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ACCEPTED MANUSCRIPT

Modelling the influence of metabolite diffusion on non-starter lactic acid bacteria growth in ripening Cheddar cheese

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ABSTRACT

The influence of metabolite diffusion within the cheese matrix on growth of non-starter lactic acid bacteria (NSLAB) during Cheddar cheese ripening was mathematically modelled. The model was calibrated at a realistic range of diffusion of metabolites and the decay and growth parameters of immobilised starter LAB (SLAB) and NSLAB colonies, respectively. Metabolite diffusion is the limiting factor for NSLAB growth only if essential metabolite molecules are extremely large or otherwise immobilised in the matrix. For relatively small molecules diffusion cannot be a limiting factor; the diffusive replenishment of small molecule nutrients around the NSLAB colonies consuming them is generally faster than the release rate from all possible sources within the curd. Assuming that the only nutrient source limiting NSLAB growth is the release of metabolites from lysed SLAB colonies, the decay rate of SLAB, rather than metabolite diffusion, most probably determines the rate of NSLAB growth during Cheddar cheese ripening.

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1. Introduction

Cheese microbiota is pivotal to nearly all processes taking place during cheese production. Starter lactic acid bacteria (SLAB) are responsible for the conversion of lactose to lactate during the fermentation of milk and results in a pH decrease. The species of SLAB used for the manufacture of cheese depends on the cheese type, with mesophilic species such as *Lactococcus lactis* subsp. *lactis* and subsp. *cremoris* used for the production of Cheddar and cottage cheese types. These mesophilic species can be supplemented with citrate fermenting *Lactococcus lactis* and various *Leuconostoc* species for the production of Gouda and Danbo cheese types. In addition to the mesophilic SLAB, thermophilic SLAB, such as *Streptococcus thermophilus*, *Lactobacillus delbrueckii* subsp. *bulgaricus*, and *Lactobacillus helveticus* are used for the production of pasta-filata and Swiss-type cheeses. Frequently, *S. thermophilus* and/or *Lb. helveticus* cultures may be added to the core mesophilic cultures used for Cheddar and Gouda cheese types to produce a meso-thermo blend. Such meso-thermo blends give improved phage robustness and increased flavour properties. Besides the fermentation of lactose, SLAB are also critical for degradation of casein into peptides and free amino acids, and in the biotransformation of these free amino acids into a very diverse range of aroma compounds (McSweeney, 2017; Yvon, Thirouin, Rijnen, Fromentier, & Gripon, 1997). The SLAB used for the manufacture of cheese are carefully selected and controlled by the cheese producer, and normally obtained from specialist suppliers in freeze-dried or frozen format.

In contrast to the SLAB, the non-starter lactic acid bacteria (NSLAB) are not controlled due to the non-aseptic nature of industrial cheese production. Pasteurisation of the cheese milk only lowers NSLAB levels, but does not eliminate them from the cheese milk (De Angelis et al., 2004). The NSLAB isolated from cheese belong to a very heterogeneous

group, frequently they are members of the *Lactobacillus* species and include *Lactobacillus casei, Lactobacillus paracasei, Lactobacillus plantarum, Lactobacillus pentosus, Lactobacillus curvatus, Lactobacillus rhamnosus, Lactobacillus fermentum, Lactobacillus parabuchneri* and *Lactobacillus brevis*. The non-*Lactobacillus* species of NSLAB commonly isolated from cheese comprise *Pediococcus acidilactici*, *Pediococcus pentosaceus*, *Enterococcus durans*, *Enterococcus faecalis*, and *Enterococcus faecium* (Settanni & Moschetti, 2010). NSLAB originate from the cheese milk and the cheese making environment, and may vary significantly from one dairy plant to another (Banks & Williams, 2004; Settanni & Moschetti, 2010; Sgarbi et al., 2013).

The conversion of milk to cheese can be considered as a two-step process. In the first step, milk is converted into a fresh curd, while in the second step the fresh curd is converted into ripened cheese. In the first step, SLAB grow rapidly in the milk due to the abundance of available substrate (lactose), and after approximately 24 hours reach levels of 10^9 cfu g^{-1} in the fresh curd. No further growth of SLAB occurs in the fresh curd due to the hostile environment encountered (absence of a fermentable carbohydrate, high salt concentration, and low pH). In the second step, which occurs over several months, the SLAB numbers begin to decline, while the NSLAB numbers begin to increase. At the beginning of ripening the NSLAB start at rather low levels of 10^1–10^3 cfu g^{-1}, and may in fact be undetectable using conventional plating techniques. As ripening progresses their number increases to approximately 10^7–10^8 cfu g^{-1} (De Dea Lindner et al., 2008; Fitzsimons, Cogan, Condon, & Beresford, 2001; Gatti et al., 2008; McMahon et al., 2014). The SLAB and NSLAB all grow as immobilised colonies in the cheese during ripening, making them dependent on diffusion of metabolites in the cheese matrix. The distribution of the immobilised bacteria cells in the cheese matrix is random and therefore the mean distance between the colonies is strongly affected by the initial inoculation levels (Jeanson et al., 2011).

The substrate source(s) and how they migrate to the regions in which the NSLAB cells are sparsely distributed within the cheese matrix is not fully elucidated (Gobbetti, De Angelis, Di Cagno, Mancini, & Fox, 2015). Considering the fact that sugars such as lactose, glucose and galactose are rapidly depleted after a few days ripening (Budinich et al., 2011), then other substrate sources must be present. In particular, SLAB derived substrate sources as a result of cell death have been considered as potential carbon and nitrogen sources for NSLAB growth. A typical SLAB cell composition, based on percentage of total dry weight, is approximately 45% protein, 12–15% polysaccharide, 10% teichoic acid, 6–8% RNA, 7% inorganic ions, 5.5% amino sugars, 4–4.3% lipid and 3–3.3% DNA (Novák & Loubiere, 2000). Other possible substrate sources include either more complex milk-derived carbohydrates from κ-casein or the milk fat globular membrane (Moe, Faye, Abrahamsen, Østlie, & Skeie, 2012) and free amino acids and small peptides from the caseins. Strong evidence supports the theory that it is the SLAB derived carbon that the NSLAB use as a growth source. In several studies (Adamberg et al., 2005; Sgarbi et al., 2013; Thomas, 1987; Williams, Withers, & Banks, 2000) it has been demonstrated in vitro that NSLAB are able to grow on dead SLAB material such as ribose and cell-wall originating sugars. Furthermore, it has been confirmed that NSLAB grew faster in Cheddar cheese manufactured with a fast lysing SLAB than a slow lysing SLAB (Lane, Fox, Walsh, Folkertsma, & McSweeney, 1997). More recently (Moe et al., 2012), it has been demonstrated that NSLAB can utilise likely sources of nitrogen to support the growth of NSLAB during cheese ripening are free amino acids and peptides released from the casein due to the action of the rennet, as well as SLAB cell-wall associated proteinase and intracellular peptidases (Cotter & Beresford, 2017). These sources of nitrogen are abundantly available in the ripening cheese.

Very little is known about how and at what rate the SLAB cell components, in particular the limited carbohydrate substrate sources (polysaccharide, teichoic acids, RNA,

DNA and amino sugars) migrate from the SLAB regions of the cheese matrix to the NSLAB regions. Hydrolysis of the polymeric SLAB cell components (polysaccharide, teichoic acids, RNA, DNA) into their constituent monomers (N-acetylglucosamine, N-acetylmuramic acid, ribose and deoxyribose) would be a necessary step prior to diffusion in the cheese matrix. Furthermore, high concentrations of casein-derived free amino acids and peptides are expected to be localised in the SLAB regions, and it is unknown how and at what rate these components migrate to the NSLAB regions. Recently, Floury et al. (2015) reported that milk proteins (bovine serum albumin, lactoferrin and α_{S1}-casein) could not penetrate the inside of bacterial colonies immobilised in a model cheese system. Interestingly, the effect of the spatial distribution of *L. lactis* colonies (small colonies or large colonies) in the same model cheese system was shown to influence the rate of degradation and production of various cheese metabolites such as caseins, free amino acids and volatiles (Le Boucher et al., 2016).

This study, through the use of mathematical modelling, seeks to address the key question regarding how the SLAB and NSLAB grow as immobilised colonies in the cheese matrix during ripening. The micro-ecological approach we take here has not often been taken in dairy research yet, even though ripening cheese provides an ideal setting for both theoretical and experimental studies in microbial ecology (Wolfe & Dutton, 2014). The essential features of the three component processes of cheese ripening – SLAB decay, nutrient substrate diffusion and NSLAB growth – are concisely represented in a simple reaction-diffusion system. Studying numerical realisations of the model with known values of the rates of SLAB decay, nutrient diffusion and NSLAB growth, the potential effects of the component processes on the course of ripening can be assessed separately, providing experimentally testable predictions on the ripening process itself. Specifically, the model examines metabolite diffusion rates and their influence on the space-time dynamics of SLAB death and NSLAB growth. Furthermore, it seeks to examine the effect of a slow lysing SLAB versus a

fast lysing SLAB on the growth rate of the NSLAB population. The model proposed here is applicable for dry-salted cheeses such as Cheddar, in which the SLAB are immediately present in a high salt environment post manufacture.

2. Material and methods

2.1. The model

The presented mathematical model simulates the dynamics of SLAB lysis and NSLAB growth during cheese ripening, based on a typical Cheddar cheese ripening scenario. In the model the SLAB can be set to start decaying within the curd immediately after salting or at any time after that. Upon SLAB lysis, nutrients are directly released from the cytosol or produced by the hydrolytic enzymes of the decaying SLAB cells. These nutrients comprise SLAB cell wall monomer components (N-acetylglucosamine, N-acetylmuramic acid), sugars from hydrolysed nucleic acids (ribose and deoxyribose), free amino acids, and small peptides (10–15 amino acid residues). All these are assumed to diffuse from the localised, lysed SLAB cells within the cheese matrix. The model is set to predict the growth of NSLAB colonies utilising the diffusing material as their nutrient source. We assume that the nutrients actually limiting NSLAB growth in the curd (whether they are nucleotides, nucleobases, sugars or essential co-factors) originate from lysed SLAB cells and need to diffuse to the localised NSLAB colonies. For simplicity we assume that both the SLAB and the NSLAB are homogeneous with respect to their dynamical properties, i.e., all SLAB strains have the same probability of death/lysis, d_{SLAB}, and the expected maximum growth rates of NSLAB strains, r_{NSLAB}, are also the same for all NSLAB bacteria in the cheese matrix. If diffusion is limiting

for NSLAB growth it is the diffusion parameter *D* that is the critical parameter for NSLAB growth. Fig. 1 explains the details of the corresponding dynamics.

With these simplifying assumptions, the model is implemented as a system of partial differential equations in two spatial dimensions, one equation for each of SLAB (*S*), nutrient (*L*) and NSLAB (*N*):

$$\frac{\partial S(x,y,t)}{\partial t} = -d_{SLaB}\, S(x,y,t) \quad [1]$$

$$\frac{\partial L(x,y,t)}{\partial t} = D\left(\frac{\partial^2 L(x,y,t)}{\partial x^2} + \frac{\partial^2 L(x,y,t)}{\partial y^2}\right) + d_{SLaB}\, S(x,y,t) - r_{NSLaB}\frac{L(x,y,t)}{L(x,y,t)+1} N(x,y,t) \quad [2]$$

$$\frac{\partial N(x,y,t)}{\partial t} = r_{NSLaB}\frac{L(x,y,t)}{L(x,y,t)+1} N(x,y,t) \quad [3]$$

where *t* is time from salting, *x* and *y* are coordinates in two spatial dimensions, representing spatial positions in the perpendicular projection of the 3D cheese volume onto a plane. The reduction of the number of spatial dimensions to two is necessary for computational reasons (diffusion in 3D is extremely slow to simulate), and it does not affect the conclusions in the qualitative sense. The decay coefficient d_{SLaB} of the SLAB population implies that SLAB decay is a random process, with each bacterium carrying the same risk of death and lysis within any small period of time, resulting in an exponential decay curve for the SLAB population. The constancy of the decay rate implies that the environment within the curd (salt concentration, pH, temperature) is essentially constant during the ripening process. Nutrients released from lysed SLAB cells move within the matrix following Fick's second law of diffusion, and they are locally consumed by NSLAB, which have a saturating consumption response to local nutrient concentration, i.e., the higher the nutrient concentration *L* around an

NSLAB colony the closer the actual growth rate of that colony to its maximum. We assume, again without a loss of generality in the qualitative sense, that the conversion factor is 1, meaning that the limiting component of the cell material from the lysis of a single SLAB cell is sufficient for the production of one NSLAB cell. Note that nutrient sources other than SLAB lysate (e.g., amino acids and small peptides from the proteolysis of the casein matrix) may be present in excess, but in the model we assume that the nutrients supporting NSLAB growth are released from lysed SLAB cells. Changing the conversion factor to any arbitrary number less than 1 would just decrease the stationary density of NSLAB accordingly.

We follow the time course of the total masses of *S*, *L* and *N* within a small, square shaped region (10 mm side length) of the cheese, using periodic boundary conditions. Defining periodic boundaries amounts to assuming that the focal square region, and those of the same size and shape adjacent to it, are identical, which means that the curd consists of an infinite repetition (lattice) of the focal region in both spatial dimensions. Fig. 2 is an illustration of the model dynamics within the focal region, with arbitrary parameters. Digital Supplement 1 is a video showing the time course of the same process.

The initial patterns of both the SLAB and the NSLAB colonies are random, and the number of NSLAB colonies per focal region is a parameter of the model. Since the cell count of SLAB at salting is about 10^9 cfu g^{-1} and it is evenly distributed within the curd, it is safe to assume that the actual initial SLAB distribution is continuous and uniform – so this is what we assume in all the simulations. The initial NSLAB distribution is still random and discrete, since NSLAB is present in the curd at very low density, represented by only a few individuals within the focal region (about 10 cfu g^{-1}) initially. The founders of NSLAB colonies are implemented as very narrow and very low Gaussian shaped $N(t,x,y)$ initial density curves representing single colony forming units. The height, width and the number of these colonies

within the focal region are parameters of the model. Fig. 3 shows the time course of the log total densities of *S*, *L* an *N* within the same sample.

2.2. Model calibration

We have determined a biologically/chemically feasible range for each of the three key parameters of the model (SLAB decay rate: d_{SLAB}; Nutrient diffusion rate from lysed SLAB: *D*; and NSLAB population growth rate under excess resource supply: r_{NSLAB}) based on fundamental empirical data from the literature. SLAB decay rates and NSLAB growth rates are calculated from known lysis times of different SLAB cultures and replication times of NSLAB at different temperatures, respectively (see below). The metabolite diffusion rates used in the simulations include the range from the diffusivity of small molecules in water to that of large peptides in gels. These parameter ranges have been scanned for sections at which SLAB decay, diffusion or NSLAB growth would be the limiting factor of NSLAB growth and thus of the speed of cheese ripening. The calibrated parameter ranges are the following:

2.2.1. SLAB decay rate (d_{SLAB})

One of the features by which starter cultures are specified is their characteristic time to complete lysis during the ripening process. For Cheddar this may extend from about 3 months (Fox, Guinee, Cogan, & McSweeney, 2017) or 2160 h (using a "fast lysing" SLAB culture like *L. lactis* AM1 or AM2) to approximately 9 months or 6480 h ("slowly lysing" SLAB culture). Assuming exponential decay of the SLAB culture,

$$N(t) = N(0) \times e^{-d_{\mathrm{SLAB}}t} \qquad [4]$$

the relation between the time for 99% of SLAB to lyse, $t_{1\%}$, and the decay rate, d, is

$$\frac{N(t)}{N(0)} = e^{-d_{\mathrm{SLAB}} t_{1\%}} = 0.01 \quad [5]$$

from which

$$-d_{\mathrm{SLAB}} t_{1\%} = \ln 0.01 = -4.6 \quad [6]$$

that is,

$$d_{\mathrm{SLAB}} = \frac{4.6}{t_{1\%}} \quad [7]$$

This yields $d_{\mathrm{SLAB}} = 0.00213$ (h^{-1}) for "fast" SLAB cultures ($t_{1\%}$ = 2160 h), and $d_{\mathrm{SLAB}} = 0.00071$ (h^{-1}) for "slow" ones ($t_{1\%}$ = 6480 h). We have used d_{SLAB} = 0.0025 (h^{-1}) and d_{SLAB} = 0.0007 (h^{-1}) for the simulations.

2.2.2. Diffusion rate of nutrients from lysed SLAB (D_{Lys})

The sample of cheese that we simulate is 10 × 10 mm in size; therefore, the diffusion rates of small-molecule nutrients (like monosaccharides or amino acids) from the literature are rescaled to $mm^2\,h^{-1}$ dimensions for convenience. Literature data for the diffusion rates of the smallest mono- and disaccharides in water at 25 °C range from 5×10^{-10} to 6×10^{-10} $m^2\,s^{-1}$ (Ziegler, Benado, & Rizvi, 1987), which translates to 1.8 to 2.16 $mm^2\,h^{-1}$. Bovine serum albumin (BSA), a peptide with 583 amino acid residues and of 66.5 kDa molecular mass has a diffusion rate of 0.28 $mm^2\,h^{-1}$ at 25 °C and pH = 7 in water (Torres, Komiya, Okajima, & Maruyama, 2012). Notice that the difference between the diffusivities of a small sugar molecule and a rather large peptide is less than an order of magnitude, at least in water. We consider 2.0 $mm^2\,h^{-1}$ an upper limit for the rate of nutrients diffusion in the curd, and scan the range decreasing across 4 orders of magnitude to 0.0002 $mm^2\,h^{-1}$ during the simulations.

Note that we do not take the effect of the casein matrix as a physical barrier to the free diffusion of molecules into account here. It is obvious that very large molecules, just as the bacteria themselves, could be stuck within the matrix and thus immobilised. However,

Chapeau, Silva, Schuck, Thierry, and Floury (2016) and Silva, Lortal, and Floury (2015) show that even very large dextrans (with molecular masses of 2000 kDa) diffuse freely within the casein matrix of ripening cheese without being trapped in it. Therefore, we assume that all molecules of the size readily ingested and metabolised by NSLAB also diffuse freely within the aqueous phase of the curd. However, it should be noted here that the diffusion in cheese depends on the actual water activity of the curd, which is in the range a_w = 0.950–0.975 in ripening Cheddar (Schmidt & Fontana Jr., 2007), implying about an order of magnitude decrease in diffusion rate compared with that in water. Until we have better data available on the diffusion of different small-molecule metabolites (monosaccharides, amino acids) and oligopeptides of different sizes (experimental work in progress), shapes and surface charges in Cheddar we will use this approximation for model calibration.

Considering these facts, the estimated realistic range of diffusion for metabolites available as nutrients for NSLAB growth is about 0.2–0.02 $mm^2\,h^{-1}$ in ripening Cheddar with a water activity around 0.95.

2.2.3. NSLAB growth rate

Under ideal conditions (i.e., for unlimited food supply allowing for maximum population growth rate, at optimal temperature, pH, etc.) the generation time of a typical lactic acid bacteria strain is about half an hour (t_{gen} = 0.5 h) (Kunji, Slotboom, & Poolman, 2003). Calculations similar to Eqs. 4–7 show that the maximum rate of population growth corresponding to this generation time is r_{NSLAB} = 1.386 h^{-1}. Note that at ripening temperature (9–10 °C) the growth rate is about an order of magnitude lower than at the temperature optimal for population growth. Assuming nutrient limitation, we have defined the nutrient-dependent growth rate to be of the form:

$$r(L) = r_{NSLaB} \times \frac{L}{L+1} \qquad [8]$$

We have set r_{NSLAB} = 1.4 h^{-1} to be the highest possible growth rate (at optimum conditions in all respects), and assumed that at ripening conditions (lower temperature and pH) the growth rate is at least an order of magnitude lower (r_{NSLAB} = 0.14 h^{-1}). The simulations have been carried out using these two values of the NSLAB growth parameter.

2.3. Parameter range of model simulations

The part of the parameter space covered by the model simulations is shown in Table 1. The model simulations are focussed on four combinations of two characteristic SLAB decay rates and two characteristic NSLAB growth rates, corresponding to fast and slowly lysing SLAB (d_{SLAB} = 0.0025 and 0.0007 h^{-1}, respectively) providing nutrients to fast and slowly growing NSLAB (r_{NSLAB} = 1.4 and 0.14 h^{-1}, respectively). Each of the four possible (d_{SLab}, r_{NSLAB}) combinations was simulated at nutrient diffusion rates varying across four orders of magnitude (at D = 2.0000, 0.2000, 0.0200, 0.0020 and 0.0002 $mm^2\,h^{-1}$). The time courses of changes in SLAB (*S*), lysed SLAB (nutrient; *L*) and NSLAB (*N*) density during the first 3 months of the ripening process are shown on Figs. 4–7. The curves on all these figures are obtained by numerical integration with respect to the spatial dimensions *x* and *y* of the corresponding $S(x,y,t)$, $L(x,y,t)$ and $N(x,y,t)$ functions across the 2D cheese sample:

$$\bar{S}(t) = \iint_{-5}^{5} S(x,y,t)\,dx\,dy$$

$$\bar{L}(t) = \iint_{-5}^{5} L(x,y,t)\,dx\,dy$$

$$\bar{N}(t) = \iint_{-5}^{5} N(x,y,t)\,dx\,dy$$

where $\bar{S}(t)$, $\bar{L}(t)$and $\bar{N}(t)$ are the total masses of SLAB, nutrients and NSLAB, respectively, within the 10 × 10 mm^2 cheese samples, at time *t*.

The ripening process strongly depends on the activity of NSLAB that produces many of the aromatic compounds responsible for flavour development. Therefore, we can use the time integral of $\bar{N}(t)$, i.e.,

$$\bar{\bar{N}}(T) = \int_0^T \bar{N}(t)\, dt$$

as an approximate measure of the ripening accomplished within the cheese sample by time *T* from salting. In other words, $\bar{\bar{N}}(T)$ is the total microbial activity provided by the growing NSLAB population on ripening the curd. This is the target function of the model: the faster the NSLAB population grows, the shorter the time *T* needed to achieve a certain level of ripeness $\bar{\bar{N}}$.

3. Results

Simulation results for four different parameter scenarios are considered below: (i) fast decaying SLAB and fast growing NSLAB; (ii) slowly decaying SLAB and fast growing NSLAB; (iii) fast decaying SLAB and slowly growing NSLAB; (iv) slowly decaying SLAB and slowly growing NSLAB.

3.1. Fast decaying SLAB and fast growing NSLAB

This case corresponds to using a fast decaying SLAB culture and ripening Cheddar cheese at room temperature (about 20 °C). Diffusion is almost completely irrelevant with regard to NSLAB growth during ripening: the NSLAB growth curves corresponding to different diffusion rates almost coincide, suggesting that there is no considerable diffusion limitation on the ripening process at this parameter setting. Lysed SLAB density (i.e., the concentration of the limiting metabolite) is close to zero almost all along the process, indicating prompt consumption of the metabolites upon lysis (Fig. 4).

3.2. Slowly decaying SLAB and fast growing NSLAB

This case is essentially the same as the previous one, except that the SLAB population lyses slower, and thus the ripening process also proceeds slower (Fig. 5). Diffusion does not really make a substantial difference here either. Also, for these scenarios, ripening is limited by SLAB decay rather than diffusion.

3.3. Fast decaying SLAB and slowly growing NSLAB

This is the most "realistic" scenario set to simulate the ripening of Cheddar inoculated with a fast lysing starter culture such as *L. lactis* AM1 and AM2 (Fox et al., 2017) and ripened at 9–10 °C (Fig. 6). The conspicuous difference in the dynamics relative to that of the first case (which represents optimum conditions for NSLAB growth) is that diffusion is obviously much more important in determining NSLAB growth in this case: the growth curves are quite different at different nutrient diffusion rates. Slowly growing NSLAB seem to be more sensitive to the rate of nutrient replenishment into the depletion zone around the NSLAB colonies. However, the essential difference is confined to very low diffusion rates

(0.0200–0.0002 $mm^2\ h^{-1}$ – which is most probably below the realistic range for the nutrients that bacteria can readily utilise). At feasible diffusion rates (0.0200–0.2000 $mm^2\ h^{-1}$) the difference in the growth curves is small, meaning that moderate diffusion limitation is to be expected only at the lowest realistic rate of metabolite diffusion, where the time integral of the growth curve of NSLAB, $\overline{\overline{N}}(T)$ is somewhat smaller than at faster diffusion rates. Note that within the realistic range of diffusion rates the differences in total ripening accomplished during the three months of simulated time are still very limited. In other words, the diffusion of small molecules (like amino acids or monosaccharides, with diffusion rates in the range 0.2 to 0.3) is not limiting the speed of ripening, whereas the possibly limited accessibility of larger molecules like oligopeptides may have some effect on NSLAB growth, and thereby also on ripening time.

3.4. Slowly decaying SLAB and slowly growing NSLAB

Another "realistic" scenario set to simulate the ripening of Cheddar assuming that the SLAB inoculum is a slowly lysing starter culture such as *L. lactis* Z8, ML1 and HP (Fox et al., 2017), and the cheese is stored at 9–10 °C during the ripening phase (Fig. 7). The dynamics are similar to that of case (iii): the NSLAB growth curves are quite similar within the realistic range of nutrient diffusion rates (at D = 0.0200 and 0.2000 $mm^2\ h^{-1}$), while at lower rates the growth of NSLAB is significantly affected.

4. Discussion

Establishment of NSLAB flora during cheese ripening is considered essential for the normal flavour development in long ripened cheeses such as Cheddar, Gouda and Grana type

cheeses (Crow, Curry, & Hayes, 2001; Santarelli, Bottari, Lazzi, Neviani, & Gatti, 2013). Therefore, to control the ripening process it is important to understand how the NSLAB population develops over time. In this study we have used mathematical modelling to elucidate some of the possible limiting factors for NSLAB growth in a dry-salted cheese such as Cheddar.

The only possible scenarios in which diffusion can limit the speed of cheese ripening are either (i) some nutrient molecules essential for NSLAB growth are prevented from moving freely within the Cheddar cheese matrix and, therefore, diffuse much slower than measured in other systems, or (ii) NSLAB growth is dependent on the supply of some nutrient consisting of rather large molecules like a large peptide, the diffusion rate of which could be significantly lower than that of small metabolites.

Scenario (i) is not completely unrealistic, considering small metabolites possibly immobilised on the casein matrix by covalent or strong secondary (e.g., ionic) bonds, but we have no examples of such cases in mind. With respect to (ii), we do not know examples of cultivated NSLAB strains requiring such large molecules for their growth either; therefore, we see no reason why this should be the case when growing in the cheese matrix. Thus, we see none of the above suggested scenarios realistic and, therefore, on the basis of the simulation studies performed with the model, we conclude that diffusion is most probably not limiting NSLAB growth. This applies to systems with slow or fast lysing SLAB cultures alike.

It is important to stress that the model is built on the assumption that nutrients released from autolysed SLAB cells diffuse into the surroundings immediately after lysis. During this process the lysed cell carbohydrate components such as polysaccharides, teichoic acids, RNA and DNA are expected to be rapidly hydrolysed into their constituent monomers (N-acetylglucosamine, N-acetylmuramic acid, ribose and deoxyribose), which means that it is

most probably this pool of small-molecule nutrients that is released into the cheese matrix. Similarly, released peptidases are expected to rapidly hydrolyse the casein and large casein-derived peptides into small peptides and free amino acids, which will also rapidly diffuse into the surrounding cheese matrix.

The simulation results indicated that for all four scenarios of the three component processes (SLAB decay, limiting nutrient diffusion and NSLAB population growth) it is the decay rate of the SLAB culture that is the main determining factor for the population growth of NSLAB. Considering that the development of typical cheese flavour in Cheddar is correlated with the development of NSLAB (Coolbear et al., 2008), then the model presented here suggests that the supply of small-molecule nutrients (provided by lysed SLAB cells) to the NSLAB, rather than their rate of diffusion in the cheese matrix that is the predominant parameter for NSLAB growth. The model predicts that within the realistic small molecule nutrient diffusivity ranges of 0.02 to 0.20 $mm^2 h^{-1}$ the rate of NSLAB growth is sufficiently high to keep the limited SLAB carbohydrate sources concentration close to zero in the cheese matrix even at the suboptimal conditions (9–10 °C, pH 4–5) of cheese ripening. The model assumes that free amino acids and small peptides are in abundance. It is only at extremely (and for the relatively small molecules of typical nutrients of NSLAB, unrealistically) low diffusion rates that nutrient diffusion become limiting to such an extent that the limited carbohydrate sources released due to SLAB lysis will not be immediately accessible to NSLAB. In this situation, the limiting carbohydrate concentrations within the curd would markedly exceed zero during the ripening period, which is not what we see: carbohydrates disappear from the curd very early during ripening.

Thus, although we can conclude from these modelling studies that diffusion limitations are most probably not limiting NSLAB growth, it should be emphasised that we cannot exclude that other parameters rather than SLAB lysis could influence NSLAB growth.

For example, carbohydrates from κ-casein or the milk fat globular membrane present in the cheese matrix, as previously suggested (Adamberg et al., 2005; Moe et al., 2012) could also be important for NSLAB growth, and this needs to be still fully elucidated. Furthermore, the situation may be more complex than that modelled here, as it has been shown (Hickey, Fallico, Wilkinson, & Sheehan, 2018) that some starter cells may die but not lyse, and thus prevent the release of cellular material into the cheese matrix. Another scenario not accounted for in this model could be that the starter cells may be non-culturable but still alive. Nevertheless, if these molecules are important for NSLAB growth it is most probably the release rate of these molecules into the Cheddar cheese matrix that is determining NSLAB growth rather than their ability to diffuse to the immobilised NSLAB colonies in the matrix.

Comparison of the simulation results at optimum growth temperature (25 °C) for NSLAB (Figs. 4 and 5) with the corresponding results (Figs 6 and 7) for the temperature of Cheddar cheese ripening (9–10 °C) indicates that the difference in the effect of very small nutrient diffusions is conspicuous, and somewhat puzzling at first glance. One would expect the effect of nutrient diffusion to be even weaker on a population of lower growth rate than on a fast growing one, but in fact it is just the opposite: very slow nutrient diffusion affects slowly growing NSLAB colonies considerably more than fast ones. This effect may be explained by spatial constraints: slowly growing colonies cannot decrease the nutrient concentration at their boundary as efficiently as the fast growing cells. Therefore, at any given (very low) diffusion rate the concentration gradient at the boundary of slow growing NSLAB colonies is less steep than that of fast growing NSLABs, resulting in slower nutrient replenishment and, consequently, slower population growth. Note that this difference is evident only at unrealistically low diffusion rates: changing nutrient diffusivity within the realistic range does not have a significant dynamical effect either on a fast or a slowly growing NSLAB population.

5. Conclusions

Mathematical modelling based on realistic assumptions and modelling scenarios of the component processes of ripening of a dry-salted cheese such as Cheddar and applying parameters for SLAB decay, nutrient diffusion and NSLAB growth taken from the literature has shown that nutrient diffusion most probably cannot be the bottleneck for NSLAB growth during ripening. Neither could the growth potential of the NSLAB colonies be the limiting factor, not even at the suboptimal conditions at which they persist during ripening. The component process determining the rate of NSLAB growth (and thus also the rate of the ripening process) seems to be the supply of the nutrient that is present at limiting density within the cheese, and that in its turn depends on the decay rate of SLAB cells. Which type of nutrient is the one limiting NSLAB growth remains an open question that calls for further experimental work.

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Figure legends

Fig. 1. Schematic representation (a) and detailed explanation (b) of the component processes of the cheese ripening model. SLAB death and autolysis provides diffusible nutrients for NSLAB population growth. The nutrient supply of the NSLAB colonies, which are fixed within the curd, depends on the speed of SLAB decay and the diffusivity in the curd of the nutrients released from autolysed SLAB cells. The key parameters of the three component processes are: SLAB decay: d_{SLAB}; nutrient diffusion: D_{Lys}; NSLAB growth: r_{NSLAB}.

Fig. 2. Graphical demonstration of the space-time dynamics of the Cheddar ripening model with random initial patterns of 10 SLAB and 10 NSLAB colonies and arbitrary parameters: SLAB decay rate d_{SLAB} = 0.01 h^{-1}, diffusion rate of nutrients from lysed SLAB D_{Lys} = 0.18 $mm^2\,h^{-1}$, NSLAB population growth rate r_{NSLAB} = 0.1 h^{-1}. Each row of panels shows the spatial density distribution of SLAB, lysed SLAB (nutrient source) and NSLAB colonies, respectively, at the corresponding time (t = 0, 60, 160 and 500).

Fig. 3. The dynamics of the total densities of SLAB (blue), lysed SLAB (orange) and NSLAB (green) within the 10 × 10 mm sample of curd, obtained by integration of local densities across the sample at every 4^{th} hour. Data points are means and standard errors for 10 replicate simulations (produced with the same parameter set but different random number sequences). Parameters are the same as in Fig. 2; the graph represents the demo dynamics shown on Fig. 2.

Fig. 4. Simulated total densities of fast decaying SLAB (blue), lysed SLAB (orange) and fast growing NSLAB (green) cells at different nutrient diffusivities within the 10 × 10 mm cheese sample. Calibrated parameters of the model: SLAB decay rate d_{SLAB} = 0.0025 h^{-1}; NSLAB growth rate r_{NSLAB} = 1.4 h^{-1}; the diffusion rate of the nutrients released from lysed SLAB cells within the cheese matrix are D = 2.0, 0.2, 0.02, 0.002 and 0.0002 $mm^2\,h^{-1}$.

Fig. 5. Simulated total densities of slowly decaying SLAB (blue), lysed SLAB (orange) and fast growing NSLAB (green) cells at different nutrient diffusivities within the 10 × 10 mm cheese sample. Parameters are the same as in Fig. 4, except for SLAB decay rate: d_{SLAB} = 0.0007 h^{-1}.

Fig. 6. Simulated total densities of fast decaying SLAB (blue), lysed SLAB (orange) and slowly growing NSLAB (green) cells at different nutrient diffusivities within the 10 × 10 mm cheese sample. Parameters are the same as in Fig. 4, except for NSLAB growth rate: r_{NSLAB} = 0.14 h^{-1}.

Fig. 7. Simulated total densities of slowly decaying SLAB (blue), lysed SLAB (orange) and slowly growing NSLAB (green) cells at different nutrient diffusivities within the 10 × 10 mm cheese sample. Parameters are the same as in Fig. 6, except for SLAB decay rate: d_{SLAB} = 0.0007 h^{-1}.

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Table 1

The parameter range of the simulations.

Parameter	Lower limit	Upper limit	Dimension
SLAB decay rate, d_{SLAB}	0.0007	0.0025	h^{-1}
Lysed SLAB (nutrient) diffusion rate, D_{Lys}	0.0002	2.0000	$mm^2\ h^{-1}$
NSLAB maximum growth rate, r_{NSLAB}	0.0140	1.4000	h^{-1}

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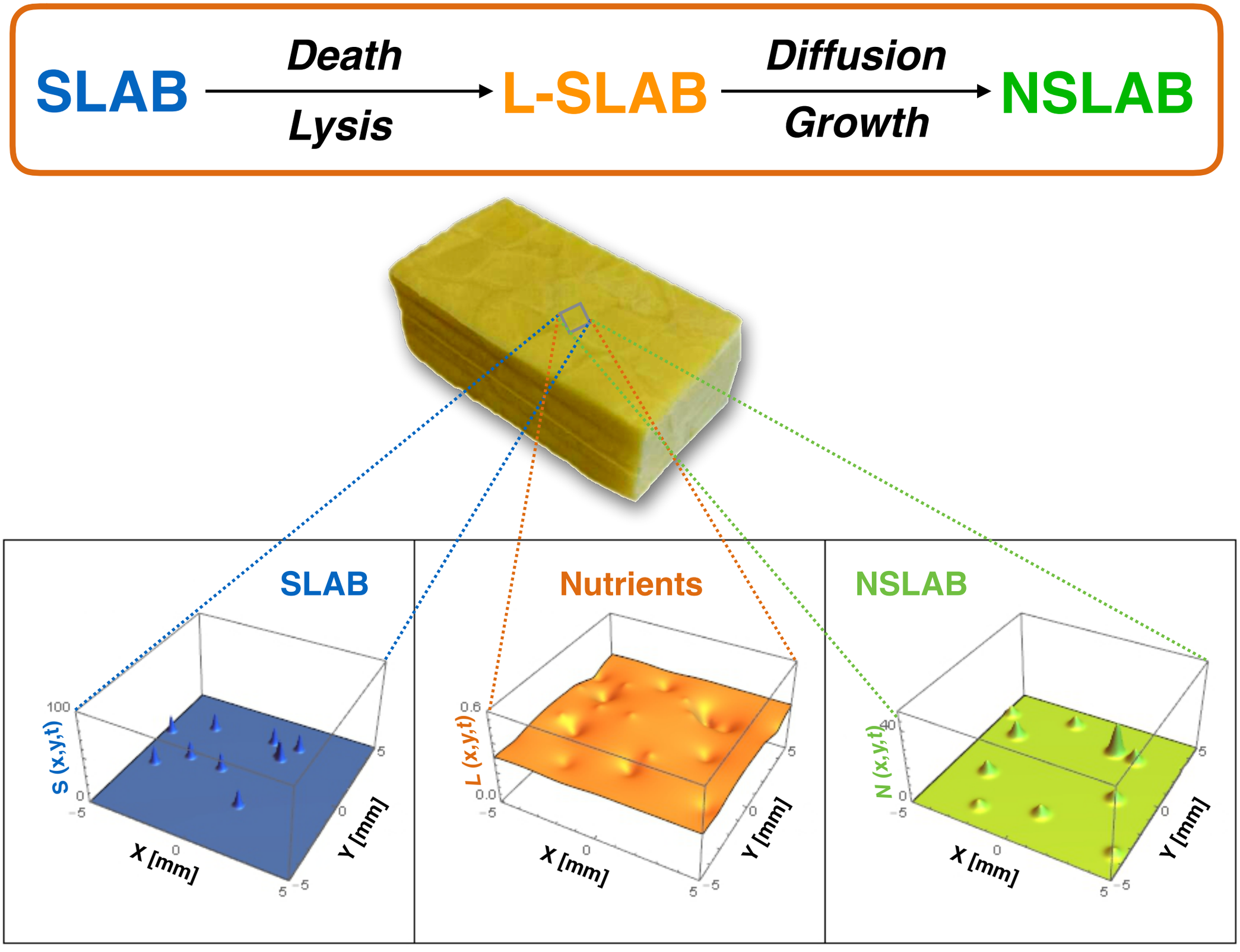
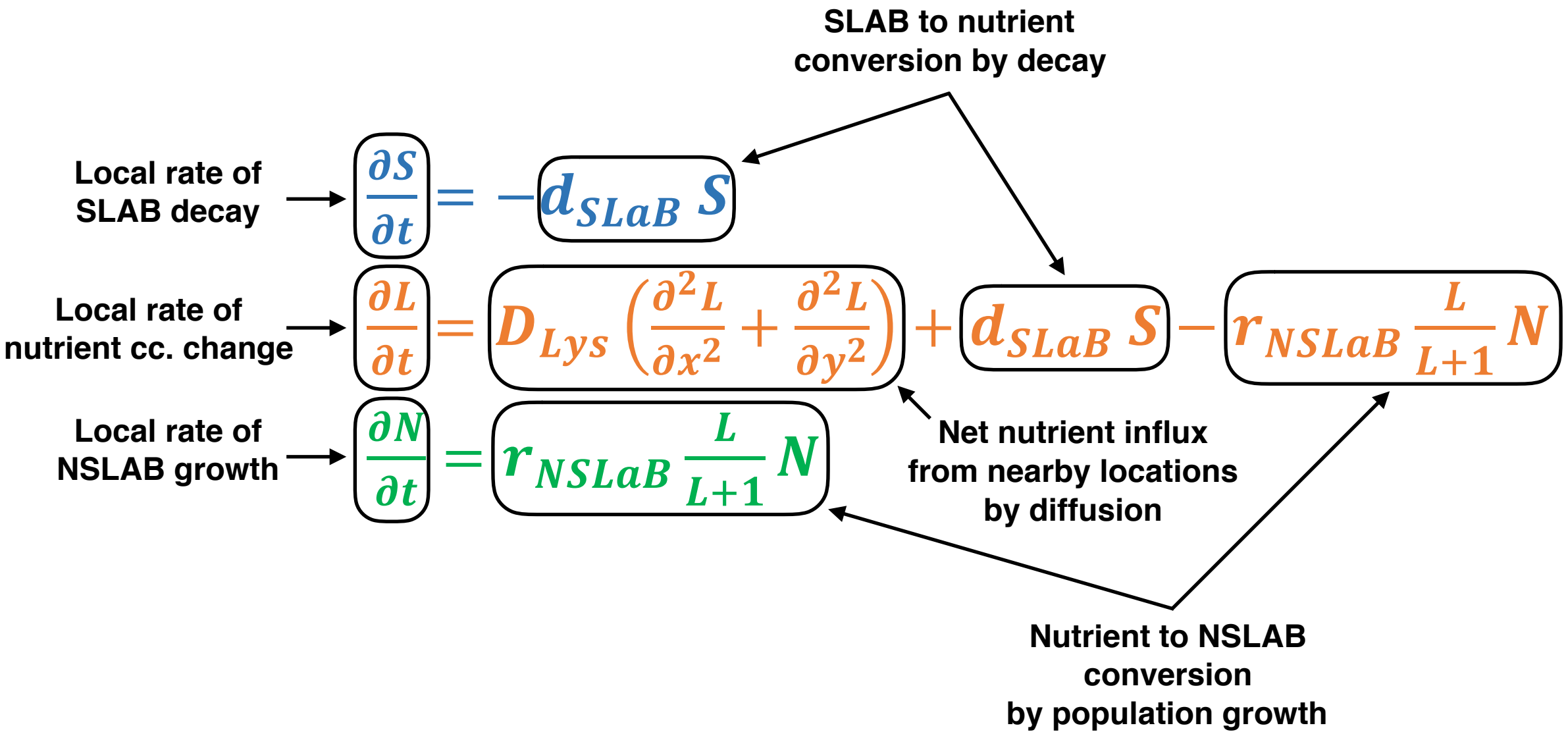
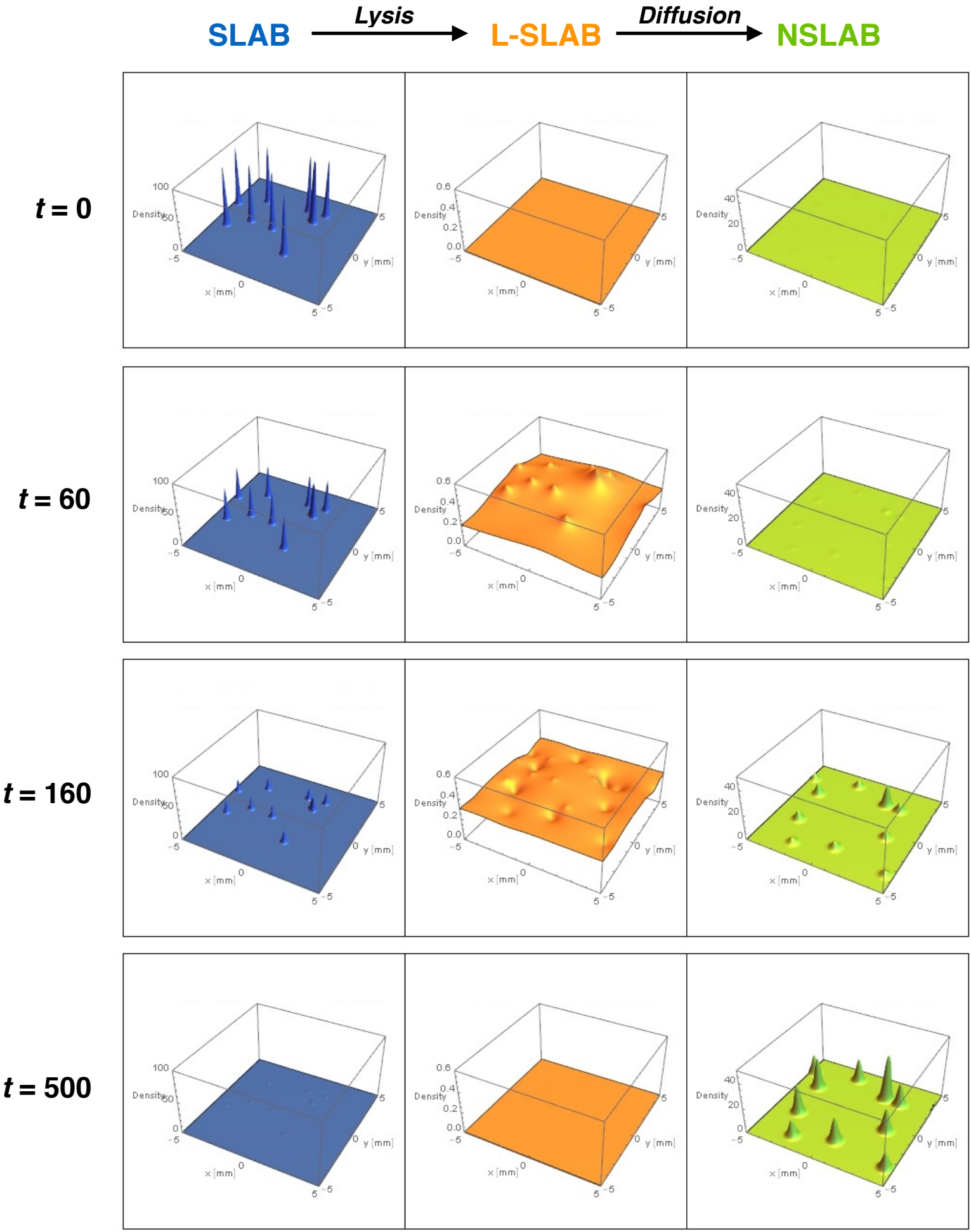
(a)

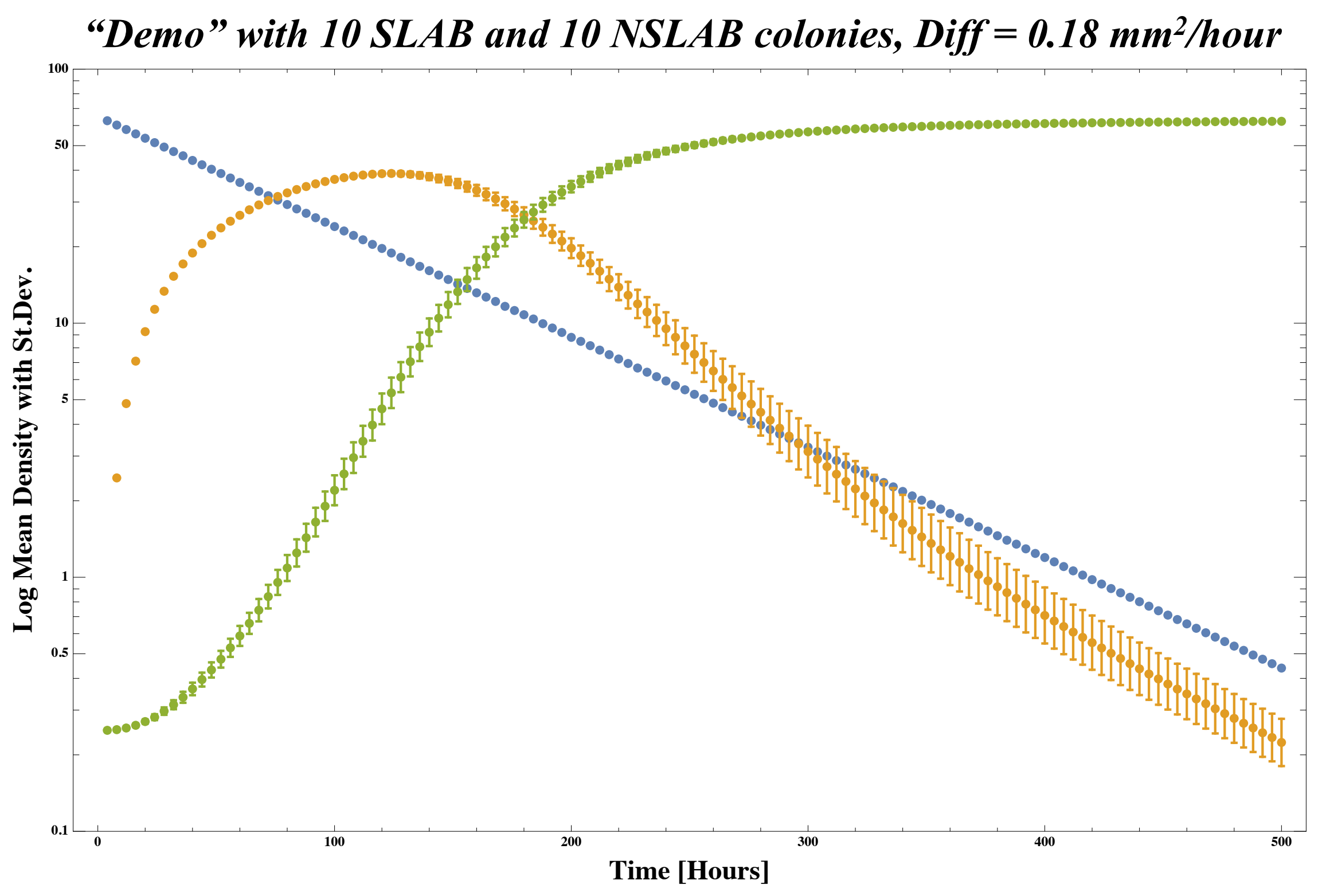

SLAB
Death
Lysis
L-SLAB
Diffusion
Growth
NSLAB
SLAB
Nutrients
NSLAB
S (x,y,t)
L (x,y,t)
N (x,y,t)
X [mm]
Y [mm]

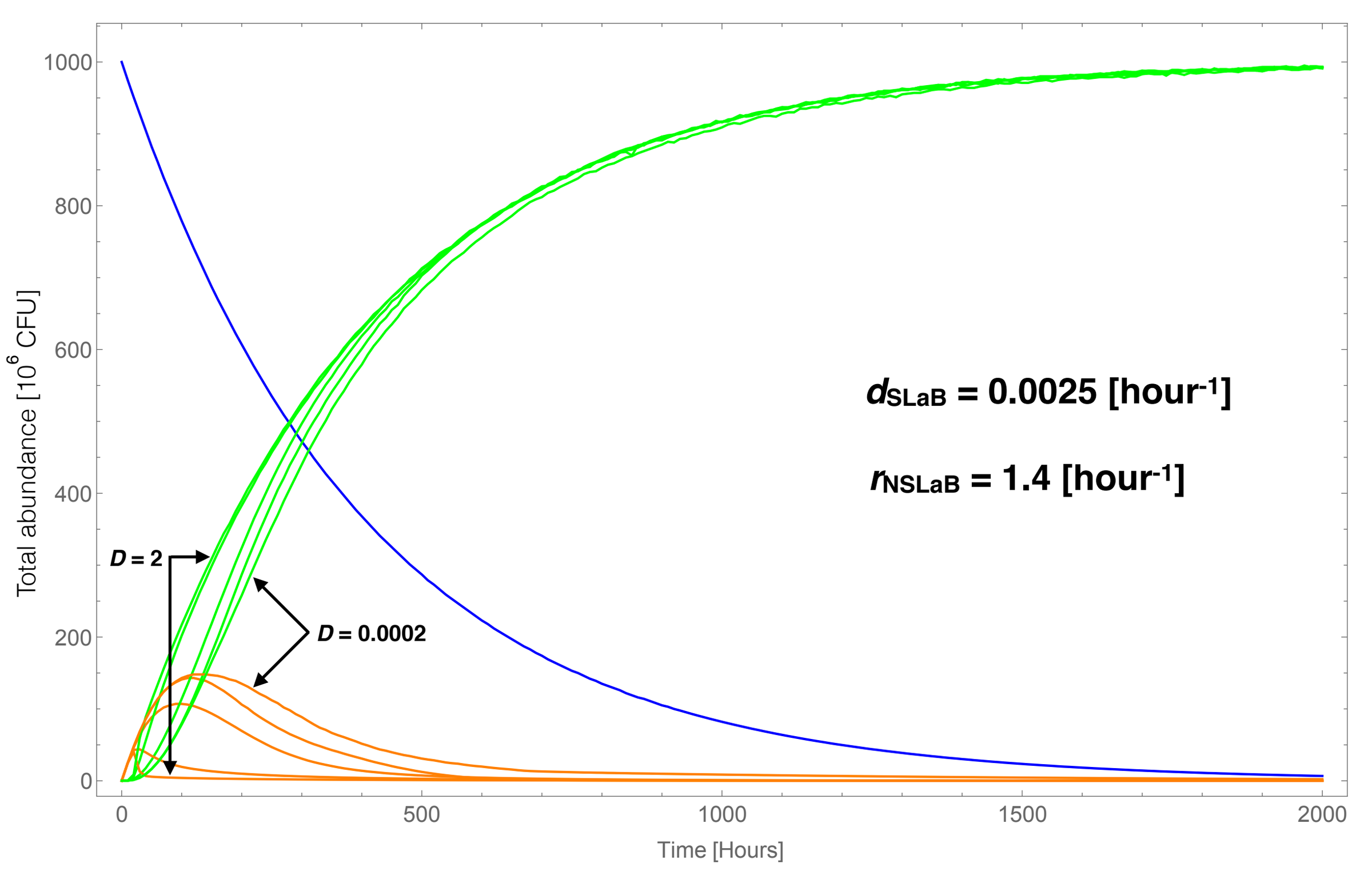
***t* = 160**
[hours]

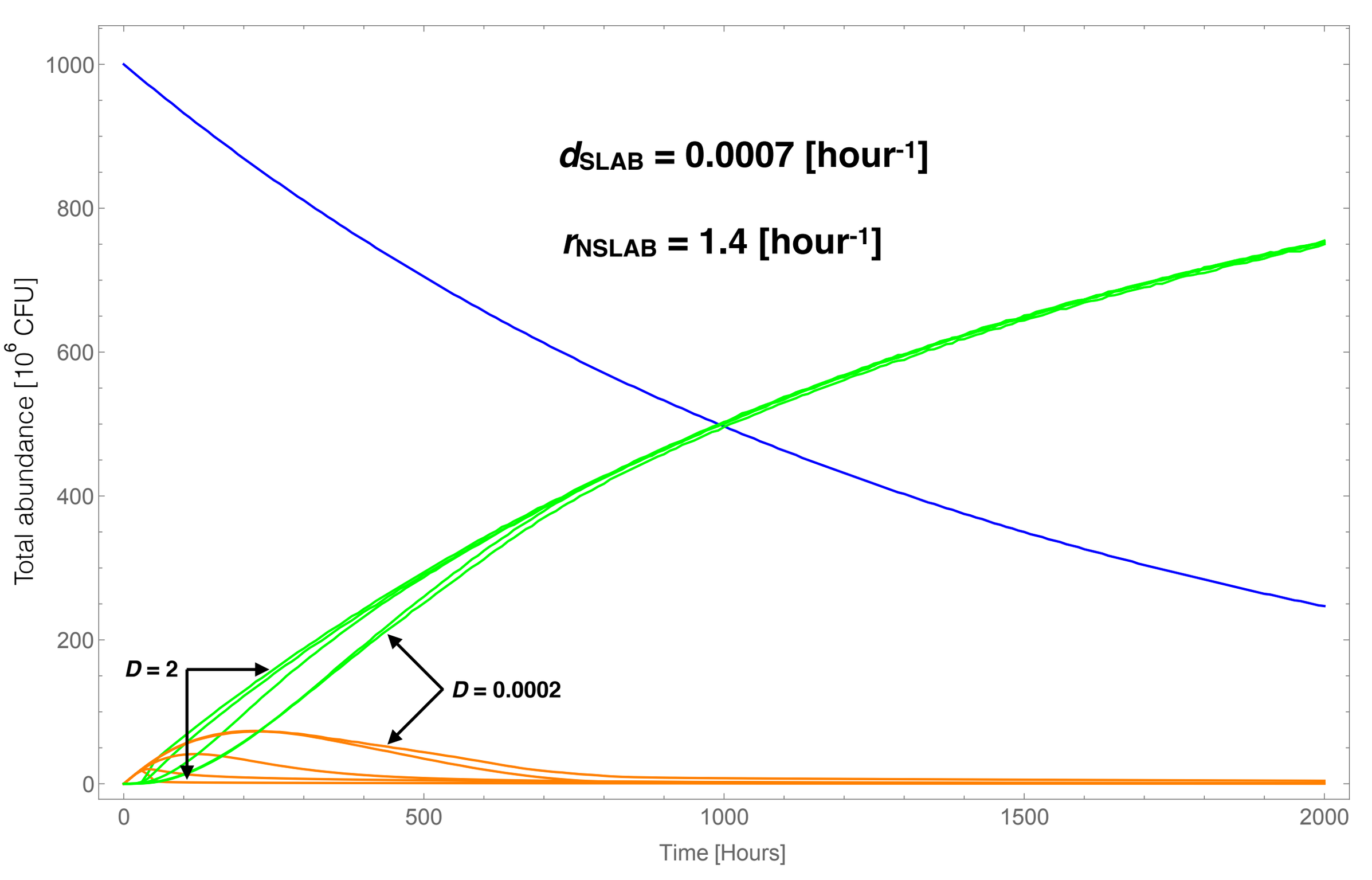
(b)

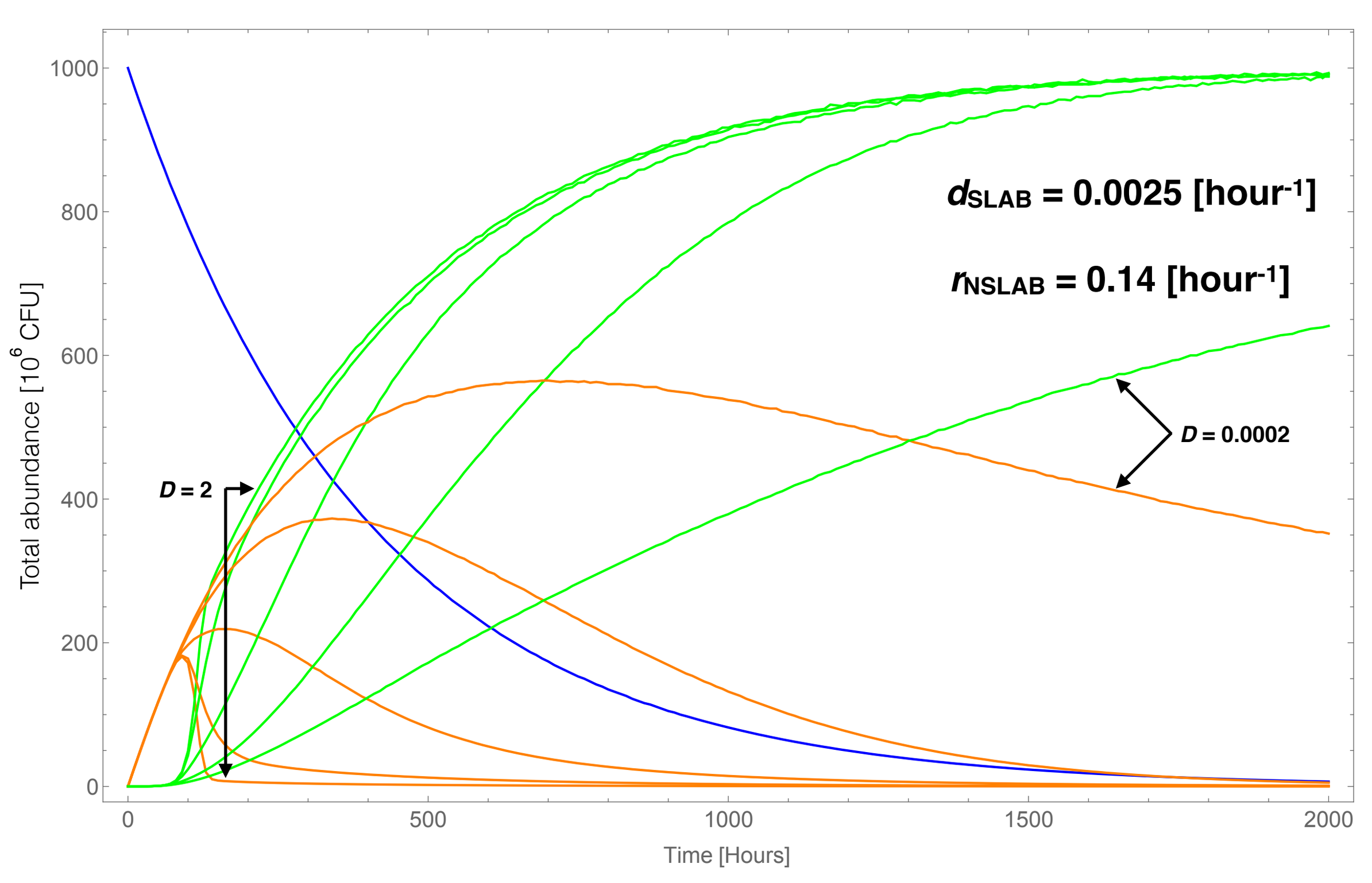

SLAB to nutrient conversion by decay
Local rate of SLAB decay
$\frac{\partial S}{\partial t} = -d_{SLaB}\, S$
Local rate of nutrient cc. change
$\frac{\partial L}{\partial t} = D_{Lys}\left(\frac{\partial^2 L}{\partial x^2} + \frac{\partial^2 L}{\partial y^2}\right) + d_{SLaB}\, S - r_{NSLaB}\frac{L}{L+1} N$
Local rate of NSLAB growth
$\frac{\partial N}{\partial t} = r_{NSLaB}\frac{L}{L+1} N$
Net nutrient influx from nearby locations by diffusion
Nutrient to NSLAB conversion by population growth

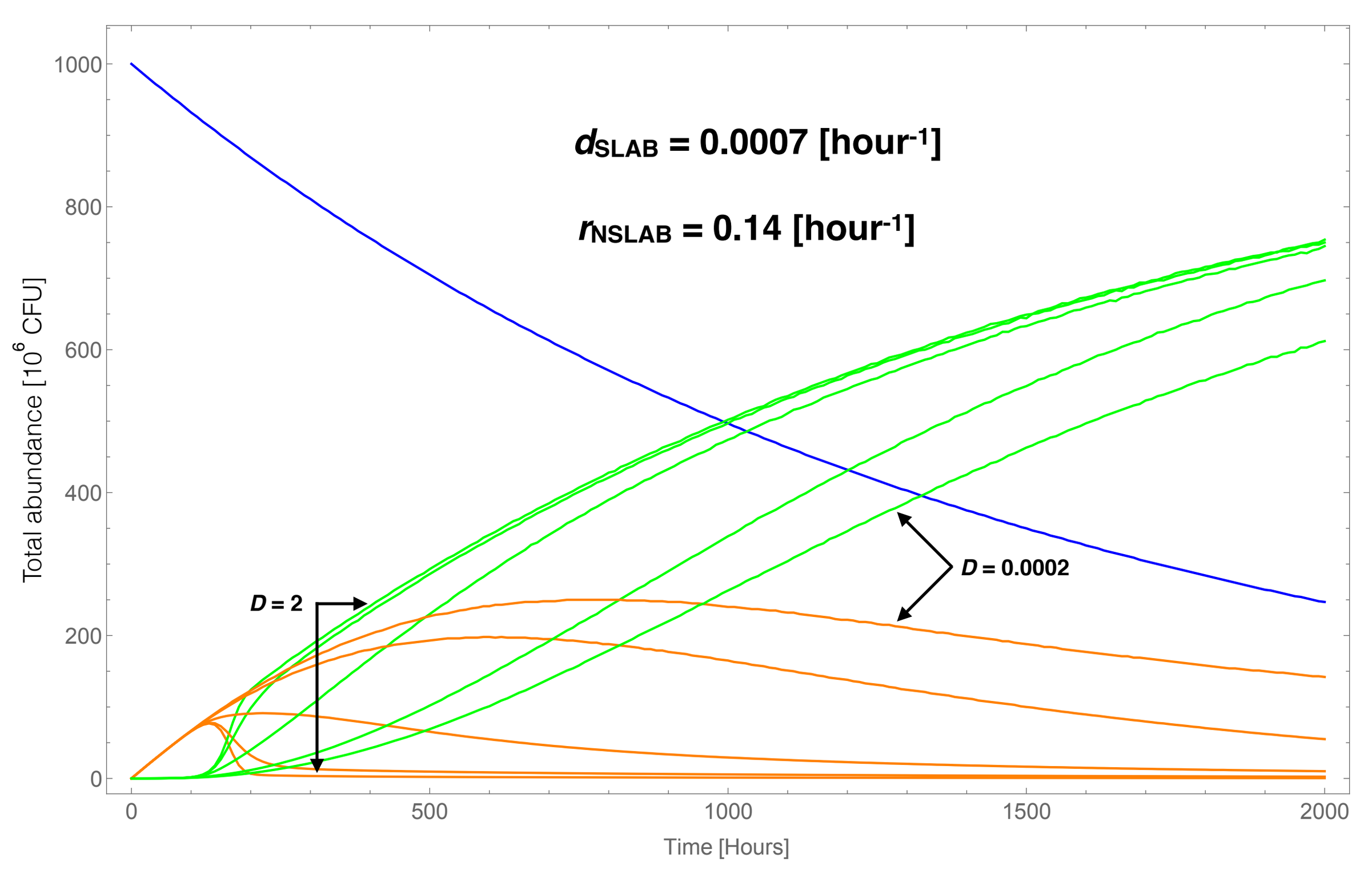

SLAB
Lysis
L-SLAB
Diffusion
NSLAB
t = 0
t = 60
t = 160
t = 500
Density
x [mm]
y [mm]


"Demo" with 10 SLAB and 10 NSLAB colonies, Diff = 0.18 mm²/hour
Log Mean Density with St.Dev.
100
50
10
5
1
0.5
0.1
0
100
200
300
400
500
Time [Hours]


1000
800
600
400
200
0
Total abundance [10^6 CFU]
0
500
1000
1500
2000
Time [Hours]
D = 2
D = 0.0002
dSLaB = 0.0025 [hour-1]
rNSLaB = 1.4 [hour-1]


d_{SLAB} = 0.0007 [hour^{-1}]
r_{NSLAB} = 1.4 [hour^{-1}]
D = 2
D = 0.0002
Total abundance [10^6 CFU]
Time [Hours]
0
200
400
600
800
1000
0
500
1000
1500
2000


d_{SLAB} = 0.0025 [hour^{-1}]
r_{NSLAB} = 0.14 [hour^{-1}]
D = 2
D = 0.0002
Total abundance [10^6 CFU]
Time [Hours]
0
200
400
600
800
1000
0
500
1000
1500
2000


d_{SLAB} = 0.0007 [hour^{-1}]
r_{NSLAB} = 0.14 [hour^{-1}]
D = 2
D = 0.0002
Total abundance [10^6 CFU]
Time [Hours]
0
200
400
600
800
1000
0
500
1000
1500
2000