Microbial Interactions from a New Perspective: Reinforcement Learning Reveals New Insights into Microbiome Evolution

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Abstract

Microbes play a vital role in diverse ecosystems, influencing material flow and shaping the dynamics of their surroundings. Understanding the function of microbial life within ecosystems is crucial for tackling modern challenges. Metagenomics studies provide valuable insights into the potential functions of microbial communities but predicting the phenotype of these communities from on their genotype remains a complex endeavor. Trophic interactions between microbes further complicate the prediction of emergent properties in microbiomes. Mathematical modeling, particularly Flux Balance Analysis (FBA) approaches, have been employed to forecast phenotypes and explain experimental findings. However, FBA solutions often lack uniqueness and assume a steady-state condition. While Dynamic Flux Balance Analysis (DFBA) addresses some of these limitations, it still relies on instantaneous biomass maximization assumptions and faces challenges related to non-uniqueness solutions of linear programming problems. In this article, a novel modeling approach is proposed that integrates deep reinforcement learning into DFBA. This framework views microbial metabolism as a decision-making process, where each microbial agent evolves by learning and adapting metabolic strategies to enhance long-term fitness. Reinforcement learning algorithms facilitate the discovery of optimal strategies through iterative trial and error, while considering the consequences of actions within a dynamic context. This approach diverges from traditional FBA assumptions and provides insights into evolutionary stable strategies, requiring minimal reliance on predefined strategies. The proposed method holds promise for elucidating the behavior and mechanisms of microbial systems, including phenomena like quorum sensing and other interactions that can be explained by considering the long-term consequences of metabolic regulation strategies. The modeling algorithm demonstrates success in predicting microbial interactions in simple communities, surpassing the capabilities of existing models, and exhibits potential for scalability when applied to Genome-scale Metabolic Models (GEMs).

Introduction

Microbes are present in almost all known biotic environments and their metabolism affects the flow of materials in their ecosystems. Microbes form intricate networks of interacting cells from various taxonomic branches with distinct functional traits which makes predicting their behavior challenging. However, determining the role of microbial life in their ecosystems can be a key to solving numerous challenges that we face today. Imbalance in human gut microbiome is consistently linked with diseases such as IBD [1]. At a larger scale, microbial metabolism is a major player in geochemical cycles on earth [2].

Metagenomics studies provides detailed information about the membership and biochemical functions of microbiomes. However, Predicting the phenotype of microbial communities from their genotype is by nature a complex problem and has been an ongoing effort

for the past few decades [3]–[6]. Trophic interactions between microbes is an important factor that significantly contributes to the evolution of microbiome composition and function in various ecosystems [7], [8] and it further complicates the prediction of emergent properties of microbiomes.

Understanding and predicting the dynamics of microbial systems has remained largely unknown despite the enormous growth in multi-omics techniques and it requires a wholistic modeling approach [9]. Mathematical models at different abstraction levels have been developed with the goal of making predictions that can explain the experimentally observed phenotypes [3], [6], [10]–[18]. GEnome-scale metabolic Models (GEMs) provide a detailed view of the biochemical networks of cells that are inferred from the genome of the organism of interest. GEMs generally contain up to thousands of biochemical reactions. Predicting the emergent properties of microbial communities by merely determining flux through such biochemical reactions is one of the main challenges in systems biology that yet remains to be addressed [5], [6]. One solution to this problem is to define kinetic expressions for all the biochemical reactions. However, finding such expressions along with the required parameters in many cases is not feasible and the data required for this goal is not likely to be available. Flux balance analysis (FBA) is a bottom-up approach that provides a scalable method for simulating cellular metabolism in the absence of reaction kinetic parameters [19]. FBA converts the system of differential equations resulting from mass balance across a cell to a linear programming problem by assuming steady state condition across the cells and defining a biologically relevant objective function[19]. Despite the defined objective function and the constraints on the flux values, FBA solutions are rarely unique, and feasible solutions form a large space where distinct phenotypes can coexist. Different approaches exist to alleviate this problem. One way is to provide more strict ranges for flux values by collecting more experimental data [20]–[27]. Another way is to make simplifying assumptions that are based on observed biological phenomena. Resource allocation constraint is an example of a such assumptions that has shown success in predicting several real scenarios [28]–[30]. Dynamic Flux Balance Analysis (DFBA) is a framework by which FBA is performed in each timepoint, and using the calculated extracellular fluxes, changes in extracellular metabolites with time are calculated. These rates of changes are used in turn to form a system of differential equations that describe the concentration profiles of different species in the system over time [10], [31]–[40]. However, the problem of lack of a unique solution in FBA propagates through time. Consequently, in the cases where the attempt is to model the dynamics of a heterogenous microbial community, one is faced with an extremely open solution space where different solutions can resemble significantly different phenotypes while all phenotypes can satisfy FBA requirements. Even in the presence of time-resolved metabolomics data, determining the role of individual microbes is challenging due to the functional redundancies inherent in microbiomes [41]–[46]. More importantly, DFBA relies on instantaneous biomass maximization assumption. Although in simple cases this assumption might result in realistic simulations [10], in many other cases it fails to predict the observed behavior of microbial systems [12] because depending on the environment, maximizing instantaneous growth rate can result in low fitness in future or even extinction. For example, cells that excrete extracellular amylase to breakdown starch are spending energy to do so and lower their instantaneous fitness in turn. However, secreting amylase is required for degrading starch to smaller molecules such as glucose for the cells' future use. Instantaneous biomass maximization will naturally predict that in this scenario no extracellular amylase secretion should happen, unless previously set as a constraint on the model, while amylase secretion has been frequently observed in nature [47]–[49].

The issue becomes even more significant when it comes to modeling microbial interactions. A simple example is microbial cells that are auxotrophs for certain amino acid. Previous results showed that DFBA fails to predict any exchange between two different auxotrophs that can experimentally complement each other because during DFBA each model is trying to maximize their instantaneous fitness as ATP is required in biosynthetic pathways that produce amino acids However, since none of such organisms can live without the presence of the other, evolution favors exchanging the required amino acids which is supported by observing growth between auxotrophic E. coli cells on minimal medium [50]. Therefore, it is important to put the concept of Nash equilibria and evolutionary stability in this context of metabolic interactions [9], [12]. A remedy proposed by Zomorrodi and Segre [51] was to determine Nash equilibria of the systems with several metabolic strategies of interest pre-defined. It beautifully captures the experimental results of some well-known microbial games of metabolic interactions. However, in general, it is virtually impossible to enumerate all possible metabolic strategies because of the high dimensional and continuous nature of the solution space defined by the mass balance constraints, directionality constraints, and nutrient availability. Therefore, an algorithm that can explore the entire possible solution space to a satisfactory extent when determining these stable interactions, and meanwhile does not rely on the instantaneous biomass maximization assumption, will greatly improve capability of predicting stable microbial interactions.

In this article we aim to address these challenges by introducing a new modeling approach that integrates deep reinforcement learning into DFBA to model microbial metabolism in a microbiome as a decision-making process. From this perspective, microbial cells evolve by trying different metabolic strategies and learning how to improve their long-term fitness by tuning their behavior using a reinforcement learning algorithm. In this framework, each GEM is modeled as an agent capable of making decisions. The decisions in this context are flux regulations in the metabolic network and the agents make these decisions using the observable environment states. Assuming that "bad decisions" are filtered through the natural selection process, we use reinforcement learning algorithms to find the strategies that lead to the long-term optimal behavior of microbes in the system that they are interacting with. In other words, microbial models learn how to interact by trial and error in their environment through self-play mechanism [52], without the need to pre-define metabolic and regulatory strategies. Reinforcement learning has shown great promise in solving very complex problems in the past decade [53]–[55] and have been used with success in different fields of science and engineering [56]–[60]. Although we still rely on FBA, this approach is fundamentally different from biomass maximizing agents assumed commonly in traditional FBA and DFBA as the long-term consequences of actions are also considered in a dynamic context to find strategies that are also performing well in future rather than only an instance of time. In several cases, discussed shortly, greedily optimizing for biomass production will lead to early community extinction. Rationally, such strategies should be eliminated by the natural selection process. The strategies taken by RL agents after training can be useful to understand why certain types of behaviors are observed in real microbial systems.

Additionally, in this approach the agents could also make their decisions by sensing other agents' concentrations which can shed light on the evolution of mechanisms such as quorum sensing. Thinking about microbial interactions from this perspective allows us to generalize from currently existing hypotheses such as electron donor exchange [61] to basically any interaction that leads to higher long-term survival of the microbial agents involved in a community. This method also relaxes the need to define pairwise and higher order interactions, similar to DFBA, that could lead to exponential computational complexity. Also, it does not force any community

level objectives that are used in other algorithms such as OptCom [18] or cFBA [62] to predict microbial exchange of compounds. We show that without using any sort of parameter fitting or pre-defining interactions this modeling algorithm can predict how interactions shape in simple microbial communities while other models fail to make such predictions.

Methods

Reinforcement Learning

In a reinforcement learning problem, one or more agents interact with their environment. An agent is an entity that is learning by interacting with the environment and is capable of *making decisions* in a given state. An environment can be defined as the collection of all entities that are surrounding the agent. Depending on the problem of interest, the agents can observe the entire or a part of their environment and based on a mapping called **policy** decide what actions to take in each state. Through this interaction with the environment the agents receive a reward and go to the next state according to the environment dynamics until it reaches the terminal state, that is when one **episode** is completed. The final goal of an agent is to maximize return during an episode. Return at time t is defined as the discounted sum of the collected reward after time t until the end of the episode [63]:

$$G_t = \sum_{k=t+1}^{t} \gamma^{k-t-1} R_k \qquad \text{(eqn. 1)}$$

Here γ is the discounting factor which determines the importance of future rewards and R_t is the reward at time t.

Policy is a function that describes the behavior of an agent in each state. Policy function π is a mapping that outputs the probability of taking action a_t when the agent is in state s_t [64]:

$$\pi: A \times S \rightarrow [0,1] \qquad \text{(eqn. 2)}$$
$$\pi(s,a) = P(a_t, s_t) \qquad \text{(eqn. 3)}$$

Another important definition is the **value function** under policy π which is defined as [63]:

$$v_\pi(s) = E_\pi[G_t | \, S = s \,] \qquad \text{(eqn. 4)}$$

Value for state s under policy π is the expected return of being in state s and following policy π for the upcoming states [63]. Let's consider two states s_1, s_2. If $v_\pi(s_1) > v_\pi(s_2)$ then this means that if the agent is following policy π, the *expected* return is higher when the agent is in state s_1 than when in s_2. In other word the value function tells us how valuable it is to be in state s when following a specific policy function.

Depending on the type of problem at hand, there are families of RL algorithms that can be used [63], [65]. In the proposed framework (described in detail in '*Self-Playing Microbes in Dynamic Flux Balance Analysis (SPM-DFBA)*'), the states are observable extracellular metabolite concentrations which are continuous variables. More generally, any quantity that is tracked during an episode can be optionally selected as an observable state variable. For example, the agent itself or other agents' concentrations can be defined as observable state variable as well. On the other hand, actions are the reaction fluxes which are also continuous variables. This means that both the

action and state space are continuous and multidimensional. For this type of problem, *policy gradient* family of algorithms is a good choice [63]. Among them, Proximal Policy Optimization [66], PPO, is an interesting policy gradient algorithm which has been used with success in many Artificial Intelligence problems recently [67]–[70] and is appropriate for modeling microbial communities.

Proximal Policy Optimization

In a RL problem, an agent tries to maximize total return during an episode by improving its policy. The policy is a function that maps the observable states to actions. Policy gradient algorithms is a family of reinforcement learning algorithms by which the agent directly improves the policy function by interacting with its environment. In policy gradient algorithms, the policy can be defined as a parameterized function, here a neural network, and the parameters of this function are tuned in a way so that the agent takes actions that lead to higher return as a result of the policy gradient theorem [71]. One issue with using reinforcement learning algorithms is that the underlying mathematical operation in our algorithm is a linear programming problem. The suggested actions by the policy function can easily form a solution that is not in the feasible region of the underlying LP problem and infeasible region can be in the proximity of areas where the return is maximum. This complicates the training process and we observed that many of the RL algorithms that we tested, failed on simple test cases. PPO addresses this issue by avoiding abrupt changes in the policy space. In the PPO algorithm, the agent tries to maximize the following surrogate objective function:

$$L^{CLIP}(\theta) = E_t\left[\, min(r_t(\theta)\,\hat{A}_t\,, clip(\,r_t(\theta), 1-\epsilon,\ 1+\epsilon)\,\hat{A}_t\,)\right] \quad \text{(eqn. 5)}$$

Where $\quad r_t(\theta) = \frac{\pi_\theta(a_t|s_t)}{\pi_{\theta_{old}}(a_t|s_t)}$

and $\quad \hat{A}_t = R_t - v(s_t)$

Setting this objective function (L^{CLIP}) causes the policy function to increase the probability of actions that lead to higher return during an episode. In eqn. 5, $r_t(\theta)$ represents the ratio of the probability of taking action a_t at state s_t under the improved and the old policy. $\hat{A}_t$ is called the advantage function which is the difference between reward collected at state s and the expected return of state s. The $clip$ function does not allow for $r_t(\theta)$ to go beyond $1-\epsilon$ and $1+\epsilon$. This objective definition has two implications on the policy function:

1- Probability of actions that result in more positive advantage will increase and the reverse will happen in the case of low advantage values.
2- The parameters of the policy function will only change if the change in the policy space is in a limited range.

Using this technique, PPO effectively stabilizes the training process. ϵ is a hyper parameter that depends on the problem of interest. In all of our simulations we used $\epsilon = 0.1$.

The value function and the policy function (called critic and actor networks respectively.) in all of our experiments are neural networks with 10 linear layers followed by hyperbolic tangent activation function [72] with Adam optimizer as the network optimizer in both cases. In all simulation cases we used 0.001 and 0.0001 as learning rates for critic and actor network respectively.

Dynamic Flux Balance Analysis

In DFBA, at each timepoint, uptake fluxes are constrained based on defined kinetics and the concentration of the extracellular metabolites for each GEM. FBA is then preformed to determine all exchange fluxes and growth rate. The exchange fluxes determine the rate of change of extracellular metabolites by a set of ordinary differential equations (ODE) based on mass balance. In this implementation, we used the forward Euler method which is commonly used for DFBA as the ODE solver.

Self-Playing Microbes in Dynamic Flux Balance Analysis (SPM-DFBA)

To incorporate PPO inside DFBA, first the agents are defined. An agents' metabolism is defined by a COBRA model. The agents observe a part of their environment, by sensing the concentrations of some of the extracellular metabolites as well as other agents' concentrations when applicable. According to their policy function, these agents regulate a subset of fluxes through their metabolic network in each state by posing constraints on the reaction flux bounds. For example, lower bounds for metabolite production fluxes can be one way to define the action space. The main difference between SPM-DFBA and the standard DFBA is that during FBA for an agent at each timepoint, additional constraints returned by the agent's policy function are imposed. After the FBA step, a reward is given to the agent based on the rewarding strategy and the environment goes to the next state using the extracellular fluxes calculated with FBA, similar to DFBA.

In our implementation, the reward function has two components: a negative reward as penalty for infeasible flux distributions and a positive reward for growth/biomass production. When the policy network generates flux values that are outside of the feasibility range, the agent will get a negative reward to learn to stay away from infeasibility. For the positive reward for the growth rate determined by FBA at each time point, we emphasize that it is different from the maximization of biomass production for the immediate time point in that future rewards also affect the decision made by the agent in any timepoint. As a result, an action with low immediate reward but high future reward might be favored over immediate biomass optimization strategy, Figure1.

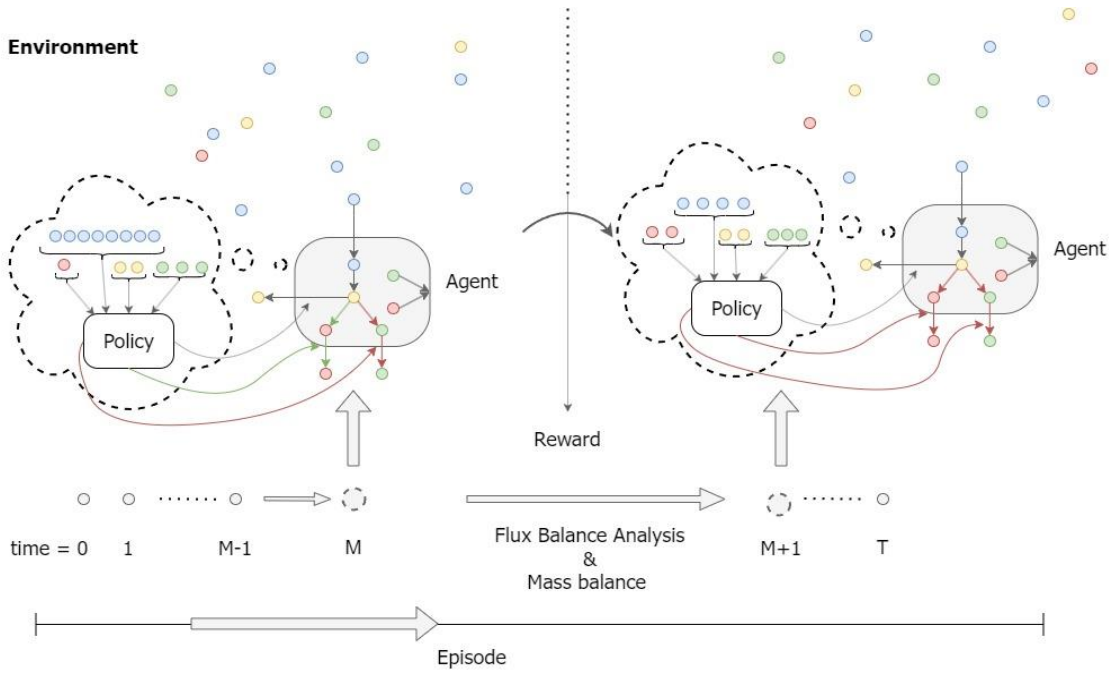

Figure 1 A schema of dynamic microbiome modeling viewed as a reinforcement learning problem. At each time step, the "agents" take an action according to their policy function without having a model of the environment dynamics and go to the next state. The actions in this sense are the constraints on the flux values.

In SPM-DFBA, each episode contains a specific number of time points, and the agents start with a random policy in the beginning of the training process. The policy function in our algorithm is a neural network with observable states as input and flux constraints as the output. The actor network (i.e., the policy function) outputs a tensor of real numbers at each state as flux, one for each action dimension. These flux values are fed as mean to a normal distribution with a predefined standard deviation. Later each action is drawn from this distribution and is later fed as constraints to the LP problem underneath as a constraint. The reason for generating this normal distribution is to add randomness to the action of the agents for the exploration purpose. The variance of this normal distribution is a problem-specific hyper-parameter. In our cases we used 0.1 as the variance for all distribution after testing the performance.

For example if the action space includes the exchange flux for A and B, then the actor network outputs a tensor [a,b]. The actual action will be an action that is drawn from normal distribution with mean values of a and b with standard deviation of 0.1 for A and B respectively. Using these actions, the environment goes to the next state by solving one timestep of DFBA, and the actions and states and observations are recorded in an array until the end of the episode. Depending on the resources available, parallel episodes are simulated for the current policy at the same time. After one batch of episodes is finished, the critic network, the value function, gets updated based on the states and reward values. This step in reinforcement learning training process is called policy evaluation [64]. Next, according to the PPO objective function (eqn. 5) and using the information collected during the batch of episodes, the actor function is updated using gradient ascent to improve the policy, Figure 2. This step is called policy improvement. As a result of this iterative process, the agents become better at taking actions that maximize their own return by trial and error in the feasible solution space.

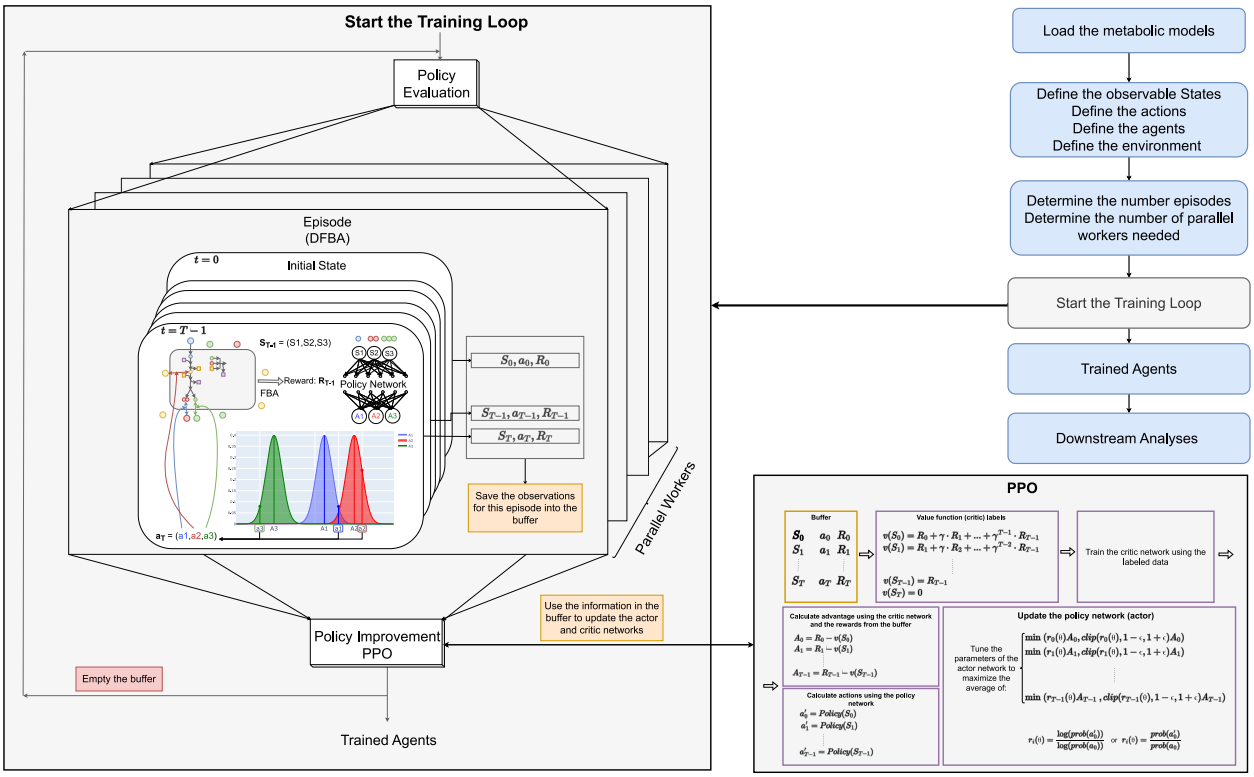

Figure 2 Schematic of SPM-DFBA algorithm and a detailed view of using PPO in DFBA: Parallel workers run episodes of defined length, and collect the observations (States, rewards, and actions) made by the agent. The collected observations enter a buffer that lasts for 1 batch of episodes. The information in this buffer is used to first update the critic network. Afterwards, the policy network is updated in a way to maximize the PPO's surrogate objective. The agents learn to act optimally though iterating this cycle until the policy improvement stops.

Mass Transfer Limitations

Simple DFBA assumes a homogenous environment by default. In the cases where we applied mass transfer limitation in DFBA, we did not change the structure of the algorithm. For example, we implemented mass transfer limitations in the Toy-Exoenzyme-Five-Agents-Mass-Transfer environment by using an indirect approach. In each environment an indefinite number of extracellular reactions can be defined. To incorporate mass transfer limitations, we made each organism secrete amylase with a specific index. For example, agent_1 secretes amylase_1. Now amylase_1 can carry the following reaction: Starch ⇒ Glucose_1. Glucose_1 can *only be taken up by agent 1*. However, Glucose_1 can get converted reversibly to a general glucose, Glucose, in the environment according to a simple diffusion kinetic. Same can happen for all other agents. In this way if diffusion rate (The rate of Glucose_x ⇔ Glucose) is low, each agent can utilize most of the glucose that it has produced and little is diffused to the community pool. For more detailed explanation in the implementation please refer to https://github.com/chan-csu/Reinforcement_Learning_Modeling/tree/main/PPO.

Implementation

All the scripts for generating the results are written in python and the python scripts and a Jupiter notebook is provided in the GitHub repository for this project that explains the procedure to examine these test cases step by step. Python v3.10, and COBRApy v0.25.0[73] was used to generate the simulation results. GLPK solver v0.4.7 in python was used to perform FBA in the toy communities and Gurobi optimizer[74] with academic license was used to perform FBA on the GEMs using COBRApy interface. Pytorch v1.12.0[75] was used for building and training the neural networks. Same network structure and hyper parameters were used for all three cases to illustrate the robustness of this method with respect to the hyperparameters. Ray library was used to perform parallel computing[76]. Plotly v5.9.0[77] was used to generate all the plots. The scripts for generating the simulations are written in a way to make studying new cases as simple as possible and are provided in:
https://github.com/chan-csu/Reinforcement_Learning_Modeling/tree/main/PPO.

Results

We created various toy microbial communities inspired by those discussed in NECom [12] to evaluate our algorithm. The following subsections will provide a detailed description of these biologically relevant toy communities.

Amylase secretion without mass transfer considerations

This group of toy communities were designed to emulate a case that microbial cells are grown on a mixture of starch and glucose in a chemostat with a very low dilution rate of 0.0001, practically a batch system, Figure 3A-C, The strains are capable of secreting amylase to degrade the available starch. However, producing amylase is an energy-consuming step in the organism's metabolism and it requires ATP that would otherwise be used in biomass production. Figure 3-A depicts a schematic view of this environment. 3 different environments belong to this family of communities: Toy-Exoenzyme-Single-Agent, Toy-Exoenzyme-Two-Agents, and Toy-Exoenzyme-Five-Agents which represent same environment but with different number of agents, 1, 2, and 5 respectively. Figure 3 D-F shows the learning curve of the agents in the corresponding environment during 5000 batch of training. Each batch contained 4 parallel episodes. In all cases, the agents converge to a stable policy which cannot be further improved. Figure 3 G-I show the concentration profiles of starch and glucose in the mentioned environments. Starch utilization significantly decreases when going from single agent environment and further decreases from two to the five-agent community. This trend shows that when more agents are present in the environment, they become more conservative in terms of secreting amylase. Figure 4 compares the policy of the trained agent in the single-agent environment and the two-agent environment.

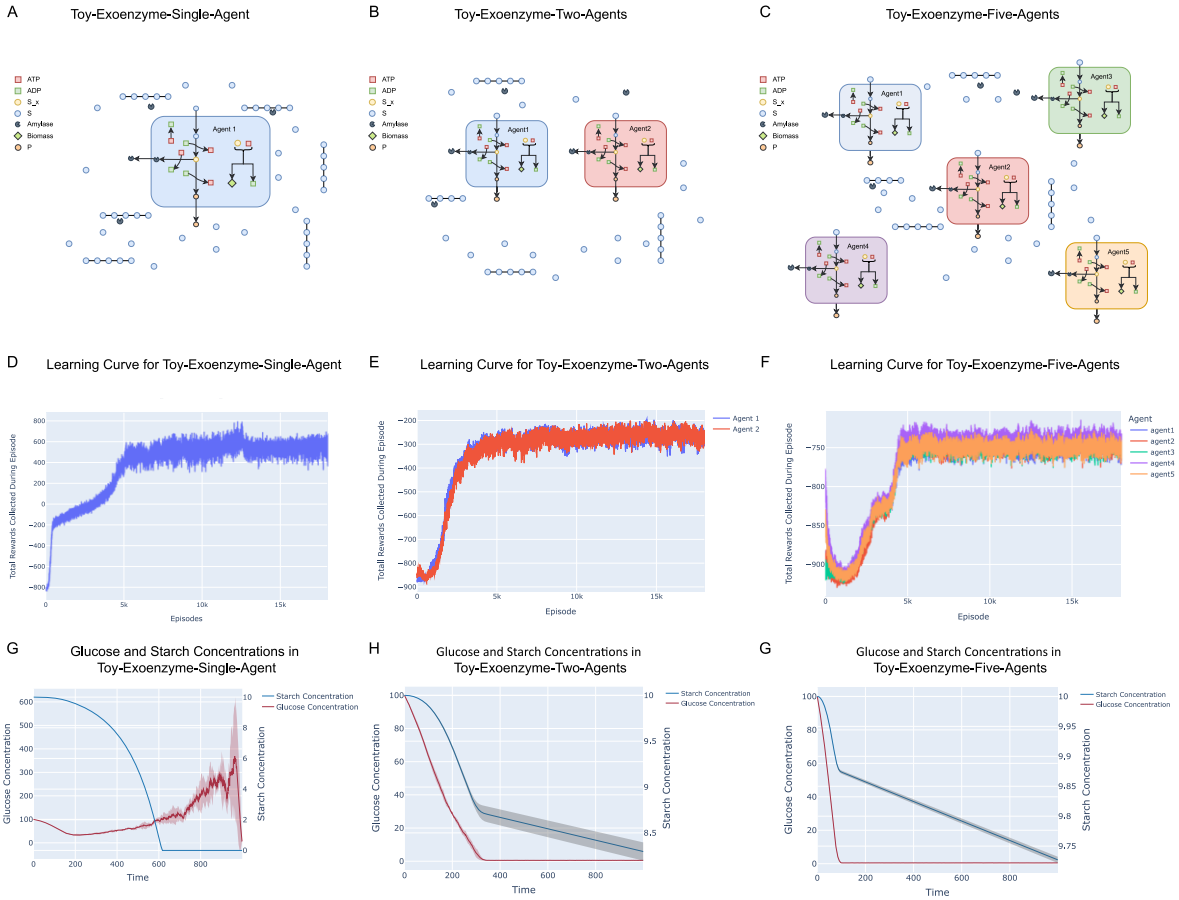

Figure 3 Training agents in a chemostat with starch and glucose initially present in the system. A-C) Schemas for the toy communities: Toy-Exoenzyme-Single-Agent, Toy-Exoenzyme-Two-Agents, and Toy-Exoenzyme-Five-Agents, respectively. D-F) Learning curve of the agents in Toy-Exoenzyme-Single-Agent, Toy-Exoenzyme-Two-Agents, and Toy-Exoenzyme-Five-Agents after training on 5000 batches of 4 episodes, respectively G-I) Starch and Glucose concentration over time in Toy-Exoenzyme-Single-Agent, Toy-Exoenzyme-Two-Agents, and Toy-Exoenzyme-Five-Agents, respectively. The solid lines represent the mean value across all episodes in a batch and the shades represent 1 standard deviation across all episodes in a batch. Note that each actor acts randomly around the mean of the actor network output with standard deviation of 0.1. The left axis in each plot shows glucose concentrations and the right axes show the starch concentrations.

Figure 4 proves our point which we hypothesized from Figure 3 G-I. The policy of the agent in Toy-Exoenzyme-Single-Agent is significantly different from the agents in Toy-Exoenzyme-Two-Agents. When the agent is trained in Exoenzyme-Single-Agent, the glucose resulted from breaking down starch can be utilized only by the agent itself. However, when other agents exist in the environment this is not the case. Other agents can learn to cheat because of the randomness in their behavior and take up the available glucose without paying the cost for building the amylase molecules. As a result, in the single-agent environment amylase secretion is negatively correlated with the glucose level, the optimal policy instructs the agent to secrete amylase when the glucose level is low, so that the low glucose concentration is compensated by breaking down the available starch. On the other hand, secreting amylase when the glucose level is low in environments with more than one agent is risky. In this case, at low glucose level cheating can have a more

deteriorative effect on the amylase producer organism. For this reason, in environments with more than one agent amylase production increases with glucose level.

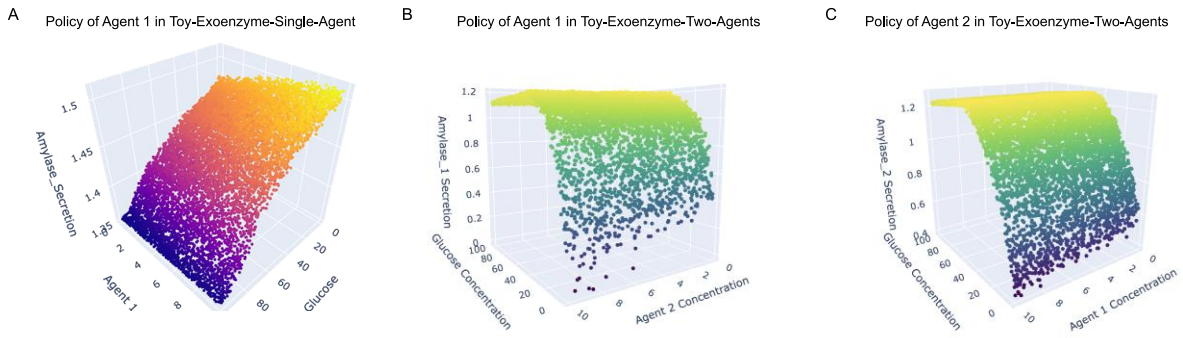

Figure 4 The policy profiles of agents in Toy-Exoenzyme-Single-Agent and Toy-Exoenzyme-Two-Agents environments after training on 5000 batches of 4 episodes. These plots are created by randomly generating 10000 points in the policy space of the trained agents. A) Policy of Agent 1 in Toy-Exoenzyme-Single-Agent with respect to glucose and Agent 1's concentration B) Policy of Agent 1 in Toy-Exoenzyme-Two-Agents with respect to glucose and Agent 2's concentration C) Policy of Agent 2 in Toy-Exoenzyme-Two-Agents with respect to glucose and Agent 1's concentration.

Another interesting trend in Figure 4 is that in Toy-Exoenzyme-Two-Agents the maximum amylase secretion is the highest when the glucose level is the highest and the other agent's concentration is lowest. This means that each agent tries to minimize the risk of cheating by other agents in a homogenous environment by secreting lower amylase when the concentration of the other agent is higher and can benefit higher from the released glucose from starch degradation.

Logically, in a multi-agent environment, the agents that are trained in multi-agent environments should perform better than when trained in a single-agent environment as they have experienced different aspects of coexisting with other agents, such as cheating by other agents. To test this statement, we created a new two-agent environment, Toy-Exoenzyme-Single-Two-Comb. One agent is selected from Toy-Exoenzyme-Single-Agent and the other from Toy-Exoenzyme-Two-Agents. Figure 5 shows the result of this simulation. Note that there is no training happening in this new environment, and the agents are trained in their own corresponding environments.

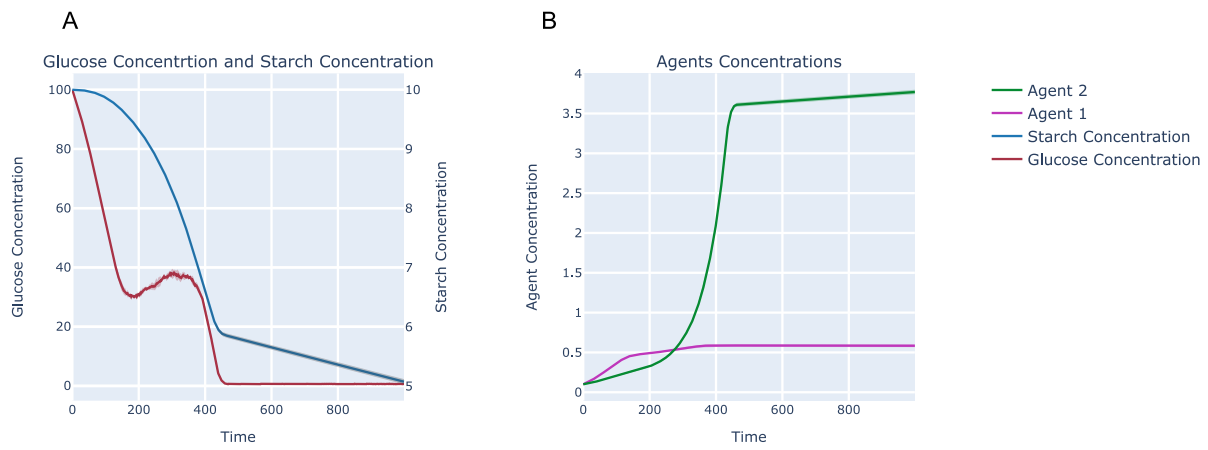

Figure 5 *Concentration profiles in Toy-Exoenzyme-Single-Two-Comb environment. A) Concentration of glucose and starch over time. B) Concentration of Agent 1 and Agent 2 over time. Agent 1 is trained in Toy-Exoenzyme-Single-Agent and Agent 2 is trained in Toy-Exoenzyme-Two-Agents.*

Figure 5 shows that agent 1 that is trained in Toy-Exoenzyme-Single-Agent achieves significantly lower return compared to agent 2 that is trained in Toy-Exoenzyme-Two-Agents. Furthermore, the level of starch utilization is higher than Toy-Exoenzyme-Two-Agents but lower than Toy-Exoenzyme-Single-Agent which points to the fact that high amylase production by agent 1 is exploited by agent 2.

Amylase secretion with mass transfer considerations

In this case we explored the effect of spatial heterogeneity on starch utilization on the environment like all previous cases. We simulated mass transfer limitation effect as is explained in the methods. see methods: *Mass Transfer Limitations*. For this environment we tried two different "mass transfer coefficients" to simulate high and low mass transfer rates. For this environment we explored the degree of starch utilization and the effect of mass transfer coefficient value on the degradation process. Supplementary Figure 1 describes the training process and the concentration profiles in this environment for both high and low mass transfer rates. The results of these simulation matched our expectation: In the environment with higher "mass transfer coefficient" cheating will receive more positive signal. Lower "mass transfer coefficient" imposes the reverse trend in the training process. Both amylase secretion and starch utilization is higher in this environment as the products are utilized by the producer at a higher rate.

Toy-NECOM-Auxotrophs

This community was created to emulate auxotrophic exchange of metabolites in a community of two microbial strains. The schematic description of this environment is provided in figure 6-A. In this case agent 1 and agent 2 have similar metabolic network with only one difference. Agent 1 cannot produce the biomass precursor A and the Agent 2 cannot produce the precursor B. Although they cannot survive on their own, the agents can grow by exchanging A and B. Each agent can sense its own concentration, the concentration of the other agent,

concentration of S, concentration of A, and the concentration of B. Figure 7 shows the result of training the agents in this environment.

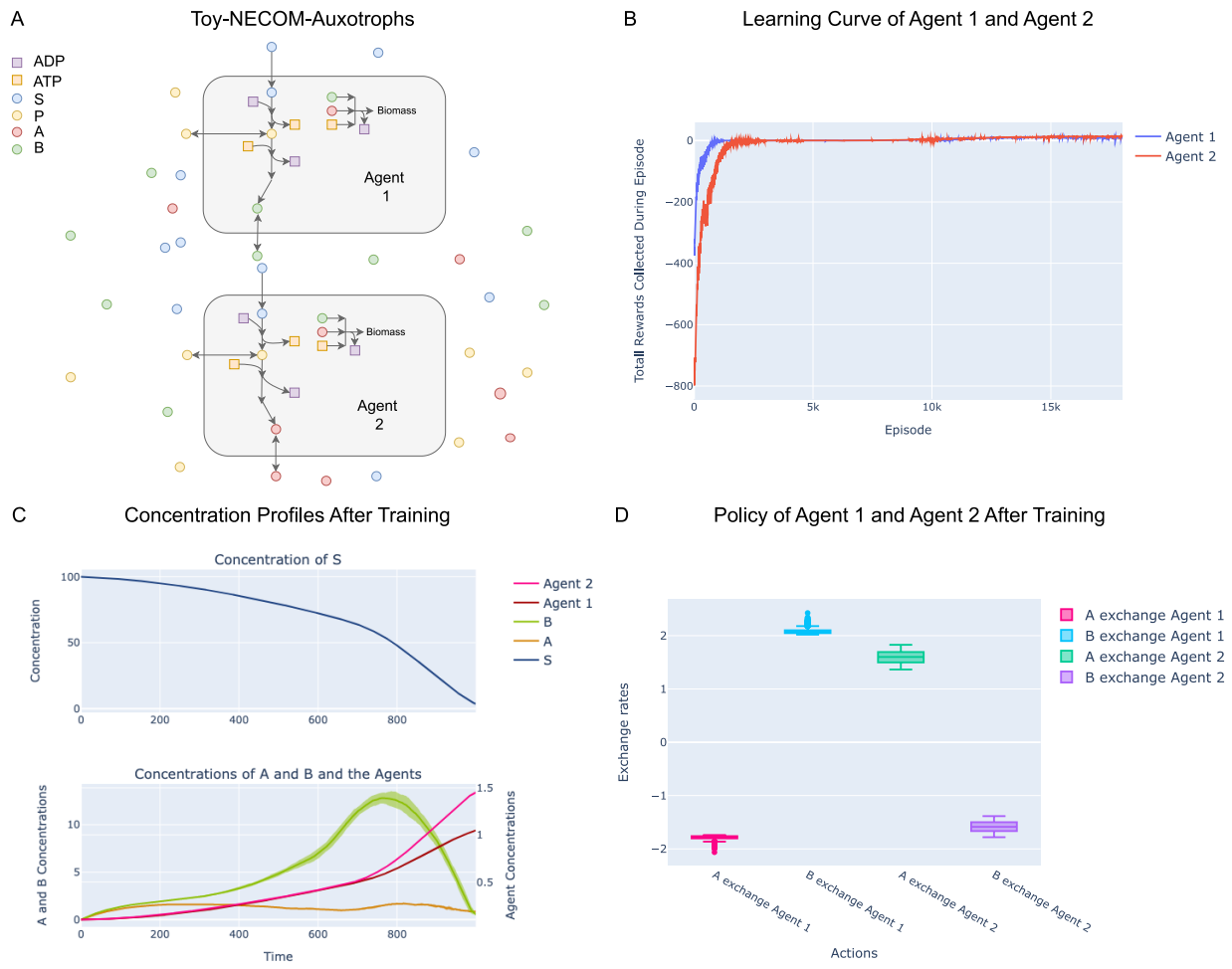

Figure 6 Training two agents in Toy-NECOM-Auxotrophs environment. A) A schematic view of the environment. B) Learning curve of the two agents during 5000 batches of training. C) Concentration profile of the species in this environment over time in a batch of 4 episodes. D) Policies learned by the agents. Negative sign for fluxes means uptake and positive sign means secretion. Agent 1 learns to uptake whatever A that exists in the environment while secreting B for agent 2. Agent 2 has learned the opposite strategy which agrees with their mutation.

After training in this environment, the agents learn to increase their own long-term fitness by exchanging precursors A and B. Although the metabolic capabilities of Agent 1 and Agent 2 are symmetric, Agent 2 achieves superior fitness compared to Agent 1. We re-executed these simulations multiple time and each time one of the agents found superior growth randomly. This means that perfectly equal presence of Agent 1 and Agent 2 is an exploitable situation and any agent that can take advantage of this situation first, will achieve higher return and will learn to grow higher by secreting less of A or B.

The observed vulnerability of the symmetric policies for Agent 1 and Agent 2 is in part due to resource limitation in this environment. A random mutation that causes one agent to secrete

less biomass precursor will result in less growth for the other agent and this in turn leaves more S to be consumed by the mutant, and as a result this behavior will be reinforced in the mutant because it leads to higher return during an episode. However, not secreting any precursor is not optimal because this strategy would hinder the growth of the other agent which is required for the mutant's growth.

Depending on each other for survival is the key factor that shapes metabolite exchange between agents. We created a new environment to test this hypothesis: Toy-NECOM-Facultative-Exchange. This toy ecosystem is similar to Toy-NECOM-Auxotrophs. However, the metabolic networks of the agents are slightly different. In this case both agents can produce both A and B. However, Agent 1 produces B more efficiently, while Agent 2 produces A more efficiently. In this case we were interested in seeing that whether differences in metabolism efficiency can result in exchange of metabolites between the agents. Figure 7 shows the learning curve, and the learned strategies as well as the concentration profiles after training the agents in this environment for 5000 iteration batches.

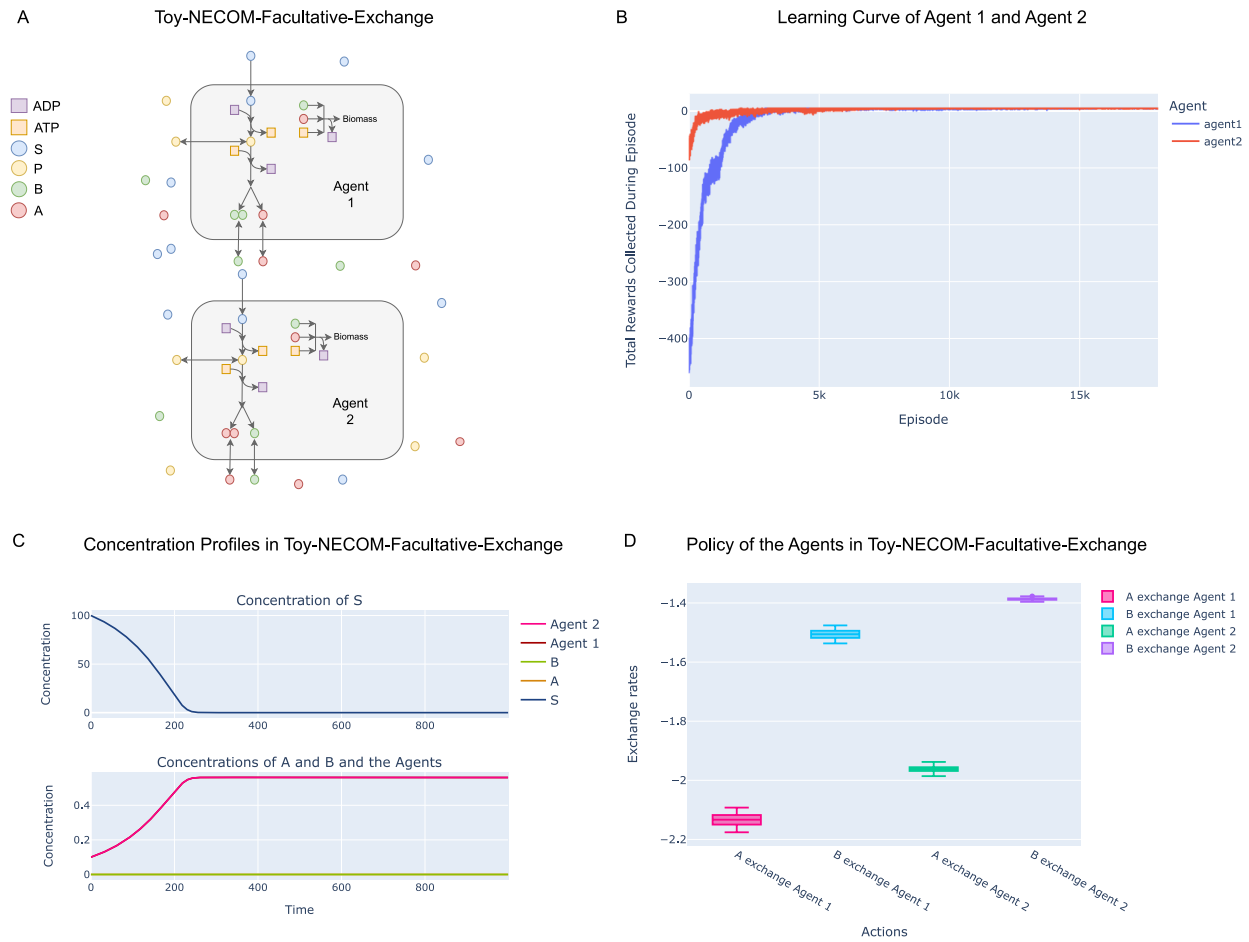

Figure 7 Training two agents in Toy-NECOM-Facultative-Exchange environment. A) A schematic view of the environment. B) Learning curve of the two agents during 5000 batches of training. C) Concentration profiles after training for 5000 batches of 4 episodes. D) The policies learned by the agents. Negative sign means uptake. Both agents learn to avoid any exchange of A or B and selfishly take up any A or B that is present in this environment. Note that if the absolute value of the flux for a reaction is larger than what the

uptake kinetics allow, then the flux value gets clipped to match the highest value that is kinetically possible, which is the case for all exchange reactions here. This case yields exactly same results as DFBA.

In this scenario the optimal policy exactly matches the simple DFBA prediction, each agent selfishly takes up any A and B that is present in the environment and avoids any metabolic exchange. This further confirms our hypothesis and matches the prediction of NECom as well [12].

If metabolite exchange in this environment is dictated merely by the fact that cells rely on each other for survival, what happens if we supplement the Toy-NECOM-Auxotrophs environment with A and B externally? To answer this question, we created a new environment, Toy-NECOM-Auxotrophs-Shift. We used the two auxotroph agents from Toy-NECOM-Auxotrophs and simulated a scenario where A and B initially is supplemented in the environment. This case is designed to predict how changes in environment can shape the trophic behavior of microbial communities. Figure 7 describes the policy shift for the agents in this new environment after 3000 iteration batches of 4 episodes.

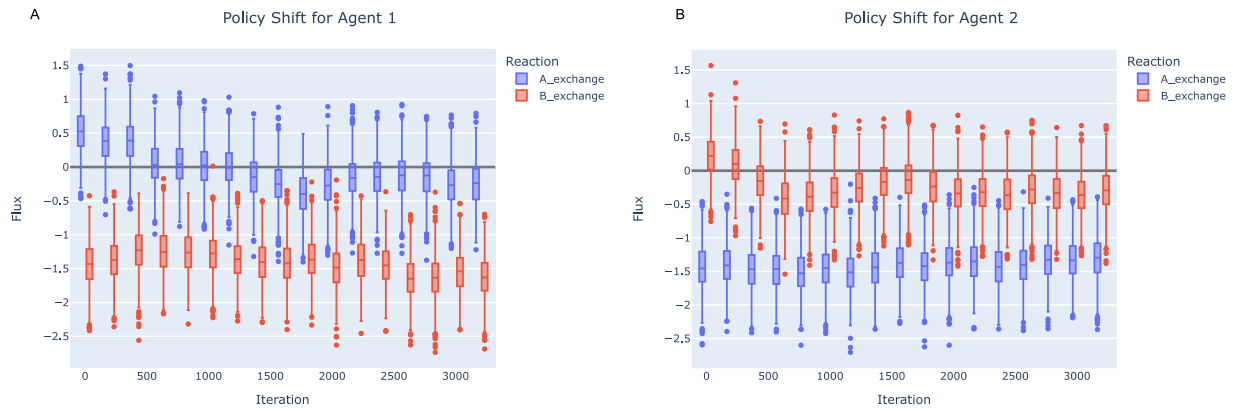

Figure 8 Policy shift after 3000 iteration of training agents of Toy-NECOM-Auxotrophs environment in Toy-NECOM-Auxotrophs-Shift. Both agents shift their policy from cross-feeding to taking up both A and B as much as possible. Here, the box plots show the range of actions across randomly generated states to represent the policy function.

Figure 8 shows that the auxotrophic agents shift their policy from metabolic exchange to selfishly taking up A and B when A and B is supplemented externally.

E. coli Auxotrophs

IJO1366-Tyr-Phe-Auxotrophs environment was created to prove the scalability of our approach to a community of genome-scale models, IJO1366. In this environment two E. coli auxotrophs were made. One mutant could not synthesize tyrosine and the other could not

synthesize phenylalanine. Neither of the mutants could grow alone on M9 minimal medium.

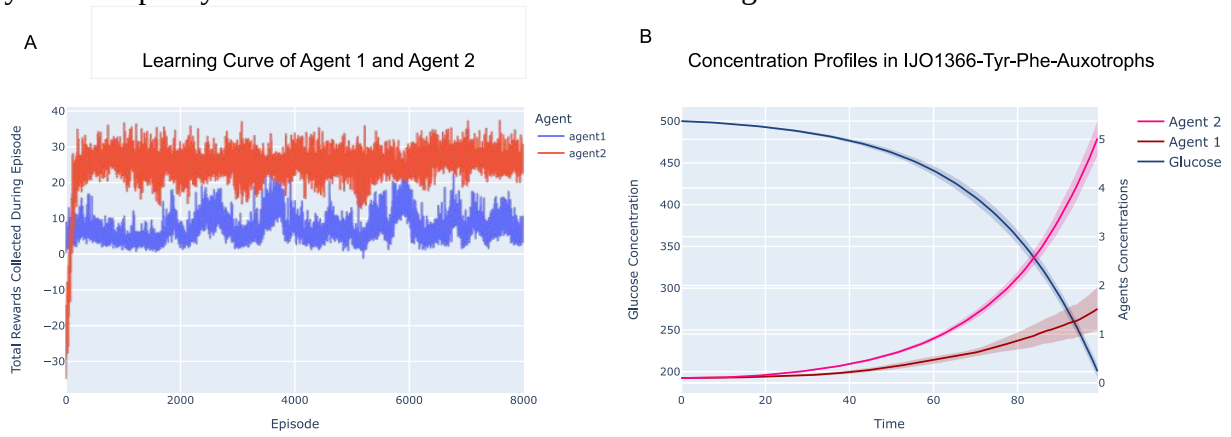

Figure 9 Training E. coli mutants in IJO1366-Tyr-Phe-Auxotrophs environment. Agent 1 is Tyrosine mutant and Agent 2 is Phenylalanine mutant. A) Learning curve of Agent 1 and Agent 2. Agent 2 receives higher return than Agent 1 although it starts from worse policy. B) Concentration profiles for glucose and the agents. It is also obvious from figure B that Agent 2, phenylalanine, achieves superior growth compared to Agent 1, tyrosine agent.

However, after training for 2000 episodes, the agents converged to a policy that they would exchange the amino acid that they can produce and grow by using the amino acid secreted by the other agent. In this environment phenylalanine mutant grew significantly higher than the tyrosine mutant. This observation is experimentally validated in [50] while no phenotypic data from this or other experiments was used during the training process, which shows the promise of the assumption that evolution favors traits at individual level that leads to high long-term fitness of the cells. Figure 9 shows the result of training the agents on 2000 batches of 4 episodes. We verified that this trend is not random by running 4 independent runs of 2000 batches.

Discussion

In this article we presented a novel algorithm that provides insights into microbial interactions by allowing the microbial agents to freely explore flux regulation strategies and select strategies that lead to their higher long-term fitness of the agents. The immediate advantage of this method is that it circumvents the need to predefine strategies for metabolic regulation. Defining this problem in a FBA frame work forces the strategies to be inside a space where mass balance and flux constraints are still satisfied. Another advantage of using FBA is that the underlying LP problems can be solved efficiently. We tested this algorithm on multiple test scenarios that emulate biologically relevant scenarios.

First set of environments were cases that multiple agents coexist with each other in an environment with starch and glucose and all agents are capable of producing amylase to breakdown the extracellular starch to glucose. What makes this toy case interesting is that secreting amylase in in contrast with immediate biomass optimization objective as amylase production requires energy that would be otherwise used for growth. In all scenarios, DFBA simulates zero amylase secretion in all situations. Another interesting aspect of these environment is the fact that when multiple agents exist this environment and one agent can stop secreting amylase and use the glucose released as the result of the activity of the amylase secreted by other agents. In this context,

an agent means a metabolic model that can independently make decisions in the environment. Depending on the number of agents present in the environment, the strategy of optimal agents is different, Figure 4. This is mainly because of cheating by the other agent. In presence of other agents, the optimal policy instructs the agents to produce the highest amylase when glucose concentration is the highest and the concentration of the other agent is the lowest. Consequently, the level of starch utilization is significantly lower in five-agent case when compared to the two and single agent case. Although these results are from toy communities, but they point to a common issue that is observed in real microbial systems. Cheating in microbial communities can significantly affect the amount which large molecules such as starch and xylan are degraded in hydrolysis [70], [78]–[88]. It should be noted that that the assumption behind these simulations so far is that the environment is homogenous. As a result, the degraded glucose is readily available to all members of the community. Taking spatial heterogeneity into consideration revealed that in communities with higher mass transfer limits, the agents secrete more amylase and starch utilization becomes higher, Supplementary Figure 1. The reason behind this observation is that low mass transfer implies that the glucose that is produced by an agent will stay away from the other agent that could possibly cheat, and in turn, the agents will see more positive signal by secreting amylase. However, the amount of utilized starch is still low compared to the single agent environment because the produced glucose still can diffuse away from the producers and used by other agents with a rate that is dictated by the physical properties of the environment. It should be noted that these simulations do not imply that mixing in real systems have deteriorative effect on starch utilization. Microbial cells have already developed strategies that creates the required spatial structures to keep their product close to themselves such as attaching to the solid surfaces [87], [88]. In fact, the selection of such traits could be interpreted as evidence of the hypothesis that preserving special structure in the environments with large molecules like starch leads to higher fitness for the cells in the long run.

With this algorithm we were able to explore other types of microbial interactions. One problem that we were interested in was that whether we can explain metabolite exchange between auxotrophic strains through this framework [50], [61]. The reason we asked this question is that basic DFBA simulations dictate that each GEM should maximize its immediate fitness. This results in imposing zero flux on any reaction that decreases the fitness, and in turn, avoid predicting any interaction if the secretion of metabolites is costly. However, thinking about this problem from an evolutionary perspective, exchanging costly metabolites can lead to higher fitness in future since the auxotrophic cells cannot achieve growth unless the required compounds are provided from outside. This type of exchange is observed frequently in nature as well [50], [89]. In SPM-DFBA, future rewards are also considered in strategy improvements, and as a result, we hypothesized that without any predetermined exchange strategies or community level objective, the optimal agents can find metabolite exchange strategy to maximize their own long-term fitness. Optimal agents in Toy-NECOM-Auxotrophs learned that exchanging A and B will increase their long-term fitness. However, in this case the agents passed from the symmetric policy and moved towards an imbalanced policy where one agent gained superior fitness compared to the other which shows exploitability of a symmetric policy when considering resource limitation.

Since SPM-DFBA is based on RL, it is implied that a large number of DFBA simulations is required for the agents to improve their policy. This might restrict the capability of this algorithm in simulating microbiomes with multiple GEMs. However, the required time per batch scales linearly with the number of GEMs in the environment as this method is based on DFBA. To see if this algorithm can be used for genome-scale model we created an environment of two E. coli

auxotrophs, tyrosine and phenylalanine, inspired by the experiments in [50]. Although we did not use any sort of experimentally observed phenotypic data, the agents learned to exchange the amino acid that they can produce, and the other agent cannot. Interestingly, our simulations indicate that the phenylalanine mutant achieves superior growth compared to the tyrosine mutant, Figure 9, which follows the same trend as is experimentally observed and reported in [50]. Being able to predict such emergent behaviors of microbiomes by purely relying on metabolic capability of the cells and ecological first principles is what distinguishes SPM-DFBA from the other existing algorithms.

Several cases for instability of auxotrophic interactions have been reported [90]. In an interesting study [91], reported the behavior change of auxotrophs when inserted in an environment that supplies all the components that they need for growth. In this scenario they shift their exchange strategy to uptake all the compounds from the environment and stop secreting the metabolites further. Our simulations, Figure 8, showed similar shift for auxotrophic agents which reflects that the agents adapt their strategies according to the changes in the environment, and shows assuming that cells are *maximizing their own long-term fitness* can reproduce several real scenarios is missed by simple DFBA.

Previous cases revealed that agents that depend on each other for survival will evolve to exchange metabolites with each other and when this strict dependence doesn't exist anymore selfish behaviors emerge. Toy-NECOM-Facultative-Exchange provides more evidence for this trend we changed the agents in Toy-NECOM-Auxotrophs so that they can produce both A and B but with different efficiencies. In this case, if a community level objective such as, total community biomass maximization, is defined then A and B exchange between agent 1 and agent 2 is logical [12], [62]. However, this is not what SPM-DFBA predicts. In this case, A and B exchange strategy is exploitable by the agents. Since the agents do not rely on each other for survival, any exchange of A and B is exploitable by either of the agents. Consequently, the agents finally adhere to taking up any A and B, limited by the kinetic rules, that exist in the environment which is shown in Figure 8. This is similar strategy to what is described in NECom [12] and exactly matches simple DFBA prediction.

As we discussed, our algorithm can predict scenarios that are observed in nature while the current models fail to do so in some of the mentioned cases. Also, one particularly interesting advantage of this algorithm is that the learned policies can be interpreted easily to infer what would an optimal agent do in this environment at each state. But what gives this framework such flexibilities and what are the challenges that are faced using this algorithm?

First, this is a dynamic framework, and the environment changes such as resources limitations can be simulated while this is not directly possible when assuming steady state. This is an important consideration that can significantly shape microbial interactions [92]. Steady state assumption in the best-case scenario can be valid when there is a continuous flux in and out of the environment. If such conditions exist, it will be the outcome of this algorithm as well.

Second, the phenotypes of the community of interest are predicted by using information at biochemical reaction level which helps the interpretation of the emergent properties of microbiomes from the biochemical reaction fluxes. This means that we can predict emergent property of microbiomes by relying only on the metabolic network and fundamental laws of nature that are assumed to be valid in biological systems such as the law of mass and energy conservation in contrast with commonly used ecological models such as Generalized Lotka-Volterra [93]–[95], which do not base their predictions on the metabolic network of the microbes.

Third, optimization is done at individual model level. This means that unrealistic interactions discussed in detail in [12] are avoided. This is supplemented with randomness in the behavior of the agents for exploration purpose. This means that if an agent can exploit a scenario by randomly changing its strategy it has a chance of happening. When many state-action pairs are simulated, the chance of such exploitations is high. If a particular random action is advantageous to the long-term fitness of an agent, this behavior gets reinforced in the policy of the agent using the PPO algorithm. We believe that this has a lot of similarities to the process of natural selection. We would like to emphasize that we do not wish to imply that the microbial cells are intelligently seeking the optimal behavior in their environment. However, it is the resemblance of this algorithm to the process of natural selection that results in more realistic predictions for a given environment.

We think there are several areas for improvement in future studies that can make this approach suitable for large-scale problems. When it comes to communities of genome-scale models, runtime required to train the agents can get intractable. However, the time required to run an episode is almost the same as DFBA because of efficient libraries that exist for training neural networks which makes the runtime for training part of the process negligible when compared to DFBA step. The architecture of the suggested algorithm allows for parallelization which increases the efficiency of the algorithm. However, there are several modifications that can make the training process more efficient in future. One way is to completely remove the need to use a linear programming formulation by letting the agent control the linear combination of the basis of the null space of the stoichiometric matrix. This approach guaranties that all suggested strategies still support the conservation of mass inside the cell and reduces the LP problem to a single matrix multiplication step which is significantly faster. However, a direct method for tractably making the policy network to abide by the flux constraints on the metabolic network was not obvious to us. This challenge is even more obvious when scaling up to genome-scale models since the feasible flux ranges for many reactions can be extremely small, while it can be very large for others. It is worth mentioning that there is no limitation regarding the selection of action or state spaces. In terms of state and action space choices, one particularly interesting approach is to make growth rate an action as well, and this can shed light on some of the growth inhibition strategies that is observed in microbial cells from an evolutionary perspective [96]. Another approach to speed up this algorithm is to use the power of neural networks as LP solver [97]. This approach will result in an accurate and fast way to substitute common LP solver and make the process more efficient.

A more general analysis that could reveal important information about an ecosystem of interest is to try many agents in one environment and statistically assess the policy profiles of each agent in this case the more relevant question is that what is the distribution of the exchange policies in such environment. We also mentioned a few simplified cases where spatial variations were considered. We believe that this algorithm can be used in more general frameworks that consider concentration gradients in the environment. However, these suggested improvements were out of the scope of this article.

Here we just showed the potentials of the algorithm and made the script for running this algorithm on a new environment easy and understandable to pave the way for other researchers to improve this approach with the hope that it can be used to further demystify the complicated world of microorganisms.

Conclusion

A novel modeling framework based on flux balance analysis is introduced. In this framework microbes are modeled as agents capable of making decisions, i.e., regulate their metabolism. Initially, agents start with random behavior. However, through trial and error, they learn what type of strategies lead to their long-term survival. This way of thinking about microbial actions allows for explaining interactions that are observed in nature with minimal previous knowledge about these environments by relying on first principles, and successfully predict scenarios where well-adapted frameworks fail.

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