MODELLING CONTINUOUS CULTURES OF MICROALGAE COLIMITED WITH NITROGEN AND PHOSPHORUS

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Abstract. The culture of microalgae is currently more and more developed at the industrial scale. In many applications, a substrate limitation is necessary to induce the production of a specific metabolite. The question of a better yield when inducing a stress by two nutrients simultaneously motivates a better modelling of microalgae colimited by two nutrients.

We present a new model that represents growth of microalgae colimited both by nitrogen and phosphorus. We show that the key point in modeling this complex biological system is the expression for the absorption rate. Phosphorus uptake must be a decreasing function of the phosphorus internal quota. The situation for the nitrogen uptake is different and we show that it must be an increasing function of the phosphorus quota to be able to represent experimental observations. Finally we end up with a model that explains the *a priori* paradoxical opposite response of nitrogen and phosphorus to dilution rate. The proposed model is compared with data of *Selenastrum minutum* and is validated both qualitatively and quantitatively.

1 Introduction

The culture of microalgae is currently in a strong growing phase with many new fields of application, like fine chemical production, hatcheries feeding or bioenergy [28, 9]. In many applications, like *e.g.* astaxanthin production [1], a substrate limitation is necessary to induce the production of a specific metabolite. These metabolic inductions can often be obtained by a nutrient stress, so that the question of a better yield when inducing a stress by two nutrients simultaneously arises.
The most popular model to reproduce growth of microalgae under substrate limitation is the Droop model [11, 8, 14]. It is widely used since it reproduces the ability of microalgae to uncouple substrate absorption and growth. This model has been deeply studied (see [24, 5, 35]), validated and proved to be relevant [14, 31, 6] to represent conditions of nitrogen or phosphorus limitations even in dynamical influent conditions [7]. However, when both nitrogen and phosphorus are suboptimal at the same time, as it can be the case for some biotechnological applications, a new model must be designed.
Nitrogen and phosphorus play a major role in the cell metabolism as they are part of many biochemical compartments of the cell. Cell nitrogen is mainly involved in proteins, amino acids and nucleic acids while phosphorus is mostly constitutive of the nucleic acids and phospholipids [19]. The possible existence of a physiological state where both nitrogen and phosphorus are suboptimal (called colimitation) is not clear and it is still a matter of debate. Because nitrogen and phosphorus interact at many biochemical levels and no clear biochemical process of interaction could be identified, both [3] and [30] suggested to consider these two nutrients as operationally independent nutrients.
On the other hand N-P colimitation has frequently been invoked in natural environment by several workers that demonstrated that N and P had to be supplied together in order to promote the community phytoplankton growth [32, 26, 36]. Some laboratory experiments on monospecific cultures also gave evidence of more complex N-P interactions. This is the case of the work of [10] that demonstrated that carbon fixation of N and P starved *Skeletonema costatum* could be significantly increased only when both N and P were simultaneously added. [33] also pointed out that while the minimum law prevailed for most of the N:P ratio a synergetic effect on *Pavlova lutheri* could occur between the two nutrients in a narrow range of N:P ratio.

Here we propose a Droop-based model designed on the idea that colimitation between nitrogen and phosphorus could arise from the uptake of nutrients rather than from the strict growth function and we demonstrate the ability of the model to properly represent both qualitative and quantitative behaviour of the experimental data that [17] obtained on *Selenastrum minutum*.

2 Model development

2.1 Presentation of the Droop model

The Droop model is widely used since its reproduces the ability of microalgae to uncouple substrate uptake ρ and growth rate μ [11, 14]. When considering nitrogen the Droop model can be writen as follows :

$$\begin{cases} \dot{n} = Dn_{in} - \rho_N(n)x - Dn \\ \dot{q_N} = \rho_N(n) - \mu(q_N)q_N \\ \dot{x} = \mu(q_N)x - Dx \end{cases} \tag{1}$$

where q_N is the internal carbon-based quota of nitrogen, D is the dilution rate of the chemostat, n is the ambient concentration of nitrogen and x the carbon-biomass.
Or with phosphorus :

$$\begin{cases} \dot{p} = Dp_{in} - \rho_P(p)x - Dp \\ \dot{q_P} = \rho_P(p) - \mu(q_P)q_P \\ \dot{x} = \mu(q_P)x - Dx \end{cases} \tag{2}$$

In this model the uptake function ρ and growth rate function μ are generally taken as Michaelis-Menten and Droop functions respectively :

$$\begin{array}{l} \rho(s) = \rho_m \frac{s}{s+K_s} \\ \mu(q) = \bar{\mu}(1 - \frac{q_0}{q}) \end{array} \tag{3}$$

Where K_s is the half saturation constant for uptake, ρ_m is the maximum uptake rate, q_0 the minimal quota that the algae can reach and $\bar{\mu}$ the growth rate when the quota q of the limiting nutrient tends toward infinity.

It can be proved [5])that the internal quota will stay between two bounds:

$$q_0 \leq q \leq q_{\max} \tag{4}$$

Where it can be mathematically shown that $q_{\max} = q_0 + \frac{\rho_m}{\bar{\mu}}$ represents the maximum quota obtained in conditions of non limiting nutrients, associated to $\mu_{\max} = \mu(q_{\max})$.

2.2 Extension of Droop's model to deal with colimitation

Now the problem consists in modifying the Droop's model to deal with colimitation by nitrogen and phosphorus. [22] proposed to allocate ressouces to two broad classes of cellular functional machinery : the acquisition and assembly machineries. The acquisition machinery that combines pigments and membrane porters is quantitatively N-rich but P-poor ; nevertheless phosporus is qualitatively involved into the active uptake of both nitrogen (NO_3) and inorganic phosphorus. Conversely, the assembly machinery that builds the growth rate can be represented by the nucleic acids and is N and P-rich. Consequently, we now assume that uptake of a nutrient is function of the ambient nutrient under consideration and the internal quota of both nutrients as well. We also assume that growth rate can be driven by either the nitrogen and the phosphorus quota. The generic model wich results is the following:

$$\begin{cases} \dot{n} = Dn_{in} - \rho_N(n, q_N, q_P)x - Dn \\ \dot{p} = Dp_{in} - \rho_P(p, q_N, q_P)x - Dp \\ \dot{q_N} = \rho_N(n, q_N, q_P) - \mu(q_N, q_P)q_N \\ \dot{q_P} = \rho_P(p, q_N, q_P) - \mu(q_N, q_P)q_P \\ \dot{x} = \mu(q_N, q_P)x - Dx \end{cases} \tag{5}$$

The question consists now in finding appropriate expressions for ρ_N, ρ_P and μ.

2.3 Modelling of the growth rate

We based our growth rate modelling approach on the assumption that N and P are both involved in a constant ratio in nucleic acids and particularly in RNA. We therefore assume that N or P can either limit growth rate depending on which of the two quota is the most limiting. Actually this assumption is supported by the findings of [12, 13] and [29]. This is also supported by the results of [4] on *Heterocapsa* sp. who found that both N and P starvation affected the RNA content while the DNA content remained approximately constant.
Since the 70's, several studies focused on how interaction between nutrients can affect the growth of phytoplankton. [12, 13] that worked on the interaction between vitamin B12 and phosphorus on *Monochrysis lutheri* first demonstrated that growth rate was limited by the most limiting nutrient in a strict Liebig fashion, where the ambient nutrient in least supply is that which limits growth. [29] achieved the same conclusion on *Scenedesmus* sp. with nitrogen and phosphorus. [33], [17] and [25] also argued in favour of a growth rate modelling based on the so-called minimum law, where growth rate depends on the most limiting extracellular nutrient. These works were

extended to the concept of the critical ratio (Rc) corresponding to a threshold model of nutrient limitation computed from the intracellular quota [2].
We therefore assume that the most limiting nutrient will drive the growth rate. Thus the growth rate results from a *min* function:

$$\mu = \min(\mu_N(q_N), \mu_P(q_P)) \tag{6}$$

For sake of simplicity, we choose an expression:

$$\mu = \mu_{\max} \min\left(\frac{1-\frac{q_{N0}}{q_N}}{1-\frac{q_{N0}}{q_{NL}}}, \frac{1-\frac{q_{P0}}{q_P}}{1-\frac{q_{P0}}{q_{PL}}}\right) \tag{7}$$

Where parameters q_{NL} and q_{PL} represent maximal values of nitrogen and phosphorus quota.

As a consequence, in the steady-state analysis we will assume that a N-limited culture has a growth rate of the form $\mu = \bar{\mu}(1-\frac{q_{N0}}{q_N})$ while a P-limited culture has a growth rate: $\mu = \bar{\mu}(1-\frac{q_{P0}}{q_P})$.

2.4 Modelling of the uptake rate

The classical modelling for uptake of an external nutrient is the Michelis-Menten based modelling of [16]. For sake of illustration, let us assume that N is the limiting nutrient at steady state of the chemostat, where $\mu = D$, then the phosphorus quota q_P is given by the following equation :

$$\rho(P) = Dq_P \tag{8}$$

Assuming that P is not limiting ($P >> K_{SP}$), we have $\rho(P) = \rho_{P\max}$ This leads to a steady state value $q_P^\star = \frac{\rho_{P\max}}{D}$ and shows that if the dilution rate tends toward zero, the phosphorus internal quota will tend toward infinity. This demonstrates that a regulation mechanism must be included in the model in order to guaranty that the quota will stay bounded between reasonable bounds. This mathematical results is supported by biological arguments. Indeed, it is usual to consider that uptake of a nutrient can be down regulated by its own internal quota [27]. This reasoning lead to a slighlty different uptake modelling, as developped by [21]. They demonstrated that under both N and P-limitations their model gave satisfying fits for several species.
This reasoning of course holds for any nutrient.

We have thus, for the phosphorus:

$$\rho(p, q_P) = \rho_{Pmax}\frac{p}{p+K_p}\frac{q_{PL}-q_P}{q_{PL}-q_{P0}} \tag{9}$$

As [18] pointed out there is no particular reason for the critical level q_{PL} to equal the Droop-maximum quota Qmax (and this obviously holds for q_{NL} too). Indeed, the data of [17] showed that the maximum achievable quota of a non limiting nutrient could exceed the observed Qmax when the same nutrient was limiting growth. This was particularly true for P that could be stored up to 16 times the actual needs of *Selenastrum minutum*, while N luxury consumption didn't exceed 4. Therefore q_{PL} is chosen larger than the maximal internal quota.

In conditions of limitations with phosphorus, we have thus

$$\dot{q_P} = \rho_{Pmax}\frac{q_{PL}-q_P}{q_{PL}-q_{P0}} - Dq_P \tag{10}$$

and when steady-state is achieved, it follows that

$$q_P^\star = \frac{\rho_{Pmax}q_{PL}}{\rho_{Pmax}+D(q_{PL}-q_{P0})} \tag{11}$$

Therefore, $q_P^\star$ is a decreasing function of the dilution rate, and is thus a decreasing function of the limiting nutrient N.

Actually this is what has been observed by [17] who showed that the phosphorus quota was decreasing for N-limited culture. Besides, our model assumes that there is no interaction between the N status and P-uptake and therfore P-uptake is solely governed by the internal P-quota. Indeed, as it will be discuted in the following, the model fitting to the experimental data of [17] showed a satisfying adequation leading to the conclusion that a model with such an interaction is not necessary to support the available experimental data.

However the situation is different for nitrogen. Indeed, [17] showed that the nitrogen quota of phosphorus limited culture is increasing with the dilution rate. This proves that the uptake rate for nitrogen cannot solely be regulated by the internal nitrogen quota.

Now we explore another mechanism based on the idea that the transport of nitrate is active and therefore needs energy and cofactors associated to the phosphorus pool. A major feature of the porters involved in NO_3 and PO_4

Parameter	Value	Unit
$\tilde{\mu}$	1.8	day^{-1}
q_{n0}	$6\ 10^{-2}$	molN. $molC^{-1}$
q_{p0}	$1.8\ 10^{-3}$	molP. $molC^{-1}$
q_{NL}	0.22	molN. $molC^{-1}$
q_{PL}	0.06	molP. $molC^{-1}$
$\rho_{N\max}$	9.6	molN.$molC^{-1}$.day^{-1}
$\rho_{P\max}$	$4\ 10^{-2}$	molP.$molC^{-1}$.day^{-1}
K_{S_N}	$0.22\ 10^{-6}$	molN.L^{-1}
K_{S_P}	$0.06\ \ 10^{-6}$	molP.L^{-1}

Table 1: Parameter values used for model of N-P colimitation with *Selenastrum minutum*

transport is that they require energy (i.e. ATP) for their activity that could be related in some extent to the P-pool status of the cell. Thus the modelling of N-uptake must ensure that as cells are getting P-starved, their P-quota lowers and consequently the transport rate of N tends to decrease. Indeed [10] demonstrated that when N and P-starved cultures of *Skeletonema costatum* were resupplied with N the C-fixation was inhibited. They assumed that the addition of N led to an enhanced demand of ATP and NADH and to mobilization of the limited phosphorus quota for N-uptake and this was detrimental to the carbon fixation.

As a consequence we assume that the uptake of nitrogen can be represented as follows:

$$\rho(n,q_N,q_P) = \rho_{Nmax}\frac{n}{n+K_n}\frac{q_{NL}-q_N}{q_{NL}-q_{N0}}\frac{q_P-q_{P0}}{q_{PL}-q_{P0}} \tag{12}$$

From that, we can infer that, in transient situations where P becomes limiting, the P-status of the cells could induce a N-uptake limitation leading to N-P colimitation. Conversely, when q_P is high, the P-driven term allow a maximum potential of N-uptake.

Let us compute the non limiting quota $q_N^\star$ in conditions of P-limitation. From (7) the growth rate can be represented by a Droop model:

$$\mu(q_P) = \bar{\mu}(1-\frac{q_{P0}}{q_P}) \tag{13}$$

At steady state in the chemostat, the non limiting nitrogen concentration is much greater than K_n, such that:

$$\dot{q_N} = \rho_{Nmax}\frac{q_{NL}-q_N}{q_{NL}-q_{N0}}\frac{q_P-q_{P0}}{q_{PL}-q_{P0}} - \bar{\mu}(1-\frac{q_{P0}}{q_P})q_N \tag{14}$$

It follows that the steady state value of the nitrogen quota for P-limited cultures is then given by:

$$q_N^\star = \frac{\rho_{Nmax}q_P^\star}{\bar{\mu}(q_{NL}-q_{N0})(q_{PL}-q_{P0})+\rho_{Nmax}q_P^\star}q_{NL} \tag{15}$$

Thus $q_N^\star$ is an increasing function of $q_P^\star$. Since, from Droop model, $q_P^\star$ is itself an increasing function of the dilution rate, it follows that $q_N^\star$ is increasing with the dilution rate. It is worth remarking that this conclusion is opposite to the decreasing behaviour of $q_P^\star$ in condition of nitrogen limitation.

3 Discussion

3.1 Model prediction at physiological limit boundaries

The results of [17] on *Selenastrum minutum* were obtained with extreme N:P ratios ($n:p=200$ and $n:p=1$) that greatly differed from the critical ratio [2]. In these extreme conditions we can assume that the non limiting C-quota were saturated in both experiments. Besides, this assumption is supported by the high ambient concentrations of the non-limiting nutrient in the two experiments.

In conditions where N is limiting and P is in large excess (*i.e.* P-uptake is maximum), the model fits the data with a q_P decreasing trend as growth rate increases (figure 2), according to equation 11. When defining the luxury consumption as the ratio between the maximum achievable quota of a non limiting nutrient and the quota of the same but limiting nutrient, it can be seen (figure 2) that the capacity of P-luxury consumption also decreases with growth rate.

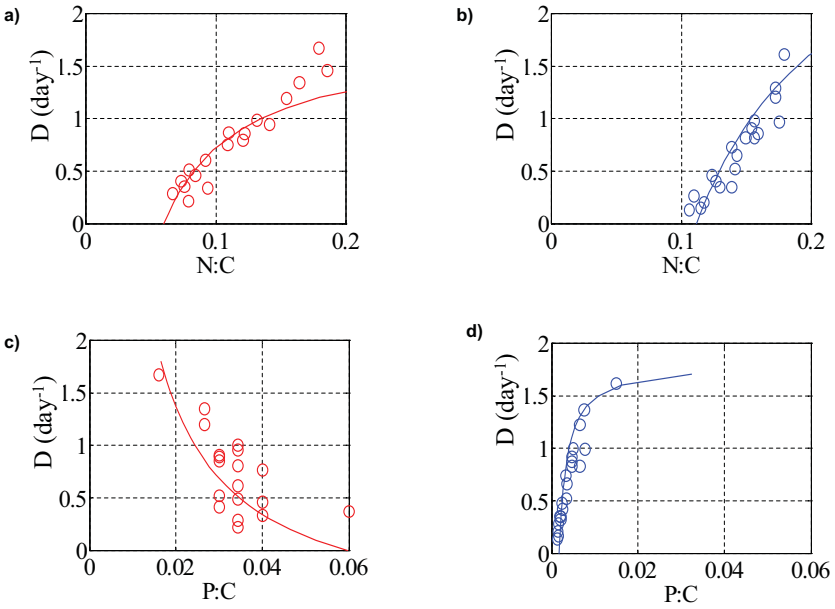

Figure 1: Comparison of experimental results for the nitrogen quota (*a* and *b*) and the phosphorus quota (*c* and *d*) obtained at steady state with *Selenastrum minutum* under nitrogen limitation (*a* and *c*), and phosphorus limitation (*b* and *d*).

Opposite to the trend of q_P under N-limitation the experimental data of saturated q_N under P-limitation turned out to be an increasing function of the growth rate for *Selenastrum minutum*. Under the assumption of [15] that a non-limiting quota follows the Droop equation with a higher apparent q_0, the Droop model predicts that q_N is an increasing function of the growth rate under P-limitation. Conversely a model that would only integrate the inhibiting effect of the internal quota would describe a decreasing trend of the N-saturated quota with growth rate as it was previously observed for the P-saturating quota under N-limiting conditions (see equation 11). Therefore if our model was neglecting the limiting effect of q_P on N-uptake it would fail to fit the physiological boundaries correctly (indeed, an equation similar to (11) would have been obtained, leading to a decrease of q_N with respect to μ). The asymmetrical functions of N (structural) and P (functional) and particularly the energetic role of P at the acquisition level were therefore critical in our modelling approach. Thus although P is quantitatively low in the acquisition machinery it appears to play a major role in the uptake of NO_3.

Finally it can also be observed from figure 2 that the capacity of N-luxury consumption decreases with growth rate as for P-luxury consumption. Moreover the capacity of N-luxury consumption has often been reported to be lower than that of P [17, 34] and the model succeeded in describing this difference.

3.2 Model prediction inside the physiological limit boundaries

In the case where more balanced N:P input ratio were used (*i.e.* ratio close to the Redfield ratio) one may suspect different kinetics for the non-limiting quota as they may not reach saturation. Indeed simulations of the model show different trends for the two non-limiting quota depending on the growth rate ranges under consideration.
Under conditions where P is limiting and in the range of low growth rates, the non-limiting q_N exhibits an increasing trend with growth rate until the physiological limit boundary is reached. From the model theory it can be assumed that at low growth rates where P is highly limiting growth the limited available energy (ATP) highly constrains N transport even if ambient N concentration is high. For higher growth rates the limiting control of P on N-uptake is decreased and q_N can build- up to the limit boundary where the inhibition of NO_3-uptake by the N-internal quota prevails. Kinetics of the non-limiting q_P are roughly similar to that observed for q_N. It can also be noticed that in situations where P-is sublimiting (figure 1b) the non-limiting q_P exhibits a very similar kinetic to that of the limiting q_P but with a slightly higher apparent q_{P0}. This Droop-like kinetic of the non limiting quota was already assumed by [13] and experimentally observed by [20] under N:P ratios close to the critical ratio.
Figure 3 shows simulation results for the kinetic of $q_N : q_P$ versus growth rate under different input N:P ratio ranging from 1 to 100. At low growth rates $q_N : q_P$ remains equivalent to the input ratio as long as the input ratio

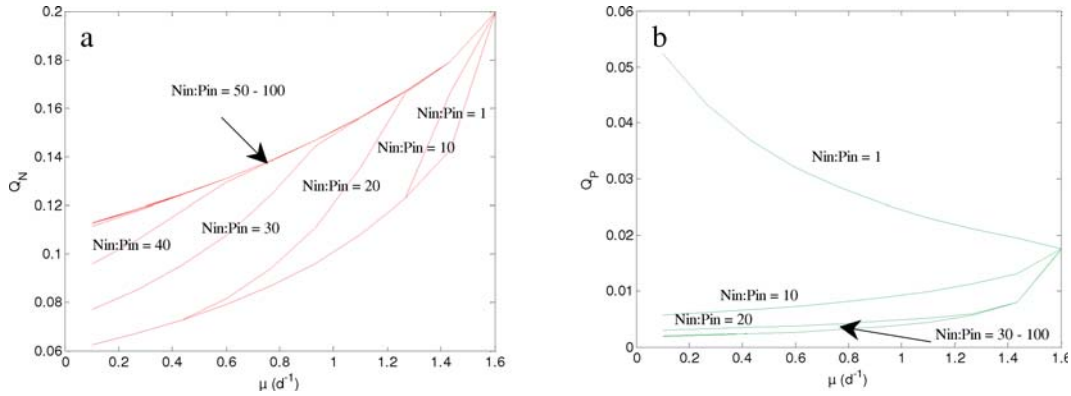

Figure 2: Model simulation of N-quota (*a*) and P-quota (*b*) versus growth rate under different N:P input ratio ranging from 1 to 100.

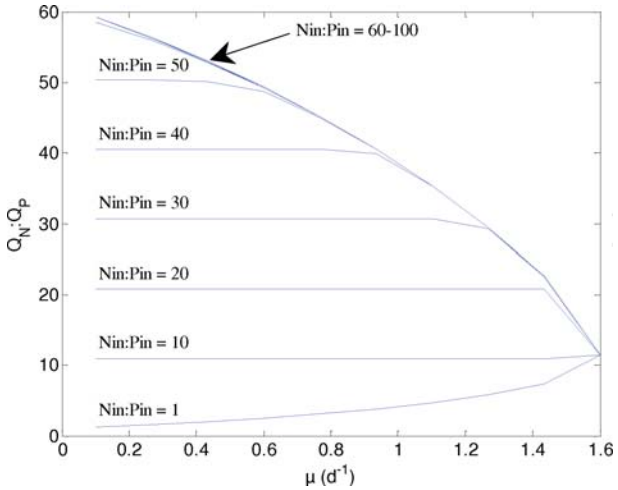

Figure 3: Model simulation of the cell-N:P versus growth rate under different N:P input ratio ranging from 1 to 100.

falls within the physiological limit boundaries and none of the two nutrients is limiting. As growth rates increases $q_N : q_P$ remains constant until the physiological boundaries are reached and uptake inhibition by the internal quota takes place. Further increase in growth rate results in alteration of the $q_N : q_P$ ratio driven by the physiological limit boundaries of the quota as we described above under extreme N:P input ratios.

4 Conclusion

We proposed a new modelling approach that focus on the N-P colimitation concept. Our Droop-based model suggests that the interaction between N and P should be considered at the acquisition rather than at the assembly level. Indeed we feel that the Droop function between growth rate and quota remains unaffected by the N-P interaction while the N-quota construction is tightly tied to the P-status of the cell. The combination of the uptake-limiting effect of P through energy availability needed by NO_3 porters and the concept of uptake-inhibition by the internal quota allowed the model to reproduce the experimental data of the few studies dealing with the two nutrients and particularly under extreme N:P input ratios. We therefore suggest that regarding the colimitation concept, N and P would better be considered as dependent nutrients rather than operationally independent ones. As several authors already pointed out [23, 18, 25] there is a critical need of new experimental data considering N and P under different conditions of input ratios and growth rates.

Acknowledgements: This paper presents research results supported by the ANR-06-BIOE-014 Shamash project.

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