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.

1 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], [8] 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 [9] and experimentally verified using a NRI-NRI* PD cycle [8]. 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 3 describes a model of the

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system based on the reaction rate ODEs of the system. Section 4 states and proves the mathematical result for designing the insulation device based on the model. Section 5 discusses the implications of this result and verifies these implications based on simulations.

2 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].

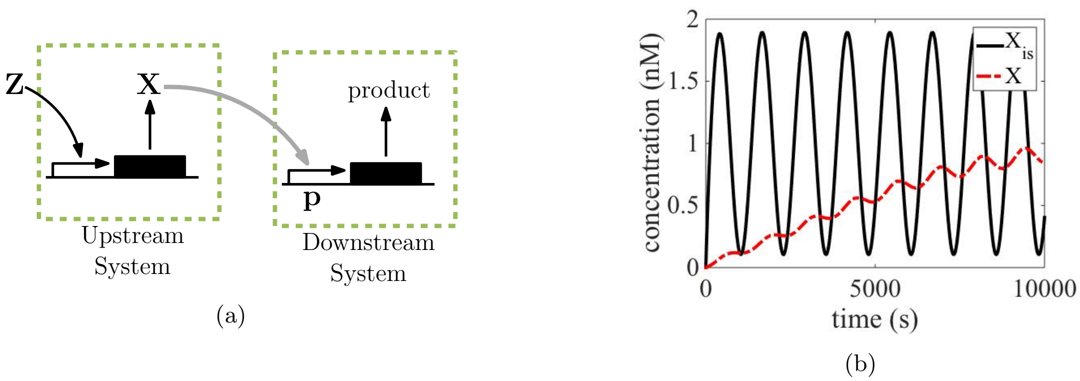

Figure 1: (a) The upstream system produces a gene product, the protein X; when the upstream system is connected with the downstream system, X acts as a transcription factor for downstream promoter sites p (b) The response of X to a periodic input Z is shown when the upstream system is not connected to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

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 $u_{\min}$ to $u_{\max}$, i.e., $u \in [u_{\min}, u_{\max}]$ and the output by y. The retroactivity to the input is $r(u, x)$ and the retroactivity to the output is $s(x, v)$. We define the ideal input, u_{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 $s = 0$.

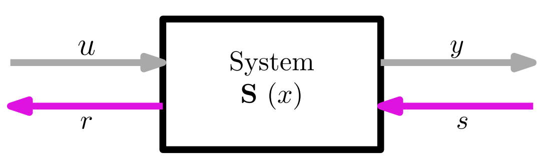

Figure 2: A system with state x, input u and output y, with retroactivity to the input r and retroactivity to the output s.

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 $u \in [u_{\min}, u_{\max}]$ with gain $G = 1$, i.e., $|u(t) - y_{is}(t)| \ll 1$.

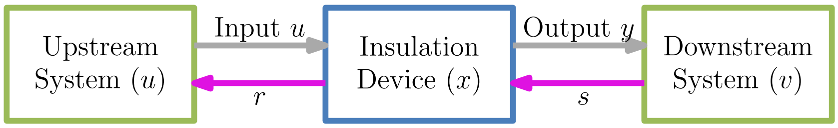

Figure 3: Insulation device connected between two systems: (i) minimizes r, (ii) attenuates the effect of s on x, and (iii) shows a linear relationship between u and y.

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

$$\begin{aligned} \dot{u} &= f_0(u,t) + G_1 A r(u,x), \\ \dot{x} &= G_1 B r(u,x) + G_1 f_1(u,x,\eta v) + C s(x,v), \\ \dot{v} &= D s(x,v). \end{aligned} \tag{1}$$

Here, the variables $t \in [t_i, t_f]$, $x \in \mathcal{D}_x \subset \mathbb{R}^n_+$, $u \in [u_{\min}, u_{\max}] \subset \mathbb{R}_+$, $y \in \mathcal{D}_y \subset \mathbb{R}_+$, $v \in \mathcal{D}_v \subset \mathbb{R}_+$, $r(u,x) \in \mathbb{R}_+$, $s(x,v) \in \mathbb{R}_+$. 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 η 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* $f_0(u,t) + G_1 A r(u,x)$ *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* $t \in [t_b, t_f]$*.*

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.

3 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 1^{st} cycle. The output of this device is X_N^*, the phosphorylated protein of the N^{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 $2 \leq i \leq N$. 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_{ideal} as

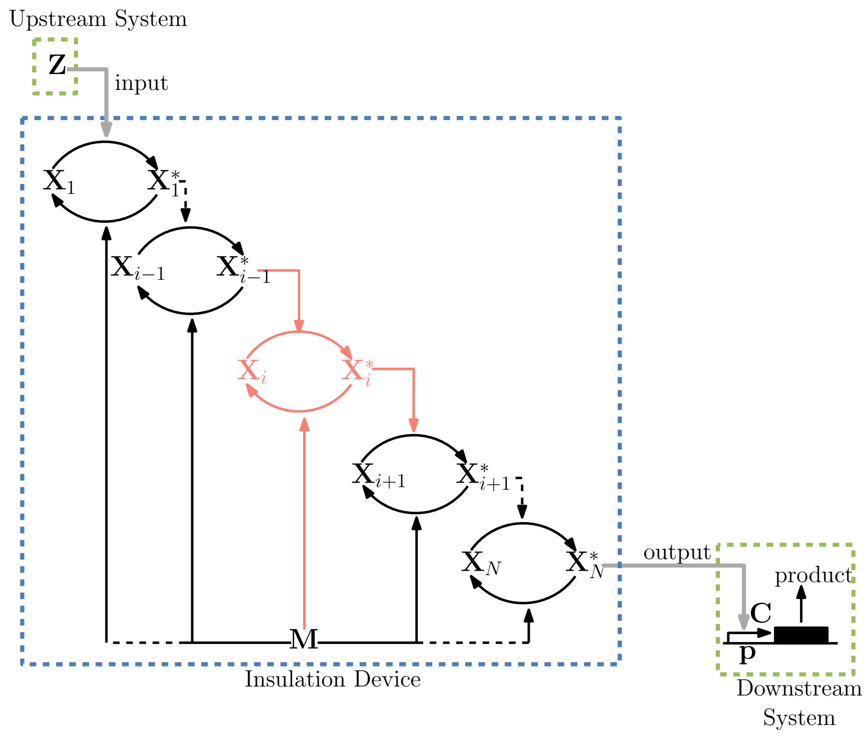

Figure 4: The i^{th} cycle is highlighted in a cascade of N cycles that together act as an insulation device; for the i^{th} cycle, X_i is phosphorylated by X_{i-1}^* to produce X_i^*, which is the kinase for the $(i+1)^{\text{th}}$ cycle; M is the common phosphatase for all cycles; for $i = 1$ the kinase 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 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:

$$\phi \underset{\delta}{\overset{k(t)}{\rightleftharpoons}} Z, \qquad X_1 + Z \underset{\alpha_{21}}{\overset{\alpha_{11}}{\rightleftharpoons}} C_{11} \xrightarrow{k_{11}} X_1^* + Z,$$

$$X_1^* + M \underset{\beta_{21}}{\overset{\beta_{11}}{\rightleftharpoons}} C_{21} \xrightarrow{k_{21}} X_1 + M,$$

$$X_1^* + X_2 \underset{\alpha_{22}}{\overset{\alpha_{12}}{\rightleftharpoons}} C_{12} \xrightarrow{k_{12}} X_1^* + X_2^*.$$

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

$$X_i + X_{i-1}^* \underset{\alpha_{2i}}{\overset{\alpha_{1i}}{\rightleftharpoons}} C_{1i} \xrightarrow{k_{1i}} X_i^* + X_{i-1}^*,\ K_{m1i} = \frac{\alpha_{2i} + k_{1i}}{\alpha_{1i}},$$

$$X_i^* + M \underset{\beta_{2i}}{\overset{\beta_{1i}}{\rightleftharpoons}} C_{2i} \xrightarrow{k_{2i}} X_i + M,\ K_{m2i} = \frac{\beta_{2i} + k_{2i}}{\beta_{1i}},$$

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

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:

$$X_{Ti} = X_i + C_{1i} + X_i^* + C_{2i} + C_{1_{i+1}}, \quad \text{for } i \in [1, N-1],\ p_T = p + C,$$

$$X_{TN} = X_N + C_{1N} + X_N^* + C_{2N} + C, \quad M_T = M + \sum_{i=1}^{N} C_{2i}.$$

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

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

For the first cycle,

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

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

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

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

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

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

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

For the last, N^{th}, cycle:

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

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

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

For the downstream system,

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

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

Assumption 3. Input is bounded, i.e., $0 < |Z(t)| \leq Z_B$.

Assumption 4. The time derivatives of the input Z and of the ideal input Z_{ideal}, i.e., $\frac{dZ}{dt}$ and $\frac{dZ_{\text{ideal}}}{dt}$ are bounded, i.e., $\left|\frac{dZ}{dt}(t)\right|, \left|\frac{dZ_{\text{ideal}}}{dt}(t)\right| \leq Z_{DB}$.

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. $\forall t$ and $\forall i \in [1,N]$, $K_{m2} \gg X_i^*(t)$.

Assumption 7. Protein PD reactions, typically measured in second^{-1} [16], are much faster than gene expression, typically measured in min^{-1} [17]. Define $\epsilon_{TS} = \max\{\sqrt{\frac{\delta}{\alpha_2+k_1}}, \sqrt{\frac{k_2}{k_1}\frac{\delta}{\beta_2+k_2}}, \frac{\delta}{\beta_2+k_2}, \sqrt{\frac{\delta}{\beta_1 K_{m1}}}, \frac{\delta}{\beta_2}, \frac{\delta}{k_1}\}$. Then, $\epsilon_{TS} \ll 1$. We also assume that $\bar{k}$ and K_{m1} are such that $\epsilon_{TS} \leq \frac{\bar{k}}{K_{m1}} \leq 1$.

Assumption 8. The Jacobian of the set of equations (14)-(22) describing the cascade has all eigenvalues with negative real parts.

To bring the system of equations (2)-(12) to a non-dimensional form, we define the following variables:
$\bar{k} = \max \frac{k(t)}{\delta}, \quad \tilde{k}(t) = \frac{k(t)}{\delta \bar{k}}, \quad z = \frac{Z}{\bar{k}}, \quad x_i^* = \frac{X_i^*}{X_{T1}},$
$c_{1i} = \frac{C_{1i}}{X_{T1}}, \quad c_{2i} = \frac{C_{2i}}{X_{T1}}, \quad c = \frac{C}{p_T}, \quad \tau = \delta t.$

The reaction rate equations (2)-(12) are rewritten for the non-dimensional system as follows, where $\dot{x} = \frac{dx}{d\tau}$.

$$\dot{z} = \tilde{k}(t) - z \underbrace{- \frac{\alpha_1 X_{T1} z}{\delta}(1 - c_{11} - x_1^* - c_{21} - c_{12}) + \frac{(\alpha_2+k_1)X_{T1}}{\delta \bar{k}} c_{11}}_{r}, \tag{13}$$

$$\dot{c}_{11} = \frac{\alpha_1 \bar{k}}{\delta} z(1 - c_{11} - x_1^* - c_{21} - c_{12}) - \frac{\alpha_2+k_1}{\delta} c_{11}, \tag{14}$$

$$\dot{c}_{21} = \frac{\beta_1 M_T}{\delta} x_1^* - \frac{\beta_1 X_{T1}}{\delta} x_1^* \sum c_{2i} - \frac{\beta_2+k_2}{\delta} c_{21}, \tag{15}$$

$$\begin{aligned}\dot{x}_1^* &= \frac{k_1}{\delta} c_{11} - \frac{\beta_1 M_T}{\delta} x_1^* + \frac{\beta_1 X_{T1}}{\delta} x_1^* \sum c_{2i} + \frac{\beta_2}{\delta} c_{21} \\ &- \frac{\alpha_1 X_{T2}}{\delta} x_1^* + \frac{\alpha_1 X_{T1}}{\delta} x_1^*(c_{12} + x_2^* + c_{22} + c_{13}) + \frac{(\alpha_2+k_1)}{\delta} c_{12}.\end{aligned} \tag{16}$$

$$\dot{c}_{1i} = \frac{\alpha_1 X_{Ti}}{\delta} x_{i-1}^* - \frac{\alpha_1 X_{T1}}{\delta} x_{i-1}^*(c_{1i} + x_i^* + c_{2i} + c_{1_{i+1}}) - \frac{\alpha_2+k_1}{\delta} c_{1i}, \tag{17}$$

$$\dot{c}_{2i} = \frac{\beta_1 M_T}{\delta} x_i^* - \frac{\beta_1 X_{T1}}{\delta} x_i^* \sum c_{2i} - \frac{\beta_2+k_1}{\delta} c_{2i}, \tag{18}$$

$$\begin{aligned}\dot{x}_i^* &= \frac{k_1}{\delta} c_{1i} - \frac{\beta_1 M_T}{\delta} x_i^* + \frac{\beta_1 X_{T1}}{\delta} x_i^* \sum c_{2i} + \frac{\beta_2}{\delta} c_{2i} - \frac{\alpha_1 X_{T_{i+1}}}{\delta} x_i^* + \frac{\alpha_1 X_{T1}}{\delta} x_i^*(c_{1_{i+1}} + x_{i+1}^* + c_{2_{i+1}} + c_{1_{i+2}}) \\ &+ \frac{(\alpha_2+k_1)}{\delta} c_{1_{i+1}}.\end{aligned} \tag{19}$$

$$\dot{c}_{1N} = \frac{\alpha_1 X_{TN}}{\delta} x_{N-1}^* - \frac{\alpha_1 X_{T1}}{\delta} x_{N-1}^*(c_{1N} + x_N^* + c_{2N} + \frac{p_T}{X_{T1}} c) - \frac{\alpha_2+k_1}{\delta} c_{1N}, \tag{20}$$

$$\dot{c}_{2N} = \frac{\beta_1 M_T}{\delta} x_N^* - \frac{\beta_1 X_{T1}}{\delta} x_N^* \sum c_{2i} - \frac{\beta_2+k_2}{\delta} c_{2N},, \tag{21}$$

$$\dot{x}_N^* = \frac{k_1}{\delta} c_{1N} - \frac{\beta_1 M_T}{\delta} x_N^* + \frac{\beta_1 X_{T1}}{\delta} x_N^* \sum c_{2i} + \frac{\beta_2}{\delta} c_{2N} \underbrace{- \frac{p_T k_{\text{on}}}{\delta}(1-c)x_N^* + \frac{p_T k_{\text{off}}}{\delta X_{T1}} c}_{s}, \tag{22}$$

$$\dot{c} = \frac{k_{\text{on}} X_{T1}}{\delta}(1-c)x_N^* - \frac{k_{\text{off}}}{\delta} c. \tag{23}$$

4 RESULTS

For designing the N-stage cascade of PD cycles described in Section 3 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 (13)-(23), under Assumptions 3-8, $\forall p_T > 0$, $\forall \epsilon : 0 < \epsilon_{TS} < \epsilon \ll 1$, there exists a Θ, a $Z_{max} > 0$ and a $t_b \in (t_i, t_f)$ which decreases with ϵ_{TS}, such that:*

(a) $|\frac{dZ}{dt}(t) - \frac{dZ_{ideal}}{dt}(t)| \leq k_1\epsilon_{TS} + \epsilon Z_{DB}, \forall t \in [t_b, t_f]$,

(b) $|X_N^*(t) - X_{N,is}^*(t)| \leq k_2 \frac{\epsilon}{1-\epsilon}\epsilon_{TS} + \epsilon Z_B, \forall t \in [t_b, t_f]$,

(c) $|Z(t) - X_{N,is}^*(t)| \leq k_3 \frac{\epsilon}{1-\epsilon}\epsilon_{TS} + \epsilon Z_B$, *for* $Z(t) \leq Z_{max}$, $\forall t \in [t_b, t_f]$.

Here, $k_1, k_2, k_3 > 0$ are independent of ϵ_{TS} and ϵ.
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_{max} is maximized over $N \in [2, \infty)$ for a fixed ϵ.*

These properties will be further illustrated in Section 5.

Lemma 1. *The system defined by the equations (13) - (23), under Assumptions 3-8 is of the form of system (1), and satisfies Assumptions 1 and 2, with $G_1 \gg 1$ such that $G_1 \geq \frac{1}{\epsilon_{TS}}$ for $X_{T1} \geq \frac{K_{m1}\epsilon_{TS}}{1-\epsilon_{TS}}$, $M_T = \lambda X_{T1}^{\frac{1}{N}}$ and $X_{TN} > X_{T1}$. Theorem 1 and Corollary 1 can thus be applied to this system.*

Proof. We see that in the system described by equations (13)-(23), the first cycle applies a retroactivity r to the input, seen in equation (13). Retroactivity to the output s is applied to the N^{th} cycle, as seen in equation (22). To bring the system to form (1), we define: $u(t) = z(t), x(t) = [\ c_{11}(t) \ \ .. \ \ c_{1i}(t) \ \ c_{2i}(t) \ \ x_i^*(t) \ \ .. \ \ x_N^*(t)\ ]^T$, $v(t) = c(t)$. Further, $f_0(u,t) = \tilde{k}(t) - z$, $A = 1$, $B = \frac{\bar{k}}{X_{T1}}[\ -1 \ \ 0 \ \ .. \ \ 0\ ]^T_{3N\times1}$, $C = [\ 0 \ \ .. \ \ 0 \ \ 1\ ]^T_{3N\times1}$ and $D = -\frac{X_{T1}}{p_T}$.

Define $G_1 = \max\{\frac{\alpha_1 X_{T1}}{\delta}, \frac{(\alpha_2+k_1)X_{T1}}{\delta\bar{k}}, \frac{\alpha_1\bar{k}}{\delta}, \frac{(\alpha_2+k_1)}{\delta}, \frac{\beta_1 M_T}{\delta}, \frac{\beta_1 X_{T1}}{\delta}, \frac{\beta_2+k_2}{\delta}, \frac{k_1}{\delta}, \frac{\beta_2}{\delta}\}$. We see that for $X_{T1} \geq \frac{K_{m1}\epsilon_{TS}}{1-\epsilon_{TS}}$, $M_T = \lambda X_{T1}^{\frac{1}{N}} X_{TN}^{\frac{N-1}{N}}$ and $X_{TN} > X_{T1}$, under Assumption 7, each of these terms is greater than/equal to $\frac{1}{\epsilon_{TS}}$. Thus, $G_1 \geq \frac{1}{\epsilon_{TS}}$, which implies that $G_1 \gg 1$.

Further, we define $r = -\frac{1}{G_1}\frac{\alpha_1 X_{T1}}{\delta} z(1 - c_{11} - x_1^* - c_{21} - c_{12}) + \frac{1}{G_1}\frac{(\alpha_2+k_1)X_{T1}}{\delta k} c_{11}$, $s = \frac{-p_T k_{\text{on}}(1-c)x_N^*}{\delta} + \frac{p_T k_{\text{off}} c}{\delta X_{T1}}$ and function $f_1 = \frac{1}{G_1}[\ 0 \ \ \dot{c_{21}} \ \ .. \ \ \dot{x_N^*}\ ]^T_{3N\times1}$. Then, we have invertible matrices $T = \frac{\bar{k}}{X_{T1}}$ and $P = \frac{p_T}{X_{T1}}$

and matrices $M = [\ 1 \quad 0 \quad .. \quad 0\ ]_{1\times 3N}$ and $Q = [\ 0 \quad .. \quad 0 \quad 1\ ]_{1\times 3N}$, such that $TA + MB = 0$, $Mf_1 = 0$, $MC = 0$ and $QC + PD = 0$. Under Assumption 8, the eigenvalues of $\frac{\partial(Br+f_1)}{\partial x}$ have negative real parts.

These definitions show that the system defined by equations (13)-(23) are of the form of system (1) and satisfy Assumptions 1 and 2 for the system. Thus, Theorem 1 and Corollary 1 can be applied for this system. □

Lemma 2. *For the system defined by equations (13)-(23), 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* $|X_N^*(t) - X_{N,is}^*(t)| \leq k_2 \frac{\epsilon}{1-\epsilon}\epsilon_{TS} + \epsilon Z_B$, *for* $t \in [t_b, t_f]$, *where* $t_b \in (t_i, t_f)$ *which decreases with* ϵ_{TS}, *and* $k_2 > 0$ *is independent of* ϵ_{TS} *and* ϵ.

Proof. For input $u(t) = z(t)$, states $x(t) = [\ c_{11}(t) \quad .. \quad c_{1i}(t) \quad c_{2i}(t) \quad x_i^*(t) \quad .. \quad x_N^*(t)\ ]^T$ and downstream state $v(t) = c(t)$, the system defined by equations (13)-(23) is of the form of system (1), as shown in Lemma 1. Theorem 1 can then be applied to obtain $||x(t) - \gamma(u(t), \eta v(t))|| = \mathcal{O}(\frac{1}{G_1})$ for $t \in [t_b, t_f]$, where $G_1 \geq \frac{1}{\epsilon_{TS}}$, i.e., $||x(t) - \gamma(u(t), \eta v(t))|| = \mathcal{O}(\epsilon_{TS})$. The function $\gamma(u, \eta v)$ is found by setting $Br + f_1 = 0$, where $B = \frac{\bar{k}}{X_{T1}}[\ -1 \quad 0 \quad .. \quad 0\ ]^T_{3N\times 1}$, $r = -\frac{1}{G_1}\frac{\alpha_1 X_{T1}}{\delta} z(1 - c_{11} - x_1^* - c_{21} - c_{12}) + \frac{1}{G_1}\frac{(\alpha_2+k_1)X_{T1}}{\delta \bar{k}} c_{11}$ and $f_1 = \frac{1}{G_1}[\ 0 \quad \dot{c_{21}} \quad .. \quad \dot{x_N^*}\ ]^T_{3N\times 1}$. We describe the states found by a bar, for example, the expression of x_i^* found by setting $Br + f_1 = 0$ is denoted by $\bar{x}_i^*$. Thus, by Theorem 1, $||x_i^* - \bar{x}_i^*|| = \mathcal{O}(\epsilon_{TS})$. Note that these are the non-dimensionalized forms of the original concentration variables, $X_i^*(t)$ and $\bar{X}_i^*$. We redimensionalize the equations $Br + f_1$ to find results in terms of the original variables. Since $X_i^* = X_{T1}x_i^*$, we have $||X_i^* - \bar{X}_i^*|| = X_{T1}\mathcal{O}(\epsilon_{TS})$. For $X_{T1} \leq K_{m1}\frac{\epsilon}{1-\epsilon}$ and by the definition of $\mathcal{O}$, we have a constant k_3 independent of ϵ_{TS} and ϵ such that $||X_i^* - \bar{X}_i^*|| \leq k_3 \frac{\epsilon}{1-\epsilon}\epsilon_{TS}$. The same argument can be made for the other state variables. The solution to the redimensionalized equations $Br + f_1 = 0$ is found as shown below:

$$\frac{k_{1i}}{k_{2i}}\bar{C}_{1i}(t) = \bar{C}_{2i}(t) \approx \frac{M_T}{K_{m2i}}\bar{X}_i^*(t), \text{ under Assumption 6,} \tag{24}$$

$$\bar{X}_i^*(t) \approx \frac{X_{Ti}\bar{X}_{i-1}^*(t)}{\frac{M_T}{\lambda} + ((\frac{k_2}{k_1}+1)\frac{M_T}{K_{m2}} + \frac{\bar{X}_{i+1}(t)}{K_{m1}} + 1)\bar{X}_{i-1}^*(t)}, \text{ for } i \in [1, N-1], \tag{25}$$

and

$$\bar{X}_N^*(t) \approx \frac{X_{TN}\bar{X}_{N-1}^*(t)\left(1 - (\frac{p_T}{X_{TN}})c(t)\right)}{\frac{K_{m1N}k_{2N}}{K_{m2N}k_{1N}}M_T + \left(\left(1 + \frac{k_{2N}}{k_{1N}}\right)\frac{M_T}{K_{m2N}} + 1\right)\bar{X}_{N-1}^*(t)}. \tag{26}$$

Let $\eta = \frac{p_T}{X_{TN}}$, $a_i(t) = \left(\frac{\bar{X}_{i+1}(t)}{K_{m1}} + (\frac{k_2}{k_1}+1)\frac{M_T}{K_{m2}} + 1\right)$ 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 (25) and (26):

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

and similarly:

$$\bar{X}_N^*(t) \approx \frac{\prod_{i=1}^N X_{Ti}Z(t)\left(1 - \eta c(t)\right)}{b^N + (\sum_{i=1}^N (b^{N-i}a_i(t)\prod_{j=1}^{i-1}X_{Tj}))Z(t)}. \tag{27}$$

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 obtain the following expression for M_T to achieve unit gain:

$$M_T = \lambda b = \lambda X_{T1}^{\frac{1}{N}} X_{TN}^{\frac{N-1}{N}}. \tag{28}$$

Expression (27) can then be rewritten as:

$$\bar{X}_N^* \approx \frac{Z(t)\,(1-\eta c(t))}{1+(\sum_{i=1}^{N}(b^{-i}a_i(t)\prod_{j=1}^{i-1}X_{Tj}))Z(t)}. \tag{29}$$

The output when a load $p_T = 0$ is $X^*_{N,is}(t)$. Substituting $\eta = \frac{p_T}{X_{TN}} = 0$ in equation (29), we find $\bar{X}^*_{N,is}(t)$:

$$\bar{X}^*_{N,is} \approx \frac{Z(t)}{1+(\sum_{i=1}^{N}(b^{-i}a_i(t)\prod_{j=1}^{i-1}X_{Tj}))Z(t)}. \tag{30}$$

By the triangular inequality:

$$\begin{aligned} &|X_N^*(t) - X^*_{N,is}(t)| \le |X_N^*(t) - \bar{X}_N^*(t)| \\ &+ |X^*_{N,is}(t) - \bar{X}^*_{N,is}(t)| + |\bar{X}_N^*(t) - \bar{X}^*_{N,is}(t)|. \end{aligned} \tag{31}$$

We have shown that for some $k_1' > 0$ and $k_2' > 0$ independent of ϵ_{TS} and ϵ, $|X_N^*(t) - \bar{X}_N^*(t)| \le k_1' \frac{\epsilon}{1-\epsilon}\epsilon_{TS}$ and $|X^*_{N,is}(t) - \bar{X}^*_{N,is}(t)| \le k_2' \frac{\epsilon}{1-\epsilon}\epsilon_{TS}$. Thus we have:

$$|X_N^*(t) - \bar{X}_N^*(t)| + |X^*_{N,is}(t) - \bar{X}^*_{N,is}(t)| \le k_2 \frac{\epsilon}{1-\epsilon}\epsilon_{TS}, \tag{32}$$

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

We now evaluate $|\bar{X}_N^*(t) - \bar{X}^*_{N,is}(t)|$ from equations (29) and (30) to obtain:

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

Note from equation (30) 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:

$$\left|\bar{X}_N^*(t) - \bar{X}^*_{N,is}(t)\right| \le \epsilon Z_B. \tag{33}$$

Using equations (32) and (33) to re-evaluate the inequality in (31), we prove the required inequality:

$$|X_N^*(t) - X^*_{N,is}(t)| \le k_2 \frac{\epsilon}{1-\epsilon}\epsilon_{TS} + \epsilon Z_B, \forall t \in [t_b, t_f].$$

□

Lemma 3. *For the system (13)-(23), 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* ϵ_{TS}*.*

Proof. We proceed as shown in the proof of Lemma 2 to obtain the redimensionalized form of $x = \gamma(u, \eta v)$, the solution of equation $Br(u, x) + f_1(u, x, \eta v) = 0$. In particular, we have:

$$\bar{C}_{11} \approx \tfrac{k_2}{k_1}\tfrac{M_T \bar{X}_1^*}{K_{m2}}, \quad \bar{X}_1^* \approx \tfrac{X_{T1} Z}{b + a_1 Z}, \tag{34}$$

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

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

$$\left|\frac{dZ}{dt}(t) - \frac{dZ_{\text{ideal}}}{dt}(t)\right| \leq \left|\frac{dZ}{dt}(t) - \frac{d\bar{Z}}{dt}(t)\right| + \left|\frac{d\bar{Z}}{dt}(t) - \frac{dZ_{\text{ideal}}}{dt}(t)\right|. \tag{35}$$

Note that $\frac{dZ}{dt} = (\bar{k}\delta)\dot{z}$. By Corollary 1 of Theorem 1, we know that $|\dot{z} - \dot{\bar{z}}| = \mathcal{O}(\epsilon_{TS})\ \forall t \in [t_b, t_f]$. Thus, $\left|\frac{dZ}{dt} - \frac{d\bar{Z}}{dt}\right| = \bar{k}\delta\mathcal{O}(\epsilon_{TS})$. By the definition of $\mathcal{O}$, we have:

$$\left|\frac{dZ}{dt}(t) - \frac{d\bar{Z}}{dt}(t)\right| \leq k_1\epsilon_{TS},\ \forall t \in [t_b, t_f], \tag{36}$$

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

The dynamics of Z_{ideal}, i.e., $\frac{dZ}{dt}(t, Z(t), 0)$ is computed from equation (13) as:

$$\frac{dZ_{\text{ideal}}}{dt}(t) = (\bar{k}\delta)\dot{z}_{\text{ideal}} = k(t) - \delta Z. \tag{37}$$

Finally, we compute $\frac{d\bar{Z}}{dt}(t)$. Define $Z_s = Z(t, \gamma(t)) + \bar{C}_{11}(t)$. Then from equations (13) and (14), we have:

$$\frac{dZ_s}{dt} = (\bar{k}\delta)\dot{z} + (X_{T1}\delta)\dot{\bar{c}}_{11} = k(t) - \delta Z. \tag{38}$$

$\frac{dZ_s}{dt}$ can also be expressed as:

$$\frac{dZ_s}{dt} = \frac{d\bar{Z}}{dt} + \frac{d\bar{C}_{11}}{dt} = \frac{d\bar{Z}}{dt}\left(1 + \frac{\partial\bar{C}_{11}}{\partial Z}\right). \tag{39}$$

From equations (38) and (39), we obtain:

$$\frac{d\bar{Z}}{dt} = \frac{k(t) - \delta Z}{1 + \frac{\partial\bar{C}_{11}}{\partial Z}}. \tag{40}$$

Using equation (34), and Assumption 5 to compute $\frac{\partial\bar{C}_{11}}{\partial Z}$, we obtain:

$$\frac{\partial\bar{C}_{11}}{\partial Z} = \frac{\partial\bar{C}_{11}}{\partial\bar{X}_1^*}\frac{\partial\bar{X}_1^*}{\partial Z} = \frac{k_2M_T}{k_1K_{m2}}\frac{X_{T1}b}{(b + a_1Z)^2},$$

where $b = \frac{M_Tk_2K_{m1}}{k_1K_{m2}}$ which gives $\frac{k_2M_T}{k_1K_{m2}} = \frac{b}{K_{m1}}$. Thus,

$$\frac{\partial\bar{C}_{11}}{\partial Z} = \frac{b}{K_{m1}}\frac{X_{T1}b}{(b + a_1Z)^2} = \frac{X_{T1}}{K_{m1}}\frac{1}{(1 + \frac{a_1Z}{b})^2} \leq \frac{X_{T1}}{K_{m1}}. \tag{41}$$

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 $\left|\frac{d\bar{Z}}{dt} - \frac{dZ_{\text{ideal}}}{dt}\right|$ using equations (37) and (40) as follows:

$$\left|\frac{d\bar{Z}}{dt}(t) - \frac{dZ_{\text{ideal}}}{dt}(t)\right| = \left|\frac{k(t) - \delta Z}{1 + \frac{\partial\bar{C}_{11}}{\partial Z}} - k(t) - \delta Z\right| = \frac{\frac{\partial\bar{C}_{11}}{\partial Z}}{1 + \frac{\partial\bar{C}_{11}}{\partial Z}}\left|\frac{dZ_{\text{ideal}}}{dt}(t)\right|.$$

We then have:

$$\left|\frac{d\bar{Z}}{dt}(t) - \frac{dZ_{\text{ideal}}}{dt}(t)\right| \leq \epsilon \left|\frac{dZ_{\text{ideal}}}{dt}(t)\right| \leq \epsilon Z_{DB}, \tag{42}$$

by Assumption 4. Using equations (36) and (42), we re-evaluate the inequality in (35) to achieve:

$$\left|\frac{dZ}{dt}(t) - \frac{dZ}{dt}_{\text{ideal}}(t)\right| \leq k_1\epsilon_{TS} + \epsilon Z_{DB} \;\forall t \in [t_b, t_f].$$

□

Lemma 4. *For the system (13)-(23), 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* $Z(t) \leq Z_{max}$, $|Z(t) - X^*_{N,is}(t)| \leq k_3\frac{\epsilon}{1-\epsilon}\epsilon_{TS} + \epsilon Z_B$, *for* $t \in [t_b, t_f]$. *Here,* $k_3 > 0$ *is not dependent on* ϵ_{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,

$$\left|Z(t) - X^*_{N,is}(t)\right| \leq \left|Z(t) - \bar{X}^*_{N,is}(t)\right| + \left|X^*_{N,is}(t) - \bar{X}^*_{N,is}(t)\right|. \tag{43}$$

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

$$\bar{X}^*_N \approx \frac{Z(t)\,(1-\eta c(t))}{1 + (\sum_{i=1}^{N}(b^{-i}a_i(t)\prod_{j=1}^{i-1}X_{Tj}))Z(t)}, \tag{44}$$

$$\bar{X}^*_{N,is} \approx \frac{Z(t)}{1 + (\sum_{i=1}^{N}(b^{-i}a_i(t)\prod_{j=1}^{i-1}X_{Tj}))Z(t)}, \tag{45}$$

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, $|\bar{X}^*_{N,is}(t) - X^*_{N,is}(t)| = X_{T1}\mathcal{O}(\epsilon_{TS})$. Since $X_{T1} \leq K_{m1}\frac{\epsilon}{1-\epsilon}$, by the definition of $\mathcal{O}$ we have a $k_3 > 0$ of ϵ_{TS} and ϵ such that:

$$|\bar{X}^*_{N,is}(t) - X^*_{N,is}(t)| \leq k_3\frac{\epsilon}{1-\epsilon}\epsilon_{TS}, \forall t \in [t_b, t_f]. \tag{46}$$

From equation (45), 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 achieve $\left|Z(t) - \bar{X}^*_{N,is}(t)\right| \leq \epsilon Z(t)$, 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:

$$\frac{(\sum_{i=1}^{N}(b^{-i}a_i(t)\prod_{j=1}^{i-1}X_{Tj}))Z(t)}{1 + (\sum_{i=1}^{N}(b^{-i}a_i\prod_{j=1}^{i-1}X_{Tj}))Z(t)} \leq \epsilon, \text{ i.e., } (\sum_{i=1}^{N}(b^{-i}a_i\prod_{j=1}^{i-1}X_{Tj}))Z(t) \leq \frac{\epsilon}{1-\epsilon}. \tag{47}$$

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 ϵ_3 as shown above. Then,

$$(\sum_{i=1}^{N}(b^{-i}a_i\prod_{j=1}^{i-1}X_{Tj}))Z \leq \epsilon_3 Z_{\max}. \tag{48}$$

Substituting the value of $b = X_{T1}^{\frac{1}{N}} X_{TN}^{\frac{N-1}{N}}$ into the expression for ϵ_3, we obtain:

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

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

(49)

From equations (47) and (48), 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 $Z(t) \leq Z_{\max}$:

$$|Z(t) - \bar{X}^*_{N,is}(t)| \leq \epsilon Z_B. \tag{50}$$

Using equations (46) and (50), we re-evaluate the inequality (43) to achieve the required inequality for $Z(t) \leq Z_{\max}$:

$$|Z(t) - X^*_{N,is}(t)| \leq k_3\frac{\epsilon}{1-\epsilon}\epsilon_{TS} + \epsilon Z_B, \forall t \in [t_b, t_f].$$

We return to $g(N)$, which was $\frac{1}{\epsilon_3}$ for ϵ_3 defined by equation (49). 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 ϵ_3 is continuous, and therefore we have the following property of $g(N)$: $\lim_{N\to\infty} g(N) = 0$. □

5 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 ϵ_{TS}, which limits how small ϵ can be made. ϵ_{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 ϵ_{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 achieve 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 $N = 2$, 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 ϵ, 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.

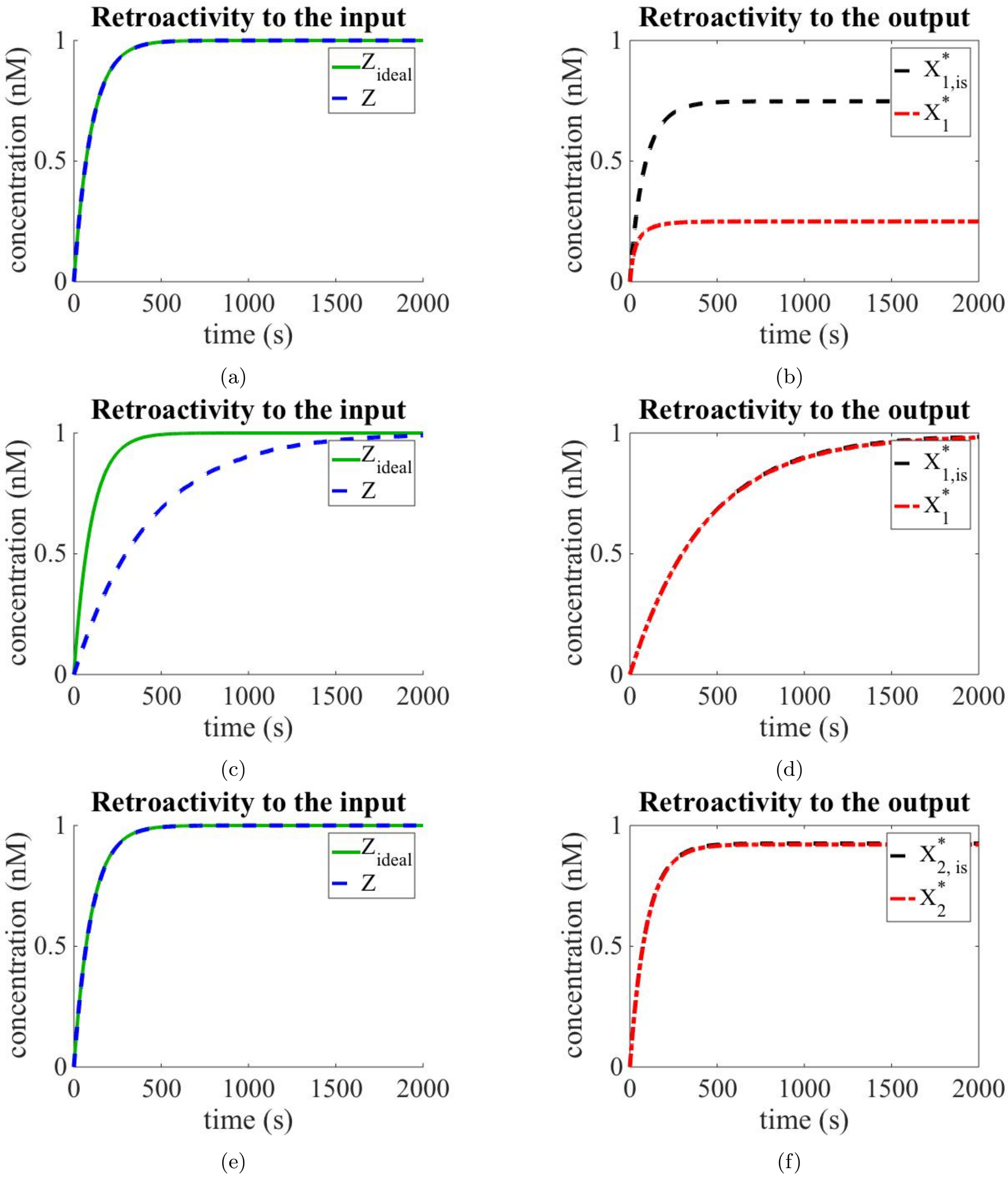

Figure 5: Simulation results that show how two cycles (e)-(f) overcome the tradeoff present in a single cycle (a)-(d). Simulation parameters: $k(t) = 0.01nM.s^{-1}, \delta = 0.01s^{-1}, \alpha_1 = \beta_1 = 6(nM.s)^{-1}, \alpha_2 = \beta_2 = 1200s^{-1}, k_1 = k_2 = 600s^{-1}$. We choose $\epsilon = 0.01$ 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

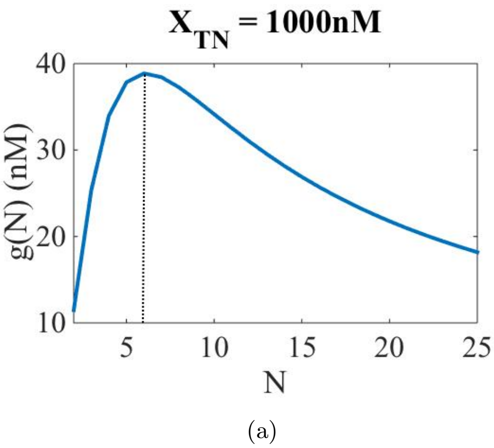

Figure 6: Figures showing the variation of $g(N)$ with N, for $\epsilon = 0.01$ with different loads p_T. Parameter values are: $K_{m1} = K_{m2} = 300nM$, $k_1 = k_2 = 600s^{-1}$, $\lambda = 1$, (a) $p_T = 10nM$, where resulting $\bar{N} = 6$ and (b) $p_T = 1200nM$, where resulting $\bar{N} = 10$.

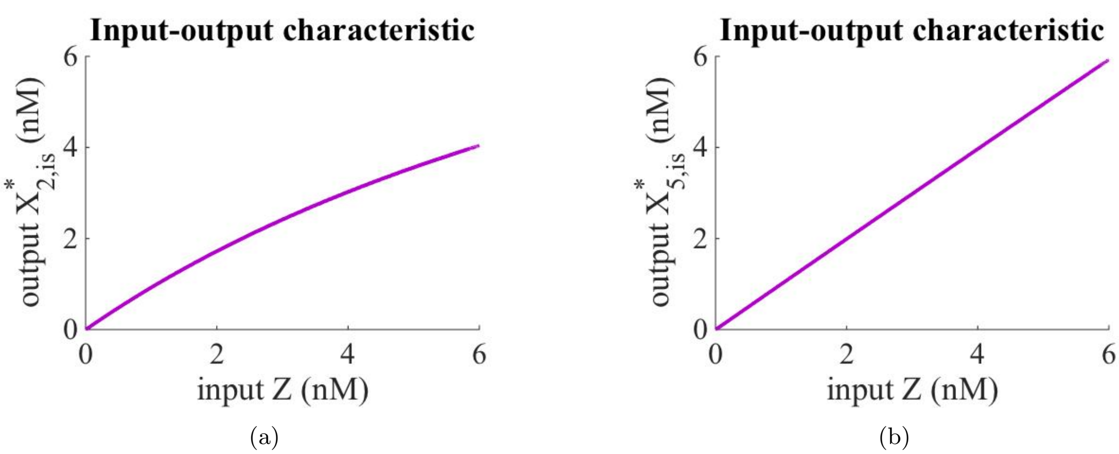

Figure 7: Figures comparing the input-output characteristic for two cascades with different N's. Simulation parameters: $k(t) = 0.01nM.s^{-1}, \delta = 0.01s^{-1}, \alpha_1 = \beta_1 = 6(nM.s)^{-1}, \alpha_2 = \beta_2 = 1200s^{-1}, k_1 = k_2 = 600s^{-1}$. We choose $\epsilon = 0.01$ and load $p_T = 10nM$,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

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