unit or more, depending on the glucose concentration. In the second scenario it was tested whether the control in highly buffered medium is effective enough. Cultivation parameters can be found in

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section 2.1. The adaptive P controller start parameters are listed in Table 5. The PID controller parameters are listed in supplementary section 1.8, Table S1.

Table 5: Adaptive controller- and medium parameters implemented for the microbial cultivation.

parameter	LB medium	ZYP-31 medium
glucose [$g \cdot L^{-1}$]	10, 20	-
glycerol [$g \cdot L^{-1}$]	-	10
set point pH	7.5	7.5
hysteresis ±pH	0.05	0.05
waiting time [s]	90	90
actuation range [µL]	10-300	5-300
calib. intercept y (*b*)	0.02	0
calib. gradient (*a*)	0.0020	0.00015
waiting time correlation [s]	70	85
minimum datapoints	5	5
maximum age [s]	3600	3600
minimum gradient (*a*)	0	0,00005
exclude nonsensical correlations	true	true

3 Results and Discussion

3.1 Polymer material tests

SLS and stereolithography (SL) polymer materials intended to use for the SFC backbone were tested concerning their ability to withstand autoclaving process and exposure to strong mineral acid and base (supplementary section 1.2). PA12 (PA 2000i, PA 1800) proved to be appropriate for heat sterilization and inert towards 5 M NaOH solution as well as 10 M hydrochloric acid (supplementary Figure S1 and S2). The tested SL resins were not suitable in terms of both criteria. Regarding the mechanical integrity of the lower part of the SFC (Figure 2A) no changes were observed after 30 autoclaving cycles.

3.2 Construction and prototyping progress

Before the SLS printed prototype shown in this manuscript was used a CNC machined pre-prototype made of polyvinylchloride (PVC) and polyether ether ketone (PEEK) was used to

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evaluate the actuators. During the early experiments it was found out that the mp6 pumps were not completely sealing during standby. The leakage was approx. 1 ml · 14 h^{-1} using water. Different custom made passive valves were tested to enable flow in presence of a sufficient liquid pressure (pumping) and close at low liquid pressure (standby). The working principle of these valves is shown in supplementary Figure S11. Unfortunately the force of the micropumps was not sufficient to compress the spring just by the produced flow and provide sealing at the same time. In the next step active solenoid valves were tested successfully and integrated into the final system (section 2.5). Since it was not possible to produce hose nozzles for small tubing (1 mm diameter inside) by SLS, 5 mm pieces of PEEK capillary were attached to these parts. Tygon® tubing stubs were put into the holes (1.5 mm deep) and the capillary stubs were plunged in. Other methods like gluing resulted in leakage after a certain time. This method was applied to the valve mounts and the tanks. The electrical parts on the circuit board showed to suffer from HCl fumes which were caused by the aforementioned leakage. The circuit board was preventively coated by a thin layer of solder lacquer. Concerning flow performance there was no loss observed after shifting from the CNC machined pre-prototype to the SLS printed prototype.

3.3 Adaptive P controller performance test

In LB medium the adaptive P controller algorithm was able to restore the setpoint (7.00 ± pH 0.02) after a pulse perturbation (by manual dosage of the SFC) of 0.20 pH units within a short time span of approx. 360 s (

Figure 3). In total a release of 256 µL 3 M HCl and 290 µL 3 M NaOH solution was recorded by the software for balancing. The observed difference of 34 µL could be caused by small amount of droplets which sticked to the nozzle after each dosage. In the case of MRS medium a higher buffer concentration led to a much higher dosage volume but not to a lower response time (200 s for a deviation of ΔpH 0.2 or 0.3).

3.4 PID controller performance test

The PID controller showed a significant slower response behavior (900 s vs. 360 s for balancing) compared to the adaptive P controller (

Figure 4). One explanation could be given by a non-optimal parametrization of the PID controller. Another more obvious explanation lies in the algorithm structure; Different from P controllers the PID controller could not be equipped with a pH hysteresis, i.e. the PID must reach the exact setpoint. While the pH can be measured by electrodes within seconds a pH sensor spots needs approx. 60 s for a stable value. Under these conditions a P controller is more advantageous due to its ambition to balance deviation with one actuation instead of approaching

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to the setpoint. By using the same controller parameters for MRS medium it was not possible to balance the perturbations in a realistic period of time. The biggest differences between both algorithms persisted in the approx. 2.5 times higher response time of the PID controller. It also tended to need more time for bigger deviations while the dosage time was independent from the dosage volume when using the adaptive P controller. It could also be confirmed that an adaptive controller is more robust against changes concerning the medium or the operation point as shown previously [30]. A disadvantage using P controller can exist in overshooting which might occur using poor start parameters for the calibration. Since hysteresis and adaptive recalibration was provided for the P controller this effect could be minimized.

3.5 Controller algorithm implementation progress

Apart from the controller algorithms described in section 2.4 a simple 3 point controller and a P controller without adaptive behavior was tested beforehand. The 3 point controller using a constant dosage showed to be able to control the pH during cultivation according to the given setpoint (supplementary Figure S9). However, the dosage had to be adjusted very specifically. High dosages (50 µL) led to overshooting at the beginning of the cultivation, while small dosages were not able to control the pH during the exponential growth phase. So the 3 point controller was replaced by a P controller. This controller was able to manage big and small deviations from the setpoint just as well but could not control the pH during the exponential growth phase (supplementary Figure S10), since the buffering properties of the liquid were changing over time. This led to the continuous recalibration of parameter *a* by the control software which will be described in section 3.7. Using this adaptive P controller it was possible to manage the changing conditions during cultivation which is presented in the following section.

3.6 pH control during microbial cultivation

The SFC was evaluated with *E. coli* K12 which is the most frequently used microorganism among biotechnical cultivations. It was cultivated in buffered LB medium without active pH control (Figure 5) and unbuffered LB Medium controlled by the SFC in combination with an adaptive P controller (Figure 6). In low buffered LB medium with 10 $g \cdot L^{-1}$ glucose the pH decreased by 1.2 pH units within 8 h cultivation resulting in a pH 6.2. This behavior was reported before [7,31]. The monitoring of DO gives evidence when the exponential growth phase started and stopped. While the observed growth rate (µ) of 0,468 h^{-1} within the first hour of oxygen limitation is common for shake flask cultivations, the continuous decrease towards 0.09 h^{-1} and less indicates the crucial influence of pH for optimal growth. 8 hours after inoculation at an OD_{600} of 3.55 the increase of DO clearly marked the stationary phase of the

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cultivation. In contrast, the pH could be kept in a range of at least 0.15 pH units around the setpoint using the SFC (Figure 6) which is even more precise compared to 0.2 reported in the literatures [2] (stationary control system) and [32] (BioLector®, microtiterplate). A growth rate of at least 0.2 h^{-1} was maintained over the whole cultivation time. A significant higher optical density of OD_{600} 4.95 was reached 8 hours after inoculation (+ 39 %) which was also reported in [7]. The end of the glucose based growth phase could be detected by the stop of the highly frequent base dispensing and a stopped increase of OD_{600} signal. After an adaption phase the culture switched to basification of the medium due to amino acid degradation [33]. This could be prevented by the control as well. Nevertheless the usage of 2 M base in this experiment was predominant and all available NaOH solution was depleted. That gave a hint of the requirement of even more concentrated correction solution. In another experiment a glucose concentration of 20 $g \cdot L^{-1}$ was used in combination with 3 M correction solution. Substrate inhibition occurred which led to a lower growth rate. Though, a higher final OD_{600} of 5.56 was reached. Like demonstrated before, the pH could be kept in a range of 0.1-0.15 pH units (Figure 7) while the NaOH solution was depleted up to 40%(v/v) of the total filling. The real capacity of the control system strongly depends on the organism, medium, substrate, setpoint and the molarity of the correction solution (with can be set from 0 up to 3 M). Concentrations > 3 M led to a significant performance loss of the micropumps and were not used subsequently. The experiments so far demonstrated that a tank capacity of 11.5 mL in combination with 3 M correction solutions and a culture volume of 100 mL is sufficient to control cultivations for at least 24 h. Nevertheless a refill during the process is possible. For the usage of bigger flask and culture volumes an upscaling of the tanks is possible as well. Concerning the mechanical stability no fluctuating of the flask with the SFC mounted on top was observed during shaking at 170 rpm.

A PID controller was applied in addition showing a very precise pH control in a range of at least 0.14 pH units (supplementary Figure S8) which is comparable to the P controller, as well as similar growth rates observed. The metabolic shift (glucose depletion) was determined after 8 h at an OD_{600} of 4.34 which further increased up to 5.49 after 19 h. In general the controller could balance the pH at range of 0.05 pH units (5x maximal pH measurement resolution). The uncontrolled cultivation performed in strong buffered ZYP-31 medium using 10 $g \cdot L^{-1}$ glycerol as substrate generally results in a pH decrease from pH 7.5 to pH 6.6-6.5. In this case pH control within a range of 0.1 pH units could be achieved with a very low dosage count (8 times) during acidification (Figure 8). In total only 2.29 mL base were used for acidification balance and 3.30 mL acid within 23 h showing that the SFC is also compatible for strong buffered defined media.

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3.7 Controller behavior and controlling parameter trend during cultivation

During the cultivation shown in Figure 6 the adaptive P controller dispensed 146 times 2 M base (MEAN 82 µL) at a concentration of 10 $g \cdot L^{-1}$ glucose in LB medium within 8 h. The correlation between dosage volume and pH shift was assumed to be linear (Equation 3, section 2.4). The adaptive part is given by the gradient *a*, which adapted within the first 4 dosages to a value of 0.0007 during the lag phase (Figure 9). In the following growth phases it could be observed that the parameter was adapting continuously, especially during the exponential growth phase (maximum glucose metabolization) between 1.5 and 7.5 h after inoculation. Dosages ranged between 50 and 130 µL at which they were increasing towards the end of the exponential growth phase just as the dosage frequency. The explanation of the slight increase of dosage can be found in the increasing culture volume (caused by the addition of correction solutions) and the release of acetate into the medium. The higher gradient *a* in the stationary growth phase might be due to an asymmetric, direction depended pH shift caused by dosage, since the controller currently uses the same function and parameters for acid and base.

In comparison with a PID controller (supplementary section 1.8) it was found that both controller algorithms worked similar in view of pH control in a definable range around the setpoint. The main differences were found in the actuation frequency during process. In the case of the cultivation type shown in Figure 6 the PID controller dispensed 266 times 3 M base (MEAN 16 µL) in within 8 h. Under consideration of the higher molarity the PID controller acted more economical in terms of correction solution usage, but accepts a higher mechanical load. A less frequent actuation could be achieved by changing the actuation range from 4-300 µl to 10-300 µl. In the presented case this method was not applied to the PID in order to prevent strong oscillation effects as reported in [30].

4 Conclusions

In these investigations it was shown that precise, online pH control in shake flask by a cap integrated 3D printed control unit is possible avoiding the use of high buffer concentrations or buffer loaded polymers. Material tests showed a fully long term autoclavability of printed PA12 and compatibility against concentrated hydrochloric acid and NaOH solution. Also commercial available micropumps and valves proved to be resistant against these liquids. For fast response control an adaptive and simple adjustable P controller proved to be more adequate compared to a PID controller. During cultivation of *E. coli* K12 in 500 mL shake flasks the pH could be controlled in a very narrow range independent of the buffer capacity. Controlled cultivations exhibit a significant higher growth rate when the substrate dependent pH shift led to growth inhibition. The process using the PID controller was proved to work most economical in terms

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of correction solution usage. Some limitations of the used technologies are apparent in the rough material surface which may cause cleaning and biosafety issues.

As an outlook, the autoclavability of all fluidic actuators would simplify sterilization process. Another goal is to replace up to 100 % of the tubing by material integrated channels. Regarding different applications the control unit enables the setup of individual pH gradients which are necessary for certain production processes or pH dependent induction of protein production [34]. The autoclavability of all fluidic actuators would also enable the dosage of non-self-sterilizing solutions (feeds, inducer, dyes, growth factors, inhibitors, trace elements etc.). The roughness of the SLS printed parts can be reduced to a certain degree by optimization of the printing parameters (laser power, scan spacing, hatch length, bed temperature, layer thickness) [35,36]. Especially the first two parameters are crucial. Optionally exterior surfaces could be completely sealed with coatings based on epoxy adhesives [37]. Material alternatives to be evaluated are given by new heat stable SLA polymers which are commercially available on the market (e.g. Accura® PEAK™, VisiJet® SL HiTemp). Further non cell based applications consist in the regulation of enzymatic reactions by titration or dispensing inhibitors at a certain pH. Finally, the successful miniaturization supports the concept of light-weight, functional labware. It is very likely that intelligent, compact, portable, flexible and easy adaptable control units like the SFC will be established for bioprocess engineering encouraged by advances in rapid prototyping, MEMS and disposable sensor technology in this decade.

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6 Figure captions

Figure 1: Functional diagram of one dosage working unit (A). Two working units are integrated for acid and base respectively. The nozzles are autoclavable while the rest was treated by sterilization in place (SIP). A jet of correction solution is dispensed by the SFC (B).

Figure 2: Exploded drawing of the SFC shown as CAD model. No capillaries, tubing or electrical connections are shown (A). The SFC is compatible with 500 mL standard corning shake flasks (B). Assembled SFC is mounted on a disposable 500 ml WB shake flask with sensor spots. The flask is placed on the multisensory platform.

Figure 3: Adaptive P controller performance in LB medium.

Figure 4: PID controller performance in LB medium.

Figure 5: Monitoring of pH, DO and OD_{600} during growth of *E. coli* K12 in LB medium with 10 $g \cdot L^{-1}$ glucose.

Figure 6: Application of the SFC on cultivation of *E. coli* K12 in LB medium with 10 $g \cdot L^{-1}$ glucose using an adaptive P controller.

Figure 7: Application of the SFC on cultivation of *E. coli* K12 in LB medium with 20 $g \cdot L^{-1}$ glucose using an adaptive P controller.

Figure 8: Application of the SFC on cultivation of *E. coli* K12 in ZYP-31 medium with 10 $g \cdot L^{-1}$ glycerol using an adaptive P controller.

Figure 9: Continuous recalibration of the adaptive P controller of an *E. coli* K12 cultivation in LB medium with 20 $g \cdot L^{-1}$ glucose.

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Highlights

- We investigated the possibility to perform online pH control in shake flask
- A combination of a SLS printed case and micropumps was tested
- pH measurement was achieved by pH chemosensors "sensorspots"
- pH is controlled very precisely during cultivation for at least 24 hours
- The progressive miniaturization prevents a severe top-heaviness of the system

Christian Ude studied Life Science at the Leibniz University of Hannover, Germany. He graduated with a Master of Science in the working group of Thomas Scheper in 2012. From 2012 to 2015, he continued working in that working group in the context of his PhD thesis. During his thesis he is working on the development of a multisensory platform and an online pH control unit for shake flask cultivations. Additionally, he developed an enzyme based, ceramic capillary reactor system to produce bioactive peptides from various protein sources.

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Patrick Lindner is a member of the scientific staff at the Institute of Technical Chemistry, Leibniz University of Hannover since 2007. He graduated with a diploma degree in chemistry in 2003 and received his PhD in 2006. His main topics of research activities include Bioanalytics, Microscopy, Bioinformatics, Statistics, Multivariate Data Analysis, Artificial Neural Networks and Software Development.

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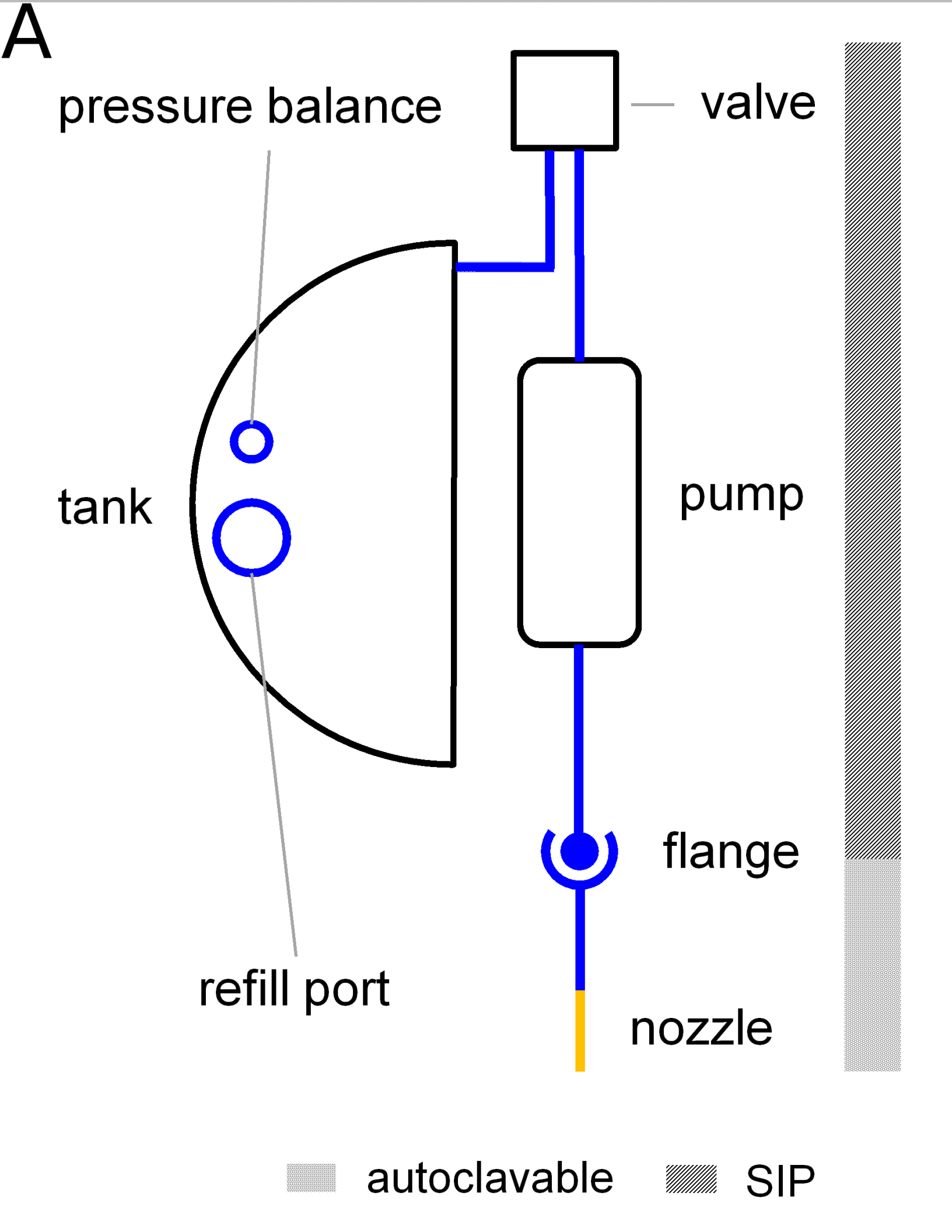
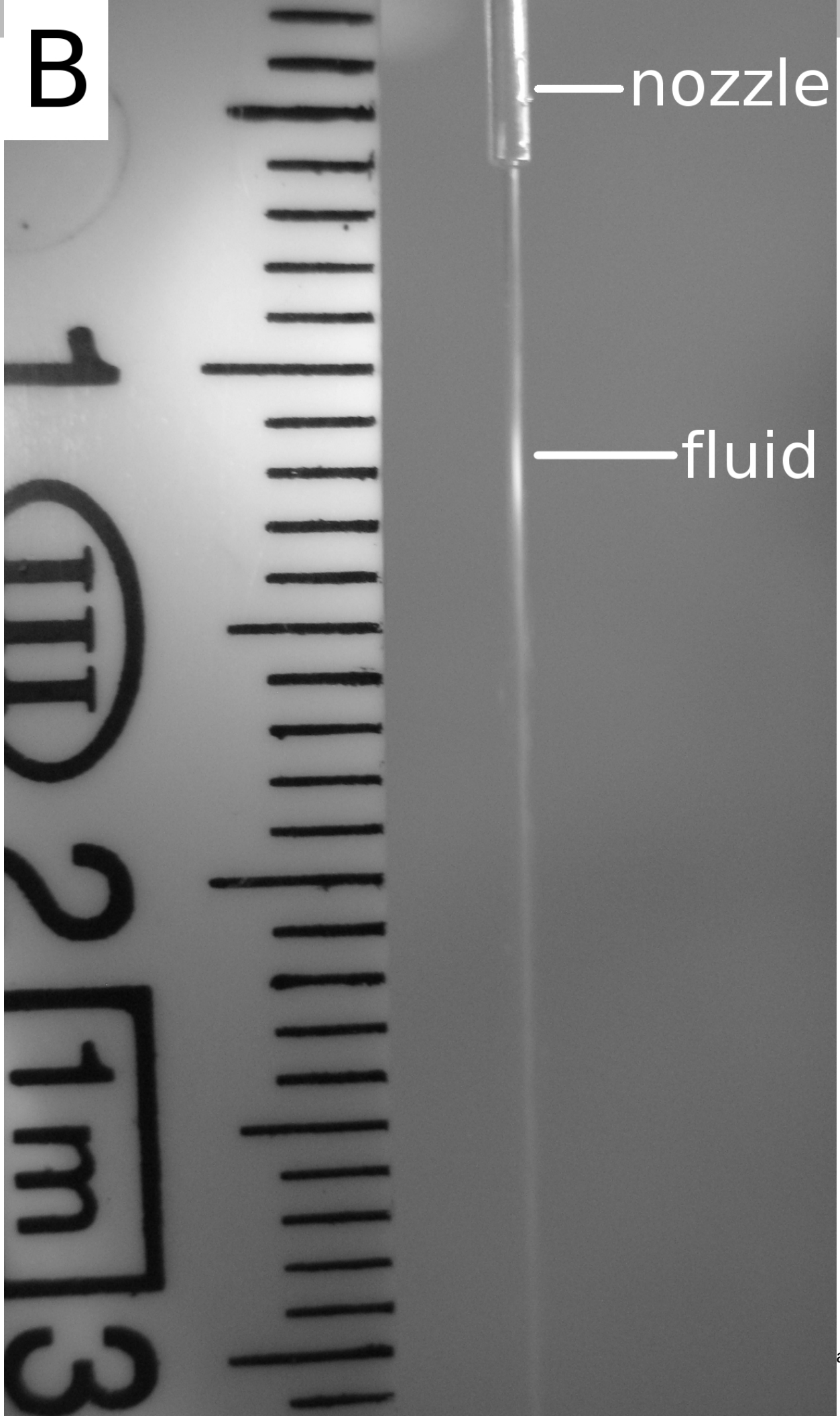

A
pressure balance
valve
tank
pump
flange
refill port
nozzle
autoclavable
SIP

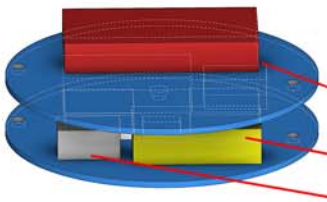

B
nozzle
fluid

A

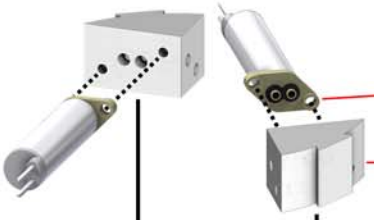
cap nut

stage 3:

valves and electronics:
(physically forms
one part with stage 2)

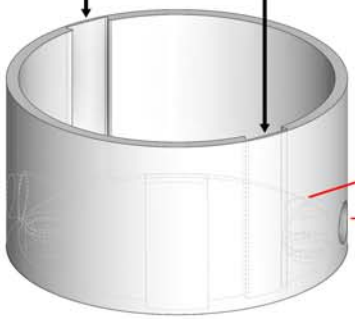


a) sandwich circuit board
- processor
- OEM controllers
for pumps
- relays



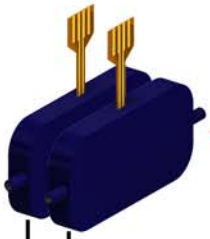
c) miniature valves
LFNA 1250

d) valve mount



e) refill port

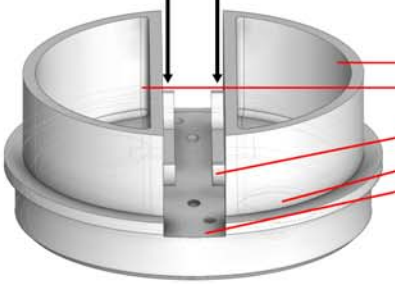
f) pressure balance

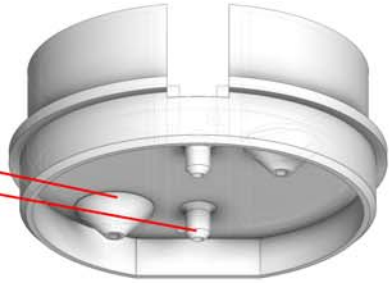


2x mp-6 micropumps

stage 2:

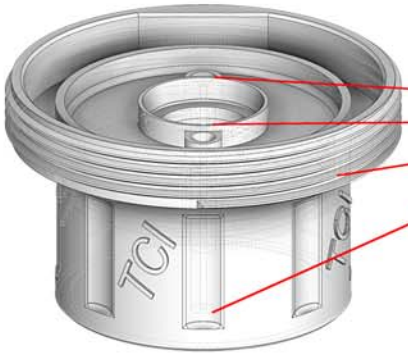
tank module with integrated:
a) tank 11.5 mL (acid)
b) tank 11.5 mL (base)
c) micropump mount
d) collection cone
e) docking connector





stage 1:

cap with integrated:
a) flanges for stage 2
b) fiber-reinforced PTFE membrane
c) gas inlets (under the thread)
d) pipe + nozzle (not printed)



10mm

B

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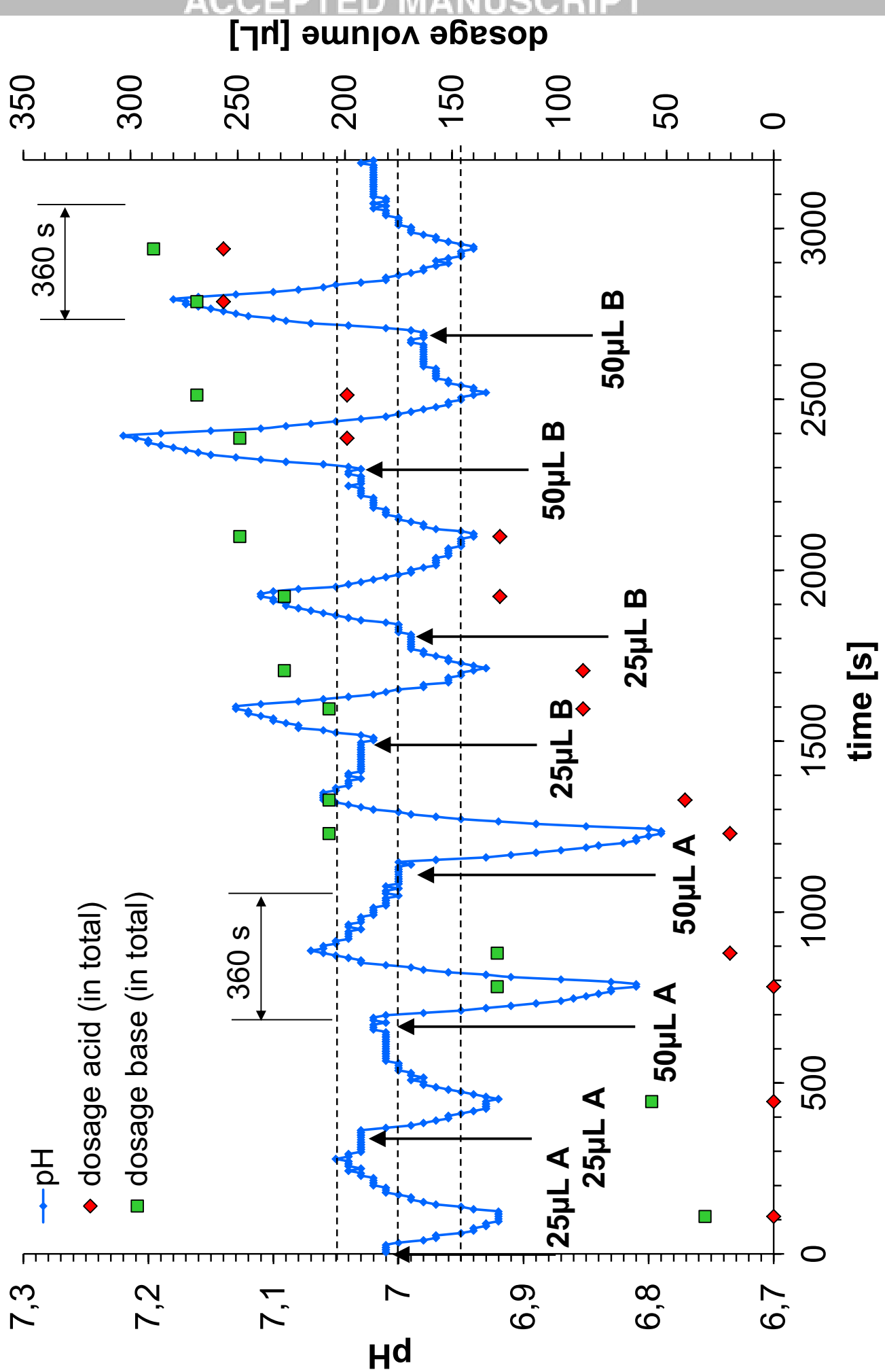

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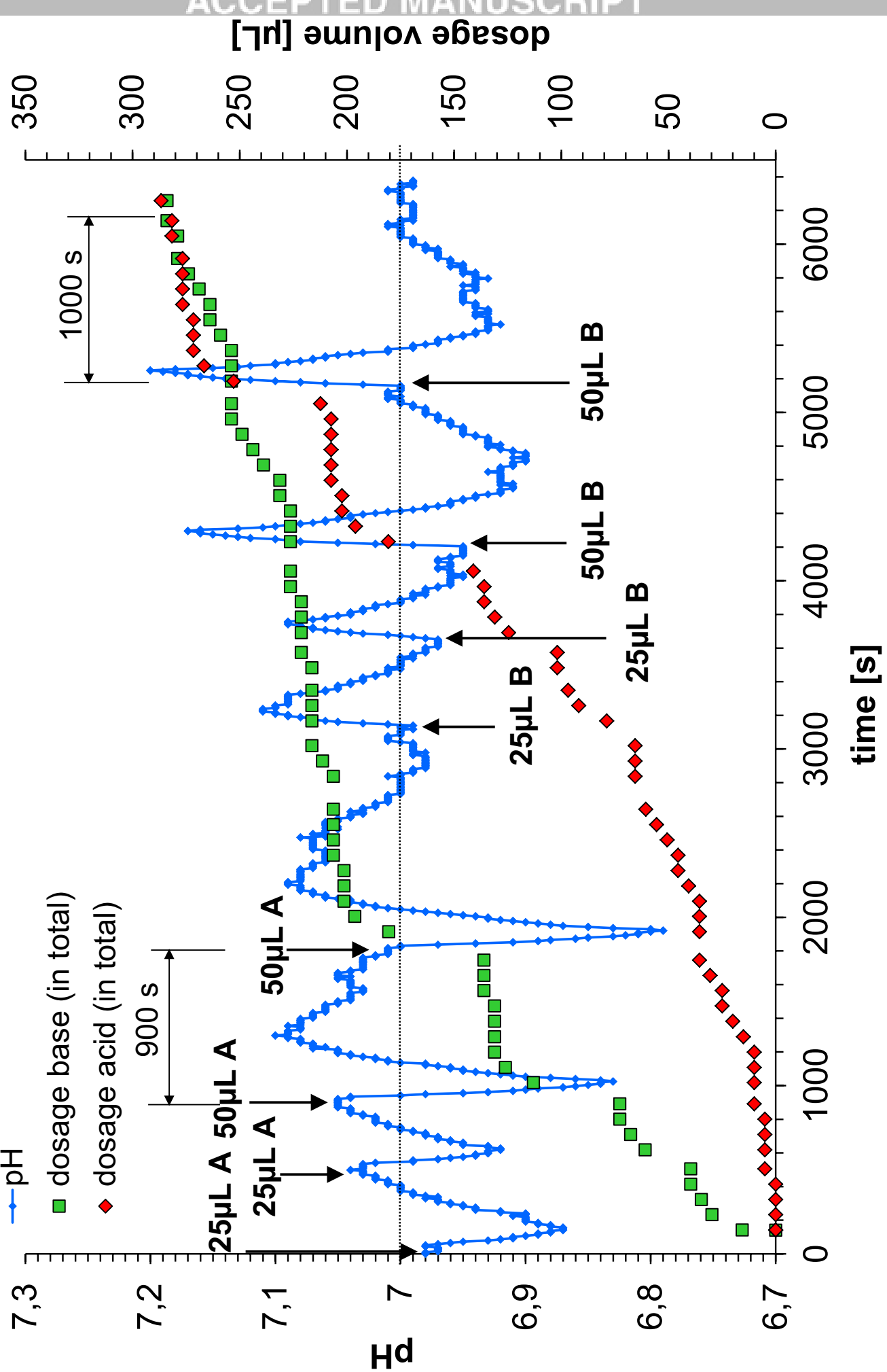

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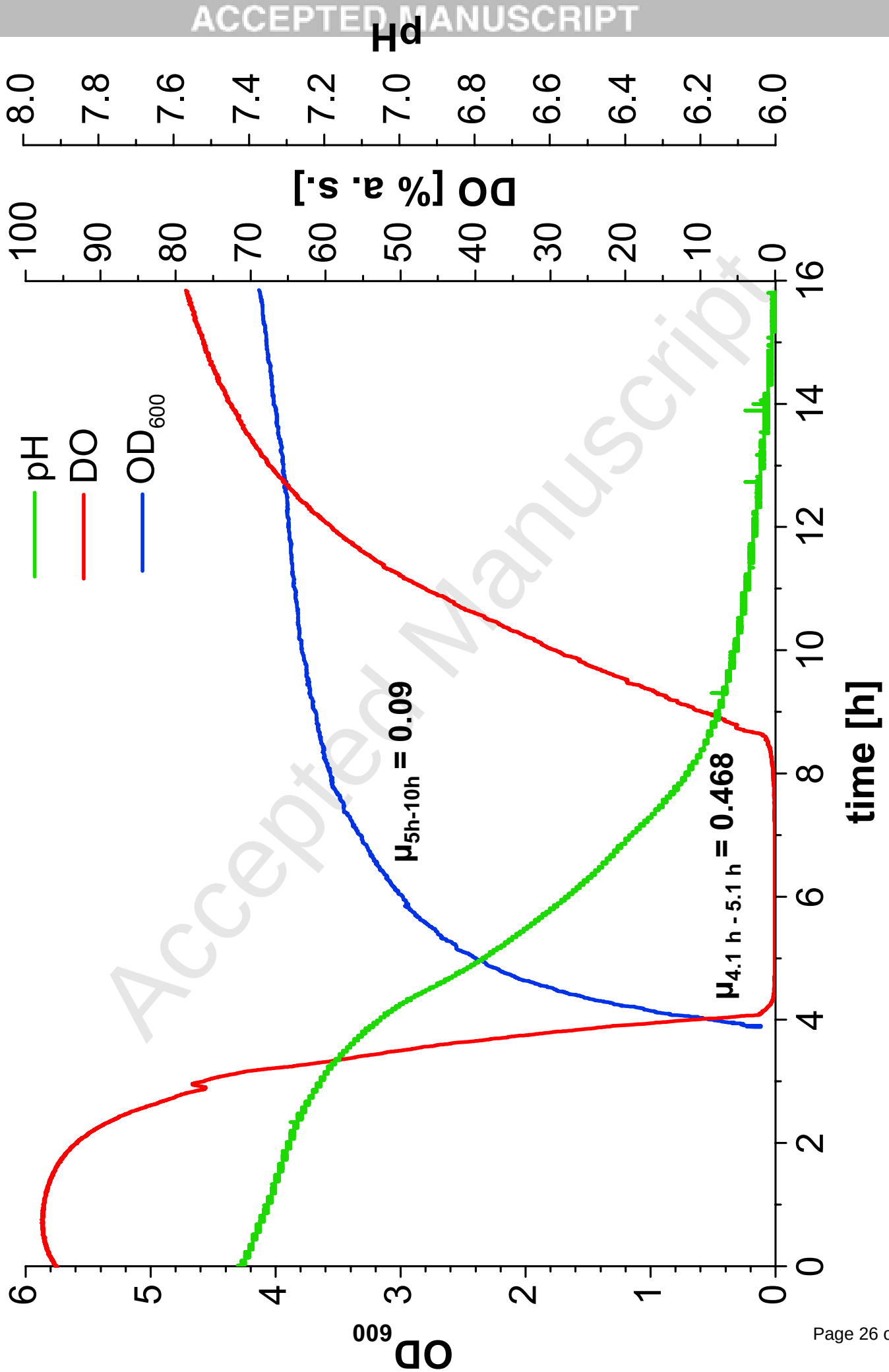

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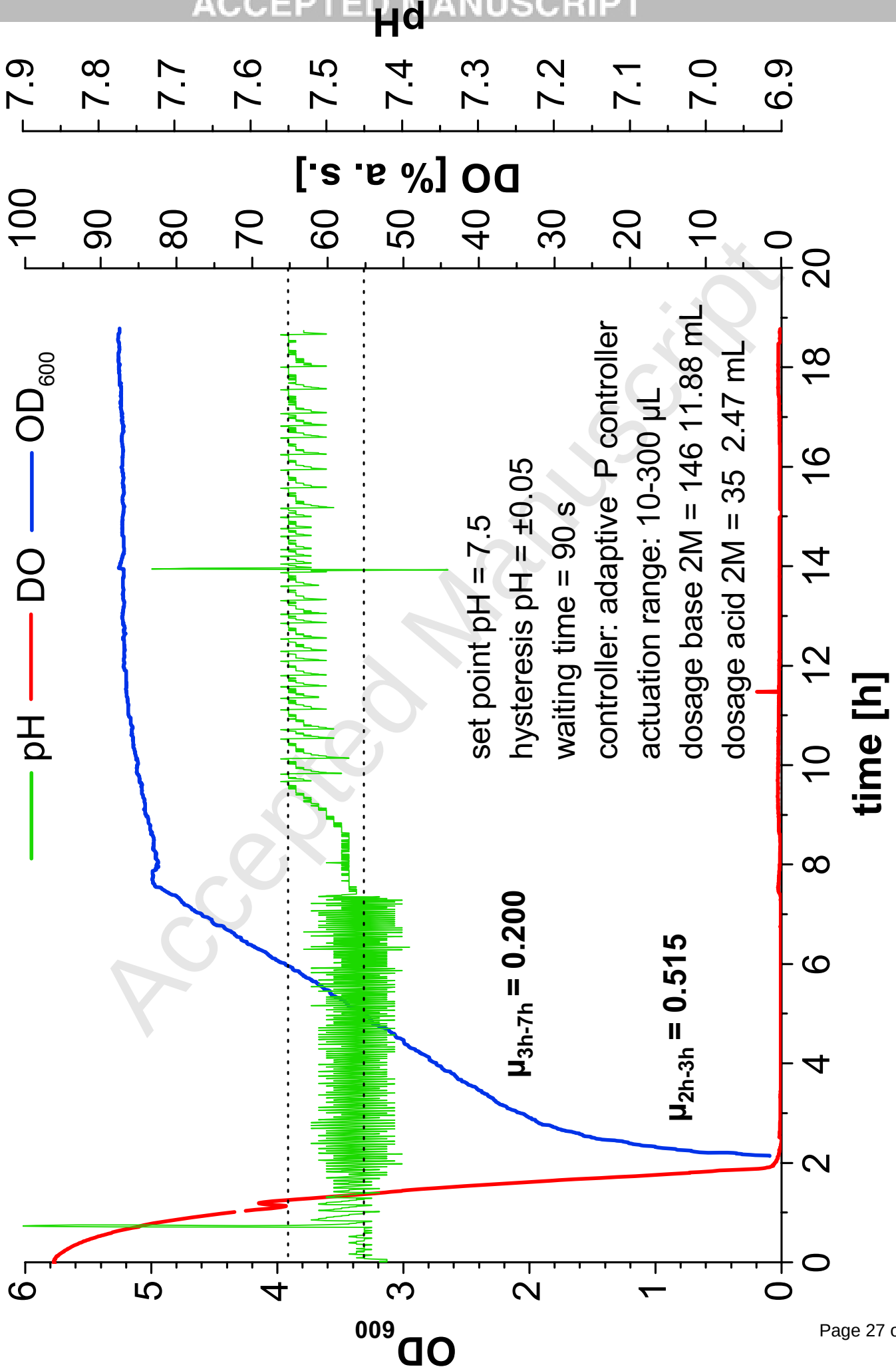

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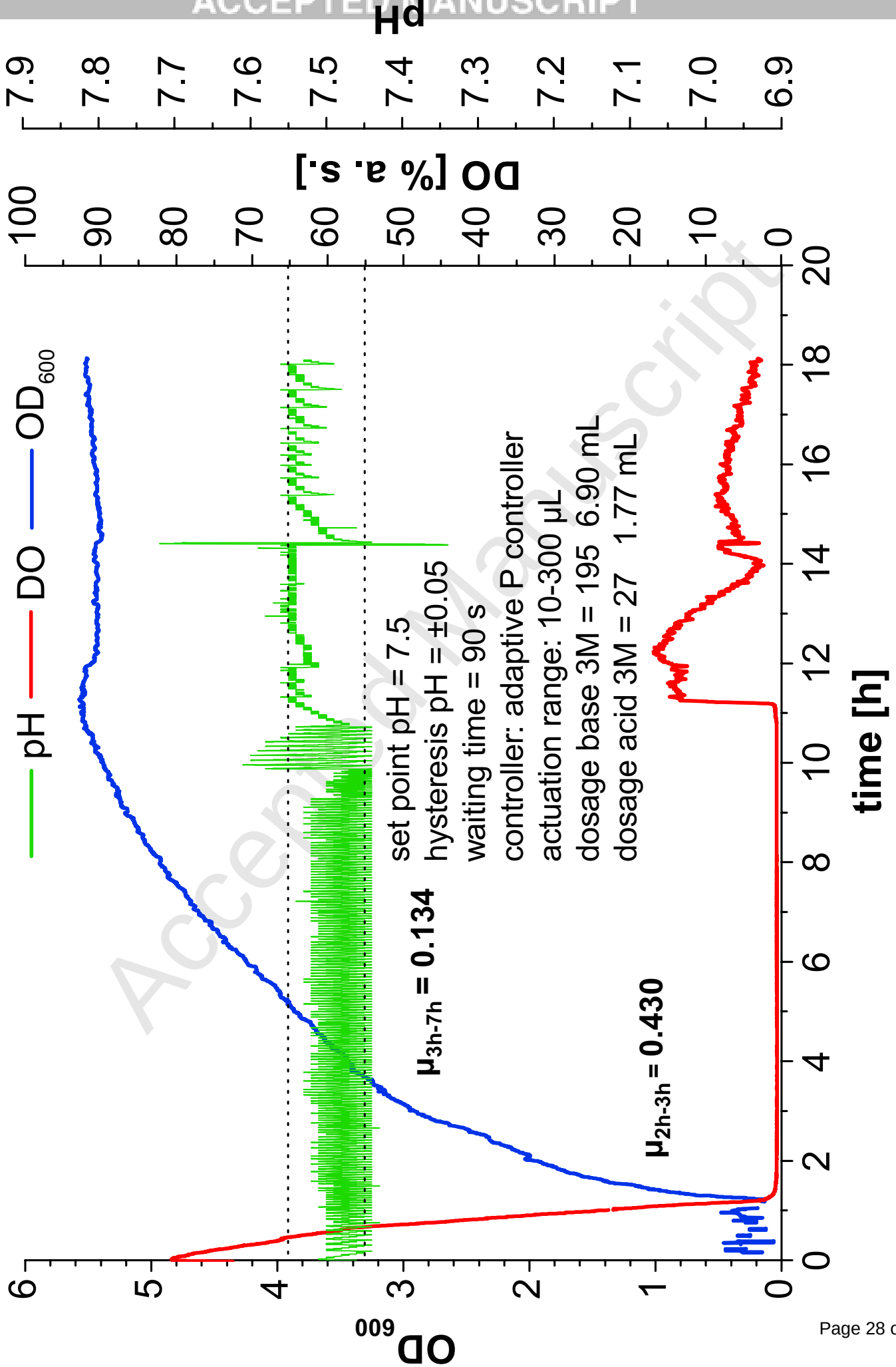

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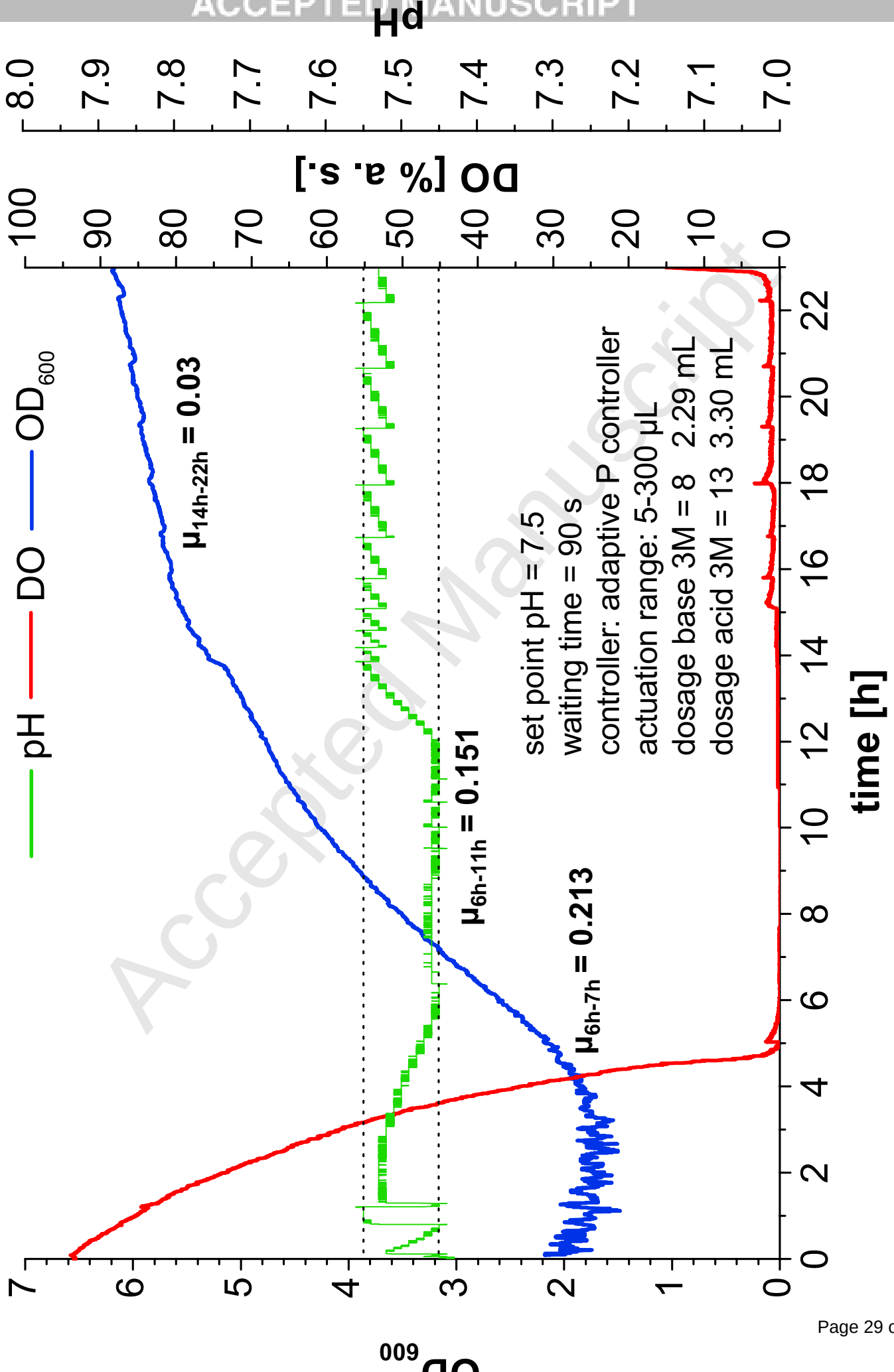

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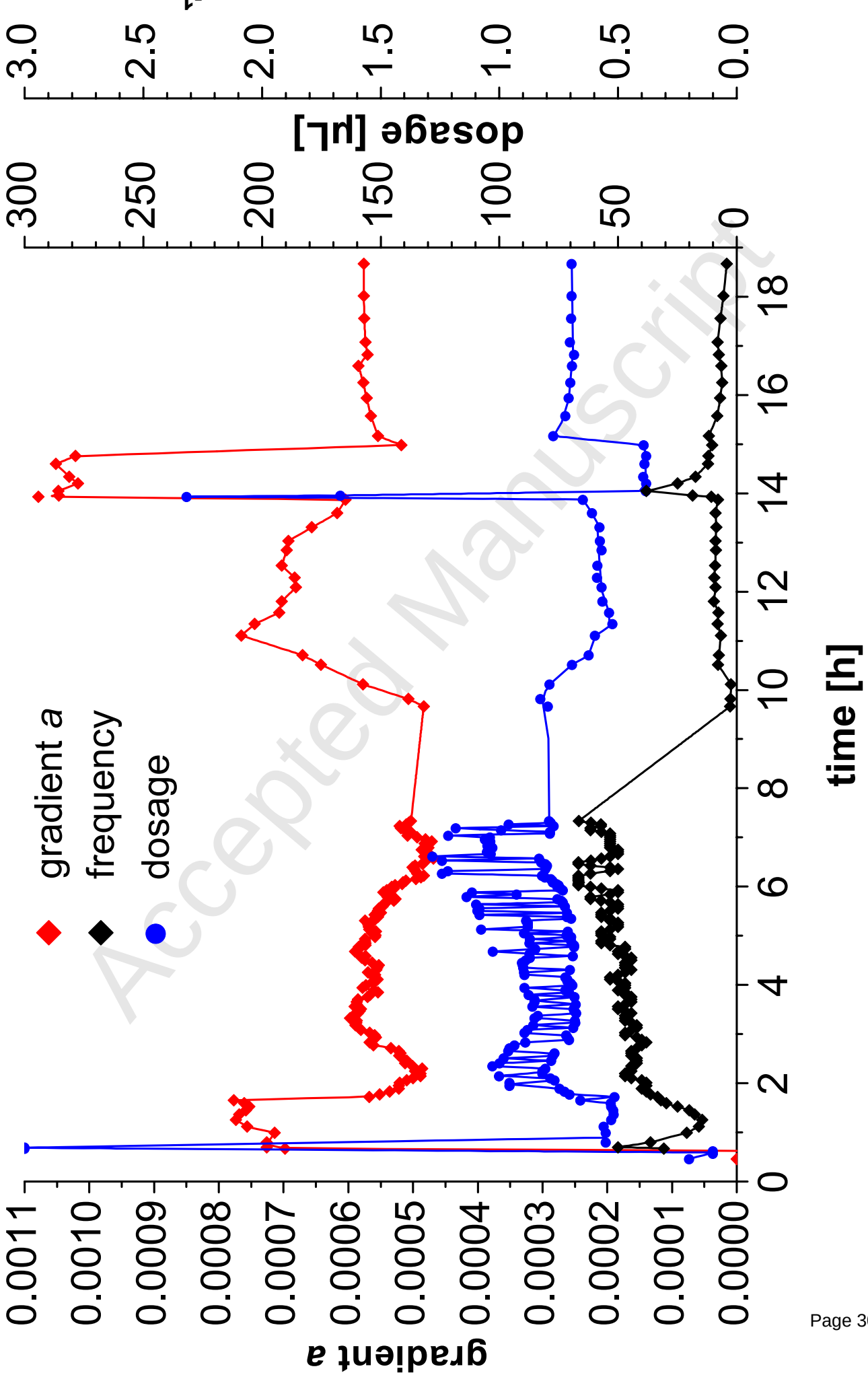

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