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The steady-state assumption in oscillating and growing systems

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HIGHLIGHTS

- New mathematical foundation of steady-state assumption based on averages.
- Applies to oscillating and growing systems.
- Does not require quasi-steady-state assumption.
- Pinpoints unintuitive effects in the integration of metabolite concentrations.
- Can be used to approximate growth maximization in dynamic metabolic network models.

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ABSTRACT

The steady-state assumption, which states that the production and consumption of metabolites inside the cell are balanced, is one of the key aspects that makes an efficient analysis of genome-scale metabolic networks possible. It can be motivated from two different perspectives. In the time-scales perspective, we use the fact that metabolism is much faster than other cellular processes such as gene expression. Hence, the steady-state assumption is derived as a quasi-steady-state approximation of the metabolism that adapts to the changing cellular conditions.

In this article we focus on the second perspective, stating that on the long run no metabolite can accumulate or deplete. In contrast to the first perspective it is not immediately clear how this perspective can be captured mathematically and what assumptions are required to obtain the steady-state condition.

By presenting a mathematical framework based on the second perspective we demonstrate that the assumption of steady-state also applies to oscillating and growing systems without requiring quasi-steady-state at any time point. However, we also show that the average concentrations may not be compatible with the average fluxes.

In summary, we establish a mathematical foundation for the steady-state assumption for long time periods that justifies its successful use in many applications. Furthermore, this mathematical foundation also pinpoints unintuitive effects in the integration of metabolite concentrations using nonlinear constraints into steady-state models for long time periods.

1. Introduction

A rather frequently used assumption for metabolic network modelling is that the production and consumption of internal metabolites must balance (steady-state assumption). This assumption lies at the core of many metabolic network analysis techniques such as flux balance analysis (FBA) (Varma and Palsson, 1994; Orth et al., 2010), elementary flux mode analysis (Schuster and Hilgetag, 1994), metabolic control analysis (Heinrich and Schuster, 1998) or gene intervention studies (Hädicke and Klamt, 2011; Burgard et al., 2003).

Given the stoichiometric matrix S of a metabolic network, we call a vector of reaction rates (fluxes) $\mathbf{w}$ a steady-state flux if it satisfies

$$S\mathbf{w} = \mathbf{0}. \tag{SS}$$

In this paper we provide a new, mathematically sound derivation of the steady-state condition using flux averages over time. This derivation does not require any underlying theory on

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dynamics, like oscillations, in metabolic networks. While the biological motivation of our approach, as detailed in Section 1.2, is well known (Fell, 1997; Steuer and Junker, 2009; Knoke et al., 2008; Schuster and Fell, 2007; Palsson, 2015), the mathematical foundation presented here strengthens the existing approaches that study metabolism using steady-state fluxes.

The steady-state assumption, as used in metabolic network analysis, is usually mathematically derived from a quasi-steady-state perspective. This perspective is however not always applicable, as pointed out in Song and Ramkrishna (2009). Therefore, our mathematical derivation presented here does not use the quasi-steady-state argument. We nevertheless outline the quasi-steady-state perspective below for the sake of comparison.

1.1. Classical derivation based on the quasi-steady-state assumption

To illustrate the differences between the existing theory and our new derivation, we first recall how the steady-state assumption is mathematically derived in the quasi-steady-state perspective.

Given a kinetic model

$$\dot{\mathbf{c}}(t) = S\mathbf{v}(t), \quad \mathbf{v}(t) = f(\mathbf{e}(t), \mathbf{c}(t)) \tag{KM1}$$

that describes the dynamics of the internal metabolite concentrations $\mathbf{c}$, reaction rates $\mathbf{v}$ and enzyme concentrations $\mathbf{e}$, we assume that the dynamics of the metabolism can be approximated by a quasi-steady-state solution with respect to the enzyme dynamics. A quasi-steady-state solution of (KM1) is a tuple of time-dependent functions $(\mathbf{c}, \mathbf{v}, \mathbf{e})$ such that

$$\mathbf{0} = S\mathbf{v}(t), \quad \mathbf{v}(t) = f(\mathbf{e}(t), \mathbf{c}(t)) \quad \text{for all } t \geq 0. \tag{QSS}$$

Note that in the QSS solution the enzyme and metabolite concentrations can still change over time (the constraint on the metabolite concentrations $\dot{\mathbf{c}}(t) = S\mathbf{v}(t)$ is dropped) while fluxes transition from one metabolic steady-state to another, and are therefore not constant.

Indeed, as Varma and Palsson put it, "this assumption is based on the fact that metabolic transients are typically rapid compared to cellular growth rates and environmental changes. The consequence of this assumption is that all metabolic fluxes leading to the formation and degradation of any metabolite must balance" (Varma and Palsson, 1994, p. 994). Similar reasons for assuming a quasi-steady-state for metabolism are obtained by comparing the time scale of metabolic processes (fast) to those of e.g. transcriptional regulation or cell cycle (slow) (Almquist et al., 2014; Heinrich and Schuster, 1996; Moreira dos Santos et al., 2004). Hence, it is assumed that at every time point the metabolite concentrations have converged to a steady-state and thus the quasi-steady-state assumption (QSS) follows (Schilling et al., 1999; Voss et al., 2003; Waldherr et al., 2015).

The quasi-steady-state assumption found successful applications in dynamic simulation models like dynamic flux balance analysis (dynamic FBA) (Mahadevan et al., 2002) and dynamic enzyme-cost flux balance analysis (Waldherr et al., 2015).

There are, however, situations when the quasi-steady-state assumption cannot be applied (Song and Ramkrishna, 2009; Behre and Schuster, 2009), which means the derivation above cannot be used. Therefore, the main result of this paper is a derivation that does not need this assumption.

Before we continue with our new mathematical approach, it is worth noting the difference between the steady-states in (QSS) and the global steady-state used in classical metabolic network analysis tools such as FBA.

Given (QSS), for every time point t, $\mathbf{v}(t)$ is a steady-state flux. Therefore, we consider the quasi-steady-state assumption a *time-local* property. From this the steady-state condition $S\mathbf{w} = 0$ as used in classical metabolic network analysis is derived. This simplification allows for an efficient analysis of metabolic networks, since metabolite concentrations and time do not need to be modelled anymore. For example, the constraint $S\mathbf{w} = \mathbf{0}$ is used in methods such as FBA to predict biomass yields and growth rates.

In FBA we use only one steady-state flux to describe the whole growth cycle. This is what we call a *time-global* steady-state flux. However, metabolic fluxes are not constant in time. For instance, during the cell cycle the cell goes through different phases (G_1, S, G_2 and M) during which the metabolic activity is different. Therefore, the metabolism can be considered to use different time-local steady-state fluxes that follow the division cycle. Since the sum of steady-state fluxes yields another steady-state flux (i.e., if $S\mathbf{w} = \mathbf{0}$ and $S\mathbf{v} = \mathbf{0}$, then $S(\mathbf{w} + \mathbf{v}) = \mathbf{0}$), by combining the time-local steady-state fluxes we can obtain a time-global steady-state flux for the whole growth cycle.

1.2. The perspective based on long time periods

However, we do not need time-local steady-states to obtain a time-global steady-state. For example the steady-state assumption is also often motivated by stating that no metabolite can accumulate or deplete on the long run (Fell, 1997). The aim of this paper is to provide a general mathematical framework based on this idea. In particular, we will generalize the approach used in Steuer and Junker (2009), and Knoke et al. (2008, 2010). They observe that, if after a time T no net change $\Delta\mathbf{c}(T) = \mathbf{0}$ has occurred in the metabolite concentrations, we obtain $S\int_0^T \mathbf{v}(t)dt = \mathbf{0}$. Hence, in this case, the average flux

$$\bar{\mathbf{v}}(T) := \frac{1}{T}\int_0^T \mathbf{v}(t)dt \tag{AVGV}$$

is also a steady-state flux. In contrast to the fluxes derived via the quasi-steady-state assumption, it applies globally over the time interval $[0, T]$. In particular, in cases where the quasi-steady-state assumption is not entirely justified (see e.g. Song and Ramkrishna, 2009), one can still obtain a time-global steady-state.

Building upon the ideas in Section 1.5.2 of Steuer and Junker (2009), we observe that, if we consider a long enough time period T, we do not necessarily need to come back to the same concentration. In order to obtain an average steady-state flux we only require that the concentrations stay bounded (see Fig. 1). While this is implied by physical laws, it should also happen because accumulation of metabolites in very high amounts is toxic for a cell. Therefore, on the long run, to avoid such toxicity, every metabolite should be produced, on average, at the same rate at which it is consumed (Fell, 1997). Moreover, even if deterministic chaos is rare in metabolic systems (Goldbeter et al., 2001), it is worth noting that the theory developed here is also applicable to chaotic and quasi-periodic systems if the attractor is bounded. Some ideas in this direction can be found in Knoke et al. (2008).

As already pointed out in Eker and Krummenacker (2013), if we consider long time periods, we also have to model the fact that molecule counts per cell change because of cell growth. Therefore, in the differential equation that models the change of concentrations in time we also need to consider an additional term that represents dilution via cell growth. Schuster et al. (2004) propose to neglect this term since it is anyway "small" compared to the intracellular fluxes.

Based on these observations, we present in Section 3 a mathematical perspective on the steady-state assumption that does not need the quasi-steady-state argument, but instead considers flux averages over time. Using this model we compute for how long we have to observe the system to obtain a sufficiently good

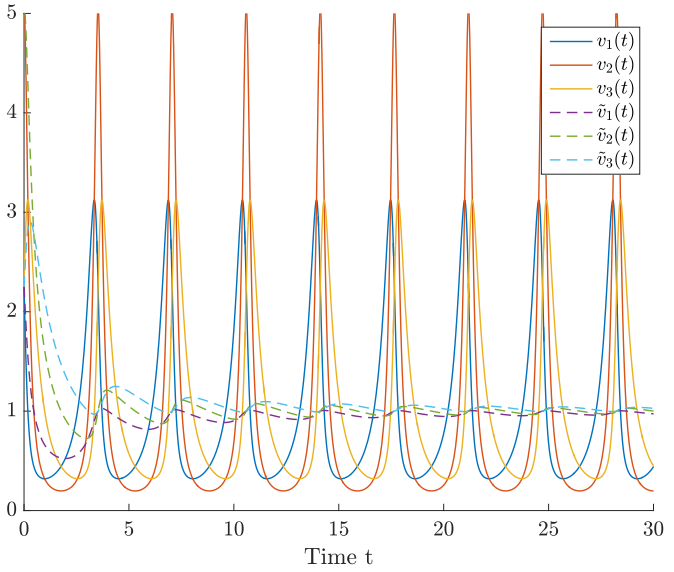

Fig. 1. Fluxes v_i and average fluxes $\tilde{v}_i$ for the example system discussed in Section 6. While the fluxes continue oscillating indefinitely, the average fluxes converge to a steady-state.

steady-state distribution on the example of three model organisms. In Section 4 we include dilution of metabolites via cell growth into the steady-state model. We also estimate the error that we make by neglecting metabolite dilution via cell growth. From these two aspects we conclude that indeed the steady-state assumption can be considered satisfied in practice even for oscillating and growing systems. The proofs of our mathematical results can be found in Appendix A.

The mathematical framework presented here is not only another justification for the steady-state assumption, but can also be used to mathematically show when FBA gives upper bounds on yield and growth rate as shown in Section 5.

However, there are also some caveats when dealing with the steady-state assumption for long time periods. In Section 6 we present a simple, artificial mass-action system, where the constraints implied by kinetic rate laws are violated by the average concentrations and fluxes. We conclude by posing the question whether a metabolic system can be more efficient by utilizing oscillations than with simple steady-state fluxes.

2. Notation

In the following we use $\mathcal{M} = \{1, \ldots, m\}$ to denote the set of metabolites, $\mathcal{R} = \{1, \ldots, r\}$ to denote the set of reactions, $\mathcal{E} = \{1, \ldots, k\}$ to denote the set of enzymes, and S to denote the stoichiometric matrix of a given metabolic network model. We use $\mathbf{v} = (v_1, \ldots, v_r)$ to denote the fluxes (reaction rates), and $\mathbf{c} = (c_1, \ldots, c_m)$ to denote the metabolite concentrations. Furthermore, we use $\mathbf{e} = (e_1, \ldots, e_k)$ to denote enzyme concentrations, $f: \mathbb{R}^k \times \mathbb{R}^m \to \mathbb{R}^r$ to denote deterministic kinetic rate laws, and μ to denote the growth rate.

Note that the fluxes $\mathbf{v}$, the concentrations $\mathbf{c}$ and $\mathbf{e}$, and the growth rate μ can depend on time. Furthermore, $\mathbf{v}(t) \in \mathbb{R}^r$, $\mathbf{c}(t) \in \mathbb{R}^m_{\geq 0}$, and $\mathbf{e}(t) \in \mathbb{R}^k_{\geq 0}$ are vectors and thus written in boldface, as all other vectors appearing in this paper.

3. The steady-state assumption for long time periods

Since no metabolite can accumulate or deplete indefinitely, it follows intuitively that production and consumption of all metabolites must balance. We will now formulate this argument mathematically.

3.1. Modelling assumptions (without dilution)

Our result applies to a very general setting. We essentially only ignore stochastic effects and thus require the following modelling assumptions:

- In this section we assume that the volume stays constant and changes in concentrations are only reaction-driven, i.e., we do not yet consider dilution of metabolites due to cell growth. The case when the volume can change is discussed in Section 4. While enzyme concentrations can be varied arbitrarily (e.g. due to regulatory control), metabolite concentrations and fluxes have to satisfy the following relationship, mentioned already in the Introduction, for every $t \geq 0$:

$$\dot{\mathbf{c}}(t) = S\mathbf{v}(t), \quad \mathbf{v}(t) = f(\mathbf{e}(t), \mathbf{c}(t)). \tag{KM1}$$

- Concentrations are measured in number of molecules per fixed volume. Hence, enzyme and metabolite concentrations are bounded, i.e., there exist $c^{\max}$ and $e^{\max}$ with $\| \mathbf{c}(t)\| \leq c^{\max}$, $\| \mathbf{e}(t)\| \leq e^{\max}$ for every time $t \geq 0$, where $\| \mathbf{x} \|$ denotes the Euclidean norm of the vector $\mathbf{x}$.
- We assume that the function f that represents the kinetic rate laws is continuous.
- We assume that $\mathbf{c}$ is differentiable and $\mathbf{e}$ is a continuous function of time.

Since f is continuous, and $\mathbf{c}$ and $\mathbf{e}$ are bounded and continuous, it follows that $\mathbf{v}$ must also be bounded and continuous.

3.2. Average fluxes

For a given time period T, we define the average fluxes $\tilde{\mathbf{v}}$, as introduced above, as:

$$\tilde{\mathbf{v}}(T) := \frac{1}{T}\int_0^T \mathbf{v}(t)dt. \tag{AVGV}$$

To mathematically analyse long time periods, we consider the case when $T \to \infty$. Unfortunately, it can happen that

$$\bar{\mathbf{v}} := \lim_{T\to\infty} \tilde{\mathbf{v}}(T) \tag{1}$$

does not exist (see Appendix B for an example). For simplicity, we assume in the following that the limit exists. Even in the case when the limit does not exist, the results hold in a similar fashion as described in Appendix A.

In the following we observe that average fluxes are steady-state fluxes:

Theorem 1. $S\bar{\mathbf{v}} = 0$.

Proof. The proof follows directly from Theorem 3 in Appendix A, since there is exactly one accumulation point of $\tilde{\mathbf{v}}(T)$ because we assume here that the limit exists. □

3.3. Violation of the steady-state condition for finite time T

For obtaining the statement of Theorem 1 we have assumed that $T \to \infty$. However, in practice we do not run the experiments for infinitely long time. We are therefore interested in how large do we have to choose T so that the fluxes violate the steady-state condition by at most ε.

We observe that with $T := \frac{c^{\max}}{\varepsilon}$ we get

$$\| S\bar{\mathbf{v}}(T)\| = \left\| \frac{1}{T}\int_0^T S\mathbf{v}(t)dt \right\| = \left\| \frac{1}{T}\int_0^T \dot{\mathbf{c}}(t)dt \right\| = \frac{\| \mathbf{c}(T) - \mathbf{c}(0)\|}{T} \leq \frac{c^{\max}}{T} = \varepsilon, \tag{2}$$

where $\| \mathbf{x} \|$ denotes the Euclidean norm of the vector $\mathbf{x}$.

We consider the three organisms *Escherichia coli*, *Saccharomyces cerevisiae* and *Homo sapiens* (HeLa cells) and compute in each case the averaging time T so that we obtain a relative violation of the steady-state condition of at most 1% in the approximation of fluxes.

3.4. Escherichia coli

For *E. coli* the average glucose uptake flux is $1.63{\cdot}10^{-18}$ mol/(s·cell) (Jain and Srivastava, 2009; Loferer-Krößbacher et al., 1998). Since we would like to have a violation of at most 1%, our ε is then $0.01{\cdot}10^{-18} = 10^{-20}$ mol/(s·cell). The maximum metabolite concentration measured in this organism is 96 mM (Bennett et al., 2009). We will therefore consider $c^{\max}$ as

$$c^{\max} = 100\,\frac{\text{mmol}}{\text{L}} = 10^{-1}\,\frac{\text{mol}}{\text{L}}{\cdot}0.6{\cdot}10^{-15}\,\frac{\text{L}}{\text{cell}} = 0.6{\cdot}10^{-16}\,\frac{\text{mol}}{\text{cell}},$$

where $1\text{ cell} = 0.6{\cdot}10^{-15}$ L is the volume of an *E. coli* cell (Kubitschek, 1990). Therefore, if we average the fluxes of this organism over a period $T = \frac{c^{\max}}{\varepsilon} = 6000$ s $\simeq 2$ h the steady-state condition will be violated by at most 1%.

Note that this means that we would have to average over six to eight generations in the case of *E. coli*. This is reasonable considering the fact that we need to average out fluctuations arising from the cell cycle.

3.5. Saccharomyces cerevisiae

In the case of *S. cerevisiae*, the average intracellular fluxes are around $1.38{\cdot}10^{-18}$ mol/(s·cell) (Stewart et al., 2010; Mitchison, 1958). Our ε in this case is therefore again 10^{-20} mol/(s·cell). According to Canelas et al. (2008) and Finka and Goloubinoff (2013), we can choose $c^{\max} = 10^{-16}$ mol/cell. Thus, the minimum time period for averaging so that we violate the steady-state condition by at most 1% is $T = 10\,000$ s $\simeq 3$ h.

3.6. Homo sapiens (HeLa cells)

Finally, for HeLa cells we have a glucose uptake flux of about $4.5{\cdot}10^{-17}$ mol/(s·cell) (Mojena et al., 1985), and thus we choose $\varepsilon = 10^{-19}$ mol/(s·cell). By the findings of Mojena et al. (1985), we can choose $c^{\max} = 10^{-15}$ mol/cell. Thus, in this case $T = 10^4$ s $\simeq 3$ h.

4. Dilution

We recall that concentration is defined as the number of molecules n of a substance present in a certain volume V of a solution, i.e., $c(t) = \frac{n(t)}{V(t)}$. While in Section 3 we assumed a constant volume, we now allow the volume to change over time. This happens for example in the case of cell growth, when the total volume of all cells grows.

The metabolic network typically consumes and produces metabolites as described in the stoichiometric matrix S. The product $S\mathbf{v}(t)$ then gives the change in metabolite concentrations when the volume stays constant. This product reflects the net production of each metabolite by the metabolic network. In case the volume changes, we also obtain a dilution term. Following the derivation in Goelzer et al. (2011), which we repeat for the reader's convenience in Appendix C, we get

$$\dot{\mathbf{c}}(t) = S\mathbf{v}(t) - \mu(t)\mathbf{c}(t), \tag{KM2a}$$

where the growth rate $\mu(t)$ is defined as

$$\mu(t) := \frac{\dot{V}(t)}{V(t)}. \tag{3}$$

Note that this is the same definition as the one used in Kacser and Beeby (1984), Heinrich and Schuster (1996) and Goelzer et al. (2011). We observe that

$$\bar{\mu}(T) := \frac{1}{T}\int_0^T \mu(t)dt. \tag{AVGM}$$

is the average growth rate:

Proposition 1. *It holds for all* $T \geq 0$ *that*

$$V(T) = V(0)\exp(\bar{\mu}(T){\cdot}T).$$

Proof. We define $W(t) := \ln(V(t))$ for all $t \geq 0$. It follows that

$$\dot{W}(t) = \frac{\dot{V}(t)}{V(t)} = \mu(t) \tag{4}$$

$$\Rightarrow \int_0^T \mu(t)dt = \int_0^T \dot{W}(t)dt = W(T) - W(0) \tag{5}$$

$$\Rightarrow \frac{V(T)}{V(0)} = \exp\Big(W(T) - W(0)\Big) = \exp\left(\int_0^T \mu(t)dt\right). \tag{6}$$

The proposition follows by definition of $\bar{\mu}$. □

4.1. Modelling assumptions (with dilution)

Following the derivation from above based on (KM2a), we now consider the new kinetic model

$$\dot{\mathbf{c}}(t) = S\mathbf{v}(t) - \mu(t)\mathbf{c}(t), \quad \mathbf{v}(t) = f(\mathbf{e}(t), \mathbf{c}(t)), \tag{KM2}$$

which now also models dilution of internal metabolites via cell growth. The rest of the assumptions are the same as in Section 3.1. In addition we assume that the growth rate $\mu(t)$ is positive, bounded and continuous for all time points $t \geq 0$.

4.2. Average fluxes and average concentrations

For a given time period T, we additionally define the average concentrations $\tilde{\mathbf{c}}$ as:

$$\tilde{\mathbf{c}}(T) := \frac{1}{T}\int_0^T \frac{\mu(t)}{\bar{\mu}(T)}\mathbf{c}(t)dt. \tag{AVGC}$$

Note that we scale the concentrations by the growth rate μ. The motivation for this is that, in order to avoid depletion of metabolite pools, it is much more important to overproduce metabolites in fast-growing periods than in slow-growing ones. We observe that $\tilde{\mathbf{c}}(T)$ can be considered an average over growth rates rather than over time since

$$\tilde{\mathbf{c}}(T) = \frac{\int_0^T \mu(t)c(t)dt}{\int_0^T \mu(t)dt}. \tag{7}$$

As in the case for the average fluxes $\bar{\mathbf{v}}$, it can happen that

$$\bar{\mathbf{c}} := \lim_{T\to\infty} \tilde{\mathbf{c}}(T), \tag{8}$$

$$\bar{\mu} := \lim_{T\to\infty} \tilde{\mu}(T) \tag{9}$$

do not exist. Hence we again assume, for simplicity, that the limits $\bar{\mathbf{v}}$, $\bar{\mathbf{c}}$, and $\bar{\mu}$ exist and detail in Appendix A the case where they do not exist.

We observe in the following that, if we consider dilution, the steady-state condition for the average fluxes changes slightly:

Theorem 2. $S\bar{\mathbf{v}} = \bar{\mathbf{c}}\bar{\mu}$.

Proof. The proof follows directly from Theorem 3 in Appendix A because we assume here that all limits exist. □

One way to understand Theorem 2 is to think of $\bar{\mathbf{c}}$ as the composition of the biomass reaction, which is typically used in constraint-based metabolic network analysis to mimic the consumption of key-metabolites for growth purposes (Feist and Palsson, 2010). $\bar{\mu}$ is then the flux through the biomass reaction. Because $\bar{\mathbf{c}}$ includes in our case all metabolites that are present in the cell, these metabolites also have to be duplicated upon cellular division. If DNA, RNA, lipids, and proteins are not explicitly modelled in the metabolic network, we can understand them as represented in $\bar{\mathbf{c}}$ in the form of precursors such as nucleic acids, amino acids, etc. This way, the dilution term also enforces the production of all macromolecules present in the cell.

We observe here a shortcoming in the construction of typical biomass reactions. The biomass reactions in FBA models do not usually include all metabolites, but only those that are needed to build macromolecules. This is done because the concentrations of internal metabolites are typically unknown and their overproduction is neglected in order to avoid overconstraining the solution space.

4.3. Violation of the steady-state condition by dilution

Since biomass reactions in FBA models do not involve all metabolites, they give rise to violations of the steady-state condition in Theorem 2. In the following we estimate the order of magnitude of this violation relative to the fluxes. For this purpose let us assume that the FBA model uses a biomass function $\mathbf{b}$ that approximates $\bar{\mathbf{c}}$ with an error of 1 mM, i.e., $|\mathbf{b} - \bar{\mathbf{c}}| = 1$ mM. We again use the three organisms *Escherichia coli*, *Saccharomyces cerevisiae* and *Homo sapiens* as examples.

The detailed calculations for the three organisms are presented in Appendix D. Other examples where the same trend can be observed can be found in Stephanopoulos et al. (1998).

4.4. Escherichia coli

E. coli has an average cell volume of 0.6 μm^3 (Kubitschek, 1990), a dry weight of 0.489 pg (Loferer-Krößbacher et al., 1998), an average growth rate on glucose of 0.9 h^{-1} (Andersen and Von Meyenburg, 1980), and a glucose uptake rate of 12 mmol/(gDW·h) (Jain and Srivastava, 2009). Using these values it follows that an approximation error of 1 mM for $\bar{\mathbf{c}}$ implies a violation of the steady-state condition in the order of 10^{-4} relative to the fluxes.

4.5. Saccharomyces cerevisiae

A similar result is obtained in the case of *S. cerevisiae*, which has an average growth rate on glucose of 0.4 h^{-1} (Waldron and Lacroute, 1975), average intracellular fluxes of 0.5 mmol/(gDW·h) (Stewart et al., 2010), a dry weight of approximately 10^{-11} g = 10 pg (Mitchison, 1958), and a volume of 20 μm^3 (Tyson et al., 1979). These values imply that an approximation error of 1 mM for $\bar{\mathbf{c}}$ leads to a violation of the steady-state condition in the order of 10^{-3} relative to the fluxes.

4.6. Homo sapiens (HeLa cells)

In the case of HeLa cells we obtain a similar order of magnitude for the violation. HeLa cells have an average growth rate of 0.06 h^{-1} (Kumei et al., 1989), glucose uptake flux of about 18 $\frac{\text{nmol}}{\text{min·mg protein}}$ (Mojena et al., 1985), approximately 150 pg protein (Finka and Goloubinoff, 2013), and a volume of 2600 μm^3 (Luciani et al., 2001; Finka and Goloubinoff, 2013). With these values, an approximation error of 1 mM for $\bar{\mathbf{c}}$ implies a steady-state condition violation in the order of 10^{-3} relative to the fluxes.

5. Applications to yield optimization

In the previous sections we have seen that, given a kinetic model, the average fluxes satisfy the steady-state assumption. This also applies to optimal control problems, which might be very hard or impossible to solve directly. However, since we know that the average flux is a steady-state flux we can build a much simpler FBA model to bind the results of the optimal control problem. Let us consider the optimum of the following FBA problem:

$$\begin{aligned} v^*_{FBA} := \max_{\mathbf{v}} \quad & v_{biomass} \\ \text{s.t.} \quad & S\mathbf{v} = \mathbf{0} \\ & \mathbf{l} \le \mathbf{v} \le \mathbf{u}, \end{aligned} \tag{FBA1}$$

where $v_{biomass}$ is the flux through the biomass reaction. Consider in addition the optimum of the following optimal control problem formulated based on the kinetic model (KM1):

$$\begin{aligned} \bar{v}^* := \max_{\mathbf{v},\mathbf{c},\mathbf{e}} \quad & \lim_{T\to\infty} \frac{1}{T} \int_0^T v_{biomass}(t)dt \\ \text{s.t.} \quad & \dot{\mathbf{c}}(t) = S\mathbf{v}(t) && \forall\, t \ge 0 \\ & \mathbf{v}(t) = f(\mathbf{e}(t), \mathbf{c}(t)) && \forall\, t \ge 0 \\ & \mathbf{l} \le \mathbf{v}(t) \le \mathbf{u} && \forall\, t \ge 0 \end{aligned} \tag{OCP1}$$

We observe that the FBA optimum is an upper bound for all steady-state solutions and hence also for average fluxes:

Corollary 1. $v^*_{FBA} \ge \bar{v}^*$.

Proof. We recall from (1) that

$$\lim_{T\to\infty} \frac{1}{T} \int_0^T v_{biomass}(t)dt = \bar{v}_{biomass}. \tag{10}$$

If the limit $\bar{\mathbf{v}}$ in (1) exists, then by Theorem 1, $\bar{\mathbf{v}}$ satisfies $S\bar{\mathbf{v}} = \mathbf{0}$. It is also easy to see that $\bar{\mathbf{v}}$ also satisfies $\mathbf{l} \le \bar{\mathbf{v}} \le \mathbf{u}$. Hence $\bar{\mathbf{v}}$ is a feasible solution of (FBA1).

If $\bar{\mathbf{v}}$ does not exist, we instead can use Theorem 3 and any accumulation point will be a feasible solution of (FBA1). □

A similar result holds in the case when we take dilution via cell growth into account. Let μ^*_{FBA} denote the optimum of the following FBA problem using as biomass function $\mathbf{b}$:

$$\begin{aligned} \mu^*_{FBA} := \max_{\mathbf{v},\mu} \quad & \mu \\ \text{s.t.} \quad & S\mathbf{v} = \mathbf{b}\mu \\ & \mathbf{l} \le \mathbf{v} \le \mathbf{u}, \end{aligned} \tag{FBA2}$$

Consider in addition $\bar{\mu}^*$ to be the optimum of the following optimal control problem formulated based on the kinetic model (KM2), where we enforce that the biomass composition ($\bar{\mathbf{c}}$) of the organism is $\mathbf{b}$:

$$\bar{\mu}^* := \max_{\mathbf{v},\mathbf{c},\mathbf{e},\mu} \lim_{T\to\infty} \frac{1}{T}\int_0^T \mu(t)dt$$
$$\text{s.t.} \quad \dot{\mathbf{c}}(t) = S\mathbf{v}(t) - \mu(t)\mathbf{c}(t) \qquad \forall\, t \geq 0$$
$$\mathbf{v}(t) = f(\mathbf{e}(t), \mathbf{c}(t)) \qquad \forall\, t \geq 0 \qquad \text{(OCP2)}$$
$$\mathbf{b} = \lim_{T\to\infty} \frac{1}{T}\int_0^T \frac{\mu(t)}{\bar{\mu}(t)}\mathbf{c}(t)dt$$
$$\mathbf{l} \leq \mathbf{v}(t) \leq \mathbf{u} \qquad \forall\, t \geq 0$$

In this case it also holds that the FBA optimum gives an upper bound for the growth rate if the correct biomass function $\mathbf{b}$ is used:

Corollary 2. *For every* $3\mathbf{b}$ *it holds that* $\mu^*_{FBA} \geq \bar{\mu}^*$.

Proof. Since the optimization problem for $\bar{\mu}^*$ is feasible, it follows that $\lim_{T\to\infty} \frac{1}{T}\int_0^T \frac{\mu(t)}{\bar{\mu}(t)}\mathbf{c}(t)dt$ has to exist. Thus, by the definition of $\bar{\mathbf{c}}$, $\mathbf{b} = \bar{\mathbf{c}}$. The rest of the argument follows as in the proof of Corollary 1, by interpreting μ as the flux through the biomass reaction. □

6. Kinetic constraints

In many cases additional constraints next to the steady-state condition are employed (Shlomi et al., 2011; Waldherr et al., 2015; Beard et al., 2002; Müller et al., 2014; Wortel et al., 2014). For example, let us assume we want to use the actual kinetic rate laws encoded by f to also constrain the average steady-state solution by the average enzyme and substrate concentrations. Our kinetically constrained steady-state model will then have the form:

$$S\mathbf{w} = \mathbf{0}, \quad \mathbf{w} = f(\mathbf{e}, \mathbf{c}), \quad \mathbf{e}, \mathbf{c} \geq \mathbf{0} \qquad \text{(KSS)}$$

Can the results from the previous sections also applied to this model?

Let us assume we have measured average fluxes $\bar{\mathbf{v}}$ and enzyme concentrations $\bar{\mathbf{e}}$ in an experiment (or from a simulation of the dynamic model). In the previous sections we have found that $S\bar{\mathbf{v}} = 0$. Can we also always find concentrations $\mathbf{c}'$ such that we get a feasible solution to the kinetically constrained steady-state model (KSS)? If we cannot, then kinetically constrained steady-state models may be overconstrained. The answer is not easy, since in the next subsection we will observe that $\mathbf{c}' = \bar{\mathbf{c}}$ does not always give a feasible solution.

To formulate the problem mathematically precisely we define the average enzyme concentrations

$$\tilde{\mathbf{e}}(T) := \frac{1}{T}\int_0^T \mathbf{e}(t)dt. \qquad \text{(AVGE)}$$

Again, the average enzyme concentrations might not exist. But for simplicity, we assume here that

$$\bar{\mathbf{e}}(T) := \lim_{T\to\infty} \tilde{\mathbf{e}}(T) \qquad (11)$$

exists. For the general case we refer the reader to Appendix A.2.

We can now formulate the problem as:

Problem 1. Does there always exist a $\mathbf{c}' \in \mathbb{R}^m_{\geq 0}$ such that $\bar{\mathbf{v}} = f(\bar{\mathbf{e}}, \mathbf{c}')$?

In the next subsection we illustrate the difficulties posed by Problem 1 using a toy example.

6.1. Average concentrations can be inconsistent with average fluxes

We consider the following toy metabolic network.

$$r_1\colon\ B + 2A_1 \rightarrow 3A_1$$
$$r_2\colon\ 2A_1 + 2A_2 \rightarrow A_1 + 3A_2$$
$$r_3\colon\ 2A_2 \rightarrow A_2 + C$$

For simplicity we assume that the system is subject to mass-action kinetics and enzyme concentrations have no effect. B and C are boundary metabolites and are kept at a constant concentration of 1. Considering all kinetic constants to be 1, we get the following system of ordinary differential equations:

$$\dot{c}_1 = v_1 - v_2 \quad v_1 = c_1^2$$
$$\dot{c}_2 = v_2 - v_3 \quad v_2 = c_1^2 c_2^2 \qquad \text{(Ex1)}$$
$$v_3 = c_2^2,$$

where c_1 and c_2 denote the concentrations of metabolites A_1 and A_2 respectively, and v_1, v_2, v_3 denote the fluxes through r_1, r_2, r_3, respectively.

The only steady-state solutions of this system are $c_1 = 0 = c_2$ and $c_1 = 1 = c_2$. If we do not start in such a steady-state, the system will oscillate. This can be seen as follows. The function

$$H(c_1, c_2) = c_1 + \frac{1}{c_1} + c_2 + \frac{1}{c_2} \qquad (12)$$

is a Hamiltonian of the considered ODE system, since its derivative $\frac{d}{dt}H(\mathbf{c}(t))$ is zero for all $t \geq 0$. We observe that the system cannot explode since for any $(c_1, c_2) > \mathbf{0}$ the Hamiltonian has a finite constant value and hence both $c_1(t)$ and $c_2(t)$ stay bounded for all $t \geq 0$. Furthermore, we observe that, for any other starting point that is not a steady-state, the Hamiltonian has a value different from 4, which is the minimum achieved at $c_1 = c_2 = 1$.

The trajectory of this system (obtained from numerical integration) with the starting point $(c_1, c_2) = (2, 2)$ is shown in Fig. 2. As (unweighted) average concentrations and fluxes we approximated numerically:

$$\bar{\mathbf{c}} = \lim_{T\to\infty} \frac{1}{T}\int_0^T \mathbf{c}(t)dt \approx (0.82, 0.82) \qquad (13)$$

$$\bar{\mathbf{v}} = \lim_{T\to\infty} \frac{1}{T}\int_0^T \mathbf{v}(t)dt \approx (1.00, 1.00, 1.00) \qquad (14)$$

We therefore conclude that in the toy example, the average concentrations are not compatible with the average fluxes, i.e., $\bar{\mathbf{v}} \neq f(\bar{\mathbf{c}})$ where f denotes the kinetic rate laws of the toy system. In

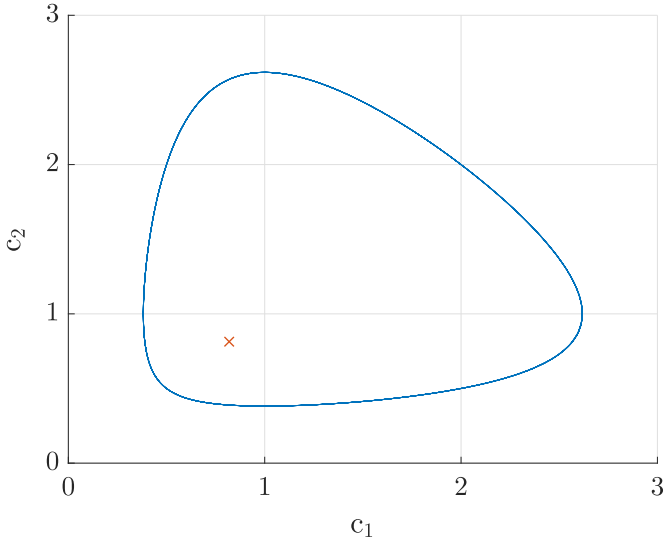

Fig. 2. Trajectory of our toy model with starting point $c_1(0) = 2$, $c_2(0) = 2$. The cross marks the average concentration for $T \to \infty$.

particular, the average concentrations do not even correspond to any steady-state flux distribution. This has also been observed and mathematically analysed by Knoke et al. (2010) for oscillations of Ca^{2+} in non-excitable cells using Jensen's inequality (Jensen, 1906).

Note that Problem 1 remains open since there exists a concentration vector $(c_1, c_2) = (1, 1)$ in the toy example that is consistent with the average fluxes.

6.2. Linear kinetic constraints remain consistent

Because of the problem described above, we consider kinetic constraints as used in Shlomi et al. (2011) and Waldherr et al. (2015). As an example, consider the simple reaction

$$A \xrightarrow{e_i} B$$

that is subject to Michaelis–Menten kinetics. Then the flux through this reaction is bounded as

$$v_i \leq \frac{k_{cat}^{+} e_i c_A}{K_M + c_A}, \tag{MM}$$

where k_{cat}^{+} and K_M are kinetic constants. Note that we use here an inequality rather than an equality to account for regulatory effects resulting in only part of the enzyme being available for catalysis. The argument below works however even if the equality version of the rate law is used.

We observe that $\frac{c_A}{K_M + c_A} \leq 1$ always. Our bound can thus be simplified as

$$v_i \leq k_{cat}^{+} e_i. \tag{ubMM}$$

For reversible reactions, we get the additional bound

$$v_i \geq -k_{cat}^{-} e_i, \tag{lbMM}$$

where k_{cat}^{-} is the turnover rate for the reverse direction of the reaction.

Therefore, we assume in the following that for a set of reactions $\mathcal{K} \subseteq \mathcal{R}$ we have constants k_i^{+}, k_i^{-} such that

$$-k_i^{-} e_i(t) \leq v_i(t) \leq k_i^{+} e_i(t) \quad \text{for all } t \geq 0 \text{ and } i \in \mathcal{K}. \tag{15}$$

This form gives us enough flexibility to also constrain the average fluxes using the average enzyme concentrations:

Proposition 2. *It holds that* $-k_i^{-}\bar{e}_i \leq \bar{v}_i \leq k_i^{+}\bar{e}_i$ *for all* $i \in \mathcal{K}$.

Proof. The result follows directly from Proposition 4 in Appendix A.2.

Therefore, results similar to those in Section 5 can also be obtained in this setting for the growth maximization case.

7. Discussion

One of the main arguments against using the steady-state assumption in models of biochemical reaction networks is that, if one assumes steady-state, oscillations that are biologically important will not be observed in the simulation results (Goldbeter, 1997; Sowa et al., 2014).

7.1. Average fluxes satisfy the steady-state assumption

However, in many cases we might not be interested in these oscillations, because they increase the complexity of the model, or even make it computationally intractable. In these cases, where only average fluxes over long time periods are of interest (e.g. if we are interested in predicting the lethality of gene knockout experiments), we have shown that the steady-state assumption can still be applied, i.e., we still get a reasonably good description of the metabolic system by computing a steady-state flux. In particular, it is also valid for oscillating systems that are not at steady-state at any point in time. The only condition is that the system is averaged over a long enough time period.

For example, FBA computes an upper bound on the biomass yield, which applies to all steady-state solutions. Hence it also applies to the average fluxes. Therefore, the system cannot obtain a higher yield using oscillations.

We showed that an average over 3 h is sufficient to obtain fluxes that only slightly violate the steady-state assumption for *E. coli*, *S. cerevisiae*, and HeLa cells. Since the estimate was rather pessimistic, much shorter averaging times might be sufficient in practice. Furthermore, the violation we obtained lies within the error range of current measurement technology for concentrations and fluxes.

7.2. Dilution of metabolites via cell growth and the biomass reaction

We observed that, depending on the organism, the steady-state condition gets violated by 0.01–0.1% for an error of 1 mM in the biomass composition. Since amino acid concentrations (including the amino acids in proteins) are in the order of 100 mM (Bennett et al., 2009), this implies that neglecting amino-acid overproduction would also only violate the steady-state condition by 1%. However, ignoring their overproduction during growth would mean that we would completely disregard protein production, which cannot lead to any biologically meaningful results. Therefore, the fact that the error we make by neglecting dilution via cell growth is small is not a sufficient argument for not taking dilution into account.

Furthermore, there are metabolites that appear in similarly high concentrations but are not part of the biomass reaction. An example is citrate, which according to Bennett et al. (2009) is present in *E. coli* at concentrations that are very close to those of amino acids. Therefore, this metabolite should in fact take part in the biomass reaction, which is not always the case in current genome-scale metabolic network reconstructions. We would like to point out that which metabolites are present at high concentration strongly depends on the growth conditions (Bennett et al., 2009). This is one more reason to always consider biomass compositions that are dependent on the growth medium.

We conclude that, in theory, the model should be capable of overproducing all metabolites in order to account for dilution via cell growth. While this condition can be qualitatively enforced as done in Eker and Krummenacker (2013), we have observed that quantitative effects can also play an important role as in the case of citrate.

7.3. Pitfalls of averaging

We have seen that, if only average fluxes over long time periods are of interest, the steady-state assumption, if combined with an appropriate biomass function, is clearly a good model. While this adds another argument why methods like FBA can indeed predict the growth rates of some organisms accurately (Edwards et al., 2001; Harcombe et al., 2013), the integration of nonlinear constraints should be done with care.

For instance, in methods like dynamic FBA (Mahadevan et al., 2002) constraints including metabolite concentrations are often integrated into the model. In dynamic FBA this is possible because time-local steady-states are used. However, we might encounter inconsistencies for time-global steady-state models, because average concentrations can be inconsistent with average fluxes.

We cannot exclude that there exists a chemical reaction system

(candidates are described in Knoke et al., 2010; Sowa et al., 2014; Gottstein et al., 2014) where an oscillation can induce a higher average flux than the flux that would be possible by assuming steady-state. We have, however, shown that linear constraints, such as those imposed by enzyme availability and enzyme capacity, do not introduce inconsistencies for average concentrations and fluxes.

With the formalisms defined in this work, it is now possible to mathematically analyse if and when oscillations can enhance metabolic capabilities.

8. Conclusion

In the present paper we have introduced a new way of mathematically deriving the steady-state assumption as flux averages over time. This approach does not require the quasi-steady-state approximation, which is typically used as a motivation for using the steady-state assumption in metabolic network analysis. Thus, even results where the use of the quasi-steady-state approximation might not be entirely justified can now be strengthened since this step is no longer needed in the argumentation. Every result based on FBA is hence put on a stronger theoretical foundation.

However, the motivation behind our approach is different from that of the quasi-steady-state approximation. Thus, when using the approach we described here with the intuition of the quasi-steady-state approximation in mind, one can run into pitfalls or unintuitive effects.

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Appendix A. Mathematical theory

A.1. Average fluxes and concentrations

We observe that the modelling assumptions of Section 3 are a special case of the assumptions in Section 4, where $\mu = 0$. However, $\tilde{\mathbf{c}}(T)$ as defined in Eq. (AVGC) is not well defined if $\tilde{\mu}(T) = 0$. Since the proofs of Theorem 1 and 2 are rather similar, we prove here a slightly more general result (for $\mu(t) \geq 0$ for all $t \geq 0$) from which these two theorems follow immediately. Therefore, we define

$$\lambda_T(t) := \begin{cases} \dfrac{\mu(t)}{\tilde{\mu}(T)} & \text{if } \tilde{\mu}(T) > 0 \\ 1 & \text{if } \tilde{\mu}(T) = 0 \end{cases} \tag{A.1}$$

$$\tilde{\mathbf{c}}(T) := \frac{1}{T}\int_0^T \lambda_T(t)\mathbf{c}(t)dt. \tag{A.2}$$

We observe that for $\tilde{\mu}(T) > 0$ this definition of $\tilde{\mathbf{c}}(T)$ coincides with the definition given in Eq. (AVGC) of Section 4. For $\tilde{\mu}(T) = 0$ this definition gives an unweighted average over time. Therefore, $\tilde{\mathbf{c}}(T)$ is always well defined. However, $\tilde{\mathbf{c}}(T)$ might not be continuous, even if μ, $\mathbf{v}$, $\mathbf{c}$, and $\mathbf{e}$ are.

Since it can happen that $\lim_{T\to\infty}\tilde{\mathbf{v}}(T)$, $\lim_{T\to\infty}\tilde{\mu}(T)$, and $\lim_{T\to\infty}\tilde{\mathbf{c}}(T)$ do not exist (see Appendix B for an example), we consider the sets of accumulation points $\bar{V}$, $\bar{M}$, and $\bar{C}$ of $\tilde{\mathbf{v}}(T)$, $\tilde{\mu}(T)$, and $\tilde{\mathbf{c}}(T)$ for $T \to \infty$, respectively:

$$\bar{C} := \left\{ \bar{\mathbf{c}} \in \mathbb{R}^m_{\geq 0} \middle| \forall\, T_0 > 0,\, \varepsilon > 0\ \exists\, T > T_0 \colon \| \bar{\mathbf{c}} - \tilde{\mathbf{c}}(T)\| < \varepsilon \right\},$$

$$\bar{V} := \left\{ \bar{\mathbf{v}} \in \mathbb{R}^r \middle| \forall\, T_0 > 0,\, \varepsilon > 0\ \exists\, T > T_0 \colon \| \bar{\mathbf{v}} - \tilde{\mathbf{v}}(T)\| < \varepsilon \right\},$$

$$\bar{M} := \left\{ \bar{\mu} \in \mathbb{R} \middle| \forall\, T_0 > 0,\, \varepsilon > 0\ \exists\, T > T_0 \colon |\bar{\mu} - \tilde{\mu}(T)| < \varepsilon \right\}.$$

Proposition 3. *$\bar{C}$, $\bar{M}$ and $\bar{V}$ are not empty.*

Proof. Concentrations $\mathbf{c}$, $\mathbf{e}$ and growth-rate μ are bounded by assumption for every time $t \geq 0$. Since the fluxes $\mathbf{v}$ are given by a continuous function of $\mathbf{c}$ and $\mathbf{e}$, the fluxes are also bounded.

The result follows, because every bounded sequence in $\mathbb{R}^d$ (with finite d) has accumulation points in $\mathbb{R}^d$. □

We show in the following that every $\bar{\mathbf{v}} \in \bar{V}$ satisfies the steady-state assumption perturbed by dilution. The following theorem is a generalization of Theorems 1 and 2:

Theorem 3. *For all $\bar{\mathbf{v}} \in \bar{V}$ there exists a $\bar{\mathbf{c}} \in \bar{C}$ and a $\bar{\mu} \in \bar{M}$ such that $S\bar{\mathbf{v}} = \bar{\mathbf{c}}\bar{\mu}$.*

Note that if the limits $\bar{\mathbf{c}}$ in (8) and $\bar{\mu}$ in (9) exist, then $\bar{C}$ and $\bar{V}$ each contain only one element. Then Theorem 3 implies that for all $\bar{\mathbf{v}} \in \bar{V}$, for all $\bar{\mathbf{c}} \in \bar{C}$, and for all $\bar{\mu} \in \bar{M}$ we have $S\bar{\mathbf{v}} = \bar{\mathbf{c}}\bar{\mu}$.

Proof. (Theorem 3) Since the concentrations are non-negative and bounded, i.e., $\mathbf{0} \leq \mathbf{c}(t)$ and $\|\mathbf{c}(t)\| \leq c^{\max}$ for all $t \geq 0$, it follows by the main theorem of integration that

$$\left\| \int_0^T \dot{\mathbf{c}}(t)dt \right\| = \|\mathbf{c}(T) - \mathbf{c}(0)\| \leq c^{\max} \tag{A.3}$$

$$\Rightarrow \left\| \frac{1}{T}\int_0^T \dot{\mathbf{c}}(t)dt \right\| \leq \frac{c^{\max}}{T} \tag{A.4}$$

$$\Rightarrow \lim_{T\to\infty} \left\| \frac{1}{T}\int_0^T \dot{\mathbf{c}}(t)dt \right\| \leq \lim_{T\to\infty} \frac{c^{\max}}{T} = \mathbf{0}. \tag{A.5}$$

Since the norm is only zero for the zero point, it follows that

$$\lim_{T\to\infty} \frac{1}{T}\int_0^T \dot{\mathbf{c}}(t)dt = \mathbf{0}. \ \square \tag{A.6}$$

Lemma 1. $\lim_{T\to\infty}\left(S\tilde{\mathbf{v}}(T) - \tilde{\mathbf{c}}(T)\tilde{\mu}(T)\right) = \mathbf{0}$

Proof. If there exists a $T > 0$ with $\tilde{\mu}(T) > 0$, it follows that $\tilde{\mu}(T') > 0$ for all $T' \geq T$. In this case, it follows by the definition (KM2) of $\dot{\mathbf{c}}(t)$ that

$$\begin{aligned} \lim_{T\to\infty}\left(S\tilde{\mathbf{v}}(T) - \tilde{\mathbf{c}}(T)\tilde{\mu}(T)\right) &= \lim_{T\to\infty}\frac{1}{T}\int_0^T S\mathbf{v}(t) - \mu(t)\mathbf{c}(t)dt \\ &= \lim_{T\to\infty}\frac{1}{T}\int_0^T \dot{\mathbf{c}}(t)dt = \mathbf{0} \end{aligned} \tag{A.7}$$

If $\tilde{\mu}(T) = 0$ for all $T \geq 0$, we conclude because $\mathbf{c}$ is bounded that

$$\int_0^T \mu(t)\mathbf{c}(t)dt = \mathbf{0} \quad \text{for all } T \geq 0. \tag{A.8}$$

This implies

$$\lim_{T\to\infty}\left(S\tilde{\mathbf{v}}(T) - \tilde{\mathbf{c}}(T)\tilde{\mu}(T)\right) = \lim_{T\to\infty} S\tilde{\mathbf{v}}(T) \tag{A.9}$$

$$= \lim_{T\to\infty} \frac{1}{T} \int_0^T S\mathbf{v}(t)dt = \lim_{T\to\infty} \frac{1}{T} \int_0^T S\mathbf{v}(t) - \mu(t)\mathbf{c}(t)dt \tag{A.10}$$

$$= \lim_{T\to\infty} \frac{1}{T} \int_0^T \dot{\mathbf{c}}(t)dt = \mathbf{0}, \tag{A.11}$$

and concludes the lemma. □

Let $\bar{\mathbf{v}} \in \bar{V}$ be arbitrary but fixed. By the definition of accumulation point, it follows that there exists a sequence $(T_n')_{n\in\mathbb{N}} \in \mathbb{R}_{\geq 0}$ with $\lim_{n\to\infty} T_n' = \infty$ and $\| \bar{\mathbf{v}} - \tilde{\mathbf{v}}(T_n')\| < \frac{1}{n}$ for all $n \in \mathbb{N}$. We observe that, given $(T_n')_{n\in\mathbb{N}} \in \mathbb{R}_{\geq 0}$, there exists an accumulation point $(\bar{\mathbf{c}}, \bar{\mu}) \in \bar{C} \times \bar{M}$ of $(\tilde{\mathbf{c}}(T_n'), \tilde{\mu}(T_n'))_{n\in\mathbb{N}}$. Thus, there exists a subsequence $(T_n)_{n\in\mathbb{N}}$ of $(T_n')_{n\in\mathbb{N}}$ with

$$\lim_{n\to\infty} \tilde{\mathbf{v}}(T_n) = \bar{\mathbf{v}}, \quad \lim_{n\to\infty} \tilde{\mathbf{c}}(T_n) = \bar{\mathbf{c}}, \quad \lim_{n\to\infty} \tilde{\mu}(T_n) = \bar{\mu}. \tag{A.12}$$

It follows by Lemma 1 and by the existence of $\bar{\mathbf{v}}$, $\bar{\mu}$, and $\bar{\mathbf{c}}$ that

$$\begin{aligned} S\bar{\mathbf{v}} - \bar{\mathbf{c}}\bar{\mu} &= S \lim_{n\to\infty} \tilde{\mathbf{v}}(T_n) - \lim_{n\to\infty} \tilde{\mathbf{c}}(T_n) \lim_{n\to\infty} \tilde{\mu}(T_n) \\ &= \lim_{n\to\infty} S\tilde{\mathbf{v}}(T_n) - \tilde{\mathbf{c}}(T_n)\tilde{\mu}(T_n) = \mathbf{0}, \end{aligned} \tag{A.13}$$

which concludes the proof of Theorem 3. □

A.2. Kinetic constraints

Since the average enzyme concentrations might not exist, we use the set of accumulation points

$$\bar{E} := \left\{ \bar{\mathbf{e}} \in \mathbb{R}^r_{\geq 0} \middle| \forall\, T_0 > 0,\, \varepsilon > 0\ \exists\, T > T_0 \colon \| \bar{\mathbf{e}} - \tilde{\mathbf{e}}(T)\| < \varepsilon \right\}.$$

For the linear kinetic constraints as used in Section 6.2, we get the following result:

Proposition 4. *It holds for all $\bar{\mathbf{v}} \in \bar{V}$ that there exists an $\bar{\mathbf{e}} \in \bar{E}$ such that*

$$-k_i^- \bar{e}_i \leq \bar{v}_i \leq k_i^+ \bar{e}_i \quad \text{for all } i \in \mathcal{K}. \tag{A.14}$$

Proof. Let $\bar{\mathbf{v}} \in \bar{V}$ be arbitrary but fixed. Using the same arguments as in the proof of Theorem 3, it follows that there exists a sequence $(T_n)_{n\in\mathbb{N}} \in \mathbb{R}_{\geq 0}$ with $\lim_{n\to\infty} T_n = \infty$ and an $\bar{\mathbf{e}} \in \bar{E}$ with

$$\lim_{n\to\infty} \tilde{\mathbf{v}}(T_n) = \bar{\mathbf{v}}, \quad \lim_{n\to\infty} \tilde{\mathbf{e}}(T_n) = \bar{\mathbf{e}}. \tag{A.15}$$

We conclude for all $i \in \mathcal{K}$ that

$$\begin{aligned} k_i^+ \bar{e}_i - \bar{v}_i &= \lim_{n\to\infty} \left(k_i^+ \tilde{e}_i(T_n) - \tilde{v}_i(T_n) \right) \\ &= \lim_{n\to\infty} \frac{1}{T_n} \int_0^{T_n} k_i^+ e_i(t) - v_i(t)dt \geq 0 \end{aligned} \tag{A.16}$$

because $v_i(t) \leq k_i^+ e_i(t)$ for all $t \geq 0$. Thus, $\bar{v}_i \leq k_i^+ \bar{e}_i$. The same argument also applies to show that $-k_i^- \bar{e}_i \leq \bar{v}_i$. □

Appendix B. Average fluxes and concentrations might not exist

Let us consider the following toy network consisting of two reactions r_1 and r_2 without taking metabolite dilution into account. Reaction r_1 is producing a metabolite A, which is consumed by reaction r_2. Hence, the change in the concentration c of A is determined by

$$\dot{c} = v_1 - v_2, \tag{Ex2a}$$

where v_1 and v_2 are the fluxes through r_1 and r_2 respectively.

Furthermore, we assume that reaction r_1 is catalysed by an enzyme with enzyme concentration e and reaction r_2 is a spontaneous reaction subject to mass-action kinetics, i.e.,

$$\begin{aligned} v_1 &:= e, \\ v_2 &:= c. \end{aligned} \tag{Ex2b}$$

To obtain multiple accumulation points, we make the assumption that the enzyme concentrations are as follows (note that we choose a non-continuous e to make the analysis easier. e can be smoothened without significantly changing the dynamics of the system):

$$e(t) = \begin{cases} 1 & \text{if } 2^{2n} \leq t \leq 2^{2n+1},\ n \in \mathbb{N} \\ 0 & \text{otherwise} \end{cases} \tag{Ex2c}$$

We remark that such enzyme concentrations might be quite unrealistic in a biological context, but they are not excluded by our modelling assumptions.

Before we show that the average enzyme concentration $\tilde{e}(T)$ and average metabolite concentration $\tilde{c}(T)$ have multiple accumulation points for $T \to \infty$, we observe some useful properties. We would like to point out that, by the definitions of v_1 and v_2, it will also follow that the average fluxes will have multiple accumulation points.

Proposition 5. *If $0 \leq c(0) \leq 1$, then $0 \leq c(t) \leq 1$ for all $t \geq 0$.*

Proof. Since $0 \leq e(t) \leq 1$ for all $t \geq 0$, this follows immediately from the definition of $\dot{c}$, which implies $\dot{c}(t) = e(t) - c(t)$ for all $t \geq 0$. □

In the following we assume that $0 \leq c(0) \leq 1$.

Proposition 6. *Let n be odd. Then it holds for $2^n < t < 2^{n+1}$ that $c(t) = c(2^n)e^{2^n - t}$.*

Proof. By definition it follows that $e(t) = 0$ for all t with $2^n < t < 2^{n+1}$. Thus, we get $\dot{c} = -c$ and the result follows immediately. □

Proposition 7. *Let n be even. Then it holds for $2^n < t < 2^{n+1}$ that $c(t) = 1 - (1 - c(2^n))e^{2^n - t}$.*

Proof. By definition it follows that $e(t) = 1$ for all t with $2^n < t < 2^{n+1}$. Thus, we get $\dot{c} = 1 - c$ and the result follows immediately. □

Proposition 8. *Let n be odd. Then it holds for $2^n < t < 2^{n+1}$ that*

$$\int_{2^n}^{2^{n+1}} c(t)dt < 1. \tag{B.1}$$

Proof. By Proposition 6 it follows that

$$\begin{aligned} \int_{2^n}^{2^{n+1}} c(t)dt &= \int_{2^n}^{2^{n+1}} c(2^n)e^{2^n - t}dt = c(2^n) \int_0^{2^n} e^{-t}dt \\ &= c(2^n)\left(1 - e^{-2^n}\right). \end{aligned} \tag{B.2}$$

We observe by Proposition 5 that $c(2^n)\left(1 - e^{-2^n}\right) < 1$, which concludes the proof. □

Proposition 9. *Let n be even. Then it holds for $2^n < t < 2^{n+1}$ that*

$$\int_{2^n}^{2^{n+1}} c(t)dt > 2^n - 1. \tag{B.3}$$

Proof. By Proposition 7 it follows that

$$\int_{2^n}^{2^{n+1}} c(t)dt = \int_{2^n}^{2^{n+1}} 1 - \left(1 - c(2^n)\right)e^{2^n - t}dt \tag{B.4}$$

$$=2^n-\left(1-c(2^n)\right)\int_0^{2^n}e^{-t}dt \tag{B.5}$$

$$=2^n-\left(1-c(2^n)\right)\left(1-e^{-2^n}\right). \tag{B.6}$$

We observe by Proposition 5 that $\left(1-c(2^n)\right)\left(1-e^{-2^n}\right)<1$, which concludes the proof. □

Theorem 4. $\lim_{T\to\infty}\tilde{c}(T)$ *does not exist.*

Proof. We observe for all $n\in\mathbb{N}$ with $n\geq 3$ that

$$\begin{aligned}
\tilde{c}(2^{2n+1}) &\geq \frac{1}{2^{2n+1}}\left(\int_{2^{2n}}^{2^{2n+1}}c(t)dt+\int_{2^{2n-2}}^{2^{2n-1}}c(t)dt\right)\\
&> \frac{1}{2^{2n+1}}\left(2^{2n}+2^{2n-2}-2\right) && \text{(by Prop. 9)}\\
&= \frac{1}{2}+\frac{1}{8}-\frac{1}{2^{2n}}>\frac{1}{2}+\frac{1}{16}\\
\tilde{c}(2^{2n}) &= \frac{1}{2^{2n}}\left(\int_{2^{2n-1}}^{2^{2n}}c(t)dt+\int_{2^{2n-2}}^{2^{2n-1}}c(t)dt\right)\\
&\quad+\left(\int_{2^{2n-3}}^{2^{2n-2}}c(t)dt+\int_{0}^{2^{2n-3}}c(t)dt\right)\\
&\leq \frac{1}{2^{2n}}\int_{2^{2n-1}}^{2^{2n}}c(t)dt+2^{2n-2}\\
&\quad+\frac{1}{2^{2n}}\int_{2^{2n-3}}^{2^{2n-2}}c(t)dt+2^{2n-3} && \text{(by Prop. 5)}\\
&\leq \frac{1}{2^{2n}}\left(2^{2n-2}+2^{2n-3}+2\right) && \text{(by Prop. 8)}\\
&= \frac{1}{4}+\frac{1}{8}+\frac{1}{2^{2n-1}}<\frac{1}{2}-\frac{1}{16}.
\end{aligned}$$

Clearly there exist two sequences $(T_n)_{n\in\mathbb{N}}$ and $(T'_n)_{n\in\mathbb{N}}$ such that $\lim_{n\to\infty}\tilde{c}(T_n)\neq\lim_{n\to\infty}\tilde{c}(T'_n)$, which concludes the proof. □

Theorem 5. $\lim_{T\to\infty}\tilde{e}(T)$ *does not exist.*

Proof. The proof is analogous to the proof of Theorem 4. □

Appendix C. Derivation of dilution term

As pointed out in Goelzer et al. (2011), during exponential growth the volume of a bacterial cell population grows exponentially, following the law

$$V(t)=V_0e^{\mu\cdot t},\quad \dot{V}(t)=\mu V(t), \tag{C.1}$$

where V_0 is the initial volume of the population at time 0 and μ is the growth rate.

The concentration of a metabolite i is defined as $c_i(t):=\frac{n_i(t)}{V(t)}$, where $n_i(t)$ is the number of moles of metabolite i. Therefore, the change of the concentration c_i is given by

$$\begin{aligned}
\frac{dc_i(t)}{dt} &= \frac{d}{dt}\frac{n_i(t)}{V(t)}=\frac{dn_i}{dt}\frac{1}{V(t)}-\frac{dV(t)}{dt}\frac{n_i(t)}{V^2(t)}\\
&= \frac{dn_i}{dt}\frac{1}{V(t)}-\frac{\dot{V}(t)}{V(t)}c_i(t).
\end{aligned} \tag{C.2}$$

The first term in the difference in (C.2) corresponds to the production of metabolite i by the metabolic network, while the second term corresponds to the dilution effect from growth.

We therefore obtain that the growth rate μ is given by

$$\mu(t)=\frac{\dot{V}(t)}{V(t)}. \tag{C.3}$$

Appendix D. Calculation of dilution fluxes

All equalities in this section should be considered as rough approximations.

Calculation for E. coli

Volume: $1\text{cell}=0.6\,\mu\text{m}^3=0.6\cdot10^{-15}\,\text{dm}^3$
$=0.6\cdot10^{-15}\,\text{L}$

Dry weight: $1\text{cell}=0.489\text{pgDW}=4.89\cdot10^{-13}\text{gDW}$

Flux:
$$\begin{aligned}
|v| &= 12\tfrac{\text{mmol}}{\text{gDW}\cdot\text{h}}=12\cdot10^{-3}\tfrac{\text{mol}}{\text{gDW}\cdot\text{h}}\\
&= 12\cdot10^{-3}\cdot4.89\cdot10^{-13}\tfrac{\text{mol}}{\text{cell}\cdot\text{h}}\\
&= \frac{12\cdot4.89\cdot10^{-16}\text{mol}}{3600\text{s}\cdot\text{cell}}\\
&= \mathbf{1.63\cdot10^{-18}}\tfrac{\text{mol}}{\text{s}\cdot\text{cell}}
\end{aligned}$$

Concentration:
$$\begin{aligned}
1\text{ mM} &= 10^{-3}\tfrac{\text{mol}}{\text{L}}\\
&= 10^{-3}\tfrac{\text{mol}}{\text{L}}\cdot0.6\cdot10^{-15}\tfrac{\text{L}}{\text{cell}}\\
&= 0.6\cdot10^{-18}\tfrac{\text{mol}}{\text{cell}}
\end{aligned}$$

Growth:
$$\mu=0.9\text{h}^{-1}=\tfrac{0.9}{3600\text{s}}=2.5\cdot10^{-4}\text{s}^{-1}$$
$$\mu\cdot1\text{ mM}=\mathbf{1.5\cdot10^{-22}}\tfrac{\text{mol}}{\text{s}\cdot\text{cell}}$$

Calculation for S. cerevisiae

Volume: $1\text{cell}=20\mu\text{m}^3=2\cdot10^{-14}\text{L}$

Dry weight: $1\text{cell}=10^{-11}\text{gDW}$

Flux:
$$\begin{aligned}
|v| &= 0.5\tfrac{\text{mmol}}{\text{h}\cdot\text{gDW}}\\
&= 0.5\cdot10^{-3}\tfrac{\text{mol}}{\text{h}\cdot\text{gDW}}\\
&= 0.5\cdot10^{-3}\cdot10^{-11}\tfrac{\text{mol}}{\text{h}}\\
&= \frac{0.5\cdot10^{-3}\cdot10^{-11}}{3600}\frac{\text{mol}}{\text{s}\cdot\text{cell}}\\
&= \mathbf{1.38\cdot10^{-18}}\tfrac{\text{mol}}{\text{s}\cdot\text{cell}}
\end{aligned}$$

Concentration :
$$\begin{aligned}
1\text{ mM} &= 10^{-3}\tfrac{\text{mol}}{\text{L}}\\
&= 10^{-3}\tfrac{\text{mol}}{\text{L}}\cdot2\cdot10^{-14}\tfrac{\text{L}}{\text{cell}}\\
&= 2\cdot10^{-17}\tfrac{\text{mol}}{\text{cell}}
\end{aligned}$$

Growth:
$$\begin{aligned}
\mu &= 0.4\text{h}^{-1}=\tfrac{0.4}{3600}\text{s}^{-1}\\
&= 1.11\cdot10^{-4}\text{s}^{-1}
\end{aligned}$$
$$\mu\cdot1\text{ mM}=\mathbf{2.22\cdot10^{-21}}\tfrac{\text{mol}}{\text{s}\cdot\text{cell}}$$

Calculation for H. sapiens (HeLa)

Volume: $1\text{ cell}=2600\,\mu\text{m}^3=2.6\cdot10^{-12}\text{L}$

Protein weight:
$$\begin{aligned}
1\text{cell} &= 150\text{ pgProtein}\\
&= 1.5\cdot10^{-7}\text{mgProtein}
\end{aligned}$$

Flux:
$$\begin{aligned}
v &= 18\tfrac{\text{nmol}}{\text{min}\cdot\text{mgProtein}}\\
&= 18\cdot10^{-9}\tfrac{\text{mol}}{\text{min}\cdot\text{mgProtein}}
\end{aligned}$$

$$= 18\cdot10^{-9}\cdot1.5\cdot10^{-7}\frac{\text{mol}}{\text{min}\cdot\text{cell}}$$

$$= \frac{18\cdot10^{-9}\cdot1.5\cdot10^{-7}}{60}\frac{\text{mol}}{\text{s}\cdot\text{cell}}$$

$$= \mathbf{4.5\cdot10^{-1}}\ \frac{\text{mol}}{\text{s}\cdot\text{cell}}$$

Concentration:

$$1\text{ mM} = 10^{-3}\frac{\text{mol}}{\text{L}}$$

$$= 10^{-3}\frac{\text{mol}}{\text{L}}\cdot2.6\cdot10^{-12}\frac{\text{L}}{\text{cell}}$$

$$= 2.6\cdot10^{-15}\frac{\text{mol}}{\text{cell}}$$

Growth:

$$\mu = 0.06\text{h}^{-1} = \frac{0.06}{3600}\text{s}^{-1}$$

$$= 1.67\cdot10^{-5}\text{s}^{-1}$$

$$\mu\cdot1\text{ mM} = \mathbf{4.34\cdot10^{-20}}\frac{\text{mol}}{\text{s}\cdot\text{cell}}$$

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