



Small doses, big troubles: Modeling growth dynamics of organisms affecting microalgal production cultures in closed photobioreactors

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HIGHLIGHTS

- ▶ Model realistically simulates growth of contaminants in a production photobioreactor (PBR).
- ▶ “Sudden” onset of contamination can be attributed to exponential growth.
- ▶ PBR management protocols can reduce the risks of serious contamination.
- ▶ Small numbers of sufficiently fast-growing contaminants can lead to loss of algal cultures in days.
- ▶ A simple and cheap protocol for short-term prediction of severe contaminants in PBRs is presented.

ARTICLE INFO

Article history:

Received 13 June 2012

Received in revised form 14 November 2012

Accepted 19 November 2012

Available online 29 November 2012

Keywords:

Microalgae culture management

Grazers

Growth prediction

ABSTRACT

The destruction of mass cultures of microalgae by biological contamination of culture medium is a pervasive and expensive problem, in industry and research. A mathematical model has been formulated that attempts to explain contaminant growth dynamics in closed photobioreactors (PBRs). The model simulates an initial growth phase without PBR dilution, followed by a production phase in which culture is intermittently removed. Contaminants can be introduced at any of these stages. The model shows how exponential growth from low initial inocula can lead to “explosive” growth in the population of contaminants, appearing days to weeks after inoculation. Principal influences are contaminant growth rate, PBR dilution rate, and the size of initial contaminant inoculum. Predictions corresponded closely with observed behavior of two contaminants, *Uronema* sp. and *Neoparamoeba* sp., found in operating PBRs. A simple, cheap and effective protocol was developed for short-term prediction of contamination in PBRs, using microscopy and archived samples.

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1. Introduction

Just like terrestrial plant crops, microalgae in large-scale biomass cultures can be beset by pests and weeds. Grazing organisms, including protozoa and microinvertebrate animals, can be devastating pests. Complete destruction of microalgal crops has been recorded in as little as 48 h from first detection of an aggressive grazer (Moreno-Garrido and Cañavate, 2001). This phenomenon is consistent with what has been observed for microalgae in nature (Sherr and Sherr, 2002; Narwani and Mazumder, 2010). Fast-growing non-target algal weeds may degrade the quality of the product and can even displace the target strain entirely, as has been observed in natural populations (Sieracki et al., 1993).

Despite the obvious risks, little has been published on this aspect of microalgal cultivation, and the research and development needs are considerable (Day et al., 2012a). Most grazers have been identified to the genus level at best, with many known to no greater accuracy than (for example) ‘amoeba’, ‘ciliate’, or ‘rotifer’ (Post et al., 1983; Moreno-Garrido and Cañavate, 2001). Without an accurate identification, information on the distribution in nature, life history, growth rate, and prey choice of the contaminating organism is not accessible – assuming that such information even exists – leaving a production team to guess whether a particular contaminant poses a risk to cultivation, and if it does, how grave is the situation. Chemical control of an established, aggressive contaminant is problematic (Moreno-Garrido and Cañavate, 2001), and other means of control have hardly been investigated (Day et al., 2012a).

Given the large number of contaminant species that can infest algae production cultures (Post et al., 1983), and the lack of information on the biology and control of practically all of these

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contaminants, a production unit currently is unlikely to be able to “cure” a mass algal culture of an established contaminant. At present, mitigation strategies attempt to prevent contaminant entry, and manage cultures to minimize growth of contaminants present relative to target algae. To accomplish this mitigation for any given production system requires the following: information on the means by which a contaminant enters the production system (vector), the amount (rate) of contaminant entry via the relevant vector(s) (inocula), the time (latency) it takes for a contaminant, from a given inoculum (or inoculation rate) to reach a set threshold – typically, a contaminant detection limit or a product quality control metric.

Latency, is likely a function of the specific growth rate of the contaminant in the production system. Therefore, given an inoculum and a specific growth rate, it should be possible to produce a simple mathematical model that will predict the behavior of a contaminant in culture and thereby provide a tool for culture management where contamination is present, without the need for an accurate taxonomic identification of the contaminant(s). The objective of this research was to develop such a model.

2. Methods

2.1. Context and assumptions

As the starting condition for the model, a closed cultivation system or photobioreactor (PBR) of the type commonly used in microalgal production systems to maintain cultures at large volumes for extended periods of time was postulated. The target alga is kept under nutrient-replete conditions, and therefore is in exponential-phase growth throughout. The PBR is operated in semi-continuous culture mode, with a portion of the culture volume being removed and replaced with fresh medium as needed to keep the target alga in exponential-phase growth. A key characteristic of all PBR designs is the isolation of the target culture from contaminants, but we postulate that the air and water supplies for our PBR are slightly leaky, and this leakiness provides the vector for contaminant entry. The lower bound for the initial inoculum via these vectors (combined) was set at 1 cell per 1000 L of culture medium – an empirically derived figure. A contaminant-free starter culture of the target alga used to initiate the PBR at time $t=0$ was assumed. The threshold value for contaminant cell numbers (for the purposes of determining latency) was set at 2×10^4 cells L^{-1} – also an empirically-derived figure representing the lowest contaminant cell number that could consistently be detected in a microscopy-based screening system.

Microscopy of PBR cultures was performed daily by trained and experienced personnel, using a Zeiss AxioObserver A1 inverted microscope at a magnification of up to $400\times$. Freshly collected samples of culture were viewed both live and after fixing with Lugol's Iodine.

Simple exponential cell growth of the contaminant was assumed with no resource limitation for most of the culture's life. Growth of the contaminant was assumed to not be significantly influenced by growth of the target alga, except as noted for Eq. (2) below.

Amoebae (*Uronema* sp.) were obtained from KPF operations (Cellana LLC, Kona Pilot Facility, Kailua-Kona, Hawaii, USA), and transported to Friday Harbor Laboratories (Friday Harbor, Washington, USA), where they were isolated into uniprotist cultures by single-cell picking. The cultures were maintained in sterile seawater medium solidified with 1.5% agar with mixed bacteria. The bacteria were also obtained from KPF operations and grown on medium 2216 (Difco) solidified with 1.5% agar. Cell counts were achieved by placing single amoebal cells in the wells of 24-well

spot plates and incubating them with sterilized seawater to which bacteria from the agar cultures were added. Ciliate cell growth was monitored daily with an inverted microscope. Samples were taken from individual wells daily over a 5-day period, fixed with Lugol's iodine, and ciliates were counted with a hemocytometer.

2.2. Terms of the model

The concentration of the contaminants after the time interval $\Delta t = t_1 - t_0$ is given by:

$$n_1 = n_0 e^{\mu(t_1 - t_0)} \quad (1)$$

where μ is the specific growth rate of the contaminant in units of d^{-1} , n_0 and n_1 denote the concentration (in cells L^{-1}) of the contaminant at times, t_0 and t_1 .

Contaminants were assigned a reduced rate of growth during the initial grow-up period following inoculation of the PBR Eq. (2). For heterotrophic contaminants (grazers), this equation represented resource-limited growth due to the small rate of encounters with algal prey in a sparse culture. For autotrophic contaminants (algae), the reduced growth rate represented the lag period before exponential growth. The growth rate increased each day during the grow-up period:

$$\mu_{su}(t) = \mu \frac{t}{t_{su}} \quad (2)$$

where $\mu_{su}(t)$ is the specific growth rate of the contaminant on day t ; μ is the (resource sufficient) specific growth rate of the contaminant; and t_{su} is the number of days with no dilution for grow-up. Calculation of contaminant population density was performed for each hour. During grow-up:

$$CC_{(h)} = e^{\frac{\mu_{su}(t)}{24} C_{(h-1)}} \quad (3)$$

where $C_{(h)}$ is the cell density at the end of the current hour; and $C_{(h-1)}$ the cell density at the end of the previous hour.

In normal production mode, the growth rate of the contaminant was assumed to be maximal and growth was continuous over 24 h. Food resources for the contaminant in the dense culture of microalgae was assumed to be limitless. Each production day was divided into an initial 12-h period without a change in volume, where:

$$C_{(h)} = e^{\frac{\mu}{24} C_{(h-1)}} \quad (4)$$

followed by 6 h where a number of cells were removed during the removal of some fraction of the medium containing the culture:

$$C_{(h)} = e^{\frac{\mu}{24} (C_{(h-1)} - rC_{(h-1)})} \quad (5)$$

where r is the hourly rate of drain/refill. The actual volume of culture medium was not used in these calculations. $C_{(h)}$ is the concentration of the population of cells in the total volume of the PBR.

For a further 6 h, the lost volume was replaced with fresh (cell free) medium and the population was again calculated by using equation (Eq. (4)). Once the refill was complete, the volume of the culture was left unchanged until the next drain/refill cycle. To simulate an ineffective water filtration system, a term for the addition of contaminants at a particular concentration (a) with the refill was added:

$$C_{(h)} = e^{\frac{\mu}{24} (C_{(h-1)} + a)} \quad (6)$$

The model was coded and graphs generated in the R environment (R Development Core Team, 2011).

3. Results and discussion

3.1. Case studies

3.1.1. Case 1: Undiluted PBRs

Under this scenario, the PBR is operating in “batch” culture mode. Three contaminant types were tested, one with a specific growth rate in the culture system of 4 d^{-1} (i.e. over 50-fold increase each day), one with a specific growth rate of 2 d^{-1} and one with a specific growth rate of 0.7 d^{-1} . For each contaminant type, the initial inocula were set at 0.001 cell L^{-1} or 1.0 cell L^{-1} .

When the inocula were 0.001 cell L^{-1} , the latency for the fastest-growing contaminant was 6 d (Fig. 1A). For a contaminant with an intermediate-growth rate (2 d^{-1}), latency was 10 d, while that for the slowest-growing contaminant was 26 days (Fig. 1B). Latency was shorter with inocula of 1 cell L^{-1} : 4 d, 5 d, and 16 d respectively. In all cases, contaminant cell numbers remained low until near the end of the latency period, at which point they increased dramatically.

The model for the fastest-growing contaminant closely followed empirical observations of bacterivorous scuticociliates (*Uronema* spp.) that typically appeared 5–12 d after inoculation. Growth rates up to 4 d^{-1} have been measured for *Uronema* species under optimal growth conditions (Parker, 1976; Pérez-Uz, 1995,1996). These measurements are also consistent with estimates of scuticociliate growth based on uniprotist cultures of *Uronema* sp. isolated from algal mass culture stocks and reared in sterile seawater with added mixed bacteria, also isolated from mass culture stocks (data not shown). No empirical observations matched the model for the slower-growing contaminants; these scenarios are unrealistic in production PBRs, as no target algae growing at a useful rate will persist in batch mode for as long as 12 days without encountering limiting nutrients and entering stationary phase.

3.1.2. Case 2: 50% daily PBR dilution

Under this scenario, the PBR is operating in “semi-continuous” culture mode, with 50% dilution of the culture daily after day 5. For this test, the contaminant growing at the rate of 4 d^{-1} was omitted (as, from Case 1, its density reaches threshold values before dilutions begin), but added two others, one with a specific growth rate of 0.6 d^{-1} , and one with a specific growth rate of 0.51 d^{-1} .

For the two contaminants also considered in Case 1, latency was increased in diluted PBRs in all cases. When the inoculum was

0.001 cell L^{-1} , latency of the culture growing at 0.7 d^{-1} increased from 26 to 86 d (Fig. 2A and B), while that of the culture growing at 2 d^{-1} increased from 10 to 12 d (Fig. 3A). For contaminants growing at slower growth rates, latency tended to infinity. The contaminant with a growth rate of 0.6 d^{-1} achieved a density of only 0.39 cells L^{-1} after 60 d., while the one with a growth rate of 0.51 d^{-1} achieved a density of only $0.002\text{ cells L}^{-1}$. With denser inocula (1 cell L^{-1}), contaminants with a growth rate of 2 d^{-1} exhibited a reduced latency period of 7 d; with a growth rate of 0.7 d^{-1} , it was 47 d. With denser inocula and a growth rate of 0.6 d^{-1} , the contaminants reached a density of 440 cells L^{-1} after 60 d, for 0.51 d^{-1} , a density of 2.4 cells L^{-1} was obtained. The size of the initial inoculum of contaminants was important in determining the useful life of the PBR. The effect of the size of the inoculum increased as the growth rate of the contaminant decreased.

The model indicated that, at least at low inocula, contaminant cells in the refill water did not make a significant contribution to contaminant cell density at any point in the life of the PBR. The latency period (12 d) remained unchanged if the medium used to refill the PBRs daily contained contaminants with a growth rate of 2 d^{-1} at a concentration of 0.001 cell L^{-1} . For those with a growth rate of 0.7 d^{-1} and the same concentration, latency decreased by a day, from 85 to 86 d. Contaminants with a rate of 0.6 d^{-1} grew to 0.92 cells L^{-1} after 60 d; those that grew at 0.51 d^{-1} reached $0.024\text{ cells L}^{-1}$. When contaminants in the inoculation and refill were at a concentration of 1 cell L^{-1} ; latency for a growth rate of 2 d^{-1} remained 7 d; for 0.7 d^{-1} , it decreased by one day to 46 d. The concentration of contaminants with a growth rate of 0.6 d^{-1} reached a density of 922 cells L^{-1} after 60 d, those with a rate of 0.51 d^{-1} grew to 24 cells L^{-1} . The latency period changed little with changes in the concentration of contaminants in the medium used to refill the PBR, even a thousand-fold increase. The relative importance of the contaminants in the refill water increased as the growth rate of the contaminant decreased.

The model for the fastest-growing contaminant followed the empirical observations of algivorous amoebae (*Neoparamoeba* sp.), which typically appeared 3–4 weeks after inoculation (data not shown). Earlier detection of these organisms was probably complicated by their benthic habit. Growth rates of up to 2 d^{-1} have been measured for a marine algivorous amoeba isolated from Florida under optimal growth conditions (Bunt, 1970). This amoeba was provisionally assigned to the genus *Vexillifera*, a close relative of *Neoparamoeba* (Kudryavtsev et al., 2011), and its measured growth rate is consistent with estimates of *Neoparamoeba* sp. growth in laboratory batch culture (data not shown).

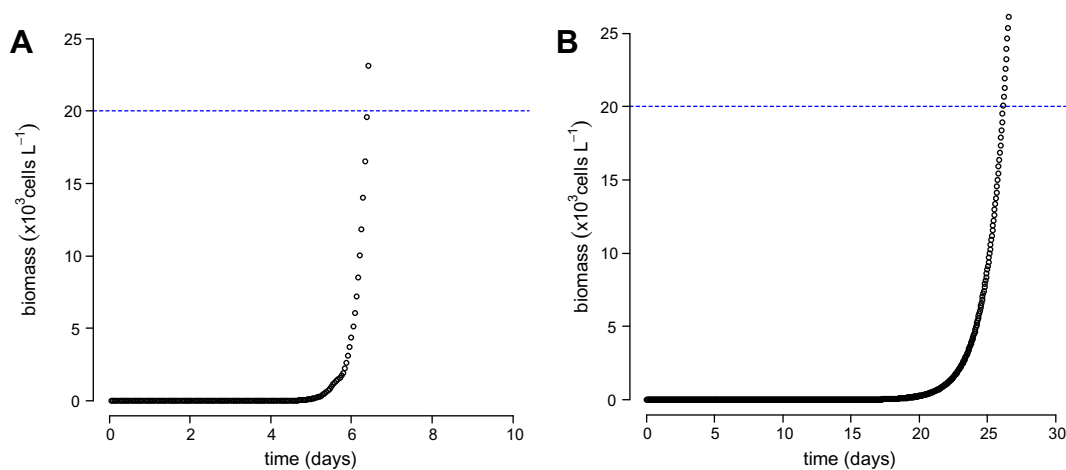


Fig. 1. Plots of the population of contaminant cells over time with no dilution of the host microalgal culture, at an initial inoculum density of $1\text{ cell }1000\text{ L}^{-1}$. (A) Specific growth rate of contaminant = 4 d^{-1} , detection limit (dashed line) was reached in 5 d. (B) Contaminant has the same growth rate as the culture i.e. 0.7 d^{-1} (one doubling per day). The population reached the detection limit in 26 d.

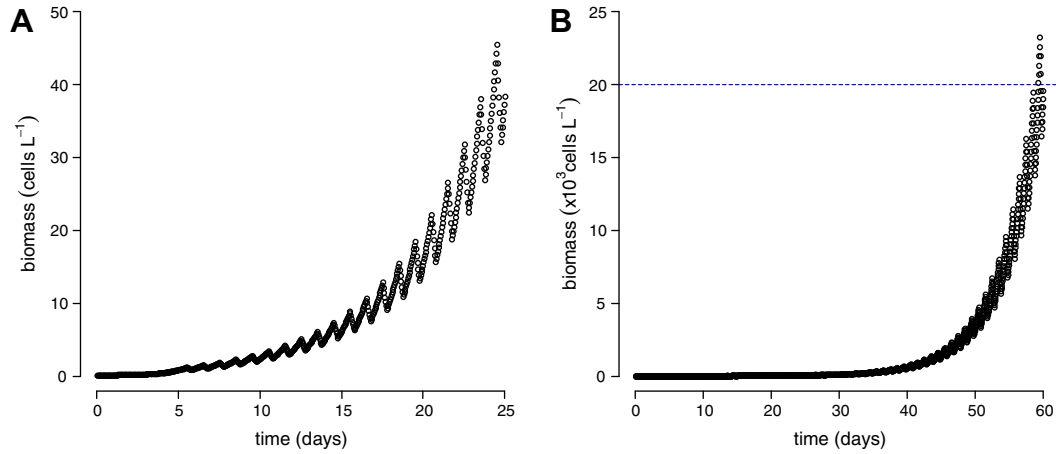


Fig. 2. Plots of contaminant cells against time for a contaminant with the same growth rate as the culture, 0.7 d^{-1} (a doubling per day); the culture was diluted by 50% each day after day 5. Other parameters were the same as for Fig. 1. (A) detail showing the effect of daily dilutions (note scale) on the y-axis (B) full 60 d run, detection limit reached on day 59.

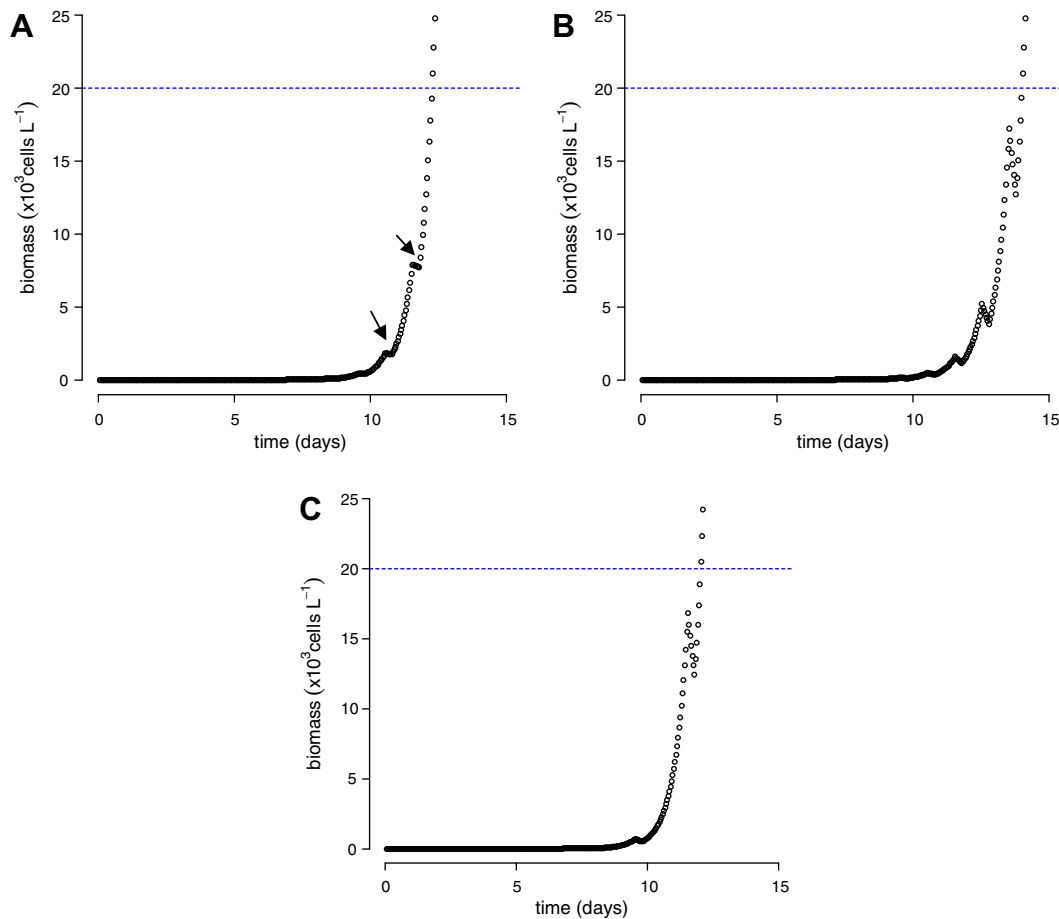


Fig. 3. A contaminant with a specific growth rate of 2 d^{-1} , inoculation rate of $1 \text{ cell } 1000 \text{ L}^{-1}$. (A) 50% dilution daily, reached detection limit in 12 d. Note only a slight deferment of increase in population with serial dilutions (arrows). (B) 75% dilution every day, reached detection limit in 14 d. (C) 75% dilution every 2nd day, also reached detection limit in 12 d, though 5 h earlier than (A).

3.1.3. Case 3: 75% daily PBR dilution

Under this scenario, the PBR is operating in “semi-continuous” culture mode, with 75% dilution of the culture daily after day 5. For this test, which was conducted only on the contaminant growing at 2 d^{-1} , it was hoped to observe whether an increased dilution rate would yield an increase in contaminant latency. Indeed, when

inoculum = $0.001 \text{ cell L}^{-1}$, the latency of the contaminant increased from 12 to 14 days (Fig. 3B). For a concentration of contaminants in the inoculum of 1 cell L^{-1} , latency increased from 7 to 8 d.

3.1.4. Case 4: 75% PBR dilution every second day

Under this scenario, the PBR is operating in “semi-continuous” culture mode, with 75% dilution of the culture every second day

after day 5. For this test, which was also conducted only on the contaminant growing at 2 d^{-1} , it was hoped to observe whether the contaminant amelioration observed in Case 3 was due to the larger percentage dilution or to the periodicity of dilution. When inoculum = $0.001 \text{ cell L}^{-1}$, the latency of the contaminant was the same 12 days as predicted for 50% daily PBR dilutions; indeed, the growth of the contaminant was slightly faster in the 75% alternate-days dilution than in the 50% daily dilution (Fig. 3C).

The model was run for multiple growth rates over 60 d, to determine the growth rate at which the contaminant reached the detection limit. Using a 50% dilution (0.7 d^{-1} specific growth rate of culture), a growth rate of 0.76 d^{-1} was required for the contaminant to become established. With dilutions every second day, a rate of only 0.62 d^{-1} was needed (Fig. 4).

3.2. Model implications

This model has several implications for the interpretation and management of contamination events in microalgal mass culture. Perhaps the most significant of these pertains to the dynamics of sudden, explosive contaminant outbreaks leading to catastrophic production losses. The most likely cause for such outbreaks, according to the model, is low-level contamination of production units days or weeks before the first detection of a problem. This explanation was verified in a production setting, in which the predictions of the model corresponded with the direct observations of vector and inocula, and with measurements of contaminant growth rates in laboratory culture on prey available in the production unit (data not shown). Discrepancies between model and observations can be explained in terms of variability in the inoculum due to variations in the populations of contaminants in nature and the performance of the aquaculture system, and natural variations in the growth rate of the contaminants (Pérez-Uz, 1995,1996).

The most likely cause of the loss of a microalgal pond culture to contamination is inoculation with previously (and undetected) contaminated culture or culture medium (e.g., from a PBR), unless that pond is maintained in the open for long periods of time or the contaminant has an extremely rapid growth rate. Unless the contaminant has a very fast growth rate and is introduced in large concentrations with the medium, contaminant latency will usually be longer than the residence time of the culture batch in the pond.

Frequent PBR dilution also confers significant advantages in contaminant mitigation; however, the frequency is constrained

by the growth rate of the target alga. The optimal strategy for delaying the onset of contamination, according to these data, is to dilute the culture as frequently and as heavily as the algal culture allows (i.e.: leaving a sufficient concentration of cells to allow the culture to recover) in the context of production schedule and targets. Basically, the faster the rate of growth of a production culture, the lower the threat of culture contamination – yet another argument for including ‘fast growth rate’ as a screening criterion for microalgal strain candidates.

3.3. Model application

The presented model confirms the principle, stated elsewhere (Day et al., 2012a,b) that early detection of contamination is essential to mitigation and minimizing the risk of catastrophic loss; however, procedures for such early detection are still in early phases of development, and the known ones make use of, or have proposed, expensive equipment and procedures (Day et al., 2012b). Contamination with multiple species of contaminants is more likely than inoculation with a monoculture. In such situations, the model can be used to back-calculate the timing of the contamination event following early detection of a relatively harmless, fast-growing species such as *Uronema* spp. Then the potential time of onset of a more problematic species can be estimated. In the event of the contaminant species with the greatest growth rate being detrimental to the culture, a slower-growing contaminant may not reach the detection limit before the culture is lost.

A protocol for early contaminant detection based on the longer latencies of contaminants in diluted vs. undiluted cultures, as represented in our model, was developed. In this protocol, daily, small aliquots of culture are removed from active PBRs and maintained in the laboratory as small-volume batch (i.e., never diluted) cultures. Without dilutions that retard the onset of contamination in the PBRs from which they came, the aliquots allow any problematic organisms to grow more rapidly. Prior to inoculation of large pond cultures from PBRs, the daily aliquots are examined for the presence of contaminants. If contamination is found, preventive measures can be implemented. Identification of the contaminant and determination of its growth rate allows the time course of contaminant growth to be predicted and the culture to be managed accordingly. This procedure, while less sensitive than methods that employ DNA signatures or flow cytometry, is simple and inexpensive, and has provided up to a 7-day advance warning of crop failure using only optical microscopy.

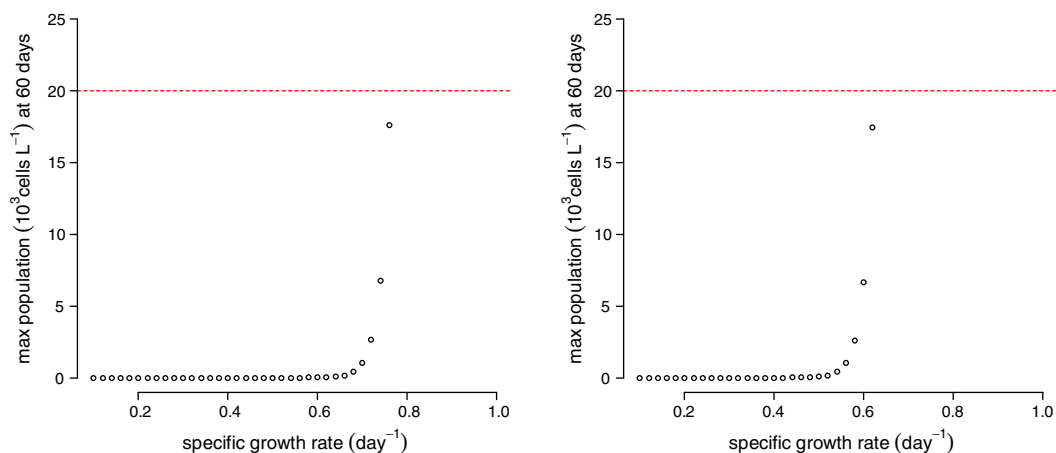


Fig. 4. Plot of population of contaminant after 60 d growth at various specific growth rates, at initial inoculum concentration of $1 \text{ cell } 1000 \text{ L}^{-1}$. (A) with daily 50% dilutions, a growth rate of 0.76 d^{-1} was required for the population of contaminants to reach the detection limit ($2 \times 10^4 \text{ cells L}^{-1}$). (B) With 75% dilutions every second day, the growth rate of the contaminant only needed to be 0.62 d^{-1} to thrive.

4. Conclusions

The presented model suggests strongly that most explosive, sudden onset appearances of contaminants in algal mass cultures result from low-level inoculation of the culture, days or weeks before contaminants become suddenly obvious. In hypothetical scenarios, the most important factors governing contaminant growth dynamics in algal mass cultures are the growth rate of the contaminant, the rate of dilution of the bioreactor containing the culture, and the size of the initial inoculum, usually in that order of significance. This model will serve as a useful starting point for understanding the behavior of contaminants in algal mass culture.

Acknowledgements

The authors wish to thank: W.P. Cochlan for the initial calculation that inspired this model, the excellent team of scientists at Cellana LLC, Kona, Hawai'i who made the work possible, and Royal Dutch Shell Petroleum plc for funding. Thanks also to J.P. Obbard and three anonymous reviewers whose suggestions greatly improved this manuscript.

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