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Vitamin B_{12} and marine ecology

V. Continuous culture as an approach to nutritional kinetics

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KURZFASSUNG: **Vitamin B_{12} und Meeresökologie. V. Dauerkultur als eine Methode zur Erforschung der Kinetik ernährungsphysiologischer Prozesse.** Der Einsatz von Turbidostaten und Chemostaten zur Dauerkultur von Algen kann auch einer Analyse der Kinetik von Prozessen dienen, die durch die Konzentration bestimmter Nähr- und Wirkstoffe limitiert werden. Die Parameter des Vitamin-B_{12}-Bedarfs von *Skeletonema costatum* wurden im Kulturversuch nach dem Chemostat-Prinzip bestimmt und mit denen von *Monochrysis lutheri* verglichen; sie stimmen bei beiden Arten weitgehend überein. Es ließ sich zeigen, daß für die Vermehrungsrate die Konzentration an Vitamin B_{12} im Medium nicht unmittelbar bestimmend ist, sondern vielmehr die in der Algenzelle enthaltene Konzentration dieses Wirkstoffs.

INTRODUCTION

Continuous cultivation is a method of keeping a population of organisms in a steady-state with respect to parameters both of the population itself and the environment. Continuous algal systems have, in the main, been used either as a means of obtaining the greatest possible algal output (in mass cultures) or of obtaining physiological material of constant mean composition. They have not, until quite recently, been used to explore the mechanisms of nutrient limitation. This is surprising in view of the fact that theoretical descriptions of continuous systems always involve postulates concerning nutrient limitation.

In essence, the apparatus consists of a constant volume growth vessel through which culture medium is made to pass, the rate of outflow being the same as the inflow and, of course, the culture has to be perfectly mixed. The cells multiply and, provided the flow rate is below a certain limit (see equation [4]), they will come to equilibrium; and the specific growth rate (μ), when measured in natural units, will be numerically equal to the dilution rate in the growth vessel, the dilution rate (D) being the quotient of the flow rate and culture volume.

The specific growth rate is also related to the concentration of the limiting nutrient in the medium (s), the generally accepted approximation having the form of a second order reaction,

$$\frac{\mu}{\mu_m} = \frac{s}{K_s + s} \qquad (1)$$

(μ_m and K_s being constants). The total nutrient in a given volume is the sum of the cell

and medium components and will equal the concentration in the inflowing medium, unless there is loss to the gas phase. We have

$$s_R = C + s \tag{2}$$

furthermore, the cell quota (Q) and cell population (x) are related by definition

$$C = Qx \tag{3}$$

after by combining the three equations:

$$x = \frac{1}{Q}\left[s_R - \frac{K_s}{\frac{\mu_m}{\mu} - 1}\right] \tag{4}$$

In the simplest instance, when Q is constant, it is readily seen that in equation (4) the solution for any one of the three variables, x, μ, and s_R, is unique in terms of the other two, indicating that a steady state can be maintained by controlling either x or μ together with s_R. Thus to keep x and s_R constant is to keep μ constant (the so-called 'turbidostat'), whereas to keep μ and s_R constant is to keep x constant (the so-called 'chemostat'). Q is not always constant, as we shall see, and the value of μ then appears to be independently dependent upon it in a complex fashion. In both systems, the true controlling factor is the concentration of the limiting nutrient. Under conditions of saturation with respect to all nutrients other factors come into play; these in any case determine the saturation levels for the various nutrients. A similar set of empirical equations can also be written for light-limited and CO_2-limited cultures (DROOP 1969).

In the turbidostat x is kept constant by some sort of population sensing device operating the flow mechanism, whereas in the chemostat the flow is kept constant by a metering pump. Chemostats operate more sensitively at slow flow rates, while turbidostats are most sensitive at fast rates (HERBERT 1958).

KINETICS OF VITAMIN B_{12} LIMITATION

The many arguments for and against the ecological importance of vitamin B_{12} to marine phytoplankton convinced me of the need for kinetic data on vitamin B_{12} limitation and, indeed, on nutrient limitation in general. The details of the simple chemostats and the methods used in my attempts to meet this need have been published (DROOP 1966, 1968) and need not be repeated. The results with the flagellate *Monochrysis lutheri* and ^{57}Co-labelled vitamin B_{12} were quite unexpected and, I think, very illuminating and I discussed them in some detail. Some of this work I have since repeated with the pelagic diatom *Skeletonema costatum*, which also has a vitamin B_{12} requirement (DROOP 1955). I shall use this experiment (Table 1) to illustrate my use of the chemostat and also to introduce the model of nutrient limitation developed for *Monochrysis lutheri*.

The object of the chemostat experiment was to measure two of the parameters associated with nutrient limitation, namely Q (which is the reciprocal of what bacteriologists term the "yield constant", Y) and K_s, the saturation constant for growth,

Table 1

Equilibrum states of two chemostats operated continuously between 2nd February and 26th May 1969, with *Skeletonema costatum*. Input concentration of vitamin B_{12}, 10 pg/ml. *D* dilution rate (volumes/day); *x* population volume (μl/ml); *s* medium vitamin (pg/ml); *C* cell vitamin (pg/ml). Calculated parameters: Q cell vitamin quota (*C*/*x*, pg/μl); K^1_s apparent saturation constant (pg/ml)

D	*x*	*s*	*C*	*Q*	K^1_s
0.250	0.226	1.670	8.330	36.91	6.769
0.251	0.299	1.780	8.220	27.46	7.204
0.254	0.237	1.578	8.422	35.61	6.287
0.257	0.291	1.624	8.376	28.75	6.344
0.356	0.215	1.419	8.581	40.00	3.627
0.441	0.285	1.305	8.695	30.50	2.440
0.528	0.238	1.573	8.427	35.35	2.198
0.599	0.235	1.323	8.677	36.90	1.470
0.601	0.163	2.028	7.972	48.91	2.242
0.673	0.168	2.032	7.968	47.57	1.787
0.678	0.203	1.196	8.804	43.35	1.037
0.698	0.180	1.453	8.547	47.36	1.180
0.746	0.159	1.111	8.889	55.98	0.774
0.770	0.201	1.087	8.913	44.29	0.699
0.797	0.187	1.121	8.879	47.54	0.659
0.825	0.199	1.214	8.786	44.17	0.648
0.848	0.108	1.480	7.870	72.79	0.728
0.863	0.113	1.309	8.691	76.65	0.611
0.980	0.086	2.064	7.936	91.95	0.600
0.998	0.076	1.857	8.143	107.40	0.496

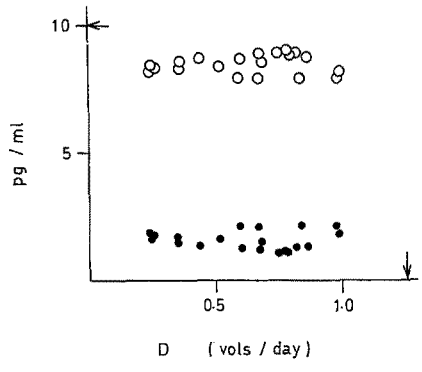

Fig. 1: *Skeletonema costatum*. Chemostat steady-states with vitamin B_{12} limiting. Open circles: cell vitamin (C); filled circles: medium vitamin (*s*) as function of dilution rate (*D*). Arrows indicate values of s_R and D_m

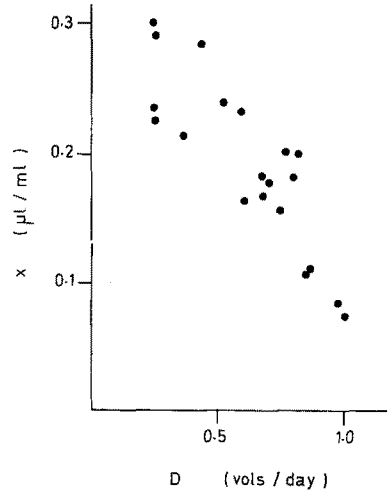

Fig. 2: *Skeletonema costatum*. Chemostat steady-states with vitamin B_{12} limiting. Cell population (*x*) as a function of dilution rate (*D*)

[see equations (1) and (3)]. Both Q and K_s proved illusory; the former because it simply was not a constant, the latter because of interference from a vitamin B_{12}-binding protein excreted by the cells.

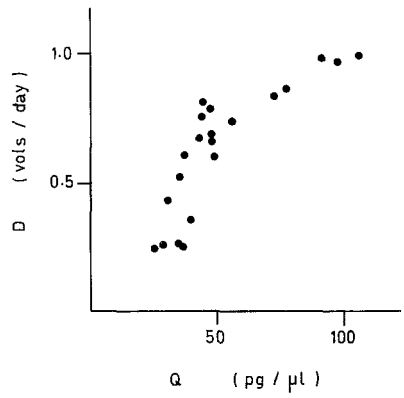

Fig. 3: *Skeletonema costatum.* Chemostat steady-states with vitamin B_{12} limiting. Relation between dilution rate (D) and cell quota (Q)

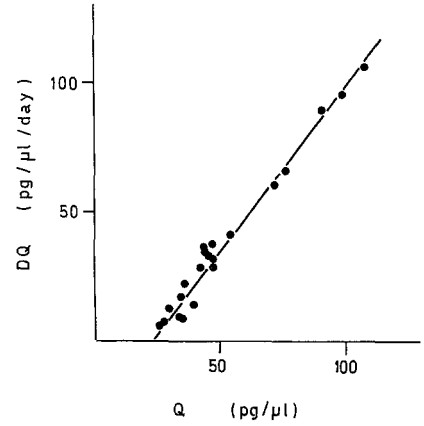

Fig. 4: *Skeletonema costatum.* Chemostat steady-states with vitamin B_{12} limiting. Regression of DQ on Q

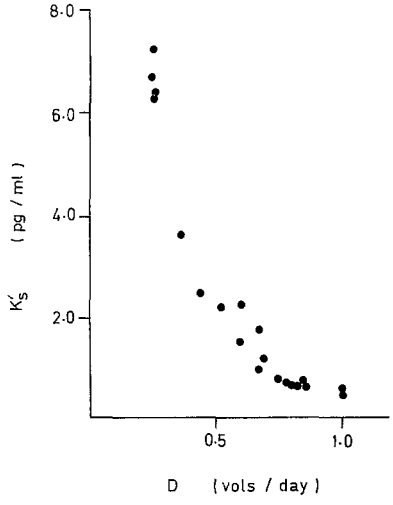

Fig. 5: *Skeletonema costatum.* Chemostat steady-states with vitamin B_{12} limiting. Apparent saturation constant for growth (K^1s) as a function of dilution rate (D)

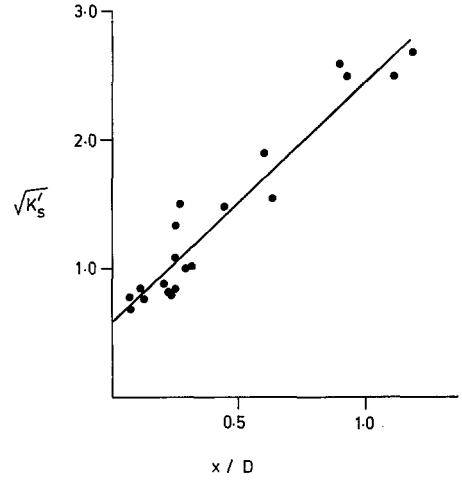

Fig. 6: *Skeletonema costatum.* Chemostat steady-states with vitamin B_{12} limiting. Regression of $\sqrt{K^1s}$ on $\frac{x}{D}$

The effect of the binding factor is seen in the way the concentration of the vitamin in the growth chamber fails to approach zero as the dilution rate decreases (Fig. 1). Similarly, the effect of the variable Q is seen in the rather linear manner in

which the population density falls as the wash-out point is approached (Fig. 2). Figure 3 illustrates the relation between Q and the dilution rate. This is a hyperbolic curve with D_m the asymtote. The Q intercept, which we term k_Q, may be regarded as the subsistence quota, below which no growth will take place. The equation for this hyperbola is

$$D = D_m \left(1 - \frac{k_Q}{Q}\right) \tag{5}$$

that is, the regression of DQ on Q is linear (Fig. 4). From it we can obtain, statistically, both k_Q and D_m. Thus, k_Q was 23.2 pg/μl (95 % fiducial limits ± 2.97). This is somewhat smaller than the equivalent figure for *Monochrysis lutheri*, which was 64 pg/μl. D_m, the slope of this regression, works out at 1.27 volumes per day (95 % limits ± 0.02).

The significance of the relationship between D and Q is that it can be written

$$\mu = \mu_m \left(1 - \frac{k_Q}{Q}\right) \tag{6}$$

and since vitamin B_{12} is limiting* the conclusion must be that μ is the dependent variable; in other words, the amount of vitamin in the cells controls the rate of growth.

The *Monochrysis lutheri* experiment (Droop 1968) was taken a good deal further than this and a relationship was established between nutrient uptake and medium concentration in batch cultures growing from minute inocula; which, when combined with equation (6), yielded a convenient expression for the growth saturation constant. Normally K_s should be derivable from equation (1), since we have both μ_m and s. However, working this out for the various steady-states of the *Skeletonema costatum* experiment produced the curve shown in Figure 5 and K_s is anything but constant. With *M. lutheri* this effect was proved, quite conclusively, to be due to the excretion of a heat-labile compound of large molecular weight that combined with the vitamin and prevented it from being assimilated. It looks very much as if the same thing is happening here, and one is probably correct in assuming that most of the vitamin in the growth chamber in this experiment was likewise protein-bound though, unfortunately, I did not have time to put this to the test before the symposium.

If a constant relative rate of release is assumed, the concentration of an excretory product is proportional to $\frac{x}{\mu}$. It is clear that K^1_s, the apparent constant, will then only equal the true constant when x is zero, i. e. at the wash-out point. Hence the K^1_s intercept of the regression of K^1_s on F ($\frac{x}{D}$) should give the true constant. The best spread of points and linearity was obtained with a square root transformation, $\sqrt{K^1_s}$ against $\frac{x}{D}$ .Accordingly, we get for K_s the value 0.323 pg/ml with 95 % fiducial limits 0.172 and 0.521 (Fig. 6).

The value for *Monochrysis lutheri* previously obtained using the dynamic uptake rate (Droop 1968) was 0.142 pg/ml, (limits 0.130 and 0.154) whereas, calculated by

* x increased $2^1/_2$ fold when s_R was increased by a like amount (D being 0.5).

the present method (Fig. 7), one gets for *M. lutheri* a K_s of 0.218 pg/ml (limits 0.074 and 0.439) which is not significantly different from the *Skeletonema costatum*

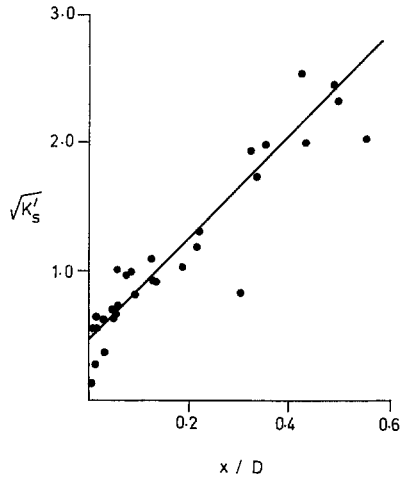

Fig. 7: *Monochrysis lutheri.* Chemostat steady-states with vitamin B_{12} limiting. Regression of $\sqrt{K_s'}$ on $\frac{x}{D}$
(Data from DROOP 1968)

result. The two organisms seem to be rather similar in their requirements for vitamin B_{12}. Even the difference in the k_Q is not much more than two-fold and could be due to systematic errors inherent in the Coulter Counter estimates of population volume.

DISCUSSION

The two main conclusions arising from the vitamin B_{12} work – namely, the dependence of the specific growth rate on the cell vitamin quota and the effect of excretory proteins – must influence our ecological thinking about this vitamin. However, at present the more important question is: how trivial are these conclusions?

Short-term experiments on the adsorption of vitamin B_{12} by *Monochrysis lutheri* (DROOP 1968) showed that the initial very high uptake of the vitamin (far in excess of the specific growth rate, in fact 'luxury consumption') was no more than an initial priming process of relatively short duration, which gave way to dynamic levels related to the specific growth rate, thus

$$u = \mu Q \tag{7}$$

EPPLEY & STRICKLAND (1968) had already suggested that the different levels of cell phosphorus and cell nitrogen reported in the literature corresponded to those necessary to support different rates of growth. We have seen that this is true of vitamin B_{12}. Indeed, it is reasonable since this vitamin functions as the prosthetic part of an enzyme

and it is not difficult to imagine a rate of synthesis controlled by the number of units operating. But it is not so certain that such arguments could apply to major nutrients

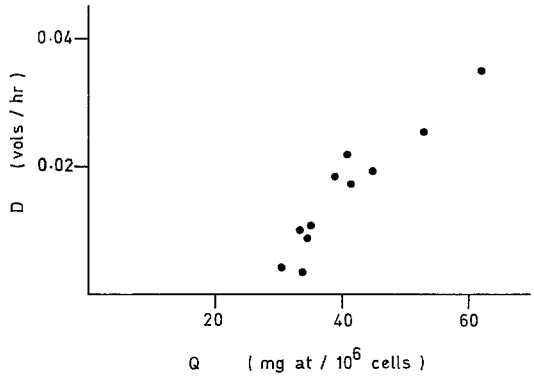

Fig. 8: *Isochrysis galbana.* Chemostat steady-states with nitrate limiting. Relation between dilution rate (D) and cell quota (Q). (Data from CAPERON 1968)

supplying structural materials. On the contrary, MONOD's original premise that the rates of nutrient utilization and increase in cell mass were in constant proportion,

$$\frac{dx}{ds} = -Y$$

which became central to chemostat theory, seemed to be true of major substrates such as glucose or glycerol for bacteria (MONOD 1942, HERBERT et al. 1956).

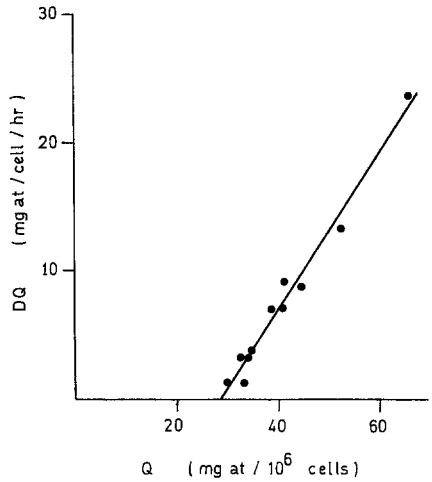

Fig. 9: *Isochrysis galbana.* Chemostat steady-states with nitrate limiting. Regression of DQ on Q. (Data from CAPERON 1968)

There are few data which appertain to major nutrients from continuous cultures in the algal field; none for phosphorus, but CAPERON (1968) has studied the response

of *Isochrysis galbana* to nitrate. His data are shown in Figure 8*. We observe precisely the same picture here for nitrate as we had for vitamin B_{12}. Moreover, equation (5) fits the curve quite satisfactorily (Fig. 9).

Two nutrients and three organisms do not prove that my model for nutrient limitation is of general application but it is surely significant that the model can apply to a major structural nutrient just as well as to a vitamin.

SUMMARY

1. Parameters of vitamin B_{12} requirement of *Skeletonema costatum* were measured in continuous culture and compared with those of *Monochrysis lutheri*.
2. The specific growth rate depended on the cell quota according to the relation

$$\mu = \mu_m \left(1 - \frac{k_Q}{Q} \right)$$

rather than directly on the medium concentration.

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* Data from CAPERON's two experiments are here combined because they were statistically not significantly different.