SIAM J. APPL. MATH.
Vol. 80, No. 3, pp. 1123–1152

UNIFORM ASYMPTOTIC EXPANSIONS BEYOND THE tQSSA FOR THE GOLDBETER–KOSHLAND SWITCH*

A. M. BERSANI†, A. BORRI‡, A. MILANESI§, G. TOMASSETTI¶, AND P. VELLUCCI‖

Abstract. In this paper we study the mathematical model of the Goldbeter–Koshland switch, or futile cycle, which is a mechanism that describes several chemical reactions, in particular the so-called phosphorylation-dephosphorylation cycle. We determine the appropriate perturbation parameter ϵ (related to the kinetic constants and initial conditions of the model) for the application of singular perturbation techniques. We also determine the inner and outer solutions and the corresponding uniform expansions, up to the first order in ϵ, beyond the total quasi-steady state approximation (tQSSA). These expansions, in particular the inner ones, can be useful for the estimation of the kinetic parameters of the reaction by means of the interpolation of experimental data. Some numerical results are discussed. Moreover, in a study case, we determine the center manifold of the system and show that, at zero order, it is asymptotically equivalent to the tQSSA of the system.

Key words. Goldbeter–Koshland switch, covalent modification, Michaelis–Menten kinetics, singular perturbations, asymptotic expansions, total quasi-steady state approximation, center manifold

AMS subject classifications. 34D15, 34E10, 37L10, 92C40, 92C45

DOI. 10.1137/19M1254416

1. Introduction.

The *Michaelis–Menten kinetics* gives a very good description of the dynamics of the different enzymes involved in a reaction and represents a staple of chemical and physiological reaction theory. It inspired much research into the modeling of intracellular signal transduction networks [6, 13].

The model considers a reaction where a substrate S binds to an enzyme E reversibly to form an unstable molecular complex C. The complex can then decay irreversibly to a product P and the enzyme, which is then free to bind to another molecule of the substrate.

A scheme of this process is given by

$$S + E \underset{d}{\overset{a}{\rightleftharpoons}} C \xrightarrow{k} P + E, \tag{1.1}$$

where a, d, k are kinetic parameters (depending on temperature but assumed constant during the reactions) related to the reaction rates. For notational convenience we will use variable names to denote both a chemical species and its concentration.

The reaction can be described using the mass action principle, which states that the growth rate of each reactant is proportional to the instantaneous concentration

*Received by the editors April 4, 2019; accepted for publication (in revised form) January 21, 2020; published electronically May 11, 2020. The first author is a member of the Gruppo Nazionale per la Fisica Matematica (GNFM) of the Istituto Nazionale di Alta Matematica (INdAM).
https://doi.org/10.1137/19M1254416

†Department of Mechanical and Aerospace Engineering, Sapienza University, 00184 Rome, Italy (alberto.bersani@sbai.uniroma1.it).

‡Institute for System Analysis and Computer Science "Antonio Ruberti" (IASI-CNR), 00185 Rome, Italy (alessandro.borri@iasi.cnr.it).

§Department of Basic and Applied Sciences for Engineering, Sapienza University, 00161 Rome, Italy (alessandro.milanesi@sbai.uniroma1.it).

¶Ministry of Education, Universities and Research, 00153 Rome, Italy (giovanna.tomassetti@uniroma1.it).

‖Department of Economics, Roma Tre University, 00145 Rome, Italy (pierluigi.vellucci@uniroma3.it).

of the reactants themselves, and using conservation laws. This approach leads to the following (full) system:

$$\begin{cases} \dfrac{dS}{dt} = -a(E_T - C)S + dC, \\ \dfrac{dC}{dt} = a(E_T - C)S - (d+k)C, \end{cases} \tag{1.2}$$

with the initial conditions

$$S(0) = S_T, \qquad C(0) = 0 \tag{1.3}$$

and the conservation laws

$$E + C = E(0) = E_T, \qquad S + C + P = S_T, \tag{1.4}$$

where $K_M = \frac{k+d}{a}$ is called the Michaelis constant or affinity constant.

The Michaelis–Menten reaction is characterized by two phases: a short transient phase of rapid increase of the complex C, and a second, slower phase called the *quasi-steady state phase*, where the complex is considered substantially in equilibrium.

The hypothesis of quasi-steady state simplifies the reaction, leading to an ordinary differential equation (ODE) for the substrate, with initial condition $S(0) = S_T$, while the complex is assumed to be in a quasi-steady state, i.e., $\frac{dC}{dt} \approx 0$.

The equations of system (1.2) become

$$\begin{cases} C(t) \approx -\dfrac{E_T \cdot S(t)}{K_M + S(t)}, \\ \dfrac{dS}{dt} \approx -kC(t) \approx -\dfrac{V_{max} S(t)}{K_M + S(t)}, \\ S(0) = S_T, \end{cases} \tag{1.5}$$

where $V_{max} = k\, E_T$.

The hypothesis of quasi-steady state has been widely discussed in the literature (see, for example, the review [6]) because this hypothesis has led to many misinterpretations of (1.5). See [22] for the correct mathematical interpretation of the standard quasi-steady state assumption (sQSSA).

In the past few decades, other researchers have introduced and explored a new approximation, called total quasi-steady state approximation (tQSSA), which has been shown to be always roughly valid in the case of an isolated reaction (as in [6]).

Let us consider again the classical Michaelis–Menten kinetics (1.2). Introducing the total substrate $\overline{S}(t) = S(t) + C(t)$, we see that (1.2) then becomes

$$\begin{cases} \dfrac{d\overline{S}}{dt} = -kC, \\ \dfrac{dC}{dt} = a[C^2 - (E_T + \overline{S} + K_M)C + E_T\overline{S}], \\ \overline{S}(0) = S_T, \\ C(0) = 0, \end{cases} \tag{1.6}$$

with conservation laws

$$E + C = E_T, \quad \overline{S} + P = S_T \ . \tag{1.7}$$

Assuming that the complex is in a quasi steady-state $\left(\frac{dC}{dt} \approx 0\right)$, it yields the *total QSSA* (tQSSA) [9], which is valid for a broader range of parameters covering both high and low enzyme concentrations,

$$d\overline{S} \approx -k\, C_-(\overline{S}), \quad \overline{S}(0) = S_T, \tag{1.8}$$

where

$$C_-(\overline{S}) = \frac{(E_T + K_M + \overline{S}) - \sqrt{(E_T + K_M + \overline{S})^2 - 4E_T\overline{S}}}{2} \tag{1.9}$$

is the only biologically allowed solution of $\frac{dC}{dt} = 0$ in the second equation (1.6).

Borghans, de Boer, and Segel [9] determine a necessary condition for the validity of the tQSSA, imposing the condition that the two timescales of the transient phase t_C and of the QSSA phase $t_{\overline{S}}$ must be well separated: $t_C \ll t_{\overline{S}}$. Following reasoning very similar to [42, 43, 44], they obtain

$$t_C = \frac{1}{a(E_T + S_T + K_M)}; \quad t_{\overline{S}} = \frac{E_T + S_T + K_M}{kE_T}$$

and show that the necessary condition is satisfied when

$$\frac{t_C}{t_{\overline{S}}} = \frac{KE_T}{(E_T + S_T + K_M)^2} \ll 1 \ , \tag{1.10}$$

where $K = \frac{k}{a}$ is the *Van Slyke–Cullen constant.*

It is easy to show that the left-hand side in (1.10) is always less than 1/4. This means that the tQSSA is always at least roughly valid. Moreover, since (1.10) can be rewritten in the form

$$\left(1 + \frac{E_T + S_T}{K} + \frac{d}{k}\right)\left(1 + \frac{S_T + K_M}{E_T}\right) \gg 1 \ ,$$

as discussed in [9], this approximation is valid, for example, when $E_T + S_T \gg K$, when $a \gg k$, and, more importantly, at low enzyme concentrations $E_T \ll S_T + K_M$, which implies that when the sQSSA is valid, then so is the tQSSA.

The total approximation has been applied to more complex mechanisms [48, 40, 36]. Among others, we mention the double phosphorylation [36]; the Goldbeter–Koshland switch, which models the single phosphorylation-dephosphorylation cycle [2, 38, 11, 35]; the double phosphorylation-dephosphorylation cycle; and the ubiquitous mitogen-activated protein kinase (MAPK) cascade [37, 28, 17, 15, 8], which is one of the most important mechanisms in the great majority of reaction networks in eukaryotic cells [26].

A reliable mathematical modeling of protein networks can be a valid tool for pharmaceutical preclinical research, because its theoretical study can help to identify the dose and chemical structure of any potential drug acting on the network itself.

It is then important to break up the reaction networks into simpler modules, which are applied to many processes affecting protein concentrations, e.g., MAPK cascade

of intracellular signal transduction, gene expression, protein degradation, and RNA metabolism.

In this paper, in the tQSSA framework, we want to study the asymptotic properties of the Goldbeter–Koshland (GK) switch, or futile cycle, where a substrate S is modified to the product P and, vice versa, P is transformed back to S:

$$\begin{cases} S + E \underset{d_1}{\overset{a_1}{\rightleftharpoons}} C_1 \xrightarrow{k_1} P + E, \\ P + F \underset{d_2}{\overset{a_2}{\rightleftharpoons}} C_2 \xrightarrow{k_2} S + F. \end{cases} \tag{1.11}$$

An example of this process is given by the phosphorylation-dephosphorylation cycle, where the substrate S is activated by phosphorylation to the form S^*, and the latter is transformed back (i.e., inactivated by dephosphorylation) to S. This reaction describes the ubiquitous mechanism of covalent modification cycles and is very important in every intracellular pathway, because the process of phosphorylation and dephosphorylation is one of the most important for activating and deactivating enzymes. Actually, the mechanism provides the building blocks of several intracellular reactions, such as the well-known MAPK cascade.

The fundamental step is to consider (and model) the contribution from intermediate complexes using mass action and conservation laws. In this way an ODE is obtained for each involved complex and substrate, where the concentration variation for each reactant is proportional to the reactant concentrations. We refer to this as the full system.

We wish to quote, among others, the following contributions from the literature on the GK switch.

Papers [11, 38] independently extended the tQSSA for the first time to the GK switch. Moreover, in [11] the authors coupled two and three GK switches together to study the effects of feedback in networks of protein kinases and phosphatases. In [35] it is shown that the tQSSA reproduces zero-order ultrasensitivity in the GK switch, while the sQSSA, for a wide range of parameter values, is not able to yield ultrasensitivity whenever it is expected by the theory.

In the 1960s, mathematicians (see, in particular, [22]) interpreted the sQSSA as the leading order of an asymptotic expansion with respect to a perturbation parameter ϵ, which must be assumed small. Heineken, Tsuchiya, and Aris [22] used $\epsilon_{HTA} = \frac{E_T}{S_T}$ (where S_T and E_T are the initial concentration values of the substrate and the enzyme, respectively), because in the literature it is widely used to impose the condition that the initial concentration of the enzyme E is much less than the concentration of the substrate S.

The parameter can also arise by virtue of a biochemical condition imposing the separation between the two timescales t_c and t_s characterizing the reaction (see also [27, 42, 43, 44, 34]). In this way, Segel and Slemrod [44] showed that the sQSSA can be obtained also as the leading order of an asymptotic expansion in terms of $\epsilon_{SS} = \frac{E_T}{S_T + K_M}$, enlarging the parameter range of validity of the sQSSA.

Dell'Acqua and Bersani [16] proved that in the case of a single reaction, the tQSSA approximation can also be viewed as the leading order of an asymptotic expansion with respect to a suitable perturbation parameter $\epsilon = \frac{E_T K}{(E_T + S_T + K_M)^2}$.

That parameter, which corresponds to (1.10), and was introduced in [9], naturally arises also from the studies by Palsson and collaborators (in particular, see [33, 34]), where the authors are able to determine a sufficient condition for the validity of any QSSA, based on the timescale separation, in terms of the trace and the determinant—

or, equivalently, the eigenvalues—of the Jacobian matrix J of the system of nonlinear ODEs, governing the mechanism, with J evaluated in its stationary point.

Taking into account that the perturbation parameter is always less than $1/4$, its introduction in terms of timescale separation appears much more natural than the previous parameters. This result gives a theoretical mathematical foundation for the choice of the parameter in the tQSSA.

In previous literature the different QSSAs are approached by means of two different tools: Tikhonov's theorem [45, 46, 50, 51, 23] (see Appendix A), which studies the asymptotic stability of systems of differential equations characterized by the presence of small perturbation parameters, and center manifold theory, which is one of the most powerful tools for studying the dimensional reduction of differential systems. On the one hand, Heineken, Tsuchiya, and Aris [22] and Dvořák and Šiška [19] quote Tikhonov's theorem in order to justify the sQSSA, while Khoo and Hegland [24] refer to this theorem to apply the tQSSA; on the other hand, other authors [32, 25] interpret the sQSSA and the tQSSA, respectively, as the slow manifold of the Michaelis–Menten kinetics. These techniques are well related by Fenichel's fundamental paper [20], which includes results on the connection between geometric singular perturbation theory [39, 5] and center manifolds.

Moreover, taking inspiration from [36], in [5] the authors study the chemical reaction of inhibition and determine the appropriate parameter for the application of Tikhonov's theorem, compute explicitly the equations of the center manifold of the system, and find sufficient conditions to guarantee that in the phase space the curves which relate the behavior of the complexes to the substrates by means of the tQSSA tend asymptotically to the center manifold of the system. In other words, paper [5] gives another example of connections among Tikhonov's theorem, center manifold, and tQSSA, after the fundamental article [20].

In order to prove the validity of the tQSSA in the case of successive reactions, where more parameters appear, in this work we need the generalization of Tikhonov's theorem [45, 51] to the case of more parameters, as in [46]: Let us consider below the case of $\{\epsilon_j\}_{j=1}^m$, all of the same order, namely, $\epsilon_j = \rho_{j-1}\epsilon$ for each $j = 1, \dots, m$ and $\rho_j > 0$ ($\rho_0 = 1$). We obtain a system of the form

$$\begin{aligned} \frac{dx}{dt} &= f(x, \mathbf{y}; t), \\ \epsilon \frac{d\mathbf{y}}{dt} &= \mathbf{g}(x, \mathbf{y}; t), \end{aligned} \tag{1.12}$$

where $\mathbf{y} = (y_1, \dots, y_m)^t$, $\mathbf{g} = (g_1, \frac{1}{\rho_1} g_2, \dots, \frac{1}{\rho_{m-1}} g_m)^t$. Then it is possible to apply, for the system (1.12), Tikhonov's theorem.

The theorem allows us to list in decreasing order the different values of ϵ_j, so that we can use the greatest among them as perturbation parameter ϵ.

In this paper, following the suggestions given in [33, 41, 9, 18, 16], we propose a parameter ϵ which is always less than $1/4$ and consequently is a very good candidate for asymptotic expansions. This parameter naturally arises from an appropriate scaling of the variables, for the adimensionalization, based on a suitable equation balancing, as in [44, 16].

We want to apply perturbation expansions beyond the tQSSA in order to determine the corrections of order ϵ. These corrections, though cumbersome, can be very useful for describing the behavior of the reactants, mainly during the early stages of the reaction, in order to determine the kinetic parameter values, by means of the interpolation with experimental data.

The paper is organized as follows. In section 2 we describe the mathematical model of the GK switch, introduce the perturbation parameter, and adimensionalize the equations governing the model. Depending on the parameter values, we study two different cases (subsections 2.1 and 2.2), determining in both cases the inner and outer solutions up to the first order in ϵ and, by means of appropriate matching conditions, to the uniform expansions. These matching conditions, at first order, are not usual in singular perturbation problems. Thus, we have to adopt a different approach, as suggested in [31]. Some numerical results are shown. In section 3 we build the center manifold for a study case, where all of the parameters are pairwise equal. Finally, we show that the center manifold, at order 0, is asymptotically equivalent to the tQSSA of the system that corresponds to our asymptotic expansion at order 0. In section 4 we state some conclusions and perspectives for future research.

2. The mathematical model of the GK switch. As already observed, a *Goldbeter–Koshland* (GK) reaction, or futile cycle, is summarized by the scheme (1.11). By the law of mass action, we get the following system of equations [21]:

$$\begin{cases} \dfrac{dS}{dt} = -a_1 SE + d_1 C_1 + k_2 C_2, \\ \dfrac{dC_1}{dt} = a_1 SE - (d_1 + k_1) C_1, \\ \dfrac{dP}{dt} = k_1 C_1 - a_2 PF + d_2 C_2, \\ \dfrac{dC_2}{dt} = a_2 PF - (d_2 + k_2) C_2. \end{cases} \tag{2.1}$$

with $S(0) = S_T,\ C_i(0) = 0,\ P(0) = 0$. Introducing the total substrates $\overline{S} = S + C_1$, $\overline{P} = P + C_2$ by conservation laws $E + C_1 = E_T$, $F + C_2 = F_T$, $S + P + C_1 + C_2 = S_T$, we have $\overline{S} + \overline{P} = S_T$. Thus the system reduces to three independent equations, and we can write the following Cauchy problem (see, for example, [11, 38, 2, 35]):

$$\begin{cases} \dfrac{d\overline{S}}{dt} = k_2 C_2 - k_1 C_1, \\ \dfrac{dC_1}{dt} = a_1[(\overline{S} - C_1)(E_T - C_1) - K_{1M} C_1], \\ \dfrac{dC_2}{dt} = a_2[(S_T - \overline{S} - C_2)(F_T - C_2) - K_{2M} C_2], \\ \overline{S}(0) = S_T, \quad C_1(0) = 0, \quad C_2(0) = 0. \end{cases} \tag{2.2}$$

where $K_{iM} = \frac{d_i + k_i}{a_i}$ are the Michaelis constants.

In [1], in the framework of the monotone systems, it was shown that the unique equilibrium point of an enzymatic futile cycle has a global convergence property. Thus it is simple to show that all of the hypotheses of Tikhonov's theorem (see Appendix A) are fulfilled. Since Tikhonov's theorem is only the first step in the asymptotic solution of initial value problems of the singular perturbation type in the form of a series in powers of ϵ, the main goal of our paper is to approximate the solutions of the system by asymptotic expansions in terms of a suitable perturbation parameter. In [14] Dell'Acqua proposes a perturbation parameter which seems not to satisfy the need for a parameter small for a wide range of parameters.

In order to fix an appropriate perturbation parameter, on the one hand we could impose Palsson's conditions [33, 34], which guarantee the separation between the timescales characterizing the fast transient phase and the QSSA phase, respectively. These conditions are based on the consideration that in the Jacobian of the system, we can observe the separation of the eigenvalues of each reaction, which are related to the timescales, as in [4, 3].

On the other hand, we could follow the method proposed in [27, 42, 43, 44], where the timescales of the complex formation t_C and of the total substrate depletion $t_{\overline{S}}$ are estimated in a different way.

Unfortunately, when we try to apply the same technique to the futile cycle, we run into several difficulties, due to the fact that on the one hand, in the Jacobian the eigenvalues of the direct and reverse reactions cannot be separated, while on the other hand, the usual techniques as in [27, 42] cannot give interesting results, due to the fact that in the futile cycle not one of the reactants is depleted, thus it is difficult to estimate $\Delta\overline{S}$ and $\Delta\overline{P}$ with this technique, even if it is possible to obtain the parametrization of the asymptotic values of the reactants and of the complexes, as in [21, 12].

However, let us recall that these methods allow us to obtain at least a rough estimate of the timescales t_C and $t_{\overline{S}}$.

In the framework of the sQSSA, Segel and Slemrod [44] showed that the same parameter ϵ_{SS} can be determined also with an appropriate scaling of the equations, based on a suitable balancing of all the terms in the equations. This technique was applied also for the adimensionalization in [16], in the framework of the tQSSA. In the single reaction the two scaling parameters γ for the adimensionalization of t in the inner and outer systems represent, in fact, the two timescales in [44, 16], respectively.

Let us remark that, using different techniques, both [9] and [16] arrive at the same perturbation parameter,

$$\epsilon = \frac{t_C}{t_{\overline{S}}} = \frac{K E_T}{(E_T + S_T + K_M)^2} \ ,$$

which was used also in [33, 41, 18] and arises as the most natural perturbation parameter.

Thus, in order to determine the asymptotic inner and outer expansions of the solutions, following the balancing technique used in [44, 16], we propose

(2.3)
$$\epsilon = \max\left\{\epsilon_1 := \frac{K_1 E_T}{(E_T + K_{1M} + S_T)^2} \ , \ \epsilon_2 := \frac{K_2 F_T}{(F_T + K_{2M} + S_T)^2} \ \ i = 1, 2\right\} < \frac{1}{4}$$

(where $K_i = \frac{k_i}{a_i}$ are the Van Slyke–Cullen constants) as the perturbation parameter.

This choice will imply the study of two different cases, which may appear a bit tedious. We could decide to choose a different parameter such as, for example, the average of ϵ_1 and ϵ_2. However, taking in each case the maximum between them allows us to better test the validity of the approximation and to stress the values of the kinetic parameters and the initial conditions in order to obtain values of ϵ even very close to the upper bound $1/4$. Moreover, since the structure of the equation does not change in a significant way, we decided to study the two cases separately.

Since, in contrast to the mechanism studied in [36, 5, 4], the situation between the two reactions in (1.11) is not symmetric, because of the asymmetry of the initial

conditions for $\overline{S}$ and $\overline{P}$, we have to focus our attention on the following two cases:

$$(2.4)\qquad (\mathbf{A})\ \epsilon = \epsilon_1 = \frac{K_1 E_T}{(E_T + K_{1M} + S_T)^2}\,; \quad (\mathbf{B})\ \epsilon = \epsilon_2 = \frac{K_2 F_T}{(F_T + K_{2M} + S_T)^2}\,.$$

2.1. Case (A). $\epsilon = \epsilon_1 = \frac{K_1 E_T}{(E_T + K_{1M} + S_T)^2}$.

The first step is the adimensionalization of the system. Following [22, 27, 30, 44, 16], let us consider the change of variables $\overline{S} = \alpha s$, $C_i = \beta_i c_i$ $(i = 1, 2)$, and $t = \gamma\tau$, with

$$(2.5)\qquad \gamma = \frac{1}{a_1(E_T + K_{1M} + S_T)};\ \alpha = S_T;\ \beta_1 = \frac{E_T S_T}{E_T + K_{1M} + S_T};\ \beta_2 = \frac{F_T S_T}{F_T + K_{2M} + S_T}.$$

Furthermore, we introduce

$$(2.6)\qquad \begin{aligned} \sigma_1 &= \frac{S_T}{E_T + K_{1M} + S_T}; \quad \eta_1 = \frac{E_T}{E_T + K_{1M} + S_T}; \quad \kappa_{1M} = \frac{K_{1M}}{E_T + K_{1M} + S_T}; \\ \sigma_2 &= \frac{S_T}{F_T + K_{2M} + S_T}; \quad \eta_2 = \frac{F_T}{F_T + K_{2M} + S_T}; \quad \kappa_{2M} = \frac{K_{2M}}{F_T + K_{2M} + S_T}\,, \end{aligned}$$

where

$$(2.7)\qquad \sigma_i + \eta_i + \kappa_{iM} = 1\,, \quad i = 1, 2\,.$$

This parameter set provides an *adimensionalization* of the model equations (see [16]), thanks to which we obtain the following system of equations for the *inner solutions*:

$$(2.8)\qquad \begin{cases} \dfrac{ds}{d\tau} &= \epsilon\left[\dfrac{\eta_2 k_2}{\eta_1 k_1} c_2 - c_1\right], \\ \dfrac{dc_1}{d\tau} &= (s - \eta_1 c_1)(1 - \sigma_1 c_1) - \kappa_{1M} c_1 = \eta_1\sigma_1 c_1^2 - (\sigma_1 s + 1 - \sigma_1)c_1 + s, \\ \dfrac{dc_2}{d\tau} &= h[(1 - s - \eta_2 c_2)(1 - \sigma_2 c_2) - \kappa_{2M} c_2] = h\left[\eta_2\sigma_2 c_2^2 - (1 - s\sigma_2)c_2 + (1 - s)\right] \end{cases}$$

(where $h = \frac{a_2}{a_1}\frac{\sigma_1}{\sigma_2}$), together with the initial conditions $s(0) = 1, c_1(0) = 0, c_2(0) = 0$.

We now write the system of equations that gives the *outer solutions*. To this aim we set $\overline{\gamma} := \frac{1}{k_1\eta_1}$, and, putting $T = \frac{t}{\overline{\gamma}}$, we see that

$$(2.9)\qquad T = \frac{\gamma}{\overline{\gamma}}\tau = \frac{k_1\eta_1}{a_1(E_T + K_{1M} + S_T)}\tau = \frac{K_1 E_T}{(E_T + K_{1M} + S_T)^2}\tau = \epsilon\tau\,.$$

So we obtain $\frac{ds}{d\tau} = \epsilon\frac{ds}{dT}$ and, hence, the *outer system*

$$(2.10)\qquad \begin{cases} \dfrac{ds}{dT} = \dfrac{\eta_2 k_2}{\eta_1 k_1} c_2 - c_1, \\ \epsilon\dfrac{dc_1}{dT} = \eta_1\sigma_1 c_1^2 - (\sigma_1 s + \eta_1 + \kappa_{1M})c_1 + s, \\ \epsilon\dfrac{dc_2}{dT} = h\left[\eta_2\sigma_2 c_2^2 - (\eta_2 + (1 - s)\sigma_2 + \kappa_{2M})c_2 + (1 - s)\right]. \end{cases}$$

Let us observe that, applying Tikhonov's theorem [45, 46, 50, 51, 23] and putting $\epsilon = 0$, we obtain the following reduced system of differential algebraic equations (DAEs):

$$\begin{cases} \dfrac{ds}{dT} = \dfrac{\eta_2 k_2}{\eta_1 k_1} c_2 - c_1, \\ \eta_1\sigma_1 c_1^2 - (\sigma_1 s + \eta_1 + \kappa_{1M})c_1 + s = 0, \\ \eta_2\sigma_2 c_2^2 - (\eta_2 + (1-s)\sigma_2 + \kappa_{2M})c_2 + 1 - s = 0, \end{cases}$$

which corresponds to the tQSSA of the system in [11, 38, 2, 35]. However, in this paper we want to apply perturbation expansions beyond the tQSSA and determine the corrections of order ϵ.

Let us remark that, following the considerations made in [44], the two parameters γ and $\overline{\gamma}$ can be considered rough estimates of the two timescales t_{C_1} and $t_{\overline{S}}$.

2.1.1. Asymptotic expansions. The parameter ϵ, appearing in the left-hand side of the second and third equations of (2.10), arises as the natural perturbation parameter of our asymptotic expansions.

Let us first focus our attention on the *inner solutions* expressed by (2.8). Let us expand the solutions of (2.8) to the form $s = s_0 + \epsilon s_1 + o(\epsilon)$, $c_1 = c_{10} + \epsilon c_{11} + o(\epsilon)$, $c_2 = c_{20} + \epsilon c_{21} + o(\epsilon)$. Upon substitution in (2.8), we find

$$\begin{cases} \dfrac{ds_0}{d\tau} = 0 \qquad \Rightarrow \qquad s_0 = 1, \\ \dfrac{ds_1}{d\tau} = \dfrac{\eta_2 k_2}{\eta_1 k_1} c_{20} - c_{10}\,. \end{cases} \tag{2.11}$$

At leading order, for c_1, we obtain

$$\frac{dc_{10}}{d\tau} = \eta_1\sigma_1 c_{10}^2 - (\sigma_1 s_0 + \eta_1 + \kappa_{1M})c_{10} + s_0,$$

and, since $s_0 = 1$, $\sigma_1 + \eta_1 + \kappa_{1M} = 1$, it follows that

$$\frac{dc_{10}}{d\tau} = \eta_1\sigma_1 c_{10}^2 - c_{10} + 1 = \eta_1\sigma_1(c_{10} - c_{10}^+)(c_{10} - c_{10}^-), \tag{2.12}$$

where $c_{10}^{\pm} = \frac{1 \pm \sqrt{1 - 4\eta_1\sigma_1}}{2\eta_1\sigma_1} > 0$. Note that

$$c_{10}^+ + c_{10}^- = \frac{1}{\eta_1\sigma_1}\,, \quad c_{10}^+ - c_{10}^- = \frac{\sqrt{\Delta}}{\eta_1\sigma_1}\,, \quad c_{10}^+ c_{10}^- = \frac{1}{\eta_1\sigma_1}\,, \quad \frac{c_{10}^+ - c_{10}^-}{c_{10}^+} = \sqrt{\Delta}\, c_{10}^-, \tag{2.13}$$

where $\Delta = 1 - 4\eta_1\sigma_1$.

The solution of (2.12) is the following:

$$c_{10}(\tau) = \frac{1}{\eta_1\sigma_1}\left[\frac{e^{\sqrt{\Delta}\tau} - 1}{c_{10}^+ e^{\sqrt{\Delta}\tau} - c_{10}^-}\right]. \tag{2.14}$$

If $\tau \ll 1$, we have $c_{10}(\tau) \sim \tau$. This means that for $t \ll 1$, $C_1(t) \sim \frac{\beta_1}{\gamma} t = a_1 E_T S_T t$. This result can be useful in order to estimate a_1 by means of experimental data at the very beginning of the reaction (see, for example, [22, 7] for similar considerations).

As to $\frac{dc_{20}}{d\tau}$, from $s_0 = 1$, we have

$$\begin{cases} \dfrac{dc_{20}}{d\tau} = h c_{20}[\eta_2\sigma_2 c_{20} - (\eta_2 + \kappa_{2M})], \\ c_{20}(0) = 0, \end{cases}$$

with the singular solution $c_{20}(\tau) = 0$. In conclusion, at order 0 the terms of the asymptotic expansions of the inner solutions are

$$\begin{cases} s_0 = 1, \\ c_{10} = \dfrac{1}{\eta_1\sigma_1}\left[\dfrac{e^{\sqrt{\Delta}\tau}-1}{c_{10}^{+}e^{\sqrt{\Delta}\tau}-c_{10}^{-}}\right], \\ c_{20} = 0. \end{cases}$$

Let us now expand the solutions of the outer system (2.10) to the form $s = \Sigma_0 + \epsilon\Sigma_1 + o(\epsilon)$, $c_1 = \Gamma_{10} + \epsilon\Gamma_{11} + o(\epsilon)$, $c_2 = \Gamma_{20} + \epsilon\Gamma_{21} + o(\epsilon)$. Substituting in (2.10), we find at order 0 that

$$\begin{cases} \dfrac{d\Sigma_0}{dT} = \dfrac{\eta_2 k_2}{\eta_1 k_1}\Gamma_{20} - \Gamma_{10}, \\ 0 = \eta_1\sigma_1\Gamma_{10}^2 - (\sigma_1\Sigma_0 + \eta_1 + \kappa_{1M})\Gamma_{10} + \Sigma_0, \\ 0 = \eta_2\sigma_2\Gamma_{20}^2 - (1-\sigma_2\Sigma_0)\Gamma_{20} + (1-\Sigma_0) \end{cases} \tag{2.15}$$

(which corresponds to the tQSSA of the system). The initial conditions are given by the matching conditions which are commonly prescribed in singular perturbation theory (see [22, 30, 29, 27, 31, 16]) by $\lim_{\tau\to+\infty} s_0(\tau) = 1 = \Sigma_0(0)$, and by the following equalities, which are, interestingly, automatically yielded:

$$\lim_{\tau\to+\infty} c_{10} = \frac{1}{c_{10}^{+}\eta_1\sigma_1} = c_{10}^{-} = \Gamma_{10}(0); \quad \lim_{\tau\to+\infty} c_{20} = 0 = \Gamma_{20}(0)\,. \tag{2.16}$$

From the two algebraic equations in (2.15) we get

$$\begin{cases} \Gamma_{10} = \dfrac{(\sigma_1\Sigma_0 + 1 - \sigma_1) - \sqrt{(\sigma_1\Sigma_0 + 1 - \sigma_1)^2 - 4\eta_1\sigma_1\Sigma_0}}{2\eta_1\sigma_1}, \\ \Gamma_{20} = \dfrac{(1-\sigma_2\Sigma_0) - \sqrt{(1-\sigma_2\Sigma_0)^2 - 4\eta_2\sigma_2(1-\Sigma_0)}}{2\eta_2\sigma_2}\,, \end{cases} \tag{2.17}$$

where we have considered only the biologically significant roots (as done, for example, in [36, 38, 35]). We thus obtain

$$\begin{aligned} \frac{d\Sigma_0}{dT} = \frac{\eta_2 k_2}{\eta_1 k_1}&\left[\frac{(1-\sigma_2\Sigma_0) - \sqrt{(1-\sigma_2\Sigma_0)^2 - 4\eta_2\sigma_2(1-\Sigma_0)}}{2\eta_2\sigma_2}\right] \\ &- \frac{(\sigma_1\Sigma_0 + 1 - \sigma_1) - \sqrt{(\sigma_1\Sigma_0 + 1 - \sigma_1)^2 - 4\eta_1\sigma_1\Sigma_0}}{2\eta_1\sigma_1}\,. \end{aligned} \tag{2.18}$$

The DAE system (2.15), together with its initial conditions, can be numerically solved. The procedure for obtaining a uniform approximation consists of adding the inner and the outer approximations and subtracting their common part [30, 27, 31, 16].

In conclusion, we can write the (adimensional) uniform expansions at zero order as

$$
\begin{aligned}
s_0^{un}(\tau) &= \Sigma_0(\epsilon\tau) + s_0(\tau) - 1 = \Sigma_0(\epsilon\tau),\\
c_{10}^{un}(\tau) &= \Gamma_{10}(\epsilon\tau) + c_{10}(\tau) - c_{10}^{-}\\
&= \frac{(\sigma_1\Sigma_0(\epsilon\tau) + 1 - \sigma_1) - \sqrt{(\sigma_1\Sigma_0(\epsilon\tau) + 1 - \sigma_1)^2 - 4\eta_1\sigma_1\Sigma_0(\epsilon\tau)}}{2\eta_1\sigma_1}\\
&\quad + \frac{1}{\eta_1\sigma_1}\left[\frac{e^{\sqrt{\Delta}\tau} - 1}{c_{10}^{+}e^{\sqrt{\Delta}\tau} - c_{10}^{-}}\right] - c_{10}^{-},\\
c_{20}^{un}(\tau) &= \Gamma_{20}(\epsilon\tau) + c_{20}(\tau)\\
&= \frac{(1 - \Sigma_0(\epsilon\tau)\sigma_2) - \sqrt{(1 - \Sigma_0(\epsilon\tau)\sigma_2)^2 - 4(1 - \Sigma_0(\epsilon\tau))\eta_2\sigma_2}}{2\eta_2\sigma_2}.
\end{aligned}
$$

Let us now consider the first-order approximation, starting from system (2.8) for the inner solutions. We have

$$
\frac{ds_1}{d\tau} = -c_{10}(\tau) = -\frac{1}{\eta_1\sigma_1}\left[\frac{e^{\sqrt{\Delta}\tau} - 1}{c_{10}^{+}e^{\sqrt{\Delta}\tau} - c_{10}^{-}}\right], \quad s_1(0) = 0, \tag{2.19a}
$$

(2.19b)

$$
\begin{cases}
\frac{dc_{11}}{d\tau} = 2\eta_1\sigma_1 c_{10}c_{11} - [\sigma_1 s_1 c_{10} + (\sigma_1 s_0 + 1 - \sigma_1)c_{11}] + s_1\\
= 2\eta_1\sigma_1 c_{10}c_{11} - [\sigma_1 s_1 c_{10} + c_{11}] + s_1 = (2\eta_1\sigma_1 c_{10} - 1)c_{11} + s_1(1 - \sigma_1 c_{10}),\\
c_{11}(0) = 0,
\end{cases}
$$

(2.19c)

$$
\begin{cases}
\frac{dc_{21}}{d\tau} = h[(\sigma_2 - 1)c_{21} - s_1],\\
c_{21}(0) = 0.
\end{cases}
$$

The solution of (2.19a) is the following:

$$
s_1(\tau) = -c_{10}^{+}\tau + \frac{1}{\eta_1\sigma_1}\ln\left[\frac{\left(c_{10}^{+}e^{\sqrt{\Delta}\tau} - c_{10}^{-}\right)\eta_1\sigma_1}{\sqrt{\Delta}}\right]. \tag{2.20}
$$

For $\tau \ll 1$, through a Maclaurin expansion in τ up to second order, we obtain $s_1(\tau) \sim -\frac{1}{2}\tau^2$. This means that, at the very beginning of the reaction,

$$
\overline{S}(t) \sim S_T\left[1 - \frac{1}{2}a_1^2 K_1 E_T t^2\right].
$$

Again, this asymptotic result can be useful in the experimental data interpolation in order to determine the kinetic parameters of the reaction. Let us underline that the computation of the first-order correction allows us to determine a much more precise approximation of $\overline{S}(t)$ in the early stages of the transient reaction, when experimental data can be used to determine the kinetic parameters. Let us observe that the approximation at zero order, $\overline{S}(t) \cong S_T$, gives too little information. Apart from the effect of improving the effectiveness of the approximations, which is useful in itself, the possibility of giving numerical tools to determine estimates of the kinetic parameters is one of the main reasons for the computation of the first-order corrections, mainly of the inner solutions.

Moreover, we get

$$s_1(\tau) \sim_{\tau\to+\infty} -c_{10}^{+}\tau + \frac{1}{\eta_1\sigma_1}\ln\left[\frac{c_{10}^{+}\eta_1\sigma_1}{\sqrt{\Delta}}e^{\sqrt{\Delta}\tau}\right] = -c_{10}^{-}\tau - \frac{1}{\eta_1\sigma_1}\ln(\sqrt{\Delta}c_{10}^{-}) \tag{2.21}$$

(i.e., a straight line), which we denote by $s_1^{as}(\tau)$, such that

$$s_1^{as}(0) = -\frac{1}{\eta_1\sigma_1}\ln(\sqrt{\Delta}c_{10}^{-}) \tag{2.22}$$

because $\frac{c_{10}^{+}\eta_1\sigma_1}{\sqrt{\Delta}} = \frac{1+\sqrt{\Delta}}{2\sqrt{\Delta}}$ and $c_{10}^{+} = \frac{1}{c_{10}^{-}\eta_1\sigma_1}$. Let us now solve (2.19b) for c_{11}. The steps are shown in Appendix B. Asymptotically, we obtain from (B.1)

$$\begin{aligned} c_{11}(\tau) &\sim_{\tau\to+\infty} \frac{c_{10}^{-}(\sigma_1 c_{10}^{-}-1)}{\sqrt{\Delta}}\tau \\ &+ \frac{1}{\eta_1\sqrt{\Delta}}\left[\frac{c_{10}^{-}}{2}\left(1-\frac{1-2\eta_1}{\sqrt{\Delta}}\right) + \frac{1-\sigma_1 c_{10}^{-}}{\sigma_1}\ln\left(\frac{c_{10}^{+}}{c_{10}^{+}-c_{10}^{-}}\right)\right] =: c_{11}^{as}(\tau) \end{aligned} \tag{2.23}$$

such that

$$c_{11}^{as}(0) = \frac{-1}{2\Delta\eta_1\sigma_1}[c_{10}^{-}\sigma_1(1-2\eta_1-\sqrt{\Delta}) - 2\sqrt{\Delta}(c_{10}^{-}\sigma_1-1)\ln(\sqrt{\Delta}c_{10}^{-})]. \tag{2.24}$$

This means that also c_{11} behaves asymptotically as a straight line for $\tau \to +\infty$.

As to $c_{21}(\tau)$, the solution is obtained in Appendix B.

Also in this case, we can consider the following asymptotic behavior: We have

$$\begin{aligned} c_{21}(\tau) &\sim_{\tau\to+\infty} \frac{c_{10}^{-}}{(1-\sigma_2)}\tau \\ &- \left[\frac{c_{10}^{-}}{h(1-\sigma_2)^2} + \frac{1}{\eta_1\sigma_1(1-\sigma_2)}\ln\left(\frac{c_{10}^{+}}{c_{10}^{+}-c_{10}^{-}}\right)\right] =: c_{21}^{as}(\tau) \end{aligned} \tag{2.25}$$

(again a straight line) and

$$c_{21}^{as}(0) = \frac{-1}{h(\sigma_2-1)^2}\left[c_{10}^{-} + \frac{h(\sigma_2-1)}{\eta_1\sigma_1}\ln(\sqrt{\Delta}c_{10}^{-})\right]. \tag{2.26}$$

Concerning the first-order corrections of the outer solutions, we have

$$\begin{cases} \dfrac{d\Sigma_1}{dT} = \dfrac{\eta_2 k_2}{\eta_1 k_1}\Gamma_{21} - \Gamma_{11}, \\ \dfrac{d\Gamma_{10}}{dT} = 2\eta_1\sigma_1\Gamma_{10}\Gamma_{11} - [(\sigma_1\Sigma_0 + 1 - \sigma_1)\Gamma_{11} + \sigma_1\Sigma_1\Gamma_{10}] + \Sigma_1, \\ \dfrac{d\Gamma_{20}}{dT} = h\left\{2\eta_2\sigma_2\Gamma_{20}\Gamma_{21} - [(1-\sigma_2\Sigma_0)\Gamma_{21} - \sigma_2\Sigma_1\Gamma_{20}] - \Sigma_1\right\}, \end{cases} \tag{2.27}$$

with suitable initial conditions, determined in Appendix B.

This is a DAE system, where Σ_1 satisfies a differential equation, while Γ_{11} and Γ_{21} solve two algebraic equations. The solutions can be found by numerical integration.

Let us remark that, in this case, the inner solutions diverge for $\tau \to \infty$, as shown in (2.21), (2.23), (2.25). This behavior, which can be observed only passing

to the first-order corrections, is not common in the literature. Thus, in order to determine the initial conditions for the outer corrections, we have to adopt a more general strategy, as suggested in the following very elegant and simple way in [31]: "The inner expansion of the outer expansion equals the outer expansion of the inner expansion."

Thus, we are now able to obtain the first-order corrections of the uniform approximation, by adding the inner and the outer solutions and subtracting the common parts:

$$
\begin{aligned}
s_1^{un}(\tau) &= s_1(\tau) + \Sigma_1(\epsilon\tau) + c_{10}^- \tau + \frac{1}{\eta_1\sigma_1}\ln(\sqrt{\Delta}c_{10}^-) \\
&= \Sigma_1(\epsilon\tau) + \frac{1}{\eta_1\sigma_1}\ln\left(1 - \frac{c_{10}^-}{c_{10}^+}e^{-\sqrt{\Delta}\tau}\right), \\
c_{11}^{un}(\tau) &= c_{11}(\tau) + \Gamma_{11}(\epsilon\tau) - \frac{c_{10}^-(\sigma_1 c_{10}^- - 1)}{\sqrt{\Delta}}\tau \\
&+ \frac{1}{2\Delta\eta_1\sigma_1}[c_{10}^-\sigma_1(1 - 2\eta_1 - \sqrt{\Delta}) - 2\sqrt{\Delta}(c_{10}^-\sigma_1 - 1)\ln(\sqrt{\Delta}c_{10}^-)], \\
c_{21}^{un}(\tau) &= c_{21}(\tau) + \Gamma_{21}(\epsilon\tau) - \frac{c_{10}^-}{(1-\sigma_2)}\tau + \frac{1}{h(\sigma_2-1)^2}\left[c_{10}^- + \frac{h(\sigma_2-1)}{\eta_1\sigma_1}\ln(\sqrt{\Delta}c_{10}^-)\right].
\end{aligned}
$$

2.2. Case (B). $\epsilon = \epsilon_2 = \frac{K_2 F_T}{(F_T + K_{2M} + S_T)^2}$.

Taking inspiration from the case (A), let us consider the change of variables $\overline{S} = \alpha s$, $C_i = \beta_i c_i$ $(i = 1, 2)$, $t = \gamma\tau$ with $\gamma = \frac{1}{a_2(F_T + K_{2M} + S_T)}$, $\alpha = S_T$, $\beta_1 = \frac{E_T S_T}{E_T + K_{1M} + S_T}$, and $\beta_2 = \frac{F_T S_T}{F_T + K_{2M} + S_T}$, recalling that in this case,

$$
\epsilon = \epsilon_2 = \frac{F_T K_2}{(F_T + K_{2M} + S_T)^2}. \tag{2.28}
$$

Let us introduce, as in case (A), σ_i, η_i, and κ_{iM}. This parameter set provides an *adimensionalization* of the model equations, thanks to which we obtain the following system of equations for the *inner solutions*:

$$
\begin{cases}
\dfrac{ds}{d\tau} = \epsilon\left[c_2 - \dfrac{\eta_1 k_1}{\eta_2 k_2}c_1\right], \\
\dfrac{dc_1}{d\tau} = \dfrac{1}{h}\left[\eta_1\sigma_1 c_1^2 - (s\sigma_1 + 1 - \sigma_1)c_1 + s\right], \\
\dfrac{dc_2}{d\tau} = \eta_2\sigma_2 c_2^2 - (1 - \sigma_2 s)c_2 + 1 - s.
\end{cases} \tag{2.29}
$$

As in case (A), in order to obtain the system of equations that gives the *outer solutions,* we set $\overline{\gamma} := \frac{1}{k_1\eta_1}$ and note that putting $T = \frac{t}{\overline{\gamma}}$, we see that $T = \frac{\gamma}{\overline{\gamma}}\tau = \epsilon\tau$. Let us observe that in this case, γ and $\overline{\gamma}$ can be considered a rough estimation of the timescales t_{C_2} and $t_{\overline{P}}$, respectively.

In the end, we obtain the *outer system*

$$
\begin{cases}
\dfrac{ds}{dT} = c_2 - \dfrac{\eta_1 k_1}{\eta_2 k_2}c_1, \\
\epsilon\dfrac{dc_1}{dT} = \dfrac{1}{h}\left[\eta_1\sigma_1 c_1^2 - (s\sigma_1 + 1 - \sigma_1)c_1 + s\right], \\
\epsilon\dfrac{dc_2}{dT} = \eta_2\sigma_2 c_2^2 - (1 - \sigma_2 s)c_2 + 1 - s.
\end{cases} \tag{2.30}
$$

2.2.1. Asymptotic expansions. Let us expand the inner solutions (i.e., the solutions of (2.29)) to the form $s = s_0 + \epsilon s_1 + o(\epsilon)$, $c_1 = c_{10} + \epsilon c_{11} + o(\epsilon)$, $c_2 = c_{20} + \epsilon c_{21} + o(\epsilon)$. The results for the zero- and first-order approximations can be summarized as follows (the procedure to obtain them is the same as that in case (A), so we will only sketch it).

Zero order. As in case (A), it is easy to see that

$$\begin{cases} \frac{ds_0}{d\tau} = 0 \quad \Longrightarrow \quad s_0(\tau) = 1, \\ \frac{dc_{10}}{d\tau} = \frac{\sigma_2 a_1}{\sigma_1 a_2}\left(\eta_1\sigma_1 c_{10}^2 - c_{10} + 1\right), \\ \frac{dc_{20}}{d\tau} = \eta_2\sigma_2 c_{20}^2 - (1-\sigma_2)\, c_{20} \quad \Longrightarrow \quad c_{20}(\tau) = 0, \end{cases}$$

$$\frac{dc_{10}}{d\tau} = \frac{\sigma_2 a_1 \eta_1}{a_2}(c_{10} - c_{10}^+)(c_{10} - c_{10}^-),$$

where $c_{i0}^{\pm}$ are defined as in (2.13).

Since $c_{10}(0) = 0$, and calling $A := \frac{\sigma_2 a_1}{\sigma_1 a_2}\sqrt{\Delta}$, we obtain by integration

$$c_{10}(\tau) = c_{10}^+ c_{10}^- \left[\frac{e^{A\tau} - 1}{c_{10}^+ e^{A\tau} - c_{10}^-}\right] = \frac{1}{\eta_1\sigma_1}\left[\frac{e^{A\tau} - 1}{c_{10}^+ e^{A\tau} - c_{10}^-}\right]. \tag{2.31}$$

If $\tau \ll 1$, we have $c_{10}(\tau) \sim \frac{A}{\sqrt{\Delta}}\tau = \frac{\sigma_2 a_1}{\sigma_1 a_2}\tau$. This implies that as in case (A), for $t \to 0$, $C_1(t) \sim a_1 E_T S_T t$. Finally, we can see that $\lim_{\tau\to\infty} c_{20}(\tau) = 0$, $\lim_{\tau\to\infty} s_0(\tau) = 1$, $\lim_{\tau\to\infty} c_{10}(\tau) = c_{10}^-$. We now turn our attention to the outer solutions. Let us expand the solutions of (2.30) to the form $s = \Sigma_0 + \epsilon\Sigma_1 + o(\epsilon)$, $c_1 = \Gamma_{10} + \epsilon\Gamma_{11} + o(\epsilon)$, $c_2 = \Gamma_{20} + \epsilon\Gamma_{21} + o(\epsilon)$.

At zero order we have

$$\begin{cases} \frac{d\Sigma_0}{dT} = \Gamma_{20} - \frac{\eta_1 k_1}{\eta_2 k_2}\Gamma_{10}, \\ 0 = \eta_1\sigma_1\Gamma_{10}^2 - (\Sigma_0\sigma_1 + 1 - \sigma_1)\Gamma_{10} + \Sigma_0, \\ 0 = \eta_2\sigma_2\Gamma_{20}^2 - (1 - \sigma_1\Sigma_0)\Gamma_{20} + 1 - \Sigma_0, \end{cases} \tag{2.32}$$

from which Γ_{i0} are as in (2.17), while $\Sigma_0(0)$ and $\Gamma_{i0}(0)$ are identical to case (A).

The uniform expansion at zero order is then given by

$$\begin{cases} s_0^{un}(\tau) = s_0(\tau) + \Sigma_0(\epsilon\tau) - 1 = \Sigma_0(\epsilon\tau), \\ c_{10}^{un}(\tau) = c_{10}(\tau) + \Gamma_{10}(\epsilon\tau) - c_{10}^- \\ = \frac{1}{\eta_1\sigma_1}\left[\frac{e^{A\tau}-1}{c_{10}^+ e^{A\tau} - c_{10}^-}\right] \\ + \frac{(\sigma_1\Sigma_0(\epsilon\tau) + \eta_1 + \kappa_{1M}) - \sqrt{(\sigma_1\Sigma_0(\epsilon\tau) + \eta_1 + \kappa_{1M})^2 - 4\eta_1\sigma_1\Sigma_0(\epsilon\tau)}}{2\eta_1\sigma_1}, \\ c_{20}^{un}(\tau) = c_{20}(\tau) + \Gamma_{20}(\epsilon\tau) = \Gamma_{20}(\epsilon\tau) \\ = \frac{(1 - \sigma_2\Sigma_0(\epsilon\tau)) - \sqrt{(1 - \sigma_2\Sigma_0(\epsilon\tau))^2 - 4\eta_2\sigma_2(1 - \Sigma_0(\epsilon\tau))}}{2\eta_2\sigma_2}. \end{cases} \tag{2.33}$$

First order. For the corrections of the inner solutions, we have

$$\begin{cases} \frac{ds_1}{d\tau} = c_{20} - \frac{\eta_1 k_1}{\eta_2 k_2} c_{10} = \frac{-k_1}{\eta_2 k_2 \sigma_1}\left[\frac{e^{A\tau}-1}{c_{10}^+ e^{A\tau} - c_{10}^-}\right], \\ \frac{dc_{11}}{d\tau} = \frac{\sigma_2 a_1}{\sigma_1 a_2}\left(2\eta_1\sigma_1 c_{11} c_{10} - c_{11} - \sigma_1 s_1 c_{10} + s_1\right) \\ = \frac{\sigma_2 a_1}{\sigma_1 a_2}\left[(2\eta_1\sigma_1 c_{10} - 1)c_{11} + s_1(1 - \sigma_1 c_{10})\right], \\ \frac{dc_{21}}{d\tau} = (\sigma_2 - 1)\, c_{21} - s_1, \\ s_1(0) = c_{11}(0) = c_{21}(0) = 0. \end{cases} \tag{2.34}$$

This system can be numerically integrated, but, again, we need to know the asymptotic behavior of the solutions for $\tau \to \infty$.

As in case (A), we formally obtain

$$\begin{cases} s_1(\tau) = \frac{\eta_1 k_1}{\eta_2 k_2}\left[-c_{10}^+\tau + \frac{\sqrt{\Delta}}{A\eta_1\sigma_1}\ln\left(\eta_1\sigma_1\frac{c_{10}^+e^{A\tau}-c_{10}^-}{\sqrt{\Delta}}\right)\right], \\ c_{21}(\tau) = \frac{\eta_1 k_1}{\eta_2 k_2}c_{10}^-\left[\frac{1}{1-\sigma_2}\tau + \frac{1}{(1-\sigma_2)^2}\left(e^{(\sigma_2-1)\tau}-1\right)\right], \\ -\frac{\eta_1 k_1}{\eta_2 k_2}\left(\frac{c_{10}^+-c_{10}^-}{A}\right)\left[\frac{1}{1-\sigma_2}\ln\left(\frac{c_{10}^+}{c_{10}^+-c_{10}^-}\right)\left(1-e^{(\sigma_2-1)\tau}\right)\right. \\ \left.+\int_0^\tau e^{(1-\sigma_2)t}\ln\left(1-\frac{c_{10}^-}{c_{10}^+}e^{-At}\right)dt\right], \\ c_{11}(\tau) = \frac{\sigma_2 a_1}{\sigma_1 a_2}e^{\frac{\sigma_2 a_1}{\sigma_1 a_2}\int_0^\tau[2\eta_1\sigma_1 c_{10}(t)-1]dt}\left\{\int_0^\tau[s_1(\tau)(1\right. \\ \left.+-\sigma_1 c_{10}(t))]e^{\frac{\sigma_2 a_1}{\sigma_1 a_2}\int_0^t[1-2\eta_1\sigma_1 c_{10}(s)]ds}dt\right\}. \end{cases}$$

Developing $s_1(\tau)$ in Maclaurin expansion with respect to τ up to the second order, after tedious but very simple computations, it is possible to show that for $\tau \ll 1$, $s_1(\tau) \sim -\frac{1}{2}\frac{k_1\eta_1\sigma_2 a_1}{k_2\eta_2\sigma_1 a_2}\tau^2$. This means that at the very beginning of the reaction, $\overline{S}(t) \sim S_T[1-\frac{1}{2}a_1^2K_1E_Tt^2]$ as in case (A). As a consequence, no matter what the greatest value between ϵ_1 and ϵ_2, we obtain the same formula for the approximation of $\overline{S}(t)$. This can be very useful for the determination of the parameters by means of experimental data, because biochemists can rely on a unique formula.

For $\tau \to +\infty$, a straightforward computation shows that

(2.35a)
$$s_1(\tau) \sim s_1^{as}(\tau) := \frac{\eta_1 k_1}{\eta_2 k_2}\left[-c_{10}^-\tau - \frac{a_2}{\sigma_2 a_1\eta_1}\ln\left(\sqrt{\Delta}c_{10}^-\right)\right],$$

(2.35b)
$$c_{11}(\tau) \sim c_{11}^{as}(\tau) := \frac{\eta_1 k_1}{\eta_2 k_2\sqrt{\Delta}}\left(\sigma_1 c_{10}^- - 1\right)\left[c_{10}^-\tau - \frac{c_{10}^-}{A} + \frac{a_2}{\sigma_2 a_1\eta_1}\ln\left(\sqrt{\Delta}c_{10}^-\right)\right],$$

(2.35c)
$$c_{21}(\tau) \sim c_{21}^{as}(\tau) := \frac{\eta_1 k_1}{\eta_2 k_2(1-\sigma_2)}\left\{c_{10}^-\tau - \left[\frac{c_{10}^-}{1-\sigma_2} - \frac{a_2}{\sigma_2 a_1\eta_1}\ln\left(\sqrt{\Delta}c_{10}^-\right)\right]\right\}.$$

Passing to the corrections of the outer solutions, at first order we have

$$\text{(2.36)} \qquad \begin{cases} \frac{d\Sigma_1}{dT} = \Gamma_{21} - \frac{\eta_1 k_1}{\eta_2 k_2}\Gamma_{11}, \\ \frac{d\Gamma_{10}}{dT} = \frac{1}{h}\left[2\eta_1\sigma_1\Gamma_{10}\Gamma_{11} - (\Sigma_0\sigma_1 + 1 - \sigma_1)\Gamma_{11} - \Sigma_1\sigma_1\Gamma_{10} + \Sigma_1\right], \\ \frac{d\Gamma_{20}}{dT} = 2\eta_2\sigma_2\Gamma_{20}\Gamma_{21} - (1-\sigma_2\Sigma_0)\Gamma_{21} + \sigma_2\Sigma_1\Gamma_{20} - \Sigma_1. \end{cases}$$

From (2.35a) we have $\Sigma_1(0) = s_1^{as}(0) = -\frac{a_2 k_1}{\sigma_2 a_1\eta_2 k_2}\ln(\sqrt{\Delta}c_{10}^-)$, while from (2.36),

$$\Gamma_{11}(T) = \frac{h\frac{d\Gamma_{10}}{dT} - (1-\sigma_1\Gamma_{10})\Sigma_1}{2\eta_1\sigma_1\Gamma_{10} - \Sigma_0\sigma_1 - 1 + \sigma_1}, \quad \Gamma_{21}(T) = \frac{\frac{d\Gamma_{20}}{dT} + (1-\sigma_2\Gamma_{20})\Sigma_1}{2\eta_2\sigma_2\Gamma_{20} - 1 + \sigma_2\Sigma_0},$$
$$\Gamma_{11}(0) = \frac{h\frac{d\Gamma_{10}}{dT}(0) - [1-\sigma_1\Gamma_{10}(0)]\Sigma_1(0)}{2\eta_1\sigma_1\Gamma_{10}(0) - 1}, \ \Gamma_{21}(0) = \frac{\frac{d\Gamma_{20}}{dT}(0) + [1-\sigma_2\Gamma_{20}(0)]\Sigma_1(0)}{2\eta_2\sigma_2\Gamma_{20}(0) - 1 + \sigma_2}.$$

Since $\Gamma_{10}(0) = c_{10}^-$, $\Gamma_{20}(0) = 0$, $\frac{d\Sigma_0}{dT}(0) = -\frac{\eta_1 k_1}{\eta_2 k_2} c_{10}^-$, and, differentiating (2.17), $\frac{d\Gamma_{10}}{dT}(0) = \frac{-k_1 c_{10}^- \eta_1}{\eta_2 k_2 \sqrt{\Delta}}(1 - \sigma_1 c_{10}^-)$, $\frac{d\Gamma_{20}}{dT}(0) = \frac{\eta_1 k_1 c_{10}^-}{\eta_2 k_2 (1-\sigma_2)}$, one obtains, after tedious but simple algebra, $\Gamma_{11}(0) = c_{11}^{as}(0)$, $\Gamma_{21}(0) = c_{21}^{as}(0)$, as expected from the matching conditions.

It follows that the uniform correction at first order is given by

$$
\begin{aligned}
s_1^{un} &= s_1(\tau) + \Sigma_1(\epsilon\tau) - s_1^{as}(\tau) \\
&= s_1(\tau) + \Sigma_1(\epsilon\tau) - \left\{ \frac{\eta_1 k_1}{\eta_2 k_2} \left[-c_{10}^- \tau - \frac{a_2}{\sigma_2 a_1 \eta_1} \ln\left(\sqrt{\Delta} c_{10}^- \right) \right] \right\} \\
&= \Sigma_1(\epsilon\tau) + \left(\frac{a_2 k_1}{\sigma_2 a_1 \eta_2 k_2} \right) \ln\left(1 - \frac{c_{10}^-}{c_{10}^+} e^{-A\tau} \right), \\
c_{11}^{un} &= c_{11}(\tau) + \Gamma_{11}(\epsilon\tau) - c_{11}^{as}(\tau) \\
&= c_{11}(\tau) + \Gamma_{11}(\epsilon\tau) - \left\{ \frac{\eta_1 k_1}{\eta_2 k_2 \sqrt{\Delta}} \left(\sigma_1 c_{10}^- - 1 \right) \left[c_{10}^- \tau - \frac{c_{10}^-}{A} + \frac{a_2}{\sigma_2 a_1 \eta_1} \ln\left(\sqrt{\Delta} c_{10}^- \right) \right] \right\}, \\
c_{21}^{un} &= c_{21}(\tau) + \Gamma_{21}(\epsilon\tau) - c_{21}^{as}(\tau) \\
&= c_{21}(\tau) + \Gamma_{21}(\epsilon\tau) - \left\{ \frac{\eta_1 k_1}{\eta_2 k_2 (1 - \sigma_2)} \left\{ c_{10}^- \tau - \left[\frac{c_{10}^-}{1 - \sigma_2} - \frac{a_2}{\sigma_2 a_1 \eta_1} \ln\left(\sqrt{\Delta} c_{10}^- \right) \right] \right\} \right\}.
\end{aligned}
$$

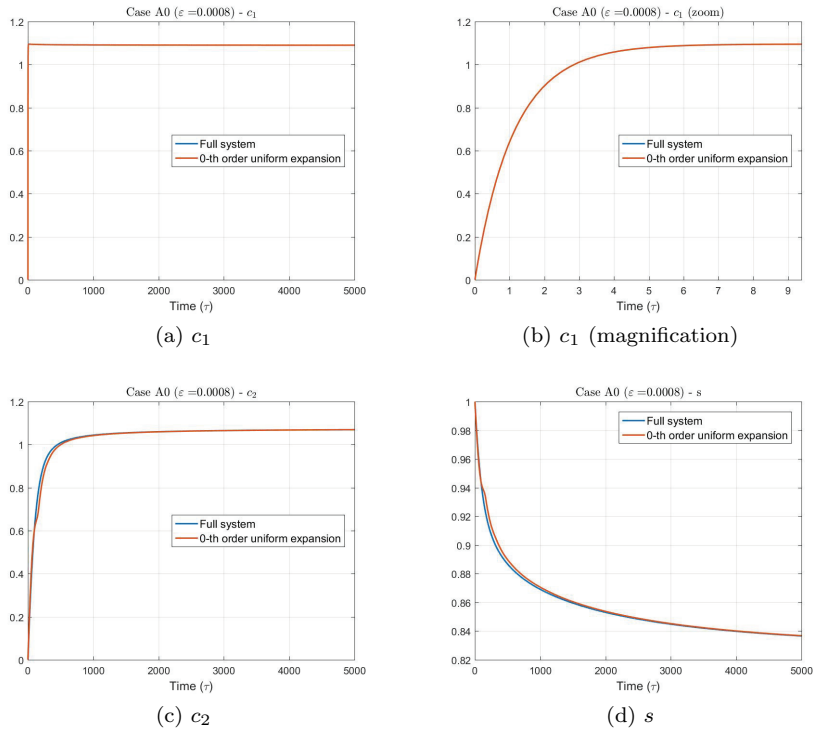
2.3. Numerical results. In Figures 1, 2, 3 (case (A)) and Figures 4, 5 (case (B)) we show the behavior of the concentrations of c_1, c_2, and s in the GK switch. In Figure 1, since $\epsilon = \epsilon_1 = 0.0008$ is very small, it is sufficient to use the zero-order approximation. In Figure 4, since the parameter is still small ($\epsilon = \epsilon_2 = 0.0059$), the first-order approximation is sufficiently satisfactory. In Figure 2, where $\epsilon = \epsilon_1 = 0.082$, the approximation begins to not be completely satisfactory in reproducing the matching region for c_1, though we can appreciate the improvement of the approximation from the zero order to the first order and the very good agreement of the matching time, that is, the time when the reaction passes from the transient phase to the quasi-steady state phase.

In Figure 3 ($\epsilon = \epsilon_1 = 0.12$) and Figure 5 ($\epsilon = \epsilon_2 = 0.25$), the approximation is shown to fail in reproducing, in particular, the matching region. However, let us observe that in both cases the values of the parameters were stressed ad hoc in order to give high and comparable values of ϵ_1 and ϵ_2 (in Figure 3, $\epsilon = \epsilon_1 = 0.12$, $\epsilon_2 = 0.11$; in Figure 5, $\epsilon = \epsilon_2 = 0.25$, $\epsilon_1 = 0.22$), which implies the prediction of some difficulties in the efficiency of the approximation.

This result may not seem satisfactory. However, let us recall that in Figure 3 we would have $\epsilon_{SS} \cong 1$, $\epsilon_{HTA} = 2$ and, mainly, in Figure 5, we would have $\epsilon_{SS} \cong 2$, $\epsilon_{HTA} = 200$, and any sQSSA would have dramatically failed. We can conclude that, in any case, even in critical cases, the tQSSA is definitely much more reliable than the sQSSA.

3. Center manifold—a case study. Tikhonov's theorem (see Appendix A) allows us to determine a stable root of (A.1). In his seminal paper [20], Fenichel showed that it corresponds to a center manifold of the full system. As shown in [20, 10], the center manifold is not unique, and there are several ways to obtain it, as shown in [45, 10, 39, 53].

Since Tikhonov's theorem is only the first step in the asymptotic solution of initial value problems of singular perturbation type in the form of a series in powers of ϵ, in this section we compare the asymptotic expansions up to zero order, obtained in



(a) c_1 (b) c_1 (magnification)

(c) c_2 (d) s

FIG. 1. *GK switch. Case* A. *zero order.* $\epsilon = 0.0008$*: Comparison between the full system and its uniform asymptotic expansion beyond the tQSSA. Since* $\epsilon = 0.0008$*, it is sufficient to use only the zero-order term. Parameters:* $a_1 = k_1 = 0.1, d_1 = 0.1, K_1 = 1, K_{1M} = 2, a_2 = 1, k_2 = d_2 = 0.1, K_2 = 0.1, K_{2M} = 0.2, E_T = F_T = 10, S_T = 100, \epsilon = \epsilon_1 = 0.0008, \epsilon_2 = 0.00008, \epsilon_{SS} \cong 0.1, \epsilon_{HTA} = 0.1$.

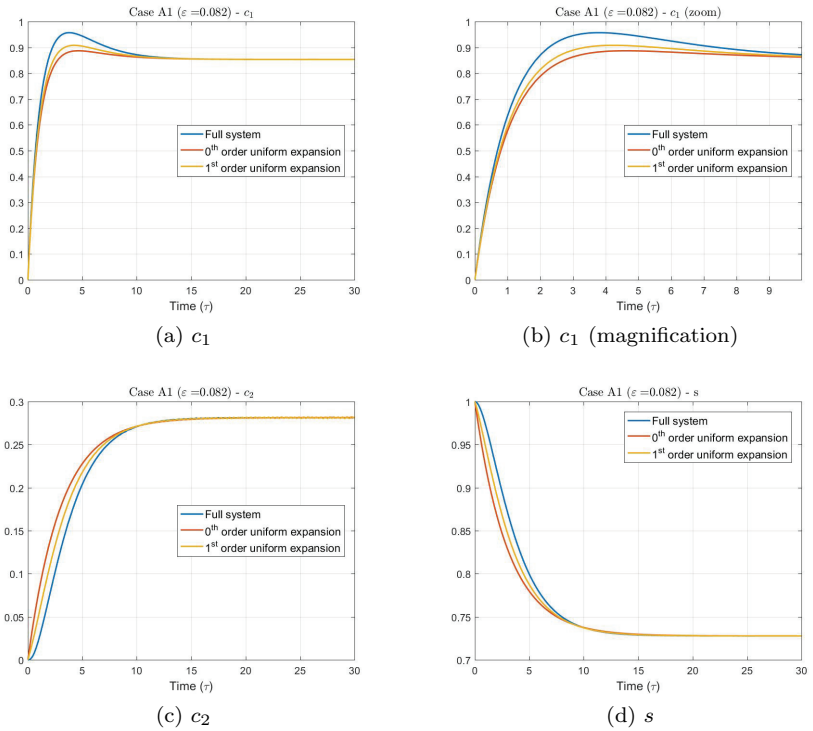
the previous section, with the center manifold of the system, which will be obtained according to [52, 53].

Let

$$\begin{cases} \dfrac{ds}{d\tau} = \epsilon \left[\dfrac{\eta_2 k_2}{\eta_1 k_1} c_2 - c_1 \right], \\ \dfrac{dc_1}{d\tau} = \eta_1 \sigma_1 c_1^2 - (\sigma_1 s + 1 - \sigma_1) c_1 + s, \\ \dfrac{dc_2}{d\tau} = h \left[\eta_2 \sigma_2 c_2^2 - (1 - s\sigma_2) c_2 + (1 - s) \right], \end{cases} \tag{3.1}$$

and, for the sake of simplicity, consider the *model case* where $\eta_1 = \eta_2 = \eta$, $k_1 = k_2 = k$, $\kappa_1 = \kappa_2 = \kappa$, $a_1 = a_2 = a$, and $\sigma_1 = \sigma_2 = \sigma$. This is the case of the parameters that are pairwise equal. It implies that $E_T = F_T$, $K_{1M} = K_{2M} = K_M$, and $\epsilon_1 = \epsilon_2 = \epsilon$.

The adimensionalization $p = \frac{P}{S_T}$ brings us to the relation $p = 1 - s$, from which



(a) c_1 (b) c_1 (magnification)

(c) c_2 (d) s

FIG. 2. *GK switch. Case* (A). *First order.* $\epsilon = 0.082$*: Comparison between the full system and its uniform first-order asymptotic expansion beyond the tQSSA. In the plots we can clearly observe the effects of the first-order correction. Parameters:* $a_1 = k_1 = 1, d_1 = 0.5, K_1 = 1, K_{1M} = 1.5, a_2 = k_2 = 1, d_2 = 2, K_2 = 1, K_{2M} = 3, E_T = 1, F_T = 26, S_T = 1, \epsilon = \epsilon_1 = 0.082, \epsilon_2 = 0.03\ , \epsilon_{SS} = 0.4, \epsilon_{HTA} = 26$.

$\frac{dp}{d\tau} = -\frac{ds}{d\tau}$. Then

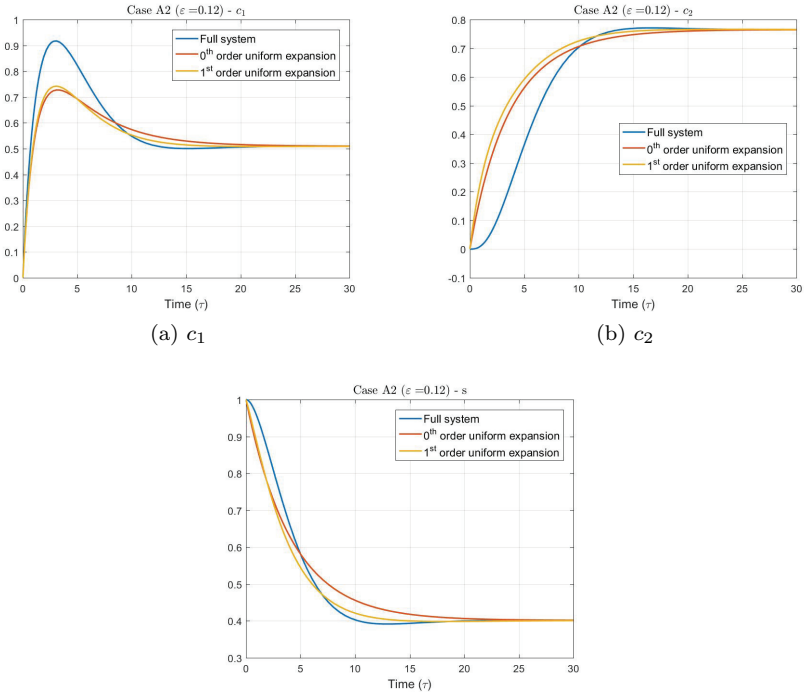
$$\text{(3.2)} \qquad \begin{cases} \dfrac{ds}{d\tau} = \epsilon\,(c_2 - c_1)\,, \\ \dfrac{dp}{d\tau} = -\epsilon\,(c_2 - c_1)\,, \\ \dfrac{dc_1}{d\tau} = (\sigma - 1)c_1 + s - \sigma s c_1 + \eta\sigma c_1^2, \\ \dfrac{dc_2}{d\tau} = (\sigma - 1)c_2 + p - p\sigma c_2 + \eta\sigma c_2^2\,. \end{cases}$$

Let us single out the fixed points of the system (3.2). Setting the derivatives of the first two equations equal to zero, we get $c_1 = c_2$, which, substituted in the third and fourth equations, gives

$$\text{(3.3)} \qquad \begin{cases} \eta\sigma c_1^2 - (\sigma s + 1 - \sigma)c_1 + s = 0, \\ \eta\sigma c_1^2 - (1 - s\sigma)c_1 + (1 - s) = 0, \end{cases}$$

where we used the fact that $p = 1 - s$.

Now, subtracting term by term the two equations in (3.3), we have $(2s - 1)(1 - \sigma c_1) = 0$. We can discard the solution $c_1 = \frac{1}{\sigma}$ because substituting it in (3.3) leads



(a) c_1 (b) c_2

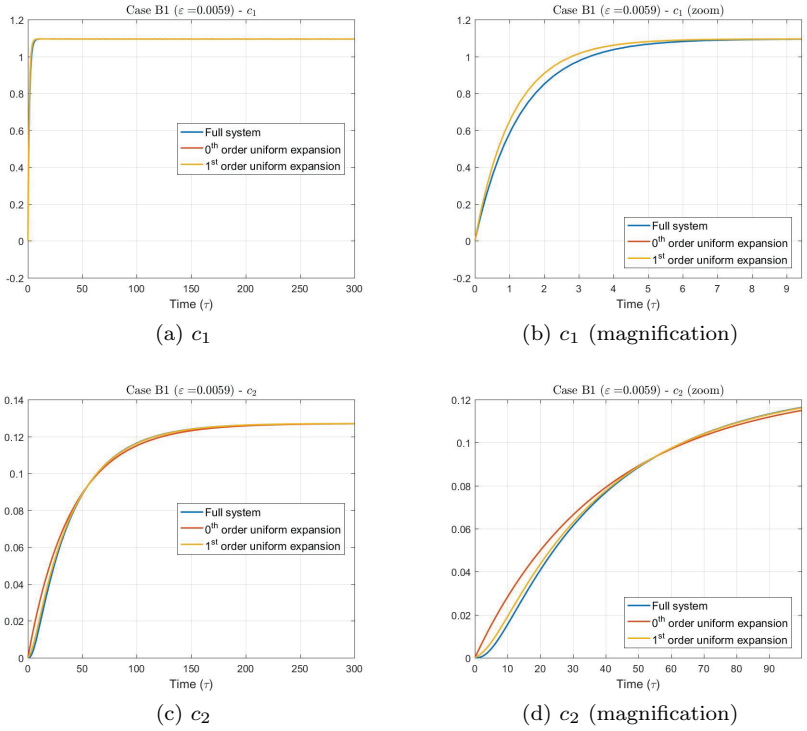
(c) s

FIG. 3. *GK switch. Case* A. *First order.* $\epsilon = 0.12$*: Comparison between the full system and its uniform first-order asymptotic expansion beyond the tQSSA. Since in this case* $\epsilon = 0.12$*, in the plots we can clearly observe that the first-order corrections are not sufficient to guarantee a satisfactory approximation of the numerical solutions. Parameters:* $a_1 = k_1 = 1, d_1 = 0.01, K_1 = 1, K_{1M} = 1.01, a_2 = k_2 = 1, d_2 = 0.01, K_2 = 1, K_{2M} = 1.01, E_T = 2, F_T = 1, S_T = 1, \epsilon = \epsilon_1 = 0.12, \epsilon_2 = 0.11, \epsilon_{SS} \cong 1, \epsilon_{HTA} = 2$.

to $\eta + \sigma = 1$, which cannot hold, since it would mean, by (2.7), that $\kappa_{1M} = 0$, which has no physical meaning. Then we obtain $s = \frac{1}{2}$ (from which $p = \frac{1}{2}$), which means $S + C_1 = \frac{S_T}{2}$, $P + C_2 = \frac{S_T}{2}$. It follows that $C_1 < \frac{S_T}{2}$. Since $C_1 = \beta_1 c_1$, from (2.5) we have $\frac{E_T S_T}{E_T + K_{1M} + S_T} c_1 < \frac{S_T}{2}$ and then $c_1 < \frac{1}{2\eta_1} = \frac{1}{2\eta}$, where in the latter we used the expression of coefficient η_1 in (2.6).

Substituting $s = \frac{1}{2}$ in (3.3), we get $\eta\sigma c_1^2 - (1 - \frac{\sigma}{2})c_1 + \frac{1}{2} = 0$. The roots are $c_1^{\pm} = \frac{2-\sigma \pm \sqrt{(2-\sigma)^2 - 8\eta\sigma}}{4\eta\sigma}$. We discard c_1^+ because $c_1^+ > \frac{1}{2\eta}$. In fact, $c_1^+ < \frac{1}{2\eta}$ if $2 - \sigma + \sqrt{(\sigma-2)^2 - 8\eta\sigma} < 2\sigma$, i.e., if $\sqrt{(\sigma-2)^2 - 8\eta\sigma} < 3\sigma - 2$. It may occur only if $\sigma > \frac{2}{3}$; in this case, squaring both members of the inequality, we get $8\sigma(\sigma + \eta - 1) > 0$, which is impossible because $\sigma + \eta < 1$. We can then conclude that the only acceptable fixed point of (3.2) is $(s, p, c_1, c_2) = (\frac{1}{2}, \frac{1}{2}, c_1^-, c_1^-)$.

Before proceeding with center manifold calculations, we center the system (3.2) in the fixed point $(s, p, c_1, c_2) = (\frac{1}{2}, \frac{1}{2}, c_1^-, c_1^-)$ through the substitution $(s, p, c_1, c_2) = \left(\tilde{s} + \frac{1}{2}, \tilde{p} + \frac{1}{2}, \tilde{c}_1 + c_1^-, \tilde{c}_2 + c_1^-\right)$. Let us observe that, since $p + s = 1$, then $\tilde{s} = -\tilde{p}$.



(a) c_1 (b) c_1 (magnification)

(c) c_2 (d) c_2 (magnification)

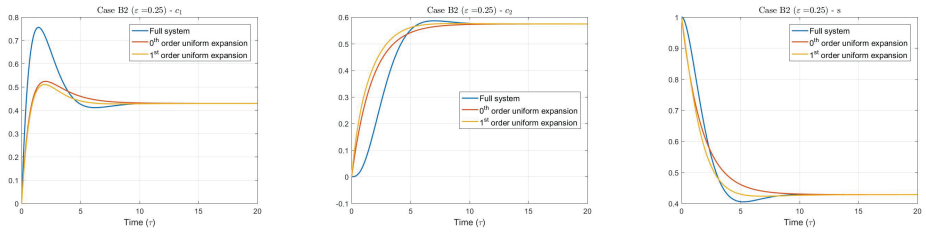
FIG. 4. *GK switch. Case* (B). *First order.* $\epsilon = 0.0059$*: Comparison between the full system and its uniform first-order asymptotic expansion beyond the tQSSA. In the plots we can clearly observe the effects of the first-order correction. Parameters:* $a_1 = k_1 = 0.1, d_1 = 0.1, K_1 = 1, K_{1M} = 2, a_2 = 0.1, k_2 = d_2 = 1, K_2 = 10, K_{2M} = 20, E_T = 10, F_T = 10, S_T = 100, \epsilon = \epsilon_2 = 0.0059, \epsilon_1 = 0.0008, \epsilon_{SS} \cong 0.1, \epsilon_{HTA} = 0.1$.

Accordingly, the system (3.2) becomes

$$
\begin{cases}
\dfrac{d\tilde{s}}{d\tau} = \epsilon\left(\tilde{c}_2 - \tilde{c}_1\right), \\
\dfrac{d\tilde{p}}{d\tau} = -\epsilon\left(\tilde{c}_2 - \tilde{c}_1\right), \\
\dfrac{d\tilde{c}_1}{d\tau} = -\dfrac{1}{2}\sqrt{(\sigma-2)^2 - 8\eta\sigma}\,\tilde{c}_1 + \tilde{s}\left(1 - \sigma c_1^-\right) - \sigma\tilde{s}\tilde{c}_1 + \eta\sigma\tilde{c}_1^2, \\
\dfrac{d\tilde{c}_2}{d\tau} = -\dfrac{1}{2}\sqrt{(\sigma-2)^2 - 8\eta\sigma}\,\tilde{c}_2 + \tilde{p}\left(1 - \sigma c_1^-\right) - \sigma\tilde{p}\tilde{c}_2 + \eta\sigma\tilde{c}_2^2,
\end{cases}
\tag{3.4}
$$

where we have used the identities $(\sigma-1)c_1^- + \frac{1}{2} - \sigma\frac{c_1^-}{2} + \eta\sigma(c_1^-)^2 = 0$ and $\frac{\sigma}{2} - 1 + 2\eta\sigma c_1^- = -\frac{1}{2}\sqrt{(\sigma-2)^2 - 8\eta\sigma}$. Now, we rewrite (3.4), operating the substitutions $y_1 = -\frac{1}{2}\sqrt{(\sigma-2)^2 - 8\eta\sigma}\,\tilde{c}_1 + \tilde{s}\left(1 - \sigma c_1^-\right)$, $y_2 = -\frac{1}{2}\sqrt{(\sigma-2)^2 - 8\eta\sigma}\,\tilde{c}_2 + \tilde{p}\left(1 - \sigma c_1^-\right)$, and then

$$
\tilde{c}_1 = 2\frac{\tilde{s}\left(1 - \sigma c_1^-\right) - y_1}{\sqrt{(\sigma-2)^2 - 8\eta\sigma}}, \qquad \tilde{c}_2 = 2\frac{\tilde{p}\left(1 - \sigma c_1^-\right) - y_2}{\sqrt{(\sigma-2)^2 - 8\eta\sigma}}.
\tag{3.5}
$$



(a) c_1 (b) c_2 (c) s

FIG. 5. *GK switch. Case* (B). *First order.* $\epsilon = 0.25$*: Comparison between the full system and its uniform first-order asymptotic expansion beyond the tQSSA. Since in this case,* $\epsilon = 0.25$*, in the plots we can clearly observe that the first-order corrections are not sufficient to guarantee a satisfactory approximation of the numerical solutions. Parameters:* $a_1 = k_1 = 1, d_1 = 0.01, K_1 = 1, K_{1M} = 1.01, a_2 = k_2 = 1, d_2 = 0.01, K_2 = 1, K_{2M} = 1.01, E_T = 2, F_T = 1, S_T = 0.01, \epsilon = \epsilon_2 \cong 0.25, \epsilon_1 = 0.22, \epsilon_{SS} \cong 2, \epsilon_{HTA} = 200.$

From (3.5) we have, respectively,

$$\frac{dy_1}{d\tau} = -\frac{1}{2}\sqrt{(\sigma-2)^2 - 8\eta\sigma}\,\frac{d\tilde{c}_1}{d\tau} + \left(1-\sigma c_1^-\right)\frac{d\tilde{s}}{d\tau}, \tag{3.6a}$$

$$\frac{dy_2}{d\tau} = -\frac{1}{2}\sqrt{(\sigma-2)^2 - 8\eta\sigma}\,\frac{d\tilde{c}_2}{d\tau} + \left(1-\sigma c_1^-\right)\frac{d\tilde{p}}{d\tau}. \tag{3.6b}$$

Substituting in (3.6a) and (3.6b) the right-hand sides of the third and fourth equations of the system (3.4)—where $\tilde{c}_1$, $\tilde{c}_2$ are replaced by (3.5)—and applying the technique described in [39, 53, 52], the system (3.4) becomes

$$\begin{cases} \frac{d\tilde{s}}{d\tau} &= \frac{2\epsilon}{\sqrt{(\sigma-2)^2-8\eta\sigma}}\left[(\tilde{p}-\tilde{s})\left(1-\sigma c_1^-\right)+y_1-y_2\right], \\ \frac{d\tilde{p}}{d\tau} &= -\frac{2\epsilon}{\sqrt{(\sigma-2)^2-8\eta\sigma}}\left[(\tilde{p}-\tilde{s})\left(1-\sigma c_1^-\right)+y_1-y_2\right], \\ \frac{dy_1}{d\tau} &= -\frac{1}{2}\sqrt{(\sigma-2)^2-8\eta\sigma}\,y_1 + f_1(\tilde{s},\tilde{p},y_1,y_2,\epsilon), \\ \frac{dy_2}{d\tau} &= -\frac{1}{2}\sqrt{(\sigma-2)^2-8\eta\sigma}\,y_2 + f_2(\tilde{s},\tilde{p},y_1,y_2,\epsilon), \\ \frac{d\epsilon}{d\tau} &= 0, \end{cases} \tag{3.7}$$

where we have isolated the linear part in $(\tilde{s},\tilde{p},y_1,y_2,\epsilon)$ from the nonlinear parts, i.e.,

$$\begin{aligned} f_1(\tilde{s},\tilde{p},y_1,y_2,\epsilon) &:= \sigma\tilde{s}\left(\tilde{s}\left(1-\sigma c_1^-\right)-y_1\right) - 2\eta\sigma\frac{\left(\tilde{s}\left(1-\sigma c_1^-\right)-y_1\right)^2}{\sqrt{(\sigma-2)^2-8\eta\sigma}} \\ &\quad + \frac{2\left(1-\sigma c_1^-\right)\epsilon}{\sqrt{(\sigma-2)^2-8\eta\sigma}}\left[(\tilde{p}-\tilde{s})\left(1-\sigma c_1^-\right)+y_1-y_2\right], \end{aligned} \tag{3.8a}$$

$$\begin{aligned} f_2(\tilde{s},\tilde{p},y_1,y_2,\epsilon) &:= \sigma\tilde{p}\left(\tilde{p}\left(1-\sigma c_1^-\right)-y_2\right) - 2\eta\sigma\frac{\left(\tilde{p}\left(1-\sigma c_1^-\right)-y_2\right)^2}{\sqrt{(\sigma-2)^2-8\eta\sigma}} \\ &\quad + -\frac{2\left(1-\sigma c_1^-\right)\epsilon}{\sqrt{(\sigma-2)^2-8\eta\sigma}}\left[(\tilde{p}-\tilde{s})\left(1-\sigma c_1^-\right)+y_1-y_2\right]. \end{aligned} \tag{3.8b}$$

In the end, we obtain *the center manifold* of (3.7),

$$y_1(\tilde{s},\tilde{p},\epsilon) = \frac{4\sigma(1-\eta-\sigma)}{(\sigma-2)^2-8\eta\sigma}\tilde{s}^2 + \frac{4\left(1-\sigma c_1^-\right)^2}{(\sigma-2)^2-8\eta\sigma}(-\tilde{s}\epsilon+\tilde{p}\epsilon) + o\left(|\xi|^2\right), \tag{3.9a}$$

$$y_2(\tilde{s},\tilde{p},\epsilon) = \frac{4\sigma(1-\eta-\sigma)}{(\sigma-2)^2-8\eta\sigma}\tilde{p}^2 + \frac{4\left(1-\sigma c_1^-\right)^2}{(\sigma-2)^2-8\eta\sigma}(\tilde{s}\epsilon-\tilde{p}\epsilon) + o\left(|\xi|^2\right). \tag{3.9b}$$

THEOREM 3.1. *The center manifold obtained in* (3.9a) *and* (3.9b) *is asymptotically equivalent to the roots of the equations obtained by applying the tQSSA (which corresponds to imposing* $\epsilon = 0$*) to the model case of* (2.10)*, that is,*

$$\begin{cases} (\sigma-1)c_1 + s - \sigma s c_1 + \eta\sigma c_1^2 = 0, \\ -c_2 + 1 - s + s\sigma c_2 + \eta\sigma c_2^2 = 0\,. \end{cases} \tag{3.10}$$

Proof. Just solve (3.10) in c_1 and c_2 and consider $\epsilon \ll 1$. ∎

4. Conclusion. In order to increase our understanding of intracellular mechanisms, it is necessary to deepen our study of the interactions between molecules and pathways. In this context, mathematical modeling is essential for biologists, as it allows them to analyze complex processes.

The involved mathematical modeling consists of systems of first-order nonlinear ordinary differential equations (ODEs). These systems represent kinetic models of basic *modules* that make up more complex intracellular mechanisms. Actually, a widely used approach to modeling intracellular mechanisms is to break up the mechanism network into simpler subnetworks (the *modules*), which represent simpler mechanisms interconnected by inflows and outflows. Examples of these modules are the phosphorylation-dephosphorylation cycle, the double phosphorylation linear reaction, inhibition, etc.

The long-term goal of these studies is the reproduction of the global behavior of a specific cell type, writing the description of the dynamics and control characteristics of living organisms on physico-chemical bases. To do this, the integration of different modules is a fundamental step.

In this paper we have studied the asymptotic properties of the Goldbeter-Koshland (GK) switch, or futile cycle, a very important module, which models several crucial intracellular phenomena, in particular the phosphorylation-dephosphorylation cycle. Our studies have been carried out in the tQSSA framework, which has proven to be always roughly valid for a broad range of parameter values covering both high and low enzyme concentrations.

Since the sQSSA and tQSSA can also be related to the *asymptotic expansion* of the solutions of the ODEs governing the process with respect to an appropriate parameter [30, 22, 44, 16], the main goal of this paper has been to approximate the solutions of the system by asymptotic expansions. With the choice of a suitable perturbative parameter ϵ, we have applied Tichonov's theorem to the GK switch, determining the asymptotic expansions up to the first order in ϵ for the inner and outer solutions and the corresponding uniform expansions.

We have also given numerical results for different values of the perturbative parameter ϵ used in the uniform approximations. Numerical results show the influence of the initial concentration of dynamical molecular species and the kinetic constants and guarantee that the predictions made by our analysis are sufficiently accurate.

For a case study where the parameters of the two reactions are identical, we have determined the center manifold of the system and shown that it is asymptotically equivalent to the tQSSA of the system.

The values of the kinetic parameters are, in general, not easy to find. The explicit expressions of some approximations obtained here—in particular the inner zero-order approximations—can be very useful for fitting the experimental data in the above described mechanism. Actually, as a *descriptive* tool, the analytic approach can be used as an alternative to simulation but, as a *prescriptive* tool, it can support the *design* of intracellular mechanisms with desired features because the analytic formulations can be used to identify the actual values of the kinetic parameters so that the intracellular mechanism behave as intended.

In order to guarantee a good approximation of the time course of the concentrations, for *in vitro* experimental purposes, it would be sufficient to take values of E_T and S_T such that the corresponding values of ϵ are sufficiently small. This condition is certainly fulfilled by our choice of the expression giving the perturbation parameter ϵ, which assumes very small values for very large parameter ranges. Roughly speaking, the results obtained via numerical integration of the equations are typically influenced by the actual values of the parameters used in simulation. On the contrary, analytic results can significantly help to capture qualitative characteristics of the networks.

Then, since the uniform expansions provide such a reliable approximation of the full system, it would be helpful to find the explicit formulas of all of them, up to the first order. A possible approach might be to use the coordinate transformations introduced in [49], based on generalized Lambert functions, in order to obtain explicit formulas also for the outer expansions—at least for the zero order (which is the most important, from an experimental viewpoint).

Appendix A. Tikhonov theorem. In this appendix we will refer to the well-known book by Wasow [51] and, in particular, to its relevant section on singular perturbations. A systematic study of the qualitative aspects of such singular perturbation problems can be found in a series of papers by Tikhonov [45, 46, 47].

We consider differential systems of the form

$$\begin{cases} \dfrac{dx}{dt} = f(x,y), \\ \epsilon \dfrac{dy}{dt} = g(x,y), \end{cases} \tag{A.1}$$

where x is a c-dimensional vector and y an s-dimensional vector. All variables are real, and ϵ is positive.

We assume the following:

(A) The functions f and g in (A.1) are continuous in an open region Ω of the (x,y)-space.

(B) There is an s-dimensional vector function $\phi(x)$ continuous in $\xi_1 \le x \le \xi_2$ such that the points $(x, \phi(x))$, for all $\xi_1 \le x \le \xi_2$, are in Ω and

$$g(x, \phi(x)) \equiv 0.$$

(C) There exists a number $\eta > 0$, independent of x, such that the relations

$$\|y - \phi(x)\| < \eta, \quad y \neq \phi(x) \quad \text{in} \quad \xi_1 \le x \le \xi_2$$

imply

$$g(x,y) \neq 0 \quad \text{in} \quad \xi_1 \le x \le \xi_2.$$

The function $\phi(x)$ will be referred to as a root of the equation $g(x,y)=0$, which may have other roots besides $\phi(x)$. A root $\phi(x)$ that satisfies condition (C) will be called *isolated* in $\xi_1 \le x \le \xi_2$.

DEFINITION A.1. *The system of differential equations*

$$\epsilon \frac{dy}{dt} = g(x,y), \tag{A.2}$$

in which x is a parameter, will be called the boundary layer equation belonging to the system (A.1).

To (A.1) *there corresponds the reduced (or degenerate) system*

$$\begin{cases} \dfrac{dx_0}{dt} = f(x_0,y_0), \\ 0 = g(x_0,y_0). \end{cases} \tag{A.3}$$

The solutions of (A.1) *and* (A.3) *define trajectories* $(x(t,\epsilon),y(t,\epsilon))$ *and* $(x_0(t),y_0(t))$ *in the* (x,y)*-space.*

We also assume the following:

(D) The singular point $y=\phi(x)$ of the boundary layer equation (A.2) is asymptotically stable for all $\xi_1 \le x \le \xi_2$.

The root $\phi(x)$ will be called, briefly, a stable root in $\xi_1 \le x \le \xi_2$ if assumption (D) is satisfied.

In accordance with our previous terminology we refer to the problem consisting of equations (A.1), together with the initial condition

$$x=\alpha, \quad y=\beta \quad \text{for } t=0, \tag{A.4}$$

as the full problem. The reduced problem is here defined by

$$\begin{cases} \dfrac{dx}{dt} = f(x,\phi(x)), \\ y=\phi(x), \end{cases} \tag{A.5}$$

$$x=\alpha \quad \text{for } t=0. \tag{A.6}$$

The differential equation in (A.5) is, of course, obtained by setting $\epsilon=0$ in (A.1) and determining the root $y=\phi(x)$ of the equation $g(x,y)=0$. Moreover, we assume the following:

(E) The full problem, as well as the reduced one, has a unique solution in an interval $0 \le t \le T$.

(F) The asymptotic stability of the singular point $y=\phi(x)$ is uniform with respect to x in $\xi_1 \le x \le \xi_2$.

Let $\mu > 0$. The set of points in the (x,y)-space for which the inequalities

$$\|y-\phi(x)\| < \mu, \quad \xi_1 \le x \le \xi_2$$

hold will be called a "μ-tube." The set

$$\|y-\phi(x)\| = \mu, \quad \xi_1 \le x \le \xi_2$$

constitutes the "lateral boundary" of the μ-tube.

LEMMA A.2. *Suppose assumptions* (A)–(F) *are satisfied. Let* $\mu > 0$ *be arbitrary but so small that the closure of the* μ*-tube lies in* Ω*. Then there exist two numbers* $\gamma(\mu)$ *and* $\epsilon(\mu)$ *such that for* $\epsilon < \epsilon(\mu)$ *the following is true: Any solution of the full equation that is in the interior of the* μ*-tube for some value* $\tilde{t}$ *of* t*,* $0 \le \tilde{t} \le T$*, and in the closure of the* μ*-tube for all* t *in* $\tilde{t} \le t < T$*, does not meet the lateral surface of the* μ*-tube for* $\tilde{t} \le t < T$*.*

The lemma states that, for small ϵ, any solution that comes close to the curve $y = \phi(x)$ in $\xi_1 \le x \le \xi_2$ remains close to it, as long as $\xi_1 \le x \le \xi_2$.

For a convenient formulation of Tikhonov's theorem, according to [51], we introduce one more term.

DEFINITION A.3. *A point* $(\alpha, \beta) \in \Omega$*,* $\xi_1 \le \alpha \le \xi_2$ *is said to lie in the domain of influence of the stable root* $y = \phi(x)$ *if the solution of the problem*

$$dy/d\tau = g(\alpha, y), \quad y(0) = \beta$$

exists and remains in Ω *for all* $\tau > 0$*, and if it tends to* $\phi(\alpha)$*, as* $\tau \to +\infty$*.*

THEOREM A.4 (Tikhonov's theorem). *Let Assumptions* (A)–(F) *be satisfied and let* (α, β) *be a point in the domain of influence of the root* $y = \phi(x)$*. Then the solution* $x(t, \epsilon)$*,* $y(t, \epsilon)$ *of the full initial value problem* (A.1)*,* (A.4) *is linked with the solution* $(x_0(t)$*,* $y_0(t) = \phi(x_0(t)))$ *of the reduced problem* (A.5)*,* (A.6) *by the limiting relations*

$$\begin{aligned} &\lim_{\epsilon \to 0} x(t, \epsilon) = x_0(t), && 0 \le t \le T_0, \\ &\lim_{\epsilon \to 0} y(t, \epsilon) = y_0(t) = \phi(x_0(t)), && 0 < t \le T_0. \end{aligned} \tag{A.7}$$

Here T_0 *is any number such that* $y = \phi(x_0(t))$ *is an isolated stable root of* $g(x_0(t), y) = 0$ *for* $0 \le t \le T_0$*. The convergence is uniform in* $0 \le t \le T_0$*, for* $x(t, \epsilon)$*, and in any interval* $0 < t_1 \le t \le T_0$ *for* $y(t, \epsilon)$*.*

Appendix B. Computation of the first-order corrections. Let us solve equations (2.19b) for c_{11}, (2.19c) for c_{21}, and (2.27) for the first-order corrections of the outer solutions.

The structure of (2.19b) is similar to equation (18) in [16], so we can directly write down the solution with the appropriate symbol changes:

$$\begin{aligned} c_{11}(\tau) = N(\tau) \Bigg\{ & \left[e^{\sqrt{\Delta}\tau}(\sqrt{\Delta}\tau - 1) + 1 \right] (\eta_1 c_{10}^+ - 1) c_{10}^+ + \frac{\Delta \tau^2}{2} (c_{10}^+ + c_{10}^- - 2 c_{10}^+ c_{10}^- \eta_1) \\ & + c_{10}^- (\eta_1 c_{10}^- - 1) \left[-e^{-\sqrt{\Delta}\tau}(\sqrt{\Delta}\tau + 1) + 1 \right] \Bigg\} \\ & + Q(\tau) \Bigg\{ c_{10}^+ (\eta_1 c_{10}^+ - 1) \left[e^{\sqrt{\Delta}\tau} \ln \left(\frac{c_{10}^+ e^{\sqrt{\Delta}\tau} - c_{10}^-}{c_{10}^+ - c_{10}^-} \right) - e^{\sqrt{\Delta}\tau} + 1 \right] \\ & + (c_{10}^- - c_{10}^+) \ln \left(\frac{c_{10}^+ e^{\sqrt{\Delta}\tau} - c_{10}^-}{c_{10}^+ - c_{10}^-} \right) \\ & + c_{10}^- (\eta_1 c_{10}^- - 1) \left[-e^{-\sqrt{\Delta}\tau} \ln \left(\frac{c_{10}^+ e^{\sqrt{\Delta}\tau} - c_{10}^-}{c_{10}^+ - c_{10}^-} \right) - \frac{c_{10}^+}{c_{10}^-} \sqrt{\Delta}\tau \right] \\ & + (c_{10}^+ + c_{10}^- - 2\eta_1 c_{10}^+ c_{10}^-) \int_1^{e^{\sqrt{\Delta}\tau}} \frac{1}{z} \ln \left(\frac{c_{10}^+ z - c_{10}^-}{c_{10}^+ - c_{10}^-} \right) dz \Bigg\}, \end{aligned} \tag{B.1}$$

where

$$N(\tau) = -\frac{e^{\sqrt{\Delta}\tau} c_{10}^{+}}{\Delta\eta_1 (c_{10}^{+} e^{\sqrt{\Delta}\tau} - c_{10}^{-})^2}; \quad Q(\tau) = \frac{e^{\sqrt{\Delta}\tau}}{\sqrt{\Delta}\eta_1 \sigma_1^2 (c_{10}^{+} e^{\sqrt{\Delta}\tau} - c_{10}^{-})^2}\,.$$

Though formula (B.1) does not have a closed form, we can consider its asymptotic behavior. Since

$$\int_1^{e^{\sqrt{\Delta}\tau}} \frac{1}{z} \ln\left(\frac{c_{10}^{+} z - c_{10}^{-}}{c_{10}^{+} - c_{10}^{-}}\right) dz = \frac{\Delta\tau^2}{2} + \sqrt{\Delta}\tau \ln\left(\frac{c_{10}^{+}}{c_{10}^{+} - c_{10}^{-}}\right) + \mathcal{R}_0(\tau),$$

where $\mathcal{R}_0(\tau)$ is a converging term for $\tau \to +\infty$, we obtain from (B.1)

$$\begin{aligned} c_{11}(\tau) \sim_{\tau\to+\infty} & \frac{c_{10}^{-}(\sigma_1 c_{10}^{-} - 1)}{\sqrt{\Delta}}\tau \\ & + \frac{1}{\eta_1\sqrt{\Delta}}\left[\frac{c_{10}^{-}}{2}\left(1 - \frac{1-2\eta_1}{\sqrt{\Delta}}\right) + \frac{1-\sigma_1 c_{10}^{-}}{\sigma_1} \ln\left(\frac{c_{10}^{+}}{c_{10}^{+} - c_{10}^{-}}\right)\right] =: c_{11}^{as}(\tau) \end{aligned}$$

(see equation (42) in [16]) such that

$$c_{11}^{as}(0) = \frac{-1}{2\Delta\eta_1\sigma_1}[c_{10}^{-}\sigma_1(1 - 2\eta_1 - \sqrt{\Delta}) - 2\sqrt{\Delta}(c_{10}^{-}\sigma_1 - 1)\ln(\sqrt{\Delta}c_{10}^{-})]. \tag{B.2}$$

This means that also c_{11} behaves asymptotically as a straight line for $\tau \to +\infty$.

As to $c_{21}(\tau)$, the solution of (2.19c) is

$$c_{21}(\tau) = e^{h(\sigma_2 - 1)\tau}\left[\mathcal{I}_1 - \frac{h}{\eta_1\sigma_1}\mathcal{I}_2\right], \tag{B.3}$$

where

$$\begin{aligned} \mathcal{I}_1 &= \int_0^{\tau} e^{\int_0^u h(1-\sigma_2)ds} h c_{10}^{+} u du = \frac{c_{10}^{+}}{(1-\sigma_2)}\left[\left(\tau - \frac{1}{h(1-\sigma_2)}\right)e^{h(1-\sigma_2)\tau} \right. \\ &\quad \left. + \frac{1}{h(1-\sigma_2)}\right], \\ \mathcal{I}_2 &= \int_0^{\tau} e^{h(1-\sigma_2)u} \ln\left(\frac{c_{10}^{+} e^{\sqrt{\Delta}u} - c_{10}^{-}}{\sqrt{\Delta}}\eta_1\sigma_1\right) du \\ &= \ln\left(\frac{c_{10}^{+}}{c_{10}^{+} - c_{10}^{-}}\right)\frac{e^{h(1-\sigma_2)\tau} - 1}{h(1-\sigma_2)} + \frac{\sqrt{\Delta}}{h(1-\sigma_2)}\left[e^{h(1-\sigma_2)\tau}\tau - \frac{e^{h(1-\sigma_2)\tau} - 1}{h(1-\sigma_2)}\right] + \mathcal{I}_3, \\ \mathcal{I}_3 &= \int_0^{\tau} e^{h(1-\sigma_2)u} \ln\left(1 - \frac{c_{10}^{-}}{c_{10}^{+} e^{\sqrt{\Delta}u}}\right) du. \end{aligned}$$

Also in this case, we can consider the asymptotic behavior. Let us start from $\mathcal{I}_3$,

$$\mathcal{I}_3 \sim_{\tau\to+\infty} -\frac{c_{10}^{-}}{c_{10}^{+}}\left[\frac{e^{(h(1-\sigma_2)-\sqrt{\Delta})\tau} - 1}{h(1-\sigma_2) - \sqrt{\Delta}}\right] + \mathcal{R}(\tau)\,,$$

where $\mathcal{R}(\tau)$ is a converging term for $\tau \to +\infty$. Asymptotically, we have

$$\begin{aligned} c_{21}(\tau) \sim_{\tau\to+\infty} & \frac{c_{10}^{-}}{(1-\sigma_2)}\tau \\ & - \left[\frac{c_{10}^{-}}{h(1-\sigma_2)^2} + \frac{1}{\eta_1\sigma_1(1-\sigma_2)} \ln\left(\frac{c_{10}^{+}}{c_{10}^{+} - c_{10}^{-}}\right)\right] =: c_{21}^{as}(\tau) \end{aligned}$$

(again a straight line) and

$$c_{21}^{as}(0) = \frac{-1}{h(\sigma_2-1)^2}\left[c_{10}^- + \frac{h(\sigma_2-1)}{\eta_1\sigma_1}\ln\left(\sqrt{\Delta}c_{10}^-\right)\right]. \tag{B.4}$$

Let us now determine the first-order corrections of the outer solutions (see system (B.3)). In order to determine the initial conditions, we must observe that, in this case, the inner solutions diverge for $\tau \to \infty$, as shown in (2.21), (2.23), (2.25). This behavior, which can be observed only passing to the first-order corrections, is not common in the literature. Thus we have to adopt a more general strategy, as suggested in the following very elegant and simple way in [31]: "The inner expansion of the outer expansion equals the outer expansion of the inner expansion." Thus, from (2.21) we obtain the initial condition for Σ_1: $\Sigma_1(0) = s_1^{as}(0) = -\frac{1}{\eta_1\sigma_1}\ln(\sqrt{\Delta}c_{10}^-)$. From (2.27) we get

$$\Gamma_{11} = \frac{\frac{d\Gamma_{10}}{dT} + (\sigma_1\Gamma_{10}-1)\Sigma_1}{2\eta_1\sigma_1\Gamma_{10} - (\sigma_1\Sigma_0 + 1 - \sigma_1)}; \quad \Gamma_{21} = \frac{\frac{d\Gamma_{20}}{dT} + h\Sigma_1(1-\sigma_2\Gamma_{20})}{h[2\eta_2\sigma_2\Gamma_{20} + \sigma_2\Sigma_0 - 1]}, \tag{B.5}$$

where $\frac{d\Gamma_{10}}{dT}$ and $\frac{d\Gamma_{20}}{dT}$ can be obtained by differentiating (2.17). Let us recall that $\Sigma_0(0) = 1$, $\frac{d\Sigma_0}{dT}(0) = -c_{10}^-$, $\Gamma_{10}(0) = c_{10}^-$, and $\Gamma_{20}(0) = 0$. Thus we have

$$\frac{d\Gamma_{10}}{dT}(0) = \frac{c_{10}^-}{2\eta_1\sqrt{\Delta}}(1 - 2\eta_1 - \sqrt{\Delta}); \quad \frac{d\Gamma_{20}}{dT}(0) = \frac{c_{10}^-}{1-\sigma_2}. \tag{B.6}$$

Hence, from (B.5), we can see that the following matching conditions for Γ_{11} and Γ_{21} are automatically satisfied:

$$\Gamma_{11}(0) = \frac{-1}{2\Delta\eta_1\sigma_1}[c_{10}^-\sigma_1(1-2\eta_1-\sqrt{\Delta}) - 2\sqrt{\Delta}(c_{10}^-\sigma_1 - 1)\ln\left(\sqrt{\Delta}c_{10}^-\right)] = c_{11}^{as}(0) \tag{B.7}$$

and

$$\Gamma_{21}(0) = \frac{-1}{h(\sigma_2-1)^2}\left[c_{10}^- + \frac{h(\sigma_2-1)}{\eta_1\sigma_1}\ln\left(\sqrt{\Delta}c_{10}^-\right)\right] = c_{21}^{as}(0). \tag{B.8}$$

This passage can be considered as a very useful tool for testing the correctness of the computations.

As to Σ_1, it only remains to numerically solve the following Cauchy problem:

$$\begin{cases} \dfrac{d\Sigma_1}{dT} = \dfrac{\eta_2 k_2}{\eta_1 k_1}\Gamma_{21} - \Gamma_{11}, \\ \Sigma_1(0) = -\frac{1}{\eta_1\sigma_1}\ln\left(\sqrt{\Delta}c_{10}^-\right), \end{cases} \tag{B.9}$$

where Γ_{11} and Γ_{21} are as given in (B.5).

Thus, we are now able to obtain the first-order corrections of the uniform approximation by adding the inner and outer solutions and subtracting the common

parts:

$$
\begin{aligned}
s_1^{un}(\tau) &= s_1(\tau) + \Sigma_1(\epsilon\tau) + c_{10}^{-}\tau + \frac{1}{\eta_1\sigma_1}\ln(\sqrt{\Delta}c_{10}^{-}) \\
&= \Sigma_1(\epsilon\tau) + \frac{1}{\eta_1\sigma_1}\ln\left(1 - \frac{c_{10}^{-}}{c_{10}^{+}}e^{-\sqrt{\Delta}\tau}\right), \\
c_{11}^{un}(\tau) &= c_{11}(\tau) + \Gamma_{11}(\epsilon\tau) - \frac{c_{10}^{-}(\sigma_1 c_{10}^{-} - 1)}{\sqrt{\Delta}}\tau \\
&\quad + \frac{1}{2\Delta\eta_1\sigma_1}[c_{10}^{-}\sigma_1(1 - 2\eta_1 - \sqrt{\Delta}) - 2\sqrt{\Delta}(c_{10}^{-}\sigma_1 - 1)\ln(\sqrt{\Delta}c_{10}^{-})], \\
c_{21}^{un}(\tau) &= c_{21}(\tau) + \Gamma_{21}(\epsilon\tau) - \frac{c_{10}^{-}}{(1-\sigma_2)}\tau + \frac{1}{h(\sigma_2 - 1)^2}\left[c_{10}^{-} + \frac{h(\sigma_2 - 1)}{\eta_1\sigma_1}\ln(\sqrt{\Delta}c_{10}^{-})\right].
\end{aligned}
$$

REFERENCES

[1] D. Angeli and E. D. Sontag, *Translation-invariant monotone systems, and a global convergence result for enzymatic futile cycle*, Nonlinear Anal. Real World Appl., 9 (2008), pp. 128–140, https://doi.org/10.1016/j.nonrwa.2006.09.006.

[2] A. Bersani, E. Bersani, and L. Mastroeni, *Deterministic and stochastic models of enzymatic networks—applications to pharmaceutical research*, Comput. Math. Appl., 55 (2008), pp. 879–888, https://doi.org/10.1016/j.camwa.2006.12.092.

[3] A. Bersani, A. Borri, A. Milanesi, G. Tomassetti, and P. Vellucci, *Asymptotic analysis of the double phosphorylation mechanism, in a tqssa framework*, J. Math. Anal. Appl., submitted.

[4] A. Bersani, A. Borri, A. Milanesi, G. Tomassetti, and P. Vellucci, *A study case for the analysis of asymptotic expansions beyond the tQSSA for inhibitory mechanisms in enzyme kinetics*, Comm. Appl. Ind. Math., 10 (2019), pp. 162–181, https://doi.org/10.1515/caim-2019-0019.

[5] A. Bersani, A. Borri, A. Milanesi, and P. Vellucci, *Tihonov theory and center manifolds for inhibitory mechanisms in enzyme kinetics*, Comm. Appl. Ind. Math., 8 (2017), pp. 81–102.

[6] A. M. Bersani, E. Bersani, G. Dell'Acqua, and M. G. Pedersen, *New trends and perspectives in nonlinear intracellular dynamics: One century from Michaelis–Menten paper*, Contin. Mech. Thermodyn., 27 (2015), pp. 659–684.

[7] A. M. Bersani and G. Dell'Acqua, *Is there anything left to say on enzyme kinetic constants and quasi-steady state approximation?*, J. Math. Chem., 50 (2012), pp. 335–344, https://doi.org/10.1007/s10910-010-9770-7.

[8] A. M. Bersani, G. Dell'Acqua, and G. Tomassetti, *On stationary states in the double phosphorylation-dephosphorylation cycle*, AIP Conf. Proc., 1389 (2011), pp. 1208–1211, https://doi.org/10.1063/1.3637833.

[9] J. Borghans, R. de Boer, and L. Segel, *Extending the quasi-steady state approximation by changing variables*, Bull. Math. Biol., 58 (1996), pp. 43–63.

[10] J. Carr, *Applications of Center Manifold Theory*, Springer-Verlag, New York, 1981.

[11] A. Ciliberto, F. Capuani, and J. J. Tyson, *Modeling networks of coupled enzymatic reactions using the total quasi-steady state approximation*, PLoS Comput. Biol., 3 (2007), e45, https://doi.org/10.1371/journal.pcbi.0030045.

[12] C. Conradi and A. Shiu, *Dynamics of posttranslational modification systems: Recent progress and future directions*, Biophys. J., 114 (2018), pp. 507–515, https://doi.org/10.1016/j.bpj.2017.11.3787.

[13] A. Cornish-Bowden, *One hundred years of Michaelis–Menten kinetics*, Perspect. Sci., 4 (2015), pp. 3–9, https://doi.org/10.1016/j.pisc.2014.12.002.

[14] G. Dell'Acqua, *Leading order asymptotics in the Goldbeter–Koshland switch*, J. Math. Chem., 51 (2013), pp. 1300–1309, https://doi.org/10.1007/s10910-013-0146-7.

[15] G. Dell'Acqua and A. M. Bersani, *Bistability and the complex depletion paradox in the double phosphorylation-dephosphorylation cycle*, in BIOINFORMATICS 2011 - Proceedings of the International Conference on Bioinformatics Models, Methods and Algorithms, Rome, Italy, 2011, pp. 55–65.

[16] G. Dell'Acqua and A. M. Bersani, *A perturbation solution of Michaelis–Menten kinetics in a "total" framework*, J. Math. Chem., 50 (2012), pp. 1136–1148.
[17] G. Dell'Acqua and A. M. Bersani, *Quasi-steady state approximations and multistability in the double phosphorylation-dephosphorylation cycle*, in Biomedical Engineering Systems and Technologies, A. Fred, J. Filipe, and H. Gamboa, eds., Springer, Berlin, 2013, pp. 155–172.
[18] J. W. Dingee and A. B. Anton, *A new perturbation solution to the Michaelis-Menten problem*, AIChE J., 54 (2008), pp. 1344–1357.
[19] I. Dvořák and J. Šiška, *Analysis of metabolic systems with complex slow and fast dynamics*, Bull. Math. Biol., 51 (1989), pp. 255–274.
[20] N. Fenichel, *Geometric singular perturbation theory for ordinary differential equations*, J. Differential Equations, 31 (1979), pp. 53–98.
[21] A. Goldbeter and D. E. Koshland, *An amplified sensitivity arising from covalent modification in biological systems*, Proc. Natl. Acad. Sci. USA, 78 (1981), pp. 6840–6844.
[22] F. G. Heineken, H. M. Tsuchiya, and R. Aris, *On the mathematical status of the pseudo-steady state hypothesis of biochemical kinetics*, Math. Biosci., 1 (1967), pp. 95–11.
[23] F. C. Hoppensteadt, *Singular perturbations on the infinite interval*, Trans. Amer. Math. Soc., 123 (1966), pp. 521–535.
[24] C. F. Khoo and M. Hegland, *The total quasi-steady state assumption: Its justification by singular perturbation and its application to the chemical master equation*, ANZIAM J., 50 (2008), (C), pp. C429–C443.
[25] A. Kumar and K. Josić, *Reduced models of networks of coupled enzymatic reactions*, J. Theoret. Biol., 278 (2011), pp. 87–106.
[26] C. Kwang-Hyun, S. Sung-Young, K. Hyun-Woo, O. Wolkenhauer, B. McFerran, and W. Kolch, *Mathematical modeling of the influence of RKIP on the ERK signaling pathway*, in Computational Methods in Systems Biology, C. Priami, ed., Springer, Berlin, 2003, pp. 127–141.
[27] C. C. Lin and L. A. Segel, *Mathematics Applied to Deterministic Problems in the Natural Sciences*, Classics Appl. Math. 1, SIAM, Philadelphia, 1988, https://doi.org/10.1137/1.9781611971347.
[28] S. MacNamara and K. Burrage, *Krylov and steady-state techniques for the solution of the chemical master equation for the mitogen-activated protein kinase cascade*, Numer. Algorithms, 51 (2009), pp. 281–307, https://doi.org/10.1007/s11075-008-9239-y.
[29] J. Murray, *Asymptotic Analysis*, Springer-Verlag, New York, 1984.
[30] J. Murray, *Mathematical Biology: An introduction*, Springer-Verlag, New York, 2002.
[31] A. Nayfeh, *Perturbation Methods*, reprint of the 1973 original, Wiley Classics Library, Wiley-Interscience [John Wiley & Sons], New York, 2000.
[32] A. H. Nguyen and S. J. Fraser, *Geometrical picture of reaction in enzyme kinetics*, J. Chem. Phys., 91 (1989), pp. 186–193, https://doi.org/10.1063/1.457504.
[33] B. O. Palsson, *On the dynamics of the irreversible Michaelis-Menten reaction mechanism*, Chem. Engrg. Sci., 42 (1987), pp. 447–458.
[34] B. O. Palsson and E. N. Lightfoot, *Mathematical modelling of dynamics and control in metabolic networks* I: *On Michaelis-Menten kinetics*, J. Theoret. Biol., 111 (1984), pp. 273–302.
[35] M. G. Pedersen and A. M. Bersani, *Introducing total substrates simplifies theoretical analysis at non-negligible enzyme concentrations: Pseudo first-order kinetics and the loss of zero-order ultrasensitivity*, J. Math. Biol., 60 (2010), pp. 267–283, https://doi.org/10.1007/s00285-009-0267-6.
[36] M. G. Pedersen, A. M. Bersani, and E. Bersani, *The total quasi-steady-state approximation for fully competitive enzyme reactions*, Bull. Math. Biol., 69 (2006), pp. 433–457.
[37] M. G. Pedersen, A. M. Bersani, and E. Bersani, *Quasi steady-state approximations in complex intracellular signal transduction networks – a word of caution*, J. Math. Chem., 43 (2008), pp. 1318–1344, https://doi.org/10.1007/s10910-007-9248-4.
[38] M. G. Pedersen, A. M. Bersani, E. Bersani, and G. Cortese, *The total quasi-steady-state approximation for complex enzyme reactions*, Math. Comput. Simul., 79 (2008), pp. 1010–1019, https://doi.org/10.1016/j.matcom.2008.02.009.
[39] A. J. Roberts, *Model Emergent Dynamics in Complex Systems*, Math. Model. Comput. 20, SIAM, 2014.
[40] M. Sabouri-Ghomi, A. Ciliberto, S. Kar, B. Novak, and J. J. Tyson, *Antagonism and bistability in protein interaction networks*, J. Theoret. Biol., 250 (2008), pp. 209–218, https://doi.org/10.1016/j.jtbi.2007.09.001.
[41] S. Schnell and P. Maini, *Enzyme kinetics far from the standard quasi-steady-state and equi-*

librium approximations, Math. Comput. Model., 35 (2002), pp. 137–144.
[42] L. A. Segel, *Modeling Dynamic Phenomena in Molecular and Cellular Biology*, Cambridge University Press, Cambridge, UK, 1984.
[43] L. A. Segel, *On the validity of the steady state assumption of enzyme kinetics*, Bull. Math. Biol., 50 (1988), pp. 579–593.
[44] L. A. Segel and M. Slemrod, *The quasi steady-state assumption: A case study in perturbation*, Siam Rev., 31 (1989), pp. 446–477, https://doi.org/10.1137/1031091.
[45] A. Tikhonov, *On the dependence of the solutions of differential equations on a small parameter*, Mat. Sb. (N.S.), 22 (1948), pp. 193–204 (in Russian).
[46] A. Tikhonov, *On a system of differential equations containing parameters*, Mat. Sb. (N.S.), 27 (1950), pp. 147–156 (in Russian).
[47] A. Tikhonov, *Systems of differential equations containing small parameters in the derivatives*, Mat. Sb. (N.S.), 31 (1952), pp. 575–586 (in Russian).
[48] A. Tzafriri and E. R. Edelman, *The total quasi-steady-state approximation is valid for reversible enzyme kinetics*, J. Theoret. Biol., 226 (2004), pp. 303–313, https://doi.org/10.1016/j.jtbi.2003.09.006.
[49] A. R. Tzafriri and E. R. Edelman, *Quasi-steady-state kinetics at enzyme and substrate concentrations in excess of the Michaelis-Menten constant*, J. Theoret. Biol., 245 (2007), pp. 737–748, https://doi.org/10.1016/j.jtbi.2006.12.005.
[50] A. B. Vasil'eva, *Asymptotic behaviour of solutions to certain problems involving non-linear differential equations containing a small parameter multiplying the highest derivatives*, Russian Math. Surveys, 18 (1963), pp. 13–84.
[51] W. Wasow, *Asymptotic Expansions for Ordinary Differential Equations*, Pure Appl. Math. XIV, Interscience Publishers, John Wiley & Sons, Inc., New York, 1965.
[52] S. Wiggins, *Normally Hyperbolic Invariant Manifolds in Dynamical Systems*, Appl. Math. Sci. 105, Springer-Verlag, New York, 1994.
[53] S. Wiggins, *Introduction to Applied Nonlinear Dynamical Systems and Chaos*, Texts Appl. Math. 2, Springer-Verlag, New York, 2003.