A Simulator-Assisted Workshop for Teaching Chemostat Cultivation in Academic Classes on Microbial Physiology†

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Understanding microbial growth and metabolism is a key learning objective of microbiology and biotechnology courses, essential for understanding microbial ecology, microbial biotechnology and medical microbiology. Chemostat cultivation, a key research tool in microbial physiology that enables quantitative analysis of growth and metabolism under tightly defined conditions, provides a powerful platform to teach key features of microbial growth and metabolism.

Substrate-limited chemostat cultivation can be mathematically described by four equations. These encompass mass balances for biomass and substrate, an empirical relation that describes distribution of consumed substrate over growth and maintenance energy requirements (Pirt equation), and a Monod-type equation that describes the relation between substrate concentration and substrate-consumption rate. The authors felt that the abstract nature of these mathematical equations and a lack of visualization contributed to a suboptimal operative understanding of quantitative microbial physiology among students who followed their Microbial Physiology B.Sc. courses.

The studio-classroom workshop presented here was developed to improve student understanding of quantitative physiology by a set of question-guided simulations. Simulations are run on Chemostatus, a specially developed MATLAB-based program, which visualizes key parameters of simulated chemostat cultures as they proceed from dynamic growth conditions to steady state.

In practice, the workshop stimulated active discussion between students and with their teachers. Moreover, its introduction coincided with increased average exam scores for questions on quantitative microbial physiology. The workshop can be easily implemented in formal microbial physiology courses or used by individuals seeking to test and improve their understanding of quantitative microbial physiology and/or chemostat cultivation.

INTRODUCTION

Acquiring a quantitative insight into the interaction of microorganisms with their growth environment and, specifically, the way in which nutrient availability affects microbial growth kinetics and biomass yields is an essential learning objective in academic microbiology programs. Understanding this key aspect of microbial physiology is important across many domains of microbiology, including the design of experiments for isolating novel microorganisms from nature, the understanding and optimization of antibiotic therapies, and the optimization of microbial product formation in industrial bioreactors. In view of the latter application, biotechnology and bioengineering curricula have historically emphasized the importance of mass balancing in microbial processes, as well as of the ensuing (biomass-specific) rates and microbial growth kinetics.

The chemostat is a continuous cultivation device that is especially suitable for quantitative physiological comparison of microorganisms under highly defined conditions (1–6). The power of the chemostat lies in the fact that, after inoculation and an initial dynamic phase (here referred to as non-steady state), the system approaches a state in which not only the physicochemical environment but also all rates of production and consumption remain constant in time (hence called steady state). In ideally
mixed, steady-state chemostat cultures, the specific growth rate of the microorganisms equals the dilution rate of the system, which can be set by the experimenter.

Growth and substrate consumption of microorganisms in chemostat cultures can be described by a set of four equations: the mass balances of biomass and growth-limiting substrate, an equation describing the distribution of the growth-limiting substrate over growth and maintenance processes, and an equation describing the specific substrate consumption rate as a function of substrate consumption (see the “Prerequisite student knowledge” section for an extensive description of the system). Operational knowledge of and insight into these equations is important for experimental design of chemostat experiments, to correctly interpret experimental data and, in general, to understand the impact of growth conditions on microbial growth and performance.

Quantitative microbial physiology in chemostat cultures is an integral part of the Microbial Physiology course that, for the past 10 years, two of us (JTP and AJAvM) taught together as part of a second-year BSc curriculum in Life Science and Technology offered jointly by the Delft University of Technology and Leiden University (the Netherlands). Based on their experience in classroom teaching and evaluation of written exams, all three authors attributed the lower-than-desired operational knowledge on this topic to the somewhat abstract nature of the mathematical equations and, in particular, insufficient visualization of the ways in which growth conditions affect the growth of microorganisms in chemostat cultures.

Several models have been described in the literature that explain chemostat cultivation processes. However, these models are generally aimed at users with an advanced understanding of the subject and are therefore suboptimal for use in educational settings (7, 8). To help students come to grips with the key quantitative aspects of non-steady-state and steady-state growth phases in chemostat cultures, we developed a simple, robust simulator that specifically visualizes the time-dependent dynamics that ultimately result in steady-state chemostat cultures. Around this simulator, we designed a question-guided workshop in which students explore how individual experimental design parameters and/or key characteristics of the microorganism itself influence the non-steady-state and steady-state behavior of chemostat cultures. The workshop was held in a studio-classroom learning environment but can, in principle, be run on standalone computers.

Here, we describe the simulator, the accompanying lecture material, the questions used in the workshop and our experiences with the implementation of this workshop.

Intended audience

The simulator-assisted workshop is intended for students who have proceeded in microbiology or (bio)chemical engineering majors with a focus on microbial physiology and/or microbial biotechnology. These students should have had general microbiology classes prior to the workshop and should have been introduced to the theory described below in “Prerequisite student knowledge,” preferably no longer than two weeks prior to the workshop. The workshop is most easily integrated into courses that already have a focus on microbial physiology.

Higher-level students and researchers aiming to improve their knowledge and understanding of quantitative physiology can also use the simulator without the accompanying workshop.

Prerequisite student knowledge

In a standard chemostat culture, fresh medium is continuously added to a cultivation vessel, the (bio)reactor, while continuous removal of the spent medium containing biomass is controlled to maintain a constant volume. The fresh medium is typically designed in such a way that a single nutrient will limit growth, while all other medium components are in excess. This workshop focuses on chemostat cultures in which the energy substrate is the growth-limiting nutrient which, in organoheterotrophs, also acts as the carbon source. Growth in such chemostat cultures can be described according to two mass balances: a mass balance for the substrate (a non-volatile carbon and energy source) and a mass balance for biomass.

Description of chemostat cultures with these simple mass balance equations requires that three important criteria be met: 1) the culture is ideally mixed, i.e., concentrations of biomass and substrate within the bioreactor should be identical to those in the outflow, 2) the culture volume remains constant over time, and 3) the inflow and outflow rates are equal. The characteristic parameter that can be fixed in a chemostat culture is the dilution rate (D, h^-1).

Substrate is added to the bioreactor as part of the fresh, sterile inlet medium. Once inside the reactor, it can either be consumed by the microbe in the bioreactor or be removed with the spent medium (Fig. 1). The microbe in the bioreactor will grow and at the same time be removed with the spent broth.

\[
\frac{dM_s}{dt} = \frac{dC_s}{dt} = V \frac{dC_v}{dt} = F_{in}C_{in} - F_{out}C_{out} - q_sC_sV
\]

\[
\frac{dM_l}{dt} = \frac{dC_l}{dt} = V \frac{dC_v}{dt} = -F_{out}C_{out} + \mu C_sV
\]

The distribution of energy substrate over growth and maintenance energy requirements, in the absence of ATP-requiring product formation, can be described according to the Pirt equation (Eq. 3), an empirical relation that assumes a growth rate-independent energy requirement for maintaining cellular viability and integrity (9, 10). An important consequence of this assumption is that, as the growth rate
maximum biomass yield ($Y_{x/s,\, \text{max}}$, g $x$·g $s$ $^{-1}$), the biomass-specific maintenance-energy requirement ($m_s$, g $x$·g $s$ $^{-1}$·h$^{-1}$), the substrate concentration in the fresh inflowing medium ($C_{s,\, \text{in}}$, g L$^{-1}$), the initial biomass concentration ($C_{x,\, \text{in}}$, g L$^{-1}$), the initial substrate concentration ($C_{s,\, \text{in}}$, g L$^{-1}$), and the total time for the model to run (Max time, days).

$$q_s = \frac{\mu}{Y_{x/s,\, \text{max}}} + m_s$$

$$q_s = q_{s,\, \text{max}} \frac{C_s}{K_S + C_s}$$

Learning time

The entire workshop takes approximately four hours to complete. It starts with an introductory part, consisting of a set of introductory questions followed by a presentation by a teacher or course assistant. Subsequently, the students start working with the simulation program and systematically tackle the guided questions. Finally, results are discussed with all students present. It is crucial that students should be allowed sufficient time to “wrestle” with the questions themselves.

- The workshop starts with the students answering the introductory questions provided in Appendix 3 (25 minutes)
- An introductory presentation recapitulates the answers to the introductory questions and the operation of chemostat cultures. It also explains how the non-steady-state dynamics can be analyzed. The PowerPoint presentation in Appendix 2 can be used for this purpose (30 minutes)
- Students start the MATLAB program and familiarize themselves with the functions (20 minutes)
- Students answer the guiding questions about non-steady-state and steady-state dynamics in chemostat cultures provided in Appendix 3 (120 minutes)
- The workshop ends with an interactive plenary discussion on the answers to the questions (45 minutes)
- Students are encouraged to use the simulator to individually explore quantitative physiology outside of the workshop

Learning objectives

Upon completion of the simulator-assisted workshops about the physiological concepts of energy-source limited chemostat cultivation, students will be able to:

1. Report the mass balances for substrate and biomass that describe a continuous cultivation
2. Report the assumptions that are required to describe steady-state conditions
3. Explain and describe the non-steady-state dynamics in biomass concentration and substrate concentration

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Learning objectives

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1. Report the mass balances for substrate and biomass that describe a continuous cultivation
2. Report the assumptions that are required to describe steady-state conditions
3. Explain and describe the non-steady-state dynamics in biomass concentration and substrate concentration
in chemostat cultivations that ultimately lead to steady-state conditions
4. Describe the relation between the specific growth rate ($\mu$) and the specific substrate uptake rate ($q_s$) through the Pirt equation
5. Describe the relation between the specific substrate uptake rate ($q_s$) and extracellular substrate concentrations ($C_s$) through Monod-type kinetics
6. Identify the effect on the biomass yield $Y_{x/s}$ and the residual substrate concentrations ($C_s$) in steady-state conditions of the following parameters:
   - Maintenance energy requirements ($m_s$)
   - Maximum biomass yield ($Y_{x/s, max}$)
   - Saturation constant for substrate ($K_s$)
   - Maximum substrate uptake rate ($q_s, max$)
   - Dilution rate ($D$)
   - Substrate concentration in the inflowing medium ($C_{s,in}$)

All these concepts are addressed in the workshop questions and can be tested with exam questions, for which examples are provided (Appendix 5).

PROCEDURE

Materials

- Student computers that can either work with MATLAB (.m) files or that have the Chemostatus simulation program installed as a stand-alone module (we have extensively tested this stand-alone version on Windows 7 64 bit). An installation manual is provided in Appendix 1. The MATLAB files as well as the stand-alone version can be requested by sending an email to chemostatus@gmail.com. Chemostatus cannot be installed as a stand-alone version on Mac or Linux computers.
- A studio classroom (i.e., a classroom with PCs or laptops on which Chemostatus simulator and Microsoft Excel can be run). The studio classroom should ideally enable students to work in pairs and have easy access for course assistants.
- Printed student questions, with introductory and guiding questions on separate sheets.
- A projector and screen.
- The Power Point presentation supplied in Appendix 2 or equivalent teaching material.
- A whiteboard or blackboard for the interactive discussion and explanation by teachers/course assistants at the end of the workshop.

Student instructions

Students are advised to work in pairs as this stimulates discussion of the observations. A key aspect to improve the learning experience in this workshop is to address each question in a systematic order, answering three sub-questions: 1) which (qualitative) changes are to be expected based on the answers to the introductory questions, 2) use the Chemostatus simulator and/or simulation data exported to Microsoft Excel to answer the question quantitatively, and 3) combine knowledge of quantitative physiology and numerical answers to qualitatively understand the changes.

Chemostatus allows the user to change each of the input parameters at will and evaluate the output for six consecutive calculations. Time-dependent simulations provide insight into how steady-state conditions are reached. Its outputs consist of plots of biomass concentration, substrate concentration, biomass-specific substrate consumption rate, specific growth rate, and actual biomass yield on substrate, from the start ("virtual inoculation") of the experiment until a predefined time point. The simulated data that are used to generate the plots can be exported to a Microsoft Excel file for further calculations.

Faculty instructions

It is important that the studio classroom not only enable students to work on computers, but also enable them to follow instructions by teachers and/or course assistants (using beamer, blackboard, and/or whiteboard). The required software (Chemostatus simulator, Microsoft Excel) should be installed and tested on the computers prior to the workshop. A manual for installation is available in Appendix 1. Note that, due to user administrator restrictions, installation of software may require involvement of professional support staff.

Prior to the workshop, the prerequisite knowledge should be explained during classroom lectures. The workshop starts by answering a set of (refresher) questions about steady-state chemostat cultures (Appendix 3, “Introductory questions”) without using a computer. Preferably, the underlying concepts of chemostat cultivation should previously have been discussed in regular classroom teaching. The introductory set of questions aims to bring students to the entry level required for the rest of the workshop. Subsequently, the answers are discussed. A PowerPoint presentation for teachers/course assistants has been supplied (Appendix 2) to be used during the workshop.

After discussing the relevant growth parameters, equations, and assumptions as presented above, students are given 20 minutes to familiarize themselves with the Chemostatus simulator. After this, they are supplied with a set of questions (Appendix 3, “guiding questions”) that guide them through simulations of the physiological impacts of changes in different parameters. As stated above, we recommend that students work in pairs to facilitate discussion and peer learning. During this part of the workshop, the teacher is available to address questions from students. Obviously, the goal here is not to answer the questions for students but to encourage them to find the answers themselves. Typically, this involves asking additional "guiding" questions.
Depending on the group size, the teacher might require support from course assistants. In our experience, a group of 50 to 60 students can be guided by three experienced teachers and/or teaching assistants.

After students have had sufficient time to answer the questions, the answers are discussed in a plenary session. The teacher can explain the answers to each of the questions and can also point out other interesting observations during the non-steady-state or steady-state phases of the simulated cultures. Preferably, this involves running the Chemostatus on a computer connected to a large screen.

**Suggestions for determining student learning**

A stepwise approach to the questions, starting out with a formulation of the expected qualitative outcome of the simulations is, in our experience, a major success factor for this workshop. Teachers and course assistants can stimulate and verify this approach by engaging students in conversations themselves and by actively encouraging discussions about the questions among students.

At the end of the workshop, the answers to the questions are discussed by the teachers in an interactive plenary session. The feedback from the students during this session provides the teacher with a clear view of student learning results.

Written exam questions provide an effective way of testing the extent to which students master this subject. Quantitative physiology is a recurring topic in exams of our Microbial Physiology course. Examples of exam questions are provided in Appendix 5, with corresponding learning objectives.

**Sample data**

Answers to the student questions of the workshop are provided in Appendix 4.

**Safety issues**

The workshop does not involve (biological) safety hazards (with the possible exception of student exposure to computer keyboards (15)).

**DISCUSSION**

**Field testing**

- Prior to its use in the regular teaching program, the authors and two student volunteers tested the workshop for user friendliness and clarity.
- The workshop was used twice by the authors as an integral part of their Microbial Physiology course at the Delft University of Technology (December 2015). The workshops, each attended by 60 to 70 students, were supervised by the three authors.
- The workshop was as an optional workshop in addition to a course on chemostat cultivation taught to eight PhD students by Professor J. G. Kuenen (emeritus professor at Delft University of Technology) and hosted by Professor K. N. Nealson of the Geobiology group in the Department of Earth Sciences, University of Southern California, Los Angeles. This course was held in April 2016.

**Evidence of student learning**

During the workshop, students showed great involvement in the topic and came up with meaningful questions. Spontaneous, enthusiastic discussions occurred about the explanation of complex simulation results (e.g., “overshoots” of biomass concentrations during non-steady-state simulations due to a lower impact of maintenance energy requirements during fast growth). We consider this active involvement and feedback a good indicator that the workshop enhanced student learning.

Two of the authors have taught quantitative microbial physiology for a full decade as part of their Microbial Physiology course, which they teach in a “duo presentation” mode. Quantitative aspects of microbial growth is the subject of one of four questions in the final written exam. This question typically consists of five sub-questions, each with a possible score of 0 to 10. In 2014 and 2015, chemostat theory was only explained during lectures. The teachers were not satisfied with student scores for the quantitative physiology question and decided to implement the workshop described in this paper. To analyze the effect of the workshop, the average scores per student for the sub-questions specifically dealing with steady-state chemostat cultures were evaluated for the two years before introduction and for the year of introduction of the workshop. The selected sub-questions and corresponding learning objectives are shown in Appendix 5. Upon introduction of the workshop in 2016, the average grade for these sub-questions increased significantly relative to previous years for both the regular exam (Student’s t-test; 2014 to 2016 p < 0.001; 2015 to 2016 p < 0.001) and the retake exam (Student’s t-test; 2014 to 2016 p < 0.05; 2015 to 2016 p < 0.001). More importantly the fraction of students who now grasp these quantitative physiological concepts has increased with the introduction of the workshop, as shown by the higher 75% percentile compared with the previous two years (Fig. 2, Panel A and B) and the increased percentage of students who passed the exam (Fig. 2, Panel C and D).

At the Delft University of Technology, all courses are evaluated via student surveys and oral evaluation. The course that contained this workshop as a new element was evaluated very positively and students specifically indicated the usefulness of the workshop on quantitative physiology.

**Possible modifications**

This workshop is a complete activity that is ready for use as presented here. In its current format, the workshop
is held in one session but can be split into two shorter sessions in which the first session focuses on the concepts and introductory questions and the second session provides students with the opportunity to focus on the “guiding” questions. To meet specific requirements of different student groups or courses, new sets of questions can be designed. Furthermore, the original MATLAB (.m) files can be requested by sending an email to chemostatus@gmail.com, providing the opportunity to implement new functions. The following modifications can contribute to additional learning:

- Without the accompanying questions and the structure of the workshop, advanced students can use the Chemostatus simulator outside the context of the workshop to deepen their understanding of key concepts of microbial physiology.
- The current version of the workshop is based on simulated data only. The accompanying questions could be extended with an exercise in which experimental chemostat data of a well-known microorganism (for which the input parameters of the model are known or can be estimated based on simulations as part of the exercise) are compared with simulations.
- In microbial ecology, chemostat cultivation is a powerful tool to study competition for a limiting nutrient (16–18). This concept could be implemented by introducing a second set of parameters, equations, and mass balances. This would require the coding of the MATLAB program to be adapted. We would recommend not adding microbial competition as an extra subject in the current four-hour workshop but, instead, making it the subject of a separate simulation workshop. Similarly, the occurrence of mutants with altered growth kinetics might be simulated to gain a deeper understanding of (laboratory) evolution.
- Microbial product formation, which is of special interest in industrial biotechnology, could be implemented by introducing an additional mass balance for product and by providing the model with a relation between product formation and growth rate.

**SUPPLEMENTAL MATERIALS**

Appendix 1: Chemostatus manual
Appendix 2: PowerPoint presentation Chemostatus
Appendix 3: Student questions
Appendix 4: Student data
Appendix 5: Exam questions and Learning Objectives

The files of the Chemostatus simulator can be requested by sending an e-mail to chemostatus@gmail.com.
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XDVH designed the MATLAB program and prepared the PowerPoint presentations. All three authors contributed to the design of the workshop, tested it in practice and wrote the paper together. The authors would like to thank Prof. J. G. Kuenen for fruitful discussions on the contents and for field testing the model at the University of Southern California (Los Angeles). We thank Leonor Guedes da Silva for valuable stimulating discussions on the MATLAB coding. The authors thank Maaike Voskamp and Joachim van Renselaar for testing the workshop for user-friendliness. In particular, we thank the 2016 class of our Microbial Physiology course at the Delft University of Technology for acting as highly constructive guinea pigs for this workshop. The authors declare that there are no conflicts of interest.

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