# An $N$-stage Cascade of Phosphorylation Cycles as an Insulation Device for Synthetic Biological Circuits*

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***Abstract*— Single phosphorylation cycles have been found to have insulation device abilities, that is, they attenuate the effect of retroactivity applied by downstream systems and hence facilitate modular design in synthetic biology. It was recently discovered that this retroactivity attenuation property comes at the expense of an increased retroactivity to the input of the insulation device, wherein the device slows down the signal it receives from its upstream system. In this paper, we demonstrate that insulation devices built of cascaded phosphorylation cycles can break this tradeoff allowing to attenuate the retroactivity applied by downstream systems while keeping a small retroactivity to the input. In particular, we show that there is an optimal number of cycles that maximally extends the linear operating region of the insulation device while keeping the desired retroactivity properties, when a common phosphatase is used. These findings provide optimal design strategies of insulation devices for synthetic biology applications.**

## I. INTRODUCTION

A multitude of functional units have been developed in synthetic biology: genetic switches [1], oscillators [2] and digital gates [3]. The aim of synthetic biology is to connect these different functional units to design larger circuits for various applications [4], [5]. One of the problems faced when connecting such units is that of retroactivity [6]. Retroactivity is the change in dynamics in the upstream system due to the interconnection of a downstream system. When two units are interconnected, predicting the behaviour of the system is made easy by a property called modularity, i.e., when the properties of the individual units do not change on connection. However, the effect of retroactivity interferes with this property. This introduces the need for insulation: a way to connect these units such that the effect of retroactivity is negligible. Functional units that attenuate the effects of retroactivity are called insulation devices [6].

A single phosphorylation-dephosphorylation (PD) cycle has been theoretically [6] and experimentally [7], [9] shown to behave as an insulation device due to a high-gain feedback mechanism. In these works, the total substrate and phosphatase concentration of the cycle is increased to attenuate the effect of retroactivity on the output due to the presence of load. The output is thus made independent of the presence of load, however, such a device slows down the dynamics of the input. This tradeoff was theoretically characterized in [8] and experimentally verified using a NRI-NRI* PD cycle [9]. The results of [10] suggest that this tradeoff may be overcome by using multiple stages of PD cycles. In [11], a cascade of PD cycles are analyzed for the propogation of downstream disturbances to the input, and sufficient conditions for attenuating these disturbances are provided. This motivates the current work, which analyzes the insulation properties of an $N$-stage cascade of PD cycles with a common phosphatase. We find that the tradeoff present in a single PD cycle is overcome by cascading two cycles. Furthermore, increasing the number of cycles $N$ up to an optimal $\bar{N}$ increases the linear operating region of the insulation device. Thus, based on the total amount of load, the Michaelis-Menten constants of the cycles and the operating range of the input, the cascade can be designed to be an insulation device for various applications in synthetic biology.

This paper is organized as follows. The next section formally defines retroactivity and insulation, and provides a mathematical framework to analyze the cascade of PD cycles. Section III describes a model of the system based on the reaction rate ODEs of the system. Section IV states and proves the mathematical result for designing the insulation device based on the model. Section V discusses the implications of this result and verifies these implications based on simulations.

## II. RETROACTIVITY AND INSULATION

As introduced in the previous section, retroactivity is the change in dynamics in the upstream system due to its interconnection with a downstream system. For example, consider the behaviour of a simple module with an activator Z, which activates the production of a transcriptional component X, shown in Fig. 1a. Throughout this paper, species are referred to in Times New Roman, such as X and Z, and their concentrations are referred to in the corresponding italics, such as $X$ and $Z$. For this system, then, $Z$ acts as a periodic input, and $X$ is the output. The response of $X$ when the downstream system is not present is shown by the black plot in Fig. 1b. However, when X is used to activate the downstream system, its response to the same input $Z$ changes dramatically, as shown by the dashed red plot in Fig. 1b. This loading phenomenon has been experimentally shown both *in vivo* and *in vitro* in bacteria and yeast [12], [7], [10].

Fig. 2 shows a system **S** that formally captures this loading effect through retroactivity signals [6], [12], [7]. The state of **S** is described by $x$, the input by $u$, which ranges from $u_{\text{min}}$ to $u_{\text{max}}$, i.e., $u \in [u_{\text{min}}, u_{\text{max}}]$ and the output by $y$. The

*This work is supported by the NIH P50 GMO98792 grant.

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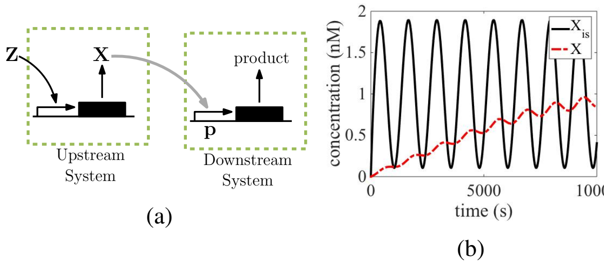


Fig. 1: (a) The upstream system produces a gene product, the protein X; when the upstream system is connected with the downstream system, X acts as a transcription factor for downstream promoter sites p (b) The response of $X$ to a periodic input $Z$ is shown when the upstream system is not connected to the downstream system in black; the red dotted graph shows the response of $X$ when it is connected to a downstream system.

retroactivity to the input is $r(u,x)$ and the retroactivity to the output is $s(x,v)$. We define the ideal input, $u_{\text{ideal}}$, as the input received from the upstream system when nothing is connected to it downstream, i.e., $u_{\text{ideal}} = u$ when $r = 0$. The ideal output, $y_{is}$, is the output of **S** when it has no downstream load, i.e., $y_{is} = y$ when $s = 0$.

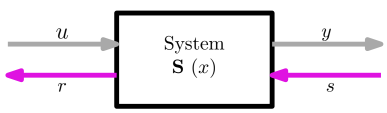


Fig. 2: A system with state $x$, input $u$ and output $y$, with retroactivity to the input $r$ and retroactivity to the output $s$.

Retroactivity effects make it difficult to design interconnected systems. The problem of retroactivity can be solved by an intermediate module, connected between the upstream and downstream systems to act as an insulation device, as shown in Fig. 3.

*Definition 1:* (Adapted from [13]) System **S** is called an insulation device when it satisfies the following properties:

(i) Small retroactivity to the input $r$: here, the effect of $r$ is characterized by the change in the dynamics of the input due to $r$, i.e., $|\dot{u}_{\text{ideal}}(t) - \dot{u}(t)| \ll 1$.

(ii) Attenuation of retroactivity to output $s$: the effect of $s$ on $x$, the state, and therefore $y$, the output, is attenuated, i.e., $|y_{is}(t) - y(t)| \ll 1$.

(iii) Linearity: the input-output response is approximately linear for $u \in [u_{\text{min}}, u_{\text{max}}]$ with gain $G = 1$, i.e., $|u(t) - y_{is}(t)| \ll 1$.

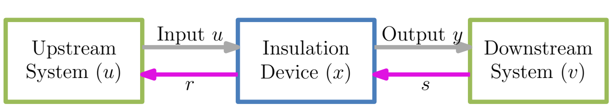


Fig. 3: Insulation device connected between two systems: (i) minimizes $r$, (ii) attenuates the effect of $s$ on $x$, and (iii) shows a linear relationship between $u$ and $y$.

Referring to Fig. 3, the model for the system is:

$$\begin{aligned}\dot{u} &= f_0(u,t) + G_1 Ar(u,x),\\ \dot{x} &= G_1 Br(u,x) + G_1 f_1(u,x,\eta v) + Cs(x,v),\\ \dot{v} &= Ds(x,v).\end{aligned} \quad (1)$$

Here, the variables $t \in [t_i, t_f]$, $x \in \mathcal{D}_x \subset \mathbb{R}^n_+$, $u \in [u_{\text{min}}, u_{\text{max}}] \subset \mathbb{R}_+$, $y \in \mathcal{D}_y \subset \mathbb{R}_+$, $v \in \mathcal{D}_v \subset \mathbb{R}_+$, $r(u,x) \in \mathbb{R}_+$, $s(x,v) \in \mathbb{R}_+$. The matrices $A \in \mathbb{R}^{1\times 1}$, $B \in \mathbb{R}^{n\times 1}$, $C \in \mathbb{R}^{n\times 1}$ and $D \in \mathbb{R}^{1\times 1}$.

The positive scalar $G_1$ depends on parameters of the insulation device, and $\eta$ is a constant that depends on parameters of the downstream system and the insulation device.

*Assumption 1:* $G_1 \gg 1$ and eigenvalues of $\frac{\partial(Br+f_1)}{\partial x}$ have negative real parts.

*Assumption 2:* There exist invertible matrices $T$ and $P$, and matrices $Q$ and $M$, such that $TA + MB = 0$, $Mf_1 = 0$, $QC + PD = 0$ and $MC = 0$.

For this system, we state the following Theorem, adapted from [14].

*Theorem 1: For system (1), under Assumptions 1 and 2, $||x(t) - \gamma(u(t), \eta v(t))|| = \mathcal{O}(\frac{1}{G_1})$, for $t \in [t_b, t_f]$, where $x = \gamma(u, \eta v)$ is the solution to $f_1(u,x,\eta v) + Br(u,x) = 0$ and $t_b$ is such that $t_i < t_b < t_f$ and $t_b - t_i$ decreases as $G_1$ increases.*

*Corollary 1: If $f_0(u,t) + G_1 Ar(u,x)$ is Lipschitz continuous in $x$, then under Assumptions 1 and 2, $||\dot{u}(u(t), x(t), t) - \dot{u}(u(t), \gamma(u(t), \eta v(t)), t)|| = \mathcal{O}(\frac{1}{G_1})$, for $t \in [t_b, t_f]$.*

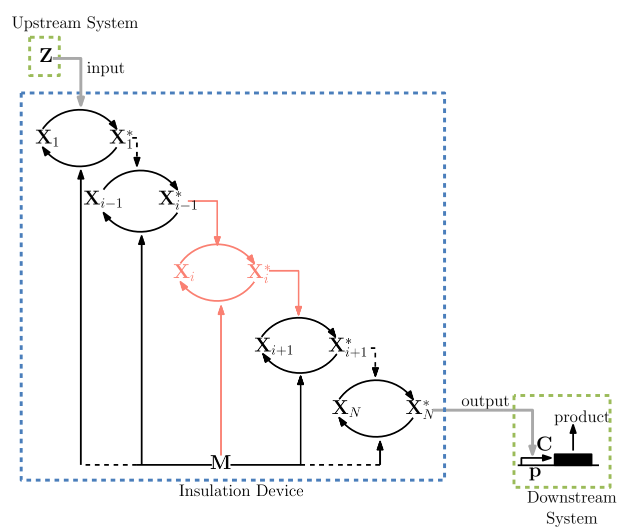


Fig. 4: The $i^{\text{th}}$ cycle is highlighted in a cascade of $N$ cycles that together act as an insulation device; for the $i^{\text{th}}$ cycle, $X_i$ is phosphorylated by $X^*_{i-1}$ to produce $X^*_i$, which is the kinase for the $(i+1)^{\text{th}}$ cycle; M is the common phosphatase for all cycles; for $i = 1$ the kinase is the input Z; for $i = N$ the phosphorylated product $X^*_N$ is the output of the insulation device, which is the transcription factor for downstream promoters.

The next section describes the system model for an $N$-stage cascade of PD cycles. The section after that uses the framework described by Theorem 1 to analyze this system.

## III. SYSTEM MODEL

We consider a cascade of $N$ PD cycles, shown in Fig. 4. We denote the substrate of each cycle by $X_i$ and the phosphorylated product as $X_i^*$, where $i$ is the number of the cycle in the cascade. The input to this device is Z, the kinase of the 1st cycle. The output of this device is $X_N^*$, the phosphorylated protein of the $N^{\text{th}}$ cycle, which acts as a transcription factor for a number of downstream sites. The phosphorylated protein of each cycle but the last is the kinase for the next cycle, i.e., $X_{i-1}^*$ is the kinase that phosphorylates $X_i$ to form $X_i^*$, for $2 \le i \le N$. For simplicity, we sometimes denote Z by $X_0^*$, since it is the kinase for the first cycle. The common phosphatase for each cycle is M, which dephosphorylates $X_i^*$ to $X_i$ for all $i$. The input signal $u$ to the insulation device is concentration $Z$ and the output signal $y$ is concentration $X_N^*$. We define $Z_{\text{ideal}}$ as the input when no downstream cascade is connected to it and $X_{N,is}^* = X_N^*$ when there are no downstream sites.

The kinase Z is assumed to be the only molecule to undergo degradation, due to attached degradation tags. Complexes that the kinase forms with other molecules, as well as the substrate and the phosphorylated protein are assumed to not undergo degradation, and are only removed from the system by dilution. Dilution rates for non-degrading compounds are governed by the cell growth rate, typically measured in hour$^{-1}$ [15], which is much smaller than PD rates, typically measured in second$^{-1}$ [16]. Dilution can therefore be neglected compared to PD. Apart from Z, the other species in the system are conserved. The total substrate concentration of each cycle is denoted by $X_{Ti}$ and the total phosphatase concentration is denoted by $M_T$. The number of downstream sites are $p_T$ (load).

The two-step reactions for the cascade are shown below. The reactions involving species of the first cycle are given by:

$$\phi \underset{\delta}{\overset{k(t)}{\rightleftharpoons}} Z, \quad X_1 + Z \underset{\alpha_{21}}{\overset{\alpha_{11}}{\rightleftharpoons}} C_{11} \xrightarrow{k_{11}} X_1^* + Z,$$
$$X_1^* + M \underset{\beta_{21}}{\overset{\beta_{11}}{\rightleftharpoons}} C_{21} \xrightarrow{k_{21}} X_1 + M,$$
$$X_1^* + X_2 \underset{\alpha_{22}}{\overset{\alpha_{12}}{\rightleftharpoons}} C_{12} \xrightarrow{k_{12}} X_1^* + X_2^*.$$

The reactions involving species of the $i^{\text{th}}$ cycle, for $i \in [2, N-1]$, are given by:

$$X_i + X_{i-1}^* \underset{\alpha_{2i}}{\overset{\alpha_{1i}}{\rightleftharpoons}} C_{1i} \xrightarrow{k_{1i}} X_i^* + X_{i-1}^*,$$
$$X_i^* + M \underset{\beta_{2i}}{\overset{\beta_{1i}}{\rightleftharpoons}} C_{2i} \xrightarrow{k_{2i}} X_i + M,$$
$$X_i^* + X_{i+1} \underset{\alpha_{2_{i+1}}}{\overset{\alpha_{1_{i+1}}}{\rightleftharpoons}} C_{1_{i+1}} \xrightarrow{k_{1_{i+1}}} X_i^* + X_{i+1}^*.$$

And those for the final cycle are given by:

$$X_N + X_{N-1}^* \underset{\alpha_{2N}}{\overset{\alpha_{1N}}{\rightleftharpoons}} C_{1N} \xrightarrow{k_{1N}} X_N^* + X_{N-1}^*,$$
$$X_N^* + M \underset{\beta_{2N}}{\overset{\beta_{1N}}{\rightleftharpoons}} C_{2N} \xrightarrow{k_{2N}} X_N + M,$$
$$X_N^* + p \underset{k_{\text{off}}}{\overset{k_{\text{on}}}{\rightleftharpoons}} C.$$

The conservation laws for the system are:

$$X_{Ti} = X_i + C_{1i} + X_i^* + C_{2i} + C_{1_{i+1}}, \quad \text{for } i \in [1, N-1],$$
$$X_{TN} = X_N + C_{1N} + X_N^* + C_{2N} + C, \quad M_T = M + \sum_{i=1}^{N} C_{2i}.$$

The Michaelis-Menten constants for the system are:

$$K_{m1i} = \tfrac{\alpha_{2i} + k_{1i}}{\alpha_{1i}}, \quad K_{m2i} = \tfrac{\beta_{2i} + k_{2i}}{\beta_{1i}}.$$

The reaction rate equations for the system are then given below, for time $t \in [t_i, t_f]$. For the first cycle,

$$\dot{Z} = k(t) - \delta Z \underbrace{-\alpha_{11}(X_{T1} - C_{11} - X_1^* - C_{21} - C_{12})Z + (\alpha_{21} + k_{11})C_{11}}_{r}, \tag{2}$$

$$\dot{C}_{11} = \alpha_{11}(X_{T1} - C_{11} - X_1^* - C_{21} - C_{12})Z - (\alpha_{21} + k_{11})C_{11}, \tag{3}$$

$$\dot{C}_{21} = \beta_{11}X_1^*(M_T - \sum_{i=1}^{N} C_{2i}) - (\beta_{21} + k_{21})C_{21}, \tag{4}$$

$$\dot{X}_1^* = k_{11}C_{11} - \beta_{11}X_1^*(M_T - \sum_{i=1}^{N} C_{2i}) + \beta_{21}C_{21} - \alpha_{12}X_1^*(X_{T2} - C_{12} - X_2^* - C_{22} - C_{13}) + (\alpha_{22} + k_{12})C_{12}. \tag{5}$$

For the $i^{\text{th}}$ cycle, where $i \in [2, N-1]$:

$$\dot{C}_{1i} = \alpha_{1i}(X_{Ti} - C_{1i} - X_i^* - C_{2i} - C_{1_{i+1}})X_{i-1}^* - (\alpha_{2i} + k_{1i})C_{1i}, \tag{6}$$

$$\dot{C}_{2i} = \beta_{1i}X_i^*(M_T - \sum_{i=1}^{N} C_{2i}) - (\beta_{2i} + k_{2i})C_{2i}, \tag{7}$$

$$\dot{X}_i^* = k_{1i}C_{1i} - \beta_{1i}X_i^*(M_T - \sum_{i=1}^{N} C_{2i}) + \beta_{2i}C_{2i} - \alpha_{1_{i+1}}X_i^*(X_{T_{i+1}} - C_{1_{i+1}} - X_{i+1}^* - C_{2_{i+1}} - C_{1_{i+2}}) + (\alpha_{2_{i+1}} + k_{1_{i+1}})C_{1_{i+1}}. \tag{8}$$

For the last, $N^{\text{th}}$, cycle:

$$\dot{C}_{1N} = \alpha_{1N}(X_{TN} - C_{1N} - X_N^* - C_{2N} - C)X_{N-1}^* - (\alpha_{2N} + k_{1N})C_{1N}, \tag{9}$$

$$\dot{C}_{2N} = \beta_{1N}X_N^*(M_T - \sum_{i=1}^{N} C_{2i}) - (\beta_{2N} + k_{2N})C_{2N}, \tag{10}$$

$$\dot{X}_N^* = k_{1N}C_{1N} - \beta_{1N}X_N^*M + \beta_{2N}C_{2N} + \underbrace{p_T(-k_{\text{on}}(1-c)X_N^* + k_{\text{off}}c)}_{r}, \tag{11}$$

$$\dot{c} = k_{\text{on}}(1-c)X_N^* - k_{\text{off}}c, \text{ where } c = \frac{C}{p_T} \in [0, 1]. \tag{12}$$

We make the following Assumptions 3-8 for the system:

*Assumption 3:* The input is bounded, i.e., $0 < |Z(t)| \le Z_B$.

*Assumption 4:* The time derivatives of the input $Z$ and of the ideal input $Z_{\text{ideal}}$, i.e., $\dot{Z}$ and $\dot{Z}_{\text{ideal}}$ are bounded, i.e., $|\dot{Z}(t)|, |\dot{Z}_{\text{ideal}}(t)| \le Z_{DB}$.

*Assumption 5:* All cycles have the same reaction constants, i.e., $\forall i \in [1, N]$, $k_{1i} = k_1, k_{2i} = k_2, \alpha_{1i} = \alpha_1, \beta_{1i} = \beta_1, \alpha_{2i} = \alpha_2, \beta_{2i} = \beta_2$. Then, $K_{m1i} = K_{m1}, K_{m2i} = K_{m2}$. Define $\lambda = \frac{k_1 K_{m2}}{k_2 K_{m1}}$.

*Assumption 6:* $\forall t$, $K_{m2} \gg X_i^*(t)$.

*Assumption 7:* Protein PD reactions, typically measured in second$^{-1}$ [16], are much faster than gene expression, typically measured in min$^{-1}$ [17]. Define $\epsilon_{TS} = \max\{\sqrt{\frac{\delta}{\alpha_2+k_1}}, \sqrt{\frac{k_2}{k_1}\frac{\delta}{\beta_2+k_2}}, \frac{\delta}{\beta_2+k_2}\}$. Then, $\epsilon_{TS} \ll 1$.

*Assumption 8:* The Jacobian of the set of equations (3)-(11) describing the cascade has all eigenvalues with negative real parts.

## IV. RESULTS

For designing the $N$-stage cascade of PD cycles described in Section III as an insulation device according to Definition 1, we now state the following theorem:

*Theorem 2: Let $\Theta = (X_{T1}, X_{T2}, ..., X_{TN}, M_T)$, $N \geq 2$. For the system defined by equations (2)-(12), under Assumptions 3-8, $\forall p_T > 0$, $\forall 0 < \epsilon_{TS} < \epsilon \ll 1$, there exists a $\Theta$, a $Z_{max} > 0$ and a $t_b \in (t_i, t_f)$ which decreases with $\epsilon_{TS}$, such that:*

*(a) $|\dot{Z}(t) - \dot{Z}_{ideal}(t)| \leq k_1\epsilon_{TS} + \epsilon Z_{DB}, \forall t \in [t_b, t_f]$,*

*(b) $|X_N^*(t) - X_{N,is}^*(t)| \leq k_2\epsilon_{TS} + \epsilon Z_B, \forall t \in [t_b, t_f]$,*

*(c) $|Z(t) - X_{N,is}^*(t)| \leq k_3\epsilon_{TS} + \epsilon Z_B$, for $Z(t) \leq Z_{max}$, $\forall t \in [t_b, t_f]$.*

*Here, $k_1, k_2, k_3 > 0$ are independent of $\epsilon_{TS}$.*

*One such parameter tuple $\bar{\Theta}$ is given by:*

*(i) $X_{T1} : \frac{\epsilon_{TS}}{1-\epsilon_{TS}} \leq \frac{X_{T1}}{K_{m1}} \leq \frac{\epsilon}{1-\epsilon}$;*

*(ii) $X_{TN} : X_{TN} \geq \tilde{X}_{TN}$ where $\tilde{X}_{TN} > \max\{\frac{p_T}{\epsilon}, X_{T1}\}$;*

*(iii) $X_{Ti} = X_{TN}, i \in [2, N-1]$;*

*(iv) $M_T = \lambda X_{T1}^{\frac{1}{N}} X_{TN}^{\frac{N-1}{N}}$.*

*In particular, $Z_{max} = g(N)\frac{\epsilon}{1-\epsilon}$, where $g(N) > 0$ is a continuous function of $N \in [2, \infty)$, such that $\lim_{N\to\infty} g(N) = 0$.*

*Proof:* Follows from Lemmas 1, 2, 3 and 4 given below. ■

*Remark 1:* The tradeoff encountered in the single cycle (requiring a large substrate concentration $X_T$ to attenuate retroactivity to the output versus requiring a small $X_T$ for a small retroactivity to the input) is overcome by picking a small $X_{T1}$ to ensure a small retroactivity to the input and a large $X_{TN}$ to attenuate the retroactivity to the output.

*Remark 2:* Since $g(N) > 0$ is continuous on $N \in [2, \infty)$ and $\lim_{N\to\infty} g(N) = 0$, there exists an $N = \bar{N}$ such that $g(N)$, and therefore $Z_{\text{max}}$ is maximized over $N \in [2, \infty)$ for a fixed $\epsilon$.

These properties will be further illustrated in Section V.

*Lemma 1: Define $\frac{1}{G_1} = \max\{\frac{\delta}{\alpha_2+k_1}, \frac{\delta}{\beta_2+k_2}, \max_i \frac{\delta}{\alpha_1 X_{Ti}}, \frac{\delta}{\beta_1 M_T}\}$. If $X_{T1} \leq X_{Ti}$ for $i \in [2, N]$, $M_T = \lambda X_{T1}^{\frac{1}{N}} X_{TN}^{\frac{N-1}{N}}$, and $\frac{\epsilon_{TS}}{1-\epsilon_{TS}} \leq \frac{X_{T1}}{K_{m1}}$ then, under Assumption 7, $\frac{1}{G_1} \leq \epsilon_{TS} \ll 1$.*

*Proof:* Since $\frac{1}{G_1}$ is the maximum of $\{\frac{\delta}{\alpha_2+k_1}, \frac{\delta}{\beta_2+k_2}, \max_i \frac{\delta}{\alpha_1 X_{Ti}}, \frac{\delta}{\beta_1 M_T}\}$ by definition, to prove that $\frac{1}{G_1} \leq \epsilon_{TS}$, we prove that each of these terms is less than or equal to $\epsilon_{TS}$.

(a) $\frac{\delta}{\alpha_2+k_1} \leq \epsilon_{TS}^2 \leq \epsilon_{TS}$ under Assumption 7.

(b) $\frac{\delta}{\beta_2+k_2} \leq \epsilon_{TS}$ under Assumption 7.

(c) $\frac{\delta}{\alpha_1 X_{T1}} = \frac{\delta}{\alpha_1 K_{m1}} \frac{K_{m1}}{X_{T1}} = \frac{\delta}{\alpha_2+k_1} \frac{K_{m1}}{X_{T1}} \leq \epsilon_{TS}^2 \frac{1-\epsilon_{TS}}{\epsilon_{TS}} < \epsilon_{TS}$

Then, $\frac{\delta}{\alpha_1 X_{Ti}} \leq \frac{\delta}{\alpha_1 X_{T1}} < \epsilon_{TS}$ as shown above, since $X_{T1} < X_{Ti}$.

Thus, $\max_i \frac{\delta}{\alpha_1 X_{Ti}} \leq \epsilon_{TS}$.

(d) $\frac{\delta}{\beta_1 M_T} = \frac{\delta}{\beta_1 \lambda X_{T1}^{\frac{1}{N}} X_{TN}^{\frac{N-1}{N}}} = \frac{\delta}{\beta_1 \lambda X_{T1}^{\frac{1}{N}} X_{TN}^{\frac{N-1}{N}}} \frac{X_{T1}}{K_{m1}} \frac{K_{m1}}{X_{T1}}$

$= \frac{\delta}{\beta_1 \frac{k_1}{k_2} K_{m2}} \frac{K_{m1}}{X_{T1}} \left(\frac{X_{T1}}{X_{TN}}\right)^{\frac{N-1}{N}} < \frac{k_2}{k_1} \frac{\delta}{\beta_2+k_2} \frac{K_{m1}}{X_{T1}}$

$\leq \epsilon_{TS}^2 \frac{1-\epsilon_{TS}}{\epsilon_{TS}} < \epsilon_{TS}$.

Thus, $\frac{1}{G_1} \leq \epsilon_{TS} \ll 1$ under Assumption 7. ■

*Lemma 2: For the system defined by equations (2)-(12), under Assumptions 3-8, for any $0 < \epsilon \ll 1$, if $X_{TN} \geq \frac{p_T}{\epsilon}$, for $i \in [2, N-1] : X_{Ti} = X_{TN} > X_{T1}$ and $M_T = \lambda X_{T1}^{\frac{1}{N}} X_{TN}^{\frac{N-1}{N}}$, we have $|X_N^*(t) - X_{N,is}^*(t)| \leq k_2\epsilon_{TS} + \epsilon Z_B$, for $t \in [t_b, t_f]$, where $t_b \in (t_i, t_f)$ which decreases with $\epsilon_{TS}$, and $k_2 > 0$ is independent of $\epsilon_{TS}$.*

*Proof:* In the system described by equations (2) - (12), the first cycle applies a retroactivity $r$ to the input, seen in equation (2). Retroactivity to the output $s$ is applied to the $N^{\text{th}}$ cycle, as seen in equation (11). We now proceed by bringing the system to form (1). To this end, we define $u(t) = Z(t)$, $x(t) = \begin{bmatrix} C_{11}(t) & .. & C_{1i}(t) & C_{2i}(t) & X_i^*(t) & .. & X_N^*(t) \end{bmatrix}^T$ for all $i$, $v(t) = c(t)$ and the constant $\eta = \frac{p_T}{X_{TN}}$. We define $G_1$ as in Lemma 1. Under Assumption 7, $G_1 \gg 1$. As a demonstration of how the system can be brought to form (1), we show how the $i^{th}$ cycle can be brought to the desired form. Using Assumption 5, we now assume equal reaction rates for the system. Define constants $q_1 = \frac{\alpha_1 X_{Ti}}{\delta G_1}$, $q_2 = \frac{\beta_1 M_T}{\delta G_1}$, $q_3 = \frac{k_1+\alpha_2}{\delta G_1}$ and $q_4 = \frac{\alpha_1 X_{T_{i+1}}}{\delta G_1}$. By the definition of $G_1$, $q_1, q_2, q_3, q_4 \geq 1$. We rewrite the dynamics of the $i^{th}$ cycle as follows (here again, we do not explicitly state that the signals are functions of time):

$$\dot{C}_{1i} = G_1\delta q_1 \left(1 - \frac{C_{1i}}{X_{Ti}} - \frac{X_i^*}{X_{Ti}} - \frac{C_{2i}}{X_{Ti}} - \frac{C_{1_{i+1}}}{X_{Ti}}\right) X_{i-1}^* - G_1\delta q_1 K_{m1} \frac{C_{1i}}{X_{Ti}},$$

$$\dot{C}_{2i} = G_1\delta q_2 \left[X_i^* \left(1 - \frac{\sum_{j=1}^N C_{2j}}{M_T}\right) - K_{m2}\frac{C_{2i}}{M_T}\right],$$

$$\dot{X}_i^* = G_1\delta \left[q_3 C_{1i} \frac{k_1}{k_1+\alpha_2} - q_2 X_i^* \left(1 - \frac{\sum_{j=1}^N C_{2j}}{M_T}\right)\right] + G_1\delta \left[q_2 K_{m2}\frac{C_{2i}}{M_T} + q_4 K_{m2} \frac{C_{1_{i+1}}}{X_{T_{i+1}}}\right] - G_1\delta \left[q_4 X_i^* \left(1 - \frac{C_{1_{i+1}}}{X_{T_{i+1}}} - \frac{X_{i+1}^*}{X_{T_{i+1}}} - \frac{C_{2_{i+1}}}{X_{T_{i+1}}} - \frac{C_{1_{i+2}}}{X_{T_{i+1}}}\right)\right].$$

Similarly, the first and last cycles can also be brought to this form, and the system is in form (1) with $A \in \mathbb{R}^{1\times1}$,

$B \in \mathbb{R}^{3N\times 1}, C \in \mathbb{R}^{3N\times 1}, D \in \mathbb{R}^{1\times 1}, f_0, f_1, r, s$ defined as:

$$A = 1, \qquad B = \begin{bmatrix} -1 & 0 & .. & 0 \end{bmatrix}^T_{1\times 3N},$$
$$C = \begin{bmatrix} 0 & .. & 0 & 1 \end{bmatrix}^T_{1\times 3N}, \qquad D = -1,$$

$$r = \frac{(-\alpha_1 X_1 Z + (\alpha_2 + k_1)C_{11})}{G_1},$$
$$s = p_T\left(-k_{\text{on}}(1-c)X_N^* + k_{\text{off}}c\right),$$
$$f_0 = k(t) - \delta Z,$$
$$f_1 = \begin{bmatrix} 0 & \frac{1}{G_1}\dot{C}_{22}...\frac{1}{G_1}\dot{C}_{1i} & \frac{1}{G_1}\dot{C}_{2i} & \frac{1}{G_1}\dot{X}_i^*...\frac{1}{G_1}\dot{X}_N^* \end{bmatrix}^T_{1\times 3N}.$$

Matrices $T = 1$, $M = \begin{bmatrix} 1 & 0 & . & . & . & 0 \end{bmatrix}_{1\times 3N}$, $Q = \begin{bmatrix} 0 & . & . & . & 0 & 1 \end{bmatrix}_{1\times 3N}$, $P = 1$ then satisfy $TA + MB = 0$, $Mf_1 = 0$, $QC + PD = 0$ and $MC = 0$, and, using Assumption 8, $\frac{\partial(Br+f_1)}{\partial x}$ has eigenvalues with negative real parts. Theorem 1 can then be applied to get $||x(t) - \gamma(u(t), \eta v(t))|| = \mathcal{O}(\frac{1}{G_1})$ for $t \in [t_b, t_f]$. The function $\gamma(u, \eta v)$ is found by setting $Br + f_1 = 0$. We describe the states thus found by a bar, for example, the expression of $X_i^*(t)$ found by setting $Br + f_1 = 0$ is denoted by $\bar{X}_i^*(t)$. These are found as shown below:

$$\frac{k_{1i}}{k_{2i}}\bar{C}_{1i}(t) = \bar{C}_{2i}(t) \approx \frac{M_T}{K_{m2i}}\bar{X}_i^*(t), \text{ under Assumption 6,} \tag{13}$$

$$\bar{X}_i^*(t) \approx \frac{X_{Ti}\bar{X}_{i-1}^*(t)}{\frac{M_T}{\lambda} + ((\frac{k_2}{k_1}+1)\frac{M_T}{K_{m2}} + \frac{\bar{X}_{i+1}(t)}{K_{m1}} + 1)\bar{X}_{i-1}^*(t)}, \text{ for } i \in [1, N-1], \tag{14}$$

and

$$\bar{X}_N^*(t) \approx \frac{X_{TN}\bar{X}_{N-1}^*(t)\,(1-\eta c(t))}{\frac{K_{m1N}k_{2N}}{K_{m2N}k_{1N}}M_T + \left(\left(1+\frac{k_{2N}}{k_{1N}}\right)\frac{M_T}{K_{m2N}} + 1\right)\bar{X}_{N-1}^*(t)}. \tag{15}$$

Let $a_i(t) = \left(\frac{\bar{X}_{i+1}(t)}{K_{m1}} + (\frac{k_2}{k_1}+1)\frac{M_T}{K_{m2}} + 1\right)$ for $i \in [1, N-1]$, $a_N = \left((\frac{k_2}{k_1}+1)\frac{M_T}{K_{m2}} + 1\right)$ and $b = \frac{M_T}{\lambda}$. We have from equations (14) and (15):

$$\bar{X}_1^* \approx \frac{X_{T1}Z}{b + a_1 Z},$$
$$\bar{X}_2^* \approx \frac{X_{T2}\bar{X}_1^*}{b + a_2\bar{X}_1^*} = \frac{X_{T2}\frac{X_{T1}Z}{b+a_1Z}}{b + a_2\frac{X_{T1}Z}{b+a_1Z}} = \frac{X_{T2}X_{T1}Z}{b^2 + (ba_1 + a_2X_{T1})Z},$$
$$\bar{X}_3^* \approx \frac{X_{T3}\bar{X}_2^*}{b + a_3\bar{X}_2^*} = \frac{X_{T3}X_{T2}X_{T1}Z}{b^3 + (b^2a_1 + ba_2X_{T1} + a_3X_{T2}X_{T1})Z},$$

and similarly:

$$\bar{X}_N^*(t) \approx \frac{\prod_{i=1}^N X_{Ti}Z(t)\,(1-\eta c(t))}{b^N + (\sum_{i=1}^N (b^{N-i}a_i(t)\prod_{j=1}^{i-1} X_{Tj}))Z(t)}. \tag{16}$$

To achieve unit gain, we have $b^N = \prod_{i=1}^N X_{Ti} = X_{T1}X_{TN}^{N-1}$, given $X_{Ti} = X_{TN}$, for $i \in [2, N]$. Since $b$ was defined as $\frac{M_T}{\lambda}$, we then get the following expression for $M_T$ to achieve unit gain:

$$M_T = \lambda b = \lambda X_{T1}^{\frac{1}{N}} X_{TN}^{\frac{N-1}{N}}. \tag{17}$$

Expression (16) can then be rewritten as:

$$\bar{X}_N^* \approx \frac{Z(t)\,(1-\eta c(t))}{1 + (\sum_{i=1}^N (b^{-i}a_i(t)\prod_{j=1}^{i-1} X_{Tj}))Z(t)}. \tag{18}$$

The output when a load $p_T = 0$ is $X_{N,is}^*(t)$. Substituting $\eta = \frac{p_T}{X_{TN}} = 0$ in equation (18), we find $\bar{X}_{N,is}^*(t)$:

$$\bar{X}_{N,is}^* \approx \frac{Z(t)}{1 + (\sum_{i=1}^N (b^{-i}a_i(t)\prod_{j=1}^{i-1} X_{Tj}))Z(t)}. \tag{19}$$

By the triangular inequality:

$$\begin{aligned} |X_N^*(t) - X_{N,is}^*(t)| &\le |X_N^*(t) - \bar{X}_N^*(t)| \\ &+ |X_{N,is}^*(t) - \bar{X}_{N,is}^*(t)| + |\bar{X}_N^*(t) - \bar{X}_{N,is}^*(t)|. \end{aligned} \tag{20}$$

By Theorem 1, $|X_N^*(t) - \bar{X}_N^*(t)| = \mathcal{O}(\frac{1}{G_1})$ and $|X_{N,is}^*(t) - \bar{X}_{N,is}^*(t)| = \mathcal{O}(\frac{1}{G_1})$. By the definition of $\mathcal{O}$ and $\frac{1}{G_1}$, we have some $k_1' > 0$ and $k_2' > 0$ independent of $\frac{1}{G_1}$ and therefore $\epsilon_{TS}$ such that $|X_N^*(t) - \bar{X}_N^*(t)| \le k_1'\frac{1}{G_1} \le k_1'\epsilon_{TS}$ and $|X_{N,is}^*(t) - \bar{X}_{N,is}^*(t)| \le k_2'\frac{1}{G_1} \le k_2'\epsilon_{TS}$. Thus we have:

$$|X_N^*(t) - \bar{X}_N^*(t)| + |X_{N,is}^*(t) - \bar{X}_{N,is}^*(t)| \le k_2\epsilon_{TS}, \tag{21}$$

for $t \in [t_b, t_f]$. Here, $k_2 = k_1' + k_2'$ is independent of $\epsilon_{TS}$.

We now evaluate $|\bar{X}_N^*(t) - \bar{X}_{N,is}^*(t)|$ from equations (18) and (19) to get:

$$\left|\bar{X}_N^*(t) - \bar{X}_{N,is}^*(t)\right| = |\eta c(t)\bar{X}_{N,is}^*(t)| \le |\eta\bar{X}_{N,is}^*(t)|.$$

Note from equation (19) that $|X_{N,is}^*(t)| \le Z(t) \le Z_B$ by Assumption 3. Thus,

$$\left|\bar{X}_N^*(t) - \bar{X}_{N,is}^*(t)\right| \le \eta Z_B.$$

Thus, for $\eta = \frac{p_T}{X_{TN}} \le \epsilon$, i.e., $X_{TN} \ge \frac{p_T}{\epsilon}$, we have:

$$\left|\bar{X}_N^*(t) - \bar{X}_{N,is}^*(t)\right| \le \epsilon Z_B. \tag{22}$$

Using equations (21) and (22) to re-evaluate the inequality in (20), we prove the required inequality:

$$|X_N^*(t) - X_{N,is}^*(t)| \le k_2\epsilon_{TS} + \epsilon Z_B, \forall t \in [t_b, t_f].$$

■

*Lemma 3: For the system (2)-(12), under Assumptions 4-8, for any $\epsilon : 0 < \epsilon_{TS} < \epsilon \ll 1$, if $X_{Ti} \ge X_{T1}$, $M_T = \lambda X_{T1}^{\frac{1}{N}} X_{TN}^{\frac{N-1}{N}}$ and for $\frac{\epsilon_{TS}}{1-\epsilon_{TS}} \le \frac{X_{T1}}{K_{m1}} \le \frac{\epsilon}{1-\epsilon}$, we have $\left|\dot{Z}(t) - \dot{Z}_{ideal}(t)\right| \le k_1\epsilon_{TS} + \epsilon Z_{DB}$, for $t \in [t_b, t_f]$, and $k_1 > 0$ is not dependent on $\epsilon_{TS}$.*

*Proof:* We proceed with the system expressed in the form of system (1), under Assumptions 6-8, with $G_1$ as defined in Lemma 1 as shown in the proof of Lemma 2, to get $x = \gamma(u, \eta v)$. In particular, we have:

$$\bar{C}_{11} \approx \frac{k_2}{k_1}\frac{M_T\bar{X}_1^*}{K_{m2}}, \quad \bar{X}_1^* \approx \frac{X_{T1}Z}{b + a_1Z}, \tag{23}$$

where $b = \frac{M_T}{\lambda}$ and $a_1 = \left(\left(\frac{k_2}{k_1}+1\right)\frac{M_T}{K_{m2}} + \frac{\bar{X}_2}{K_m1} + 1\right)$.

$Z_{\text{ideal}}$ is the input without the cycle present. Thus, if the dynamics of the input $Z$ are given by: $\dot{Z}(t, Z(t), x(t))$, the dynamics of $Z_{\text{ideal}}$ are given by $\dot{Z}(t, Z(t), 0)$. We define $\dot{\bar{Z}}$ as the dynamics of the system where $x = \gamma(Z, \eta c)$, i.e., $\dot{\bar{Z}} = \dot{Z}\left(t, Z(t), \gamma(Z(t), \eta c(t))\right)$. By the triangular inequality,

$$\begin{aligned} &|\dot{Z}(t) - \dot{Z}_{\text{ideal}}(t)| \\ &\leq |\dot{Z}(t) - \dot{\bar{Z}}(t)| + |\dot{\bar{Z}}(t) - \dot{Z}_{\text{ideal}}(t)|. \end{aligned} \tag{24}$$

By Corollary 1 of Theorem 1, we have:

$$|\dot{Z}(t) - \dot{\bar{Z}}(t)| = \mathcal{O}(\frac{1}{G_1}),\ \forall t \in [t_b, t_f].$$

By the definition of $\mathcal{O}$, we have:

$$|\dot{Z}(t) - \dot{\bar{Z}}(t)| \leq k_1 \frac{1}{G_1} \leq k_1 \epsilon_{TS},\ \forall t \in [t_b, t_f], \tag{25}$$

where $k_1 > 0$ is independent of $\epsilon_{TS}$.

The dynamics of $Z_{\text{ideal}}$, i.e., $\dot{Z}(t, Z(t), 0)$ is computed from equation (2) as:

$$\dot{Z}_{\text{ideal}}(t) = k(t) - \delta Z. \tag{26}$$

Finally, we compute $\dot{\bar{Z}}(t)$. Define $z = Z(t, \gamma(t)) + \bar{C}_{11}(t)$, from equations (2) and (3), we have:

$$\dot{z} = \dot{\bar{Z}} + \dot{\bar{C}}_{11} = k(t) - \delta Z. \tag{27}$$

$\dot{z}$ can also be expressed as:

$$\dot{z} = \dot{\bar{Z}} + \dot{\bar{C}}_{11} = \dot{\bar{Z}} \left(1 + \frac{\partial \bar{C}_{11}}{\partial Z}\right). \tag{28}$$

From equations (27) and (28), we get:

$$\dot{\bar{Z}}(t) = \frac{k(t) - \delta Z}{1 + \frac{\partial \bar{C}_{11}}{\partial Z}}. \tag{29}$$

Using equation (23), and Assumption 5 to compute $\frac{\partial \bar{C}_{11}}{\partial Z}$, we obtain:

$$\frac{\partial \bar{C}_{11}}{\partial Z} = \frac{\partial \bar{C}_{11}}{\partial \bar{X}_1^*} \frac{\partial \bar{X}_1^*}{\partial Z} = \frac{k_2 M_T}{k_1 K_{m2}} \frac{X_{T1} b}{(b + a_1 Z)^2},$$

where $b = \frac{M_T k_2 K_{m1}}{k_1 K_{m2}}$ which gives $\frac{k_2 M_T}{k_1 K_{m2}} = \frac{b}{K_{m1}}$. Thus,

$$\frac{\partial \bar{C}_{11}}{\partial Z} = \frac{b}{K_{m1}} \frac{X_{T1} b}{(b + a_1 Z)^2} = \frac{X_{T1}}{K_{m1}} \frac{1}{(1 + \frac{a_1 Z}{b})^2} \leq \frac{X_{T1}}{K_{m1}}. \tag{30}$$

Thus, if $\frac{X_{T1}}{K_{m1}} \leq \frac{\epsilon}{1-\epsilon}$, then the following is true:

$$\frac{\frac{\partial \bar{C}_{11}}{\partial Z}}{1 + \frac{\partial \bar{C}_{11}}{\partial Z}} \leq \epsilon,\ \text{i.e.,}\ \frac{\partial \bar{C}_{11}}{\partial Z} \leq \frac{\epsilon}{1 - \epsilon},$$

We now compute $\left|\dot{\bar{Z}}(t) - \dot{Z}_{\text{ideal}}\right|$ using equations (26) and (29) as follows:

$$\begin{aligned} \left|\dot{\bar{Z}}(t) - \dot{Z}_{\text{ideal}}(t)\right| &= \left|\frac{k(t) - \delta Z}{1 + \frac{\partial \bar{C}_{11}}{\partial Z}} - k(t) - \delta Z\right| \\ &= \frac{\frac{\partial \bar{C}_{11}}{\partial Z}}{1 + \frac{\partial \bar{C}_{11}}{\partial Z}} \left|\dot{Z}_{\text{ideal}}(t)\right|. \end{aligned}$$

We then have:

$$\left|\dot{\bar{Z}}(t) - \dot{Z}_{\text{ideal}}(t)\right| \leq \epsilon \left|\dot{Z}_{\text{ideal}}(t)\right| \leq \epsilon Z_{DB}, \tag{31}$$

by Assumption 4. Using equations (25) and (31), we re-evaluate the inequality in (24) to get:

$$|\dot{Z}(t) - \dot{Z}_{\text{ideal}}(t)| \leq k_1 \epsilon_{TS} + \epsilon Z_{DB}\ \forall t \in [t_b, t_f].$$

■

*Lemma 4: For the system (2)-(12), under Assumptions 3-8, with $X_{Ti} = X_{TN} > X_{T1}$ for $i \in [2, N-1]$ and $M_T = \lambda X_{T1}^{\frac{1}{N}} X_{TN}^{\frac{N-1}{N}}$, if $Z_{max} = g(N)\frac{\epsilon}{1-\epsilon}$, then for $Z(t) \leq Z_{max}$, $|Z(t) - X_{N,is}^*(t)| \leq k_3 \epsilon_{TS} + \epsilon Z_B$, for $t \in [t_b, t_f]$. Here, $k_3 > 0$ is not dependent on $\epsilon_{TS}$. Here, $g(N) > 0$ is continuous over $N \in [2, \infty)$ and such that $\lim_{N \to \infty} g(N) = 0$.*

*Proof:* By the triangular inequality,

$$\begin{aligned} &\left|Z(t) - X_{N,is}^*(t)\right| \leq \\ &\left|Z(t) - \bar{X}_{N,is}^*(t)\right| + \left|X_{N,is}^*(t) - \bar{X}_{N,is}^*(t)\right|. \end{aligned} \tag{32}$$

Proceeding with the system expressed in the form of system (1) as shown in the proof of Lemma 2, under Assumptions 6-8, we get:

$$\bar{X}_N^* \approx \frac{Z(t)\,(1 - \eta c(t))}{1 + (\sum_{i=1}^N (b^{-i} a_i(t) \prod_{j=1}^{i-1} X_{Tj})) Z(t)}, \tag{33}$$

$$\bar{X}_{N,is}^* \approx \frac{Z(t)}{1 + (\sum_{i=1}^N (b^{-i} a_i(t) \prod_{j=1}^{i-1} X_{Tj})) Z(t)}, \tag{34}$$

for $X_{Ti} = X_{TN} > X_{T1}$ for $i \in [2, N-1]$ and $M_T = \lambda X_{T1}^{\frac{1}{N}} X_{TN}^{\frac{N-1}{N}}$. Here, $a_i(t) = \left(\frac{\bar{X}_{i+1}(t)}{K_{m1}} + (\frac{k_2}{k_1} + 1)\frac{M_T}{K_{m2}} + 1\right)$ for $i \in [1, N-1]$, $a_N = \left((\frac{k_2}{k_1} + 1)\frac{M_T}{K_{m2}} + 1\right)$ and $b = \frac{M_T}{\lambda}$. As previously defined, $\bar{X}_{N,is}^*(t)$ is $\bar{X}_N^*(t)$ when $p_T = 0$.

By Theorem 1, $|\bar{X}_{N,is}^*(t) - X_{N,is}^*(t)| = \mathcal{O}(\frac{1}{G_1})$. By the definition of $\mathcal{O}$, then, we have a $k_3 > 0$ independent of $\frac{1}{G_1}$ and therefore of $\epsilon_{TS}$, such that:

$$|\bar{X}_{N,is}^*(t) - X_{N,is}^*(t)| \leq k_3 \frac{1}{G_1} \leq k_3 \epsilon_{TS}, \forall t \in [t_b, t_f]. \tag{35}$$

From equation (34), we have:

$$|Z(t) - \bar{X}_{N,is}^*(t)| = \frac{(b^{-i} a_i(t) \prod_{j=1}^{i-1} X_{Tj})) Z(t)^2}{1 + (\sum_{i=1}^N (b^{-i} a_i(t) \prod_{j=1}^{i-1} X_{Tj})) Z(t)}.$$

To get $\left|Z(t) - \bar{X}_{N,is}^*(t)\right| \leq \epsilon Z(t)$, we must have:

$$\frac{(\sum_{i=1}^N (b^{-i} a_i(t) \prod_{j=1}^{i-1} X_{Tj})) Z(t)^2}{1 + (\sum_{i=1}^N (b^{-i} a_i \prod_{j=1}^{i-1} X_{Tj})) Z(t)} \leq \epsilon Z(t).$$

By Assumption 3, $Z(t) \neq 0$. Thus, we must have:

$$\begin{aligned} &\frac{(\sum_{i=1}^N (b^{-i} a_i(t) \prod_{j=1}^{i-1} X_{Tj})) Z(t)}{1 + (\sum_{i=1}^N (b^{-i} a_i \prod_{j=1}^{i-1} X_{Tj})) Z(t)} \leq \epsilon, \\ &\text{i.e.,}\ (\sum_{i=1}^N (b^{-i} a_i \prod_{j=1}^{i-1} X_{Tj})) Z(t) \leq \frac{\epsilon}{1 - \epsilon}. \end{aligned} \tag{36}$$

Note that $b$ and $\prod_{j=1}^{i-1} X_{Tj}$ are constants. The upper bound for $a_i(t) = \left(\frac{\bar{X}_{i+1}(t)}{K_{m1}} + (\frac{k_2}{k_1}+1)\frac{M_T}{K_{m2}} + 1\right), i \in [1, N]$, is given by seeing that the maximum value for $\bar{X}_{i+1}$ is $X_{T_{i+1}}(t) = X_{TN}, i \in [1, N-1]$. Let the maximum value of $Z(t)$ for which $\left|Z(t) - \bar{X}^*_{N,is}(t)\right| \leq \epsilon Z(t)$ be $Z_{\max}$. We then have:

$$(\sum_{i=1}^{N}(b^{-i}a_i\prod_{j=1}^{i-1}X_{Tj}))Z(t)$$
$$\leq \underbrace{\left(\sum_{i=1}^{N}(b^{-i}\left(\frac{X_{TN}}{K_{m1}} + (\frac{k_2}{k_1}+1)\frac{M_T}{K_{m2}} + 1\right)\prod_{j=1}^{i-1}X_{Tj})\right)}_{\epsilon_3} Z_{\max}.$$

We define $\epsilon_3$ as shown above. Then,

$$(\sum_{i=1}^{N}(b^{-i}a_i\prod_{j=1}^{i-1}X_{Tj}))Z \leq \epsilon_3 Z_{\max}. \tag{37}$$

Substituting the value of $b = X_{T1}^{\frac{1}{N}} X_{TN}^{\frac{N-1}{N}}$ into the expression for $\epsilon_3$, we get:

$$\epsilon_3 = \frac{\frac{X_{TN}}{K_{m1}} + (\frac{k_2}{k_1}+1)\frac{M_T}{K_{m2}} + 1}{X_{T1}^{\frac{1}{N}} X_{TN}^{\frac{N-1}{N}}} + \frac{\left((\frac{k_2}{k_1}+1)\frac{M_T}{K_{m2}} + 1\right)}{X_{TN}}$$
$$+ \left(\frac{X_{TN}}{K_{m1}} + (\frac{k_2}{k_1}+1)\frac{M_T}{K_{m2}} + 1\right)\left(\frac{X_{T1}}{X_{TN}^2}\right)\sum_{i=2}^{N-1}\left(\frac{X_{TN}}{X_{T1}}\right)^{\frac{i}{N}}.$$

Substituting $M_T = \lambda X_{T1}^{\frac{1}{N}} X_{TN}^{\frac{N-1}{N}}$, and using the geometric series sum, we get the following expression for $\epsilon_3$:

$$\epsilon_3 = \underbrace{\frac{1}{K_{m1}}\left(\frac{X_{TN}}{X_{T1}}\right)^{\frac{1}{N}} + \frac{1}{X_{T1}^{\frac{1}{N}} X_{TN}^{\frac{N-1}{N}}}}_{(1)} + (\frac{k_2}{k_1}+1)\frac{\lambda}{K_{m2}}$$
$$+ \underbrace{\left(\frac{X_{T1}}{X_{TN}K_{m1}} + (\frac{k_2}{k_1}+1)\frac{\lambda}{K_{m2}}\left(\frac{X_{T1}}{X_{TN}}\right)^{1+\frac{1}{N}} + \frac{X_{T1}}{X_{TN}^2}\right)}_{(2a)}.$$
$$\underbrace{\left(\frac{\frac{X_{TN}}{X_{T1}} - \left(\frac{X_{TN}}{X_{T1}}\right)^{\frac{2}{N}}}{\left(\frac{X_{TN}}{X_{T1}}\right)^{\frac{1}{N}} - 1}\right)}_{(2b)} + \underbrace{\frac{\lambda(\frac{k_2}{k_1}+1)}{K_{m2}}\left(\frac{X_{T1}}{X_{TN}}\right)^{\frac{1}{N}}}_{(2c)} + \frac{1}{X_{TN}},$$

where (2a).(2b) are being multiplied. (38)

From equations (36) and (37), and the corresponding discussion, we have that when $\epsilon_3 Z_{\max} = \frac{\epsilon}{1-\epsilon}$, $|Z(t) - \bar{X}^*_{N,is}(t)| \leq \epsilon Z(t) \leq \epsilon Z_B$, by Assumption 3 for $Z(t) \leq Z_{\max}$. We define $g(N)$ as $\frac{1}{\epsilon_3}$, since $\epsilon_3 > 0$. Then, for $Z_{\max} = g(N)\frac{\epsilon}{1-\epsilon}$ we have, for $Z(t) \leq Z_{\max}$:

$$|Z(t) - \bar{X}^*_{N,is}(t)| \leq \epsilon Z_B. \tag{39}$$

Using equations (35) and (39), we re-evaluate the inequality (32) to get the required inequality for $Z(t) \leq Z_{\max}$:

$$|Z(t) - X^*_{N,is}(t)| \leq k_3\epsilon_{TS} + \epsilon Z_B, \forall t \in [t_b, t_f].$$

We return to $g(N)$, which was $\frac{1}{\epsilon_3}$ for $\epsilon_3$ defined by equation (38). Starting from $N = 2$, we see that since $X_{T1} < X_{TN}$, term (1) decreases with $N$, terms (2a), (2b) and (2c) increase with $N$ and as $N \to \infty$, $\epsilon_3 \to \infty$. The function $\epsilon_3$ is continuous, and therefore we have the following property of $g(N)$: $\lim_{N\to\infty} g(N) = 0$. ■

## V. IMPLICATIONS AND SIMULATION RESULTS

We first note that, for $\epsilon_{TS}, \epsilon \ll 1$, the properties (a), (b) and (c) of the cascade as described in Theorem 2 imply the properties (i), (ii) and (iii) of an insulation device as given in Definition 1. We motivated the above analysis by the tradeoff faced when the single PD cycle was used as an insulation device. As mentioned in Remark 1 this tradeoff can be broken by cascading PD cycles. The first and last cycles decouple the requirements for the first two properties in Definition 1 of an insulation device and break the tradeoff that was faced in the case of a single cycle.

We note, however, that there is a limit to which $r$ and $s$ can be made small. This is governed by $\epsilon_{TS}$, which limits how small $\epsilon$ can be made. $\epsilon_{TS}$ represents the degree of timescale separation between the dynamics of the input and that of the PD reactions. For realistic cases, however, since PD reactions are much faster than gene expression, it is possible to make $\epsilon_{TS}$ small enough to achieve small retroactivity.

The above discussion is verified in Fig. 5. Figs. 5a-5d show the tradeoff in the case of a single cycle, while Figs. 5e and 5f show this tradeoff being overcome with a two-cycle cascade. When the total substrate concentration for a single cycle is low, the retroactivity to the input is small (Fig. 5a) but the retroactivity to the output is not attenuated (Fig. 5b). When the total substrate concentration of this cycle is increased, the retroactivity to the output is attenuated (Fig. 5d) but the input, and therefore the output, slow down due to an increase in the retroactivity to the input (Figs. 5c, 5d). When the same two cycles are cascaded, with the low substrate concentration cycle being the first and the high substrate concentration cycle being the second, retroactivity to both the input as well as the output are attenuated (Figs. 5e, 5f).

The final condition that the cascade must satisfy to qualify as an insulation device is (iii) linearity between the input and output with unit gain. While two cycles are enough to satisfy conditions (i) and (ii), as seen from Theorem 2(i) and (ii), more than two cycles might be required to achieve linearity for a larger input range, $Z_{\max}$, as established by $g(N)$. As $g(N)$ increases, the operating input range $Z_{\max}$ increases, as seen in Theorem 2. As stated in Remark 2, there is an optimal $N = \bar{N}$ at which $g(N)$ is maximized, and therefore so is $Z_{\max}$. The change in $\bar{N}$ as the amount of load $p_T$ increases is shown in Fig. 6. We see that with load, the number of cycles needed increase. Note that, it may not be necessary to have $\bar{N}$ cycles to get a desirable result, i.e., a sufficiently large operating range. However, it is possible that no $N$ is capable of producing linearity for the desired operating range, since $g(N)$ is bounded above.

The above discussion is captured in the input-output characteristics shown in Fig. 7. As shown in Fig. 7a, for

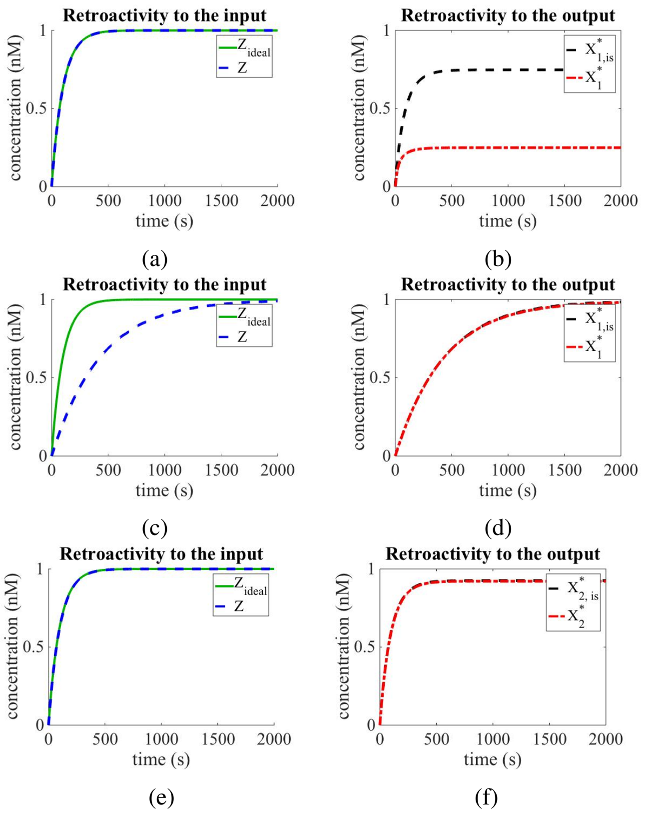


Fig. 5: Simulation results that show how two cycles (e)-(f) overcome the tradeoff present in a single cycle (a)-(d). Simulation parameters: $k(t) = 0.01nM.s^{-1}, \delta = 0.01s^{-1}, \alpha_1 = \beta_1 = 6(nM.s)^{-1}, \alpha_2 = \beta_2 = 1200s^{-1}, k_1 = k_2 = 600s^{-1}$. We choose $\epsilon = 0.01$ and load $p_T = 10nM$.
(a) Comparison of response of input $Z$ with and without the $1^{st}$ cycle: $X_T = 3nM$ (b) Comparison of the output response $X^*$ with and without load with just the $1^{st}$ cycle as insulation (c) Comparison of response of input $Z$ with and without just the $2^{nd}$ cycle: $X_T = 1000nM$ (d) Comparison of the input response $X^*$ with and without load with just the $2^{nd}$ cycle as insulation (e) Comparison of input response $Z$ with and without the cascaded system with $X_{T1} = 3nM$ and $X_{T2} = 1000nM$ (f) Comparison of the output response $X_2^*$ with and without load with the cascaded system as insulation

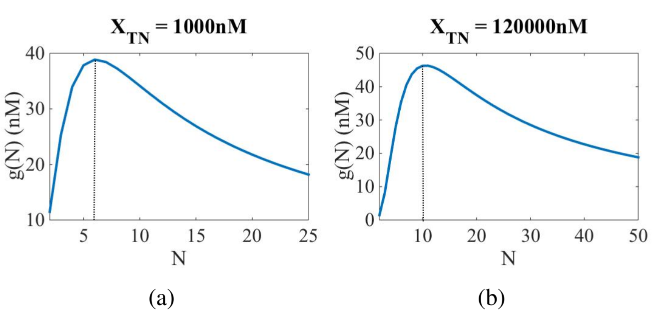


Fig. 6: Figures showing the variation of $g(N)$ with $N$, for $\epsilon = 0.01$ with different loads $p_T$. Parameter values are: $K_{m1} = K_{m2} = 300nM$, $k_1 = k_2 = 600s^{-1}$, $\lambda = 1$, (a) $p_T = 10nM$, where resulting $\bar{N} = 6$ and (b) $p_T = 1200nM$, where resulting $\bar{N} = 10$.

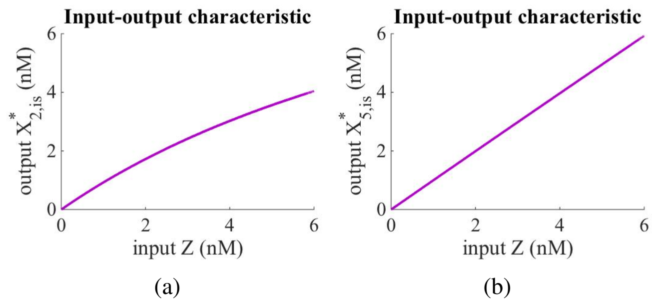


Fig. 7: Figures comparing the input-output characteristic for two cascades with different $N$'s. Simulation parameters: $k(t) = 0.01nM.s^{-1}, \delta = 0.01s^{-1}, \alpha_1 = \beta_1 = 6(nM.s)^{-1}, \alpha_2 = \beta_2 = 1200s^{-1}, k_1 = k_2 = 600s^{-1}$. We choose $\epsilon = 0.01$ and load $p_T = 10nM$, (a) $N = 2$ and (b) $N = 5$.

$N = 2$, the operating input range over which the input-output characteristic is linear with unit gain is low. When $N$ is increased to 5, for the same $\epsilon$, $p_T$ and reaction rates, the operating range of the input increases dramatically. The retroactivity to the input and to the output are both attenuated, and are similar to the results shown in Figs. 5e and 5f. Thus, this system, with $N = 5$, now satisfies all the three requirements of the Definition 1 of an insulation device.

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