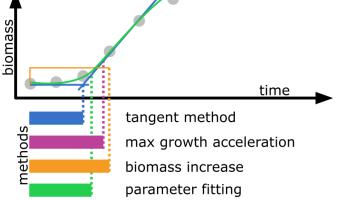
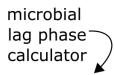
- 1 TITLE:
- 2 How to determine microbial lag phase duration?
- 3
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- 10
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- 12

13 GRAPHICAL ABSTRACT

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- 15

WHAT IS THE LAG PHASE DURATION?







16 ABSTRACT

17 The lag phase is a temporary non-replicative period observed when a microbial population is 18 introduced to a new nutrient-rich environment. Its duration can have a pronounced effect on 19 population fitness, and it is often measured in laboratory conditions. However, calculating the lag 20 phase length may be challenging and method and parameters dependent. Moreover, the details of these 21 methods and parameters used throughout experimental studies are often under-reported. Here we 22 discuss the most frequently used methods in experimental and theoretical studies, and we point out 23 some inconsistencies between them. Using experimental and simulated data we study the performance 24 of these methods depending on the frequency of population size measurements, and parameters 25 determining the growth curve shape, such as growth rate. It turns out that the sensitivity to each of 26 these parameters depends on the lag calculation methods. For example, lag duration calculation by 27 parameter fitting to a logistic model is very robust to low frequency of measurements, but it may be 28 highly biased for growth curves with low growth rate. On the contrary, the method based on finding 29 the point where growth acceleration is the highest, is robust to low growth rate, but highly sensitive to 30 low frequency of measurements and the level of noise in the data. Based on our results, we propose a 31 decision tree to choose a method most suited to one's data. Finally, we developed a web tool where the 32 lag duration can be calculated based on the user-specified growth curve data, and for various explicitly 33 specified methods, parameters, and data pre-processing techniques.

34

36 INTRODUCTION

37 Microbial planktonic populations grown in a batch culture follow a predictable pattern in terms of how 38 the population size changes in time. Such growth kinetics can be represented by a growth curve which 39 is typically divided into the following phases: (1) lag phase, when cells adjust to a new environment 40 before they start dividing, (2) exponentially growing phase (or logarithmical growth), when cells 41 divide with a maximum rate and population's density doubles regularly, (3) stationary phase, when 42 cells cease divisions due to nutrients depletion, and, if the measurements are conducted long enough, (4) the decline/death phase when population's density drops due to the cell death. Such pattern of 43 44 growth is typical for planktonic prokaryotic and eukaryotic microorganisms. 45 The lag phase was first described in 1895 by Müller as a temporary non-replicative period after bacteria are introduced to new media [1]. Indeed, adjustment to a new environment after growth-arrest 46 47 requires broad cellular reorganizations, which are universal for prokaryotes and eukaryotes. The cells 48 need to adjust their transcriptome, and proteome (e.g. in S. cerevisiae [2-4]) and rearrange cellular 49 components that are necessary for nutrient uptake and biomass accumulation. These processes are 50 activated shortly after environment change - for example, it was reported that gene expression in 51 Salmonella enterica bacteria was affected as soon as 4 minutes after transfer into a fresh media, and 52 within 20 minutes, almost a thousand genes were upregulated [5]. Proliferation restart requires 53 induction of a broad range of processes, which include: glycolysis, nutrient sensing, amino acid

- 54 metabolism, nucleotide biosynthesis, protein processing (including translation, folding, modification,
- 55 translocation, and degradation), coenzyme and cell wall biosynthesis, transcription of genes involved
- 56 in stress response, respiration, cell cycle control, and division. For more details about biological

57 processes during the lag please see the review by Bertrand (2019) [6].

- 58 While many studies focus on the exponential phase and use the exponential growth rate as a measure
- of population fitness [7], the quantification of the lag duration is equally important to assess the stress
- or fitness of microbial populations [8,9]. Classically, fitness is defined by the number of progeny,
- 61 which in microbiology is translated into the growth rate (i.e. the rate at which the population size
- 62 doubles). However, the lag phase duration also affects fitness because it shows how quickly a given
- 63 cell or population can adapt to an environmental change. Shorter lags enable earlier divisions, which
- 64 might allow a cell to produce higher number of progeny within a fixed period of time. Such growth
- 65 dynamics would be especially important in case of competition for limited resources or a limited time
- 66 frame when resources can be used. Thus, the short lag phase is generally believed to be beneficial, and
- 67 therefore populations in favourable conditions may be expected to evolve toward decreased lag
- duration [10]. However, the opposite strategy was observed in presence of antibiotics. In particular,
- 69 when bacterial populations were exposed to antibiotics before a transfer to new growth media, they
- round towards increased lag duration which matched the duration of antibiotic exposure [11].

71 The variation in lag phase duration can be influenced by genetic, epigenetic, and environmental factors 72 (e.g. [12–14]). For Saccharomyces cerevisiae, both nuclear and mitochondrial genes are important 73 during the lag phase, and their expression patterns correlate with the lag phase length [13]. Besides genetics, it has been shown that the lag phase duration is history-dependent. Namely, previous cells' 74 75 exposure to given conditions shortens the time needed to adapt if the conditions are reintroduced [14– 76 17]. What's even more interesting - this effect has also been observed in daughter cells that had never 77 experienced the initially introduced conditions what suggests epigenetic inheritance. [15]. Lags of 78 individual cells may differ even within a clonal population grown in unaltered conditions. For 79 example, older cells experience longer lags than their younger clones [18,19]. Such heterogeneity may 80 be beneficial for the population, as it enables a bet-hedging strategy where a fraction of cells assures 81 fitness advantage by fast growth in favourable conditions, while the other fraction provides survival in 82 stressful conditions by extending their lag time [19–21].

83 There are multiple definitions of the lag phase and multiple ways of measuring its duration. One 84 common definition is based on microscopic observations and it uses the first morphological signs of 85 cell division as markers of the lag phase end (e.g. [15,22,23]). Within the studies on population-level, 86 the lag phase can be defined as the time before any detectable increase in the cell abundance (biomass) 87 [19], or as the time delay before a population reaches exponential growth [5]. To measure how the 88 population's density (cell abundance) changes in time standard laboratory methods can be used. 89 Usually, one of the following methods is applied: spectrophotometry, colony counting on agar plates (CFU, Colony Forming Unit), and flow-cytometry. Viability counts (CFU) provide precise estimates 90 91 of cell abundance even in a small population, however, this technique is time-consuming, requires 92 previously determined culture dilutions, and is difficult to automatize and scale up. Nevertheless, due 93 to its high sensitivity, it's broadly used in food safety control [24,25]. Flow cytometry has a broad 94 detection spectrum and it enables simultaneous measurements of various cell properties e.g. cell size 95 or DNA content [26]. Spectrophotometry (optical density (OD) or absorbance) has narrower detection 96 limits, but it is convenient, fast, cost-effective, and can be easily adapted for high-throughput testing

97 via automatic measurements in constant time intervals.

98 Spectrophotometry is currently the standard way of obtaining microbial growth curves. However, one 99 of its limitations is the fact that optical density is an indicator of biomass rather than cell counts 100 (discussed by Swinnen *et al.* (2004) and Rolfe *et al.* (2012) [5,27]). To add to this problem, the optical 101 density may be also affected by dead or lysed cells, or even by the cell shape which may change 102 during the lag phase [28]. Additionally, if the inoculum size is small, exponential growth may start 103 before the biomass reaches the OD detection level, and thus the observed (apparent) lag duration may

- 104 be overestimated. This problem has been tackled by Baranyi (1999) [29] who proposed a so-called
- 105 time to detection (TTD) method which has been applied in some experimental studies (e.g. [30]). The
- time to detection (11D) method which has been applied in some experimental studies (e.g. [50]). The
- 106 problem of initial biomass being below the detection level has also been tackled by Pierantoni *et al.*

(2019) [31] who have proposed a new calculation method that uses the apparent lag phases to measure
the initial population biomass. More broadly, the problem of density-based detection of growth for
small populations could be mitigated by assuming certain model growth curve shapes below the OD
detection level and/or by knowing the exact starting density of cells capable to proliferate (excluding
dead and senescent cells) [32].

112 Mathematical models can be used to overcome some methodological limitations. In order to know 113 when exactly cells started duplicating (i.e. the end of the lag phase), we would need to continuously 114 monitor the number of cells. This is, however, not possible with methods such as spectrophotometry. 115 As outlined above, the spectrophotometry measurements not only are taken in intervals, but also may 116 be inaccurate if the cells change their mass or shape, or if their initial amount is below the detection 117 level [5]. In such cases, some assumptions are needed to calculate the lag phase duration with 118 sufficient accuracy. If one assumes that there is no population growth during the lag phase and then 119 cells start synchronically dividing at a constant growth rate, the end of the lag phase can be calculated 120 as the intersection between the tangent line to the point of maximum growth rate and the y = log(N0)121 line, where N0 is the inoculation density (hereinafter "tangent method" [6]; see for example: 122 [12,15,24]). This is in fact the most frequently used method of calculating the lag duration. However, 123 there are also other methods, for example: defining the end of lag as the point of the growth curve 124 where the second derivative of the population size in time is maximal (hereinafter "max growth 125 acceleration", e.g. [33,34]), determining when the biomass increased from the initial value by some 126 predefined threshold (minimal detectable increase, hereinafter "biomass increase", e.g. [19]), or fitting 127 experimental data to a mathematical model (hereinafter "parameter fitting to a model", e.g. [8]). 128 Various mathematical models have been proposed to account for the lag phase [27], and there are tools 129 and packages which use those to estimate the lag duration from the experimental data (for example R 130 package *nlsMicrobio* [35]). However, none of these tools is strictly focused on calculating the 131 population lag duration. Moreover, they require a good knowledge of the models and R programming 132 skills, which may make them difficult to use. Finally, as discussed in Baty et al. (2004) [32], the data 133 quality impacts the lag duration measurements to a higher extent than the choice of a model. Although 134 Baty et al. (2004) [32] investigated the insufficient number of data points as a potential problem in lag 135 duration calculation, experimental biologists may face other problems with the data quality such as 136 noisiness or growth curve shape that deviates from mathematical models [36]. Interestingly, 137 technicalities related to dealing with such 'unideal' data tend to be omitted in methodologies described 138 by empirical studies that measure lags. They have also not been discussed in theoretical studies which 139 focus on the mathematical formulations and biological assumptions rather than the reality and 140 limitations of laboratory experiments. These facts make a knowledge transfer between theoretical and 141 experimental biologists difficult.

- 142 Within this study, we describe the methods most frequently used to measure the lag duration and
- 143 discuss how these methods stem from various mathematical models describing microbial growth
- 144 curves. We highlight the advantages and limitations of each lag calculating method. We further use
- both empirical and simulated growth curve data to show how the lag duration estimate may depend on
- 146 the data quality (i.e. noisiness), the shape of the growth curve, and the lag duration calculation method.
- 147 We propose a decision tree that may be useful in choosing the lag calculating method best suited to
- 148 one's data. We aim to emphasise that chosen methodology, parameters, and data pre-processing can
- strongly influence the results, which is especially visible for less typically shaped growth curves.
- 150 Finally, we develop a publicly available web server MICROBIAL LAG PHASE DURATION
- 151 CALCULATOR (https://microbialgrowth.shinyapps.io/lag_calulator/) which allows calculating the
- 152 population lag duration according to various state-of-art methods. The calculator allows for fast and
- 153 easy data analysis and direct comparison of different methods.

155 THE LAG PHASE IN MATHEMATICAL MODELS

156 There are many mathematical models that describe the microbial population growth depicted by a 157 growth curve. They include the traditional simple models such as exponential [37], logistic [38] or 158 Monod growth [39], as well as more modern stochastic or agent-based models (see the review by 159 Charlebois and Balázsi (2019) [40]). While the pure exponential model is parametrised only by the 160 growth rate, the other models may depend on multiple parameters. For example, the logistic model 161 introduces the *carrying capacity* parameter which represents the maximum population size, and 162 Monod model introduces the so-called *half-velocity constant* (which quantifies the relationship 163 between nutrient concentration in media and population growth rate). Another version of this model 164 may be parametrised by the *maximum resource uptake rate* and the *saturation constant* as derived by 165 Michaelis-Menten.

166 Please note, that within this publication "method" refers to the way in which the lag phase is

167 determined, while "model" refers to a set of equations that reproduce the entire microbial growth

168 curve.

169 Out of the three typically described growth phases (lag, exponential and stationary phase), the

170 exponential and stationary phases are recovered by most of the models discussed in Charlebois *et al.*

171 (2019) [40]. One exception is the pure exponential model which only describes the exponential phase.

172 However, the lag and death phases require additional assumptions and are rarely described with

173 sufficient detail. The models that aim to capture or predict the lag phase are well summarised in the

two extensive reviews [27,32]. Some models describe the lag only phenomenologically. For example,

the Baranyi (1993) and Baranyi and Roberts (1994) [41,42] models assume that there is some

adjustment function that describes the population's adaptation to a new condition. On the other hand,

177 there are models that attempt to make specific assumptions regarding what happens in the lag phase.

178 The Hills and Wright (1994) [43] assume that certain biomass needs to be reached for the cell to start 179 the chromosomal replication. Consequently, their model independently tracks in time the amount of

180 biomass and chromosomal material. The model proposed by McKellar (1997) [44] assumes there is

181 some heterogeneity in the population, and that one part of the population grows exponentially from the

182 very beginning, whereas the other part does not replicate. A more realistic version of this model was

183 proposed by Baranyi (1998) [45] where the non-replicating cells are assumed to transform at a

184 constant rate into the replicating ones, meaning that they exit the lag phase. Finally, a model by Yates

185 (2007) [46] adds another compartment, namely cells that die at a constant rate. This specific

assumption allowed the model to reproduce the initial biomass decline which is sometimes observed

187 during the lag phase. Interestingly, as noticed by Baty (2004) [32], some of the models described

above are equivalent, in spite of being based on distinct biological assumptions.

190 THE LAG PHASE CALCULATION METHODS

191 The most popular and intuitive method of calculating the lag duration ("tangent method") defines the

- 192 lag phase end at the intersection point of log(N0) (where N0 denotes the initial biomass) and a line
- 193 tangent to the logarithm of population size in the exponential phase [6,39]. Why bother about more
- 194 complicated mathematical models?
- 195 The definition above is in fact a consequence of the pure exponential growth assumption. If cells do

196 not divide for some time λ and then start growing with a constant growth rate, then the time λ perfectly

197 corresponds to the lag as measured by the intersection between the lines described above (Table 1, Fig.

- 198 1: exponential model & tangent method).
- 199 However, microbial populations in a batch culture do not grow with a constant growth rate. According

200 to the Monod model assumptions [39], the growth rate depends on the availability of the resources,

- and therefore it decreases over time. The growth rate may also depend on other factors, e.g. the
- 202 population density [47]. In the case of cancer cells or in populations that reproduce sexually the
- 203 growth rate may be lower at low population density, which is known as the Allee effect (e.g. [48,49]).
- 204 This is why, in order to assess the most exact time when cells start divisions, there is a need for a
- 205 mathematical model that is likely to represent the empirical growth curve and to fit the lag time
- 206 together with other growth parameters. In particular, note that when there is some growth detection
- 207 threshold, the time by which we notice any growth (i.e. apparent lag) may be impacted not only by the
- 208 lag length but also by other growth parameters. For example, when the initial biomass and growth
- 209 rates are very low, the slow growth may not be detected and treated as lag. This problem has been
- 210 extensively discussed in Pierantoni *et al* (2019) [31].
- 211 The summary of the most popular methods of calculating the lag duration, the assumptions underlying
- each of the methods as well as possible challenges related to each method are given in Table 1.

Name	Lag end calculation method	Underlying model assumptions	derlying model assumptions Challenges		
Biomass increase	The increase of OD or biomass by a certain value from the beginning of the growth.	The population size does not increase its biomass during the lag and then starts growing with any growth rate that may be variable or hard to measure. The increase by the threshold value is the minimal increase possible to detect with high confidence.	Choice of the threshold value is arbitrary (not 'biologically- relevant')	Data where the initial size of the viable population cannot be determined with high confidence.	Opalek <i>et al.</i> 2022 [19]
MAX GROWTH ACCELERATION	The point of the growth curve where the second derivative of population size in time is maximal.	The population size does not increase its biomass during the lag and then starts growing with a decreasing growth rate.	Noisy data may affect the detection of such a point.	Growth in a batch culture where the amount of resources decreases with time and the amount of unfavourable by-products increases with time.	Liu <i>et al.</i> 2021 [33]
Tangent method	The intersection of the initial density line and the line tangent to the growth curve at the maximal growth rate (on log. scale).	The population size does not increase its biomass during the lag and then starts growing exponentially with a constant growth rate.	If the growth rate varies, it may be challenging to find the "real" or maximal growth rate. Moreover, the initial density needs to be determined with high confidence	Growth in constant resources and environmental conditions (e.g. chemostat (at starting point), serial transfers).	Valík <i>et al.</i> 2021 [24]
Parameter fitting to the Baranyi model	Using parameter fitting procedures in order to simultaneously find all Baranyi & Roberts model parameters that provide the best fit to the entire growth curve. Then the lag is defined as $\ln(1+1/q_0)/r$ where r is the maximal growth rate and q_0 represents the physiological state of the inoculum.	The population size grows according to the logistic model adjusted by function: $\alpha = q(t)/(1 + q(t))$ where $q(t) = q_0 e^{vt}$ describes the concentration of some critical substance. The adjustment function slows down the initial growth but it does not pause it during the lag time as assumed by other methods.	The lag duration fitted to the data may depend on many technical parameters of the fitting algorithms, or it may not be found if the fitting algorithms fail to converge. Finally, the lag understood by Baranyi is not the time when cells do not divide, but the time required to adjust to the new media (see Discussion for more details)	Data that is well described by the Baranyi & Roberts model, where ideally some parameters (r, K, or q_0) are known <i>a</i> <i>priori</i> .	Kim <i>et al.</i> 2022 [50]
Parameter fitting to the logistic model	Using parameter fitting procedures in order to simultaneously estimate the lag duration, carrying capacity, and growth rate parameter values.	The population size does not increase during the lag and then starts growing according to the logistic model: i.e. with the growth rate decreasing according to the following equation: r(1-N(t)/K) where r is the maximal growth rate, K is the maximal population size, and N(t) is the population size at time t.	Assumes a very specific growth curve shape. Moreover, the lag duration fitted to the data may depend on many technical parameters of the fitting algorithms. Finally, the fitting methods may not converge to any solution.	Data that is well described by the logistic model, where ideally some parameters (r or K) are known <i>a priori</i> .	Reding- Roman <i>et al.</i> 2017 [8]

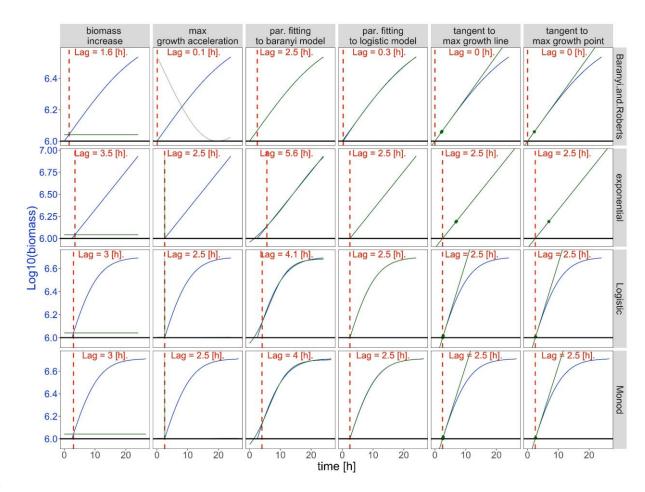
215 RESULTS

216 TESTING METHODS FOR LAG DURATION DETERMINATION ON THE SIMULATED DATASET

217 Given that the methods of calculating the lag phase duration described above (Table 1) were based on 218 theoretical assumptions, we first simulated microbial growth curves based on various well-known 219 deterministic mathematical models: (i) the simple EXPONENTIAL model which assumes that microbes 220 do not grow nor divide for some time (lag) and then start growing exponentially with a constant 221 growth rate; (ii) LOGISTIC model which assumes the microbes do not grow nor divide for some time 222 (lag) and then start growing exponentially with a decreasing growth rate; (iii) MONOD model where 223 microbes do not grow nor divide for some time (lag) and then the speed of the growth after the lag 224 phase is coupled with the decrease in resources, and (iv) BARANYI model which assumes cells do 225 grow and divide in the lag phase but that growth is slower than in the exponential phase. See the

- Appendix for the formulation of each model. We set that for all generated growth curves the lag phase
- lasts for 2.5 hours.
- 228 Then, for each of these simulated growth curves, we calculated the lag phase duration using the well-
- established methods described in Table 1. The most common tangent method was further split into:
- tangent to point (i.e. where the tangent line is drawn to the first point where the growth rate is
- maximal) [23] and tangent to line method (i.e. where the tangent line is understood as a regression line
- fitted to a number of points around those with the maximal growth rate) [7].
- 233 Max growth acceleration, parameter fitting to the logistic model, and both tangent methods found the
- correct lags (lag = 2.5 h) for data simulated under exponential, logistic, and Monod models (Fig. 1).
- Even the simplest tangent method worked well for the data simulated under these models, even though
- the data does not necessarily meet the assumption of a constant growth rate. Importantly the Baranyi
- 237 model seems to be inconsistent will all other models. Namely, the lag durations calculated for the data
- simulated under the Baranyi model are underestimated by all methods apart from the one where
- 239 parameters are fitted explicitly to the Baranyi model. Conversely, the parameter fitting to the Baranyi
- 240 model method tends to overestimate the lags for data simulated under all models apart from Baranyi.
- 241 The biomass increase method overestimates lag phase duration by one timepoint (0.5 h) for logistic
- and Monod models and two timepoints (1 h) for the exponential model. It is the consequence of this
- 243 method formulation, where lag phase end is defined as the first timepoint after detectable growth.

245



246

Fig. 1. Lag phase durations (given in red) calculated for data (blue lines) simulated under various 247 models (rows) and calculated by different methods (columns). The red dashed lines indicate the end of 248 249 the lag phase, black solid lines indicate the initial biomass (first measurement) and the green lines are 250 (left to right): the threshold of log(biomass) value at which the lag phase is assumed to end [biomass increase method]; second derivative of log(biomass) scaled to the values visualised in the plot [max. 251 growth acceleration method]; fitted growth curve [par. fitting to the Baranyi model and par. fitting to 252 253 logistic model methods]; tangent lines to the exponential growth [tangent to max. growth line and point methods]. 254

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258 TESTING METHODS FOR LAG DURATION DETERMINATION ON THE EMPIRICAL DATASET

- Having verified how the lag calculation methods work on simulated data (Fig. 1), we set off to verify
- 260 how these methods perform on real experimental data. We used 11 growth curves from
- 261 Saccharomyces cerevisiae grown in various conditions (see Methods and Supplement). Some of these
- 262 curves resemble the model data, while others are much noisier and challenge some of the model
- assumptions, for example: the biomass drops at the beginning of measurements (Fig. 2: curve_5 and
- curve_6), there is no typical exponential phase (Fig. 2: curve_11) or the lag phase is boldly prolonged
- and turbulent (Fig. 2: curve_7 and curve_11). Results obtained for empirical data show that the lag
- 266 duration estimates may vary depending on the lag calculation method (Fig. 2, Supplementary Table 2).
- 267 The lowest discrepancy between different methods is observed for curve_1, where the lag duration
- 268 calculated by all methods is 1.86 ± 0.83 h. In some cases, all but one method give similar results (e.g.
- 269 curve 7, where the biomass increase method gives an outlying result), in others, some methods fail to
- 270 find the correct lag duration (e.g. curve_7, curve_11) due to the data noisiness (Fig. 2). The highest
- discrepancy between methods was obtained for curve_11 where a minimal lag phase length of 1.5 h
- 272 was obtained by the max growth acceleration method, while both tangent methods and parameter
- 273 fitting to logistic model yielded the lag duration of ~18h.

tangent to max growth point par. fitting to baranyi model Lag = 2.6 [h]. par. fitting to logistic model Lag = 1.9 [h]. tangent to max growth line Lag = 1.6 [h]. biomass max growth acceleration Lag = 1.5 [h]. increase Lag = 2 [h] 7.7 curve 7.6 7.5 Lag = 1 [h]. Lag = 2.6 [h]. Lag = 1.9 [h]. Lag = 1.6 [h]. Lag = 1.5 [h]. Lag = 1.6 [h]. 7.75 7.70 7.65 7.60 7.55 Lag = 1 [h]. Lag = 1.5 [h]. Lag = 2 [h] Lag = 0.9 [h]. Lag = 0.7 [h]. Lag = 0.7 [h]. 7.7 curve_3 7.6 7.5 Lag = 3.5 [h]. Lag = 3.5 [h]. Lag = 6.1 [h]. Lag = 4.4 [h] Lag = 4.4 [h]. Lag = 5.2 [h]. 7.6 CUIVE 4 7.4 7.2 7.0 Lag = 2 [h]. Lag = 2.5 [h]. Lag = 1.9 [h]. Lag = 1 [h]. Lag = 1.8 [h]. Lag = 1.7 [h]. 7.7 curve_5 7.6 7.5 Lag = 2 [h]. Lag = 2.5 [h]. Lag = 2.2 [h]. Lag = 1.9 [h]. Lag = 1.9 [h]. Lag = 1 [h]. Log10(biomass) 7.7 7.6 7.5 Lag = 1.5 [h]. Lag = NA [h]. _ag = 5 [h] Lag = 11 [h]. 8.5 [h]. Lag = 8.5 [h] Lag = 7 CUIVE 6 NW 111 11 I V 1 Lag = 7.9 [h]. Lag = 6.8 [h]. Lbg = 7 [h] Lag = 3 [h] Lag = 6.6 [h]. 6.7 [h] Lag 7.70 7.65 curve 7.60 7.55 7.50 W + = 4.5 [h] = 4.4 [h]. Lag = 4 [h] Lag = 1 [h]. Lag = 5.6 [h]. Lag = 4.1 [h]. 7.70 7.65 CUIVE 7.60 7.55 7.50 Lac Lab 7.3 [h] Lag = 5.8 [h] = 4 [h] 5.1 [h] 5.1 [h 7.6 curve_10 7.4 AAA 7.2 7.5 Lag = 14.5 [h]. Lag = 1.5 [h]. Lag = 20.2 [h] Lag = 17.6 [h]. Lag = 18.2 [h]. Lag = 18.2 [h]. 7.3 curve_11 7.1 6.9 0 \$ 10 15 20 250 \$ 10 15 20 200 \$ 10 10 10 \$ 10 10 10 \$ 10 10 10 \$ 10 10 \$ 10 10 \$ 10 10 \$ 10 10

- Fig. 2. Lag phase duration (given in red) calculated for empirical growth curves (blue lines) obtained
- 277 for *S. cerevisiae* grown in various conditions (top to bottom). The red dashed lines indicate the end of
- the lag phase, black solid lines indicate the initial biomass (first measurement) and the green lines are
- 279 (left to right): threshold value at which the lag phase is assumed to end [biomass increase method];
- 280 second derivative of log(biomass) scaled to the values visualised in the plot [max. growth acceleration
- 281 method]; fitted growth curve [par. fitting to Baranyi model and par. fitting to logistic model methods];
- tangent lines to the exponential growth [tangent to max. growth line and point methods].

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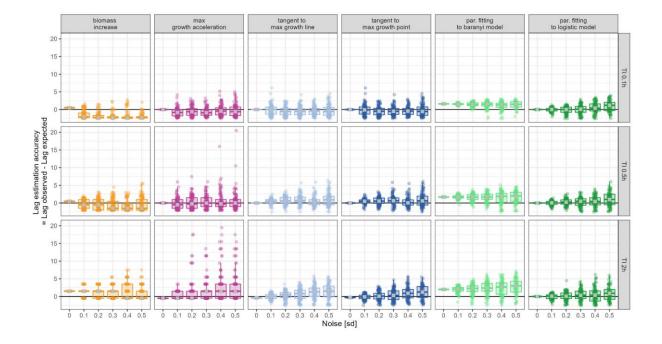
285 TESTING THE SENSITIVITY OF LAG DETERMINATION METHODS TO DATA NOISINESS

- 286 In order to understand the source of possible errors and biases in lag calculation on experimental data,
- 287 we simulated growth curves from the logistic model (which in our experience the most adequately
- 288 represents the real growth curves) and to each curve we added noise simulated from the random
- 289 distribution with mean = 0 and standard deviation dependent on the initial biomass B0 (Supplementary
- 290 Fig. 2). Thus, our simulated growth curves could be described as:

291 $B_{noisy}(t) = B(t) + N(0, sd * B(0))$, where B(t) is the solution from the deterministic logistic model

with a lag component (see Supplement for the formulation of this model).

- 293 We varied the level of noisiness (i.e. set *sd* between 0 and 0.5) together with other parameters such as:
- 294 population growth rate, lag time, and time interval between data points (which represents the
- 295 frequency of population size measurements). For each combination, we simulated 100 curves. Then
- 296 for each of these curves, we calculated the lag according to each of the lag calculation methods and we
- 297 calculated the bias i.e. the difference between observed and expected lag.
- 298 First, we tested how the frequency of population size measurement impacts the lag estimation. In
- agreement with previous reports [32], it turned out that frequent measurements improve the accuracy
- 300 of lag duration estimation. This effect can be observed within all lag calculation methods (Fig. 3: top
- 301 vs bottom row) and it is especially pronounced for data with high noise (sd = 0.5). Large time intervals
- 302 between data points (Fig. 3: bottom row) lead to increased variance in the lag duration estimation
- 303 (wide boxplot) and to high bias (median value deviates from y = 0 line). It is especially pronounced in
- 304 the biomass increase and max growth acceleration methods which overestimate lag durations even
- 305 when there is minimal amount of noise (low sd). Such bias results from the fact that these methods
- 306 operate only on the data points provided and they do not use any implicit models to interpolate307 between them.



310

Fig. 3. The impact of time intervals (TI) between subsequent measurements of population size on lag phase duration estimations. For each sd (noisiness, x-axis), measurement frequency (time interval, rows), and lag calculation method (columns) 100 simulations were conducted (points). The y-axis illustrates the difference between true (expected, set as 2.5 h) and estimated (observed) lag. Boxplots illustrate the distribution of points, and the median value (grey square). All points located above y = 0, show these calculations where lag phase length was overestimated, similarly, all points below y = 0show calculations where lag duration was underestimated.

319 Next, we checked whether the population's growth rate has an impact on lag estimation. We calculated 320 lags for three growth rates representing slow, moderate, and fast growth, and for varied levels of data 321 noisiness (Fig. 4). For most of the methods slow growth results in low accuracy of lag estimation, 322 which may additionally decreases when data is noisy. These results are consistent with our experience, 323 namely, when the growth curve is flat, it is challenging to find a point where the population starts 324 growing exponentially (Fig. 4: top row). In contrast, even for noisy data, fast growth facilitates lag 325 phase estimations (Fig. 4: bottom row). The only method which is not affected by the growth rate is 326 the biomass increase method, however it systematically underestimate lag duration, and this bias 327 increases with introduced noisiness. Conversely, the parameter fitting to the Baranyi model 328 systematically overestimates lag lengths especially when the population grows slowly. Max growth 329 acceleration and both tangent methods are not biased, and are sensitive to growth rates to a similar 330 degree, where lag estimates becoming less accurate with increasing noise. Interestingly, parameter 331 fitting to the logistic model shows good accuracy and no bias for moderate and fast growth, but it 332 suffers from low accuracy and high bias in the case of low population growth rate and high levels of 333 noise. 334 Another factor that affects the lag length estimation accuracy is the actual length of the lag phase. The

longer the lag, the easier it is to erroneously detect some noise during the lag phase as the first signs of

growth. Indeed, if a population has a long lag phase, its duration tends to be underestimated, while

337 very short lags tend to be overestimated by all methods. This bias is increasing with higher noisiness

338 (Supplementary Fig. 3), with the estimations of lag phase duration by parameter fitting to the logistic

339 model being the most robust.

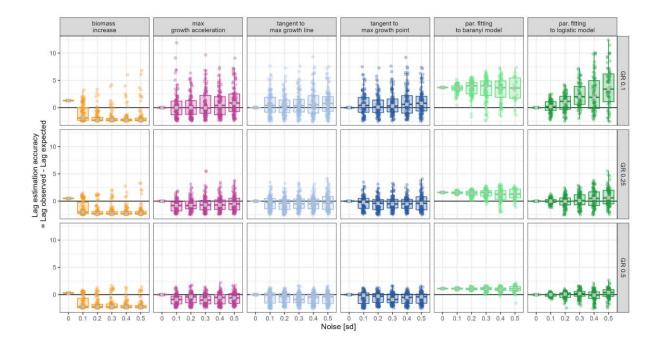
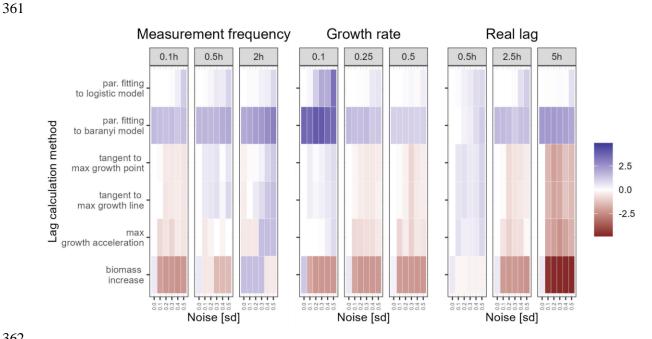


Fig. 4. Impact of growth rate (GR) on lag phase duration estimations. For each sd (noisiness, x-axis),
growth rate (rows), and lag calculation method (columns) 100 simulations were conducted (points).
The y-axis illustrates the difference between true (expected, set as 2.5 h) and estimated (observed) lag.
Boxplots illustrate the distribution of points, and the median value (grey square). All points located

346 above y = 0, show these calculations where lag phase length was overestimated, similarly, all points

347 below y = 0 show calculations where lag duration was underestimated.

- 349 The comparison of biases of all lag calculation methods are shown in Fig. 5. The methods that tend to
- 350 be systematically biased within our parameter range are the biomass increase (underestimates the lags)
- 351 and fitting to Baranyi model (overestimates the lags). The biomass increase tends to erroneously pick
- 352 random points as sudden lag phase end, which leads to underestimation of lag phase duration.
- 353 Conversely, the parameter fitting to the Baranyi model systematically overestimates lag duration. This
- is a consequence of the dataset being simulated by the logistic model which is inconsistent with
- 355 Baranyi's assumptions. The other methods are less biased within our parameter range. Both tangent
- and max growth acceleration methods have poor performance on growth curves with long lags, and
- 357 max growth acceleration method is very sensitive to high levels of noise. The parameter fitting to the
- 358 logistic model is the most robust to data noisiness, however, interestingly what affects it the most is
- the low growth rate.



362

363 Fig. 5. The median bias of lag estimation of each of the lag calculation methods applied to growth

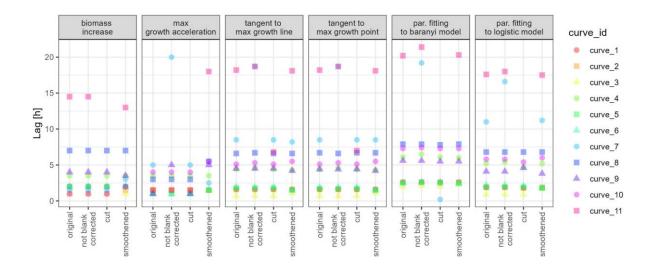
curves with varied level of noise (x-axis) and other growth curve parameters (column panels). White 364 365 squares mean the method is unbiased, blue means the lags tend to be overestimated, and red means the

366 lags tend to be underestimated.

368 TESTING THE EFFECTS OF DATA PRE-PROCESSING ON ESTIMATED LAG LENGTHS

369 Lag estimation is challenging if data is noisy. This problem could be mitigated by some data pre-370 processing techniques that allow for removing noise from the data. Therefore, we verified how manual 371 curation or data pre-processing affect the lag calculation accuracy. We applied the following pre-372 processing methods to our experimental curves (Fig.2): (i) smoothening the curve by Tukey's 373 smoothing function, (ii) cutting the data at 12h to remove the noise observed in the stationary phase. It 374 turned out that for the "typical" growth curve shapes, such as the ones presented in curve 1 and 375 curve 2 (Fig. 2), various algorithms are consistent and the data pre-processing does not influence the results. However, the noisier the data, the less consistent the results based on multiple lag calculation 376 377 methods and data pre-processing techniques (Fig. 3). We have additionally verified that adding a 378 constant to the entire growth curve (e.g. by neglecting the blank correction) affects the lag phase 379 length estimates given by all but the biomass increase method (Fig 3). Importantly, such a constant 380 value added to the growth curve may not only relate to blank correction, but also to the case when a 381 fraction of the population is dead or damaged and does not duplicate throughout the growth. We 382 further investigate how that phenomenon affects the lag calculation in Supplementary Fig 1.

383



384

Fig. 6. Distribution of lag duration estimated by six discussed methods (columns) per each curve

(colour & shape) and pre-processing algorithm (x-axis). "Original" means no pre-processing has been
applied, "not blank corrected" means data were not corrected for the blank value, "cut" means the data
curve has been shortened to 12 hours only, and "smoothened" means Tukey smoothening has been
applied.

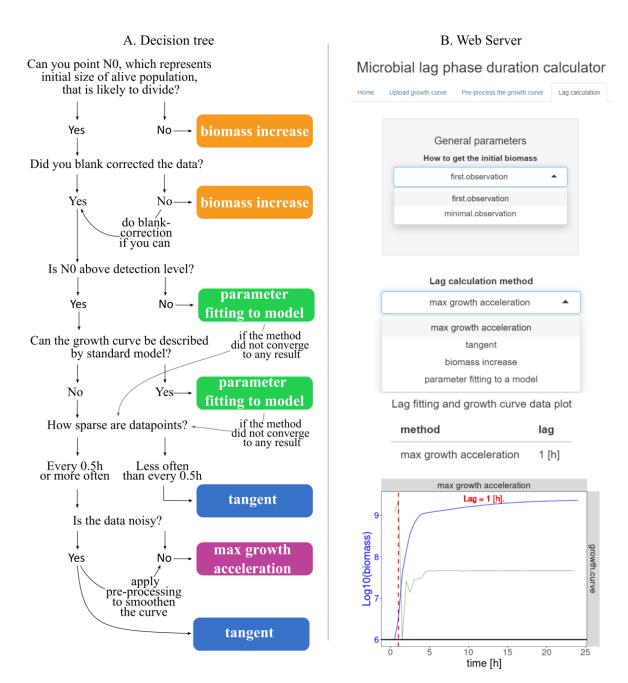
390

392 How to choose the Lag calculation method best suited to one's dataset?

- 393 We propose a decision tree to facilitate the choice of lag calculation method (Fig. 7A). The
- 394 recommendations are based on our results shown in previous sections. Altogether, we suggest trying to
- 395 estimate lag duration by parameter fitting to the logistic model in the first place. This method is the
- 396 most robust, it captures whole growth dynamics and because of that, it mitigates technical limitations
- 397 (such as a device's detection limits). On the other hand, the biomass increase method is the least
- 398 dependent on any assumptions. In particular, it is the only one that is not affected by the blank
- 399 correction or existence of dead cells in the culture. Therefore we recommend it if the other methods
- 400 cannot be applied. Additionally, we encourage to use multiple methods and to investigate possible
- 401 inconsistencies between their results.
- 402

404 THE LAG PHASE DURATION CALCULATOR

- 405 Finally, in order to allow other researchers to calculate lag duration by different methods and compare
- 406 the results, we created a web server MICROBIAL LAG PHASE DURATION CALCULATOR, where
- 407 lags can be calculated by various methods upon insertion of the growth curve data (i.e. table with time
- 408 and biomass columns). The webserver is freely available under the following address:
- 409 <u>https://microbialgrowth.shinyapps.io/lag_calulator/</u>
- 410 The MICROBIAL LAG PHASE DURATION CALCULATOR is designed to automate the lag
- 411 duration calculation process. The tool does not require any programming, nor mathematical modelling
- 412 skills. Additionally, we deposited our code and lag calculating functions on GitHub so that they can be
- 413 used locally, customised, and further improved.



416 Fig. 7. A: Decision tree to facilitate the choice of appropriate lag calculation method. B: Print-screen

- 417 from web server MICROBIAL LAG PHASE DURATION CALCULATOR where lag phase duration
- 418 can be calculated for a user-specified growth curve, and by any of the methods discussed in Table 1.

419 DISCUSSION

420 Lag phase duration is an important fitness component for microbial populations. Within this 421 publication, we discussed the most popular approaches to calculate the lag phase duration for 422 population-level data. We implemented these approaches and tested them on simulated and empirical 423 data to show that they may disagree depending on the shape of the growth curve. To further compare 424 the methods in terms of their accuracies and biases, we simulated growth curve data with varied level 425 of noise and other parameters such as growth rates, lag lengths or the time intervals between data 426 points (understood as the frequency of measurements). For each of these growth curves we estimated 427 the lag duration with each of the lag calculation methods. It turned out that the noise and other 428 parameters affect the quality of lag duration estimation, hence we tested how smoothening the growth 429 curve and other data pre-processing techniques influence the lag estimation. Since various methods 430 were sensitive to various parameters (for example the max, growth acceleration method was largely 431 affected by the high noise, while the parameter fitting to logistic model method was affected by low 432 growth rates), we used our results to propose a decision tree designed to help in choosing the lag 433 calculation method best suited to one's data. We have also developed a web tool where the lag duration 434 can be calculated based on the user-specified growth curve data, and for various explicitly specified 435 methods, parameters, and data pre-processing techniques. Additionally, we deposited our code on 436 GitHub so that the function "Calculate.Lag" can be taken directly to the R environment for local use 437 and customization.

438 Here, we chose the four most frequently used approaches to estimate lag phase duration. We compare: 439 (i) THE BIOMASS INCREASE, which uses a predefined value of a biomass (or absorbance) gain that can 440 be confidently marked as population's growth; (ii) THE MAX GROWTH ACCELERATION, which assumes 441 the lag ends at the time point where the second derivative of a population size in time is maximal, (iii) 442 THE TANGENT METHOD, where lag phase endpoint is marked as the intersection point of a line tangent 443 to maximal growth and a line y = log(N0), where N0 is population density at the inoculation; and (iv) 444 PARAMETER FITTING TO A MODEL which simultaneously finds best-fitted parameter values for the 445 entire curve (e.g. lag phase length, maximal population size, maximal growth rate) using logistic or 446 Baranyi models. All these methods are developed based on typically shaped growth curves. Therefore, 447 we tested the accuracy of their estimations using simulated (typical) and empirical (typical and 448 untypical) growth curve data. As expected, for data with typically shaped growth curves, the majority 449 of the methods showed similar results. This is in line with the previous observation, that the choice of 450 a model influences the calculated lag phase duration to a lesser extent than the data quality and 451 characteristic [32]. Interestingly the Baranyi model [51] is not consistent with other methods. This is a consequence of how the lag is defined within Baranyi model. While other models assume the lag is the 452 453 time when the population size does not grow, Baranyi defines lag as the delay between the population 454 size expected if the cells started growing immediately after inoculation with the maximum growth rate

and the observed one [32]. This is consistent with the original definition [52] which defines lag

456 duration as the difference between the time expected to take to reach the observed population size if

457 population size was growing at a maximal rate and the actual time taken to reach that population size

458 (including the lag duration). Note, however, that such definition is affected by all factors that lead to a

- 459 slower growth (e.g. nutrient depletion), and may be dependent on the time when measurements are
- 460 taken.

Taken together, the lag phase estimation cause little or no trouble when the growth curve resembles a
standard model shape, and in such cases, the choice of the lag calculation method can be driven by
one's preferences. It, however, becomes more complicated for noisy or untypical growth curves.

In line with previous research [32], we demonstrated that the frequency of measurements can strongly

influence the lag phase duration estimates (Fig. 4). We recommend taking measurements with

466 maximal 0.5h intervals, and more frequently if one expects untypically shaped growth curves. We also

467 highlight the importance of correct calculation of N0 (the initial number of alive cells, capable of

468 proliferating) and of that number being above the detection limit. In the laboratory settings, this 469 number can be relatively easily adjusted by adequate dilutions and simultaneously gives a much

469 number can be relatively easily adjusted by adequate dilutions and simultaneously gives a much
470 broader spectrum of possible analysis afterward. If N0 is below the detection level, then we are likely

- brouder speed and of possible analysis area ward. If the is below the detection level, then we are inkerg
- to overestimate the lag duration, because the first signals of growth will also be under detection level.

To overcome this problem, one can assume a certain growth curve shape below the detection limit as

done in Pierantoni *et al.* 2019 [31] and apply model fitting to estimate the lag duration. Additionally, if

- this number cannot be measured with high confidence (for example because of some dead or senescent
- 475 cells being a part of the inoculum) one can use the biomass increase method to estimate population lag
- 476 duration.

477 Although THE BIOMASS INCREASE method is simplistic, and it may be questionable if its results

478 represent the real lag length, we believe it provides a good ecological measure of how efficiently a

- 479 given population can inhabit a niche (net biomass gain). We suggest to apply this method if the growth
- 480 curve greatly deviates from model shape or when the growth curve cannot be corrected for blanks or
- 481 dead cells (i.e. if a fraction of the population size accounts for dead cells). In this case all other

482 methods do not work correctly, because their assumptions are violated (Fig. 6). Note however that an

483 important drawback of this method is that the chosen threshold value is arbitrary and may have no

484 biological meaning.

485 THE MAX GROWTH ACCELERATION is a very elegant method from mathematical point of view, and it

486 may be a good choice for growth curves with non-standard shapes. However, it is very sensitive to

- 487 noise (i.e. the calculation of the second derivative is very noise sensitive, Fig. 5). We recommend
- 488 using smoothening function before applying the max growth acceleration method (Fig. 6).

489 The most popular TANGENT METHOD works reasonably well even if the assumption about the constant 490 growth rate is violated. Additionally, it is not highly affected by data noisiness, but it performs worse 491 than the parameter fitting method if the measurements are not taken often enough (Fig. 5). The 492 challenging step may be the choice of how to draw the tangent line. If the tangent line is drawn to one 493 point only (the point where the growth rate is maximal) there is a risk its slope will be under or 494 overestimated if the data is noisy and an outlying point is chosen. This problem can be mitigated by 495 drawing the regression line around points in the exponential phase. However, it may be not obvious in 496 which time range the population grows exponentially. In fact, in order to know where the exponential 497 phase starts one needs to know where the lag phase finishes, which brings us back to the original 498 problem. Thus, the selection of data points in exponential phase often requires some manual inspection 499 or additional assumptions. Within our web tool, N points are chosen around the point with the 500 maximal growth rate, where N is a user-specified parameter. Note that the tangent method requires the 501 initial number of cells capable of proliferating (N0) to be determined with high confidence. If a 502 substantial fraction of the population is dead or senescent, what may be the case for populations that 503 had previously experienced some stress (e.g. drug exposure, long-term starvation) [19,36], the NO 504 captured by absorbance will be heavily overestimated.

505 THE PARAMETER FITTING TO THE LOGISTIC MODEL is the most robust lag calculation method. It shows 506 good accuracy even for noisy data (Fig. 5), and it can be used to overcome some technical limitations 507 (such as N0 below detection level, or a high level of noise). Importantly, the method performed well 508 not only for the curves simulated from logistic model, but also for the ones simulated from Monod 509 model (Supplementary Fig. 4). The performance of this method depend on the shape of the growth 510 curve, e.g. if the growth curve highly deviates from the standard shape, the fitting may not converge to 511 any solution (Supplementary Fig. 5). This problem can be fixed by finding a more suitable 512 optimisation algorithm, initial parameter values, or data transformation. These options are available within our web tool LAG PHASE DURATION CALCULATOR. However, we highlight the fact that the lag 513 514 phase duration estimation is dependent not only on the selected method but also on multiple 515 parameters of that method which tend to be underreported within experimental studies.

517 CONCLUSIONS

- 518 The results presented here emphasize that while determining lag phase duration, all steps (pre-
- 519 processing, choice of a method, and parameters) influence the final outcome. We would like to
- 520 highlight that all these steps should be thoughtfully reported to ensure data reproducibility and
- 521 credibility. Even the simplest tangent method requires specifying some details such as (i) if the initial
- 522 biomass is represented by the first measurement or the minimal value, or (ii) the number of data points
- 523 (or time frame) taken to draw the tangent line whether a single point (e.g. [23]) or multiple points
- from the exponential phase (e.g. [7]) were used.
- 525 Within this publication, we came out with two solutions to facilitate the process of reproducible lag
- 526 phase duration determination. First, we designed a decision tree (Fig. 7), which helps to choose the
- 527 method best suited to one's experimental conditions, taking into account various technical limitations
- 528 and data imperfections. Second, we have developed an online tool, which will help to directly compare
- 529 the lag phase duration estimated by different algorithms (i.e. combinations of methods, parameters,
- and data pre-processing techniques). The tool allows parameter adjustments and data pre-processing.
- 531 Moreover, we share our code on GitHub
- 532 [https://github.com/bognabognabogna/microbial_lag_calulator] so that it can be further developed and
- 533 customised. In particular, we share the function "Calculate.Lag" which can be taken directly to the R
- 534 environment. We perceive our tool as an initial point to further improvements made by the scientific
- 535 community so that any new potential challenges can be solved in a reproducible way.

537 METHODS

- 538 EXPERIMENTAL GROWTH CURVES:
- 539 The empirical growth curve data used here were a part of a project described in Opalek *et al.* (2022)
- 540 [19]. All growth curves were acquired by growing *Saccharomyces cerevisiae* S288C cultures in rich
- 541 YPD media at 30°C. The populations were starved in various conditions before the growth procedure
- 542 (see details in Supplement). The populations' densities were monitored by absorbance measurements
- 543 (optical density (OD), 600 nm in SpectraMax iD3) taken every half an hour. Then, the biomass was
- 544 calculated according to the equations:
- 545 For OD measurements below 0.1: 10^(1.885845 + 28.72096×OD)
- 546 For OD measurement equal or above 0.1: $3566518 \times OD + 26754296 \times OD^2 19881567 \times OD^3$
- 547 SIMULATED GROWTH CURVES:
- 548 Growth curves shown in Fig. 1 were simulated from the models described in the Supplement, and for
- 549 time points spanning every 6 minutes between 0 and 24 hours. The parameters used are listed in the
- 550 Supplementary Table 3.
- 551 LAG DURATION CALCULATION METHODS:
- 552 Let t denote time, λ denote lag duration, B denote biomass, and let t_i denote the i'th time point of the
- growth curve. If we define $N = \ln(B)$, then $\frac{dN}{dt}$ can be understood as the growth rate.
- Additionally let B_0 denote the first observation of biomass, and $N_0 = \ln(B_0)$

555 Tangent to max. growth line

556 The derivative of *lN* is approximated using to the central scheme i.e.

557
$$\frac{dN(t_i)}{dt} = \frac{N(t_{i+1}) - N(t_{i-1})}{t_{i+1} - t_{i-1}}$$

- 558 Then the maximal growth rate is defined as the maximum value of $\frac{dN}{dt}$ found within the growth curve
- 559 i.e.: $\max_{i}(\frac{dN(t_i)}{dt_i})$. The first point at which growth rate is maximal is then denoted as $(t^{\max.growth}, t)$
- 560 $N^{\text{max.grwoth}}$). Finally, a tangent line to this point is calculated.
- 561 Subsequently, lag duration is defined as the time when that tangent line crosses the N_0 line. This value 562 can be calculated as:

563
$$\lambda = (N_0 - N^{\max.growth} - \max_i (\max_i(\frac{dN(t_i)}{dt_i})) * t^{max.growth}) / \max_i(\frac{dN(t_i)}{dt_i})$$

564 **Tangent to max. growth point**

- 565 The first point at which growth rate is maximal is calculated as above and it is denoted as
- 566 $(t^{\text{max.growth}}, N^{\text{max.grwoth}})$. Then *n* points around that point are taken and a regression line is
- 567 calculated using the *lm* function from the *stats* package (R). Subsequently, lag duration is defined as
- the time when that regression line crosses the N_0 line. Within the manuscript n = 3. However, within

our web tool this value can be modified by the user. Moreover, the value of N_0 can be defined either as the first or the minimal log(biomass) value.

571 **Parameter fitting to logistic model**

- 572 All parameters of the logistic model (described in Supplement) are fitted simultaneously to the growth
- 573 curve data using the *nlsLM* function from *minpack.lm* package (R), and the Levenberg-Marquardt
- algorithm, max.iter = 100. The initial values are estimated from the data and the following initial
- 575 values: initial K = max(B), and initial lag = lag as calculated with the tangent method, and the initial
- 576 growth rate is fitted to the data points that are likely to be in exponential growth i.e. those where
- biomass is between $B_0 + 0.2(\max(B) B_0)$ and $B_0 + 0.8(\max(B) B_0)$. All these initial values
- 578 can be adjusted within the web tool.

579 Parameter fitting to Baranyi model

580 All parameters of the Baranyi and Roberts model (described in Appendix) are fitted simultaneously to

the growth curve data using the *nls* function from *stats* package (R) and the *baranyi* formula from the

nlsMicrobio package. We use the Gauss-Newton algorithm, max.iter = 100, and the following initial

values: init.LOG10Nmax = max(log₁₀(B), init.LOG10N0 = log₁₀(B_0), and the initial growth rate is

- fitted to the data points that are likely to be in exponential growth i.e. those where biomass is between $B_0 + 0.2(\max(B) - B_0)$ and $B_0 + 0.8(\max(B) - B_0)$. All these values can be adjusted within the
- 586 web tool.

587 Max. growth acceleration

588 The second derivative of *N* is calculated approximated with the central scheme i.e.

589
$$\frac{d^2 N(t_N)}{dt^2} = \frac{N(t_{N+1}) + N(t_{N-1}) - N(t_N)}{((t_{N+1} - t_{N-1})/2)^2}$$

590 Then the max. growth acceleration point is defined as the maximum value of $\frac{d^2 N(t_i)}{dt^2}$ found within the

591 growth curve i.e.: $\max_{i} \left(\frac{d^2 N(i)}{dt^2}\right)$. Consequently, lag duration λ is defined as the minimum time t_M at 592 which $\frac{d^2 N(t_i)}{dt^2} = \max_{i} \left(\frac{d^2 N(t_i)}{dt^2}\right)$.

593 **Biomass increase**

- 594 Let us define:
- $\Delta B_{t_i} = B(t_i) B_0$
- 596 Lag duration λ is defined as the minimum time t_M at which
- 597 $\Delta B_{t_M} \ge$ threshold

Finally, if the lag calculated by any of the method above turn out negative (which indeed may happenin the tangent and parameter fitting methods) we assume it is equal to 0.

- 600 Throughout this manuscript threshold = 10^5 . However, within our web tool this value can be modified
- by the user. Moreover, the value of B_0 can be defined either as the first or the minimal biomass value.
- 602 Additionally in case the lag estimate is negative, NA is returned.

603 The code used to calculate the lag according to various methods can be found on GitHub

- 604 https://github.com/bognabognabogna/microbial_lag_calulator
- 605

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617 AUTHOR CONTRIBUTION

- 618 Conceptualization M.O., B.J.S., D.W.-S.; Empirical data M.O.; Data visualization B.J.S., M.O.; B.J.S.
- 619 developed the web server MICROBIAL LAG PHASE DURATION CALCULATOR; Writing-
- 620 original draft preparation M.O, B.J.S.; Writing reviewing B.J.S., D.W.-S.
- 621 All authors have read and agreed to the published version of the manuscript
- 622
- 623 CONFLICT OF INTEREST
- 624 The authors declare no conflict of interest.
- 625

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768 SUPPLEMENT

769 FORMULATIONS OF MATHEMATICAL MODELS USED TO SIMULATE THE GROWTH CURVE DATA:

- 1. Exponential model
- 771 $\frac{dN}{dt} = \begin{cases} rN, & t \ge L\\ 0, & t < L \end{cases}$
- which can be solved as

773
$$N(t) = \begin{cases} N(0)e^{r(t-L)}, & t \ge L\\ N(0), & t < L \end{cases}$$

- where N(t) is the population size at time t, L is the lag phase duration, and r is the growth rate
 (constant in time).
- 777 2. Logistic model

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781

782 783 where N(t) is the population size at time *t*, *L* is the lag phase duration, *r* is the growth rate (constant in time), and *K* is the saturation constant (i.e. the maximum population size).

 $\frac{dN}{dt} = \begin{cases} rN\left(1 - \frac{N}{K}\right), t \ge L\\ 0, t < L \end{cases}$

3. Monod Model

784
$$\frac{dN}{dt} = \begin{cases} a \frac{VG}{K^G + G} N, t \ge L \\ 0, t < L \end{cases}$$

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786
$$\frac{dG}{dt} = \begin{cases} -\frac{VG}{K^G + G}G, t \ge L\\ 0, t < L \end{cases}$$

787where N(t) is the population size at time t, G(t) is the limiting nutrient (for example glucose)788concentration at time t, L is the lag phase duration. V denotes the maximal rate of the glucose789uptake pathway, and K^G denotes the Michaelis-Menten constant (so that if $K^G=G$, $\frac{VG}{K^G+G}=V/2$).790Moreover, the efficiency of converting glucose into biomass is described by a parameter a, which791for simplicity is assumed to be constant.

792 4. Baranyi and Roberts Model

$$\frac{dN}{dt} = r\left(1 - \frac{N}{K}\right)\frac{Q}{1+Q}N$$

794

793

795Where N(t) is the population size at time t, K is the saturation constant (i.e. the maximum796population size), and Q represents the physiological state proportional to the concentration of797some critical substrate.

 $\frac{dQ}{dt} = vQ$

798 THE GROWTH CURVE DATA:

- Supplementary Table 1 gathers information about the populations used as examples (Fig. 2, Fig. 3) for
- 800 empirical growth curves within this publication (Fig. 2, 6). We choose growth curves that demonstrate
- 801 possible challenges in analysis, such as: biomass drop at the beginning of measurements (curve_1,
- 802 curve_6), prolonged lag phase (curve_7, curve_8, curve_11), lack of easily-visible lag phase
- 803 (curve_3), slow biomass increase (curve_8, curve_9), lack of exponential growth phase (curve_11),
- turbulent measurements in biomass (curve_10).
- 805 The data was originally collected to analyse possible advantages of phenotypic heterogeneity within a
- starving clonal population and it was used in Opalek et al. 2022. In particular, the growth curves come
- 807 from three artificially prepared *S.cerevisiae* population types: Q monoculture, NQ monoculture, and
- 808 mix culture. The Q (growth-arrested quiescent cells) and NQ (non-quiescent) cells are distinct
- 809 phenotypes that can be separated from the stationary phase population (Allen *et al.* 2006). The
- 810 populations had experienced starvation in complex (spent rich YPD medium) or simple (H_{20})
- 811 environments and had been regrown in rich media afterward for fitness analysis. For details, see
- 812 Opalek et al. 2022.
- 813 The data was not analysed nor visualised in the form presented in this publication.
- 814 Supplementary Table 1. The summary of starvation conditions, chronological age, and phenotypic
- states of populations used to acquire growth curves analysed within this publication.

CURVE ID	STARVATION MEDIA	STARVATION DURATION	CELL TYPE	
curve_1	complex environment	7 days	NQ monoculture	
curve_2	complex environment	7 days	NQ monoculture	
curve_3	complex environment	7 days	natural mix culture	
curve_4	simple environment	14 days	NQ monoculture	
curve_5	complex environment	14 days	Q monoculture	
curve_6	complex environment	14 days	Q monoculture	
curve_7	simple environment	35 days	NQ monoculture	
curve_8	complex environment	35 days	NQ monoculture	
curve_9	complex environment	42 days	NQ monoculture	
curve_10	simple environment	42 days	Q monoculture	
curve_11	simple environment	42 days	NQ monoculture	

816

- 818 Supplementary Table 2. The summary of lag phase durations calculated by various methods
- 819 (columns). The table compares the similarities of results for each of the curves. The numbers are given
- 820 in hours.

821

CURVE_ID	BIOMASS INCREASE	MAX GROWTH ACCELE- RATION	PAR. FITTING TO THE BARANYI MODEL	PAR. FITTING TO THE LOGISTIC MODEL	TANGENT TO MAX GROWTH LINE	TANGENT TO MAX GROWTH POINT	AVERAGE ± SD	MAX	MIN
curve_1	2	1.5	2.6	1.9	1.6	1.6	1.87 ± 0.83	2.60	1.50
curve_2	1	1.5	2.6	1.9	1.6	1.6	1.70 ± 1.40	2.60	1.00
curve_3	1	1.5	2	0.9	0.7	0.7	1.13 ± 1.33	2.00	0.70
curve_4	3.5	3.5	6.1	5.2	4.4	4.4	4.52 ± 5.07	6.10	3.50
curve_5	2	1	2.5	1.9	1.8	1.7	1.82 ± 1.19	2.50	1.00
curve_6	2	1	2.5	2.2	1.9	1.9	1.92 ± 1.27	2.50	1.00
curve_7	1.5	5	NA	11	8.5	8.5	6.90 ± 54.70	11.00	1.50
curve_8	7	3	7.9	6.8	6.6	6.7	6.33 ± 14.43	7.90	3.00
curve_9	4	1	5.6	4.1	4.5	4.4	3.93 ± 11.95	5.60	1.00
curve_10	1	4	7.3	5.8	5.1	5.1	4.72 ± 22.47	7.30	1.00
curve_11	14.5	1.5	20.2	17.6	18.2	18.2	15.03 ± 236.77	20.20	1.50

⁸²² 823

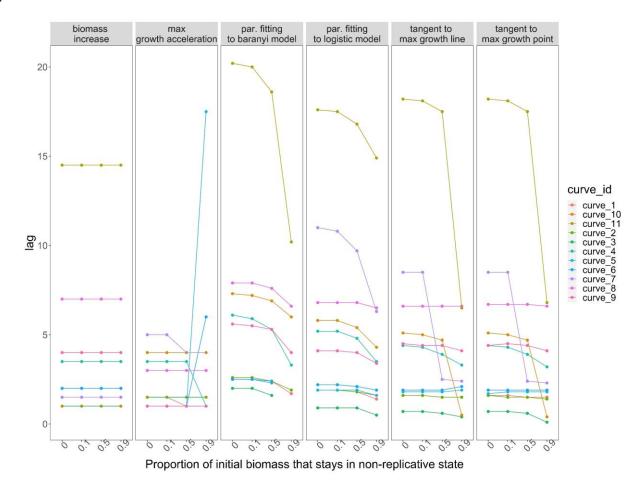
824 Supplementary Table 3. Default parameters used to simulate data from models described in section

825 FORMULATIONS OF MATHEMATICAL MODELS USED TO SIMULATE THE GROWTH CURVE DATA.

Parameter name	Default value
Growth rate r (exponential and Baranyi model)	$0.1 \ [h^{-1}]$
Max. growth rate r (logistic model)	$0.25 \ [h^{-1}]$
Carrying capacity K (logistic model)	5 * 10 ⁶ [cells/mL]
Initial biomass N ₀	10 ⁶ [cells/mL]
Lag L in logistic, exponential, and Monod model	2.5 [h]
Initial physiological state in Baranyi model Q_0	3.52 [units]
Efficiency of substrate uptake in Monod model <i>a</i>	3*10 ⁵ [cells/mmol substrate]
Maximal substrate uptake rate in Monod model V	$1.5*10^{(-5)}$ [mmol substrate / cells × h])
Michaelis-Menten constant in Monod model K^G	300 [mmol substrate]

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832 Supplementary Figure 1. Lag duration as calculated for 11 experimental growth curves with various
833 methods, assuming that a percentage of the initial population is non-replicative.

In case of growth curves, where there is no biomass increase for prolonged time (e.g. curve_11), the

835 proportion of alive vs senescent (permanently non-replicative cells) highly impact estimated lag

836 duration. If all cells are capable of proliferating (x = 0), then the observed lack of biomass increase is

837 indeed a lag phase, however if a substantial fraction of population is unable to proliferate (x = 0.9),

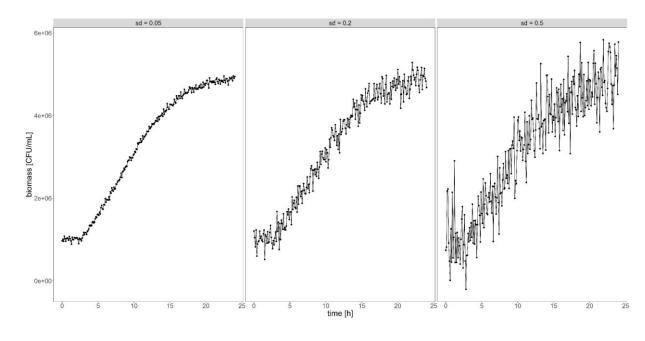
838 then the remaining 10% of alive cells start proliferate earlier (lag = 5-10 h, depending on the method

839 (columns)), however the increase in biomass is small to be marked as the lag phase end. That is why

the proportion of dead cells should be also monitored when analysing growth curves.

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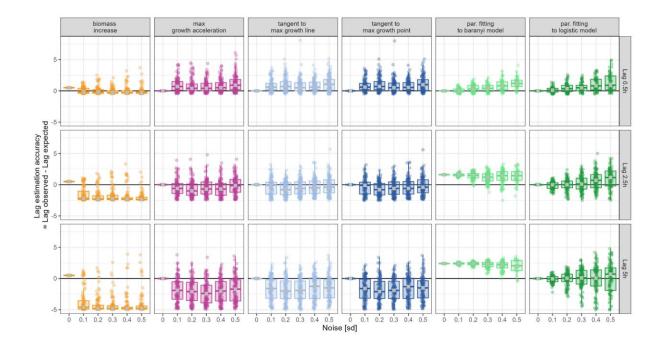
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846 Supplementary Figure 2. The visualization of the impact of introduced noisiness (sd) on growth curve

- shape. The noise was simulated from the random distribution with mean = 0 and standard deviation
- standardized by the initial biomass B0

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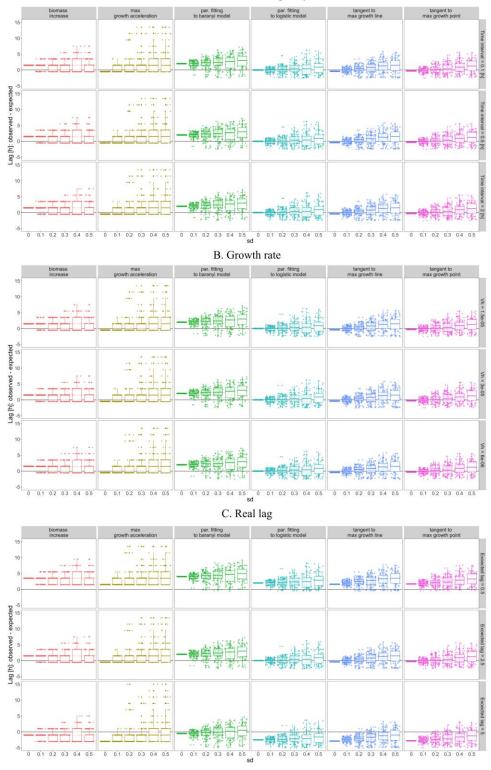


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854 Supplementary Fig. 3. Impact of real lag phase duration on calculation accuracy. For each sd 855 (noisiness, x-axis), expected lag length (rows), and lag calculation method (columns), 100 simulations were conducted (points). For each row, the true (expected) lag was set as a different value, e.g. in the 856 857 first row, the true lag was set as 0.5h, and the y = 0 correspond to lag = 0.5h, while in the second row, 858 y = 0 correspond to lag = 2.5h. Boxplots illustrate the distribution of points, and the median value 859 (grey square). All points located above y = 0, show these calculations where lag phase length was 860 overestimated, similarly, all points below y = 0 show calculations where lag duration was 861 underestimated.

Parameter fitting to Baranyi model systematically overestimates lag phase duration. For short lags (first row), all but biomass increase methods tend to overestimate lag duration when noisiness increase, while for long lags (last row), all but parameter fitting to logistic model methods underestimate lag duration when noisiness increase. Median value (grey square) for the parameter fitting to the logistic model is closest to y = 0 what indicates that this method is least biased.

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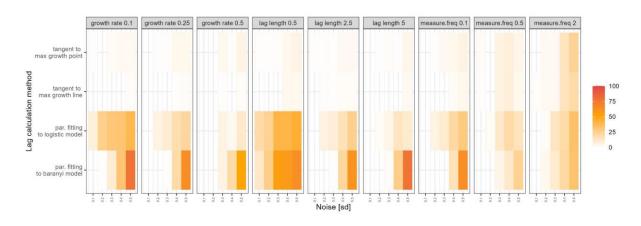


A. Measurement frequency

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Supplementary Fig. 4. Testing robustness of results for data simulated via Monod model. The biases
and accuracies of the methods show the same pattern as described in the manuscript (Fig. 3, Fig. 4,
Supp. Fig. 3), and as such, the outcomes are not driven by the model itself.





875

876 Supplementary Fig. 5. Frequency of NA value generated (indicating failure of the lag estimation) out 877 of 100 simulated growth curves (the curves described in section: TESTING THE SENSITIVITY OF LAG 878 DETERMINATION METHODS TO DATA NOISINESS). In case of parameter fitting to model NA means that the fitting didn't converge to any solution, while for tangent method NA is generated if lag was 879 880 estimated as a negative value. No filling indicates that all 100 lag estimations values were generated. 881 For biomass increase and max growth acceleration methods all lag estimations were successful and as 882 such they were excluded from the graph. The maximum of 78 failed lag estimations were generated 883 twice for parameter fitting to Baranyi model: for data simulated with slow growth rate (0.1) and long 884 lag phase (5h), both with and high noise (sd = 0.5).