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Recommendations for process engineering characterisation of single-use bioreactors and mixing systems by using experimental methods

2nd Edition

Recommendations for process engineering characterisation of single-use bioreactors and mixing systems by using experimental methods (2nd Edition)

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1. Background

1.1. Introduction

Since the mid-2000s, the use of single-use bioreactors (SUB) and single-use mixing systems (SUM) in biopharmaceutical research and production has increased enormously in terms of scope and diversity. This means that single-use technology (SUT) in all process steps – especially in laboratory and pilot scale – is now of considerable importance for biopharmaceuticals and biosimilars. SUB and SUM are mainly used in processes where protein-based biotherapeutics from mammalian cell cultures are the target product. In addition, SUT is also used for the cultivation of plant cell cultures, microorganisms and algae, as well as for special products in the food and cosmetics sector [DECHEMA 2011], [Lehmann 2014], [Eibl and Eibl 2019].

Aim of these recommendations

A variety of disposable bioreactors and disposable mixing systems with a volume of up to 6,000 litres (SUB) or 5,000 litres (SUM) are currently available. These systems differ in terms of the type of power input, mixing and gassing strategy. It is therefore not easy to compare or select a system for a planned application [Minow et al. 2014], [Delafosse et al. 2018]. Already in 2011 a systematisation and classification according to the characterisation methods of conventional bioreactors made of stainless steel or glass was carried out in a status paper [DECHEMA 2011]. In 2016, the first edition of "Recommendations for process engineering characterisation of single-use bioreactors and mixing systems by using experimental methods" was published [DECHEMA 2016]. The authors are members of the working group "Upstream processing (USP)" of the DECHEMA expert group "Single-use technology in biopharmaceutical manufacturing", in which experts from industry and academia work together to provide the community with knowledge-based guidelines. The aim of these recommendations was and is to improve the compatibility and comparability of SUB and SUM among each other and in comparison to conventional glass and stainless steel bioreactors using standardised test methods and to provide manufacturers and users with objective criteria for comparison. Based on earlier publications [Meusel et al. 2013], [Löffelholz et al. 2013a], the focus of the recommendations is on the following aspects:

- A standardised catalogue of experimental methods for the determination of relevant parameters for the characterisation of SUB and SUM
- Evaluation and validation of these methods
- Models and criteria for characterisation and scale-up of SUB and SUM, mainly in relation to mass transfer

The described methods are applicable to single-use systems (SUS) for cell culture and microbial applications (for a biological evaluation of microbial applications see also the respective recommendations of the working group "Single-use microbial" [DECHEMA 2019]). The methods can be used for SUS in different scales, with and without a visual window and under the assumption of Newtonian flow behaviour of the used media.

Improvements in this edition
This second completely revised edition of the recommendations includes a number of improvements. The main enhancement is a new method for the determination of the volumetric mass transfer coefficient (k_La value). Compared to the method described in the first edition, the determination of the k_La value is now formulated in a generally valid manner rather than just for special cases. As a result, there is now concordance between the experimental evaluation method (Section 4) and the underlying theory of mass transfer, as it is also presented in relevant textbooks on the subject.

In addition, some inconsistencies and errors in text, figures and equations were removed or corrected.

To support the users of single-use equipment in the application of the new experimental method for determining the k_La value, the working group has also developed an evaluation tool based on Microsoft Excel that can be downloaded from the DECHEMA homepage (see Section 4.6).

The "Recommendations for process engineering characterisation of single-use bioreactors and mixing systems by using experimental methods (2nd Edition)" provide manufacturers and operators of SUB and SUM with a uniform set of methods for process engineering characterisation by means of validated Standard Operating Procedures (SOPs). In addition, this guideline can also be used for the engineering characterisation of reusable systems.

1.2. Process engineering parameters for describing single-use bioreactors and mixing systems

Apart from biological information on optimal cell growth and for efficient product formation, biopharmaceutical manufacturing in SUS requires process-related knowledge of bioreactor and mixing systems. Thus, the selection, dimensioning and design of the bioreactors and mixing systems are important parts of process development and optimisation [Maischberger 2019]. Often, the scaling-up of successfully established processes in the laboratory and technical scale is the focus of engineering and/or economic considerations. An example is the expansion or adjustment of manufacturing capacities. This requires a critical analysis of the relevance of certain characteristic bioreactor dimensions and associated process parameters for the respective process. In case of scale-up, the selection of suitable scale-up criteria is necessary. The parameters mentioned need to be provided, specified and calculated. A range of process parameters, which can be classified into three groups according to [Löffelholz et al. 2013a] (Figure 1), are available for the characterisation and scaling of single-use bioreactors and mixing systems.

The **process parameters** are generally obtained on the basis of simple equations. This primarily includes statements on the flow regime (Reynolds number, laminar, turbulent and transition zone) and on characteristic velocities of the liquid phase (e.g. tip speed) and of the gaseous phase (e.g. superficial gas velocity). For the calculation of process engineering parameters which depend on mixing principle of the single-use systems (stirred, wave-mixed, shaken or mixed by oscillations), different characteristic dimensions should be used [Löffelholz et al. 2013a], [Eibl, R. et al. 2006].

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Biochemical engineering parameters		
Operation conditions	**Experimentally determined parameters**	**Numerically determined parameters (CFD)**
• Flow regime • Fluid velocity • Superficial gas velocity	• Fluid flow pattern • Fluid velocity distribution • Power consumption • Mixing time • Residence time distribution • Particle (shear) stress • Volumetric mass transfer coefficient	• Fluid flow pattern • Fluid velocity distribution • Power consumption • Mixing time • Residence time distribution • Volumetric mass transfer coefficient • Energy dissipation rate

Figure 1: Classification of process parameters for single-use bioreactors and mixing systems [Löffelholz et al. 2013a].

In contrast to this, there is a group of variables and parameters that can be determined numerically, primarily by the **Methods of Computational Fluid Dynamics (CFD)** (see Figure 1). Therewith, highly detailed information can be obtained, not only on the **spatial and time-related dependencies** of the flow velocities, the energy dissipation rate and the residence time distribution, but also on integral parameters, such as the volumetric mass transfer coefficient (k_La) or on the mixing time [Löffelholz et al. 2011], [Platas Barradas et al. 2012], [Scully et al. 2020].

Thereby, critical zones (inadequate mixing, high shear rates, oxygen limitation, etc.) inside the bioreactor can be detected very quickly. With this information improvements can be achieved by making virtual modifications in equipment and process data. Hence, the numerical implementation of the bioreactor geometry and numerical boundaries is still challenging and requires appropriate expert knowledge. Apart from the special software and hardware, appropriate expert knowledge and specialists are necessary for the effective utilisation of these methods. In any case, the issue to be clarified is the degree of scientific penetration to be used for the characterisation of the bioreactors and mixing systems, and whether the cost and effort required is justified compared to the benefits.

However, the cost and effort required with respect to the measuring equipment needed, time for measurement and personnel are very different. Thus, the measurement of flow fields and local distribution of velocity must fullfil special requirements in terms of measurement equipment, for example, Particle Image Velocimetry (PIV) and Laser Doppler Anemometry (LDA). Due to the high personnel qualification level, such analyses are generally conducted at universities or in specially-equipped research laboratories of companies. In contrast, other experimentally determined process engineering parameters with greater practical relevance (specific power input, mixing time, distribution of residence time, volumetric mass transfer coefficient) can be measured with relative ease. Hence, they can be considered so-called **routine measurements**. In several practical applications, **experimentally determined process engineering parameters** are applied for the characterisation of single-use bioreactors and mixing systems (see

Figure 1), [Kraume 2003], [Liepe et al. 1998], [Pilarek et al. 2018]. However, due to the different investigation methods and variants for their specific execution and evaluation, the results published by companies and scientific institutions are difficult to compare.

1.3. Relevant experimental parameters and their determination

For the selection and operation of single-use bioreactors or mixing systems in day-to-day practice, it is adequate to undertake process-related characterisation in the form of **specific power input** (P/V_L), **mixing time** (θ) and **volumetric mass transfer coefficient** (k_La), as well as to assume Newtonian flow behaviour of the media, otherwise see [Henzler 2007]. For this reason the following explanations, evaluations and recommendations are restricted initially to these parameters and conditions.

For the assessment on comparability and applicability for SUB and SUM, the measurement methods published have been investigated and critically evaluated in detail by the USP working group of the DECHEMA expert group on "Single-use technology in biopharmaceutical manufacturing". The USP working group has critically investigated and evaluated various methods for engineering characterisation of bioreactors in order to assess and compare different SUB and SUM systems. The focus was on:

- The specific implementation of the methods (materials, measuring equipment, sensors and measurement procedure used).
- The evaluation of the tests (statistical certainty, evaluation of replicates, mean value calculation and correction factors, etc.).

A huge wealth of experience of the academia (Zurich University of Applied Sciences, Wädenswil; Anhalt University of Applied Sciences, Köthen; Trier University of Applied Sciences, Umwelt-Campus Birkenfeld, Hoppstädten-Weiersbach; Hamburg University of Technology, Hamburg) and companies (Sartorius Stedim Biotech GmbH, Göttingen; Bayer AG, Leverkusen; Eppendorf AG Bioprocess Center, Jülich; Roche Diagnostics GmbH, Penzberg) was used to evaluate the process engineering characterisation methods.

In the course of processing, existing internal SOPs on the determination methods were adapted to the special conditions of single-use technology. Coordinated and standardised procedures were developed and proposed as SOPs (see Sections 2 to 4). The temperature influences the viscosity of a fluid (Table 1). Therefore, the power input and the mixing time increase with decreasing temperature and thus increasing viscosity for Newtonian flow behaviour. In addition the Henry constants are also temperature-dependent and the solubility of the gases decrease with increasing temperature. Therefore, the temperature for the experimental determination of the process parameters should be well considered. In order to achieve comparability of the measurement results different temperatures are recommended in this publication for the determination of the process parameters. This is based on measurement accuracy and on economic fact. Therefore, the determination of specific power input and mixing time is recommended at more or less room temperature (25 °C). The determination of volumetric mass transfer coefficient should be done at a process temperature of 37 °C.

Table 1: Viscosity of pure water as a function of the temperature at 20, 25 and 37 °C [NIST 2015]

Temperature [°C]	Viscosity [mPa·s]
20	1000
25	889
37	691

1.3.1. Power input

The specific power input (P/V_L) is one of the most important process parameters for the design, operation and scaling-up of conventional and single-use bioreactors and mixing systems. The required electrical power, the design of the stirrer and its shaft in stirred bioreactors, as well as the guarantee of certain mixing operations such as suspension, homogenisation, dispersion (gas bubbles, liquid drops), is directly dependent on the specific power input. For stirred SUB and SUM, the power input can be calculated from Eq. 1.

$$P = Ne \cdot \rho_L \cdot n^3 \cdot d^5 \qquad \textbf{Eq. 1}$$

In Eq. 1, Ne (Newton number / dimensionless power number) represents a stirrer-specific power number, which may further depend on the bioreactor geometry (configuration and the degree of baffling, stirrer type, etc.), the Reynolds number (Eq. 2), the Froude number and the aeration conditions.

$$Re = \frac{n \cdot d^2}{\vartheta_L} \qquad \textbf{Eq. 2}$$

Experimentally, the specific power input can be obtained, for example, via the torque (M) measured on the stirrer shaft (Eq. 3), (see Section 2):

$$\frac{P}{V_L} = \frac{2 \cdot \pi \cdot n \cdot (M - M_d)}{V_L} \qquad \textbf{Eq. 3}$$

The specific power input (P/V_L) related to the liquid volume must be used for process-related characterisation and especially for the scale-up.

The specific power input is a commonly used scale-up criterion in biotechnology because many process engineering parameters correlate during scale-up (e.g. mass transfer and shear stress). This criterion is proven for applications with cell culture and microorganisms [Löffelholz et al. 2013a]. As a result, the measurement of the specific power input provides manufacturers and operators with valuable information in order to characterise the power capability of SUB and SUM.

Primarily, there are two methods of measurement for bioreactors with mechanical power input which are applicable by taking practical aspects into consideration [Löffelholz et al. 2013a]:

- Torque measurement method [Wollny 2010], [Büchs 2000], [Kaiser et al. 2016].
- Temperature method [Raval et al. 2007], [Sumino et al. 1972].

According to [Kraume 2003], determining the power input by measuring the electric current and voltage for alternating current motors is mostly not suitable in laboratory scale. This motor type is most commonly used for bioreactors and consequently an alternative method is recommended in this guideline.

The **torque measurement method** is recommended for the following reasons:

- The method can be easily applied to single-use bioreactors and mixing systems (stirred, orbital shaken, rotary oscillating).
- The measurement costs are lower than with the temperature method (insulation of the SUB/SUM and highly sensitive temperature sensors, which sometimes affect the flow pattern, are required for the latter).
- In case the shaft is easy to access and the position of the motor can be changed, this method is easier to apply to SUB. If it is given, the method provides more accurate results than the temperature method.

Detailed instructions on the exact execution and evaluation of the torque measurement method are provided in the SOP (Section 2), based on several years of practical experience gained by the involved universities and companies. In particular, the following instructions should be considered for the measurement of small values of power input on the laboratory scale (measurement accuracy):

- Proper choice of the torque sensor range necessary.
- Precise installation of the torque measurement systems.
- Exact positioning and guidance of the respective drive shafts (ensuring vibration-free operation).
- Exact determination of the values of dead weight torque (torque values under operating conditions without filling the reactor).

1.3.2. Mixing time

Mixing refers to the distribution of two or more components that are different with respect to at least one property, such as concentration, temperature, colour, density, viscosity, etc. [Kraume 2003], [Storhas 1994], [Ascanio 2015]. The homogeneity of all components inside the bioreactor is one of the most important basic requirements for both conventional and single-use systems. As a result, mainly with larger equipment/bags, concentration and temperature gradients are prevented in order to avoid an unfavourable impact on cell growth and product formation [Löffelholz et al. 2013a]. Homogenisation (mixing) is characterised by the mixing quality to be achieved and the mixing time necessary [Chmiel 2011]. The mixing quality represents a preventive value (for example, macro-mixing, fine mixing or mixing up to molecular dimensions) [Liepe et al. 1998]. Additionally, the **mixing time** (θ) depends on the bioreactor geometry, the used stirrer type and size, the specific power input as well as the liquid properties [Löffelholz et al. 2013a], [Zlokarnik 1999]. Furthermore, the dimensionless **mixing number (mixing coefficient)** ($c_H = n \cdot \theta$) is a constant in the turbulent range and can be used to compare different mixing systems (SUB/SUM). In stirred bioreactors, the mixing number represents the number of stirrer revolutions necessary to achieve a sufficient mixing quality [Liepe et al. 1998].

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In principle, the following methods are applied for experimental determination of the mixing time:

- Decolourisation method [Kraume 2003].
- Sensor method [Zhang et al. 2009].
- Optical and colorimetric method [Manna 1997].

The optical and colorimetric methods are based on the fluorescence of a dye that emits light of a certain wavelength when excited by a laser beam. In fact, highly accurate results can be obtained in this way. But the high cost and effort involved (Laser Induced Fluorescence (LIF) and Particle Image Velocimetry (PIV)) and the optical accessibility of the reactors are often the obstacles to use this method in day-to-day practice.

The decolourisation methods use redox or neutralising reactions, which lead to a specific change in colour by adding suitable chemicals that are used as an indicator for homogeneous mixing right up to molecular dimensions. Thus, the mixing time is the time from the addition of the substance triggering the change in colour until complete decolourisation of the entire bioreactor volume (see Section 3). Based on the fundamental principle of the measurement, decolourisation methods require a transparent reactor wall (film), or at least a sufficiently large window, in order to detect the colour change visually. However, this is the case for most of the bag-based SUB or SUM. Otherwise, the bag may be opened in order to enable visual observation from the top of the reactor [Löffelholz et al. 2013a].

Regardless of these possible limitations, the decolourisation method using the iodometry variant (redox reaction) (see Section 3) is recommended for experimental determination of the mixing times in SUB and SUM due to the following reasons:

- It is a very simple and cost-effective method compared to the sensor method.
- Mixing times obtained are valid for the entire geometry of the bioreactor, whereas the sensor method only provides the mixing time at certain points inside the bioreactors.
- Dead zones and zones with poor mixing can be determined.
- No sensors are needed that potentially influence the flow conditions compared to normal operation.

In addition, it must be emphasised that the decolourisation method, based on the measurement principle, leads to measurement results that are suspected to be subjective in nature. This can be countered by selecting an appropriately large number of individual measurements and thus a representative mean value. In practice, it has also been proven to be useful to record the colour change / decolourisation in parallel with visual measurement, as prevalent, state-of-the-art, low cost video technology is available today. For bioreactors **without a visual window** the **sensor method** can be applied to determine the mixing time. In general, the method is based on the high time-resolved measurement of the conductivity in the liquid volume of the SUB/SUM. The mixing time is obtained as the time between the specific addition of inhomogeneity (tracer, salt solution) until homogeneity is achieved again. A homogeneity of 95 % ($\theta_{95\%}$) is generally specified to be adequate [Kraume 2003], [Liepe et al. 1998], (see Section 3).

The result achieved (mixing time) from this method depends, among others, on the position of the sensor in the SUB/SUM, the number of the sensors, the location of the tracer addition, the quantity added, as well as the sensor response time and the sampling rate of the conductivity transmitter. The pre-installed sensors in most SUBs only allow measurements at defined positions. However, the measurements do not require sterile conditions and, therefore, it is possible to modify existing sensors or insert additional sensors at desired positions. In general, the sensors have to be fixed properly in order to avoid measurement fluctuations and leakages.

Principally, the response times of most conductivity sensors are low (see also Section 3.3, Oxygen Sensors). It is recommended to use sensors with a response time ($t_{63\%}$) that are approximately five times less than the mixing time measured [Zlokarnik 1999] (see Section 3). Moreover, the sampling rate of the transmitter of the conductivity measurement system must be taken into account. A sampling rate of at least once per second is recommended. If necessary, appropriate modifications of the measurement system must be made.

1.3.3. Volumetric mass transfer coefficient

In aerobic biotechnical processes, the gassing system (sparger/stirrer, surface aeration) integrated in the bioreactor must ensure a sufficient oxygen supply to the organisms. As a consequence of the very low solubility of oxygen in the culture media used, oxygen limitations may occur very quickly. Therefore, the characterisation of the oxygen transfer rate (OTR) is one of the most important parameters for the evaluation for both conventional and single-use bioreactors. The oxygen transfer rate can be obtained in accordance with Eq. 4:

$$\mathrm{OTR} = \mathrm{k_L a} \cdot (\mathrm{DO^*} - \mathrm{DO}) \qquad \textbf{Eq. 4}$$

This contains the **volumetric mass transfer coefficient (k_La)** as critical parameter, which comprises the volumetric interfacial gas-liquid surface area (a) and the mass transfer coefficient (k_L). Since it is extremely difficult to experimentally determine the k_L and a values, both are measured only as a product in the form of k_La [Garcia-Ochoa et al. 2010], which is used for the characterisation and design of bioreactors.

The volumetric mass transfer coefficient (k_La) can be interpreted as a measure of the velocity of the oxygen entry (reciprocal of the value of the oxygen transfer time) [Löffelholz et al. 2013a], [Garcia-Ochoa et al. 2010]. It is therefore suitable for evaluating the effectivity of oxygen mass transfer within SUB and conventional bioreactors or to compare different systems with each other. The k_La value depends on equipment-specific parameters (H/D, d/D, h_R/D stirrer type, installation conditions), process parameters (agitation rate, filling, aeration rate, type of gassing device, and pressure) and properties of the liquid (density, viscosity, surface tension, and coalescence properties) [van't Riet 1979].

In biotechnology, it is general practice to evaluate the so-called k_La models in the form of dimension-afflicted mass transfer models, which also may be used for scale-up. In most cases, the specific power input (P/V_L) and the superficial gas velocity (u_G) are used as model parameters.

For the experimental determination of the volumetric mass transfer coefficient (k_La) a number of measurement methods have been published [Löffelholz et al. 2013a], [Zlokarnik 1999]. Usually, different names

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are used for one and the same method. This makes it difficult for the user to have an overview in practice. This shortcoming is meant to be overcome by the following systematisation based on the procedure and the materials used:

- Measurement method based on saturation curves **without organisms**

  Absence of oxygen (zero point) can be achieved either by gassing with nitrogen **(Gassing-out method)** [van't Riet 1979], [Garcia-Ochoa et al. 2009] or by adding a certain quantity of sodium sulphite and cobalt catalyst **(Dynamic sulphite method)** [Puskeiler 2004], [Suresh et al. 2009], [Malig et al. 2011].

- Measurement method based on the saturation curve with **organisms (Respiratory gassing-out method, Dynamic method)** [Bandyopadhyay et al. 1967], [Chmiel 2011], [Liepe et al. 1998], [Linek et al. 1996].

- Measurement method based on the determination of the exhaust gas composition **(Balancing method)** [Liepe et al. 1998].

- Measurement method using chemical model media: **Sulphite method (Static sulphite method)**, [Hermann 2001], [Liepe et al. 1998], **Hydrazine method** [Zlokarnik 1973], **Glucose oxidase method** [Linek et al. 1981].

Taking the specifics of single-use bioreactors into account and considering practical experience, it is recommended that the **saturation method without organisms with nitrogen stripping (Gassing-out method)** should be used for process-related characterisation of SUB (see Section 4).

This is due to the following reasons:

- The cost-effective method can be carried out with minimal effort in terms of measurement and materials and in a short period of time in different SUB.
- Data evaluation is easy and can be done very quickly.
- Experiments can be carried out under non-sterile conditions.
- The process parameters can be varied within a wide range without restrictions from the biological system.
- The method is predestined for the comparison of different systems.
- No environmentally hazardous chemicals are necessary.
- All experiments can be done without media change.

Regardless of the points mentioned, and in addition to the SOP (see Section 4), attention should be drawn to certain **characteristics** while executing this method.

Since this method is based on a dynamic measurement of oxygen concentration, the time behavior of the probe must be taken into account. When using unsuitable sensors (very long response time), considerable errors may occur with the determination of the k_La values.

The response time is defined as the time which is needed for the sensor to reach a defined end value (steady state) after a switch in the oxygen content of the aeration gas. The response time is influenced by inflow velocities, delays from the oxygen diffusion processes in the membrane of the Clark electrodes (amperometric probes) and delays within the measurement electronics. For other types of sensors (optical, galvanic), there are similar reasons for time delays.

In order to capture the response time quantitatively, a step response function should be recorded (see Section 4). In this test, the oxygen sensor is transferred very quickly (as far as possible without any time delay) from an oxygen-free to an oxygen-saturated liquid, and the measurement signal is recorded (see Section 4). The response function obtained is generally equivalent to a PT1 response (delay of the 1st order) and can be described mathematically by Eq. 5.

$$y(t) = C \cdot \left(1 - e^{-t/\tau}\right) \qquad \textbf{Eq. 5}$$

The parameter τ is the time constant of the sensor and is equivalent to the time at which 63.2 % ($t_{63\%}$) of the final value is reached. **This time constant should be used as the basis for comparing different sensors.** The value $t_{95\%}$ is also frequently used and is equal to three times the value of $t_{63\%}$, based on Eq. 5.

This time constant ($t_{63\%}$) of the oxygen sensor gives a first guess as to what extent k_La values can be determined. However, various opinions are provided in the literature.

Often, the criterion of [van't Riet 1979] is used, according to which the response time can be neglected if it is less than the reciprocal of the volumetric mass transfer coefficient (k_La). Thus, a critical response time ($t_{63\%,crit}$) can be calculated in accordance with Eq. 6, which should not be exceeded in order that larger measurement errors be prevented.

$$t_{63\%,crit} = \frac{1}{k_L a_{max}} \qquad \textbf{Eq. 6}$$

Based on this, reference values (Table 2) for acceptable values of the time constant $t_{63\%,crit}$ of the sensors can be specified depending on the desired measuring range of the k_La values. In contrast to this, other authors have formulated more stringent conditions. Thus, [Garcia-Ochoa et al. 2009] refer to a time constant that is ten times less than the reciprocal of the k_La value as negligible. The same statement has been made by [Liepe et al. 1998], who determined that only time constants of the same order as those specified by [Garcia-Ochoa et al. 2009] allow measurement errors below 1 % (Table 2). Contrary to this, [Zlokarnik 1999] specifies a value that is five times less than that of the van't Riet criterion (Table 2).

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Table 2: Reference values for maximum possible response times $t_{63\%,crit}$ depending on the maximum k_La value.

k_La [h^{-1}]	$t_{63\%, crit}$ [s] by van' Riet	$t_{63\%, crit}/5$ [s] by Zlokarnik	$t_{63\%, crit}/10$ [s] by Garcia-Ochoa, Liepe
10	360	72	36
50	72	14	7
120	30	6	3
200	18	4	2
300	12	2	1
500	7	1	0.7

The exact evaluation of the impact of the time delay of the sensor on the measurement accuracy can be achieved only with appropriate differential equations. One approach for this is given by [Badino et. al. 2000], who has used a first order kinetic for the oxygen sensor.
In view of the different references, it is recommended that sensors with $t_{63\%}$ for k_La measurements be used, which meet the criterion according to [Zlokarnik 1999] (Table 2). Thus, considering practical aspects, the impact of the time delay on the measurement result can be neglected.

Hence, it could be concluded that it is easily possible to realise measurement for typical cell culture application ($k_La < 15$ - 20 h^{-1}) [Eibl, D. et al. 2009] with already available sensors. However, in the field of microbial fermentation ($k_La < 300$ - 500 h^{-1}), larger k_La value errors may occur, if sensors with long response times (see Table 2) are used.

Moreover, it is pointed out that the application of the gassing out method is practically meaningful (Table 2) only for k_La values up to maximum 500 h^{-1}. Over and above this, stationary and fixed methods should be preferred.

A uniform procedure in accordance with the SOP (in Section 4, i.e. apart from the recommendations already made concerning the oxygen sensors), is recommended for comparable studies, publications, etc. that 1 x PBS solution should be used as medium. This is necessary, on the one hand, for achieving appropriate conditions (presence of ions) for the single-use sensor patches integrated in certain SUB, and, on the other hand, to adjust the usual fermentation media with respect to the coalescence conditions (coalescence-inhibited medium). The use of pure sodium chloride solutions (e.g. 10 g/L) can lead to corrosion in the stainless steel components of the bioreactors and mixers and, hence, is not recommended.

When using the gassing out method with nitrogen stripping in single-use bioreactors with surface gassing (head space aeration), other peculiarities must be taken into consideration (see Section 4). First, the zero point is lowered by nitrogen supplementation to the head space of the reactor under operating conditions (agitation necessary). After reaching DO = 0 %, the gassing with air can be started, but the nitrogen excess has to be removed from the headspace before starting the k_La determination. If the nitrogen is not removed completely, uncertainties can occur due to modified gas composition in the head space.

As a result, the driving force and thus the saturation concentration is modified, resulting in a lower k_La value [Malig et al. 2011]. Hence, it is necessary to flush the head space with air (agitation switched off) prior to the actual measurement until exhaust gas analysis ensures 21 % of oxygen and thus atmospheric conditions in the head space. If exhaust gas analysis is not available, then the head space volume should be exchanged at least three times [Malig et al. 2011]. The associated extension in time for the measurement must be taken into account when planning experiments.

1.4. Verification of the k_La determination method

The SOP for determining the volumetric mass transfer coefficient (k_La) (see Section 4) was applied by the working group on "Single-use bioreactors for microbial applications" of the DECHEMA expert group on "Single-use technology in biopharmaceutical manufacturing" in order to test feasibility under practical conditions. Bioreactor systems from different manufacturers with different volumes (laboratory/pilot scale) and different types of energy input (stirred, wave mixed) were used (see Figure 2). All measurements were carried out using an optical probe showing $t_{63\%}$ to be lower than 1.5 s according to Table 2 (PreSens Precision Sensing GmbH, Regensburg, Germany). Rocking systems used flat patch designs

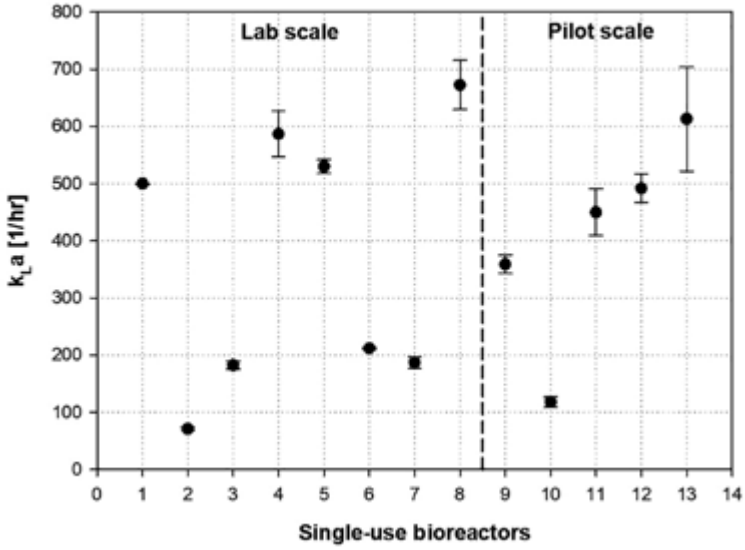

Figure 2: Volumetric mass transfer coefficients in different bioreactors at laboratory and pilot scale. The data were obtained by the bioreactor manufacturers when using maximum possible specific power input and aeration rate at 37 °C. The SOP was used and the results were categorised into laboratory (up to 50 litres of working volume) and pilot scale (beyond 50 litres). Bioreactor 1 was a glass bioreactor, which was used as reference system.

while stirred systems used PG 13.5 designs. Thus, a generalised statement about the applicability of the SOP can be derived. The aim of the investigations was primarily to get a qualitative statement on the reliability of the methods, i.e. that the standard deviation is maximum ±10 % when the k_La value is determined several times.

For this purpose, the different SUB should be operated with a maximum aeration rate and a temperature of 37 °C with the maximum possible specific power input (W/m³). A 10 g/L sodium chloride solution was used as the medium for analysis, but no significant difference was found to the non-coalescing PBS solution (see Section 4). The investigations were carried out by the respective manufacturers. The results of the investigation demonstrate that the determination of the volumetric mass transfer coefficient (k_La) of SUB from different manufacturers (2-13) provides meaningful results (Figure 2). The standard deviation for all values was within the recommended range of 10 %. This confirms the reproducibility of the tests. For established SUB of the laboratory scale (up to 50 litres of working volume), k_La values in the range of 70 to 590 h^{-1} were achieved. In the pilot scale (beyond 50 litres up to about 500 litres), k_La values above 300 to 680 h^{-1} were determined (exception: Bioreactor supplier 9 with a k_La value of 120 h^{-1}).

Based on the experiences obtained through the interlaboratory tests, it can be concluded that the SOP for determining the volumetric mass transfer coefficient (k_La) for single-use bioreactors can be applied regardless of volume, energy input and the use of optical, single-use or conventional probes. Furthermore, the methods can be transferred to conventional bioreactor systems made of glass and/or stainless steel as internal investigations have demonstrated.

1.5. Using the experimental data for the design and evaluation of single-use bioreactors and mixing systems

After the process engineering parameters, such as mixing time, specific power input and volumetric mass transfer coefficient, have been determined experimentally, the issue to be clarified is how these parameters can be applied meaningfully for design and scale-up. For calculations, comparisons and evaluations, the process engineering parameters should be displayed in dependency of the agitation rate/ principle. **It is preferred to use dimensionless analysis for scale up.**

As practice demonstrates, in exceptional cases, such as mass transport from gas to liquid or mixing time, even simplified dimensioned correlations can be applied for scale-up. Hence, the measured parameters should be used in order to create dimensioned correlations based on these that are valid across a large range of relevant processes and equipment sizes, and thus may be used as basis for design and scale-up studies.

Volumetric mass transfer coefficient (k_La):

In general, experimental data are used to create a simple **k_La model** (Eq. 7). This contains the specific power input (P/V_L) and the superficial gas velocity (u_G), a measure of the aeration intensity (Eq. 8):

$$k_La = C \cdot \left(\frac{P}{V_L}\right)^a \cdot u_G^b \qquad \textbf{Eq. 7}$$

with

$$u_G = \frac{\dot{V}_G}{A_G} \qquad \textbf{Eq. 8}$$

In certain cases, even the volumetric aeration rate $\beta = \dot{V}_G/V_L$ is used. However, this is not recommended for SUB with mechanical power input because, when scaled-up, the aeration intensity in those bioreactors is often underestimated.

The constant (C) and the exponents (a and b) must be determined from the basic measurement data. The fact that the Eq. 7 is not a homogeneously dimensioned equation needs to be taken into consideration, which means that similar geometrical conditions and identical material systems must be present. Furthermore, the properties of the liquid to be aerated, especially the coalescence properties, have a great impact on the determined k_La value. However, since this cannot easily be described by simple mathematical models, differentiation is made primarily between **two groups of k_La models**. On the one hand, there are models for coalescent media (bubble coalescence present without restriction, no surfactants and barely any salts in the medium) and, on the other hand, there are models for coalescence-inhibiting media (bubble coalescence prevented by surfactants and possibly higher concentrations of salt present in the medium). As an example, the frequently used models according to van't Riet should be mentioned [van't Riet 1979]. These were successfully applied for a large number of conventional, stirred systems with sparger aeration and large ranges of (P/V_L) in scales between 50 L and 4 m³ (Eq. 9 and Eq. 10).

$$k_La = 0.026 \cdot \left(\frac{P}{V_L}\right)^{0.4} \cdot u_G^{0.5} \quad \text{Coalescent media} \qquad \textbf{Eq. 9}$$

$$k_La = 0.002 \cdot \left(\frac{P}{V_L}\right)^{0.7} \cdot u_G^{0.2} \quad \text{Coalescence-inhibiting media} \qquad \textbf{Eq. 10}$$

For SUB, k_La models are published, among others, by [Löffelholz 2013b], [Löffelholz et al. 2013a] and [Kaiser et al. 2011]. If validated k_La models are available for a certain type of reactor, these can be used for process design and scale-up. To achieve a desired k_La value for a given superficial gas velocity, e.g. the specific power input (stirrer speed, etc.) is obtained directly from the model (Eq. 11):

$$\left(\frac{P}{V_L}\right)_{erf} = \left(\frac{k_La_{erf}}{C \cdot u_G^b}\right)^{1/a} \qquad \textbf{Eq. 11}$$

In contrast, for a defined specific power input, the required superficial gas velocity may be obtained in a similar manner (Eq. 12).

$$u_{G,erf} = \left(\frac{k_La_{erf}}{C \cdot (P/V_L)^a}\right)^{1/b} \qquad \textbf{Eq. 12}$$

1. BACKGROUND

Mixing time for single-phase, liquid material systems:

With the experimental data for the mixing time, a similar procedure as for the volumetric mass transfer coefficient is proposed. Optionally, the dimensionless mixing number (mixing coefficient (c_H), Eq. 13) can be derived.

$$c_H = n \cdot \theta \qquad \textbf{Eq. 13}$$

This is equivalent to the number of stirrer rotation necessary until homogeneity is achieved. Usually, the mixing number is correlated with the Reynolds number [Liepe et al. 1998], [Kraume 2003]. For numerous stirred systems, the mixing number in the range of turbulent flow is constant. Hence it can easily be used for scale-up. According to [Löffelholz 2013b], the numeric value of c_H for stirred SUB lies in the range of 20 to 50 and, as a result, is barely different from that of stainless steel reactors. Furthermore, a model approach based on turbulent diffusion in stirred bioreactors proves to be promising, as proposed by [Nienow 2006, 2010]. Based on this approach, [Löffelholz 2013b] could specify the following correlation on the basis of experimental and numerical data (Eq. 14).

$$\theta = 3.5 \cdot \left(\frac{P}{m_L}\right)^{-1/3} \cdot \left(\frac{d}{D}\right)^{-1/3} \cdot \left(\frac{H}{D}\right)^{2.43} \cdot D^{2/3} \qquad \textbf{Eq. 14}$$

The dimension-afflicted Eq. 14 is based on data from SUB with up to 2,000 L of production volume. Thus, it could be demonstrated that even simplified correlations may be suitable for describing the mixing time in different scales (laboratory to production scale). Corresponding correlations may be prepared in a similar manner for other types of SUB and SUM based on collected experimental data.

Generally, we recommend using dimensionless numbers (Reynolds number, Froude number, Newton number) and dimensionless geometry-releated parameters (d/D, H/D, h_R/D etc.) rather than dimension-afflicted parameters, such as the specific power input (P/V_L) and the superficial gas velocity (u_G), for the model development. This increases the general validity of the models and facilitates the design and scale-up of the SUB and SUM.

1.6. Summary and Outlook

The process-related design and characterisation of single-use bioreactors and mixing systems is a significant part of development, execution and optimisation of biotechnical processes for the manufacture of biopharmaceutical products. Experimental determined parameters are of importance because of their direct practical relevance. Additionally, these parameters can be determined with minimal effort and low costs. Furthermore, the implementation can be realised very quickly. This includes the specific power input (P/V_L), the mixing time (θ) and the volumetric mass transfer coefficient (k_La). Taking the constructive characteristics of SUB and SUM into account, the following methods are proposed for the experimental determination of existing options:

- Specific power input: Torque method
- Mixing time: Decolourisation and sensor methods
- Volumetric mass transfer coefficient: Gassing-out method

These methods are applicable for SUB and SUM of different scales. Moreover, apart from the SUB and SUM, even "Reusable Systems" made of glass or stainless steel can be characterised based on these guidelines. The specific execution of the methods, the required devices and materials to be used, as well as the evaluation and possible sources of error are shown in the SOPs provided. These are based on comprehensive research of references and experience of the companies and universities involved in characterisation of SUB and SUM.

Above all, the sensor response time must be considered when determining the volumetric mass transfer coefficient (k_La). Here, considerable measurement errors and misinterpretations may occur when inappropriate sensors (very large response time) are used. Similarly, this also applies to the conductivity sensors for determining the mixing time with the sensor method. The SOPs provided are meant to facilitate process-related studies on single-use bioreactors and mixing systems and to make the results generated in the process more comparable. This leads to greater assurance with the selection, design and operation of the bioreactors and mixing systems mentioned. This primarily concerns the more stringent requirements with respect to heat and (oxygen) mass transport. An update to these guidelines shall be provided when appropriate results become available.

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1. BACKGROUND

Abbreviations

CFD	Computational Fluid Dynamics
DO	Dissolved oxygen
KPi	Potassium phosphate buffer
LDA	Laser Doppler Anemometry
LIF	Laser Induced Fluorescence
OTR	Oxygen transfer rate
OUR	Oxygen uptake rate
PBS	Phosphate buffered saline
PIV	Particle Image Velocimetry
SOP	Standard operating procedure
SUB	Single-use bioreactors
SUM	Single-use mixing system
SUS	Single-use system
SUT	Single-use technology
USP	Up-stream processing

Symbols used

a	Volumetric specific phase boundary, constant in k_La model
A_G	Aeration cross-section of the reactor
B	Constant in k_La model
c_H	Mixing coefficient, mixing time index
C	Constant
d	Stirrer diameter
D	Container/ Bag diameter
DO	Dissolved oxygen concentration
DO*	Dissolved oxygen saturation concentration
F_{surf}	Surface aeration rate
h_R	Stirrer height
H	Filling height of the reactor
Ho	Homogeneity
k_L	Mass transfer coefficient
k_La	Volumetric mass transfer coefficient
m_L	Filling mass in the reactor
M	Torque with liquid filling
M_d	Torque in air (without liquid filling, dead weight torque)
Ne	Newton number
n	Stirrer speed
P	Power, power input
P/V_L	Specific power input
Re	Reynolds number
t	Time
T	Temperature
t_{aer}	Surface aeration time
$t_{63\%}$	Probe response time, time constant of the sensor
$t_{63\%,crit}$	Maximum possible sensor constant without the necessary correction of the measured values
t_{cl}	Time required to flush the headspace
u_G	Superficial gas velocity
u_{Tip}	Tip speed
vvm	Volume of gas per volume of liquid and per minute
$\dot{V}_G$	Gas flow rate
V_L	Reactor filling volume, working volume
V_{tot}	Total volume
y	General measured value
β	Volumetric aeration rate
η	Dynamic viscosity of liquid medium
θ	Mixing time
$\theta_{95\%}$	Mixing time for reaching 95% homogeneity
κ	Conductivity of liquid medium
ν_L	Kinematic viscosity of the liquid medium
ρ_L	Density of liquid medium
τ	Time constant, general

2. Guideline – Experimental determination of specific power input for bioreactors – Torque measurements

The following guideline describes how to determine the specific power input (P/V_L) in bioreactors, a key parameter for process design. This document focuses on torque measurements for bioreactors with a rotating axis. Besides the fact that P/V_L correlates with several process engineering parameters (e.g. mixing time, k_La value, shear forces), it is commonly used for scale-up as well as for the transfer of the processes [Storhas 1994], [Xu et al. 2017]. Many mixing operations, such as suspension of solids, homogenization of liquids and dispersion of gases, are achieved as a result of energy transferred from a stirrer (power input). Successful scale-up using a constant P/V_L has been demonstrated for cell culture applications [Minow et al. 2013], [Möller et al. 2020]. For cell culture applications, a specific power input of between 10 and 250 W/m³ is recommended [Löffelholz et al. 2013a].

2.1. Introduction

2.1.1. Power characteristic

The specific power input (P/V_L) can be determined by torque measurements. This measuring method is based on the installation of a torque sensor on the moving axis of the bioreactor (stirrer shaft). In order to carry out the measurements, the cultivation chamber is filled with liquid (commonly water). Afterwards, the rotation is started and the torque is recorded, P/V_L can then be calculated using Eq. 15.

$$\frac{P}{V_L} = \frac{2 \cdot \pi \cdot n \cdot (M - M_d)}{V_L} \qquad \text{Eq. 15}$$

In this equation, M is the torque, n is the stirrer speed and V_L is the liquid volume. In a stirred bioreactor, P/V_L depends on the impeller diameter (d), the density of the liquid used (ρ_L), the stirrer speed n and the Ne number (dimensionless Power number) (Eq. 16) [Zlokarnik 1999].

$$\frac{P}{V_L} = \frac{Ne \cdot \rho_L \cdot n^3 \cdot d^5}{V_L} \qquad \text{Eq. 16}$$

The Ne number is an important parameter and allows different impeller types and configurations to be compared with each other (see Eq. 17).

$$Ne = \frac{\frac{P}{V_L} \cdot V_L}{\rho_L \cdot n^3 \cdot d^5} = \frac{P}{\rho_L \cdot n^3 \cdot d^5} \qquad \text{Eq. 17}$$

Figure 3 shows the general characteristic of the Ne number as a function of the Re number (Re, calculated using Eq. 18) [Storhas 1994]. The Ne number depends on the stirrer configuration being used, the position where the stirrers are installed on the shaft, the number of stirrers number and geometry of the baffles used.

$$\mathrm{Re} = \frac{\rho_L \cdot n \cdot d^2}{\eta_L} \qquad \textbf{Eq. 18}$$

For Re < 100, Ne is directly proportional to the reciprocal of the Re number (Ne ~ Re^{-1}) (see Figure 3) and laminar flow patterns are present in this region. With increasing Re numbers the transition zone is achieved, where the flow becomes increasingly turbulent. For higher Re (> $1 \cdot 10^4$), the Ne number remains constant due to the fact that the influence of the viscosity can be neglected and the flow patterns are turbulent [Zlokarnik 1999].

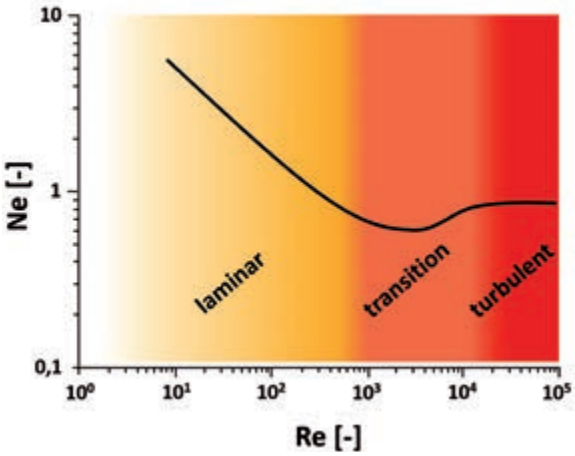


Figure 3: General Ne number characteristics for a stirred tank bioreactor depending on the Re number.

It should also be noted that aeration can have a strong influence on the Ne number. On the one hand, the relative density of the medium is changed by the gas input. Therefore, as aeration increases, the Ne number, and therefore the specific power input, decreases. On the other hand, so-called gas cavities can also form on the stirrer blades. As aeration increases, the Ne number, and therefore the specific power input, decreases. The effect of aeration on the power input is further described in [Zlokarnik 1999].

2.1.2. Factors influencing torque measurements

To perform torque measurements, a torque sensor has to be mounted between the shaft and the drive unit. The sensor is installed between the drive unit and the stirrer shaft and has to be firmly attached in order to avoid undesired displacement. The torque is determined by measuring the torsion of the sensor shaft while the stirrer rotates. These measurements are very sensitive and therefore it is very important that the sensor is installed in the correct position. Otherwise, this may lead to inaccurate results or the sensor may be damaged. The setup of motor, torque sensor and stirrer shaft must be perpendicular to the horizontal axis and any deviations in the horizontal axis must be compensated for, by bellow couplings, for example. It may be necessary to eliminate lateral forces by installing special bearings (e.g. air

bearings). Figure 4 shows potential misalignments which can occur when the torque sensor is mounted between the stirrer shaft and the drive unit.

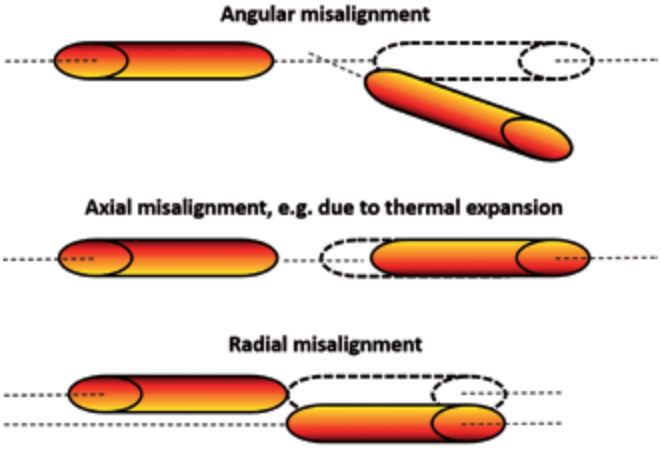


Figure 4: Potential misalignments which can occur when a torque sensor is installed (modified from [Burster 2009]). The upper image shows a misalignment where the angle between torque sensor and the stirrer shaft is not perpendicular. The middle image shows a compression, and the bottom picture shows the situation where the torque sensor and the shaft are displaced.

Note I:
The sensor should be selected based on the expected torque range. Furthermore, the upper and lower detection limit should also be noted. An oversized sensor can result in inaccuracies in the measured values. If the measurement range is too low, the sensor may get damaged.

2.2. Materials

- **Bioreactor system**
- **Control unit**
- **Computer-aided data acquisition**
- **Torque sensor**
- **Torque transducer**
- **2 couplings**
- **Air bearing**

2.3. Experimental setup

1. Mount the torque sensor between the modified stirrer shaft and the drive unit (see Figure 5).
   - The installation of two couplings is recommended for all bioreactor sizes in order to compensate for any misalignments (see Figure 4). The installation of an air bearing is particular recommended for small scale bioreactors in order to reduce friction.
2. Install a fixing point for the torque sensor on the shaft.
3. Install the modified shaft inside the bioreactor.
4. Install the control unit and the data acquisition program or software.
5. Prepare the bioreactor.
6. Initialize the control unit.
7. Connect the torque sensor to the torque transducer using the corresponding cable.

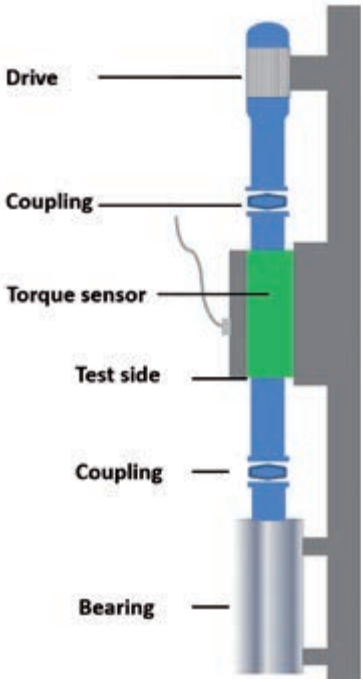


Figure 5: Example setup for torque measurements (modified from [Burster 2009]).

2.4. Measurement procedure

By varying the stirrer speed and/or the viscosity of the fluid, the power characteristics can be determined for a specific impeller type (see Figure 3). To determine the Ne number, and thus allow different stirrer designs to be compared, turbulent flow conditions should be present. This is indicated by a constant Ne number when plotted against the Re number (Figure 3). Turbulent flow conditions in bioreactors can also be assumed for a Re number (see Eq. 18) above 10,000 [Zlokarnik 1999].

2.4.1. Determining the dead weight torque

It is recommended to perform the torque measurements (M) first and then the measurements of the deadweight torque (M_d). The system, especially the bearings, require a certain running-in period.

8. Reset the torque measurement to zero before the commissioning of the sensor.
9. Start data recording of the torque transducer.

10. Start agitation.
11. Determine the torque for the empty vessel (dead weight torque).
12. Record at least 60 measuring points in 1 minute.
13. Stop data recording.
14. Stop agitation.

2.4.2. Determining the torque

15. Fill up the cultivation chamber to the maximum working volume with pure water.
16. Adjust control parameters:
    - Start agitation on the control unit and adjust a constant temperature of e.g. 25 °C (±0.5 °C; Table 1).
    - If desired, set the aeration rate on the control unit.
17. Start data recording of the torque transducer.
18. Record at least 60 measuring points in 1 minute.
19. Stop agitation.
20. Repeat steps 17 to 20 at least five times.

Note II:
Due to the fact that for microbial applications the gassing can influence specific power input, measurements including aeration are noted (see Section 2.1.1). Typical aeration rates for cell culture applications are ~ 0.1 vvm. The influence of these low aeration rates on the specific power input can be neglected [Zlokarnik 1999]. Measurements at different stirrer speeds should be performed to evaluate the power characteristics of the bioreactor (see Section 2.1.1). A sampling rate of 1 Hz should be used for the measurements. To confirm the resultant Ne number, measurements for multiple different stirrer speeds are recommended (more measurements increase accuracy).

The measurements of each parameter setting should be performed at least three times and the mean values are to be calculated (see Eq. 19). If the standard deviation is above 10 %, the measurements must be repeated. Inaccuracies can result from misalignments in the experimental setup (see Figure 4) or imbalances in the stirrer shaft.

2.5. Evaluation

21. The average torque is then calculated by the arithmetic mean of the difference between the torque and the dead weight torque (M_d) (Eq. 19).

$$\overline{M}(25°C) = \frac{1}{j}\sum_{i=1}^{j}(M_i - M_{d,i}) = \frac{(M_1 - M_{d,1}) + (M_2 - M_{d,2}) + (M_3 - M_{d,3}) + ... + (M_j - M_{d,j})}{j} \qquad \textbf{Eq. 19}$$

2.6. Appendix I

An example of how to calculate the Ne number is given below (calculation according to Eq. 17).

- Configuration: Rushton turbine + 3-blade segment impeller ⟶ stirrer diameter= 0.143 m
- V_L = 50 L = 0.050 m³
- Filled with water ρ_L = 997 kg/m³
- Temperature: 25 °C
- u_{Tip} = 1.5 m/s

Using Eq. 20, the stirrer speed can be calculated from the tip speed.

$$n = \frac{u_{Tip}}{\pi \cdot d} = 3.33\ s^{-1} \qquad \textbf{Eq. 20}$$

22. The average of at least 60 measuring points for the dead weight torque (M_d) was calculated (for the given u_{Tip}).

Measurement number [-]	Measured M_d [Nm]	Measurement number [-]	Measured M_d [Nm]
1	0.013	32	0
2	0.010	33	0.001
3	0.010	34	0.003
4	0.009	35	0.002
5	0.010	36	0.003
6	0.009	37	0.005
7	0.008	38	0.004
8	0.008	39	0.003
9	0.013	40	0.002
10	0.024	41	0.002
11	0.036	42	0.003
12	0.048	43	0.002
13	0.061	44	0.003
14	0.074	45	0.004
15	0.075	46	0.005
16	0.068	47	0.004
17	0.054	48	0.003
18	0.030	49	0.003
19	0.022	50	0.003
20	0.025	51	0.004
21	0.028	52	0.004
22	0.022	53	0.006
23	0.010	54	0.006
24	0.008	55	0.004
25	0.010	56	0.002
26	0.007	57	0.001
27	0.004	58	0.003
28	0.004	59	0.004
29	0.004	60	0.004
30	0.002		
31	0.000	**Average M_d**	**0.0133**

23. The average of at least 60 torque measuring points (M_1, M_2, M_3) was calculated.

Measurement number [-]	Run M_1 [Nm]	Run M_2 [Nm]	Run M_3 [Nm]
1	0.40	0.42	0.42
2	0.34	0.36	0.37
3	0.35	0.35	0.36
4	0.35	0.31	0.32
5	0.37	0.32	0.33
6	0.37	0.39	0.40
7	0.39	0.40	0.40
8	0.39	0.43	0.40
9	0.37	0.33	0.34
10	0.36	0.31	0.32
11	0.39	0.39	0.40
12	0.39	0.37	0.38
13	0.38	0.42	0.43
14	0.36	0.33	0.34
15	0.36	0.34	0.35
16	0.35	0.36	0.37
17	0.36	0.32	0.33
18	0.36	0.33	0.34
19	0.36	0.39	0.40
20	0.38	0.43	0.44
21	0.39	0.40	0.41
22	0.39	0.41	0.40
23	0.37	0.34	0.35
24	0.37	0.40	0.41
25	0.38	0.38	0.39
26	0.36	0.37	0.38
27	0.35	0.34	0.35
28	0.37	0.32	0.33
29	0.37	0.38	0.39
30	0.35	0.32	0.33
31	0.37	0.33	0.34
32	0.39	0.36	0.37
33	0.39	0.37	0.38

Measurement number [-]	Run M_1 [Nm]	Run M_2 [Nm]	Run M_3 [Nm]
34	0.38	0.40	0.41
35	0.37	0.36	0.37
36	0.35	0.38	0.38
37	0.35	0.38	0.38
38	0.34	0.31	0.31
39	0.36	0.38	0.38
40	0.37	0.41	0.38
41	0.37	0.34	0.35
42	0.37	0.41	0.37
43	0.37	0.35	0.36
44	0.37	0.33	0.34
45	0.37	0.34	0.35
46	0.35	0.34	0.35
47	0.36	0.34	0.35
48	0.35	0.33	0.34
49	0.35	0.38	0.39
50	0.39	0.38	0.39
51	0.39	0.34	0.35
52	0.38	0.43	0.37
53	0.35	0.38	0.39
54	0.36	0.38	0.39
55	0.37	0.38	0.39
56	0.39	0.39	0.38
57	0.39	0.39	0.38
58	0.36	0.44	0.38
59	0.34	0.31	0.32
60	0.33	0.30	0.31
61	0.34	0.31	0.32
62	0.39	0.30	0.31
Average $\overline{M}(25\ °C)$	**0.368**	**0.366**	**0.367**

2. GUIDELINE
– EXPERIMENTAL DETERMINATION OF SPECIFIC POWER INPUT FOR BIOREACTORS
– TORQUE MEASUREMENTS

24. Calculating the average torque using Eq. 19.

$$\overline{\mathrm{M}}(25^{\circ}\mathrm{C}) = \frac{(0.368 - 0.0133) + (0.366 - 0.0133) + (0.367 - 0.0133)}{3} = 0.354\ \mathrm{Nm} \qquad \textbf{Eq. 21}$$

25. The specific power input was calculated using Eq. 15.

$$\frac{\mathrm{P}}{\mathrm{V_L}} = \frac{0.354\ \mathrm{Nm} \cdot 2 \cdot \pi \cdot 3.333\mathrm{s}^{-1}}{0.05\ \mathrm{m}^3} = 148\ \frac{\mathrm{W}}{\mathrm{m}^3} \qquad \textbf{Eq. 22}$$

26. The Ne number can be calculated using Eq. 17.

$$\mathrm{Ne} = \frac{148\ \frac{\mathrm{W}}{\mathrm{m}^3} \cdot 0.05\ \mathrm{m}^3}{997\ \frac{\mathrm{kg}}{\mathrm{m}^3} \cdot (3.333\ \mathrm{s}^{-1})^3 \cdot (0.145\ \mathrm{m})^5} = 3.1 \qquad \textbf{Eq. 23}$$

3. Guideline – Experimental determination of mixing time – Decolourisation and sensor method

Efficient homogenisation and suspension of the fermentation broth is a general requirement for every bioreactor. These processes avoid the sedimentation of cells and prevent temperature and concentration gradients inside a bioreactor, which can have negative effects on cell growth and production [Storhas 1994]. Mixing in bioreactors aims to achieve a unique, homogeneous solution at the molecular level. Energy transfer (specific power input per volume) generated, for example, by a stirrer, induces a convective fluid flow inside the vessel and decreases the diffusion distances between chemical components [Zlokarnik 1999]. The mixing efficiency of a bioreactor can be described by the quality of the mixture and mixing time parameters. These parameters can be influenced by the reactor geometry, impeller design, power input and the fluid properties [Löffelholz et al. 2013a]. The mixing time is defined as the time required to achieve a certain degree of homogeneity. Two different methods for determining the mixing time, which depend on the availability of an optical window to the fluid of the bioreactor system, are commonly used:

» The decolourisation method (iodometry) is the recommended method for determining the mixing time in bioreactor systems with optical windows that allows the user to view the fluid.

» Sensor methods (conductivity method) are recommended for determining the mixing time in bioreactor systems without optical accessibility to the fluid.

3.1. Materials

Depending on optical accessibility to the fluid, the following equipment is required to determine mixing times (Table 3).

Table 3: Equipment required to determine mixing time by either the decolourisation or sensor method.

	Decolourisation method (iodometry)	Sensor method (conductivity)
Bioreactor system	x	x
Control unit	x	x
Computer-aided data acquisition	x	x
Stop watch	x	
Conductivity probe		x

3.2. Experimental setup

1. Set up bioreactor, control unit and data acquisition software. To determine the mixing time using the sensor method, connect the conductivity sensor to the control unit/data acquisition and install the sensor in the bioreactor.
2. Fill the bioreactor system up to the maximum working volume with pure water (decolourisation method) or depending on the measurement range of the sensor, pure water with conductivity solution (solution 4; Section 3.6.2; sensor method).
3. Start agitating the solution and adjust the solution to a constant temperature of e.g. 25 °C (±0.5 °C; Table 1).

3.3. Response time for the sensor method

If the response time of the conductivity measuring track is unknown, for instance after long-term storage or multiple autoclave cycles, the response time ($t_{63\%}$) should be determined using a step response.

4. Fill two beakers (beaker A and beaker B) with a known volume of pure water.
5. Add 1 mL/L conductivity solution (solution 4; Section 3.6.2) to beaker B.
6. Place the conductivity sensor into beaker A, so that the probe is covered with pure water.
7. If a constant conductivity value is obtained in beaker A, start the data acquisition and then transfer the conductivity sensor into beaker B immediately (the sensor must be covered by pure water or conductivity solution).
8. Stop data acquisition as soon as a constant conductivity value is obtained in beaker B.
9. Determine the response time by using 63 % of the step response signal ($t_{63\%}$). Analysis of the response time can be performed by plotting the normalised conductivity (Eq. 24) as a function of time (Figure 6) with a minimum of seven conductivity values.

$$\kappa_{\mathrm{perc}}(\mathrm{t}) = \frac{\kappa(\mathrm{t})}{\kappa_{\infty}} \cdot 100 \qquad \textbf{Eq. 24}$$

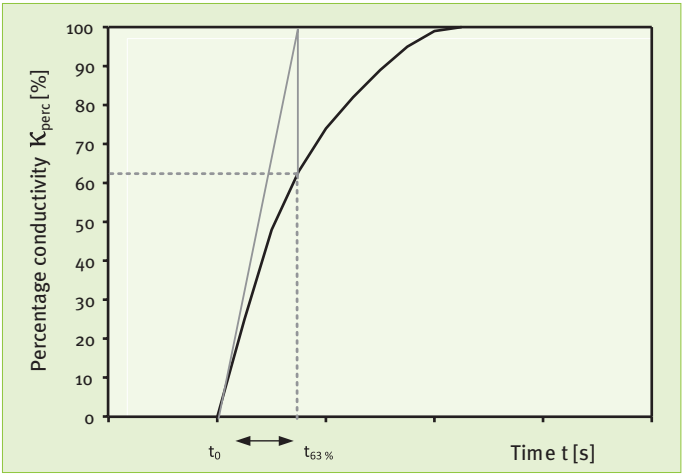


Figure 6: Experimental determination of the response time $t_{63\%}$ by plotting the normalised conductivity as a function of time (t).

Note III:
When determining the mixing time, computer-aided data acquisition with a sampling rate of maximum 1 Hz helps to improve the accuracy of the results. In general, a conductivity measuring track response time should be as low as possible because response time is limiting the maximum mixing time measurement.

3.4. Measurement procedure

Mixing time should be investigated using fluid colour change, when the bioreactor has an optical window to the fluid. These methods provide the possibility of localising zones of poor mixing. For bioreactor systems without an optical window to the fluid, sensor methods using conductivity are recommended for measuring the mixing time. Therefore, special attention must be paid to the positioning of the probes.

Note IV:
Agitation should be defined by the specific power input, with the mixing time determined using the maximum and the process relevant minimum specific power inputs. Based on this approach, typical process power inputs can be used to determine the mixing time.

The mixing time should be determined for the maximum working volume of the bioreactor. This represents the maximum challenge with regard to mixing. Afterwards, the working volume can be reduced (according to process specific requirements).

3.4.1. Decolourisation method (iodometry)

The decolourisation method (iodometry) requires a bioreactor with an optical window to the fluid. The decolourisation method is based on a redox reaction. The solution contains a dye and a complete colour change can be observed after the addition of a decolourising substance. A huge advantage of the decolourisation method is that it is very simple and cost-efficient [Löffelholz et al. 2013]. This method enables the detection of possible zones with poor mixing. Installation of sensors is not required. However, the accuracy of the measurements is determined by the performer and his subjective errors. In addition, the method can only determine values for 95 % homogeneity.

10. Set the desired agitation parameters in the bioreactor system.
11. Start agitating at a constant temperature of 25 °C (±0.5 °C; Table 1).
12. Add 2 mL/L iodine / potassium iodide (solution 1; Section 3.6.1).
13. Add 5 mL/L starch solution under agitation (solution 2; Section 3.6.1).
14. Wait at least 360 s (colour change to dark blue) in order to ensure a completely homogeneous chemical solution and a (quasi-)stationary fluid flow pattern.
15. Add 4 mL/L sodium thiosulfate solution (solution 3; Section 3.6.1) under agitation (by pouring or pumping) and then immediately start to measure the time using a stop watch.
16. Stop the time measurement when a colour change from dark blue to colourless is achieved.
17. To determine the mixing time, the solution has to be exchanged after each measurement.

Note V:
The addition of sodium thiosulfate should be carried out quickly and without a large impulse in order to avoid influences on the measured mixing time.

The visual judgment of the decolourisation process in this method leads to large deviations in results, caused by the subjectivity of the person carrying out the experiments. Therefore, it is recommended that a video recording is made in parallel.

If no colour change to dark blue is obtained in step 17, which could occur in bioreactor scales above approximately 100 L, repeat process steps 2 to 3 and 10 to 17 in order to determine the mixing time.

3.4.2. Sensor method (conductivity)

The sensor method is based on changes to a physical property of the fluid, which are measured by at least one sensor. An established sensor technique is the conductivity method. An electrolyte solution is added to the fluid inside the bioreactor and the conductivity change is recorded by a sensor. For bioreactors without optical windows, sensor methods provide a simple and practical approach to the investigation of mixing times. However, the measurements depend on the tracer type, the location of the tracer

addition, the number of sensors, their positions and response times, the sample volume and the mixing criterion [Löffelholz et al. 2013].

18. Install the conductivity probe at the defined position in the bioreactor.
19. Set the desired agitation parameters in the bioreactor system, start agitation at a constant temperature of 25 °C (±0.5 °C; Table 1) and wait at least 360 s in order to ensure a (quasi-)stationary fluid flow pattern.
20. Start data acquisition.
21. Immediately add 1 mL/L conductivity solution (solution 4; Section 3.6.2) under agitation at a defined position (by pouring or fast pumping).
22. The measurement is complete when a stable conductivity concentration value is reached.
23. The solution has to be exchanged after three measurements, depending on the measurement range of the sensor.
24. To determine the mixing time, the investigations should be performed up to ten times repeating process steps 19 to 24.

Note VI:
In order to prevent falsification of the mixing time, the conductivity solution (tracer) has to be added in one step and in a quick manner. To increase the accuracy of mixing time measurements, the response time of the sensor should be 5 times faster than the measured mixing time. Additionally, 95 % homogeneity is assumed to be adequate mixing performance [Xing et al. 2009]. For mixing times exceeding 360 s (step 19) repeat process steps 18 to 24. However, instead of waiting for 360 s to ensure a (quasi-)stationary fluid flow pattern, set the waiting period to the newly-established longer mixing time.

3.5. Evaluation

3.5.1. Decolourisation method (iodometry)

25. The mixing time values (θ_1; θ_2; θ_3; θ_j with j = count) are then averaged by the arithmetic mean (Eq. 22).

$$\overline{\theta}(25°C) = \frac{1}{j}\sum_{i=1}^{j}\theta_i = \frac{\theta_1 + \theta_2 + \theta_3 + ...\theta_j}{j} \qquad \textbf{Eq. 25}$$

26. If the mixing time measurements for a defined stirrer speed have been performed at least 5 times (steps 10 to 17), the standard deviation should be in the range of 10 %.

3.5.2. Sensor method (conductivity)

27. Determine the maximum and minimum normalised conductivity value (κ_∞ and κ_0).

28. Use Eq. 26 to calculate the homogeneity ($H_o(t)$) for each investigation.

$$H_o(t) = \frac{\kappa(t) - \kappa_0}{\kappa_\infty - \kappa_0} \qquad \textbf{Eq. 26}$$

29. Plot the homogeneities as a function of time on a line plot.

30. Add the constant lines of 0.95 (95 %) and 1.05 (105 %) as a function of time.

31. Determine the mixing time by using the value from the graph at the point where the 0.95 (95 %) line crosses the homogeneity. If the homogeneity fluctuates, the mixing time is defined as the time at which the graph crosses either the 0.95 (95 %) or the 1.05 (105 %) homogeneity line for the last time.

32. The mixing time values (θ_1; θ_2; θ_3; θ_j with j = count) are then averaged by the arithmetic average (Eq. 25).

33. In situations where the mixing time has been determined ten times [Kraume et al. 2001], the percentage deviation should be in the range of 10 %.

34. Re-check the conductivity sensor calibration and repeat steps 18 to 24.

3.6. Appendix II

3.6.1. Preparation of the solutions for the decolourisation method (iodometry)

Chemical
Starch
Sodium thiosulfate pentahydrate
Iodine
Potassium iodine

Solution 1: 1 L Iodine / potassium iodide

- Weigh out 40 g potassium iodide in a beaker,
- add a magnetic stirrer bar,
- add 300 mL pure water into the beaker,
- dissolve the solution,
- add 20 g iodine,
- add 200 mL pure water,
- dissolve the solution,
- fill up to 1 L total volume with pure water,
- transfer the solution into a 1 L glass bottle,
- protect the iodine / potassion iodide solution from light using aluminium foil.

For pilot and production scale bioreactors, the concentration of the solutions should be adapted according to the sodium thiosulfate solution.

Iodine / potassium iodide solution always must be freshly prepared [Kraume 2003].

Solution 2: Starch solution (10 g/L)

- Weigh out 10.0 g starch in beaker A,
- add 10 mL pure water into beaker A,
- mix the starch/water solution with a spatula,
- boil 80 mL pure water in a separate beaker or graduated flask (B),
- add a magnetic stirrer bar to beaker B,
- transfer the starch/water solution into beaker B,

» remove the residual starch from beaker A with 10 mL pure water and add it to beaker B (boiling water),

» dissolve the solution until it is clear,

» fill up to 1 L total volume with pure water,

» cool down the starch solution,

» transfer the starch solution into a 1 L glass bottle.

The shelf-life of the starch solution is approximately 1 week and should be stored in a fridge at 4 °C.

Solution 3: 1 L Sodium thiosulfate (0.1 M)

» Weigh out 24.8 g sodium thiosulfate pentahydrate in a beaker,

» add a magnetic stirrer bar to the beaker,

» add 500 mL pure water in a graduated flask,

» dissolve the solution,

» fill up to 1 L total volume with pure water,

» transfer the sodium thiosulfate solution into a 1 L glass bottle,

» store the solution at room temperature.

For pilot and production scale bioreactors, the concentration of the solutions should be adapted up to a concentration of 3 M sodium thiosulfate.

The shelf-life of sodium thiosulfate solution is approximately 2 weeks.

3.6.2. Preparation of the solutions for the sensor method (conductivity)

Chemical
Potassium chloride
Potassium phosphate dibasic
Potassium phosphate monobasic

Solution 4: 1 L Conductivity solution (KCl solution or KPi buffer)

Preparation of KCl solution (4 M):

» Add 700 mL pure water to a beaker,

» weigh out 298.2 g KCl,

» dissolve the solution,

» fill-up to 1 L total volume with pure water,

» transfer the buffer solution into a 1 L glass bottle.

or

Preparation of KPi buffer solution:

» Add 700 mL pure water to a beaker,

» weigh out 212.28 g potassium phosphate dibasic,

» add potassium phosphate dibasic to the beaker,

» dissolve the solution,

» weigh out 106.06 g potassium phosphate monobasic,

» add the potassium phosphate monobasic to the beaker,

» fill up to 1 L total volume with pure water,

» transfer the buffer solution to a 1 L glass bottle (shelf life of one year).

3.6.3. Example

3.6.3.1. Iodometry

The mixing time was determined in a stirred bioreactor system (2 L working volume) for cell culture applications. The bioreactor was equipped with a two-stage segment blade stirrer. The solution was added at the fluid surface.

» The bioreactor system was prepared (steps 1 to 3) using the following parameters:

- V = 2 L
- u_{Tip} = 1.2 m/s

» The measurement process for determining the mixing time (steps 10 to 17; u_{Tip} = 1.2 m/s) was performed in triplicate, recorded by a camera (Figure 7).

» Based on the experimental raw data, it was possible to determine the mixing time using steps 25 and 26, as well as Eq. 25 from the previously described evaluation process. Based on the video, the mixing time was 4 s (Table 4 and Eq. 27).

3. GUIDELINE
– EXPERIMENTAL DETERMINATION OF MIXING TIME
– DECOLOURISATION AND SENSOR METHOD

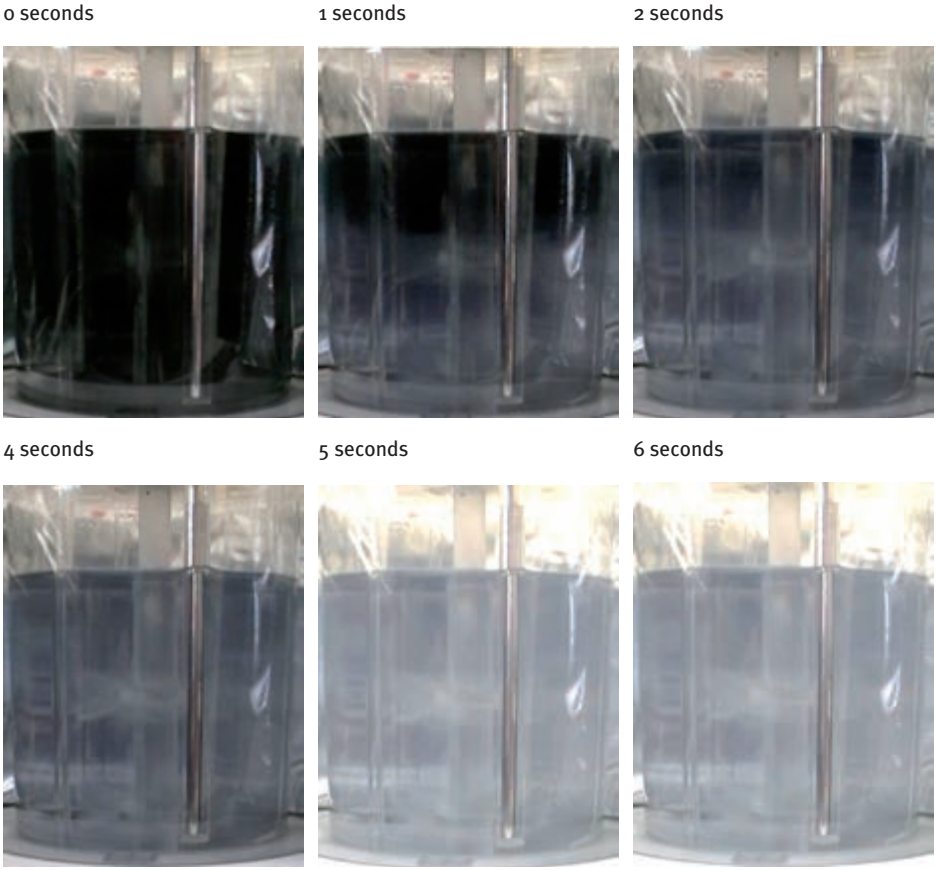


Figure 7: Determining the mixing time based on the decolourisation method using a video camera. Specified is the time after addition of sodium thiosulfate, 0 seconds is the moment of addition.

Table 4: Overview of the exerimental raw data for the decolourisation method.

Run	θ_1	θ_2	θ_3	θ_4	θ_5
Mixing time [s]	3.5	3.4	3.5	3.6	3.6

$$\overline{\theta}(25^{\circ}\mathrm{C}) = \frac{1}{\mathrm{j}}\sum_{\mathrm{i}=1}^{\mathrm{j}}\theta_{\mathrm{i}} = \frac{3.5\ \mathrm{s} + 3.4\ \mathrm{s} + 3.5\ \mathrm{s} + 3.6\ \mathrm{s} + 3.6\ \mathrm{s}}{5} = 3.5\ \mathrm{s} \qquad \textbf{Eq. 27}$$

3.6.3.2. Conductivity method

The mixing time was determined in a stirred bioreactor system (10 L working volume) for cell culture applications. The bioreactor was equipped with a single marine impeller and the solution was added at the fluid surface.

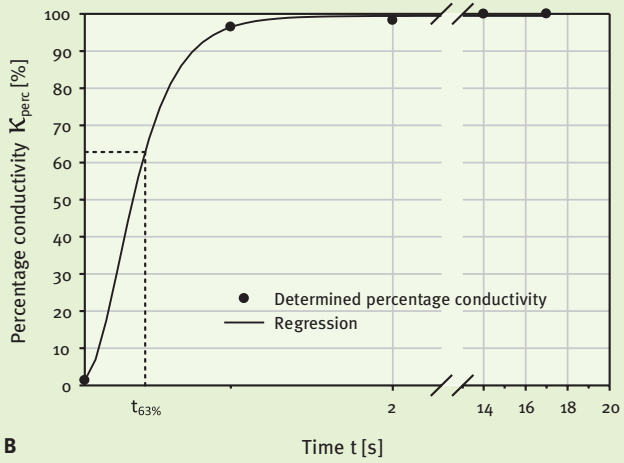
» The bioreactor system was prepared (steps 1 to 3) using the following parameter:

- V_L = 10 L
- u_{Tip} = 1.05 m/s

» The conductivity sensor response time was measured (steps 4 to 9) using the following generated data:

Time t [s]	Percentage conductivity κ_{perc} [%]
0	0
1	96.5
2	98.3
5	99.3
8	99.7
11	99.7
14	100.0
17	100.0

A



B

Figure 8: Determining the response time $t_{63\%}$ of a conductivity sensor based on raw data (A) and plotting the percentage conductivity as a function of time (B).

» The measurement process for determining the mixing time (steps 10 to 17) was performed in triplicate (see Table 5) using the following parameter:

- u_{Tip} = 1.05 m/s

3. GUIDELINE
– EXPERIMENTAL DETERMINATION OF MIXING TIME
– DECOLOURISATION AND SENSOR METHOD

Table 5: Overview of the experimental raw data for the conductivity method.

Run 1			Run 2			Run 3		
Time t_1 [s]	Conductivity κ_1 [µS/cm]	Homogeneity Ho_1 [-]	Time t_2 [s]	Conductivity κ_2 [µS/cm]	Homogeneity Ho_2 [-]	Time t_3 [s]	Conductivity κ_3 [µS/cm]	Homogeneity Ho_3 [-]
0	196.29	0.00000	0	378.67	0.00000	0	561.85	0.00000
1	196.3	0.00005	1	379.43	0.00414	1	562.07	0.00121
2	196.31	0.00011	2	378.86	0.00103	2	562.12	0.00148
3	196.32	0.00016	3	378.82	0.00082	3	562.18	0.00181
4	196.35	0.00033	4	378.83	0.00087	4	562.11	0.00143
5	196.37	0.00044	5	378.78	0.00060	5	561.98	0.00071
6	196.38	0.00049	6	378.69	0.00011	6	561.96	0.00060
7	196.41	0.00066	7	378.77	0.00054	7	561.96	0.00060
8	448.75	1.37813	8	659.13	1.52773	8	562.03	0.00099
9	365.23	0.92221	9	516.23	0.74932	9	931.75	2.03409
10	392.26	1.06976	10	638.22	1.41383	10	744.8	1.00605
11	389.94	1.05710	11	576.5	1.077623	11	753.36	1.053121
12	382.96	1.01900	12	567.07	1.02626	12	751.37	1.04218
13	383.53	1.02211	13	562.99	1.00403	13	749.08	1.02958
14	380.29	1.00442	14	563.25	1.00545	14	743.69	0.99995
15	380.21	1.00398	15	562.65	1.00218	15	744.7	1.00550
16	379.9	1.00229	16	562.72	1.00256	16	744.01	1.00170
17	379.58	1.00055	17	562.58	1.00180	17	743.98	1.00154
18	379.71	1.00126	18	562.58	1.00180	18	743.84	1.00077
19	379.53	1.00027	19	562.51	1.00142	19	743.81	1.00060
20	379.52	1.00022	20	562.44	1.00103	20	743.65	0.99973
21	379.65	1.00093	21	562.38	1.00071	21	743.7	1.00000
22	379.49	1.00005	22	562.38	1.00071			
23	379.51	1.00016	23	562.29	1.00022			
24	379.55	1.00038	24	562.26	1.00005			
25	379.48	1.00000	25	562.27	1.00011			
			26	562.41	1.00087			
			27	562.32	1.00038			
			28	562.26	1.00005			
			29	562.27	1.00011			
			30	562.16	0.99951			
			31	562.25	1.00000			

» Based on the experimental raw data, it is possible to determine the mixing time using the previously described evaluation process (steps 27 to 34)

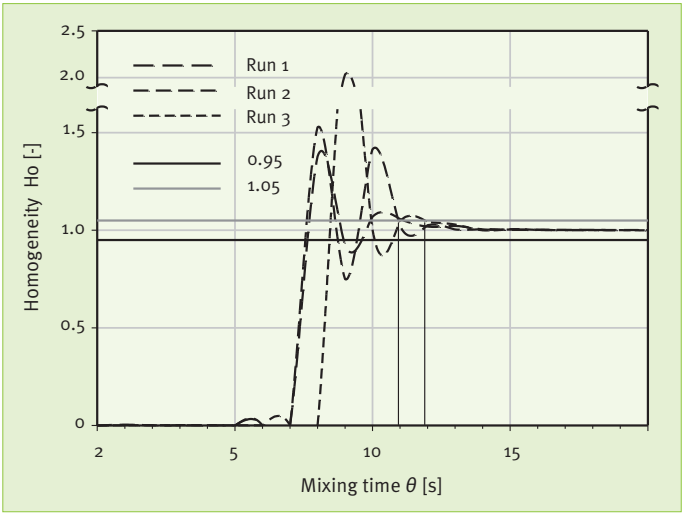


Figure 9: The evaluation was based on the homogeneity as a function of mixing time.

$$\overline{\theta}(25^{\circ}\mathrm{C}) = \frac{1}{\mathrm{j}} \sum_{\mathrm{i}=1}^{\mathrm{j}} \theta_{\mathrm{i}} = \frac{11\ \mathrm{s} + 11\ \mathrm{s} + 12\ \mathrm{s}}{3} = 11\ \mathrm{s} \qquad \textbf{Eq. 28}$$

4. Guideline – Experimental determination of the volumetric mass transfer coefficient – Gassing-out method

The volumetric oxygen mass transfer coefficient (k_La) is a key parameter in biochemical engineering and is defined as the reciprocal time for the transfer of oxygen from the gaseous to the liquid phase. The k_La value is determined using the gassing-out method with headspace exchange which measures the dissolved oxygen in a fluid, using either conventional oxygen sensors (amperometric or optical) or a single-use sensor patch. Nitrogen is used to strip oxygen from the liquid. The headspace exchange should be performed to reduce the residual nitrogen concentration in the gassing phase. This results in a more precise measurement of the oxygen transfer. Therefore, this guideline was modified concerning the headspace exchange compared to the first edition of these recommendations [DECHEMA 2016]. When determining the k_La value during the subsequent aeration phase, computer-aided data acquisition with a sampling rate of between 1 and 5 s is required in order to obtain accurate results. The use of a salty medium (1 x PBS buffer solution) is suggested for comparative studies. The PBS buffer solution is used to mimic the coalescence property of cell culture media and enables the application of single-use sensor patches, which require a minimum ion concentration.

4.1. Materials

- Bioreactor system
- Control unit
- Computer-aided data acquisition
- Nitrogen supply
- Air supply
- 2 Beakers or graduated flasks
- 1 x PBS buffer solution
- Oxygen sensor

4.2. Experimental setup

1. Set up the bioreactor, the control unit and the data acquisition software.
2. Fill up the bioreactor system to the desired working volume with 1 x PBS buffer solution (see Chapter 4.5.1).

3. Connect the dissolved oxygen (DO) sensor to the control unit. If Clark probes are used, they must be polarised for at least 6 h. When using optical, pre-installed, single-use sensors, wait at least 2 h to equilibrate the sensor patches.
4. Adjust the 1 x PBS buffer solution to a constant temperature of 37 °C (±0.5 °C; Table 1).
5. Start agitating the 1 x PBS buffer solution.

4.2.1. Calibration (two-point calibration)

The two-point calibration is performed by supplying nitrogen and air to the test medium using a gassing device or via the head space. When optical, pre-installed, single-use sensors are used, an initial calibration may be necessary and these parameters should be entered manually and step 6 followed. Since the calibration procedure depends on the controller unit, steps 7 to 10 may vary and the control unit user guide should be consulted. In most cases, a predefined automated calibration procedure is provided.

6. Start data acquisition (T = 37 °C ±0.5 °C; Table 1).
7. Start the nitrogen supply to the 1 x PBS buffer solution to strip out the dissolved oxygen (zero adjustment). Once a constant DO value is achieved, the DO value should be calibrated to 0 %.
8. Stop the nitrogen supply.
9. Start the air supply to achieve maximum DO conditions.
10. Calibrate the measured DO value to 100 % when a stable signal is achieved.

4.2.2. Response time

If the oxygen sensor response time is unknown, for instance after long-term storage or multiple autoclave cycles, the response time ($t_{63\%}$) must first be determined by a step response. However, this can only be done for conventional sensors. For pre-installed, single-use sensor patches, the response time is usually provided in the sensor manual.

11. Fill two beakers (beaker A and beaker B) with 1x PBS buffer solution and adjust solutions in both beakers to a constant temperature of 37 °C (±0.5 °C; Table 1). In order to consider the inflow velocity, beaker B should be mixed.
12. Eliminate the dissolved oxygen in beaker A using nitrogen.
13. Add air to beaker B to saturate the 1 x PBS buffer solution with oxygen.
14. Put the DO sensor into beaker A so that the probe is covered with 1 x PBS buffer solution and start data acquisition.
15. When a constant DO value is obtained in beaker A, immediately put the DO sensor into beaker B (the probe must be covered by the 1 x PBS buffer solution).

16. Stop data acquisition when a constant DO value has been obtained in vessel B.

17. Determine the response time using the step response at 63 % DO ($t_{63\,\%}$). Analysis of the response time is performed by plotting the concentration of DO as a function of time (Figure 10) with a minimum of seven DO values. For DO transmitter with a response time exceeding 1 second, add the separate transmitter response time to the sensor response time.

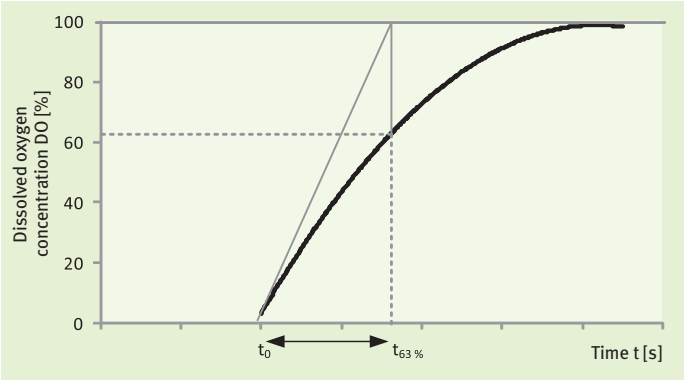


Figure 10: Experimental determination of the response time $t_{63\%}$ by plotting the concentration of DO as a function of time.

Note VII:
In general, an oxygen sensor's response time should be as low as possible. Since relatively low aeration rates (up to 0.1 vvm) and specific power input (up to 250 W/m³) are used for cell culture applications [Löffelholz et al 2013], a maximum response time of $t_{63\%,\,crit} < 30$ s is recommended, which makes it possible to accurately measure a maximum k_La ($t_{63\%,\,crit} = 5/k_La_{max}$) of 120 h^{-1} [Zlokarnik 1999]. For higher maximum k_La values, a significantly shorter response time is required. In the case of microbial fermentations, with expected k_La values above 300 h^{-1}, the response time should be less than 3 s.

4.3. Measurement procedure

Determining k_La values is based on the alternating elimination and following accumulation of dissolved oxygen in a 1 x PBS buffer solution in the bioreactor system. Nitrogen or process air is introduced into the solution via a gassing device (sparger, dip tube, open pipe, etc.) or the fluid surface. When using gassing devices, especially microspargers, attention must be paid to erroneous measurement signals, which may be caused by gas bubbles adhering to the sensor membrane.

In bioreactor systems, the head space must be flushed before the start of the k_La investigation. Determination of the time required to flush the head space (t_{cl}) is covered in Section 4.5.3, which also describes the time required to exchange a gas phase within the head space of a bioreactor. Once the head space has been flushed, continue with step 22. If the head space in bioreactor systems is not flushed properly before each k_La investigation, reduced k_La values will be obtained [Malig et al. 2011].

18. Set the desired agitation parameters and start agitating the bioreactor system at desired process temperature of e.g. 37 °C (±0.5 °C; Table 1). Introduce nitrogen to eliminate the oxygen in the 1 x PBS buffer solution (typically DO = 0 %).

19. Stop the nitrogen supply (only for surface-aerated bioreactors).

20. **Flushing the head space for surface-aerated bioreactors**
    Stop agitating. When the liquid is no longer in motion, start the process air supply to flush the head space for a period of t_{cl} (Section 4.5.3).

21. Start data acquisition.

22. **Measurement procedure for gassing device**
    Stop the nitrogen supply.
    Set the process air supply to the desired aeration rate and surface aeration rate of 0.05 vvm and desired agitation rate. Start aeration and agitation.
    Measurement procedure for surface aeration
    Set the process air supply to the desired aeration rate, start agitation and aeration.

23. The measurement is complete when a saturated oxygen concentration has been reached, which is indicated by a stable DO value of 100 %. This ensures that the sensor is still correctly calibrated. For evaluation purposes, a DO saturation rate of between 10 % and 90 % is used.

24. To determine the k_La value, the investigations should be performed at least three times, but preferably up to five times, by repeating process steps 18 to 23.

Note VIII:
Agitation should be defined by the specific power input, with the k_La value determined using the maximum and the process relevant minimum specific power inputs. Based on this approach, typical process power inputs can be used to determine the k_La value.

The k_La value should be determined for the maximum working volume of the bioreactor. Afterwards the working volume can be reduced (according to the process specific requirements).

To compare different bioreactors and sparger designs, the back pressure (stainless steel) and the superficial gas velocity (calculated from the aeration rate) should be recorded.

4.4. Evaluation

25. Measure the minimum DO ($DO(t_0)$) and the DO at saturation (DO*).

26. The k_La is then determined based on Eq. 29 to Eq. 31 with DO(t) as the measured DO during the experiment:

$$\frac{dDO}{dt} = k_La \cdot (DO^* - DO) \qquad \textbf{Eq. 29}$$

$$\int_{DO(t=0)}^{DO(t)} \frac{dDO}{(DO^* - DO)} = \int_{t=0}^{t} k_La \cdot dt \qquad \textbf{Eq. 30}$$

$$-\ln\left(\frac{DO^* - DO(t)}{DO^* - DO(t_0)}\right) = k_La \cdot (t - t_0) \qquad \textbf{Eq. 31}$$

27. The y-axis values (left side of Eq. 31) are plotted for an evaluation range between 10 and 90 % as a function of time with a minimum of seven values. Non-linear slopes may be caused by secondary effects superimposed to the first-order kinetics of the oxygen mass transfer. This may include, among others, mixing inefficiencies at low agitation and/or in large bioreactors and response effects of the DO probe at very high oxygen transfer rates.

28. Insert a linear trend line with the corresponding trend line equation (Eq. 32).

$$y = m \cdot x + b \qquad \textbf{Eq. 32}$$

29. The absolute value of the slope of the trend line represents the k_La value ("m" in Eq. 32 and red value in Figure 11).

30. If the k_La measurements for a defined stirrer speed have been performed at least 5 times, the standard deviation should be below the range of 10 %.

31. The k_La values (k_La_1, k_La_2, k_La_3, k_La_j with j = count) should be averaged by the arithmetic mean (Eq. 33).

$$k_La(37°C) = \frac{1}{j}\sum_{i=1}^{j} k_La_i = \frac{k_La_1 + k_La_2 + k_La_3 + \ldots + k_La_j}{j} \qquad \textbf{Eq. 33}$$

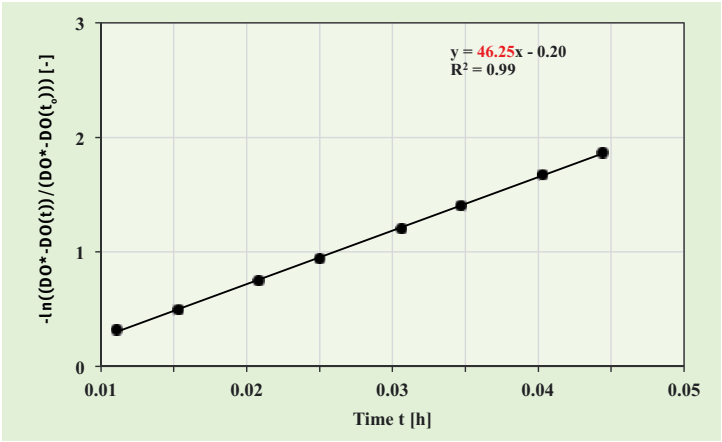


Figure 11: Determining the k_La value by plotting the left side of Eq. 31 as a function of time. The absolute slope of the graph represents the k_La value.

32. Recheck the sensor calibration for 0 and 100 % DO by first supplying nitrogen and waiting for the DO to remain constant (typically DO = 0 %) and then starting the air supply until the maximum DO concentration is achieved (typically DO = 100 %).

4.5. Appendix III

4.5.1. Preparation of 10 x PBS buffer solution

Chemicals for 10 x PBS buffer solution
80 ± 0.1 g NaCl
2 ± 0.05 g KCl
26.8 ± 0.1 g Na_2HPO_4 x 7 H_2O
or 17.8 g Na_2HPO_4 x 2H_2O
or 14.2 g Na_2HPO_4
2.4 ± 0.05 g KH_2PO_4

Preparation:

- Weigh out the chemicals in a 1 L measuring cylinder,
- add a magnetic stirrer bar,
- add approximately 800 mL pure water,
- dissolve the solution,
- remove the magnetic stirrer bar and fill-up to 1 L with pure water,
- transfer the PBS buffer solution in an 1 L glass bottle,
- for sterile applications, the PBS buffer solution should be autoclaved (121 °C, 20 min).

The buffer solution can be stored at room temperature. Minimum shelf-life: 1 year.

Usually, the **1 x PBS buffer solution** is used.

The PBS buffer solution has a pH-value of 7.4 ± 0.1 and a conductivity of 14 mS/cm.

4.5.2. Comparison of different response times using the time constant value

A disadvantage of conventional DO sensors is the time delay before the measurement signal is received. This is caused by the diffusion of oxygen through the membrane and can be described by Eq. 34.

$$y(t) = C \cdot \left(1 - e^{-t/\tau}\right) \qquad \textbf{Eq. 34}$$

Determining the time constant $\tau_{63\,\%}$ using Eq. 35.

$$\tau = \frac{-t}{\ln\left(1 - \frac{y(t)}{C}\right)} \qquad \textbf{Eq. 35}$$

Example I: A DO sensor achieves a response time of 30 s with a measurement signal of 63 % (Eq. 36).

$$\tau = \frac{-30\ \mathrm{s}}{\ln\left(1 - \frac{63\ \%}{100\ \%}\right)} = 30\ \mathrm{s} \qquad \textbf{Eq. 36}$$

Example II: A DO sensor achieves a response time of 90 s with a measurement signal of 95 % (Eq. 37).

$$\tau = \frac{-90\ \mathrm{s}}{\ln\left(1 - \frac{95\ \%}{100\ \%}\right)} = 30\ \mathrm{s} \qquad \textbf{Eq. 37}$$

4.5.3. Determining the oxygen ratio for head space flushing using off-gas analysis

Determining the k_La value in bioreactor systems requires the head space to be flushed. Therefore, off-gas analysis is necessary; meaning the off-gas analyser user guide should also be consulted in addition to steps 33 to 36.

33. Fill the bioreactor system up to its desired working volume with 1 x PBS buffer solution.
34. Connect the off-gas analyser to the exhaust line of the bioreactor.
35. Start the off-gas analyser data acquisition and the air supply. The measurement is complete when a constant oxygen concentration of 21 % is measured in the exhaust gases.
36. The head space flushing procedure must be performed before each k_La value investigation in bioreactor systems, otherwise the resulting k_La value will be either false or too low.

Note IX:
If there is no off-gas analyser available, the total gas in the head space should be exchanged three times.

Example: If the aeration rate of 0.5 L/min is used for a bioreactor with a head space of 1 L the exchange should be performed for 6 min, or with an air supply of 2 L/min for at least 90 s.

4.5.4. Example

Determining the k_La value for a 2 L stirred bioreactor system with a ring sparger using dissolved oxygen sensors for cell culture applications. The bioreactor is equipped with a two-stage segment blade stirrer.

» Prepare the bioreactor system (steps 1 to 5) using the following parameter:

- u_{Tip} = 1.8 m/s

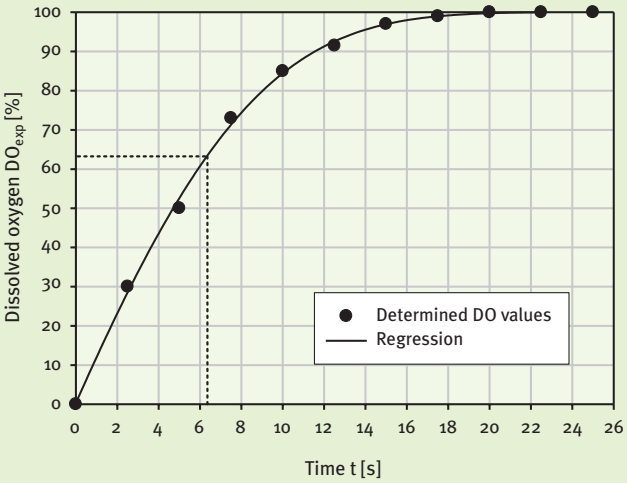
» Perform the two-point calibration (steps 6 to 10) using the following parameters:

- u_{Tip} = 1.8 m/s
- β_{N_2} : maximum nitrogen aeration (sparger aeration)
- β_{air} = 0.1 vvm (sparger aeration)

» Measure the response time of the DO sensor (steps 11 to 17) using the data depicted in Figure 12:

Time t [s]	Dissolved oxygen DO [%]
0	0
2.5	30
5	50
7.5	73
10	85
12.5	91.5
15	97
17.5	99
20	100
22.5	100
25	100

A



B

Figure 12: Determining the response time $t_{63\%}$ of a DO sensor based on the raw data (A) and plotting the dissolved oxygen as a function of time (B).

Graphical determination of the sensor response time: $t_{63\%} = \tau_{63\%} = 6.3\ s$
The time constant ($\tau_{63\%}$ = 6.3 s) determined for the sensor (sensor A) correlated to another oxygen sensor (sensor B) with a response time of 19 s and a measurement signal of 95 % (Eq. 35, Eq. 36 and Eq. 37).

» The measurement process for determining the k_La value (steps 18 to 24) was performed in triplicate using the following parameters:

- u_{Tip} = 1.2 m/s
- β_{N_2} : maximum nitrogen aeration (sparger aeration)
- β_{air} = 0.1 vvm (sparger aeration)

» Based on the experimental raw data, it was possible to determine the k_La value using the previously described evaluation process (steps 25 to 32 and Eq. 29 to Eq. 33).

Table 6: Overview of the experimental raw data for the k_La determination.

Time t [h]	$DO_{\_kLa1}$ [%]	Eq. 33 [-]	$DO_{\_kLa2}$ [%]	Eq. 33 [-]	$DO_{\_kLa3}$ [%]	Eq. 33 [-]
0.0000	0.00	0.00	0.02	0.00	0.67	0.00
0.0014	1.34	0.01	2.59	0.03	3.32	0.03
0.0028	4.46	0.05	6.07	0.06	6.57	0.06
0.0042	7.91	0.08	9.94	0.10	10.26	0.10
0.0056	11.05	0.12	13.93	0.15	14.24	0.15
0.0069	15.10	0.16	16.75	0.18	18.28	0.20
0.0083	19.18	0.21	21.99	0.25	22.44	0.25
0.0097	23.22	0.26	26.14	0.30	26.50	0.30
0.0111	27.35	0.32	30.11	0.36	30.58	0.36
0.0125	31.41	0.38	33.98	0.42	35.31	0.43
0.0139	35.33	0.44	37.77	0.47	38.76	0.48
0.0153	39.07	0.50	41.49	0.54	42.41	0.55
0.0167	42.58	0.55	44.98	0.60	45.99	0.61
0.0181	46.01	0.62	48.26	0.66	49.24	0.67
0.0194	49.97	0.69	51.50	0.72	52.34	0.73
0.0208	52.81	0.75	54.79	0.79	55.28	0.80
0.0222	55.56	0.81	57.65	0.86	58.03	0.86
0.0236	58.33	0.88	59.83	0.91	60.62	0.93
0.0250	60.93	0.94	62.34	0.98	63.03	0.99
0.0264	63.48	1.01	64.68	1.04	65.39	1.05
0.0278	65.76	1.07	66.93	1.11	67.62	1.12
0.0292	67.97	1.14	68.99	1.17	69.87	1.19
0.0306	70.01	1.20	71.01	1.24	71.84	1.26
0.0319	71.94	1.27	72.88	1.30	73.52	1.32
0.0333	73.77	1.34	74.62	1.37	75.20	1.39
0.0347	75.44	1.40	76.25	1.44	76.64	1.45
0.0361	77.16	1.48	77.80	1.50	78.14	1.51
0.0375	78.60	1.54	79.54	1.59	79.51	1.58
0.0389	80.03	1.61	80.69	1.64	80.84	1.65
0.0403	81.22	1.67	81.92	1.71	82.02	1.71
0.0417	82.33	1.73	83.08	1.78	83.22	1.78
0.0431	83.49	1.80	84.16	1.84	84.24	1.84
0.0444	84.54	1.87	85.20	1.91	85.26	1.91
0.0458	85.51	1.93	86.14	1.98	86.44	1.99
0.0472	86.39	1.99	86.99	2.04	87.26	2.05
0.0486	87.35	2.07	87.83	2.11	88.02	2.12
0.0500	88.10	2.13	88.56	2.17	88.77	2.18
0.0514	88.81	2.19	89.30	2.23	89.49	2.25

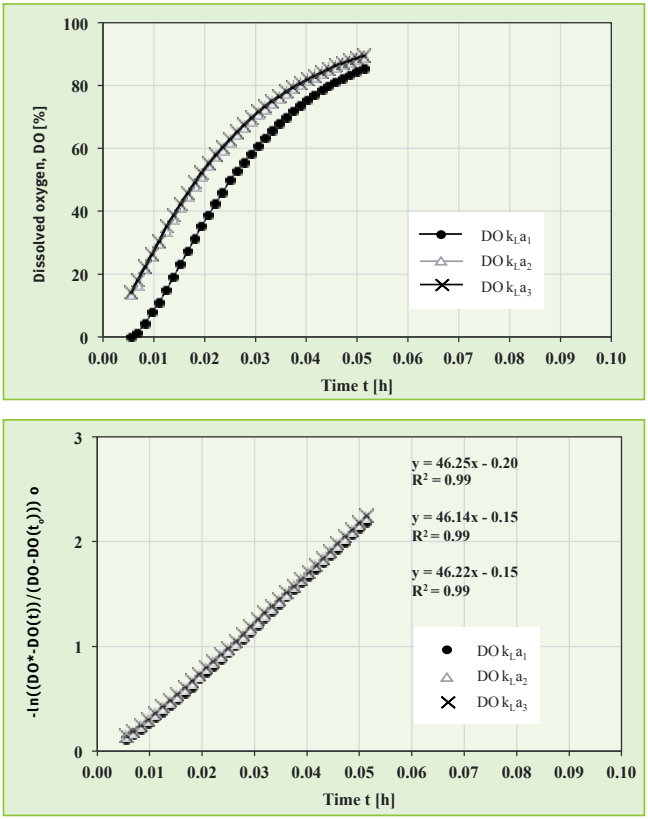


Figure 13: The k_La value is determined by measuring DO as a function of time (A). Thereafter, the left side of Eq. 31 is calculated out of the measured DO values and plotted as a function of time (B). The absolute value of the slope of the trend line in plot B represents the k_La value. DO* was previously determined to be 100 %.

$$k_La(37°C) = \frac{1}{j}\sum_{i=1}^{j} k_La_i = \frac{46.3\ h^{-1} + 46.1\ h^{-1} + 46.2\ h^{-1}}{3} = 46.2\ h^{-1} \qquad \textbf{Eq. 38}$$

4.6. Calculator sheet for k_La

For an easier and faster calculation of the k_La values according to the method described in this publication the authors developed a calculation tool. The Excel-based tool is available for download at www.dechema.de/studien.

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