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Importance of Considering the Isotonic System Hypothesis When Modelling the Self-Control of Gene Expression Regulatory Modules in Living Cells

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Abstract

Systems Biology is one of the modern tools, which uses advanced mathematical simulation models for *in-silico* design microorganisms that possess desired characteristics. The deterministic models developed to simulate the cell metabolism biochemistry, are based on a hypothetical (reduced) reaction mechanism, of known kinetics and stoichiometry. A central part of such models concerns the adequate simulation of the protein synthesis homeostatic self-regulation present in any gene expression regulatory module (GERM) that produces enzymes controlling the whole cell metabolism with negative feedback loops and rapid adjustments of the enzymatic activity. However, classical formulations by using the default Constant Volume Whole-Cell (CVWC) continuous variable ordinary differential (ODE) dynamic models do not explicitly consider the cell volume exponential increase during the cell growth leading to biased and distorted conclusions on GERM regulatory performances. This paper exemplifies the overwhelming importance of using a holistic variable-volume whole-cell (VVWC) modelling framework with explicitly including constraints accounting for the cell-volume growth while preserving a constant osmotic pressure and membrane integrity. To point-out the discrepancy between the two simulation approaches, the comparison is made in the case of a simple generic GERM from the *E. coli* cell, by mimicking the cell homeostasis and its response to dynamic perturbations.

Keywords: kinetic modelling; Cell protein synthesis control; Homeostatic regulation; Gene expression regulatory modules (GERM); Variable cell-volume approach

Introduction

Living cells are evolutionary, auto-catalytic, self-adjustable structures able to convert raw materials from environment into additional copies of themselves. Living cells are organized, self-replicating, evolvable, and responsive biological systems to environmental stimuli. The structural and functional cell organization, including components and reactions, is extremely complex, involving $O(10^{3-4})$ components, $O(10^{3-4})$ transcription factors (TF-s), activators, inhibitors, and at least one order of magnitude higher number of (bio)chemical reactions, all ensuring a fast adaptation of the cell to the changing environment [1]. Relationships between structure, function and regulation in complex cellular networks are better understood at a low (component) level rather than at the highest-level [2].

Cell regulatory and adaptive properties are based on *homeostatic* mechanisms, which maintain quasi-constant key-species concentrations and output levels, by adjusting the synthesis rates, by switching between alternative substrates, or development pathways. Cell regulatory mechanisms include allosteric enzymatic interactions and feedback in gene transcription networks, metabolic pathways, signal transduction and other species interactions [3]. In particular, protein synthesis homeostatic regulation includes a multi-cascade control of the gene expression with negative feedback loops and allosteric adjustment of the enzymatic activity [1,4].

The *in-silico* re-design of the cell metabolism is an up-to-date subject in *Synthetic Biology*. But in this effort, *Synthetic Biology* is closely assisted by the *Systems Biology* and by the *Process Biochemistry* focus on the cell organization and component interactions, the formers being one of the main tools in the *in-silico* design of genetically modified micro-organisms (GMO) with desired characteristics, with applications in medicine, industrial biosynthesis, production of biosensors, etc. According to Hood [5], *Systems Biology* is defined as "the science of discovering,

modelling, understanding and ultimately engineering at the molecular level the dynamic relationships between the biological molecules that define living organisms". In other words, *Systems Biology* "aims at understanding the dynamic interaction between components of a living system or between living systems." (http://www.erasysbio.net/). To realize these ambitious objectives, *Systems Biology* uses a wide range of tools, but mainly complex mathematical simulation models linked to -omics databanks [1].

In this context, the adequate modelling of the genetic regulatory circuits (GRC), made from linked GERMs, together with modelling the cell central carbon metabolism (CCM) remain subjects of tremendous importance on which researches have been focus over the last decades [6,7], as long as GRCs are the essential metabolic components used to re-design the whole cell metabolism [1].

To model complex GERMs, intensive efforts have been invested over the last decades, and various types of dynamic models have been proposed, both in a deterministic [8-16], or stochastic approach [15-19]. See also the reviews [1,14,20,21] concerning structured deterministic models with using continuous variables, built-up from time-series experiments [22]. However, to not complicate the resulted simulation model when coupling GERM chains in complex GRC-s, simple GERM dynamic models have been proposed and investigated by [9-12,20,23], etc., with Hill-type [24], or pseudo-Hill-type [25] activation.

A central part of any CCM model concerns self-regulation of the metabolic processes via protein (enzymes) synthesis in GERMs. Consequently, one particular application of the dynamic deterministic cell models is the study of GRC-, in order to predict ways by which biological systems respond to signals, or environmental perturbations. The emergent field of such efforts is the so-called '*gene circuit engineering*' and a large number of examples have been reported with *in-silico* re-creation / design of GRC-s conferring new properties/functions to the mutant cells (i.e. desired 'motifs' in response to external stimuli) [1,21]. Such an effort is facilitated by the use of GERM simulation models.

As mentioned by the pioneers of this field, "with the aid of recombinant DNA technology, it has become possible to introduce specific changes in the cellular genome. This enables the directed improvement of certain properties of microorganisms, such as the productivity, which is referred to as *Metabolic Engineering* [26-28]. This is potentially a great improvement compared to earlier random mutagenesis techniques, but requires that the targets for modification are known. The complexity of pathway interaction and allosteric regulation limits the success of intuition-based approaches, which often only take an isolated part of the complete system into account. Mathematical models are required to evaluate the effects of changed enzyme levels or properties on the system as a whole, using metabolic control analysis or a dynamic sensitivity analysis" [29]. In this context, GERM and GRC dynamic models are powerful tools in developing re-design strategies of modifying genome and gene expression seeking for new properties of the mutant cells in response to external stimuli [1]. Examples of such GRC modulated functions include [30]: toggle-switches, hysteretic GRC behaviour, GRC oscillator, specific treatment of external signals, GRC signalling circuits and cell-cell communicators. The development of dynamic models on a deterministic basis to adequately simulate *in detail* the cell metabolism self-regulation, cell growth, and replication for such an astronomical cell metabolism complexity is practical impossible due to lack of structured information and computational limitations. A review of some trials was presented by [31]. In spite of such tremendous modelling difficulties, development of *reduced* dynamic models to adequately reproduce the cell complex syntheses related to the CCM [29,31,32], but also the GRC[30] tightly controlling the metabolic processes reported significant progresses over the last decades in spite of the lack of structured experimental kinetic information. Even of being rather based on sparse information from various sources, and on unconventional identification lumping algorithms [1,20], such structured deterministic kinetic models have been proved to be extremely useful for *in-silico* design of novel GRC-s [1,21].

As discussed by Maria [1], the classical (default) modelling tools use Constant Volume Whole-Cell (CVWC) continuous variable ODE dynamic models which, do not explicitly consider the cell volume exponential increase during the cell growth. Such an approach may lead to biased and distorted conclusions on the GERM's performances, thus making difficult the modular constructions of GRCs by linking GERM-s. By contrast, the holistic variable-volume whole-cell (VVWC) modelling framework promoted by Maria [1,20] has been proved to be more realistic and robust, by explicitly including in the model relationships the cell-volume growth, with preserving the cell-osmotic pressure. The added isotonicity constraint by Maria [1,20] was proved to be essential for predicting more adequate performance regulatory indices of GERM and GRC.

The scope of this paper is to exemplify, in a simple way, the importance of using a VVWC modelling framework compared to the classical CVWC models when simulating some regulatory properties of GERM-s by explicitly accounting for the cell-volume growth, and system thermodynamic isotonicity (constant osmotic pressure). Exemplification is made for the case of a simple generic GERM model with characteristics taken form *E. coli* cells [20,25,33-35], by mimicking the cell homeostasis and its response to dynamic perturbations. The paper subject importance is very high, as long as a large number of cell simulators are developed and used for practical applications in the biosynthesis industry, and in medicine. The isotonicity constraint is proving to be a natural way to preserve the homeostatic properties of the cell system [1,20,36], instead of imposing others constraints, such as "the total enzyme activity" and "total enzyme concentration" constraints [37].

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Finally, it is to underline the tremendous importance of such structured (i.e. including individual or lumped cellular species), but reduced (lumped) deterministic dynamic models in the Synthetic Biology, Bioinformatics, and Bioengineering. Such deterministic (mechanistic) models are proved to be powerful tools to adequatelly represent essential metabolic processes in the living cells. In particular, kinetic models with continuous variables related to the CCM [13,29,31,32], or to the GRC [1,8,12,14-16] are to be mentioned. In fact, all these deterministic models (constructed to match the experimental kinetic data) allow *in-silico* estimating the metabolic fluxes into the cell (that is the stationary reaction rates under a balanced cell growth, that is the so-called cell homeostasis) [28,38]. These fluxes, expressed in relative terms, indicate how the substrate/nutrients are used into the cell. Such a cell metabolism rough (but quick) evaluation offers the possibility to eventually *in-silico* re-programming the cell metabolism, by removing or amplifying some reactions, { via acting on their associated enzymes, i.e. the so-called "gene knockout strategy"; see [7,38,42-44], or by cloning cells with plasmids [39,40]}. In such a manner, new microorganisms with desirable properties/functions ('motifs') are design, of practical applications in the biosynthesis industry, environmental engineering, and medicine [1,21,36]. At this point, it is to mention that dynamic cell models using continuous variables, in spite of some disadvantages, they present a large number of advantages (see the discussion of [20,31,36,41]).

The CVWC vs. VVWC Modelling Framework

CVWC formulation

For a system of chemical or biochemical reactions conducted in a (cell) closed volume V (assumed an open system of uniform content), the classical (default) formulation of the corresponding (bio)chemical kinetic model based on continuous variables (concentration vector C, or number of moles vector n) implies writing an ODE mass balance including the considered system states (biological/chemical species), in the following CVWC formulation (with referring to the whole system volume):

$$\frac{1}{V(t)}\frac{dn_j}{dt}=\sum_{i=1}^{nr}\upsilon_{ij}r_i(\boldsymbol{n}/V,\boldsymbol{k},t)=h_j(\boldsymbol{C},\boldsymbol{k},t)$$

$$\frac{d(n_j/V)}{dt}=\frac{dC_j}{dt}=\sum_{i=1}^{nr}\upsilon_{ij}r_i(\boldsymbol{n}/V,\boldsymbol{k},t)=h_j(\boldsymbol{C},\boldsymbol{k},t) \quad (1a\text{-}b)$$

The above formulation assumes a homogeneous constant volume with no inner gradients or species diffusion resistance into the cell. When continuous variable ODE dynamic models are used to model cell enzymatic/metabolic processes, the default-modelling framework Eq. (1) is that of a constant volume and, implicitly, of a constant osmotic pressure (π) in isothermal systems, according to the assumed fulfilled Pfeffer's law in diluted solutions (i.e. the cytosol system) [20,45]:

$$\pi\, V(t)=RT\sum_{j=1}^{n_S}n_j(t) \quad (2)$$

Eventually some CVWC models, accounts for the cell-growing rate as a pseudo-'decay' rate of key-species (often lumped with the degrading rate) in a so-called 'diluting' rate (denoted here by an average Ds see below for its significance). In fact, by ignoring the direct influence of the cell volume increase, the CVWC dynamic model cannot ensure the system isotonicity constraint fulfilment because the sum of species number of moles doubles over the cell cycle. Such a CVWC dynamic model might be satisfactory for modelling many cell subsystems, but not for an accurate modelling of cell regulatory / metabolic processes under perturbed conditions, or for division of cells [46], distorting the prediction quality, as reviewed by Maria [1]. Other researchers [37] tried to preserve the homeostatic properties of the cell system, not by imposing the isotonic constraint Eq. (2), but "the total enzyme activity" and "total enzyme concentration" constraints.

VVWC formulation

At this point, it is to strongly emphasize that living cells are systems of variable volume. They double their volume during the cell cycle. For chemical or biochemical systems of variable-volume, another formulation is more appropriate, being given by Aris [47] and later promoted by Maria [1,20] in modelling cell subsystems by also including the cell isotonicity constraint in the so-called VVWC modelling framework. In mathematical terms, the species mass balance Eq. (1) should be re-written in the form of:

$$\frac{dC_j}{dt}=\frac{d}{dt}\left(\frac{n_j}{V}\right)=\frac{1}{V}\frac{dn_j}{dt}-\frac{n_j}{V}\frac{dV}{V\,dt}=\frac{1}{V}\frac{dn_j}{dt}-DC_j=h_j(C,k,t) \quad (3)$$

$$D=\frac{d(\ln(V))}{dt}$$

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At this point, it is to remark two possibilities to calculate the cell dilution D necessary for solving the model Eq. (3). The simplest, but not the accurate one, is to use a value averaged over the cell cycle, that is:

$$\frac{1}{V}\frac{dV}{dt} = D_S \text{, leading to } V(t) = V_0 \exp(D_S\, t) \quad (4a)$$

By accounting the cell double volume at the end of the cell-cycle, then Ds can be a-priori evaluated by using the following relationship (for cells of known cell cycle):

$$\ln\left(\frac{2V_0}{V_0}\right) = D_S\, t_c, \quad \text{and } D_S = \ln(2)/t_c \quad (4b)$$

The second alternative to evaluate the cell dilution D is those to impose a constraint accounting for the cell-volume growth while preserving a constant osmotic pressure and membrane integrity. Thus, by derivation of the Pfeffer's law Eq. (2) in respect to V, and by division to V, one obtains the "isotonic" dilution rate Di [20]:

$$D_i = \frac{1}{V}\frac{dV}{dt} = \left(\frac{RT}{\pi}\right)\sum_j^{n_S}\left(\frac{1}{V}\frac{dn_j}{dt}\right) \quad (5)$$

It is to observe in Eq. (5) that the cell content dilution rate Di is linked to the all species (taken individually or lumped) reaction rates via the isotonicity constraint. As species reaction rates varies during the cell cycle, it clearly results that formulation Eq. (5) offers a more accurate estimation of the (variable) cell dilution at any time. Such a system isotonicity constraint is more natural and eventually includes "the total enzyme activity" and "total enzyme concentration" constraints suggested by Komasilovs et al. [37].

Table 1: Variable cell-volume whole-cell VVWC dynamic modelling framework, and its basic hypotheses (adapted from [20]).

Mass Balance and State Equations	Remarks
$\frac{dC_j}{dt} = \frac{1}{V}\frac{dn_j}{dt} - D\,C_j = g_j(\boldsymbol{C},\boldsymbol{k})$	continuous variable dynamic model representing the cell growing phase (ca. 80% of the cell cycle)
$\frac{1}{V}\frac{dn_j}{dt} = r_j(\boldsymbol{C},\boldsymbol{k})\ ;\ j = 1,\ldots,n_r$	
$V(t) = \frac{RT}{\pi}\sum_{j=1}^{n_s} n_j(t)$	Pfeffer's law in diluted solutions
$D = \frac{1}{V}\frac{dV}{dt} = \left(\frac{RT}{\pi}\right)\sum_j^{n_s}\left(\frac{1}{V}\frac{dn_j}{dt}\right)$	D = cell content dilution rate = cell volume logarithmic growing rate
$\frac{RT}{\pi} = \frac{V}{\sum_{j=1}^{n_S} n_j} = \frac{1}{\sum_{j=1}^{n_S} C_j} = \frac{1}{\sum_{j=1}^{n_S} C_{jo}}$ constant.	constant osmotic pressure (π) constraint
$\left(\sum_j^{all} C_j\right)_{cyt} = \left(\sum_j^{all} C_j\right)_{env}$	Derived from the isotonic osmolarity constraint
Hypotheses:	
a. Negligible inner-cell gradients.	
b. Open cell system of uniform content.	
c. Semi-permeable membrane, of negligible volume and resistance to nutrient diffusion, following the cell growing dynamics.	
d. Constant osmotic pressure (the same in cytosol "cyt" and environment "env"), ensuring the membrane integrity ($\pi_{cyt} = \pi_{env}$ = constant).	
e. Nutrient and overall environment species concentration remain unchanged over a cell cycle t_c.	
f. Logarithmic growing rate of average $D_s = \ln(2)/t_c$; volume growth of ; $V = V_o e^{D_s t}$; t_c = duration of the cell cycle.	
g. Homeostatic stationary growth of $\left(dC_j/dt\right)_s = g_j(C_s,k) = 0$.	
h. Perturbations in cell volume are induced by variations in species copynumbers under the isotonic osmolarity constraint: $V_{perturb}/V = \left(\sum n_j\right)_{perturb} / \left(\sum n_j\right)$.	

Notations: T = absolute temperature; R = universal gas constant; V= cell (cytosol) volume; π = osmotic pressure; C_j = cell species j concentration; n_j = species j number of moles; r_j = j-th reaction rate; t = time; k =rate constant vector; "s" index indicates the stationary state.

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The basic hypotheses of the VVWC dynamic models of type Eqs. (3-5) are briefly presented in the Table 1. These formulations are valid over ca. 80% of the cell cycle representing the balanced cell growth before its division [46].

GERM Lumped Models

As pointed-out in the literature [1], the genetic regulatory systems (GRC), that control the synthesis of all proteins (enzymes) in the cell, present a modular construction, every operon (a cluster of genes under the control of a single promoter) including a variable number of interacting GERM-s. However, it is well-known that one GERM interacts with no more than other 23-25 GERM-s [48], while most of GERM structures are repeatable. Consequently, in developing the GRC analysis, the modular approach is preferred due to several advantages: a separate analysis of the constitutive GERM-s in conditions that mimic the stationary or perturbed cell growth. The modules are then linked to construct GRC of an optimized regulatory efficiency that ensures key-species homeostasis and network holistic properties. Investigation of GERM and GRC characteristics is focus on the tight control of gene expression, the quick dynamic response, the high sensitivity to specific inducers, and the GRC robustness (i.e. a low sensitivity vs. undesired inducers). Such advanced regulatory structures must ensure the homeostasis (quasi-stationarity) of the regulated key-species, and quick recovery (with a trajectory of minimum amplitude) after a dynamic (impulse-like) or stationary (step-like) perturbation of one of the involved metabolites or nutrients [1].

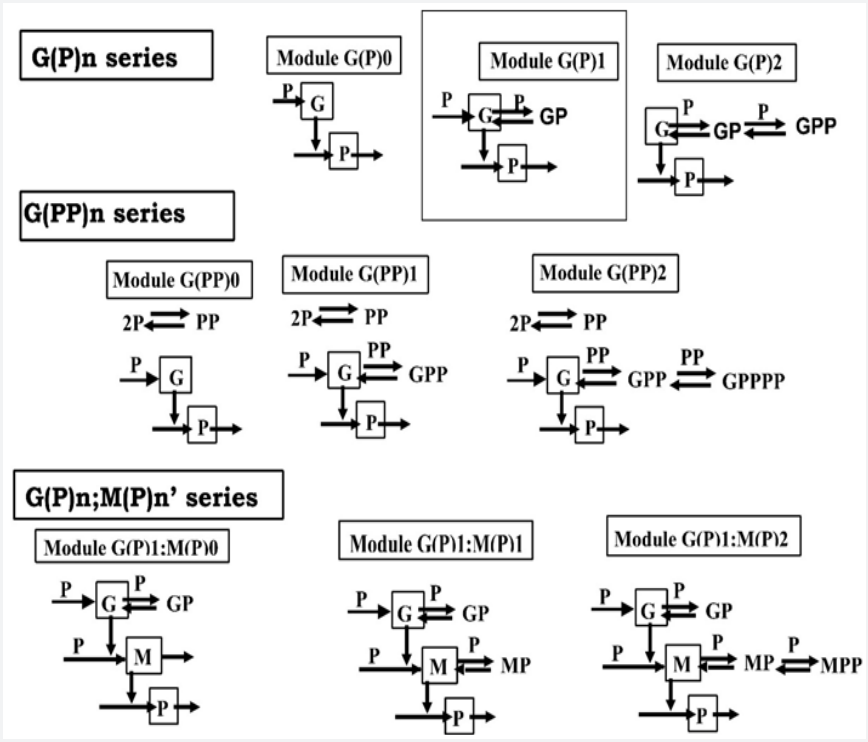

Figure 1: Simplified representations of a generic gene expression G/P regulatory module (GERM) following [20]. The horizontal arrows indicate reactions; vertical arrows indicate catalytic actions; absence of a substrate or product indicates an assumed concentration invariance of these species; G= gene encoding P; M= mRNA. Up-row: simplified representation of the gene expression model corresponding to [G(P)n] regulatory module types. The transcriptional factor is the protein P itself, the self-regulation over the transcription and translation steps being lumped together [20]. To improve the system homeostasis stability and self-regulation, despite of perturbations in nutrients Nut*, and metabolites Met*, or of internal cell changes, a very rapid buffering reaction G + P <===> GP(inactive) has been added. Middle-row. simplified representation of the gene expression model corresponding to a [G(PP)n] regulatory module types. The transcriptional factor is the dimmer PP. Down -row. simplified representation of the gene expression model corresponding to [G(P)1; M(PP)n] regulatory module types. The models account for the cascade control of the expression via the separate transcription and translation steps.

Notations: G= DNA gene encoding P; M= mRNA; P,PP= allosteric effectors of the transcription / translation. Adapted from [20,49] by courtesy of CABEQ JI.

In order to not complicate the deterministic models, lumped GERM and GRC structures have been adopted in the literature. Some of them are presented in the Figure 1. The simplest GERM structure with one regulatory element is those denoted by G(P)1, also approached in this paper. The generic G(P)1 regulatory module (also represented schematically in Figure 2), refers to the synthesis of a generic protein P and the simultaneous replication of its encoding gene G. The lumped G(P)1 model includes only one regulatory element, that is a fast "buffer" reversible reaction G + P <===> GP(inactive) (Figure 2), aiming at controlling the P synthesis rate and its homeostatic (quasi-stationary, QSS) level. In such a generic lumped construction, the protein P and its encoding gene G

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mutually catalyses the synthesis of each other. The protein P is the "control node" playing multiple roles in such a simplified lumped representation. Thus, P is a permease leading to the import of nutrients NutG, NutP in the cell, but also a metabolase converting the nutrients into precursors MetG and MetP of the G and P respectively. Protein P is also a polymerase catalysing the gene replication. And, finally, the protein P is also a transcriptional factor by dynamically adjusting the catalytic activity of the G by means of a very rapid "buffer" regulatory reaction G + P <===> GP(inactive). When P is produced in excess, it reversible inactivates more amount of G, which in turn, will slow-down the P synthesis. When P is produced in too low amounts, the regulatory process goes backwards. As proved in previous works [1], when acting as a transcriptional factor, P is a better effector if it is present in a dimeric form acting at both G and M levels of the expression (middle and down-rows of Figure 1), thus developing a cascade control scheme of the expression where transcription and translation regulatory steps are separately considered [1].

Estimate the Rate Constants and Simulate A G(P)1 Module Dynamics and its Self-Regulation after A Dynamic Perturbation using the VVWC Approach

In order to simply illustrate the predictive capabilities of VVWC dynamic models, one considers a generic GERM of G(P)1 type (with the lumped reaction scheme given in Figure 2). The reaction rate expressions for the G(P)1 model are given in Eq. (6). The species mass balance can be formulated in both number of moles (copynumbers) by using the notation *nj* = [*n*-moles] of species *j*, and concentrations $C_j = n_j(t) / V_{cyt}(t)$, [nM], where $V = V_{cyt}$.

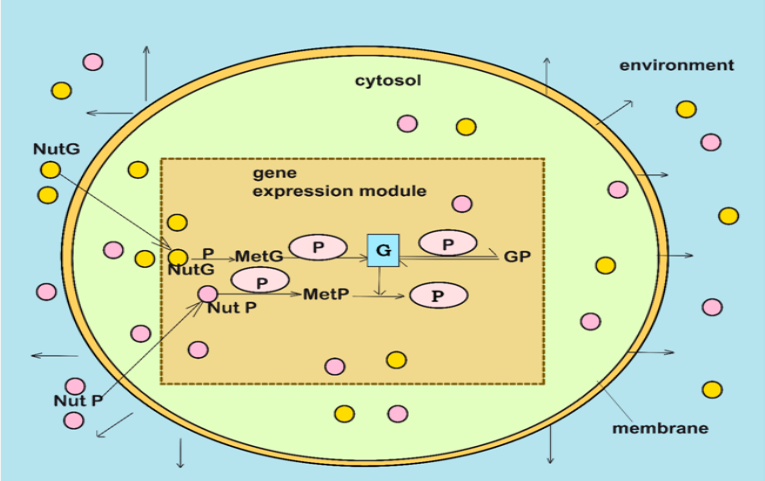

Figure 2: The simplified reaction scheme of a generic gene G expression, by using a regulatory module of G(P)1 type. The model was used to exemplify the synthesis of a generic P protein in the *E. coli* cell by [20]. To improve the system homeostasis stability, that is quasi-invariance of key species concentrations (enzymes, proteins, metabolites), despite of perturbations in nutrients Nut*, and metabolites Met*, or of internal cell changes, a very rapid buffering reaction G(active) + P <===> GP(inactive) has been added. Horizontal arrows indicate reactions; vertical arrows indicate catalytic actions; G = active part of the gene encoding protein P; GP = inactive part of the gene encoding protein P; MetG, MetP = lumped DNA and protein precursor metabolites, respectively.

$$\begin{cases} NutG + P \xrightarrow{k_1} MetG + P\,,\ r_1 = k_1 C_{NutG} C_P \\ NutP + P \xrightarrow{k_2} MetP + P\,,\ r_2 = k_2 C_{NutP} C_P \\ MetG + P \xrightarrow{k_3} G + P\,,\quad r_3 = k_3 C_{MetG} C_P \\ MetP + G \xrightarrow{k_4} P + G\,,\quad r_4 = k_4 C_{MetP} C_G \\ P + G \xrightarrow{k_5} GP\,,\quad r_5 = k_5 C_P C_G \\ GP \xrightarrow{k_6} G + P\,,\quad r_6 = k_6 C_{GP} \\ C_j = n_j(t)/V(t) \end{cases} \qquad (6)$$

The formulated CVWC model in Eq. (7-left) does not explicitly includes the dilution term *D*. However, to ensure a certain model consistency of the CVWC when it is necessary to evaluate the cell volume *V(t)*, e.g. when the model Eq. (7-left) is solved in terms of the number of moles nj , or when nj (t) are required, an average dilution *Ds* Eq. (4a-b) is assumed. By comparison, in the below VVWC

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dynamic model formulation Eq. (7-right) the cell content dilution rate *Di* is computed during the cell cycle by using Eq. (5) and the isotonicity constraint related to the all species reaction rates (taken individually or lumped):

Table 2: The nominal stationary homeostatic (QSS) lumped / individual species concentrations $C_{j,s}$ of the analysed *E. coli* cell, and species recovering times of the steady-state in a GERM of [G(P)1] type after a –10% impulse perturbation in the key-protein stationary $[P]_s$ produced at an arbitrary moment t =0. Values are adopted following the example of Maria[20]. nM= nano-molar.

Species	Homeostatic level [nM]
Lump $C_{NutG,s}$	$3*10^6$ (adopted)
Lump $C_{NutP,s}$	$3*10^8$ (adopted)
Lump $\sum_j C_{MetG_{j,s}}$ (note a)	approx. 10^6
Lump $\sum_j C_{MetP_{j,s}}$	$3*10^8$ (adopted)
$C_{P,S}$	1000 (adopted)
$C_{G,S} = C_{G,P,S} = 0.5$	(adopted; see note b)
t_c	Cell life cycle of 100 min
$D_s = \ln 2 / t_c$.	Cell-volume logarithmic growing rate (average) [1/min]
V_o, Cell (cytosol) initial volume	$1.660434503\ 10^{-15}$ (L)

Footnotes:

(a) Evaluated from the isotonicity constraint $\sum_{all\ j}^{cell} C_j = \sum_{all\ j}^{env} C_j = C_{NutG} + C_{NutP} = \Sigma_j C_{MetG_j} + \Sigma_j C_{MetP_{j,s}}$

(b) Adopted to ensure the maximum responsiveness of the GERM steady-state (see the discussion of Maria [1,20,25,30,49].

Footnotes: Max(Re(λ_j)) < 0 indicates a stable cell quasi-stady-state (QSS, homeostasis), where λ_j are the Jacobian eigenvalues of the VVWC kinetic model of GERM. Lumps NutP and NutG denote substrates used in the synthesis of metabolites MetP and MetG respectively, former being further used for the P and G synthesis. Notations: G= a generic gene (DNA) from *E. coli* cell; P= the protein encoding G; M= mRNA; GP= the inactive complex of G with the transcription factor P; Cj = species "j" concentration; Indices cyt= cytoplasma; "0"= initial; 's' refers to the QSS; NG = negligible.

(*) Species copynumbers correspond to the *E. coli* cell K-12 strain (EcoCyc [33]; Allen & Kornberg[34]; details of Maria[25]). Species concentrations in the cell are computed with the formula of Maria[20]:
$\text{Concentration} = \frac{\text{no. of copies/cell}}{N_A \times V_{cyt,0}}$, where N_A is the Avogadro number. For instance, for an *E. coli* cell with an approximate volume of $V_{cyt,0}$ = 1.66 10^{-15} L [35], concentration of one gene G copynumber value is: [G]s= (1/(6.022×10^{23})(1.66×10^{-15}) = 1nM (that is 10-9mol/L).

(**) The lump $\Sigma_j C_{\mathrm{MetG}_{j,s}}$ results from the isotonicity constraint $RT/\pi_{cyt} = RT/\pi_{env}$, which translates into: $\sum_{all\ j}^{cell} C_{j,s} = \sum_{all\ j}^{env} C_{j,s} = C_{NutG,s} + C_{NutP,s} = \Sigma_j C_{\mathrm{MetG}_{j,s}} + \Sigma_j C_{\mathrm{MetP}_{j,s}}$

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CVWC $\begin{cases} \frac{dC_{MetG}}{dt} = r_1 - r_3 \\ \frac{dC_{MetP}}{dt} = r_2 - r_4 \\ \frac{dC_P}{dt} = r_4 - r_5 + r_6 \\ \frac{dC_G}{dt} = r_3 - r_5 + r_6 \\ \frac{dC_{GP}}{dt} = r_5 - r_6 \\ r_j(C_j);\ C_j = n_j(t)/V(t) \\ V(t) = V_0 \exp(D_s\ t) \\ D_s = \ln(2)\ /\ t_c \end{cases}$ versus VVWC $\begin{cases} \frac{dC_{MetG}}{dt} = r_1 - r_3 - D \cdot C_{MetG} \\ \frac{dC_{MetP}}{dt} = r_2 - r_4 - D \cdot C_{MetP} \\ \frac{dC_P}{dt} = r_4 - r_5 + r_6 - D \cdot C_P \\ \frac{dC_G}{dt} = r_3 - r_5 + r_6 - D \cdot C_G \\ \frac{dC_{GP}}{dt} = r_5 - r_6 - D \cdot C_{GP} \\ r_j(C_j);\ C_j = n_j(t)/V(t) \\ D_i = \frac{1}{V}\frac{dV}{dt} = \left(\frac{RT}{\pi}\right)\sum_j^{n_S}\left(\frac{1}{V}\frac{dn_j}{dt}\right) \end{cases}$ (7)

By contrast, the VVWC model Eq. (7-right) explicitly includes the cell dilution term *D*, accurately evaluated any time as *Di* with the Eq. (5) which, in the present case study, translates in:

$$D_i = \frac{RT}{\pi}(r_1 + r_2 - r_5 + r_6). \quad (8)$$

The term (RT/ p) is evaluated from the cell initial state, by using the Pfeffer's law Eq. (2):

$$V(t) = \frac{RT}{\pi}\sum_{j=1}^{n_S} n_j(t) \Rightarrow \frac{RT}{\pi} = \frac{V(t)}{\sum_{j=1}^{n_S} n_j(t)} = \frac{1}{\sum_{j=1}^{n_S} C_j} = \frac{1}{\sum_{j=1}^{n_S} C_{j,0}} = \text{constant} \quad (9)$$

Table 3: Estimated rate constants for the model G(P)1 in the VVWC formulation. The units of the 1st order reactions are min^{-1} , while those of the 2nd order are $nM^{-1}min^{-1}$. Species stationary concentrations are those of Table 2. *V* denotes the cell (cytosol) volume. (*) Adopted k6 value much larger than *Ds* and the other rate constants, see the discussion of Maria [20].

Reaction	*Rate expression*	*Estimated rate constant*
$NutG + P \rightarrow MetG + P$	$r_1 = k_1(n_{NutG}/V_{env})(n_P/V)$	6.929161×10^{-6}
$NutP + P \rightarrow MetP + P$	$r_2 = k_2(n_{NutP}/V_{env})(n_P/V)$	6.931494×10^{-6}
$MetG + P \rightarrow P + G$	$r_3 = k_3(n_{MetG}/V)(n_P/V)$	2.311261×10^{-12}
$MetP + G \rightarrow G + P$	$r_4 = k_4(n_{MetP}/V)(n_G/V)$	4.623291×10^{-8}
$P + G \rightarrow GP$	$r_5 = k_5(n_P/V)(n_G/V)$	$1.000000069\times10^{+2}$
$GP \rightarrow P + G$	$r_6 = k_6(n_{GP}/V)$	(*) $1\times10^{+5}$ (*)

At this point, it is to observe that, in a VVWC model formulation, all cell species should be considered (individually or lumped), because all species net reaction rates contribute to the cell volume increase Eq. (9). In the present case, the rest of the cell content was mimicked by adopting large concentrations for the lumped NutP and NutG (Table 2). Solving the models Eq. (7) is made with using a stiff integrator ("ODE15s") of Matlab™ package due to the very fast buffering reactions no. 5-6 in Eq. (6) compared to the rest of reactions (see the discrepancy in the rate constants in the Table 3). The number of moles nj (t) can be calculated at any time with *nj(t)= Cj(t) V(t)* , by using the species concentrations Cj(t) derived from solving the ODE model.

The rate constants of the dynamic VVWC model Eq. (7-right) have been estimated from the steady-state (homeostatic) initial condition of the cell *(dCj/dt)s = gj(Cs,k)*= 0, that is:

$$\begin{cases} 0 = r_{1,s} - r_{3,s} - D_s \cdot C_{MetG,s} \\ 0 = r_{2,s} - r_{4,s} - D_s \cdot C_{MetP,s} \\ 0 = r_{4,s} - r_{5,s} + r_{6,s} - D_s \cdot C_{P,s} \\ 0 = r_{3,s} - r_{5,s} + r_{6,s} - D_s \cdot C_{G,s} \\ 0 = r_{5,s} - r_{6,s} - D_s \cdot C_{GP,s} \end{cases} \quad (10)$$

By replacing in Eq. (10) the stationary concentrations of Table 2 (taken for a GERM from *E. coli* cell K-12 strain), the resulting nonlinear algebraic set is solved analytically by using the MAPLE™ package, thus yielding the following analytical solution:

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$$k_1 = \frac{D(C_{MetG} + C_G + C_{GP})}{C_{NutG} C_P}, k_2 = \frac{D(C_{MetP} + C_P + C_{GP})}{C_{NutP} C_P}, k_3 = \frac{D(C_G + C_{GP})}{C_{MetG} C_P}, \quad (11)$$

$$k_4 = \frac{D(C_P + C_{GP})}{C_{MetP} C_G}, \quad k_5 = \frac{k_6 C_G + D C_{GP}}{C_P C_G}$$

The numerical values of the rate constants for the initial cell condition, and an average *Ds* are given in the Table 3. For the rapid buffer reaction G + P <===> GP, the reverse reaction rate constant k6 (not estimable) was adopted at a value much larger than *Ds* (see the discussion of [49]). The rate constants of the CVWC model Eq. (7-left) cannot be estimated on the same way because of singularities of the resulting nonlinear algebraic set. Consequently, the same rate constants of VVWC model were used instead.

Here it is to observe that the values of the lump $\Sigma_j C_{MetG_{j,s}}$ results from the isotonicity constraint (ensuring the membrane integrity) *(RT/ πcyt) = (RT/ πenv)* which, using Eq. (9), indicates that the sum of cell species concentrations must equal those of the environment, i.e. $\left(\sum_j^{all} C_j\right)_{cyt} = \left(\sum_j^{all} C_j\right)_{env}$. Otherwise, the osmosis will eventually lead to an equal osmotic pressure πcyt = πenv. Even if, in a real cell, such equality is approximately fulfilled due to perturbations and transport gradients, and in spite of migrating nutrients from environment into the cell, the overall environment concentration is considered to remain unchanged. On the other hand, species inside the cell transform the nutrients into metabolites and react to make more cell components. In turn, increased amounts of polymerases are then used to import increasing amounts of nutrients. The net result is an exponential increase of cellular components in time, which translates, through isotonic osmolarity assumption, into an exponential increase in volume with time [see Eq. (4a)]. The environment volume hypothetical variable *Venv* necessary to evaluate environment species concentrations when solving the cell model of Table 3, is evaluated from the same isotonicity constraint at the initial state, that is: $V_{env,0}/V_{cyt,0} = \left(\sum_j^{all} C_j\right)_{env,0} / \left(\sum_j^{all} C_j\right)_{cyt,0}$, with the environment species from the Table 2 (the "lump NutG", and the "lump NutP").

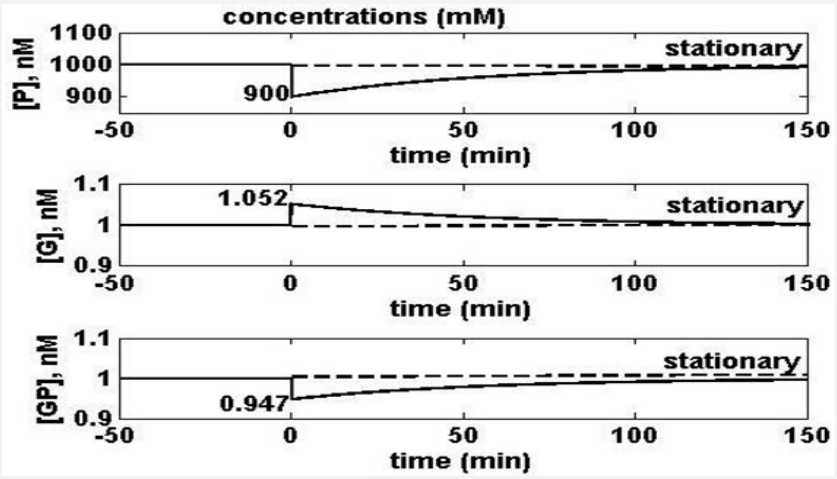

Figure 3: Exemplification of the self- and mutual G/P pair catalysis and regulation after an impulse perturbation of –10% in the steady-state $[P]_s$ = 1000 nM (from 1000nM to 900nM) at an arbitrary time t=0 for a generic GERM of [G(P)1] type. Simulated species dynamics (solid line) in a VVWC approach are generated starting from the cell nominal stationary conditions of Table 2, but with adopting $[G]_s$ = $[GP]_s$ =1nM. The dashed lines indicate steady-state concentrations. G = active part of the gene encoding the protein P; GP = inactive part of the gene G.

To exemplify how the self-control of the protein synthesis works due to the rapid *buffer* reaction G + P <===> GP, one starts from the QSS cell nominal stationary conditions of Table 2, but with adopting $[G]_s$ =$[GP]_s$ = 1 nM, and one applies a dynamic perturbation (impulse like) at an arbitrary moment t=0 (Figure 3), by diminishing the stationary [P]s from 1000nM to 900nM (for a GERM of G(P)1 type). As a result, the regulatory buffer system leads to a quickly [G]s increase from 1nM to 1.052nM (due to less P in the buffer reaction, on the expense of GP which displays an accordingly decrease of $[GP]_s$ from 1nM to 0.947nM) which, in turn, will speed-up the P synthesis enough to recover the initial [P]s in ca. 127min. (with an acceptable tolerance of 1%).The same regulatory mechanism also applies to the $[G]_s$, controlled by the same buffer reaction, the recovering time being of ca. 118 min. If one repeats the simulation, but with an initial $[G]_s$ =$[GP]_s$= 0.5nM then, as presented in the Table 2, the recovering time of [P]s is 133min., and 93 min. for [G]s , respectively. It is to be noted that in wild *E. coli* cells $[G]_s$ is around 1nM (see the proof in the footnote of Table 2), but in cloned cells with plasmids, this level can be higher.

To complete the discussion, one simulates also the dynamics of cell components present in large amounts (MetP, MetG), with using the same initial QSS of Table 2, but with adopting a stationary level of $[G]_s$= $[GP]_s$= 0.5nM (to get a maximum regulatory efficiency; see

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[20,49]). By applying a dynamic perturbation to $[P]_s$ steady-state for a generic GERM of [G(PP)1] type, that diminishes the stationary [P]s from 1000 nM to 900nM, simulation with the identified VVWC model Eqs. (7-9) is leading to the species trajectories displayed in Figure 4, proving again the good self-control of the P-synthesis produced by the lumped G(P)1 GERM model.

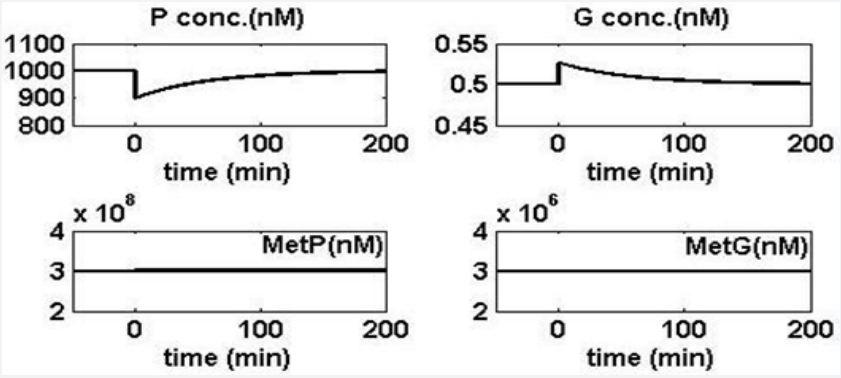

Figure 4: Exemplification of the self- and mutual G/P pair catalysis, in a VVWC approach, after an impulse perturbation in the $[P]_s$ = 1000 nM leading to a -10% decline of the steady-state at an arbitrary time t=0 for a generic [G(PP)1] model. Simulated main species dynamics in a VVWC approach starting from the nominal stationary conditions of Table 2, with adopting $[G]_s$= $[GP]_s$ =0.5nM.

It is self-understood that, as the regulatory scheme (in Figure 1) is more effective, as the GERM regulatory efficiency is better. For an extensive discussion on regulatory properties of various GERM-s the reader is referred to the review of Maria[1].

Illustrate Superiority of VVWC Vs. CVWC Models

In order to simply illustrate the discrepancy in predictive capabilities between VVWC and CVWC kinetic models, and how deceptive can the predictions of such CVWC kinetic models be, one considers the same example of a generic GERM in the *E. coli* cell of G(P)1 type illustrated in the chap. 4. The reaction scheme, reaction rate expressions for the G(P)1 gene expression module are those given in Eq. (6), while the species mass balances are given in Eq. (7) for both CVWC and VVWC formulations.

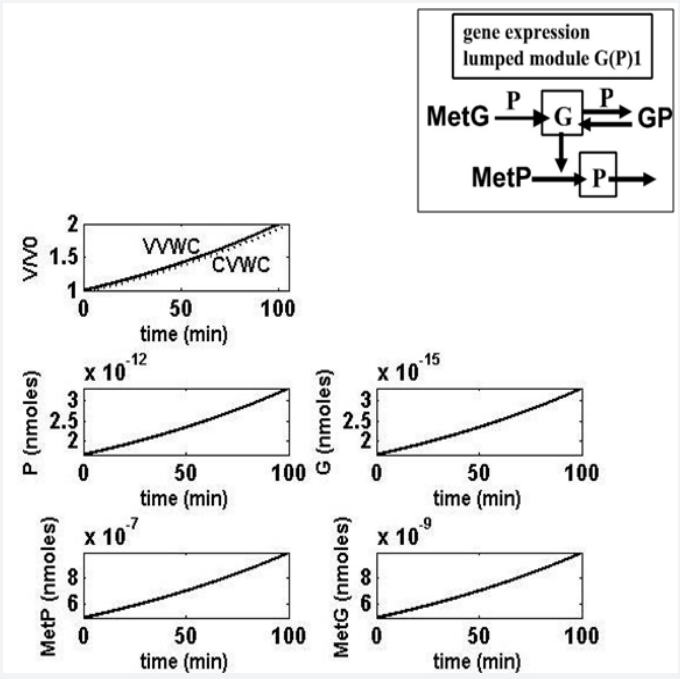

Figure 5: Dynamics of the cell volume (up-row)(_____ generated with the VVWC model and Di ; ------- generated with the CVWC model and D_s), and the dynamics of species copynumbers *nj* during the cell cycle predicted by the VVWC model for the G/P expression using a regulatory module of G(P)1 type. The *E. coli* cell species homeostatic concentrations are those of Table 2.

After rate constant estimation in the G(P)1 VVWC model by using the *E. coli* cell data with the initial homeostatic concentrations of Table 2, simulations of one cell cycle by using the VVWC model lead to obtaining the species copynumber dynamics plotted in Figure 5. It is to observe that, while the cell volume doubles, the species copynumbers double as well. However, there is a very small

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discrepancy in the predicted cell-volume dynamics because the average *Ds* used by the CVWC model is slightly different from the *Di* used by the VVWC model.

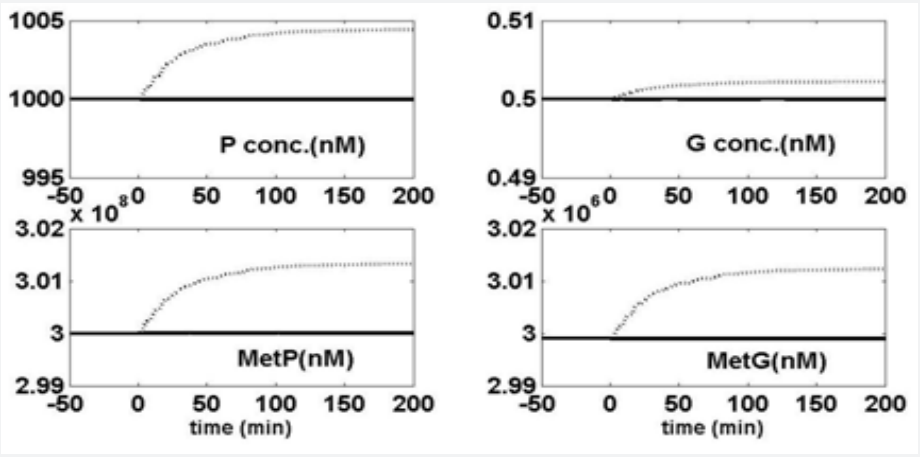

Figure 6: Dynamics of species concentrations (in nM) during the cell cycle, ***without any perturbation***, predicted by the constant volume CVWC model (------- , with an average dilution Ds in the model), compared to those predicted by the variable volume VVWC model (_____ , with dilution Di estimated from the isotonicity constraint). The generic G/P expression is using a regulatory module of G(P)1 type. The E. coli cell species homeostatic concentrations are those of Table 2. Model predictions of the two models are very close to each other (in relative values).

If the species dynamics is plotted in terms of concentrations (referred to the cell cytosol volume), the predicted trajectories for the *un-perturbed* cell growth case, given in Figure 6 by the CVWC model and the VVWC model are very close to each other, the difference being negligible (in relative terms). That is because, over a large time-domain, the formula Eq. (4a-b) satisfactorily averages the detailed formula Eq. (5). In Figure 6 both models correctly indicate how the key-species stationary values are preserved under balanced cell growth conditions. However, differences in the predictions of the two models better appear *under perturbed* growing conditions. Thus, the recovering trajectories predicted by the CVWC model and by the VVWC model after an impulse-like perturbation by diminishing the stationary $[P]_s$ from 1000nM to 900 nM, are quite different as revealed by the plots of Figure 7. The differences in model predictions are as larger as the species present a lower level in the cell (that is for P, G, and GP species).

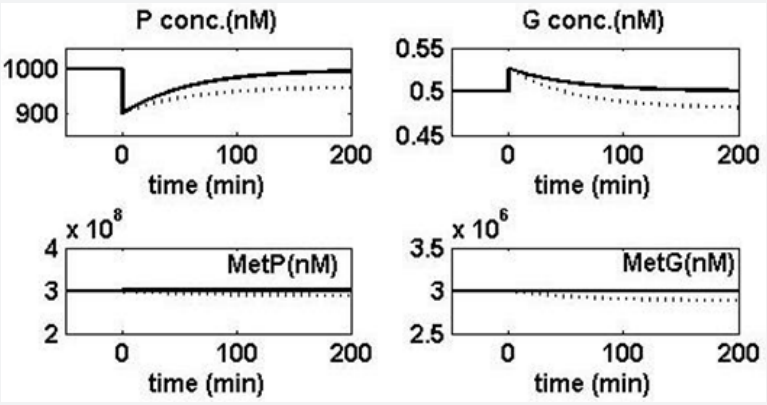

Figure 7: Dynamics of species concentrations (in nM) during the cell cycle, ***under perturbed conditions*** (after an impulse perturbation in the $[P]_s$ = 1000 nM leading to a 10% decline of the steady-state at an arbitrary time t=0), predicted by the constant volume **CVWC** model (------- , with an average dilution D_s in the model), compared to those predicted by the variable volume VVWC model (_____ , with dilution D_i estimated from the isotonicity constraint). The generic G/P expression is using a regulatory module of G(P)1 type. The *E. coli* cell species initial homeostatic concentrations are those of Table 2.

Consequently, while the VVWC model correctly reproduces the system homeostasis recover after a perturbation, species concentrations being kept quasi-constant because both nominator and denominator of the fraction *Cj (t)= nj (t) / Vcyt (t),* [nM], are doubling at the same rate. By contrast, the CVWC model predictions are inaccurate, the predicted species concentration dynamics under perturbed conditions with using an average formula for the cell content dilution being very biased. So, the CVWC models cannot be used in a satisfactory manner to simulate the regulatory properties of GERM and GRC [1]. One can conclude that the VVWC model formulation better reflects the GERM regulatory properties after dynamic (impulse-like) or stationary (step-like, not

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discussed here) internal or external perturbations.

Conclusions

As revealed by the simple but eloquent example discussed in this study, there are important issues to be considered when developing modular VVWC models of GRC-s including a variable number of GERM-s.

The reviewed simple case studies of VVWC modular kinetic models of GERM-s proved that the chemical and biochemical engineering principles, together with the control theory of the nonlinear systems are fully applicable to modelling complex metabolic cell processes, including sophisticated GRC-s controlling the cell enzyme's syntheses and metabolic fluxes. The ODE kinetic models with continuous variables are fully feasible alternatives to well describe the cell response to stationary or dynamic continuous perturbations from the environment [36].

In fact, such cell process models 'translate' from the 'language' of molecular biology to that of mechanistic chemistry and mathematics/computing languages, trying to preserve the structural, functional, and timing hierarchy of the cell components and functions. To avoid very extended ODE cell kinetic models, difficult to be identified, and to be used, lumped model structures should be used with a lumping degree chosen to ensure a satisfactory trade-off between model simplicity and its predictive quality [49].

As discussed by Maria[1], the classical (default) CVWC continuous variable ODE dynamic models are not always recommended. By not explicitly considering the relationship between the cell volume exponential increase during the cell growth, the osmotic pressure, and species reaction rates, they can lead to biased and distorted conclusions on GERM regulatory performances (i.e. the response to perturbations), thus making difficult the modular construction of GRC-s by linking GERM-s.

Symbols

Cj	-	species (lump, or 'pool') concentration
D	-	cell content dilution rate (i.e. cell-volume logarithmic growing rate)
g, h	-	kinetic model function vector
$J= dg / dC$	-	kinetic model Jacobian matrix
k	-	rate constant vector
nj	-	species j number of moles, or number of effector species binding the 'catalyst' L in a GERM
nr	-	number of reactions
ns	-	no. of species
N_A	-	the Avogadro number
rj	-	species j reaction rate
R	-	universal gas constant
t	-	time
tc	-	cell-cycle time
T	-	temperature
V	-	cell volume

Greeks -

$\lambda(J)$	-	eigenvalues of the dynamic model Jacobian
υij	-	stoichiometric coefficient of the species "j" (individual or lumped) in the reaction "i";
π	-	osmotic pressure
τj	-	species j recovering time of the steady-state

Index

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cyt - cytoplasma

env - environment

0 - initial

s - Stationary state

perturb - perturbed

Abbreviations

CCM - central carbon metabolism

G - The active Gene (DNA)

GRC - genetic regulatory circuits

GERM - gene expression regulatory module

GMO genetically modified micro-organisms

GP - the inactive complex of G with the transcription factor P (its encoding protein in the reduced model here)

L - species at which regulatory element acts

M - mRNA

Met - metabolite

nM - nmol L^{-1}, nano-molar (i.e. 10^{-9} mol L^{-1} concentration)

NG - negligible

Nut - nutrient

ODE - ordinary differential equations

P - protein

TF - transcription factor

QSS - quasi steady-state

[.] - concentration

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