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Birth, Growth and Death as Structuring Tools in Bacterial Population Dynamics

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ABSTRACT

A new model is presented that describes microbial population dynamics that emerge from complex interactions among birth, growth and death as oriented, discrete events. Specifically, birth and death act as structuring operators for individual organisms within the population, which become synchronised as age clusters (called cell-generations that are structured in age-classes) that are born at the same time and die in concert; a pattern very consistent with recent experimental data that show bacterial group death correlates with temporal population dynamics in chemostats operating at carrying capacity. Although the model only assumes "natural death" (i.e., no death from predation or antimicrobial exposure), it indicates that short-term non-linear dynamic behavior can exist in a bacterial population growing under longer term pseudo-steady state conditions (a confined dynamic equilibrium). After summarizing traditional assumptions about bacterial aging, simulations of batch, continuous-flow, and recycling bioreactors are used to show how population dynamics vary as function of hydraulic retention time, microbial kinetics, substrate level, and other factors that cause differential changes in the distribution of living and dead cells within the system. In summary, we show that population structures induced by birth and death (as discrete and delayed events) intrinsically create a non-linear dynamic system, implying that a true steady-state can never exist in growing bacterial populations. This conclusion is discussed within the context of process stability in biotechnology.

Keywords

Non-linear dynamics, Populations, Bacteria, Age-class, Programmed death

INTRODUCTION

One of the eternal questions humans have always pondered is “how life appeared”, which has both philosophical and scientific implications. However, the question of “how death appeared” and whether death is inevitable or even necessary also has broad connotations. As such, scientists and philosophers have contemplated “aging” as a sequential process between birth and death, and attempts at deciphering mechanisms and impacts of increasing age in populations have been undertaken for time eternal to pave the way for possible immortality or, at least, to understand how one might delay its apparition.

Aging, sometimes called senescence, is the gradual accumulation of cellular damage in individuals until which the extent of damage goes beyond metabolically acceptable limits and death occurs (Erjavec *et al.*, 2008; Ackermann *et al.*, 2007a; Ackermann *et al.*, 2007b; Evans and Steinholtz, 2007; Johnson and Mangel, 2006; Zamamiri *et al.*, 2002). However, until recently, it was assumed that classical aging evolved after the origin of eukaryotes (Ackermann *et al.*, 2007b; Degterev and Yuan, 2008) and many theories have been developed to explain observations. For example, “mutation accumulation theory” assumes that genetic mutations lethal later in life slowly accumulate over time, and only in “old age”, do such late-acting mutations accumulate and cause death. Alternately, “antagonistic pleiotropy theory” assumes a more active role for late-acting mutations where they benefit organisms early in life, being selected as life proceeds. Further, “the reliability theory of aging and longevity” theorizes that organisms lose irreplaceable parts over time, which accumulate and make death inevitable (Johnson and Mangel, 2006). Finally, “the disposable soma theory of aging” postulates that senescence is evolutionarily linked to the cost of maintaining functional cellular systems;

however, organisms have a finite amount of energy per life and they die at a particular time to maximize gene representation in future generations (Johnson and Mangel, 2006; Stewart *et al.*, 2005). Although these theories were conceived to explain eukaryotic aging, they were subsequently broadened to include any organism that displays asymmetric reproduction and the presence of a juvenile or differentiated phase. As an example, the bacteria *Caulobacter crescentus* starts life as a swarmer cell that swims away after division from an immobilized older stalk cell (Ackermann *et al.*, 2003; Ackermann, 2008). However, the reproductive output of the stalk cells declines continuously, whereas newborn cells are active and rejuvenated after “birth” (Ackermann, 2008; Stewart *et al.*, 2005). As such, differentiated replication constrains aging to a minority of cells (Barer and Harwood, 1999).

However, Stewart *et al.* (2005) demonstrated experimentally that morphologically symmetric dividing bacteria, like *Escherichia coli*, also display an aging parent and a rejuvenated progeny. Although no juvenile morphology is apparent, functional asymmetry between progeny and parent exists; i.e., one cell inherits more old structures (the pseudo-parent) than the other progeny cell (the pseudo-offspring) (Feldman *et al.*, 2007; Ackermann, 2008; Stewart *et al.*, 2005). Reproductive asymmetry in bacteria has evolutionary implications, such as the continual emergence of rejuvenated offspring over successive generations that retains an intact cell line (Erjavec *et al.*, 2008; Ackermann *et al.*, 2007b). However, it also might impact how they behave as dynamic entities. Specifically, if each cell division produces sub-groups of more (offspring) or less (parent) metabolically active cells, as time proceeds the population will become structured in clusters of organisms of different “age” (i.e., age clusters), which likely have different kinetic characteristics. Furthermore, recent experimental data show that bacteria tend to be born and die in groups, possibly due to programmed death in populations (Lewis 2000; Lindner *et al.*, 2008;

Maisonneuve *et al.*, 2008a; Maisonneuve *et al.*, 2008b). As an example, Figure 1 shows that the culture cell density (provided as optical density, which reflects both living and dead cells) of chemostat-grown *Escherichia coli* strain K12-MG1655 display oscillations that mirror the ratio of living versus dead cells in the system when operating at carrying capacity (Chi Fru *et al.*, in revision). Further, each live-dead cycle follows the same basic pattern; the relative live cell density starts high, but then drops suddenly associated with a rapid increase in dead cells followed by re-growth of living cells until a new crest is reached. These data show that a population becomes structured in discrete groups that are clustered by the systematic death of some cells and subsequent growth of new cells. As such, a true steady-state is never reached and confined dynamics ensue, consistent with the predictions of our new model. In summary, we show that birth and death can act as delayed, oriented events that structure bacterial populations and cause emergent non-linear dynamic behaviour. Practical implications to process stability in biotechnical applications are discussed.

Model Conceptualization

Modelling life, death, and population dynamics is challenging in bacterial systems because one must make critical assumptions that bias the model prior to construction, therefore assumptions must be as close to reality as possible. For example, there is debate about whether bacteria actually “die” or they simply enter extended dormant phases (states of low metabolic activity, incapable of dividing or forming colonies; Kell and Young, 2000). We will not enter this debate, but our first assumption is that dead and dormant cells are functionally the same because both states imply little or no metabolic function. This is reasonable given that rates of activity during dormancy are several orders of magnitudes lower than during viable activity. Second, we

presume populations are comprised of heterogeneous groups of cells that differ slightly according to the number of cell-cycles they have experienced (i.e., differ in age), possibly resulting from factors like increasing intracellular protein aggregation (Lindner *et al.*, 2008; Maisonneuve *et al.*, 2008a; Maisonneuve *et al.*, 2008b). Third, birth and death are discrete events, but these events occur simultaneously to clusters of organisms, consistent with group death observed in experimental work (Chi Fru *et al.*, in revision). Fourth, we assume each individual cell within a population has the same lifespan (Stewart *et al.*, 2005) where lifespan is the physical time from when a bacterium becomes metabolically active at birth to the moment activity disappears at death, and each bacterium experiences multiple cell-cycles that differ in number depending on growth conditions. Finally, we assume age-related differences dominate metabolic activity among age clusters in the population. We recognize this is a simplification because individuals within a population might differ metabolically for reasons other than age (Davey *et al.*, 1995), but we choose to keep the model simple to assess whether age and death alone can explain emerging population dynamics. However, bias towards age-related differences is probably legitimate because metabolic activity differs with bacterial age (e.g., Stewart *et al.*, 2005; Watve *et al.*, 2006; Erjavec *et al.*, 2008). The model will be extended to simulate other metabolic differences in future work.

We will use bioreactors as the setting for studying age-class-related responses in populations since they provide clear boundaries and precise control of inlet and outlet conditions. In fact, bioreactors (we use the term bioreactor because it is more general than chemostat) have been workhorses in experimental and modelling studies related to dynamics of biological processes for many years, although debate exists about such systems related to questions of purpose and stability (Feldman *et al.*, 2008; Ferenci, 1999; Ferenci, 2006; Hoskisson and Hobbs, 2005). For

example, gene expression patterns that affect growth kinetics can differ under subtly different conditions for same the same specie (Feldman *et al.*, 2008), and nutrient limitations can cause evolutionary change in a population over time. Such effects imply no true steady-state ever exists in bioreactors (Kovarova-Kovar and Egli, 1998); however, for early model construction, factors like evolutionally change are not included for simplicity. Further, experimental evidence indicates that bioreactors often display confined dynamics in controlled fermentations (Davey *et al.*, 1995; Chi Fru *et al.*, in revision), which show that non-linear dynamic behaviour and non-steady-state operations may intrinsically exist within bioreactor systems.

Model Options

When one considers specific model options for assessing age-related dynamics, various approaches exist. Existing models can be classified in three general categories: structured-unsegregated, unstructured-segregated, and structured-segregated (Zhang *et al.*, 2002).

Unstructured models assume detailed modelling of intracellular behaviour is not needed to describe growth, whereas structured models lump chemical components into virtual entities interacting within the cell. Unsegregated models assume that individual cells have identical physical and chemical properties, while segregated models permit the population to contain distributions of properties like cell mass or age (Zamamiri *et al.*, 2002; Zhang *et al.*, 2002).

Two general strategies exist for modelling cell cultures, with some specific variants: population balance equation (PBEM) and cell ensemble (CEM) models. In PBEM, the population is unstructured, but segregation exists based upon factors like age or mass, whereas in CEM, the population is structured, permitting more detailed descriptions of cellular metabolism and/or cell cycle progression. A drawback of the PBEM is the usual lack of a biochemical data needed to

describe physiological functions associated with single cell growth and division (Hjortso and Nielsen, 1995; Zamamiri *et al.*, 2002; Henson, 2003). Such functions include growth rates, transition rates between different cell compartments, and partitioning relationships of properties between newborn cells (Zamamiri *et al.*, 2002). Further, the solution of partial integro-differential equations that arise in PBEM is not trivial (Zamamiri *et al.*, 2002). In contrast, dynamic models of single microbial cells that describe cellular processes are essential in building a CEM, which ultimately is represented by a state vector containing concentrations of key intracellular species. In CEM, an ensemble model is usually build-up from a large number of single-cells where the state-vector is randomly perturbed from a nominal value to capture heterogeneities associated with particular processes (Grivet, 2001; Henson, 2003). Although single-cell models provide good agreement with some data, ensemble models comprised of two individual cells and extracellular balances do not always simulate system dynamics well (Henson, 2003). A few examples will elucidate the different modelling approaches.

Hjortso and Nielsen (1995) used PBEM where the cell cycle parameter was a function of ambient environmental conditions (e.g., the concentration of a substrate or product) to observe autonomous microbial oscillations. Their models are remarkably simple, although they often capture a wide range of dynamic behaviour (single synchronous population, complex periodic solutions with periods very different from the cell cycle length etc). The dependent variable in a PBEM is the distribution of states, often age, using a function that defines the frequency of cells in a given state over time. PBEM are always coupled to a mass balance on an environmental parameter (Hjortso and Nielson, 1995). Periodic solutions arise via three main feedback loops: 1) an initially synchronized or partially synchronized population leading to periodic variations in parameter concentrations as the cells proceed through cell cycles; 2) periodic changes in the

growth medium causing periodic changes in age at cell division; and 3) periodic changes in the division age that stabilize synchrony (Hjortso and Nielson, 1995). However, unlike simpler PBEM based on mass, models based on age involve partial differential equations (Zamamiri *et al.*, 2002). This happens because all newborn cells have zero age and the integral term for the rate of formation of new cells appears as a boundary condition rather than in the population balance itself. In such models, environmental conditions are often initially lumped in single variables for simplicity, such as the substrate concentration (Zamamiri *et al.*, 2002).

In contrast to classical PBEM and CEM, a slightly different approach is presented by Watve *et al.* (2006), which uses a modified Leslie matrix to examine the effects of symmetric and asymmetric division on the dynamics of bacterial growth. Instead of the dynamics of individuals in different age classes (like PBEM), Watve *et al.* (2006) adapt the Leslie matrix to model the dynamics of cell components of varying ages that differ in symmetric and asymmetric division, structuring each cell into a finite number of growth-limiting components related to age differences. Growth is assumed to behave as a Markov process; i.e., in each time unit, all pre-existing cell components pass to the next age class and all newly synthesized components form the first new class of organisms with similar age. All of the components of different classes are carried forward to the next age class, and after the demise of the oldest age class, components disappear. Further, components have age-specific efficiencies that contribute to cell growth rate and new components are added based on the net rate of cell growth; however, reproductive efficiency of each component decreases with age (Stewart *et al.*, 2005). Therefore, the rate of growth of a given cell depends upon the distribution of all cell components; cells with wholly new components will divide at maximum growth rate, although rates decline with age. The main prediction of Watve *et al.* (2006) is that truly symmetrical division theoretically exists in nature,

although data from Stewart *et al.* (2005) show this is unlikely. Therefore, refinements to Watve *et al.* (2006) are needed to better describe “reality”, which are presented herein.

METHODS

Further assumptions for the new model

The model we introduce emerged from evidence that showed non-linear dynamic behaviour directly parallels shifting demographics among living and dead cells within the same population (Chi Fru *et al.* in revision). Although the model is conceptually close to the models of Watve *et al.* (2006) and Erjavec *et al.* (2008), it has distinctively different features that make it more general and flexible. For example, neither previous model considers death as the key structuring element in the population or confined dynamics associated with it. The underlying structural assumptions will be presented first and then mathematical details will be provided.

For simplicity, we assume a virtual system in which the modelled bacterial population starts from a pool of synchronized newly born cells (ultimately a single cell). We then define the *cell-generation* as a group of individual bacteria having the same age and *cell-cycle* experience. For modelling purposes, each *cell-generation* is lumped into a single representative virtual bacteria whose mass equals the sum of the individual masses of the cells synchronized into this *cell-generation* (see Figure 2a, where the first cell-generation is the top line, the line beneath is the second cell-generation resulting from the first, the line beneath is the third cell-generation resulted from the second, etc). During the lifetime of a given cell-generation, τ_T , (Figure 2a, the overall length of each rectangle representing a cell-generation) the mass of this entity doubles during each cell-cycle which lasts τ_D (the doubling time; Figure 2a, τ_{D1} is for the first cell-cycle,

τ_{D2} is for the second and is longer due to age effects, and τ_{D3} is for the last). The mass of a cell-generation extends from an initial value to twice that value immediately before division.

To simplify analysis further, we assume that τ_T represents a *datum* for a specific single-cell organism (Stewart *et al.*, 2005; they estimate the lifetime as $100 \cdot \tau_D$ where τ_D is the doubling time when cells are under no stress) and is independent of the external factors (this isn't likely true, but we will refine this assumption in later development of the model); i.e., a bacteria will live on average τ_T time, regardless of growth conditions. However, we assume τ_D varies with local substrate availability, age, and other external limiting factors. Implicitly, the doubling time of a given cell-generation will increase over time due to age-effects; therefore older cells will have progressively longer cell-cycles as their life proceeds. Further, if we denote the number of cell-cycles as N_C , each cell-generation will have $\tau_{D,n}$ ($n=1, 2 \dots N_C$) doublings per lifetime with a new cell-generation appearing when at least one of the existing cell-generations completes its cell-cycle and divides (Figure 2a shows this process). As such, a bacterial population should be viewed as a discrete collection of cell-generations whose number depends upon their intrinsic lifetime and actual doubling time. If we denote the greatest common divisor of all doubling times for a specific cell-generation by τ_m (for a given environment), a bacterial cell population would have $N_G = \tau_T / \tau_m$ different cell-generations coexisting simultaneously, but being born at discrete consecutive time intervals of length τ_m . Finally, if one clusters cell-generations according to their particular cell-cycle and assumes steady-state conditions, $N_n = \tau_{D,n} / \tau_m$ cell-generations will belong to the same cell-cycle n , but each cell-generation will exist at different points in their cell-cycle. Therefore, as cell-generations become older and die after N_C doublings, N_G cell-generations will exist at any time with a discrete age distribution from zero to τ_T .

Figure 2a visually presents the affect of age and death on population structure: e.g., after three divisions (N_C in this case), the oldest cell-generation dies and the second cell-generation becomes the oldest. Then, after another division, the second cell-generation dies and the process repeats itself indefinitely. This actually represents the population death rate that equals the birth rate τ_T times ago (Figure 2a). In fact, death is a discrete, deterministic event happening after each cell-generation attains its lifetime, during which growth and division are non-mandatory parts of this time-oriented cycle. After a transitory period, which could last several lifetimes, the structure of the population stabilizes according to the duration of each cell-cycle, which depends upon age-effects and environmental conditions, such as the limiting nutrient. Note that the cell-cycle and age-class concepts are formally equivalent, although the latter concept disregards the existence of cell-generations and their distribution within an age-class (Figure 2a). In fact, if aging effects are ignored and the environment is steady, the number of cell-generations in a population is the same as the number of age-classes because no age-related effects are included (e.g., intracellular damage). We will not include the direct effects of aging in this initial model to examine the influence of age-class structure alone on population dynamics. This simplification is justified because it assumes that “old age” is not the only possible cause of death; it just assumes group death by whatever cause, structures the population over time, which is most consistent with the existing experimental data.

Under optimal conditions of substrate concentration (S_{op}), doubling time, τ_{Dmin} , has the minimum value of $\ln(2)/\mu$, where the specific growth rate, μ , is either μ_{max} or $\mu_{max} \cdot S_{op} / (K_S + S_{op})$, assuming Monod-like kinetics apply. The former value would be considered when the substrate level is in excess; however, this condition will rarely occur in a continuously

operating bioreactor. No matter the case, the population will have $N_{\max} = \tau_T / \tau_{D\min}$ age-classes under optimal growth conditions (Figure 2b). When the substrate concentration gradually decreases between two steady states, the duration of the consecutive cell-cycles/age-classes increases accordingly and thus their total number decreases (see Figure 2c). Moreover, since the cells live a defined lifespan, we assume that they could die before completing the final cell-cycle (i.e., doubling), which means that there could be discontinuities in living cell abundances, especially for low substrate levels with only a few age-classes. As an example, after the substrate concentration reaches a new pseudo-steady-state (Figure 2c, the pseudo-stationary regime), two cell-generations coexist, although only one reaches the end of its cell-cycle and actually doubles. The second age-class does not live through its cell-cycle and dies beforehand. Thus, only half of the cells continue activity, producing significant fluctuations in cell mass and substrate uptake.

When one analyses the behaviour displayed in Figure 2, it is possible to predict median death rates for the current population simply by knowing birth rates at the moment when the oldest generation appeared. In fact, a closer look to Figure 2 shows that birth and death can be viewed as delayed oriented irreversible processes. The process is *delayed* because there will always be time between birth and death, *oriented* due to the irreversibility of time, and *irreversible* because substrate is transformed into cellular building blocks that are not energetically equivalent to the original substrate.

THE MATHEMATICAL MODEL

Kinetics of a structured bacterial population

Before introducing the complete model, we will derive kinetic equations describing the biological process that results from the aforementioned hypotheses and Figure 2a. We index the cell-generations from 1, the oldest generation, to N_G , the youngest generation. Implicitly, when the oldest cell-generation dies, there will be a unit decrease in the index of the remaining generations. Further, the total number of coexisting generations depends upon substrate concentration and the duration of any cell-cycle (see Figure 2c); i.e. $N_G = N_G(S)$ and $\tau_{D,n} = \tau_{D,n}(S)$. For simplicity, we suppose that each generation does not experience any adaptive growth lag, so the mass balance for the k^{th} cell-generation is:

$$\frac{dX_k}{d\tau_k} = \mu(S, \tau_k) \cdot X_k, \quad 0 \leq \tau_k \leq \tau_T, k = 1, 2, \dots, N_G(S) \quad (1)$$

We assume that bacteria obey the simplest Monod-like kinetic dependency upon substrate; however, maximum specific growth rate, μ_{max} , and the substrate affinity constant, K_S , depend upon the physical time since birth of the cell, τ_k .

$$\mu(S, \tau_k) = \mu_{max}(\tau_k) \frac{S}{K_S(\tau_k) + S} \quad (2)$$

Due to the discrete nature of the damage accumulation in cells owing to asymmetric division (Erjavec *et al.*, 2008), the values of μ_{max} and K_S will change from the previous cell-cycle to the actual cell-cycle of the k^{th} cell-generation. In other words, both the maximum specific growth rate and the substrate affinity constant change in a step-wise manner in time (not continuously), which is consistent with group death shown in Figure 1. The kinetic model (equation (1)) also includes initial conditions (equation 3), cell division (equation 4) and death (equation 5); i.e.,

$$X_k|_{\tau_k=0} = \frac{1}{2} \sum_{j \in K^*} X_j, \quad K^* = \left\{ j \mid 0 < j < k \ \& \ \tau_j = \sum_{i=1}^n \tau_{D,i}, \ n = 1, 2, \dots, N_G \right\} \quad (3)$$

$$X_k|_{\tau_k=\tau_n^+} = \frac{X_k|_{\tau_k=\tau_n^-}}{2}, \quad \tau_n = \sum_{j=1}^n \tau_{D,j}, \quad n = 1, 2, \dots, N_G \quad (4)$$

$$X_k|_{\tau_k=\tau_T^+} = 0 \quad (5)$$

Note that equation (3) simply states that the mass of any new cell-generation k results from the division of some immediately previous generation, K^* , and equation (4) states that when τ_k coincides with the end of the current cell-cycle, τ_n . Finally, the mass of the k^{th} cell-generation is assumed to halve relative to the next cell-cycle due to division.

The complete mathematical model for bioreactors

To demonstrate the implications of our new death-structured model, we will examine classical bioreactor-separator systems shown in Figure 3 (Grady *et al.*, 1999). Such systems are very common in biotechnology and permit comparisons between microbial population behaviours under various environmental and operating conditions (i.e. batch, fed-batch, continuous, or continuous with external recycle). We assume the bioreactor is perfectly mixed and the “separator is ideal” (i.e. no cells escape in the liquid outlet stream). Further, when recycle is included in the model, separated cells return back to the bioreactor at a known fraction α of the inlet stream. The separator and recycle introduce an additional delay in the system with respect to the living cells in the main reactor chamber. Finally, a solids wastage line is included to remove dead cells and excess growth from the system with the withdrawal fraction assumed to

be β relative to the inlet stream. For this general system, we will derive a generic mathematical model by developing mass balance equations for living and dead cells and for the primary substrate. Using model's parameters (α and β), we will examine different topologies and then study transitory states.

Mass balance for the living cells

The mass balance for the living cells of the k^{th} cell-generation takes into account both cells recycled from the separator and the assumption that death rate is equal to a τ_T time-delayed birth rate. In dimensionless form ($x_k = X_k/X_{in}$, where X_{in} is the initial concentration of the living cells in the bioreactor), and using the age factor $\phi = \tau_T/\bar{t}$ (\bar{t} is the residence time, alias hydraulic retention time), the mass balance is:

$$\frac{dx_k}{d\tau} = \tau_T \cdot [\mu(\tau) \cdot x_k(\tau) - \mu(\tau - \tau_T) \cdot x_k(\tau - \tau_T)] + [x_k(\tau - \tau_S) - x_k(\tau)] \cdot \phi \cdot (1 + \alpha) \quad (6)$$

It must be stressed that in equation (6), τ represents the time, during which the first cell-generation starts its existence as the bioreactor starts working and lasts τ_T , and the k^{th} cell-generation appears after k cell-cycles of the oldest cell-generation.

Mass balance for the dead cells

Using the same approach, the mass balance for dead cells in dimensionless form is (i.e.,

$$x_D = X_D/X_{in}):$$

$$\frac{dx_D}{d\tau} = \tau_T \cdot \mu(\tau - \tau_T) \cdot x_k(\tau - \tau_T) + [x_D(\tau - \tau_S) - x_D(\tau)] \cdot \phi \cdot (1 + \alpha) \quad (7)$$

Mass balance for the substrate

In order for the mathematical model to be complete, we add the dimensionless mass balance for the substrate ($\sigma = S/S_0$), since both the coexisting number of generations and the number of cell-cycles a cell-generation has is a function of the food supply:

$$\frac{d\sigma}{d\tau} = -\frac{\tau_T \cdot X_{in}}{S_0} \sum_{k=1}^{N_G} \left(\frac{\mu(\tau) \cdot x_k}{Y_{XS}} + m \cdot x_k \right) + [\sigma(\tau - \tau_S) - \sigma(\tau)] \cdot \phi \cdot (1 + \alpha) \quad (8)$$

Due to the complexity of the interactions implied by the two time scales (biological lifetime of bacteria, τ_T , and residence time of the bioreactor, \bar{t}), we will solve the mathematical model and discuss the results only for non-aging populations in this initial presentation.

Model summary

Owing to the discrete nature of age-classes, birth, and death, the mathematics that underlie this new model address each virtual bacterium assigned to an age-class with respect to the mass conservation law; i.e., the number of equations mirrors the number of age-classes. Taking into account that the population has $N_A(S)$ age-classes at any given time (considering the beginning of the experiment as the starting point), this includes N_A+2 differential equations with initial, final, and time-milestone conditions (equations 1-8). Due to the non-aging assumption, the structure of the bacterial population simplifies, containing only N_A virtual bacterium (see Figure 2b) instead of N_G virtual bacterium (see Figure 2a). This means the number of cell-generations in the model, N_G , can be replaced by the number of age-classes, N_A . Further, each differential equation describes the mass variation over time of each virtual bacterium associated with an age-class.

The initial condition provides the mass of an entity at birth (equal with half of the total mass of

the population immediately before division) and the final condition states that after a lifetime the oldest age-class vanishes. The time-milestone conditions mark cell divisions (if any) of this virtual bacterium during its lifetime. The model was solved using in-house software coded in C++ based upon a combination of stiff/non-stiff Runge–Kutta algorithms for ordinary differential equations. Kinetic parameters and reactor conditions used in the simulations were typical of *E. coli* strain K12-MG1655 grown in bioreactors consistent with complimentary experiments (Kovarova-Kovar and Egli, 1998; Stewart *et al.*, 2005; Chi Fru *et al.*, in revision).

RESULTS AND DISCUSSION

Discontinuous/batch bioreactors

In a batch process, the number of age-classes, which depends upon the substrate level and affinity, should decrease gradually due to the continuous depletion of the substrate. Figure 4 shows when substrate is high, cells achieve near optimal doubling times at the beginning of the process; they grow and divide rapidly and form ten age-classes within the first 10% of their lifetime. Further, only living cells exist during early growth in the batch reactor and it isn't until after one lifetime that dead cells start to accumulate when the oldest age-class dies (Figure 4, dimensionless time greater than unity). Although birth is a discrete event, the concentration of the living cells varies continuously in the absence of death. Birth means only a change in the age distribution of population through the apparition of new age-classes, which inherit half of the population mass prior to division without changing the living cells concentration. Like birth, death is a discrete event, equal to the birth rate the population experienced earlier. Finally, death discretely affects the living cell profile, which drops as dead cell levels increase (Figure 4).

Continuous-flow chemostats

Continuous-flow chemostats theoretically operate at a steady state when the specific growth rate of the microorganisms equates with dilution rate, which should result in constant effluent cell densities. However, experimental evidence suggests that bacterial populations in chemostats do not achieve a true steady-state even under “ideal” conditions (e.g., Figure 1). We suggest that this behaviour can be explained by the structuring influence of apparent group death, which continuously occurs to the “oldest” age-class in the population. It is important to reiterate that it is not known with certainty the actual cause death, but the model assumes that the “oldest cells” will most likely die first because they are going to be most subject to the impact of aging.

Based on this assumption, we performed numerous simulations using the model to assess the impact of growth and death upon the structure and behaviour of a bacterial populations in a chemostat under various hydraulic residence times (HRT; the inverse of the dilution rate). Simulations ranged from near “washout” conditions (where dilution rate is greater than specific growth rate) to HRTs near complete life spans; however, only illustrative examples are provided of general trends.

Overall, the mathematical model suggests that chemostats operate under a pseudo-steady state, called a *confined dynamic equilibrium*, which includes small-scale oscillations embedded within longer term steady conditions. However, characteristics of the confined dynamic equilibrium vary as a function of HRT with longer HRTs generally displaying more complex behaviour than shorter HRTs. A typical pattern is shown in Figure 5, which shows confined dynamic equilibrium after dimensionless times >1.5 when a HRT of 4.5 h is assumed. Upon closer inspection, it is apparent that observed cyclic patterns result from the sequential loss (i.e., death)

and re-growth of live cells over time, which closely resembles experimental results shown in Figure 1 (Note: Figure 1 used HRT of 3.5 h in experiments). Further, the model shows that dead cell dynamics are more influential than live cell dynamics on cyclic patterns, presumably because live cells are self-replenishing. In reality, the amplitude of variation of living cell densities in the model is relatively small ($4e-3$ in dimensionless units), therefore visually appears near steady-state (Figure 5). However, observed variations were consistent among all mid-range HRT simulations and also confirmed by physical experiments when measurement detection limits low (Chi Fru *et al.*, in revision). It should be noted that there is one dilution rate (HRT of 0.32 h) beyond which the only attainable steady state is trivial and corresponds with washout (Figure 6). In this case, population densities cannot be confined within the system's boundaries and washout occurs because the bacterial cells do not have the physical time to replicate themselves.

For HRTs higher than 4.5 h, the amplitude of variation of the confined dynamic equilibrium increases and its boundaries start displaying a double periodicity (Figure 7). Depending upon the relationship between the process time scale (doubling time) and the bioreactor's time scale (residence time), boundaries are nearly constant with symmetric population dynamics in the confined equilibrium (Figure 7a-1&2) or in a more periodic manner with asymmetric but periodic behaviour over short intervals, but more symmetric and cyclic behaviour over longer intervals (Figure 7b-1&2). In both cases, the amplitude of variation of the living cell densities is sufficiently high so that a time-series of state parameter readings from the bioreactor might display pseudo-chaotic behaviour, especially when the sampling time of the probe has no relationship with the timescale of the system; i.e. when their quotient is not a rational number.

Analysing the phase-plane trajectories (Fig. 7a-2 and 7b-2), emerging pseudo-chaotic population behaviour is suggested. The process is repetitive, but without a fixed period; i.e. the time trajectories do not overlap even for the smaller HRT of 10 h, for which the system has rather constant boundaries. The variable timescale of the periodicity is responsible for different values of the state variables. Furthermore, the apparent pseudo-chaotic behaviour increases as the HRT increases (HRT of 42 h) due to the higher amplitude of variations of living and dead cells concentrations (see Figure 7a-1 and 7b-1 for comparison). This happens because the number of age-classes diminishes as the HRT increases and the impact of death of the oldest age-class is greater. Ultimately, for a sufficiently high HRT, there will be only one fully developed age-class; i.e., the second age-class which cannot reach division since its lifetime ends before the reactor HRT. This is the result of decreasing substrate concentrations which cause doubling times to approach residence times. The system has a complex attractor (Figure 7b-2) around washout, represented by the centre of the circle. Increasing residence times will result in extinction, since the level of the substrate concentration drops under the maintenance threshold. Consequently, the doubling time becomes greater than the lifetime and bacteria will die before multiplying.

Bioreactor-separator systems

In order to prevent washout and stabilize the system, external recycle lines are sometimes used in bioreactors. Recycle systems return cells that have exited the bioreactor back into the influent (often via a solids separator). Further, cells also sometimes are wasted from the recycle line, which is designed to counterbalance accumulation of cells in the system and discard excess cell solids when cell yields are high in the system. The inclusion of external recycle makes the model

more complex, since another time-scale is added (i.e., the recycle delay, which is the time for the cell mass to leave reactor and then return back as recycle).

Figure 8 shows the system with recycle is still in a confined dynamic equilibrium with conditionally constant or periodic boundaries defined by complex relationships among the three time scales: doubling time, residence time, and recycle delay. Despite the apparent complexity, the amplitude of variation of living cells concentration still depends upon the bioreactor's HRT, but results in narrower or wider attractors (see Figure 8a-2 & 8b-2). When the attractor is narrow, the system appears in a pseudo-steady state, with distributed living cell concentrations around 0.938 and a dispersion of 0.043 (for the case presented in Figure 8a-2). However, as the attractor becomes wider due to the increased value of the amplitude (0.182 for the case presented in Figure 8b-2), the system behaves more dynamically since dispersion widens.

Even with external recycle, an actual steady state never seems to exist and the population always operating in a confined dynamic equilibrium. In fact, the model shows that by simply considering that organisms in a population have different ages and are subject to death, complex population behaviour emerges in an apparently simple system. The model shows that death and growth structure populations over time and can cause dynamics to shift from a pseudo-steady state to periodic to possibly chaotic conditions, which has significant ramifications to population (and community) dynamics and stability in all living systems.

Implications

The mathematical model presented here assumes that bacterial populations are not comprised of identical individuals, but include individuals of different age at different points in their lifetime

at any moment in time. The model further assumes that individuals pass through their lives in groups (age-classes) that are segregated by group death events that occur among a sub-set of organisms within the system, probably the oldest age-classes (Erjavec *et al.*, 2008; Chi Fru *et al.*, in revision). Finally, this group death is “programmed”, possibly by some quorum sensing mechanism (Rice and Bayles, 2008), but this needs to be proved. Regardless of cause, the model broadly shows that emergent complex dynamics, including conditional pseudo-chaotic behaviour, result from these simple, but experimentally verified assumptions. Although this version of the model does not include explicit differences in kinetics among age-classes, it shows if individuals are born and die at different points in time, intrinsic complex behaviour results that closely follow observations in recent experiments (Figure 1).

The basis if this complex behaviour varies from case to case and varies as function of HRT, but it appears to largely result from delayed-oriented birth, growth, and death of different age-classes in the system that result in different cycling patterns of live and dead cells (Figures 2 and 7). As age-classes are born, live, and die, the distribution of age-classes continuously varies over time, triggering temporal changes in population density that is influenced by microbial kinetics, substrate availability, lifespan, the operational mode of the bioreactor, and other factors. However, regardless of details, the age-class concept clearly results is a dynamic system, which in fact, contradicts classical reactor theory (that has traditionally assumed a steady-state), but also better matches common experience with reactor systems.

Although basically simple, we contend this new model may demand a paradigm shift in how we view bacterial population dynamics in reactors (and other systems) because it explains complex behaviour at both a qualitative and quantitative level. This model’s predictions and this alternate

worldview may of general academic interest, it should be of particular interest to those that study and operate reactors for population growth in biotechnology. Process stability is a continual concern with such systems, but such systems also are known to become transiently unstable and even fail unpredictably (Knapp and Graham, 2007). Therefore, this new model provides a baseline for new studies on transient instability, even in pure cultures, which might allow, first through refined simulations but followed by further laboratory experiments, a new direction for both predicting and designing away from instability. As such, we hope that this new paradigm will be the catalyst for future research related to this subject, especially larger-scale experimental studies that test and validate the model's predictions.

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FIGURES

Figure 1. Temporal patterns of cell density (measured every minute as optical density, OD, at 600 nm wavelength) and the ratio of live versus dead *Escherichia coli* strain K12-MG1655 cells in an operating chemostat. Operating conditions included: hydraulic retention time = 3.5 h; temperature = 27°C; mixing rate = 200 rpm; growth media = 0.1% wt/wt glucose plus essential mineral nutrients; pH = 7.0. Live and dead cell densities were determined using the LIVE/DEAD® BacLight Bacterial Viability Kit, L13152 (Molecular Probes, Invitrogen, Paisley, UK). Results typical of operations for hydraulic retention times ranging from 3 to 10 h.; system washout retention time was 2.6 h. Data adapted from Chi Fru *et al* (in revision) where further details are available.

Figure 2. Dynamic relationship between cell-generations, life time, doubling time, and number of age-classes present in the system with respect to the substrate concentration; a) substrate is plenty and at the optimal concentration, aging affects bacteria (see the text for the meaning); b) the same conditions as in a), but aging does not affect bacteria; c) the substrate is consumed during the biological process, decreasing to the limit where the doubling time becomes approaches the lifetime and a single fully active age-class exists (the bacteria from the older age-class die prior to divide).

Figure 3. Chemostat model including wastage line.

Figure 4. Typical batch profile for living and dead cells dimensionless concentrations to illustrate the influence of time delay between birth and death ($S_0 = 8000$ mg/L, *E. coli*, $\mu_{max} = 3$ h⁻¹, $K_S = 3.96$ mg/L, $\tau_D = 0.231$ min, $\tau_T = 23.1$ min, dimensionless time = t/τ_T).

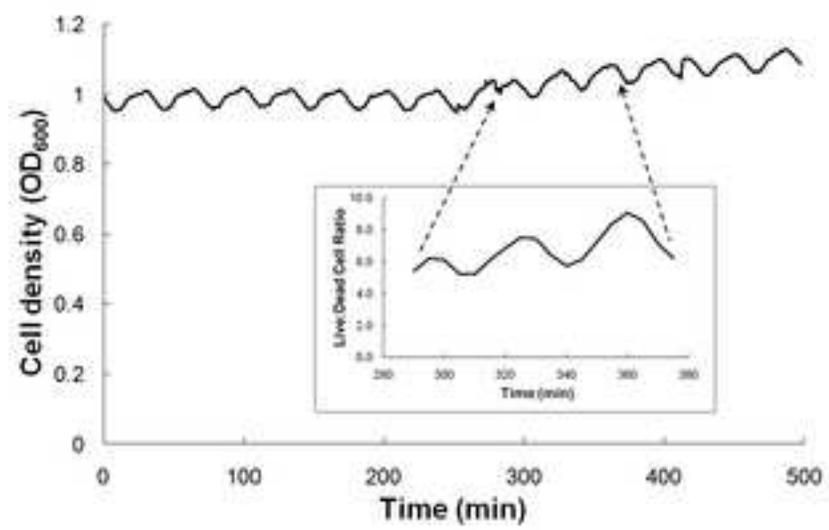
Figure 5. Dimensionless concentration profiles for a continuous-flow chemostat where confined dynamic equilibrium is achieved: number of age-classes = 6-7; residence time = 4.5 h; $\tau_D = 3.13$ h; chemostat substrate concentration = 0.315 mg/L; mean dimensionless steady-state living cells concentration = 0.99 (actual chemostat concentration divided by the maximum exit concentration); mean ratio of living to dead cell concentrations = 188.3 (the kinetic parameters are the same as in Figure 3).

Figure 6. Washout state for residence time = 0.32 h (the kinetic parameters are the same as in Figure 3).

Figure 7. Confined dynamic equilibrium concept: a-1) constant boundaries for the amplitude of variation, number of age-classes: 2-3, residence time = 10 h, chemostat substrate concentration = 0.155 mg/L; mean dimensionless steady-state living cells concentration = 0.879; mean ratio of living to dead cell concentrations = 7.8; a-2) phase-plane representation of the time trajectory for the living cells concentration – the attractor has regular boundaries, b-1) periodic boundaries for the amplitude of variation, number of age-classes: 1, residence time = 42 h, chemostat substrate concentration = 0.082 mg/L; mean dimensionless steady-state living cells concentration = 0.418; mean ratio of living to dead cell concentrations = 0.74; b-2) phase-plane representation of the time trajectory for the living cells concentration – the attractor has irregular boundaries; on average, the chemostat has steady-state concentration, but within shorter time scales, this varies between two limits and can be asymmetric (the kinetic parameters are the same as in Figure 3).

Figure 8. Implications of the external recycle upon the confined dynamic equilibrium. Possible interactions displayed when the recycle time-delay is under the doubling time (a-1&2, residence time = 0.5 h, delay time = 1.0 h) or over the doubling time (b-1&2, residence time = 1.5 h, delay

time = 1.0 h), for the same operating parameters: recycle fraction = 0.5, sludge waste ratio = 0.05. a-1) number of age-classes: 10, chemostat substrate concentration = 0.538 mg/L; mean dimensionless steady-state living cells concentration = 0.938; mean ratio of living to dead cell concentrations = 11.7; b-1) number of age-classes: 3, chemostat substrate concentration = 0.206 mg/L; mean dimensionless steady-state living cells concentration = 0.896; mean ratio of living to dead cell concentrations = 3.23 (the kinetic parameters are the same as in Figure 3). The influence of the recycle time-delay is readily observable for the dead cells concentration profile – the decline due to the withdrawal is dumped by the dead cells entering the system after this delay (the slope of the profile changes).



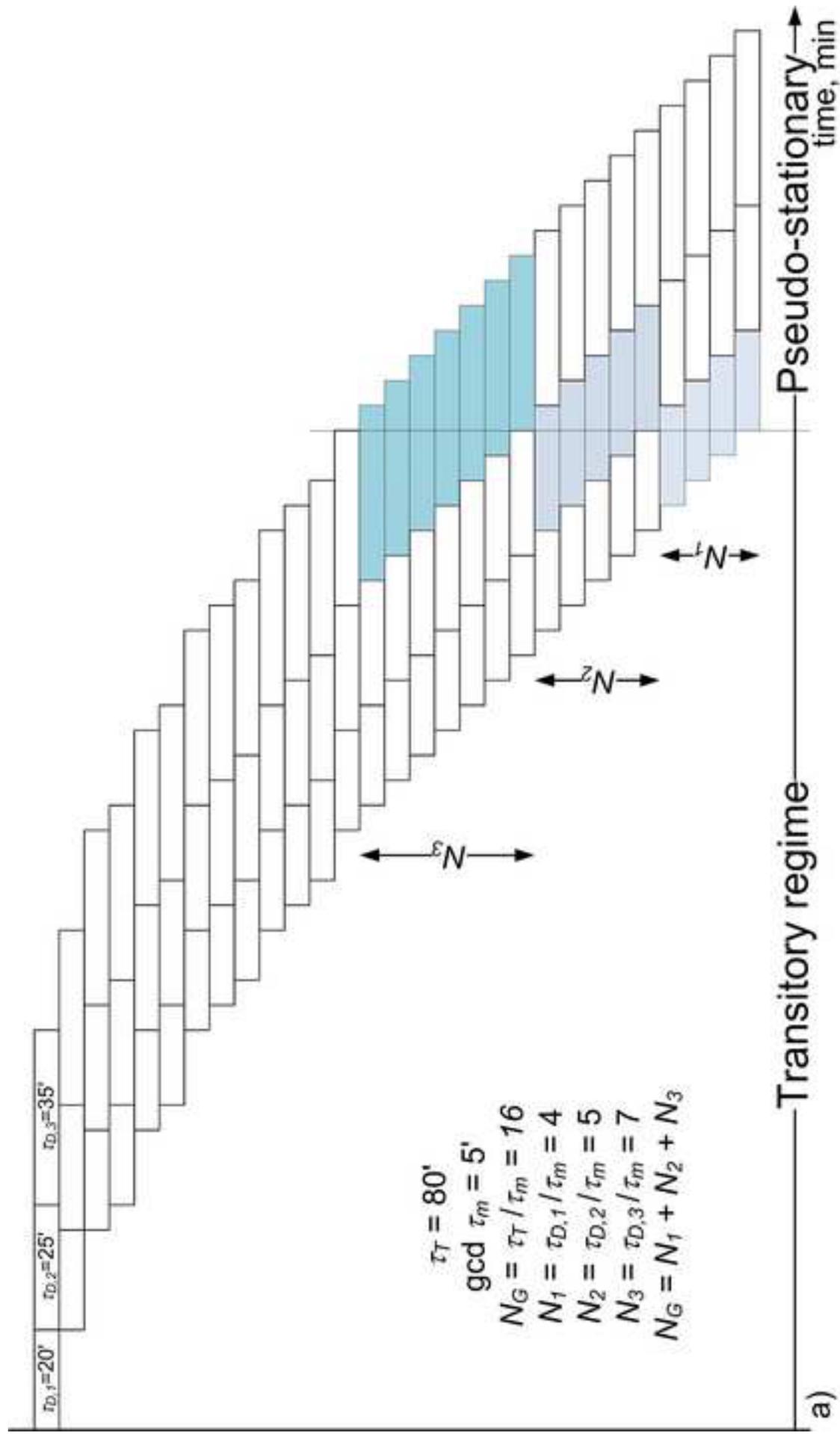
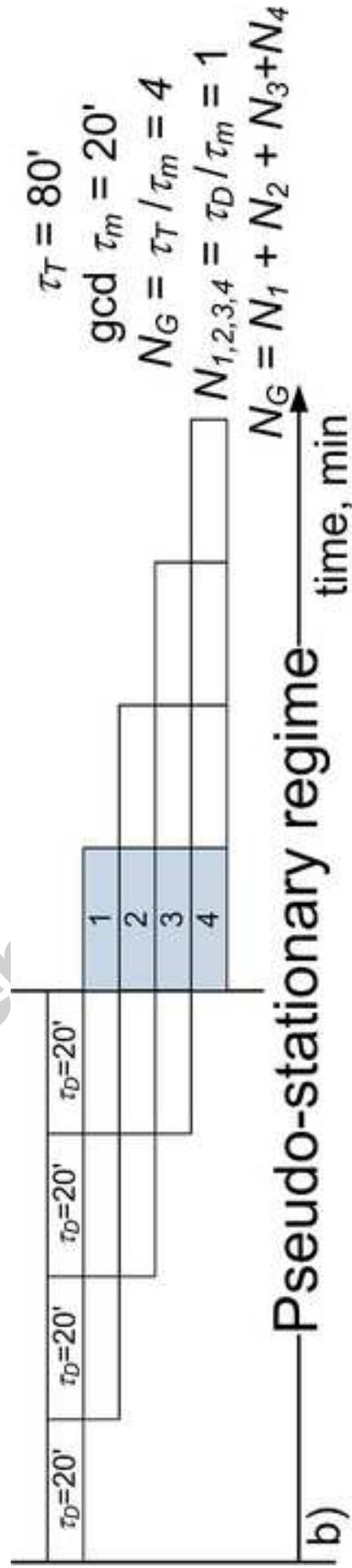
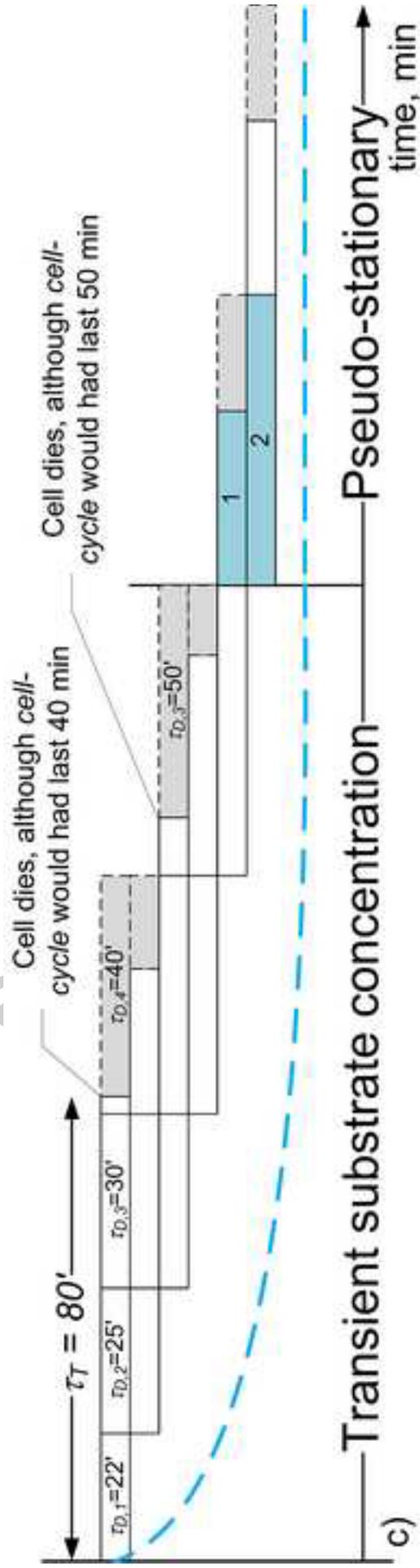


Figure 2a





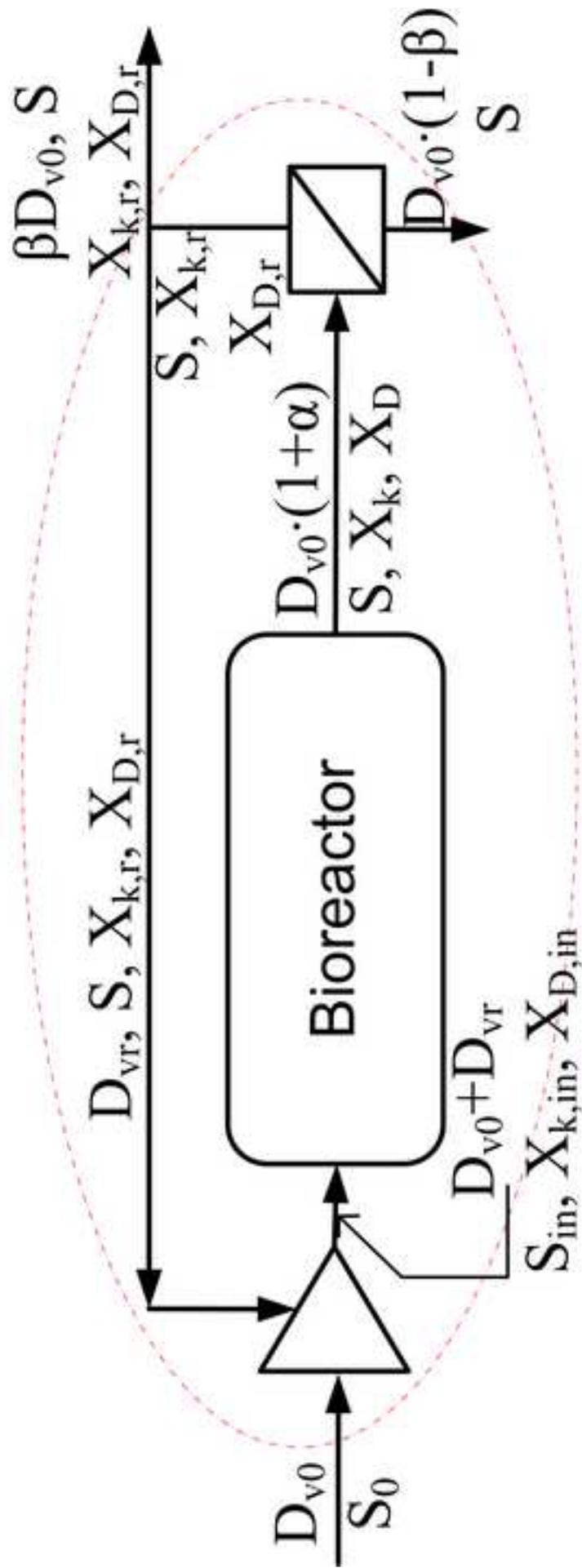


Figure 3

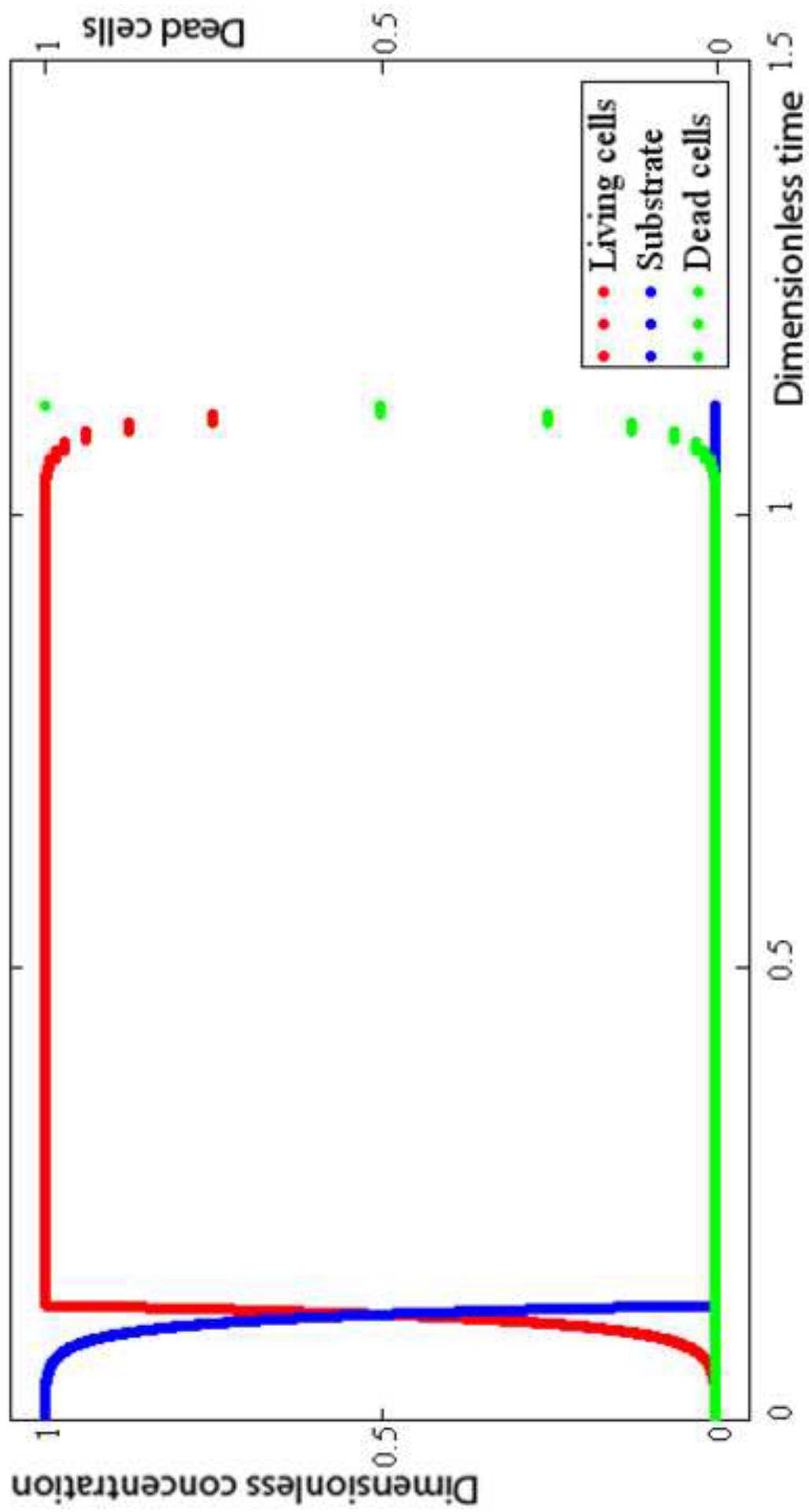


Figure 4

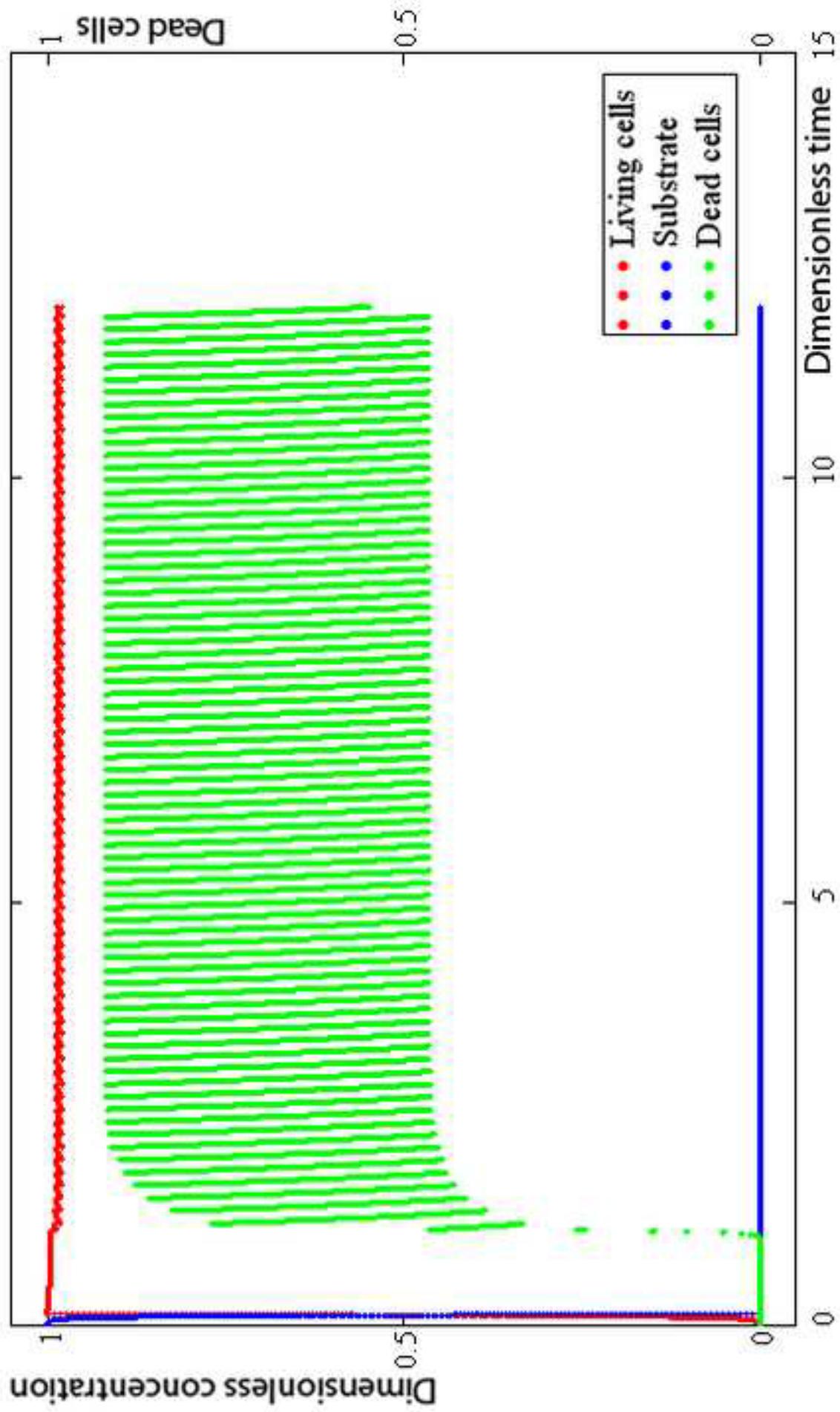


Figure 5

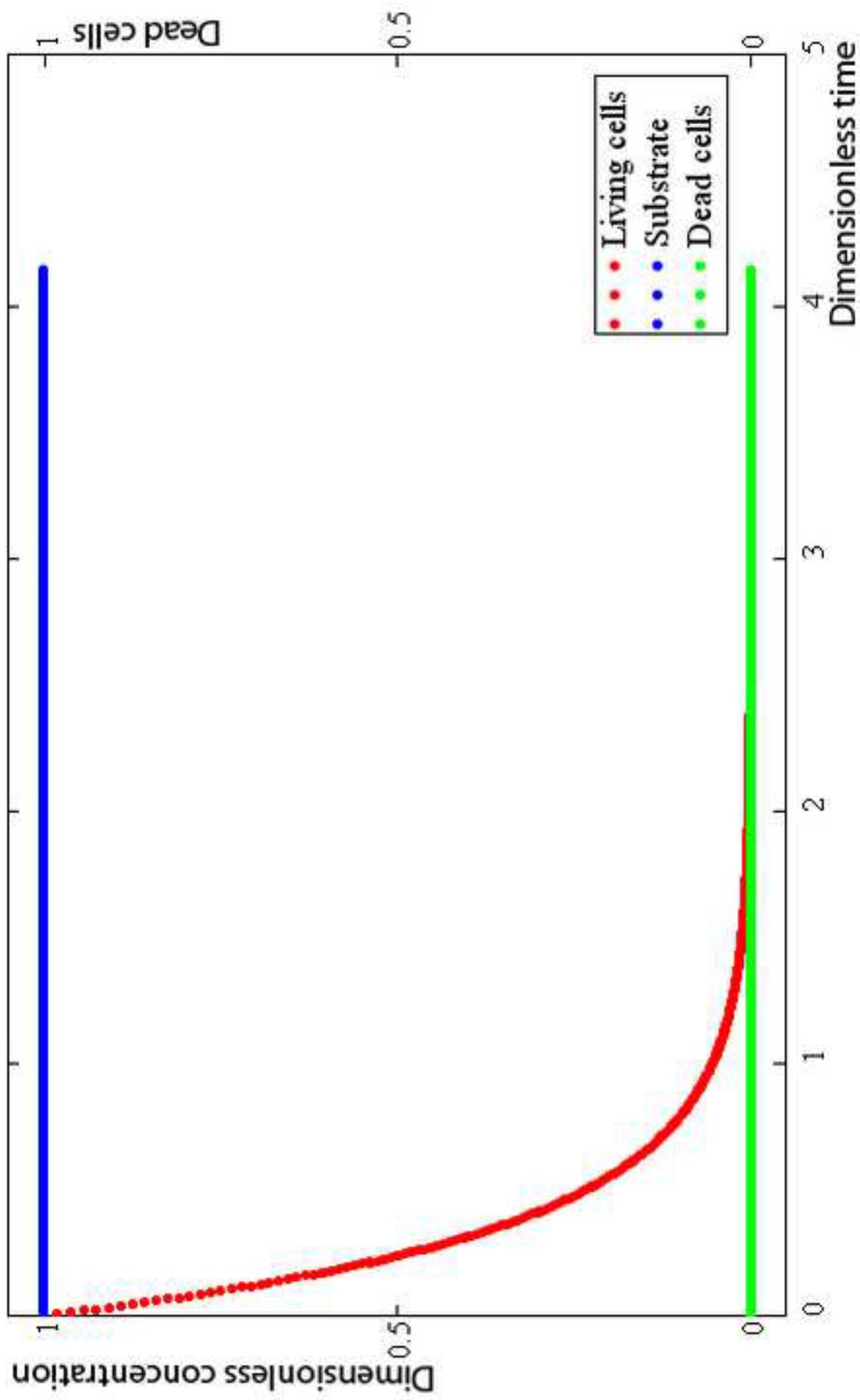
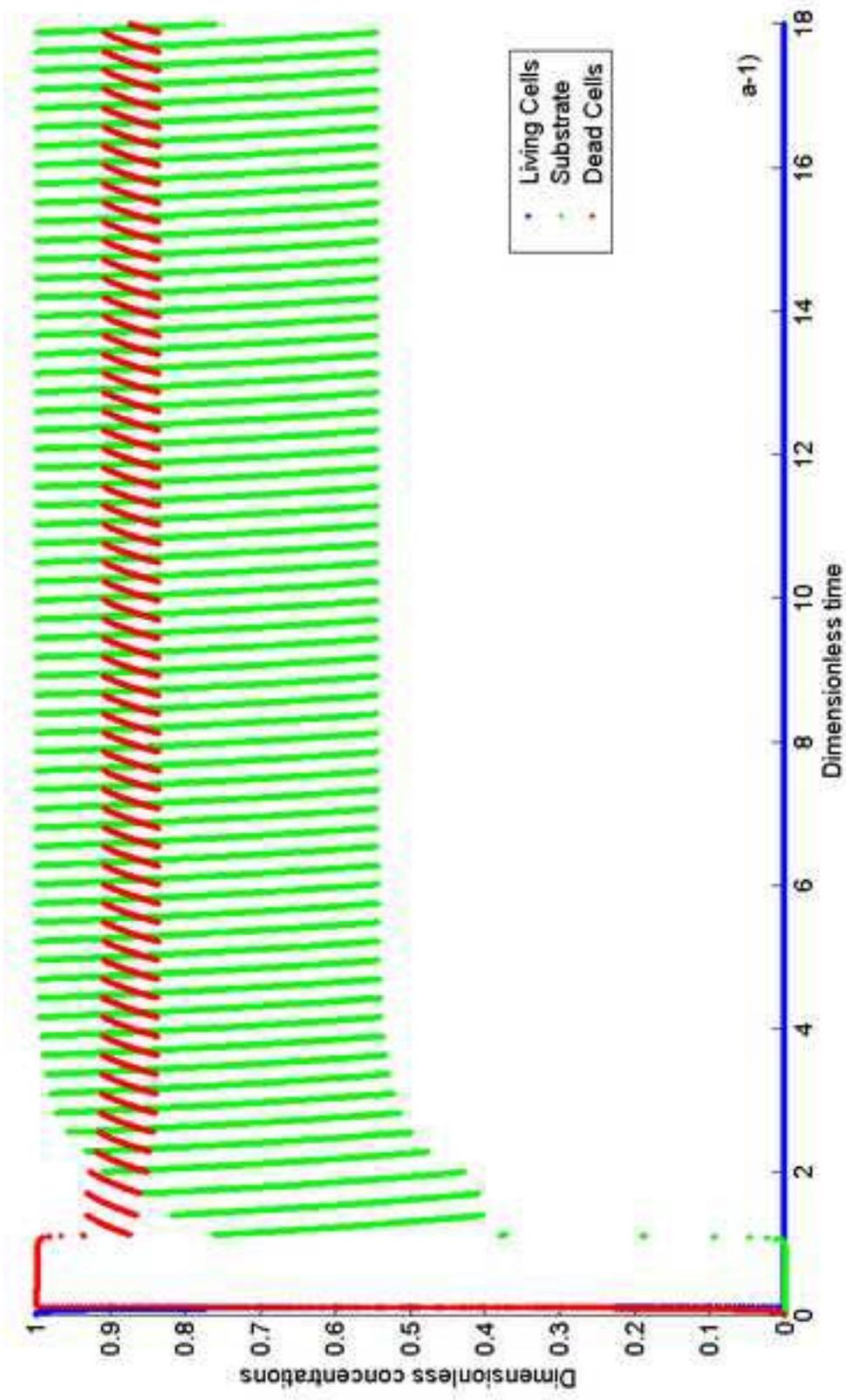
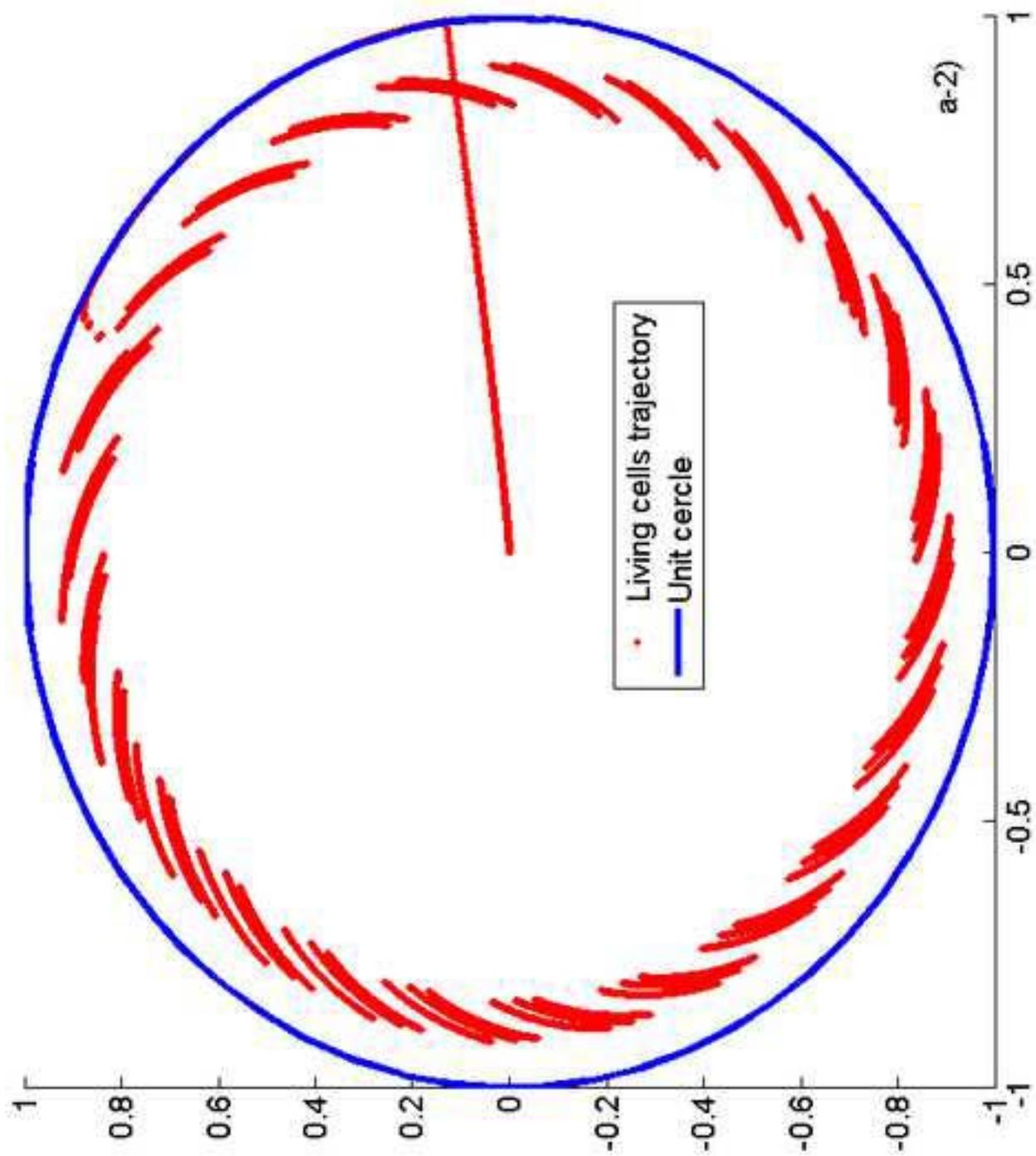
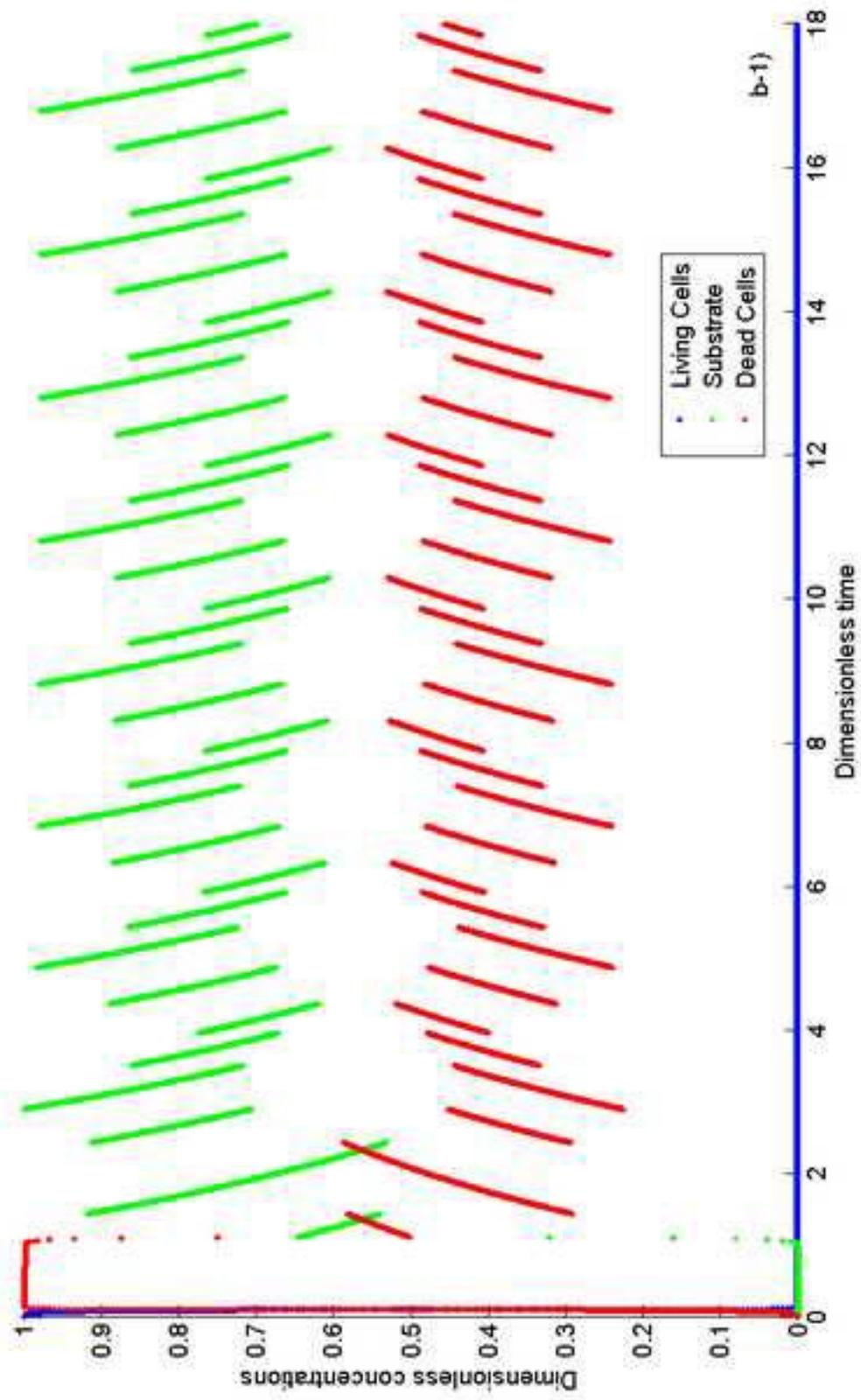
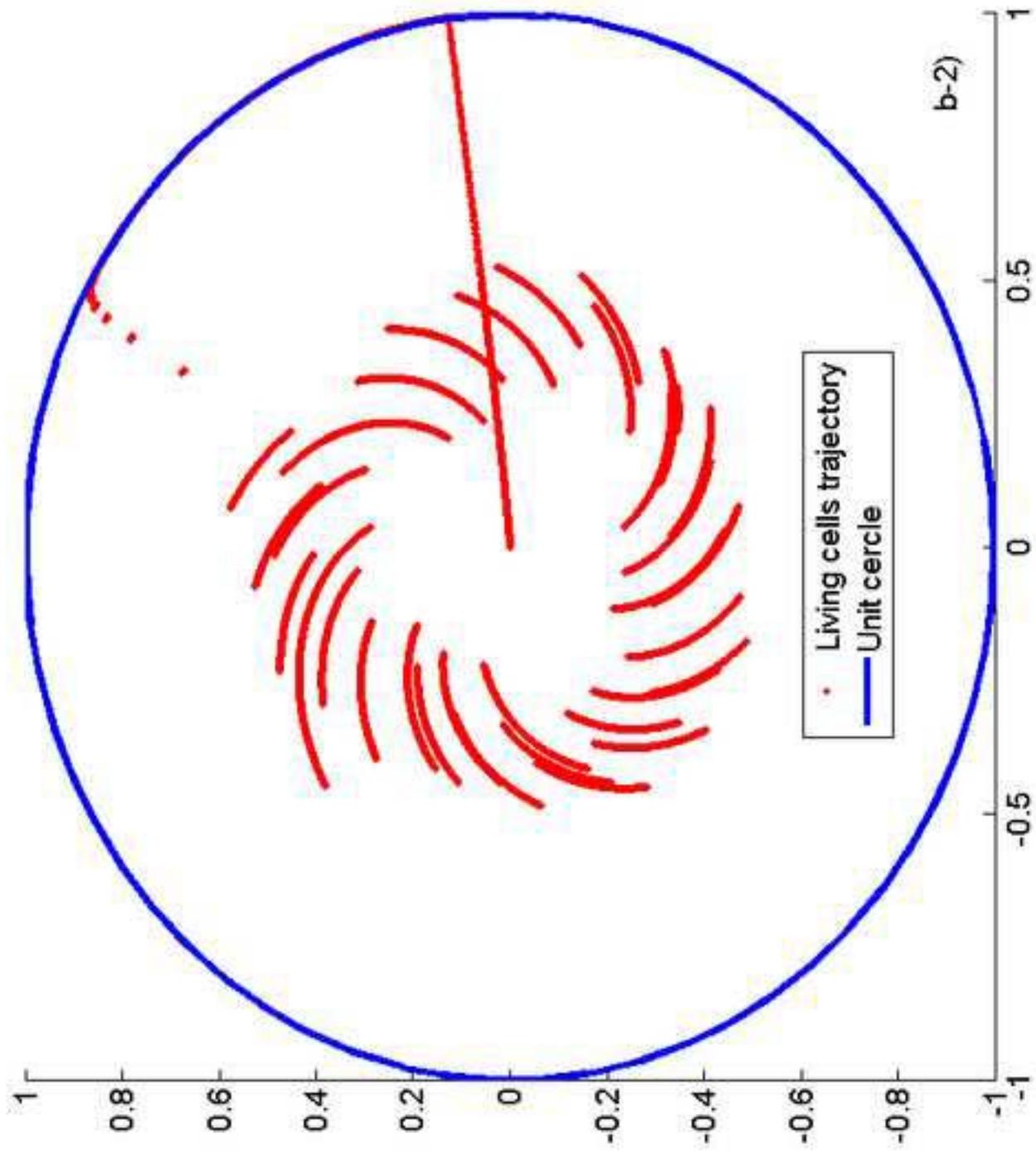


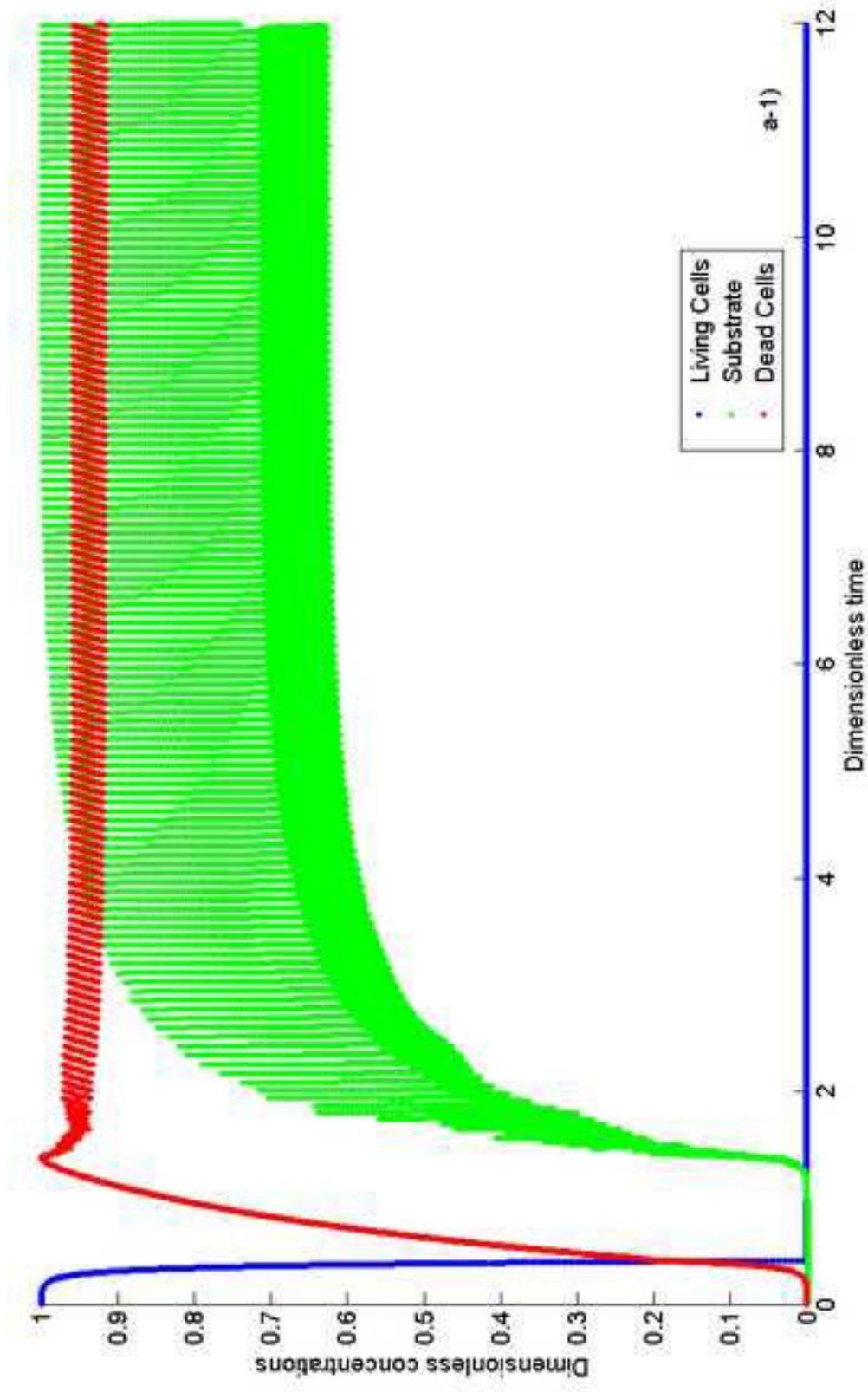
Figure 6

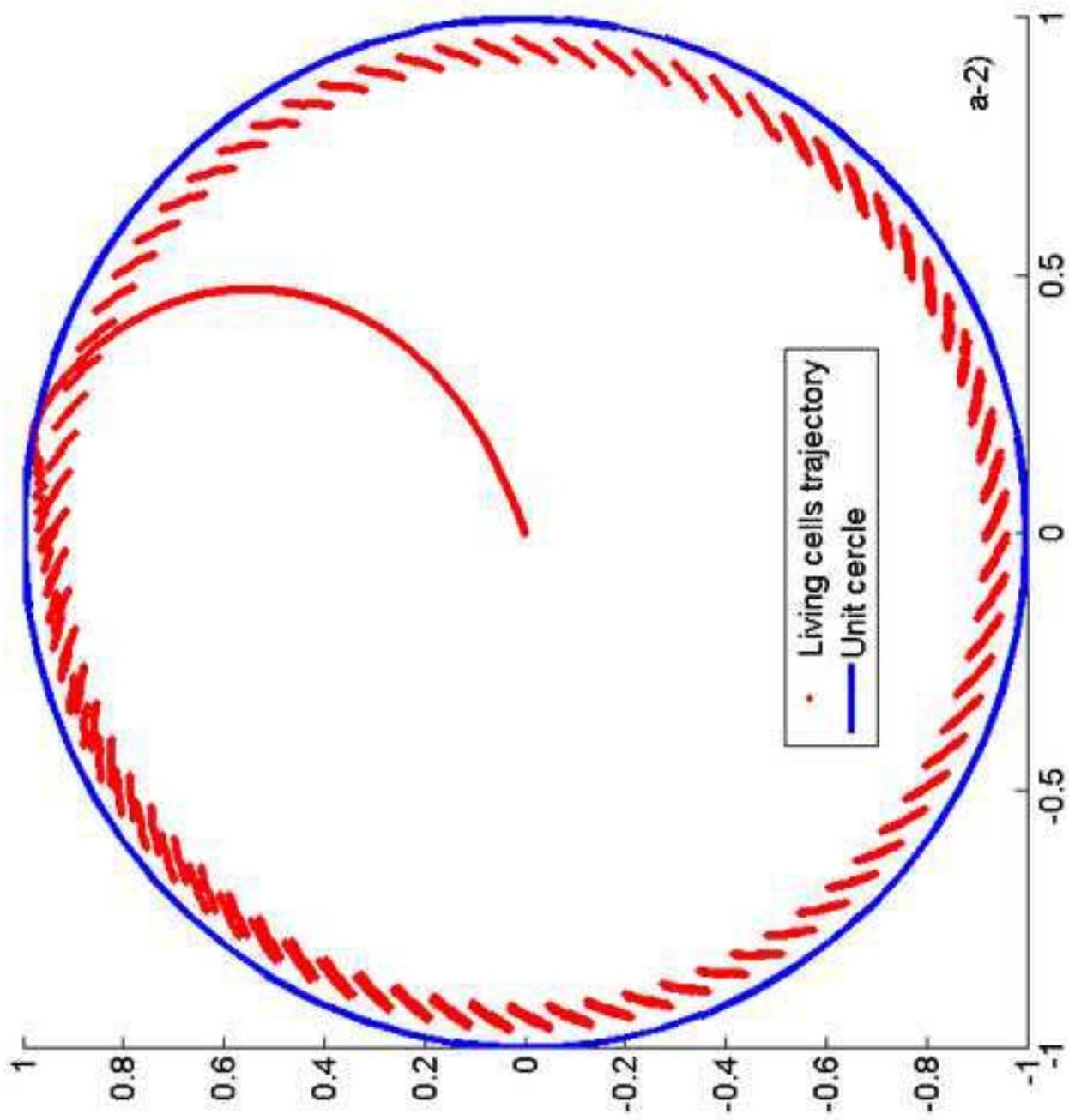












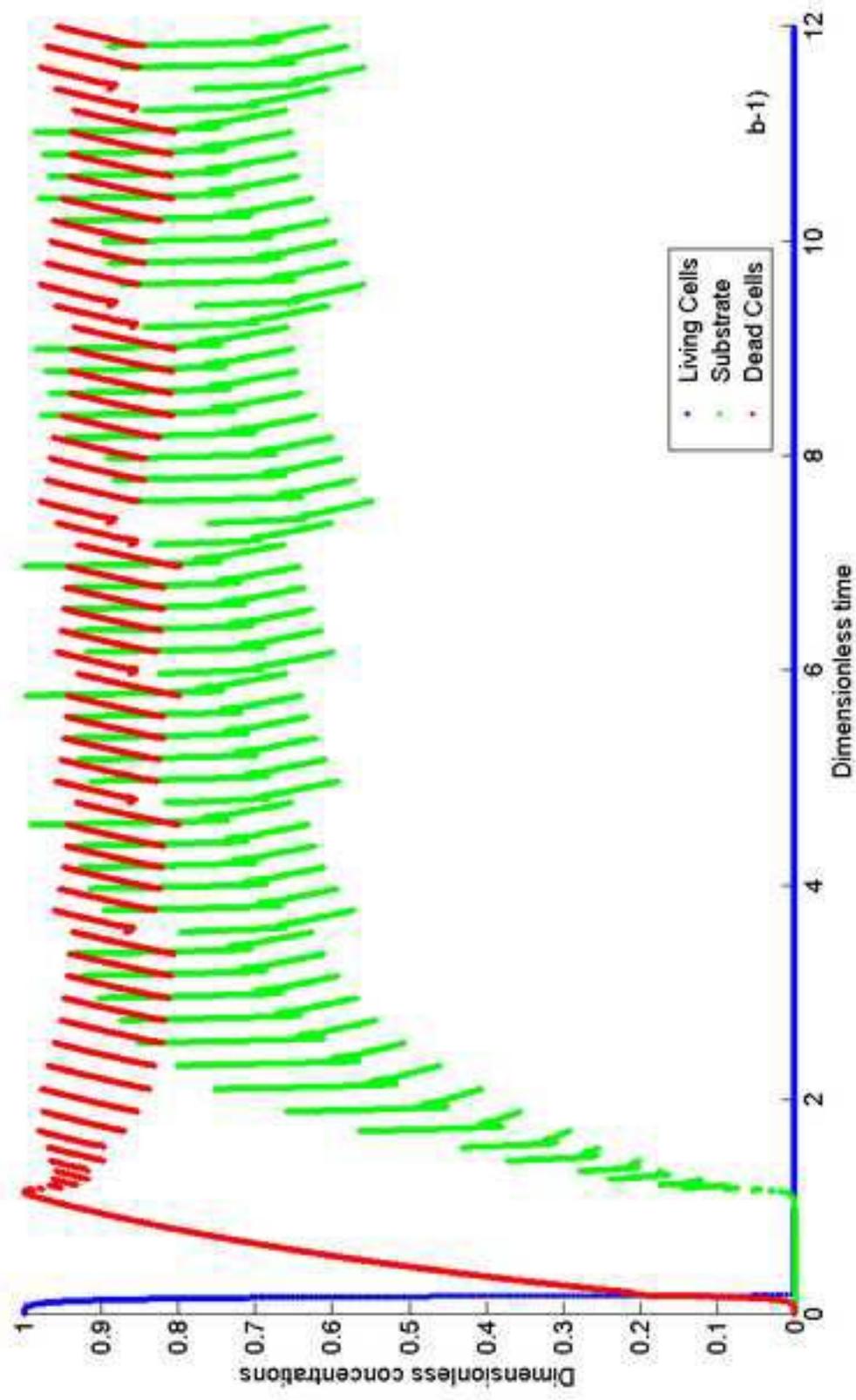


Figure 8b-1

