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HORIZONS

Vitamins, phytoplankton and bacteria: symbiosis or scavenging?

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The conclusion that over 25% of global primary production depends on direct algal/bacterial symbiosis involving vitamin B_{12} [Croft et al.*, (2005) Algae acquire vitamin B_{12} through a symbiotic relationship with bacteria.* Nature, **438**, *90–93] is patently false, for it is based on a misconception of the probable level of the vitamin B_{12} requirement in marine pelagic algae. A review of the various published attempts at measuring this requirement suggests that it is likely to be so low that oceanic and coastal concentrations of the vitamin would usually be sufficient to sustain the populations that occur without the assistance of direct algal/bacterial symbiosis. The levels measured are discussed in relation to method (batch or continuous culture) and protocols used. Requirement values considered by the author to be acceptable range from 0.1 to 0.3 pM for the vitamin growth saturation constant (K_S) and from 30 to 100 μL algal biomass $pmol^{-1}$ vitamin for the yield.*

INTRODUCTION

The recent renewal of interest in the marine ecology of vitamin B_{12} has included a paper (Croft *et al.*, 2005), the hyperbole and implication of whose title warrants careful scrutiny. The authors describe a neat experiment demonstrating that algae and certain bacteria can form a loose symbiotic association involving exchange of photosynthetically produced carbon for vitamin B_{12}. However, I question the global inference drawn from the results, suggesting that this is quite unwarranted. The idea that the amount of free vitamin in the sea and fresh waters is not sufficient to support the phytoplankton without the involvement of symbiosis rests on a misconception of the level of requirement in plankton algae. Croft *et al.* appear to have so misinterpreted the literature on the topic that one may even question whether they were aware of the full extent of it. I refer to work on pelagic genus *Thalassiosira* (Carlucci and Silbernagel, 1969; Swift and Taylor, 1974) and to some of mine (Droop, 1968, 1970) on *Skeletonema costatum* and the haptophyte *Pavlova* (*Monochrysis*) *lutheri*, all of which, incidentally, bears a modicum of responsibility for the general lack of interest in vitamin B_{12} on the part of marine ecologists. The thrust of my previous two communications on this topic (Droop, 1968, 1970) was centred on the kinetics of nutrient uptake and growth *per se*, using vitamin B_{12} as a model system. While the relation between the level of requirement of the vitamin and its availability in the sea was not emphasized, the published data contained the

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relevant information. It now appears that a pointed interpretation of the data from that continuous culture work, together with a summary of work leading up to it, would be useful, and could also be usefully discussed in relation to other work in the area.

REVIEW

The yield relation

The yield in batch culture of *P. lutheri* (cell vol. 200 μm^3) worked out at $\sim 1/3\ \mu m^3$ algal biomass per vitamin molecule (201 $\mu L\ pmol^{-1}$, since 1 μL is equivalent to $10^9\ \mu m^3$ and 1 mole to 6.02×10^{23} molecules) over a range of vitamin concentrations from 0.07 to 14 pM (Droop, 1957). This, however later proved to be an overestimate that only applied to cells well into the stationary phase of growth. A 'subsistence quota', the reciprocal of yield, of ~2.25 fmol per million cells obtained in continuous culture by extrapolation to zero specific growth rate indicated a yield of 89 $\mu L\ pmol^{-1}$, a more realistic estimate than the previous one, since it referred to cells with their metabolic machinery still largely intact (Droop, 1968). The previous calculations should therefore be decreased by a factor 89/201. It may be argued that *P. lutheri*, an inhabitant of supralittoral rock pools, may not be a good model for truly marine species, but the subsistence quota of the pelagic diatom *S. costatum* (cell vol. 250 μm^3), similarly culled from chemostat data, was 65 $\mu L\ pmol^{-1}$, a result similar to the *Pavlova* figure (Droop, 1970). This may be a pure chance, but similar reasoning points to requirements of the same order in *Euglena gracilis* (cell vol. 2000 μm^3) (Hutner and Provasoli, 1951), *Stichococcus* (cell vol. 12 μm^3) (Lewin, 1954) and *Thalassiosira pseudonana* (cell vol. 200 μm^3) (Swift, 1984), one might be tempted to hazard that this figure may represent the yield requirement in algae generally, namely that each pmol of vitamin can yield 30–100 μL of algal biomass. (Assuming a relationship of 200 gC L^{-1} cell volume, this equates to ~1 pmol B_{12} supporting 6–20 mgC biomass.) If so, judging by the size of oceanic populations quoted by Bainbridge (1957), it seems unlikely, even given the variability in C-biomass per unit of cell volume are taken into account, that more vitamin than 0.1 pM would be required to support them.

The rate relation

Aside from the above, there is some justification in supposing that the relation between specific growth rate and substrate concentration is of greater ecological and

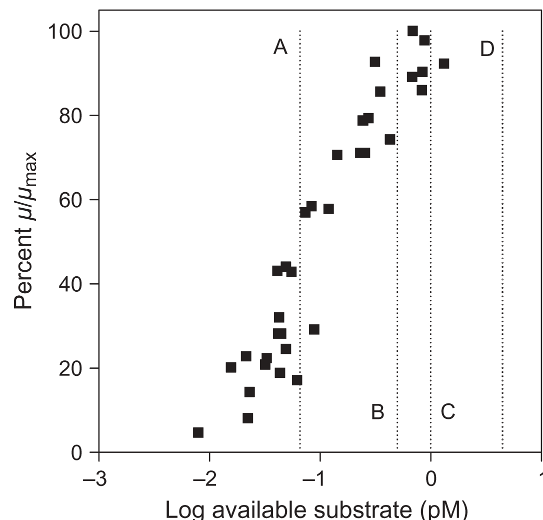

Fig. 1. *Pavlova lutheri.* Chemostat steady-state: dilution rate expressed as a percentage of the curve-fitted washout rate ($\equiv\%\mu/\mu_{max}$) against concentration of free vitamin, as log pM. The vertical lines, the means of some assays of marine waters. A: North Atlantic and north North Sea, summer. B: north North Sea, autumn. C: Butt of Lewis, Scotland, spring (Cowey, 1956). D: Firth of Clyde, Scotland, incoming tide (Droop, 1955), spring, and La Jolla, California, spring and summer (Carlucci, 1970). Reviews of some more recent vitamin B_{12} assays of marine and fresh waters will be found in Provasoli and Carlucci, (1974) and in Swift (1984).

competitive significance than is the yield relation. Accordingly, again with batch cultures of *P. lutheri* (Droop, 1961), the rate-limiting concentrations proved to be below 0.07 pM, a figure I found hard to believe in view of Ford's rate data for *Ochromonas malhamensis* (Ford, 1958) but I could not fault it. *O. malhamensis* is an obligate chemotroph and it is possible that chemotrophy in the dark and the high temperature require more vitamin B_{12} than phototrophy. I concluded, having regard for the levels of vitamin being recorded in the sea (see the legend to Figs 1 and 2), that it was unlikely that the vitamin was of ecological importance, particularly in view of the general dearth of nitrogen. Batch cultures however do not leave sufficient room to manoeuvre when the interesting concentrations are vanishingly small. Continuous culture offers a way out of this impasse, but it has problems of its own. Paramount among these is the vitamin binding phenomenon, which is very relevant to the interpretation of the steady-state data that follows.

The binding protein

Proteins released by cultured algal cells that combined reversibly with vitamin B_{12} and rendered it unavailable, were first reported by Kristensen (1956) in cultures of *E. gracilis* and by Ford (1958) with *O. malhamensis*. These

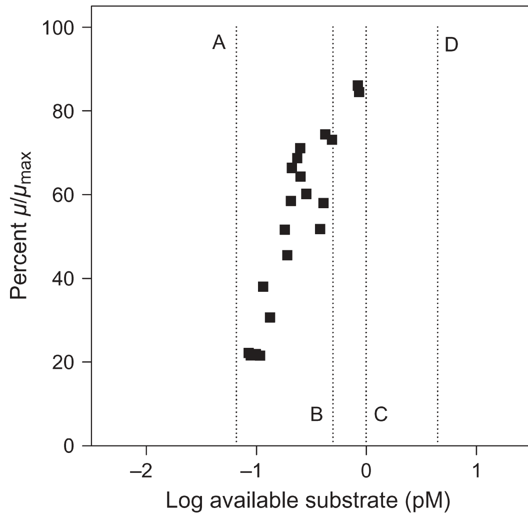

Fig. 2. *Skeletonem costatum.* Chemostat steady-state: dilution rate expressed as a percentage of the curve-fitted washout rate ($\equiv\%\mu/\mu_{max}$) against concentration of free vitamin, as log pM. The vertical lines, the means of some assays of marine waters. A: North Atlantic and north North Sea, summer. B: north North Sea, autumn. C: Butt of Lewis, Scotland, spring (Cowey, 1956). D: Firth of Clyde, Scotland, incoming tide (Droop, 1955), spring, and La Jolla, California, spring and summer (Carlucci, 1970). Reviews of some more recent vitamin B_{12} assays of marine and fresh waters will be found in Provasoli and Carlucci, (1974) and in Swift (1984).

authors observed that the phenomenon, if general, could interfere with attempts to establish the levels of requirement. So it has proved, for in steady-state situations the kinetics are such that interference is so obvious that the classical equations of chemostat operation (Monod, 1950; Herbert *et al.*, 1956) cannot be applied without modification (Droop, 1968). [Symptoms of the interference are first, that the curve of growth rate on apparent substrate concentration is C-shaped, rather than a rectangular hyperbola passing through the origin and second, that the saturation constant (K_S, the substrate concentration producing half maximal growth rate) appears to increase with increasing biomass and decreasing growth rate].

The modification I adopted, which is entirely successful in regard to chemostat operation, is based on the single assumption of constant rate of protein excretion per cell. This leads directly from the mass-action equation (Dixon and Webb, 1958) to a simple expression for the ratio of total to free (i.e. available) substrate in the chemostat, namely

$$\frac{S_T}{S_A} = 1 + \frac{(\tau/\kappa)x}{\mu}. \qquad (1)$$

[x, biomass; μ, specific growth rate; and (τ / κ), a composite constant being the ratio of the rate of excretion of inhibitor to the dissociation constant of the complex]. The ratio S_T/S_A defines the ratio of the spurious to the true saturation constant, i.e. (in the chemostat), $(K_S)_{x=i}/(K_S)_{x=0}$. The value of (τ/κ) is obtained from the chemostat data and is given by the regression

$$\log\left[\frac{(K_S)_{x=i}}{(K_S)_{x=0}} - 1\right] = \log\left[\left(\frac{\tau}{\kappa}\right)\right] + \log\left[\frac{x}{\mu}\right] \qquad (2)$$

solved for zero $\log(x/\mu)$. Details of the work on the binding factor, carried out with *P. lutheri* with ^{57}Co-labelled vitamin in continuous culture, and of my derivation of the two equations, are to be found in my 1968 paper (Droop, 1968). Failure to take into account the binding protein, if present in continuous cultures with vitamin B_{12}, will show total concentration which, at lower dilution rates, can reach a 100-fold that of the available vitamin.

In the application to batch cultures

$$\frac{S_T}{S_A} = 1 + \int_{x=i}^{x=j} \left(\frac{\tau}{\kappa}\right) x \, dt, \qquad (3)$$

and for exponential growth from a small origin,

$$\frac{S_T}{S_A} = 1 + \frac{e(\tau/\kappa)x}{\mu}. \qquad (4)$$

In the open sea, on the other hand, the importance of the binding phenomenon is likely to be negligible. One can obtain an idea of the scale of sequestration from the *S. costatum* data (Droop, 1970). The value of (τ/κ) in the experiment upon which Fig. 2 is based is 16 mL medium μL^{-1} biomass day^{-1} and the ratio of total to free vitamin in the environs of an exponential population will be $1 + e(16x/\mu)$, (x in $\mu L\,mL^{-1}$, μ in day^{-1}). *S. costatum*, a dominant member of the spring bloom of phytoplankton in British coastal waters, can reach 10^4 cells mL^{-1} in a good year. Assuming a cell volume of 250 μm^3, this population amounts to 0.0025 μL biomass mL^{-1}. It gives the S_T/S_A ratio as $1 + 0.04e$ (=1.108), showing that protein binding is of no consequence here; but matters could be different when populations reach red tide proportions.

The *Pavlova and Skeletonema* chemostat data

Figures 1 and 2 are based, respectively, on *P. lutheri* (Droop, 1968) and *S. costatum* (Droop, 1970), and use available B_{12} concentration, computed as detailed above, rather than total as entered in the published tables. The abscissa scale is logarithmic for the sake of

clarity; on a linear scale, the points follow a typical Monod hyperbola. Any conclusions drawn from the figures strictly apply to organisms of dimension similar to the subjects and one would expect, for any particular substrate concentration, uptake to vary as the cell surface area and the growth rate to vary inversely as the cell volume, the latter therefore as the reciprocal of the linear dimension. However, since the restraint applies equally to the maximum as to the lower rates, specific growth rate expressed as a percentage of the maximum should be independent of cell size.

I remarked above that subsistence quotas of the two organisms, *P. lutheri* and *S. costatum*, indicate that each pmol of vitamin was sufficient to yield ~70−90 μL of biomass. Figures 1 and 2 show that both species grow at over 80% of their maximum rates in a concentration of 1.0 pM, while in a concentration of 0.1 pM (i.e. a concentration that might be expected in the open sea) the equivalent rates are 60 and 20%, respectively. It appears that in the open sea an organism like *S. costatum*, if not limited by another nutrient, would reach a population of 26 million cells (=6.5 μL) L^{-1}, while growing at 20% of its maximum rate, whereas in coastal waters, with vitamin titres 1.0 pM or above there is enough vitamin to produce populations growing at their maximum rate to a size more than an order of magnitude greater than that normally recorded. Thus, the results from continuous culture go some way in confirmation of the previous batch data, both as to yield and rate. The diatom apparently has a growth saturation constant (K_S, from the curve fit of μ on S_A with standard error in parentheses) about double that of the haptophyte [0.19 (25%), 0.086 (12%) pM, respectively].

Therefore, I suggest that, at any rate on the evidence of these two species, the supposed general need for symbiosis rests on a misconception of the level of requirement. In the sea, there is no mystery about the provenance of the vitamin in outline; it follows the route taken by other nutrients, namely decay and sinking of spent plankton, bacterial regeneration and upwelling.

COMMENT

The level of requirement

These conclusions from work, now some 35 years old, are at such variance with those reported by Croft *et al.* (Croft *et al.*, 2005) that some comment is in order. The work was sufficiently focused at least to warrant some discussion; accordingly, I invite them to reconsider the global inference they make on a very doubtful premise. They may wish to consider the relative relevance of simple enrichment experiments of the type they report, *vis-à-vis* detailed quantitative work, and also consider the influence of the physiological state of the inoculum on their results as well as the methodological implications of the binding protein and its carryover with the inoculum. Incidentally, Croft *et al.* (Croft *et al.*, 2005) include in their argument the figure 10 μg L^{-1} as the amount of vitamin that most cultures contain. This is hardly relevant; moreover that figure is a 100-fold the highest quantity I ever used when requiring a medium with ample excess vitamin.

To give an example on the effect of the binding protein, a 3 week old source culture of *P. lutheri* with, say, 8 million cells mL^{-1} would contain some 1600 pM of binding protein (in vitamin equivalents), which after a 100-fold dilution on transfer, the ratio of total to free and available vitamin would be something of the order of 6.3.

$$\frac{S_T}{S_A} = \left(\frac{1}{d}\right) \cdot \left(\frac{\tau}{\kappa}\right) \cdot \left(\sum_{0}^{t} x \Delta t\right) + 1 \qquad (5)$$

The two constants of the mass-action relation, τ and κ, were measured by Davies and Leftley (Davies and Leftley, 1985) for *P. lutheri*, and later by Sahni *et al.* (Sahni *et al.*, 2001) for *T. pseudonana* with similar result. The example above is rather extreme, but equation (5) underlines the need to keep biomass (x) as small, and the dilution factor (d), as large as possible.

I may be accused of generalizing from results gathered from a mere two species, and Provasoli and Carlucci (Provasoli and Carlucci, 1974) are correct to caution that algae have been found to differ from one another in the level of requirement for vitamin B_{12}. They suggest that *P. lutheri* may not be typical, but the two examples they cite are unfortunate. The first, dark growth of *O. malhamensis*, an obligate chemotroph whose normal mode of nutrition is phagotrophy (Ford, 1958), is not, as I mentioned, a good example of phototrophic phytoplankton. The second, an unpublished chemostat study (Wood, 1962) of *S. costatum*, did not take sequestration into account and would show substrate concentrations in error of up to two orders of magnitude at lower chemostat dilution rates. In my hands, the growth saturation constant (of free substrate) for *S. costatum* was less than double the mean of my *P. lutheri* entries in Table I and compares with those of *T. pseudonana* (Swift and Taylor, 1974) and *T. oceanica* (Carlucci and Silbernagel, 1969), which ranged from 0.13 to 0.26 pM.

Some measurements of the six species listed by Swift (1984) are shown in Table I. The 20-fold discrepancy in the *P. lutheri* batch entries can be attributed to protocol: an

Table I: Vitamin B_{12} growth saturation constants (K_S, in pM) of the six species for which rate data exist

Species	Method	K_S	Reference
O. malhamensis	Batch	8.7	Ford, 1958[a]
P. lutheri	Batch	<0.1	Droop, 1961
P. lutheri	Batch	0.072	Droop, 1968
P. lutheri	Chemostat	0.086	Droop, 1968 data reworked
P. lutheri	Chemostat	0.18	Droop, 1974, Expt. II
P. lutheri	Batch	1.87	Swift and Taylor, 1974[a]
P. lutheri	Chemostat	0.11	Droop *et al.*, 1982, Expt. VIC
I. galbana	Batch	1.13	Swift and Taylor, 1974[a]
S. costatum	Chemostat	≈5.0?	Wood, 1962[a]
S. costatum	Chemostat	0.19	Droop, 1970 data reworked
T. pseudonana	Batch	0.26	Swift and Taylor, 1974
T. pseudonana	Chemostat	0.17	Swift and Taylor, 1974
T. oceanica	Batch	0.13–0.26	Carlucci and Silbernagel, 1969

[a]See comment in the text.

initial count of 100 exponential phase cells mL^{-1} used in 1961 versus one of 20 000 possibly stationary phase cells mL^{-1} in 1974. It would appear that inoculum size affects the result. This is most likely due to interference by the yield relation when the inoculum is heavy. For example, with an inoculum of 20 000 cells mL^{-1} of *P. lutheri*, with a K_S even as small as 0.1 pM vitamin, growth would have virtually ceased in 4 days in any culture having initially less than 1.4 pM vitamin. As I remarked earlier, a batch system leaves little room for manoeuvre. Also, possible hysteresis in the cell metabolism at the sudden change in substrate concentration and the need to allow time for its avoidance cannot be ruled out, while on the other hand, the effect of binding protein in the 1974 experiment would not be great, with S_T/S_A at the very most 2.0. The same considerations apply to the *Isochrysis galbana* entry. *P. lutheri* chemostat data indicate that the lower figure for K_S is the more likely. Indeed, the figure 0.086 pM indicated by the curve fit of the chemostat μ on S_A data is confirmed by the independent batch uptake data (Droop, 1968). This is possible because the Monod and Cell Quota equations are equivalent in the steady state, and they reveal the following identities when the Michaelis equation for uptake is combined with them (Droop, 1973).

$$K_S = \frac{\mu_{max} k_S k_Q}{U_m} \tag{6}$$

and

$$\frac{k_Q}{U_m} = \frac{1}{\mu_{max}} - \frac{1}{\mu'_m} \tag{7}$$

(K_S and k_S the growth and uptake saturation constants, k_Q the subsistence quota. μ_{max}, U_m and μ'_m the asymptotes of respectively the Monod, uptake and Cell Quota equations). Clothing equation (6) with the parameter values obtained from the 1968 uptake and cell quota data, one can obtain

$$K_S = \frac{0.85(3.0\%) \times 1.73(8.1\%) \times 2.25(4.5\%)}{45.7(15\%)} \text{ pM}$$

$$K_S = 0.072(18\%) \text{ pM}$$

(Standard errors in parentheses). It is inconceivable that there could be a 20-fold error in this value for K_S.

Swift and Taylor (1974) point out that the chemostat-derived K_S has not the same interpretation as that derived directly from the Monod equation. Very true, the one is based on S_A, the other on S_T. However, S in the Monod equation is *necessarily* always S_A, and I note that the validity of my derivation of S_A was not questioned. Swift and Taylor (1974) do not appear to have thought the matter through, for if there is protein binding of the vitamin then $S_A < S_T$, so that

$$\mu = \frac{\mu_{max} S_A}{S_A + K_S} \neq \frac{\mu_{max} S_T}{S_T + K_S}. \tag{8}$$

In batch cultures, the effect of substituting S_T for S_A may not be obvious; in a continuous system, the effect, as I mentioned earlier, is catastrophic. But chemostats do not have the disadvantage of dependence on the immediate previous history of the inocula and are more reliable than batch techniques for this reason; their

operation does not require ultra clean technique and the ^{57}Co label is very convenient. Moreover, they can distinguish between total and available substrate and they can extend the range of the latter to regions inaccessible in batch cultures.

The inocula of the *Thalassiosira* experiments were probably sufficiently small (1800 cells mL^{-1}) for the conclusions concerning them to be reliable. In which case four of the entries in Table I, three of which are pelagic marine diatoms, are very similar in their vitamin B_{12} requirement, both on a yield and a rate basis, and it is very low; they would not generally be limited by the amounts of vitamin occurring in the sea. Other pelagic organisms may have greater requirements, but where is the evidence?

Some wider implications

Of course more is likely to be involved in the control of phytoplankton than simply the level of requirement as measured in the laboratory. It is known that clones cultivated for long periods may adapt to the conditions in which they are kept, and with a plentiful nutrient supply, they might be expected eventually to loose an ability to grow at high rates with low nutrient concentrations and hence come to need higher levels than they did in the wild. I have not considered the provenance and requirement for the various variants of the vitamin molecule, nor of temperature effects. Even the meaning of 'requirement' becomes blurred: Swift and Guillard (1978), for instance, found significant stimulation of a number of non-auxotrophic diatoms by the addition of vitamin B_{12}. Another example that also clouds the issue is a recent paper by Sañudo-Wilhelmy *et al.* (2006) demonstrating that a natural population, brought into the laboratory and enriched with 500 pM vitamin, can result in a 10-fold increase in biomass, but only in the $>5\ \mu m$ fraction, a result that is difficult to interpret in view of the enormous quantity of vitamin employed.

Provenance of the vitamin

Regarding the source of vitamin B_{12}, that it is eventually bacterial is not in question (Hall *et al.*, 1950; Haines and Guillard, 1974), nor is the fact that aquatic bacteria are normally associated with solid matter, nor that some live in close association with algae and can be regarded as symbiotic (Cole, 1982). This, however is a long step from most, especially pelagic, algae being necessarily in symbiotic association as suggested by Croft *et al.* (Croft *et al.*, 2005) on the evidence of one experimental example (of a facultative terrestrial species). Furthermore, regarding the measured marine concentrations of vitamin B_{12}, Andersen (Andersen, 2005) points out, quoting Menzel and Spaeth (Menzel and Spaeth, 1962), that 'there is strong circumstantial evidence that this vitamin is produced on the ocean floor at depths where darkness makes it unlikely that an algal-bacterial symbiosis can exist'.

In the 50s and 60s of the last century interest, engendered by the ideas of Lucas (Lucas, 1949) and Lefèvre *et al.* (Lefèvre *et al.*, 1952) in the possible importance of micronutrients and so-called 'ectocrines' in marine and fresh waters, was strong and the question of close association and even symbiosis with bacteria was very much in our minds. In one attempt at an answer I and my colleague Keith Elson (Droop and Elson, 1966) looked for and tested for bacteria associated with several hundred cells of the large centric diatom *Coscinodiscus concinnus* from tow-nettings over a period of several months in the winter of 1963. We saw no bacteria on the cells and only 12 individuals out of 533 tested positive for bacteria. We also took chains of *S. costatum*, also directly from tow-nettings, in the following spring and examined them for attached bacteria under phase contrast. We found that during the exponential phase of the spring bloom (∼14 days) the bacterial count remained constant at from 3 to 5 per 100 diatom cells and remained so for the first week of the stationary phase, then quite suddenly the count began a rapid increase and was accompanied by signs of diatom lysis. These observations indicate, quite conclusively, that for these two species at least symbiosis was not involved during the growth phase. One cannot of course generalize from two, albeit typical and common, species, but the finding is not in contradiction of the general impression that healthy pelagic diatoms are remarkably clean (Waksman *et al.*, 1937; Cole, 1982).

CONCLUSION

Having regard for the protocols used for measuring the level of vitamin B_{12} requirement in the few marine phytoplankton for which there are data, it would appear that the requirement, on the present evidence, and in the light of marine vitamin available, is sufficiently low for there not to be a general need for direct bacterial symbiosis involving the vitamin to account for the populations occurring.

'Algae acquire vitamin B_{12} through a symbiotic relationship with bacteria' (Croft *et al.*, 2005). Symbiosis certainly, but in the sense that mankind is in symbiotic association with wheat.

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