Retroactivity Attenuation in Bio-molecular Systems Based on Timescale Separation

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***Abstract*—As with several engineering systems, bio-molecular systems display impedance-like effects at interconnections, called *retroactivity*. In this paper, we propose a mechanism that exploits the natural timescale separation present in bio-molecular systems to attenuate retroactivity. Retroactivity enters the dynamics of a bio-molecular system as a state dependent disturbance multiplied by gains that can be very large. By virtue of the system structure, retroactivity can be arbitrarily attenuated by internal system gains even when these are much smaller than the gains multiplying retroactivity terms. This result is obtained by employing a suitable change of coordinates and a nested application of the singular perturbation theorem on the finite time interval. As an application example, we show that two modules extracted from natural signal transduction pathways have a remarkable capability of attenuating retroactivity, which is certainly desirable in any (engineered or natural) signal transmission system.**

I. Introduction

MODULARITY is a fundamental property that allows the prediction of the behavior of a system from the behavior of its components, guaranteeing that the input/output behavior of a component does not change upon interconnection. This property is often taken for granted and tacitly exploited in several engineering areas, such as electrical engineering. Modularity is usually a fair assumption because mechanisms such as operational amplifiers in suitable feedback configurations are employed so that impedance effects at interconnections can be neglected [1]. As a result, systems can be conveniently composed by simple static output-to-input assignments. Modularity has been more recently advocated also in systems biology and in synthetic biology, in which networks of bio-molecular interactions between species, such as proteins, enzymes, DNA sites, and signaling molecules take place. In particular, in systems biology one seeks to understand the behavior of a natural bio-molecular network from the behavior of the composing modules or motifs [2]–[4]. Complementary to systems biology, researchers in synthetic biology aim at constructing complex networks of interactions between genes and proteins in living cells with the ultimate goal of controlling cell behavior. A key approach in doing so is the design and construction of simple bio-molecular systems, such as oscillators [5], [6] and toggles [7], which are then interconnected in a modular fashion to design bio-molecular circuits with more complex functionalities [8], [9].

A fundamental systems engineering issue that arises when interconnecting systems with each other is how the dynamic state of the sending system (upstream system) is affected by the dynamic state of the receiving system (downstream system). The effect of downstream loads has been well characterized and accounted for in electrical, mechanical, and hydraulic systems. It has been recently argued that similar problems appear in bio-molecular systems. In particular, Alon states that bio-molecular modules, just like engineering modules, should have special features that make them easily embedded in almost any system. For example, output connections should have "low impedance" so that connecting additional downstream clients should not change the output to existing clients up to some limit [10]. A recent theoretical study has shown, however, that output connections in bio-molecular systems do not always have low impedance. Instead, they can be affected by large impedance-like effects that dramatically distort the dynamics of a system in the face of downstream loads [11]. These impedance-like effects have been called *retroactivity* to extend the notion of impedance to non-electrical systems and in particular to bio-molecular systems.

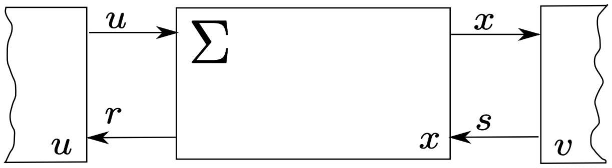

Figure 1. A system Σ with input and output signals, along with the interconnection structure with its upstream and its downstream systems. The retroactivity to the output s accounts for the change in the system Σ dynamics when it is connected to downstream systems. The retroactivity to the input r accounts for changes that Σ causes on upstream systems when it connects to receive the information u.

From a systems biology point of view, one method to deal with retroactivity is to partition large networks into "modules" for which retroactivity effects are minimal, by employing graph and information theoretic approaches [12]–[16]. By contrast, the studies in [11], [17] consider fixed modules, which is more aligned with the synthetic biology perspective. Specifically, [11] characterizes retroactivity, and investigates interconnection mechanisms that provide arbitrary retroactivity attenuation. To this end, it proposes an alternative model to the standard input/output model employed in virtually every systems engineering book [18] (a notable exception to the standard system input/output model is Willem's work [19], which blurs the distinction between inputs, states, and outputs). Within this alternative modeling framework, an input/output system model is augmented with two additional signals: the

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retroactivity to the input r and the retroactivity to the output s (Figure 1).

In this formalism, achieving low output impedance becomes the problem of attenuating retroactivity to the output. The problem of arbitrarily attenuating the retroactivity to the output is in turn conceptually similar to problems of disturbance attenuation and decoupling [20], [21]. *Insulation devices* are then designed in such a way to (a) arbitrarily attenuate the retroactivity to the output (thus they can keep the same output independently of the downstream systems connected to such an output) and (b) have low retroactivity to the input (thus they do not affect the upstream system from which they receive the signal). Insulation devices can be placed between the upstream system sending the signal and the downstream one receiving the signal to insulate these systems from retroactivity. One design method for bio-molecular insulation devices has been illustrated in [11]. It employs a large amplification gain in a negative feedback loop (in analogy to the design of non-inverting amplifiers in electronics) to attenuate the retroactivity to the output.

In this paper, we show that a special interconnection structure found in bio-molecular systems enables a different mechanism for retroactivity attenuation. Retroactivity to the output enters the system dynamics as a state dependent disturbance, which is often multiplied by very large gains. These gains are large due to the fact that bio-molecular system interconnection often occurs through processes that can be among the fastest processes in bio-molecular systems [10], [22]. We show that, for a class of systems with this interconnection structure, whenever the dynamics of a system Σ evolves on a timescale faster than that of its upstream system, the retroactivity to the output of Σ can be arbitrarily attenuated. We also show that this attenuation property is independent of the gains multiplying retroactivity and that the faster the timescale of system Σ with respect to its upstream system, the better the retroactivity to the output attenuation achieved. As a consequence, one can arbitrarily attenuate state dependent disturbances even when these enter the dynamics of system Σ through gains that are orders of magnitude higher than the gains internal to Σ itself. In order to show this retroactivity attenuation capability enabled by timescale separation, we employ singular perturbation techniques for systems with one and multiple small parameters [23]–[25].

Singular perturbation arguments have been used in biochemical applications to show the validity of the quasi-steady state approximation for enzyme kinetics [26]–[31]. In these studies, the timescale separation stems from large initial conditions of either substrate or enzyme, or due to large values for the Michaelis-Menten constant. The separation of timescales in the systems studied in this paper are also due to differences in the order of magnitude of the reaction rates of the processes considered. In bacterial systems, for example, the timescale of gene expression and protein dilution is of the order of minutes [10], [32], the one of post-translational modification processes range from the order of milliseconds to seconds [33], [34], that of proteins binding to small signaling molecules can be in the sub-second timescale [10], and the timescale of transcription factor-DNA interactions can be as fast as few milliseconds [22], [35], [36].

Despite several timescales being present in the processes here considered, the resulting models are not in standard singular perturbation form. This issue arises because the states involved in the interconnection are shared by systems with dynamics in different timescales. This problem is often encountered also in chemical reaction systems [37], [38] and in biochemical systems [27], [29]. A common solution is to employ a change of variables for which the system is in standard singular perturbation form. In this study, we provide sufficient conditions for the existence of a linear coordinate transformation that takes the original system to standard singular perturbation form. Then, we perform a nested application of Tikhonov singular perturbation theorem on the finite time interval as it appears in standard references [25]. Finally, by taking the reduced system back to the original coordinates, we find that the dynamics of the original system on the slow manifold is independent of the retroactivity to the output.

As an application example, we show how modules extracted from natural signal transduction systems can attenuate the retroactivity to the output based on the separation of time scale mechanism illustrated in the paper. It is also shown that the capacity of attenuating retroactivity holds independently of the timescale of the downstream interconnection. The examples in this paper employ a phosphorylation cycle and a phosphotransfer module, both of which are ubiquitous in natural signal transduction systems [39], [40].

This paper is organized as follows. In Section II, we introduce the bio-molecular system model and the retroactivity to the output attenuation problem. The main result is provided in Section III, in which a change of coordinates and a nested application of Tikhonov singular perturbation theorem is performed. Section IV shows the application of the theory to two motifs extracted from natural signal transduction systems: a phosphorylation system and a phosphotransfer system.

II. System Model and Problem Formulation

In this paper, we consider the system model depicted in Figure 1. In addition to the usual input and output signals, we add two additional signals traveling from downstream to upstream: a retroactivity to the output s and a retroactivity to the input r. The retroactivity to the output s is a signal (which may depend on x and on the internal variables v of the downstream system) that appears in the dynamics of Σ whenever Σ is connected to the downstream system. The retroactivity to the input r (which may depend on u and on x) is a signal that system Σ applies to its upstream system as an input whenever Σ connects to the upstream system to receive the information u. The system Σ is said *isolated* when it is not connected to the downstream system. In such a case, $s = 0$.

From an engineering point of view, signals s and r do not necessarily carry information. They are present only because of the physics of the interconnection between system components. For example, if Σ is a voltage generator with voltage V and internal resistance R_0, the value x of its output when Σ is isolated is exactly equal to V. However, when Σ is connected to a downstream load, a voltage drop is caused by current flowing through the internal resistance R_0 so that the new value x of its output voltage will be smaller than what we had in the isolated configuration. In this case, s is due to the non-zero current flowing through R_0 upon interconnection with the downstream load. A similar situation is found in bio-molecular systems. When a synthetic bio-molecular oscillator, such as those of [5], [6], is employed as a signal generator and connected to downstream clients to, for example, synchronize them, the oscillator dynamics can be dramatically affected [11].

A. Bio-molecular System Model

In this section, we specialize the general interconnection structure of Figure 1 to the case of bio-molecular systems so that $u \in D_u \subset \mathbb{R}^q_+$, $x \in D_x \subset \mathbb{R}^n_+$, and $v \in D_v \subset \mathbb{R}^p_+$ are vectors whose components denote concentrations of chemical species, such as proteins, enzymes, DNA sites, etc. We employ a model similar from a formal point of view to that of metabolic networks [41]. Let $r(x,u) \in \mathbb{R}^r$ and $s(x,v) \in \mathbb{R}^s$ be reaction rate vectors modeling the interaction of species in the vector u with species in the vector x and of species in the vector x with species in the vector v, respectively. Let $A \in \mathbb{R}^{r\times q}$, $B \in \mathbb{R}^{r\times n}$, $C \in \mathbb{R}^{s\times n}$, and $D \in \mathbb{R}^{s\times p}$ be constant matrices. Let $f(x,u) \in \mathbb{R}^n$, $l(v) \in \mathbb{R}^p$, and $h(v,t) \in \mathbb{R}^p$ be vector fields and G_1, G_2 be positive constants. The model that we consider for Σ in the interconnection of Figure 1 is thus the following:

$$\begin{aligned}\dot{u} &= g(u,t) + G_1 A r(x,u)\\ \dot{x} &= G_1 B r(x,u) + G_1 f(x,u) + G_2 C s(x,v)\\ \dot{v} &= G_2 D s(x,v) + G_2 l(v) + h(v,t),\end{aligned} \tag{1}$$

with initial conditions $u(t_0), x(t_0), v(t_0)$. The model of Σ when it is isolated from the downstream system becomes ($s(x,v) = 0$)

$$\begin{aligned}\dot{u}_{\text{is}} &= g(u_{\text{is}},t) + G_1 A r(u_{\text{is}}, x_{\text{is}})\\ \dot{x}_{\text{is}} &= G_1 B r(u_{\text{is}}, x_{\text{is}}) + G_1 f(x_{\text{is}}, u_{\text{is}}),\end{aligned} \tag{2}$$

with initial conditions $u_{\text{is}}(t_0) = u(t_0),\ x_{\text{is}}(t_0) = x(t_0)$.

System (1) is a general model for a bio-molecular system. Interconnections always occur through reactions, whose rates (r and s, in this case) appear in both the upstream and the downstream systems with different coefficients (captured by matrices A, B, C, and D). Constants G_1 and G_2 explicitly model the fact that some of the reactions may be several orders of magnitude faster than others. Constant G_1 models the timescale of system Σ. In this paper, we are interested in those cases in which Σ evolves on a faster timescale than its input, that is, $G_1 \gg 1$. This situation is encountered, for example, when Σ models protein modification processes (such as phosphorylation, allosteric modification, dimerization, etc.), while its upstream system models slower processes such as protein production and decay or signaling from outside the cell (here modeled by $g(u,t)$) [10], [33], [34]. Constant G_2 models the timescale of the interconnection mechanism of Σ with its downstream system. For example, when this downstream system models gene expression, s models the binding and unbinding process of transcription factors to DNA binding sites. This reaction is faster than expression and degradation of proteins and therefore, $G_2 \gg 1$ [10], [22]. Additionally, it is possible for the protein modification processes to be in the same range as, much faster than, or much slower than DNA-transcription factor binding and unbinding [33]–[36]. Therefore, it is important to consider the cases in which $G_1 = G_2$, $G_1 \gg G_2$, and $G_1 \ll G_2$.

B. Retroactivity Attenuation Problem

In this paper, we are interested in determining conditions that allow Σ to attenuate the retroactivity to the output and in quantifying the retroactivity to the input. To this end, we define the retroactivity to the output attenuation property of system Σ in the interconnection structure of Figure 1 as follows. Let $u(t, 1/G_1, 1/G_2), x(t, 1/G_1, 1/G_2), v(t, 1/G_1, 1/G_2)$ and $u_{\text{is}}(t, 1/G_1), x_{\text{is}}(t, 1/G_1)$ be the unique solutions for $t \in [t_0, \bar{t}_f]$ with $\bar{t}_f > t_0$ to systems (1) and (2), respectively.

Definition 1. System Σ has the *retroactivity to the output attenuation property* provided there are constants $t_b \in (t_0, \bar{t}_f]$, $G_1^* > 0$, $G_2^* > 0$, and a compact set $\Omega \subset D_x \times D_u \times D_v$ such that the following properties hold for $G_1 > G_1^*$ and $(x(t_0), u(t_0), v(t_0)) \in \Omega$:

(i) $x(t, 1/G_1, 1/G_2) - x_{\text{is}}(t, 1/G_1) = O\left(\frac{1}{G_1}\right) \forall t \in [t_b, \bar{t}_f]$ when $(G_2/G_1) \to \{O(1), 0\}$ as $G_1 \to \infty$;

(ii) $x(t, 1/G_1, 1/G_2) - x_{\text{is}}(t, 1/G_1) = O\left(\frac{G_1}{G_2}\right) \forall t \in [t_b, \bar{t}_f]$ when $(G_2/G_1) \to \infty$ as $G_1 \to \infty$ and $G_2 > G_2^*$.

If a system Σ enjoys the retroactivity to the output attenuation property, its dynamics are not affected by the retroactivity to the output as G_1 grows, independent of the value of G_2. In particular, independently of whether G_2 is smaller than, much larger than, or of the same order as G_1, state dependent disturbances $G_2 s(x,v)$ can be arbitrarily attenuated by a sufficiently large G_1. Furthermore, one can achieve arbitrary retroactivity attenuation by properly adjusting the system parameter G_1.

The remainder of this paper focuses on providing sufficient conditions under which system Σ enjoys the retroactivity to the output attenuation property. Furthermore, we quantify the retroactivity to the input of Σ by determining the impact of Σ on the dynamics of u.

III. Problem Solution

System (1) models processes occurring at multiple timescales. Specifically, since $G_1 \gg 1$, there are at least two timescales and when $G_2 \gg G_1$ there are three timescales. However, there may not be a separation of timescales as the system is not in standard singular perturbation form [25]. This situation is typical of bio-molecular and chemical systems. Such systems often display multiple timescales but there is no explicit separation between fast and slow variables [29], [37]. However, when the interconnection occurs trough binding processes, faster reaction rates appear in the dynamics of both upstream and downstream systems multiplied by integers related to the stoichiometric coefficients [41]. Therefore it is possible to extract the slow variables of a system through a linear combination of the states of the upstream and downstream systems. This motivates an approach that employs a linear coordinate transformation to take the original system to standard singular perturbation form.

In what follows, we first determine conditions for the existence of a linear coordinate transformation independent of G_1 and G_2 that transforms systems (1) and (2) to standard singular perturbation form. Then, we employ Tikhonov singular perturbation theorem to study the dynamics of the system on the slow manifold. To this end, we restrict the class of systems (1) to those with the two following central properties:

P1 There is an invertible matrix $T \in \mathbb{R}^{q\times q}$ and a matrix $M \in \mathbb{R}^{n\times q}$ such that *(i)* $TA + MB = 0$; *(ii)* $Mf(x,u) = 0$ for all (x,u); and *(iii)* $MC = 0$.

P2 There is an invertible matrix $Q \in \mathbb{R}^{n\times n}$, and a matrix $P \in \mathbb{R}^{p\times n}$ such that *(i)* $QC + PD = 0$; *(ii)* $Pl(v) = 0$ for all v.

Let $\epsilon_1 := 1/G_1$, $\epsilon_2 := 1/G_2$, and

$$\bar{h}(y,v) := D\ s(Q^{-1}(y - Pv), v) + l(v) \tag{3}$$

$$\begin{aligned}\bar{f}(z,y,v) := Q\Big[&Br\Big(Q^{-1}(y-Pv), T^{-1}(z - MQ^{-1}(y-Pv))\Big)\\ &+ f\left((Q^{-1}(y-Pv), T^{-1}(z - MQ^{-1}(y-Pv))\right)\Big]\end{aligned} \tag{4}$$

$$\tilde{f}(z,x) := Br(x, T^{-1}(z - Mx)) + f(x, T^{-1}(z - Mx)). \tag{5}$$

Then, we prove the following proposition.

Proposition 1. *Under properties P1 and P2, the linear change of coordinates*

$$z = Tu + Mx,\ y = Qx + Pv \tag{6}$$

takes systems (1) and (2) respectively to the standard singular perturbation forms

$$\begin{aligned}\dot{z} &= Tg\left(T^{-1}(z - MQ^{-1}(y-Pv)), t\right)\\ \epsilon_1\dot{y} &= \bar{f}(z,y,v) + \epsilon_1 Ph(v,t)\\ \epsilon_2\dot{v} &= \bar{h}(y,v) + \epsilon_2 h(v,t)\end{aligned} \tag{7}$$

and

$$\begin{aligned}\dot{z}_{is} &= Tg\left(T^{-1}(z_{is} - Mx_{is}), t\right)\\ \epsilon_1\dot{x}_{is} &= \tilde{f}(z_{is}, x_{is}).\end{aligned} \tag{8}$$

Proof: From the linear coordinate transformation (6), we have that $\dot{z} = T\dot{u} + M\dot{x}$ and $\dot{y} = Q\dot{x} + P\dot{v}$. By substituting in these relations the expressions of $\dot{u}$, $\dot{x}$, and $\dot{v}$ from system (1) (from system (2)), writing $u = T^{-1}(z - Mx)$, and $x = Q^{-1}(y - Pv)$, one obtains system (7) (system (8)). ■

Conditions $TA + MB = 0$ and $Mf(x,u) = 0$ from property P1 and the conditions from property P2 ensure the existence of a linear coordinate transformation that takes the system to standard singular perturbation form. Additionally, condition $MC = 0$ from property P1 is necessary to ensure that once $\epsilon_1 = \epsilon_2 = 0$, the dynamics of u do not depend on v, and thus that the retroactivity to the output does not directly propagate to the input. Properties P1 and P2 give sufficient conditions on the interconnection structure that allows for insulation employing separation of timescales. For low dimensional systems, matrices T, M, Q and P that satisfy properties P1 and P2 can be easily determined by inspection of matrices A, B, C and D. This is illustrated in Section IV with two five-dimensional application examples. For more general cases, prior work has focused on the existence and construction of non-linear coordinate transformations that bring a system to standard singular perturbation form [37].

For $(u,x,v) \in D_u \times D_x \times D_v$, define the domains D_z and D_y to be the images of D_u, D_x, D_v through transformations (6). We also define the map $\mathcal{F} : D_u \times D_x \to D_z \times D_x$ for all $(u,x) \in D_u \times D_x$ as $\mathcal{F}(u,x) := (Tu + Mx, x)$. Note that this map is continuous and invertible. Similarly, define the map $\mathcal{G} : D_u \times D_x \times D_v \to D_z \times D_y \times D_v$ for all $(u,x,v) \in D_u \times D_x \times D_v$ as $\mathcal{G}(u,x,v) := (Tu + Mx, Qx + Pv, v)$. Note that this map is also continuous and invertible.

A. Technical Assumptions

In the following sections, a nested application of Tikhonov singular perturbation theorem, as found in standard references, is employed in systems (7) and (8). To assure validity of the theorems, we pose technical assumptions which are considered valid on the domains D_u, D_x, D_v, D_z, and D_y. In what follows, we say that a square matrix $A(x)$ depending on $x \in D \subset \mathbb{R}^n$ is Hurwitz uniformly for $x \in D$ if there is a real $c > 0$ such that $\Re\{\lambda(A(x))\} < -c$ for all $x \in D$.

A1 The functions g, f, h, r, s are smooth;

A2 The functions g, f, h, r are Lipschitz continuous for all $t \in \mathbb{R}_+$;

A3 The function $v = \phi_1(y)$ is the unique solution of $\bar{h}(y,v) = 0$, it is Lipschitz continuous and smooth;

A4 The function $y = \phi_2(z)$ is the unique solution of $\bar{f}(z,y,\phi_1(y)) = 0$ and it is Lipschitz continuous;

A5 The function $x = \phi_x(z)$ is the unique solution of $\tilde{f}(z,x) = 0$ and it is Lipschitz continuous;

A6 We have that $\frac{\partial}{\partial v}\bar{h}(y,v)\Big|_{v=\phi_1(y)}$ is Hurwitz uniformly for $y \in D_y$;

A7 We have that $\frac{\partial}{\partial y}\bar{f}(z,y,\phi_1(y))\Big|_{y=\phi_2(z)}$ is Hurwitz uniformly for $z \in D_z$;

A8 We have that $\frac{\partial}{\partial(y,v)}\begin{bmatrix}\bar{f}(z,y,v)\\ \bar{h}(y,v)\end{bmatrix}\Bigg|_{y=\phi_2(z), v=\phi_1\circ\phi_2(z)}$ is Hurwitz uniformly for $z \in D_z$;

A9 We have that $\frac{\partial}{\partial x}\tilde{f}(z,x)\Big|_{x=\phi_x(z)}$ is Hurwitz uniformly for $z \in D_z$.

Assumptions A1 and A2 guarantee existence and uniqueness of the solutions of systems (1) and (2). As a consequence, assumptions A1 and A2 also guarantee the existence and uniqueness of the solutions of systems (7) and (8). Assumptions A3, A4, A6 and A7 guarantee the stability of the boundary layer systems obtained when employing a nested application of Tikhonov theorem to system (7) for the case in which $G_1 \ll G_2$. Along with assumption A8, assumptions A3 and A4 are also employed to guarantee the stability of the boundary layer system in the application of Tikhonov theorem to system (7) for the case in which G_1 and G_2 are of the same order of magnitude. Assumptions A5 and A9 guarantee the stability of the boundary layer system when employing Tikhonov theorem to system (8) and to system (7) when $G_1 \gg G_2$.

Proposition 2. *Let* $(y,v) = (\varphi_y(z), \varphi_v(z))$ *be a solution to* $(\bar{f}(z,y,v), \bar{h}(y,v)) = 0$. *Then, such a solution is unique. Furthermore* $\varphi_y(z) = \phi_2(z)$ *and* $\varphi_v(z) = \phi_1 \circ \phi_2(z)$.

Proof: Since $(y, v) = (\varphi_y(z), \varphi_v(z))$ is a solution to equation $(\bar{f}(z, y, v), \bar{h}(y, v)) = 0$, we have that $\bar{h}(\varphi_y(z), \varphi_v(z)) = 0$. By A3, this implies that $\varphi_v(z) = \phi_1 \circ \varphi_y(z)$. This along with $\bar{f}(z, \varphi_y(z), \varphi_v(z)) = 0$ imply that $\bar{f}(z, \varphi_y(z), \phi_1 \circ \varphi_y(z)) = 0$. This along with A4 imply that $\varphi_y(z) = \phi_2(z)$. As a consequence, we have that $\varphi_v(z) = \phi_1 \circ \varphi_y(z) = \phi_1 \circ \phi_2(z)$. ■

B. Main Result

The main result of the paper is based on the two following lemmas, which employ Tikhonov singular perturbation theorem in the form presented in [25]. Specifically, Lemmas 1 and 2 provide approximations of the isolated and connected system trajectories, respectively, when we consider as small parameters $\epsilon_1 := 1/G_1$ and $\epsilon_2 := 1/G_2$. These approximations are then compared with each other to obtain the retroactivity to the output attenuation property, which is the main result of the paper.

Before giving the first lemma, we define the two following sets. For any $\alpha > 0$, define the set $R_{u,x}(\alpha) \subset D_u \times D_x$ by

$$R_{u,x}(\alpha) := \{(u, x) \in D_u \times D_x \mid \|x - \phi_x(Tu + Mx)\| < \alpha\} \qquad (9)$$

and let $\Omega_{u,x}(\alpha)$ be any compact subset of $R_{u,x}(\alpha)$. For $\alpha > 0$, define the set $R_{u,x,v}(\alpha) \subset D_u \times D_x \times D_v$ by

$$R_{u,x,v}(\alpha) := \left\{ (u, x, v) \in D_u \times D_x \times D_v \,\middle|\, \left\| \begin{matrix} v - \phi_1(Qx + Pv) \\ Qx + Pv - \phi_2(Tu + Mx) \end{matrix} \right\| < \alpha \right\}, \qquad (10)$$

and let $\Omega_{u,x,v}(\alpha)$ be any compact subset of $R_{u,x,v}(\alpha)$. The next proposition shows the relationship between the sets $R_{u,x}$ and $R_{u,x,v}$.

Proposition 3. *Consider the sets defined in equations (9) and (10). Then, for all* $\alpha > 0$ *there is* $\beta > 0$ *such that* $(u, x, v) \in R_{u,x,v}(\beta)$ *implies that* $(u, x) \in R_{u,x}(\alpha)$.

Proof: Since $x = \phi_x(z)$ is the unique solution of $\tilde{f}(z, x) = 0$ and $\bar{f}(z, \phi_2(z), \phi_1 \circ \phi_2(z)) = 0$, it follows from the definition of $\bar{f}$ (equation (4)) that $Q^{-1}[\phi_2(z) - P\phi_1 \circ \phi_2(z)] = \phi_x(z)$. Since Q is invertible, for all $\alpha > 0$ there is $\beta_2 > 0$ such that $\|Qx - \phi_2(Tu + Mx) + P\phi_1 \circ \phi_2(Tu + Mx)\| < \beta_2$ implies $\|x - \phi_x(Tu + Mx)\| < \alpha$. By applying the triangular inequality, one can show that for all $\beta_2 > 0$ there is $\beta_1 > 0$ such that $\|v - \phi_1 \circ \phi_2(Tu + Mx)\| < \beta_1$ and $\|Qx + Pv - \phi_2(Tu + Mx)\| < \beta_1$ imply $\|Qx - \phi_2(Tu + Mx) + P\phi_1 \circ \phi_2(Tu + Mx)\| < \beta_2$. Finally, the continuity of ϕ_1 along with the triangular inequality imply that for all $\beta_1 > 0$ there is $\beta_0 > 0$ such that $\|v - \phi_1(Qx + Pv)\| < \beta_0$ and $\|Qx + Pv - \phi_2(Tu + Mx)\| < \beta_0$ imply $\|v - \phi_1 \circ \phi_2(Tu + Mx)\| < \beta_1$. Let $\beta := \min(\beta_0, \beta_1)$. ■

As a consequence of this proposition, if $\Omega \subset R_{u,x,v}(\beta)$ is compact, then the set $\{(u, x) \mid (u, x, v) \in \Omega\}$ is a compact subset of $R_{u,x}(\alpha)$.

Under properties P1-P2 and assumptions A1-A9, we give the two following lemmas.

Lemma 1. *Let* $u_{is}(t, 1/G_1), x_{is}(t, 1/G_1)$ *be the unique solution of system (2) for* $t \in [t_0, t_f]$ *with initial condition* $u_{is}(t_0) \in D_u$ *and* $x_{is}(t_0) \in D_x$. *Let* $\bar{u}(t)$ *be the unique solution of system*

$$\dot{\bar{u}} = \left(T + M\frac{d\gamma_x(\bar{u})}{d\bar{u}}\right)^{-1} Tg(\bar{u}, t) \qquad (11)$$

for $t \in [t_0, \bar{t}_f]$ *with initial condition* $\bar{u}(t_0) = T^{-1}(z_{is}(t_0) - \phi_x(z_{is}(t_0)))$ *where* $z_{is}(t_0) = Tu_{is}(t_0) + Mx_{is}(t_0)$ *and* $x = \gamma_x(u)$ *is the locally unique solution of* $Br(x, u) + f(x, u) = 0$. *Then, there is* $\alpha > 0$ *such that for all* $t_b \in (t_0, \bar{t}_f]$ *there exists* $G_1^* > 0$ *such that* $u_{is}(t, 1/G_1) - \bar{u}(t) = O\left(\frac{1}{G_1}\right)$ *and* $x_{is}(t, 1/G_1) - \gamma_x(\bar{u}(t)) = O\left(\frac{1}{G_1}\right)$ *hold uniformly for* $t \in [t_b, \bar{t}_f]$ *provided* $G_1 > G_1^*$ *and* $(u_{is}(t_0), x_{is}(t_0)) \in \Omega_{u,x}(\alpha)$.

Proof: For convenience, define $\bar{g}(z_{\rm is}, x_{\rm is}) := Tg\left(T^{-1}(z_{\rm is} - Mx_{\rm is}), t\right)$ and denote the solution of system (8) by $z_{\rm is}(t, \epsilon_1)$, $x_{\rm is}(t, \epsilon_1)$ for $t \in [t_0, t_f]$ with $z_{\rm is}(t_0) = Tu_{\rm is}(t_0) + Mx_{\rm is}(t_0)$. Let $x = \phi_x(z)$ be the unique solution of the algebraic equation $\tilde{f}(z, x) = 0$ and denote by $\bar{z}_{\rm is}(t)$ the unique solution of the reduced system

$$\dot{\bar{z}}_{\rm is} = \bar{g}(\bar{z}_{\rm is}, \phi_x(\bar{z}_{\rm is})) \qquad (12)$$

for $t \in [t_0, \bar{t}_f]$ and $\bar{z}_{\rm is}(t_0) = z_{\rm is}(t_0)$ (the uniqueness of the solution follows from the fact that $\bar{g}$ is Lipschitz continuous on its domain by Assumptions A2 and A5). Assumption A9 further guarantees that the boundary layer system is locally exponentially stable. The region of attraction thus contains the set of x such that $\|x - \phi_x(z(t_0))\| < \beta$ for some $\beta > 0$ sufficiently small. Define the set $R_{z,x}(\beta) = \{(z, x) \mid \|x - \phi_x(z)\| < \beta\}$. Let $\Omega_{z,x}(\beta)$ be any compact subset of $R_{z,x}(\beta)$. By Tikhonov theorem, for all $t_b \in (t_0, \bar{t}_f]$, there exists $\epsilon_1^* > 0$ such that

$$\begin{aligned} z_{\rm is}(t, \epsilon_1) - \bar{z}_{\rm is}(t) &= O(\epsilon_1) \text{ and} \\ x_{\rm is}(t, \epsilon_1) - \phi_x(\bar{z}_{\rm is}(t)) &= O(\epsilon_1) \text{ uniformly for } t \in [t_b, \bar{t}_f] \end{aligned} \qquad (13)$$

provided $\epsilon_1 < \epsilon_1^*$ and $(x_{\rm is}(t_0), z_{\rm is}(t_0)) \in \Omega_{z,x}(\beta)$.

To obtain these approximations in the original coordinate system, define

$$\bar{u}_{\rm is} := T^{-1}\left(\bar{z}_{\rm is} - M\phi_x(\bar{z}_{\rm is})\right). \qquad (14)$$

We seek to show that $\bar{u}_{\rm is}(t)$ satisfies the differential equation (11). Since $x = \phi_x(z)$ is the locally unique solution of $\tilde{f}(z, x) = 0$, we must have that

$$\tilde{f}(z, \phi_x(z)) = 0. \qquad (15)$$

Since $\tilde{f}(z, x) = Br\left(x, T^{-1}(z - Mx)\right) + f\left(x, T^{-1}(z - Mx)\right)$, equation (15) implies that

$$\begin{aligned} &Br\left(\phi_x(\bar{z}_{\rm is}), T^{-1}\left(\bar{z}_{\rm is} - M\phi_x(\bar{z}_{\rm is})\right)\right) \\ &\quad + f\left(\phi_x(\bar{z}_{\rm is}), T^{-1}\left(\bar{z}_{\rm is} - M\phi_x(\bar{z}_{\rm is})\right)\right) = 0. \end{aligned} \qquad (16)$$

From the assumptions of the lemma, we have that $x = \gamma_x(u)$ is the locally unique solution of $Br(x, u) + f(x, u) = 0$. This along with equation (16) imply that $\phi_x(\bar{z}_{\rm is}) = \gamma_x(T^{-1}\left(\bar{z}_{\rm is} - M\phi_x(\bar{z}_{\rm is})\right)) = \gamma_x(\bar{u}_{\rm is})$. As a consequence, we can re-write equation (14) as $\bar{z}_{\rm is} = T\bar{u}_{\rm is} + M\gamma_x(\bar{u}_{\rm is})$. Taking the time derivative of both sides of this expression, we obtain $\dot{\bar{z}}_{\rm is} = T\dot{\bar{u}}_{\rm is} + M\frac{d\gamma_x(\bar{u}_{\rm is})}{d\bar{u}_{\rm is}}\dot{\bar{u}}_{\rm is}$. Employing equation (12) on the left-hand side and re-arranging the terms, we obtain that $\dot{\bar{u}}_{\rm is} = \left(T + M\frac{d\gamma_x(\bar{u}_{\rm is})}{d\bar{u}_{\rm is}}\right)^{-1}\bar{g}(\bar{z}_{\rm is}, \phi_x(\bar{z}_{\rm is}))$, in which (by equations (8)) we have that $\bar{g}(\bar{z}_{\rm is}, \phi_x(\bar{z}_{\rm is})) = Tg\left(T^{-1}(\bar{z}_{\rm is} - M\phi_x(\bar{z}_{\rm is})), t\right) = Tg(\bar{u}_{\rm is}, t)$, leading to $\bar{u}_{\rm is}(t)$ satisfying the differential equation (11) for $t \in [t_0, \bar{t}_f]$ with $\bar{u}_{\rm is}(t_0) = T^{-1}(\bar{z}_{\rm is}(t_0) - M\phi_x(\bar{z}_{\rm is}(t_0)))$. Since $u_{\rm is}(t, \epsilon_1) = T^{-1}[z_{\rm is}(t, \epsilon_1) - Mx_{\rm is}(t, \epsilon_1)]$ and equations (13) hold, we have that $u_{\text{is}}(t,\epsilon_1) = T^{-1}[\bar{z}_{\text{is}}(t)+O(\epsilon_1)-M(\phi_x(\bar{z}_{\text{is}}(t))+O(\epsilon_1))]$, which, by employing equation (14) and the fact that $\phi_x(\bar{z}_{\text{is}}) = \gamma_x(T^{-1}(\bar{z}_{\text{is}} - M\phi_x(\bar{z}_{\text{is}}))) = \gamma_x(\bar{u}_{\text{is}})$, leads to

$$\begin{aligned} u_{\text{is}}(t,\epsilon_1) - \bar{u}_{\text{is}}(t) &= O(\epsilon_1) \text{ and} \\ x_{\text{is}}(t,\epsilon_1) - \gamma_x(\bar{u}_{\text{is}}(t)) &= O(\epsilon_1) \text{ uniformly for } t \in [t_b, \bar{t}_f] \end{aligned} \tag{17}$$

provided that $\epsilon_1 < \epsilon_1^*$ and $(z_{\text{is}}(t_0), u_{\text{is}}(t_0)) \in \Omega_{z,x}(\beta)$. Since $R_{z,x}(\beta)$ is the image of $R_{u,x}(\beta)$ under the continuous map $\mathcal{F}$, we have that for any compact set $\Omega_{z,x}(\beta) \subset R_{z,x}(\beta)$, there is a compact subset $\Omega_{u,x}(\beta) \subset R_{u,x}(\beta)$ such that $\Omega_{z,x}(\beta) = \mathcal{F}(\Omega_{u,x}(\beta))$. As a consequence, equations (17) hold provided $\epsilon_1 < \epsilon_1^*$ and $(u_{\text{is}}(t_0), x_{\text{is}}(t_0)) \in \Omega_{u,x}(\beta)$ with $\Omega_{u,x}(\beta) := \mathcal{F}^{-1}(\Omega_{z,x}(\beta))$. Set $\alpha = \beta$ and $G_1^* := 1/\epsilon_1^*$. ■

The following lemma provides approximations to the solution of system (1) in a way similar to what was performed in Lemma 1 for system (2). The main technical difference between Lemma 1 and Lemma 2 is that system (1) has two small parameters, that is, $\epsilon_1 = 1/G_1$ and $\epsilon_2 = 1/G_2$, which can take different relative values. The proof of the lemma thus considers three different cases: $G_2/G_1 \to O(1)$ as $G_1 \to \infty$ (i.e., G_1 and G_2 are of the same order of magnitude); $G_2/G_1 \to 0$ as $G_1 \to \infty$ (i.e., G_1 is orders of magnitude larger than G_2); $G_2/G_1 \to \infty$ as $G_1 \to \infty$ (i.e., G_2 is orders of magnitude larger than G_1). In particular, in the latter case the system has three different timescales and therefore it is treated by performing a nested application of Tikhonov singular perturbation theorem.

Lemma 2. *Let $x(t, 1/G_1, 1/G_2)$, $u(t, 1/G_1, 1/G_2)$, $v(t, 1/G_1, 1/G_2)$ be the unique solution of system (1) for $t \in [t_0, t_f]$ with initial conditions $(x(t_0), u(t_0), v(t_0)) \in D_x \times D_u \times D_v$. Let $\bar{u}(t)$ be the unique solution of system*

$$\dot{\bar{u}} = \left(T + M\frac{d\gamma_x(\bar{u})}{d\bar{u}}\right)^{-1} Tg(\bar{u}, t), \tag{18}$$

for $t \in [t_0, \bar{t}_f]$ with initial condition $\bar{u}(t_0) = T^{-1}(z(t_0)-\phi_x(z(t_0)))$ with $z(t_0) = Tu(t_0) + Mx(t_0)$ and $x = \gamma_x(u)$ the locally unique solution of $f(x,u) + Br(x,u) = 0$. Then, there is $\alpha > 0$ such that for all $t_b \in (t_0, \bar{t}_f]$ there are $G_1^* > 0$ and $G_2^* > 0$ such that the following properties hold for $(u(t_0), x(t_0), v(t_0)) \in \Omega_{u,x,v}(\alpha)$ and $G_1 > G_1^*$:

- *(i) $x(t,1/G_1,1/G_2)-\gamma_x(\bar{u}(t)) = O\left(\frac{1}{G_1}\right)$ and $u(t,1/G_1,1/G_2)-\bar{u}(t) = O\left(\frac{1}{G_1}\right)$ uniformly for $t \in [t_b, \bar{t}_f]$ when $G_2/G_1 \to \{O(1), 0\}$ as $G_1 \to \infty$;*
- *(ii) $x(t,1/G_1,1/G_2)-\gamma_x(\bar{u}(t)) = O\left(\frac{G_1}{G_2}\right)$ and $u(t,1/G_1,1/G_2)-\bar{u}(t) = O\left(\frac{G_1}{G_2}\right)$ uniformly for $t \in [t_b, \bar{t}_f]$ when $G_2/G_1 \to \infty$ as $G_1 \to \infty$ and $G_2 > G_2^*$.*

Proof:

Define for convenience the function $\bar{g}(z,y,v,t) := Tg(T^{-1}(z - MQ^{-1}(y - Pv)), t)$ and let $z(t,\epsilon_1,\epsilon_2)$, $y(t,\epsilon_1, \epsilon_2)$, $v(t,\epsilon_1,\epsilon_2)$ be the unique solution of system (7) for $t \in [t_0, t_f]$ with initial conditions $z(t_0) = Tu(t_0)+Mx(t_0)$, $y(t_0) = Qx(t_0)+Pv(t_0)$, and $v(t_0)$. There are three cases: $\epsilon_2/\epsilon_1 \to 0$ as $\epsilon_1 \to 0$, $\epsilon_2/\epsilon_2 \to O(1)$ as $\epsilon_1 \to 0$, and $\epsilon_2/\epsilon_1 \to \infty$ as $\epsilon_1 \to 0$.

Case 1: $\epsilon_2/\epsilon_1 \to 0$ as $\epsilon_1 \to 0$. We perform a nested application of Tikhonov singular perturbation theorem. Define the new small parameters $\mu_1 := \epsilon_1$ and $\mu_2 := \epsilon_2/\epsilon_1$ and re-write system (7) as

$$\begin{aligned} \dot{z} &= \bar{g}(z,y,v,t) \\ \mu_1\dot{y} &= \bar{f}(z,y,v) + \mu_1 Ph(v,t) \\ \mu_2\mu_1\dot{v} &= \bar{h}(y,v) + \mu_2\mu_1 h(v,t). \end{aligned} \tag{19}$$

Set $\mu_2 = 0$ and let $v = \phi_1(y)$ be the locally unique solution of $\bar{h}(y,v) = 0$. Let also $\bar{z}(t,\epsilon_1)$ and $\bar{y}(t,\epsilon_1)$ be the unique solution of the reduced system obtained once $\mu_2 = 0$

$$\begin{aligned} \dot{z} &= \bar{g}(z,y,\phi_1(y),t) \\ \mu_1\dot{y} &= \bar{f}(z,y,\phi_1(y)) + \mu_1 Ph(\phi_1(y),t) \end{aligned} \tag{20}$$

for $t \in [t_0, T_a]$, $\bar{z}(t_0) = z(t_0)$, and $\bar{y}(t_0) = y(t_0)$ (uniqueness of the solution follows from Assumptions A2 and A3). Assumption A6 further guarantees that the boundary layer system is locally exponentially stable. For some $\beta > 0$ sufficiently small, the region of attraction contains the set of v such that $\|v-\phi_1(y(t_0))\|$ is sufficiently small. Define the set $R_{y,v}(\beta) := \{(y,v) \in D_y \times D_v \mid \|v - \phi_1(y)\| < \beta\}$ and let $\Omega_{y,v}(\beta) \subset R_{y,v}(\beta)$ be compact. Then, by Tikhonov theorem, for all $t_b > 0$ there is $\mu_2^* > 0$ such that

$$\begin{aligned} &z(t,\mu_1,\mu_1\mu_2) - \bar{z}(t,\mu_1) = O(\mu_2) \text{ and} \\ &y(t,\mu_1,\mu_1\mu_2) - \bar{y}(t,\mu_1) = O(\mu_2) \text{ uniformly for } t \in [t_0, T_a] \\ &v(t,\mu_1,\mu_1\mu_2) - \phi_1(\bar{y}(t,\mu_1)) = O(\mu_2) \text{ uniformly for } t \in [t_b, T_a] \end{aligned} \tag{21}$$

hold provided $\mu_2 < \mu_2^*$ and $(\bar{y}(t_0), v(t_0)) \in \Omega_{y,v}(\beta)$.

System (20) is also in standard singular perturbation form with small parameter μ_1. Set $\mu_1 = 0$ and let $y = \phi_2(z)$ be the locally unique solution of $\bar{f}(z,y,\phi_1(y)) = 0$. Let $\tilde{z}(t)$ be the unique solution of the resulting reduced system when $\mu_1 = 0$

$$\dot{\tilde{z}} = \bar{g}(\tilde{z}, \phi_2(\tilde{z}), \phi_1 \circ \phi_2(\tilde{z}), t) \tag{22}$$

for $t \in [t_0, \bar{t}_f]$ with $\tilde{z}(t_0) = \bar{z}(t_0)$ (uniqueness of the solution follows from Assumptions A2, A3, and A4). Furthermore, Assumption A7 guarantees that the boundary layer system is locally exponentially stable. For some $\delta > 0$ sufficiently small, the region of attraction contains the set of y such that $\|y - \phi_2(z(t_0))\| < \delta$. Define the set $R_{z,y}(\delta) := \{(z,y) \in D_z \times D_y \mid \|y-\phi_2(z)\| < \delta\}$ and let $\Omega_{z,y}(\delta) \subset R_{z,y}(\delta)$ be compact. Then, from Tikhonov theorem, for all $t_b > 0$, there is $\mu_1^* > 0$ such that

$$\begin{aligned} \bar{z}(t,\mu_1) - \tilde{z}(t) &= O(\mu_1) \text{ uniformly for } t \in [t_0, \bar{t}_f] \\ \bar{y}(t,\mu_1) - \phi_2(\tilde{z}(t)) &= O(\mu_1) \text{ uniformly for } t \in [t_b, \bar{t}_f] \end{aligned} \tag{23}$$

hold provided $\mu_1 < \mu_1^*$ and $(\tilde{z}(t_0), \bar{y}(t_0)) \in \Omega_{z,y}(\delta)$. As a consequence of relations (23), for $\mu_1 < \mu_1^*$ the solution of system (20) is uniquely defined for $t \in [t_0, \bar{t}_f]$. We can thus let $T_a = \bar{t}_f$ so that for $\mu_2 < \mu_2^*$, with μ_2^* sufficiently small, also the solution of system (19) is uniquely defined for $t \in [t_0, \bar{t}_f]$. Let $\eta := \min(\beta,\delta)$ and define $R_{z,y,v}(\eta) := \left\{(z,y,v) \in D_z \times D_y \times D_v \mid \left\| \begin{matrix} v - \phi_1(y) \\ y - \phi_2(z) \end{matrix} \right\| < \eta \right\}$. Let $\Omega_{z,y,v}(\eta) \subset R_{z,y,v}(\eta)$ be any compact set. Combining expression (21) with

$T_a = \bar{t}_f$ and expression (23), the solution of system (1) satisfies

$$\begin{aligned} &v(t,\mu_1,\mu_1\mu_2) - \phi_1 \circ \phi_2(\tilde{z}(t)) = O(\mu_1) + O(\mu_2) \\ &y(t,\mu_1,\mu_1\mu_2) - \phi_2(\tilde{z}(t)) = O(\mu_1) + O(\mu_2) \\ &z(t,\mu_1,\mu_1\mu_2) - \tilde{z}(t) = O(\mu_1) + O(\mu_2) \text{ uniformly for } t \in [t_b, \bar{t}_f], \end{aligned} \tag{24}$$

in which we have used that $\phi_1(\phi_2(z)+O(\mu_1)) = \phi_1\circ\phi_2(z)+O(\mu_1)$ since ϕ_1 is smooth. In order to return to the original coordinate system, define

$$\bar{u} = T^{-1}\left(\tilde{z} - MQ^{-1}(\phi_2(\tilde{z}) - P\phi_1 \circ \phi_2(\tilde{z}))\right). \tag{25}$$

We seek to show that $\bar{u}(t)$ satisfies equation (11). Since $y = \phi_2(z)$ is the locally unique solution of $\bar{f}(z,y,\phi_1(y)) = 0$, by the definition of $\bar{f}$ (equation (4)), we have that $Br(Q^{-1}(\phi_2(\tilde{z})-P\phi_1\circ\phi_2(\tilde{z})),\bar{u})+f(Q^{-1}(\phi_2(\tilde{z})-P\phi_1\circ\phi_2(\tilde{z})),\bar{u}) = 0$. This equation along with the fact that $x = \gamma_x(u)$ is the locally unique solution of $Br(x,u) + f(x,u) = 0$ lead to

$$\begin{aligned} &Q^{-1}(\phi_2(\tilde{z}) - P\phi_1 \circ \phi_2(\tilde{z})) \\ &\quad = \gamma_x(T^{-1}(\tilde{z} - MQ^{-1}(\phi_2(\tilde{z}) - P\phi_1 \circ \phi_2(\tilde{z})))) = \gamma_x(\bar{u}). \end{aligned} \tag{26}$$

Substituting this into equation (25) and re-arranging the terms, we obtain the equation $\tilde{z} = T\bar{u} + M\gamma_x(\bar{u})$. Taking the time derivative both sides, we obtain that $\dot{\tilde{z}} = T\dot{\bar{u}} + M\frac{d\gamma_x(\bar{u})}{d\bar{u}}\dot{\bar{u}}$. Employing equation (22) on the left-hand side and re-arranging the terms, we obtain $\dot{\bar{u}} = \left(T + M\frac{d\gamma_x(\bar{u})}{d\bar{u}}\right)^{-1}\bar{g}(\tilde{z},\phi_2(\tilde{z}),\phi_1 \circ \phi_2(\tilde{z}),t)$, in which we have that $\bar{g}(\tilde{z},\phi_2(\tilde{z}),\phi_1 \circ \phi_2(\tilde{z}),t) = Tg\left(T^{-1}(\tilde{z} - MQ^{-1}(\phi_2(\tilde{z}) - P\phi_1 \circ \phi_2(\tilde{z}))),t\right)$ with $T^{-1}(\tilde{z} - MQ^{-1}(\phi_2(\tilde{z}) - P\phi_1 \circ \phi_2(\tilde{z}))) = \bar{u}$ from equation (25). Therefore, $\bar{u}(t)$ is the unique solution of (18) for $t \in [t_0, \bar{t}_f]$ and $\bar{u}(t_0) = T^{-1}\left(\tilde{z}(t_0) - MQ^{-1}[\phi_2(\tilde{z}(t_0)) - P\phi_1 \circ \phi_2(\tilde{z}(t_0))]\right)$, in which $\tilde{z}(t_0) = z(t_0)$. Since $x = \phi_x(z)$ is the unique solution of $\tilde{f}(z,x) = 0$ and $\bar{f}(z,\phi_2(z),\phi_1 \circ \phi_2(z)) = 0$, it follows from the definition of $\bar{f}$ (equation (4)) that $Q^{-1}[\phi_2(z) - P\phi_1 \circ \phi_2(z)] = \phi_x(z)$. Thus, $\bar{u}(t_0) = T^{-1}[z(t_0) - M\phi_x(z(t_0))]$.

From the coordinate transformation (6), we have $x(t,\mu_1,\mu_1\mu_2) = Q^{-1}(y(t,\mu_1,\mu_1\mu_2) - Pv(t,\mu_1,\mu_1\mu_2))$. Employing the relations for y and v from (24), we obtain $x(t,\mu_1,\mu_1\mu_2) = Q^{-1}[\phi_2(\tilde{z}(t))+O(\mu_1)+O(\mu_2)-P(\phi_1 \circ \phi_2(\tilde{z}(t)) + O(\mu_1) + O(\mu_2))]$. By employing equations (25) and (26), one obtains that $x(t,\mu_1,\mu_1\mu_2) = \gamma_x(\bar{u}(t)) + O(\mu_1) + O(\mu_2)$. Similarly, from the change of variable $u(t,\mu_1,\mu_1\mu_2) = T^{-1}(z(t,\mu_1,\mu_1\mu_2) - MQ^{-1}[y(t,\mu_1,\mu_1\mu_2) - Pv(t,\mu_1,\mu_1\mu_2)])$, (24), and (25), we obtain that $u(t,\mu_1,\mu_1\mu_2) = \bar{u}(t) + O(\mu_1) + O(\mu_2)$. Hence, we have that

$$\begin{aligned} u(t,\mu_1,\mu_1\mu_2) - \bar{u}(t) &= O(\mu_1) + O(\mu_2) \text{ and} \\ x(t,\mu_1,\mu_1\mu_2) - \gamma_x(\bar{u}(t)) &= O(\mu_1) + O(\mu_2) \end{aligned} \tag{27}$$

uniformly for $t \in [t_b, \bar{t}_f]$ provided $\mu_1 < \mu_1^*$, $\mu_2 < \mu_2^*$, and $(\tilde{z}(t_0), \bar{y}(t_0), v(t_0)) \in \Omega_{z,y,v}(\eta)$. Since $R_{z,y,v}(\eta)$ is the image of $R_{u,x,v}(\eta)$ under the continuous map $\mathcal{G}$, we have that for any compact set $\Omega_{z,x,v}(\eta) \subset R_{z,x,v}(\eta)$, there is a compact set $\Omega_{u,x,v}(\eta) \subset R_{u,x,v}(\eta)$ such that $\Omega_{z,x,v}(\eta) = \mathcal{G}(\Omega_{u,x,v}(\eta))$. As a consequence, equations (27) hold provided $\mu_1 < \mu_1^*$, $\mu_2 < \mu_2^*$, and $(u(t_0), x(t_0), v(t_0)) \in \Omega_{u,x,v}(\eta)$ with $\Omega_{u,x,v}(\eta) = \mathcal{G}^{-1}(\Omega_{z,x,v}(\eta))$. Define $\epsilon_1^{\text{case 1}} := \mu_1^*$ and $\alpha^{\text{Case 1}} := \eta$.

Case 2: $\epsilon_2/\epsilon_1 = O(1)$ as $\epsilon_1 \to 0$. Letting $a := \epsilon_1/\epsilon_2$, system (7) becomes

$$\begin{aligned} \dot{z} &= \bar{g}(z,y,v,t) \\ \epsilon_1\dot{y} &= \bar{f}(z,y,v) + \epsilon_1 Ph(v,t) \\ \epsilon_1\dot{v} &= a\bar{h}(y,v) + \epsilon_1 h(v,t). \end{aligned} \tag{28}$$

Denote the solution of system (28) by $z(t,\epsilon_1)$, $y(t,\epsilon_1)$, and $v(t,\epsilon_1)$ for $t \in [t_0, t_f]$. By Proposition 2, $(y,v) = (\phi_2(z), \phi_1 \circ \phi_2(z))$ is the locally unique solution of $(\bar{f}(z,y,v), \bar{h}(y,v)) = 0$. Define $\phi_3(z) := \phi_1 \circ \phi_2(z)$ to simplify notation. Let $\bar{z}(t)$ be the unique solution of the reduced system

$$\dot{z} = \bar{g}(z,\phi_2(z),\phi_3(z),t) \tag{29}$$

for $t \in [t_0, \bar{t}_f]$ and $\bar{z}(t_0) = z(t_0)$ (uniqueness of the solution follows from Assumptions A2, A3, and A4). Furthermore, Assumption A8 guarantees that the boundary layer system is locally exponentially stable. For some $\beta > 0$ sufficiently small, the region of attraction contains the set of all (y,v) such that $\|(y,v) - (\phi_2(z(t_0)), \phi_3(z(t_0)))\| < \beta$. Define the set $R_{z,y,v}(\beta) := \{(z,y,v) \in D_z \times D_y \times D_v \mid \|(y,v) - (\phi_2(z), \phi_3(z))\| < \beta\}$ and let $\Omega_{z,y,v}(\beta) \subset R_{z,y,v}(\beta)$ be compact. Then, by Tikhonov theorem, for all $t \in (t_0, \bar{t}_f]$ there is $\epsilon_1^{\text{Case 2}} > 0$ such that

$$\begin{aligned} &z(t,\epsilon) - \bar{z}(t) = O(\epsilon_1) \text{ uniformly for } t \in [0, \bar{t}_f] \\ &y(t,\epsilon) - \phi_2(\bar{z}(t)) = O(\epsilon_1) \text{ and} \\ &v(t,\epsilon) - \phi_3(\bar{z}(t)) = O(\epsilon_1) \text{ uniformly for } t \in [t_b, \bar{t}_f] \end{aligned} \tag{30}$$

provided $\epsilon_1 < \epsilon_1^{\text{Case 2}}$ and $(z(t_0), y(t_0), v(t_0)) \in \Omega_{z,y,v}(\beta)$.

Define

$$\bar{u} := T^{-1}(\bar{z} - MQ^{-1}(\phi_2(\bar{z}) - P\phi_3(\bar{z}))). \tag{31}$$

We seek to determine the differential equation that $\bar{u}(t)$ obeys. Since $\bar{f}(z,\phi_2(z),\phi_3(z)) = 0$, we have by the definition of $\bar{f}$ (equation (4)) that $Br\Big(Q^{-1}(\phi_2(z) - P\phi_3(z)), T^{-1}(z - MQ^{-1}(\phi_2(z) - P\phi_3(z)))\Big) + f\Big(Q^{-1}(\phi_2(z) - P\phi_3(z)), T^{-1}(z - MQ^{-1}(\phi_2(z) - P\phi_3(z)))\Big) = 0$. Given that by assumption $x = \gamma_x(u)$ is the locally unique solution of $Br(x,u) + f(x,u) = 0$, we must have that $Q^{-1}(\phi_2(\bar{z}) - P\phi_3(\bar{z}))) = \gamma_x\left(T^{-1}[\bar{z} - MQ^{-1}(\phi_2(\bar{z}) - P\phi_3(\bar{z}))]\right) = \gamma_x(\bar{u})$. Substituting this in equation (31), we obtain that $\bar{z} = T\bar{u} + M\gamma_x(\bar{u})$. Computing the time derivative both sides of this equation, employing equation (29) and equation (31), one obtains that $\bar{u}(t)$ is the solution of (18) for $t \in [t_0, \bar{t}_f]$ with $\bar{u}(t_0) = T^{-1}\Big(\bar{z}(t_0) - MQ^{-1}[\phi_2(\bar{z}(t_0)) - P\phi_3(\bar{z}(t_0))]\Big)$ and $\bar{z}(t_0) = z(t_0)$. Since, as for Case 1, we have that $Q^{-1}[\phi_2(\bar{z}(t_0)) - P\phi_3(\bar{z}(t_0))] = \phi_x(\bar{z}(t_0))$, then $\bar{u}(t_0) = T^{-1}[\bar{z}(t_0) - M\phi_x(\bar{z}(t_0))]$.

Finally, employing the change of coordinates (6) and approximations (30), we obtain that

$$\begin{aligned} u(t,\epsilon_1) - \bar{u}(t) &= O(\epsilon_1) \text{ and} \\ x(t,\epsilon_1) - \gamma_x(\bar{u}(t)) &= O(\epsilon_1) \end{aligned} \tag{32}$$

hold uniformly for $t \in [t_b, \bar{t}_f]$ provided $(z(t_0), y(t_0), v(t_0)) \in \Omega_{z,y,v}(\beta)$ and $\epsilon_1 < \epsilon_1^{\text{Case 2}}$. Define the new region $R_{z,y,v}(\eta) := \{(z,y,v) \mid \|(y,v) - (\phi_2(z), \phi_1(y))\| < \eta\}$. By the continuity of

ϕ_1 and the triangular inequality, it follows that for all $\beta > 0$ there is $\eta > 0$ such that $\bar{R}_{z,y,v}(\eta) \subset R_{z,y,v}(\beta)$. Since $\Omega_{z,y,v}(\beta)$ is an arbitrary compact subset of $R_{z,y,v}(\beta)$, it can be chosen such that $\Omega_{z,y,v}(\beta) = \bar{\Omega}_{z,y,v}(\eta)$ for $\bar{\Omega}_{z,y,v}(\eta)$ a suitable compact subset of $\bar{R}_{z,y,v}(\eta)$. Since $\bar{R}_{z,y,v}(\eta) = \mathcal{G}(R_{u,x,v}(\eta))$ and $\mathcal{G}$ is a continuous mapping, we have that for all compact sets $\bar{\Omega}_{z,y,v}(\eta) \subseteq \bar{R}_{z,y,v}(\eta)$ there is a compact set $\Omega_{u,x,v}(\eta) \subset R_{u,x,v}(\eta)$ such that $\bar{\Omega}_{z,y,v}(\eta) = \mathcal{G}(\Omega_{u,x,v}(\eta))$. As a consequence, equations (32) hold provided $\epsilon_1 < \epsilon_1^{\text{Case 2}}$ and $(u(t_0), x(t_0), v(t_0)) \in \Omega_{u,x,v}(\eta)$ with $\Omega_{u,x,v}(\eta) = \mathcal{G}^{-1}(\bar{\Omega}_{z,y,v}(\eta))$. Define $\alpha^{\text{Case 2}} := \eta$.

Case 3: $\epsilon_2/\epsilon_1 \to \infty$ as $\epsilon_1 \to 0$. In this case, only the change of coordinates $z = Tu + Mx$ is applied to system (1), leading to the system in the new coordinates

$$\begin{aligned} \dot{z} &= g(T^{-1}(z - Mx), t), \\ \epsilon_1 \dot{x} &= \tilde{f}(z, x) + \frac{\epsilon_1}{\epsilon_2} C s(x, v), \\ \epsilon_2 \dot{v} &= D s(x, v) + l(v) + \epsilon_2 h(v, t). \end{aligned} \tag{33}$$

Let $x = \phi_x(z)$ be the locally unique solution to the equation $\tilde{f}(z, x) = 0$ (in which we have that $\phi_x(z) = Q^{-1}[\phi_2(z) - P\phi_1 \circ \phi_2(z)]$) and let $\bar{z}(t), \bar{v}(t, \epsilon_2)$ be the unique solution of the reduced system

$$\begin{aligned} \dot{z} &= g(T^{-1}(z - M\phi_x(z)), t) \\ \epsilon_2 \dot{v} &= D s(\phi_x(z), v) + l(v) + \epsilon_2 h(v, t) \end{aligned} \tag{34}$$

for $t \in [t_0, \bar{t}_f]$ with $\bar{z}(t_0) = z(t_0)$ and $\bar{v}(t_0) = v(t_0)$ (uniqueness of the solution follows from Assumptions A2 and A5). Assumption A9 further guarantees that the boundary layer system is locally exponentially stable. For some $\beta > 0$ sufficiently small, the region of attraction contains the set of x such that $\|x - \phi_x(z(t_0))\| < \beta$. Define the set $R_{z,x}(\beta) := \{(z, x) \in D_z \times D_x \mid \|x - \phi_x(z)\| < \beta\}$. Let $\Omega_{z,x}(\beta)$ be any compact set contained in $R_{z,x}(\beta)$. From Tikhonov theorem, there is $\beta > 0$ such that for all $t_b \in (0, \bar{t}_f]$, there exist $\epsilon_1^{\text{Case 3}} > 0$ such that

$$\begin{aligned} &z(t, \epsilon_1, \epsilon_2) - \bar{z}(t) = O(\epsilon_1) \text{ and} \\ &v(t, \epsilon_1, \epsilon_2) - \bar{v}(t, \epsilon_2) = O(\epsilon_1) \text{ uniformly for } t \in [0, \bar{t}_f] \\ &x(t, \epsilon_1, \epsilon_2) - \phi_x(\bar{z}(t)) = O(\epsilon_1) \text{ uniformly for } t \in [t_b, \bar{t}_f] \end{aligned} \tag{35}$$

provided $\epsilon_1 < \epsilon_1^{\text{Case 3}}$ and $(z(t_0), x(t_0)) \in \Omega_{z,x}(\beta)$. In order to obtain the approximations in the original coordinate system, define $\bar{u} = T^{-1}(\bar{z} - M\phi_x(\bar{z}))$. Since $x = \gamma_x(u)$ is the locally unique solution of $Br(x, u) + f(x, u) = 0$ and $x = \phi_x(z)$ is the locally unique solution of $Br(x, T^{-1}(z - Mx)) + f(x, T^{-1}(z - Mx)) = 0$, we have that $\phi_x(\bar{z}) = \gamma_x(T^{-1}(\bar{z} - M\phi_x(\bar{z}))) = \gamma_x(\bar{u})$. Then, we can write $\bar{z} = T\bar{u} + M\gamma_x(\bar{u})$ and conclude that $\bar{u}(t)$ is the unique solution to system (11) for $t \in [t_0, \bar{t}_f]$ with $\bar{u}(t_0) = T^{-1}[\bar{z}(t_0) - M\phi_x(\bar{z}(t_0))]$. By employing the coordinate change $z = Tu + Mx$ as performed in Case 1, we finally obtain that

$$\begin{aligned} &u(t, \epsilon_1, \epsilon_2) - \bar{u}(t) = O(\epsilon_1) \text{ and} \\ &x(t, \epsilon_1, \epsilon_2) - \gamma_x(\bar{u}(t)) = O(\epsilon_1), \end{aligned} \tag{36}$$

uniformly for $t \in [t_b, \bar{t}_f]$ provided $\epsilon_1 < \epsilon_1^{\text{Case 3}}$ and $(z(t_0), x(t_0)) \in \Omega_{z,x}(\beta)$. Since $R_{z,x}(\beta)$ is the image of $R_{u,x}(\beta)$ under the continuous map $\mathcal{F}$, for any compact set $\Omega_{z,x}(\beta) \subset R_{z,x}(\beta)$, there is a compact set $\Omega_{u,x}(\beta) \subset R_{u,x}(\beta)$ such that $\Omega_{z,x}(\beta) = \mathcal{F}(\Omega_{u,x}(\beta))$. As a consequence, equations (36) hold provided $\epsilon_1 < \epsilon_1^{\text{Case 3}}$ and $(u(t_0), x(t_0)) \in \Omega_{u,x}(\beta)$. By Proposition 3, for all $\beta > 0$ there is $\eta > 0$ such that $(u, x, v) \in R_{u,x,v}(\eta)$ implies $(u, x) \in R_{u,x}(\beta)$. Let $\Omega_{u,x,v}(\eta) \subset R_{u,x,v}(\eta)$ be any compact set. Then, $(u, x, v) \in \Omega_{u,x,v}(\eta)$ implies $(u, x) \in \Omega_{u,x}(\beta)$ for some compact set $\Omega_{u,x}(\beta) \subset R_{u,x}(\beta)$. As a consequence, equations (36) hold provided $(u(t_0), x(t_0), v(t_0)) \in \Omega_{u,x,v}(\eta)$. Let $\alpha^{\text{Case 3}} := \eta$.

By combining Case 1, Case 2, and Case 3, the result of the theorem follows with $\alpha = \min(\alpha^{\text{Case 1}}, \alpha^{\text{Case 2}}, \alpha^{\text{Case 3}})$, $G_1^* = 1/\epsilon_1^*$ with $\epsilon_1^* = \min(\epsilon_1^{\text{Case 1}}, \epsilon_1^{\text{Case 2}}, \epsilon_1^{\text{Case 3}})$, and $G_2^* := 1/(\epsilon_1^* \mu_2^*)$. ■

By combining the results of the lemmas, we can obtain the main result of the paper.

Theorem 1. *Under Properties P1-P2 and Assumptions A1-A9, system Σ has the retroactivity to the output attenuation property.*

Proof: By virtue of Lemma 1, we have that there is $\alpha_1 > 0$ such that for all $t_b \in (t_0, \bar{t}_f]$ there exists $G_1^a > 0$ such that $x_{\text{is}}(t, 1/G_1) - \gamma_x(\bar{u}(t)) = O(\frac{1}{G_1})$ hold uniformly for $t \in [t_b, \bar{t}_f]$ whenever $G_1 > G_1^a$ and $(u_{\text{is}}(t_0), x_{\text{is}}(t_0)) \in \Omega_{u,x}(\alpha_1)$. Similarly, Lemma 2 shows that there is $\alpha_2 > 0$ such that for all $t_b \in (t_0, \bar{t}_f]$ there are $G_1^b > 0$ and $G_2^* > 0$ such that *(i)* and *(ii)* hold uniformly for in $t \in [t_b, \bar{t}_f]$ whenever $G_1 > G_1^b$ and $(u(t_0), x(t_0), v(t_0)) \in \Omega_{u,x,v}(\alpha_2)$. By Proposition 3, for all $\alpha_1 > 0$ there is $\eta > 0$ such that $(u, x, v) \in R_{u,x,v}(\eta)$ implies $(u, x) \in R_{u,x}(\alpha_1)$. Let $\Omega_{u,x,v}(\eta) \subset R_{u,x,v}(\eta)$ be any compact set. Then, $(u, x, v) \in \Omega_{u,x,v}(\eta)$ implies $(u, x) \in \Omega_{u,x}(\alpha_1)$ for some compact set $\Omega_{u,x}(\alpha_1) \subset R_{u,x}(\alpha_1)$. Letting $G_1^* := \max(G_1^a, G_1^b)$ and $\alpha := \min(\eta, \alpha_2)$, we obtain the desired result. ■

Remark 1. *(Retroactivity to the input)* An immediate consequence of Lemma 2 is the quantification of the retroactivity to the input of system Σ, that is, the impact of Σ on the dynamics of the upstream system. Specifically, one can make the retroactivity to the input small by choosing the parameters of Σ in such a way to make $d\gamma_x(\bar{u})/d\bar{u}$ small. Therefore, $d\gamma_x(\bar{u})/d\bar{u}$ can be considered as a measure of the retroactivity to the input of system Σ.

IV. Application Examples

In this section, we show how the interconnection structure of system (1) is found in bio-molecular systems extracted from natural signal transduction pathways and how it can be used to build insulation devices. In particular, we consider as system Σ two post-translational modification systems which are recurrent motifs in signal transduction: phosphorylation cycles and phosphotransfer systems. In both examples, the system output is connected to the downstream system through the binding of transcription factors to DNA. Studies show that this type of interaction can be much faster than, much slower than or of the same order as the post-translational modification processes analyzed here. For example, [35] gives a first-order reaction rate of 40s^{-1} for DNA-protein interaction, while [33], [34] give first-order reaction rates ranging from 10^{-1}s^{-1} to 10^4s^{-1} for phosphorylation cycles and phosphotransfer systems. Therefore, in this application, it is important to show that the retroactivity to the output attenuation property holds when the downstream interconnection dynamics are of the same order as, much faster or much slower than the dynamics of system Σ. Phosphorylation cycles are among the most common intracellular signal transduction mechanisms. They have been observed in virtually every organism, carrying signals that regulate processes such as cell motility, nutrition, interaction with environment and cell death [42]. In this paper, we describe a phosphorylation system extracted from the MAPK cascade [33], similar to the device proposed in [11]. While in [11] the timescales of the downstream interconnection and that of the phosphorylation cycle are the same, here we consider the situation in which the timescale of the downstream interconnection is much faster than that of the phosphorylation cycle reactions. Phosphotransfer systems are also a common motif in cellular signal transduction [43], [44]. These structures are composed of proteins that can phosphorylate each other. By contrast to kinase-mediated phosphorylation, in which the phosphate donor is usually ATP, in phosphotransfer the phosphate group comes from the donor protein itself. Each protein carrying a phosphate group can donate it to the next protein in the system through a reversible reaction. In this paper, we describe a module extracted from the phosphotransferase system [45]. In this example, we consider all the three possible relationships between the timescale of the downstream interconnection and that of the phosphotranfer device.

A. Example 1: Phosphorylation

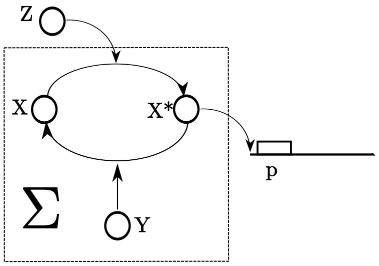

Figure 2. System Σ is a phosphorylation cycle. Its product X* activates transcription through the reversible binding of X* to downstream DNA promoter sites p.

In this section, we analyze the dynamics of a system Σ modeling a phosphorylation cycle as shown in Figure 2. This system takes as input a kinase Z that phosphorylates a protein X. The phosphorylated form of X, denoted X^*, is a transcription factor, which binds to downstream DNA promoter binding sites p. Therefore, the downstream system in terms of Figure 1 is the binding and unbinding process to DNA sites. The phosphorylated protein X^* is converted to the original dephosphorylated form by phosphatase Y. A standard two-step reaction model for the phosphorylation and dephosphorylation reactions is given by $\mathrm{Z} + \mathrm{X} \underset{\beta_2}{\overset{\beta_1}{\rightleftharpoons}} \mathrm{C}_1 \xrightarrow{k_1} \mathrm{X}^* + \mathrm{Z}$ and $\mathrm{Y} + \mathrm{X}^* \underset{\alpha_2}{\overset{\alpha_1}{\rightleftharpoons}} \mathrm{C}_2 \xrightarrow{k_2} \mathrm{X} + \mathrm{Y}$, respectively, in which C_1 and C_2 are the complexes of protein Z with substrate X and of protein Y with protein X^*, respectively [46]. The binding reactions of transcription factor X^* with downstream binding sites p are given by $\mathrm{X}^* + \mathrm{p} \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} \mathrm{C}$, in which C is the complex of X^* bound to site p. In this system, the total amounts of proteins X and Y and the total amount of promoter p are conserved. Their total amounts are denoted X_T, Y_T, and p_T, respectively, so that the conservation laws are given by $X_T = X + X^* + C_1 + C_2 + C$, $Y_T = Y + C_2$, and $p_T = X^* + p$. Assuming Z is expressed at time-varying rate $k(t)$ and decays at rate δ, the differential equations for the concentrations of the various species of system Σ when connected to the downstream system are given by

$$\begin{aligned}
\dot{Z} &= k(t) - \delta Z - \beta_1 Z(X_T - X^* - C_1 - C_2 - C) \\
&\quad + (\beta_2 + k_1)C_1 \\
\dot{C}_1 &= \beta_1 Z(X_T - X^* - C_1 - C_2 - C) - (\beta_2 + k_1)C_1 \\
\dot{C}_2 &= -(k_2 + \alpha_2)C_2 + \alpha_1 X^*(Y_T - C_2) \\
\dot{X}^* &= k_1 C_1 + \alpha_2 C_2 - \alpha_1 X^*(Y_T - C_2) + k_{off} C \\
&\quad - k_{on} X^*(p_T - C) \\
\dot{C} &= -k_{off} C + k_{on} X^*(p_T - C).
\end{aligned} \tag{37}$$

A common approach to take a system to the standard singular perturbation form is to rewrite it in terms of non-dimensional variables [25], [30]. To this end, let $\bar{k} := \max_t k(t)/\delta$ and define the non-dimensional input $\tilde{k}(t) := k(t)/(\delta k)$. Define also the new variables $u := \frac{Z}{k}$, $x_1 := \frac{C_1}{X_T}$, $x_2 := \frac{C_2}{X_T}$, $x_3 := \frac{X^*}{X_T}$, $v := \frac{C}{p_T}$ and $\tau = \delta t$. For a variable x, denote $\dot{x} := dx/d\tau$. The system (37) in these new variables becomes

$$\begin{aligned}
\dot{u} &= \tilde{k}(t) - u - \frac{\beta_1 X_T}{\delta} u \left(1 - x_1 - x_2 - x_3 - \frac{p_T}{X_T} v\right) \\
&\quad + \frac{(\beta_2 + k_1) X_T}{\delta \bar{k}} x_1 \\
\dot{x}_1 &= \frac{\beta_1 \bar{k}}{\delta} u \left(1 - x_1 - x_2 - x_3 - \frac{p_T}{X_T} v\right) - \frac{\beta_2 + k_1}{\delta} x_1 \\
\dot{x}_2 &= -\frac{k_2 + \alpha_2}{\delta} x_2 + \frac{\alpha_1 Y_T}{\delta} x_3 \left(1 - \frac{X_T}{Y_T} x_2\right) \\
\dot{x}_3 &= \frac{k_1}{\delta} x_1 + \frac{\alpha_2}{\delta} x_2 - \frac{\alpha_1 Y_T}{\delta} x_3 \left(1 - \frac{X_T}{Y_T} x_2\right) \\
&\quad + \frac{p_T k_{off}}{X_T \delta} v - \frac{k_{on} p_T}{\delta} x_3 (1 - v) \\
\dot{v} &= -\frac{k_{off}}{\delta} v + \frac{k_{on} X_T}{\delta} x_3 (1 - v).
\end{aligned} \tag{38}$$

In this example, we assume the parameter k_{off} to be much larger than k_1, k_2, $\alpha_1 Y_T$, α_2, $\beta_1 X_T$, β_2, which are in turn much larger than δ [10], [32], [33], [35]. This timescale differences can be made explicit by defining the large parameters $G_1 := \frac{k_1}{\delta}$ and $G_2 := \frac{k_{off}}{\delta}$, in which $G_2 \gg G_1 \gg 1$. Define also the non-dimensional constants $a_1 := \frac{\alpha_1 Y_T}{k_1}$, $a_2 := \frac{\alpha_2}{k_1}$, $b_1 := \frac{\beta_1 X_T}{k_1}$, $b_2 := \frac{\beta_2}{k_1}$, $\rho := \frac{X_T}{Y_T}$ and $c_2 := \frac{k_2}{k_1}$. Define also the dissociation constant $k_d := k_{off}/k_{on}$. By employing these constants, system (38) can be re-written as

$$
\begin{aligned}
\dot{u} &= \tilde{k}(t) - u - G_1 b_1 u \left(1 - x_1 - x_2 - x_3 - \frac{p_T}{X_T} v\right) \\
&\quad + G_1 \frac{X_T(b_2+1)}{\bar{k}} x_1 \\
\dot{x}_1 &= G_1 \frac{b_1 \bar{k}}{X_T} u \left(1 - x_1 - x_2 - x_3 - \frac{p_T}{X_T} v\right) - G_1(b_2+1)x_1 \\
\dot{x}_2 &= -G_1(c_2 + a_2)x_2 + G_1 a_1 x_3 (1 - \rho x_2) \\
\dot{x}_3 &= G_1 x_1 + G_1 a_2 x_2 - G_1 a_1 x_3 (1 - \rho x_2) + G_2 \frac{p_T}{X_T} v \\
&\quad - G_2 \frac{p_T}{k_d} x_3 (1 - v) \\
\dot{v} &= -G_2 v + G_2 \frac{X_T}{k_d} x_3 (1 - v).
\end{aligned}
\tag{39}
$$

The domains for the variables of this system are given by $D_u := \mathbb{R}_+$, $D_x := [0,1] \times [0,1] \times [0,1]$, and $D_v := [0,1]$. Compare system (39) with the structure of model (1). The retroactivity to the input term $r = -b_1 u (1 - x_1 - x_2 - x_3 - (p_T/X_T)v) + (X_T(b_2+1)/\bar{k})x_1$ is a function of the downstream system state v. This implies that the retroactivity to the output of impacts directly the retroactivity to the input. In order to remove this effect, and therefore, match the structure of system (1), in which r does not depend on v, we require the ratio p_T/X_T to be small enough so that the term $(p_T/X_T)v$ becomes negligible with respect to one, since $v \in [0,1]$. This assumption gives a limit to the amount of load that can be added to the system for any fixed value of X_T. Under this assumption, the system fits the structure (1) with $g(u,t) = \tilde{k}(t) - u$, $r(x,u) = b_1 u (1 - x_1 - x_2 - x_3) - \frac{(b_2+1)X_T}{k} x_1$, $[f(x,u) = \begin{bmatrix} 0 \\ -(c_2 + a_2)x_2 + a_1 x_3 (1 - \rho x_2) \\ x_1 + a_2 x_2 - a_1 x_3 (1 - \rho x_2) \end{bmatrix}$, $s(x,v) = -\frac{p_T}{X_T} v + \frac{p_T}{k_d} x_3 (1 - v)$, $l(v) = 0$, $h(v,t) = 0$, $A = -1$, $B = \begin{bmatrix} \bar{k}/X_T & 0 & 0 \end{bmatrix}^T$, $C = \begin{bmatrix} 0 & 0 & -1 \end{bmatrix}^T$ and $D = \frac{X_T}{p_T}$. By inspection of the matrices A, B, C and D, we can choose matrices $T = 1$, $M = [\frac{X_T}{k} \;\; 0 \;\; 0]$, $Q = \mathbb{I}_3$ (3 by 3 identity matrix) and $P = \begin{bmatrix} 0 & 0 & \frac{p_T}{X_T} \end{bmatrix}^T$ that satisfy properties P1 and P2. This can be verified by checking that indeed $TA + MB = 0$, $Mf(x,u) = 0$, $MC = 0$, $QC + PD = 0$ and, trivially, $Pl(v)$=0. The linear coordinate transformation that takes this system to the standard singular perturbation form is, thus, given by $z := Tu + Mx = u + \frac{X_T}{k} x_1$ and $y = (y_1, y_2, y_3) := Qx + Pv = \left(x_1, x_2, x_3 + \frac{p_T}{X_T} v\right)$.

Since we are considering the case in which $G_1 \ll G_2$, it is necessary to show that technical assumptions A1-A7 and A9 are satisfied. For brevity, we show the properties A3, A6 and A7 only. Expression $\bar{h}(y,v) = 0$ leads to $p_T v^2 - v(X_T y_3 + p_T + k_d) + X_T y_3 = 0$ which leads to the unique isolated solution $v = \phi_1(y) = \frac{X_T y_3 + p_T + k_d - \sqrt{(X_T y_3 + p_T + k_d)^2 - 4 p_T X_T y_3}}{2 p_T}$ in the domain $D_v = [0,1]$. This function is Lipschitz continuous as the argument of the square root is bounded away from zero and thus A3 is satisfied. The Jacobian matrix $\frac{\partial \bar{h}(y,v)}{\partial v}$ evaluated at $v = \phi_1(y)$ is given by $\left.\frac{\partial \bar{h}(y,v)}{\partial v}\right|_{v=\phi_1(y)} = -\sqrt{(X_T y_3 + p_T + k_d)^2 - 4 p_T X_T y_3}$, in which the argument of the square root is always bounded away from zero. Therefore, A6 is satisfied. The Jacobian $\frac{\partial \bar{f}}{\partial y}$ gives $\frac{\partial \bar{f}}{\partial y} = \begin{bmatrix} -\tilde{A} & -\tilde{B} & -\eta \tilde{B} \\ 0 & -\tilde{C} & \tilde{D} \\ 1 & -c_2 + \tilde{C} & -\tilde{D} \end{bmatrix}$, in which $\eta = 1 - \frac{p_T}{X_T} \frac{d\phi_1(y)}{dy_3}$, $\tilde{A} = b_2 + 1 + (1 - y_1 - y_2 - y_3 + (p_T/X_T)\phi_1(y)) + (\bar{k}/X_T)z - y_1$, $\tilde{B} = (\bar{k}/X_T)z - y_1$, $\tilde{C} = c_2 + a_2 + a_1 \rho (y_3 - (p_T/X_T)\phi_1(y))$, $\tilde{D} = a_1 \eta (1 - \rho y_2)$. We show that this Jacobian matrix is Hurwitz by employing the Routh-Hurwitz criterion. Note first that $\tilde{A}$, $\tilde{B}$, $\tilde{C}$ and $\tilde{D}$ are all positive terms. The characteristic equation of the Jacobian is given by $\Delta(\lambda) = \lambda^3 + \lambda^2(\tilde{A} + \tilde{C} + \tilde{D}) + \lambda(\tilde{A}\tilde{C} + \tilde{A}\tilde{D} + c\tilde{D} + \eta\tilde{B}) + c\tilde{A}\tilde{D} + \tilde{B}(\eta\tilde{C} + \tilde{D})$. Employing Routh-Hurwitz method, the terms in the first column of the Routh-Hurwitz table are given by $\mu_0 = 1$, $\mu_1 = \tilde{A} + \tilde{C} + \tilde{D}$, $\mu_2 = (\tilde{A} + \tilde{C} + \tilde{D})(\tilde{A}\tilde{C} + \tilde{A}\tilde{D}) + c\tilde{D}(\tilde{C} + \tilde{D}) + \eta\tilde{B}(\tilde{A} + \tilde{D}) - \tilde{B}\tilde{D}$, and $\mu_3 = (c\tilde{A}\tilde{D} + \eta\tilde{B}\tilde{C} + \tilde{B}\tilde{D})$. Provided that X_T is large enough, all the coefficients are positive and, therefore, the real part of all eigenvalues of $\frac{\partial \bar{h}(y,v)}{\partial v}$ is negative and property A7 is satisfied. Similarly, it is possible to show that assumptions A4, A5 and A9 are satisfied.

Figure 3 shows that, for low values of G_1, the system does

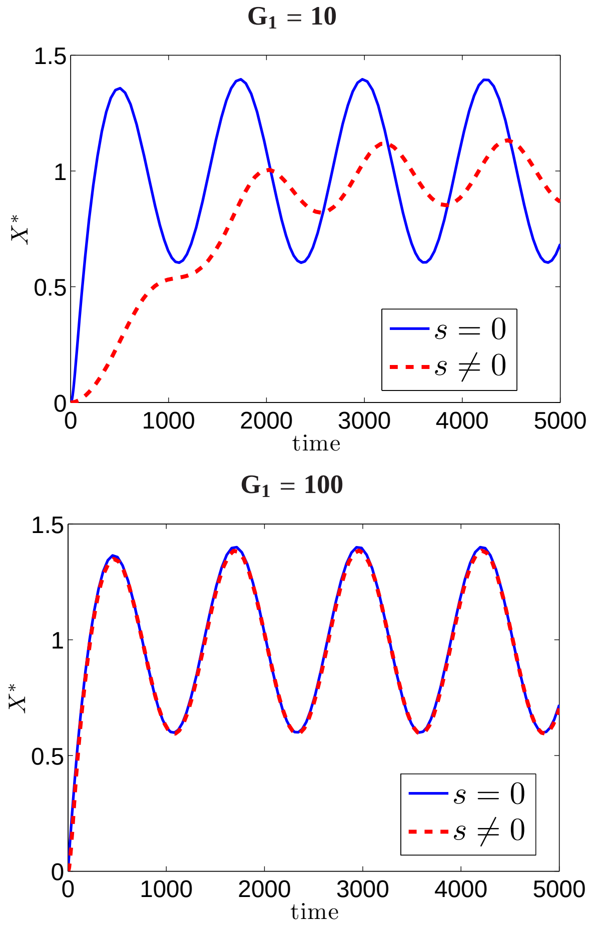

Figure 3. Output response to a sinusoidal signal $k(t) = \delta(1 + 0.5 \sin \omega t)$ of the phosphorylation system Σ. The parameter values are given by $\omega = 0.005$, $\delta = 0.01$, $X_T = 5000$, $Y_T = 5000$, $\alpha_1 = \beta_1 = 2 \times 10^{-6} G_1$, and $\alpha_2 = \beta_2 = k_1 = k_2 = 0.01 G_1$, in which $G_1 = 10$ (left-side panel), and $G_1 = 1000$ (right-side panel). The downstream system parameters are $k_{on} = 100$, $k_{off} = 100$ and, thus, $G_2 = 10000$. Simulations for the connected system ($s \neq 0$) correspond to $p_{TOT} = 100$ while simulations for the isolated system ($s = 0$) correspond to $p_{TOT} = 0$.

not attenuate the retroactivity to the output s as the permanent behavior of the isolated and connected systems are different. By contrast, and in accordance to the theory, large values of G_1 lead to retroactivity to the output attenuation. Note also that this property is achieved even if the gain G_2 multiplying the state-dependent disturbance $s(x, v)$ is much larger than G_1.

In practice, while reactions rates k_1, k_2, α_2 and β_2 are often much larger than δ, constants α_1 and β_1 may not achieve such high values [33]. It is, however, possible to compensate for this and obtain the desired timescale separation by having larger amounts of X_T and Y_T. Large values of X_T and Y_T are also instrumental in removing the direct effect of retroactivity to the ouput on the retroactivity to the input. Finally, large values of X_T and Y_T are also necessary to guarantee the stability of the boundary layer system, as concluded when showing that property A7 holds. In a synthetic bio-molecular system, expression level of proteins X and Y can be tuned by having their respective genes under the control of inducible promoters. It is therefore possible to tune this system so that the retroactivity to the output attenuation property holds.

B. *Example 2: Phosphotransfer*

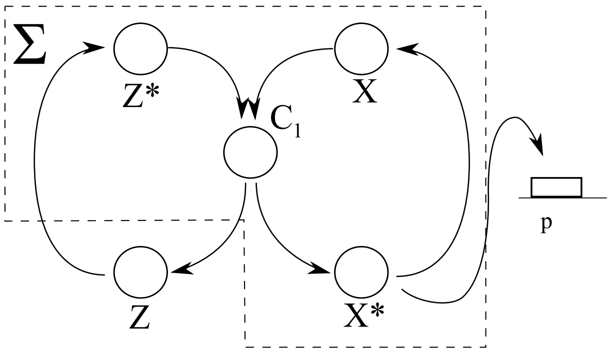

Figure 4. System Σ is a phosphotransfer system. The output X* activates transcription through the reversible binding of X* to downstream DNA promoter sites p.

In this section, we model the phosphotransfer module shown in Figure 4. Let X be a transcription factor in its inactive form and let X* be the same transcription factor once it has been activated by the addition of a phosphate group. Let Z* be a phosphate donor, that is, a protein that can transfer its phosphate group to the acceptor X. The standard phosphotransfer reactions [34] can be modeled according to the two-step reaction model $\mathrm{Z}^* + \mathrm{X} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \mathrm{C}_1 \underset{k_4}{\overset{k_3}{\rightleftharpoons}} \mathrm{X}^* + \mathrm{Z}$, in which C$_1$ is the complex of Z bound to X bound to the phosphate group. Additionally, protein Z can be phosphorylated and protein X* dephosphorylated by other phosphotransfer interactions. These reactions are modeled as one step reactions depending only on the concentrations of Z and X*, that is, $\mathrm{Z} \xrightarrow{\pi_1} \mathrm{Z}^*$, $\mathrm{X}^* \xrightarrow{\pi_2} \mathrm{X}$. Protein X is assumed to be conserved in the system, that is, $X_{TOT} = X + C_1 + X^* + C$. We assume that protein Z is produced with time-varying production rate $k(t)$ and decays with rate δ. The active transcription factor X* binds to downstream DNA binding sites p with total concentration p_{TOT} to activate transcription through the reversible reaction $\mathrm{p} + \mathrm{X}^* \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} \mathrm{C}$. Since the total amount of p is conserved, we also have that $C + p = p_{TOT}$. The ODE model corresponding to this system is thus given by the equations

$$
\begin{aligned}
\dot{Z} &= k(t) - \delta Z + k_3 C_1 - k_4 X^* Z - \pi_1 Z \\
\dot{C}_1 &= k_1 X_T \left(1 - \frac{X^*}{X_T} - \frac{C_1}{X_T} - \frac{C}{X_T}\right) Z^* - k_3 C_1 - k_2 C_1 \\
&\quad + k_4 X^* Z \\
\dot{Z}^* &= \pi_1 Z + k_2 C_1 - k_1 X_T \left(1 - \frac{X^*}{X_T} - \frac{C_1}{X_T} - \frac{C}{X_T}\right) Z^* \\
\dot{X}^* &= k_3 C_1 - k_4 X^* Z - k_{on} X^* (p_T - C) + k_{off} C - \pi_2 X^* \\
\dot{C} &= k_{on} X^* (p_T - C) - k_{off} C.
\end{aligned}
\tag{40}
$$

As performed in Example 1, we introduce non-dimensional variables for this system. Let $\bar{k} := \max_t k(t)/\delta$ and define the non-dimensional input $\tilde{k} := k(t)/(\delta \bar{k})$. Define also the non-dimensional variables $u := \frac{Z}{\bar{k}}$, $x_1 = \frac{C_1}{X_T}$, $x_2 = \frac{Z^*}{\bar{k}}$, $x_3 = \frac{X^*}{X_T}$, $v = \frac{C}{p_T}$ and $\tau := \delta t$. For a variable x, denote $\dot{x} := dx/d\tau$. System (40) in these new variables becomes

$$
\begin{aligned}
\dot{u} &= \tilde{k}(t) - u + \frac{k_3 X_T}{\delta \bar{k}} x_1 - \frac{k_4 X_T}{\delta} x_3 u - \frac{\pi_1}{\delta} u \\
\dot{x}_1 &= \frac{k_1 \bar{k}}{\delta} \left(1 - x_1 - x_3 - \frac{p_T}{X_T} v\right) x_2 - \frac{k_3}{\delta} x_1 - \frac{k_2}{\delta} x_1 \\
&\quad + \frac{k_4 \bar{k}}{\delta} x_3 u \\
\dot{x}_2 &= \frac{\pi_1}{\delta} u + \frac{k_2 X_T}{\delta \bar{k}} x_1 - \frac{k_1 X_T}{\delta} \left(1 - x_1 - x_3 - \frac{p_T}{X_T} v\right) x_2 \\
\dot{x}_3 &= \frac{k_3}{\delta} x_1 - \frac{k_4 \bar{k}}{\delta} x_3 u - \frac{k_{on} p_T}{\delta} x_3 (1 - v) + \frac{k_{off} p_T}{\delta X_T} v \\
&\quad - \frac{\pi_2}{\delta} x_3 \\
\dot{v} &= \frac{X_T k_{on}}{\delta} x_3 (1 - v) - \frac{k_{off}}{\delta} v.
\end{aligned}
\tag{41}
$$

Phosphotranferase reactions are much faster than gene expression and protein decay rates [34]. To make this timescale separation explicit, we define the large parameter $G_1 := \frac{k_2}{\delta} \gg 1$ and define the non-dimensional constants $\bar{k}_1 := \frac{k_1 X_T}{k_2}$, $\bar{k}_3 := \frac{k_3}{k_2}$, $\bar{k}_4 := \frac{k_4 X_T}{k_2}$, $\bar{\pi}_1 := \frac{\pi_1}{k_2}$ and $\bar{\pi}_2 := \frac{\pi_2}{k_2}$. The fact that the process of protein binding and unbinding to promoter sites is much faster than protein production and decay [10], [32] is made explicit by the ratio $G_2 := \frac{k_{off}}{\delta} \gg 1$. In this example we do not make any assumption on the relationship between G_1 and G_2. Let also the dissociation constant be $k_d := k_{off}/k_{on}$. By using these constants, system (41) can be written as

$$\begin{aligned}
\dot{u} &= \tilde{k}(t) - u + G_1\frac{\bar{k}_3 X_T}{\bar{k}}x_1 - G_1\bar{k}_4 x_3 u - G_1\bar{\pi}_1 u\\
\dot{x}_1 &= G_1\frac{\bar{k}_1\bar{k}}{X_T}\left(1 - x_1 - x_3 - \frac{p_T}{X_T}v\right)x_2 - G_1\bar{k}_3 x_1 - G_1 x_1\\
&\quad + G_1\frac{\bar{k}_4\bar{k}}{X_T}x_3 u\\
\dot{x}_2 &= G_1\bar{\pi}_1 u + G_1\frac{X_T}{\bar{k}}x_1 - G_1\bar{k}_1\left(1 - x_1 - x_3 - \frac{p_T}{X_T}v\right)x_2 \qquad (42)\\
\dot{x}_3 &= G_1\bar{k}_3 x_1 - G_1\frac{\bar{k}_4\bar{k}}{X_T}x_3 u - G_1\bar{\pi}_2 x_3 - G_2\frac{p_T}{k_d}x_3(1-v)\\
&\quad + G_2\frac{p_T}{X_T}v\\
\dot{v} &= G_2\frac{X_T}{k_d}x_3(1-v) - G_2 v.
\end{aligned}$$

The domain for the states of this system are given by $D_z = \mathbb{R}_+$, $D_x = [0,1]\times\mathbb{R}_+\times[0,1]$ and $D_v = [0,1]$. Compare system (42) with system (1). In system (42), the internal dynamics term is given by $f = \begin{bmatrix} \frac{\bar{k}_1\bar{k}}{X_T}\left(1 - x_1 - x_3 - \frac{p_T}{X_T}v\right)x_2 - x_1\\ \frac{X_T}{\bar{k}}x_1 - \bar{k}_1\left(1 - x_1 - x_3 - \frac{p_T}{X_T}v\right)x_2\\ \bar{k}_3 x_1 - \frac{\bar{k}_4\bar{k}}{X_T}u x_3 - \bar{\pi}_2 x_3 \end{bmatrix}$ and it depends on output term v. Therefore, in order for system (42) to fit the structure of system (1), we require that the ratio p_T/X_T to be small enough so that $(p_T/X_T)v$ becomes negligible with respect to 1 in the term $(1-x_1-x_3-(p_T/X_T)v)$, as $v\in[0,1]$. This assumption, in practice, limits the amount of load this insulation device can accommodate for a given amount of X_T. Under this assumption, system (42) fits the structure of model (1) with $g(u,t) = \tilde{k}(t) - u$, $r(x,u) = \begin{bmatrix} \frac{\bar{k}_3 X_T}{\bar{k}}x_1 - \bar{k}_4 x_3 u\\ -\bar{\pi}_1 u \end{bmatrix}$, $f(x,u)\begin{bmatrix} \frac{\bar{k}_1\bar{k}}{X_T}(1 - x_1 - x_3)x_2 - x_1\\ \frac{X_T}{\bar{k}}x_1 - \bar{k}_1(1 - x_1 - x_3)x_2\\ \bar{k}_3 x_1 - \frac{\bar{k}_4\bar{k}}{X_T}u x_3 - \bar{\pi}_2 x_3 \end{bmatrix}$, $s(x,v) = -\frac{p_T}{k_d}x_3(1-v) + \frac{p_T}{X_T}v$, $l(v) = 0$, $h(v,t) = 0$, $A := [1\ \ 1]$, $B = \begin{bmatrix} -\frac{\bar{k}}{X_T} & 0\\ 0 & -1\\ 0 & 0 \end{bmatrix}$, $C = \begin{bmatrix} 0\\ 0\\ 1 \end{bmatrix}$ and $D = -\frac{X_T}{p_T}$. By inspecting matrices A, B, C and D it is possible to choose matrices $T = 1$, $M = \left[\frac{X_T}{\bar{k}}\ 1\ 0\right]$, $Q = \mathbb{I}_{3\times3}$ and $P = \begin{bmatrix} 0 & 0 & \frac{p_T}{X_T} \end{bmatrix}^T$, which satisfy properties P1 and P2. This can be verified by checking that indeed $TA + MB = 0$, $Mf(x,u) = 0$, $MC = 0$, $QC + PD = 0$ and, trivially, $Pl(v) = 0$. By applying the linear coordinate transformation given by $z = Tu + Mx$ and $y = Qx + Pv$, we obtain the system

$$\begin{aligned}
\dot{z} &= k(t) - \left(z - \frac{X_T}{\bar{k}}y_1 - y_2\right)\\
\epsilon_1\dot{y}_1 &= \frac{\bar{k}_1\bar{k}}{X_T}\left(1 - y_1 - y_3 + \frac{p_T}{X_T}v\right)y_2 - \bar{k}_3 y_1 - y_1\\
&\quad + \frac{\bar{k}_4\bar{k}}{X_T}\left(y_3 - \frac{p_T}{X_T}v\right)\left(z - \frac{X_T}{\bar{k}}y_1 - y_2\right)\\
\epsilon_1\dot{y}_2 &= \bar{\pi}_1\left(z - \frac{p_T}{X_T}y_1 - y_2\right) + y_1 - \bar{k}_1\left(1 - y_1 - y_3 + \frac{p_T}{X_T}v\right)y_2\\
\epsilon_1\dot{y}_3 &= \bar{k}_3 y_1 - \frac{\bar{k}_4\bar{k}}{X_T}\left(y_3 - \frac{p_T}{X_T}v\right)\left(z - \frac{X_T}{\bar{k}}y_1 - y_2\right)\\
&\quad - \bar{\pi}_2\left(y_3 - \frac{p_T}{X_T}v\right)\\
\epsilon_2\dot{v} &= \frac{X_T}{k_d}\left(y_3 - \frac{p_T}{X_T}v\right)(1-v) - v.
\end{aligned} \qquad (43)$$

In this example, we do not claim any relationship between G_1 and G_2. In this the situation it is necessary to show that all assumptions A1-A9 are satisfied to prove that the retroactivity to the output property holds. For brevity we restrict to show that assumptions A3, A6 and A7 hold.

As in the phosphorylation system, we have that $\bar{h}(y,v) = \frac{1}{k_d}(X_T y_3 - p_T v) - v$. Therefore, A3 and A6 are satisfied as it was for the phosphorylation system.

Since the function $\phi_1(y) = \frac{y_3+p_T+k_d-\sqrt{(y_3+p_T+k_d)^2-4p_T y_3}}{2}$ is sufficiently smooth (the argument of the square root is bounded away from zero) we define the diffeomorphism $w := \Psi(y) = \begin{bmatrix} y_1 & y_2 & y_3 - \phi_1(y) \end{bmatrix}^T$. Define $\hat{f}(z,w) := \bar{f}(z,y,\phi_1(y))\big|_{y=\Psi^{-1}(w)}$. Since under a diffeomorphism the linearization of a nonlinear system is invariant [47], it is sufficient to show that $\frac{\partial\hat{f}(z,w)}{\partial w}\Big|_{w=\Psi(\phi_2(z))}$ is Hurwitz. We have that $\frac{\partial\hat{f}(z,w)}{\partial w} = \begin{bmatrix} -\tilde{E} - \rho\tilde{F} & \rho\tilde{B} - \tilde{A} & \tilde{D} - \tilde{C}\\ -\frac{\bar{\pi}_1}{\rho} + \tilde{F} & -\bar{\pi}_1 - \tilde{B} & \frac{\tilde{C}}{\rho}\\ \tilde{E} & \tilde{A} & -\tilde{D} - \bar{\pi}_2 \end{bmatrix}$, in which $\rho = \frac{\bar{k}}{X_T}$, $\tilde{A} = \bar{k}_4\bar{k}w_3$, $\tilde{B} = X_T(1-w_1-w_3)$, $\tilde{C} = \bar{k}w_2$, $\tilde{D} = \bar{k}_4\bar{k}(z-\frac{w_1}{\rho}-w_2)$, $\tilde{E} = \bar{k}_3 + \frac{\tilde{A}}{\rho}$ and $\tilde{F} = \frac{\bar{k}_2}{\rho} + \frac{\tilde{C}}{\rho}$. The characteristic equation of this Jacobian is given by $\Delta(\lambda) = \lambda^3 + \lambda^2(\tilde{E} + \rho\tilde{F} + \bar{\pi}_1 + \bar{\pi}_2 + \tilde{B} + \tilde{D}) + \lambda(\bar{\pi}_1\bar{\pi}_2 + \tilde{A}\bar{k}_2/\rho + \bar{\pi}_1\bar{k}_3 + \bar{\pi}_1\tilde{D} + \bar{\pi}_2\tilde{B} + \tilde{B}\tilde{D} + \rho\bar{\pi}_1\tilde{F} + \tilde{B}\tilde{E} + \rho\tilde{D}\tilde{F} + \bar{\pi}_1\tilde{B} + \tilde{E}\tilde{C} + \bar{\pi}_2\tilde{E} + \rho\bar{\pi}_2\tilde{F}) + \bar{\pi}_1\bar{\pi}_2\bar{k}_3 + \bar{\pi}_1\tilde{C}\bar{k}_3 + \rho\bar{\pi}_1\bar{\pi}_2\tilde{F} + \rho\bar{\pi}_1\tilde{D}\tilde{F} + \bar{\pi}_2\tilde{B}\tilde{E} + \bar{\pi}_1\bar{\pi}_2\tilde{B} + \bar{\pi}_1\tilde{B}\tilde{D} + \bar{\pi}_2\tilde{A}\tilde{F}$. Write the characteristic equation as $\Delta(\lambda) = \lambda^3 + \alpha_2\lambda^2 + \alpha_1\lambda + \alpha_0$ where α_i are implicitly defined. The terms on the first column of the Routh-Hurwitz table are given by 1, α_2, $(\alpha_1\alpha_2 - \alpha_0)/\alpha_2$ and α_0. Since all α_i are positive, we are guaranteed to have only positive terms on the first column of the Routh-Hurwitz table if $\alpha_2\alpha_1 - \alpha_0 > 0$. In particular, the term $\alpha_2\alpha_1 - \alpha_0$ can be reduced to $\alpha_2\alpha_1 - \alpha_0 = \mu + \bar{\pi}_1\bar{k}_2\bar{k}_4\bar{k}\left(z - \frac{w_1}{\rho} - w_2\right) - \frac{\bar{\pi}_2\bar{k}_2\bar{k}_4 w_3}{\rho}$, in which the term $\mu > 0$. It remains to show that $\bar{k}_2\bar{k}_4[\bar{\pi}_1(z - w_1/\rho - w_2) - \bar{\pi}_2 w_3/\rho] \geq 0$ on the manifold $w = \Psi(\phi_2(z))$. From the system of equations $\bar{f}(x,y,\phi_1(y)) = 0$, one can obtain the identity $\bar{\pi}_2(y_3 - p_T\phi_1(y)/X_T) = \rho\bar{\pi}_1(z - y_1/\rho - y_2)$. Substituting $y = \Psi^{-1}(w)$ in this identity, we obtain that $\bar{\pi}_2 w_3 - \bar{\pi}_1(z - w_1/\rho - w_2) = 0$. As a result, $\alpha_2\alpha_1 - \alpha_0 = \mu > 0$ and thus, the Jacobian matrix $\frac{\partial\hat{f}(z,w)}{\partial w}\Big|_{w=\Psi(\phi_2(z))}$ is Hurwitz satisfying

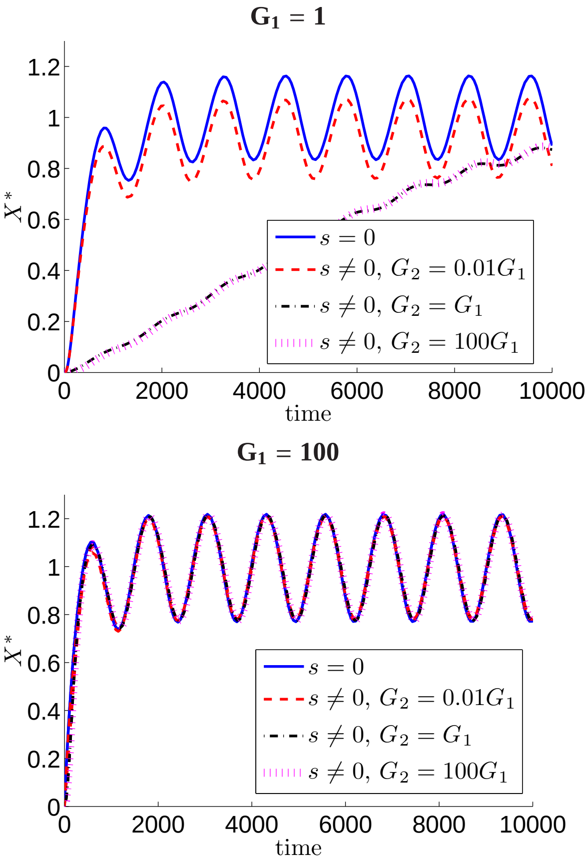

Figure 5. Output response of the phosphotransfer system with a periodic signal $k(t) = \delta(1{+}0.5 sin\omega t)$. The parameters are given by $\delta = 0.01$, $X_T = 5000$, $k_1 = k_2 = k_3 = k_4 = \pi_1 = \pi_2 = 0.01G_1$ in which $G_1 = 1$ (left-side panel), and $G_1 = 100$ (right-side panel). The downstream system parameters are given by $k_d = 1$ and $k_{off} = 0.01G_2$, in which G_2 assumes the values indicated on the legend. The isolated system ($s = 0$) corresponds to $p_{TOT} = 0$ while the connected system ($s \neq 0$) corresponds to $p_{TOT} = 100$.

condition A7.

We illustrate the retroactivity to the output attenuation property of this system using simulations for the cases in which $G_1 \gg G_2$, $G_1 = G_2$, and $G_1 \ll G_2$. Figure 5 shows that, for a periodic input $k(t)$, the system with low value for G_1 suffers the impact of retroactivity to the output. However, for a large value of G_1, the permanent behavior of the connected system becomes similar to that of the isolated system, whether $G_1 \gg G_2$, $G_1 = G_2$ or $G_1 \ll G_2$. Notice that, in the bottom panel of Figure 5, when $G_1 \gg G_2$, the impact of the retroactivity to the output is not as dramatic as it is when $G_1 = G_2$ or $G_1 \ll G_2$. This is due to the fact that s is scaled by G_2 and it is not related to the retroactivity to the output attenuation property. This confirms the theoretical result that, independently of the order of magnitude of G_2, the system can arbitrarily attenuate retroactivity for large enough G_1.

V. CONCLUSIONS

In this paper, we have proposed a mechanism for attenuating the retroactivity to the output of a bio-molecular system which is based on timescale separation. A special structure found in bio-molecular systems allows the attenuation of state dependent disturbances that enter the dynamics through arbitrarily large gains. This attenuation can be achieved even when the internal system gains are several orders of magnitude smaller than the gains that multiply the disturbance. One structural assumption at the basis of our result is that the retroactivity to the input r of the system and the vector field f do not explicitly depend on the variables v of the downstream system. In future work, we will investigate how the retroactivity to the output attenuation property may be relaxed when both the retroactivity r and the function f depend on v.

We illustrated this mechanism by presenting two instances of bio-molecular systems that have the capability of attenuating the retroactivity to the output based on timescale separation. These are a phosphorylation cycle and a phosphotransfer system, which are ubiquitous in natural signal transduction systems. Our finding suggests that a reason why these systems are fundamental building blocks of natural signal transduction systems is that, in addition to their well recognized signal amplification capability, they can attenuate retroactivity to the output and therefore enforce unidirectional signal propagation. This property is certainly desirable in any signal transmission system, natural or engineered. More interestingly, this finding suggests that phosphorylation and phosphotransfer systems can be employed in synthetic bio-molecular circuits to attenuate retroactivity and to thus allow modular interconnection of synthetic circuit components.

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